AN ECOLOGICAL STUDY ON THE BLOOD PARASITES OF THE AUSTRALIAN SKINK, *EGERNIA STOKESII*

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by

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Meinen Eltern

To my parents

Abstract

AN ECOLOGICAL STUDY ON THE BLOOD PARASITES OF THE AUSTRALIAN SKINK, *EGERNIA STOKESII*

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In the course of the present study, field populations of the South Australian skink *E. stokesii* were found to be frequently infected by an unknown number of apicomplexan blood parasite species. A field survey over three consecutive years examined the demography of the lizard, its ectoparasites and the blood parasites at six populations in the Flinders Ranges. Laboratory experiments focussed on the taxonomic descriptions of the blood parasite species, on the identification of their vectors and their impact on ectoparasites of *E. stokesii*, the ticks *Amblyomma vikirri* and *Amblyomma limbatum*. A molecular biological study aimed to develop a diagnostic test for blood parasite infections of *E. stokesii*, and also on a comparative analysis of the 18ssrDNA-nucleotide sequence of each blood parasite species. It was found that *E. stokesii* was infected by six species of apicomplexan blood parasites. The prevalence of the blood parasites and the ectoparasites found varied between the six populations of *E. stokesii*. The adeleorine blood parasites *Hemolivia mariae* and *Hemolivia biplicata* affected the fitness of infected starving nymphs of both tick species. The diagnostic molecular biological assay detected blood parasites from a parasitemia of 0.1% and above. The comparative analysis of the 18 ssrRNA gene supported the taxonomic findings of this study.

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Chapter One

1. GENERAL INTRODUCTION

Contemporary ecological investigations usually aim to describe aspects of the complex interactions of life from a model organism. Critics of this approach claim, that all we will ever achieve through this is a large collection of examples, and that ecological modelling is only wishful thinking. However, considering the little ecological knowledge we have today, attempts to model life seem as presumptuous as the attempts to deny such a possibility. The main obstacle in ecological studies is that with the materials and methods currently available to ecologists, it is only possible to focus on a subset of organisms and environmental factors. Under these circumstances, the choice of a suitable model organism becomes very important. Parasites, and in particular blood-parasites have some significant advantages over many other organisms as study objects, because often their life is closely linked to only one or very few host organisms, and the living conditions when inside a host are often relatively stable. Both of these points substantially reduce the number of variable factors that have to be taken into consideration.

In spite of their epidemiological significance, studies on the ecology of parasites are only a small part of ecological research. During the last decade, a range of models for parasite-host relationships has been produced with the hope to create a better theoretical understanding of the population dynamics of parasites and thus more adequate epidemiological predictions (May & Nowak 1994; Nowak & May 1994; Claessen & De Roos, 1995; Lipsitch et al., 1995; May & Nowak, 1995; van Baalen & Sabelis 1995; Lipsitch *et al.*, 1996; Marschner, 1997). Yet, it is not the lack of models, but the lack of field data that severely hampers further development (Bull, 1994). This is even more true for the complex relationships in parasite-vector-host systems. Risk assessment or attempts to control the spread of the tick transmitted Lyme borreliosis in Europe and the USA is largely impeded by the number of potential tick-hosts and the probably high number of reservoir competent vertebrates (Spielman *et al.*, 1985; Kahl, 1991; Tällekint & Jaenson, 1995; Gray *et al.*, 1998). A detailed study of a natural parasite-vector-host system with a more discernible number of species involved could produce valuable information for both practical and theoretical epidemiological questions.

Due to their territoriality and their often long life span, reptiles are easy to mark and recapture; thus parasitic micro-organisms of reptiles are excellent study objects to investigate the evolution and epidemiology of natural infections (Ayala, 1977). The micro-parasites most frequently reported to infect reptiles belong to the protozoan phylum Apicomplexa (Levine, 1988). Among the Apicomplexa, many species of blood parasites have been described from reptiles. However, in order to identify the proper generic position of blood parasites, in most cases the gametogenesis and sporogony within a definitive host have to be described (Garnham, 1954; Ball, 1967; Paterson & Desser, 1976; Levine 1982; Siddall & Desser, 1990). So far only a few life cycles have been examined completely (Desser, 1997), due to the often laborious task of finding a vector and describing the development of a given micro-parasite. As a result, the species diversity of the blood parasites is poorly understood, and more experimental work is needed to identify species boundaries (Adlard & O'Donoghue, 1998).

In contrast to the many blood parasites of medical and veterinary importance, little is known about the pathogenicity of blood parasites on non-mammals (Desser, 1993). Nevertheless, this may have substantial implications for the epidemiology of vectored diseases and the many taxa parasitising lower vertebrates and invertebrates may hold the key to effective biological control agents for arthropod vectors of disease.

Beginning in 1995, several research projects at the Flinders University of South Australia focused on the skink *Egernia stokesii* Gray (Reptilia: Scincidae), mainly to investigate the ecology and behaviour of a lizard that reportedly lived in social groups. Extensive laboratory and field studies stretched from the ethology over the molecular genetics to the blood parasites of the lizard. A preliminary investigation on the blood of *E. stokesii* from the Flinders Ranges in South Australia indicated an abundant and a possibly diverse community of coccidian (Protozoa: Apicomplexa) parasites. At the starting point of this study, the extent of diversity of the micro-parasite fauna in *E. stokesii* was not known. Since it has been estimated that less than one third of the Australian parasitic protozoa in vertebrates have been described (Adlard & O'Donoghue, 1998), it was possible that undescribed species of blood parasites were involved. It was obvious that on the one hand a proper identification of the blood parasites was essential for the planned comparative demographic field-study, but on the other hand it was not predictable whether eg. a vector could be identified for each parasite species involved. Thus it was necessary to establish the morphological features to at

least differentiate between all blood stages of parasites in **mixed infections**[1]. A host of methods was employed in order to strengthen or reject the taxonomic validity of these then hypothetical features, ranging from histological studies with the light microscope and transmission electron microscope (TEM) to transmission experiments to molecular biological techniques.

1.1 The light microscope (LM)

Morphological examinations with the light microscope have been the basis for the vast majority of all surveys and taxonomic publications of blood parasites in the past. The light microscope was the fastest and most economic tool to identify and quantify micro-parasites, yet with the few discernible features of the vertebrate stages of blood parasites it is often of very limited use to differentiate between species in mixed infections (Levine, 1982). Because of the planned survey on blood smears of lizard populations, it was crucial for this study to find features that allowed the author to discriminate between the species of blood parasites by light microscopy. A comparison of the taxonomic results of this study derived from light microscopy are presented in chapter five. The results of the survey on the population dynamics of the blood parasites and their vertebrate host and, where known, invertebrate host were described in chapter fourteen.

1.2 The transmission electron microscope (TEM)

The TEM reveals the so-called ultra-structure of a parasite, ie. structural features at the sub-cellular level. Because TEM allows study of the three dimensional structure of objects in a much better way than LM, it helps to reveal the true shape of objects. Many TEM studies on coccidian blood parasites have been conducted in the past, eg. (Stehbens & Johnston, 1967; Sterling & Aikawa, 1973; Klein *et al.*, 1988a; Siddall & Desser, 1992; Lowichik *et al.*, 1993; Tegoshi *et al.*, 1993; Desser *et al.*, 1995; Paperna & Finkelman, 1996; Smallridge & Paperna, 1997), thus permitting a comparative analysis of the ultrastructural morphology. Nevertheless, the preparations for TEM are laborious. Because of the brittle structure of some blood stages the preparations are often poor quality (Desser *et al.*, 1995) and the taxonomic use is limited. Still, the results can be of use when combined with data gathered from other methods. Where available, the taxonomic results of this study derived from TEM are presented separate for each parasite species investigated in the chapters six to ten.

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[1] Glossary terms were printed in bold (find glossary on p. 359).

1.1 The transmission experiments

Transmission experiments mainly serve the dual purpose of identifying a vector, and to document the complete life cycle of a parasite. As explained above, in many cases the developmental stages and their location within a vector are important characters in the taxonomic identity of a parasite. The identification of a vector species is of fundamental importance for the understanding of parasite epidemiology. In this study, it was of particular concern to follow up new blood infections and to identify all **semaphoronts** in the life cycle of a blood parasite in order to establish the simple discriminative features for mixed infections. The results of the transmission experiments were included in the chapters six to ten. The vector capacity of in particular the ticks *Amb. vikirri* and *Amb. limbatum*, both ectoparasites of *E. stokesii*, were presented in chapter twelve.

1.2 The evaluation of tick fitness

As mentioned before, little or nothing is known about the impact of micro-parasites on their arthropod vectors. In a comparative analysis, the fitness of the tick species *Amb. limbatum* and *Amb. vikirri* was examined in regard to the influence of blood parasite infections of their lizard host, *E. stokesii.* Five aspects of tick fitness were evaluated: the number of successfully engorging ticks, the time they spent attached to the host, their detachment mass, their body mass loss and their mortality. The results are presented in chapter thirteen.

1.3 The molecular biological study

The two main tasks of this part of the thesis project was to develop a sensitive diagnostic test for the lizard blood parasites by the polymerase chain reaction (PCR) and to support the taxonomic data gathered by the vector studies, by comparison of assumed homologous ssrRNA gene-sequences from the blood parasites infecting *E. stokesii.* There was no intent to carry out a phylogenetic analysis of the taxa investigated, although the data gathered hopefully may serve as the starting point for any such study in the future. The results of this investigation are summarised in chapter eleven.

1.4 Study aims

The aims of this study were to identify the micro-organisms and their life cycles, to elucidate their pathogenicity, to develop a diagnostic molecular biological assay for the blood parasites and to describe the demographic relationships of lizards, vectors and blood parasites at six field sites over a period of three years.

Chapter Two

2. THE TAXONOMY OF BLOOD PARASITES

A preliminary survey of peripheral blood smears of *E. stokesii* indicated the presence of eucariotic, apparently intraerythrocytic parasites. The only eucariotes known to enter red blood cells of vertebrates are protozoans from the apicomplexan subclass Coccidia (Wakelin, 1984). Thus a preliminary identification of the blood parasites found in *E. stokesii* was made using their obvious eucariotic features. The following paragraphs of this chapter will give a short overview of the higher taxa within the coccidia, emphasising the genera that were of relevance for this study.

On two occasions, lizard blood from outside the regular study sites was found to be infected with a microfilaria (Nematoda) (Figure 7/D, p. 31). Because of the very low prevalence, no further attempts to identify this parasite have been made. Most lizards in this study were monitored for parasites by blood smears only. In addition, eight faecal flotations were inspected and nine lizards were killed for histological examinations. Faecal flotations of *E. stokesii* housed in the Animal Care Unit (ACU) of the Flinders University of South Australia (FUSA) showed infections with a species of *Eimeria* (Figure 7/A), an oxyurid nematode (Figure 7/B) and a parasite of uncertain taxonomic status (Figure 7/C). Dr. Sam Telford Jr., to whom some parts of the material were sent, confirmed these results. No attempts were made to determine their prevalence in the field. Hopefully their ecology will be the subject of future research projects.

2.1 The Apicomplexa

About 4600 species of the entirely parasitic protozoan phylum Apicomplexa have been named so far, with probably ten times as many to be described (Levine, 1988). The phylum contains organisms that are considered to have no detrimental effect on their hosts as well as the most lethal protozoan diseases of humans and animals.

2.1.1 Basic taxonomic considerations

With the systematics of the phylum Apicomplexa under constant revision, it is impossible to display a taxonomic scheme without joining or rejecting certain schools of thought. The author would like to emphasise that the main accent of this thesis was not to rank any of these suggestions, though hopefully some of the data presented will contribute to the taxonomic discussion. The classification used here (Figure 1) is based largely on the publication of Levine (1988), but partly from other authors as well. Figure 1 lists all major taxa relevant for the taxonomic reflections in this study. I have followed the phylogenetic structure of the taxon Haemogregarinidae proposed by Barta (1989) but included a recently described genus relevant for this study, *Hemolivia* (Petit *et al.*, 1990). For the family Plasmodiidae, the classification suggested by Telford (1988; 1994) was used.

The main difficulties in the systematics do not arise with the taxonomic classification of the Apicomplexa, but with the lack of consistency of the nomenclature (Cox, 1993). The nomenclature adopted for this thesis was derived mainly from those publications that shaped the structure of the displayed taxonomic scheme. Wherever an author is quoted and the nomenclature used for this thesis differs from the original quotation, the terminology of the original author is added in brackets.

The nomenclature for the life cycle stages the family Karyolysidae, which is one of the haemogregarine taxa described in the following, has been recently discussed (Barta & Desser, 1998; Siddall & Desser, 1991). Those authors suggested to adopt a nomenclature based on the terms generally used in the developmental sequence of the coccidia, rather than on the unique terms proposed by Reichenow in the original description of the Karyolysidae (Reichenow, 1921). This thesis followed the original terms proposed by Reichenow, in order to keep the text easier to read.

Phylum **APICOMPLEXA** Levine, 1970
- Class **SPOROZOEA**
  - Subclass **COCCIDIA** LEUCKART, 1879
    1. Order **COCCIDIASINA**
    2. Order **EUCOCCIDIORIDA** Léger and Duboscq, 1910
       - Suborder **ADELEORINA** Léger, 1911
         - Family **HAEMOGREGARINIDAE** Neveu-Lemaire, 1901
           - *Haemogregarina*
         - Family **KARYOLYSIDAE** Wenyon 1926
           - *Hemolivia* Petit et al., 1990
           - *Karyolysus* Labbé, 1894
         - Family **HEPATOZOIDAE** Wenyon 1926
           - *Hepatozoon* Miller, 1908
       - Suborder **EIMERIORINA** Léger, 1911
         - Family **EIMERIIDAE**
           - *Eimeria* Schneider, 1875
         - Family **LANKESTERELLIDAE**
           - *Lankesterella*
         - Family **DACTYLOSOMATIDAE** Labbé, 1894
           - *Schellackia* Reichenow, 1919
    3. Order **HAEMOSPORORIDA** Danilewsky, 1885
       - FAMILY **PLASMODIIDAE** Mesnil, 1903
         - *Haemoproteus*
         - *Leucocytozoon*
         - *Plasmodium* Marchiafava and Celli, 1885
           - Subgenus *Asiamoeba*
           - Subgenus *Carinamoeba*
           - Subgenus *Fallisia*
           - Subgenus *Lacertamoeba* Telford, 1988
           - Subgenus *Garnia*
           - Subgenus *Ophidiella*
           - Subgenus *Paraplasmodium*
           - Subgenus *Sauramoeba*
         - *Saurocytozoon*
    4. Order **PIROPLASMORIDA**

Figure 1. The phylogenetic scheme of the Coccidia adopted for this thesis (see also 2.1.1). The relevant taxa are listed with their authors.

2.1.2 Life cycle

A characteristic of the phylum Apicomplexa is a life cycle consisting of three replicative phases: A) **merogony/schizogony**[2], B) **gamogony** and C) **sporogony** (Levine, 1980; Mehlhorn and Walldorf, 1988; Barta, 1989). This basic pattern has been modified several times throughout the phylum, and it is mainly the special features of the life cycle that are diagnostic for the phylogenetic position of a blood parasite. Most stages within the life cycle are haploid. The diploid zygote undergoes meiotic division soon after its formation to become haploid again.

2.1.3 Ultrastructure

There is a high degree of uniformity in ultrastructure within the phylum Apicomplexa For instance the fine structure of *Plasmodium* and the "haemogregarines" (see 2.2.1) is very similar (Kim *et al.*, 1998). The autapomorphies for the phylum Apicomplexa include the apical complex, which is an ultrastructural feature observable during some phases of the life cycle. This consists of a conoid, a polar ring, apical rings and rhoptries (Siddall, 1995). The function of the apical complex is unknown, but it is believed to be of special importance during the penetration of a host cell wall (Siddall & Desser, 1992). Other characteristic sub-cellular structures are a tri-laminar pellicle, micropores, sub-pellicular microtubules and micronemes (Siddall, 1995). Some of the taxa have flagellated sexual stages, but the motility is believed to depend on structures of the cell membrane, longitudinal ridges, and their connection to intracellular microtubules.

2.2 The Coccidia

2.2.1 Taxonomy

The generally intracellular apicomplexan subclass of Coccidia comprises the four orders Coccidiasina, Eucoccidiorida, Haemospororida and Piroplasmorida (Levine, 1988)(Figure 1), The Coccidiasina are parasites of marine invertebrates and the Piroplasmorida are important blood parasites of livestock, but do not parasitise reptiles. Their biology is not explained in more detail here. The Eucoccidiorida and the Haemospororida are known to contain taxa that frequently infect reptilian blood. The Eucoccidiorida are formed by two suborders: the

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[2] In the literature, the term schizogony it is often used interchangeably with merogony. Because the term schizogony is historically so tightly interwoven with the description of *Plasmodium* parasites, it was preferred in this thesis for the *Plasmodium* life cycle; the now more widely accepted term merogony was bestowed on the haemogregarines.

Adeleorina and the Eimeriorina. The Haemospororida contain only the single family Plasmodiidae.

In order to identify the proper generic position of a coccidian parasite, the gametogenesis and sporogony within a definitive host have to be described (Siddall & Desser, 1992; Garnham, 1954; Ball, 1967; Paterson & Desser 1976; Levine, 1982; Siddall & Desser, 1990; Desser, 1997). However, most species descriptions in the literature are based entirely on the morphology of one semaphoront of their life cycle, mostly the intraerythrocytic gamont (Siddall, 1995). In practice, almost all apicomplexan parasites found in vertebrate blood cells are labelled with the colloquial name "haemogregarines", unless they can be identified as piroplasmids or malarial parasites (Levine, 1982). The qualifier "sensu lato" has been proposed as a label for all haemogregarines where the sexual stages and the sporogony are unknown (Mohammed & Mansour, 1959). As a result, the haemogregarines are "a taxonomic morass" (Levine, 1982).

2.2.2 Life cycle

The typical life cycle of a coccidian parasite consists of several asexual generations (sporogony and merogony/schizogony) followed by a sexual generation (gamogony). Generally, sporozoites enter an epithelial cell of a host and undergo merogony/schizogony, where the nucleus of the parasite (now a meront/schizont) experiences a rapid succession of mitoses. The resulting merozoites are released and enter a new cell of the same host. The number of times this cycle is repeated may vary among taxa and is used in some cases as a taxonomic feature. Eventually some merozoites develop into the sexual forms, the gametocytes, which initiate the sexual cycle of development (gametogenesis). The sexual stages enter new cells, become rounded, and differentiate into the male microgametocytes and the female macrogametocytes. The microgametocytes grow, undergo reduction division and develop into several microgametes. The macrogametocytes increase in size as well and experience a single reduction division. During fertilisation, a single microgamete enters a macrogamete, while the remaining microgametes are finally absorbed from the resulting zygote. The zygote undergoes sporogony, forming sporoblasts that each subsequently develop into a hard-walled sporocyst. The life cycle is completed with the development of sporozoites, the infective stages inside the sporocysts.

2.2.3 Parasite-host relationships

The development of the Coccidia can be restricted to a single host (monoxenous) or it may depend on two or more hosts (heteroxenous). In parasites that develop an obligate heteroxenous life cycle, the place where each of the developmental steps take place can be different. In some genera, eg. *Plasmodium*, merogony occurs in the vertebrate host and the gamogony and the sporogony occurs in the invertebrate vector. In contrast, parasites from the genus *Schellackia* have their merogony, gamogony and sporogony in the vertebrate host and no development takes place in the vector.

The host animals of heteroxenous parasites can be separated into the definitive host, where the sexual phase proceeds, and the intermediate host. Thus, for example the definitive host for parasites from the genus *Plasmodium* is the invertebrate vector species. Some parasites have parathenic hosts, where no development occurs but only the accumulation of infectious stages takes place (Mehlhorn & Walldorf, 1988). In ecological terms, the latter is also part of the group of reservoir hosts, comprising all organisms in which the parasite is able to persist.

2.2.4 The suborder Adeleorina

Typical for the suborder Adeleorina is a temporal association of the sexual stages during the phase of reproduction labelled as syzygy and a microgamont that produces one to four microgametes (Levine, 1988). Barta (1989) concluded from his detailed phylogenetic analysis of the class Sporozoea that the Adeleorina (Haemogregarinidae) are not a monophyletic group, and his data supported the split of the group Adeleorina into three families: the Haemogregarinidae, the Karyolysidae and the mono-generic Hepatozoidae, as suggested earlier by Wenyon (1926, as cited in Barta, 1989). The genus *Hemolivia* was described after these reviews were written (Petit *et al.*, 1990), yet in a soon to be published taxonomic revision *Hemolivia* will be placed within the family Karyolysidae (Barta, in press).

2.2.4.1 The family Karyolysidae

The gamonts of the family Karyolysidae have been found primarily in erythrocytes. Merogony takes place in the internal organs of the vertebrate host. Syngamy, meiosis and sporogony occur in the invertebrate vector. The main taxonomic feature of this family is the development of motile sporokinetes within the oocyst. The sporokinetes enter new cells of the same vector individual to form sporocysts, which after maturation contain up to 40

infective sporozoites. The vertebrate host becomes infected by the ingestion of the invertebrate vector (Levine, 1988).

2.2.4.1.1 Karyolysus

The genus *Karyolysus* was given its name because the first described species was reported to break up the nucleus of the parasitised erythrocyte. Levine (1982) stated that this was not only the wrong translation for the described process (it should be *Karyohexis*), but it was actually not appropriate because many other blood parasites from diverse genera described since then have also been reported to break up the host cell nucleus (e.g. Kim *et al.*, 1998). Moreover, some other *Karyolysus* species described since then do not split the erythrocyte nucleus at all (Dörflein & Reichenow, 1953; Svahn, 1975).

Karyolysus has been found exclusively in lacertid lizard-hosts (Levine, 1988). Six of the ten species of *Karyolysus* known today were described between 1894 and 1921. In all cases where a vector has been identified, the micro-parasites are transmitted by the lizard ingesting haematophagous mites (Levine, 1988). The most detailed description to date of the life cycles of five *Karyolysus* species in the lizard *Lacerta muralis* was done in 1921 (Reichenow, 1921). Figure 2 displays the life cycle of *Karyolysus lacertae* in the lizard host, *Lacerta muralis*, and in the invertebrate vector, the mite *Liponyssus saurarorum* (drawing from Mehlhorn & Walldorf, 1988). Sporozoites (1) enter the lizard host when it ingests an infected mite. They penetrate the gut epithelium of the lizard and invade endothelial cells of the blood capillaries in the liver and other internal organs (2). After a first round of merogony (3)(4) the resulting large merozoites undergo one additional merogonic cycle in which smaller merozoites are produced. The small type of merozoites may undergo more merogonic cycles or they may enter the red blood cells of the lizard (5), where they form the gamonts. During a blood meal on an infected lizard, a mite can ingest infected erythrocytes. The gamonts are released into the midgut lumen of the mite (6). The further development divides the genus into two groups: 1. the species that develop within the digestive system of the mite (eg. *K. bicapsulatus, K. lacertae*) described in Figure 2, and 2. the species that develop within the mite coelom (eg. *K. biretortus, K. lacazei*), which are not displayed in the figure. Both groups form an elongated, spindle shaped bi-cellular syzygy (7). The gamonts of group 1. either associate in the lumen or enter singly the non-digestive, juvenile epithelial cells to undergo syzygy there. The pairs of gamonts in the gut lumen become engulfed by the digestive epithelial cells of the mite midgut (8). The gamonts of group 2 penetrate the midgut wall and enter the coelom of the

mite. They develop in the muscle, hypodermis, tracheal cells, salivary glands and the seminal receptacle of the mite. Once inside a host cell, further development of both groups is very similar. The macrogamont increases in size (9). The nucleus of the microgamont divides once and finally produces two bi-flagellated microgametes (10). One microgamete fertilises the macrogamete (11), and the resulting zygote grows considerably (12)(13). After nuclear division (14), up to 64 (*K. bicapsulatus)* sporokinetes are formed within a round oocyst (15). The shape of the oocyst may be distorted to oval or irregular shapes. The sporokinetes leave the oocyst (16) and if a female mite has produced eggs, they enter the eggs (17) and form sporocysts, which after further development contain 14-40 infectious sporozoites (18)(19). Otherwise their development in female mites is halted until eggs are produced. In male mites, sporokinetes do not develop into sporocysts (Reichenow, 1921).

Oviposition in the mite *L. saurarorum* occurs 1-2 days after a female has taken a blood meal. Although one egg may be infected by hundreds of sporokinetes, the development of the larva is not affected. The larvae hatch after 5 days; they live on yolk reserves from the eggs and do not take up food. The sporokinetes begin to form the sporocysts, which early on develop a strong cyst wall. After 1-2 days the larvae moult to nymphs. At that time the development of the infective sporozoites within the sporocysts is completed. Sporocysts occur intracellularly in the epithelial cells of the nymphal midgut. Within a few days a nymph takes its first blood meal, whilst it stays attached to the host for one to four days. The engorged nymphs need approximately 24 h until the blood meal is digested. During this time the mites often lose the inherited infection, probably due to the excretion of void digestive cells, which harboured the sporocysts. The nymphs moult to adults and mate immediately afterwards. Infections acquired during the nymphal blood meal usually do not persist into the adult stage. Only *K. biretortus* has been described to develop within engorging nymphs. The first blood meal as an adult occurs over night, two to three days post mating and usually lasts only a few hours. Females lay their first batch of eggs within a few days; a second and third blood meal occurs after six to eight and ten to fourteen days respectively, always followed by oviposition. Where a female has had its first blood meal on an infected host, the first batch of eggs may be already infected.

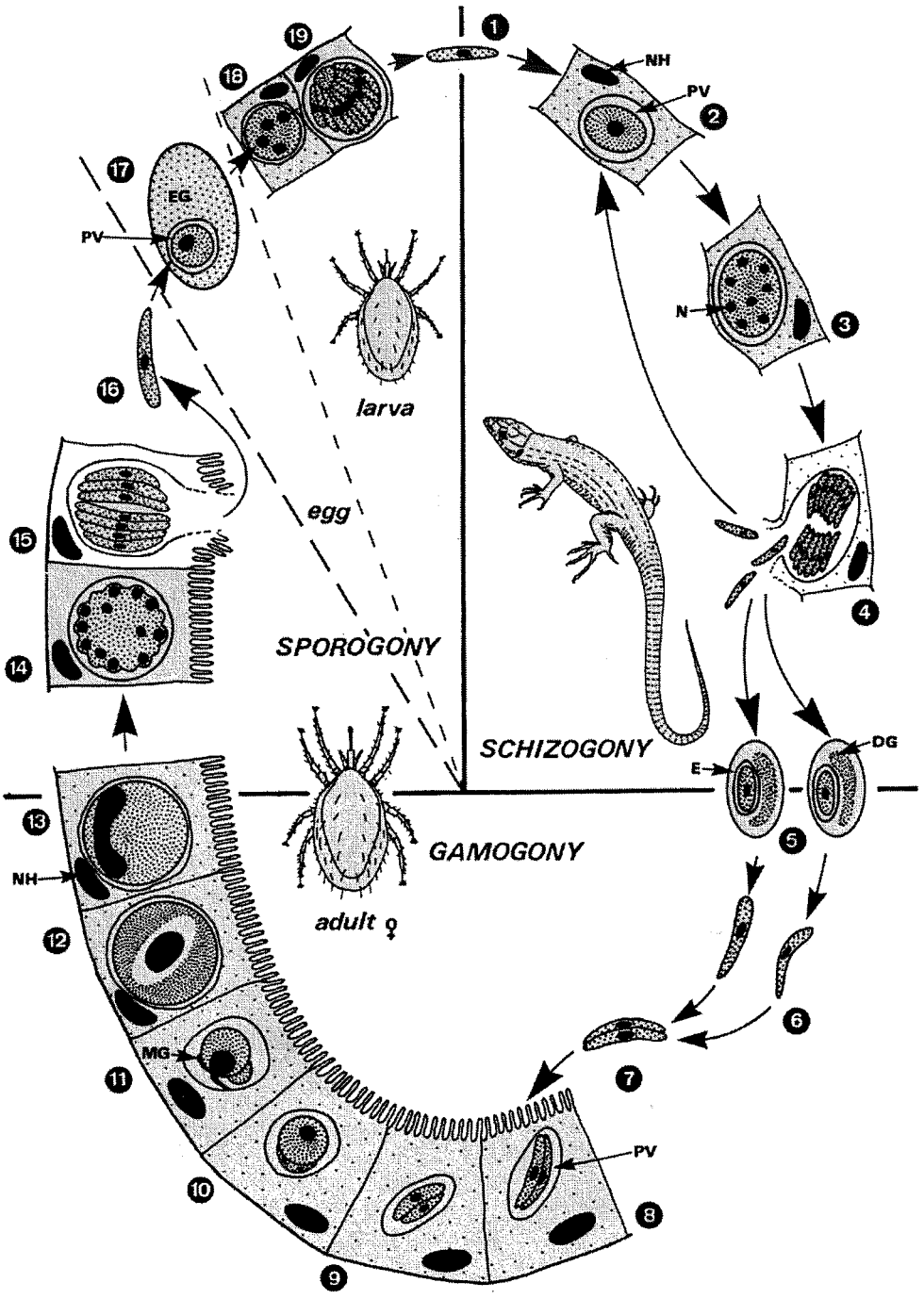

Figure 2. The life cycle of *Karyolysus lacertae* inside lizards and mites (from Mehlhorn & Walldorf, 1988); for explanation see p. 12).

In total one female may lay 50 - 60 eggs before it eventually dies. Males can feed and mate several times before they die. These observations were made in temperature conditions between 25°C-30°C. Reichenow reported two negative implications of high *Karyolysus*-infections on the mite *L. saurarorum*: adults may lose their motility and sometimes a new blood meal can not be digested. Otherwise there were no observable negative effects of *Karyolysus* on the vector or the vertebrate host. In laboratory experiments, unfed mites had to be burst open before they were fed to the lizard in order to achieve a transmission (Reichenow, 1921).

2.2.4.1.2 Hemolivia

The genus *Hemolivia* within the family Karyolysidae has only recently been proposed. It originates from the description of *Hem. stellata* infecting the South American toad, *Bufo marinus*, and the vector tick, *Amblyomma rotondatum* (see Petit *et al.*, 1990). Since then, two other species of this genus have been described. *Hem. mariae* from the Australian lizard *Tiliqua rugosa* is transmitted by two species of ticks, *Amblyomma limbatum* and *Aponomma hydrosauri* (see Smallridge & Paperna, 1997). *Hemolivia mauritanica* was re-described from *Hepatozoon mauritanicum*, a blood parasite of the north African tortoise, *Testudo graeca*, and the vector, *Hyalomma aegyptium* (see Landau & Paperna, 1997). Petit *et al.* (1990) postulated that *Hemolivia* is the sister taxon to *Karyolysus*.

Figure 3 displays the life cycle of *Hemolivia stellata* in the Brazilian toad, *Bufo marinus*, and in the vector tick *Amblyomma rotondatum* (drawing from (Smith, 1996). Gamonts (1), ingested during a blood meal, enter the midgut epithelium of the tick (2) to form a syzygy (3). During gametogenesis, two microgametes and one macrogamete are formed (4). One microgamete mates with the macrogamete (5)(6). The developing zygote (7) becomes star shaped (8)(9) and motile sporokinetes (10) develop inside. Bursting epithelial cells release large oocysts into the midgut lumen of the tick (11) where the sporokinetes leave the oocyst and either re-enter epithelial cells (12a) or enter exhausted, dispatched digestive cells (12b). Sporocysts were sometimes found excreted with the faeces (15). In the laboratory, *H. stellata* can be transmitted to toads by force feeding them with infected tick nymphs, or, hypothetically, by smears from infected tick faeces. Sporozoites are released into the gut lumen of the toads (18)(19). Sporozoites then penetrate the epithelial cells of the digestive tract to form meronts, either in the reticulo-endothelial cells of the blood capillaries (20)(22)(25) or in various tissue cells of the inner organs (21)(23).

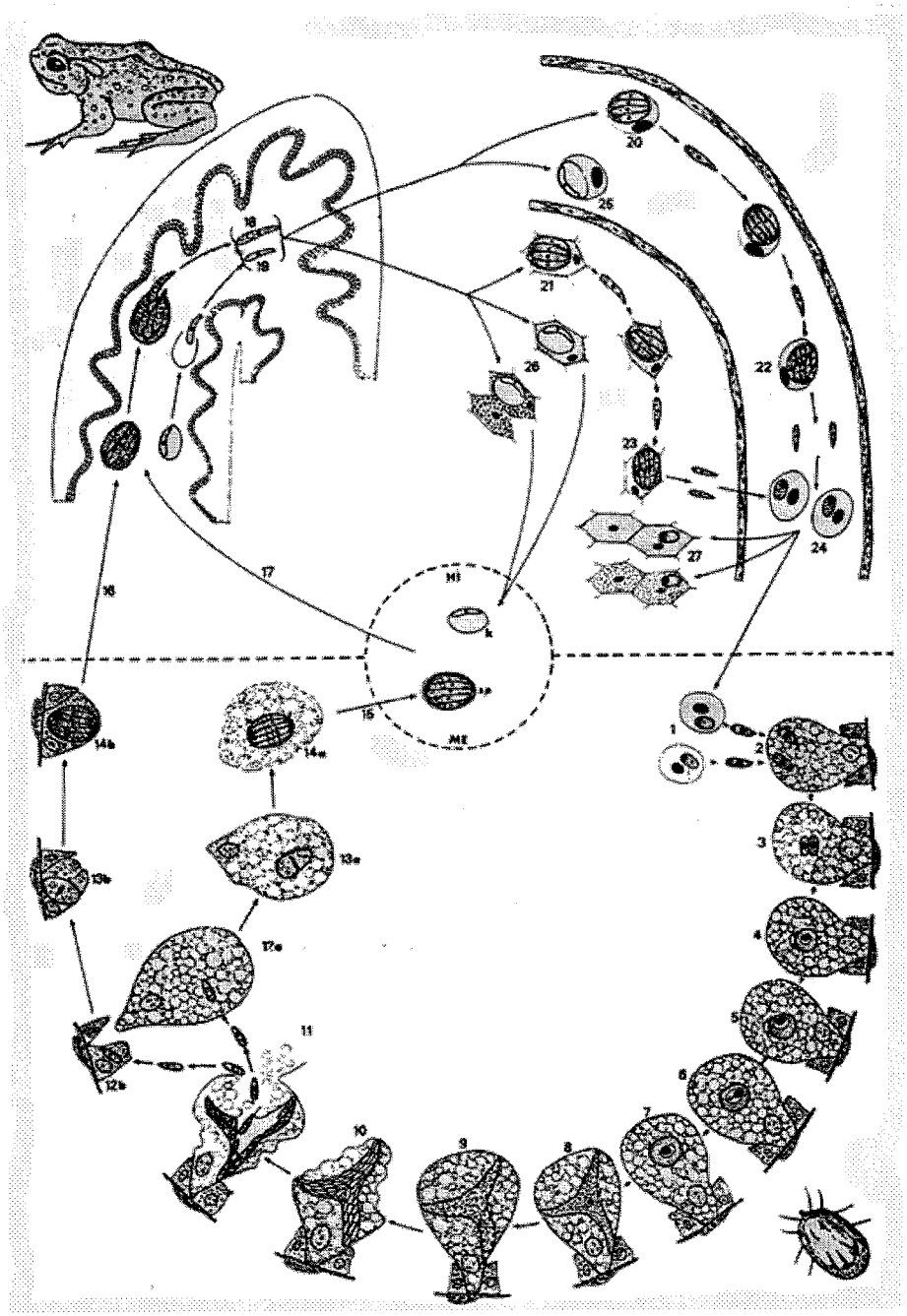

Figure 3. The life cycle of *Hemolivia stellata* in the toad *Bufo marinus* and the tick *Amblyomma rotondatum* (from Petit *et al.*, 1990); for explanation see p. 15.

Mature merozoites enter the red blood cells of the host (24) where they might re-infect other tissue cells (27) or form the gametocytes (25). Petit *et al.* suggested that sporozoites that had entered tissue cells (26) might be excreted with the faeces of the toad and might re-infect other *B. marinus* (1990). Normally the cycle is repeated when ticks engorge on infected toad erythrocytes.

The life cycles of species in the genus *Hemolivia* is very similar to those of *Karyolysus* that have been documented. *Hem. stellata* was placed in a new genus for the following reason: "In contrast to *Karyolysus*, in which sporokinetes are formed in the adult mite and sporozoites in the offspring, in *Hemolivia*, sporokinetes and sporozoites are formed in the same individual tick" (Petit *et al.*, 1990). In the same publication, several "peculiarities" of the life cycle of *Hem. stellata* were noted:

1. merogony (schizogony) and cyst formation in the erythrocytes and reticulo-endothelial cells
2. sporogony inside the gut cells of the tick, with the formation of a star-shaped oocyst
3. sporokinetes migrating from the oocysts to new digestive cells in the same tick individual, where they divide and give rise to sporocysts containing sporozoites.

2.2.4.2 The family Hepatozoidae

The third family in the suborder Adeleorina, and the second to contain species to infect reptiles, is the family Hepatozoidae. However, one recent study suggested that the genus *Hepatozoon* within this family is not a monophyletic group (Smith & Desser, 1997). The phylogenetic relationships within the family have not been established yet. For the latest review of the genus *Hepatozoon* see Smith (1996).

2.2.4.2.1 Hepatozoon

Relatively few *Hepatozoon* life cycles have been elucidated (Desser, 1993). The life cycle resembles those of the *Karyolysidae*, but with some differences. Sporogonic development results in the formation of large round oocysts that can contain hundreds of sporocysts. Each of the sporocysts embodies 4-16 or more of the infective sporozoites. This multi-sporocystic oocyst marks the distinct difference in the life cycle between *Hepatozoon* and the Karyolysidae, where the sporokinetes move out of the oocyst and form the sporocysts that develop outside of the oocyst.

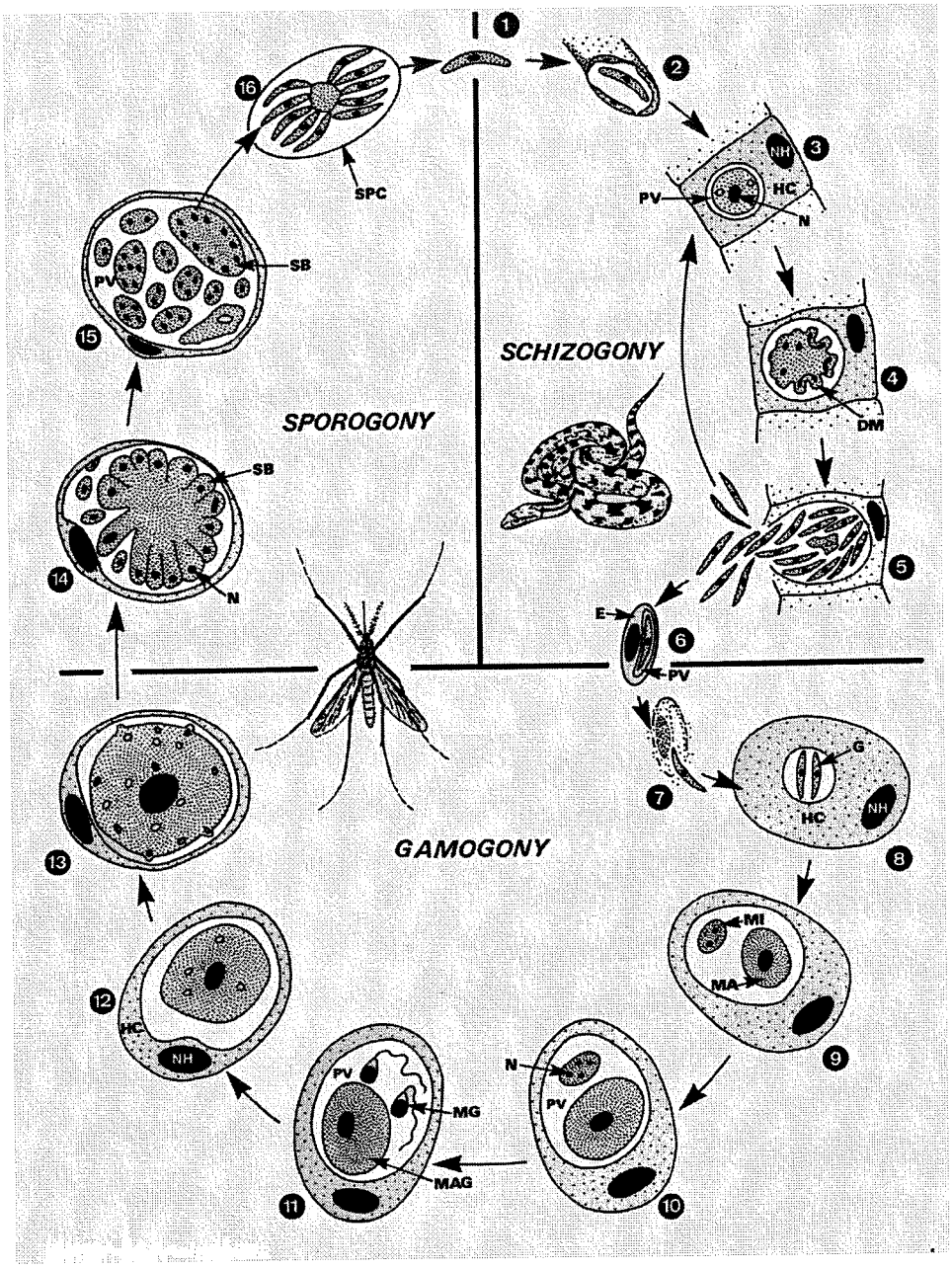

Figure 4. The life cycle of *Hepatozoon aegypti* inside snakes and mosquitoes (from Mehlhorn & Walldorf, 1988); for explanation see p. 19.

The basic life cycle of a range of species within the genus *Hepatozoon* was found to be very similar (Smith, 1996). As an example, the development of *Hepatozoon aegypti* inside the snake, *Spalerosopsis diadema*, and the mosquito vector, *Culex pipiens molestus* is displayed in Figure 4 (drawing from Mehlhorn & Walldorf, 1988). Sporozoites (1) enter the snake by ingestion of a female mosquito and move into the lung capillaries (2). After penetration of the endothelial cells of the capillaries (3), they develop into schizonts (4), that contain merozoites (5). After the first round of merogony the resulting large merozoites undergo additional merogonic cycles within various organs before they finally give rise to small type merozoites. The small merozoites enter the erythrocytes and sometimes leucocytes of the snake, where they form the gamonts (6). These are ingested by mosquitoes feeding on snakes. Once inside the mosquito (7), the development can occur in the gut lumen, the coelom or the malphigian tubules (Wenyon, 1909) The gamonts form a syzygy in a parasitophorous vacuole (8) and subsequently they differentiate into micro- and macro-gamonts (9). Nuclear division(s) (9)(10) form two to four uni- or biflagellate microgametocytes (12). One microgametocyte enters the macrogametocyte to form the zygote (13), the remaining microgametocyte(s) stay adhered to the zygote wall. The large developing oocyst (14) finally contains up to several hundred sporocysts (15) and remains intact even when the sporozoites (16) have matured.

A great variety of haematophagous invertebrates have been identified as vectors for species of *Hepatozoon* including ticks, mites, reduviid bugs, sandflies, tsetse flies, fleas, lice, leeches and mosquitoes (Smith, 1996). The vertebrates found infected include mammals, birds, lizards, snakes, turtles and amphibians (Levine, 1988). Only one Australian species of *Hepatozoon* from reptiles has been described from varanid lizards in Queensland, vectored by *Culex pipiens fatigans* (see Mackerras, 1961).

2.2.5 The suborder Eimeriorina

Most species of this suborder inhabit the intestinal epithelium of their host. The macrogamete and microgamont develop independently and syzygy does not occur. A few genera also infect blood cells. The life cycle of the majority of parasites from this group resembles that of the genus *Eimeria*, which is briefly described in the next paragraph (2.2.5.1). Although *Eimeria* is not a blood parasite, its was mentioned in this chapter because it was of further relevance to the taxonomic descriptions in following chapters. Depending on the genus, the Eimeriorina may be monoxenous or heteroxenous. In a single-host life cycle, the parasite is usually transmitted by the ingestion of sporulated oocysts in faeces. Most species expel unsporulated oocysts with the faeces of the host, and the oocysts develop exogenously. In many reptilian Eimeriorina, the oocysts are already sporulated when they are set free with the faeces. Most eimeriorine genera are commonly reported to infect reptiles. A brief description of the life cycle for the genus *Eimeria* and a detailed description of the life cycle for the genus *Schellackia* are presented because they were of further relevance for this thesis.

2.2.5.1 *Eimeria*

Parasites from this entirely homoxenous genus are undoubtedly the most common coccidian affecting vertebrates and more than 120 species have been described from reptiles alone (Barnard & Upton, 1994). In the typical life cycle, sporozoites from sporulated oocysts infect a host through the oral route and enter the intestinal epithelial cells, where all development takes place. During merogony, multinucleate meronts give rise to motile merozoites, which may undergo another round of merogony or form the gamonts. Multinucleate microgamonts produce many flagellated microgametes. Uninucleate macrogamonts form the macrogametocytes. After fertilisation the zygote develops into the oocyst, which is set free via the faeces.

2.2.5.2 *Schellackia*

The life cycle of species from the genus *Schellackia* may be heteroxenous or homoxenous. The vectors are, where known, mites and dipterans. Merogony, gamogony and sporogony take place in the small intestine of the vertebrate host. Typical for the species in this genus that infect reptiles and amphibians is an oocyst that produces eight sporozoites. Very similar in its life cycle is the genus *Lankesterella,* but in contrast to *Schellackia, Lankesterella* forms 32 or more sporozoites. In some species of *Schellackia*, the parasite enters blood cells of their vertebrate host (Levine, 1988), but in contrast to the adeleorine taxa, it is the sporozoites and

not the sexually differentiated gamonts that do so in the eimeriorine taxa. The fact that no development of the sporozoites occurs in the vector is of epidemiological importance, because as a result, the blood stages of one vertebrate host-individual are infective for new vertebrate host-individuals. The eimeriorine taxa that occupy blood cells can be transmitted from vertebrate to vertebrate by blood inoculation.

Figure 5 shows the diagrammatic life cycle of *S. landauae* in the lizard *Polychrus marmoratus* and in the experimental vector *Culex pipiens fatigans* (drawing from Lainson *et. al.*, 1976). After a blood meal, imbibed sporozoites (A) remain unchanged in the epithelial cells of a mosquito until the vector is ingested by the lizard. The sporozoites then penetrate the epithelial cells of the small intestine, become round and enlarge and form a parasitophorous vacuole (B). In the first round of merogony, multinucleate micromeronts (microschizonts) (C) produce micromerozoites (D). The micromerozoites may enter monocytes and lymphocytes in the spleen and liver to perform an asexual division, apparently by endodyogeny (G) or undergo a second merogonic cycle in a new host cell, where the resulting multinucleate macromeronts (macroschizonts) (E) give rise to macromerozoites (F). The latter develop into the micro- (H) and macrogametocytes (J), that mate intracellularly to form the zygote. The developing oocysts enter the lamina propria (K), a layer of connective tissue the gut epithelium is attached to. The eight motile sporozoites that develop inside a single oocyst are liberated when they have matured and enter the reticulo endothelial cells and capillaries (L). From there they may infect the blood cells of their host, mainly the erythrocytes (N), but also the leucocytes (M), where they can be taken up by a haematophagous vector. Infective, dormant cells can be found in the reticulo endothelial cells of the liver, lung and other viscera (O).

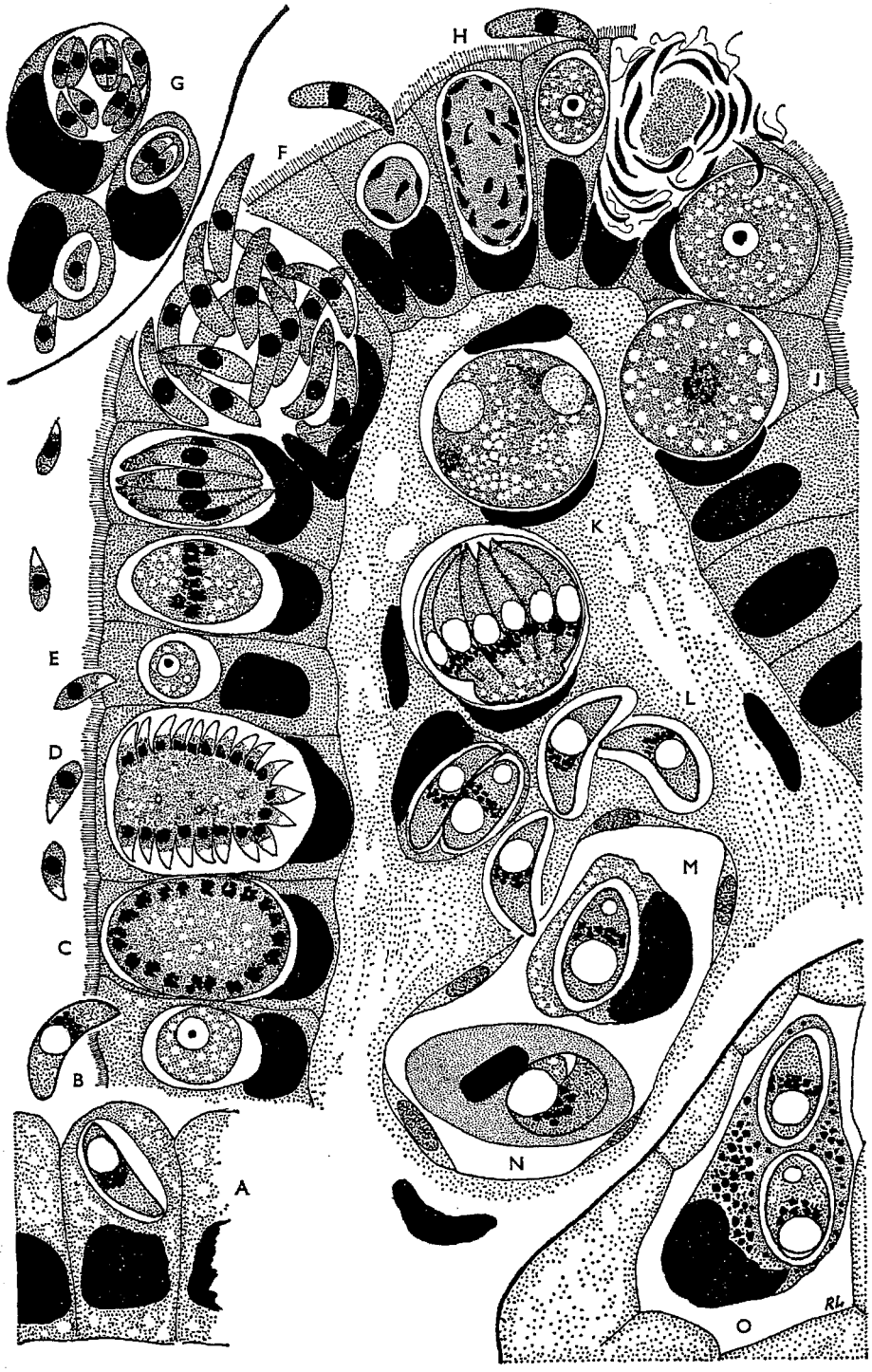

Figure 5. The life cycle of *Schellackia landauae* inside the lizard *Polychrus marmoratus* (from Lainson *et al.*, 1976); for explanation see p. 21.

2.2.6 The order Haemospororida

This order contains a single family, Plasmodiidae (Figure 1) which comprises species that infect mammals, birds and reptiles (Levine, 1988). The family consists of the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Saurocytozoon* (Telford, 1994). The genus most frequently reported to infect reptiles is *Plasmodium.*

2.2.6.1 *Plasmodium*

Reptilian malarias were first described in 1909 by Wenyon from reptiles in Sudan (Wenyon, 1909; as cited in Telford, 1994) and in the same year by Aragão and Neiva in Brazilian lizards (1909; as cited in Telford, 1994). Approximately 90 reptile malaria species have been recognised up to 1999, with Sam R. Telford Jr. as the most prolific malaria taxonomist describing almost half of them. During the last four decades, Telford authored more than three-quarters of the taxonomic descriptions of reptilian malarias.

Reptilian malarias are considered very good models for the study of host parasite relationships (Telford, 1994), and although there are still great gaps in the knowledge of their biology, they are by far better studied than the "haemogregarines". The ecology of the plasmodia of reptiles was reviewed by Telford (1994) and the diversity and ecology of lizard malaria in particular was reviewed by Schall (1996). In the following, the main research findings relevant for this thesis will be briefly summarised.

2.2.6.1.1 Taxonomy

Until the late 1960's most species of reptilian malaria were poorly described. Telford (1988) established the now widely accepted taxonomic framework for the classification of reptile malaria (Schall, 1996). Based on 18 characteristics, Telford (1994) created seven subgenera within the genus *Plasmodium* (Figure 1). An eighth subgenus, *Fallisia,* was included later. The species diversity of malaria is greatest in reptiles (90 species) (Telford, 1997), compared to 32 species in birds (Bennett *et al.*, 1982) and 51 species in mammals (Levine, 1988).

2.2.6.1.2 Life cycle

A general life cycle of a lizard *Plasmodium* is illustrated in Figure 6 (drawing from Mehlhorn & Walldorf, 1988; modified). Sporozoites (1) are released into the vertebrate host by the bite of a vector. In mammalian malarias, the sporozoites enter the parenchymal cells of the liver (2), whereas in most avian malarias they can be found in the reticulo-endothelial cells. The pathway of sporozoites injected into the capillaries of a lizard host is not known. In a cycle of asexual division (schizogony), large exoerythrocytic (EE) schizonts, containing hundreds of merozoites, are formed (3)(4). Depending on the species of *Plasmodium*, the merozoites either begin a new EE-schizogony in lizard liver cells, or enter the blood cells (6) after only one schizogonic cycle.

In the red blood cells of the lizard, the merozoites increase in size and become trophozoites, which then undergo another asexual division (intraerythrocytic schizogony) (6)(7)(8). The progeny of these divisions re-enter more red blood cells and can either undergo a new cycle of intraerythrocytic schizogony (9.2)(10.2) or form the gamonts, the sexual stages of *Plasmodium* (9.1)(10.1).

When a potential vector ingests blood containing male and female gametocytes, the micro-parasites leave the ingested blood cells and the male gametocytes undergo exflagellation (11), a process where several microgametes are formed. Single microgametes (12) enter the matured macrogametes (12) and their nuclei fuse, resulting in a motile elongated zygote named an ookinete (14). The ookinete then penetrates the midgut epithelium (15) of the insect vector and begins a profuse asexual division (sporogony) leading to the formation of a rounded oocyst on the outside of the midgut which contains many motile sporozoites (16)(17)(18). From rupturing oocysts (19), sporozoites find their way to the salivary glands (20) of the vector where they are released into a new vertebrate host during the next blood meal.

Most stages of the life cycle of *Plasmodium* in the vertebrate host can undergo asexual reproduction, thus all of them are infective stages for other vertebrates. Consequently, transmission experiments can be conducted by intracardial (IC) or intravenous (IV) injection or intraperitoneal (IP) inoculation of peripheral blood from one infected lizard to another.

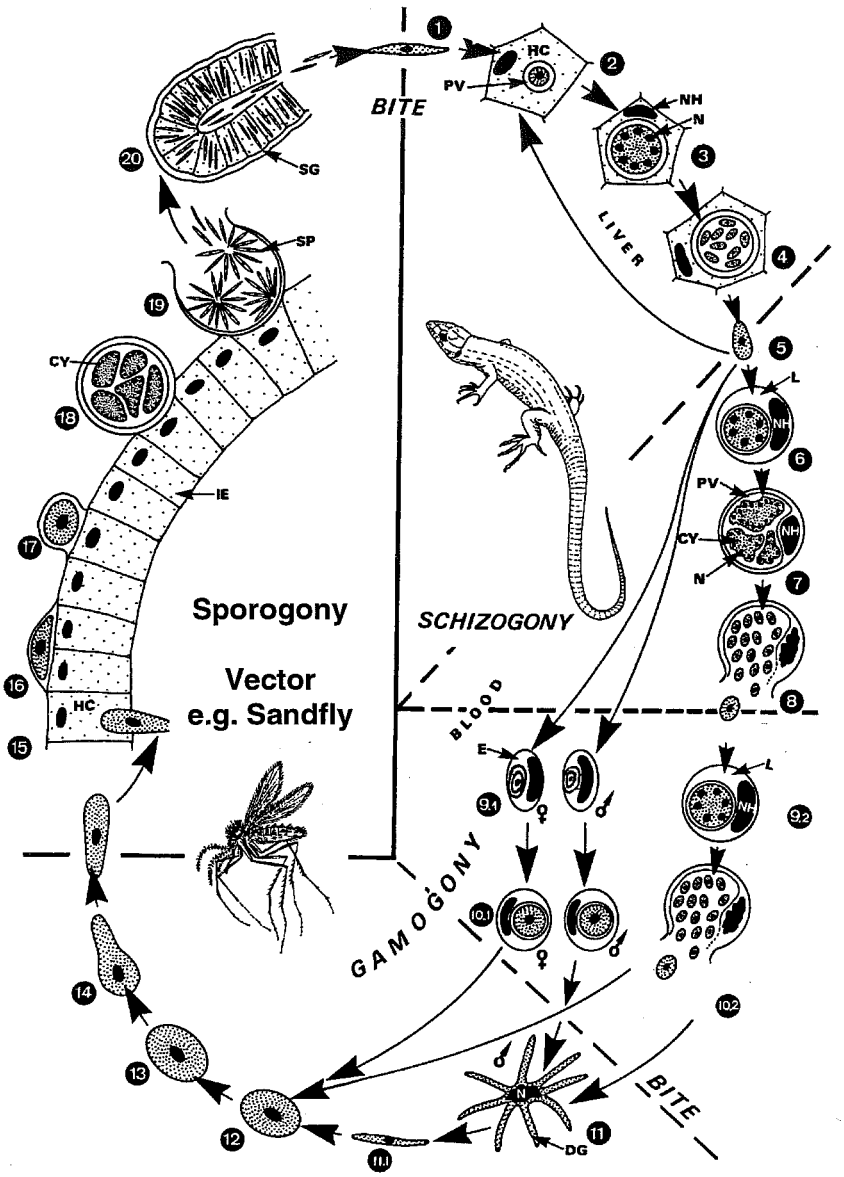

Figure 6. The life cycle of *Plasmodium* inside lizards and phlebotomine sand flies (from Mehlhorn &Walldorf, 1988; changed); for explanation see p. 24.

Blood infections of the genus *Plasmodium* are known to recur or relapse (eg. Krotoski, 1985). This effect has also been described from lizard plasmodia, but the mechanism and the life cycle stages responsible are poorly understood. Using the nomenclature that emerged from the recent findings of the life cycle of mammalian plasmodia, Telford (1989) summarised his hypothesis, as follows: "Sporozoites enter hepatic parenchymal cells where some may become dormant as hypnozoites, and others form cryptozoic schizonts. The stages formed in the latter, the cryptozoites, parasitise hepatic macrophages and form metacryptozoic schizonts. Metacryptozoites produce phanerozoic schizonts in the capillary endothelium and connective tissue of the lung and other organs. Phanerozoites and possibly metacryptozoites then invade the erythrocytes to begin the erythrocytic cycle. Some of the phanerozoites in endothelium, connective tissue and skeletal muscle become encysted as chronozoic schizonts, and their progeny, chronozoites, renew the erythrocytic cycle throughout the life of the host and produce seasonal relapses of gametocytemia, in spring, at the end of hibernation by the lizard."

2.2.6.1.3 Vectors

So far, vectors for saurian malarias have only been found among the haematophagous dipteran insects. Sporogony has been described from phlebotomine sandflies (Ayala & Lee 1970; Ayala, 1971; Klein *et al.*, 1987; Kimsey, 1992), ceratopogonid midges (Petit *et al.*, 1983), and mosquitoes (Klein *et al.*, 1987). Avian malarias have been found to be transmitted by ingestion of their vectors (Sergent, 1937; Young, 1941; as cited in Telford, 1994).

2.2.6.1.4 Ultrastructure

Ultrastructural studies have shed a new light on the host parasite relationships of malarial parasites (Smyth *et al.*, 1976). In particular, it has been shown that the parasite does not actually penetrate the vertebrate blood cell but only forms an invagination in it (Ladda *et al.*, 1969), where an inner and an outer layer is formed by two inner membranes and two outer membranes. The inner membranes are formed by the parasite and the outer membranes are host cell derivatives. Thus it has been suggested that malarial parasites are actually inter-cellular and not intra-cellular parasites of blood cells (Ladda *et al.*, 1969), a finding that still remains to be generally accepted.

The most notable changes during the development of the blood stages occur after it is engulfed by the host cell, where the parasite changes from a merozoite to a trophozoite that

feeds on the haemoglobin of the erythrocytic cytoplasm. The apical complex disappears, a cytostome with two supporting rings is formed (Scorza, 1971a; Klein *et al.*, 1988b) and double layered invaginations emerge containing engulfed cytoplasm at different stages of digestion (Sterling & Aikawa, 1973). Further changes become obvious after the development of trophozoites and gametocytes, but because ultrastructural images of these stages were not obtained in this study, a description will not be given here.

2.2.6.1.5 Epidemiology

Parasites of the genus *Plasmodium* are definitely known to occur only in lizards and snakes among the cold blooded vertebrates, with more species known from lizards than from snakes (Telford, 1994). In contrast to avian plasmodia, host specificity of lizard malaria is high. Most species have been described to occur only in a single host species. One of the few polytopic exceptions is *P. colombiense*, which is known to infect over 20 *Anolis* and *Sceloporus* species in North and Middle America and the Caribbean (Telford, 1994). Cross infections of saurian malaria between vertebrate host family lines are rare (Telford, 1994). Mixed infections of two or more *Plasmodium* species within a single host are common and have been recorded in 43 species of lizard malaria (Telford, 1994).

Despite the discovery of lizard malaria in 1909 and an intensive phase of research to establish lizard malaria as a model for antimalarial drug testing (Thompson, 1946a; Thompson, 1946b), the first sporogony of a saurian malaria was not described until 1970 (Ayala & Lee, 1970). Only two studies have investigated the course of infections induced by the transmission of sporozoites (Ayala, 1970; Klein, 1985), the developmental stage considered to be the only source for new infections for saurian malarias under field conditions.

Four saurian malaria species have been investigated for the course of the erythrocytic infection(Thompson, 1944; Thompson & Huff 1944; Goodwin & Stapleton 1952; Jordan, 1970; Telford, 1972; Jordan, 1975; Klein, 1985). With the exception of Klein, the lizards in these studies were infected by intracardial (IC) or intravenous (IV) injection or intraperitoneal (IP) inoculation of lizard blood stages. Most of the remaining literature consists of taxonomic studies or surveys (Telford, 1994). A complete life cycle of saurian malaria has never been described. Therefore, a problem with the diagnosis of saurian malaria is that most studies have to rely on blood smears to determine the **prevalence** of the

parasite. As discussed earlier, saurian P*lasmodium* species can persist, probably for the lifetime of the lizard, as subpatent infections (Telford, 1989; Telford, 1998) or at a very low **parasitemia** (Perkins *et al.*, 1998). Hence, studies using blood smears as the only diagnostic basis will usually underestimate the prevalence of the parasite (Telford, 1994).

2.2.6.1.6 Pathogenicity

In this thesis, the term pathogenicity was used instead of the term virulence, because the latter is usually used to define the mortality a pathogen inflicts on a host. Studies investigating the impact of reptilian malaria on lizard hosts have concluded that, in contrast to mammalian plasmodia, there is little observable negative effect on the natural vertebrate host (Schall, 1990). Thus the use of the term virulence would be inappropriate. The effects of a lizard malaria on the behaviour and the fitness of its vertebrate host and the vector have been investigated by Schall and his coworkers (Schall, 1982; Schall, 1983; Schall & Sarrni, 1987; Schall & Dearing, 1987; Schall, 1990, Fialho & Schall, 1995) using the western fence lizard *Sceloporus occidentalis*, the sand fly *Lutzomyia vexator* and *P. mexicanum* as a model. Infected female *S. occidentalis* from Californian field populations were found to store less fat that their uninfected counterparts, thus the infection reduced their reproductive capacity (Schall, 1982; Schall, 1983). In addition, male *S. occidentalis* had testes that were an average of 37% smaller than uninfected males. It was speculated that the infection impacts on the genetic contribution of infected lizards to the population, but Schall concluded that "the population consequences for lizards of malarial infection thus appear absent or slight" (Schall, 1990). Reptile malarias have rarely been reported to kill their host, although there is one documented case where a *Plasmodium* species did occasionally cause the death of its natural lizard host (Jordan, 1975). Lizards with *Plasmodium* infections can develop anaemia (Ayala, 1970; Scorza, 1971; Telford 1972; Ayala & Spain 1976). The *Plasmodium* parasite digests the haemoglobin of the red blood cells. The end product of the haemoglobin metabolised by the parasite is haemozoin, the malaria pigment, which can be found as little highly refractile granules inside the parasite. Heavily infected lizards were found to have extremely low haemoglobin concentrations (Scorza, 1971b). A reduction of the competitive ability and reproductive capacity of the lizard host has been shown (Schall, 1983). Telford pointed out that because the infection-status was determined by the examination of blood films, the experimental groups compared by Schall and his coworkers were lizards with active infections on the one side and lizards with no or patent infections on the other side (Telford, 1994).

2.2.6.1.7 The host response

From the studies on four species of lizard malaria where lizards have been infected by IP inoculation Telford (1994) separated two general patterns for the course of erythrocytic infections. In the first one, a rise of the parasitemia to a peak occurs, followed by a phase of decline. The second pattern does not show steep increases or declines of parasitemias, but the lizard develops a chronic infection of the red blood cells that lasts for its lifetime. It has been speculated that the two patterns are caused by intrinsic differences of the vertebrate hosts, either in their immune response (Thompson, 1944; Jordan, 1975), or their haematopoietic response (Ayala, 1970; Scorza, 1971; Ayala, 1977). A *Plasmodium* infection can alter the temperature preferences of the lizard hosts in that infected lizards prefer cooler temperatures than uninfected lizards (Scorza, 1970; as cited in Telford, 1994), or it may affect the mobility of the hosts (Bromwich & Schall, 1986).

2.2.6.1.8 Evolution

Two main hypotheses exist to explain the evolution of *Plasmodium* (Malagon, 1992; Telford 1994). One model suggests that the modern order Haemospororida evolved from ancestral intestinal coccidia of vertebrates and probably their invertebrate ancestors eg. (Manwell, 1955). The other model suggests that malaria parasites were derived from monoxenous parasites of dipterans (Huff, 1938). The latter model was supported by a phylogenetic analysis of the Sporozoea (Barta, 1989).

2.2.6.1.9 Zoogeography

Reptile malarias occur mainly throughout the tropics and the subtropics, although occasionally they have been found in temperate regions (Telford, 1997). From the few data available, studies on African lizard plasmodia revealed two main patterns of distribution, one where the parasite occurred over wide areas and the other where groups of parasites with very restricted host and geographical distributions occurred in localised endemic zones (Telford, 1986; Telford, 1988; Telford, 1994). With a total of six species described from Australasia, four of which are found only in Australia, little or nothing is known about the distribution pattern of saurian malaria in the Australasian region (Telford, 1994).

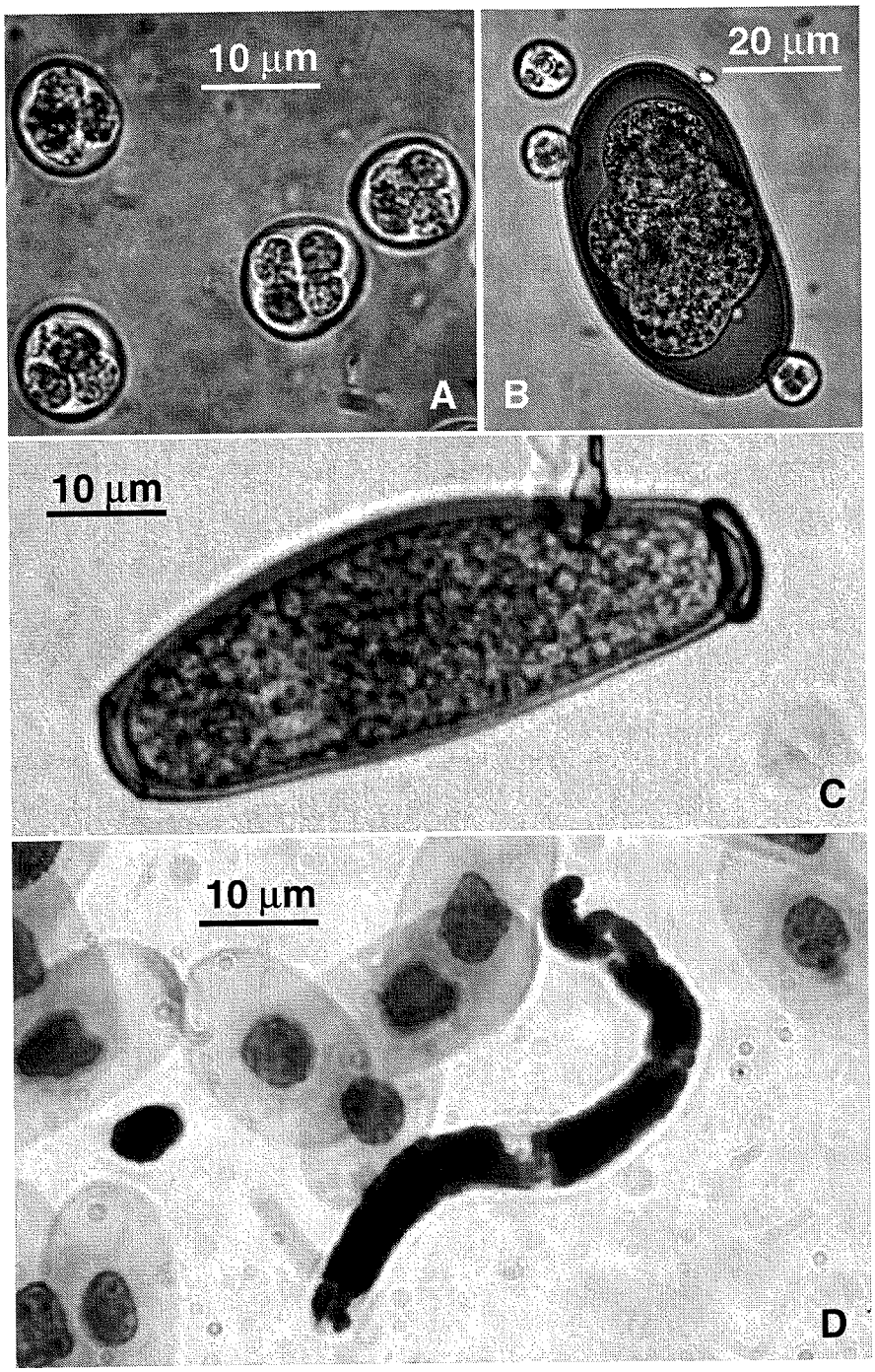

Figure 7. A-C: parasitic organisms found during faecal flotations of *E. stokesii*. A: sporulated oocysts of *Eimeria* sp.. B: Nematode egg (with *Eimeria* oocysts). C: organism of unknown taxonomic position. D: Microfilaria in giemsa stained blood smear (b&w).

Chapter Three

3. THE LIZARD HOST *E. STOKESII*

3.1 Introduction

The gidgee skink, *E. stokesii* Gray 1845 (Reptilia: Scincidae) (Figure 132/B, chapter 14), is distributed from the central coast of Western Australia into the arid interior and from South Australia to southwestern Queensland and northwestern New South Wales (Cogger, 1996). In the Flinders Ranges of South Australia, this viviparous, diurnal lizard lives in isolated populations on rocky outcrops and stony hills (Duffield & Bull, 1996a), with individuals within a population often found aggregating in rock crevices and under boulders (Greer, 1989). Adults have a snout-vent length of approximately 16-21 cm and a body mass between 150 - 310 g. The species feeds largely on plants (Duffield & Bull, 1998) and uses particular defecation foci where small piles of faeces accumulate (Cogger, 1996).

As a more advanced evolutionary form of parental care, vivipary evolved to a different degree several times independently in lizards (Shine, 1983; Shine, 1985; Shine, 1987). The viviparous birth of *E. stokesii* was described by Duffield & Bull (1996a). Data from 29 litters over three years from a population near Hawker, South Australia, were presented. Females produced an average 5.07 young per litter in late summer. Individual litters were produced over an average 2.8 days. Baby lizards were observed to ingest their placenta directly post partum (G. Duffield, pers. comm.). The placenta of viviparous reptiles has a variety of functions, including supplemental nourishment to the yolk reserves of the embryo, and gas and water exchange (Stewart, 1992). Similar to mammals, the ontogenetic origin of the placenta of reptiles is embryonic and proves an efficient barrier for most pathogens between mother and embryo before parturition (J. Stewart, pers. comm.). However, when a neonate ingests its placenta it may ingest also maternal blood and associated parasites, which could be of epidemiological significance. Three major types of placenta have been described in reptiles (Stewart & Blackburn, 1988; Stewart, 1993), but the placental structure of *E. stokesii* is unknown.

Like all lizards, *E. stokesii* is an ectothermic organism that does not maintain its body temperature by metabolic processes like mammals and birds. Like all non-mammalian vertebrates, it has nucleated blood cells. Most other aspects of the biology of this lizard remain unknown, and that is probably due to its secretive and well-sheltered lifestyle inside narrow and deep rock crevices. Capture of *E. stokesii* is a difficult task even for an experienced lizard collector. Although they can be extracted from some crevices with experience and the right tools (for details see chapter 14), their keeled scales and the spiked tail in combination with their muscular body prevents extraction from many other crevices.

This study mainly focused on the blood parasites of *E. stokesii*, thus few new insights into the biology of this lizard were gained during its course. However, some physiological and behavioural aspects of *E. stokesii* were observed that were of an imminent epidemiological significance. Particularly important were the findings concerning reproductive activities of *E. stokesii* and observations of its behaviour in close proximity to potentially disease-carrying vector species. With the onset of yolk deposition in female lizards, giemsa stained blood smears stain a deep pink or purple and are easily distinguished by the naked eye from the normal blue-grey stain of non-reproductive lizards (Telford, 1971). It has been speculated that this reddish precipitation adhering to the glass slide in between the blood cells results from lipid materials mobilised during vitellogenesis and that its appearance demonstrates the onset of vitellogenesis of the ovarian follicles in sexually mature females (Telford, 1971). The presence of this particular staining reaction during the successive regular bleedings was recorded in this study and was used as a method to predict potentially reproducing wild females for further observations.

One set of experiments was designed to study the behaviour of *E. stokesii* in close contact with ticks, but only preliminary results are presented in this thesis. The main focus of the experiments was to investigate whether *E. stokesii* would eat ticks. These experiments were initiated by the author, but the practical work and parts of the experimental setup were maintained by Ms. Clare Griffin. Although the results were clearly the product of a joint effort, they are reported here because of their epidemiological significance for other findings within this thesis.

3.2 Materials and Methods - reproductive cycles and tick eating

E. stokesii were captured at field sites over much of South Australia (for details see chapter fourteen). The lizards were housed in the Animal Care Unit (ACU) at the Flinders University of South Australia (FUSA). The following housing conditions were used:

1) glass terraria (15-60 L) in rooms with constant 25°C and a light regime of 12 hours (h) light and 12 h darkness. Adults were kept either as single individuals or as a pair. Subadults lived in groups up to five individuals, depending on their size. Each terrarium had a single 60 W light bulb as heat source hanging approximately 20 cm above a basking rock, thus supplying a temperature gradient in the terrarium. Newspaper sheets that were changed twice per week were placed at the bottom of each terrarium to prevent the smearing of faeces on the glass.
2) outdoor pens (9 m^2), made from approximately 60 cm high galvanised, metal sheets. Lizards were protected from predators by a large wire cage (mesh width 3-cm) that covered all pens. Shelter was provided by three artificial crevice-setups per pen, made of three concrete slabs (40 x 40 x 4 cm) respectively. The slabs were stacked on top of each other and tilted by one 3 cm by 3 cm wooden stick between each two slabs, placed alternating at opposite ends (Figure 132/D, chapter fourteen). Lizards were kept in outdoor pens in groups of up to 12 individuals.
3) indoor pens (3 m^2) with one shelter per pen made of three concrete slabs as described above, and another shelter in each pen made from two hollow building bricks (24 x 17 x 9 cm and 24 x 17 x 16 cm). Each pen had a single 100 W light bulb as heat source hanging approximately 20 cm above a basking rock (30 x 30 x 4 cm).

Food was provided twice a week, water was provided *ad libitum*. The body mass of each lizard was monitored at 1-10 week intervals. Most pairs in the terraria comprised of one male and one female lizard. All pens contained both male and female lizards. *E stokesii* were sexed by expressing the hemipenes of males (Szidat, 1968), which allowed a reliable sex determination for lizards with body mass exceeding approximately 50 g. The lizards were regularly inspected by an independent veterinarian. All experiments conducted were approved by the Animal Welfare Committee of the Flinders University.

Blood smears taken from *E. stokesii* in the field and in the laboratory were stained with giemsa (for details of the bleeding procedure and the staining protocol see 5.2.1.1). To monitor birth and certain behavioural aspects relating to parasite transmission, some lizards within the ACU were filmed in their terraria. At the end of the field study in 1998, female

field-lizards in which blood smears showed the pink staining indicating vitellogenesis, were removed to the ACU in January. No other criterion was available to predict or diagnose a gravid female; the body mass and shape of gravid females was not noticeably different from non-gravid females. A video camera was mounted approximately 1.5 m above a set of two to four occupied terraria and the lizard behaviour was taped with a video recorder. Pieces of cardboard were fitted in between the terraria, to prevent visual contact between the lizards from different terraria. Single females were placed into individual terraria that were filled with an approximately 3-cm layer of dry soil. A set of five terraria was continually filmed over several consecutive weeks during February and March 1998. Lizards that had given birth under camera surveillance were replaced by other females that were suspected to be gravid. The resulting videotapes were studied for the behaviour of *E. stokesii* during and shortly after birth. Special emphasis was laid on the documentation of the anecdotally reported ingestion of the placenta by newborn *E. stokesii.*

In the second set of behavioural experiments, unengorged tick adults and engorged larvae were exposed to three subadult lizards that were kept together in one terrarium. The lizards were born in the laboratory in February 1997 and the experiments were conducted between November 1998 and January 1999. The lizards were starved for three weeks before an experiment was started. The ticks were placed into an empty plastic dish (10 cm x 15 cm x 1 cm) in the terrarium. The upper rim of the terrarium was taped with double-sided sticky tape to prevent the ticks from escaping. The behaviour of the lizards was observed for up to two hours after an experiment was started.

3.3 Results

3.3.1 The occurrence of pink stained blood smears

From a total of 4041 blood smears obtained between July 1995 and July 1998, the smears of 58 individual female *E. stokesii* were found to stain pink (Table 1). The majority of these pink smears were obtained from females caught in the field, but a total of nine individual *E. stokesii* kept in the ACU also produced blood smears that stained pink. One *E. stokesii* female bled at weekly intervals in the ACU for a transmission experiment was found to generate pink smears over three consecutive weeks. The occurrence of pink stained blood smears from lizards caught and bled in the field showed a strong seasonality over the 36 month study, with the majority of cases occurring during November (Figure 8; Figure 9; see also Table 3). The appearance of pink stains derived from blood smears of lizards kept in the

ACU had the same average date but emerged over a broader spread of time (Figure 10). All of these *E. stokesii* were kept in the ACU at least one year before the unusual staining reaction was detected. The blood smears of a total of eight *E. stokesii* individuals stained pink twice within this three year study (Table 2). Seven *E. stokesii* individuals were observed to have blood smears staining pink in two consecutive years; one of these females was housed in the ACU from 1994, all others were field lizards. Twelve *E. stokesii* females were brought into the ACU after the pink staining reaction of blood smears was observed during the field study in the spring of 1997 Ten of those gave birth during February and March of 1998. All six *E. stokesii* where the unusual staining reaction occurred while they were in long time care in the ACU gave birth within the following five months. Two *E. stokesii* gave birth in the ACU in March 1998 when previously a pink staining reaction had not been detected. One of those two lizards was born in the ACU, the other one was held there since January 1996. Both lizards were bled four times during the relevant phase preceding birth, on the 18th of July 1997, on the 22nd of September 1997, on the 7th of November 1997 and on the 14th of January 1998. The lab-born lizard was the youngest mother found, giving birth to two babies approximately two years after it was born itself. This is probably abnormal for this species, but can be explained by the continual high temperature and food which accelerates growth compared to field populations. Blood smears of two other *E. stokesii* that were born in the ACU were found to stain pink 29 and 31 months after they were born. Each of them gave birth to a single baby approximately three years after they were born. A total of three *E. stokesii* generated blood smears with a pink stain although they were kept single in one terrarium for at least one year before the staining reaction occurred; none of them gave birth.

Table 1. The number of individual *E. stokesii* with pink blood smears found between July 1995 and July 1998.

Season	Blood smears produced in total	Pink smears in total	Field lizards with pink smears	ACU lizards with pink smears
1995/96	1073	8	6	2
1996/97	1325	23	22	1
1997/98	1643	27	21	6
Σ	4041	58	49	9

Table 2. *E. stokesii*, which showed pink, stained blood smears in two of the three seasons investigated.

E. stokesii	Site of bleeding	Pink smear in season 1995/96	Pink smear in season 1996/97	Pink smear in season 1997/98
5	ACU	+	+	-
101	Field	+	+	*
210	Field	-[1]	+	+
251	Field	*	+	+
266	Field	+	*	+
325	Field	*	+	+
388	Field	*	+	+
558	Field	*	+	+

* = Lizard individual not caught during the relevant time frame (Sept.-Dec.).

-[1] = Lizard individual caught once during the relevant time frame (Sept.-Dec.).

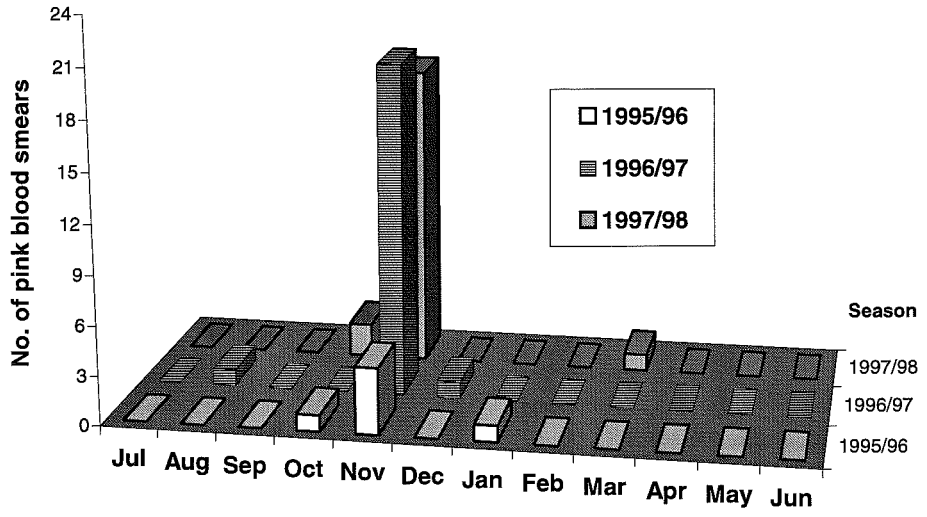

Figure 8: The seasonal occurrence of pink blood smears from *E. stokesii* caught in the field (n=49).

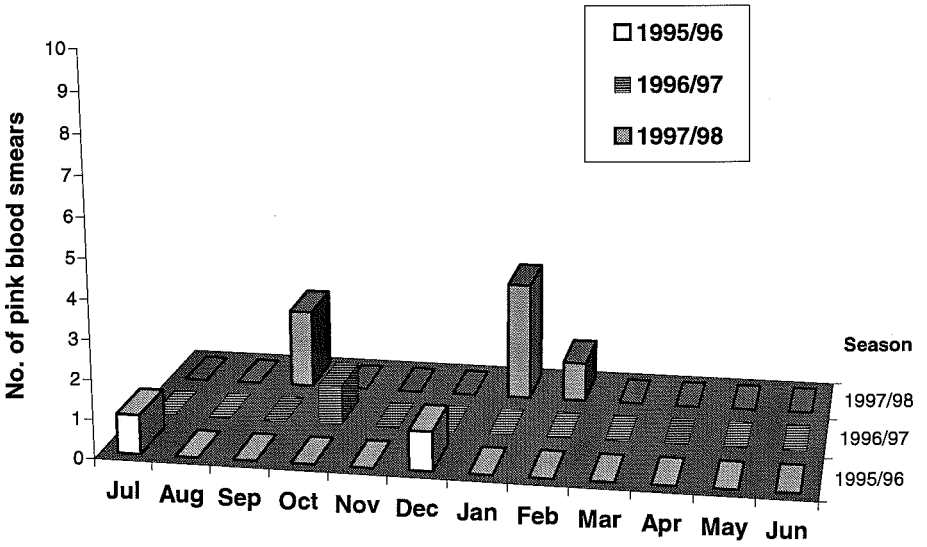

Figure 9: The seasonal occurrence of pink blood smears from *E. stokesii* kept and bled in the ACU (n=9).

Table 3: The seasonal occurrence of pink-stained blood smears.

Season	Site of bleeding	Pink smears in total	Day of the year pink smears were found on average		
			Arithmetic mean	Standard error	Standard deviation
1995/96	Field	8	318	14.1	34.5
1996/97	Field	22	310	4.6	21.4
1997/98	Field	21	312	5.5	25.3
1995-98	ACU	9	320	24.7	74.1

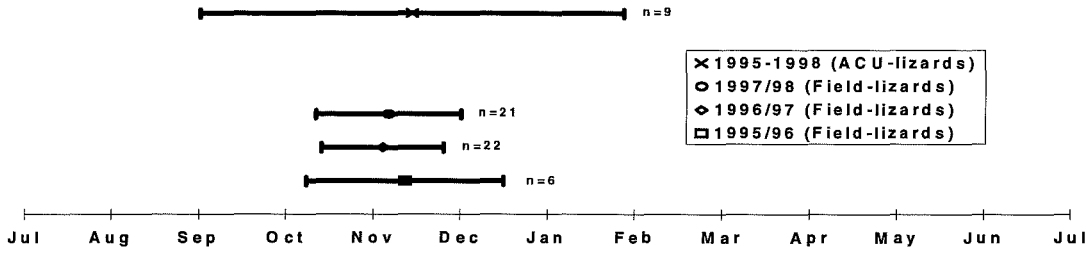

Figure 10: The mean and the standard deviation of the seasonal occurrence (day of the year) of pink blood smears from *E. stokesii* kept and bled in the ACU compared to *E. stokesii* caught in the field.

3.3.2 The filming of the birth of *E. stokesii* in the ACU

The birth of 15 of the 51 *E. stokesii* born in the ACU in 1998 was taped in February of that year. The filming documented that newborns ingest their placenta immediately after birth, an observation that has been made previously by G. Duffield (G. Duffield, pers. comm.). Observations and the filming of births in the ACU were made from mid February to mid April 1998, the phase when *E. stokesii* is known to give birth (Duffield & Bull, 1996a). During this time, the births of fourteen lizards from seven females were filmed. Nine of the fourteen newborns ate their placenta as a whole within seconds post partum. Two placentae were eaten in part and their remains were found within the terraria. Two placentae were withheld from the newborns by seizing the newborns directly after birth. The placentae were collected and examined histologically. On one occasion the recorded birth took place on the hand of the author. The newborn lizard was still attached to its placenta by the umbilical cord. The new baby moved within the hands and around the fingers of the observer, thereby pulling the placenta out of the cloaca of its mother. With the arrival of the placenta, the newborn vigorously bent and struggled and approached it with jaws sequentially opening wide and closing. The author removed the placenta before the newborn could reach it.

3.3.3 The filming of the behaviour of *E. stokesii* when exposed to ticks

Sets of five to ten engorged larvae of *Amb. limbatum* were placed into plastic petridishes in three terraria, each holding three subadult *E. stokesii,* during December 1998. The lizards had not been fed for ten days. The lizards readily took the ticks into their mouth, but they did not always swallow them. The lizards were attracted by the movement of the ticks. Thus, some ticks were taken up by a lizard outside of the plastic dish. In each case where a lizard took a tick into its mouth, it chewed on the tick, but some lizards then spat the ticks out or smeared the crushed tick onto the wall or the bottom of the terrarium. The smearing was accomplished either by wiping with a paw alongside the side of the mouth where pieces of the ticks were sticking out, or by pressing and wiping the head against the terrarium wall. Twice lizards were observed to tongue flick and ingest remains of ticks squashed by other lizard individuals. Although only two of the nine lizards ingested ticks they had picked up, six of them chewed at ticks they had taken into their mouth. The results are summarised in Table 4.

Table 4. The observations made when engorged larvae of *Amb. limbatum* were placed into a terrarium with starved subadult *E. stokesii*.

E. stokesii individual	No. of ticks attempted to take into mouth	No. of ticks taken into mouth	No. of ticks chewed on	No. of ticks smeared into terraria	No. of ticks swallowed*
1	1	1	1	1	
2	2				
3	4	4	4	2	4
4	2	2	2	2	
5	2	2	2	2	
6					
7	6	6	6		6
8	3	3	3	3	
9					

*: partly or as a whole

Table 5: The observations made when unengorged adults of *Amb. limbatum* were placed into a terrarium with starved subadult *E. stokesii*.

E. stokesii individual	No. of ticks attempted to take into mouth	No. of ticks taken into mouth	No. of ticks chewed on	No. of ticks smeared into terraria	No. of ticks swallowed*
10	2	2	2	2	
11	1	1	1	1	
12					
13	1	1	1	1	1
14					
15					

*: partly or as a whole

The observations made when unengorged adult *Amb. limbatum* were placed into the aquarium were very similar to those made with engorged larvae (Table 5). One noticeable difference was that lizards would bite adult ticks into half, drop one half and smear the other half into the terrarium. Twice it was observed that the remains of ticks were taken up by a lizard again, once by the same individual that killed it, in which case the tick remains then were eaten, and once by another lizard individual. Several lizards tongue flicked at the remains of smeared ticks and one lizard made contact with the smeared tick, "licking" at the remains.

3.4 Discussion

The results of the seasonal occurrence of pink blood smears from female *E. stokesii* caught in the field, and the high number of lizards giving birth after the staining reaction was noted, support the theory that this phenomenon is related to reproductive activity of female lizards. The noticeable differences between the occurrence of pink blood smears from field lizards when compared to *E. stokesii* that were housed long term in the ACU may indicate a loss of seasonal entrainment of their physiological cycles after *E. stokesii* were kept for more than a year under the constant environmental conditions of the ACU. Whether the obvious seasonal occurrence of the reproductive cycle of *E. stokesii* is influenced by extrinsic factors, like the photoperiod, remains to be investigated. The occurrence of pink smears from blood of *E. stokesii* kept single, indicates that the onset of the reproductive cycle does not require close contact of females with males; nor does it require an act of mating.

The behavioural peculiarity of newborn *E. stokesii* to eat its placenta is possibly of epidemiological significance. Some parasites within the coccidia are known to be transmitted by the digestion of infected blood, eg. *Plasmodium*, where the inoculation of infected blood cells is a standard method of transmission experiments, or eimeriorine blood parasites from the genus *Schellackia* and *Lankesterella* (for details see 2.2.5.2). Similar to mammals, the ontogenetic origin of the placenta of reptiles is embryonic and proves an efficient barrier for most pathogens (J. Stewart, pers. comm.), thus coccidians should not be transmitted by the ingestion of placenta-tissue. Nevertheless, maternal bleeding is likely to occur during the dislocation of the placenta from the uterus and blood was observed in abundance on the freshly expelled placentae. No blood smears obtained from fresh placentae were found infected, but in each of the three cases where a placenta was obtained the mother lizard had an extremely low parasitemia of blood parasites.

The observed behaviour of subadult *E. stokesii* to ingest or chew on engorged larvae or unengorged adults of *Amb. limbatum* has significant epidemiological implications, because many haemogregarine blood parasites are known to be transmitted to the vertebrate host by the ingestion of their vector. The behaviour of *E. stokesii* to lick on the remains of freshly killed ticks increases the risk of infections of orally transmitted pathogens and suggests that one infected adult tick has the potential to infect several lizards of one population. Particularly in a species like *E. stokesii*, where there is social aggregation of lizards, both at basking sites and in crevice refuges, this behaviour might explain high prevalences of orally transmitted pathogens from tick reservoir hosts.

It was remarkable that in each case a tick definitely was killed after a lizard paid attention to it, but only a few of the lizards actually swallowed the tick or larger parts of it. On the one hand it can be speculated that the uptake of the ticks was aimed at the control of the tick population in lizard shelters and thus was more related to grooming behaviour, rather than foraging behaviour. On the other hand, the extremely high prevalence and parasitemias of blood parasites in *E. stokesii* at remote and barren study sites, eg. at the shore of a salt lake, indicated that at field sites with scarce vegetation the uptake of ticks might become a more important part of the lizard's diet (for details see chapter fourteen).

Chapter Four

4. THE ECTOPARASITES OF *E. STOKESII*

Field populations of *E. stokesii* in the Flinders Ranges were monitored for ectoparasites over a period of three years. The lizards were found to be infested by two species of ticks, *Amblyomma limbatum* and *Amblyomma vikirri* (Figure 11/E&F; p. 55). The vector experiments conducted within this thesis focused on these two tick species, thus their biology will be discussed in more detail here. The results of the tick dissections and of the histological investigation are placed within the taxonomic chapters six to ten. The impact of the blood parasites on the tick species is described in chapter thirteen.

A phlebotomine sand fly found in abundance engorging on blood of *E. stokesii* was identified as *Australophlebotomus dycii*. Transmission experiments were not conducted because the maintenance of a sandfly colony proved to be too elaborate for this study, and the biology and the results of the dissection of the phlebotomines will be addressed only briefly.

Mosquitoes were observed occasionally on one of the field sites in the Flinders Ranges (Duffield, pers. comm.), but several attempts to trap them for this investigation failed. Two species of mosquitoes found naturally in South Australia, *Aedes notoscriptus* and *Culex quinquefasciatus*, were used for transmission experiments in the laboratory, but no conclusive development of a parasite or successful transmission with mosquitoes as a vector were shown in this study. Thus, the biology of the mosquitoes and the results of the vector study will be discussed only briefly.

4.1 The ticks

4.1.1 Taxonomy and zoogeography

Ticks are obligate haematophagous ectoparasites of terrestrial vertebrates that form the entirely parasitic suborder Ixodida within the arachnid subclass Acari (mites). Three families with a total of around 850 species have been described: the Ixodidae or hard ticks, the Argasidae or soft ticks and the monotypic Nuttalliellidae (Hoogstraal & Aeschlimann, 1982). There are more than 650 species of hard ticks presently known, with a worldwide distribution ranging from the tropics to sub-arctic areas and to sub-Antarctica (Oliver, 1989). The Ixodidae are split into two groups: the Prostriata, containing only the genus *Ixodes* with approximately 212 species described, and the Metastriata, with about 440 species in 16 genera. The two tick species investigated in this study belong to the metastriate genus *Amblyomma*, which comprises 103 species of ticks; 37 of them are known to infest reptiles (Oliver, 1989).

4.1.2 Life cycle

In contrast to the several nymphal instars of argasids, the Ixodidae have only one nymphal instar, which leads to a four-stage life cycle: eggs, larvae, nymphs and adults. Each motile instar takes a single blood meal. The increase in volume from that blood meal may result in an increase of body mass by over 200 times. Eggs hatch into the six-legged larvae, which attach to their first host. After the blood meal, the engorged larvae drop off the host and moult into eight legged nymphs. Following a further blood meal, the nymphs develop to adults. Only at this stage a ventral genital opening appears. Adults show a prominent sexual dimorphism of larger females and smaller males. The male dorsum is covered completely by a non-expandable sclerotized scutum. This scutum is comparably small in females (Balashov, 1968). Mating occurs on the third host. Following the blood meal on the third host, mated females drop off the host and after a pre-oviposition period they lay one mass of approximately 1000-3000 eggs, usually in sheltered places on the ground or in animal burrows, and die. Males are attached to a host for extended periods between matings, but the repeated intake of host fluid is small. They may mate with several females until they eventually die.

The time required for a blood meal depends on the instar and on the host. Larvae and nymphs feeding on warm blooded animals usually need between 3-7 (larvae) and 4-8 (nymphs) days to repletion respectively; females require between 7-14 days to repletion

(Oliver, 1989). As a rule, the feeding of ticks on reptiles takes longer than on mammalian hosts (Bull *et al.*, 1978; Bull, 1996). The time required for the off-host development of an engorged tick until ecdysis depends on the temperature of its environment and an optional developmental diapause (morphogenetic diapause[3]). The latter can delay the developmental process of moulting and egg-laying and can occur within the life cycle of many tick species (Belozerov, 1982). After moulting, a continuation of development occurs. Thus freshly moulted ticks require some time before they can start host seeking activity (Balashov, 1968). For example in the European hard tick *Ixodes ricinus*, the commencement of activity of the adult tick after ecdysis is entrained to the European spring by the occurrence of a behavioural diapause (Stein, 1993). The duration of the life cycle of ixodid ticks may vary between several broods per year in tropical one-host ticks, eg. in the cattle tick *Boophilus microplus*, to several years, eg. in *I. ricinus*. The span of a life cycle may vary considerably even within one tick species and depends on many factors like the micro-climate, the photoperiod and the availability of suitable hosts in the tick habitat or the occurrence of dormancy phenomena within the tick life cycle (Oliver, 1989).

Most ixodid ticks species mate on their host. In contrast to the Prostriata and all Argasida, in adult metastriate ticks, meiosis and sperm production does not occur before engorgement, thus adults do not mate unless they have taken a blood meal. Histologic examinations of larvae and nymphs show genital primordia that differentiate gradually by mitosis into a genital mass (Roshdy, 1969). Early meiosis can be observed in engorging nymphs, but the process is halted and can only be completed after another blood meal, when the female has fed (Kahlil, 1972).

4.1.3 Tick-host relationships

About 625 of the approximately 650 species of hard ticks have a three host life cycle, where each instar needs a different individual host (Needham & Teel, 1991). Usually the three hosts are not conspecific, and larvae and nymphs that feed on mammals prefer smaller animal species than the adults do (Oliver, 1989). In cases where all tick stages are able to parasitise one host species, larvae, nymphs and adults are often reported to prefer different places on the host for attachment. Tick species that have developed a one-host or two-host life cycle

[3] The creator of the term morphogenetic diapause, Prof V. Belozerov, today prefers the term developmental diapause (Belozerov, pers. comm.).

are rare: there, subadult ticks moult once or twice on the host respectively, but with only a few exceptions, the engorged females drop off the host to lay the eggs (Oliver, 1989).

Two main ecological categories of tick-host relationships have been described (Sonenshine, 1991). Most tick species drop off a host somewhere within its habitat. They have to find a place to develop within close range of the point of detachment. After moulting to the next stage, one group of ticks sits and wait until another host passes by close enough to cling on to it. This has been labelled the ambush strategy. In contrast, the second group of tick species actively searches for hosts.

Many argasids, but also some ixodid tick species are nidicolous, ie. they develop inside the nest or burrow of their host, which has the advantage of a shelter and greatly reduces the need to search for a host. This close adaptation to the host can often be found for soft tick species, because they only feed for a short period, mostly for less than one hour. However, nidicolous hard ticks are attached to a host for days. In order to assure repletion within the host shelter, they need a drop off rhythm that is entrained to the activity pattern of the host.

4.1.4 Physiological adaptations to parasitism

All ticks examined so far conserve water or energy to extend their life for months or years (Knülle & Rudolph, 1982; Jaworski *et al.*, 1984; Kahl, 1989). Most ticks spend more than 90% of their life cycle off the host, where they have to adapt to conditions of long term starving, with sometimes dramatic diurnal or seasonal periodic changes of the abiotic conditions. The ability to cope with the abiotic environmental stress in the non-parasitic part of their life cycle increases with each motile developmental instar. Larvae are usually most vulnerable to desiccation, heat or chill (eg. Dautel & Knülle, 1996). Nymphs can survive longer than larvae, and the adults are the most durable instars. This is partly due to the increasingly larger energy reserves of each life cycle stage (Steele & Randolph, 1985). Ticks use their energy reserves very economically. In order to find a host, most species sit and wait in the motionless "questing" pose at well situated positions until a host passes by, a behaviour oddly labelled as "activity" (Lees, 1948). The energy reserves also determine the longevity of ticks mainly by influencing the ability to maintain a body water equilibrium (Knülle & Rudolph, 1982). Furthermore, the whole body permeability for water vapour, which is determined by structural features of the tick cuticle, decreases with each developmental instar.

In all tick species that have been investigated in this respect, unfed larvae, nymphs and adults have the ability to absorb water from sub-saturated atmospheres by an energy consuming process where a highly hygroscopic fluid is produced by the salivary glands (Knülle & Rudolph, 1982). The mechanism in ticks is still not fully understood. It has been shown that the saliva is expectorated on sections of the mouth parts and reabsorbed by the tick after some time to extract the water gains (Rudolph & Knülle, 1979). The ability to gain water from sub-saturated atmospheres is a common phenomenon within arthropods and has evolved several times independently, with a remarkably variability of mechanisms and physiological adaptations involved (Wharton, 1985). The exact relative humidity (RH)-threshold at which arthropods are able to gain water from the surrounding atmosphere is often species specific and is called the critical equilibrium humidity (CEH) (Wharton, 1985). Most ticks are able to gain body water from CEH's above 75% (Knülle & Rudolph, 1982). These conditions often occur in the micro-climate of small fissures in rocks or in soil, even in habitats of otherwise life-hostile macro-climatic conditions (Edney, 1977). Ticks can also gain water by metabolic processes during the digestion of their storage fat (Sonenshine, 1991) but this is believed not to contribute significantly to the water balance of a starving tick. Atmospheric water seems to be the main source for ticks to maintain their water equilibrium off the host. Even a tick species most vulnerable to cuticular water loss does not drink liquid water (Kahl & Alidousti, 1997). As a result of their remarkable energy and water management strategies, ticks often outlive their hosts, eg. the small mammals they parasitise (Needham & Teel, 1991), although they neither drink nor feed during most of their lifetime.

To find a host, ticks rely on an array of optical, locomotory and chemical sensory organs. Many tick species have eyes, but all of them have a complex of sensilla close to the second joint of the first pair of legs, the Haller's organ. Ticks wave the first pair of legs in the air in order to identify the spatial origin of host stimuli like CO_2. Thus functionally the first pair of legs is often compared to antennae. Ticks have extraordinary abilities to cling to a host, even before they are finally attached to the skin by their mouthparts. Once on the host, they search for a suitable spot to engorge by probing with sensory organs located at the tip of their pedipalps, a process that may take several hours.

During engorgement, the tick is challenged by the necessity of a rapid and selective uptake of large amounts of substances from blood, where the benefits of being subjected to the more benign biotic conditions on a host meet a trade off in the growing possibility to be groomed off during the advancing engorgement. Also the host may have a number of immunological responses to the presence of a feeding tick, which the tick needs to counter - usually by products within its salivary gland secretions. At first, the tick prepares for the high volume fluid uptake by growing, stretching and unfolding the soft integument of the cuticle (Hackman & Filshe, 1982). In female ticks, after the cuticle has been prepared to expand for the large blood meal, the final engorgement takes only one or two days for most species, thus reducing the risk to be detected or accidentally brushed off by the host. During the final feed, ticks concentrate the proteins and cells of their meal in their midgut and re-inject water and ions back into the host through the salivary glands. After a fully engorged female detaches, it has to find a suitable habitat to lay the eggs, which can be a difficult task for a creature with such limited motile abilities.

Like the haematophagous mites, ticks lack extracellular digestion. With the start of the feeding process, some cells of the midgut epithelium expand and ingest parts of the blood meal by phago- or pinocytosis. The meal can be metabolised only intracellularly. The total digestion of a blood meal can last several months and it is often not completed after ecdysis to the next stage (Balashov, 1968). Thus, molecular biological methods can often identify the host species an unfed tick nymph has fed on as a larva (Kirstein & Gray, 1996). In order to ensure that the adults find their conspecific mating partner, many tick species, in particular the males, produce pheromones (Sonenshine, 1991).

4.1.5 Pathogenicity

Ticks transmit a greater variety of infectious agents than any other group of blood-feeding arthropod (Hoogstraal, 1985). Ticks can have other harmful effects on their hosts than passing on microorganisms. They also can inject toxic saliva that can cause paralysis to its host (Gothe & Neitz, 1991) or, in large numbers, they can irritate or even exsanguinate hosts that are already affected in their fitness, for example by disease or old age. Furthermore, the epidermis of a host may be permanently scarred and secondary infections can develop at skin-lesions caused by feeding ticks.

4.1.6 Amblyomma limbatum Neumann

The hard tick *Amb. limbatum* is an obligate reptile parasite (Roberts, 1970) and can be found throughout many parts of Australia, in particular the arid and semi arid areas in the centre, but sometimes its range extends into the tropical north (Smyth, 1973). During the last three decades, the ecology of *Amb. limbatum* has been the subject of a range of investigations, mainly concerning its basic ecology in comparison to sympatric and parapatric reptile-tick species within South Australia, eg. (Bull & Smyth, 1973; Andrews, 1982; Andrews *et al.*, 1982; Andrews *et al.*, 1986; Bull *et al.*, 1987; Bull *et al.*, 1989; Bull & Burzacott, 1994; Chilton & Bull, 1994). Some of the results relevant for this study will be summarised here. *Amb. limbatum* is a three host tick, each motile instar infests the same wide range of host species (Belan & Bull, 1995). In South Australia, the tick mainly parasitises the sleepy lizard *Tiliqua rugosa* (Bull, 1996), but among other hosts it was also found to infest *E. stokesii* at field sites in the Flinders Ranges (Duffield & Bull, 1996b). While attached to a host, the tick is protected from heat stress by the thermo-regulatory behaviour of the host (Bull & Smyth, 1973; Chilton *et al.*, 1992a; Chilton *et al.*, 1992b). Attached female ticks emit a pheromone to stimulate males to search on the host (Andrews & Bull, 1982). *Amb. limbatum* detaches in the refuge sites of *T. rugosa*, (Andrews, 1982) and stays immobile when detached (Petney *et al.*, 1983). The engorged stages are prone to ant predation (Dawes-Gromadzki & Bull, 1997). Adults and nymphs can only locate a host from 20 cm or closer, the host detection range of larvae is even smaller (Belan & Bull, 1991).

4.1.7 Amblyomma vikirri Keirans et al.

Because *Amb. vikirri* has been described only recently (Keirans *et al.*, 1996), not much is known about the biology of this tick. It has been assumed that the basic biology of *Amb. vikirri* is similar to that of *Amb. limbatum* (Duffield & Bull, 1996b). *Amb. vikirri* is endemic to South Australia. Its major host is *E. stokesii*, but it has been found engorging occasionally on *Tiliqua rugosa* (Duffield, 1993), though only in close proximity to populations of *E. stokesii* (Duffield & Bull, 1996b; Keirans *et al.*, 1996). Larvae, nymphs and adults were found engorging at different places on the lizard host (Duffield & Bull, 1996b). Under laboratory conditions, *Amb. vikirri* larvae showed a higher susceptibility to water loss and a higher mortality under water stress than *Amb. limbatum* larvae (Duffield & Bull, 1996b). In microhabitat choice experiments, *Amb. vikirri* preferred rocks rather than leaf litter (Duffield & Bull, 1996b).

4.2 The sand fly

4.2.1 Taxonomy and zoogeography

Phlebotomine sandflies are haematophagous dipterans from the family Psychodidae. This rather inconspicuous, usually only 2-3 mm long group of insects is widespread throughout the tropics and subtropics (Günther *et al.*, 1989). Although adults prefer moist and shady places and are rarely seen on the wing (Colless & McAlpine, 1991), many phlebotomines have been found in arid and semi arid areas, frequently parasitising small vertebrates (Chaniotis, 1967). Many species are nocturnal. Characteristics of the adults are a hairy body, the upward-slanting position of the oblong, pointed, hairy wings, the rather large, round eyes and a short but robust proboscis (Figure 11/D). Tropical phlebotomines are often yellowish-brown. Their colloquial name, sandflies, originating from their resemblance to sand grains, often leads to confusion because the group of simuliid flies are labelled sandflies as well. The occurrence of phlebotomine sandflies in Australia was first recorded by Hill (1923). Until around 1970 it was believed that phlebotomines were relative uncommon in Australia. Since then, detailed studies have found 35 species in three genera that occur in abundance in most parts where the annual rainfall exceeds 750 mm (Dyce, 1971; Lewis & Dyce, 1988) or in protected habitats like caves (Dyce & Wellings, 1991).

4.2.2 Life cycle

Most phlebotomines breed in burrows, caves, crevices or fissures where organic material is decomposing (Chaniotis, 1967). The eggs are laid usually about two days after the last blood meal. The minimum average temperature required for egg development is 21°C (Günther *et al.*, 1989). The larvae hatch after 6-12 days; they need a high relative humidity and detritus to feed on (Chaniotis, 1964). The 4th instar larva often climbs vertical objects and the developing pupa attaches to the object with its shed larval cuticle. Adults emerge after 6-7 weeks, but a developmental diapause in the 4th instar may occur in some species (Chaniotis, 1967). Only the reproducing, adult females engorge on blood, males live on nectar or other plant juices. Females can take several blood meals, and it is only then that they gain a role in vectoring diseases. Once taken up by a phlebotomine, if they are to be transmitted to a new host, micro-parasites have to develop to the infective stage and enter the salivary ducts before the sand fly feeds again (Fialho & Schall, 1995).

4.2.3 Pathogenicity

Phlebotomines are known to transmit a variety of etiologic agents that cause serious diseases in humans. *Phlebotomus papatasi* can transmit a virus causing papatase-fever, a disease occurring around the Mediterranean Sea, the Middle East and North India. Various species of the parasitic protozoan genus *Leishmania*, which can cause lethal, debilitating or disfiguring infections to humans, are recognised to be transmitted by phlebotomines throughout the tropics. Furthermore, phlebotomine sandflies have been shown to transmit lizard malaria (Ayala & Lee, 1970). In Australia, a virus has been isolated from phlebotomines in Queensland (Doherty *et al.*, 1973). Where they occur in large numbers, sandflies can irritate their hosts, eg. livestock. The bite of a sandfly can be itchy, but no further negative effects have been reported.

At some of the study sites in the Central Flinders Ranges phlebotomine sandflies were observed feeding in abundance on *E. stokesii*. A series of sandflies mounted for microscopic study were identified by Dr. Alan Dyce as *Australophlebotomus dycii* Seccombe *et al.*, 1993. Some phleboto-mines were dissected for the presence of coccidian blood parasites or used in an attempt to breed this species in the laboratory.

4.2.4 The field observations

On nine occasions from February to April of 1997, 1998 and 1999 respectively (Table 39, p. 181), up to 100 or more sandflies were seen at one time in close proximity to a single host animal. The flies were avidly intent on obtaining a blood meal, flying and hopping over the host and back and forth from their resting places in rock fissures and in small openings between the soil and rock boulders. On each occasion, phlebotomines were collected with an aspirator, mostly from *E. stokesii* and the crevice the lizard was sheltering in. Some sandflies were caught emerging from crevice entrances during the evening hours. Other lizard species found, eg. *Tiliqua rugosa* Gray, *Pogona vitticeps* Ahl, *Gehyra variegata* Duméril and Bribon or *Ctenotus robustus* Storr, were never observed to be affected by sandflies, even when they were occasionally found inside crevices previously inhabited by *E. stokesii*.

During February and March of all three years, many flies were observed to feed to repletion as indicated by the red blood showing through the pale wall of the swollen abdomen. Midguts of all freshly engorged females contained nucleated erythrocytes and some were carrying life cycle stages of blood parasites found in *E. stokesii*. The parasitic stages found

and their identification are listed Table 39 (p. 181). From early April onwards, there were very few freshly fed *A. dycii*. Dissected flies were carrying between 39 and 64 eggs. An attempt was made to breed *A. dycii* in the laboratory, using the standard methods described in the literature (Young *et al.*, 1981; Endris *et al.*, 1982; Ward, 1990). Attempts to feed these adults on *E. stokesii* failed.

Prior to the present observations there are only two records of natural host attack by Australian sandflies; the record of Dyce (1971) involving *Australophlebotomus brevifilis* Tonnoir (recorded as *Phlebotomus brevifiloides* Fairchild) feeding to repletion on the upper arm of the human observer at 21:00 h in a lit porch at Moree, New South Wales, 03/02/1952; and the record of Lewis and Dyce (1988). That "In 1964 Mr. M.R.L Johnson sent to the British Museum (Natural History) females of *Sergentomyia englishi* Tonnoir found on the gecko *Gehyra variegata* Duméril and Bribon, in the Baradine State Forest, New South Wales. One fly was observed feeding in early morning after frost on 24.VII.1964, and two more at 10:00 on 12/07/1964". All other evidence of host acceptance has been derived from experimental feeding (Tonnoir, 1935), precipitin tests of wild caught engorged females (Lee *et al.*, 1962), or by inference from trophic morphological comparison (Lewis & Dyce, 1982). Thus the present observations are the first of natural attack by substantial numbers of sandflies under field conditions in Australia. They add to the contention of Lewis & Dyce (1982) that "In Australia lizards largely take the place occupied by rodents in other regions as host of sandflies".

4.3 The mosquitoes

4.3.1 Taxonomy and zoogeography

Most of the approximately 2500 members of the family Culicidae, better known as mosquitoes, are cosmopolitan haematophagous parasites of land vertebrates; occasionally they even attack fish (Colless & McAlpine, 1991). Characteristics of the adults are the venation of the wings and scales alongside the veins and posterior margin of the wing. Numerous mosquito species are very host specific; many do not attack humans. Adults normally rest during the day in humid, shady places. The flight activity mainly starts at sundown, but some species are active during the day (Colless & McAlpine, 1991). All species have a niche for their larval development. In Australia for example, *Aedes australis* breeds in concentrated sea water in rock pools, where other species prefer fresh water habitats like running streams, tree holes or permanent pools (Colless & McAlpine, 1991). The Australian mosquito species used in this study were *Aedes notoscriptus* and *Culex quinquefasciatus*. *Aedes notoscriptus* larvae thrive in any small water holding container like bottles, cans or old car tires. *C. quinquefasciatus* is a member of the *C. pipiens fatigans* species complex, which occurs mainly around human settlements (Colless & McAlpine, 1991), breeding in larger water bodies like water tanks or dams. *C. quinquefasciatus* were frequently found in the township of Hawker, SA, which was the geographical and logistical centre of the field study.

4.3.2 Life cycle

The culicoid larva is always aquatic, thus oviposition takes place on the surface of water bodies or wet surfaces. After hatching, the larvae take in atmospheric oxygen at the surface by spiracles on their abdomen. After further development into the short-lived motile pupae stage, they hatch as winged adults (Günther *et al.*, 1989). Females require a blood meal before the eggs can mature, males live on sugary plant juices. Mating occurs often on the wing. Hungry females pierce the skin of a host with their elongated proboscis, and they usually feed and lay eggs several times before they die.

4.3.3 Pathogenicity

Many mosquito species are important vectors of disease, and some genera are notorious for transmitting certain diseases, for example the genus *Anopheles* for malaria and the genus *Aedes* for filariasis. Even some of the Australian members of these genera have been shown to act as vectors, eg. the *Anopheles farauti* species complex for malaria, *Culex fatigans* for filarial nematodes and *Aedes aegypti* for the viral Dengue fever (Colless & McAlpine, 1991). Apart

from their vector role, mosquitoes can be serious pests, affecting the productivity of livestock and the well being of humans.

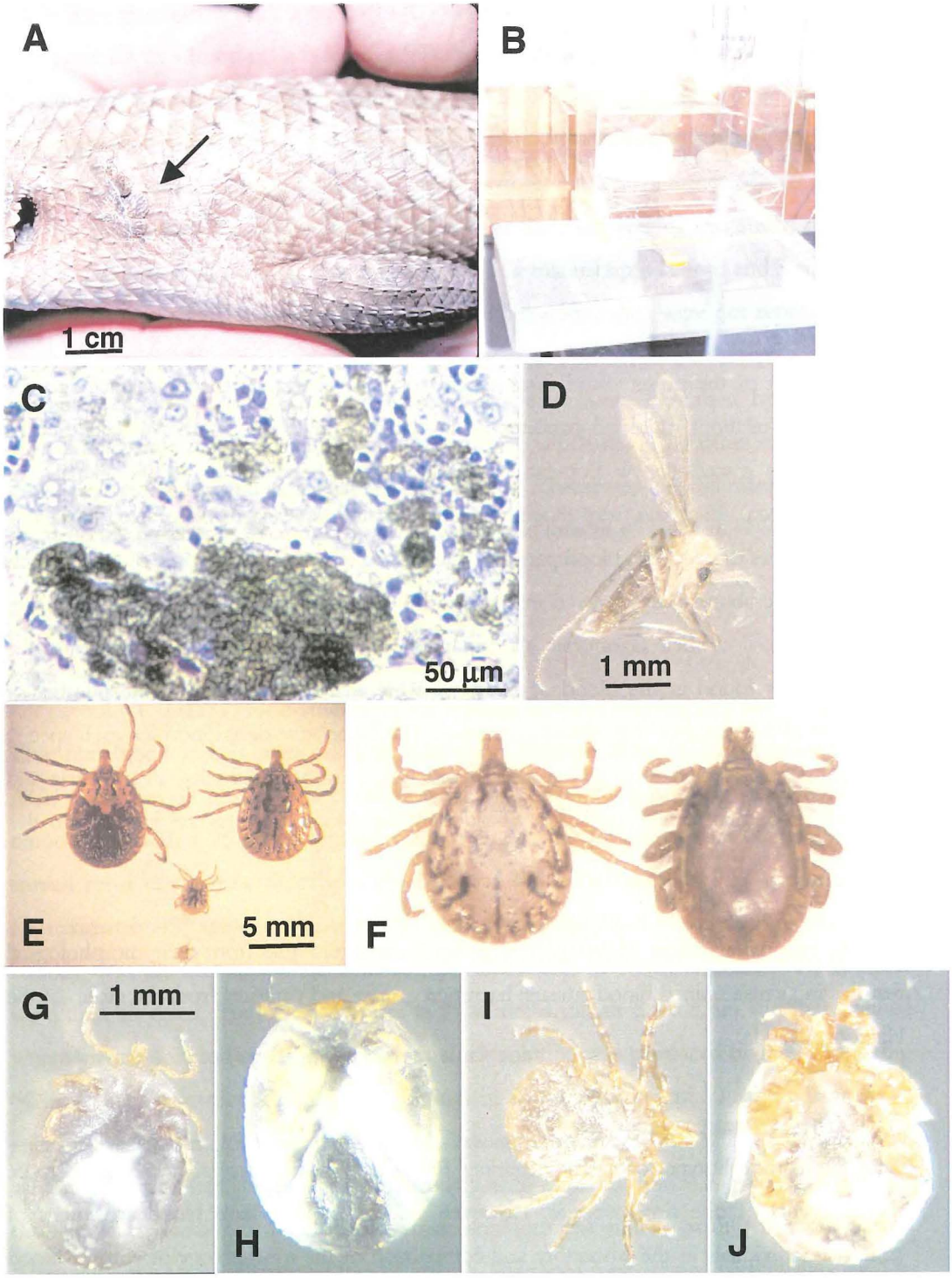

Figure 11. A: adult *Amb. vikirri* engorging on *E. stokesii*. B: tick detachment cage. C: accumulation of pigment in hepatic tissue of *E. stokesii* (giemsa). D: the phlebotomine sand fly *Australophlebotomus dycii*. E: *Amb. vikirri*, female, male, nymph and larva. F: male *Amb. vikirri* (left) and male *Amb. limbatum* (right). G-J: *Amb. limbatum* (photo: G. Duffield) G: engorged larva, healthy. H: engorged larva, excessive guanin. I: moulted nymph, healthy. J: partly moulted nymph, crippled legs.

Chapter Five

5. THE DETERMINATION OF THE NUMBER OF BLOOD PARASITE SPECIES FOUND

5.1 Introduction

A component of the present study was a field survey investigating the prevalence and the demographic relations of blood parasites in the lizard *E. stokesii* in South Australia. Special emphasis was placed on six populations in the central Flinders Ranges (for details see chapter fourteen). *E. stokesii* from those populations were suspected to be infected by several different species of coccidian blood parasites. Due to the resemblance of some of the life cycle stages and the abundance of mixed infections, the determination of the number of species involved as well as their taxonomic allocation proved to be intricate. In this chapter, the parasite stages found in the blood of *E. stokesii* were analysed in regard to the number of species of blood parasites they actually represent. The taxonomic description for each species will be presented in chapters six to ten. In order to prevent repetitions, all materials and methods used for the taxonomic identification of the blood parasites are included in this chapter.

Initially, two main groups of parasites were easily distinguishable from their morphological features in giemsa stained blood smears: haemogregarines and parasites from the group of the Haemospororida.

5.1.1 The haemogregarines

The various morphotypes of haemogregarines found in *E. stokesii* were at first believed to be semaphoronts of one single species. However, analysis of long term blood screenings of *E. stokesii* individuals in the laboratory and comparison of the morphology of chronic blood stages, suggested the presence of four distinctly different chronic blood parasite stages (Figure 13), labelled in this chapter as the types 1, 2, 3 and 4. For taxonomic descriptions for those four types of haemogregarines, a detailed look at their life-cycle features was obligatory.

5.1.2 The Haemospororida

The presence of a lizard malaria species (Haemospororida: Plasmodiidae) was demonstrated by numerous intraerythrocytic trophozoites, schizonts and gametes (Figure 74). It was concluded that this species belonged to the genus *Plasmodium* because intraerythrocytic merogony of haemozoin-pigmented parasites was observed. The presence of a second lizard malaria species was discovered at the beginning of the third year of this study, due to the help of Dr. Sam R. Telford Jr.. The final version of a manuscript (Telford and Stein 2000, in press) has been added as an appendix, and those taxonomic data were not repeated in this thesis. However, all noteworthy results that were not included in that publication are presented in chapter ten. The two *Plasmodium* species were described by comparative morphology only of stages in the lizard blood cells, which made a further examination of those two species within this chapter unnecessary. Therefore, the following analysis will focus on the number of haemogregarine species found infecting *E. stokesii*.

5.2 Materials and Methods

5.2.1 Lizard handling

5.2.1.1 Lizard blood-smears, tissue-smears, fixation, staining and mounting

Thin-film blood smears were taken at 1-10 week intervals from all lizards housed in the ACU and from all field lizards on the day of capture. A drop of approximately 20 µl of blood taken with a 25 gauge sterile syringe needle from the caudal vein of the lizard was spread on a clean glass slide by a second glass slide. The second slide was held in an approximately 45° angle and after a brief contact with the fresh blood drop it was moved steadily over the first slide, thus smearing the blood cells in the desired mono-layer. Slides were then air dried, fixed for 5 minutes in 99% methanol, air dried again and then stained with a giemsa solution for 1 h. The giemsa stock solution was produced by dissolving 0.8 g "Giemsa stain" powder (Probing & Structure) in 50 ml glycerin and 50 ml methanol. The solution was shaken for three days on a shaker and then filtered through filter paper (542, ashless). A phosphate buffer was produced (5.47 g KH_2PO_4 and 3.8 g Na_2HPO_4 in 1 l distilled water) and one part giemsa stock solution was diluted with ten parts buffer. Afterwards the stain was titrated to a ph of 7.2. The slides were stained with giemsa for 1 h, then it was washed off with flowing tap water. The slide was air dried and mounted with Zeiss™ mounting medium and a glass cover-slip.

5.2.1.2 Lizard killing and dissection

For this study, five *E. stokesii* were killed by intra-peritoneal (IP) injection of 0.3 ml Lethabarb™. All major organs, brain, muscle, blood vessels and the digestive tract were dissected immediately after death and parts of them were prepared for the histological examinations by light microscopy (LM) or transmission electron microscopy (TEM). Tissue smears were made by dissecting approximately 0.3 mm^3 blocks of fresh tissue that were then placed on a clean glass slide. With a second glass slide the tissue block was firmly pressed until it was smeared.

5.2.1.3 Lizard tissue fixation, staining and mounting

E. stokesii tissues from dissections were fixed and stored in Zamboni's fluid (Stefanini *et al.*, 1967). For further preparations, the tissues were gradually dehydrated in 70% ethanol over night, then in 80%, 90% and twice in 100% ethanol for 1 h respectively. The tissues were kept in chloroform over night, and afterwards submerged in paraffin at 45°C for 1 h. The paraffin was changed twice and a vacuum was applied for 45 minutes after each change. The tissues were then embedded in fresh paraffin and cooled down until the paraffin had hardened. The embedded tissues were sectioned at 5 µm with an ultra microtome and the resulting sections were carefully spread on ethanol cleaned glass slides. The tissue sections were dried over night in a 45°C incubator and stained after Kimsey (1992). Briefly, a 0.2 m stock citrate-phosphate buffer was produced by adding 28.39 g/l sodium phosphate (anhydrous, mol. wt. 142) and 19.21 g/l citric acid (anhydrous, mol. wt. 192.1). The final working buffer contained 20 ml stock buffer and 80 ml acetone diluted in 400 ml distilled water (dH_2O). For the giemsa stain, 3.6 ml giemsa stock was added to 40 ml working buffer.

To stain the sections, they were dewaxed twice in xylene and then subsequently rehydrated in 100%, 90%, 80%, and 70% ethanol, for 3 minutes respectively. Afterwards slides were submerged in the giemsa stain for 1 h and then differentiated by dipping three times into 95% and 100% (2x) ethanol respectively, until no giemsa stain was visible in the ethanol blotted off the slides. Then they were kept in xylene (2x) for 3 minutes each. Finally slides were mounted with Zeiss™ mounting medium and a glass cover-slip. Some additional samples were stained with haematoxylin & eosin.

5.2.1.4 Lizard faecal samples

Faecal flotation's were performed with an Ovatector™ disposable faecal diagnostic system, produced by BGS Medical Products INC., Florida, USA. Fresh faeces of *E. stokesii* were placed into a small collection cylinder that was then filled with the flotation solution provided by the manufacturer. The faeces were manually dispersed in the solution. The sample was allowed to settle for 15 minutes, after that time a glass cover slip was dipped once on the surface of the solution at the top of the cylinder. The collected liquid was placed on a glass slide and inspected for parasite stages on a compound microscope.

5.2.1.5 The parasite load evaluation of blood smears

Blood smears and tissue sections were screened under a Olympus-CH compound microscope at 1000x magnification. Parasitic stages were measured with an ocular micrometer, and documented photographically on a Leitz Dialux 20 EB microscope. Best results were obtained with an Ectachrome Kodak-Tungsten™ film. The infected erythrocytes found in blood smears were counted in microscope-fields containing approximately 100 red blood cells (RBC's) and were expressed as infected erythrocytes per 10 000 (10^4) RBC's or as percentage of cells infected. Multiple infected RBC's were counted as one infected RBC, mixed infected RBC's were counted as one infected RBC per parasite species found. Data were typed directly into a Microsoft Access™ form created specifically for this purpose, thus keeping the time to read in the data to a minimum. Because a reliable identification of the species of blood parasites was not possible for all stages found, the different morphotypes of parasites as they appeared in a giemsa stain were counted. An overview of the morphotypes found is presented in Figure 73.

A maximum of 100 microscope fields was examined for each blood smear. Due to mixed infections in individual lizards and because some blood parasites had extremely high parasitemias in *E. stokesii*, it was impractical to count the parasitemia of all parasite species in 100 microscope fields. Depending on the parasitemia of each species found during the examination of the first ten fields of a smear, a graded counting of fields, according to. Table 6 was used instead.

Table 6. The number of microscope fields on a slide evaluated for blood parasites depended on the parasitemia of the blood parasite species.

Parasitemia	Number of parasites in 100 fields	No. of fields evaluated per slide for each species
0%-0.1%	0-10	100
0.1%-5%	11-500	50
> 5%	>500	10

The method of estimating parasitemias by counting parasites from microscope fields has been criticised by Godfrey *et al.* (1987) and Fedynich *et al.* (1995), who pointed out that estimated data were not comparable between different studies and also were not readily suitable for statistical analysis (Fedynich *et al.*, 1995). Instead of counting parasites in fields that contained an estimate of 100 blood cells, they suggested evaluating at least 2000 blood cells that were counted exactly. This would take about 30 minutes per slide by an experienced examiner, given a single infection with a (0.2-4.2 %) parasitemia. The method suggested by Godfrey *et al.* (1987) was not used for this study, because its benefits did not justify the extra time necessary to examine each slide. One emphasis of this thesis was to compare the prevalence and parasitemia of blood parasites of different populations of *E. stokesii* rather than to make these results comparable to other studies. In addition, it became apparent that many lizards in this study had a parasitemia lower than 0.2%, and the number of lizard erroneously diagnosed as uninfected (ie. false negatives) would have been increased by counting only 2000 erythrocytes.

5.2.1.6 Blood inoculation for transmission experiments

Blood inoculations were performed either via intra-peritoneal (IP) or oral routes. In all experiments, a standardised amount of 0.2 ml of blood was applied to an *E. stokesii.* One of three types of anticoagulants were used to coat the 25 G injection needle:

1. a citrate buffer (40 g sodium citrate dihydrate and 8.5 g NaCl filled to 1 litre with distilled water and then autoclaved for sterilisation).
2. heparin
3. a reptilian ringer solution (6 g NaCl, 0.075g KCl, 0.1 g $CaCl_2$ and 0.1 g $NaHCO_3$ filled to 1 l with distilled water and then autoclaved for sterilisation).

The syringe-needle was coated by filling and emptying the syringe once through a sterile needle with either one of the anticoagulants. Blood was drawn from the caudal vein of a

lizard and was then applied immediately to a new lizard. The needle was inserted ventrally by pushing it carefully under one of the scales in the lower right section of the abdomen, then the blood was carefully injected.

5.2.2 Tick handling

5.2.2.1 Tick collection and housing

Engorged larvae, nymphs and adults of both tick species, *Amb. vikirri* and *Amb. limbatum*, were obtained from *E. stokesii* and *T. rugosa* at the six study sites in the Flinders Ranges. The lizards were taken to the ACU where they were placed into especially designed detachment cages (Figure. 11/B). The lizards stayed on a metal grid within a 36 cm x 18 cm perspex cage. The grid mesh-width was chosen depending on the stage of the ticks attached to the lizards: 0.5 cm for larvae or nymphs, or 1 cm for female ticks. In the cage, a shelter and a basking rock (10 x 10 x 4 cm) was provided. Each detachment cage had a single 60 W light bulb as heat source hanging approximately 20 cm above the basking rock, thus supplying a temperature gradient according to general practice for reptile husbandry. Lizards were kept in these cages until the ticks had dropped off.

Detached subadult ticks were kept single in 1.5 ml Eppendorf vials, with each lid pierced three times by a 25 gauge syringe needle to allow the exchange of air and humidity. Adult ticks were kept in 60ml plastic vials with an approximately 1 cm hole punched through the lid. The hole was covered by a fine gauze that was glued to the lid. All ticks were stored together in the same desiccator over a saturated $MgCl_2$ solution, in a temperature controlled room at 25°C, keeping the relative humidity (RH) at 95% (Winston & Bates, 1960). The light regime was set to 12 hours (h) light and 12 h darkness.

5.2.2.2 Tick feeding and transmission experiments

Only larvae derived from females that were caught in the field were used for this study. Larvae were used 4-12 weeks after oviposition, because in preliminary experiments the feeding and moulting success of older larvae was poor. The life history of all previous instars of the nymphs and adult ticks used for transmission experiments was recorded. For each tick, the identity and infection status of all previous hosts were known, including the host for the female tick of the parent generation.

Two different types of transmission experiments were performed, depending on the host that was to be infected. The first group of experiments aimed to infect the ectoparasites by feeding on the lizard host, the second group of experiments aimed to infect the lizard host by the ingestion of the vector tick.

In order to infect ticks, single infected *E. stokesii* and unfed ticks were placed into a cotton bag (20 x15 cm) and kept over night in a room with constant 37°C. The mouth of the lizard was taped closed, to prevent the lizard ingesting the ticks. If the ticks did not attach, the lizard was kept in the 37°C room for a second night. When ticks did not attach after two nights in the 37°C room, an experiment was terminated. *E. stokesii* with ticks attached were placed immediately into the detachment cages. Food was provided twice a week, water ad libitum. During the first weeks after the attachment the detachment cages were monitored daily and the detached ticks were counted and collected. When no ticks dropped off for 7 days, lizards were inspected and ticks found to be still attached were removed with fine forceps. The lizards were then placed back into their original housing conditions (see 3.2).

In order to infect lizards, vectors (mainly ticks, but on one occasion mosquitoes) were force fed to *E. stokesii.* The lizards usually did not open their mouth voluntarily, so during the short periods where they would open their jaws a little, a 1.5 ml Eppendorf tube, where the tip had been cut off with a scalpel blade, was swiftly but carefully placed between the jaws. The vectors were placed on a small piece of banana and manipulated down the tube into the mouth of the lizard with a small paint brush. After the pieces were placed in the mouth, the tube was carefully removed and the lizards were set back into their housing containers. The cuticle of the unfed tick vectors was always cut at least once with a scalpel blade before offering it to the lizard, to enhance the release of the infective stages from the tick midgut.

5.2.2.3 Tick killing, dissection, fixation, staining and mounting

Ticks were dissected on a clean glass slide into a drop of distilled water (dH_2O) or a reptilian ringer solution under a dissection microscope. A cut was made with a sterile scalpel blade through the distal end of the opistosoma, severing the gnathosoma together with the mouth parts from the opistosoma at an imaginary line through both eyes. The midgut was then carefully squeezed out of the opistosoma and the remaining cuticle was removed from the slide. A glass cover-slip was placed onto the tick midgut immediately after the dissection This type of preparation is known as a "wet mount".

Some slides with midguts where parasitic stages could be identified in the wet mount or slides with midguts of an uncertain infection status were air dried, fixed for 5 minutes in 99% methanol, air dried again and then stained with a giemsa solution for 10 minutes. The giemsa stain was carefully washed off by dipping the slides several times into a 3 l container filled with tap water, the slide was air dried and mounted with Zeiss™ mounting medium and a glass cover-slip.

5.2.2.4 Tick parasite-load evaluation

Wet mount or giemsa stained slides were placed under a compound microscope and screened at 400x. Parasitic stages were measured by an ocular micrometer. The presence of coccidian life cycle stages was recorded.

5.2.3 Sand fly handling

5.2.3.1 Sand fly collection and housing

Australophlebotomus dycii were collected with an aspirator directly from *E. stokesii* or from crevices frequently visited by *E. stokesii*. The delicate phlebotomines were carefully blown into a 100 ml collection vial with a 1 cm hole in the lid where a moist cotton pad was inserted. The collection vials were kept in a Styrofoam™ box and were covered with wet towels to provide the appropriate temperature and RH for the survival of *A. dycii* (for review see Ward, 1990).

At the FUSA, all instars of *A. dycii* were kept in autoclaved terra-cotta pots with a 2 cm layer of plaster of paris at the bottom and a fine gauze held by rubber bands over the top opening. The pots were held in plastic dishes filled with approximately 3 cm of de-ionised water. The water was topped up daily, and changed once per week.

5.2.3.2 Sand fly feeding

First instar larvae, hatched from eggs laid into the terra-cotta pots, were transferred into new pots and fed with an aged mix of rabbit food and rabbit faeces (Young *et al.*, 1981). The aging of the food prevented excessive fungal growth within the pots. Briefly, 0.1 kg of commercially available rabbit food (pellets) and 0.1 kg of rabbit faeces obtained from the ACU of the Flinders Medical Centre (FMC) were air dried and ground down to a fine dust with a mortar and pestle. The mix was filled into a 21 cm wide dish, moistened with dH_2O and placed into a desiccator filled at the bottom with dH_2O, thus creating a saturated relative humidity. A piece of bread mould was placed onto the mix, and the desiccator was kept at constant 25°C for 3 months. The mix was stirred once a month. After three months all fungal growth had ceased and the mix was dried at 45°C and portioned and stored in clean 5 ml screw-top Eppendorf vials. The mix was sprinkled sparingly onto the plaster of paris in the sand fly containers.

Emerging adults were transferred into new pots and introduced to *E. stokesii* several times between 1-6 weeks after moulting in the "diptera box" (see 5.2.4.2) for up to 24 h. Adult *A. dycii* were fed on a 50:50 solution of honey and de-ionised water. Approximately 50 µl of the solution was placed as a single drop on the gauze, the drops were changed every two days, and the remaining old drop was blotted away with a paper tissue.

5.2.3.3 Sand fly killing, dissection, fixation, staining and mounting

A. dycii midguts and salivary glands were dissected using the method described by Furman & Catts (1982). Slides were air dried, fixed for 5 minutes in 99% methanol, air dried again and then stained with a giemsa solution for 10 minutes. The giemsa stain was carefully washed off by dipping the slides several times into a 3 l container filled with tap water, the slide was air dried and mounted with Zeiss™ mounting medium and a glass cover-slip.

5.2.3.4 Sand fly parasite-load evaluation

Wet mount or giemsa stained slides were placed under a compound microscope and screened at 400x. The presence of coccidian life cycle stages was recorded, parasitic stages were measured by an ocular micrometer and documented photographically.

5.2.4 Mosquito handling

5.2.4.1 Mosquito collection and housing

The techniques used are documented by Trembley (1955) if not stated otherwise. Two mosquito species, *Aedes notoscriptus* and *Culex quinquefasciatus*, were used in the experiments. Most of these were provided by Mr. Craig Williams from the mosquito breeding facilities at the School of Pharmacy and Medical Sciences of the University of South Australia (UniSA). Attempts to collect mosquitoes from the study sites in the Flinders Ranges usually failed. Techniques that were attempted included egg sampling by a pool trap, and trapping with a light source and CO_2 (dry ice) as bait. The latter was used over several nights at the Glenlyle study site in the Flinders Ranges between September and December of 1997. On one occasion three adult mosquitoes were collected with an aspirator from a house wall within the township of Hawker.

At the University of South Australia, mosquitoes were kept in cubic boxes (60 x 60 x 60 cm) made from plywood with a glass front. One opening in the glass front, covered by a sleeve made of a fine gauze, allowed access into the box, the other opening was in the bottom of the box and gave the mosquitoes access to a tray filled with de-ionised water. The water provided the high humidity necessary for the long term survival of the mosquitoes and an opportunity for the females to lay the eggs. The room was kept at constant 25°C, the relative humidity was around 40 %. A reversed photoperiod of 12 h dark (in the day) and 12 h light (in the night) allowed more convenient working hours during the mosquitoes' main phase of biting activity, which is at dusk for *A. notoscriptus* and around midnight for *C. quinquefasciatus*.

Two batches of *A. notoscriptus* larvae were bred at the FUSA. The larvae were obtained by Mr. Williams and his colleagues in the parks of the city of Adelaide by trapping with a 2 l plastic bottle filled with water and a small amount of soil. Freshly engorged female *A. notoscriptus* laid their eggs onto the surface of the water. The *A. notoscriptus* larvae were raised to pupae in a tray filled with de-ionised water at 25°C and a non-reversed photoperiod of 12 h light and 12 h darkness. Emerging pupae were transferred by a wide-mouth pipette (0.8 cm) into a plastic bottle within the glass cage with a neck width of 1.5 cm, wide enough for the hatching mosquitoes to fly out of the bottle, but narrow enough to prevent mosquitoes flying back into it, thus preventing drowning. The holding cage for the emerging adult mosquitoes was made from a glass cage (60 x 40 x 40 cm) with a saturated salt solution

($MgCl_2$) standing in two trays at the bottom. The salt solution was separated from the rest of the cage by a perspex sheet with a 12 cm hole in the centre, covered by fine gauze.

5.2.4.2 Mosquito feeding

For egg production, female mosquitoes were allowed to feed on human blood, otherwise males and females were provided with a 10% sucrose solution *ad libitum*. Larvae were fed on a 1:1 mixture of ground fish food (Tetra™) and ground dry dog food (Good-O™). Most feeding experiments were conducted at the University of South Australia, where *E. stokesii* were placed into an Elliot-trap with both trap doors kept open by a piece of cardboard that covered the whole inner bottom of the trap. The two openings were covered by wire mesh (mesh width 3 cm) fixed by a strong rubber band and the trap, containing a single *E. stokesii*, was placed into the mosquito holding cage in the dark. Experiments at the University of South Australia were always started around midday and the light was turned on briefly 1 h after introducing the lizard into the cage, to see whether a sufficient number of mosquitoes had fed. On two occasions lizards were kept in the cages for 24 h, because the number of mosquitoes in the cage was low and no mosquitoes had fed after 1 h.

Feeding experiments at the FUSA were conducted only with *A. notoscriptus*. Female *A. notoscriptus* from the holding cage were collected with an aspirator and together with a single *E. stokesii* they were introduced into the "diptera-box" (18 x 18 x 35 cm). The diptera-box was made of perspex with one of the square surfaces made as a door and one rectangular surface made with two 11 cm holes and one 1 cm hole. The large holes were sealed with one rubber glove each, attached at the wrist side, thus allowing manipulations within the box. The small hole was covered by a sliding piece of perspex attached at one side with a screw and provided an entry for the aspirator-hose, in case engorged Diptera were to be removed from the box. The door had a 5 cm hole sealed with a fine gauze to allow airflow. The diptera-box was kept in a temperature controlled room at 25°C or 37°C for up to 4 h, and engorged mosquitoes were removed with an aspirator and gently blown back into the holding cage.

5.2.4.3 Mosquito killing, dissection, fixation, staining and mounting

Mosquito midguts and salivary glands were dissected using the method described by Furman & Catts (1982). Slides were air dried, fixed for 5 minutes in 99% methanol, air dried again and then stained with a giemsa solution for 10 minutes. The giemsa stain was carefully

washed off by dipping the slides several times into a 3 l container filled with tap water. The slide was air dried and mounted with Zeiss™ mounting medium and a glass cover-slip.

5.2.4.4 Mosquito parasite-load evaluation

Wet mount or giemsa stained slides were placed under a compound microscope and screened at 400x. The presence of coccidian life cycle stages was recorded, parasitic stages were measured by an ocular micrometer and documented photographically.

5.2.5 Transmission Electron Microscopy (TEM)

5.2.5.1 Fixation and embedding

The preparation of the specimen was conducted at the FUSA. For lizard tissues and blood samples, best results were obtained with the following protocol: fresh lizard tissues (smaller than 4 mm^3) or lizard blood (less than 50 µl) were fixed over night in a solution of 2.5% glutaraldehyde and 0.1 mol cacodylate-buffer (pH 7.4) in mold capsules provided by ProSciTech™, P.O. Box 111, Thuringowa, Qld. 4817, Australia. The fluid exchange at each step was done by pipetting the used liquids carefully from the vials and fresh liquids into the vials. The tissues were washed three times in 0.1 mol cacodylate buffer and transferred into a solution of 1% osmium tetroxide and 0.1 mol cacodylate buffer for 1 h. Specimens were washed three times in dH_2O and subsequently dehydrated in 50%, 70%, 80%, 95%, and 100% (2x) ethanol for 5 minutes each. The first three ethanol solutions were kept a 4°C, and all others were kept at room temperature. The ethanol was exchanged with propylene oxide (2x) for 15 minutes each, then the tissues were embedded in a 25:75 mix of epoxy-resin and propylene oxide for 2 h. The resin was produced from a mixture of 10.8 ml Procure™, 9.1 ml DDSA™, 3.8 ml NMA™ and 0.2 ml BDMA™, available from ProSciTech™. In three steps, increasing concentrations of resin (50:50, 75:25 and finally the pure resin) were applied every 2 h. After the last exchange the vials were left unsealed at 4°C over night and the resin was replaced with fresh resin the next morning. The tissues were placed into an incubator at 65°C where the resin polymerised completely within 48 h. The protocol for lizard blood differed from the one described above inasmuch as until the propylene oxide was applied, each vial was vortexed and then spun down for approximately 20 seconds respectively, to allow a more thorough mixing of the substances.

5.2.5.2 Trimming, sectioning and mounting

The resulting mold blocks were trimmed and sectioned at the Microscopic Imaging Facility of the FMC. Trimming was done with a fresh razor blade, until a less than 0.1 mm^2 plane face of a specimen was obtained. Semi-thin and ultra-thin (stretched colour silver to gold) sections were prepared on a Reichert ultra-microtome and transferred onto butvar coated or non coated copper grids (mesh width 200 µm or 300 µm) or single slot specimen holders, coated with a butvar-film.

The butvar solution was produced by dissolving 0.66 g butvar in 100 ml chloroform. Three quarters of a thoroughly cleaned glass slide was dipped into the solution and pulled out at a steady speed by a clamp attached to a string pulled by an electric motor. The slide was left to air dry for 60 seconds, leaving a clear, thin film on both sides of the lower three quarters of the slide. Fine cuts were made on one side of the slide with a fresh scalpel blade alongside the sides of the slide and the upper rim of the film. The slide was then breathed upon twice and then slowly submerged into dH_2O, which in most cases resulted in the detachment of a rectangular piece of butvar film floating on top of the water. Grids were carefully placed onto the film, with the shiny side facing upwards. A piece of parafilm, slightly larger than the butvar-film square, was dropped onto the butvar-film and then pulled out with forceps. In most cases the butvar-film with the grids would stay attached to the parafilm and after air drying, the grids could be picked up singly by fine forceps.

5.2.5.3 Staining and screening

Grids containing ultra-thin tissue sections were placed onto a single drop of uranyl acetate for 8 minutes and then washed briefly by dipping it ten times into dH_2O. Each grid was then placed onto a single drop of lead citrate for 10 minutes. Grids were washed thoroughly in dH_2O by dipping into three flasks 30 times respectively. After the grids were air dried or dried under a 60 W lamp (approximately 20 cm from the light bulb), the specimen were screened at a 5000x magnification under a transmission electron microscope. The presence of coccidian life cycle stages was recorded. Parasitic stages were documented photographically and measured from the scale printed on the negative.

5.2.5.4 The nomenclature for the viewing-aspects of parasites

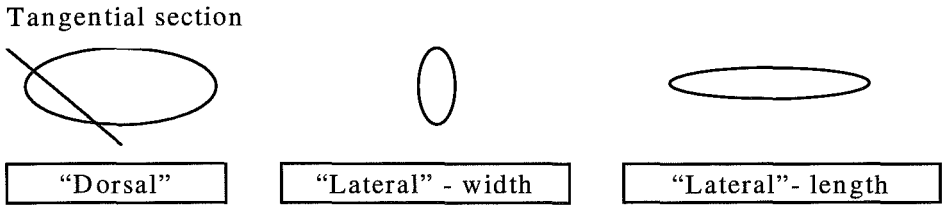

Figure 12. Illustration of the viewing aspects of intraerythrocytic blood parasites in histological sections. The ellipsoid objects represent schematic blood parasites. "Dorsal" view: aspect as it appears through a compound microscope; "Lateral" views: aspect from a 90° angle from the optical axis of a microscope. A tangential section is indicated by the straight line.

In order to keep the descriptions of the three dimensional structure of the blood parasites comprehensible, the nomenclature used must be briefly explained. Following the laws of gravity, structures shaped other than spherical will generally come to lie on one side. Because the three dimensional structure of the taxonomically important chronic blood parasites species was not spherical or evenly cylindrical, the photographs of parasites in blood smears generally showed only one viewing-aspect of the parasitic stages. For the present study, this viewing-aspect was labelled as "dorsal" (see Figure 12), although the shortcomings of this term in connection with protozoan morphology were obvious. In contrast to the rather uniform positioning of the parasites in blood smears, parasites in histological preparations come to lie in random orientation. As a result, histological sections were produced for any angle or level through the micro-parasite. In addition to the "dorsal" view of parasites in blood smears, histological sections showed blood parasites "from the side", with a viewing-aspect rotated by an angle of 90° to the optical axis in a microscope. Due to the elongated shape of the chronic blood parasite stages, the "lateral" view can show either their narrow (width) or their wide (length) side. In this thesis, the descriptions of histological sections of parasites on TEM images will refer to the viewing aspects outlined in Figure 12.

5.2.6 Material published outside the framework of this thesis

Parts of the material used in this thesis were published with Dr. Sam R. Telford Jr. (Telford & Stein 2000, see appendix). Drawings or photographs of Dr. Telford were used in this thesis with the permission of Dr. Telford and are labelled accordingly. Copied or altered drawings from textbooks or publications and maps are labelled in the same manner.

5.3 Results

The survey on *E. stokesii* populations in the field revealed that certain morphotypes of blood parasites were only found at particular locations (for details see chapter 14). A long term study on the blood of field caught *E. stokesii* in the ACU revealed a total of four distinctly different morphotypes chronically infecting lizard erythrocytes. It was therefore suspected that these four morphotypes represent the chronic stages of four species of blood parasites. The four chronic stages were described in the following as types 1, 2, 3 and 4 (Figure 13). A common characteristic for three of the four chronic stages was the development of an encapsulating sheath that hampered the penetration of giemsa stain. Morphological measurements (Table 7) showed statistically significant differences between the four types (Table 8). A more detailed analysis revealed that the length of the blood parasite types was significantly different between each of the four types (Table 9), and that except for types 1 and 2, parasite width was also statistically different between the four types (Table 10). Measurements of lizard erythrocytes showed that the chronic stage of three out of the four haemogregarines altered the size of infected erythrocytes or the size of the erythrocyte nucleus significantly (Table 11). No morphological alterations were detected between erythrocytes infected with the type 1 parasite and uninfected erythrocytes of the same lizard individual. However, the average width of a host erythrocyte was significantly larger and the average width of the host cell nucleus was significantly smaller when erythrocytes from a lizard singly infected with the type 2 blood parasite were compared to uninfected erythrocytes of the same lizard individual. Similarly, erythrocytes from an *E. stokesii* singly infected with the type 3 parasite were on average significantly wider when infected and the average width of the nucleus was significantly smaller. The nucleus of erythrocytes infected with the type 4 parasite was on average significantly shorter when compared to nuclei from uninfected erythrocytes of the same lizard individual.

Besides the chronic stages, a wide range of other morphotypes of haemogregarines occurred in each blood infection (for an overview see Figure 73). Due to their morphological resemblance, they could not be reliably separated into different genera when present in mixed infections. In order to produce comparable data throughout this thesis, these stages have been generally listed as "unspecified morphotypes", even if the lizard was only single infected. The unspecified morphotypes were grouped into the types A, B and C by their morphological features visible in a giemsa stain (Figure 73). Transmission experiments suggested that, in the course of an infection, the unspecified morphotypes are the first

parasite stages that infect the blood of a lizard host, and that they later encapsulate within the erythrocyte, thus developing into the chronic stage. The unspecified morphotypes A, B and C were counted separately. The term abundance was used where they were quantified. In the following, the parasitemia for a given blood parasite species always refers to the number of the chronic stage exclusively.

Spontaneous blood infections with the type 4 parasite occurred in the vast majority of all laboratory born lizards monitored for this study. This finding was analysed in more detail within the taxonomic description of *Schellackia* sp. in chapter nine. No spontaneous infections were found for the other three haemogregarine blood parasite types. No spontaneous infections with *Plasmodium* occurred in *E. stokesii* born in the ACU. Spontaneous *Plasmodium*-infections noted in few *E. stokesii* held in the ACU after they were captured in the field, were interpreted as relapses of previous infections.

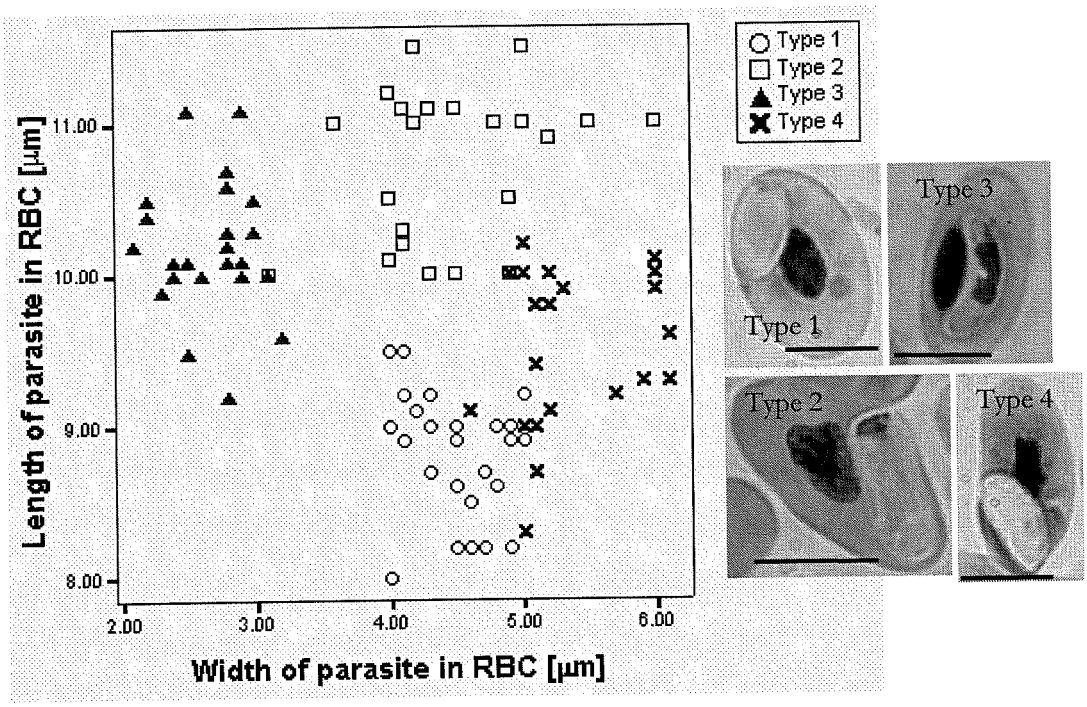

Figure 13. The proportions of the four chronic intraerythrocytic morphotypes found in infected *E. stokesii* [n = 4 x 25; images from giemsa stained blood smears, b&w, bar = 10 µm].

Table 7. Average length and width (n=25) comparing the chronic stages of four types of haemogregarines. Each type was measured from one *E. stokesii* with a single chronic infection [$\bar{X}$ = arithmetic mean; SD: standard deviation].

Type of blood parasite	Length of chronic stage in erythrocyte		Width of chronic stage in erythrocyte	
	$\bar{X}$	SD	$\bar{X}$	SD
Type 1	8.808	0.411	4.492	0.335
Type 2	10.68	0.519	4.508	0.623
Type 3	10.192	0.429	2.656	0.318
Type 4	9.556	0.524	5.308	0.446

Table 8. One way analysis of variance (ANOVA) of the length and the width in µm between the four haemogregarines from within red blood cells of four single infected *E. stokesii* [df.: degrees of freedom; F: variance-ratio distribution; Sig.: significance].

Feature		Sum of Squares	df.	Mean Square	F	Sig.
Length	Between Groups	49.283	3	16.428	73.221	**0.00**
	Within Groups	21.538	96	0.224		
	Total	70.822	99			
Width	Between Groups	94.625	3	31.542	157.571	**0.00**
	Within Groups	19.217	96	0.200		
	Total	113.842	99			

Table 9. Post-hoc (Tukey) analysis of the variances of the length of the erythrocytic stage between the four types of blood parasites in chronic infections of *E. stokesii* [SD: standard deviation; Sig.: significance].

Type of blood parasite		Type of blood parasite	Mean Difference	SD	Sig. (2-tailed)
1	&	2	-1.872	0.134	**0.000**
1	&	3	-1.384	0.134	**0.000**
1	&	4	-0.748	0.134	**0.000**
2	&	3	0.488	0.134	**0.002**
2	&	4	1.124	0.134	**0.000**
3	&	4	0.636	0.134	**0.000**

Table 10. Post-hoc (Tukey) analysis of the variances of the <u>width</u> of the erythrocytic stage between the four types of blood parasites in chronic infections of *E. stokesii* [SD: standard deviation; Sig.: significance].

Type of blood parasite		Type of blood parasite	Mean Difference	SD	Sig. (2-tailed)
1	&	2	-0.016	0.127	0.999
1	&	3	1.836	0.127	**0.000**
1	&	4	-0.816	0.127	**0.000**
2	&	3	1.852	0.127	**0.000**
2	&	4	-0.8	0.127	**0.000**
3	&	4	-2.652	0.127	**0.000**

Table 11. Mean length and width [µm] of red blood cells (RBC's) and RBC nuclei (Nucl.) compared between infected [n=25] and uninfected RBC's [n=25] of the same lizard individual for four lizard hosts with different single infections. Significantly different features in bold [*E. s.*: *E. stokesii*; $\bar{X}$: arithmetic mean; SD: standard deviation; t: paired t-test value; Sig.: significance].

			Infected		Uninfected			
E. s.	Infection	Feature	$\bar{X}$	SD	$\bar{X}$	SD	t	Sig. (2-tailed)
620	Type 1	RBC length	17.5	1.27	17.8	1.40	0.32	0.75
		RBC width	9.9	0.59	10.1	0.62	1.42	0.17
		Nucl. length	6.5	0.71	6.5	0.64	-0.93	0.36
		Nucl. width	4.0	0.63	4.1	0.37	-1.11	0.28
112	Type 2	RBC length	17.1	1.10	16.5	1.32	-1.89	0.07
		RBC width	8.2	0.57	7.9	0.48	-2.31	**0.03**
		Nucl. length	6.2	0.74	6.3	0.98	0.50	0.62
		Nucl. width	3.1	0.35	3.4	0.36	2.62	**0.01**
25	Type 3	RBC length	16.9	0.92	16.7	1.56	-0.71	0.49
		RBC width	9.4	0.60	8.8	0.84	-3.53	**0.00**
		Nucl. length	6.9	0.42	6.6	1.07	-1.21	0.24
		Nucl. width	2.8	0.38	3.2	0.55	2.29	**0.03**
272	Type 4	RBC length	17.1	1.17	17.1	1.09	-0.01	0.99
		RBC width	9.1	0.97	9.3	0.69	0.80	0.43
		Nucl. length	5.6	0.76	6.4	0.73	3.96	**0.00**
		Nucl. width	3.7	0.64	3.8	0.26	0.76	0.45

5.4 Discussion

The data presented in this chapter suggest the presence of four different species of haemogregarines in *E. stokesii.* Because the number of haemogregarine species was assessed only by the morphological comparison of chronic stages as they appeared in giemsa stained blood smears and their visible effect on lizard-host erythrocytes, further analysis was necessary to confirm those results. The following taxonomic investigations relied on several methods including transmission experiments, histological examinations and a molecular biological study. The histological results and the results of the transmission experiments are given within the taxonomic description of each blood parasite. The results of the molecular biological study are presented separately as chapter eleven. The order in which the results are given for each species follows the life cycle of the micro-parasites, starting from the stages found in *E. stokesii* and continuing as far as it was elucidated.

Chapter Six

6. *HEMOLIVIA MARIAE* (TYPE 1)

6.1 Introduction

This chapter describes the haemogregarine parasite described as type 1 in chapter five. The first description of the adeleorine *Hemolivia mariae* (Coccidia: Karyolysidae) came from South Australian populations of the sleepy lizard, *Tiliqua rugosa* (Reptilia: Scincidae) (Smallridge, 1998). Apart from the hosts, the life cycle described for *Hem. mariae* was identical to the life cycle of *Hem. stellata* (Figure 3, p. 16), as far as both were investigated. *Hem. mariae* was described to be transmitted by ingestion of the reptile tick species *Amb. limbatum* and *Aponomma hydrosauri.* Mt. Mary, where *Hem. mariae* was discovered, lies about 250 km south-east of the closest site investigated for this thesis project (Figure 129, chapter fourteen). In the present study, no significant differences were detected between the previously described *Hem. mariae* and one of the blood parasites frequently infecting *E. stokesii.* Thus it was concluded that they belong to the same adeleorine species.

In the following, *Hem. mariae* was described from *E. stokesii* and *Amb. limbatum. Hem. mariae* was also found to develop in *Amb. vikirri* ticks. No differences were found between the development of *Hem. mariae* in *Amb. limbatum* and *Amb. vikirri.* Vector experiments with the sand fly *Australophlebotomus dycii* or the mosquitoes *Culex quinquefasciatus* and *Aedes notoscriptus* were not conducted. Encapsulated gamonts of *Hem. mariae* were found in abundance in two *A. dycii* on one of the regular study sites (Table 39, p. 181) but no evidence of development of *Hem. mariae* was found in any other dissections of *A. dycii.*

In order to confirm that one of the coccidian blood parasites found regularly in blood smears of *E. stokesii* at field sites in the Flinders Ranges was *Hem. mariae,* five transmission experiments were conducted. Transmission from lizard to lizard was accomplished by force feeding infected *Amb. limbatum* ticks to the lizards. The experiments are described in detail later, but are summarised below.

1. In experiment I, *Hem. mariae* was successfully transmitted from two infected *T. rugosa*[4] from the Mt. Mary region to two *E. stokesii* that were born and raised in the ACU. The presence of *Hem. mariae* was documented by the appearance of *Hem. mariae* gamonts in giemsa stained blood smears of the newly infected lizards.
2. In experiment II, parasites believed to be *Hem. mariae* infecting two *E. stokesii* from field sites in the Flinders Ranges were successfully transmitted to two *E. stokesii* born and raised in the ACU. The successful transmission was documented by the appearance of gamonts that belonged to the parasite suspected to be *Hem. mariae* in the blood of the newly infected lizards. One of the newly infected lizards was killed and dissected shortly after the onset of the blood infection and various tissues were prepared for histological examinations by LM and TEM.
3. In experiment III, the development and the time course of infections in *Amb. limbatum* were evaluated by a series of dissections of **xenodiagnostic** ticks that had fed on *E. stokesii* infected with the parasite believed to be *Hem. mariae.* Most dissected ticks were monitored for parasitic life cycle stages in a wet mount with LM, but two ticks were examined histologically by TEM.
4. In experiment IV, the trans-stadial transmission of *Hem. mariae* from larval to adult *Amb. limbatum* was demonstrated by examining adult *Amb. limbatum* that had fed as larvae on infected *E. stokesii* and as nymphs on uninfected *E. stokesii.* Dissected unfed adults were examined for parasites in a wet mount with LM.
5. In experiment V, the sites of development of *Hem. mariae* in engorged female *Amb. limbatum* were investigated. Results could only be obtained from two engorged female ticks that had fed on a lizard with a mixed blood infection of *Hem. mariae* and another adeleorine parasite, *Hemolivia biplicata* n. sp. (see chapter seven).

In addition to these five experiments, several batches of *Amb. limbatum* and *Amb. vikirri* larvae were attached to lizards infected with *Hem. mariae* or with mixed infections during the course of this thesis project (see chapter twelve and thirteen). Some of those ticks were always dissected to search for coccidian developmental stages. In case where images in this section were obtained from a tick outside of experiment I-V, a brief summary of their life history will be given. The life history of the ticks and lizards used in experiment I-V will be presented in detail with each experiment. Finally, the developmental sequence and the

[4] Kindly supplied by Dr. Cathy Smallridge.

morphology of the life cycle stages found in all experiments were compared to the data available from *Hem. mariae* infections in *T. rugosa* and *Amb. limbatum.*

6.1.1 The life cycle of *Hem. mariae*

Figure 14 displays the life cycle of *Hem. mariae* in the lizard *E. stokesii* and the tick *Amb. limbatum.* The images were obtained by LM or TEM; the morphology of the life cycle stages are described in more detail in 6.1.1.2. Infective sporozoites (A) enter the lizard host by ingestion of an infected tick and leave their sporocyst to enter host cells. Meronts (B) form in a parasitophorous vacuole in the spleen, liver, heart, lung, testes and throughout the intestinal tract. Numerous meronts can occur in reticulo-endothelial cells (C) attached to the capillary walls. In one extremely high infection, a meront was found in the peripheral blood. Merozoites, which represent the unspecified morphotypes in this life cycle, enter the erythrocytes (D). Many multiply infected RBC's are observable at this stage. The merozoites develop into gamonts and encapsulate in a characteristically shaped sheath (E), which is the chronic stage. A sexual dimorphism does not occur. Gamonts that are taken up by a feeding tick leave their protective capsule and aggregate in the lumen of the tick midgut. The gamonts stretch and formed a syzygy (F), where a slight sexual dimorphism is noticeable. The syzygy was observed inside and outside of the epithelial cells of the tick midgut. The micromeronts divide once to form two micromerozoites (G), one of them enters the macromerozoite and mates. Both, micromerozoites and mating were only observed inside the epithelial cells of the tick midgut. The zygote develops in a parasitophorous vacuole in the epithelial cells of the midgut and undergoes multiple nuclear divisions, where the nuclei move to the periphery (H). Large protrusions develop on the surface of the oocyst, which leads to a star shaped appearance (I). Large oocysts are usually found extracellular, floating in the midgut lumen. Starting from the very tip of the oocyst, sporoblasts (J) are formed that develop into the motile sporokinetes (K). In engorging tick larvae, sporokinetes enter new epithelial cells of the midgut to develop into sporocysts, which eventually form the infective sporozoites (L2). In engorging tick females, the sporokinetes leave the midgut to enter the reproductive tract. Few sporokinetes have been found in the ovary, many developing sporocysts were observed in cuboid epithelial cells of the common oviduct (L1). No infected eggs were noticed.

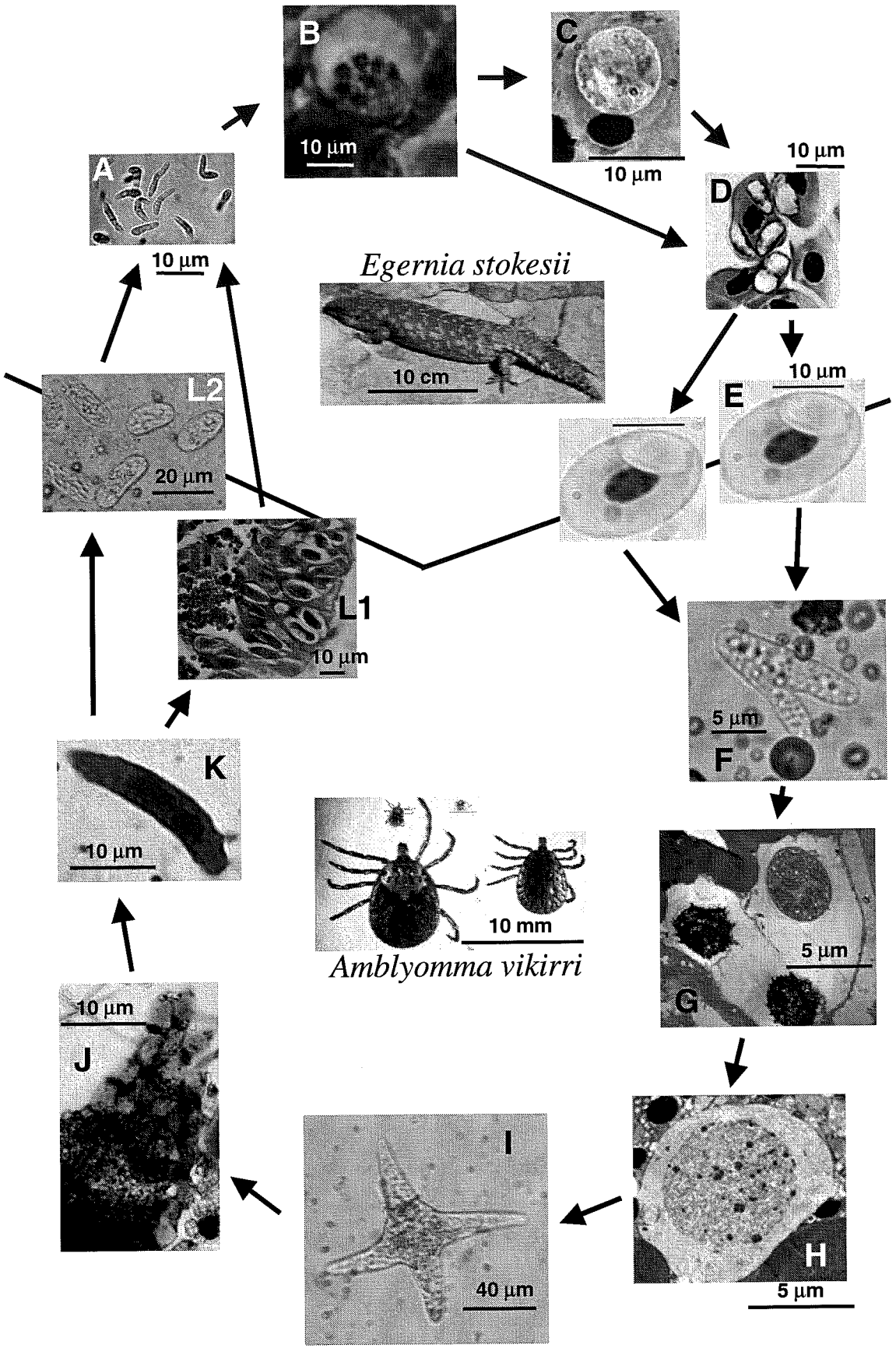

Figure 14. The life cycle of *Hemolivia mariae*, compiled from images produced for this thesis (for explanation see p. 79).

6.1.2 The morphology of *Hem. mariae*

6.1.2.1 The developmental stages in *E. stokesii*

The data presented here were gathered from the dissection and tissue preparations of the experimentally infected *E. stokesii 30*. Meronts were frequently found in the heart, the liver, the lung and in the spleen. Many meronts were exoerythrocytic, some within the lung endothelium, the hepatocytes, macrophages and throughout the lizard spleen tissue. Scattered, encysted, erythrocytic meronts of *Hem. mariae* were found in lizard muscle, urinary bladder, lower small intestine. The histological findings were confirmed by Dr. S. R. Telford Jr., who received copies of the material. In addition, blood samples from the chronically infected *E. stokesii 620* and 1377 were examined with TEM. Apart from the encapsulated gamonts and their host cells, only the maximum and minimum size measured from all stages were presented, because the taxonomic value of these data was expected to be limited.

The size of meronts (Figure 15/A&B) was variable, the diameter ranging from 5.5-6.5 μm in round forms to 4.5-12 μm in ovoid forms. A parasitophorous vacuole (Figure 15/B) was not always present but where present, measured between 7-9 μm in diameter. Mature meronts contained between 6-11 merozoites; the merozoites were always densely packed side by side.

The shape of young gamonts in giemsa stained blood (Figure 73) was either:

A. short and straight,
B. elongated and curved or
C. ovoid to round.

TEM images of young gamonts are shown in Figure 16. The size of the early blood stages was highly variable and ranged between 5-11 μm in length and 2-8 μm in width. The nucleus was positioned terminally or centrally. In a giemsa stain, the chromatin was scattered throughout parts of the parasite cytoplasm, but sometimes it appeared condensed to a granular mass. The ultrastructure of the early blood stages revealed the typical apicomplexan features such as the apical complex, rhoptries, micronemes and microtubules (Figure 16/E). Chromatin was often found dispersed within the nucleus. The three dimensional structure of the parasite was rod-, banana-, bean- or egg-shaped. Two double layered sets of membranes were observed, an inner- or plasma membrane and an outer membrane (Figure 16/E). During the process of encapsulation, the parasite shrank considerably in size and showed membranous folds (Figure 21/ B) that disappeared with the development of the protective sheath. In giemsa stained blood smears, the terminal nucleus of the consistently oval,

encapsulated gamont, was sometimes stained slightly blue, but in most cases the capsule was impenetrable for the stain. At this stage, the parasite was found most often at one end of the red blood cell, slanting diagonally from one end of the host cell to one of its sides (Figure 14/E). The host cell nucleus was not displaced and only in a few cases it was stained slightly darker than in uninfected cells. The TEM-results showed the three dimensional structure of encapsulated *Hem. mariae* gamonts to be an elongated, relatively thin, oval platelet. Lateral-width[5] (Figure 17/A) and lateral-length sections (Figure 17/C) showed that the platelet had a narrow centre and a wider rim. Two double layered sets of membranes were visible, the plasma membrane and an outer membrane (Figure 17/B), separated by a brittle cyst wall. A peculiar ornamentation, created by a protuberance of the cyst wall, completely girdled the gamont lateral-horizontally. An approximately 20 nanometer (nm) wide and 100 nm long structure interpreted by the author as a micro-canal was regularly found permeating the protuberance (Figure 17/B).

6.1.2.2 The developmental stages in *Amb. limbatum*

Numerous gamonts freed from their protective cyst were observed in wet mounts of freshly engorged tick larvae (Figure 24/A). Free gamonts were 8-14 µm long and 1-4 µm wide. Some gamonts were already forming a syzygy. TEM images show a common membrane formed around both parasites during syzygy (Figure 18/A); two flagellae were apparent inside this membrane. On the TEM images, syzygy was only observed within the midgut epithelium of ticks. During the final phase of gametogenesis, two round microgametocytes (diameter approximately 3-4 µm) and one ovoid or round macrogametocyte (diameter approximately 4-5 µm) were formed. The ultrastructure of the microgametocytes differed greatly from that of the macrogametocytes. The latter was relatively large in diameter, had a smooth outer layer and contained many lipid and amylopectin inclusions, whereas the macrogametocytes were relatively small, had a rippled appearance and stained almost black in the TEM preparations (Figure 19/A). Stages believed to be solitary macrogametocytes featured an array of plasma folds and microtubules (Figure 19/B). Uninucleate round or ovoid zygotes (8-15 µm) developed in a parasitophorous vacuole (Figure 18/B) and were found at the base of digestive epithelial cells. Early oocysts (Figure 18/C) contained multiple nuclei advancing to its periphery. Late oocysts grew very large and developed 3-6 arms that protruded into all three spatial dimensions and gave the oocyst a star shaped appearance

[5] For an explanation of the terminology used here see 5.2.5.4.

(Figure 20/B,C,&G). In rare cases, only two fat arms would grow in opposite directions resulting in an oocyst of an elongated diamond shape resembling a pickaxe-head. One arm of a star could measure up to 180 μm in length and 11 μm in width. The widest stars observed measured more than 350 μm over all. Large numbers of star shaped oocysts were observed floating inside the midgut lumen (Figure 20/E). Starting from the tip of the arms, maturing oocysts formed sporoblasts that developed within the oocyst into motile sporokinetes (Figure 20/D). The elongated, uninuclear sporokinetes contained 1-2 crystalloid bodies (Figure 20/F), and the former presence of an apical complex was indicated by the conoid depicted in Figure 21/A. Free sporokinetes were observed in the lumen of the midgut. Movement could only be observed when the ticks were dissected in ringer solution, distilled or tap water bloated the sporokinetes and no movement could be observed. The size of sporokinetes varied between 18-33 μm in length and 3-5 μm in width. Sporokinetes developing into sporocysts were found only intracellularly. Inside the sporocyst (length 15-18 μm, width 7-9 μm) (Figure 20/F&I), slim uninucleate sporozoites containing one or two crystalloid bodies were formed (Figure 22/A). The site of the sporocyst development depended on the instar of the tick vector, with sporocysts in engorging larvae and nymphs developing in the midgut epithelium and sporocysts in engorging females developing exclusively in the reproductive tract. In heavily infected unfed nymphs, sporocysts could fill almost the entire midgut lumen (Figure 23/B). Some sporocysts were seen inside a fine hull. In engorged females, sporokinetes were found in the ovary, but developing sporocysts were seen in the lumen of the common oviduct and packed densely in the cuboid cells in the inner epithelium of the area where the two oviducts merge, specified in the literature as the "common oviduct" (Roshdy, 1969) (Figure 24/C&D). Infected eggs were not observed, but due to their shell and their brittle yolk mass they were not easy to investigate histologically. The infective motile sporozoites (Figure 14/A) were variable in size (16-27 μm x 1-2 μm) and were only observed free when sporocysts were squashed during tick dissections. The firm sporocyst wall proved to be impenetrable for giemsa stain and sporozoite measurements were only taken from burst sporocysts.

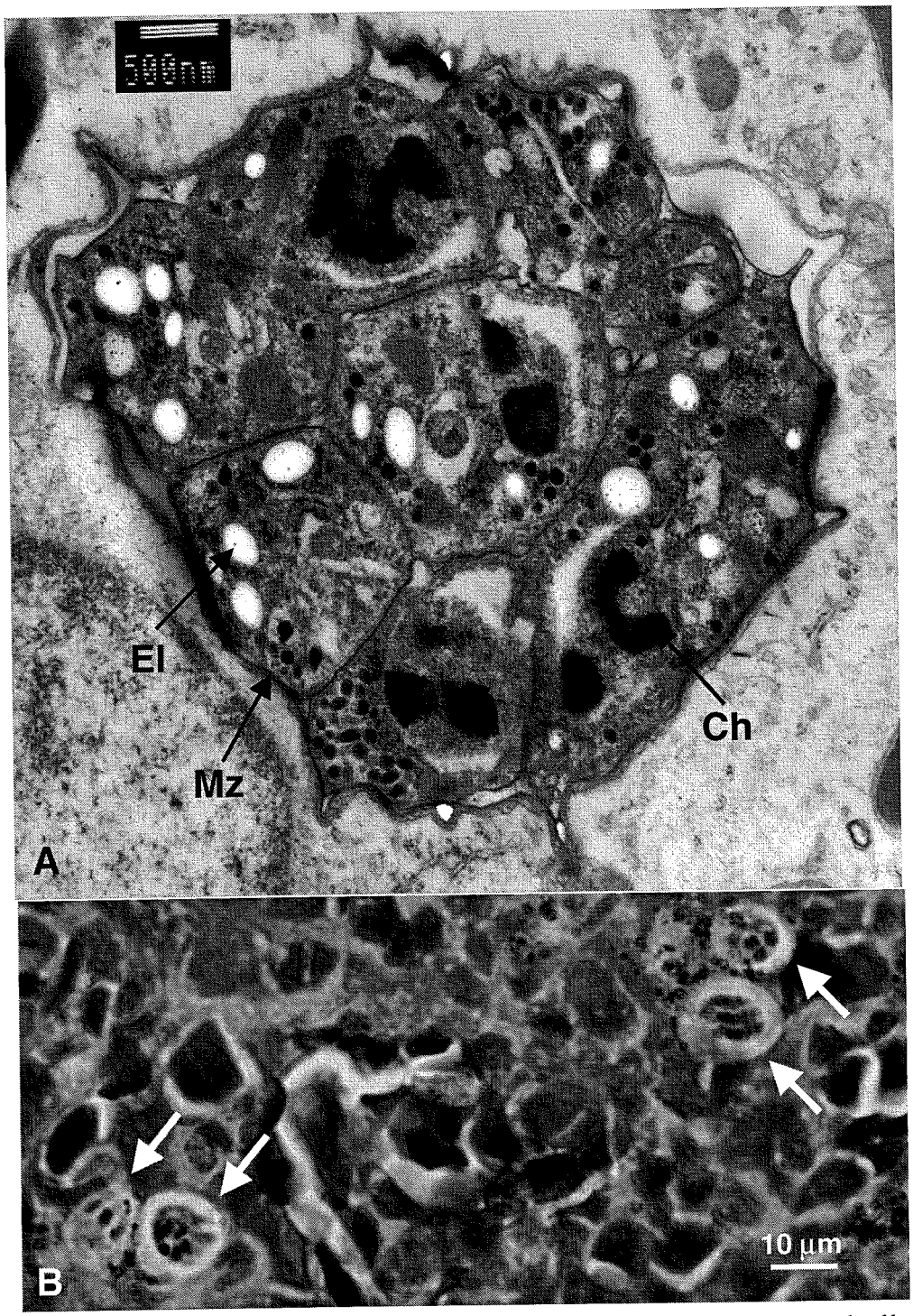

Figure 15. A+B: meronts of *Hemolivia mariae* in the spleen of *E. stokesii*. A: mature meront, containing eight merozoites (TEM) [Ch: chromatin, El: electron lucid body, Mz: merozoite]. B: white arrows mark meronts in spleen tissue (LM, giemsa, b&w).

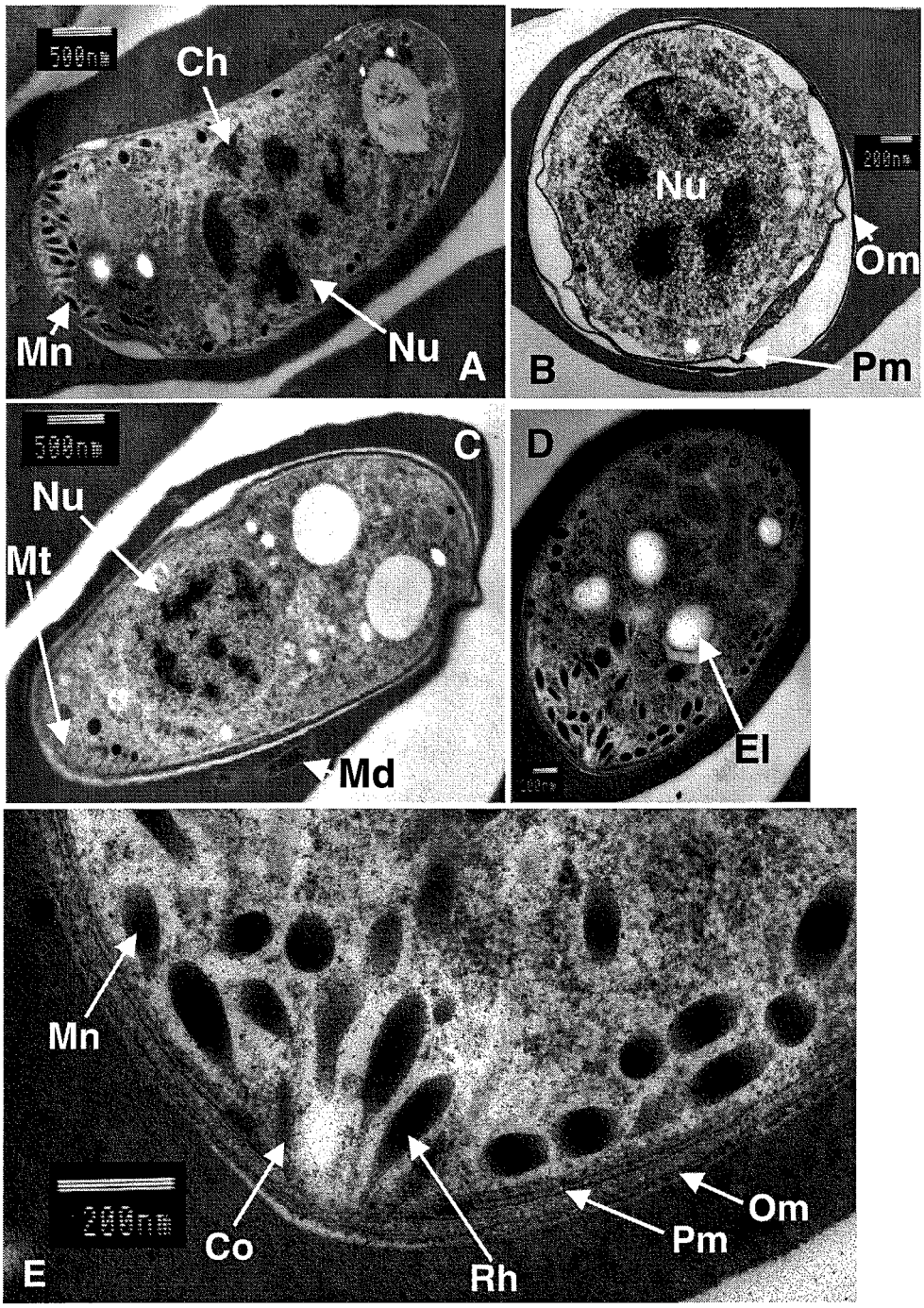

Figure 16. A-E: young gamonts of *Hem. mariae* in erythrocytes of *E. stokesii* (TEM; A, C, D: dorsal, B: lateral-width, E: magnification of D) [Ch: chromatin, Co: conoid, El: electron lucid body, Md: host mitochondrion, Mn: microneme, Mt: microtubule, Nu: nucleus, Om: outer membrane, Pm: plasma membrane, Rh: rhoptries].

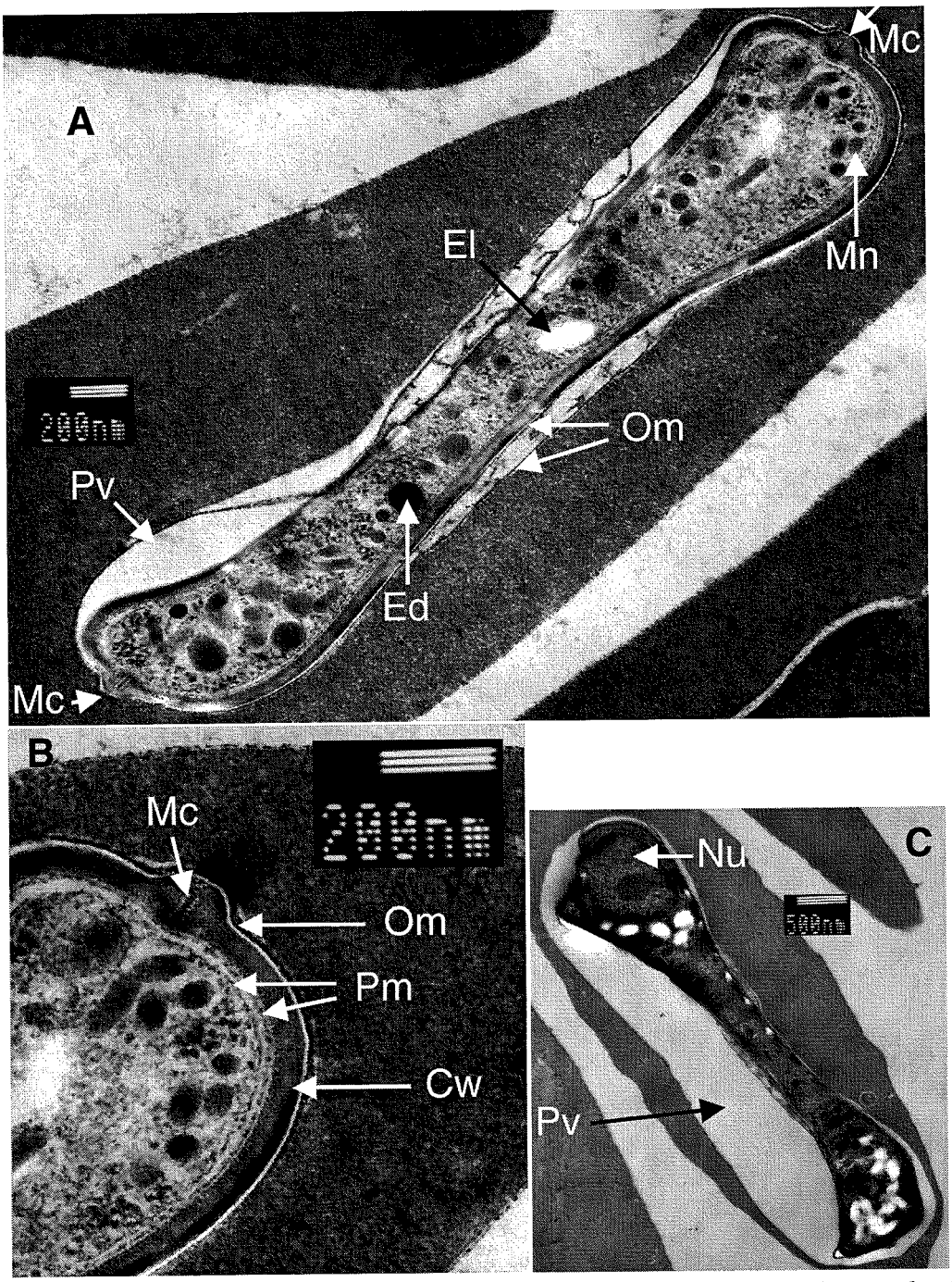

Figure 17. A-C: mature gamonts of *Hem. mariae* in erythrocytes of *E. stokesii* (TEM, lateral-width); B: magnification of A; C: lateral-lenght [Ed: electron dense body, El: electron lucid body, Mc: micro-canal, Mn: microneme, Nu: nucleus, Om: outer membrane, Pm: plasma membrane, Pv: parasitophorous vacuole].

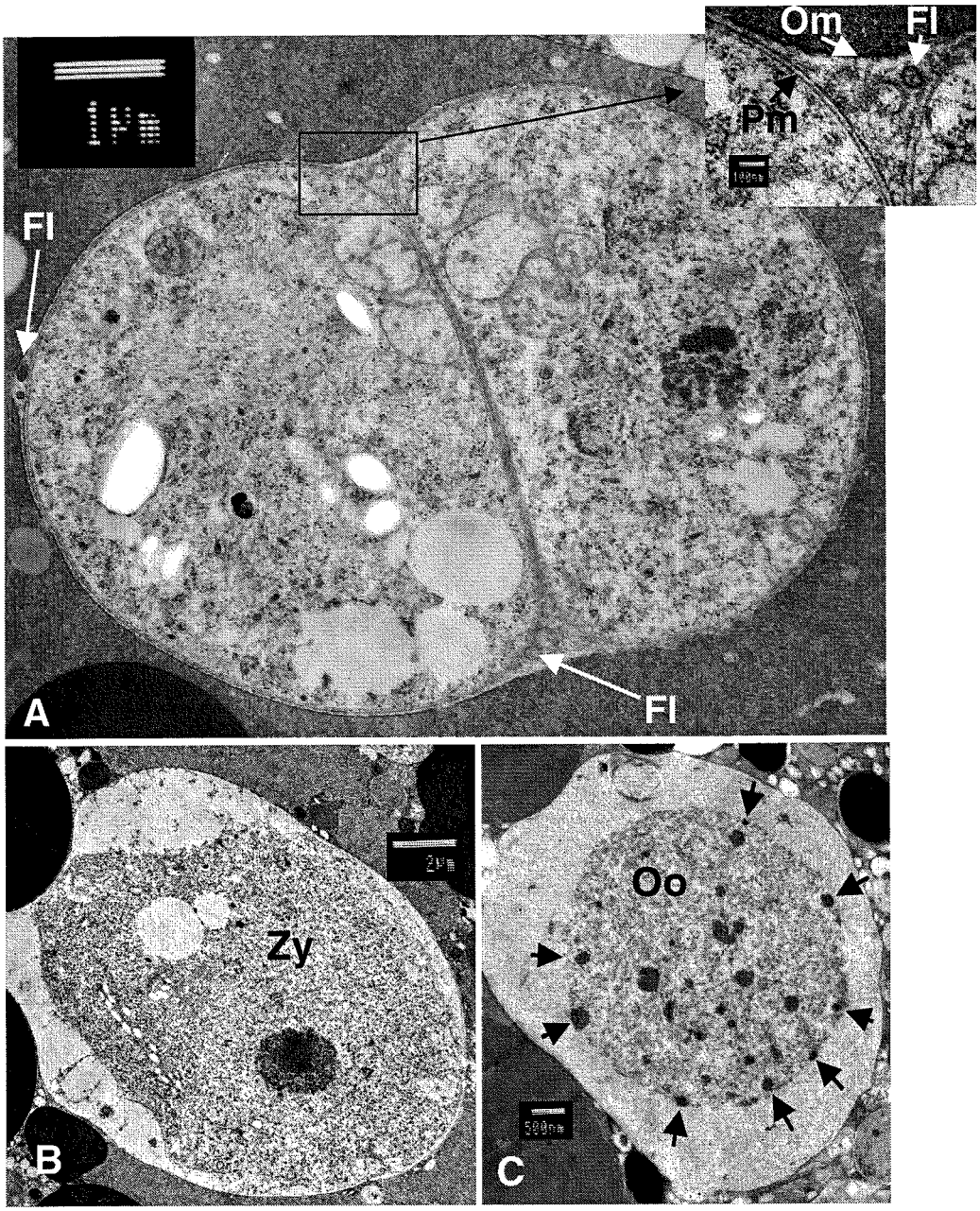

Figure 18. A-C: *Hem. mariae* developing in the midgut of engorged *A. limbatum* larva (TEM) [Fl: flagellum, Om: outer membrane, Oo: oocyst, Pm: Plasma membrane, Zy: zygote]. A: syzygy in midgut lumen; B: zygote in midgut epithelium; C: young oocyst, arrows mark marginal nuclei.

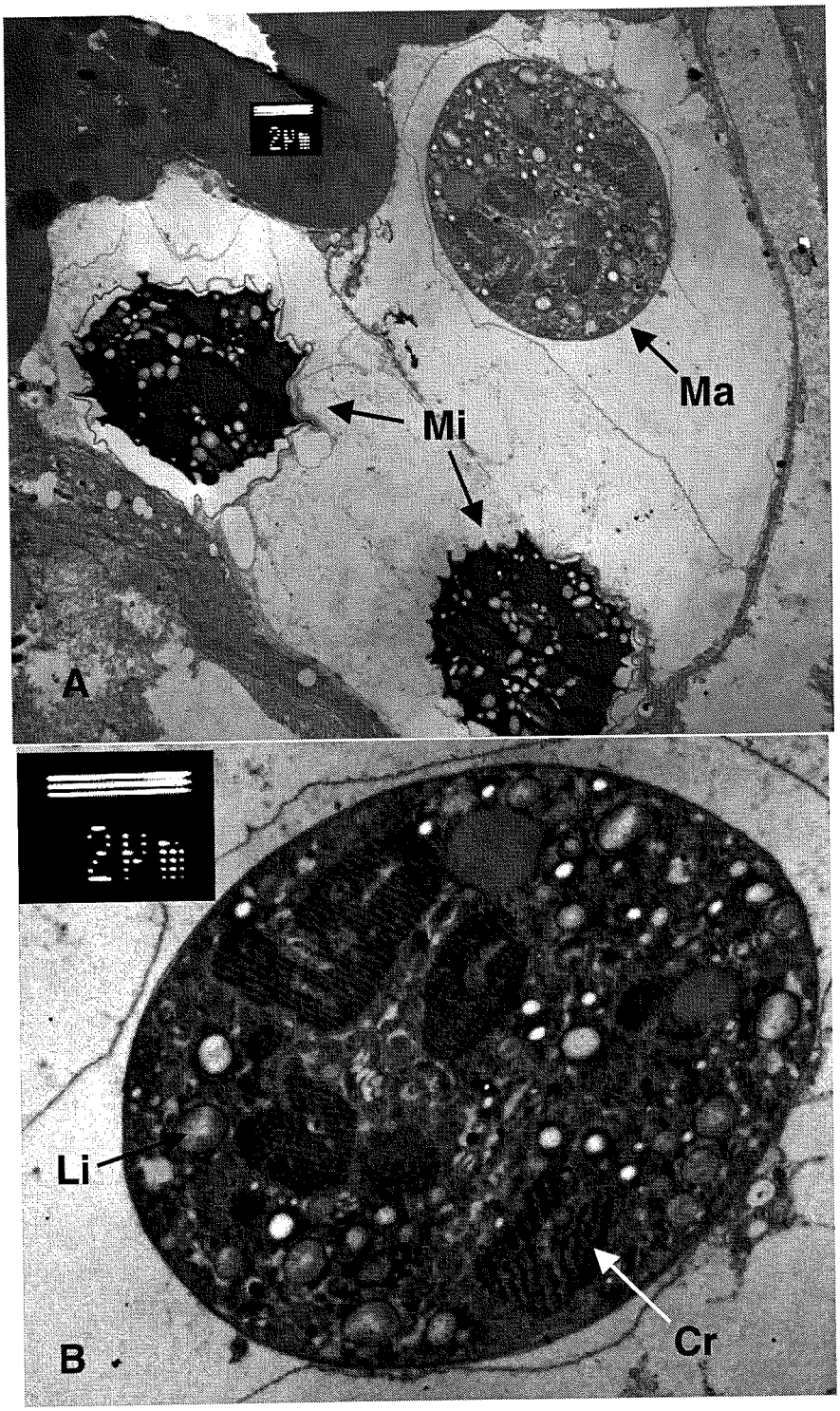

Figure 19. A+B: syngamy of *Hem. mariae* in the midgut epithelium of engorged *Amb. limbatum* larva (TEM)[Cr: chromatin, Li: lipid body, Ma: macrogametocyte, Mi: microgametocyte]. B: macrogametocyte, partial magnification of A.

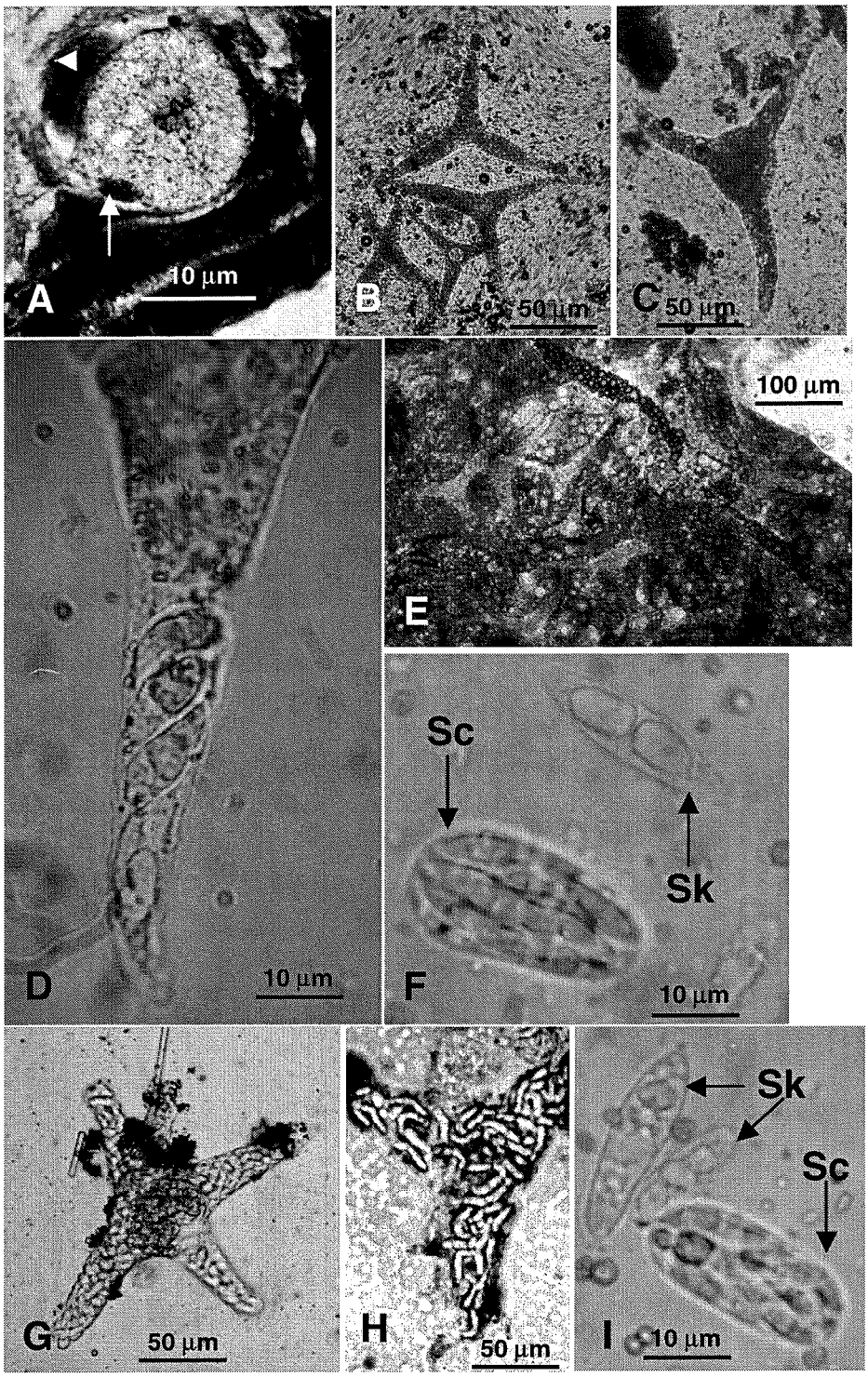

Figure 20. A-I: developmental stages of *Hem. mariae* in the midgut of *A.limbatum*(A: section of midgut epithelium, LM, giemsa, b&w; B-I: midgut lumen, LM, wet mount) [Sc: sporocyst, Sk: sporokinete]. A: mating in the midgut epithelium, arrows mark microgametocytes; B&C: immature oocysts; D: oocyst with developing sporokinetes; E: midgut lumen filled with oocysts; F&I: sporokinetes and sporocysts containing sporozoites; G: maturing oocyst. H: mature oocyst containing sporokinetes.

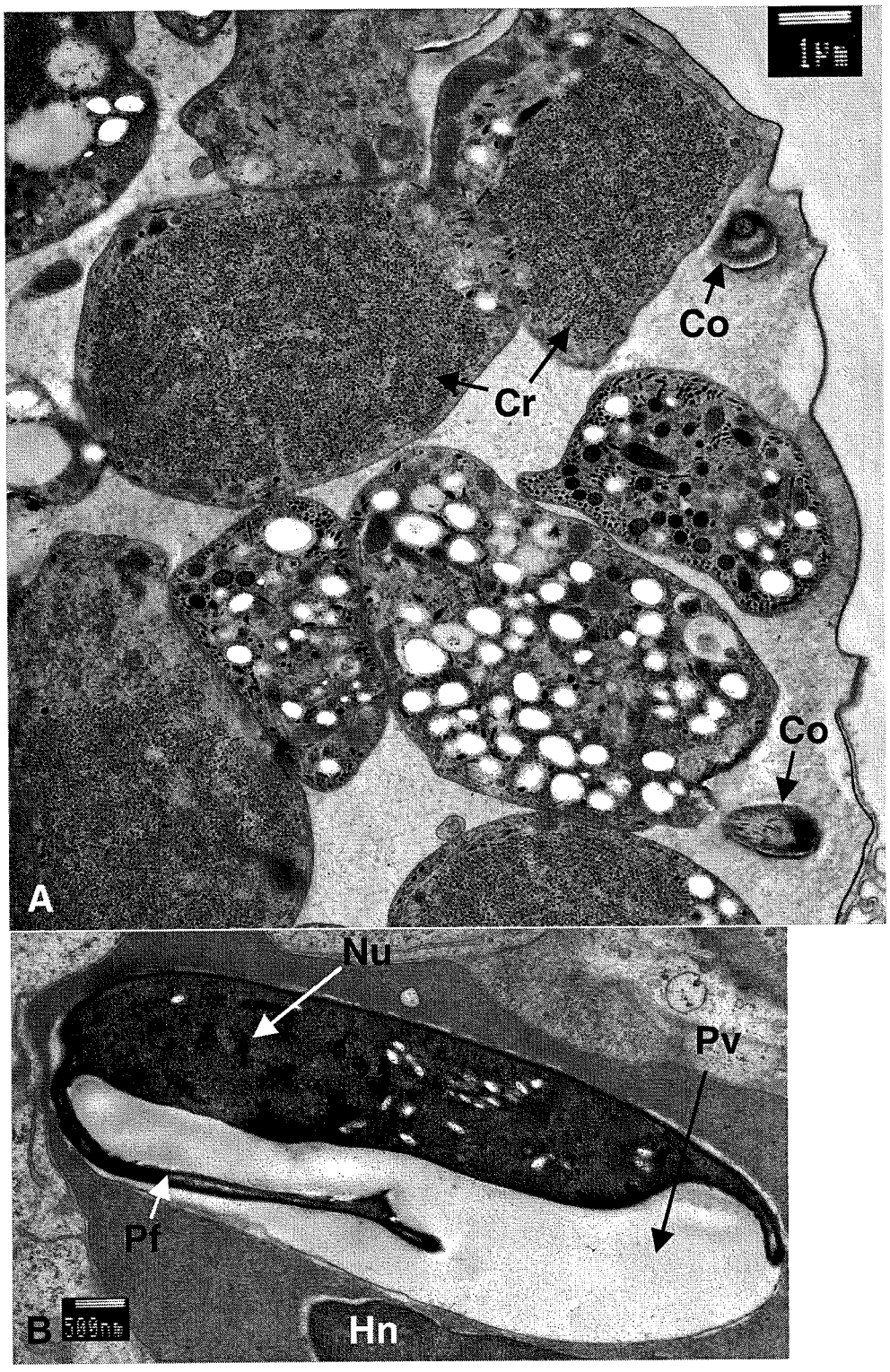

Figure 21. A&B: Life cycle stages of *Hem. mariae* (TEM) [Co: conoid, Cr: crystalloid body, Nu: nucleus, Pf: plasma-membrane fold, Pv: parasitophorous vacuole]. A: maturing oocyst containing sporokinetes from the midgut lumen of *Amb. limbatum*. B: encapsulating gamont (dorsal) in hepatic erythrocyte of *E. stokesii*.

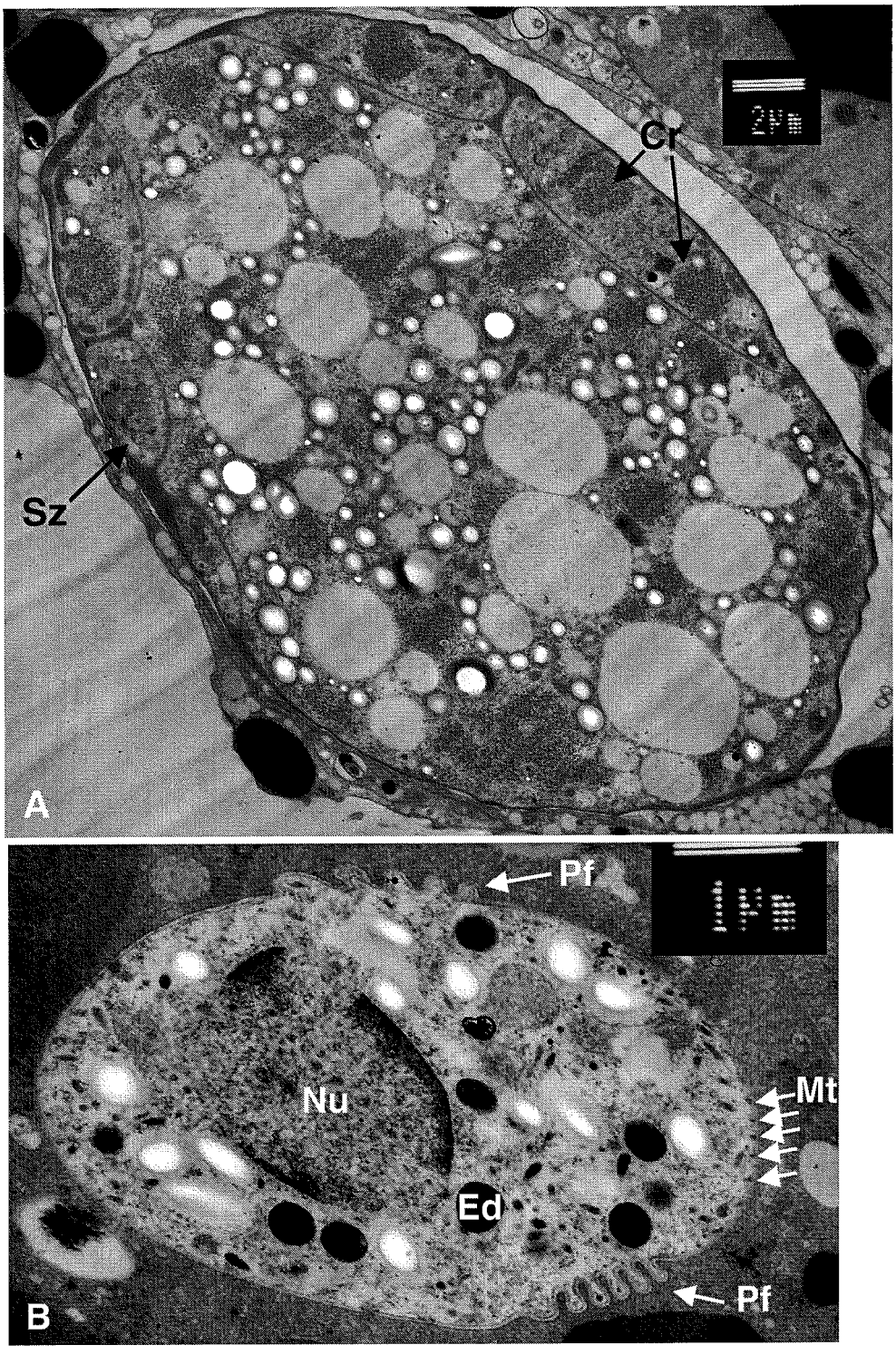

Figure 22. A&B: Life cycle stages of *Hem. mariae* in *Amb. limbatum* (TEM) [Cr: crystalloid body, Ed: electron dense body, Mt: microtubules, Nu: nucleus, Pf: plasma-membrane fold, Sz: sporozoite]. A: maturing sporocyst containing sporozoites from the midgut epithelium of a freshly molted *Amb. limbatum* nymph; B: solitary macrogametocyte with peculiar plasma-membrane folds.

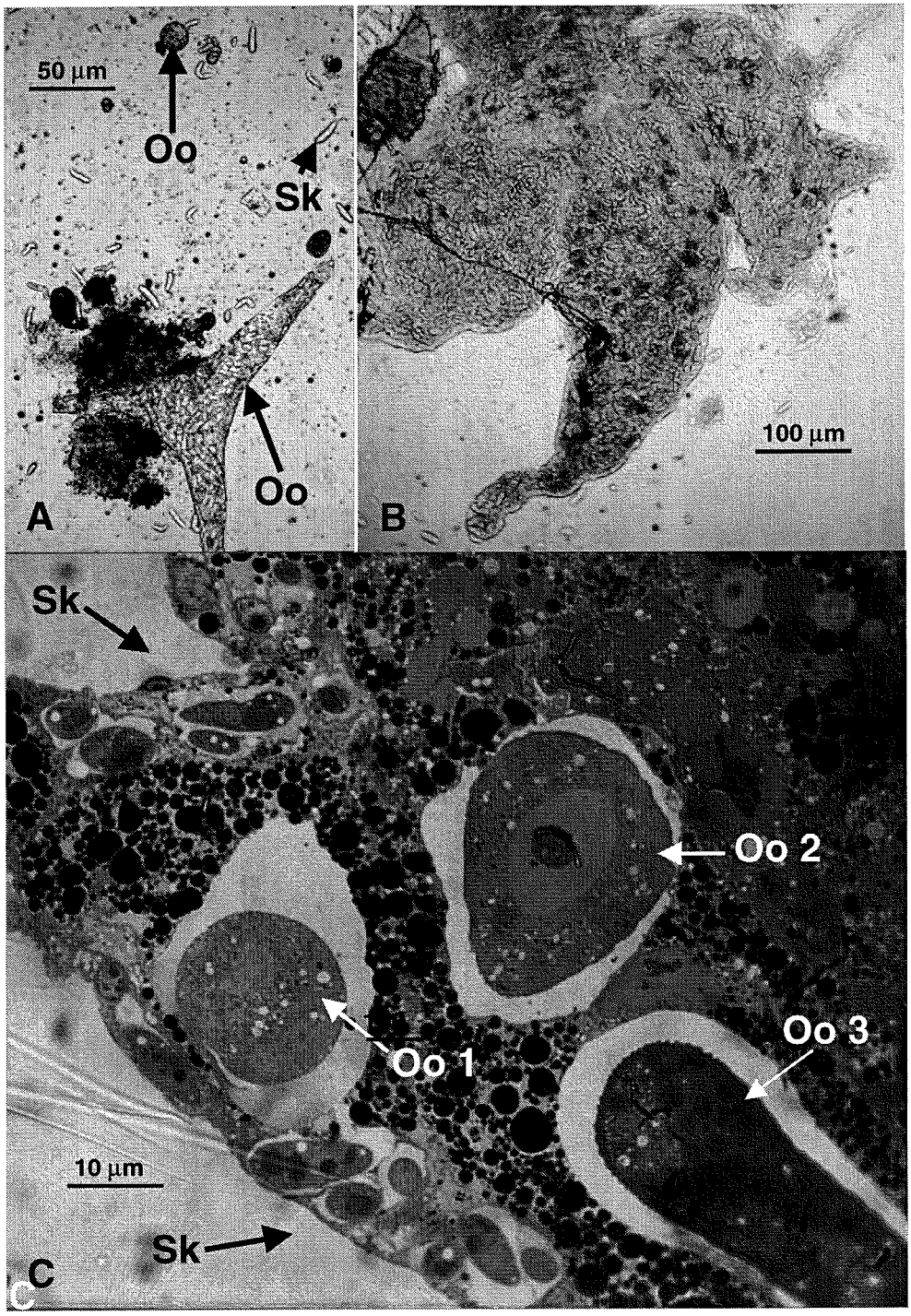

Figure 23. A-C: midgut preparations of unengorged *Amb. limbatum* nymphs with mixed infection of *Hem. mariae* and *Hem. biplicata* (A&B: wet mount, LM; C: semi-thin TEM section, LM, toluene blue) [Oo: oocyst, Sk: sporokinete]. A: star shaped oocyst of *Hem. mariae* and spherical, thickly encysted oocyst of *Hem. biplicata* from midgut lumen; B: midgut lumen filled with sporocysts; C: developing oocycts and sporokinetes in midgut epithelium.

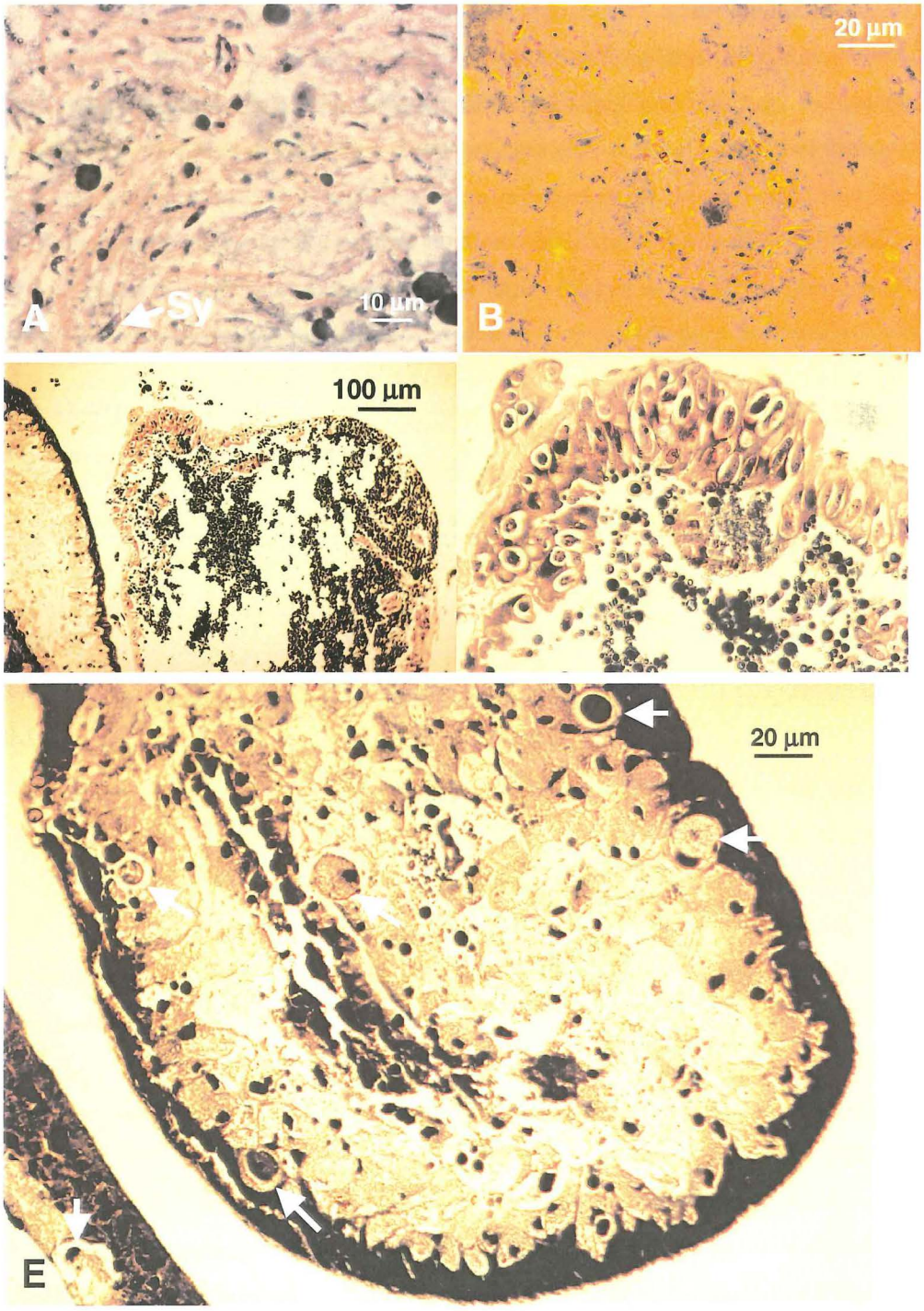

Figure 24. A-E: histological sections of two engorged female *Amb. limbatum*, infected with both, *Hem. mariae* and *Hem. biplicata* (LM, giemsa) [Sy: syzygy]. A: freshly engorged female, syzygy; B: freshly engorged female, aggregation of gamonts; C&D: egg laying female, developing sporocysts in cuboid cells of common oviduct (D: enlargement of C); E: egg laying female, developing oocysts.

6.1.3 The localities where *Hem. mariae* was found

All localities in South Australia where *E. stokesii* infected with *Hem. mariae* were found during this thesis project are listed in Table 12 (for a comparative analysis of the spatial distribution of the blood parasite species found see chapter fourteen).

Table 1. The coordinates of the sites where *Hem. mariae* was found infecting *E. stokesii* during the field survey (regular study sites in bold).

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [Σ] with *H. mariae* and its prevalence [%]	
	S	W		Σ	%
Camel Hill (CH)	**31°53'**	**138°25'**	**148**	*	**0.9**
Chace Range	31°45'	138°35'	7	1	14.3
Drakes Nob (DN)	**32°05'**	**138°09'**	**27**	*	**24.4**
Gawler Ranges[1]	32°30'	136°50'	12	6	50.0
Genlyle (GL)	**31°50'**	**138°35'**	**62**	*	**32.1**
Hawker North (HN)	**31°53'**	**138°25'**	**97**	*	**15.0**
Hawker South (HS)	**31°53'**	**138°25'**	**159**	*	**19.8**
Island Lagoon	31°42'	136°68'	14	13	92.9
Jarvis Hill	32°55'	138°21'	10	2	20.0
Mernamerna	31°45'	138°22'	2	1	50.0
Neuroodla (NR)	**31°50'**	**138°08'**	**68**	*	**44.9**
Partacoona	32°00'	138°10'	6	1	16.7
Pichi Richi	32°20'	138°00'	27	2	7.4
Point Lowly[1]	32°50'	137°45'	8	3	37.5
Quorn	32°15'	138°05'	8	1	12.5
The Four Mile	31°50'	138°30"	1	1	100.0
Willow Springs	31°25'	138°50'	4	2	50.0
Yapalla	31°50'	138°20"	4	1	25.0

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

* = Data of *E. stokesii* individuals were pooled over three years (for details see chapter fourteen)

6.1.4 The transmission experiments

6.1.4.1 Experiment I: transmission from *T. rugosa* to *E. stokesii*

The *Amb. limbatum* nymphs used in this transmission experiment had been fed as larvae on *T. rugosa* that were uninfected with blood parasites (Dr. C. Smallridge, pers. comm.). The nymphs were fed on two *T. rugosa* that were captured at Mt. Mary, South Australia. The lizards were diagnosed as infected only by *Hem. mariae* by Dr. Smallridge. The two *T. rugosa* were held in the ACU and were regularly monitored for blood parasites by Dr. Smallridge until the ticks for the transmission experiment were attached in September 1997 and December 1997 respectively (Table 13). The nymphs fed, detached and moulted to adults and were held in the adult stage for several months. One male and one female *Amb. limbatum* were force fed to each of two *E. stokesii* on the 23/11/98. Body fluids flowing out of the ticks due to the deliberate laceration of the cuticle (see 5.2.2.2) were examined for parasitic stages. In three out of the four adults, the fluids were found to be infected with *Hem. mariae* sporocysts that contained the infective sporozoites (Table 13).

The two *E. stokesii* used in this experiment, *101* and *102,* were born in the ACU in March 1996. Their blood was monitored at six to 10 week intervals up to the start of the experiment. Approximately five months after they were born in the ACU, blood from both these *E. stokesii* was found to be infected with *Schellackia* sp. (see chapter nine) and the lizards stayed infected at a low parasitemia until the transmission experiment began (Table 14; Figure 25 & Figure 26). Unspecified morphotypes were found in low frequencies typical for *Schellackia* sp. (for details on the occurrence of unspecified morphotypes in infections with *Schellackia* sp. see chapter nine).

After ingesting the ticks, the lizards were bled each week for eight weeks, and then at 2-6 week intervals until the experiment was terminated (Table 14). Five weeks after the ticks were ingested, the first blood parasite stages that could be reliably identified as the encapsulated gamonts of *Hem. mariae* were detected in blood smears of both lizards. One week before that, there was a sudden increase of unspecified morphotypes that continued throughout the early period of the blood infection (Figure 27 & Figure 28). Figure 29 and Figure 30 show the abundance of the morphotypes A, B, and C as outlined in Figure 73, for both lizards during the transmission experiment. In both cases, the peak of abundance of the unspecified morphotypes consists mainly of the type A. After its peak, type A rapidly decreased in numbers and almost completely disappeared out of the blood about eight

weeks after the beginning of the experiment. The broader and less abundant type B were found in the blood about a week longer than type A. The round type C was relatively uncommon. Towards the end of the experiment, mainly encapsulated gamonts of *Hem. mariae* were found in the blood cells. Encapsulated sporozoites of *Schellackia* sp. were occasionally detected throughout the blood infection with *Hem. mariae*, but their numbers were relatively low when compared to the time before the transmission experiment (Table 14). The results described here show that *Hem. mariae* infecting *T. rugosa* from the Mt. Mary region can be transmitted by *Amb. limbatum* to *E. stokesii*. They also suggest that the unspecified morphotypes of *Hem. mariae* are merozoites, which later encapsulate and form the gamonts, labelled as the chronic type 1 in chapter five.

Table 13. Cross transmission of *Hem. mariae* from *T. rugosa* to *E. stokesii* by force feeding infected adult *Amb. limbatum*.

Lizard host the tick nymphs were fed on	Parasitemia in 10^4 RBC's of lizards at the time of tick engorgement	Date of nymphal blood meal	Lizard host resulting tick adults were fed to	Number and sex of tick adults fed to lizard	Adult body-fluid residue found to be infected
T. r. 4517	15	10/97	*E. s. 101*	1♀+ 1♂	+/+
T. r. 4522	9	12/97	*E. s. 102*	1♀+ 1♂	-/+

Table 14. Blood smear counts of the infections of the *E. stokesii 101* and *102* as parasite stages per 10^4 RBC's. Ticks were fed to both lizards on the 23/11/98. The cross transmission experiment was shaded for its duration. (A,B,C: unspecified morphotypes A,B,C; U. m.: sum of unspecified morphotypes; *S.* sp.: *Schellackia* sp.; *H. m.*: *Hem. mariae*)

Date of blood smear	E. stokesii 101						E. stokesii 102					
	A	B	C	U. m.	S. sp.	H. m.	A	B	C	U. m.	S. sp.	H. m.
27/03/96	0	0	0	0	0	0	0	0	0	0	0	0
08/05/96	0	0	0	0	0	0	0	0	0	0	0	0
30/06/96	0	0	0	0	0	0	0	0	0	0	0	0
19/08/96	0	12	11	23	2	0	0	1	2	3	0	0
13/10/96	0	17	12	29	4	0	0	4	4	8	0	0
22/11/96	0	1	15	16	9	0	0	6	9	15	6	0
09/01/97	0	8	8	16	12	0	1	22	8	31	42	0
04/03/97	0	2	8	10	5	0	0	18	12	30	27	0
22/04/97	0	2	4	6	4	0	2	2	4	8	10	0
17/07/97	0	4	6	10	7	0	0	3	4	7	8	0
22/09/97	0	9	9	18	9	0	0	3	3	6	7	0
07/11/97	0	4	5	9	5	0	0	4	3	7	11	0
14/01/98	0	3	1	4	1	0	0	6	12	18	8	0
26/03/98	0	1	1	2	1	0	0	3	2	5	4	0
17/07/98	0	1	4	5	4	0	0	2	0	2	3	0
30/09/98	0	2	2	4	3	0	3	1	3	7	0	0
23/11/98	0	3	1	4	1	0	1	3	4	8	2	0
30/11/98	0	2	1	3	1	0	0	2	3	5	2	0
07/12/98	0	1	1	2	1	0	1	4	1	6	1	0
14/12/98	2	3	2	7	1	0	0	0	7	7	2	0
21/12/98	10	15	0	25	0	0	0	12	6	18	3	0
29/12/98	54	53	32	139	0	13	45	11	4	60	0	30
04/01/99	280	60	20	360	0	150	190	20	0	210	0	210
10/01/99	128	60	20	208	2	340	180	20	0	200	0	330
28/01/99	0	20	0	20	0	280	1	4	0	5	0	83
11/02/99	0	0	0	0	1	68	0	4	0	4	1	49
23/03/99	0	0	0	0	0	6	4	0	0	4	0	16

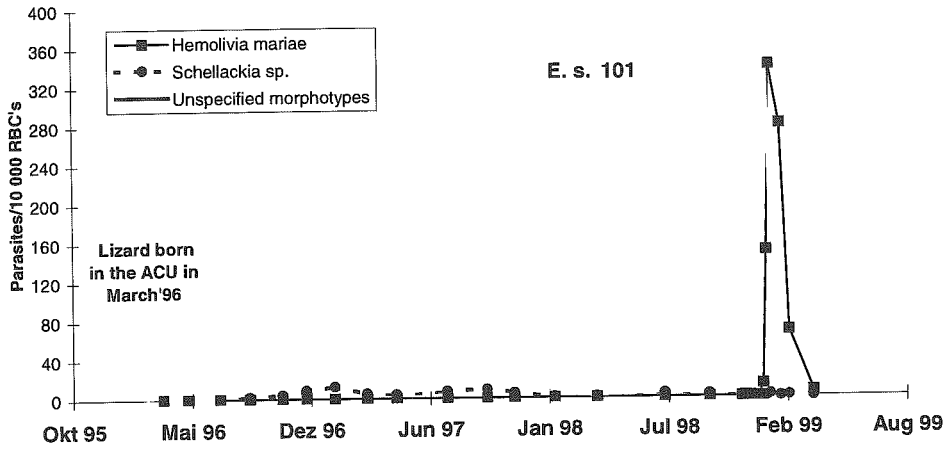

Figure 25. The presence of blood parasite stages in *E. stokesii 101* throughout the time span it was monitored.

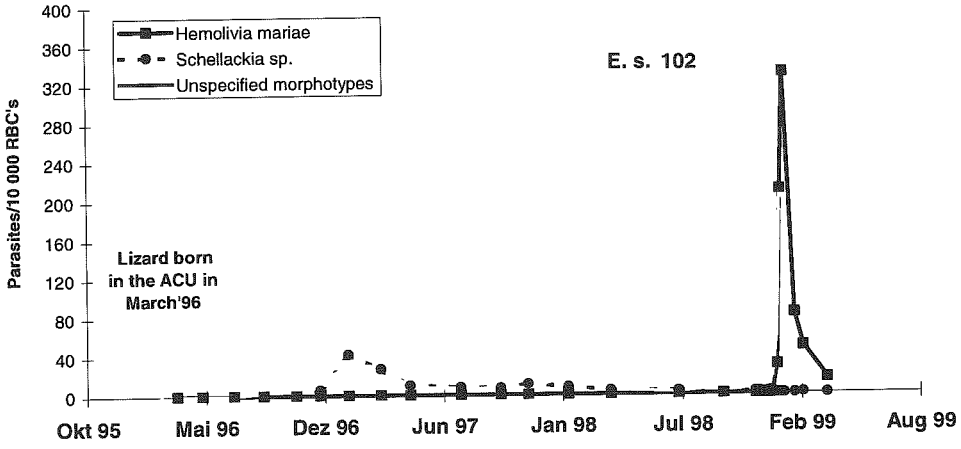

Figure 26. The presence of blood parasite stages in *E. stokesii 102* throughout the time span it was monitored.

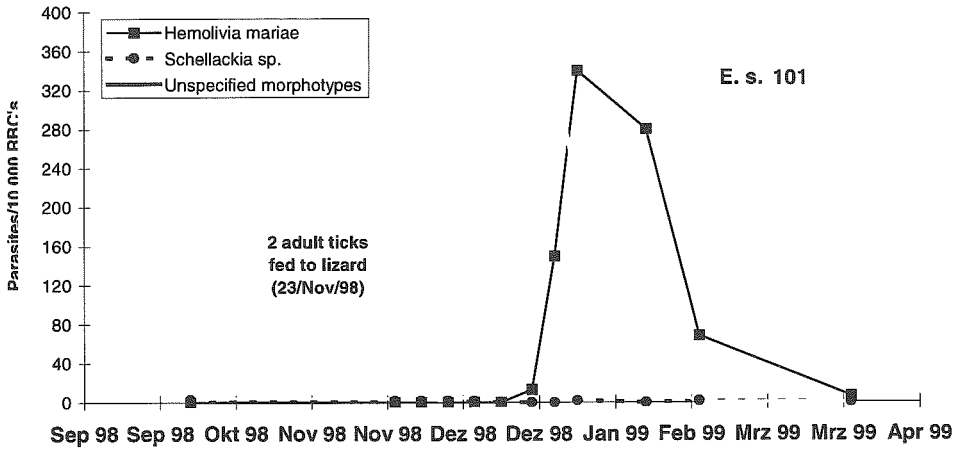

Figure 27. The presence of blood parasite stages in *E. stokesii 101* during the transmission experiment.

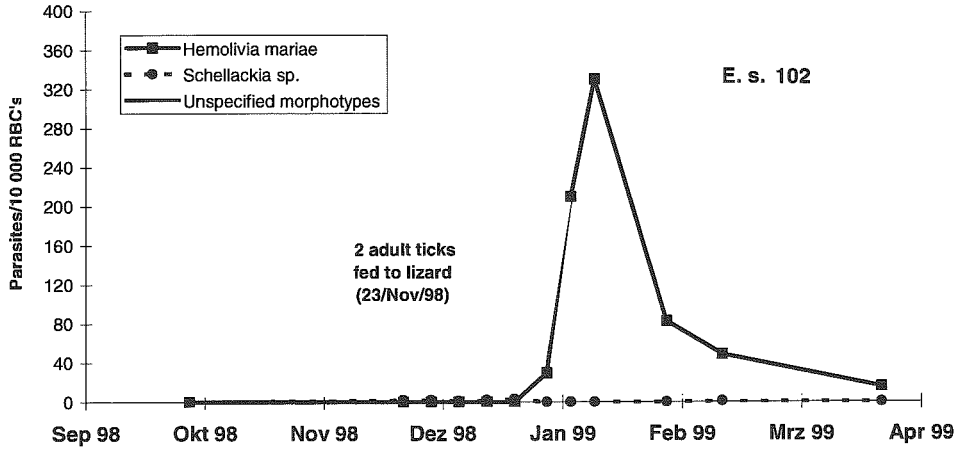

Figure 28. The presence of blood parasite stages in *E. stokesii 102* during the transmission experiment.

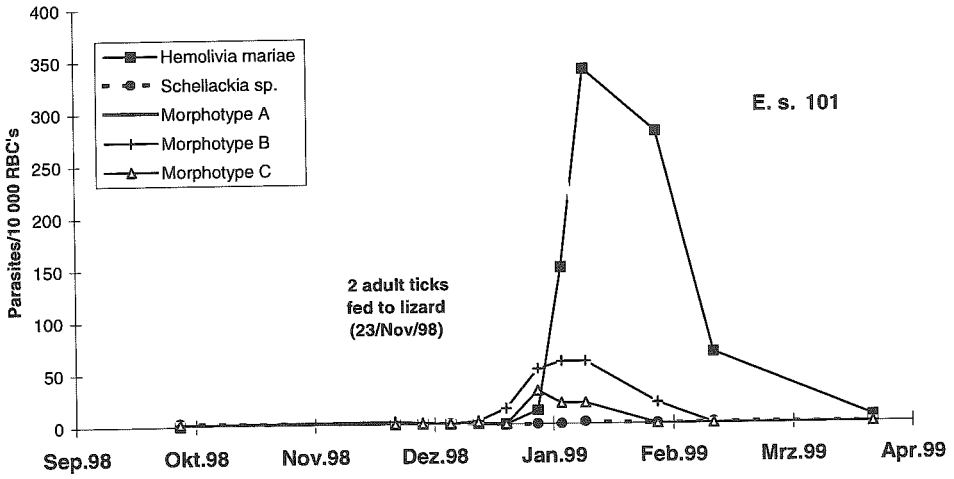

Figure 29. The presence of blood parasite stages in *E. stokesii 101* during the transmission experiment. The unspecified morphotypes were subdivided into type A, B and C.

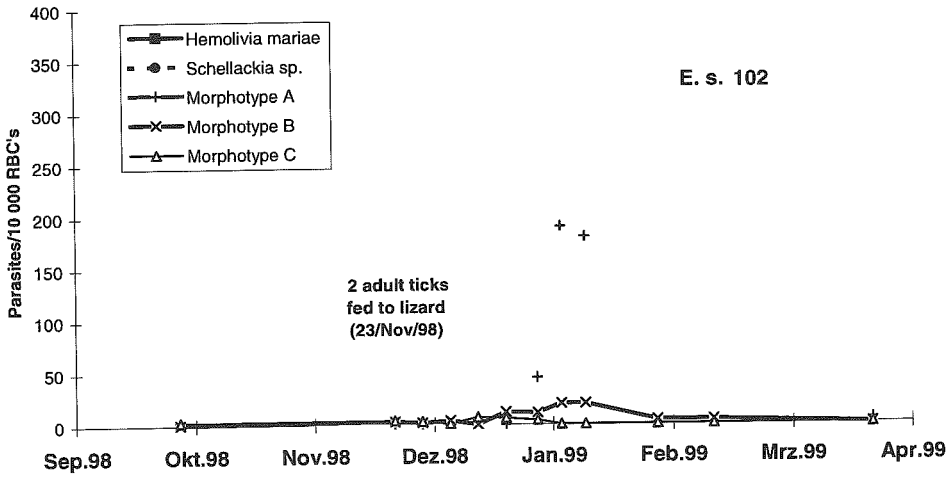

Figure 30. The presence of blood parasite stages in *E. stokesii 101* during the transmission experiment. The unspecified morphotypes were subdivided into type A, B and C.

6.1.4.2 Experiment II: transmission from *E. stokesii* to *E. stokesii*

In this experiment, two lizards caught at the regular study sites in the Flinders Ranges infected with blood parasites believed to be *Hem. mariae* were held in the ACU and their blood was monitored regularly (Figure 31 and Figure 33). Both lizards had mixed infections at the time of tick attachment. Additional to *Hem. mariae*, *E. stokesii 620* was infected with *P. mackerrasae* (see chapter ten; Figure 31) and *E. stokesii 41* was infected with *Schellackia* sp. (Figure 33). No other parasite species were detected. Tick larvae derived from one female *A. limbatum* were allowed to engorge on those two lizards on the 17/09/98. After detachment and development to nymphs, the ticks were fed to *E. stokesii 24* (n=10) and *E. stokesii 30* (n=5) on the 20/11/98 (Table 15).

Table 15. Transmission of *Hem. mariae* from feral *E. stokesii* to lab born *E. stokesii* by force feeding infected *Amb. limbatum* nymphs.

Lizard host tick larvae were fed on	Field site lizard was captured	Lizard host resulting tick nymphs were fed to	Number of nymphs fed to lizard	Nymphal body-fluid residue found to be infected
E. s. 620	HN	*E. s. 24*	10	+
E. s. 41	GL	*E. s. 30*	5	+

The two *E. stokesii 24* and *30* were born in the ACU in February 1995. Their blood was monitored from July 1995 at six to 10 week intervals up to the start of the experiment (Figure 32 and Figure 34). Three years after *E. stokesii 24* was born in the ACU, its blood spontaneously became infected with *Schellackia* sp. in March 1998, but the infection was not detectable when the transmission experiment started (Table 16). No blood infection was detectable in *E. stokesii* 30 from its birth to the time the experiment started.

During the experiment, the lizards were bled weekly for eight weeks, and then at 2-6 week intervals until the experiment was terminated (Table 16). Four and six weeks after the ticks were ingested, the first blood parasite stages that were reliably identified as the encapsulated gamonts of *Hem. mariae* were detected in blood smears of *E. stokesii 30* and *24* respectively. *E. stokesii 24* maintained a relatively low parasitemia throughout the experiment with only a few unspecified morphotypes present. No chronic stages of *Schellackia* sp. were detectable. In *E. stokesii 30*, the first unspecified morphotypes appeared two weeks after tick ingestion.

One week before the detection of the first *Hem. mariae* gamonts, there was a sudden increase of unspecified morphotypes. During the first phase of the blood infection, morphotype A was most abundant, but was quickly surpassed in numbers by morphotype B, at which stage the lizard was killed and dissected. The round type C was relatively uncommon.

This experiment confirmed that the parasite thought to be *Hem. mariae* can be transmitted from an *E. stokesii* host to another by ingestion of infected *Amb. limbatum* ticks, similar to the mode of transmission previously described for *Hem. mariae* infecting *T. rugosa* (Smallridge and Paperna, 1997).

6.1.4.3 Experiment III: time course of development in *Amb. limbatum*

In this experiment, *Amb. limbatum* larvae derived from an egg batch of one female tick were fed on four *E. stokesii* infected with *Hem. mariae* on 11/03/98. Fifty five tick individuals were subsequently dissected at various times from 6-42 days after detachment and screened for parasitic developmental stages in a wet mount. The results are summarised in Figure 35 and Table 17. All ticks that were dissected more than 30 days after their detachment as a larva had moulted to nymphs. The first visible developmental stage of *Hem. mariae* in a wet mount were star shaped oocysts that were found in a midgut of an engorged larvae 11 days after the tick had detached. The last oocysts were seen in the midgut of a moulted nymph 38 days after the tick had dropped off its host as an engorged larva. Oocysts containing sporokinetes were detected in engorged larvae from 25 to 38 days post detachment. The first sporocysts were found in engorged larvae 28 days after they had detached. Sporocysts containing sporozoites were present wherever sporocysts were detected, but their number were very low at first. Following moulting to nymphs and with increasing days after detachment, the ratio of sporocysts containing the sporozoites increased to nearly 100%. This experiment showed that the time course and the sequence of development were very similar to those reported for *Hem. mariae* in *T. rugosa* (Smallridge, 1988).

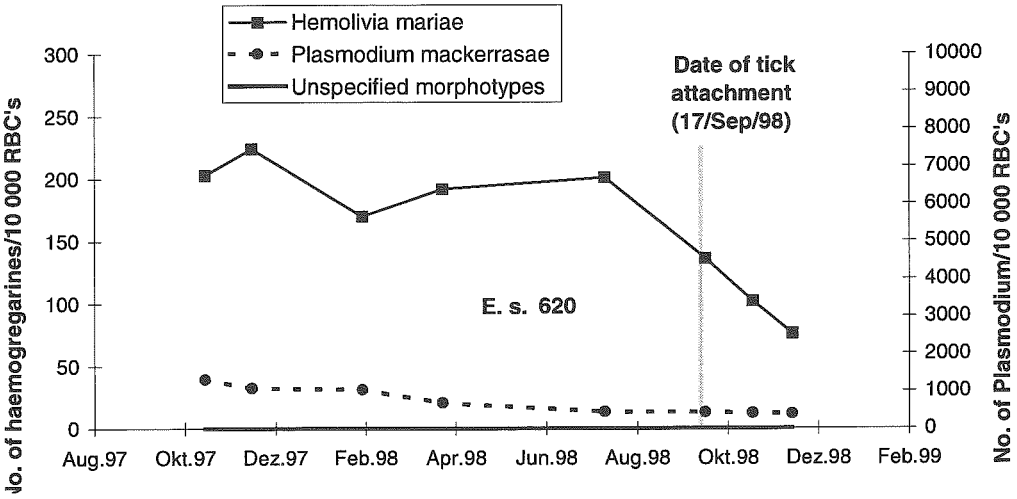

Figure 31. The presence of blood parasite stages in *E. stokesii 620* throughout the time span it was monitored in the ACU. Five unfed nymphs that developed from the detached *Amb. limbatum* larvae were fed to *E. stokesii 24*.

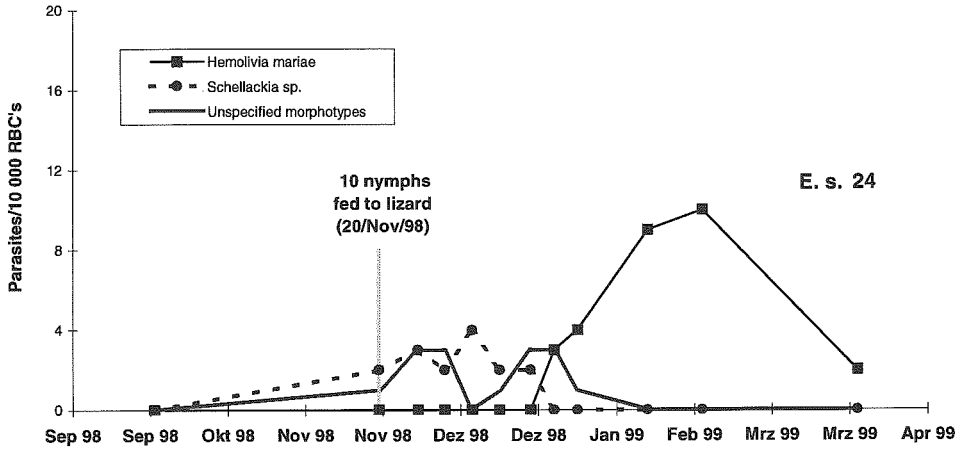

Figure 32. The presence of blood parasite stages in *E. stokesii 24* during the transmission experiment.

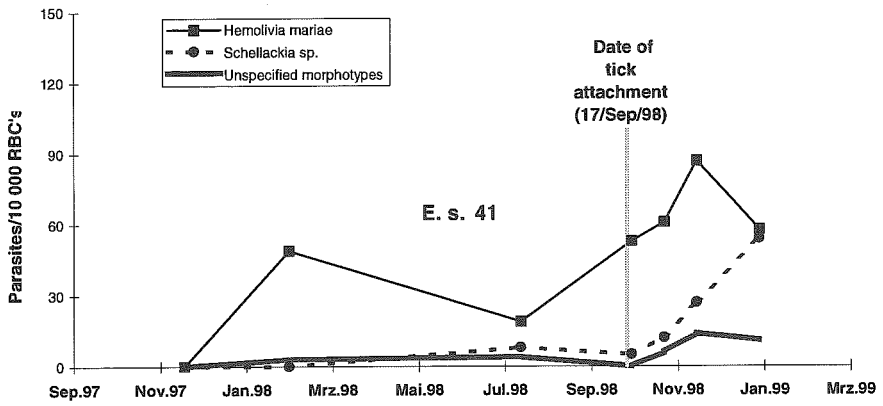

Figure 33. The presence of blood parasite stages in *E. stokesii 41* throughout the time span it was monitored in the ACU. Five unfed nymphs that developed from the detached *Amb. limbatum* larvae were fed to *E. stokesii 30*.

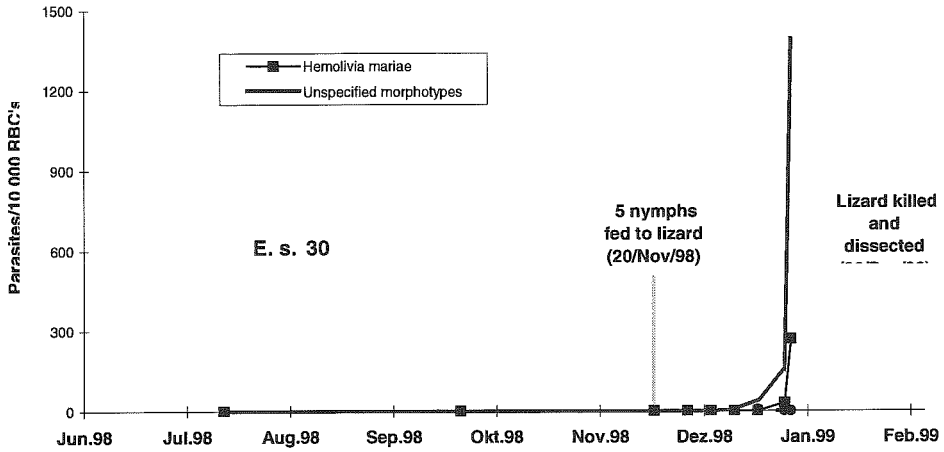

Figure 34. The presence of blood parasite stages in *E. stokesii 30* during the transmission experiment.

Table 16. Blood smear counts of the infections of the *E. stokesii 24* and *30* as parasite stages per 10^4 RBC's. Ticks were fed to both lizards on the 20/11/98. The transmission experiment was shaded for its duration (A,B,C: unspecified morphotypes A,B,C; U. m.: sum of unspecified morphotypes; *S.* sp.: *Schellackia* sp.; *H. m.*: *Hem. mariae*).

Date of	E. stokesii 24						E. stokesii 30					
blood smear	A	B	C	U. m.	S. sp.	H. m.	A	B	C	U. m.	S. sp.	H. m.
11/07/95	0	0	0	0	0	0	0	0	0	0	0	0
20/09/95	0	0	0	0	0	0	0	0	0	0	0	0
04/11/95	0	0	0	0	0	0	0	0	0	0	0	0
07/12/95	0	0	0	0	0	0	0	0	0	0	0	0
03/01/96	0	0	0	0	0	0	0	0	0	0	0	0
28/01/96	0	0	0	0	0	0	0	0	0	0	0	0
28/03/96	0	0	0	0	0	0	0	0	0	0	0	0
08/05/96	0	0	0	0	0	0	0	0	0	0	0	0
30/06/96	0	0	0	0	0	0	0	0	0	0	0	0
19/08/96	0	0	0	0	0	0	0	0	0	0	0	0
13/10/96	0	0	0	0	0	0	0	0	0	0	0	0
22/11/96	0	0	0	0	0	0	0	0	0	0	0	0
09/01/97	0	0	0	0	0	0	0	0	0	0	0	0
04/03/97	0	0	0	0	0	0	0	0	0	0	0	0
22/04/97	0	0	0	0	0	0	0	0	0	0	0	0
18/07/97	0	0	0	0	0	0	0	0	0	0	0	0
22/09/97	0	0	0	0	0	0	0	0	0	0	0	0
14/10/97	0	0	0	0	0	0	0	0	0	0	0	0
07/11/97	0	0	0	0	0	0	0	0	0	0	0	0
08/12/97	0	0	0	0	0	0	0	0	0	0	0	0
14/01/98	0	0	0	0	0	0	0	0	0	0	0	0
26/03/98	2	4	8	14	4	0	0	0	0	0	0	0
15/06/98	1	4	11	16	5	0	0	0	0	0	0	0
14/07/98	0	2	14	16	6	0	0	0	0	0	0	0
23/09/98	0	0	0	0	0	0	0	0	0	0	0	0
20/11/98	0	1	0	1	2	0	0	0	0	0	0	0
30/11/98	0	1	2	3	3	0	0	0	0	0	0	0
07/12/98	0	2	1	3	2	0	0	2	0	2	0	0
14/12/98	0	0	0	0	4	0	0	3	2	5	0	0
21/12/98	0	1	0	1	2	0	0	25	15	40	0	5
29/12/98	0	2	1	3	2	0	93	50	10	153	0	30
04/01/99	2	1	0	3	0	3	600*	750*	40*	1390*	0*	270*
10/01/99	0	1	0	1	0	4						
28/01/99	0	0	0	0	0	9						
11/02/99	0	0	0	0	0	10						
23/03/99	0	0	0	0	0	2						

*= Date of dissection (31/12/98)

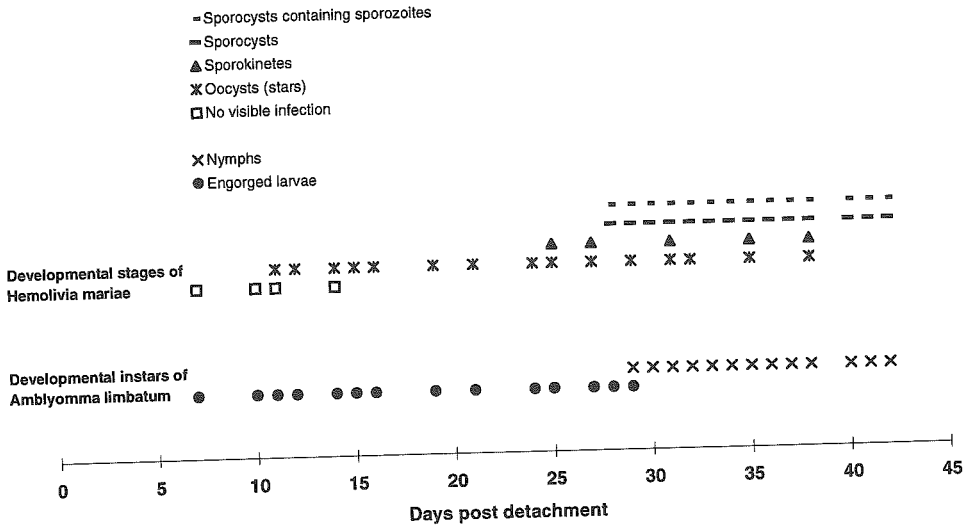

Figure 35. Life cycle stages of *Hem. mariae* found in developing *Amb. limbatum* ticks that had engorged as larvae (n=55) on infected *E. stokesii* (n=4). Due to overlaying of some symbols, not all results are visible.

Table 17: Developmental stages of *H. mariae* found in developing *Amb. limbatum* ticks that had engorged as larvae (n=54) on infected *E. stokesii* (n=4), [+= developmental stage present; Ooc.: oocyst, Spk.: sporokinete, Spc.: sporocyst, Spz.: sporozoite].

No. of ticks dissected in total	No. of ticks dissected per lizard	Lizard individual	Days post attachment	Days post detachment	Engorged larva	Nymph	No visible infection	Ooc. (stars)	Spk.	Spc.	Spc. containing spz.
1	1	46	25	16	+			+			
2	2	46	35	21	+			+			
3	3	46	31	24	+			+			
4	4	46	32	25	+			+			
5	5	46	42	29	+					+	+
6	6	46	42	29	+					+	+
7	7	46	42	29		+		+			
8	8	46	42	29	+					+	+
9	9	46	50	30		+				+	+
10	10	46	42	32		+		+	+		
11	11	46	42	32		+				+	+
12	12	46	42	32		+				+	+
13	13	46	42	32		+				+	+
14	14	46	42	32		+				+	+
15	15	46	52	37		+				+	+
16	16	46	52	38		+				+	+
17	17	46	50	40		+				+	+
18	18	46	52	40		+				+	+
19	19	46	52	40		+				+	+
20	20	46	52	40		+				+	+
21	21	46	52	41		+				+	+
22	22	46	52	42		+				+	+
23	23	46	52	42		+				+	+
24	24	46	52	42		+				+	+

Continued on next page

Continuation of Table 17 (see previous page).

No. of ticks dissected in total	No. of ticks dissected per lizard	Lizard individual	Days post attachment	Days post detachment	Engorged larva	Nymph	No visible infection	Ooc. (stars)	Spk.	Spc.	Spc. containing spz.
25	1	170	25	14	+		+				
26	2	170	35	19	+			+			
27	3	170	36	25	+			+	+		
28	4	170	42	28	+					+	+
29	5	170	42	29	+			+			
30	6	170	50	31		+		+	+	+	+
31	7	170	50	33		+				+	+
32	8	170	52	34		+				+	+
33	9	170	42	35		+		+	+		
34	10	170	50	38		+		+	+		
35	11	170	50	38		+		+	+		
36	1	620	27	7	+		+				
37	2	620	27	10	+		+				
38	3	620	27	11	+			+			
39	4	620	25	11	+		+				
40	5	620	27	12	+			+			
41	6	620	37	14	+			+			
42	7	620	25	15	+			+			
43	8	620	25	15	+			+			
44	9	620	25	16	+			+			
45	10	620	27	19	+			+			
46	11	620	42	29		+		+			
47	12	620	50	32		+				+	+
48	13	620	50	34		+				+	+
49	14	620	50	35		+				+	+
50	1	1377	25	14	+			+			
51	2	1377	36	24	+			+			
52	3	1377	42	27	+			+	+		
53	4	1377	50	30		+				+	+
54	5	1377	50	36		+				+	+

6.1.4.4 Experiment IV: trans-stadial transmission in *Amb. limbatum*

For this experiment, *Amb. limbatum* larvae that originated from a single egg batch were attached to uninfected *E. stokesii 21* and to infected *E. stokesii* 71 on 17/03/98. *E. stokesii 71* was infected with *Hem. mariae* and had a relatively low parasitemia with *Schellackia* sp. at the time of tick attachment. *E. stokesii 21* was born in February 1995 and raised in the ACU and no blood infection was detected from when it was first monitored in July 1995 until the end of this study in December 1998. After the resulting engorged larvae had developed into nymphs, 10 from each lizard were dissected and studied for the presence of blood parasites in a wet mount between 39 and 124 days post detachment. Nine nymphs from *E. stokesii 71* and none from *E. stokesii 21* were found infected (Table 18).

Table 18. Dissection results of *Amb. limbatum* nymphs that had attached as larvae on the 17/03/98 [*H. m.*: *Hem. mariae* ; *S.* sp.: *Schellackia* sp.].

Larval host	Parasitemia (in 10^4 RBC's)		Nymphs found infected (from 10)	Days post detachment
	H. m.	*S.* sp.		
E. stokesii 71	212	4	9	41-124
E. stokesii 21	-	-	0	39-87

The 15 remaining unengorged nymphs that fed as larvae on *E. stokesii 71* were attached to uninfected *E. stokesii 21* on the 24/06/98. Nine of those engorged and moulted to six male and three female ticks. These were subsequently dissected and screened for developmental stages of *Hem. mariae*. Five males and two females were found to be infected (Table 19). Of the 28 remaining unengorged nymphs that fed as larvae on uninfected *E. stokesii 21*, 20 were attached to the same *E. stokesii 21* on the 28/07/98, after all the previous nymphs had detached. Twelve of them engorged and moulted to four males and eight females. Of these, two males and two females were dissected between 44 and 56 days post attachment; none of them were found to be infected. The remaining two males and six females, presumed also to be uninfected, were used in experiment V, described below. These results show that ticks that become infected with *Hem. mariae* by feeding on infected *E. stokesii* as larvae, retain that infection as adults when fed on uninfected *E. stokesii* as nymphs. This result mirrors the report of transstadial transmission of *Hem. mariae* in *Amb. limbatum* fed on *T. rugosa* (Smallridge, 1998).

Table 19. Dissection results of adult *Amb. limbatum* that fed as larvae on *E. stokesii 71* infected with *Hem. mariae* and as nymphs on the uninfected *E. stokesii 21* [+= developmental stage present].

Dissected ticks		Days post detachment	Detected parasite stages	
♂♂	♀♀		Oocysts	Sporocysts
1		38	-	+
2		55	-	+
3		63	-	+
4		65	-	+
5		68	-	+
6		68	-	-
	7	53	-	-
	8	58	-	+
	9	65	-	+

6.1.4.5 Experiment V: development in female *Amb. limbatum*

To investigate the development of *Hem. mariae* in female *Amb. limbatum*, two females that had engorged on an infected lizard were dissected and prepared for sectioning and a histological examination at two different times after detachment (Table 20). Numerous serial sections were produced throughout the entire body of the engorged ticks. Three male and two female *Amb. limbatum* were placed into cotton bags with *E. stokesii 657* on the 09/02/99. The female ticks have been fed previously as larvae and nymphs on uninfected lizards. Other ticks with the same feeding history remained uninfected at the unfed adult stage (see experiment IV). Two of the male ticks were also derived from experiment IV, and the other had also only fed on uninfected lizards. The results came from two engorged female ticks that fed on *E. stokesii 657*, that was born in the ACU in February 1995 and was raised there. *E. stokesii 657* had an experimentally induced mixed blood infection of *Hem. mariae* and *Hem. biplicata* (Figure 36). *E. stokesii 657* had a relatively high parasitemia for both parasites at the time. In particular the parasitemia of infected *E. stokesii 657* was ten times higher than levels found adequate to infect engorging *Amb. limbatum* larvae with *Hem. mariae* from blood of *T. rugosa*. This was the last experiment that could be conducted within the time frame of this project, and because of the elaborate preparations it was not possible to repeat this experiment which investigated the site of development in single infected female ticks.

Despite these problems the results of this experiment were included in this chapter, because a careful analysis revealed some facts of taxonomic significance for both *Hem. mariae* and *Hem. biplicata.*

Table 20. The time course of transmission experiment V: two previously uninfected female *Amb. limbatum* were attached to infected *E. stokesii 657.*

Amb. limbatum	Attachment date	Detachment date	Dissection date	Days post detachment	Days post attachment
I	09/02/99	28/02/99	06/03/99	6	25
II	09/02/99	15/03/99	18/04/99	34	68

Female I detached fully engorged from *E. stokesii 657* after 19 days of attachment. Within the next six days the tick slowly turned dark, which usually indicated that a tick was dying. It was fixed on day six post detachment and later sectioned for histological examination. At the time of fixation, the tick still had its fully engorged berry shape. The images obtained from the giemsa stained sections showed encapsulated gamonts from both species, *Hem. mariae* and *Hem. biplicata,* in abundance scattered throughout the midgut lumen. In some cases, worm like gamonts of both species were observed leaving their protective capsule. Especially conspicuous were numerous aggregations of parasites throughout the midgut lumen, each containing up to a hundred or more free floating, worm like gamonts (Figure 24/A) or adjacent gamonts that were forming a syzygy. The lizard blood cells had already disintegrated and the aggregations of free gamonts seemed to float in proximity to the top of active digestive cells of the midgut epithelium of the tick or to cells free floating in the midgut lumen (Figure 24/B). Very few early zygotes were seen in the cells of the midgut epithelium. Except for a slight dimorphism between the stages that formed a syzygy, the morphology of the stages observed did not indicate any difference between the two different species of blood parasites.

Female II detached fully engorged from *E. stokesii 657* on the 15/03/99, after 34 days of attachment. It was observed to be half engorged after 13 days post attachment, but did not further advance in size until two days before detachment. The tick began to lay eggs on the 02/04/99 and was fixed and prepared for dissection on the 18/04/99, 34 days post detachment and 68 days post attachment. At that time it had laid approximately 800 eggs,

and from the size of its still swollen body it was estimated that it would have laid at least the same amount of eggs again. During the dissection, the cuticle was carefully removed. Special caution was taken to recover the entire reproductive tract and the midgut. After processing, the glass slide the tick was dissected on was screened for parasitic stages and later air dried, fixed in methanol and stained with giemsa. Numerous parasitic stages were seen floating in the wet mount, ranging from still encapsulated gamonts to motile sporokinetes, the latter either inside or outside of oocysts. From all stages observed, only the oocysts showed a prominent difference in size and shape. Some oocysts were relatively large and star shaped, other oocysts were relatively small and spherical (Figure 23/A).

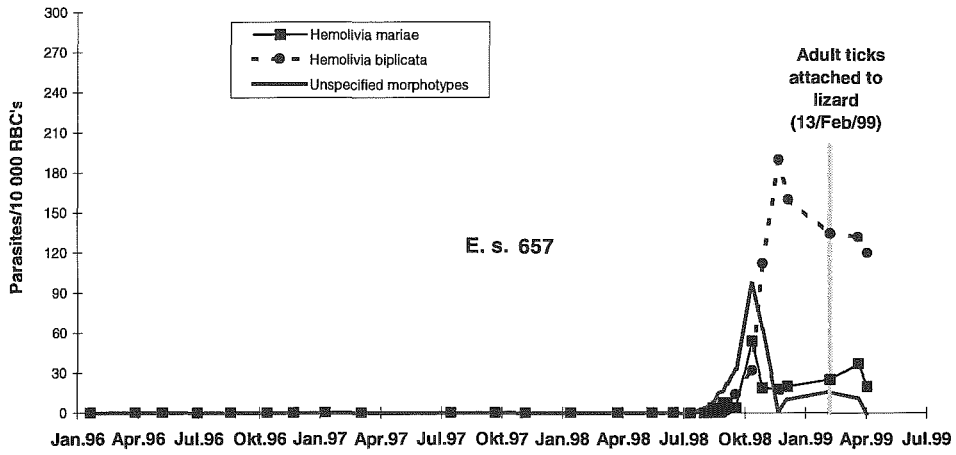

Figure 36. The presence of blood parasite stages in *E. stokesii 657* throughout the time span it was monitored.

Images of the histological sections of female II showed a heavy infection displaying the whole range of karyolysid tick-stages. Certain developmental stages were found concentrated in particular areas of the tick body. Many worm like gamonts were seen floating in those areas of the midgut lumen with low digestive activities. In these areas, haemoglobin was found to be stored as crystalline needles. Numerous zygotes and oocysts were found at the base of the digestive epithelial cells (Figure 24/E) and oocysts of various sizes and developmental age were seen either in the upper part of larger digestive cells or already floating in the midgut lumen (Figure 23/C). Two types of oocysts were observed floating in the midgut lumen: relatively large, star shaped oocysts and relatively small, round oocysts. Some specimen of both types were seen to contain sporokinetes. No intracellular sporokinetes, sporocysts or sporozoites were found in the digestive tract of the tick. Free

sporokinetes were seen in the midgut lumen or penetrating the midgut epithelium. The latter were not found within cells, but passing through the midgut wall in between the epithelial cells, sometimes with one part still embedded in between the midgut wall cells and the other part outside the midgut. A few sporokinetes were found in the ovarian duct and among the sperm. A massive concentration of sporokinetes, sporocysts and developing sporozoites were detected in the cuboidal epithelium cells of the common oviduct (Figure 24/C&D). Only very few mature eggs that were already detached from the ovary were sectioned in these preparations, and none of them were found to be infected. Approximately 100 eggs laid previously by this tick were sectioned separately, but no infected eggs were found. Due to their shell and their brittle yolk mass, eggs were difficult to investigate histologically.

6.1.5 Comparison between *Hem. mariae* from T. rugosa and E. stokesii

The data obtained from the study of the life cycle of *Hem. mariae* in *E. stokesii* were compared to the data available from *Hem. mariae* in *T. rugosa* from a previous study (Smallridge, 1998). No differences were found between the two studies in the sequence of the developmental stages or in the time course of the development within ticks and lizards. The dimensions of the chronic blood stage of *Hem. mariae*, the encapsulated gamont, were tested for significant differences using three sets of data (Table 21) by an analysis of variance (ANOVA). The test compared the gamont-dimensions in a chronic infection in *E. stokesii 620* from the study site HN, the experimentally transmitted infection from *T. rugosa* to *E. stokesii 102* and data published for *Hem. mariae* in *T. rugosa* (Smallridge, 1998). No significant differences among the data sets of *Hem. mariae* from *T. rugosa* and *E. stokesii* were found (Table 22).

Table 21. Data used for the comparison of the dimensions of the encapsulated gamont of *Hem. mariae* from blood of *E. stokesii* and *T. rugosa*.

Host	Data source	n	Origin of infection	Mean gamont length		Mean gamont width	
				$\bar{X}$	SD	$\bar{X}$	SD
E. stokesii	Present study	25	Chronic infection	8.81	0.41	4.49	0.34
E. stokesii	Present study	25	Transmitted from *T. rugosa*	8.59	0.51	4.45	0.32
T. rugosa	Dr. C. Smallridge	7 x 30	Chronic infections or transmitted from *T. rugosa*	8.714	0.30	4.69	0.15

Table 22. One way analysis of variance (ANOVA) of the length and the width [µm] between intraerythrocytic gamonts of *Hem. mariae* from three data sets as outlined in Table 21, with the degrees of freedom (df.), the variance-ratio distribution (F) and significance (Sig.).

Feature		Sum of Squares	df.	Mean Square	F	Sig.
Length	Between Groups	.584	2	.292	1.460	.241
	Within Groups	10.805	54	.200		
	Total	11.390	56			
Width	Between Groups	.300	2	.150	1.528	.226
	Within Groups	5.309	54	0.090		
	Total	5.610	56			

6.2 Discussion

The analysis of the life-cycle data presented in this study for one species of blood parasite found frequently infecting *E. stokesii* at study sites in the Flinders Ranges proved that it belonged to the family Karyolysidae by the finding of motile sporokinetes in the oocyst. The presence of a star shaped oocyst allocated this parasite into the genus *Hemolivia*. The fact that *Hem. mariae* was transmitted from *T. rugosa* to *E. stokesii* and that despite a detailed analysis no feature could be identified to distinguish this parasite found in *E. stokesii* from *Hem. mariae* described from *T. rugosa* strongly suggested that they were conspecific.

Some details of the life cycle and morphology of *Hem. mariae* were described in this thesis for the first time, namely the site and the ultrastructure of the meronts in *E. stokesii*, the structure of the membrane layers of the early blood stages, the presence of a micro-canal passing through the protrusions of the encapsulated gamonts and the ultrastructure of the micro- and macrogametocytes.

The sites where meronts were found in *E. stokesii* resembled closely those in the descriptions of *H. stellata* in *B. marinus* (Petit *et al.*, 1990), except for two points. In the male *E. stokesii 30*, meronts developed inside tissue cells of the liver, spleen, lung, intestinal tract and the testes. Whether this was normal for *Hem. mariae* or due to the very high parasitemia of *E. stokesii 30* could not be determined with certainty. In contrast to *H. stellata*, no meronts were found in erythrocytes.

The ultrastructural images of the blood stages of *Hem. mariae* closely resembled those described by Smallridge (Smallridge, 1998). From the images it was apparent that the parasite changes its shape considerably during the process of encapsulation. The highly variable forms of the often rod- bean- or banana-shaped early gamonts would transform into a highly uniform translucent, flat and oval encapsulated stage. Longitudinal and latitudinal sections through the gamonts revealed a very peculiar halteridial appearance. Apart from *Hem. mariae*, this hourglass or dumbbell shape of the encapsulated gamonts has been described from several *Karyolysus* species (Reichenow, 1921; Svahn, 1975), but those were rather vague descriptions made with LM from fresh blood or dissected mites. Only few studies have retrieved ultrastructural images of the encapsulated blood stages of adeleorine parasites successfully due to the brittle nature of the capsule wall (Desser & Weller, 1973).

The observed pattern of two double layered membranes enclosing the blood stages of *Hem. mariae* seems to be unique within the adeleorine ultrastructure. The formation of a capsule-layer in between those two double layers suggests that, like in *Plasmodium*, the outer layer might be formed by the host cell. Possibly the parasite never actually enters the red blood cell, but is only engulfed by it. These thoughts remain highly speculative, further studies are necessary to clarify this issue.

To the best knowledge of the author, no ultrastructural feature like the structure interpreted as a micro-canal has been described from adeleorine or any other group of blood parasites. Because the micro-canals were hit in many but not all sections through the distinctive capsule-protrusions, it seems probable that they represent a linear succession of narrowly set pores through the capsule wall. They might be of importance for the exchange of substances necessary for parasitic metabolism. Some evidence for this theory will be given in the following chapter.

A remarkable ultrastructural feature of stages believed to be single macrogametocytes were the array of pellicular folds as seen in (Figure 22/B). Structures like these have been described from extracellular stages of an unknown haemogregarine in the frog *Rana berlandieri* (Desser & Weller, 1973) and from macromerozoites of *Hepatozoon sipedon* (Smith & Desser, 1998). In both cases, pellicular folds were believed to play an important role in the motility of the described stages. Single macrogametocytes have been described from mites infected by a species of *Karyolysus* (Dörflein & Reichenow, 1953).

Observations of the aggregation of gamonts around cells in the tick lumen and the development of sporocysts in the epithelial cells of the common oviduct were made from ticks with mixed infections of *Hem. mariae* and *Hem. biplicata* n. sp. Definitive conclusions are difficult under these circumstances. Nevertheless it can be inferred that the described aggregation of gamonts in the midgut lumen of female I must occur in both species of blood parasites, because despite a thorough search, no other site of syzygy or parasite aggregation was detected. Gamonts, presumably of both species, were observed leaving their protective capsules, and from then on they could not be distinguished from each other by light microscopy. However, there were very many cases of syzygy observed within the large aggregations around the midgut cells and none were observed elsewhere. Many gamonts apparently aggregated around the tips of the actively digesting epithelial cells of the midgut.

Similar events have been described in detail from some mite and tick parasites in the family Karyolysidae (Reichenow, 1921; Petit *et al.*, 1990), which need to be ingested by one of these cells for further development. Other aggregations were observed around relatively small spherical cells that were floating free in the tick midgut lumen. Whether that was a common place for the further development of *Hemolivia* only due to the high intensity of the infection remains to be investigated. The nature of these targeted cells could not be determined with certainty. The cells might be exhausted digestive cells that had detached from the epithelium, but their relatively small size and the fact that they frequently occurred in the rather early stage of digestion of the blood meal of female I, and that they were not observed in the more advanced digestive stage of female II, made that assumption unlikely. It is possible that the small cells are pathologic and that they were somehow associated with the probable death of female I shortly after detachment. Whether the presence or the abundance of adeleorine parasites directly or indirectly caused the death of female I remains unclear.

The observed intracellular development of sporokinetes into sporocysts within the reproductive tract of female II certainly represents a part of the life cycle of at least one of the two blood parasites, *Hem. mariae* or *Hem. biplicata.* It is possible that this behaviour occurred in both species of blood parasites. The development of round[6] and star shaped oocysts in female II strongly suggested that a mixed infection had developed in this tick. In the wet mount and the histological preparations of female II, mature sporokinetes were found in abundance within both the spherical and the star shaped oocysts. How far each of the parasites had advanced from there in its developmental cycle was difficult to establish under these circumstances, but from the development of sporocysts within the reproductive tract of the tick it can be assumed that in both species sporocyst development had already begun.

In *Hem. mariae* infections in engorged *Amb. limbatum* larvae, sporocyst development usually took place in the midgut epithelium around 30 days post detachment (Figure 35). At the time of fixation, female II had detached from the host for 34 days, thus *Hem. mariae* had sufficient time to develop into sporocysts. Also female II spent a relatively long time (34 days) on the host, during which it had taken a relatively large amount of blood in the first

[6] The round oocyst will be illustrated in detail within the taxonomic description of *Hem. biplicata* n. sp. in chapter seven.

two weeks of attachment. Support for the hypothesis that both parasite species were developing inside the reproductive tract of female II comes from the fact that, despite the abundance of sporokinetes formed by both species and although both species had sufficient time for the production of sporocysts, not a single sporocyst was found in over 70 histological sections made throughout the entire midgut of female II. It is possible that one of the parasite species halted its development at the stage where sporokinetes would not develop any further, but because of the negative implications on the fitness of the blood parasite this hypothesis seems unlikely. For parasites transmitted solely by the ingestion of their vector, it is mandatory to develop into the stage infective for the next host before the vector is consumed, or else no transmission can occur. A halt in the development to the infective stage would reduce the chances to be transmitted. It might be argued that a female *Amb. limbatum* is a dead end for the development of one of these two parasite species, but the assumption that the parasite therefore ends its development at the sporokinete stage seems, in the opinion of the author, more complicated than to postulate that both species entered the reproductive tract for further development. Further studies are needed to elucidate the site of the sporogonic development of both *Hem. mariae* and *Hem. biplicata* in engorging female ticks.

The relatively long feeding time of female II and its seemingly interrupted feeding pattern was also observed in some other adult *Amb. limbatum* that were fed on *E. stokesii* during the course of this study, and was not always connected with the presence of blood parasites in the lizard host. The reasons for this behaviour remain to be investigated.

Only very few sporokinetes and no developing sporocysts were found inside the ovary, but due to the nature of the histological preparation, extracellular sporokinetes would have been washed away from the outside and partly from the inside of the ovary. The presence of sporokinetes in the upper part of the oviduct and the massive infection of epithelial cells in the common oviduct led to the following hypothesis. Sporokinetes probably enter the reproductive tract of engorged *Amb. limbatum* females through the ovary. They move down the ovarian duct, through the stored sperm to enter cuboid cells in the inner epithelium of the common oviduct. The latter cells have been suggested to function as an uterus (Roshdy, 1969) and perhaps the sporokinetes are somehow attracted to the substances produced there, somehow mistaking them for an egg. It remains unclear whether *Hem. mariae* or *Hem. biplicata* infected the eggs of *Amb. limbatum* ticks, but the dissections of eggs described

in experiment V and the feeding experiments of larvae described in chapter thirteen suggest that if they did, it was extremely rare. There was no evidence that any larvae were ever infected before they had attached to lizards.

The fact that sporocysts of probably both, *Hem. mariae* and *Hem. biplicata* n. sp. developed either in the midgut epithelium or in the reproductive tract, depending on the tick instar, seems surprising at first. However, metastriate ticks do not complete the development of their reproductive system before the adult has engorged. It can be postulated that whatever attracts the sporokinetes to the reproductive system of adults, has not yet developed in subadult ticks. All vector ticks described to transmit *Hemolivia* belong to the Metastriata. The reproductive tract of engorged females ticks infected with parasites of the genus *Hemolivia* has never been studied histologically so far. The findings regarding the site of development of the sporocysts have been entirely based on dissection of subadult or unengorged adult ticks. Further studies on the described species of *Hemolivia* are necessary to determine whether the parasites enter the eggs of an engorged adult tick vector.

Taking into account the original goal of this part of the project to establish the taxonomic features necessary to distinguish between the blood parasites infecting wild *E. stokesii,* the question whether the parasites do or do not develop in the reproductive tract of engorged *Amb. limbatum* females might appear irrelevant. Nevertheless, this point might have considerable taxonomic implications. The genus *Hemolivia* was erected on the basis that some features of the life cycle clearly separate it from its sister taxon *Karyolysus.* "In contrast to *Karyolysus*, in which sporokinetes are formed in the adult mite and sporozoites in the offspring, in *Hemolivia*, sporokinetes and sporozoites are formed in the same individual tick" (Petit *et al.*, 1990). Firstly, the question whether all sporozoites of *Karyolysus* develop exclusively in the offspring has, to the knowledge of the author, not been investigated. Secondly, with the strong possibility that *Hem. mariae* develops inside the reproductive tract of engorged female *Amb. limbatum* but otherwise in the midgut epithelium of the subadult tick, the question arises whether the original definition of the genus *Hemolivia* regarding the site of the development of sporocysts represents a monophyletic character of the genus *Hemolivia,* or whether it is a superficial cut through a phylogenetic continuum. It can be argued, that the essential taxonomic feature in this case is the successful transovarial transmission of a parasite. Whether or not the parasites will be successfully transmitted to

the offspring is of epidemiological importance, but is probably also highly dependent on the vector species.

7. HEMOLIVIA BIPLICATA N. SP (TYPE 2)

7.1 Introduction

At some field sites, a type of encapsulated blood parasite that was significantly different in size from *Hem. mariae* (Figure 13, and Table 9&10) was found frequently infecting *E. stokesii.* This parasite was described as type 2 in chapter five. In the following, the parasite is described as the encapsulated gamont of *Hemolivia biplicata* n. sp.; a taxonomic publication based on the results given in this chapter is in preparation. The new blood parasite was assigned to the genus *Hemolivia* because of the presence of a round oocyst that contained motile sporokinetes and the development of oocysts and sporozoites within one tick individual.

The data presented here were gathered from:

1. The dissection and blood smears of the field caught *E. stokesii 42,* which had a mixed infection of *Hem. mariae* (0.53%) and *Hem. biplicata* (0.26%). *E. stokesii 42* was caught on the 07/12/97 at Willow Springs, (31°25'S; 138°50'W) and killed and dissected on the 23/01/98.
2. Blood samples from *E. stokesii 1012* and *12.* The latter was captured in October 1995. It remained in the ACU and was constantly monitored for blood parasites (Figure 82). *E. stokesii 12* was chronically infected with *Hem. biplicata* and *Hem. mariae. E. stokesii 1012* was born in February 1998 and became infected with *Hem. biplicata* and *Hem. mariae* (Figure 37) during the course of ethological experiments outside the framework of this study by Ms. Clare Griffith in June 1998. Ms. Griffith's experiments included *Amb. limbatum* ticks, but due to the ethological focus of that project, the circumstances leading to the infection were not recorded. Routine bleedings from the 17/07/98 and the 30/09/98 showed a high parasitemia of *Hem. biplicata* in the blood of *E. stokesii 1012. Hem. mariae* was detected for the first time in a smear from the 4/01/99. Repeated attempts to feed subadult ticks on *E. stokesii 1012* during the phase when only *Hem. biplicata* could be detected in its blood failed, probably because at that time it was still a relatively young and small lizard, but blood samples were taken for the TEM study.

3. Transmission experiments, where infected ticks were force-fed to previously uninfected *E. stokesii.* Apart from the short phase in the young *E. stokesii 1012*, *Hem. biplicata* was always found in mixed infections with *Hem. mariae.* Therefore the transmission experiments had to be performed using *E. stokesii* with mixed infections. These experiments are described in transmission experiment I.
4. Dissection of ticks that fed on lizards with mixed infections. A time series of dissections was done on ticks that had fed as larvae on *E. stokesii 12*, that had a mixed chronic infection of *Hem. biplicata* and *Hem. mariae* and on ticks that fed on four *E. stokesii* with a single infection of *Hem. mariae.* The latter part of this transmission experiment was presented as transmission experiment IV in the previous chapter.

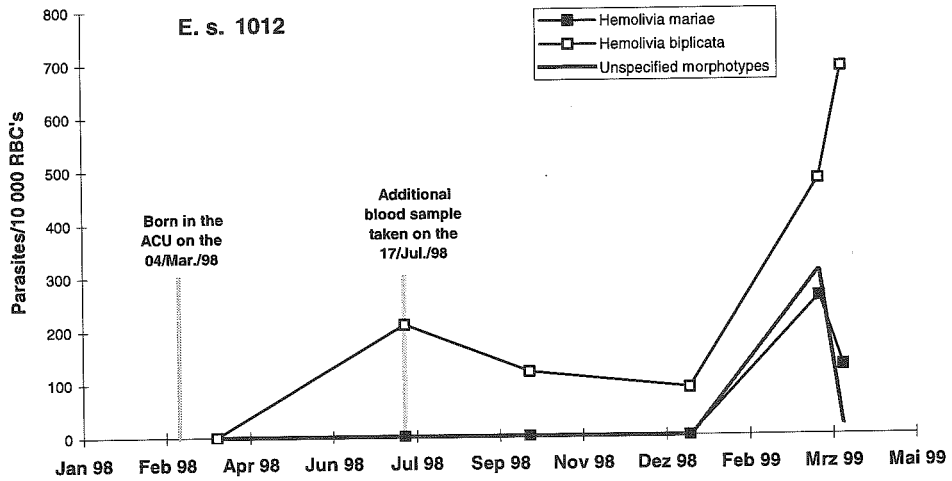

Figure 37. The presence of blood parasites in the anecdotally infected *E. stokesii 1012.* Blood samples for TEM were taken on the 17/07/98, when only a single infection with *Hem. biplicata* was detected.

7.1.1 The life cycle of *Hem. biplicata*

Like *Hem. mariae, Hem. biplicata* was found to develop in *Amb. limbatum* and *Amb. vikirri* ticks and as it will be shown in the following, was successfully transmitted between lizards by force feeding of infected tick nymphs. Except for the shape and the size of the encapsulated gamont and the oocyst, no additional or aberrant life cycle stages have been found in hosts with mixed infections compared to hosts infected with *Hem. mariae* only. Vector experiments with the sand fly *A. dycii* or the mosquitoes *C. quinquefasciatus* and *A. notoscriptus* were not conducted. It was concluded that the life cycles of *Hem. biplicata* and *Hem. mariae* are identical, apart from the shape and size of the encapsulated gamont and the oocyst.

7.1.2 The morphology of *Hem. biplicata*

7.1.2.1 The encapsulated gamont

In giemsa stained blood smears, the terminal nucleus of the consistently more rectangular, encapsulated gamont (Figure 73), was sometimes stained lightly blue, but in most cases the capsule was impenetrable for the stain. At this stage, the parasite was found mainly at one end of the red blood cell, slanting diagonally from one end of the host cell to one of its sides, often dilating the host cell membrane (Table 11, p. 75). The host cell nucleus was often displaced and in most cases it would stain darker than in uninfected cells. Blood from the chronically infected *E. stokesii 12* and the anecdotally infected *E. stokesii 1012* was examined with TEM. The results described the three dimensional structure of encapsulated *Hem. biplicata* gamonts to be an elongated, relatively thin platelet. Like with *Hem. mariae,* lateral-length[7] (Figure 38/C) and lateral-width sections (Figure 39/A) revealed that the platelet had a narrow centre and a wider rim. In contrast to *Hem. mariae*, there was a fold along both sides of the capsule rim, sometimes resulting in bizarrely shaped sections (Figure 40/A&B). The fold on each side of the encapsulated gamont determined the etymology of this newly described species: *Hemolivia biplicata.* The parasite did not fill the entire interior of the capsule and plasma protuberances resembling the plasma fold observed with one developing *Hem. mariae* gamont (Figure 21/B) were seen inside of some of the capsule folds (Figure 38/B). The peculiar capsule-protuberance with the micro-canal was situated at the base of each fold. In the host cell cytoplasm, round or ovoid bodies were seen in close proximity of the micro-canal (Figure 39/B), some were observed disintegrating in some distance of the micro-canal (Figure 38/B). The parasite nucleus was always terminal. In one case structures resembling a disintegrating apical complex were found (Figure 38/C). Similar to *Hem. mariae*, two double layered sets of membranes were visible, the plasma membrane and an outer membrane, separated by a brittle cyst wall (Figure 39/B). Frequently, host cell mitochondria were seen in close proximity to the parasite (Figure 39/A, Figure 40/B).

[7] For an explanation of the terminology used here see 5.2.5.4.

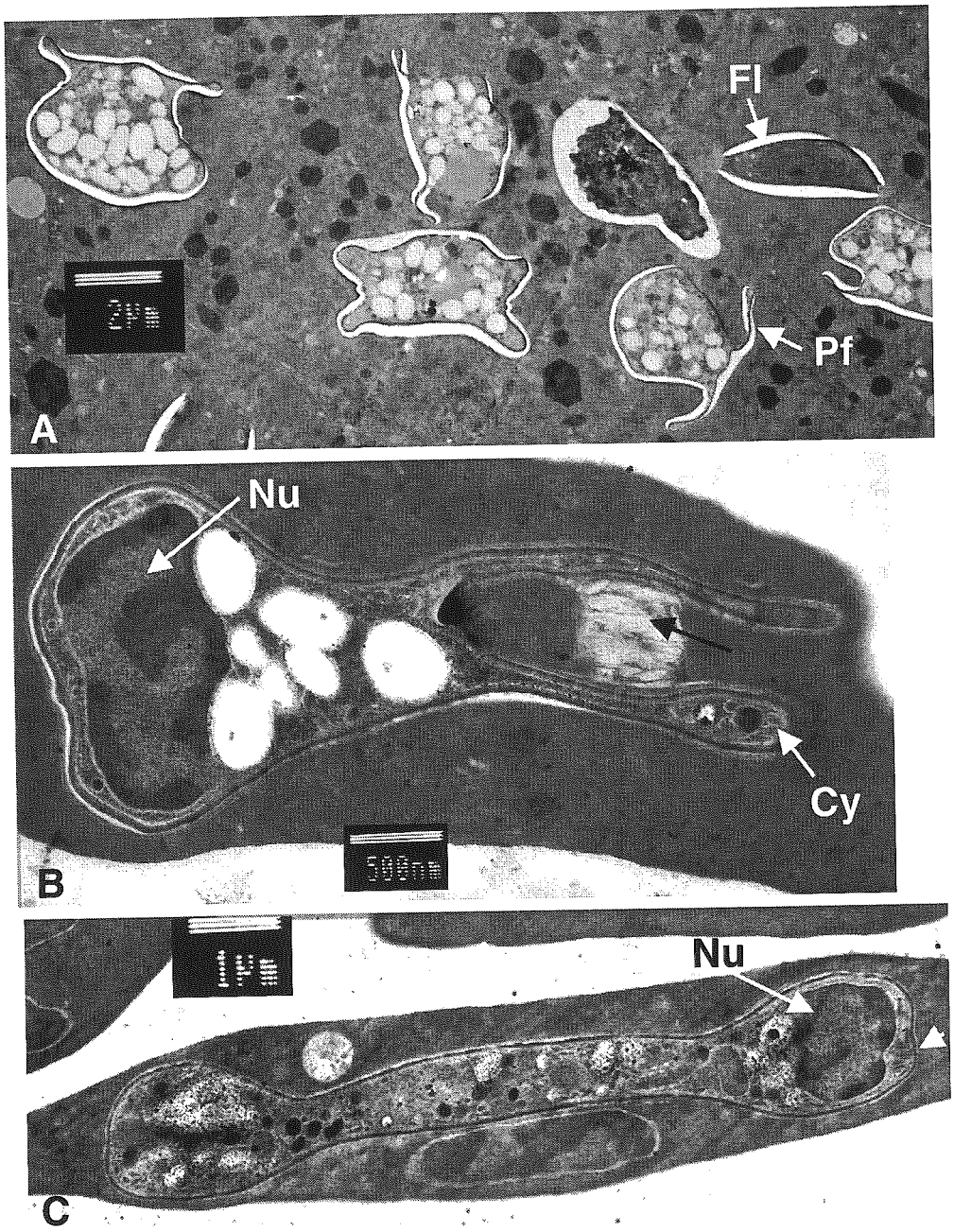

Figure 38. A-C: gamonts of *Hem. biplicata* (TEM). A: aggregating uncysted gamonts in the midgut lumen of a freshly engorged *Amb. limbatum* larva. Frequently occurring paired breaks in the sections were interpreted as indicators for the presence of flagellae; B: intraerythrocytic encapsulated gamont of *Hem. biplicata* in *E. stokesii* (lateral/tangential), black arrow marks structure interpreted as dispersing material originating from the micro-canals; C: intraerythrocytic encapsulated gamont of *Hem biplicata* in *E. stokesii* (lateral/lenght), white arrow marks remains of apical complex. [Cy: parasite cytoplasm, Fl: flagellum, Nu: nucleus, Pf: plasma membrane fold, Pv: parasitophorous vacuole].

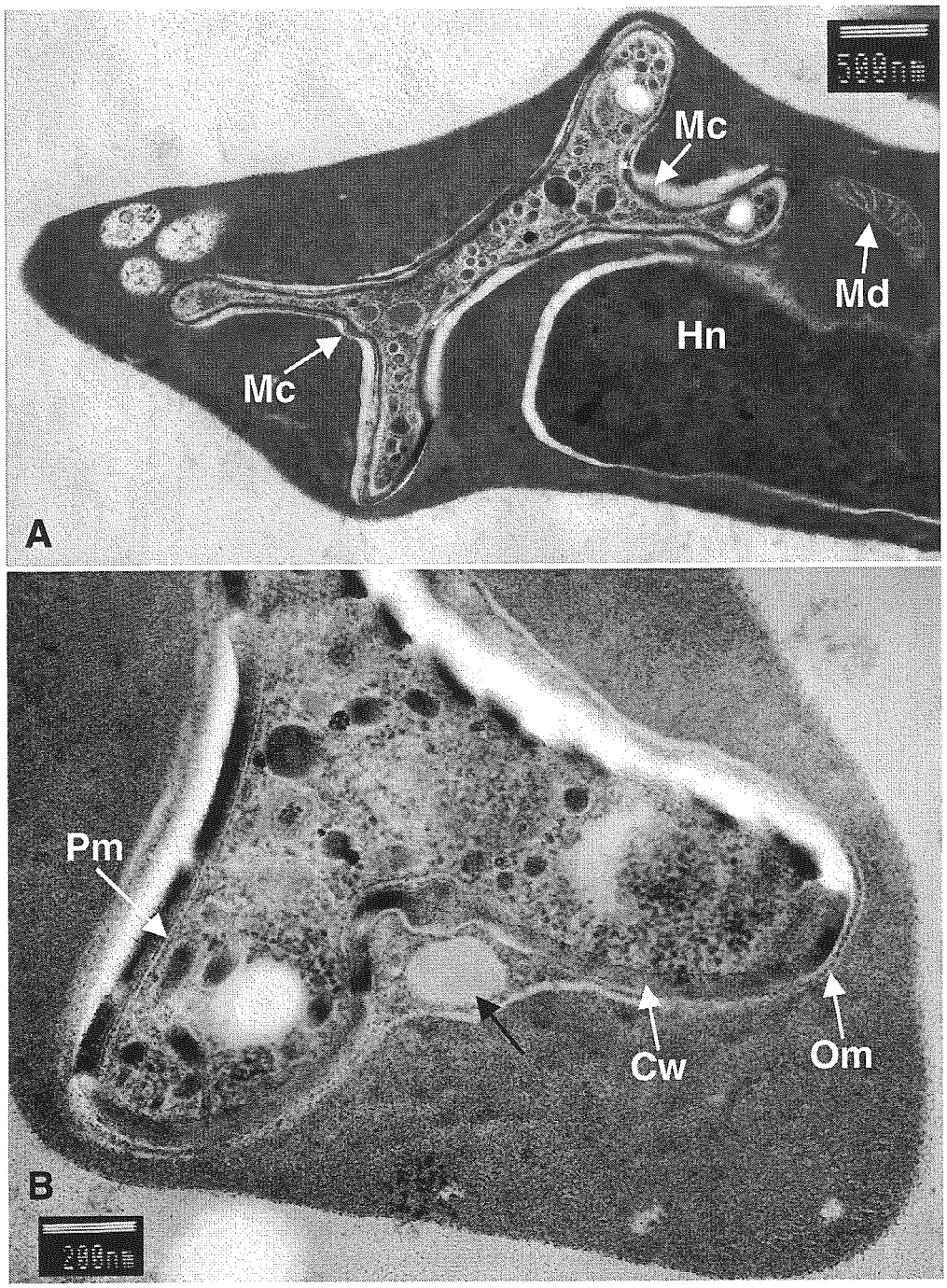

Figure 39. A&B: intraerythrocytic gamonts of *Hem. biplicata* in *E. stokesii* (TEM) [Cw: cyst wall, Hn: host cell nucleus, Mc: micro -canal, Md: host cell mitochondrion, Om: outer membrane, Pm: plasma membrane]. A: encapsulated gamont (lateral/ width); B: encapsulated gamont (lateral/width), black arrow marks structure interpreted as vesicle originating from the micro -canals.

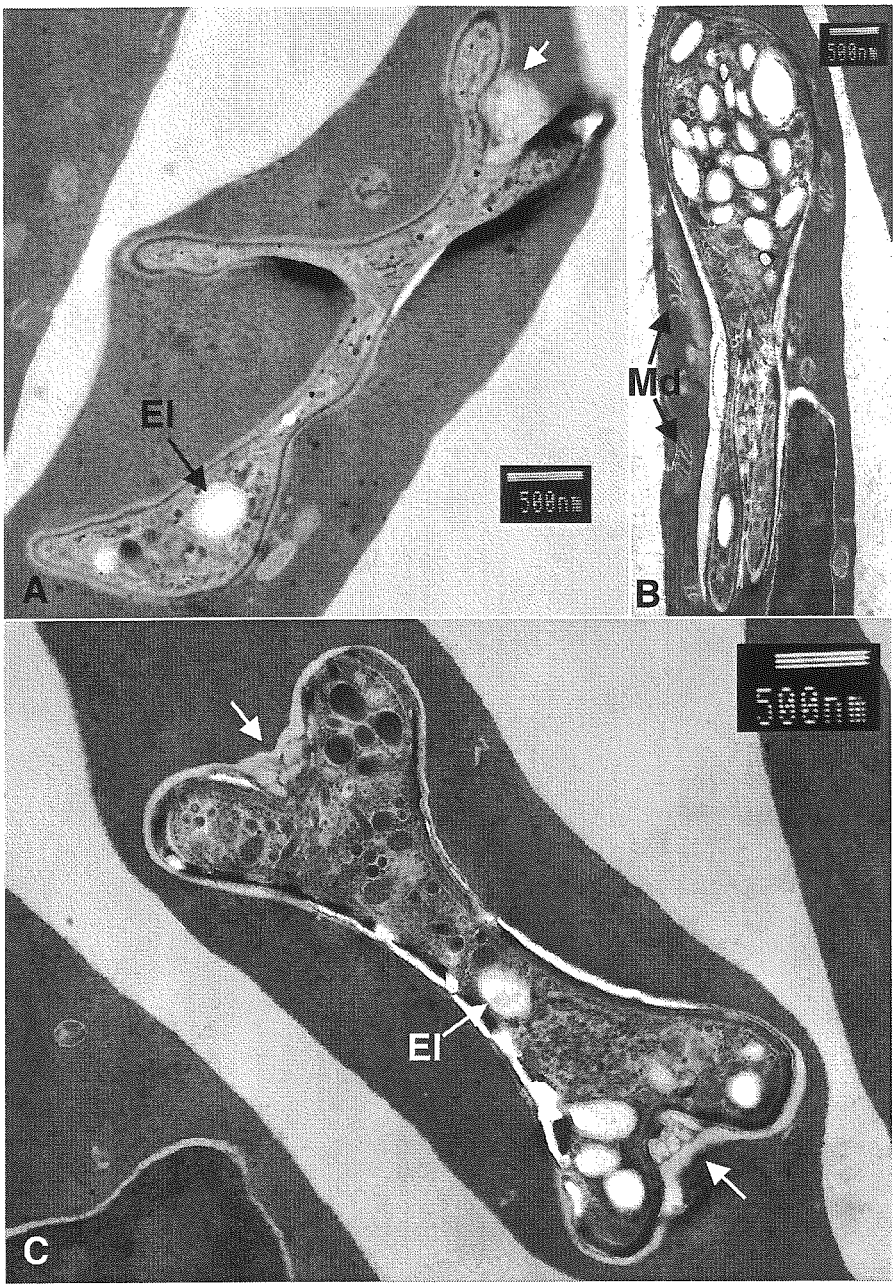

Figure 40. A-C: intraerythrocytic gamonts of *H. biplicata* in *E. stokesii* (TEM) [El: electron lucid body, Md: host cell mitochondrion]. A: encapsulated gamont, irregular shape, white arrows mark structures interpreted as dispersing vesicle originating from the micro-canals; B: encapsulated gamont (lateral/tangential); C: encapsulated gamont, (lateral/width).

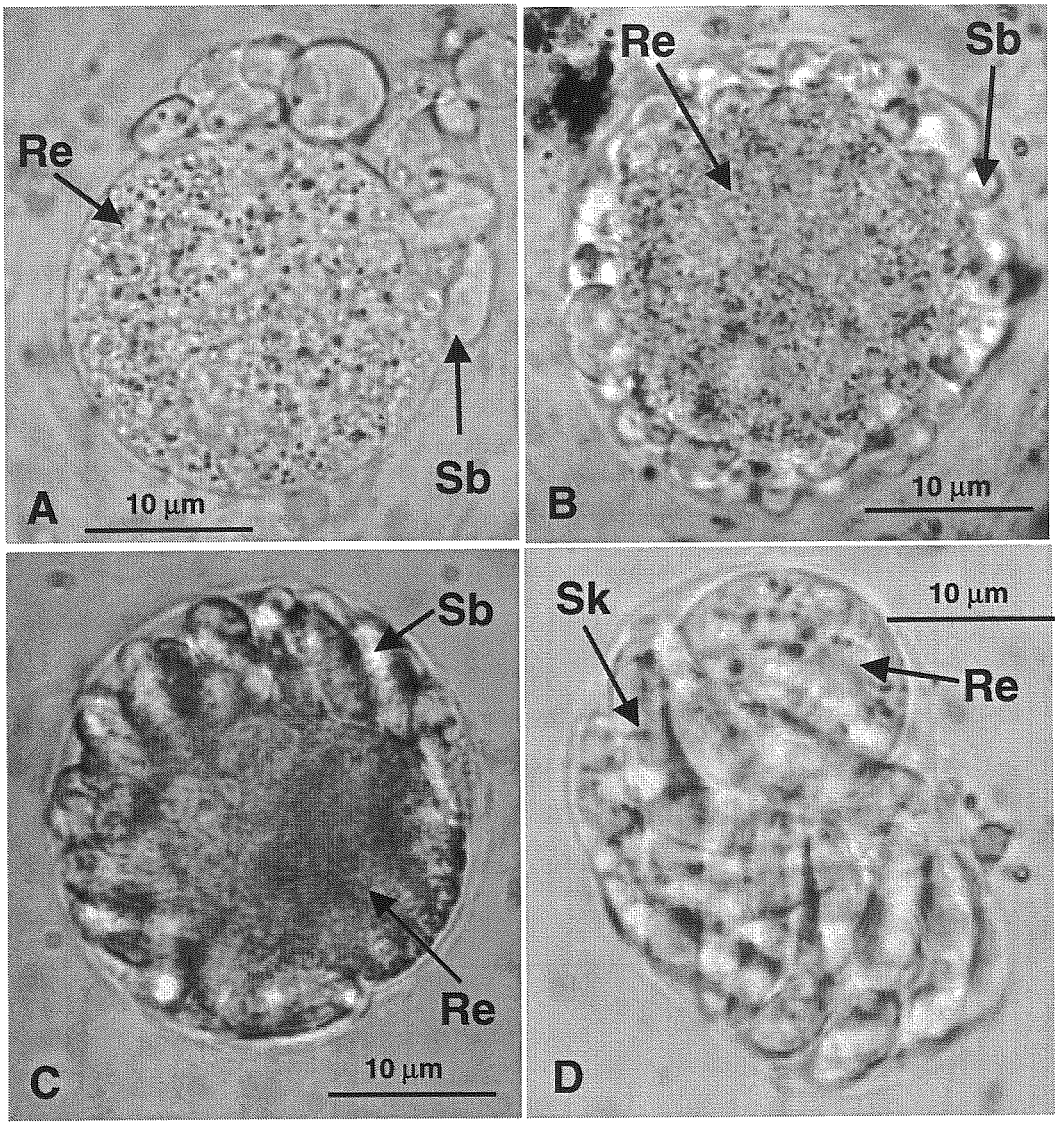

Figure 41. A-D: developing oocysts of *H. biplicata* (wet mount) [Re: residual body, Sb: sporoblast, Sk: sporokinete].

7.1.2.2 The oocyst

Early oocysts measured between 20-23 µm and comprised a large residual body plus young sporoblasts (Figure 41/A-D). With further development, the sporoblasts increased in size and the mass of the residual body was reduced. The size of the usually spherical oocyst did not change substantially during maturation, but it developed a thick cyst wall. Late oocysts grew up to 24 µm in diameter, and were observed floating inside the tick midgut lumen. Mature oocysts contained up to 30 motile sporokinetes. No differences were found between the development of *Hem. biplicata* in *Amb. limbatum* and *Amb. vikirri*.

7.1.3 The locations *Hem. biplicata* where found

All localities where *E. stokesii* infected with *Hem. biplicata* were found in the course of this study are listed in Ta. *Hem. biplicata* was found infecting lizards in far less populations than *Hem. mariae*. However, at sites where it was found *Hem. biplicata* generally had a moderate to high prevalence (for a more detailed comparative analysis of the spatial distribution of the blood parasite species found see chapter fourteen).

Table 23. The coordinates of the sites where *Hem. biplicata* was found infecting *E. stokesii* during the three year field survey. *Hem. biplicata* was not found at the regular study sites.

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [Σ] with *Hem. biplicata* and its prevalence [%]	
	S	W		Σ	%
Chace Range	31°45'	138°35'	7	1	14.3
Gawler Ranges[1]	32°30'	136°50'	12	6	50
Island Lagoon	31°42'	136°68'	14	13	92.9
Jarvis Hill	32°55'	138°21'	10	6	60
Point Lowly[1]	32°50'	137°45'	8	1	12.5
Willow Springs	31°25'	138°50'	4	4	100

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

7.1.4 The transmission experiments

7.1.4.1 Experiment I: transmission from *E. stokesii* with mixed infections

In transmission experiment I, *Amb. limbatum* and *Amb. vikirri* larvae were fed between the 21/02/97 and the 11/03/98 on ten *E. stokesii*. Two of the lizards were uninfected, the other eight had single or mixed blood infections, and six of them were infected with *Hem. biplicata*. After moulting, the resulting unfed nymphs were force fed to ten *E. stokesii* on the 16/05/98; those lizards were uninfected or had only a single blood infection of *Schellackia* sp. at that time. This experiment I was part of a set of experiments that investigated the vector capacity of *Amb. limbatum* and *Amb. vikirri*. They were presented in detail in chapter twelve. Due to the lack of ticks that engorged on *E. stokesii* that were single infected with *Hem. biplicata*, no additional transmission experiments with *E. stokesii* as donors were conducted.

Five out of six lizards that had ingested *Hem. biplicata*-infected nymphs became infected with *Hem. biplicata*. The first chronic stages were detected between 84 and 197 days after the nymphs were ingested. As with *Hem. mariae*, *Hem. biplicata* was transmitted by unengorged nymphs of both species, *Amb. limbatum* and *Amb. vikirri*. Due to the lack of *E. stokesii* with single infections of *Hem. biplicata*, transmissions were only achieved in connection with *Hem. mariae*.

7.1.4.2 Experiment II: transmission from *T. rugosa* to *E. stokesii*

A parasite resembling *Hem. biplicata* was detected in 1998 in the blood of single infected *T. rugosa* from the Mt. Mary region (C. Smallridge, pers. comm.). A transmission experiment was conducted where larvae of *Amb. limbatum* were fed on that *T. rugosa*. Then seven unfed nymphs from those larvae were force fed to one uninfected *E. stokesii*. No transmission of any *Hem. biplicata* like parasite was detected in blood tests in 1-6 week intervals during the following five months.

7.1.4.3 Experiment III: time course of development

Amb. limbatum larvae were attached to *E. stokesii 12* on the 11/03/98. The larvae were from the same egg batch as those used in transmission experiment III of the previous chapter, and they were attached on the same day as those in that experiment III. In Table 24, the results of the subsequent dissections are summarised and compared to the results of the experiment III from the previous chapter. No significant differences were found in the occurrence of

life cycle stages between the ticks that fed on *E. stokesii* infected with *Hem. mariae* and the ticks that fed on the *E. stokesii* with a mixed infection of *Hem. biplicata* and *Hem. mariae*. Only star shaped oocysts were found in experiment III from chapter six. In ticks with mixed infections, both spherical and star shaped oocysts were seen between 15 and 31 days post detachment (Table 25); mature oocysts containing sporokinetes were found towards the end of that phase. Due to their much larger size, star shaped oocysts were very easy to detect, whereas the smaller round oocysts were sometimes very difficult to find. It was therefore very difficult to compare the abundances for both types of oocysts from the tick material.

Table 24. Dissection results of *Amb. limbatum* that have fed as larvae on *E. stokesii* either infected with *Hem. biplicata* and *Hem. mariae (n=18) or* infected with *Hem. mariae* only (n=55) [Exp. III: transmission experiment III, chapter six, Ooc.: oocyst, Spk.: sporokinete, Spc.: sporocyst, Spz.: sporozoite].

	Time span when parasite stages where found during dissections (days post detachment).					
	Ooc.	**$\bar{X}$**	**SD**	**Spk.**	**Spc.**	**Spc. with spz.**
Subadult ticks infected with *Hem. biplicata* and *Hem. mariae*	15-31*	22.8	7.8	26-31	>26	>26
Ticks infected with *Hem. mariae* only (Exp. III)	11-38	23.5	5.7	25-38	>28	>28

*= Spherical and star shaped oocysts found simultaneously

Table 25: Developmental stages of *Hem. biplicata* and *Hem. mariae* found in developing *Amb. limbatum* ticks that had engorged as larvae (n=18) on infected *E. stokesii 12* [Indiv.: individual; Ooc.: oocyst; spher.: spherical; Spk.: sporokinete; Spc.: sporocyst; Spz.: sporozoite].

Days post attach-ment	Days post detach-ment	Eng. larva	Nymph	No visible infection	Ooc.	Spk.	Spc.	Spc. with spz.
26	15	+			*/O			
33	18	+			*/O			
37	19	+			*/O			
32	22	+			*/O			
38	26	+			*/O	+		
37	26	+					+	+
36	27	+			*/O	+		
40	27	+					+	+
40	30		+				+	+
39	31	+			*/O	+		
41	32		+				+	+
42	32		+				+	+
42	33		+				+	+
42	32		+				+	+
49	37		+				+	+
50	38		+				+	+
50	41		+				+	+
53	44		+				+	+

*: star shaped oocyst

O: spherical oocyst

+: instar/stage present

7.2 *Discussion*

The morphological features described for *Hem. biplicata* clearly separate this species from *Hem. mariae.* However, an overall comparison of the life cycle and the morphology of *Hem. mariae* and *Hem. biplicata* revealed more similarities than differences. Yet, despite the rather detailed information on the life cycle, it was difficult to determine the exact taxonomic position for *Hem. biplicata.* Based on the findings described in this chapter, *Hem. biplicata* belonged to the family *Karyolysidae*, because of the occurrence of motile sporokinetes in the oocyst. *Hem. biplicata* is also a newly discovered species because it does not resemble any of the 13 previously described species of this family. The real problem arose with the identification of the generic level within the family *Karyolysidae*, because when the data obtained in this study were considered, the taxonomic characters given to separate the genus *Hemolivia* from the genus *Karyolysus* became questionable.

Petit *et al.* noted several "peculiarities" (sic) of the life cycle of *Hem. stellata* (1990):

1. The occurrence of merogony (schizogony) and cyst formation in the erythrocytes and reticulo-endothelial cells.

   Merogony and cyst formation in erythrocytes have been described from the family Haemogregarinidae and the order Piroplasmorida (Siddall, 1995). Within the Karyolysidae, it was described from *Hem. stellata and Hem. mauritanica.* Erythrocytic merogony has not been described for *Hem. mariae* or *Hem. biplicata,* despite the extremely high parasitemia sometimes detected in *E. stokesii.* It has to be noted that the reticulo-endothelial cells are the preferred site of merogonic development for many adeleorine parasites, eg. *Karyolysus* and *Hepatozoon*, so that character is not of taxonomic value.
2. Sporogonic development inside the gut cells of the tick, with the formation of a star-shaped oocyst.

   Parasites from the genus *Karyolysus* develop within the midgut epithelium of mites, thus it is not the site of development but the vector that according to this definition separate the two genera *Karyolysus* and *Hemolivia.* The formation of a star shaped oocyst is, in the opinion of the author, an autapomorphy of the genus *Hemolivia* and should be used as a taxonomic feature on the generic level. However, in the definition of the creators of the genus, mature oocysts of *Hemolivia* can also be spherical (Landau & Paperna, 1997).
3. Sporokinetes migrate from the oocysts to new digestive cells in the same tick individual, where they divide and give rise to sporocysts containing sporozoites.

The optional development of *Hem. mariae* and *Hem. biplicata* sporozoites in either the midgut epithelium or the reproductive tract of *Amb. limbatum*, depending on the tick instar (according to the findings in this study), questions the taxonomic value of this feature.

In order to assess the taxonomic identity of *Hem. biplicata*, the five characters that remained as probably taxonomically distinctive between *Karyolysus* and *Hemolivia*, listed in Table 26, are discussed below.

1. All previously described species of *Karyolysus* developed in mites and all described species of *Hemolivia* developed in ticks. However, the type of vector was not considered as taxonomically important on the generic level by the authors of the descriptions. Furthermore, the "pigeon-holing" of taxa by their vectors has been criticised as not producing a classification that reflects the phylogeny of the group (Barta, 1989). Consequently, a classification of *Hem. biplicata* by its vector was regarded as inappropriate.
2. All described parasites of the genus *Karyolysus* possess a spherical oocyst. Parasites of the genus *Hemolivia* may develop either a star shaped or a spherical oocyst. *Hem. biplicata* has a spherical oocyst, thus this feature was of no taxonomic value on the generic level.
3. Oocysts and the resulting sporozoites that develop in the same vector individual have been demonstrated in ticks infected with *Hem. mariae, Hem. mauritanica* and *Hem. stellata.* In all previously described *Karyolysus*-species, sporokinetes developed into sporozoites only within the offspring of mites. On the ground of this taxonomic character the generic position of the newly described parasite *Hem. biplicata* was determined to be within *Hemolivia* rather than *Karyolysus.*
4. Sporokinetes entering the reproductive tract of their vectors have been described from all species of *Karyolysus.* As described in here, the same was observed for at least one of the two species *Hem. mariae* or *Hem. biplicata*, with a strong possibility that sporocysts of both species developed exclusively within the reproductive tract of an engorged *Amb. limbatum* female. Because no histological data were available for the previously described species of *Hemolivia* on the site of development of sporocysts in engorged female ticks, this feature could not be employed for taxonomic considerations. However, the optional development of *Hemolivia* sporozoites in the reproductive tract of *Amb. limbatum* considerably weakens the quality of the only character left (see previous point 3.) to distinguish *Hemolivia* from *Karyolysus*, because it indicates that when the option arises, *Hemolivia* sporokinetes behave very similarly to *Karyolysus* sporokinetes. Engorged females

are the only instar of relevance to investigate this behaviour, because in metastriate ticks, the reproductive tract is fully developed only after the engorgement of the female. In all previous examinations, the development of sporozoites in the midgut epithelium of the vector ticks was observed in subadult or unengorged adult stages.

5. Transovarial transmission from the mother mite to the eggs has been demonstrated for all described species of *Karyolysus*. No evidence for transovarial transmission was detected in the life cycle of *Hem. mariae, Hem. biplicata, Hem. mauritanica* or *Hem. stellata*, but to the best knowledge of the author, this topic has never been studied in detail.

Larvae of haematophagous mites do not imbibe blood. The nymphs are the first parasitic stage and they engorge and moult into adults in less than a week. Mites take relatively small blood meals compared to ticks and in contrast to ticks, the adults feed repeatedly on blood. Considering the differences between the tick and mite vectors, it can be speculated that the described differences between the site of the sporozoite development of *Hemolivia* and *Karyolysus* represent adaptations to the biology of their vector. Future research will show whether species of *Hemolivia* are able to develop in mites and whether the sites of their sporogonic development resembles that of *Karyolysus* species when *Hemolivia* develops in a mite host. After a careful analysis of the data available, the suspicion remains that on the ground of its current taxonomic definition, the genus *Hemolivia* is not a monophyletic group. Nevertheless, in this thesis, the genus *Hemolivia* will be retained to classify the two species found in *E. stokesii*.

E. stokesii infected with *Hem. biplicata* and *Hem. mariae* had relatively long prepatent and chronic phases of the infections compared to *E. stokesii* infected with *Hem. mariae* alone. *E. stokesii* experimentally infected with *Hem. mariae* had a prepatent phase between four and ten weeks. They went from a typical steep rise of parasitemia to a relatively short chronic phase; most lizards had reached a relatively low parasitemia when the experiments were terminated. *E. stokesii* that were experimentally infected with both, *Hem. mariae* and *Hem. biplicata*, showed a relatively long prepatent phase (between 12-16 weeks). In many infections a long chronic phase for both species was maintained which was still relatively high when the experiments were terminated. Some *E. stokesii* that were captured with mixed infections in the field maintained a high chronic blood infection for years. Whether these largely extended chronic phases in mixed infections were:

1. a product of the relatively small sample size,

2. influenced by the interactions of the two blood parasite species,
3. related to intrinsic factors of a lizard host with mixed infections,
4. due to the possible frequent occurrence of super infections,
5. or were triggered by the uptake of extremely high loads of infective agents, remains to be investigated.

Table 26: Five taxonomic characters that were evaluated for the differentiation between the genera *Hemolivia* and *Karyolysus* (*K.* spp.: valid for all ten described species of *Karyolysus*).

	Taxonomic character	*K. spp.*	*H. biplicata*	*H. mariae*	*H. stellata*	*H. mauritanica*
1.	Type of vector	Mite	Tick	Tick	Tick	Tick
2.	Shape of oocysts	Spherical	Spherical	Star	Star	Spherical
3.	Oocysts and resulting sporozoites develop in the same vector individual	-	+	+	+	(+[2])
4.	Sporokinetes enter reproductive tract of engorged adult female vector	+	(+[1])	(+[1])	?	?
5.	Transovarial transmission	+	?	?	?	?

(+[1]) = discovered in one *Amb. limbatum* with mixed infection of *H. biplicata* and *H. mariae*.

(+[2]) = redescribed from fixed material of the E. Brumpt collection by Landau & Paperna (1997).

Chapter Eight

8. SCHELLACKIA SP. (TYPE 3)

8.1 Introduction

A type of blood parasite that was significantly different in size from *Hem. mariae* and *Hem. biplicata* (Figure 13 and Table 9&10) was found frequently infecting wild caught *E. stokesii*. The parasite was postulated to be the encapsulated sporozoite of an eimeriorine species from the genus *Schellackia*, because not more than eight sporozoites were found in oocysts in the digestive tract of *E. stokesii* infected with *Schellackia* sp.. This *Schellackia* species was the most prevalent blood parasite found in blood smears of *E. stokesii* from the Flinders Ranges (for details see chapter fourteen). Even *E. stokesii* born and raised in the ACU frequently became spontaneously infected with *Schellackia* sp., which made the transmission experiments difficult to evaluate.

In order to identify possible pathways of parasite transmission, smears and histological sections from the placentae of two newborn lizards were studied. The placentae had to be seized immediately after birth to prevent their ingestion by the newborns (see also 3.1.3.2). Vector studies in the laboratory focused on the ticks *Amb. limbatum* and *Amb. vikirri*, but the mosquitoes *Aedes notoscriptus* and *Culex quinquefasciatus* were also investigated. Dissections of the field caught sandfly *Australophlebotomus. dycii*, ultrastructural images of blood stages and long term observations of *E. stokesii* born in the ACU were also evaluated.

In order to identify the eimeriorine parasite and its life cycle, *E.* stokesii *356* and *E. stokesii 177*, that both had a chronic blood infection with *Schellackia* sp. (Figure 42 and Figure 43), were dissected (Table 27). Both lizards were born in the ACU and were found spontaneously infected with *Schellackia* sp. during the regular bleedings of lizards kept in the ACU. In addition, the ACU-born *E. stokesii 222*, that was dissected in the course of transmission experiments with *P. mackerrasae* (for details see chapter ten), was found to be infected with *Schellackia* sp., although up to the time of euthanasia the infection had not been detected in the blood cells during routine bleedings.

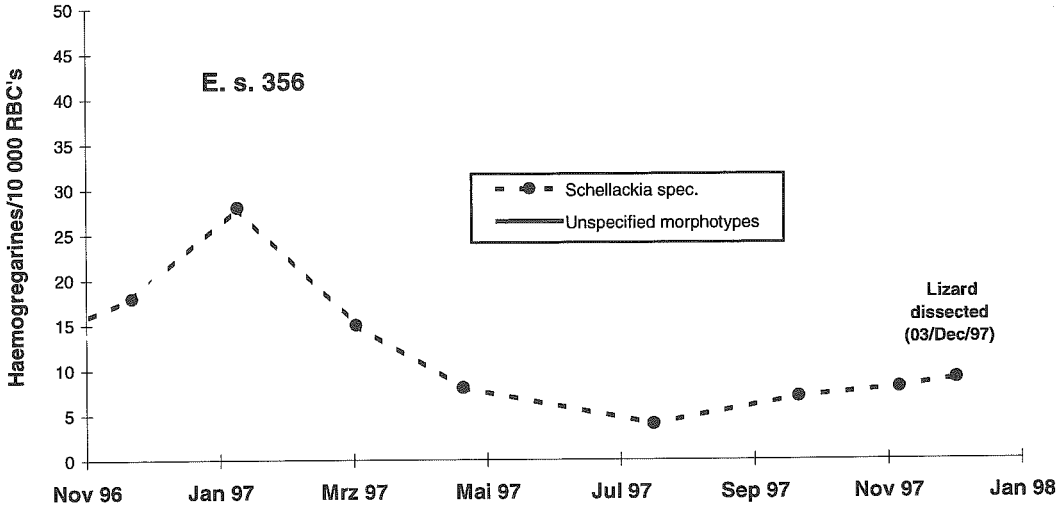

Figure 42. The presence of blood parasite stages in *E. stokesii 356*, spontaneously infected with *Schellackia* sp. in the ACU. The lizard was killed and dissected on the 03/12/97.

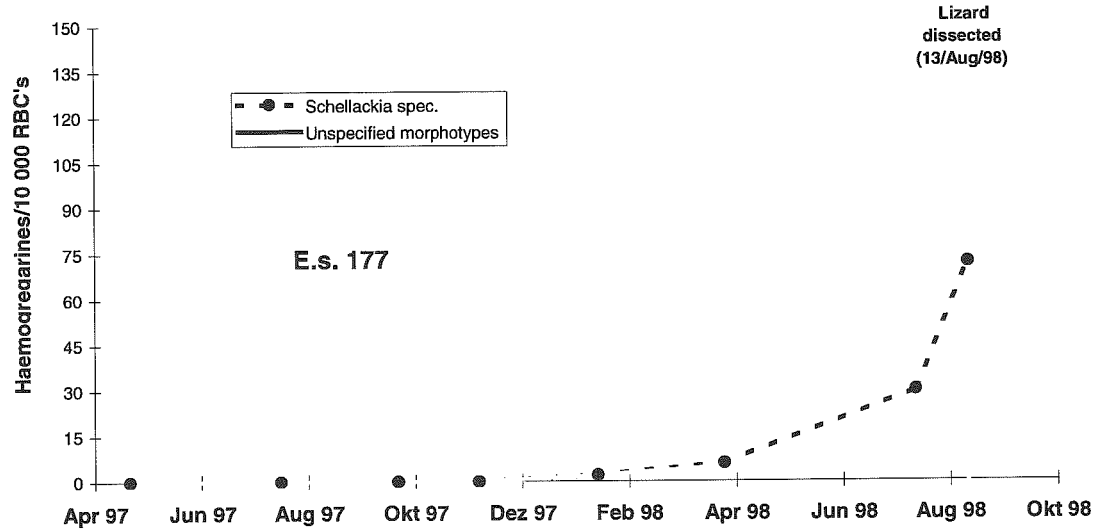

Figure 43. The presence of blood parasite stages in *E. stokesii 177*, spontaneously infected with *Schellackia* sp. in the ACU. The lizard was killed and dissected on the 13/08/98.

As a part of the comprehensive autopsy, faecal flotations of all three lizards disclosed the presence of a second eimeriorine species (Figure 7/A, p. 31). In the case of *E. stokesii 356*, an oxyurid nematode species (Figure 7/B) and a parasite of unknown taxonomic origin (Figure 7/C) was also present. The second eimeriorine parasite was suggested as belonging to the genus *Eimeria* by the presence of round oocysts that contained four sporocysts with two sporozoites respectively when sporulated. The *Eimeria* parasite was found in five out of six further faecal flotations from other individual *E. stokesii* housed in the ACU. Sporulated *Eimeria* oocysts were identified from faeces flotations of three *E. stokesii* where the other two intestinal parasites could not be detected. Because *Eimeria* parasites are transmitted by smear infections and all experimental lizards were kept in groups of two or more, the probability that all lizards kept in the ACU were infected with *Eimeria* sp. was very high. The origin of this parasite is unclear. Several other *Egernia* and *Tiliqua* lizard species were also housed in the ACU and the parasite could have come from any of them.

Table 27. Dissections of two *E. stokesii* born in the ACU that were spontaneously infected with *Schellackia* sp. and that had chronic blood infections at the time of the dissection.

E. stokesii	Date of birth (at the ACU)	Date of dissection
353	2/95	03/12/98
177	3/97	13/08/98

Eimeria parasites are postulated to be the evolutionary ancestors of the eimeriorine blood parasites. Within the intestinal tract of a vertebrate host, most of the life cycle stages of *Eimeria* and *Schellackia* species are identical and it is impossible to distinguish between the haemococcidia and the intestinal coccidia by light microscopy. Thus, apart from mature oocysts, the life cycle stages of *Schellackia* sp. in the digestive tract of *E. stokesii* could not be studied histologically without confusing them with the life cycle stages of the *Eimeria* species. For that reason, further dissections of lizards to identify the life cycle of *Schellackia* sp. were not conducted. Unsporulated oocysts of the *Eimeria* species were seen in abundance the midgut lumen of *E. stokesii 177, 356* and *222*, but only two maturing oocyst that were interpreted to belong to an eimeriorine blood parasite of the genus *Schellackia* were found.

8.1.1 The life cycle

On the basis of the parasite stages found in *E. stokesii 356* and *177* and *222*, only a few conjectures were possible. Histological sections throughout the intestinal tract of both

lizards showed eimeriorine sporozoites as well as merogonic stages and zygotes within the gut epithelium. Many zygotes were also found in the lamina propria. Developing oocysts were observed mainly in the lamina propria or in the midgut lumen. The latter obviously belonged to the *Eimeria* species, but the young oocysts and sporozoites in the lamina propria could not be differentiated into two separate species. Only a few developing oocysts in the lamina propria were seen that contained visible sporozoites, and single sporozoites were not easily distinguishable. The number of sporozoites found never exceeded eight. Two stages interpreted as developing oocysts containing four and six sporozoites respectively were found in the capillary epithelium of the heart and liver. Sections of the major organs showed many sporozoites of *Schellackia* sp. within the blood cells of the capillaries and many of thick walled cysts containing up to six sporozoites in the spleen. A few of these cystic stages were also found in the liver, but not in any other of the various tissues studied. The cysts were not intraerythrocytic, but were most commonly found in the outer layers of the spleen and were often associated with the connective tissue around the spleen.

It was assumed that the *Schellackia* parasite develops mainly in the intestinal tract, from where the resulting sporozoites enter the blood stream. The sporozoites represent the unspecified morphotypes of this life cycle; a process of sporozoite encapsulation leads to the chronic stage. Probably sporozoites can undergo an endodyogenic cycle of multiplication in the spleen. Some oocysts may develop in the epithelial cells of the capillaries of the heart and liver.

No parasites were detected in smears and dissections of two placentae that were withheld from two newborns after birth, but the parasitemia of the single infected mother lizard with *Schellackia* sp. was low around the time of birth. The mother was caught in the field only weeks before it gave birth to the babies in March 1998 and maintained a relatively low parasitemia (0.02%) with *Schellackia* sp. until this study was terminated in September 1998.

Sporozoites were found unchanged in wet mounts of freshly engorged *Amb. limbatum* and on one occasion in a freshly engorged *A. dycii* (Table 39, p. 181). No developmental stages other than sporozoites were detected in wet mounts of freshly engorged *Amb. limbatum* and *Amb. vikirri* larvae that had fed on *E. stokesii* with single infections of *Schellackia* sp.. No parasitic stages were detected in wet mounts of ticks after they moulted to the next instar. Histological sections were not conducted, because the relatively low parasitemias of

Schellackia sp. made the search for intracellular parasite stages in a vector a very laborious task. Due to the frequent occurrence of spontaneous infections, no conclusive results were obtained from transmission experiments where ticks that fed on *E. stokesii* were fed to *E. stokesii* uninfected with *Schellackia* sp.. These transmission experiments are described in more detail in chapter twelve.

7.1.4 The morphology

Most of the life cycle stages found in the dissections of the digestive tract of *E. stokesii 356*, *177* and *222* could not be distinguished with certainty into two separated eimeriorine species, thus the description of the morphology of *Schellackia* sp. will concentrate on the developmental stages that were discernible. The few spherical oocysts from lizard digestive tract that contained naked sporozoites measured 10-11 μm in diameter. The spherical to ovoid tissue cysts found in the liver and spleen always had a thick cyst wall, contained up to six sporozoites and measured 8-14 μm in diameter. The various types of blood stages (Figure 73) were interpreted as either sporozoites that were released from oocysts or as sporozoites that were derived from the cysts in the spleen and liver. In a giemsa stain, sporozoites appeared either round or elongated, with a terminal or central nucleus either condensed or scattered throughout the parasite cytoplasm (Figure 73). The round stages often displaced the host cell nucleus, but no other effects on the host cell were noted. The chronic blood stages of *Schellackia* sp. gradually lost their stainability with giemsa. TEM images revealed that the three dimensional structure of the blood stages of *Schellackia* sp. was either spherical, drop- or banana-shaped. The spherical morphotypes often contained a sporozoite within a large parasitophorous vacuole (Figure 44/A). The shape of the chronic stage was rather more cylindrical than the flat platelet of the two *Hemolivia* species. The chronic stage of *Schellackia* sp. had a highly characteristic bean shape, with a thick cyst wall developing outside the probably trilaminar plasma membrane (Figure 44/C). Structures interpreted as ducts or pores that permeated the cyst wall were visible (Figure 44/D). Numerous invaginations and folds of the plasma membrane were regularly seen at one end of the encapsulated sporozoite (Figure 44/B, C&D).

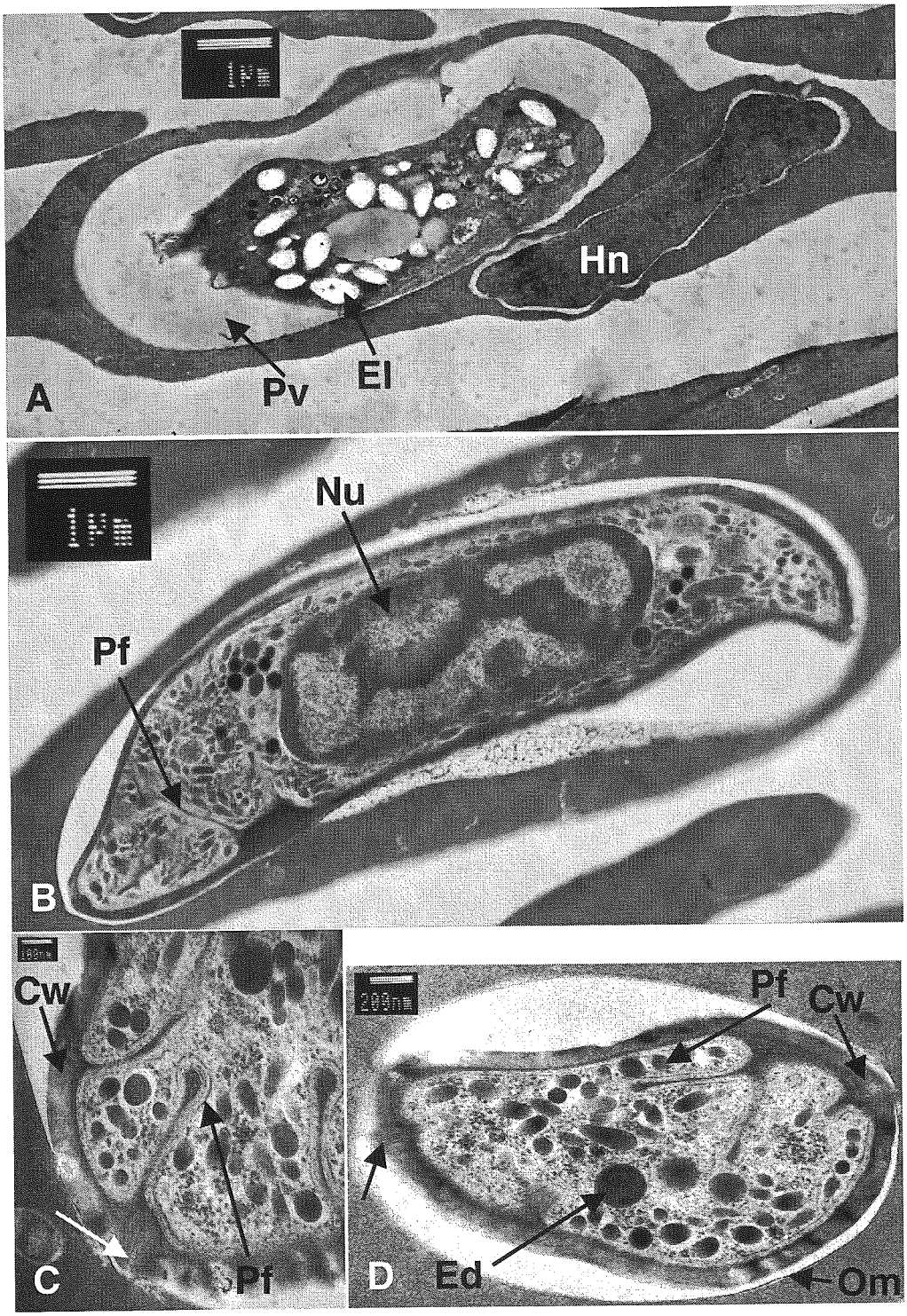

Figure 44. A-D: intraerythrocytic sporozoites of *Schellackia* sp. in *E. stokesii* (TEM) [Cw: cyst wall, Ed: electron dense body, El: electron lucid body, Hn: host cell nucleus, Om: outer membrane, Pf: plasma membrane fold Pv: parasitophorous vacuole]. A: morphotype "C", (dorsal); B: encapsulating sporozoite (dorsal); C&D: encapsulated sporozoite (ventral/tangential), unlabelled arrows mark structure permeating the cyst wall.

8.1.3 The locations were *Schellackia* sp. was found

All localities where *E. stokesii* infected with *Schellackia* sp. were found in the course of this study are listed in Table 28. *Schellackia* sp. was the most prevalent parasite found (see also chapter 14).

Table 28. The coordinates of the sites where *Schellackia* sp. was found infecting *E. stokesii* during the three year field survey (study sites in bold).

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [Σ] with *Schellackia* sp. and its prevalence [%]	
	S	W		Σ	%
Billa Kallinna	31°24'	136°57'	8	1	12.5
Camel Hill (CH)	31°53'	138°25'	**148**	*	**78.8**
Chace Range	31°45'	138°35'	7	1	14.3
Drakes Nob (DN)	32°05'	138°09'	**27**	*	**48.2**
Druid Range	31°50'	138°40'	4	1	25.0
Gawler Ranges[1]	32°30'	136°50'	12	3	25.0
Glenlyle (GL)	31°50'	138°35'	**62**	*	**43.5**
Hawker North (HN)	31°53'	138°25'	**97**	*	**58.2**
Hawker South (HS)	31°53'	138°25'	**159**	*	**67.2**
Island Lagoon	31°42'	136°68'	14	4	28.6
Jarvis Hill	32°55'	138°21'	10	4	40.0
Neuroodla (NR)	31°50'	138°08'	**68**	*	**67.9**
Partacoona	32°00'	138°10'	6	4	66.7
Pichi Richi	32°20'	138°00'	27	3	11.1
Point Lowly[1]	32°50'	137°45'	8	2	25.0
Quorn	32°15'	138°05'	8	4	50.0
Warruw. Hill	31°55'	138°25'	8	7	87.5
Willow Springs	31°25'	138°50'	4	3	75.0
Yapalla	31°50'	138°20"	4	1	25.0

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

* = Data of *E. stokesii* individuals were pooled over three years (for details see chapter 14)

8.1.4 Spontaneous infections of *E. stokesii* with *Schellackia* sp.

For the present study, a sub-sample of three cohorts of *E. stokesii* that were born and raised in the ACU were bled at regular intervals to monitor for infections. In all three cohorts, the baby lizards investigated were chosen at random. The mothers of the first cohort of lizards were captured by Mr. Glen Duffield and the babies were born in February 1995[8]. In most cases, the babies were kept with their mother in one aquarium for approximately one year, until they grew too large to be housed together. Most of the mother lizards had blood infections with *Schellackia* sp. at the time of birth. The selected newborns were bled for the first time in July 1995, when this study began. The mothers of subsequent cohorts were either caught in the field or were born in the ACU. The newborns were regularly bled from approximately six weeks after birth. *E. stokesii* born in 1995 and 1996 were bled until September 1998, newborns born in 1997 were bled until July 1998, after which most of them were used for transmission experiments. The lizards born in 1998 were not included into this analysis, because the time span until the observations were terminated was too short. A total of 53 *E. stokesii* were kept for long term observations (Table 29). The results of their blood-tests will be analysed in the following. From the 53 *E. stokesii* monitored, 44 became spontaneously infected with *Schellackia* sp. during the course of the study. The average onset of the infection occurred between 254 and 355 days after birth, depending on the cohort. Some lizards of the first cohort showed a spontaneous blood infection almost three years after they were born (Figure 46), which resulted in a high variability of the average onset of infection (Table 29). A typical infection began with the appearance of morphotypes B and C (Figure 45), which usually peaked in abundance within a few weeks after their first appearance. This peak was followed usually within two months by a peak of the chronic stage. From there on, the chronic stage was usually the most frequent one, but morphotypes B and C were also common throughout the chronic phase of the infection; morphotype A was extremely rare. Because there were no mixed infections observed in the newborns, in the following, all blood forms are assumed to be *Schellackia* sp. and the unspecified morphotypes and the chronic blood stage of *Schellackia* sp. were added up and displayed as one parasitemia-curve.

The blood of 18 *E. stokesii* born in 1995 was monitored regularly over three consecutive years. All four of their mother lizards had chronic blood infections with *Schellackia* sp.; one

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[8] The timing of birth in *E. stokesii* is relatively tightly entrained to the months February and March (Duffield & Bull, 1996).

of the mothers had a mixed chronic infection with *Hem. mariae* and *Hem. biplicata* (Table 30). Sixteen of the newborns became spontaneously infected with *Schellackia* sp., ten of them became infected within one year after birth (Figure 46). The two *E. stokesii* whose blood remained uninfected until the end of the study came from the same mother, *E. stokesii 7* (Table 30). A characteristic pattern of the blood infections was that, after the onset of infection, most of the newborns of the season 1995 developed a peak parasitemia around spring and summer 1996 (Figure 47 and Figure 48). The remaining lizards were either long term uninfected (two *E. stokesii)* or had a less defined peak of parasitemia around the spring and the summer of 1997 (Figure 49 and Figure 50). Furthermore, the first peak of parasitemia was usually followed by a steady decline during the following months. Some of the lizards that became infected within one year after birth showed a small increase in parasitemia during the spring and summer of their second year, but the blood of most of them became increasingly infected towards the third year, when the experiments were terminated. The patterns of parasitemia were less distinct in the newborns where blood became infected later than one year after birth.

The blood of 14 *E. stokesii* born in 1996 was monitored regularly over two consecutive years. All five mother lizards had a chronic blood infection with *Schellackia* sp.; one of them also had chronic infections with *Hem. mariae*, *Hem. biplicata and Hepatozoon* sp. (Table 31). Thirteen of the newborns became spontaneously infected with *Schellackia* sp., eleven of the lizards became infected within one year of birth (Figure 51). Like in the previous year, and despite the fact that they were kept under the constant environmental conditions of the ACU, most lizards of the 1996 cohort developed a peak parasitemia around summer and autumn of 1997 (Figure 52 and Figure 53). Only one lizard was found to be long term uninfected. Two *E. stokesii* became infected later than one year after birth, their parasitemia peaked during the summer and autumn of 1998 (Figure 55). Like in the previous year, the first peak of parasitemia was often followed by a steady decline during the following winter months, but in contrast to 1995, the following patterns of parasitemia were erratic. Some lizards showed a steady decline in parasitemia and some developed a second peak of parasitemia before the experiments were terminated (Figure 54).

A total of 21 *E. stokesii* was selected from the lizards born in the ACU in 1997 and their regular blood smears were monitored over the following 18 months. Fifteen of them became spontaneously infected with *Schellackia* sp. during this time. Seven out of eight

mother lizards had a chronic blood infection with *Schellackia* sp. (Table 32). One mother was negative for blood parasites at the time of birth, but a very low chronic blood infection of *Schellackia* sp. was discovered in this lizard (*E. stokesii 11*) three weeks after birth. The blood of four out of the eight mother lizards was diagnosed with various chronic mixed infections. Two of the six newborn lizards that remained uninfected until this study ended came from the mother with no detectable blood infection of *Schellackia* sp. at the time of birth (*E. stokesii 11*), but a third sibling became infected (Table 32). The onset of the spontaneous blood infections in the newborns was on average later than in the two previous years (Table 29). It was not as strongly entrained to the spring and summer of the first year after birth (Figure 56). In most cases the patterns of parasitemia of the blood infections showed a steady increase until the experiment was terminated (Figure 57 and Figure 58).

Table 29. The average time span until the onset of spontaneous infections with *Schellackia* sp. in individual *E. stokesii* born in three consecutive seasons in the ACU (median and quartiles of days since birth). The lizards were bled in 3-10 week intervals until the experiments were terminated.

Born in season	Number of *E. s.* monitored	Number of *E. s.* spontaneously infected		Onset of infection (days)	
			%	Median	Quartiles
1995	18	16	89	254	455
1996	14	13	93	226	48
1997	21	15	71	355	181
	53	44	83		

Table 30. The occurrence of spontaneous infections in long term monitored *E. stokesii* from clutches born in the ACU in February 1995 and the earliest available infection status of the mother lizards (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *Hem. b.*: *H. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp. P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A.. v.*: *Amb. vikirri*).

Infection status of the mother lizard at the beginning of the study (parasitemia in 10^4 RBC's)							Time of birth	Numbers of *E. stokesii* born in the ACU found with spontaneous infections						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*		Clutch size	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
1	*7*[1]			6				Feb. 95	3			1			
2	*310*[1]			6				Feb. 95	7			7			
3	*134*[2]	82	4	19				Feb. 95	3			3			
4	*279*[2]			6				Feb. 95	5			5			
									18			16			

[1] = first bled in July 1995
[2] = first bled in September 1995

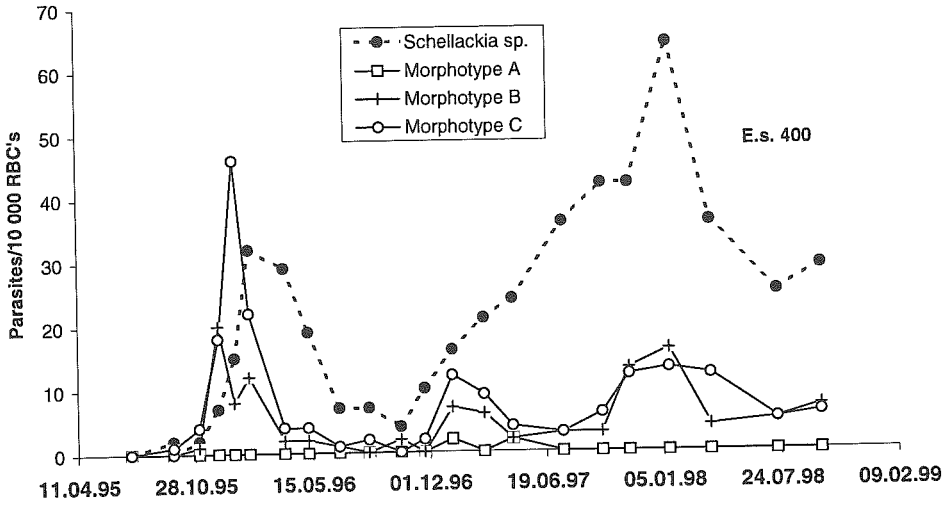

Figure 45. The presence of blood parasite stages in *E. stokesii 400*, spontaneously infected with *Schellackia* sp. in July 1995. The unspecified morphotypes were subdivided into type A, B and C.

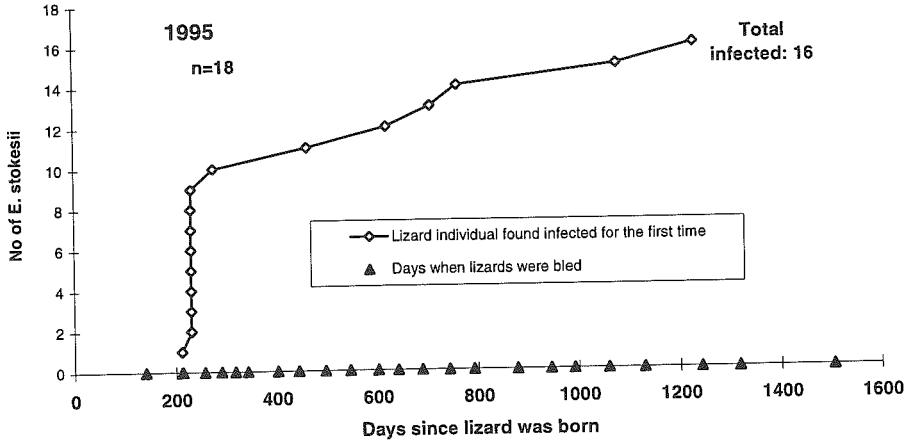

Figure 46. The onset of blood infections with *Schellackia* sp. in randomly selected lizards born in the ACU in 1995.

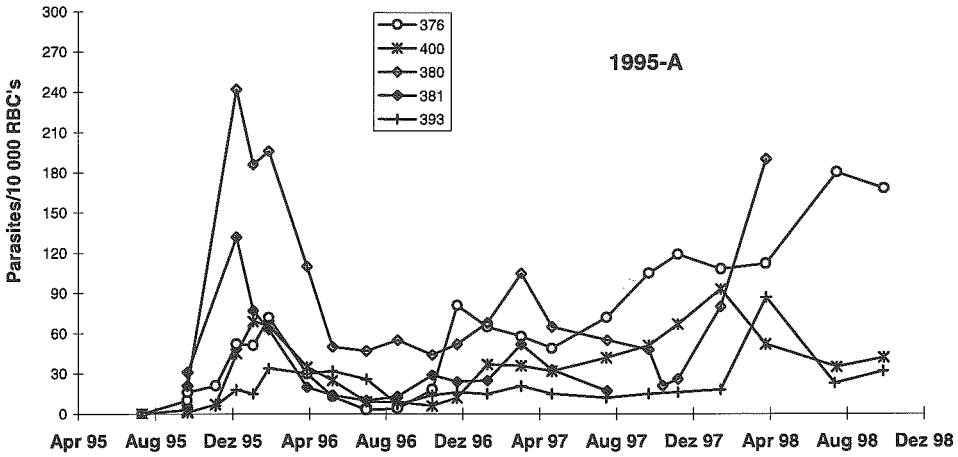

Figure 47. The patterns of parasitemia of *Schellackia* sp. in five *E. stokesii* individuals born in the ACU in 1995.

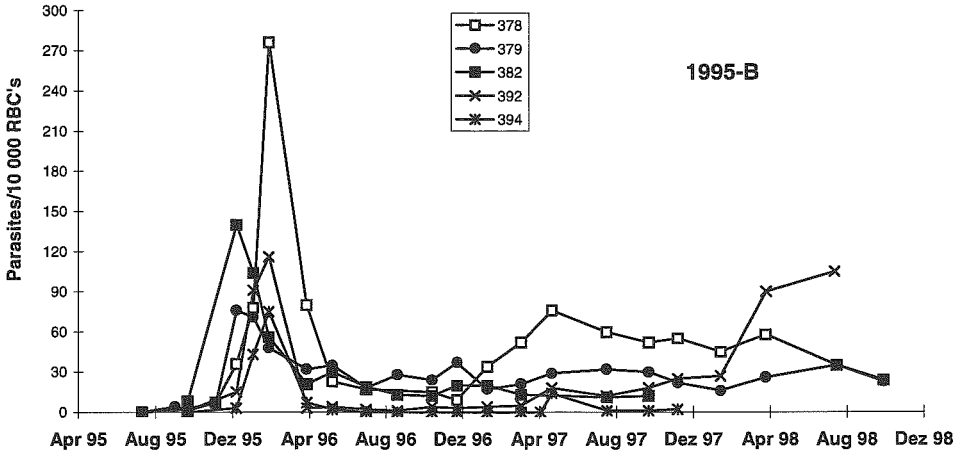

Figure 48. The patterns of parasitemia of *Schellackia* sp. in five *E. stokesii* individuals born in the ACU in 1995.

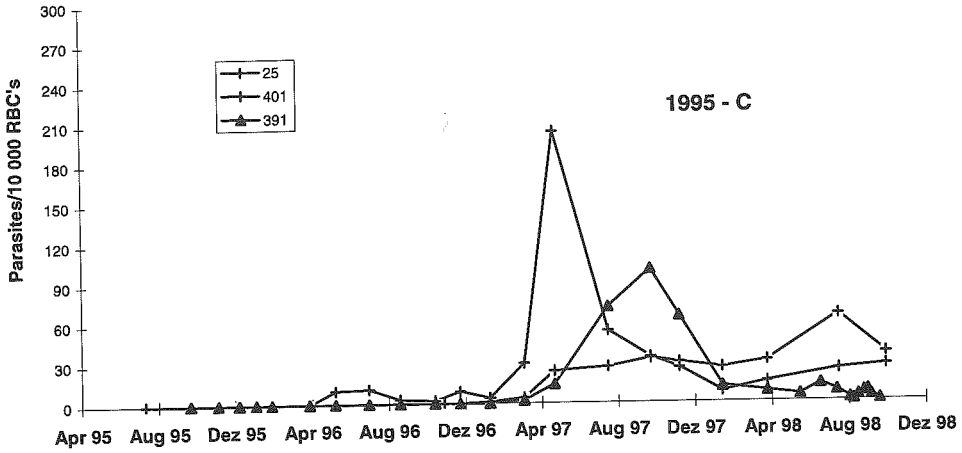

Figure 49. The patterns of parasitemia of *Schellackia* sp. in three *E. stokesii* individuals born in the ACU in 1995.

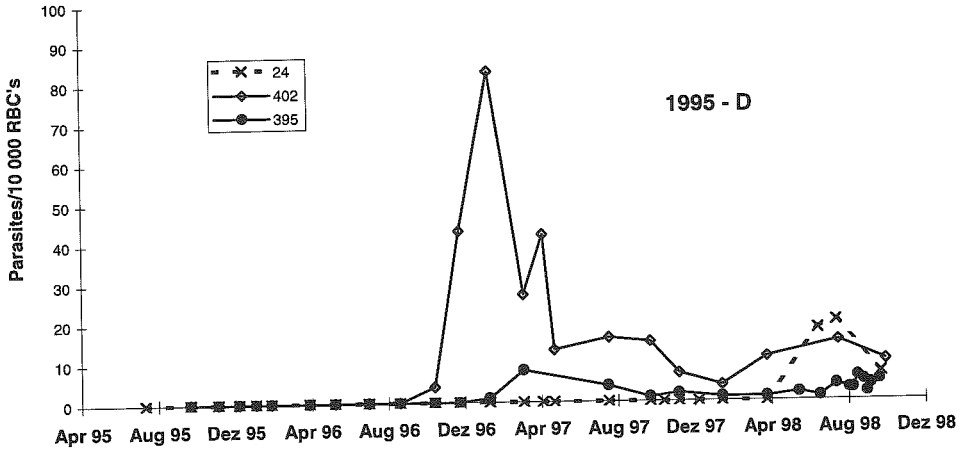

Figure 50. The patterns of parasitemia of *Schellackia* sp. in *E. stokesii* born in the ACU in 1995.

Table 31. The occurrence of spontaneous infections in long term monitored *E. stokesii* from clutches born in the ACU in February 1996 and the infection status of the mother lizards (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *H. b.*: *Hem. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp. P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A.. v.*: *Amb. vikirri*).

Infection status of the mother lizard on the 17th of January 1996 (parasitemia in 10^4 RBC's).								**Time of birth**	**Numbers of *E. stokesii* found with spontaneous infections (experiment terminated in Mar. 99)**						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*		No. of *E. stokesii* monitored	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
1	*352*	39	49	5	18			Feb. 96	2			1			
2	*100*			4				Feb. 96	2			2			
3	*655*			4				Feb. 96	4			4			
4	*700*	-1		1				Feb. 96	2			2			
5	*751*			2				Feb. 96	4			4			
									14			13			

-1 = Was found to be infected in March 1996

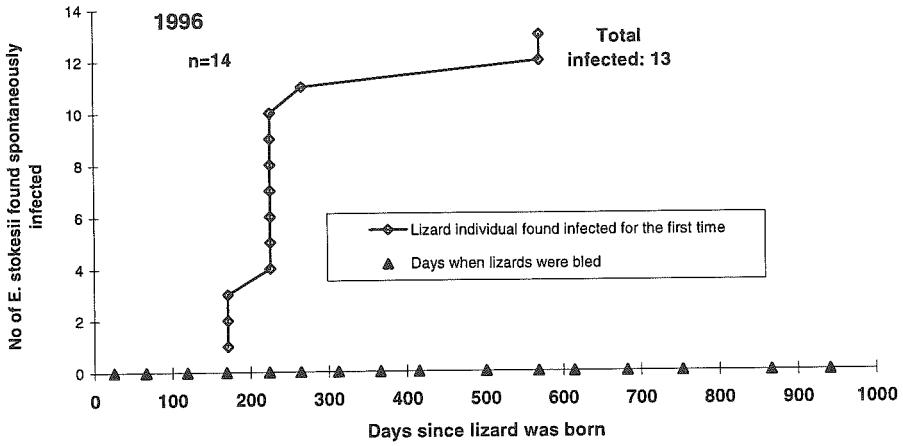

Figure 51. The onset of blood infections with *Schellackia* sp. in randomly selected lizards born in the ACU in 1996.

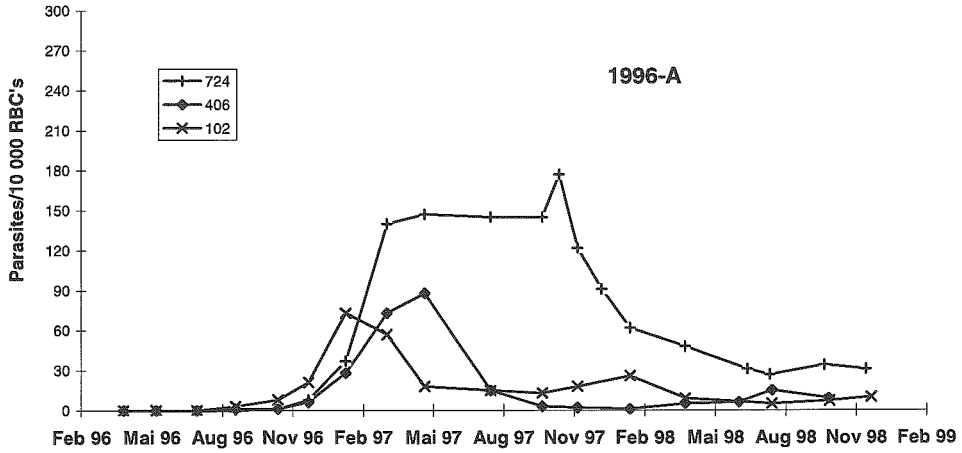

Figure 52. The patterns of parasitemia of *Schellackia* sp. in three *E. stokesii* individuals born in the ACU in 1996.

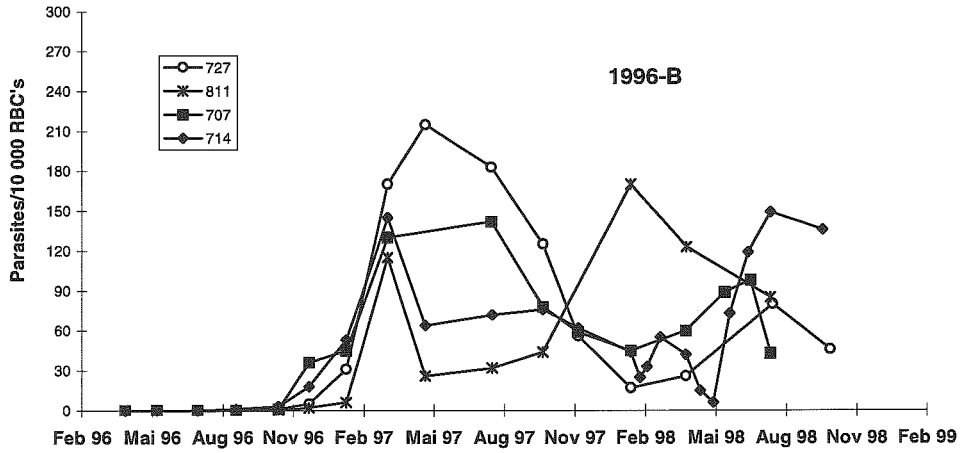

Figure 53. The patterns of parasitemia of *Schellackia* sp. in four *E. stokesii* individuals born in the ACU in 1996.

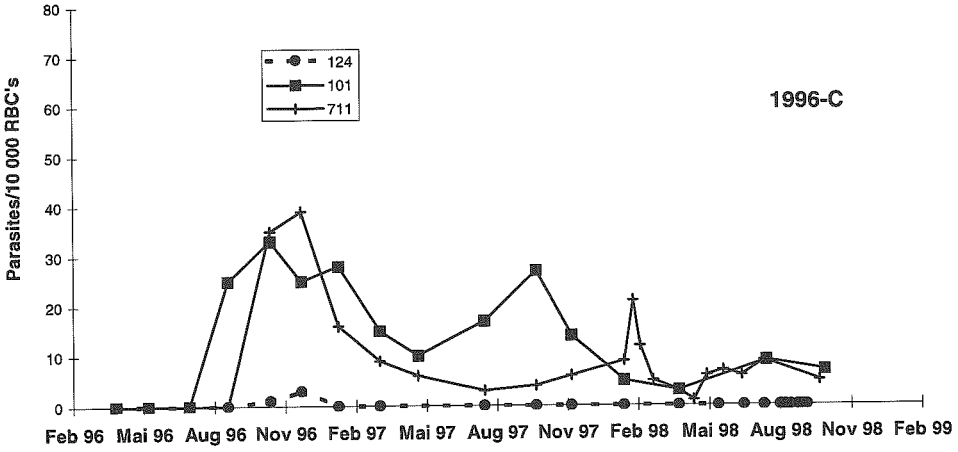

Figure 54. The patterns of parasitemia of *Schellackia* sp. in three *E. stokesii* individuals born in the ACU in 1996.

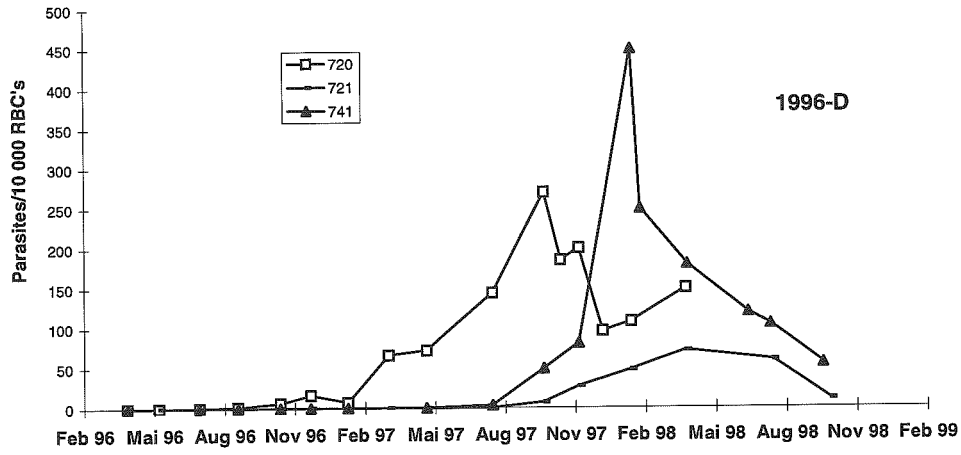

Figure 55. The patterns of parasitemia of *Schellackia* sp. in three *E. stokesii* individuals born in the ACU in 1996.

Table 32. The occurrence of spontaneous infections in long term monitored *E. stokesii* from clutches born in the ACU in February 1997 and the infection status of the mother lizards (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *H. b.*: *Hem. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp.* *P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A.. v.*: *Amb. vikirri*).

Infection status of the mother lizard on the 4th of March 1997 (parasitemia in 10^4 RBC's).								**Time of birth**	**Numbers of *E. stokesii* found with spontaneous infections (experiment terminated in Jul. 98)**						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*		No. of *E. stokesii* monitored	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
1	*7*			1				Mar. 97	1			1			
2	*11*	220	330	-1	10	-1,2		Mar. 97	3			1			
3	*200*			2				Mar. 97	5			2			
4	*202*	2		2		23		Mar. 97	1			1			
5	*207*	16		3		49		Mar. 97	3			3			
6	*210*			1				Mar. 97	5			4			
7	*400*			21				Jan. 97	1			1			
8	*442*	1	1	2				Mar. 97	2			2			
									21			15			

-1 = Was found to be infected later
-2 = A relapse occurred in June 1998

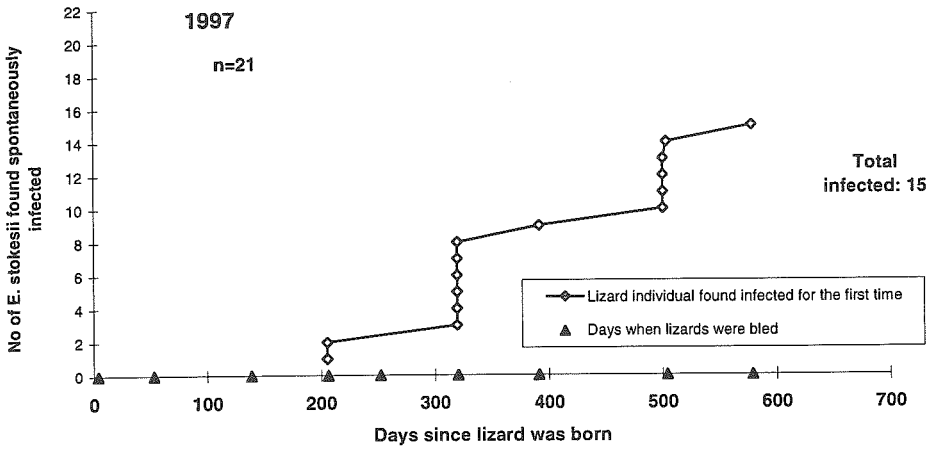

Figure 56. The onset of blood infections with *Schellackia* sp. in randomly selected lizards born in the ACU in 1997.

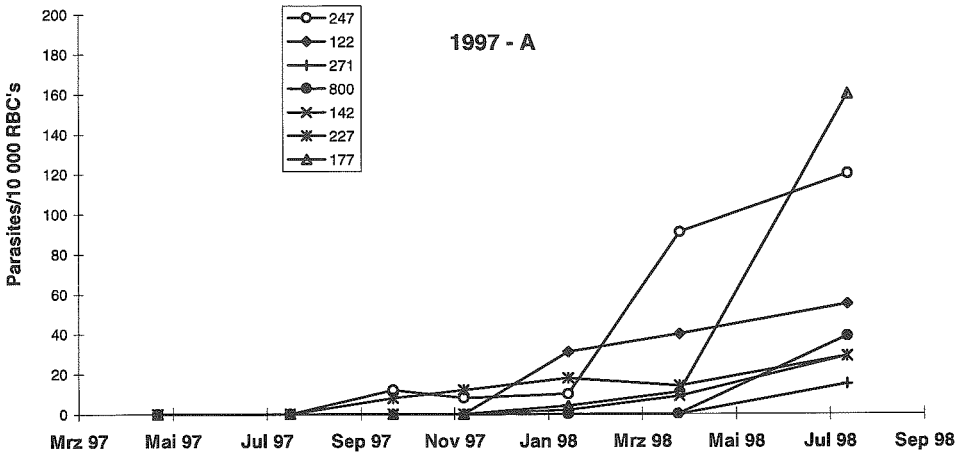

Figure 57. The patterns of parasitemia of *Schellackia* sp. in seven *E. stokesii* individuals born in the ACU in 1997.

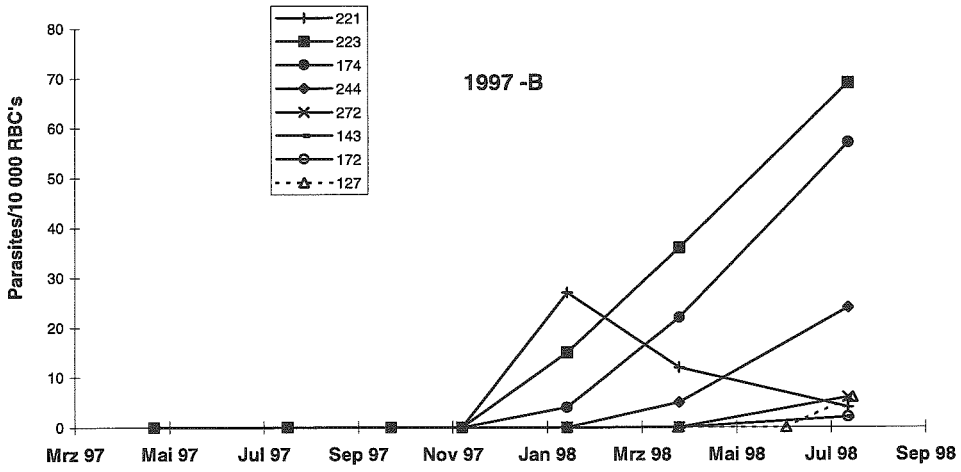

Figure 58. The patterns of parasitemia of *Schellackia* sp. in eight *E. stokesii* individuals born in the ACU in 1997.

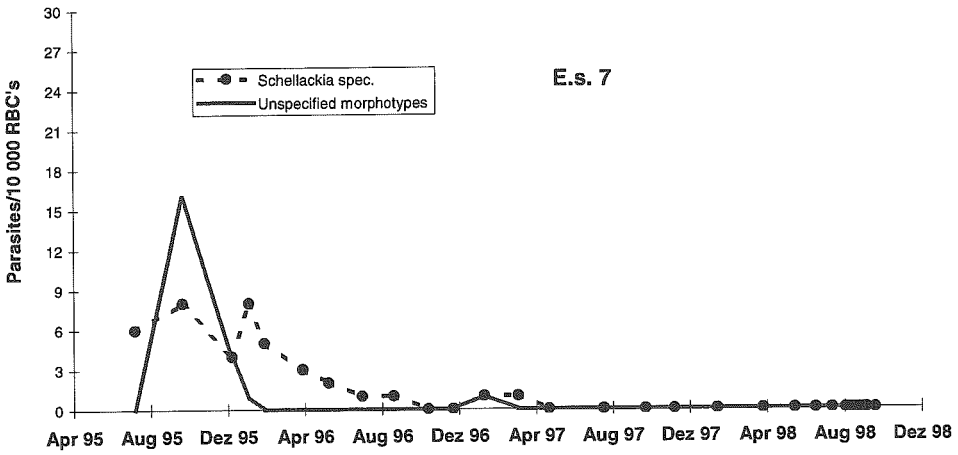

Figure 59. The presence of blood parasite stages in *E. stokesii 7*, caught single infected with *Schellackia* sp. as an adult in 1995.

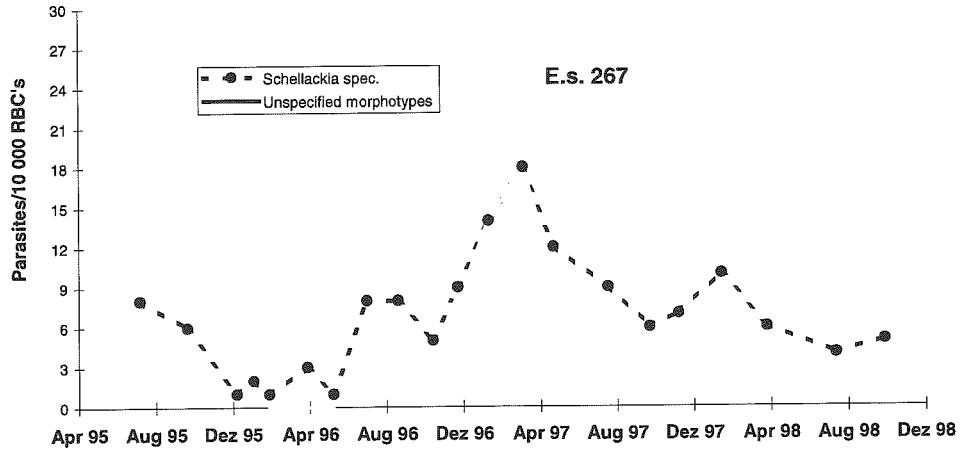

Figure 60. The presence of blood parasite stages in *E. stokesii 267*, caught single infected with *Schellackia* sp. as an adult in 1995.

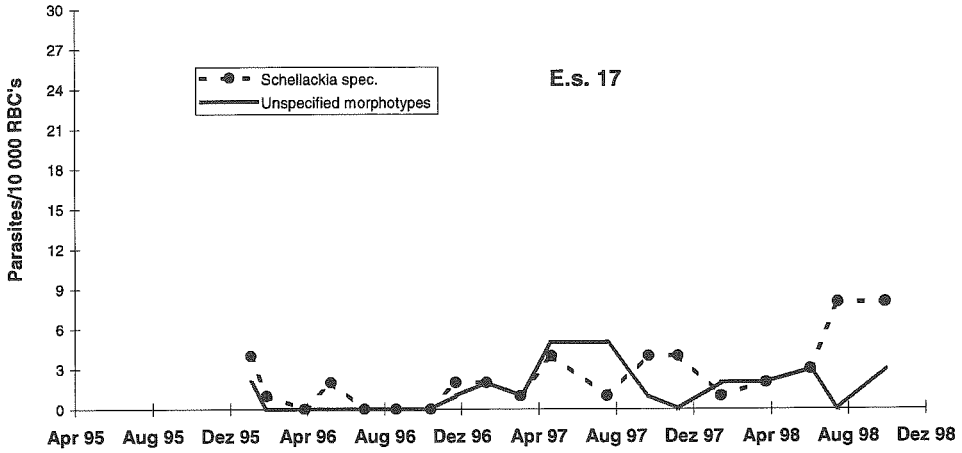

Figure 61. The presence of blood parasite stages in *E. stokesii 17*, caught single infected with *Schellackia* sp. as an adult in 1995.

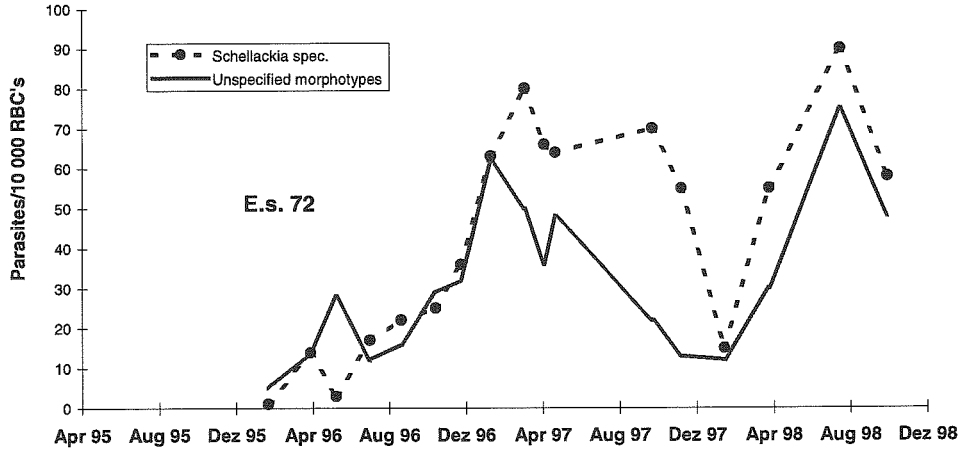

Figure 62. The presence of blood parasite stages in *E. stokesii 72*, caught single infected with *Schellackia* sp. as an adult in 1995.

8.1.5 Long term observations of *E. stokesii* with *Schellackia* sp. infections

For this part of the investigation, eight adult lizards that were single infected with *Schellackia* sp. at the time of their capture in 1995 were kept in the ACU and bled at regular intervals to monitor the course of their blood infections. More than 30 *E. stokesii* that were caught between 1995 and 1997 were subsequently included into this study. No seasonal patterns of the parasitemia of *Schellackia* sp. were detected. Figures 59-62 display examples of the rather erratic patterns of the seasonal parasitemia found. Some infected lizards lost their blood infection within the time they were studied (Figure 59), others developed an overall peak parasitemia after some months of captivity (Figure 60) or remained rather constant in their blood infection (Figure 61). Some lizards showed two or more distinct peaks of parasitemia (Figure 62). Only a few lizards had a relapse with *Schellackia* sp. after no parasites were detected in their blood for several months, but those relapses were short and the parasitemias during that phase were always relatively low. Unspecified morphotypes were always present throughout the chronic phase of the infection. The overall parasitemia of *Schellackia* sp. was relatively low compared to *Hem. mariae* or *Hem. biplicata.*

8.2 Discussion

Two main characteristics are generally used in order to identify parasites from the genus *Schellackia*, which are the absence of development inside the obligate vector, and an oocyst that contains eight sporozoites, found usually in the lamina propria of the lizard-duodenum, -colon and -rectum. In this case, the results of tick dissections suggested that there was no development of *Schellackia* sp. in the two tick species, *Amb. limbatum* and *Amb. vikirri,* but because a vector role of the ticks was questionable due to the frequent spontaneous infections, those results were inconclusive. Moreover, the oocysts found in the lizard dissections were not reliably identifiable because of the presence of a species of *Eimeria.* Thus, the taxonomic identification of this parasite could not be determined with absolute certainty. The blood parasite is postulated to belong to the genus *Schellackia* for two reasons:

1. The number of sporozoites found in the few developing early oocysts from the histological sections of *E. stokesii 356* never exceeded eight. *Eimeria* species also develop oocysts containing eight sporozoites, but the sporozoites are confined in groups of two into four sporocysts and sporulation occurs in the gut lumen of the vertebrate host. Parasites of the closely related genus *Lankesterella,* that also enter red blood cells, develop oocysts with at least 32 sporozoites.

2. Encysted parasitic stages, typically containing two sporozoites, occurred in the liver and spleen of *E. stokesii 356*. A similar phenomenon was described from *Schellackia landauae* in the Brazilian lizard *Polychrus marmoratus* (Lainson *et al.*, 1976) and is known as endodyogeny.

The spontaneous infections of *E. stokesii* with *Schellackia* sp. and the presence of the *Eimeria* parasite severely hampered the investigation of the life cycle of this parasite. The cause of the spontaneous infections with *Schellackia* sp. remained obscure. The natural pathway of transmission of an eimeriorine blood parasite has been described previously only from the ingestion of a vector species. The transmission of *Schellackia* sp. by an unidentified arthropod vector organism was highly unlikely in this case, for two reasons:

1. From the beginning of this study, the housing conditions of *E. stokesii* kept in the ACU were optimised to eliminate arthropods like ticks, mites and dipterans that could potentially act as vectors. Additionally, all vector experiments were conducted in especially equipped rooms that were separated from the long term housing rooms. All lizards were closely examined for ectoparasites every four to ten weeks. Apart from the lizards in transmission experiments, no ectoparasites have been found attached to or even close to *E. stokesii* in the ACU through the entire three year period of this study.
2. The only parasite species found to cause spontaneous infections in laboratory born *E. stokesii* was *Schellackia* sp., although there were several other species of blood parasites present. Two of those parasites, *Hem. mariae* and *Hem. biplicata*, have been described in this study to be transmitted by vectors, but there was not a single case of a spontaneous infection of either *Hemolivia* parasite for all *E. stokesii* kept in the ACU.

The cause for spontaneous *Schellackia* sp. infections of *E. stokesii* needs further investigation. On the basis of the data collected for this thesis, one possible explanation for this phenomenon was that newborn *E. stokesii* acquired an infection from their mother by the ingestion of the maternal blood adhered to the placenta. This hypothesis suggests a naturally occurring vertical transmission of *Schellackia* sp. from mother to offspring in the life cycle of *E. stokesii*, a phenomenon that, to the best knowledge of the author, has never been described before. Nevertheless this was the most likely explanation, because the blood of an *E. stokesii* infected with *Schellackia* sp. contained a life cycle stage that is infective for a new host, and where a birth had been observed, all newborns ingested their placenta or intended to do so. All newborns where spontaneous infections occurred were born from mothers that

were infected with *Schellackia* sp. around the time of birth. Mother lizards with mixed infections could not transmit adeleorine parasites, because they have to develop inside a vector to infect a new lizard. The behavioural peculiarity of the newborns to eat their placenta probably reduces the need for a vector for *Schellackia* sp. significantly and might explain its high prevalence in field populations of *E. stokesii* (described in detail in chapter fourteen). No parasitic stages were detected in histological examinations and smears of two placentae, but the mother lizard had a very low parasitemia at the time of birth (0.02%) and most of the maternal blood was lost before the surface of the placentae was smeared on glass slides. One placenta dropped onto the soil of the terrarium and had to be washed in a physiological saline solution before it was further investigated. The other placenta was expelled into the hand of the author, but most of the attached blood was smeared onto the latex glove and dried out while the baby lizard tried to ingest the placenta.

Chapter Nine

9. HEPATOZOON SP. (TYPE 4)

9.1 Introduction

A haemogregarine blood parasite (Figure 13) that was significantly different in size from *Hem. mariae* and *Hem. biplicata* and *Schellackia* sp. (Table 9 and Table 10) was found infecting some populations of wild caught *E. stokesii*. It also differed from the first three blood parasites in that its chronic stage did readily stain with giemsa. *Hepatozoon* sp. was described in chapter five as type 4. Only the chronic stage of this parasite was found and it was postulated to be the gamont of *Hepatozoon* sp. because of the selective amplification of this parasite by a primer designed to amplify the ITS-1 gene of the genus *Hepatozoon* (for details see chapter eleven). A great variety of haematophagous invertebrates have been identified as vectors for species of *Hepatozoon* (see 2.2.4.2.1). Only one Australian species of *Hepatozoon* from reptiles has been described, vectored by the mosquito *Culex pipiens fatigans* (Mackerras, 1961). Attempts to identify a vector for *Hepatozoon* sp. in this study by using *Amb. limbatum* and *Amb. vikirri* ticks and *C. quinquefasciatus* mosquitoes were unsuccessful. The life cycle of this micro-parasite remains to be investigated.

9.1.2 The morphology

In giemsa stained blood smears of infected lizard blood, this parasite had a highly consistent and distinctive morphology. It was banana shaped, with a condensed and in most cases central nucleus staining dark red, and had a clear cytoplasm (Figure 73). Sometimes the parasite nucleus showed a deep cleft, but it was never found to be split completely. The nucleus of the host blood-cell was always displaced and appeared shrunken. The TEM confirmed the three dimensional shape to be cylindrical and slightly bent (Figure 63/A). The chronic blood stage of the micro-parasite was encysted and was lying in a parasitophorous vacuole, surrounded by an outer membrane. Structures interpreted as young developing oocysts were found in the malphigian tubules of dissected *C. quinquefasciatus* mosquitoes that had fed on *E. stokesii* single infected with *Hepatozoon* sp. (Figure 63/B). The oocysts never matured, possibly due to a maladaption to this particular vector species, thus no further life cycle stages could be described.

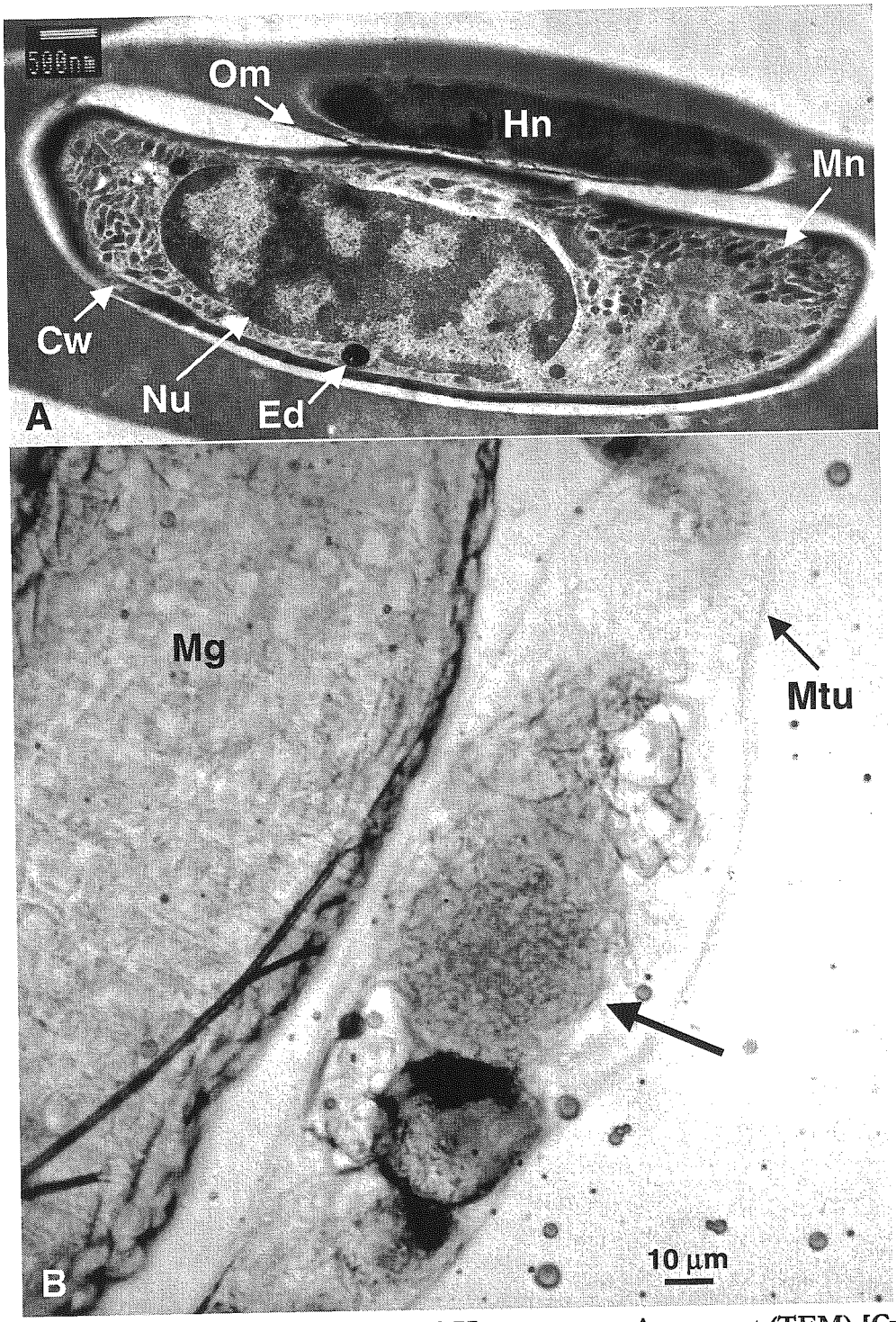

Figure 63. A+B: life cycle stages of *Hepatozoon* sp. A: gamont (TEM) [Cw: cyst wall, Ed: electron dense body, Hn: Host cell nucleus, Mn: microneme, Nu: nucleus]. B: dissected *Culex quinquefasciatus*, fat arrow marks structure interpreted as developing oocyst (LM, wet mount) [Mg: midgut, Mtu: malpighian tubule].

9.1.2 The locations where *Hepatozoon* sp. was found

The three localities where *E. stokesii* infected with *Hepatozoon* sp. were found in the course of this study are listed in Table 33. *Hepatozoon* sp. was found only at relatively few locations (for a more detailed comparative analysis of the spatial distribution of the blood parasite species found see chapter fourteen).

Table 33. The coordinates of the sites where *Hepatozoon* sp. was found infecting *E. stokesii* during the three year field survey (regular study sites in bold).

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [Σ] with *Hepatozoon* sp. and its prevalence [%]	
	S	W		Σ	%
Gawler Ranges[1]	32°30'	136°50'	12	2	16.7
Island Lagoon	31°42S	136°68'	14	7	50
Neuroodla (NR)	31°50'	138°08'	**68**	*	**36.1**

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

* = Data of *E. stokesii* individuals were pooled over three years (for details see chapter 14)

9.1.3 The transmission experiments

9.1.3.1 The force feeding of unengorged *Amb. limbatum* nymphs to *E. stokesii*

Amb. limbatum larvae were allowed to engorge on *E. stokesii 37*, a lizard that was single infected with *Hepatozoon* sp. (Figure 64). A total of five nymphs that had engorged as larvae on *E. stokesii 37* on the 17/09/98 were force fed to *E. stokesii 5* on the 20/11/98. No blood infection was detected at regular bleedings of *E. stokesii 5* until this experiment was terminated in April 1999. Additional experiments, which are described in detail in chapter twelve, investigated in detail the vector competence of the ticks *Amb. limbatum* and *Amb. vikirri*. In those experiments, no transmission was detected by feeding nine groups of *Amb. limbatum* and *Amb. vikirri* nymphs that had fed as larvae on *E. stokesii* infected with *Hepatozoon* sp. to uninfected lizards.

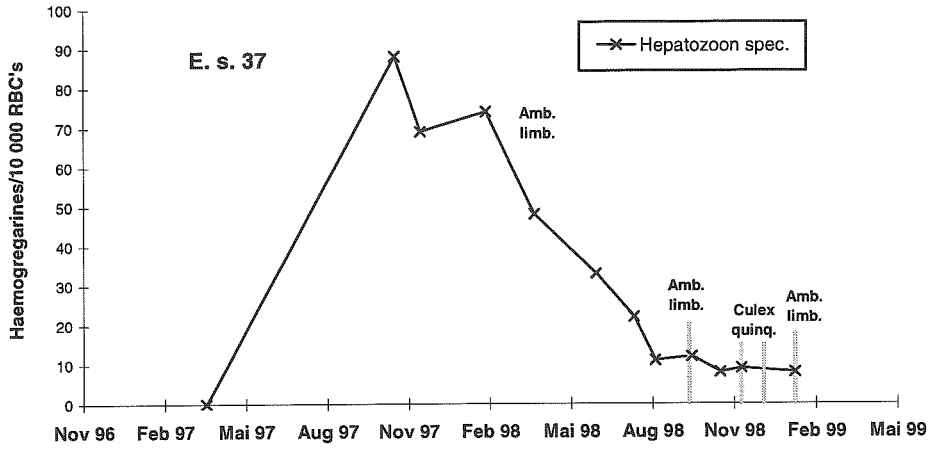

Figure 64. The parasitemia of *E. stokesii 37*, single infected with *Hepatozoon* sp., and the occasions when vectors were allowed to engorge on the lizard (Amb. limb.: *Amb. limbatum*, Culex quinq.: *C. quinquefasciatus*).

9.1.3.2 The dissections of *Amb. limbatum* ticks

Cohorts of *Amb. limbatum* larvae were allowed to engorge on *E. stokesii 37* on three occasions (Table 34). *E. stokesii 37* was single infected with *Hepatozoon* sp. (Figure 64). The resulting nymphs were dissected and their midgut, the malphigian tubules, the salivary glands and the haemocoel were studied in a wet mount for parasitic stages between two and eight weeks after moulting (Table 35). No parasitic stages were detected.

Table 34. The number of *Amb. limbatum* nymphs dissected after they fed as larvae on *E. stokesii 37*, and the parasitic stages found.

Date of larval attachment	No of nymphs dissected	Parasitic stages found
11/03/98	12	-
17/09/98	22	-
14/01/99	20	-

Table 35. Dissections of *Culex quinquefasciatus* reared at the UniSA. The mosquitoes were fed on *E. stokesii 37*, which was single infected with *Hepatozoon sp*..

Date of engorgement	Dissection of mosquitoes			
	Date	No. dissected	Days post engorgement	No. found infected
10/12/98	14/12/98	2	4	-
	18/12/98	2	8	-
	23/12/98	2	13	1*
	29/12/98	2	19	1*
	02/01/99	2	23	1*
	05/01/99	2	26	1*
	08/01/99	1	29	1*
14/01/99	16/01/99	3	2	-
	19/01/99	3	5	-
	23/01/99	3	9	-
	26/01/99	3	12	1*
	30/01/99	3	16	2*
	03/02/99	3	20	2*
	05/02/99	4	22	2*
	08/02/99	3	25	1*
	11/02/99	2	28	-
	13/02/99	2	30	1*
	18/02/99	5	35	2*

* = structures in malphigian tubules interpreted as developing oocyst

9.1.3.3 The dissections of mosquitoes

On two occasions, groups of *Culex quinquefasciatus*-mosquitoes were allowed to engorge on *E. stokesii 37*. The lizard was single infected with *Hepatozoon* sp. (Figure 64) After engorgement, the mosquitoes were dissected and the digestive tract, the haemocoel and the salivary glands were studied in a wet mount. In both groups, structures interpreted as developing oocysts were observed in the malphigian tubules of mosquitoes (Figure 72/B) from approximately two weeks post engorgement (Table 35). These structures were found in 16 of the 47 mosquitoes observed. No mature oocysts were found.

9.2 Discussion

Due to the lack of most parts of its life cycle, the taxonomic identification of this parasite had to rely entirely on the results of the molecular biological experiments described in chapter eleven, where a primer specifically designed for the highly variable ITS-1 gene created a product from blood infected with *Hepatozoon* sp., but not from any other adeleorine, eimeriorine or *Plasmodium* parasite found in this study.

The question whether the structures observed in the malphigian tubules were in fact developing oocysts of *Hepatozoon* sp. could not be answered with certainty. Malphigian tubules of mosquitoes have been described as the site of development from *Hepatozoon clamatae* infecting frogs and the mosquito *Culex territans* (Kim *et al.*, 1998). Further investigations with other vector species are necessary to elucidate the life cycle of *Hepatozoon* sp..

Although the mosquitoes were thoroughly investigated, it is possible that *Hepatozoon* sp. developed undetected into the infective sporozoites. Due to the lack of evidence for a development of *Hepatozoon* sp. in *C. quinquefasciatus,* further transmission experiments were not conducted. Additional studies are needed to investigate the possible ways of transmission.

Chapter Ten

10. P. MACKERRASAE AND P. CIRCULARIS

10.1 Plasmodium mackerrasae

Originally, *P. mackerrasae* Telford 1979 was described from blood of *Egernia cunninghami* (type host), *E. striolata* and *E. whitei* (experimental host) from Queensland, Australia (Telford, 1979). *P. mackerrasae* (Figure 74/A&B) was first described from scanty material, but has now been re-described from the data collected for this thesis. The taxonomic description was possible on the blood stages, but some lizard-tissue stages have now also been documented (Telford & Stein, 2000; in press). The final manuscript of that paper can be found in the appendix of this thesis. The following results will concentrate on the aspects of the life history that were not included therein.

In order to identify the exoerythrocytic stages of *P. mackerrasae* in *E. stokesii*, three lizards with single blood infections of *P. mackerrasae* were dissected (Table 36). Two of the lizards (*E. stokesii 10* and *204*) were field-caught adults that had an active blood infection with *P. mackerrasae* at the time of death. One lizard (*E. stokesii 222*) was born in the ACU and showed a short term and very low parasitemia of *P. mackerrasae* after it was used in a transmission experiment (for details see 10.1.5), but was found negative for blood parasites at the time of death. *E. stokesii 10* was caught on the 20/10/97 and was found to be single infected with *P. mackerrasae* in three consecutive bleedings with a steady parasitemia of about 17% until it was euthanased on the 03/12/97. The parasitemias of *E. stokesii 204* and *222* are plotted as Figure 65 and Figure 66.

Developmental stages identified as phanerozoites of a *Plasmodium* species by Dr. S. R. Telford Jr. were found in tissues of the ACU-born lizards *E. stokesii* 177 and 356 (Table 36). These lizards were also infected with *Schellackia* sp. and had been dissected to identify life stages of that parasite. Phanerozoites were present in the connective tissue and endothelium of blood vessels and in the pericardium. The lizards had been kept in the ACU for all of their life time. This finding was surprising because the blood of the field caught mothers of *E. stokesii 177* and *356* tested negative for *Plasmodium* parasites during the time they were held in captivity.

Table 36. Lizards in which *Plasmodium* phanerozoites were found in histological sections of various tissues.

E. stokesii	Origin of lizard	Date of birth	Detected blood infection at the time of death	Date of dissection
10	Field caught	?	*P. mackerrasae*	20/10/97
204	Field caught	?	*P. mackerrasae*	12/01/99
222[1]	ACU born	3/97	-	12/01/99
177[2]	ACU born	3/97	*Schellackia* sp.	13/08/98
356[2]	ACU born	2/95	*Schellackia* sp.	03/12/97

[1] = Lizard was inoculated with blood infected with *P. mackerrasae*

[2] = Lizard was never used in a transmission experiment

10.1.1 The life cycle

The life cycle of *P. mackerrasae* in *E. stokesii* is documented in detail, as far as it is known (Telford & Stein, 2000, see appendix). Briefly, parasitic stages have been found in erythrocytes and connective tissue or endothelium in circulatory, respiratory, digestive, excretory, and muscular systems. Blood infections begin with a massive emergence of exoerythrocytic (EE) schizonts in the blood plasma. The EE-schizonts separate into vast numbers of tiny merozoites that flood the blood stream (Figure 74/B). The massive flooding of the blood stream with merozoites was observed only once in *E. stokesii* housed in the ACU, and on eight occasions in field lizards, indicating that this phase is relatively short. The merozoites enter the red blood cells and develop into trophozoites, the stage that feeds on the haemoglobin of its host cell. Some trophozoites undergo an intraerythrocytic schizogony, thus giving rise to new merozoites. Others develop into gamonts (Figure 74/A). Attempts to identify the vector of *P. mackerrasae* failed, thus no further developmental stages could be described.

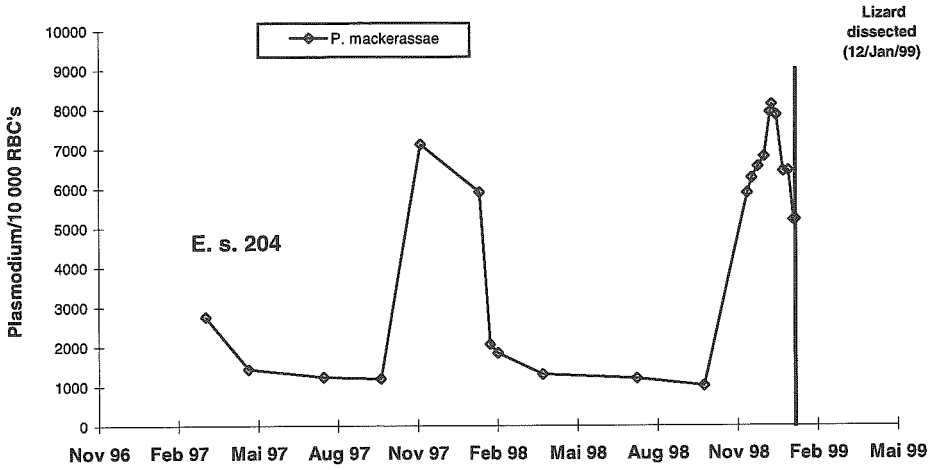

Figure 65. The occurrence of *P. mackerrasae* in the blood of the single infected *E. stokesii 204* during the time it was monitored in the ACU. The lizard was killed and dissected shortly after the second peak of the relapsing infection.

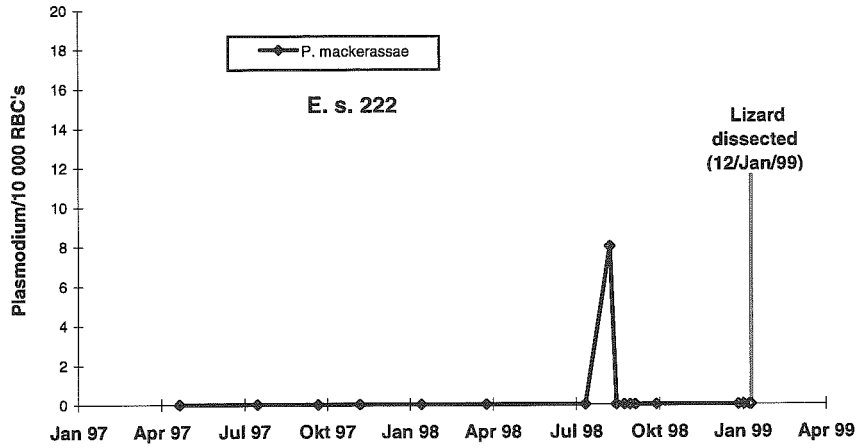

Figure 66. The occurrence of *P. mackerrasae* in the single infected *E. stokesii 222* during the time it was monitored. The lizard was born in the ACU in February 1997 and was experimentally infected in June 1998. It was killed and dissected approximately 5 months after the blood infection was detectable.

10.1.2 The morphology

The morphology of the blood stages of *P. mackerrasae* as it appeared in giemsa stained blood smears is described in detail in the taxonomic publication attached to this thesis. Not included therein were the images of *P. mackerrasae* blood-stages obtained by TEM. Figure 67 depicts an early trophozoite, as it appeared under a transmission electron microscope. Conspicuous were the remaining subpellicular microtubules (Mt) and the broken plasma membrane (Pm), both of which characterise the merozoite stage but they were still visible in this early trophozoite. Structures as small as ribosomes were discernible, as well as a prominent acristate mitochondrion. Figure 68/A shows a fully developed trophozoite, with a cytostome bordered by characteristic cytostome rings, a prominent nucleus, and an invagination interpreted as a vesicle that releases its content into the host cell cytoplasm (Iv). Double layered invaginations were frequently observed (Figure 68/C), also electron dense structures interpreted as food vacuoles (Fv) and haemozoin particles (Hp), the metabolic end-product of the digested haemoglobin. Gametocytes appeared more elongate (Figure 68/B), with few discernible structures within their cytoplasm.

Also not described in the recent taxonomic publication of *P. mackerrasae* was the finding of extensive deposits of pigment in the liver of *E. stokesii* chronically infected with *P. mackerrasae* (Figure 11/C). The pigment deposits often resembled exoerythrocytic schizonts of malaria parasites, but stained black in a giemsa stain, in contrast to the purplish red of the *Plasmodium* parasites.

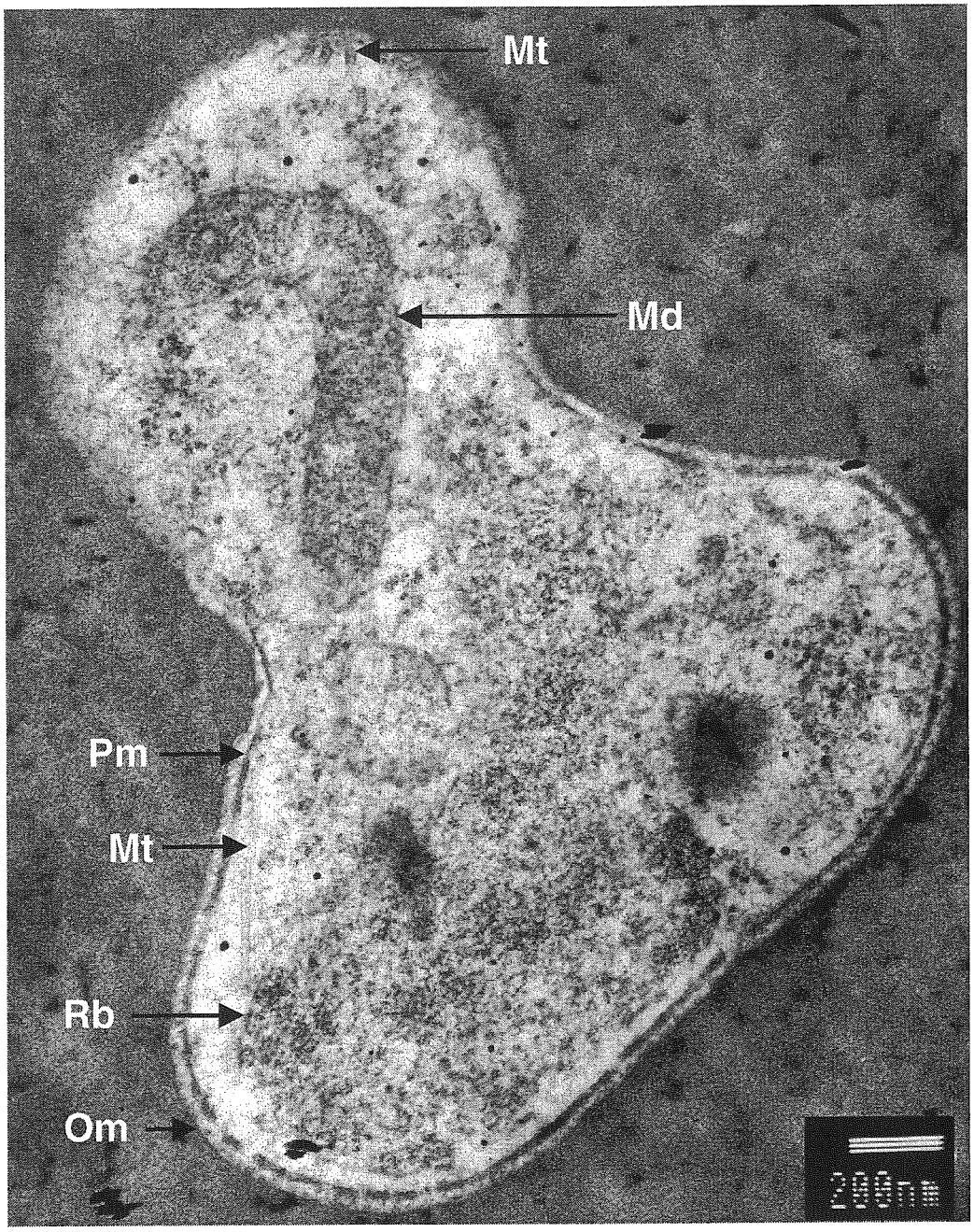

Figure 67. Early trophozoite of *Plasmodium mackerrasae* found intraerythrocytic in *E. stokesii*, with typical acristate mitochondrion, few remaining microtubules and gaps in the plasma membrane (TEM) [Md: mitochondrion, Mt: microtubule, Om: outer membrane, Pm: plasma membrane, Rb: ribosomes].

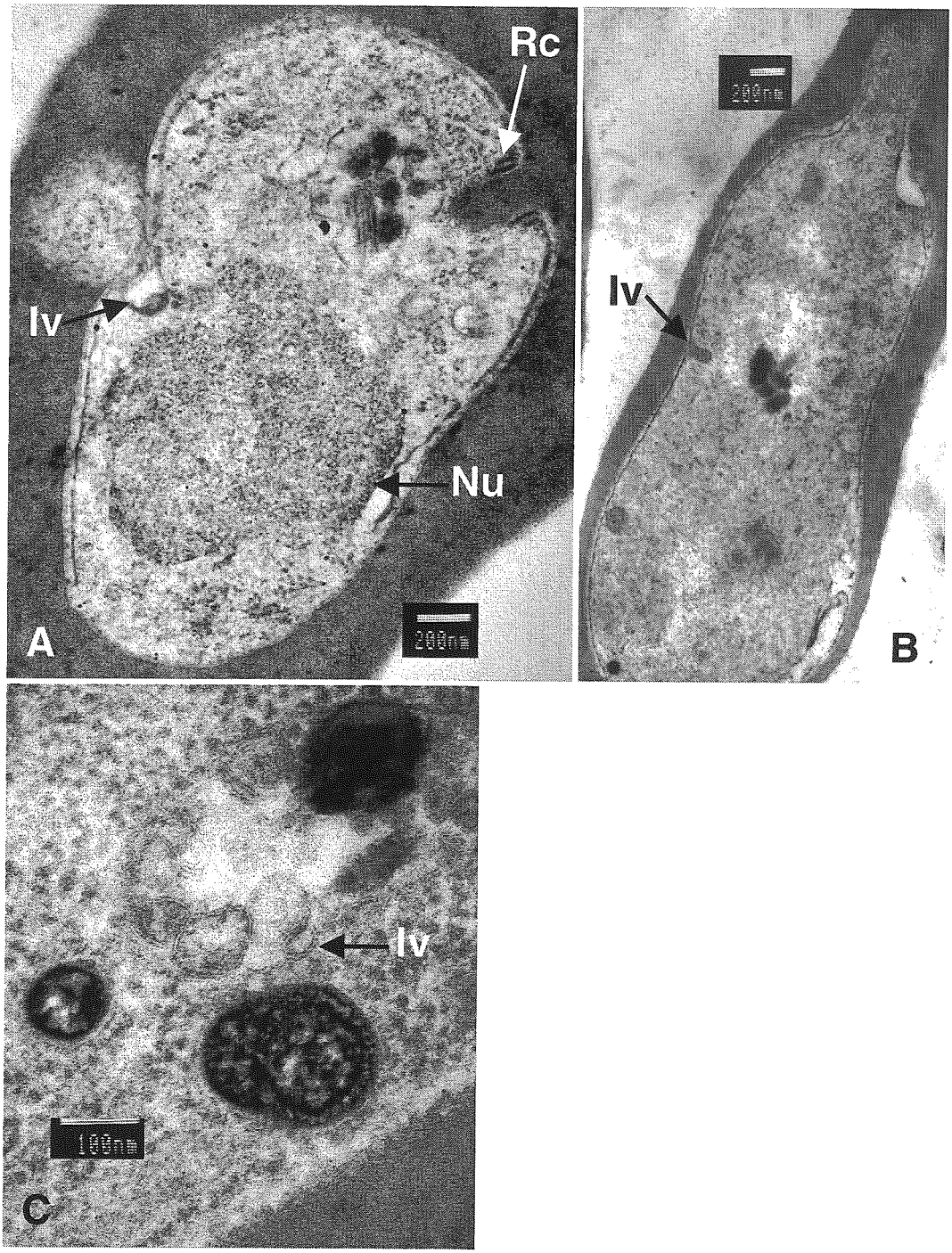

Figure 68. A-C: intraerythrocytic *P. mackerrasae* in *E. stokesii* (TEM). A: trophozoite with cytostome, vacuole (Iv) releasing content into host cell cytoplasm. B: maturing gamont. C. cluster of double membraned invaginations in parasite cytoplasm [Iv: invagination, Nu: nucleus, Rc: rings of cytostome].

10.1.3 The locations where *P. mackerrasae* was found

All localities where *E. stokesii* infected with *P. mackerrasae* were found in the course of this study are listed in Table 37. The parasite usually had a relatively moderate to high prevalence where it occurred (Details of prevalence at the main study sites are given in chapter fourteen).

Table 37. The coordinates of the sites where *P. mackerrasae* was found infecting *E. stokesii* during the three year field survey .

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [∑] with *P. mackerrasae* and its prevalence [%]	
	S	W		∑	%
Camel Hill (CH)	31°53'	138°25'	**148**	*	**5.7**
Chace Range	31°45'	138°35'	7	2	28.6
Drakes Nob (DN)	32°05'	138°09'	**27**	*	**47.0**
Gawler Ranges[1]	32°30'	136°50'	12	1	8.3
Glenlyle (GL)	31°50'	138°35'	**62**	*	**52.3**
Hawker North (HN)	31°53'	138°25'	**97**	*	**24.8**
Hawker South (HS)	31°53'	138°25'	**159**	*	**23.6**
Island Lagoon	31°42'	136°68'	14	7	50.0
Mernamerna	31°45'	138°22'	2	1	50.0
Neuroodla (NR)	31°50'	138°08'	**68**	*	**47.3**
Partacoona	32°00'	138°10'	6	5	83.3
Point Lowly[1]	32°50'	137°45'	8	1	12.5
Quorn	32°15'	138°05'	8	5	62.5
The Four Mile	31°50'	138°30"	1	1	100.0
Warruwarld. Hill	31°55'	138°25'	7	3	42.9
Willow Springs	31°25'	138°50'	4	1	25.0
Yapalla	31°50'	138°20"	4	1	25.0

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

* = Data of *E. stokesii* individuals were pooled over three years (for details see chapter fourteen)

10.1.4 The occurrence of relapses of the infection

The spontaneous infections with *P. mackerrasae* found in field caught *E. stokesii* kept in the ACU were interpreted as relapses of previous infections. Spontaneous infections of *P. mackerrasae* in laboratory born *E. stokesii* were not observed. However, phanerozoites were found in lab born *E. stokesii 177* and *356* (see page 170). Of the more than 100 field caught *E. stokesii* that were kept in the ACU for the study of blood parasites, 14 showed the spontaneous appearance of *P. mackerrasae* that was interpreted as a relapse. No seasonal pattern was detectable in the onset or the peak of the parasitemia, nor was there a pattern in regard to the time the host lizard has been held in captivity. Relapsing infections were found in hosts with mixed infections or in hosts where no other infection was detected during the time they were held in captivity. Figures 69-72 show some examples of the patterns of parasitemia found. Relapses first occurred either after several months of captivity (Figure 69) or at the beginning of captivity (Figure 70). The blood stages would appear sometimes in a single small peak with an extremely high parasitemia and a relatively short chronic phase of a few weeks (Figure 69), or in a slowly increasing peak with a chronic phase that would last several months (Figure 72). Consecutive peaks of parasitemia were also observed (Figure 71 and Figure 65).

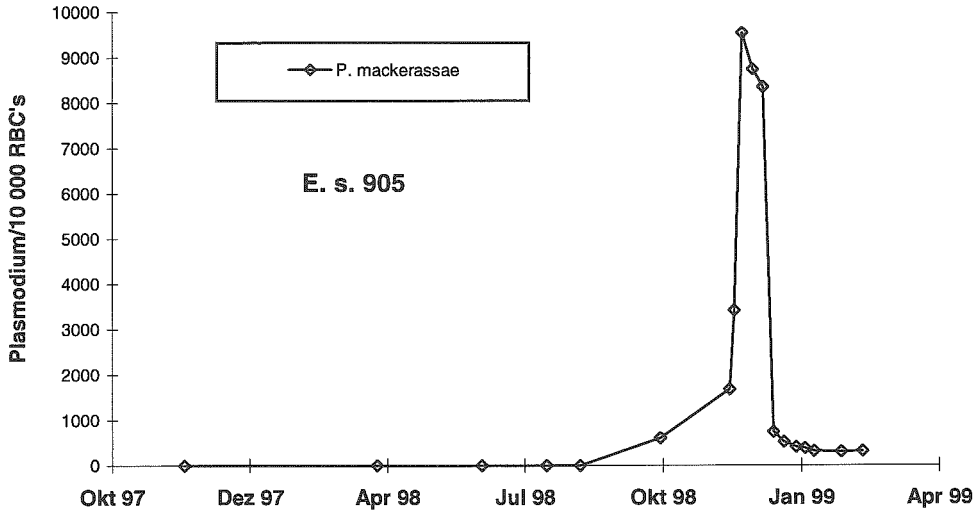

Figure 69. A relapsing infection of *P. mackerrasae* in the field caught *E. stokesii 905*.

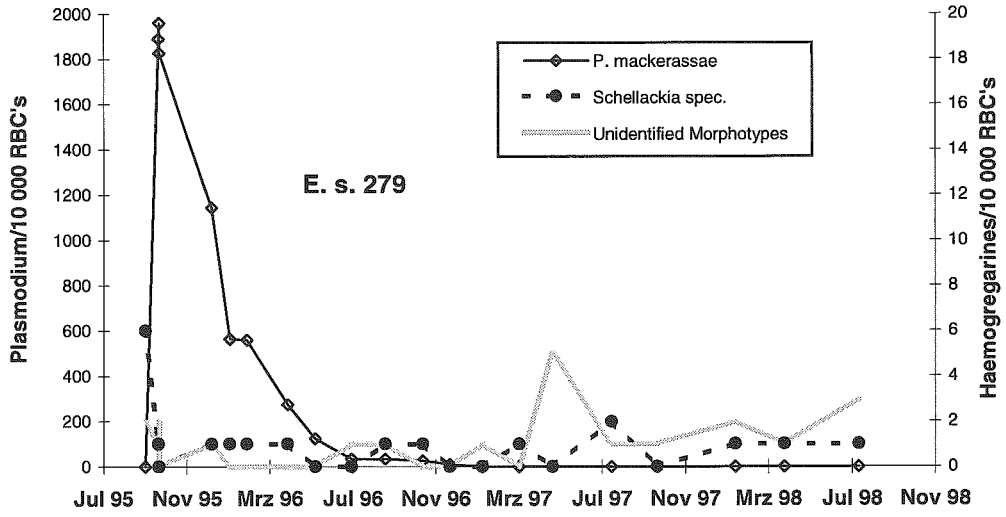

Figure 70. A relapsing infection of *P. mackerrasae* in the field caught *E. stokesii 279*.

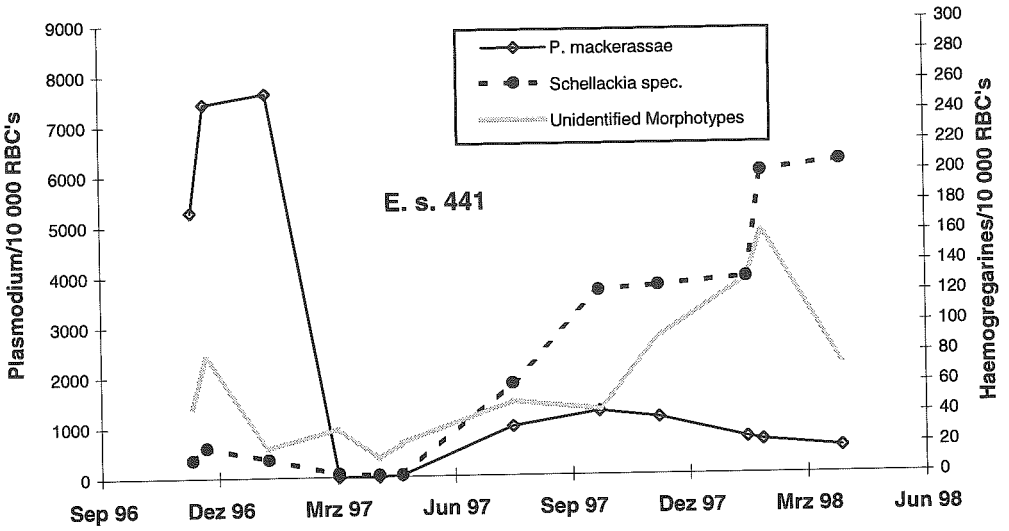

Figure 71. A relapsing infection of *P. mackerrasae* in the field caught *E. stokesii 441*.

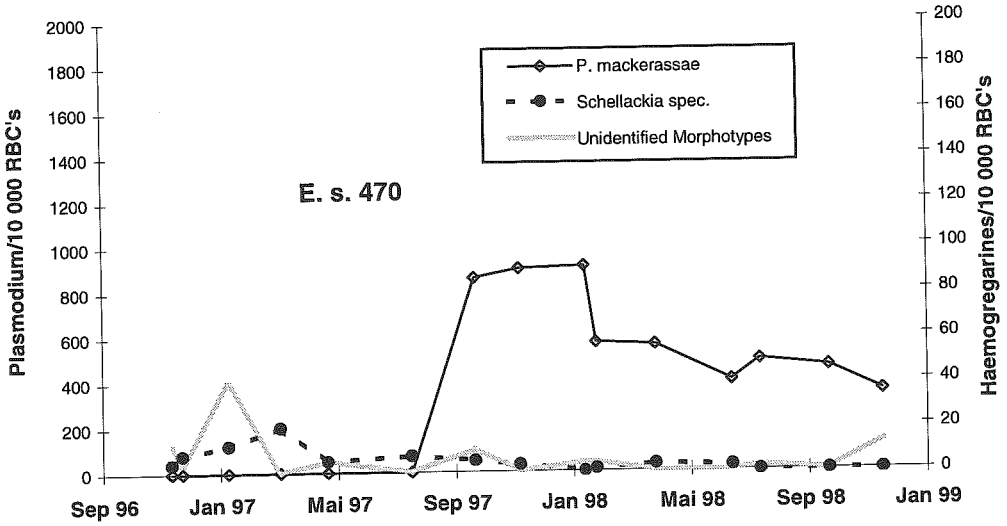

Figure 72. A relapsing infection of *P. mackerrasae* in the field caught *E. stokesii 470*.

10.1.5 The transmission experiments

10.1.5.1 Blood transmissions

A series of five experiments were run to investigate the transmission of *P. mackerrasae* from *E. stokesii* to *E. stokesii* by infected lizard blood. All lizards used as blood donors were infected with *P. mackerrasae* and had a parasitemia between 23% and 86% at the time of the experiment. For details of the methods used see 5.2.1.6. Only *E. stokesii* born in the ACU were used as receivers of the infected blood. From a total of 21 attempted transmissions in five experimental blocks, two *E. stokesii* (*222* and *244*) were diagnosed with blood infections of *P. mackerrasae* (Table 38) after the blood was transmitted. The timing of the appearance, the duration and the parasitemia of both blood infections were found to be very similar. The infection of *E. stokesii 222* was plotted as an example in Figure 66. Asexual stages of *P. mackerrasae* were found in the peripheral blood of both lizards during their first routine bleedings, eight days after the intra-peritoneal (IP) inoculation. No blood parasites were detected in any following routine bleedings. The parasitemias of 0.8% and 0.5% were extremely low for an infection with *P. mackerrasae. E. stokesii 222* was dissected approximately five months after the blood infection was detected. Other than phanerozoites found in the muscle and lower intestinal wall, no further evidence was found for the presence of a *Plasmodium* species.

10.1.5.2 Dissections of potential vector species

Sand flies (*Australophlebotomus dycii*), caught recently fed on lizard blood from crevices occupied by *E. stokesii* at study sites in the Flinders Ranges were dissected for the presence of coccidian blood parasite stages. The site Camel Hill (CH) was not examined on those field trips, because no sand flies were observed in frequent and close examinations of *E. stokesii* at that site. That lizard population was studied for five years by Mr. Glen Duffield, who also assisted with the capture of sand flies at other sites, but no sand flies were observed on Camel Hill. (G. Duffield, pers. comm.). No evidence was found for developmental stages of parasites from the genus *Plasmodium* in *A. dycii* (Table 39), but the blood stages of two haemogregarine species, *Hem. mariae* and *Schellackia* sp., were detected. No evidence of a development of the haemogregarines was detected. In a series of experiments, batches of two species of mosquitoes, *A. notoscriptus* and *C. quinquefasciatus,* were allowed to engorge on *E. stokesii* infected with *P. mackerrasae.* This was the first study to describe the feeding of the mosquito *A. notoscriptus* on a reptile. No developmental stages of *Plasmodium* were identified in the dissections of either mosquito species (Tables 40-44).

Table 38. Transmission experiments using 0.2 ml of blood infected with *P. mackerrasae* to transmit the parasite from field caught *E. stokesii* to *E. stokesii* born and raised in the ACU (IP: intra-peritoneal).

Number of *E. stokesii* attempted to be infected	Date of blood transmission	Method of blood transfer	Anticoagulant	Successful transmission
5	26/01/98	Oral	Reptile ringer	-
5	23/07/98	Oral	Citrate buffer	-
5	02/08/98	IP	Citrate buffer	2
3	24/09/98	IP	Reptile ringer	-
3	19/11/98	Oral	Heparin	-

Table 39. The number of *Australophlebotomus dycii* caught and dissected on all field trips. None of the sand flies were found to be infected with parasites from the genus *Plasmodium* (no sand flies were observed at site CH).

Sites	HS				GL				NR				DN			
	Caught		Dissected		Caught		Dissected		Caught		Dissected		Caught		Dissected	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
20/03/97	-	-	-	-	53	18	11	1	-	-	-	-	-	-	-	-
30/03/97	-	-	-	-	26	7	10	-	-	-	-	-	-	-	-	-
13/03/98	-	-	-	-	2	-	-	-	-	-	-	-	7	6	7	-
16/03/98	2[1]	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
23/03/98	-	-	-	-	-	-	-	-	13	2	4	0	2	3	2	-
06/04/98	-	-	-	-	21	7	10	1	6	-	2	-	2	-	2	-
20/04/98	-	-	-	-	1	-	-	-	*	*	-	-	*	*	-	-
23/04/98	*	*	-	-	*	*	-	-	1	-	-	-	5	1	1	-
08/04/99	*	*	-	-	43	38	23[2]	2	*	*	-	-	-	-	-	-

* = not sampled

2[1] = *H. mariae* gamonts found in both

23[2] = *Schellackia sp.* sporozoite found in one of them

Table 40. Feeding experiments of *Aedes notoscriptus* on E. stokesii at the FUSA (*E. s.*: *E. stokesii*, *P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H. sp.*: *Hepatozoon sp.*, *S. sp.*: *Schellackia sp.*, o/n = over night)

Date of experiment.	*E. s.*	Parasitemia of lizard blood parasites at the time of engorgement						No of mosqu. within diptera cage	T °C in dipt. cage	Durat. feeding	No. engorg. mosquitoes
		P. m.	*P. c.*	*H. m.*	*H. b.*	*H. sp.*	*S. sp.*				
18/02/98	*220*	5240	100	-	-	-	6	25	25	o/n	0
09/03/98	*220*	4800	100	-	-	-	12	25	37	2 h	11
19/03/98	*620*	620	60	192	-	-	9	30	37	o/n	5
20/03/98	*220*	4800	100	-	-	-	12	30	37	2 h	0

Table 41. Dissection results of *Aedes notoscriptus*

Engorgement Date	Dissection Date	No. dissected	Days post engorgement	Found infected
09/03/98	10/03/98	2	1	-
	13/03/98	2	4	-
	16/03/98	3	6	-
	20/03/98	1	8	-
19/03/98	20/03/98	1	1	-
	22/03/98	1	3	-

Table 42. Feeding experiments of *Culex quinquefasciatus* on E. stokesii at the FUSA and the UnSA (*E. s.*: *E. stokesii*, *P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H. sp.*: *Hepatozoon sp.*, *S. sp.*: *Schellackia sp.*, o/n = over night)

Date of experiment.	*E. s.*	Parasitemia of lizard blood parasites at the time of engorgement						No of mosqu. within diptera cage	T °C in dipt. cage	Durat. feeding	No. off engorg.
		P. m.	*P. c.*	*H. m.*	*H. b.*	*H. sp.*	*S. sp.*				
18/05/98	*220*	3300	80	-	-	-	8	10	25	o/n	3
11/05/98	*220*	3560	80	-	-	-	11	18	37	o/n	5
29/07/98	*220*	2430	40	-	-	-	9	12	37	2h	3
09/11/98	*220*	860	-	-	-	-	17	>200	25*	2h	17
16/11/98	*905*	1680	-	-	-	-	-	>500	25*	2h	32
10/12/98	*37*	-	-	-	-	10	-	>200	25*	2h	20
11/12/98	*204*	7620	300	-	-	-	-	>200	25*	o/n	38
14/01/99	*37*	-	-	-	-	9	-	>500	25*	o/n	86

* = engorged at the UnSA

Table 43. Dissection results of *Culex quinquefasciatus* reared and fed at the UniSA.

Engorgement Date	Dissection Date	No. dissected	Days post engorgement	Found infected
18/05/98	19/05/98	1	1	-
	22/05/98	1	4	-
	25/05/98	1	7	-
11/05/98	14/05/98	1	3	-
	16/05/98	1	5	-
	20/05/98	1	9	-
29/07/98	01/08/98	2	3	-
09/11/98	10/11/98	2	1	-
	12/11/98	2	3	-
	14/11/98	2	5	-
	17/11/98	2	8	-
	20/11/98	2	11	-
	21/11/98	1	12	-
	22/11/98	2	14	-
	25/11/98	1	17	-

Continuation of Table 43 (see previous page). Dissection results of *Culex quinquefasciatus* reared and fed at the UniSA.

Engorgement Date	Dissection Date	No. dissected	Days post engorgement	Found infected
09/11/98	10/11/98	2	1	-
	12/11/98	2	3	-
16/11/98	17/11/98	2	1	-
	18/11/98	2	2	-
	21/11/98	2	5	-
	23/11/98	2	7	-
	25/11/98	2	9	-
	28/11/98	2	12	-
	30/11/98	1	14	-
	4/12/98	3	18	-
	06/12/98	2	20	-
11/12/98	12/12/98	1	1	-
	14/12/98	3	2	-
	16/12/98	5	2	-
	18/12/98	7	2	-
	21/12/98	10	2	-
	23/12/98	12	2	-
	29/12/98	18	2	-
	31/12/98	20	2	-

10.1.6 Discussion

One of the most surprising results of this thesis was the finding of *Plasmodium* phanerozoites in *E. stokesii* that were born and raised in the ACU and that were never used for transmission experiments. *E. stokesii 177* and *356* were dissected to investigate their spontaneous infection with *Schellackia* sp.. Both lizards were born from mothers that had an active infection of *Schellackia* sp., but the mothers had no detectable blood infection of *P. mackerrasae* during the months they were kept in the ACU. *E. stokesii 177* and *356* were held with their peer group of ACU born lizards in terraria and none of the lizards born in the ACU ever developed an active infection with *P. mackerrasae*. The reason for the latent infection in the two dissected *E. stokesii 177* and *356* remains to be investigated. It is possible that the two lizards became infected by an unidentified vector during their stay in the terraria, but due to the care taken in regard to the control of vector species, this hypothesis seems highly unlikely. Another possible way of transmission of *P. mackerrasae* might be through the ingestion of the placenta by the newborns, similar to the speculations regarding the transmission of *Schellackia* sp. (see 8.1.1.4). Like in eimeriorine parasites, most blood stages of *Plasmodium* are infective for a new lizard host. Newborns might acquire a *P. mackerrasae* infection by the ingestion of infected maternal blood that is attached to the placenta. Human malaria parasites have been found in maternal blood in the placenta (Miller & Telford, 1996), and a case of a congenital transmission of human malaria was described (Thompson *et al.*, 1977). A congenital transmission of malaria in reptiles has never been reported, nevertheless it seems to be the most plausible explanation for the latent infections found *E. stokesii 177* and *356*. Although the blood of their mothers were found to be uninfected with *P. mackerrasae*, the mother lizards might have had a latent infection that they passed adhered to the placental tissue to their offspring, or they had an infection that was below the detectable threshold.

The brief appearance of asexual stages of *P. mackerrasae* in the blood of *E. stokesii* 222 and 244 after they were inoculated with infected blood is the only evidence for a possible transmission of *P. mackerrasae*. However, the short appearance of the blood stages and the low parasitemia might indicate that the blood stages observed were not derivatives of a successful transmission, but the few remaining *Plasmodium* stages of the inoculum that reached the blood stream from their original point of entry, the peritoneal cavity.

The reasons for the mainly unsuccessful attempts to transmit *P. mackerrasae* by blood inoculations might be:

1. There were not enough asexual parasites in the inoculum. This explanation seems highly unlikely, because in other studies inoculations using lesser amounts of blood with a lower parasitemia than the ones utilised here were successful.
2. The lizards did not show a blood infection because of an acquired immunity against *P. mackerrasae*, that either eliminated the infection altogether or suppressed a blood infection. Possibly many of the newborn lizards became infected through the ingestion of their placenta, but they may have received antibodies against *P. mackerrasae* from their mother as well. This has never been reported from reptiles, but an immunisation against malaria during murine pregnancy has been described (Pavia & Niederbuhl, 1991).
3. Less benign living conditions than the ones experienced in the ACU might trigger the onset of blood infections and could explain why none of the *E. stokesii* born in the ACU developed a blood infection, although at least two of them were infected with *P. mackerrasae*.

Developmental stages of *P. mackerrasae* were found in none of the vector species studied for this thesis. A possible reason for this result might be that none of the vector species used were suitable for the development of *P. mackerrasae*, and that the main vector of *P. mackerrasae* might be for example a local mosquito species. Another possible reason is, that the environmental conditions during the experiments or the physiological status of the vector species were not adequate. For example a study examining the transmission biology of *P. mexicanum*, a parasite of the fence lizard *Sceloporus occidentalis*, and its vector, the sand fly *Lutzomyia vexator*, found that there is only a small window of opportunity for the *Plasmodium* parasites to be transmitted (Fialho & Schall, 1995). Further studies are needed to identify the vector and thereby the complete life cycle of *P. mackerrasae*.

10.2 Plasmodium circularis n. sp.

This blood parasite was the most obscure species found during the course of the present study. Due to the close resemblance of the asexual blood stages of *P. circularis* to the immature gametocytes of *P. mackerrasae*, and to the fact that *P. circularis* was found almost entirely in mixed infections with *P. mackerrasae*, the existence of *P. circularis* was not discovered until the fixed material was sent to Dr. S. R. Telford Jr.. A vector for *P. circularis* was not identified and apart from the blood stages in *E. stokesii*, no further life cycle stages were found. Thus there were no further taxonomic data available than the ones presented by Telford & Stein (in press, see appendix).

Table 44. The coordinates of the sites where *P. circularis* was found infecting *E. stokesii* during the three year field survey (regular study sites in bold; for details of the prevalence data for all blood parasites found seen chapter fourteen).

Site	Location		No of *E. stokesii* captured	Number of *E. stokesii* found infected [Σ] with *P. circularis* and its prevalence [%]
	S	W		
Camel Hill	31°53'	138°25'	148	**1.7**
Chace Range	31°45'	138°35'	7	14.3
Drakes Nob	32°05'	138°09'	27	**18.2**
Glenlyle	31°50'	138°35'	62	**19.1**
Hawker North	31°53'	138°25'	97	**6.8**
Hawker South	31°53'	138°25'	159	**2.0**
Neuroodla	31°50'	138°08'	68	**26.4**
The Four Mile	31°50'	138°30"	1	100.0

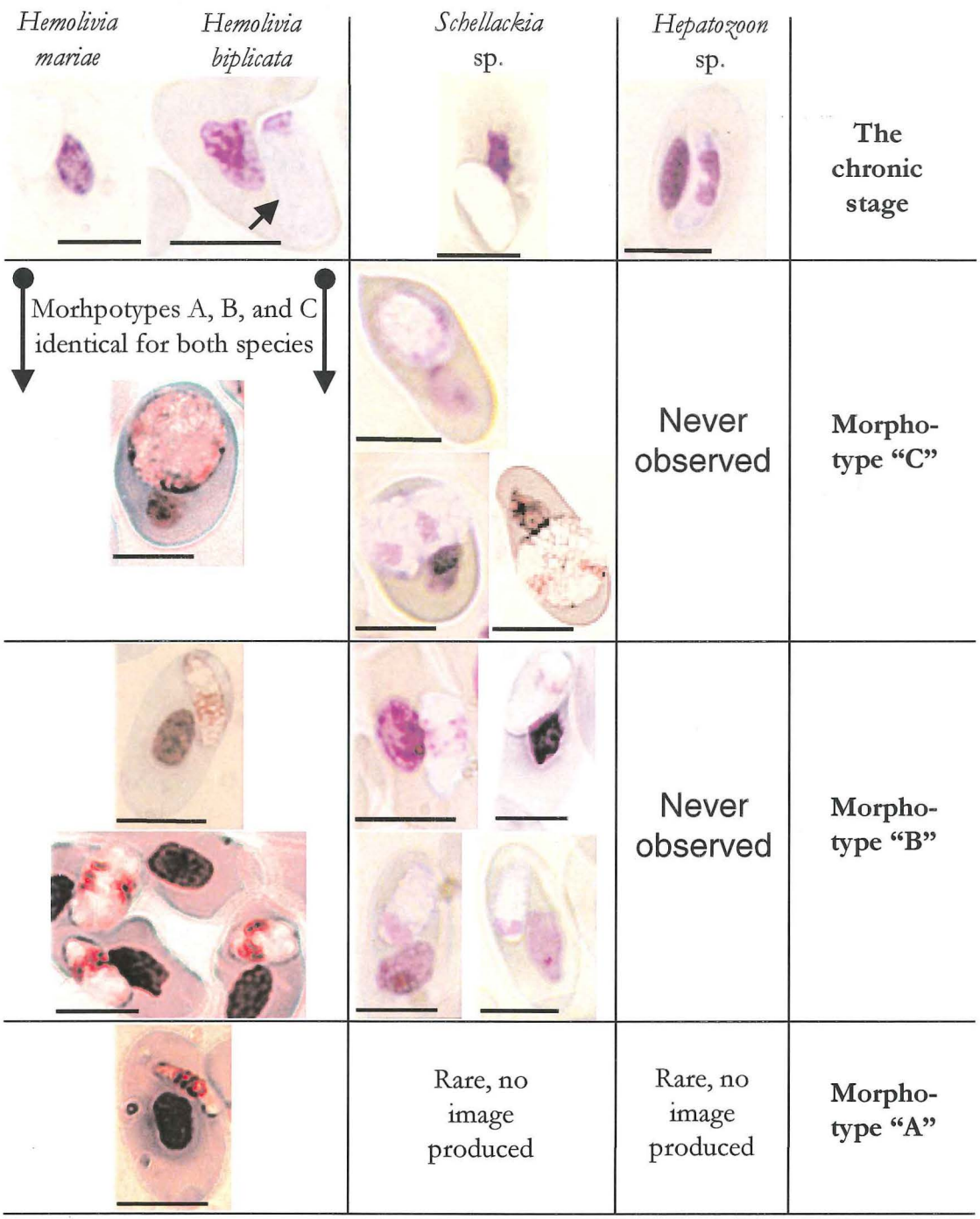

Figure 73. The diversity of haemogregarines found in giemsa stained blood smears of *Egernia stokesii*, sorted by parasite species and morphotype categories (bar= 10 µm). Due to the interspecific resemblances among the morphotypes A, B, and C, these blood stages were listed as "unspecified morphotypes" in the quantitative analysis of the blood-infection data. The short arrow marks a faintly visible fold in the gamont of *Hem. biplicata*.

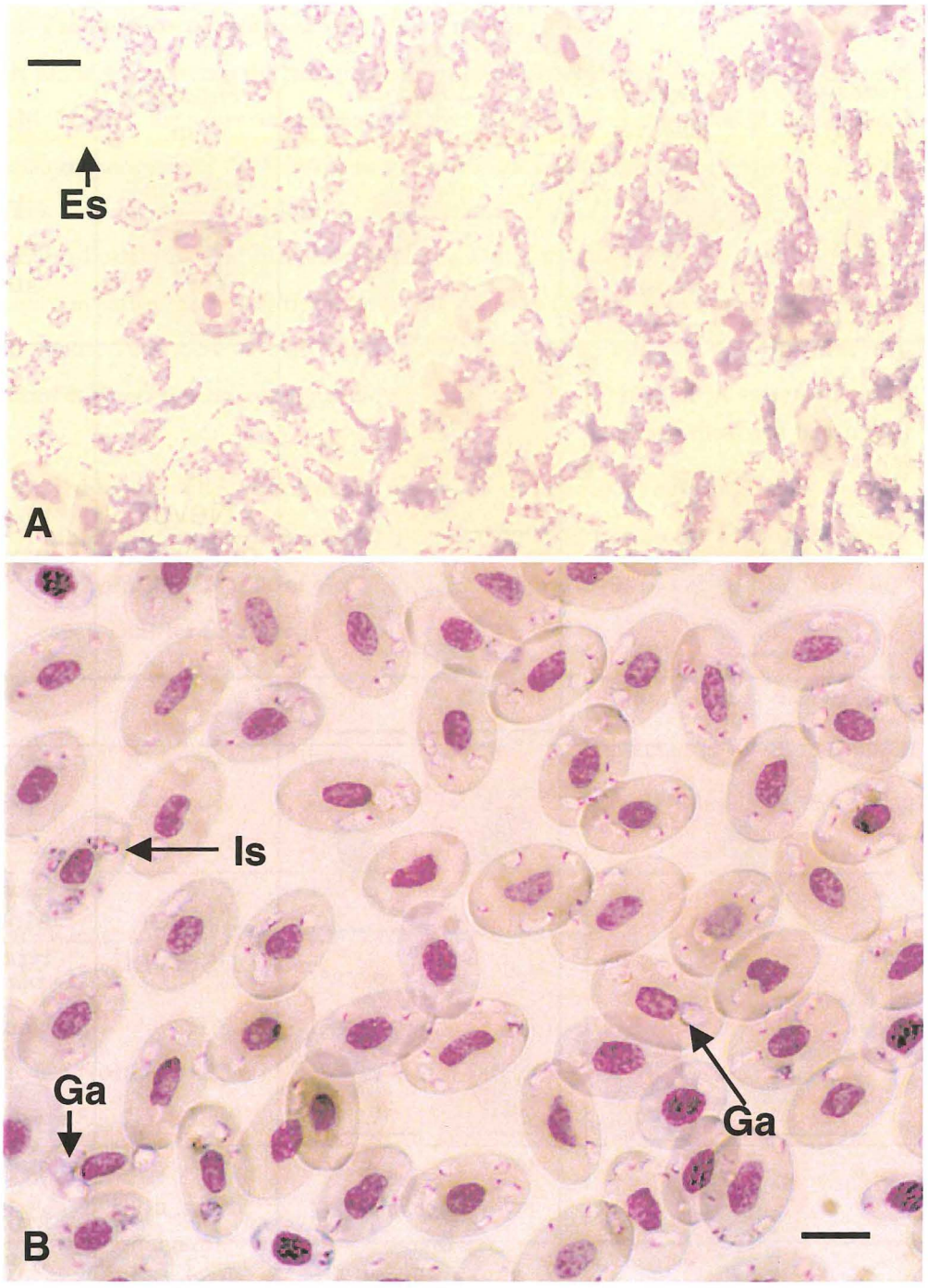

Figure 74. A+B: severe blood infections of *Egernia stokesii* with *Plasmodium mackerrasae* (bar = 10 µm). A: massive flooding with exoerythrocytic schizonts [Es]; B: chronic stage of infection [Is: intraerythrocytic schizogony, Ga: gamont].

Chapter Eleven

11. A MOLECULAR BIOLOGICAL STUDY ON THE BLOOD PARASITES OF *E. STOKESII*

11.1 Introduction

With the availability of sensitive molecular biological methods like the polymerase chain reaction (PCR) to selectively detect and amplify parasitic nucleic acid, a variety of tests have been developed for the diagnosis of many diseases of human and veterinary importance. Although the *Plasmodium* parasites that infect human blood are closely related to those infecting lizards (Qari *et al.*, 1996), the methods employed to diagnose eg. *Plasmodium falciparum* in humans did not work for the reptilian malaria parasites, mainly because the large amount of nucleic acid and proteins from the nucleated blood cells of reptiles present a major obstacle for the existing molecular approaches. Nevertheless, a series of investigations have successfully developed diagnostic molecular biological tests for coccidian parasites from nucleated blood cells (Ellis *et al.*, 1992; Procunier *et al.*, 1993; Wozniak *et al.*, 1994; Feldman *et al.*, 1995; Wozniak *et al.*, 1996; Perkins *et al.*, 1998). Three publications described the diagnosis of *Plasmodium* or haemogregarines from lizard blood (Wozniak *et al.*, 1994; Wozniak *et al.*, 1996; Perkins *et al.*, 1998), but despite precise reproduction of the methods described in the original papers, attempts by the author to repeat those results on the blood parasites of *E. stokesii* failed. Thus a new approach to diagnose the coccidian parasites by PCR was developed.

A fundamental hypothesis in the theory of evolution is the natural selection of hereditary variations. With the availability of inexpensive methods to determine the sequence of the hereditary material itself, the DNA, the comparative analysis of assumed homologous genes and their sterical structure has become a novel approach to determine phylogenetic relationships among organisms. Many studies focused on the DNA structure of the nuclear 18S small sub-unit ribosomal RNA (ssrRNA) gene and the adjacent internal transcribed spacers (ITS) because they represent highly conserved (ssrRNA) and highly variable (ITS) nucleotide sequences (eg. Kim *et al.*, 1998). During the last decade comparative analysis of the molecular structure of genes has become a promising approach to analyse the notoriously difficult phylogenetic relationship among the Apicomplexa, (eg. Ellis *et al.*, 1992;

Hotzel *et al.*, 1995; Morrison & Ellis, 1997; Kim *et al.*, 1998). Progress has been made, but new problems also arose. For example the detection of three developmentally regulated, distinct ssrRNA genes showed that homology of this gene can not be assumed throughout the Apicomplexa (Barta, 1997).

In the present study, in addition to microscopic examination of the morphology of the parasites and their life cycle, molecular techniques were attempted to help differentiate the various taxa present in lizard blood. This study was conducted in the laboratory of Dr. Warwick Grant at the FUSA, who together with Dr. Andrew Dubowsky supervised this part of the project. The two main tasks of this examination were to develop a sensitive diagnostic test for the lizard blood parasites by the polymerase chain reaction (PCR), and to support the taxonomic data gathered by the vector studies, by comparison of assumed homologous ssrRNA gene-sequences from the coccidians infecting *E. stokesii*. There was no intent to carry out a phylogenetic analysis of the coccidian taxa investigated, although the data gathered hopefully may serve as the starting point for any such study in the future. The results of this investigation are summarised in this chapter.

11.2 Materials and Methods

11.2.1 Polymerase Chain Reaction (PCR)

11.2.1.1 DNA-extraction

Approximately 0.2 ml fresh, unclotted blood from the caudal vein of an *E. stokesii* was taken with a sterile 25 gauge needle attached to a 1 ml syringe and poured on approximately 0.5 cm^2 of FTA™ paper[9]. The blood spot was air dried over night and four pieces of approximately 0.1 cm^2 blood-soaked FTA paper were punched out and placed into a 1.5 ml Eppendorf vial. The paper was washed for 30 minutes in 100 mM Tris-0.1% SDS, then the supernatant was discarded. One ml DNA-zol™ was applied for 10 minutes and the supernatant was carefully removed. The punches were washed twice with dH_2O and then soaked in methanol for 10 minutes respectively. Each time the supernatant was carefully removed. After the methanol treatment, the punches were air dried over night. Finally, 100 µl of dH_2O were added and the Eppendorf tube was then placed in boiling water for 10 minutes. The supernatant was used for the PCR reaction.

[9] Kindly supplied by Assoc. Prof. Lee Burgoyne

To investigate the tick vector, single, infected, unfed tick-nymphs were dissected and their midgut placed into DNA-zol. The midgut was taken up through a widened opening of a pipette several times. From there on the same protocol as for blood was used.

11.2.1.2 The PCR reaction-agents

The optimal reaction for both primers to amplify coccidian DNA from blood of *E. stokesii* is listed in Table 45. TAQ enzyme produced from Bresatec™, Australia was used.

Table 45. Amount and concentration of reagents used in a single optimised PCR reaction.

	µl	Reagent
	3	$MgCl_2$ (25%)
	0.6	Primer Forward (10 µ M)
	0.6	Primer Reverse (10 µ M)
	2	dNTP (4 mM)
	2	Buffer (10 x)
	7.8	H_2O
	4	Template
Σ	20µl	
	+ 1	Unit TAQ

11.2.1.3 The primers

Two sets of oligo-nucleotides targeting the adjacent ssrRNA and ITS regions were used:

1. Primers targeting parts of the parasitic ssrRNA gene, obtained from the laboratory of Dr. Warwick Grant. The use of a 'degenerate' reverse primer with an optional A or G nucleotide at mer 20 (R) allowed the annealing and amplification of a variety of related sequences and suited the primary purpose of this set of oligo-nucleotides, the diagnoses of the blood parasites.

Forw.: 5'-GAA CGA GAT CTT AAC CTG C-3'

Rev.: 5'-CAA GAT TAA TAA TTG CAA TRA TC-3'

2. Primers amplifying the complete, highly variable ITS region with a binding site of the forward primer upstream from the 3' end of the ssrRNA gene and the reverse primer binding downstream from the 5' end of the 5.8S rRNA gene (which is bordering the ITS region) (Kim *et al.*, 1998).

Forw.: 5'-CTG TAG GTG AAC CTG CGG AAG G-3'

Rev.: 5'-GCT GCG TCC TTC ATC GAT GC-3'

11.2.1.4 The temperature cycles of the PCR reaction

Best results were obtained with a "touchdown" PCR, where the annealing temperature was subsequently lowered in the first ten cycles by 0.3°C decrements from 57°C to 54°C (Table 46). This procedure increased the overall specificity of the reaction by allowing only highly specific alignments during the first cycles.

Table 46. Temperature cycle of a standard PCR after optimisation.

T °C	Duration	No. of cycles
94C	5 min	1
94C	30 sec	
57C	30 sec	9 (0.3°C decrements)
72C	60 sec	
94C	30 sec	
54C	30 sec	30
72C	60 sec	
72 C	7 min	1

11.2.1.5 Gel-electrophoresis

After the PCR was completed, 2 µl of loading dye was added to 10 µl of each reaction. The mixture was loaded on a 2% agarose gel and the electrophoresis was done at 100 mV for 40 minutes. Also loaded on the gel was the Bresatec® molecular weight marker pUC19 DNA/*Hpa* II diluted in 2x loading buffer, which produces 13 fragments between 501 and 26 base pairs. The gel was stained in ethidium bromide for 30 minutes and photographs of the product-bands were taken in the light of a strong UV lamp.

11.2.2 DNA-Sequencing

The Promega® "Wizard® Plus Minipreps DNA Purification System" was used in order to purify the DNA. Attempts to sequence products from the purified PCR product failed, thus the DNA was cloned using the "Promega pGEM-T Vector System" and vector competent *E.-coli* cells. Briefly, the product was desalted (Atrazhev & Elliott, 1996), and 5 µl were then

transferred into a ligation reaction for 5 h. The resulting ligated product was transformed into *E.-coli* bacteria by electroporation and the bacteria were subsequently grown in LB at 37°C for 2 h. Then, 300 µl of each bacterial suspension were streaked out on one ampicillin treated agar plate respectively and incubated for 12 h. In order to assure single clone colonies, minute bits of some of the resulting colonies were transferred onto fresh ampicillin-plates and incubated. A piece of these single colonies was individually placed into fresh LB on a pipette tip and incubated over night. The DNA of the resulting bacterial broth was purified with the "Wizard® Plus Minipreps DNA Purification System" and sent away for automated sequencing. The sequencing primers were T7 and SP6, which were recommended to use with the p-GEM-T system.

11.3 Results

11.3.1 The diagnosis of the blood parasites by PCR

The 18 ssrRNA gene of the genera *Hemolivia*, *Schellackia*, *Hepatozoon* and *Plasmodium* were reliably detected by the assay developed for this study from parasitemias of 0.1% and above (Figure 75/B). Table 47 lists the lizards used for the diagnostic assay and their blood infections at the time of sampling. A noticeable size difference occurred between the PCR products from the haemogregarine species and *P. mackerrasae* (Figure 75/A). This relatively large size difference between the assumed homologous 18 ssrRNA sequences generated a simple tool to diagnose haemogregarine parasites and the *P. mackerrasae* in one PCR.

Using the same protocols but a different set of primers, efforts to amplify the ITS-1 gene were only partly successful. Consistently, the primers created a PCR product for only one species of blood parasites, *Hepatozoon* sp.. Since a semi-conserved region of the 3' end of the 18S of *Hepatozoon sipedon, Hepatozoon catesbianae* and *Hepatozoon clamatae* were used to create the forward primer (Smith *et al.*, 1999), it was suspected that the ITS-1 primers used here were *Hepatozoon*-specific (Todd Smith, pers. comm.).

11.3.2 The analysis of 18 ssrRNA nucleotide sequences of the micro-parasites

During the first phase of the development of the diagnostic assay, all tests using lizard blood as template produced a band amplified from the lizard-host DNA. The nucleotide string amplified from lizard DNA was only slightly shorter than those of the adeleorine and eimeriorine products. In efforts to create a more specific PCR reaction, the host DNA

byproduct was finally eliminated, but the products were not pure enough for sequencing. Thus, the PCR product had to be cloned before sequencing. Alternatively to parasite DNA extraction from lizard blood, parasite DNA was also extracted from single unfed *Amb. limbatum* and *Amb. limbatum* nymphs that were single infected with *Hem. mariae*. PCR amplifications of infected ticks always resulted in very clean bands with no host byproduct (Figure 75/C).

Infected blood from a total of nine lizards was used to amplify the targeted coccidian DNA (Table 48). Eight of the nine lizards were single infected at the time of bleeding, one lizard had a mixed infection with *P. mackerrasae, P. circularis and Schellackia* sp.. The lizards were chosen for their relatively high parasitemia to maximise the yield of amplified DNA. Each lizard was bled once and the fresh blood was dried and stored on FTA-paper for further processing. Coccidian DNA was freshly isolated from each dried blood sample on three different occasions, resulting in a total of 27 DNA-samples from the nine lizards. The PCR product obtained from each DNA-sample was cloned and a minimum of two single-colony clones were sequenced from each product. The DNA isolated from two single *Amb. limbatum* nymphs (Table 49) that were single infected with *Hem. mariae* underwent the PCR and at least two single-colony clones from each DNA isolate were sequenced. Using either the T7 (5'-3') or the SP6 (3'-5') primer to cut the product from the bacterial plasmid, the forward and reverse sequencing data obtained from those samples gave consistent results. The length of the PCR product for the adeleorine and the eimeriorine species was found to be 241 base pairs (bp) (Figure 76); the size of the product of the *Plasmodium* species was found to be 454 bp (Figure 77). The 241 bp nucleotide sequences of the haemogregarines were compared in Figure 76. The sequences of *Hem. mariae* found in chronically infected *E. stokesii* at study sites in the Flinders Ranges was identical to *Hem. mariae* found in *T. rugosa* from the Mt. Mary region. The isolated nucleotide sequence of the 18 ssrRNA gene of *Hem. mariae*, *Schellackia* sp. and *Hepatozoon* sp. were almost identical. Only a two bp difference was found between *Schellackia* sp. and *Hem. mariae*. Eight bp were different between the sequences of *Hepatozoon* sp. and *Hem. mariae* and ten bp were different between *Hepatozoon* sp. and *Schellackia* sp.. A nucleotide sequence of *Hem. biplicata* could not be determined because for reasons unknown, repeated attempts to sequence that cloned PCR product failed to deliver useable results. Alignments of the nucleotide sequences of the haemogregarines using the GenBank database showed closest affinities to the 18 ssrRNA nucleotide sequence of the eimeriorine taxa *Neospora, Sarcocystis Toxoplasma, Frenkelia* and

Isospora. These data comprised the first published 18 ssrRNA nucleotide-sequences of adeleorine blood parasites.

The 18 ssrRNA nucleotide sequence of *P. mackerrasae* isolated in this study was 455 bp long. Alignments using the GenBank database showed the closest affinity to be the 18 ssrRNA nucleotide sequence of two "unidentified haemosporida" deposited in GenBank by Dame, J.B., Telford, S.R. Jr., Yowell, C.A., Higgins, D. and McCutchan, T.F in 1993 (unpublished). The alignments of the nucleotide sequences of *P. mackerrasae* with one of the identical sequences of the two unidentified haemosporida are presented in Figure 77. Of the 455 bp sequenced, 319 bp were identical between the two groups. The next closest matches were the 18 ssrRNA nucleotide sequences of the avian malarias *P. gallinaceum, P. relictum* and *P. lophurae*, followed by the human parasite *P. falciparum*. The sequences of the two "unidentified haemosporida" were obtained from blood of the Californian lizard *Sceloporus occidentalis* and *Anolis carolinensis*. They were identified with certainty as *P. mexicanum* (donor *Sceloporus occidentalis*) and *P. floridense* (donor *Anolis carolinensis*) by Dr. Sam R. Telford Jr., but due to disagreements in other aspects of that study they were deposited without consent of the original authors in GenBank (S. R. Telford Jr., pers. comm.). Apart from the two "unidentified haemosporida", no further 18 ssrRNA data of lizard malaria species have been published.

Table 47. Infection status of lizard blood that was used as template for the diagnostic assay, explaining the origin of the template used in the gel electrophoresis lanes presented in Figure 75/B&C. The shadings separate three types of infection [light grey: infected with *P. mackerrasae*, mostly mixed infections; not shaded: single infections with haemogregarines; dark grey: uninfected].

E. stokesii	Infection the lizard was carrying	Parasitemia at the time of blood sample [%]	Numbers represent lane(s) in the gel electrophoreses shown in Figure 75	
			A	B
220	*P. mackerrasae*	52.40	3	9
	P. circularis	1.00		
	Schellackia sp.	0.16		
905	*P. mackerrasae*	87.90	2	12
134	*P. mackerrasae*	41.00		4
	Schellackia sp.	0.35		
204	*P. mackerrasae*	0.84		2
	Schellackia sp.	1.10		
41	*Hem. mariae*	2.20	1	13
30	*Hem. mariae*	13.90		3
101	*Hem. mariae*	1.50		10
1012	*Hem. biplicata*	0.93		5
177	*Schellackia* sp.	1.30		6
272	*Schellackia* sp.	2.12		7
37	*Hepatozoon* sp.	0.74		1
				8
5	uninfected	-		11
4	uninfected	-		14

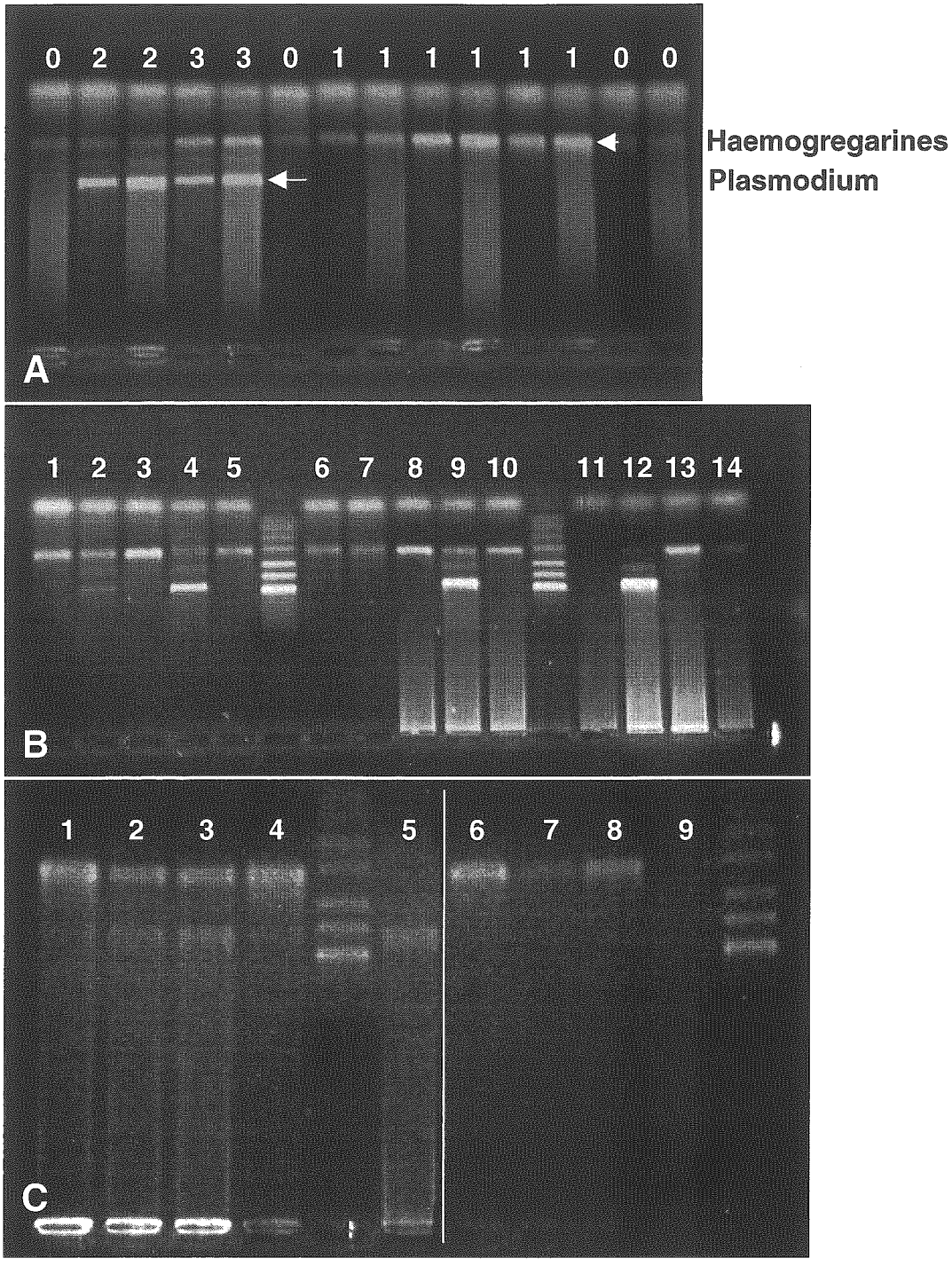

Figure 75. A-C: Gel electrophoresis of PCR products. A: diagnostic test to differentiate between haemogregarines and *Plasmodium* blood parasites from lizard blood, note that a faint band is present in uninfected samples [0: uninfected, 1: haemogregarines, 2: *Plasmodium*, 3: mixed infection of *Plasmodium* and haemogregarines]. B: diagnostic test for four different haemogregarine species (for details see Table 47). C: Clean products of the adeleorine species *Hemolivia mariae* from tick isolates (lane 6-9).

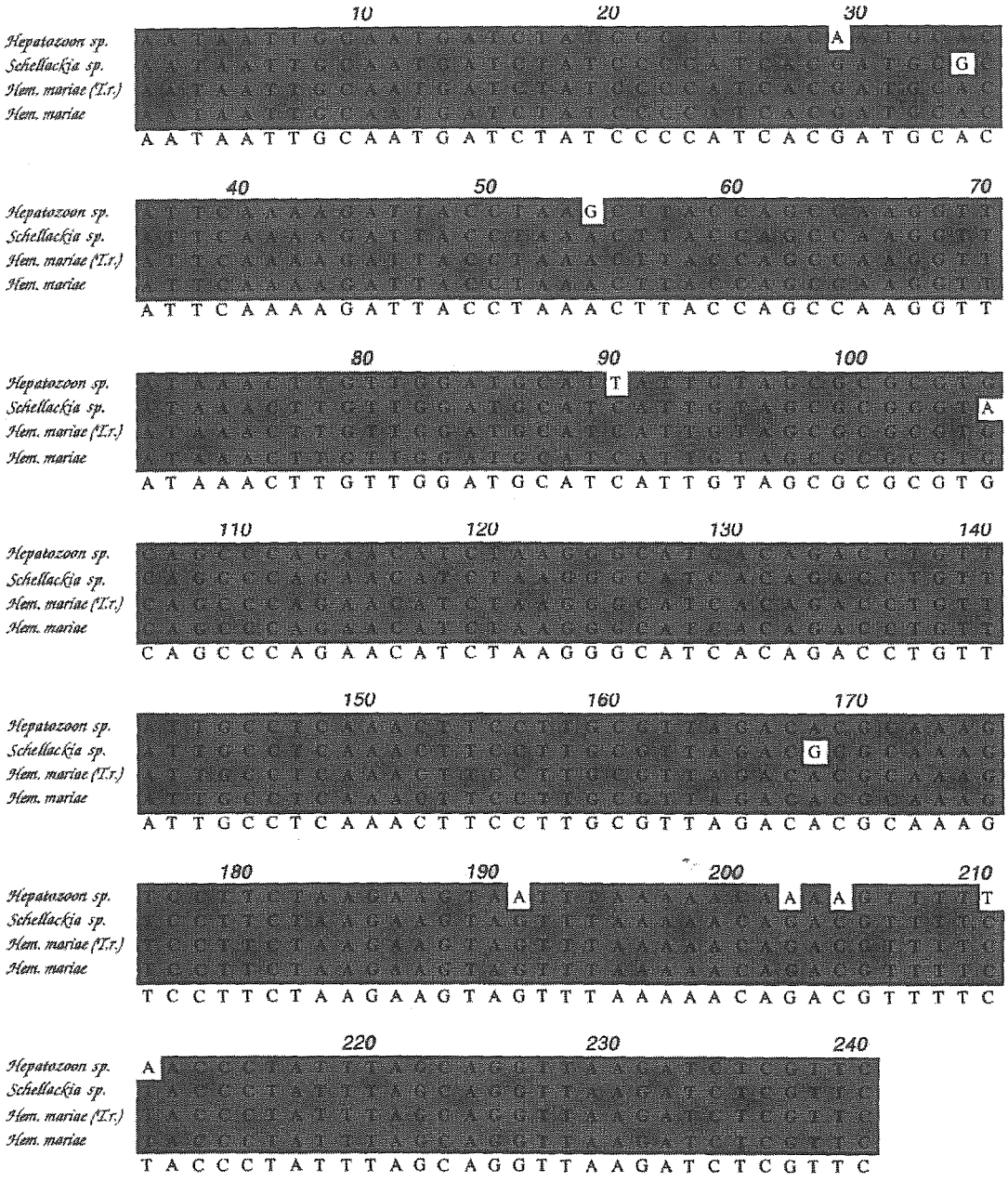

Figure 76. Nucleotide sequences of three species of haemogregarines in comparison to each other. The sequences of *Hem. mariae* isolated from the blood of *E. stokesii* and *T. rugosa* (*T. r.*) were found to be identical.

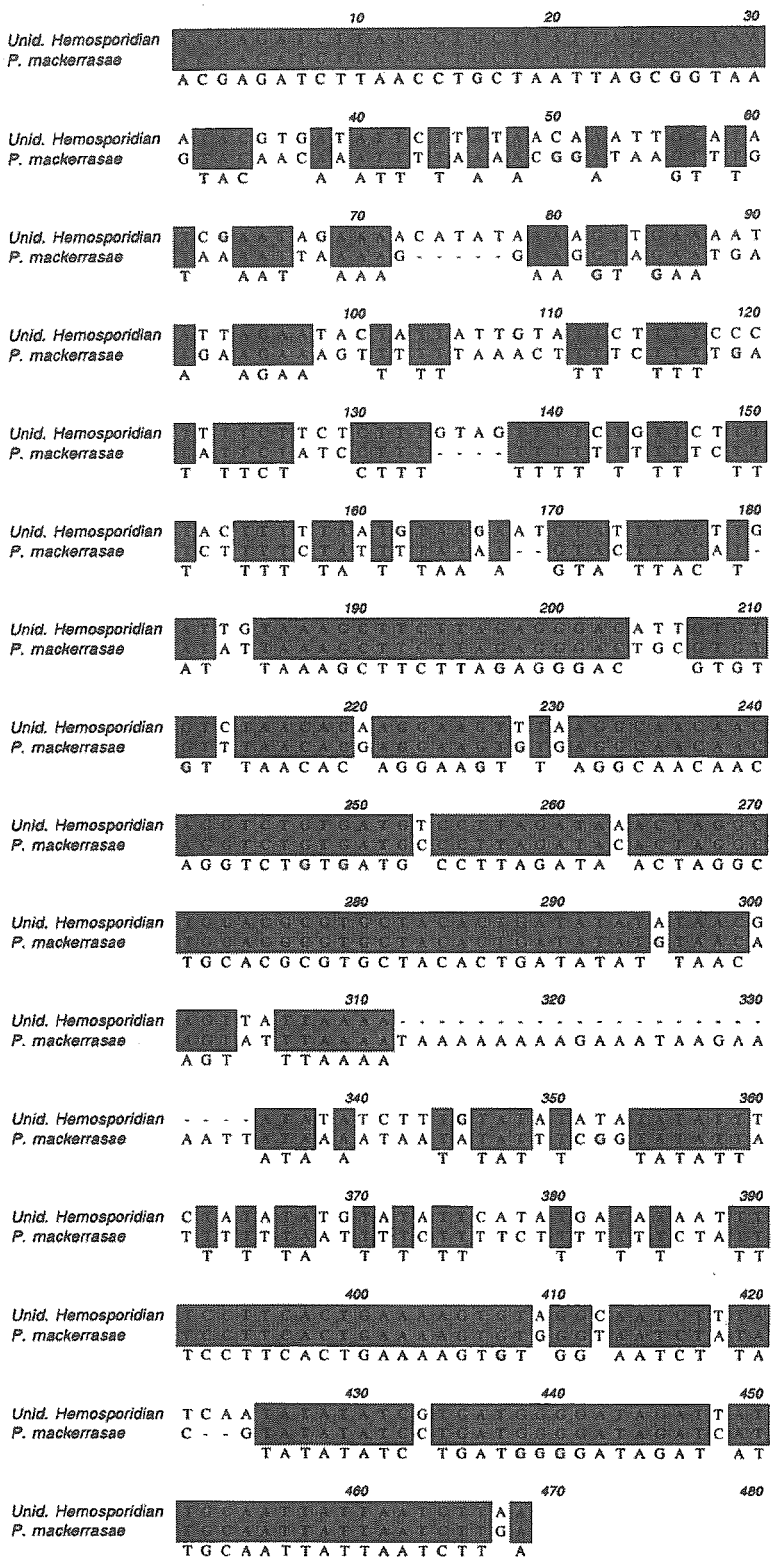

Figure 77. Nucleotide sequence of *P. mackerrasae* in comparison to its next closest match, the "unidentified haemosporida".

Table 48. Origin of lizard blood samples that were used as template in PCR reactions to amplify parts of the 18 ssrRNA gene of coccidian blood parasites.

E. stokesii	Origin of lizard	Infection the lizard was carrying	Parasitemia at the time of blood sample [%]	Type of infection
220	Field-caught	*P. mackerrasae*	52.40	Chronic
		P. circularis	1.00	Chronic
		Schellackia sp.	0.16	Chronic
905	Field-caught	*P. mackerrasae*	87.90	Chronic
41	Field-caught	*Hem. mariae*	2.20	Chronic
30[*1]	ACU-born	*Hem. mariae*	13.90	Experim. transm.
101[*2]	ACU-born	*Hem. mariae*	1.50	Experim. transm.
1012[*3]	ACU-born	*Hem. biplicata*	0.93	Anecdotally transm.
177	ACU-Born	*Schellackia* sp.	1.30	Spontaneous
272	ACU-born	*Schellackia* sp.	2.12	Spontaneous
37	Field caught	*Hepatozoon* sp.	0.74	Chronic
T. rugosa				
1[*4]	Field caught	*Hem. mariae*	0.2	Chronic

*1 = Transmitted from *E. stokesii* to *E. stokesii* (for details see 6.1.4.2).

*2 = Transmitted from *T. rugosa* to *E. stokesii* (for details see 6.1.4.1).

*3 = Transmitted during experiments outside of this study (for details see 7.1.4).

*4 = *T. rugosa* from the Mt. Mary area, kindly supplied by Dr. Cathy Smallridge.

Table 49. Origin of infected unengorged *Amb. limbatum* nymphs that were used as template in PCR reactions to amplify parts of the 18 ssrRNA gene of coccidian blood parasites.

E. stokesii that larvae used as hosts	Infection the lizard was carrying	Parasitemia at the time of larval engorgement [%]	Lane of the gel electrophoresis in Figure 75/C
1377	*Hem. mariae*	0.95	7 & 8
	P. mackerrasae	4.20	
41	*Hem. mariae*	2.20	6

11.4 Discussion

The new molecular biological assay presented in this chapter generated a relatively simple tool for the diagnosis of the blood parasites of *E. stokesii* from a parasitemia of above 0.1%. Lower parasitemias have not been tested, but are most likely to be detectable. Modifications in the procedure of DNA extraction, eg. the increase of template DNA or the reduction of water in the final step of DNA dilution would most likely increase the sensitivity of this method. Whether the assay can detect *P. circularis* parasites could not be determined because *P. circularis* was only present in mixed infections with *P. mackerrasae*. No size difference was detectable between the PCR products of *P. mackerrasae* and mixed infections of *P. mackerrasae* and *P. circularis*. However, since this assay amplified DNA from all other parasites including from single infections of *P. mackerrasae*, it was most likely that the method would work on *P. circularis* as well.

The analysis of the nucleotide sequences from *E. stokesii* and *T. rugosa* diagnosed with single infections of *Hem. mariae* found the sequences to be identical (Figure 76), thus supporting the findings of chapter six. The analysis of nucleotide sequences from *E. stokesii* diagnosed with single infections of *Hem. mariae, Schellackia* sp. and *Hepatozoon* sp. resulted in the isolation of three different nucleotide sequences, supporting the taxonomic results that these represent separate species, presented in chapter six, eight and nine. The reasons for the persistent failure to sequence clones of the PCR product of *Hem. biplicata* remain unclear.

The similarities between the three haemogregarine sequences when compared to the sequence of *P. mackerrasae* suggested a close phylogenetic relationship between the

haemogregarines. A close phylogenetic relationship between the adeleorine and eimeriorine species was postulated by Barta (1989) using a more general phylogenetic approach. This was supported by the data presented here.

In conclusion, the taxonomic analysis of the blood parasites found in *E. stokesii*, as described in chapters five to eleven, required a whole range of different methods, and each method contributed to the process to a certain extent. The two species of *Plasmodium* could be distinguished and identified taxonomically solely by comparison of the blood stages as they appear in a giemsa stain. In contrast, to distinguish between the haemogregarines and to identify them was a more complex task. One species of blood parasite that spontaneously infected lizards born and raised in the ACU was recognised by the long term monitoring of *E. stokesii* kept in the ACU. This method also elucidated the diversity of the various semaphoronts of that particular blood parasite in lizard blood. Transmission experiments proved the presence of two additional haemogregarine species that both were vectored by ticks. The long term monitoring of experimentally induced infections demonstrated high morphological resemblances between the three distinguished haemogregarine species in regard to the blood stages that were not encapsulated. Finally, the molecular biological assay presented here led to the analysis of the coccidian 18 ssrRNA sequences and confirmed the presence of a fourth haemogregarine. This species was the only haemogregarine found in *E. stokesii* that was detectable by a primer designed from genetic material of the adeleorine genus *Hepatozoon*.

Chapter Twelve

12. TRANSMISSION EXPERIMENTS USING *E. STOKESII* WITH MIXED INFECTIONS

Ticks have been identified as vectors for many adeleorine parasites (Levine, 1988), and have been suspected to transmit *Plasmodium* as well (Telford, 1994). At study sites in South Australia, the ticks *Amb. limbatum* and *Aponomma hydrosauri* were described to transmit the adeleorine *Hemolivia mariae* to the lizard *T. rugosa* (Smallridge & Paperna, 1997). In order to investigate further the vector capacity of *Amb. limbatum* and *Amb. vikirri* regarding the six species of blood parasites, a series of transmission experiments were conducted.

After two successful breeding seasons in the laboratory and additional captures of gravid *E. stokesii* in the field, a total of six long term uninfected lizards remained from over 200 *E. stokesii* held and monitored in the ACU for the transmission experiments. Because only few transmission experiments with uninfected lizards were possible, vector experiments concentrated on the hard ticks *Amb. limbatum* and *Amb. vikirri*. A series of three experiments investigated whether blood parasites could be transmitted to *E. stokesii* by:

I. the lizards ingesting infected hungry tick nymphs
II. the attachment of infected hungry tick nymphs to the lizards
III. the lizards ingesting infected engorged tick larvae.

In each experiment, *E. stokesii* were exposed to ticks that had previously fed on infected or uninfected *E. stokesii* and the blood of all lizards involved was monitored for newly emerging infections. The lizards were bled approximately every three weeks until the first successful transmission was detected. The lizards were then bled weekly for approximately five weeks and subsequently in two to three week intervals until the experiments were terminated. The results of the transmission experiments were summarised in the Tables 50-53. The results of the long-term blood parasite counts of each lizard involved are presented in the Figures 78-127. The results of experiment I that showed a successful transmissions of *Hem. biplicata* have already been presented in chapter seven. However, all figures displaying the results of experiment I are presented in this chapter (Figures 78-97).

Due to the ubiquitous presence of the *Schellackia* sp. parasite even in young, laboratory-born *E. stokesii* (see 8.1.4), few experiments could be conducted where blood parasites were transmitted to uninfected lizards. In order to make the transmission experiments as reliable as possible under these circumstances, the lizards were monitored regularly for blood parasites for at least one year before a transmission experiment was attempted. Only those lizards that had a single infection with *Schellackia* sp. or that were found to be uninfected with other species of blood parasites for longer than one year were used. The occurrence of spontaneous infections of *Schellackia* sp. were discussed within the taxonomic description for this parasite in chapter eight.

12.1.1.1 Transmission experiment I: the ingestion of hungry nymphs

Amb. limbatum and *Amb. vikirri* larvae were fed between the 14/10/97 and the 11/03/98 on ten *E. stokesii*. Two of the lizards were uninfected, the other eight had single or mixed blood infections (Table 50). After moulting, the resulting unfed nymphs were force fed to ten *E. stokesii* on the 16/05/98; the lizards were uninfected or had only a single blood infection of *Schellackia* sp. at that time.

Five out of six lizards that had ingested *Hem. mariae* infected nymphs became infected with *Hem. mariae* (*E. s. 657, 47, 391, 9, 353*; Table 50). The first chronic stages of *Hem. mariae* were detected between 84 and 151 days after the nymphs were ingested (Table 51). *E. stokesii 657*, *47*, and *391* had ingested *Amb. limbatum* nymphs, *E. stokesii 9* and *353* had ingested *Amb. vikirri* nymphs. No blood infection was detected in *E. stokesii 310*, although the ticks it ingested had engorged on a lizard infected with *Hem. mariae* (*E. stokesii 1377*; Table 50). At the time of larval engorgement, *E. stokesii 1377* had a higher parasitemia than *E. stokesii 12*, which sufficiently infected ticks to transmit *Hem. mariae* to *E. stokesii 391* (Table 50). Although *E. stokesii 9* was previously infected with *Hem. mariae* when it was caught in the field and then lost its blood-infection (Figure 95) it became infected with *Hem. mariae* again after the ingestion of the nymphs.

Five out of five lizards that had ingested *Hem. biplicata*-infected nymphs became infected with *Hem. biplicata* (Table 50). The first chronic stages were detected between 84 and 197 days after the nymphs were ingested. As with *Hem. mariae*, *Hem. biplicata* was transmitted by the ingestion of unengorged nymphs of both species, *Amb. limbatum* and *Amb. vikirri*. Due to the

lack of *E. stokesii* with single infections of *Hem. biplicata*, transmissions were only achieved in connection with *Hem. mariae*.

During the course of this experiment, three out of ten lizards became infected with *Schellackia* sp. (*E. stokesii 33*, *63* and *310*; Table 50). Two of these lizards (*E. stokesii 33* and *310*) had ingested nymphs that have fed as larvae on lizards with no detectable infection of *Schellackia* sp. (Table 50; Figure 86 and Figure 92). Only one (*E. stokesii 63*) out of four lizards that had ingested presumably *Schellackia* sp. infected nymphs became infected with *Schellackia* sp. (Table 50; Figure 91) . The spontaneous infections of *E. stokesii 33* and *310* were observed in most of the lizards kept in the ACU, whether they were born in the laboratory or caught in the field, whether they were used in transmission experiment or remained vector-naive. As a result, no conclusive evidence of transmissions of *Schellackia* sp. could be derived from all experiments conducted for this study, because it was not possible to distinguish between a spontaneous infection or an experimentally transmitted one, and eg. the occurrence of *Schellackia* sp. in the blood of *E. stokesii 63* can not be taken as evidence for a transmission by tick ingestion. The frequencies and the cause of the apparent spontaneous infections with *Schellackia* sp. have been discussed within the taxonomic description of *Schellackia* sp. in chapter eight.

In conclusion, *Hem. mariae* and *Hem. biplicata* were successfully transmitted by the ingestion of unengorged nymphs of *Amb. limbatum* and *Amb. vikirri*. In the same experiments, no transmissions of *Plasmodium* or *Hepatozoon* sp. were detected. Due to the spontaneous infections of lizards with *Schellackia* sp., for example in *E. stokesii 33* and *E. stokesii 310*, a transmission of *Schellackia* sp. from *E. stokesii 741* to *E. stokesii 63* could not be proven. The unsuccessful transmission of *Hem. mariae* from *E. stokesii 1377* to *E. stokesii 310* could be due to the relative fresh tick nymphs used, which were attached as larvae on the 11/03/98 (Table 50). Although they moulted approximately two weeks before they were fed as nymphs to *E. stokesii 310*, the time might not have been sufficient for the development of the infective stages of *Hem. mariae*. It is also possible that the ticks fed to *E. stokesii 310* were uninfected, because not all *Amb. limbatum* ticks that fed on *E. stokesii* infected with *Hem. mariae* became infected (see chapter six). This finding corresponded to the results from the original description of *Hem. mariae* in *T. rugosa* (Smallridge, 1998).

12.1.1.2 Transmission experiment II: the attachment of hungry nymphs

Amb. limbatum and *Amb. vikirri* larvae were attached to uninfected *E. stokesii* or *E. stokesii* with different mixed blood infections between the 14/10/97 and the 11/03/98 (Table 52). These ticks developed into nymphs, which then were allowed to engorge until depletion on uninfected *E. stokesii* or *E. stokesii* with only a single blood infection of *Schellackia* sp. on the 17/05/98.

No transmission of *Hem. mariae*, *Hem. biplicata*, *Hepatozoon* sp. or *Plasmodium* was detected after the fully engorged nymphs had detached. (Table 52, and Figures 98-117). During the course of this experiment, two out of ten lizards became infected with *Schellackia* sp. (*E. stokesii 91* and *1178*). In this experiment, both lizards have been the host of nymphs that have fed as larvae on lizards with no detectable infection of *Schellackia* sp. (Figure 100 and Figure 108). None out of four lizards that had hosted presumably *Schellackia* sp.-infected nymphs became infected with *Schellackia* sp. (Table 52).

12.1.1.3 Transmission experiment III: the ingestion of engorged larvae

Amb. limbatum larvae were attached to uninfected *E. stokesii* or *E. stokesii* with different mixed blood infections on the 19/04/98 (Table 53). The resulting engorged larvae were force fed to uninfected *E. stokesii* or *E. stokesii* with only a single blood infection of *Schellackia* sp. on the 16/05/98. *Amb. vikirri* ticks were not included into this experiment

No transmission of *Hem. mariae*, *Hem. biplicata*, *Hepatozoon* sp. or *Plasmodium* was detected (Table 53, and Figures 118-127). No lizard became infected with *Schellackia* sp. during the course of this experiment. *E. stokesii 21*, that had ingested presumably highly *Schellackia* sp.-infected larvae (Figure 124) did not become infected with *Schellackia* sp. (Table 53 and Figure 125). The increase in parasitemia of the *Schellackia* sp. infected *E. stokesii 357* (Figure 123) might have been due to an infection by the ingestion of the engorged larvae, but *E. stokesii* 2 (Figure 121) also showed an increase of the parasitemia although it ingested larvae presumably uninfected with *Schellackia* sp..

12.2 Discussion

Hem. mariae and *Hem. biplicata* were transmitted by the ingestion of unengorged infected nymphs of *Amb. vikirri* and *Amb. limbatum*, but not by the attachment of infected nymphs or the ingestion of engorged infected *A. limbatum* larvae. The vector role of engorged infected *A. vikirri* larvae remains to be investigated. In all experiments, no transmission by tick attachment or ingestion was detected for *Hepatozoon* sp. or *Plasmodium*. A successful transmissions of *Schellackia* sp. by the ingestion or attachment of the ticks *Amb. limbatum* and *Amb. vikirri* could not be proven, due to the frequent spontaneous infections of *E. stokesii* with the micro-parasite.

These results suggest, that of the six parasite species investigated here, the ticks *Amb. vikirri* and *Amb. limbatum* exclusively vector the blood parasites *Hem. mariae* and *Hem. biplicata*. This finding corresponded to the results of tick dissections, where no evidence for a further development of parasites except *Hem. mariae* and *Hem. biplicata* was detected. However, it can not be stated with certainty that the transmission of blood parasites other than *Hem. mariae* and *Hem. biplicata* by the ingestion of ticks did not occur. In contrast to adeleorine parasites, *Plasmodium* and *Schellackia* parasites can be transmitted by the ingestion of their vertebrate blood stages (see chapter two). Therefore it is hypothetically possible that both *Plasmodium* and *Schellackia* are transmitted by the ingestion of freshly engorged ticks, even if further development or long term survival of those parasites in the vector ticks does not occur. This particular pathway of transmission is not possible for *Hepatozoon* sp., because it needs to develop into sporozoites before it can infect a new host. It is possible that during the experiments presented in this chapter, successful transmissions of *P. mackerrasae* and *Schellackia* sp. did occur, but they might have been not detected because the lizards did not develop a blood infection. Further studies are needed to examine the vector capacity of *Amb. limbatum* and *Amb. vikirri*, and the course of new infections of *Plasmodium* and *Schellackia* sp. in *E. stokesii*.

Table 50. Transmission experiment I (A-J), the force feeding of 5 presumably infected or 5 uninfected tick nymphs to *E. stokesii*; negative controls shaded. For details see fig 78-97 (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *H. b.*: *H. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp.* *P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A. v.*: *Amb. vikirri*).

Infection status of the lizards at the time of feeding of the tick larvae (parasitemia in 10^4 RBC's)										New infections found in *E. stokesii* following the ingestion of 5 unfed nymphs on the 16/05/98.						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*	Vector species	Attachment date (larvae)	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
A	*90*	106	24	4	5	347		*A. l.*	14/10/97	*657*	+	+				
B	*11*	290	440		20			*A. l.*	14/10/97	*47*	+	+				
C	*12*	23	211					*A. l.*	14/10/97	*391*	+	+				
D	*720*			135				*A. l.*	14/10/97	*421*						
E	*24*							*A. l.*	14/10/97	*33*			+[1]			
F	*4*							*A. v.*	26/01/98	*22*						
G	*741*			100				*A. v.*	26/01/98	*63*			+			
H	*1377*	71				480		*A. v.*	11/03/98	*310*			+[1]			
I	*11*	290	423		40			*A. v.*	26/01/98	*9*	+	+				
J	*90*	91	82	1	9			*A. v.*	26/01/98	*353*	+	+				

+[1] = The lizard host of the tick larvae had no detectable blood infection with *Schellackia sp.* during the phase of engorgement.

Table 51. Transmission experiment I; the number of days from the ingestion of tick nymphs until a blood infection was detected (prepatence). Negative controls shaded.

Lizard ID	Prepatent phase of blood infections (in days)			
	Unspecified morphotypes	*Hemolivia mariae*	*Hemolivia biplicata*	*Schellackia* sp.
E. s. 657	80	93	114	
E. s. 47	127	151	151	
E. s. 391	+[1]	167	197	(+)
E. s. 421				
E. s. 33	271[2]			271[2]
E. s. 22				
E. s. 63	33			33
E. s. 310	59[2]			80[2]
E. s. 9	+[1]	127	151	(+)
E. s. 353	80	84	84	

(+) = The lizard the nymphs were fed to was already infected with *Schellackia* sp. at the time of tick ingestion.

+[1] = *Schellackia* sp. morphotypes were present throughout the experiment, thus the appearance of additional morphotypes could not be designated to *Hem. mariae* or *Hem. biplicata.*

+[2] = The lizard host of the tick larvae had no detectable blood infection with *Schellackia* sp. during the phase of tick engorgement.

Table 52. Transmission experiment II (A-J), 10 presumably infected or 10 uninfected unfed nymphs were allowed to engorge to depletion on *E. stokesii;* negative controls shaded. For details see fig 98-117 (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *H. b.*: *H. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp. P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A. v.*: *Amb. vikirri*).

Infection status of the lizards at the time of feeding of the tick larvae (parasitemia in 10^4 RBC's)								Vector species	Attachment date (larvae)	New infections found in *E. stokesii* following the attachment of 10 nymphs on the 17/05/98.						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*			*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
A	*90*	106	24	4	5	347		*A. l.*	14/10/97	*40*						
B	*16*	4	4			6100		*A. l.*	14/10/97	*91*			+[1]			
C	*12*	23	211					*A. l.*	14/10/97	*3*			(+)			
D	*720*			135				*A. l.*	14/10/97	*140*						
E	*24*							*A. l.*	04/10/97	*31*						
F	*4*							*A. v.*	26/01/98	*1178*			+[1]			
G	*741*			100				*A. v.*	26/01/98	*124*						
H	*620*	192				680		*A. v.*	11/03/98	*100*			(+)			
I	*11*	290	423		40			*A. v.*	26/01/98	*395*			(+)			
J	*90*	91	82	1	9			*A. v.*	26/01/98	*7*						

+[1] = The lizard host of the tick larvae had no detectable blood infection with *Schellackia sp.* during the phase of engorgement.
(+) = The lizard that ingested the engorged larvae was already infected with *Schellackia sp.* at that time.

Table 53. Transmission experiment III (A-E), force feeding of 5 presumably infected or 5 uninfected engorged larvae on *E. stokesii;* negative controls shaded. For details see fig 118-127 (*E. s.*: *E. stokesii*, *H. m.*: *Hem. mariae*, *H. b.*: *H. biplicata*, *S. sp.*: *Schellackia sp.*, *H. sp.*: *Hepatozoon sp. P. m.*: *P. mackerrasae*, *P. c.*: *P. circularis*, *A. l.*: *Amb. limbatum*, *A. v.*: *Amb. vikirri*).

Infection status of the lizards around the time of feeding of the tick larvae (parasitemia in 10^4 RBC's).								Vector species	Attachment date (larvae)	**New infections found in *E. stokesii* following the ingestion of 10 engorged larvae on the 16/05/98.**						
#	*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*			*E. stokesii*	*H. m.*	*H. b.*	*S. sp.*	*H. sp.*	*Pl. m.*	*Pl. c.*
A	*90*	106	109	3	9			*A. l.*	19/04/98	*210*			(+)			
B	*16*	4	28			1900		*A. l.*	19/04/98	*2*			(+)			
C	*500*	220	320	70	100			*A. l.*	19/04/98	*357*			(+)			
D	*741*			122				*A. l.*	19/04/98	*2*						
E	*5*							*A. l.*	19/04/98	*200*						

(+) = The lizard the engorged larvae were fed to was already infected with *Schellackia sp.* at the time of tick ingestion.

Figures of transmission experiment I, (A-J)

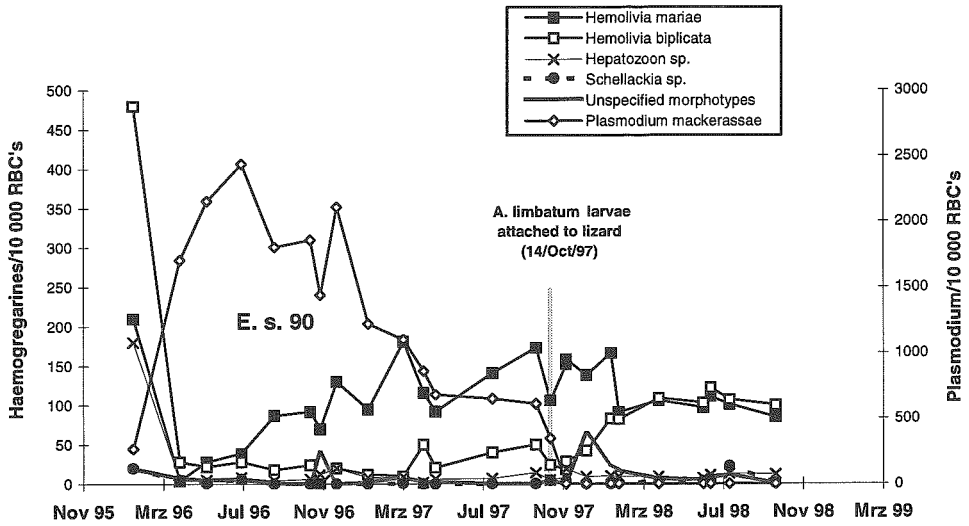

Figure 78. The presence of blood parasite stages in *E. stokesii 90* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/10/97 were force fed as unengorged nymphs (n=5) to *E. stokesii* 657 (see below) [Experiment I-A].

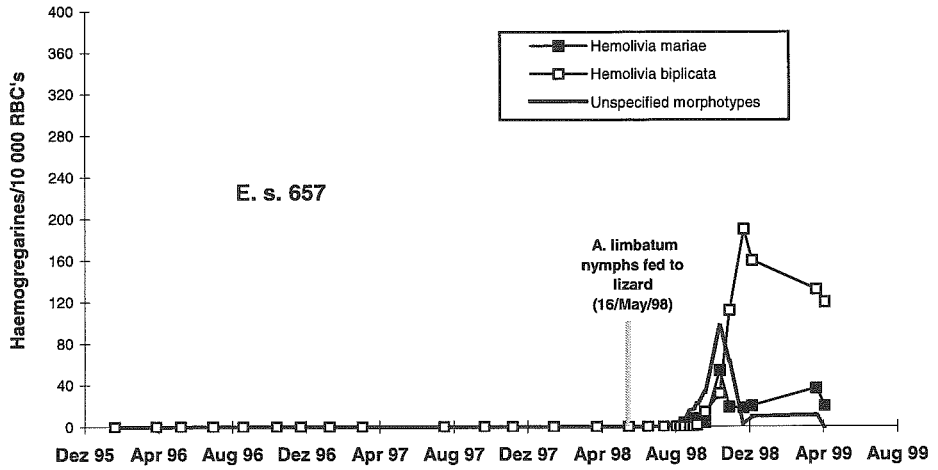

Figure 79. The presence of blood parasite stages in *E. stokesii 657* throughout the time span it was monitored. *Hem. mariae* and *Hem. biplicata* were successfully transmitted.

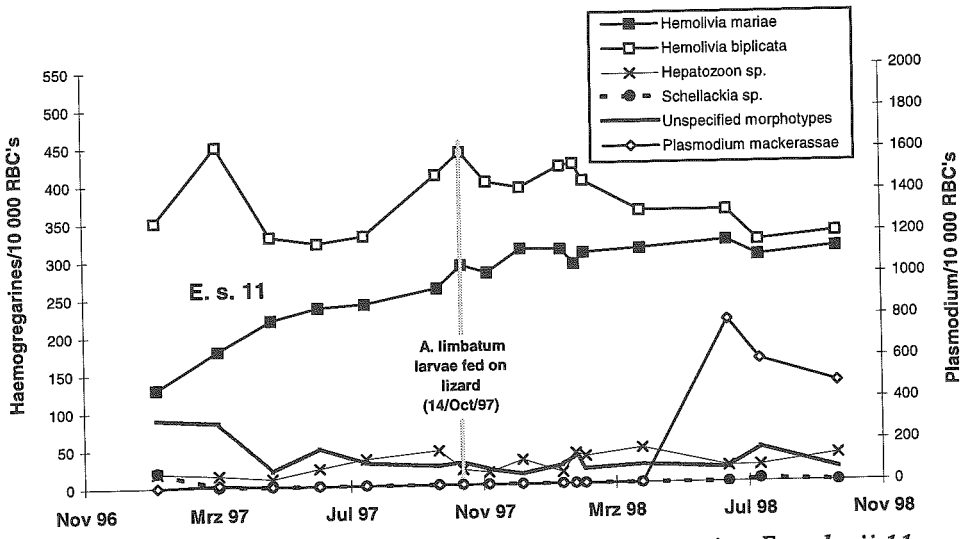

Figure 80. The presence of blood parasite stages in *E. stokesii 11* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/10/97 were force fed as unengorged nymphs (n=5) to *E. stokesii* 47 (see below) [Experiment I-B].

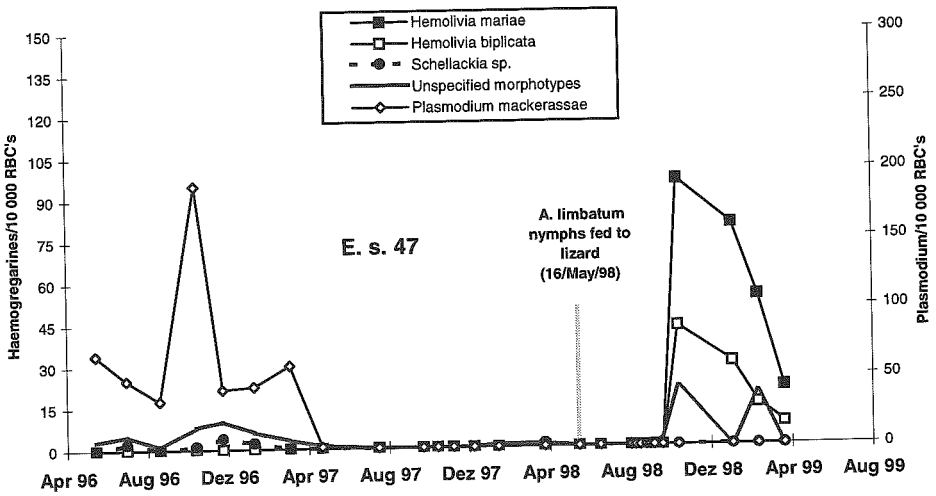

Figure 81 The presence of blood parasite stages in *E. stokesii 47* throughout the time span it was monitored. *Hem. mariae* and *Hem. biplicata* were successfully transmitted.

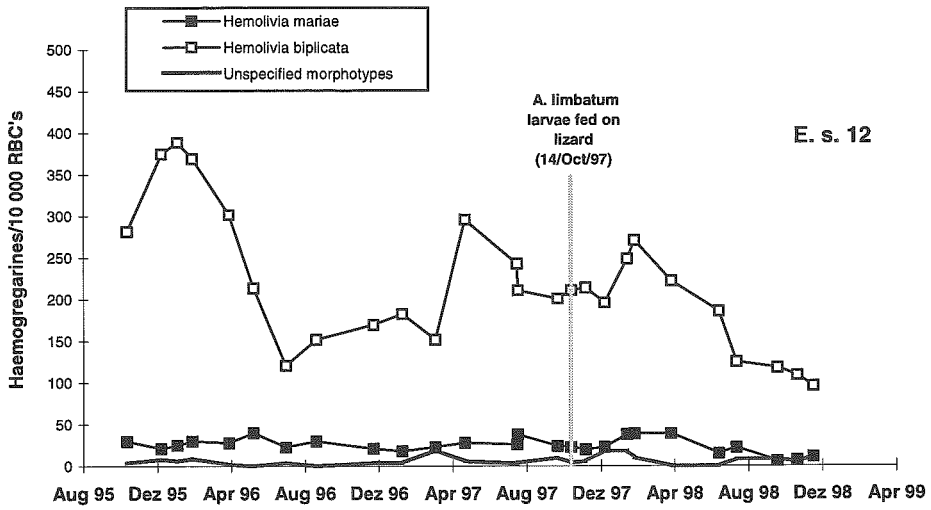

Figure 82. The presence of blood parasite stages in *E. stokesii 12* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/10/97 were force fed as unengorged nymphs (n=5) to *E. stokesii* 391 (see below) [Experiment I-C].

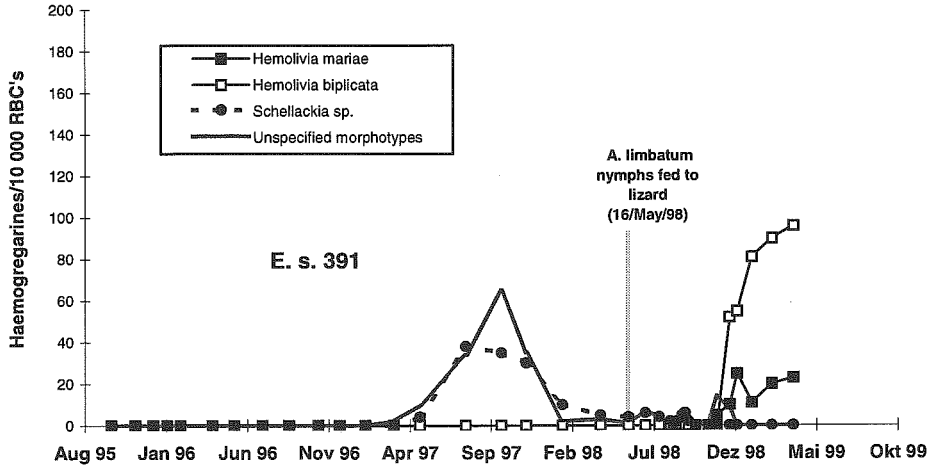

Figure 83. The presence of blood parasite stages in *E. stokesii 391* throughout the time span it was monitored. *Hem. mariae* and *Hem. biplicata* were successfully transmitted.

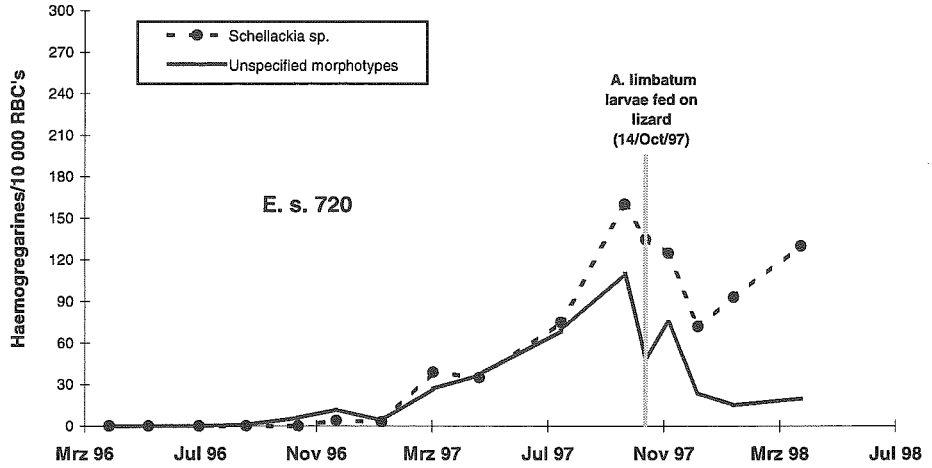

Figure 84. The presence of blood parasite stages in *E. stokesii 720* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/10/97 were force fed as unengorged nymphs (n=5) to *E. stokesii* 421 (see below) [Experiment I-D].

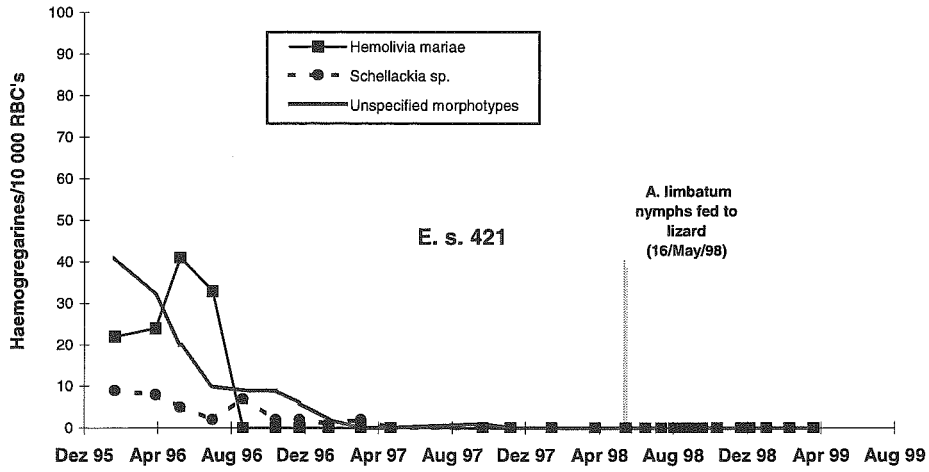

Figure 85. The presence of blood parasite stages in *E. stokesii 421* throughout the time span it was monitored. No transmission was detected.

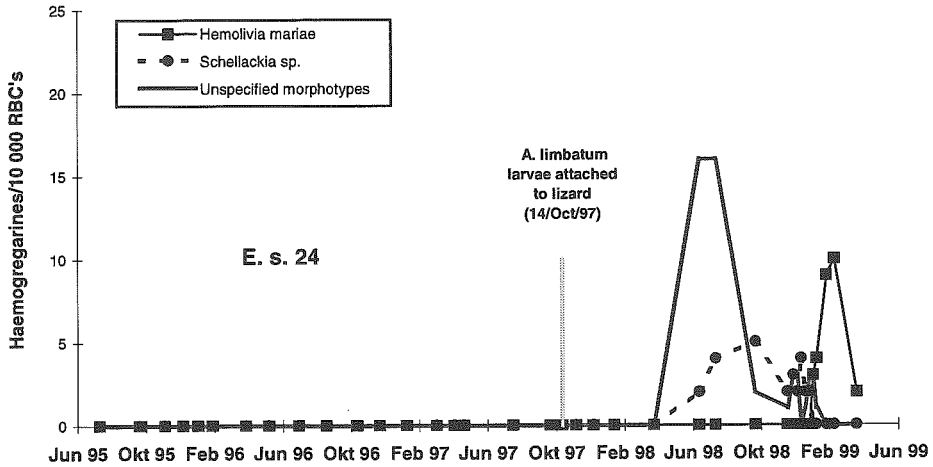

Figure 86. The presence of blood parasite stages in *E. stokesii 24* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/10/97 were force fed as unengorged nymphs (n=5) to *E. stokesii* 33 (see below) [Experiment I-E][10].

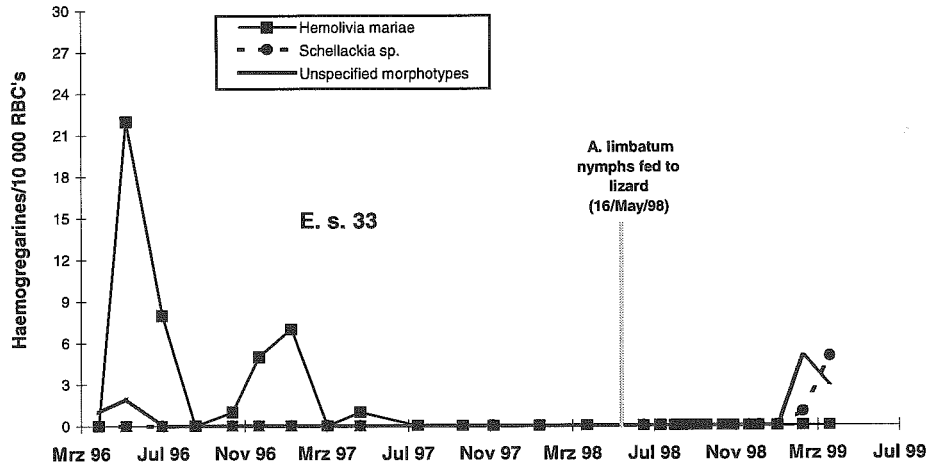

Figure 87. The presence of blood parasite stages in *E. stokesii 33* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious because the blood of *E. stokesii 24* was uninfected at the time of the engorgement of the vector ticks.

[10] *E. stokesii 24* was infected with *H. mariae* in the following experiment (20/11/98).

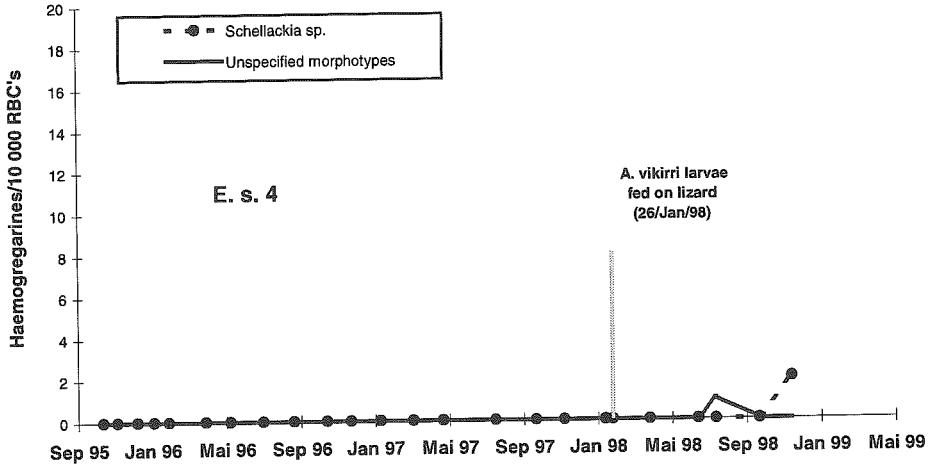

Figure 88. The presence of blood parasite stages in *E. stokesii 4* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/01/98 were force fed as unengorged nymphs (n=5) to *E. stokesii 22* (see below) [Experiment I-F].

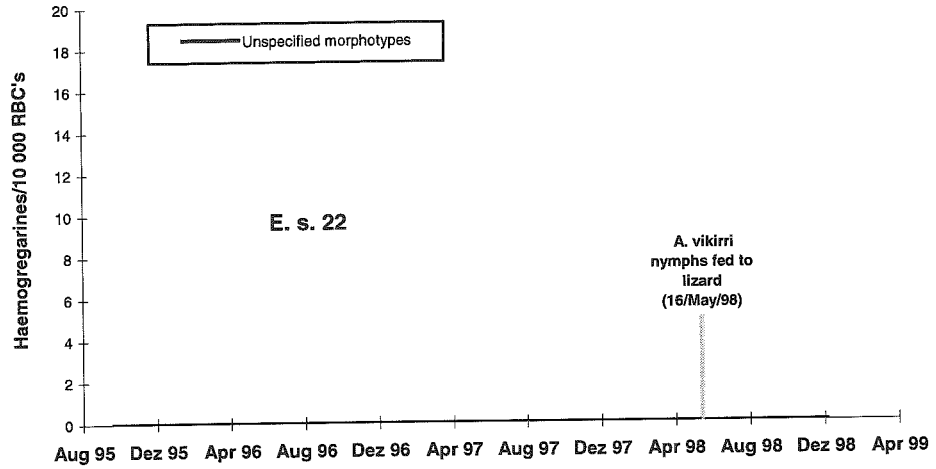

Figure 89. The presence of blood parasite stages in *E. stokesii 22* throughout the time span it was monitored. No transmission was detected.

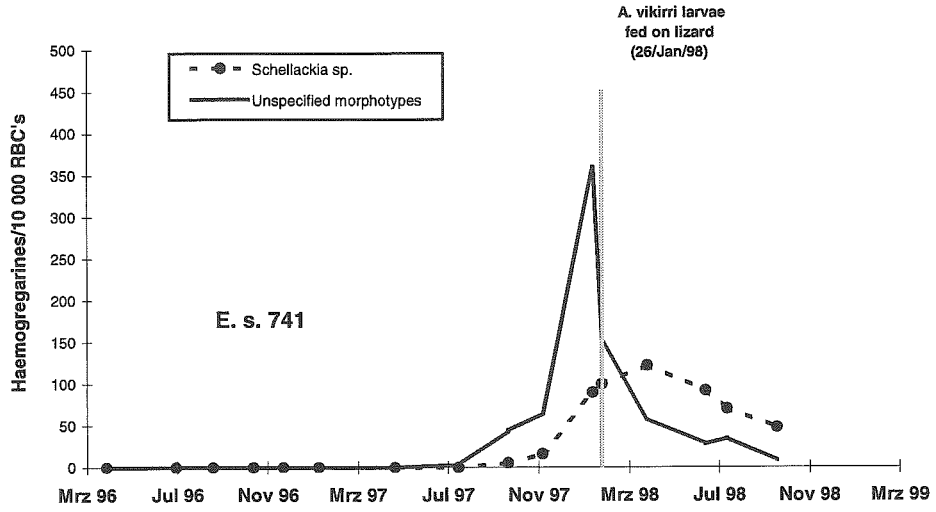

Figure 90. The presence of blood parasite stages in *E. stokesii 741* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/01/98 were force fed as unengorged nymphs (n=5) to *E. stokesii 63* (see below) [Experiment I-G].

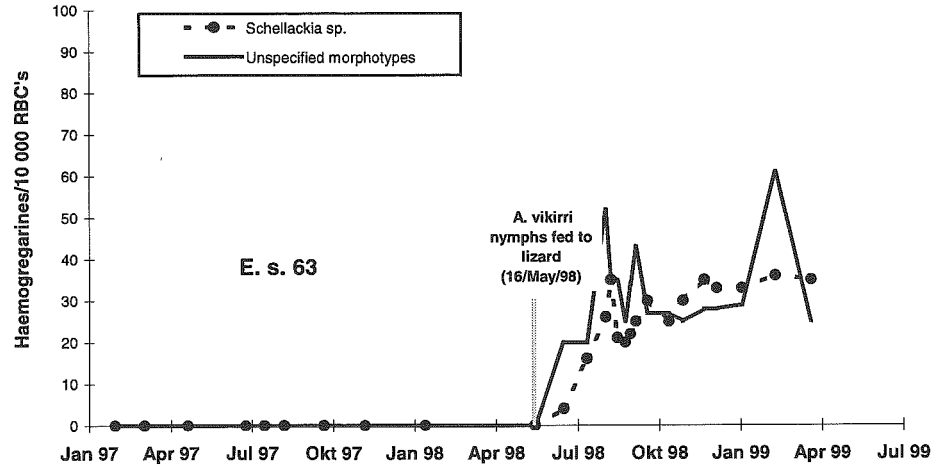

Figure 91. The presence of blood parasite stages in *E. stokesii 63* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious due to the frequent spontaneous infections of *E. stokesii* with *Schellackia* sp.

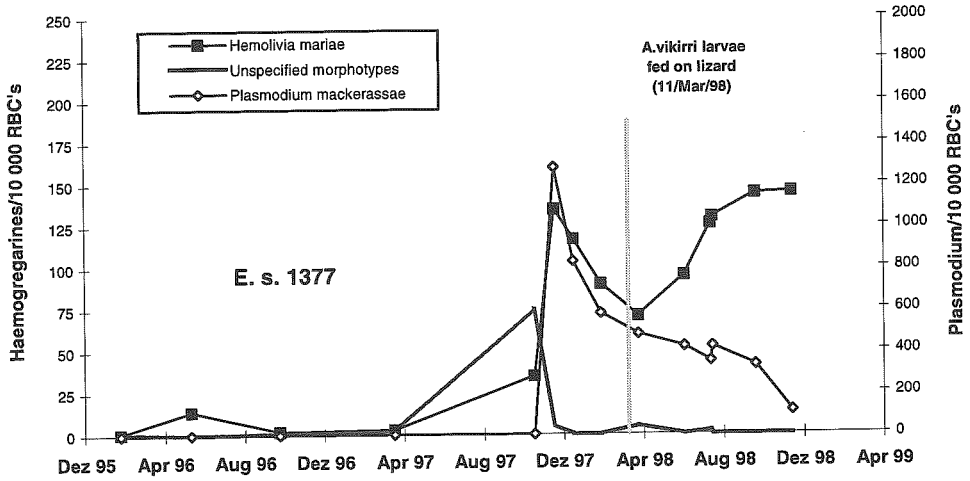

Figure 92. The presence of blood parasite stages in *E. stokesii 1377* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 11/03/98 were force fed as unengorged nymphs (n=5) to *E. stokesii 310* (see below) [Experiment I-H].

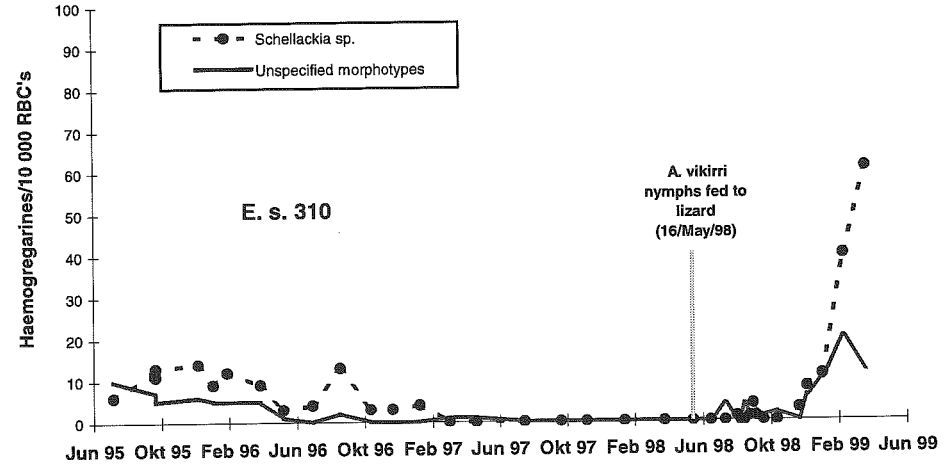

Figure 93. The presence of blood parasite stages in *E. stokesii 310* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious because the blood of *E. stokesii 1377* was uninfected with *Schellackia* sp. at the time of the engorgement of the vector ticks.

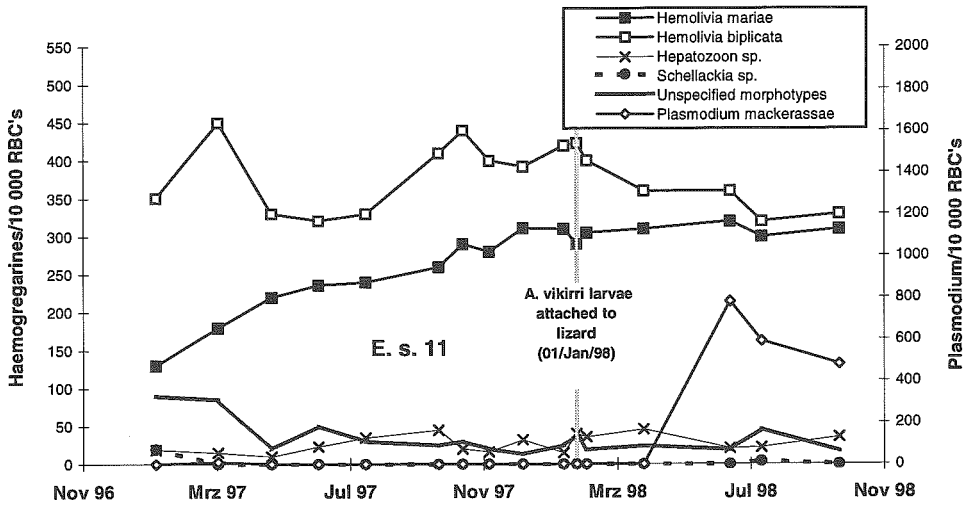

Figure 94. The presence of blood parasite stages in *E. stokesii 11* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/01/98 were force fed as unengorged nymphs (n=5) to *E. stokesii 9* (see below) [Experiment I-I].

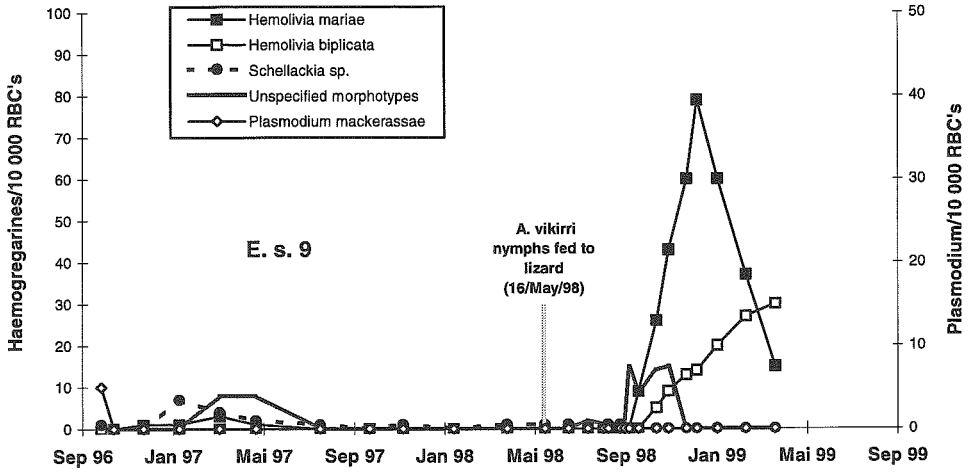

Figure 95. The presence of blood parasite stages in *E. stokesii 9* throughout the time span it was monitored. *Hem. mariae* and *Hem. biplicata* were successfully transmitted.

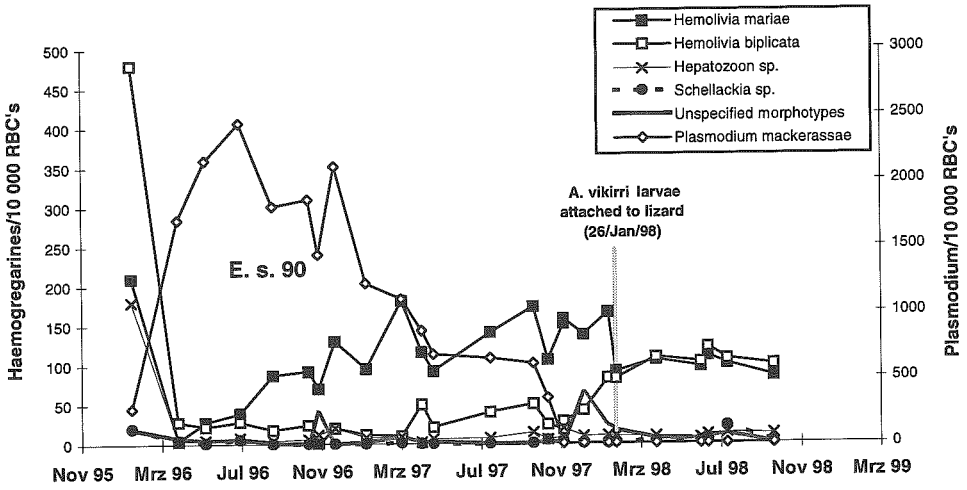

Figure 96. The presence of blood parasite stages in *E. stokesii 90* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/01/98 were force fed as unengorged nymphs (n=5) to *E. stokesii 353* (see below) [Experiment I-J].

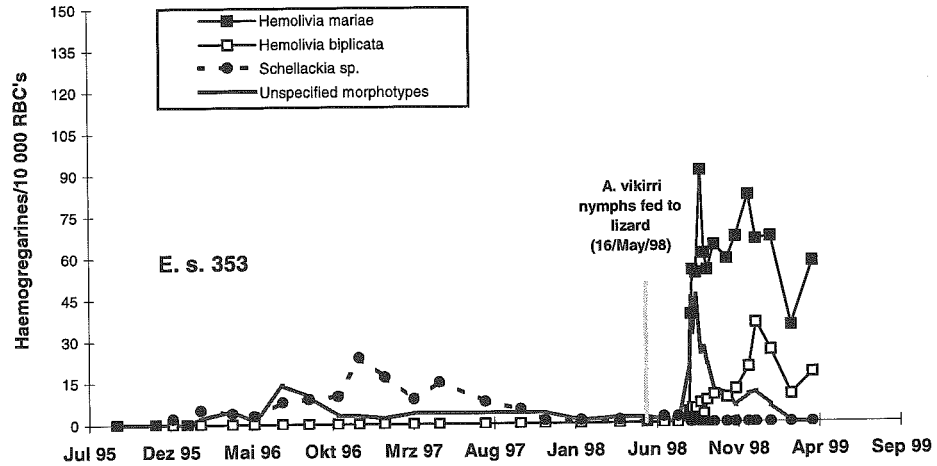

Figure 97. The presence of blood parasite stages in *E. stokesii 353* throughout the time span it was monitored. *Hem. mariae* and *Hem. biplicata* were successfully transmitted.

Figures of transmission experiment II, (A-J)

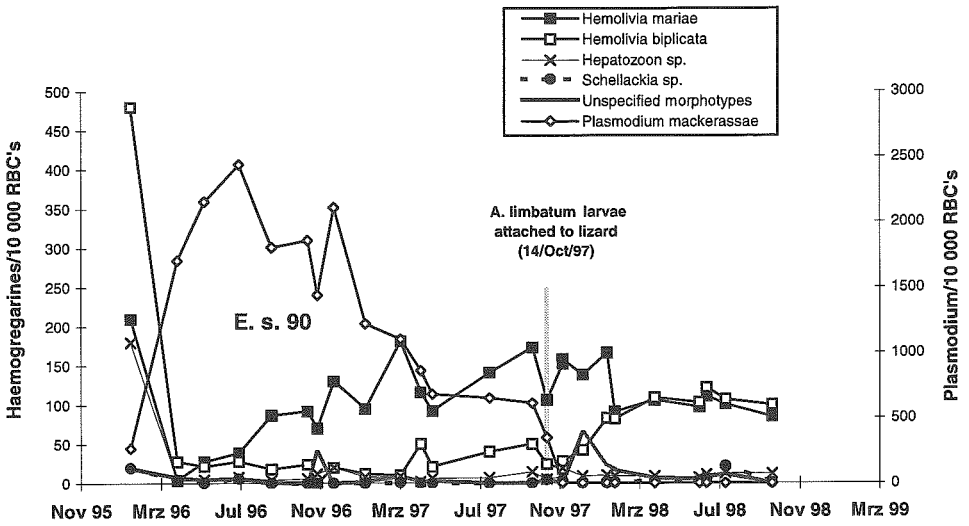

Figure 98. The presence of blood parasite stages in *E. stokesii 90* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/Oct/98 were attached as unengorged nymphs (n=10) to *E. stokesii 40* (see below) [Experiment II-A].

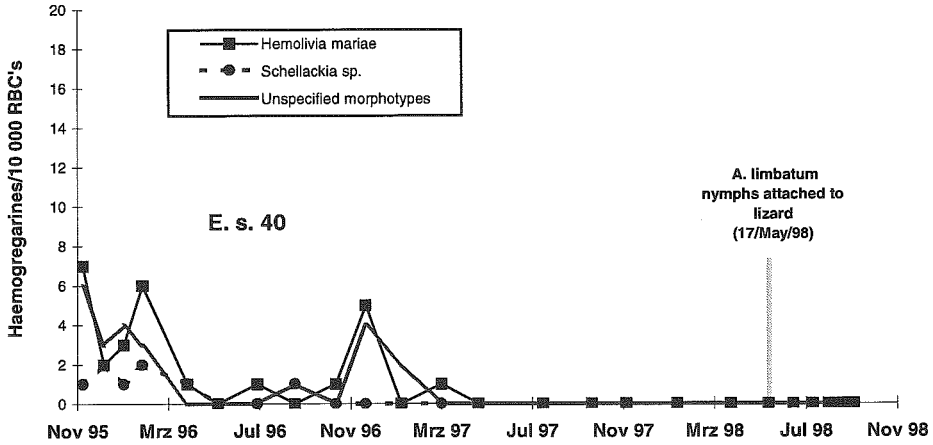

Figure 99. The presence of blood parasite stages in *E. stokesii 40* throughout the time span it was monitored. No transmission was detected.

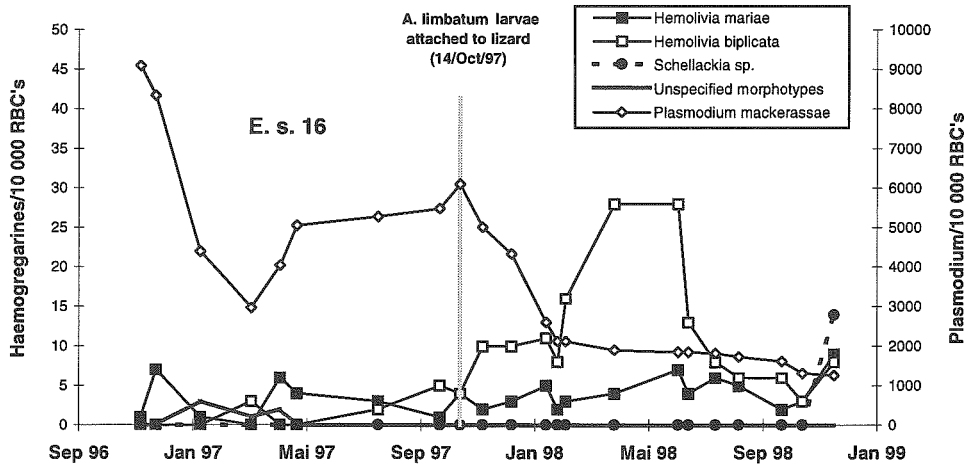

Figure 100. The presence of blood parasite stages in *E. stokesii 16* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/Oct/98 were attached as unengorged nymphs (n=10) to *E. stokesii 91* (see below) [Experiment II-B].

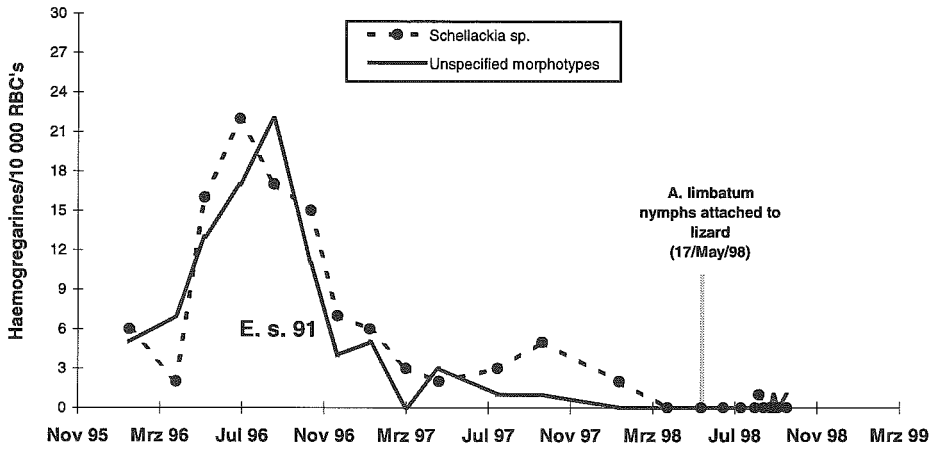

Figure 101. The presence of blood parasite stages in *E. stokesii 91* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious because the blood of *E. stokesii 16* was uninfected with *Schellackia* sp. at the time of the engorgement of the vector ticks.

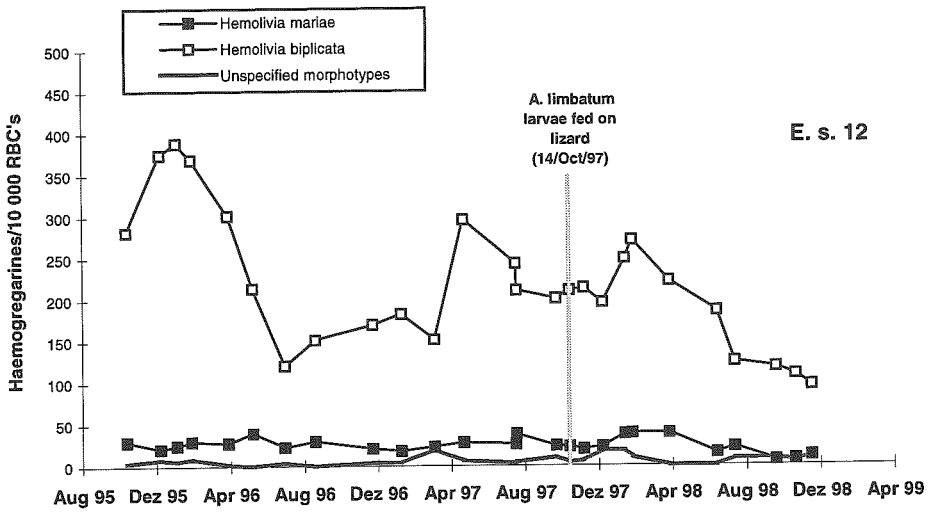

Figure 102. The presence of blood parasite stages in *E. stokesii 12* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/Oct/98 were attached as unengorged nymphs (n=10) to *E. stokesii 3* (see below) [Experiment II-C].

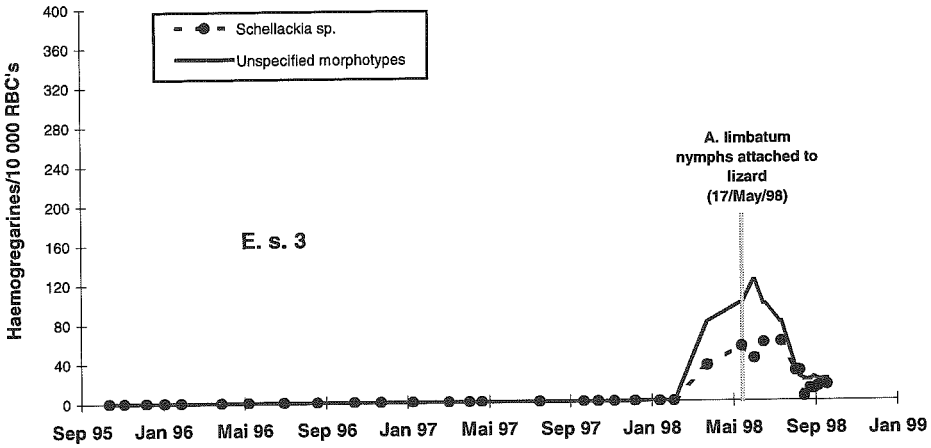

Figure 103. The presence of blood parasite stages in *E. stokesii 3* throughout the time span it was monitored. No transmission was detected.

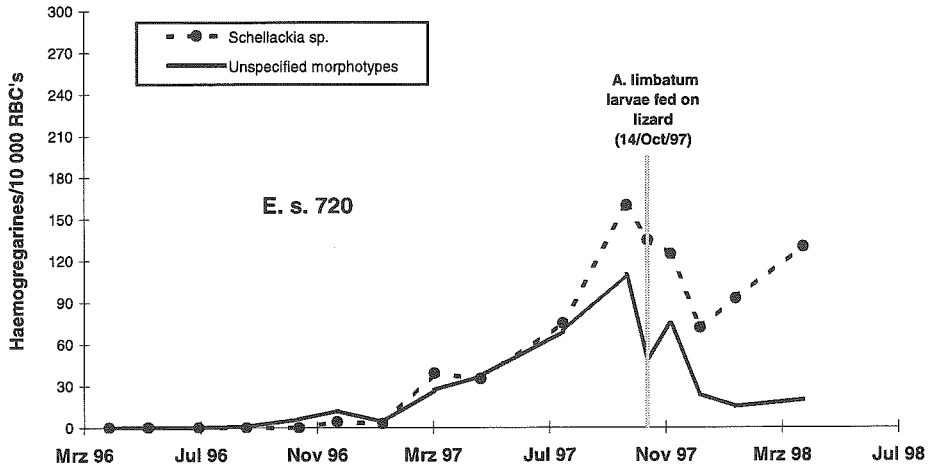

Figure 104. The presence of blood parasite stages in *E. stokesii 720* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/Oct/98 were attached as unengorged nymphs (n=10) to *E. stokesii 140* (see below) [Experiment II-D].

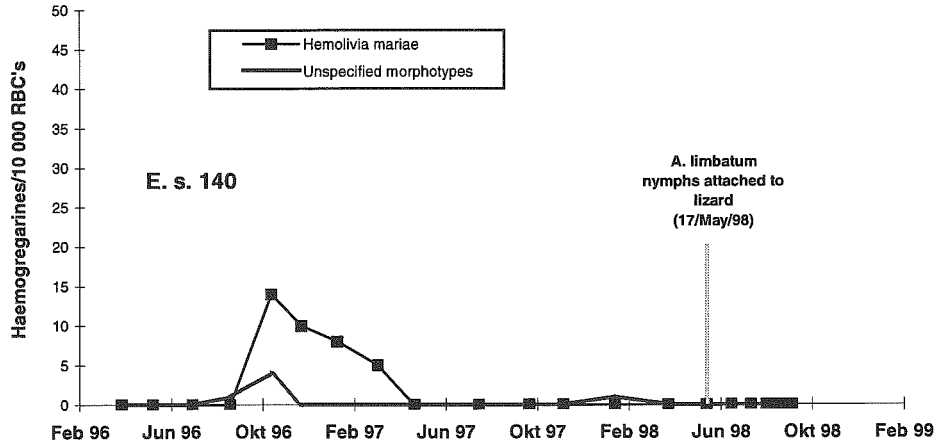

Figure 105. The presence of blood parasite stages in *E. stokesii 140* throughout the time span it was monitored. No transmission was detected.

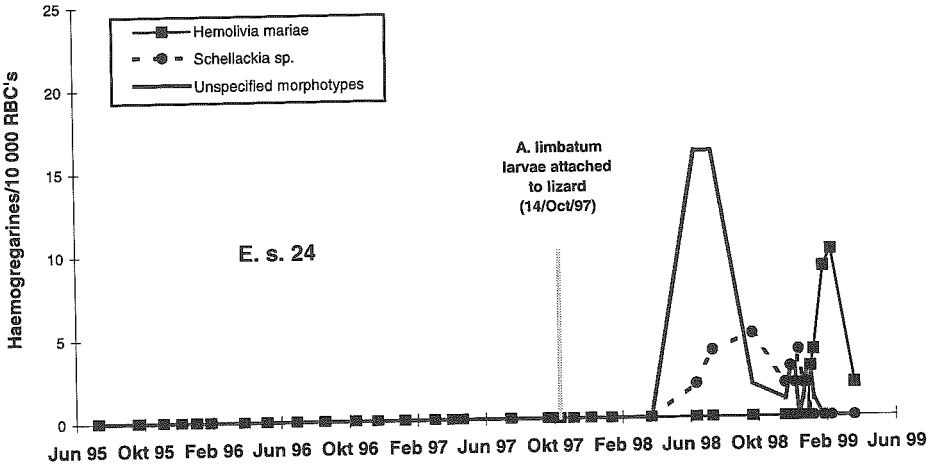

Figure 106. The presence of blood parasite stages in *E. stokesii 24* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 14/Oct/98 were attached as unengorged nymphs (n=10) to *E. stokesii 31* (see below) [Experiment II-E][11].

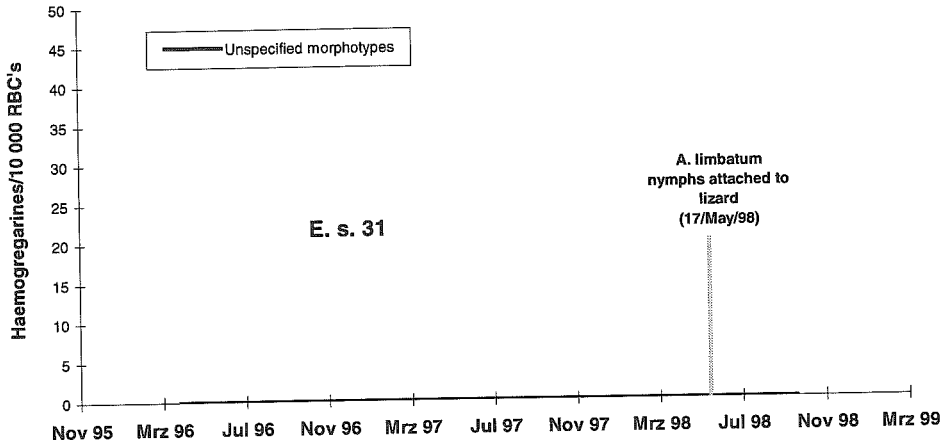

Figure 107. The presence of blood parasite stages in *E. stokesii 31* throughout the time span it was monitored. No transmission was detected.

[11] *E. stokesii 24* was infected with *H. mariae* in the following experiment (20/11/98)

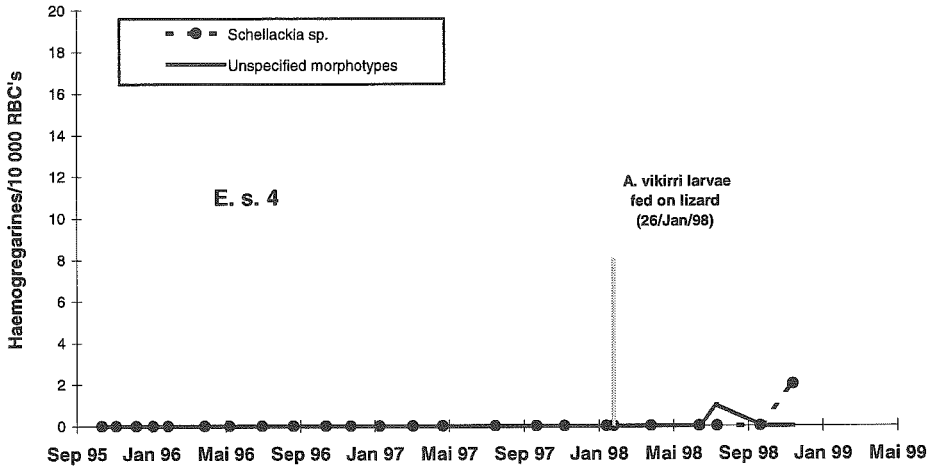

Figure 108. The presence of blood parasite stages in *E. stokesii 4* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/Jan/98 were attached as unengorged nymphs (n=10) to *E. stokesii 1178* (see below) [Experiment II-F].

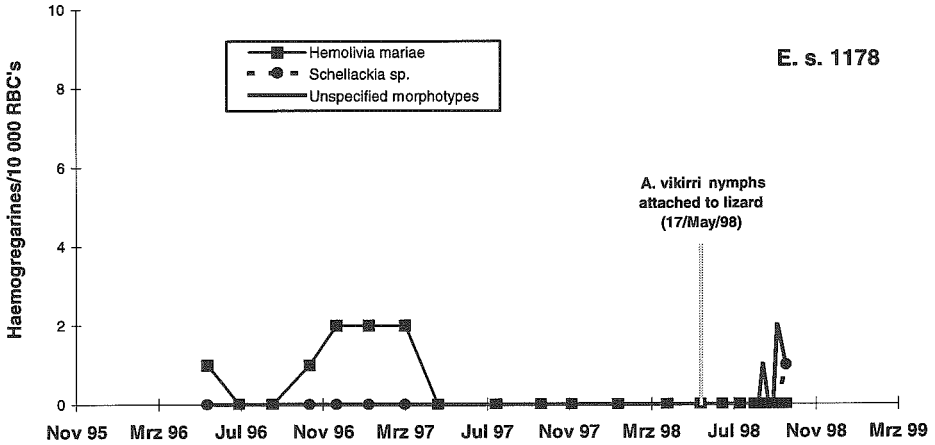

Figure 109. The presence of blood parasite stages in *E. stokesii 1178* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious because the blood of *E. stokesii 16* was uninfected with *Schellackia* sp. at the time of the engorgement of the vector ticks.

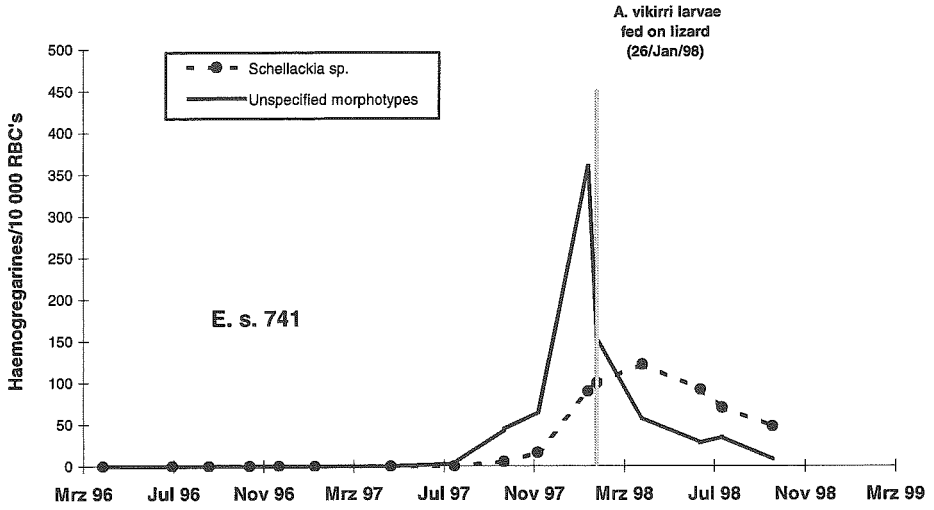

Figure 110. The presence of blood parasite stages in *E. stokesii 741* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/Jan/98 were attached as unengorged nymphs (n=10) to *E. stokesii 124* (see below) [Experiment II-G].

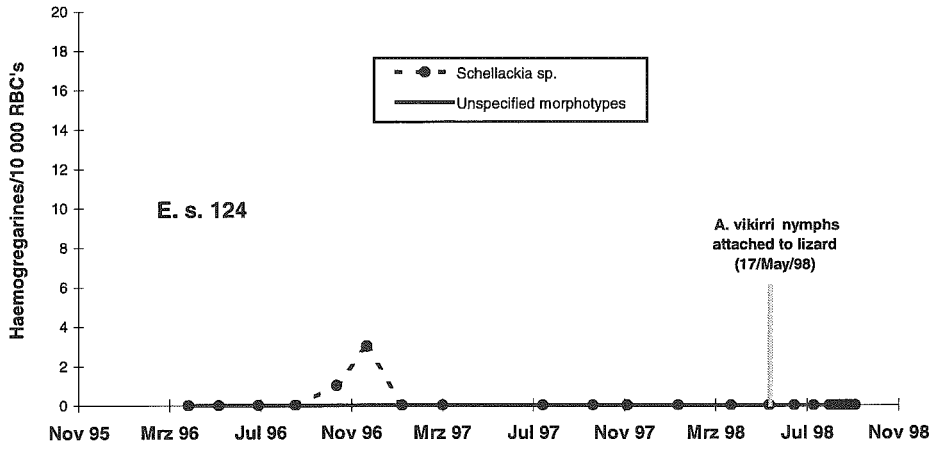

Figure 111. The presence of blood parasite stages in *E. stokesii 124* throughout the time span it was monitored. No transmission was detected.

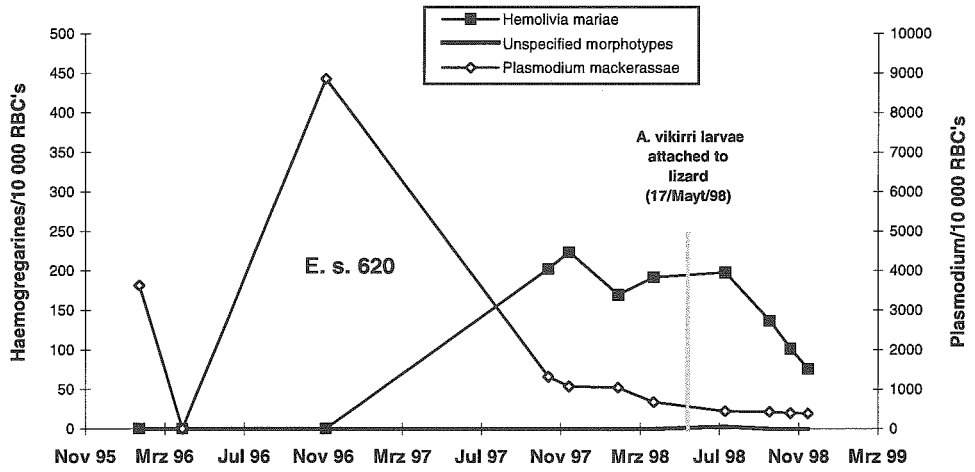

Figure 112. The presence of blood parasite stages in *E. stokesii 620* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 11/Mar/98 were attached as unengorged nymphs (n=10) to *E. stokesii 100* (see below) [Experiment II-H].

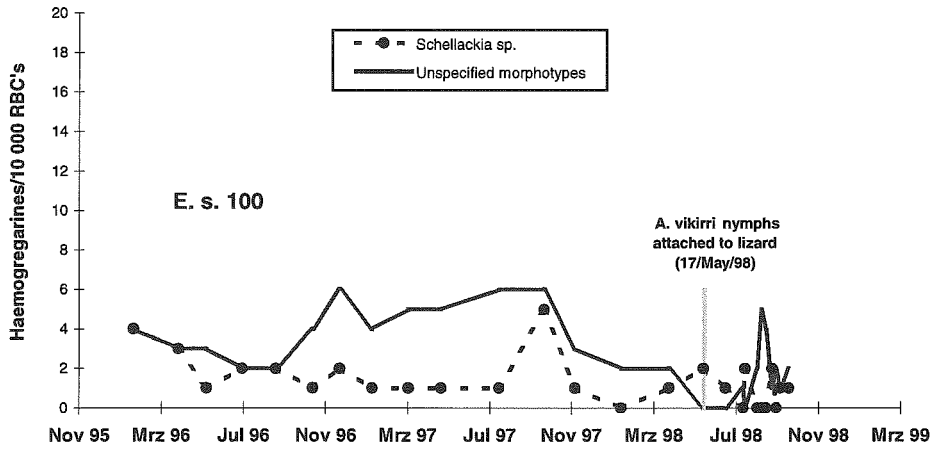

Figure 113. The presence of blood parasite stages in *E. stokesii 100* throughout the time span it was monitored. No transmission was discovered. No transmission was detected.

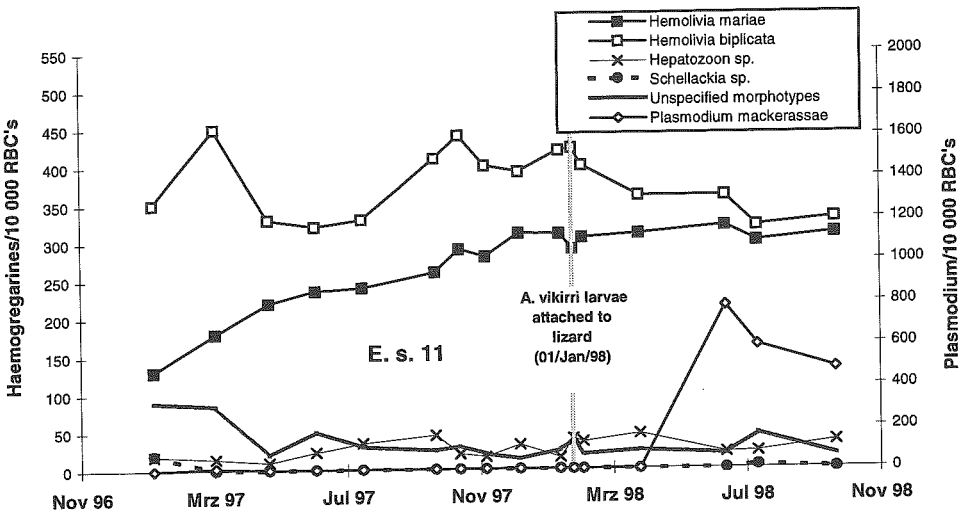

Figure 114. The presence of blood parasite stages in *E. stokesii 11* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/Jan/98 were attached as unengorged nymphs (n=10) to *E. stokesii 395* (see below) [Experiment II-I].

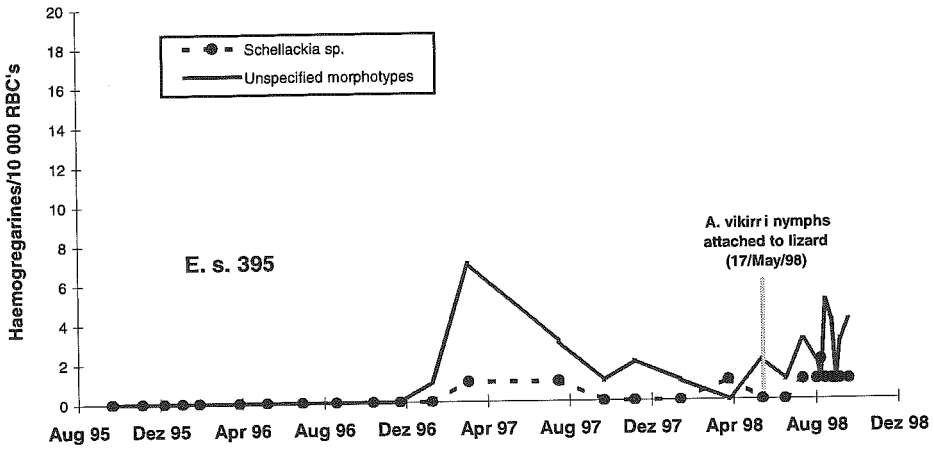

Figure 115. The presence of blood parasite stages in *E. stokesii 395* throughout the time span it was monitored. No transmission was detected.

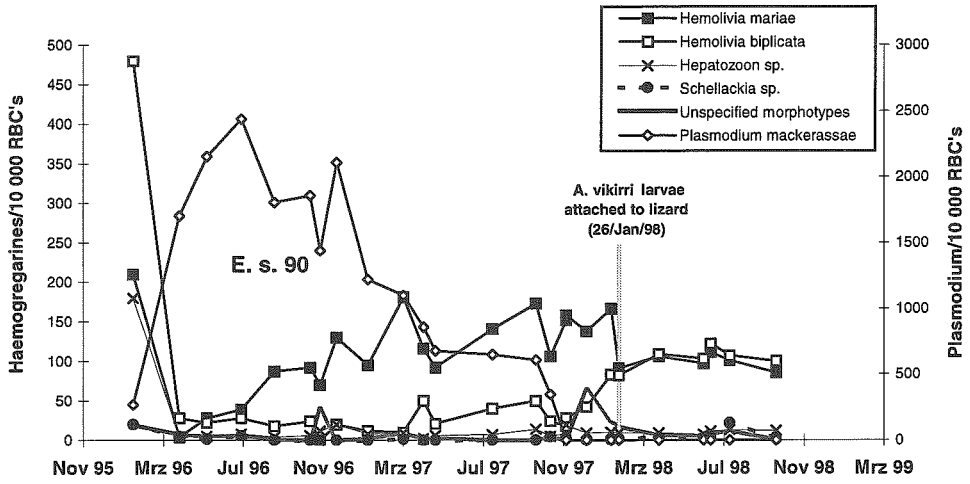

Figure 116. The presence of blood parasite stages in *E. stokesii 90* throughout the time span it was monitored. *Amb. vikirri* larvae that attached on the 26/Jan/98 were attached as unengorged nymphs (n=10) to *E. stokesii 124* (see below) [Experiment II-J].

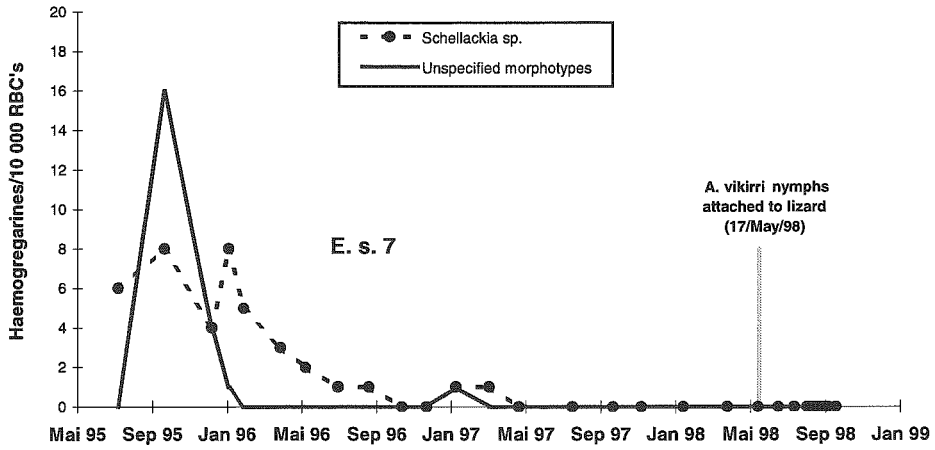

Figure 117. The presence of blood parasite stages in *E. stokesii 7* throughout the time span it was monitored. No transmission was detected.

Figures of transmission experiment III, (A-E)

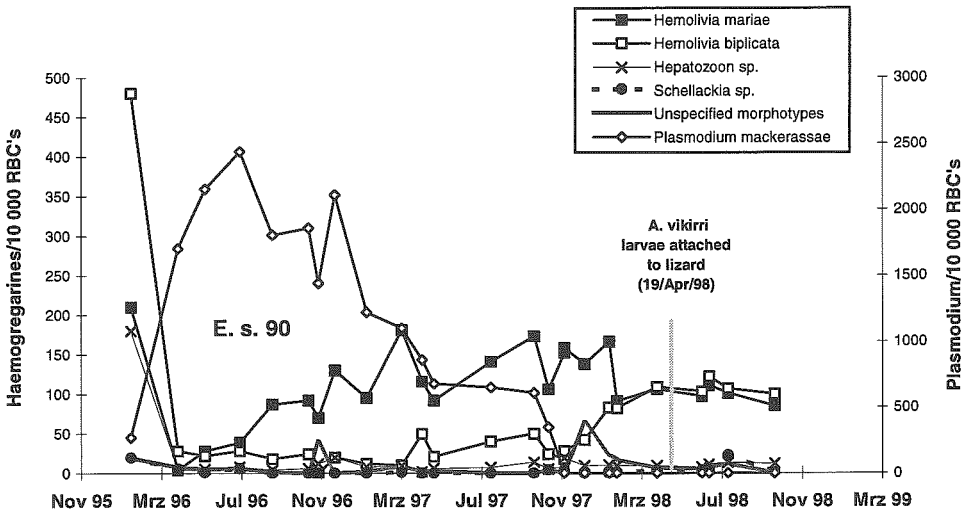

Figure 118. The presence of blood parasite stages in *E. stokesii 90* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 19/Apr/98 were fed as engorged larvae (n=10) to *E. stokesii 210* (see below) [Experiment III-A].

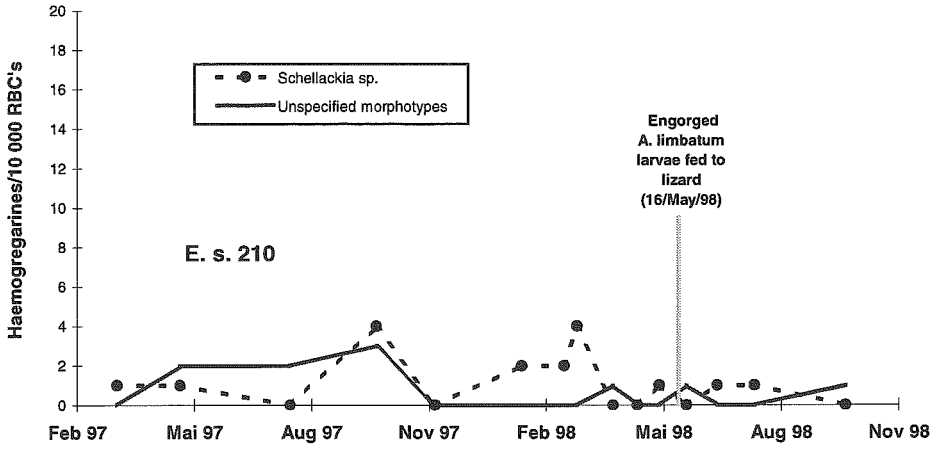

Figure 119. The presence of blood parasite stages in *E. stokesii 210* throughout the time span it was monitored. No transmission was detected.

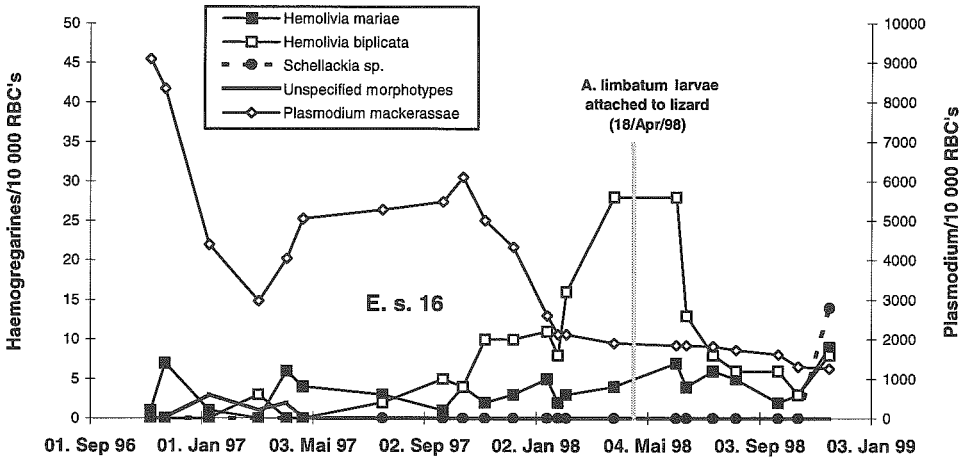

Figure 120. The presence of blood parasite stages in *E. stokesii 16* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 19/Apr/98 were fed as engorged larvae (n=10) to *E. stokesii 2* (see below) [Experiment III-B].

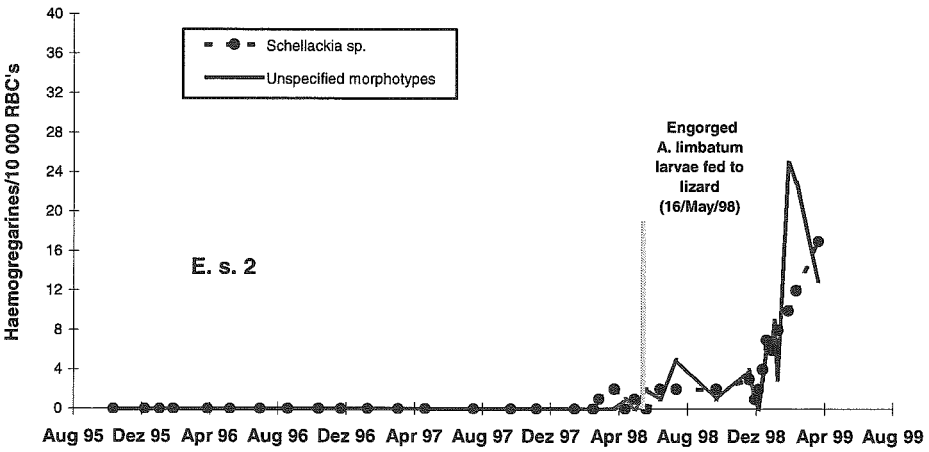

Figure 121. The presence of blood parasite stages in *E. stokesii 2* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious due to the spontaneous infection with *Schellackia* sp. before the ticks were fed to *E. stokesii 2*.

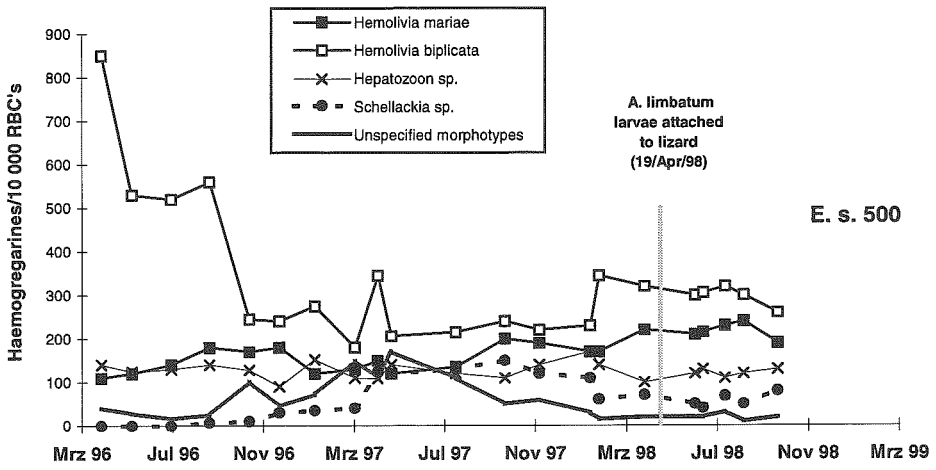

Figure 122. The presence of blood parasite stages in *E. stokesii 500* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 19/Apr/98 were fed as engorged larvae (n=10) to *E. stokesii 357* (see below) [Experiment III-C].

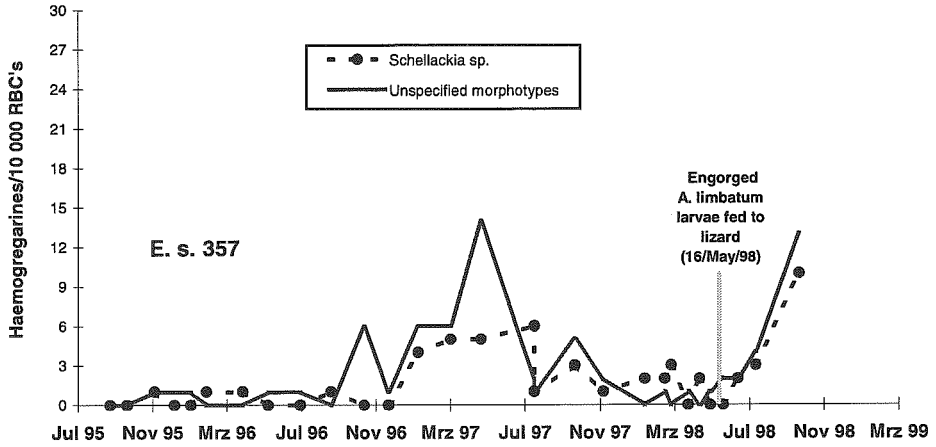

Figure 123. The presence of blood parasite stages in *E. stokesii 357* throughout the time span it was monitored. Possible transmission of *Schellackia* sp., dubious due to the spontaneous infection with *Schellackia* sp. before the ticks were fed to *E. stokesii 357*.

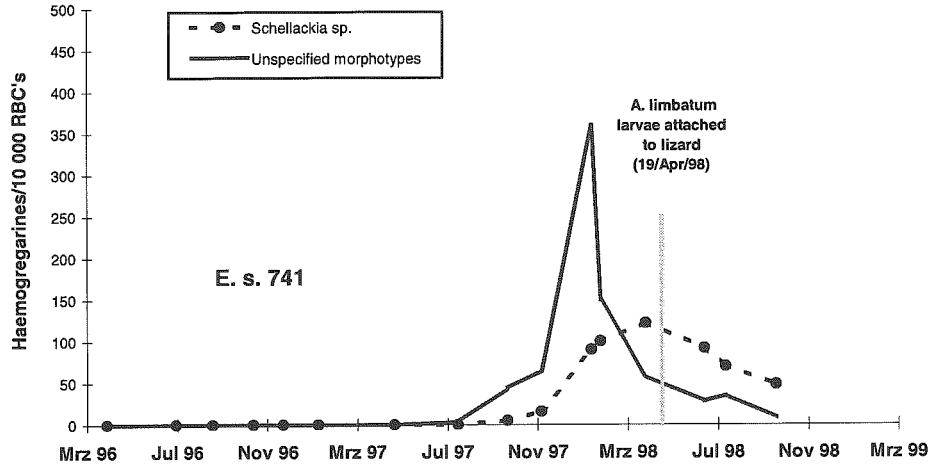

Figure 124. The presence of blood parasite stages in *E. stokesii 741* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 19/Apr/98 were fed as engorged larvae (n=10) to *E. stokesii 21* (see below) [experiment III-D].

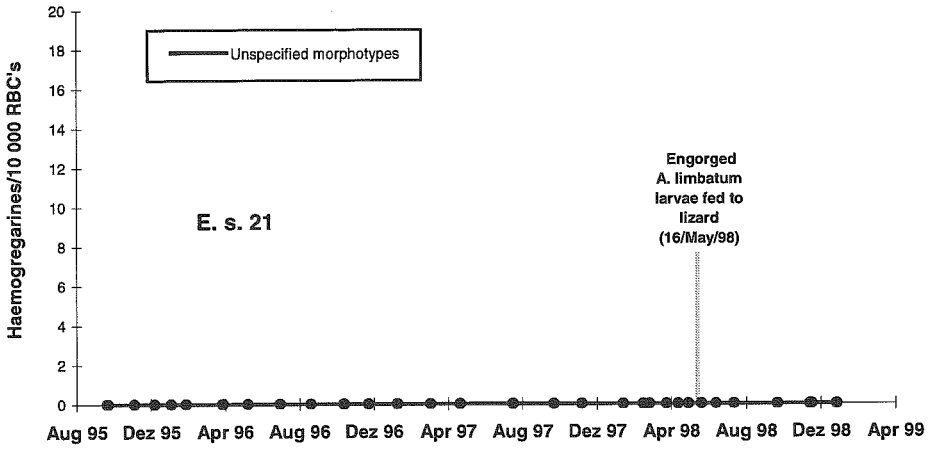

Figure 125. The presence of blood parasite stages in *E. stokesii 21* throughout the time span it was monitored. No transmission was detected.

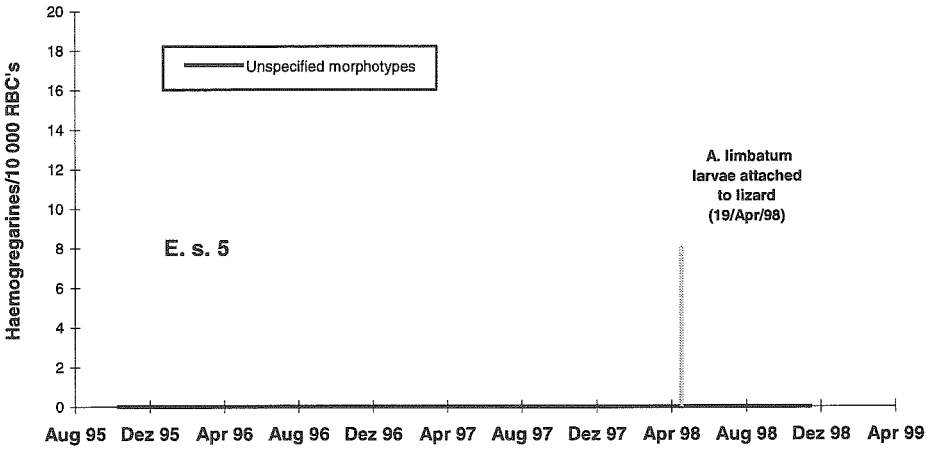

Figure 126. The presence of blood parasite stages in *E. stokesii 5* throughout the time span it was monitored. *Amb. limbatum* larvae that attached on the 19/Apr/98 were fed as engorged larvae (n=10) to *E. stokesii 200* (see below) [Experiment III-E].

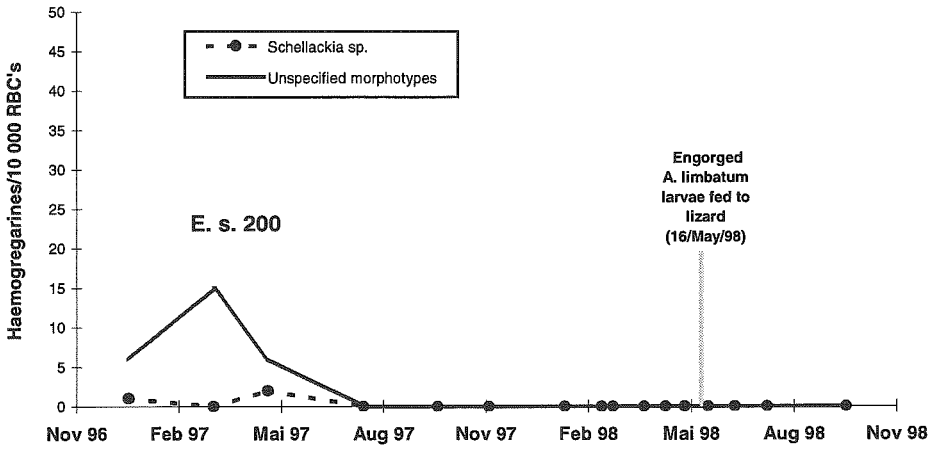

Figure 127. The presence of blood parasite stages in *E. stokesii 200* throughout the time span it was monitored. No transmission was detected.

Chapter Thirteen

13. ASPECTS OF TICK FITNESS UNDER THE INFLUENCE OF BLOOD-PARASITES

13.1 Introduction

In contrast to the many blood parasites of medical and veterinary importance, little is known about the pathogenicity of blood parasites of non-mammals (Desser, 1993). Very few reports of the pathogenicity of haemogregarine blood parasites on their vectors are available. Reichenow (Reichenow, 1921) described two negative implications of high *Karyolysus*-infections on the mite *Liponyssus saurarorum*, where some heavily infected adult mites lost their motility and in some cases a new blood meal could not be digested. *Hepatozoon balfouri* was found to increase the mortality of its mite vector (Hoogstraal, 1961), and *Hepatozoon rarefasciens* induced a higher mortality on its mosquito vector (Ball & Oda, 1971). Among the non-haemogregarine Apicomplexa, the piroplasms of the genera *Babesia* are known to increase the mortality and to affect the reproductive behaviour of their tick vectors eg. (Riek, 1964; Hoffmann, 1971; Gray, 1982). The impact of micro-parasites on their arthropod vectors might have substantial implications for the epidemiology of vectored diseases and the many taxa parasitising invertebrates may hold the key to effective biological control agents for arthropod vectors of disease. In this chapter, the impact of blood-parasites of *E. stokesii* on five aspects of the fitness of tick vectors was investigated.

In order to evaluate the pathogenicity of the blood parasites found in *E. stokesii* on the two tick species *Amb. vikirri* and *Amb. limbatum*, the fitness of engorging tick larvae and of the resulting unfed nymphs was compared between those that engorged on uninfected lizards and those that fed on lizards with different types of mixed infections. Due to the diversity of blood parasite species found, and the uncertainties regarding their taxonomic identity at the beginning of this study, the experiments focused on lizards with mixed blood infections.

In the first step of the analysis, the various associations of parasites in *E. stokesii* were condensed into three types of blood infections:

type 0: *E. stokesii* diagnosed as long term uninfected

type 1: *E. stokesii* infected with *Schellackia* sp. but not with adeleorine blood parasites. In addition, one or two species of *Plasmodium* were present in some cases.

type 2: *E. stokesii* infected with adeleorine blood parasites (single or mixed infections with *Hem. mariae* and *Hem. biplicata*; *Hepatozoon* sp. was occasionally present). In some cases, *Schellackia* sp. and one or two species of *Plasmodium* were also present.

Types 1 and 2 divided all possible combinations of mixed infections in *E. stokesii* into two groups: those that in previous experiments (see chapter six and seven) did infect both tick species (type 2), and those where despite an intensive search no infection in ticks could be proven (type 1). The data were analysed by a series of ANOVA tests. The following aspects of tick fitness were evaluated:

1. the successful detachment rate of engorging larvae
2. the time the larvae spent on the host
3. the larval body mass at the time of detachment
4. the rate of tick body mass loss over time after detachment
5. the mortality of detached ticks.

In addition, the potential influences of the gender and the body mass of the lizard hosts on the tick fitness were examined. Five attachment experiments (I-V) were conducted. In experiments I-IV, most of the type 2 infected *E. stokesii* had mixed infections with different species of adeleorine blood parasites. Attachment experiment V focused on lizards with chronic infections of the blood parasite *Hem. mariae* and omitted lizards infected with *Hem. biplicata* and *Hepatozoon* sp..

13.2 Materials and Methods

13.2.1 The lizard hosts

The lizards used for the experiments were either born and raised in the laboratory or caught in the field. Most *E. stokesii* used were captured as adults in the field. The blood infections of the field caught *E. stokesii* were regularly monitored at least for one year before an experiment began. Most lizards caught in the field had *Amb. limbatum* or *Amb. vikirri* ticks attached when they were caught. All of the field caught *E. stokesii* showed more or less severe tick scarring, indicating that adult ticks had parasitised them. Only two *E. stokesii* used

for these experiments were born and raised in the ACU (*E. stokesii 24* & *394*). They were regularly monitored for blood parasites from birth. At the time of the tick attachment experiments, none of the *E. stokesii* used were tick naive. All *E. stokesii* used for this part of the study were housed in the ACU for at least one year before the first experiment began. Regular bleedings continued throughout the entire two years during which the experiments took place. All experimental *E. stokesii* were kept indoors during the whole time span. The lizards were chosen for their stable results regarding their blood infections, either because they were found to be long term uninfected or because they had relatively high parasitemias of chronic infections with various blood parasites. All lizards used in an experiment were bled and weighed one day before the ticks were attached. Some lizards were used repeatedly in the tick attachment experiments, and their blood was regularly monitored for blood parasites throughout the time span of the experiments and beyond.

13.2.2 Tick attachments

The basic procedures used for the five experiments in this chapter concerning the attachment of tick larvae were the same as for the transmission experiments described in chapter five (see 5.2.2.2). A single experiment consisted of up to twelve lizard individuals that were each exposed to 100 tick larvae. All tick larvae in one experiment were obtained from a single engorged female tick. Only females caught as engorged larvae or nymphs in the field were used as sources of larvae. The unengorged larvae were collected by a glass pipette connected to a water tap suction pump and were counted with care. The 100 hungry tick larvae were kept inside a cotton bag with one individual *E. stokesii* in a constant 37°C room over night. The lizards were placed individually into detachment cages as soon as they were taken out of the cotton bag. The detachment cages were made from clear perspex and had the shape of a tall box (35 x 18 x 35 cm, Figure 56/B). A metal grid held firmly by bent perspex was placed near the bottom inside of the cage. The lizards were placed on top of that grid and the detaching ticks, food and faeces were funnelled into a cardboard dish underneath. The ticks would crawl out of the cardboard dish and they were funnelled from there into a tick collector vial. Each detachment cage stood on a separate perspex stand that was placed individually into one of twelve large plastic crates (90 x 50 x 12 cm) filled with water. The twelve identical detachment cages were kept in one room of the ACU that was kept at a constant 25°C with 12 h light and 12 h darkness[12]. Light bulbs (60W) provided heat

[12] During the beginning of experiment IV, the temperature control of that room was out of order for 4 days, resulting in a temperature drop to a constant 21°C.

from above and a small basking rock and a shelter were placed inside the detachment cage. The lizards were fed twice per week, water was provided *ad libitum*. During the peak phase of detachment (usually lasting about 14 days), the ticks were collected daily and they were collected every two to three days when the rate of tick detachment slowed down. During a collection, the detachment cage, the cardboard dish, the collector vial and the water tray were inspected. Most ticks were collected from the collector vial at the end of the funnel underneath the cardboard dish. A few ticks ended up in the water tray under the cage, making it necessary to carefully blot away the adhering water from the wet ticks with paper tissue. Single ticks were then placed into individually numbered 1.5 ml Eppendorf-vials with pierced lids. All vials were placed into one desiccator, containing a saturated $MgCl_2$ salt solution to keep a constant relative humidity of approximately 95% (Winston & Bates, 1960). The desiccator was placed into a room with constant 25°C, with the photoperiod set to 12 h light and 12 h darkness. Over a time of two years, five attachment experiments took place, using both, *Amb. vikirri* and *Amb. limbatum* larvae.

13.2.3 Dissection of ticks

A proportion of the ticks that detached from the lizard hosts was used for xenodiagnosis. A minimum of ten moulted, unfed nymphs per lizard was dissected where possible. On one occasion, only six nymphs were dissected because the overall detachment rate of one particular lizard was extremely low and the rest of the detached larvae were needed for further study of tick fitness. Ticks were dissected into wet mounts at least six weeks after they had detached as engorged larvae, to check for developmental stages of any of the blood parasites. For details of the dissection procedure see 5.2.2.3.

13.2.4 Weighing of ticks and determination of the time they spent on the lizard host

Tick body mass data were collected with a Mettler Toledo™ electronic microbalance that read to the nearest one microgram. The balance transferred the data directly into a Microsoft Excel™ spreadsheet via a cable connection to a portable computer and the BalanceLink™ software provided by Mettler. Most ticks were weighed for the first time one day after they dropped off the lizard host. A few ticks detached from the host outside of the peak drop off phase and were collected every two to three days. These might have spent up to 72 hours in the collection vial in the experimental room before they were placed into the desiccator. For calculation of the average duration of larval attachment to individual lizard hosts, the number of ticks collected from one lizard individual on each day was distributed evenly over

the number of days since the ticks were collected from that lizard beforehand. All experimental lizards were inspected on the same day. All ticks spent approximately 24 h before weighing in the desiccator in order to standardise as much as possible the conditions under which the detachment mass was taken. Body mass data were taken immediately after the removal of the ticks from the storage desiccator. Ticks from an experimental group were always weighed on the same day. Tick individuals were weighed repeatedly over a period of up to 18 month. To allow a comparison between the attachment experiments, the date of the subsequent weighings were expressed as the number of days since the larvae came into contact with the lizards. Where a studied feature of tick fitness was expressed as a percentage, an arcsine transformation was performed on the data before statistical analysis.

In each experiment, a few unengorged larvae were found off the lizards in the detachment cage. These were omitted from the data evaluation, whether dead or alive. Some engorged larvae were squashed during the process of collection or handling, either by the lizards or by the investigator. They were included in the counts of the total of detaching ticks per lizard individual and in the study of the duration of tick attachment, but for obvious reasons they could not be evaluated for tick body mass data. At the end of some experiments, a few ticks were found that had already developed into the nymphal instar. They had detached and moulted undetected within the detachment cage. They also were included into the counts of detaching ticks in total, but they were excluded from the body mass evaluations and the study of the duration of the tick attachment. For calculations of tick body mass loss over time, the actual body mass of an individual surviving tick at a given time was subtracted from its detachment mass. Then the individual body mass loss was calculated as a percentage. The arithmetic mean and standard deviation of the percentage of tick body mass loss was then calculated for each lizard host. The experiment continued for many months after the ticks had detached. By the end of each experiment the numbers of ticks that could be evaluated had diminished due to the increasing mortality of the ticks over time. Calculation of mortality of ticks was based on the number of ticks detaching in total from each lizard host, which included the ticks that were found already developed into nymphs. However, the ticks that were squashed during the collection and the ticks that were dissected were subtracted from the sum of detached ticks before mortality was calculated.

The dry mass of nymphs of one attachment experiment (III) was taken after the ticks were starved for approximately one year in the desiccator. The surviving nymphs were dried over

a period of 14 days at constant 37°C in an approximately 3 L desiccator containing 50g of silica gel, thus keeping the relative humidity close to 0%. The final readings for those ticks were made on a Mettler AE 163, which read to the nearest 100 micrograms. The original balance was no longer available at that time. After the desiccation, the dried out ticks were pooled for each lizard host, but despite the pooling, the dry mass of the tick nymphs was too low to allow further analysis.

13.3 Results

13.3.1 Tick attachment experiment I

The first experiment was started on the 21/02/1997, when twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larvae. The body mass and the blood infections of the *E. stokesii* at the start of this experiment were listed in Table 54. Four of the lizards were diagnosed to be long term uninfected with blood parasites (type 0), three lizards were infected, but not with adeleorine parasites (type 1) and five lizards were infected with adeleorine parasites, among others (type 2). Dissections of the xenodiagnostic ticks during the experiment showed that only ticks that fed on type 2 infected lizards were diagnosably infected (Table 54). The infectivity of the lizards towards the tick vector *Amb. vikirri* ranged from 50%-73%. *E. stokesii 16* had the lowest parasitemia regarding the adeleorine parasites (*Hem. biplicata,* 3 in 10^4 RBC's,) and also had the lowest percentage of infected xenodiagnostic nymphs (50%). However, there was no evidence for a significant correlation between the parasitemia of a lizard host and its infectivity toward the tick vector. Table 55 lists the average number of ticks detaching from each lizard host, the average time the larvae spent attached to each host and the average detachment mass of the engorged tick larvae from each host. Table 56 displays the average percentage of tick body mass loss and the average mortality per lizard host on day 256 post attachment. A series of ANOVA's on the data presented (Table 57) identified significant ($p<0.05$) differences between ticks that engorged on lizards that were infected with adeleorine blood parasites (type 2) and ticks that fed on type 0 or type 1 lizards only for one of the five studied aspects of tick fitness, the average tick body mass loss in percent (sig.=.022). A post-hoc analysis (Tukey) identified a significant difference (sig.=.019) between the body mass loss in percent between ticks from type 0 and type 2 infected lizards (Table 58). No significant differences were found between type 0, 1 or 2 infected lizards in regard to the number of ticks detaching from each lizard, the average time the ticks spent on their hosts, the average detachment mass and the average mortality in percent. No

correlations between the body mass and the sex of the lizard host were observed in respect to the studied features of tick fitness.

Table 54. Tick attachment experiment I: the lizard hosts, their body mass [g] their infections and parasitemias [10^4 RBC's] at the time of the tick attachments, and their infectivity toward the attached tick larvae [*P. m.*: *P. mackerassae*, P. c.: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H.* sp.: *Hepatozoon* sp., *S.* sp.: *Schellackia* sp., U. m.: unspecified morphotypes]. Type of infection: 0 = uninfected, 1 = infected, but not with adeleorine species, 2 = infected with at least one adeleorine species (types separated by the shadings).

Tick larvae attached to *E. stokesii*			Infections and parasitemia of lizard blood								Xenodiagnosis on vector *Amb. vikirri*	
			Plasmodium		Adeleorina			Eimeriorina				
ID	Sex	Body mass	*P. m.*	*P. c.*	*H. m.*	*H. b.*	*H.* sp.	*S.* sp.	U. m.	Type of infection	Number dissected	Found infected [%]
3	F	110.8	0	0	0	0	0	0	0	0	11	0
5	F	172.0	0	0	0	0	0	0	0	0	10	0
24	M	118.8	0	0	0	0	0	0	0	0	10	0
394	M	119.2	0	0	0	0	0	0	0	0	10	0
72	F	216.4	0	0	0	0	0	80	50	1	10	0
402	M	156.0	0	0	0	0	0	9	18	1	10	0
604	M	238.5	210	0	0	0	0	6	0	1	10	0
6	M	193.6	0	0	3	145	0	0	0	2	25	52
16	F	178.4	2970	0	0	3	0	0	1	2	10	50
90	M	119.3	1100	0	181	10	9	1	9	2	12	58
441	M	101.9	0	0	8	103	0	2	37	2	12	73
500	M	153.5	0	0	130	180	110	40	150	2	11	73

Table 55. Tick attachment experiment I. Twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larva on the 21/02/97 and the detaching engorged ticks were collected and kept individually (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	Sex	Type of Infection	No. of ticks detached	No. of engorged larvae evaluated*	Mean date of detachment [in days after attachment]		Mean detachment mass [mg]	
					$\bar{X}$	SD	$\bar{X}$	SD
3	F	0	73	69	19.6	8.06	1.32	0.32
5	F	0	70	70	19.3	7.86	1.35	0.29
24	M	0	80	79	15.1	4.61	1.49	0.26
394	M	0	77	73	15.9	5.40	1.30	0.30
72	F	1	71	63	18.9	7.31	1.40	0.29
402	M	1	80	76	17.9	6.12	1.38	0.28
604	M	1	74	72	23.1	7.87	1.37	0.29
6	M	2	61	61	16.2	6.31	1.33	0.29
16	F	2	66	62	17.0	6.72	1.40	0.21
90	M	2	56	51	16.0	5.81	1.46	0.30
441	M	2	78	74	12.3	6.76	1.51	0.23
500	M	2	55	51	17.6	6.19	1.25	0.27

* = squashed ticks or ticks found already molted to nymphs were omitted.

Table 56. Tick attachment experiment I. Twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larvae on the 21/02/97 and the individually kept ticks were weighted repeatedly (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	No. of detached ticks	No. of surviving nymphs weighed 256 days post attachment	Average body mass loss [%] since detachment		Mortality [%]
			$\bar{X}$	SD	
3	73	41	64.02	9.10	33.9
5	70	42	61.86	7.79	30.0
24	80	46	67.19	7.72	34.3
394	77	39	65.85	11.29	41.8
72	71	42	66.96	8.35	31.2
402	80	39	69.24	4.89	44.3
604	74	44	64.28	7.60	31.3
6	61	12	71.75	5.29	66.7
16	66	38	68.81	5.07	32.1
90	56	26	67.09	11.65	40.9
441	78	35	69.63	5.24	47.0
500	55	13	71.87	7.53	70.5

Table 57. List of ANOVA's for selected features studied in the tick attachment experiment I, searching for significant differences between the means of tick batches that engorged *E. stokesii* with type 0, 1 and 2 blood infections (for details of the types of infection see introduction of this chapter).

Studied feature	Type of blood infection	No. of lizard individuals	$\bar{X}$	SD	F	Sig. (2-tailed)
Average number of ticks detaching	0	4	75.0	4.40	3.832	0.063
	1	3	75.0	4.58		
	2	5	63.2	9.36		
	Total	12	70.1	8.83		
Average time ticks spent on a lizard host [days]	0	4	17.5	2.31	2.978	0.102
	1	3	20.0	2.74		
	2	5	15.8	2.08		
	Total	12	17.4	2.70		
Average detachment mass of ticks [mg]	0	4	1.36	0.08	0.114	0.893
	1	3	1.38	0.02		
	2	5	1.39	0.10		
	Total	12	1.38	0.08		
Average tick body mass loss [%]	0	4	64.7	2.31	5.966	**0.022**
	1	3	66.8	2.48		
	2	5	69.8	2.03		
	Total	12	67.4	3.07		
Average tick mortality [%]	0	4	35.0	4.93	2.645	0.125
	1	3	35.6	7.56		
	2	5	51.4	16.56		
	Total	12	42.0	13.65		

Table 58. Tick attachment experiment I; post-hoc analysis (Tukey) of the variances between the average body mass loss [%] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	- 0.028	0.023	0.476
0	&	2	- 0.069	0.020	**0.019**
1	&	2	- 0.041	0.022	0.203

13.3.2 Tick attachment experiment II

In this experiment, twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae on the 14/10/1997. Three *E. stokesii* were found to be long term uninfected with blood parasites (type 0), four lizards were infected, but not with adeleorine parasites (type 1) and five lizards were infected with adeleorine parasites, among others (type 2; Table 59). Dissections of the xenodiagnostic ticks found only those infected that engorged on type 2 infected lizards. The infectivity of the lizards towards the tick vector ranged from 40%-100%. *E. stokesii 16* had the lowest parasitemia regarding the adeleorine parasites (4 *Hem. biplicata* and 4 *Hem. mariae* in 10^4 RBC's) which coincided with the lowest percentage of infected xenodiagnostic nymphs (40%). The lizards with the highest parasitemia also showed the highest infectivity for the ticks. Table 60 lists the average number of ticks detaching from each lizard host, the average time the larvae spent attached to a host and the average detachment mass of the engorged tick larvae. Table 61 displays the average percentage of tick body mass loss and the average mortality per lizard host on day 197 post attachment. A series of ANOVA's on the data presented (Table 62) identified significant ($p<0.05$) differences between ticks that engorged on lizards that were infected with adeleorine blood parasites (type 2) and ticks that fed on type 0 or type 1 lizards for two of the five studied aspects of tick fitness, the average time the ticks were attached to a host (sig.=.045) and the average tick body mass loss in percent (sig.=.019). A post-hoc analysis (Tukey) identified a significant difference (sig.=.025) regarding the body mass loss in percent between ticks from type 1 and type 2 infected lizards (Table 64) The test could not detect significant differences in regard to the time the ticks were attached to the lizards, although the difference between type 0 and type 2 was close to significant (Table 63). No significant differences were found between type 0, 1 or 2 infected lizards in regard to the number of ticks detaching from each lizard, the average detachment mass and the average mortality in percent. No correlations between the body mass and the sex of the lizard host were observed in respect to the studied features of tick fitness.

Table 59. Tick attachment experiment II: the lizard hosts, their body mass [g] their infections and parasitemias [10^4 RBC's] at the time of the tick attachments, and their infectivity toward the attached tick larvae [*P. m.*: *P. mackerassae*, P. c.: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H.* sp.: *Hepatozoon* sp., *S.* sp.: *Schellackia* sp., U. m.: unspecified morphotypes]. Type of infection: 0 = uninfected, 1 = infected, but not with adeleorine species, 2 = infected with at least one adeleorine species (types separated by the shadings).

Tick larvae attached to *E. stokesii*			Infections and parasitemia of lizard blood								Xenodiagnosis on vector *Amb. limbatum*	
			Plasmodium		Adeleorina			Eimeriorina				
ID	Sex	Body mass	*P. m.*	*P. c.*	*H. m.*	*H. b.*	*H.* sp.	*S.* sp.	U. m.	Type of infection	Number dissected	Found infected [%]
3	F	154.4	0	0	0	0	0	0	0	0	16	0
24	M	158.8	0	0	0	0	0	0	0	0	10	0
47	F	222.9	0	0	0	0	0	0	0	0	10	0
604	M	238.0	21	0	0	0	0	2	0	1	10	0
651	F	276.9	75	0	0	0	0	20	15	1	10	0
720	F	148.7	0	0	0	0	0	135	50	1	10	0
764	F	127.5	0	0	0	0	0	105	72	1	10	0
11	F	157.1	0	0	290	440	20	0	30	2	10	100
12	M	208.9	0	0	23	211	0	0	4	2	10	90
16	F	203.3	6100	0	4	4	0	0	0	2	10	40
34	M	189.5	0	0	26	24	0	7	3	2	11	91
90	M	135.2	347	0	106	0	5	4	4	2	10	100

Table 60. Tick attachment experiment II. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larva on the 14/10/97 and the detaching engorged ticks were collected and kept individually (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	Sex	Type of Infection	No. of ticks detached	No. of engorged larvae evaluated*	Mean date of detachment [in days after attachment]		Mean detachment mass [mg]	
					$\bar{X}$	SD	$\bar{X}$	SD
3	F	0	45	45	14.51	2.26	0.99	0.19
24	M	0	46	45	15.60	2.38	1.08	0.33
47	F	0	49	47	15.47	2.12	0.95	0.21
604	M	1	43	39	16.36	2.72	0.99	0.22
651	F	1	61	60	15.42	2.53	0.97	0.20
720	F	1	51	50	14.40	1.59	1.03	0.27
764	F	1	85	83	15.89	2.61	1.09	0.21
11	F	2	23	21	18.62	2.75	1.02	0.36
12	M	2	68	66	16.91	2.86	1.09	0.22
16	F	2	36	32	16.88	3.20	0.98	0.23
34	M	2	50	50	15.54	2.38	0.99	0.30
90	M	2	38	37	16.97	2.81	1.15	0.30

* = squashed ticks or ticks found already molted to nymphs were omitted.

Table 61. Tick attachment experiment II. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae on the 14/10/97 and the individually kept ticks were weighted repeatedly (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	No. of detached ticks	No. of surviving nymphs weighed 256 days post attachment	Average body mass loss [%] since detachment		Mortality [%]
			$\bar{X}$	SD	
3	45	39	65.47	3.85	13.3
24	46	37	68.02	4.22	19.6
47	49	29	66.97	4.63	40.8
604	43	30	67.35	4.30	30.2
651	61	50	68.04	4.05	18.0
720	51	35	59.67	2.94	31.4
764	85	70	69.31	3.85	17.7
11	23	5	76.00	2.62	78.3
12	68	40	69.55	3.05	41.2
16	36	10	72.66	3.11	72.2
34	50	35	72.54	3.75	30.0
90	38	25	71.79	3.53	34.2

Table 62. List of ANOVA's for selected features studied in the tick attachment experiment II, searching for significant differences between the means of tick batches that engorged *E. stokesii* with type 0, 1 and 2 blood infections (for details of the types of infection see introduction of this chapter).

Studied feature	Type of blood infection	No. of lizard individuals	$\bar{X}$	SD	F	Sig. (2-tailed)
Average number of ticks detaching	0	3	46.7	2.08	1.406	0.294
	1	4	60.0	18.22		
	2	5	43.0	16.94		
	Total	12	49.6	16.04		
Average time ticks spent on a lizard host [days]	0	3	15.2	0.59	4.562	**0.043**
	1	4	15.5	0.84		
	2	5	17.0	1.09		
	Total	12	16.1	1.18		
Average detachment mass of ticks [mg]	0	3	1.01	0.07	0.368	0.702
	1	4	1.02	0.05		
	2	5	1.05	0.07		
	Total	12	1.03	0.06		
Average tick body mass loss [%]	0	3	66.8	1.28	6.368	**0.019**
	1	4	66.1	4.36		
	2	5	72.5	2.32		
	Total	12	69.0	4.17		
Average tick mortality [%]	0	3	24.6	14.41	3.409	0.079
	1	4	24.3	7.50		
	2	5	51.2	22.43		
	Total	12	35.6	20.63		

Table 63. Tick attachment experiment II; post-hoc analysis (Tukey) of the variances between the average time the ticks spent attached to the host [days] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	-0.32	0.70	0.891
0	&	2	-1.79	0.67	0.061
1	&	2	-1.47	0.62	0.095

Table 64. Tick attachment experiment II; post-hoc analysis (Tukey) of the variances between the average body mass loss [%] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	0.009	0.031	0.957
0	&	2	- 0.080	0.030	0.059
1	&	2	- 0.089	0.027	**0.025**

13.3.3 Tick attachment experiment III

In this experiment, twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larvae on the 26/01/1998. Two of the *E. stokesii* were found to be long term uninfected with blood parasites (type 0), two lizards were infected, but not with adeleorine parasites (type 1) and eight lizards were infected with adeleorine parasites, among others (type 2; Table 65). Dissections of the xenodiagnostic ticks showed that only ticks that fed on type 2 infected lizards were detectably infected. The infectivity of the lizards towards the tick vector ranged from 40%-100%. Table 66 lists the average number of ticks detaching from each lizard host, the average time the larvae spent attached to a host and the average detachment mass of the engorged tick larvae. Table 67 displays the average percentage of tick body mass loss and the average mortality per lizard host on day 254 post attachment. The analysis of the impact of the lizard blood infection types 0, 1 and 2 on the selected features of tick fitness revealed significant differences in the average detachment mass (sig.=.045) and the average body mass loss in percent (sig.=.027; Table 68). A post-hoc analysis (Tukey) could not detect significant differences in the average detachment mass, although the difference between type 1 and type 2 was almost significant (Table 69), but identified a significant (sig.=.031) difference of the body mass loss in percent between ticks from type 1 and type 2 infected lizards (Table 70). No significant differences were found between type 0, 1 or 2 infected lizards in regard to the number of ticks detaching from each lizard, the average time the ticks spent on a host and the average mortality in percent. It must be explained that in this experiment the calculation of the percentage of the body mass loss and other four observed features of tick fitness were not based on exactly the same data set. All ticks that detached from *E. stokesii 11* were dead at the time of weighing on day 254 (Table 67). Although their detachment mass and their mortality were available for calculations, the percentage of body mass loss could not be determined. Thus the ticks from *E. stokesii 11* were omitted from the analysis of the percentage of tick body mass loss and only seven type 2 lizards were included in the analysis. It was noteworthy that the blood of *E. stokesii 11* was chronically infected with the adeleorine *Hem. biplicata and Hem. mariae*, carrying extremely high parasitemias of both parasites at the time of tick attachment (Table 65). No correlations between the body mass and the sex of the lizard host were observed in respect to the studied features of tick fitness.

Table 65. Tick attachment experiment III: the lizard hosts, their body mass [g] their infections and parasitemias [10^4 RBC's] at the time of the tick attachments, and their infectivity toward the attached tick larvae [*P. m.*: *P. mackerassae*, P. c.: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H.* sp.: *Hepatozoon* sp., *S.* sp.: *Schellackia* sp., U. m.: unspecified morphotypes]. Type of infection: 0 = uninfected, 1 = infected, but not with adeleorine species, 2 = infected with at least one adeleorine species (types separated by the shadings).

Tick larvae attached to *E. stokesii*			Infections and parasitemia of lizard blood								Xenodiagnosis on vector *Amb. vikirri*	
			Plasmodium		Adeleorina			Eimeriorina				
ID	Sex	Body mass	*P. m.*	*P. c.*	*H. m.*	*H. b.*	*H.* sp.	*S.* sp.	U. m.	Type of infection	Number dissected	Found infected [%]
4	F	122.3	0	0	0	0	0	0	0	0	10	0
5	F	187.3	0	0	0	0	0	0	0	0	10	0
700	F	114.4	0	0	0	0	0	8	5	1	10	0
741	F	280.7	0	0	0	0	0	100	150	1	10	0
11	F	150.4	0	0	290	423	40	0	37	2	10	100
12	M	216.2	0	0	39	271	0	0	10	2	10	50
16	F	204.4	1620	500	2	8	0	0	0	2	10	40
90	M	142.9	0	0	91	82	9	1	17	2	10	80
117	F	174.7	0	0	50	138	60	60	22	2	10	90
441	M	143.8	660	16	28	172	0	200	160	2	10	80
470	M	214.5	570	0	30	195	0	1	4	2	10	80
500	M	155.6	0	0	170	345	140	60	15	2	10	80

Table 66. Tick attachment experiment III. Twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larva on the 26/01/98 and the detaching engorged ticks were collected and kept individually (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	Sex	Type of Infection	No. of ticks detached	No. of engorged larvae evaluated*	Mean date of detachment [in days after attachment]		Mean detachment mass [mg]	
					$\bar{X}$	SD	$\bar{X}$	SD
4	F	0	60	59	12.97	2.94	1.53	0.34
5	F	0	57	57	13.12	3.04	1.41	0.27
700	F	1	87	83	15.00	5.12	1.53	0.23
741	F	1	65	63	16.13	5.41	1.47	0.40
11	F	2	37	36	13.97	1.52	1.37	0.23
12	M	2	64	62	12.98	2.33	1.45	0.31
16	F	2	53	51	16.98	5.35	1.39	0.32
90	M	2	58	57	13.60	3.25	1.35	0.36
117	F	2	70	68	15.76	5.11	1.34	0.30
441	M	2	77	73	16.01	6.33	1.46	0.34
470	M	2	71	71	14.00	3.89	1.26	0.34
500	M	2	62	62	14.32	2.63	1.31	0.36

* = squashed ticks or ticks found already molted to nymphs were omitted.

Table 67. Tick attachment experiment III. Twelve *E. stokesii* were each exposed to 100 *Amb. vikirri* larvae on the 26/01/98 and the individually kept ticks were weighted repeatedly (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	No. of detached ticks	No. of surviving nymphs weighed 254 days post attachment	Average body mass loss [%] since detachment		Mortality [%]
			$\bar{X}$	SD	
4	60	20	58.19	5.03	16.6
5	57	44	62.38	5.87	6.4
700	87	65	59.09	3.90	15.6
741	65	26	57.04	3.47	52.7
11	37	0			100.0
12	64	42	64.54	5.19	22.2
16	53	36	61.83	5.11	16.3
90	58	24	62.25	4.81	50.0
117	70	50	65.23	4.90	16.7
441	77	48	60.38	4.42	28.4
470	71	48	65.47	5.10	21.3
500	62	46	65.42	4.34	11.5

Table 68. List of ANOVA's for selected features studied in the tick attachment experiment III, searching for significant differences between the means of tick batches that engorged *E. stokesii* with type 0, 1 and 2 blood infections (for details of the types of infection see introduction of this chapter).

Studied feature	Type of blood infection	No. of lizard individuals	$\bar{X}$	SD	F	Sig. (2-tailed)
Average number of ticks detaching	0	2	58.5	2.12	1.641	0.247
	1	2	76.0	15.56		
	2	8	61.5	12.50		
	Total	12	63.4	12.56		
Average time ticks spent on a lizard host [days]	0	2	13.0	0.11	2.172	0.170
	1	2	15.6	0.80		
	2	8	14.7	1.38		
	Total	12	14.6	1.38		
Average detachment mass of ticks [mg]	0	2	1.47	0.08	4.485	**0.045**
	1	2	1.50	0.04		
	2	8	1.37	0.07		
	Total	12	1.41	0.09		
Average tick body mass loss [%]	0	2	60.3	2.97	5.848	**0.027**
	1	2	58.1	1.45		
	2	7	63.6	2.07		
	Total	11	62.0	3.02		
Average tick mortality [%]	0	2	11.5	7.27	0.355	0.711
	1	2	34.2	26.26		
	2	8	33.3	29.42		
	Total	12	29.8	26.29		

Table 69. Tick attachment experiment III; post-hoc analysis (Tukey) of the variances between the average detachment mass [mg] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	-0.035	0.067	0.866
0	&	2	0.104	0.053	0.178
1	&	2	0.139	0.053	0.066

Table 70. Tick attachment experiment III; post-hoc analysis (Tukey) of the variances between the average of tick body mass loss [%] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	0.028	0.027	0.589
0	&	2	- 0.042	0.022	0.195
1	&	2	- 0.070	0.022	**0.031**

13.3.4 Tick attachment experiment IV

The fourth experiment was started on the 19/04/98, when twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae. The body mass and the blood infections of the *E. stokesii* at the start of this experiment are listed in Table 71. Three of the *E. stokesii* long term uninfected with blood parasites (type 0), two lizards were infected, but not with adeleorine parasites (type 1) and seven lizards were infected with adeleorine parasites, among others (type 2). Dissections of the xenodiagnostic ticks showed that only ticks that fed on type 2 infected lizards were detectably infected. From 33%-70% of the xenodiagnostic ticks were found to be infected. Table 72 lists the average number of ticks detaching from each lizard host, the average time the larvae spent attached to a host and the average detachment mass of the engorged tick larvae. Table 73 displays the average percentage of tick body mass loss and the average mortality per lizard host on day 271 post attachment. A series of ANOVA's on the data presented (Table 74) showed significant differences (sig.=.001) in regard to the average tick body mass loss in percent between the lizard blood infection types 0, 1 and 2. A post-hoc analysis (Tukey) identified a significant (sig.=.001) difference of the body mass loss in percent between ticks from type 0 and type 2 infected lizards (Table 75). No significant differences were found between type 0, 1 or 2 infected lizards in regard to the number of ticks detaching from each lizard, the average time the ticks spent on their hosts, the average detachment mass and the average mortality in percent. No correlations between the body mass and the sex of the lizard host were observed in respect to the studied features of tick fitness.

Table 71. Tick attachment experiment IV: the lizard hosts, their body mass [g] their infections and parasitemias [10^4 RBC's] at the time of the tick attachments, and their infectivity toward the attached tick larvae [*P. m.*: *P. mackerassae*, P. c.: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H.* sp.: *Hepatozoon* sp., *S.* sp.: *Schellackia* sp., U. m.: unspecified morphotypes]. Type of infection: 0 = uninfected, 1 = infected, but not with adeleorine species, 2 = infected with at least one adeleorine species (types separated by the shadings).

Tick larvae attached to *E. stokesii*			Infections and parasitemia of lizard blood								Xenodiagnosis on vector *Amb. limbatum*	
			Plasmodium		Adeleorina			Eimeriorina				
ID	Sex	Body mass	*P. m.*	*P. c.*	*H. m.*	*H. b.*	*H.* sp.	*S.* sp.	U. m.	Type of infection	Number dissected	Found infected [%]
4	F	118.3	0	0	0	0	0	0	0	0	10	0
5	F	170.3	0	0	0	0	0	0	0	0	10	0
24	M	179.1	0	0	0	0	0	0	0	0	10	0
741	F	102.5	0	0	0	0	0	92	28	1	10	0
764	F	160.6	0	0	0	0	0	23	8	1	10	0
11	F	144.1	780	0	320	360	21	0	19	2	13	64
12	M	198.2	0	0	15	186	0	0	0	2	10	40
16	M	202.7	1350	500	7	28	0	0	0	2	10	70
90	M	134.6	0	0	111	122	11	2	7	2	10	50
117	F	163.2	0	0	26	170	75	42	17	2	12	33
470	M	223.8	400	0	28	116	0	2	0	2	15	57
500	M	141.3	0	0	210	300	120	50	20	2	18	47

Table 72. Tick attachment experiment VI. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larva on the 19/04/98 and the detaching engorged ticks were collected and kept individually (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	Sex	Type of Infection	No. of ticks detached	No. of engorged larvae evaluated*	Mean date of detachment [in days after attachment]		Mean detachment mass [mg]	
					$\bar{X}$	SD	$\bar{X}$	SD
4	F	0	59	57	25.75	6.47	1.06	0.19
5	F	0	65	62	21.63	6.01	1.02	0.21
24	M	0	56	44	18.48	4.56	0.86	0.28
741	F	1	50	49	21.39	5.96	1.02	0.27
764	F	1	50	43	19.74	5.59	0.88	0.19
11	F	2	57	48	21.83	5.72	0.95	0.22
12	M	2	49	46	26.20	6.01	1.03	0.21
16	M	2	40	38	23.32	6.89	0.98	0.22
90	M	2	44	36	26.44	6.35	0.91	0.25
117	F	2	67	59	22.41	5.39	0.89	0.20
470	M	2	78	74	25.62	6.02	0.94	0.17
500	M	2	73	67	24.81	5.89	0.96	0.23

* = squashed ticks or ticks found already molted to nymphs were omitted.

Table 73. Tick attachment experiment IV. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae on the 19/04/98 and the individually kept ticks were weighted repeatedly (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	No. of detached ticks	No. of surviving nymphs weighed 271 days post attachment	Average body mass loss [%] since detachment		Mortality [%]
			$\bar{X}$	SD	
4	59	41	59.83	6.46	16.3
5	65	47	59.99	4.08	14.5
24	56	30	64.19	3.87	25.0
741	50	13	63.10	7.47	67.5
764	50	26	65.69	8.06	40.9
11	57	24	65.57	6.78	38.5
12	49	16	66.48	6.38	46.7
16	40	17	63.52	6.44	63.0
90	44	13	66.40	5.00	61.8
117	67	34	64.19	3.99	38.2
470	78	43	66.64	8.86	31.8
500	73	34	65.35	5.13	38.2

Table 74. List of ANOVA's for selected features studied in the tick attachment experiment IV, searching for significant differences between the means of tick batches that engorged on *E. stokesii* with type 0, 1 and 2 blood infections (for details of the types of infection see introduction of this chapter).

Studied feature	Type of blood infection	No. of lizard individuals	$\bar{X}$	SD	F	Sig. (2-tailed)
Average number of ticks detaching	0	3	60.0	4.58	0.450	0.651
	1	2	50.0	0.00		
	2	7	58.3	14.76		
	Total	12	57.3	11.62		
Average time ticks spent on a lizard host [days]	0	3	22.0	3.65	2.597	0.129
	1	2	20.6	1.16		
	2	7	24.4	1.86		
	Total	12	23.2	2.64		
Average detachment mass of ticks [mg]	0	3	0.98	0.11	0.187	0.833
	1	2	0.95	0.10		
	2	7	0.95	0.05		
	Total	12	0.96	0.07		
Average tick body mass loss [%]	0	3	61.1	2.08	15.301	**0.001**
	1	2	63.7	0.77		
	2	7	65.8	0.86		
	Total	12	64.3	2.34		
Average tick mortality [%]	0	3	19.0	6.22	0.455	0.060
	1	2	46.3	30.05		
	2	7	42.3	9.66		
	Total	12	40.2	17.36		

Table 75. Tick attachment experiment IV; post-hoc analysis (Tukey) of the variances between the average of tick body mass loss [%] comparing type 0, 1 and 2 infected *E. stokesii*.

Type of blood infection		Type of blood infection	Mean difference	SE	Sig. (2-tailed)
0	&	1	- 0.032	0.015	0.120
0	&	2	- 0.060	0.011	**0.001**
1	&	2	- 0.028	0.013	0.128

13.3.5 The comparison of the tick attachment experiments I-IV

Throughout attachment experiment I-IV, only those ticks became infected that attached to lizards with adeleorine blood infections (Table 76). The average infectivity of the type 2 infected lizards within the four experiments ranged from 52% to 85%. No significant differences of infectivity were detected between the tick species, *Amb. vikirri* and *Amb. limbatum* (Table 77).

In a further analysis of the experiments I-IV, a series of two way ANOVA's investigated the effect of the two tick species, the three types of infections and the between-subject effects on tick fitness (Table 78). A highly significant difference of the number of ticks detaching was detected between the two tick species (Table 79). The average number of detaching *Amb. vikirri* larvae was significantly higher than the average number of detaching *Amb. limbatum* larvae (Table 78). However, this aspect of tick fitness was not influenced by the type of lizard-infection, and it was also not influenced by between-subject effects (infection type & tick species, Table 79).

The duration of the blood meal was significantly longer for *Amb. limbatum* larvae than for *Amb. vikirri* larvae (Table 78 and Table 79). This result was probably due to the relatively long time the *Amb. limbatum* larvae in experiment IV needed to engorge (Table 72). During the course of experiment IV, the temperature control of the experimental room failed, which led to an undulating temperature pattern with an average of approximately 21°C over a period of nine days. This temperature drop is most likely the cause for the relatively long time those ticks spent attached to the lizard hosts, and apparently this delay of detachment in experiment IV was responsible for the detected differences between the two tick species. No significant differences of the duration of tick attachment were found in regard to the three types of infection. The same data suggested a significant between-subject effect, which again was probably due to the relatively long time necessary for the larvae of experiment IV to detach.

A highly significant difference in the detachment mass occurred between the two tick species (Table 79). Fully engorged *Amb. vikirri* larvae were on average noticeably heavier than fully engorged *Amb. limbatum* larvae (Table 78). No such effect was found between the three types of infection, or between the two subjects, tick species and type of infection.

The average tick body mass loss after detachment was not significantly different between the two tick species (Table 79). However, the average body mass loss was significantly different between the three types of infection. Ticks that engorged on type 2 (adeleorine) infected lizards lost their body mass at a faster rate than the ticks that detached from type 0 and type 1 lizards (Table 78). No between-subject effects were detected.

In experiments I-IV, no significant differences could be demonstrated between the average mortality regarding the three types of infection, although the result was almost significant (0.074, Table 79). No significant differences of the mortality were detected between the two tick species, *Amb. vikirri* and *Amb. limbatum*.

No correlation between the means of the average detachment mass of the engorged larvae and the means of the average mortality in percent was found. In other words, the ticks with the lowest average detachment mass did not suffer the highest average mortality. However, a significant negative correlation (sig.=.001, Pearson correlation) was detected between the means of the average tick body mass loss in percent and the means of their average mortality in percent. In other words, the groups of ticks with the highest percentage of body mass loss suffered the highest mortality. The results presented here will be discussed in more detail later.

Table 76. Mean percentage of infected ticks comparing tick attachment experiments I-IV [type of infection: 2 = infected with at least one adeleorine species].

Tick attachment experiment	Type of infection of lizard	Tick species attached as larvae	No. of ticks dissected	Average number of ticks found to be infected [%]	SD
I	2	*Amb. vikirri*	70	61	11.0
II	2	*Amb. limbatum*	51	85	23.9
III	2	*Amb. vikirri*	80	75	17.5
IV	2	*Amb. limbatum*	88	52	12.9

Table 77. One way ANOVA comparing the percentages of infected ticks from the experiments I-IV that engorged on type 2 infected *E. stokesii* between the two tick species *Amb. limbatum* and *Amb. vikirri*.

Tick species	Number of type 2 lizards	Mean of average number of ticks infected [%]			
		$\bar{X}$	SD	F	Sig.
Amb. vikirri	13	69.69	17.99	0.011	0.919
Amb. limbatum	12	65.17	24.61		
Total	25	67.52	21.09		

Table 78. Average of the means of selected features of tick fitness studied in the tick attachment experiment I-IV, comparing tick batches that engorged on *E. stokesii* with type 0, 1 and 2 blood infections and the two tick species *Amb. vikirri* and *Amb. limbatum*.

Studied feature	Type of blood infection	*Amb. vikirri* No. of lizard individuals	$\bar{X}$	SD	*Amb. limbatum* No. of lizard individuals	$\bar{X}$	SD
Average number of ticks detaching	0	6	69.5	9.22	6	53.3	7.97
	1	5	75.4	8.44	6	56.7	15.03
	2	13	62.2	11.01	12	51.9	16.89
	Total	24	66.8	11.15	24	53.5	14.26
Average time ticks spent on a lizard host [days]	0	6	16.0	2.90	6	18.6	4.38
	1	5	18.2	3.11	6	17.2	2.74
	2	13	15.1	1.70	12	21.3	4.10
	Total	24	16.0	2.55	24	19.6	4.14
Average detachment mass of ticks [mg]	0	6	1.40	0.09	6	0.99	0.08
	1	5	1.43	0.07	6	1.00	0.07
	2	13	1.38	0.08	12	0.99	0.07
	Total	24	1.39	0.08	24	0.99	0.07
Average tick body mass loss [%]	0	6	63.3	3.20	6	64.0	3.49
	1	5	63.3	5.16	6	65.3	3.62
	2	12	66.2	3.76	12	68.6	3.80
	Total	23	64.8	4.06	24	66.6	4.09
Average tick mortality [%]	0	6	27.2	13.11	6	27.9	19.98
	1	5	35.0	14.20	6	31.6	18.51
	2	13	40.3	26.09	12	46.0	15.97
	Total	24	35.9	21.41	24	37.9	18.80

Table 79. List of two way ANOVA's for selected features of tick fitness studied in the tick attachment experiment I-IV [n=48]. Analysis of the effect of the three types of blood infection, the two tick species *Amb. vikirri* and *Amb. limbatum* and the between-subject effects [significant differences in bold].

Studied feature	Tests of between-subject effects	df	F	Sig. (2-tailed)
Average number of ticks detaching	Type of blood infection	2	2.18	0.126
	Tick species	1	14.66	**0.000**
	Infection type & tick species	2	0.69	0.508
Average time ticks spent on a lizard host [days]	Type of blood infection	2	0.36	0.700
	Tick species	1	6.76	**0.013**
	Infection type & tick species	2	4.92	**0.012**
Average detachment mass of ticks [mg]	Type of blood infection	2	0.57	0.570
	Tick species	1	286.6	**0.000**
	Infection type & tick species	2	0.437	0.649
Average tick body mass loss [%]	Type of blood infection	2	4.858	**0.01**
	Tick species	1	2.026	0.16
	Infection type & tick species	2	0.192	0.83
Average tick mortality [%]	Type of blood infection	2	2.77	0.074
	Tick species	1	0.029	0.866
	Infection type & tick species	2	0.215	0.808

13.3.6 Tick attachment experiment V

This final experiment V was designed to examine the impact of the adeleorine *Hem. mariae* alone on the fitness of *Amb. limbatum* ticks. Thus, for the infected lizards only those *E. stokesii* were chosen that were infected with *Hem. mariae*, but not with any other adeleorine parasite (*Hem. biplicata* or *Hepatozoon* sp.; Table 80). Two of the *E. stokesii* were long term uninfected with blood parasites (type 0), and four lizards were infected with *Hem. mariae* (type 2). One of the type 2 lizards had a mixed infection with *Hem. mariae*, *Schellackia* sp. and *P. mackerrasae*, one *E. stokesii* had a mixed infection with *Hem. mariae* and *Schellackia* sp.; one lizard had a mixed infection with *Hem. mariae* and *P. mackerrasae* and one lizard was single infected with *Hem. mariae*. Those six *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae on the 09/12/98. The body mass and the blood infections of the six *E. stokesii* are listed in Table 80. Dissections of the xenodiagnostic ticks showed that only ticks that fed on type 2 infected lizards became infected and the infectivity ranged between 60% and 100% (Table 80). Table 81 lists the average number of ticks detaching from each lizard host, the average time the larvae spent attached to a host and the average detachment mass of the engorged tick larvae. Table 82 displays the average percentage of tick body mass loss and the average mortality per lizard host on day 94 post attachment.

In the statistical analysis, no significant differences were found in regard to the average number of ticks detaching between ticks that engorged on *E. stokesii* infected with *Hem. mariae* and ticks that fed on uninfected lizards. However, the average number of days of attachment to the lizard host (sig.=.028), the average detachment mass (sig.=.030), the average body mass loss in percent (sig.=.000) and the average mortality (sig.=.004) all showed significant differences between ticks that engorged on uninfected lizards and ticks that engorged on lizards infected with *Hem. mariae*. Ticks on infected lizards took longer to detach, weighed less at detachment, lost more body mass after detachment (Figure 128) and had a higher mortality, than ticks that fed on uninfected lizards. No correlations between the body mass and the sex of the lizard host were observed in respect to the studied features of tick fitness.

Table 80. Tick attachment experiment V: the lizard hosts, their body mass [g] their infections and parasitemias [10^4 RBC's] at the time of the tick attachments, and their infectivity toward the attached tick larvae [*P. m.*: *P. mackerassae*, P. c.: *P. circularis*, *H. m.*: *H. mariae*, *H. b.*: *H. biplicata*, *H.* sp.: *Hepatozoon* sp., *S.* sp.: *Schellackia* sp., U. m.: unspecified morphotypes]. Type of infection: 0 = uninfected, 2 = adeleorine infection exclusively with *H. mariae*, non-adeleorine blood parasites may be present also (types separated by the shadings).

Tick larvae attached to *E. stokesii*			Infections and parasitemia of lizard blood								Xenodiagnosis on vector *Amb. limbatum*	
			Plasmodium		Adeleorina			Eimeriorina				
ID	Sex	Body mass	*P. m.*	*P. c.*	*H. m.*	*H. b.*	*H.* sp.	*S.* sp.	U. m.	Type of infection	Number dissected	Found infected [%]
5	F	210.6	0	0	0	0	0	0	0	0	10	0
21	F	153.4	0	0	0	0	0	0	0	0	10	0
41	M	255.9	0	0	59	0	0	54	11	2	10	90
170	F	193.7	495	0	13	0	0	19	7	2	10	60
620	F	188.5	0	0	70	0	0	0	0	2	6	100
1377	M	216.8	119	0	136	0	0	0	0	2	10	70

Table 81. Tick attachment experiment V. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larva on the 09/12/98 and the detaching engorged ticks were collected and kept individually (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	Sex	Type of Infection	No. of ticks detached	No. of engorged larvae evaluated*	Mean date of detachment [in days after attachment]		Mean detachment mass [mg]	
					$\bar{X}$	SD	$\bar{X}$	SD
5	F	0	42	37	18.54	8.56	1.15	0.38
21	F	0	52	48	17.31	8.31	0.96	0.24
41	M	2	69	56	17.11	6.32	0.83	0.44
170	F	2	59	48	24.69	7.24	1.05	0.26
620	F	2	16	14	20.21	9.37	1.07	0.27
1377	M	2	25	18	18.67	7.65	0.88	0.35

* = squashed ticks or ticks found already molted to nymphs were omitted.

Table 82. Tick attachment experiment V. Twelve *E. stokesii* were each exposed to 100 *Amb. limbatum* larvae on the 09/12/98 and the individually kept ticks were weighted repeatedly (The three types of infection of the lizard host were separated by the shadings).

E. stokesii	No. of detached ticks	Surviving nymphs weighted 94 days post attachment	Average body mass loss [%] since detachment	SD	Mortality [%]
5	42	25	51.48	7.40	40.5
21	52	23	51.99	7.03	55.8
	Σ 94	Σ 48	$\bar{X}$ **51.7**	$\bar{X}$ 7.22	$\bar{X}$ **48.2**
41	69	9	67.15	6.41	87.0
170	59	34	56.73	6.08	42.4
620	16	7	56.44	4.73	56.3
1377	25	6	60.86	3.31	76.0
	Σ 169	Σ 46	$\bar{X}$ **60.3**	$\bar{X}$ 5.13	$\bar{X}$ **65.4**

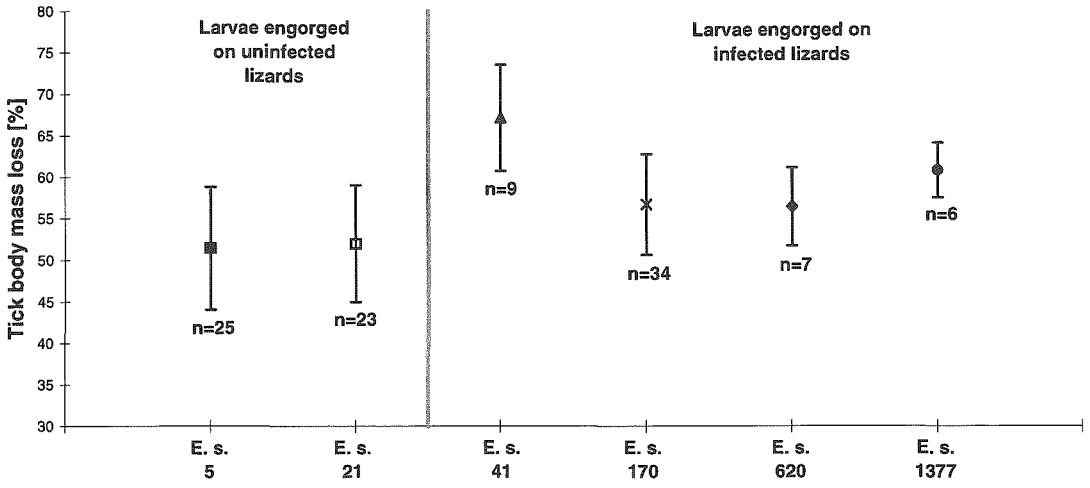

Figure 128. Tick attachment experiment V; the average tick body mass loss in percent for each lizard individual.

13.3.7 Analysis of repeated weighings of the attachment experiments I and II

As well as the weighing events previously presented for each experiment, the tick cohorts from attachment experiments I and II were weighted several other times after detachment. Table 83 lists the percent tick body mass loss for surviving *Amb. vikirri* ticks in four weighings of tick cohort I, separated into the three lizard infection-types. The first weighing after determining the detachment mass was on day 256 post attachment. Those results were described in detail in the previous analysis. Three more weighings of the surviving nymphs followed on day 361, day 432 and day 592 post attachment. With progressing time, the number of surviving ticks decreased in all three groups (types 0, 1 & 2), until on day 592 post detachment only four ticks were still alive. The average percent body mass loss remained relatively constant for the weighings on day 256, day 361 and day 592 post attachment, but was slightly higher than on the previous three weighings for the four surviving nymphs on day 592 post attachment. In Table 84, the first three weighings of the experiment were analysed for differences between lizard infection-types. A T-test detected significant differences between the ticks from type 0 and type 2 infected lizards and between ticks from type 1 and type 2 infected lizards on day 256 post attachment, but no further differences in the following weighings.

Similarly to experiment I, the tick cohort of experiment II was weighed several times after the detachment mass was determined. In contrast to experiment I, *Amb. limbatum* ticks were weighed relatively early after they were attached to the host. (Table 85) lists the four weighing events on day 44, day 131, day 197 and day 357 post detachment of tick cohort II. The data were separated into the ticks that engorged on *E. stokesii* with the three types of infection. The data from day 197 post detachment were analysed in detail at the beginning of this chapter. During the consecutive weighings, the number of surviving ticks slowly decreased while the average body mass loss of the surviving nymphs slowly increased. A T-test analysis for differences between the ticks from lizards with the three types of infection revealed significant differences at day 131 and day 197 post attachment (Table 86). On both occasions, the body mass loss of ticks that engorged on lizards infected with adeleorine blood parasites (type 2) was significantly higher than that of their siblings that engorged on type 0 or type 1 lizards. However, no significant differences were found on day 44 and day 357 post detachment.

Table 83. The average body mass loss over time of the ticks from attachment experiment I, grouped by the three types of blood infection of the lizard host.

Days since tick attachment	Infection type 0			Infection type 1			Infection type 2		
	No. of surviving ticks	Body mass loss [%]	SD	No. of surviving ticks	Body mass loss [%]	SD	No. of surviving ticks	Body mass loss [%]	SD
256	168	64.8	9.16	125	66.8	7.41	124	69.5	6.96
361	71	65.1	6.44	41	66.7	8.55	32	67.5	8.30
432	34	68.5	4.89	12	65.7	9.77	12	67.6	11.44
592	2	75.9	8.30	1	80.4	-	1	67.5	-

Table 84: Tick attachment experiment I. Analysis for significant (0.05) differences between the means of the body mass loss in percent from the three types of blood infections of the lizard host [T-tests] (significant differences in bold letters).

Days since tick attachment	Infection types 0 & 1		Infection types 0 & 2		Infection types 1 & 2	
	t	Sig. (2-tailed)	t	Sig. (2-tailed)	t	Sig. (2-tailed)
256	- 1.957	.051	- 4.524	**.000**	- 2.747	**.006**
361	- 1.160	.249	- 1.571	.119	- .363	.768
432	- 2.015	.319	- 2.015	.947	- 2.073	.376

Table 85: The average body mass loss over time of the ticks from attachment experiment II, grouped by the three types of blood infection of the lizard host.

Days since tick attachment	Infection type 0			Infection type 1			Infection type 2		
	No. of surviving ticks	Body mass loss [%]	SD	No. of surviving ticks	Body mass loss [%]	SD	No. of surviving ticks	Body mass loss [%]	SD
44	142	55.99	6.30	219	56.66	17.43	144	58.15	6.00
131	129	64.28	3.34	212	64.07	14.40	118	67.28	3.87
197	120	66.79	4.31	198	66.83	15.03	87	71.04	3.61
357	38	70.28	3.46	54	70.94	3.38	2	73.57	3.22

Table 86: Tick attachment experiment II. Analysis for significant (0.05) differences between the means of the body mass loss in percent from the three types of blood infections of the lizard host [T-tests] (significant differences in bold letters).

Days since tick attachment	Infection types 0 & 1		Infection types 0 & 2		Infection types 1 & 2	
	t	Sig. (2-tailed)	t	Sig. (2-tailed)	t	Sig. (2-tailed)
44	- 1.967	.566	- 1.969	.310	- 1.967	.081
131	- 1.967	.852	- 1.971	**.017**	- 1.968	**.013**
197	- .026	.974	- 8.728	**.000**	- 3.272	**.001**
357	- .636	.526	- 1.312	.198	-1.078	.289

13.3.8 Discussion

The results presented in this chapter indicate that some of the apicomplexan blood parasites infecting the lizard *E. stokesii* alter the fitness of the ticks *Amb. limbatum* and *Amb. vikirri*. The most consistent finding was, that infections with the adeleorine blood parasites *Hem. mariae* and *Hem. biplicata* caused starving nymphs of both tick species to lose their body mass at an accelerated rate. As a result, ticks that engorged on *E. stokesii* that were infected with *Hem. mariae* or *Hem. biplicata* died on average sooner than their siblings that engorged on uninfected lizards or lizards infected with parasites other than the two *Hemolivia* species. In the transmission experiments described in chapters six and seven, *Hem. mariae* and *Hem. biplicata* were described to be vectored by the tick species *Amb. limbatum* and *Amb. vikirri*. Both micro-parasites undergo sporogony, an asexual multiplication (see chapter two) in the vectors, which in the transmission experiments often led to a massive development of sporocysts in the midgut lumen of unengorged nymphs. The energy necessary for sporogony of the micro-parasites was almost certainly obtained from the resources inside of the tick vector, but the actual cause of the observed increased rate of body mass loss of the infected ticks remained speculative.

Some insight into the nature of the phenomenon was given by results from the repeated weighings of tick cohorts I and II. Although the sporogony in *Hem. mariae* and *Hem. biplicata* during the larval to nymphal development occurred mainly around the time of moulting, the effect of an accelerated body mass loss in infected ticks was only observed from around three months after the ticks were attached. It can be speculated that the draining of the resources of the ticks led to metabolic or physical deformations that affected the energy economy of a starving tick. A malfunction of any part of the complex tick metabolism during the development from the larval to the nymphal instar for example may lead to a less efficient usage of stored energy during the following phase of starvation. A deformation in the development of the cuticle or its wax layers for example could raise the amount of cuticular water loss, which would force the tick to gain atmospheric water more often. The energy consumption of this process could cause the observed accelerated rate of body mass loss. In an explanation favoured by the author, the accelerated rate of body mass loss may be due to the direct competition between the microparasites and the ticks for the energy of the stored blood meal. In this hypothesis the developing stages of *Hem. mariae* and *Hem. biplicata* consume energy that a starving tick would use otherwise to maintain its body mass. Naturally the mass of the multiplying microparasites contributed to the body mass of the tick, but

because metabolic processes transform biomass with a rather low efficiency, the overall body mass of an infected tick would decrease at a faster rate than that of an uninfected tick.

It was remarkable that during the repeated weighings of experiment I, the difference of the body mass loss between ticks infected with adeleorine blood parasites and uninfected ticks was statistically significant only on day 254 post attachment, but not at the weighings beyond that date. The reason for the less marked difference between the ticks from infected and uninfected lizards at the weighings from 361 days post attachment and beyond can be explained with the higher mortality of ticks that fed on *E. stokesii* infected with adeleorine blood parasites. In experiment I, like in most other experiments, a highly significant correlation existed between the average mortality of ticks from a lizard and the average body mass loss in percent of ticks from that lizard. In other words, the higher the rate of body mass loss the higher the mortality. From day 361 onwards, the ticks with the highest average rate of body mass loss within a group of detached ticks were already dead (eg. all ticks from *E. stokesii 6*), and the surviving ticks showed less marked differences in their average rate of body mass loss. In this instance it is of importance to consider that even *E. stokesii* with relatively high parasitemias of adeleorine blood parasites did not always infect all of the xenodiagnostic ticks. It was therefore plausible that from day 361 post attachment onwards, many of the surviving ticks that detached from lizards infected with *Hem. mariae* or *Hem. biplicata* were those that were less severely infected or not infected at all. The consecutive weighings demonstrate that it was important for these type of experiments to weigh the ticks at the right time, when the mortality is not yet high enough to obscure the increased body mass loss of the ticks most affected by the blood parasites by their premature death. The attachment experiments I-V have shown, that in order to investigate the impact of the blood parasites *Hem. mariae* and *Hem. biplicata* on their vectors, *Amb. limbatum* and *Amb. vikirri*, a time frame of about 100 days to 250 days post attachment was the most adequate one.

During the weighings of the five tick cohorts, two types of abnormal development of ticks were observed. In one of these abnormalities, engorged larvae showed an excess amount of guanine in large streaks throughout their body (Figure 11/G&H). The occurrence of these ticks was only quantified in attachment experiment III, where a total of 11 engorged larvae with an excess amount of guanine were found. Those ticks never moulted, but stayed alive and were very motile for up to four months. All of these larvae engorged on lizards infected with *Hem. mariae* or *Hem. biplicata*. Four of those ticks were dissected and all four were

heavily infected with adeleorine sporocysts. In the other developmental abnormality observed, physically impaired nymphs were produced that could not move on their own due to a peculiar corkscrew-deformation of their legs (Figure 11/I&J). Interestingly, the deformed legs would visibly move when the tick was agitated, for example by handling or breathing on it. Those deformed ticks survived for up to eight months in the high relative humidity of the desiccator. They too were only counted in attachment experiment III. A total of 18 cases were found, and 16 of them had engorged on lizards infected with *Hem. mariae* and *Hem. biplicata*. Two of them had engorged on uninfected lizards. Because there were only two uninfected lizards but eight lizards with mixed adeleorine infections in experiment III, this preliminary finding does not show any trend of a micro-parasitic cause for this corkscrew deformation. The reasons for these abnormalities remain unclear and further experiments are needed to investigate their occurrence. Yet, it is possible that the excessive development of guanine is due to the increased metabolic activities caused by the presence of *Hem. mariae* and *Hem. biplicata*. Furthermore and more importantly, both abnormalities, wether they are induced by parasitic activities or not, may be of epidemiological significance, because they might increase the chances that a tick is ingested by a lizard. The guanine, giving a prominent white marking may make the tick more conspicuous for a lizard. The leg-deformity certainly impedes active movements of the tick, which also may increase the probability of being preyed on by a lizard.

Chapter Fourteen

14. SPATIAL AND TEMPORAL PATTERNS OF BLOOD PARASITES AND TICKS

14.1 Introduction

The final part of this thesis analyses the results of a field survey that continued over three consecutive seasons from September 1995 until April 1998. This study focused mainly on the blood parasites of the lizard *E. stokesii*, but occasionally some other lizard species were examined as well. The main emphasis was on populations of *E. stokesii* at six field sites in the Flinders Ranges (Figure 130 & Figure 131) that were regularly monitored for blood parasites.

In the first step of the analysis, the spatial distribution of blood parasites was briefly summarised from all blood samples available, which included lizard species other than *E. stokesii* and locations that were only surveyed once. The second step of the analysis focused in detail on the blood parasites of *E. stokesii* at the six study sites in the Flinders Ranges. This part examined the **prevalence** of the different blood parasite species at the six field sites as well as within the population structure of *E. stokesii*. Furthermore, the average **parasitemia** for the blood parasites was calculated and its seasonal variations in infected lizards was investigated.

At the beginning of this study in August 1995, most of the knowledge of the rather secretive life of *E. stokesii* and how to find it was provided by Mr Glen Duffield, who also extensively contributed to the collection and the examination of *E. stokesii* for this project. Mr Duffield studied the behaviour of *E. stokesii* at one of the sites, Camel Hill (CH), that was chosen for the survey on the blood parasites, and supplied the lizards he caught from there for further bleeding. He also assisted with the laborious task of measuring, weighing and inspecting the lizards for ectoparasites, and occasionally helped catching *E. stokesii* from study sites other than CH. His study of the behavioural ecology of this lizard will be presented in another thesis from Flinders University.

14.2 Materials and Methods

14.2.1 The locations investigated

Beginning in August 1995, an extensive field survey throughout the southern and central Flinders Ranges aimed to find six suitable sized populations of *E. stokesii*. Additional excursions were conducted during the whole time of this project. Some *E. stokesii* captured outside the Flinders Ranges that were returned to the FUSA by other researches were also included into this study. The details of all study sites examined are listed in Table 87.

14.2.1.1 The study sites in the Flinders Ranges

In the belief of the native Australians of the Flinders Ranges, their ancestors and other beings created the landscape of the Flinders Ranges in a mythical period often referred to as 'dreamtime'. The features of the Ranges were formed by the actions and interactions of the beings of the 'dreamtime', and are of spiritual and navigational significance to those who know the stories.

From the geological viewpoint, the rock formations of the Flinders Ranges were formed from sediments deposited between about 1 billion to 500 million years ago. Folding of the earth crust and different resistance of rocks to erosion formed the Ranges as they appear today. Repeated flooding with sea water led to the development of salt lakes to 100 km long in the surrounding plains. In 1946, impressions of multi-cellular organisms in sediment that was about 700 million years old were found in the Flinders Ranges close to the town Ediacara. The well known Ediacara-fauna was the first evidence for metazoa from the Pre-Cambrian.

The field sites chosen for this investigation lay in the central Flinders Ranges, within an approximately 60 km radius of the township of Hawker (31°53' S 138°25' E, elevation 315.0 m) (Figure 130), about 400 km north of Adelaide. The sites were chosen following an extensive search for populations of *E. stokesii* in the spring of 1995, and they were the closest populations of *E. stokesii* that could be found at that time. Four of the sites had been used previously for a mark and recapture study of *E. stokesii* (Duffield, pers. comm.).

The climate in the Flinders Ranges is semi-arid. Most of the precipitation falls during the winter but there are occasional heavy falls of rain occurring during the spring, summer and autumn seasons. The closest meteorological station to the study sites was situated in

Hawker, where climate data were recorded over the last 90 years. The average yearly rainfall in Hawker was 310.1 mm. The mean daily maximum temperature was 25.3°C, the mean daily minimum temperature was 10.6°C. Temperatures range from -5°C in winter to 45°C in summer.

The abiotic conditions on the six study sites were very similar. All six sites were lower hills or small ridges that formed parts of long ridges. They were exposed to wind and sun and had small granite and sandstone rock formations at different stages of erosion scattered on top. The soil at the sites was a rather uniform red, sandy clay. There were pronounced differences of vegetation among the study sites, probably due to differences in grazing activities. This point will be explained in the following paragraphs in more detail for each study site.

14.2.1.2 Camel Hill (CH)

The population of *E. stokesii* at CH (Figure 131/A) was the starting point of the recent investigations on the biology of this lizard in the Flinders Ranges. Lizards in this population have been the focus of a mark and recapture study since 1993 (Duffield, pers. comm.). The site, a single hill with gentle soil slopes, lies approximately 1 km west of the town boundaries of Hawker. The crest of the hill is formed by several granite formations up to 10 m wide and 4 m high with smaller rocks of all sizes and shapes scattered in a radius of approximately 60 m. A freshwater dam, usually filled with water throughout winter and spring, was situated at the foot of the hill. In the past, the hill was occasionally grazed by cattle, sheep and horses from the adjoining paddocks, but in 1994 the fences were repaired and the "Camel Hill Lookout" was declared a nature reserve. Since then it has been used as a recreational area. Consequently the site has been grazed only by native animals and rabbits. The local rabbit population declined rapidly since the accidental release of the calicivirus in 1997. Probably due to the rather low grazing pressure of the recent past, a rather lush and diverse grassland plant community has developed, with some trees, native bushes (>2 m), herbs, vines and long grasses. Depending on the precipitation, the lower vegetation still dries out during each summer, but it returns very quickly after rain.

14.2.1.3 Hawker Ridge-South (HS)

Hawker Ridge-South (HS) is a part of a low continuous ridge with both, sand and rocky slopes that rises from the plains about 800 m north of the site CH Figure 131/B). A granite formation titled Castle Rock), which resembled the rocks at site CH, was situated at the beginning of the south side of the ridge. These rocks plus the adjacent stretch of approximately 600 m of scattered small boulders and gravel up to a fence at a gravel road formed the study site HS. Cattle and sheep from the bordering paddocks grazed on this ridge, keeping the grass and other vegetation rather short. Only a few scattered trees and shrubs were present, usually in close proximity to the larger rock boulders. Although livestock had created pathways on the ridge, the general plant-overgrowth was more dense than on the neighbouring paddocks. Lizards at this site were used for a mark and recapture study from 1994 onwards, but not as intensely as at the site CH (G. Duffield, pers. comm).

14.2.1.4 Hawker Ridge-North (HN)

The site HN (Figure 131/C) was the continuation of the ridge of the site HS. The site HN lacked a large rock formation, but otherwise the habitat was identical to HS. It was separated from HS by a fenced gravel road. The site HN began at the northern fence of the roadside and continued for approximately 800 m with loosely scattered rock boulders and gravel. The site ended at a small dip in the profile of the ridge, formed by erosion. Lizards at this site were also used for a mark and recapture study from 1994 onwards, but not as intensely as at the site CH.

14.2.1.5 Glenlyle (GL)

The site GL (Figure 131/D) was positioned about 10 km north-east of CH. It consisted of a central granite rock formation, approximately 15 m wide and 5 m high, that was situated in a 200 m stretch of loosely scattered larger boulders and dense lying rubble. The site was situated in a corner of a large, fenced paddock grazed by sheep. There were no trees and few low shrubs or herbs. The short grass was sparsely scattered on the soil.

14.2.1.6 Neuroodla (NR)

The site NR (Figure 132/C) was about 35 km west of CH It consisted of a central granite rock formation, approximately 30 m wide and 3 m high, that was situated in an 300 m stretch of scattered larger boulders and dense lying rubble. The site was once fenced, but since no livestock has been grazed there for decades, the vegetation has restored to the

predominant shrub land of the area, with scattered, low (<1 m) blue bush (*Marieana sedifolia*) and salt bush (*Atriplex nummularia*). There were no trees, and grass or herbs were scarce. Rabbit burrows and droppings were seen in abundance.

14.2.1.7 Drakes Nob (DN)

The site DN (Figure 132/A) was about 30 km south of the CH. It consisted of loosely scattered granite and sand stone boulders and rubble dispersed in a radius of approximately 100 m. The environmental factors observed at this site closely resembled those at NR. Lizards at this site were used for a mark and recapture study from 1994 onwards, but not as intensely as at the site CH.

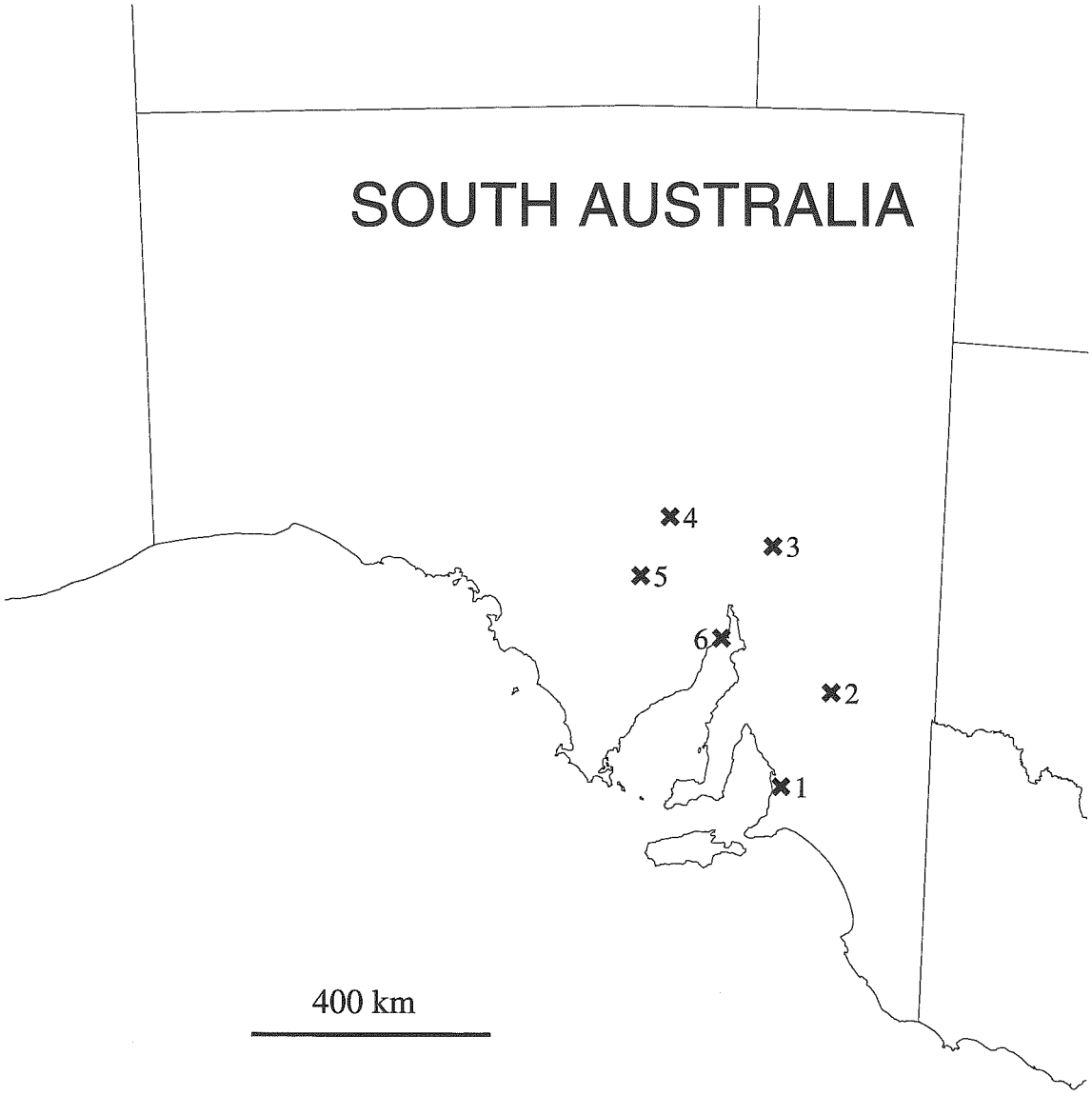

Figure 129. Map of South Australia with the city of Adelaide, the Mt. Mary area, and an overview of the locations where *Egernia stokesii* was caught during this study [1: Adelaide, 2: Mt. Mary, 3: Hawker, 4: Island Lagoon, 5: Gawler Ranges, 6: Point Lowly].

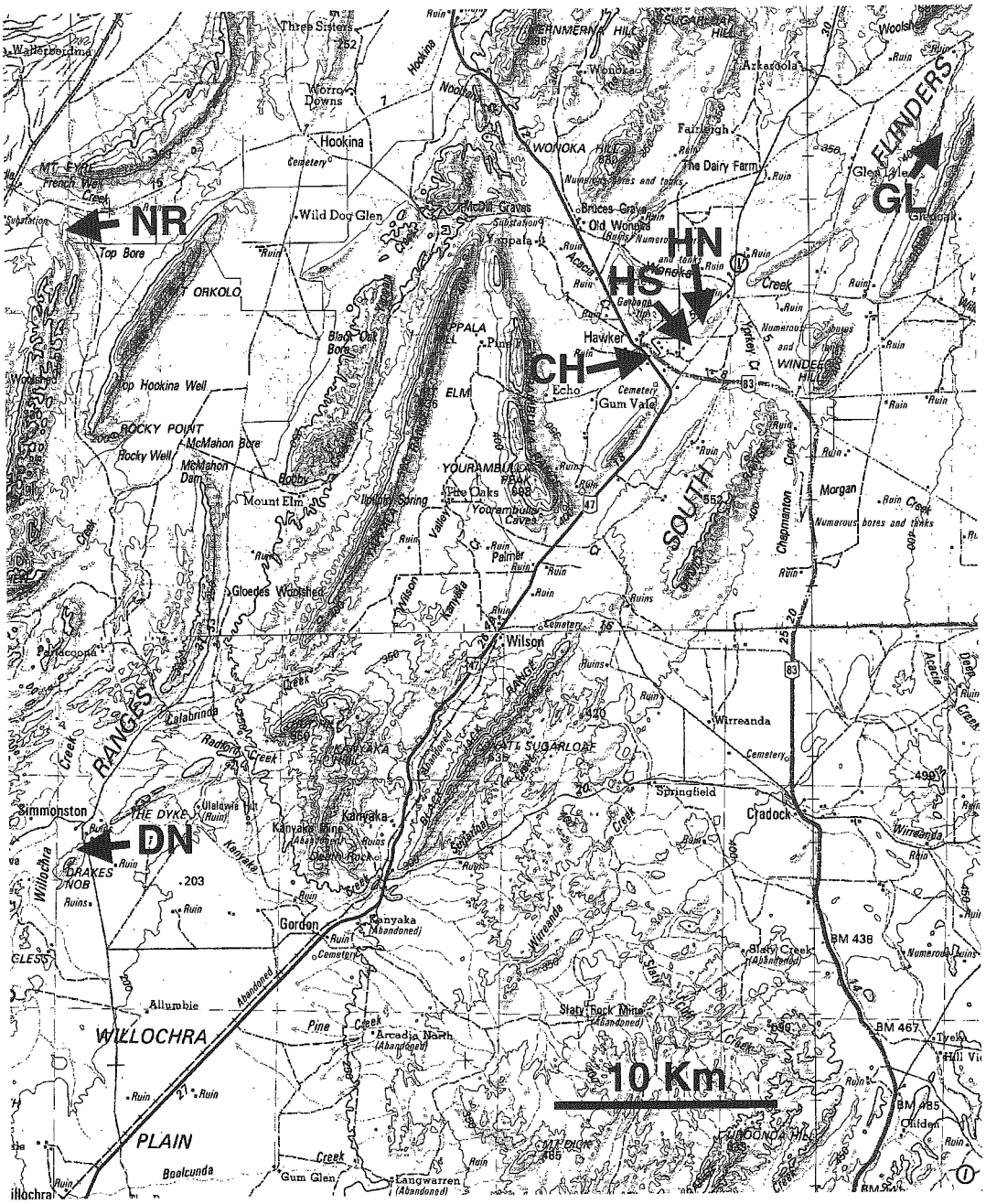

Figure 130. Map of the six study sites in the Flinders Ranges of South Australia [DN: Drakes Nob, CH: Camel Hill, GL: Glenlyle, HN: Hawker North, HS: Hawker South, NR: Neuroodla]. Underlying topographical map reproduced with permission of the Department of Lands, Mapping Branch, Australia.

Figure 131. A-D: the study sites in the Flinders Ranges.

Figure 132. A&C: the study sites in the Flinders Ranges; B&D: lizard husbandry in the ACU of the Flinders University.

14.2.2 The capture and the examination of *lizards* in the field

The *E. stokesii* populations at six study sites chosen to regularly monitor blood parasites were captured and examined at five to six week intervals during spring, summer and autumn of each year 1995, 1996 and 1997. Occasionally, *E. stokesii* were also caught during the winter. Due to the remoteness of some of the study sites, an off road motorbike was used as a field vehicle to reach them. One field trip usually lasted between four and seven days. Two to three sites were visited per day. Elliot traps were set in the morning or around midday and they were inspected on the same day or on the following morning, depending on the weather conditions. Each site was trapped for two consecutive days. The Elliot traps were placed into the suspected pathways of *E. stokesii* around the entrances of inhabited crevices and on frequently used rock ledges, identified by the typical accumulations of faeces this lizard produces.

Following setting the traps, the sites were inspected for *E. stokesii* as long as daylight was available. On each occasion, previously labelled and mapped crevices were inspected, but all other apparent lizard crevices were inspected as well. *E. stokesii* is very shy and not a single lizard was observed outside of a crevice. After a lizard was spotted in a crevice, an attempt was made to capture it with the aide of a noose or with iron rods. A noose was made from a telescopic radio antenna with a strong fishing line (diameter 0.8 mm) pushed through its hollow middle. The iron rods were approximately 1 m long and had an diameter of 8 mm, They were bent at a 90° angle at one end. Even when in rare cases the lizard was in reach of hands, it was extremely difficult to extract the lizard by hand out of the rock crevices it inhabits, because *E. stokesii* usually faces away from the collector and the keeled scales and the spiked tail in combination with the plump and muscular body successfully counteract any attempt to pull a lizard out of a crevice by the tail. Instead, the lizard had to be carefully manipulated into the noose or into the hand of a collector. This was often a time consuming and laborious process but yielded the highest capture rates. In order to prevent *E. stokesii* from becoming rigid and therefore virtually impossible to move, it was mandatory that physical contact made with the lizard with the rods or the noose was brief and very gentle.

Captured *E. stokesii* were inspected for an existing toe clip. Unmarked lizards were labelled ventrally with a black marker pen and were then kept in cotton bags; they received a permanent individual toe clip during the following examination. The crevice each *E. stokesii* was captured in was marked with an individual number by weather proof paint and the

crevices at each site were mapped. The total catch of one day was usually brought to Hawker and examined at the field station on the night of the day they were captured. On some occasions, the lizards were examined and bled at the study site and released immediately afterwards. *E. stokesii* was sexed by the method described by Szidat (1968). The body mass was taken with a set of spring scales or a battery powered electronic balance (Masscal™ 311) that read to the nearest one gram. The snout-vent length was measured with a ruler. The lizard was thoroughly examined for ectoparasites; all tick instars and the site of their attachment were recorded. Although it was relatively easy to distinguish between adult *Amb. limbatum* and *Amb. vikirri* (Figure 11/F) it was impossible to identify the nymph and larval stages of the two tick species. However, for the main part of this study this did not result in a practical problem, because at the regularly monitored study sites, adult *Amb. vikirri* were never detected. Thus, it was most likely the lizards at those sites were exclusively infested with *Amb. limbatum.* A blood sample (< 50 µl) was obtained with a sterile syringe from the caudal vein of each lizard. Details of the preparation, the staining and the evaluation of the blood smears have been described in chapter five. After examination, the lizards were placed back into cotton bags and they were returned to the exact place of capture usually on the evening of the next day. In most cases, blood smears were produced on each day of the field excursions. In order to summarise the data, each excursion received one representative date, chosen from one of the days at the centre of each excursion. All blood smears taken on one excursion were allocated to one representative date respectively.

14.2.3 The analysis of the spatial and seasonal occurrence of blood parasites

In this chapter, two separate features of blood infections, the **prevalence** and the **parasitemia** of the blood parasite species, were analysed in detail. In this analysis the term "season" refers to the period from about September to April (early spring to autumn) when lizards are active. The study covered the three seasons 95/96, 96/97 and 97/98. The investigation of the prevalence of the blood parasites of *E. stokesii* at the six study sites was conducted in four steps. In the first step, the percentage of infected lizards per season and per blood parasite species was calculated from the total number of lizards captured at each site and in each season. The mean and standard deviation of the resulting averages of three field seasons were then calculated for each parasite species. The resulting prevalence-profile of blood parasites was plotted for each study site. In the second step, possible tendencies regarding the increase or decrease of the micro-parasite prevalence were examined by comparing the average prevalence of the three seasons for each blood parasite species. The

third step analysed in detail the absolute number of blood parasite species in the various compositions of mixed infections, resulting in an index of parasite diversity for each study site. In the fourth step, the captured lizards were clustered into weight classes and the average prevalence of blood parasites was calculated for each weight group, to see if some size categories were more likely to be infected than others. In step one, two and three of this analysis, lizards that were captured several times in a season were only included once for that season - and were counted as infected if a parasite species was detected at least once. However, the fourth step of the analysis included all data of *E. stokesii* that were weighed and bled repeatedly - in order to establish uniform weight groups, it was necessary to present them as separate sets. The lizard body-mass data of the third season at the site CH were not yet available at the time this thesis was written, thus they were not included into this study.

The parasitemia of the blood parasite species was examined in two steps. In the first step, the average percentage of infected erythrocytes per lizard (from an estimate of 10^4 RBC's; for details see 5.2.1.5) was calculated for the infected *E. stokesii* at each site, each season and for each blood parasite species. The mean and standard deviation of the resulting averages of three field seasons were then determined for each parasite species. The resulting parasitemia-profile of blood parasites was plotted for each study site. In the second step, the average parasitemia of *E. stokesii* on each field trip was calculated for each blood parasite species. This part analysed the changes over an activity season of the average parasitemia of the infected *E. stokesii* for each parasite species.

As explained before, the presence of *Plasmodium circularis* was not discovered until the end of the third field season. Due to time constraints, only the blood smears of the last season were re-examined for the presence and parasitemia of *P. circularis*. However, the prevalence data collected for *P. mackerrasae* from the first two seasons were still applicable, for reasons explained in the following. During the evaluation of the recount it became apparent that *P. circularis* was relatively rare, with a low prevalence and a relatively low parasitemia compared to *P. mackerrasae*. Moreover, *P. circularis* was usually present exclusively in mixed infections with *P. mackerrasae*. Only one blood sample out of 729 samples of the last season was infected with *P. circularis* but not infected with *P. mackerrasae*. From the relative rarity of *P. circularis* in the third season, it was deduced that that species was also rare in the first two seasons. Finally, some blood smears containing the sexual stages of *P. circularis* were noted as unusual during the count of the slides from the first two seasons, although they were then

not identified as a separate species. Those slides were re-examined in the third season as well, and all of them were infected mostly with *P. mackerrasae*. Therefore the counts of the first two seasons were considered to describe accurately the prevalence of *P. mackerrasae*. In contrast, the counts of the parasitemia could not be evaluated separately for each *Plasmodium* species, because the recount-data showed both *P. circularis* and *P. mackerrasae* could reach relatively high parasitemias and each frequently infected more than 80% of erythrocytes in *E. stokesii* individuals. Therefore, the parasitemia of the two *Plasmodium* species over the three year period was labelled as *Plasmodium* spp.; the one season recount of *P. circularis* was displayed separately. It depicts a part of the data from the third season presented in the prevalence of *Plasmodium* spp..

Due to the rather small population and the stress a regular study placed on the lizards of the study site DN, this site was monitored at regular intervals only during the first season, from September 1995 to April 1996. In the following two seasons, excursions were made to the site DN at less frequent intervals, thus the decrease of the total number of *E. stokesii* captured during the second and third season did not reflect shifts in the population size.

14.3 Results

14.3.1 Spatial survey on reptilian blood parasites

Most of the blood samples investigated originated from *E. stokesii* caught in the Flinders Ranges (Table 87). Two of the locations listed in Table 87 are situated on Eyre Peninsula (Gawler Ranges, approximately 300 km west-south-west of Hawker and Point Lowly, approximately 150 km south-west from Hawker), and one site lies in the centre of South Australia (Island Lagoon, approximately 150 km west from Hawker; see Figure). At least one species of blood parasite was found at each of the 20 locations examined, with *Hem. mariae*, *Schellackia* sp. and *P. mackerrasae* the most prevalent species. No consistent pattern of the spatial distribution of blood parasites was detectable from these data.

Apart from *E. stokesii*, blood of the sleepy lizard *T. rugosa* was frequently sampled. This was because *T. rugosa* might play an important role as reservoir host and in the spread of the blood parasite between the rather isolated populations of *E. stokesii*[13]. While *E. stokesii* is largely confined to isolated rocky outcrops, *T. rugosa* inhabits the outcrops and the open non-

[13] The sleepy lizard is known to be infected by the blood parasite *Hem. mariae*, and the ticks *Amb. limbatum* and *Aponomma hydrosauri* were both proven vectors for the blood parasite (Smallridge, 1998).

rocky ground between them. Also this large, slow and conspicuous lizard was relatively easy to catch. Blood samples from *T. rugosa* were taken from eleven locations. At nine of these at least one *T. rugosa* was infected with *Hem. mariae* (Table 88). At site HN, one *T. rugosa* had an extremely low parasitemia with a blood parasite believed to be *P. mackerrasae.* Only one trophozoite was found on the entire slide, thus this finding is a preliminary indication for a possible presence of *P. mackerrasae* in *T. rugosa.* One out of three samples of the gecko *Gehyra variegata* from the Chace Range (31°45'S, 138°35'W) was infected with a species of blood parasite that probably was not one of the blood parasites described in this study, based on the morphology of the blood stages in one giemsa stained blood smear. Three more reptile species were investigated for blood parasites. None of the 24 samples from the bearded dragon, *Pogona vitticeps,* were found to be infected. Similarly, no infections were discovered in six samples of the lizard *Tiliqua scincoides* or in two samples of the brown snake *Pseudonaja textilis.* The stomach contents of the two adult snakes were examined and juvenile *E. stokesii* were found in one of them (G. Duffield, pers. comm.).

Table 87. The coordinates of the sites where *E. stokesii* were captured during the three year field study and the blood parasites found infecting the lizards [Regularly visited study sites in bold. Camel Hill: CH; Drakes Nob: DN; Glenlyle: GL; Hawker North: HN; Hawker South: HS; Neuroodla: NR].

Site	Location		No of *E. stokesii* captured	The prevalence (%) of blood parasite species in *E. stokesii*					
				H. *mariae*	H. *biplicata*	*Hepatozoon* sp.	*Schellackia* sp.	P. *mackerrasae*	P. *circularis*
	S	W		%	%	%	%	%	%
Camel Hill	**31°53'**	**138°25'**	**148**	**0.9**			**78.8**	**5.7**	**1.7**
Chace Range	31°45'	138°35'	7	14.3	14.3		14.3	28.6	14.3
Drakes Nob	**32°05'**	**138°09'**	27	24.4			48.2	47.0	18.2
Druid Range	31°50'	138°40'	4				25.0		
Gawler Ranges[1]	32°30'	136°50'	12	50.0	50	16.7	25.0	8.3	
Glenlyle	**31°50'**	**138°35'**	**62**	**32.1**			**43.5**	**52.3**	**19.1**
Hawker North	**31°53'**	**138°25'**	**97**	**15.0**			**58.2**	**24.8**	**6.8**
Hawker South	**31°53'**	**138°25'**	**159**	**19.8**			**67.2**	**23.6**	**2.0**
Island Lagoon	31°42'	136°68'	14	92.9	92.9	50	28.6	50.0	
Jarvis Hill	32°55'	138°21'	10	20.0	60		40.0		
Mernamerna	31°45'	138°22'	2	50.0				50.0	
Neuroodla	**31°50'**	**138°08'**	**68**	**44.9**		**36.1**	**67.9**	**47.3**	**26.4**
Partacoona	32°00'	138°10'	6	16.7			66.7	83.3	
Pichi Richi	32°20'	138°00'	27	7.4			11.1		
Point Lowly[1]	32°50'	137°45'	8	37.5	12.5		25.0	12.5	
Quorn	32°15'	138°05'	8	12.5			50.0	62.5	
The Four Mile	31°50'	138°30'	1	100.0				100.0	100.0
Warruw. Hill[2]	31°55'	138°25'	8				87.5	37.5	
Willow Springs	31°25'	138°50'	4	50.0	100		75.0	25.0	
Yapalla	31°50'	138°20'	4	25.0			25.0	25.0	

[1] = *E. stokesii* kindly supplied by Dr. Greg Johnston and Ms. Elvira Lanham

[2] = Warruwarldunha Hill

Table 88. The coordinates of the sites where *T. rugosa* were captured during the three year field study and the blood parasites found infecting the lizards.

Site	Location		No of *T. rugosa* captured	Number of *T. rugosa* found infected (Σ) and prevalence (%) for blood parasite species			
				H. *mariae*		*P. mackerassae*	
	S	W		Σ	%	Σ	%
CH	31°53'	138°25'	42	5	11.9		
DN	32°05'	138°09'	2	2	100		
GL	31°50'	138°35'	12	2	16.8		
HN	31°53'	138°25'	26	3	11.5	1	3.8
HS	31°53'	138°25'	29	3	10.3		
Island Lagoon	31°42'	136°68'	1	0	0		
NR	31°50'	138°08'	10	1	10		
The Four Mile	31°50'	138°30"	2	2	100		
Warruw. Hill[1]	31°55'	138°25'	6	1	16. 7		
Willow Springs	31°25'	138°50'	3	3	100		
Yapalla	31°50'	138°20"	1	0	0		

[1] = Warruwarldunha Hill

14.3.2 Numbers of *E. stokesii* captured and capture frequencies

In the course of this study, a total of 1601 blood smears were produced from 561 individual *E. stokesii* that were caught at the six study sites DN, GL, NR, CH, HS and HN. Table 89 lists the number of blood smears produced in each season and at each site and also displays the number of *E. stokesii* individuals caught and summarises these data for the entire three years. With the exception of the second and third season at the study site DN, where the *E. stokesii* were less regularly monitored, the number of smears produced and the number of lizard individuals bled probably reflected the differences of the population size between the sites. CH and HS had the largest populations of *E. stokesii*, HN, GL and NR were of medium size and DN had a relatively small population of *E. stokesii*. Consistently, there were about three times as many blood smear samples produced over the whole study as there were individuals sampled at each site. However, a comparison among sites (Table 90) showed some differences in the frequencies of captures of individual lizards between the sites. Taking into consideration the entire three year time span of this study, the highest capture rates of individual *E. stokesii* were reached at sites HN and CH, where the lizards were caught on average 3.26 times (HN) and 3.17 times (CH). The lowest frequency of average lizard recaptures was attained at the site HS (2.34). At all sites, most lizards were captured only once or twice, and very few lizards were caught more than six times during this investigation. The individual with the highest number of captures came from the site GL (14x); followed by single individuals at the site HN (13x) CH, HS & DN (11x) and NR (9x). Except for the site DN, the absolute number of captured individuals and the number of blood smears produced increased with each consecutive season. This finding was due to the improving skills of the collector and did not mirror an increase of the population sizes.

Table 89. The number of blood smears produced from *E. stokesii* from the six study sites over three consecutive seasons and the actual number of individual lizards involved in this study

Site	1995/96		1996/97		1997/98		1995-98	
	No. of bled *E. stokesii*	No. of *E. s.* individuals caught	No. of bled *E. stokesii*	No. of *E. s.* individuals caught	No. of bled *E. stokesii*	No. of *E. s.* individuals caught	No. of bled *E. stokesii*	No. of *E. s.* individuals caught
DN	44	20	16	12	12	11	72	27
GL	47	26	45	30	82	42	174	62
NR	51	28	46	33	101	53	198	68
CH	90	61	159	78	220	115	469	148
HS	91	62	107	68	174	102	372	159
HN	67	43	109	57	140	59	316	97
Σ	390	240	482	278	729	382	1601	561

Table 90. Number of *E. stokesii* individuals caught per study site and per season and the average times *E. stokesii* individuals were captured.

Site	1995/96			1996/97			1997/98			1995-1998		
	No. of E. s. individuals caught	Times lizards were recaptured		No. of E. s. individuals caught	Times lizards were recaptured		No. of E. s. individuals caught	Times lizards were recaptured		No. of E. s. individuals caught	Times lizards were recaptured	
		$\bar{X}$	SD		$\bar{X}$	SD		$\bar{X}$	SD		$\bar{X}$	SD
DN	20	2.20	1.64	12	1.33	0.65	11	1.09	0.30	27	2.67	2.25
GL	26	1.81	1.23	30	1.50	0.73	42	1.95	1.15	62	2.81	2.55
NR	28	1.82	1.06	33	1.39	0.70	53	1.91	0.99	68	2.91	2.20
CH	61	1.48	0.81	78	2.04	1.13	115	1.91	1.26	148	3.17	2.56
HS	62	1.47	0.76	68	1.57	0.94	102	1.71	1.26	159	2.34	2.12
HN	43	1.56	0.83	57	1.91	1.04	59	2.37	1.41	97	3.26	2.68

14.3.3 Prevalence of blood parasites at six study sites

The prevalence data are summarised in the Tables 97 to 101 and were statistically analysed by a series of one-way ANOVA's and post-hoc tests (Tukey), summarised in the Tables 91-96. Because the prevalences of blood parasites were in most cases statistically significant different between the study sites, only those results were referred to were the prevalences between sites were not significantly different. Statistical tests to compare the prevalence between study sites were not performed on the species *P. mackerrasae* and *Hepatozoon* sp., because the latter occurred only on one site (NR) and *P. circularis* was only counted for one season, thus those data were not comparable to the other ones.

14.3.3.1 TPrevalence-profile of blood parasites at each study site

At site CH, the average prevalences of *Hem. mariae, Schellackia* sp. and *P. mackerrasae* were significantly different from those at all other study sites (Tables 91-96).Within the three seasons investigated, on average, 78.8 of the *E. stokesii* that were bled at the study site CH were infected with *Schellackia* sp. (Figure 133). Only very few infections with *P. mackerrasae, P. circularis* and *Hem. mariae* were detected. No infection with *Hepatozoon* sp. was found.

Similar to the site CH, the average prevalence of *Schellackia* sp. at site HS was high (67.2%) the prevalence of *P. circularis* was very low (2.0%), and *Hepatozoon* sp. did not ever occur among the captured *E. stokesii* (Figure 134). In contrast to the site CH, *P. mackerrasae* (23.6%) and *Hem. mariae* (19.8%) were quite common at the site HS. The average prevalence of *Hem. mariae* at site HS was not statistically different when compared to the prevalence at the adjacent site HN and to the prevalence at site DN; exactly the same result was found for *Schellackia* sp. and *P. mackerrasae* (Tables 91-96).

The site HN was adjacent to the site HS and very similar in the character of the habitat (Figure 131/B&C). The average prevalence of the blood parasite species infecting *E. stokesii* at the site HN showed only marginal differences to the prevalence patterns at the site HS (Figure 135). On average, fewer of the lizards caught were infected with *Schellackia* sp. (58.1% compared 72.2% at HS). The average prevalence of *P. mackerrasae* was almost identical (24.7% HN; 23.6% HS) between both sites, the prevalence of *P. circularis* was slightly higher (6.8% compared to 2.0% at HS) and the average prevalence of *Hem. mariae* was slightly lower when compared to the site HS (15.0% compared to 19.8% at HS). *E. stokesii* infected with *Hepatozoon* sp. were not caught.

The prevalence patterns of the blood parasite species from *E. stokesii* that were caught at the study site GL were distinctively different from the first three sites, CH, HS and HN (Figure 136). On average, only 43.5% of the lizards were infected with *Schellackia* sp. and the average prevalence of *P. mackerrasae* was higher (52.3) than that of *Schellackia* sp. On average, 32.1% of all *E. stokesii* caught were infected with *Hem. mariae* and 19.5% were infected with *P. circularis* in the third season. No lizard infected with *Hepatozoon* sp. was found. The average prevalence of *Hem. mariae* was significantly different to most other study sites, with the exception of the site DN; the same result was found for *Schellackia* sp. and *P. mackerrasae* (Tables 91-96).

The results of the examination of blood smears of *E. stokesii* captured at the site NR produced a unique prevalence pattern of blood parasites among the six sites investigated (Figure 137). This site was the only location within the Flinders Ranges where *Hepatozoon* sp. (36.1%) was found to infect *E. stokesii* (see Table 87). The average prevalence of all parasite species was relatively high, with the highest average prevalence for *Hem. mariae* and *P. circularis* when compared to the other study sites. The average prevalences of *Hem. mariae*, *Schellackia* sp. and *P. mackerrasae* were significantly different from those at all other study sites (Tables 91-96).

The prevalence patterns of the blood parasites from *E. stokesii* caught at the site DN (Figure 139) were very similar to those from the study site GL. *P. mackerrasae* had the highest average prevalence (47.0% compared to 52.3% at GL), *Schellackia* sp. was the second most frequently found blood parasite species (48.2% compared to 44.9% at GL). *Hem. mariae* was infecting 24.4% of the lizards on average (32.1% at GL) and *P. circularis* had the lowest prevalence among the blood parasites present (18.1% compared to 19.5% at GL). The average prevalence of *Hem. mariae*, *Schellackia* sp. and *P. mackerrasae* at site DN were not significantly different from those at sites GL, HS and HN.

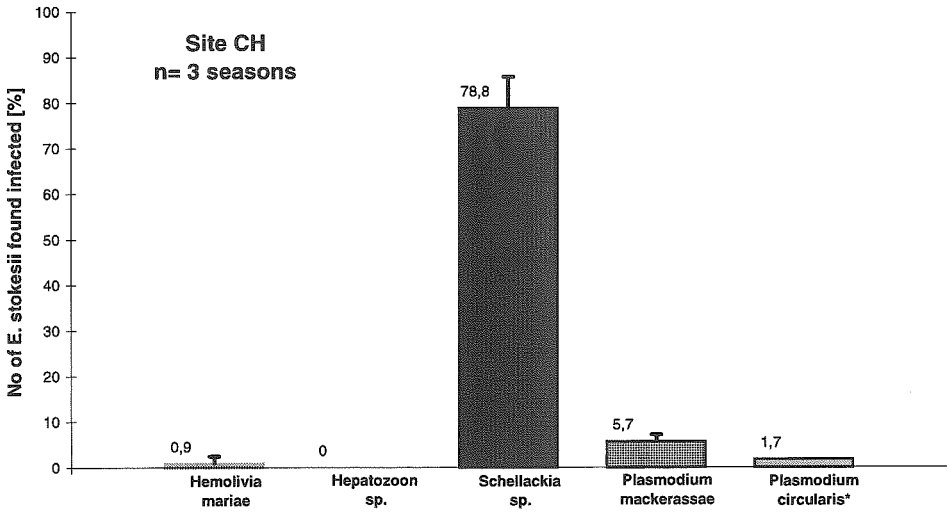

Figure 133. The means of the average prevalence per season [%] of blood parasite species at the study site CH; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

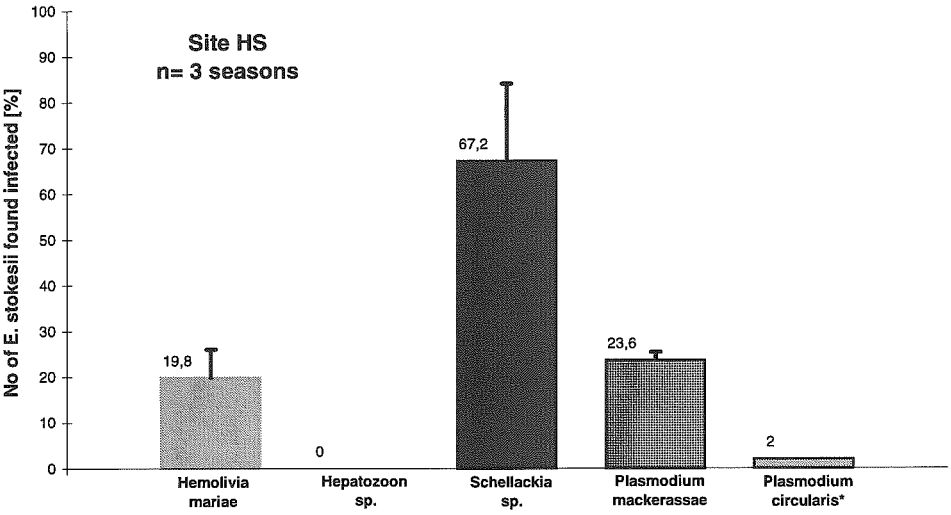

Figure 134. The means of the average prevalence per season [%] of blood parasite species at the study site HS; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

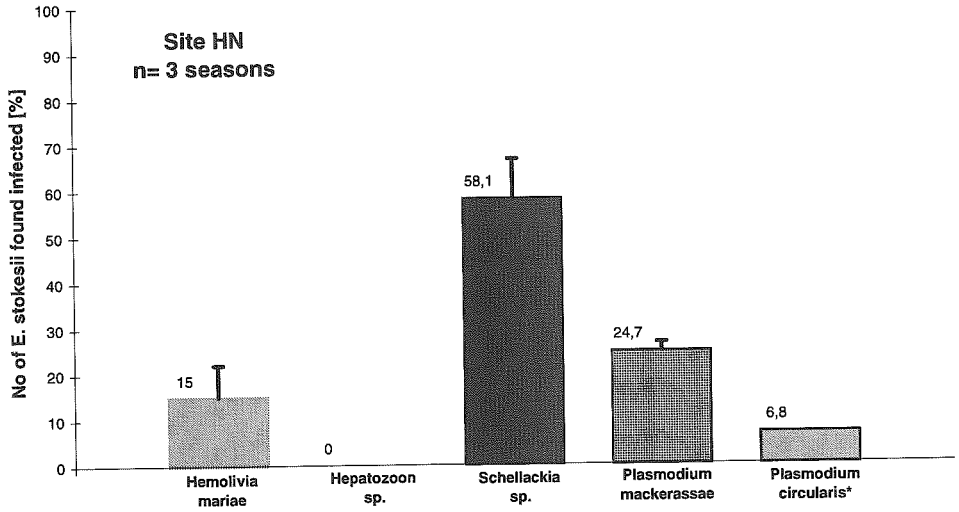

Figure 135. The means of the average prevalence per season [%] of blood parasite species at the study site HN; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

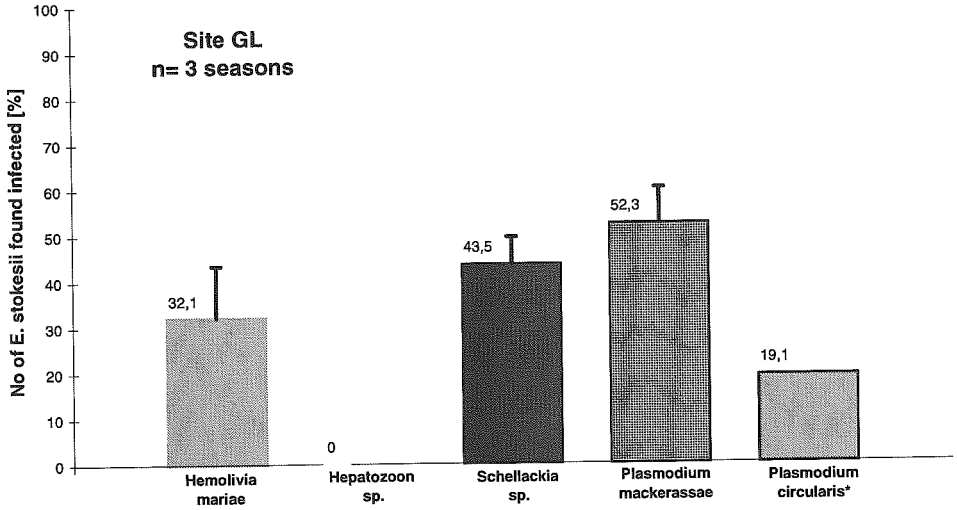

Figure 136. The means of the average prevalence per season [%] of blood parasite species at the study site GL; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

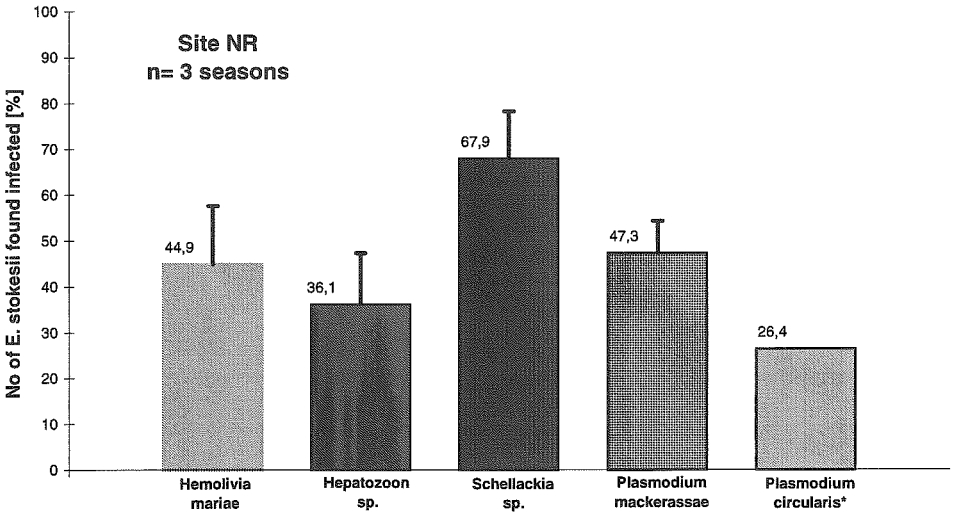

Figure 137. The means of the average prevalence per season [%] of blood parasite species at the study site NR; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

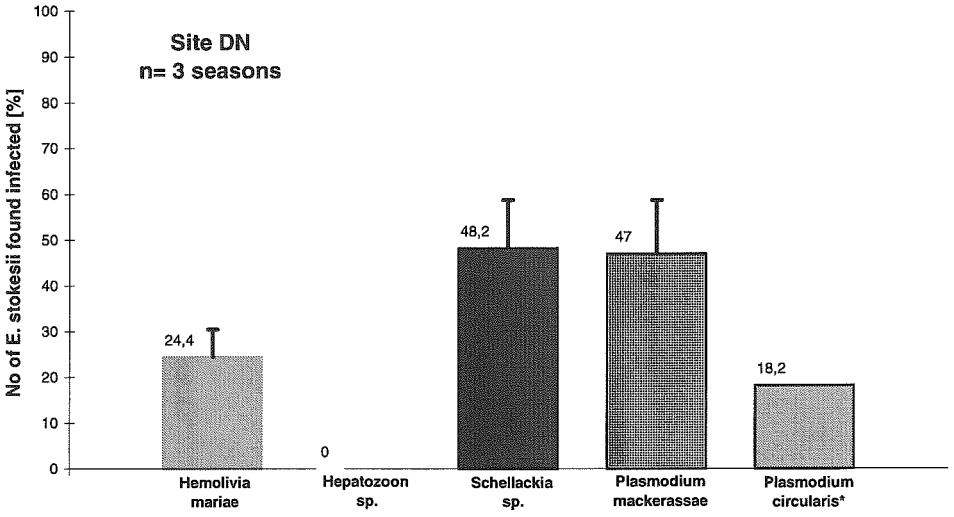

Figure 138. The means of the average prevalence per season [%] of blood parasite species at the study site DN; data of the three seasons investigated (1995-1998) were pooled [* the prevalence of *P. circularis* was only counted for the last season].

Table 91. ANOVA comparing the average prevalence of *Hem. mariae* from three seasons between the six study sites.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.6484	5	0.130	28.204	**0.000**
Within Groups	0.1103	24	0.005		
Total	0.7588	29			

Table 92. Post-hoc (Tukey) analysis of the different prevalence of *Hem. mariae* comparing individually between the six study sites.

Study site		Study site	Mean difference	SE	Sig.
DN	&	GL	-0.077	0.043	0.490
DN	**&**	**NR**	-0.214	0.043	**0.001**
DN	**&**	**CH**	0.256	0.043	**0.000**
DN	&	HS	0.064	0.043	0.677
DN	&	HN	0.108	0.043	0.160
GL	**&**	**NR**	-0.137	0.043	**0.039**
GL	**&**	**CH**	0.333	0.043	**0.000**
GL	**&**	**HS**	0.140	0.043	**0.034**
GL	**&**	**HN**	0.184	0.043	**0.003**
NR	**&**	**CH**	0.470	0.043	**0.000**
NR	**&**	**HS**	0.278	0.043	**0.000**
NR	**&**	**HN**	0.322	0.043	**0.000**
CH	**&**	**HS**	-0.192	0.043	**0.002**
CH	**&**	**HN**	-0.148	0.043	**0.022**
HS	&	HN	0.044	0.043	0.904

Table 93. ANOVA comparing the average prevalence of *Schellackia sp.* from three seasons between the six study sites.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.6198	5	0.124	11.203	**0.000**
Within Groups	0.2655	24	0.011		
Total	0.8854	29			

Table 94. Post-hoc (Tukey) analysis of the different prevalence of *Schellackia sp.* comparing individually between the six study sites.

Study site		Study site	Mean difference	SE	Sig.
DN	&	GL	0.035	0.067	0.995
DN	**&**	**NR**	-0.234	0.067	**0.019**
DN	**&**	**CH**	-0.377	0.067	**0.000**
DN	&	HS	-0.230	0.067	**0.022**
DN	&	HN	-0.103	0.067	0.639
GL	**&**	**NR**	-0.269	0.067	**0.006**
GL	**&**	**CH**	-0.412	0.067	**0.000**
GL	**&**	**HS**	-0.265	0.067	**0.006**
GL	**&**	**HN**	-0.138	0.067	0.336
NR	**&**	**CH**	-0.143	0.067	0.300
NR	**&**	**HS**	0.004	0.067	1.000
NR	**&**	**HN**	0.132	0.067	0.383
CH	**&**	**HS**	0.147	0.067	0.272
CH	**&**	**HN**	0.274	0.067	**0.005**
HS	&	HN	0.127	0.067	0.417

Table 95. ANOVA comparing the average prevalence of *P. mackerrasae* from three seasons between the six study sites.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.9577	5	0.192	67.133	**0.000**
Within Groups	0.0685	24	0.003		
Total	1.0262	29			

Table 96. Post-hoc (Tukey) analysis of the different prevalence of *P. mackerrasae* comparing individually between the six study sites.

Study site		Study site	Mean difference	SE	Sig.
DN	&	GL	-0.064	0.034	0.427
DN	**&**	**NR**	-0.005	0.034	1.000
DN	**&**	**CH**	0.434	0.034	**0.000**
DN	&	HS	0.252	0.034	**0.000**
DN	&	HN	0.241	0.034	**0.000**
GL	**&**	**NR**	0.059	0.034	0.509
GL	**&**	**CH**	0.499	0.034	**0.000**
GL	**&**	**HS**	0.316	0.034	**0.000**
GL	**&**	**HN**	0.305	0.034	**0.000**
NR	**&**	**CH**	0.439	0.034	**0.000**
NR	**&**	**HS**	0.257	0.034	**0.000**
NR	**&**	**HN**	0.246	0.034	**0.000**
CH	**&**	**HS**	-0.182	0.034	**0.000**
CH	**&**	**HN**	-0.194	0.034	**0.000**
HS	&	HN	-0.011	0.034	0.999

14.3.3.2 Comparison of prevalence per season for each blood parasite species

The highest prevalence of *Hem. mariae* was found in the captured *E. stokesii* at site NR in the third season (Figure 139 and Table 98). The lizards at the sites GL, HS and HN were also frequently infected with *Hem. mariae* in all three field seasons investigated, but almost no *E. stokesii* at the site CH were found to be infected with *Hem. mariae.*

As displayed in Figure 140, the site NR was the only one of the regularly monitored sites where *Hepatozoon* sp. was found. No trend was observed across seasons. *Schellackia* sp. was the most prevalent blood parasite of the captured *E. stokesii* from the six sites (Figure 141). A steady decrease of the prevalence per season was noticeable at the sites NR, HS and HN, whereas prevalence increased over the same time at DN. The prevalence of *P. mackerrasae* was steady in all three seasons at the three sites CH, HS and HN (Figure 142), and relatively high but without consisted trends at the sites DN, GL and NR. *E. stokesii* at the site CH consistently had the lowest prevalence with *P. mackerrasae* in all three seasons investigated. The prevalence of *P. circularis* was only evaluated for the last field season (Figure 143). This blood parasite was most prevalent in *E. stokesii* from the sites NR, DN and GL. Relatively few lizards from the sites HN, HS and CH were found infected with *P. circularis.*

No apparent patterns were observed in the more detailed analysis of the changes of the average prevalence following the seasons of the three years. The percentage of infected *E. stokesii* did not show consistent trends for the different species of blood parasites between the single excursions. The number of newly infected lizards compared among the seasons was not calculated due to the relatively low sample sizes and the relatively low recapture rates, and thus the data were not presented in more detail here.

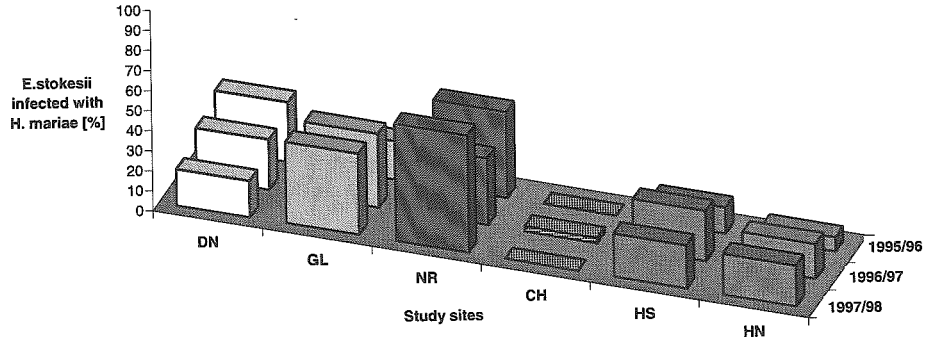

Figure 139. The prevalence per season of *Hem. mariae* compared between the six study sites.

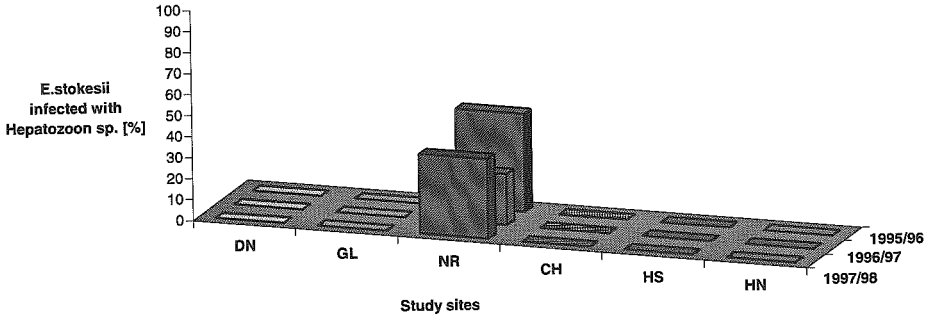

Figure 140. The prevalence per season of *Hepatozoon sp.* compared between the six study sites.

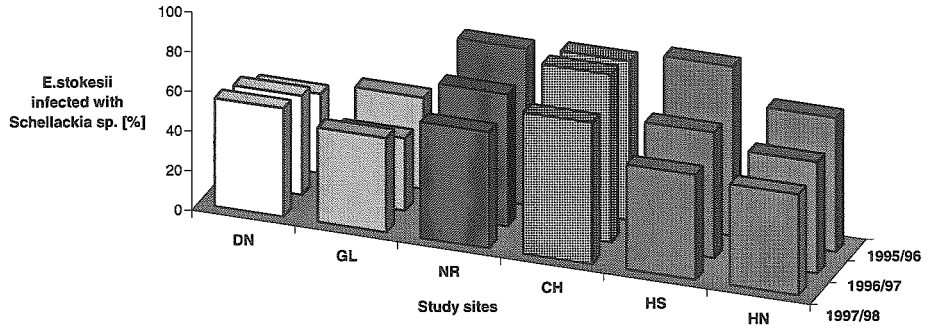

Figure 141. The prevalence per season of *Schellackia sp.* compared between the six study sites.

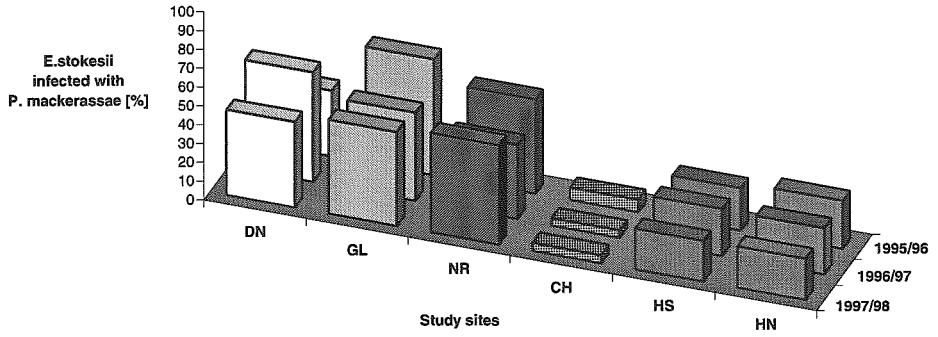

Figure 142. The prevalence per season of *P. mackerrasae* compared between the six study sites.

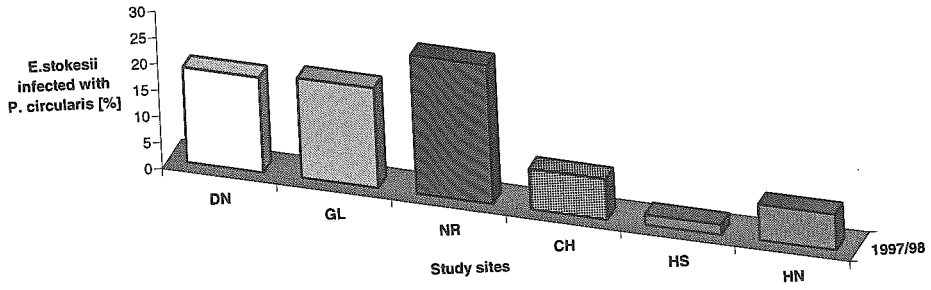

Figure 143. The prevalence of *P. circularis* from the season 1997/98 compared between the six study sites.

Table 97. The number of individual *E. stokesii* from the six study sites and the prevalence of *P. circularis*.

Site	1997/98	
	No. of *E. s.* individuals caught	Prevalence [%]
DN	11	18.18
GL	42	19.05
NR	53	26.42
CH	115	1.74
HS	102	1.96
HN	59	6.78
Σ	382	

Table 98. The number of individual *E. stokesii* from the six study sites over three consecutive seasons and the prevalence of *Hem. mariae*.

Site	1995/96		1996/97		1997/98		1995-98	
	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	Average prevalence of three seasons [%] $\bar{X}$	SD
DN	20	30.00	12	25.00	11	18.18	24.39	5.93
GL	26	19.23	30	36.67	42	40.48	32.12	11.33
NR	28	42.86	33	33.33	53	58.49	44.89	12.70
CH	61	0.00	78	2.56	115	0.00	0.85	1.48
HS	62	12.90	68	25.00	102	21.57	19.82	6.23
HN	43	6.98	57	17.54	59	20.34	14.95	7.05
Σ	240		278		382			

Table 99. The number of individual *E. stokesii* from the six study sites over three consecutive seasons and the prevalence of *Hepatozoon* sp..

Site	1995/96		1996/97		1997/98		1995-98	
	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	Average prevalence of three seasons [%] $\bar{X}$	SD
DN	20	0	12	0	11	0	0	0
GL	26	0	30	0	42	0	0	0
NR	28	46.43	33	24.24	53	37.74	36.14	11.18
CH	61	0	78	0	115	0	0	0
HS	62	0	68	0	102	0	0	0
HN	43	0	57	0	59	0	0	0
Σ	240		278		382			

Table 100. The number of individual *E. stokesii* from the six study sites over three consecutive seasons and the prevalence of *Schellackia* sp..

Site	1995/96		1996/97		1997/98		1995-98	
	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	Average prevalence of three seasons [%] $\bar{X}$	SD
DN	20	40.00	12	50.00	11	54.55	48.18	7.44
GL	26	46.15	30	36.67	42	47.62	43.48	5.95
NR	28	78.57	33	66.67	53	58.49	67.91	10.10
CH	61	80.33	78	84.62	115	71.30	78.75	6.79
HS	62	85.48	68	63.24	102	52.94	67.22	16.63
HN	43	67.44	57	56.14	59	50.85	58.14	8.48
Σ	240		278		382			

Table 101. The number of individual *E. stokesii* from the six study sites over three consecutive seasons and the prevalence of *P. mackerassae*.

Site	1995/96		1996/97		1997/98		1995-98	
	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	No. of *E. s.* individuals caught	Prevalence [%]	Average prevalence of three seasons [%] $\bar{X}$	SD
DN	20	35.00	12	58.33	11	45.45	47.02	11.69
GL	26	61.54	30	46.67	42	50.00	52.29	7.80
NR	28	50.00	33	39.39	53	52.83	47.27	7.08
CH	61	6.56	78	3.85	115	4.35	5.65	1.44
HS	62	22.58	68	25.00	102	21.57	23.64	1.76
HN	43	25.58	57	24.56	59	22.03	24.74	1.83
Σ	240		278		382			

14.3.3.3 Analysis of the number of blood parasite species in mixed infections

In this step of the analysis of the field data, the number of blood parasite species found was summarised for each individual *E. stokesii* over the entire three year period of this study. In this calculation it was not important whether the infections occurred at the same time, but only how many blood parasite species in total each *E. stokesii* was infected with over the three years. The highest average number of blood parasite species was found in the lizard population of site NR (Table 102). A relatively high average number of blood parasite species was diagnosed from the *E. stokesii* populations at the sites GL and DN. The average number of blood parasite species infecting lizards at the sites HS and HN was relatively low, and the lowest average was found in *E. stokesii* at site CH.

Table 102. Average No. of blood parasite species infecting *E. stokesii*

Study site	No. of *E. stokesii* individuals caught during three years	Average No. of blood parasite species infecting *E. stokesii*	
		8	SD
DN	27	1.37	1.11
GL	62	1.55	1.24
NR	68	2.21	1.53
CH	148	0.78	0.60
HS	159	1.08	0.89
HN	97	1.00	1.04

E. stokesii from the six study sites were grouped into lizard individuals infected with none, one, two, three, four or five species of blood parasites and displayed in Figure 144 as a frequency histogram for each study site. Over the three years of this study, the majority of the lizards at site DN were infected with one or two species of blood parasites. At site GL, more *E. stokesii* than at the site DN were diagnosed with three or four species of blood parasites. The *E. stokesii* population at site NR were most frequently infected with three species of blood parasites. *E. stokesii* infected with five species of blood parasites were only found at site NR, because only there a fifth species (*Hepatozoon* sp.) was present. Most lizards at the site CH were infected with only one species of blood parasite. Very few lizards had a mixed infection, and mixed infections composed by more than two blood parasites were extremely rare. Unlike the site CH lizards, *E. stokesii* from sites HS and HN had often mixed

infections of two parasite species. Mixed infections with three or more blood parasite species were relatively rare at those two sites when compared to sites DN, GL and NR.

An index of the average number of species infecting *E. stokesii* at the six sites was calculated on the data presented in Table 103. The mean number of blood parasites infecting *E. stokesii* was determined for each site and each season, and the average of the means of each season were plotted as Figure 145. This analysis illustrated the marked difference between the site NR and the other sites in regard to the average number of blood parasites infecting *E. stokesii* individuals. It also emphasised the relatively low average number of blood parasites infecting *E. stokesii* individuals at the site CH. A statistical analysis comparing the average number of blood parasites infecting *E. stokesii* between the six sites revealed significant differences among them (Table 104). A post-hoc analysis detected significantly different averages of the number of blood parasite species between the population at site NR and all other sites (Table 105). No further significant differences were found.

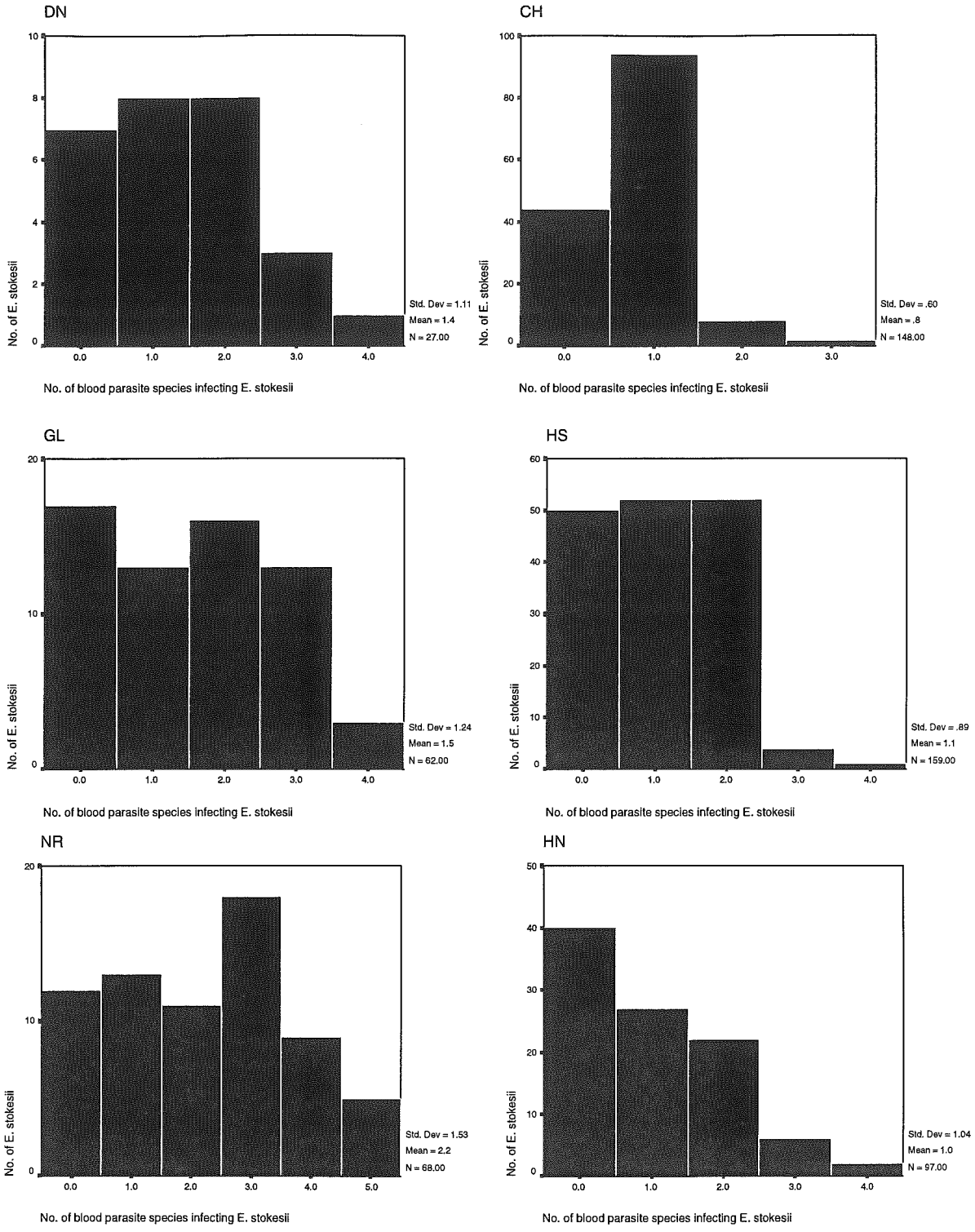

Figure 144. Histograms displaying the frequencies of the numbers of blood parasite species infecting *E. stokesii*, comparing the six regularly monitored field sites in the Flinders Ranges. The data from all three seasons were pooled (See also Table 101)

Table 103. Average numbers of blood parasite species per season infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges, and the mean of the average number of blood parasites.

Site	Mean number of blood parasite species infecting *E. stokesii*										
	1995/96			1996/97			1997/98			Average of the means of the three seasons	
	No. of *E. stokesii* individuals	$\bar{X}$	SD	No. of *E. stokesii* individuals	$\bar{X}$	SD	No. of *E. stokesii* individuals	$\bar{X}$	SD	$\bar{X}$	SD
DN	20	1.05	1.00	12	1.33	0.65	11	1.36	1.21	1.25	0.17
GL	26	1.27	0.96	30	1.20	0.96	42	1.57	1.25	1.35	0.20
NR	28	2.18	1.19	33	1.64	1.08	53	2.34	1.53	2.05	0.37
CH	61	0.87	0.46	78	0.91	0.49	115	0.77	0.53	0.85	0.07
HS	62	1.21	0.68	68	1.13	0.88	102	0.98	0.83	1.11	0.12
HN	43	1.00	0.76	57	0.98	0.86	59	1.00	1.08	0.99	0.01

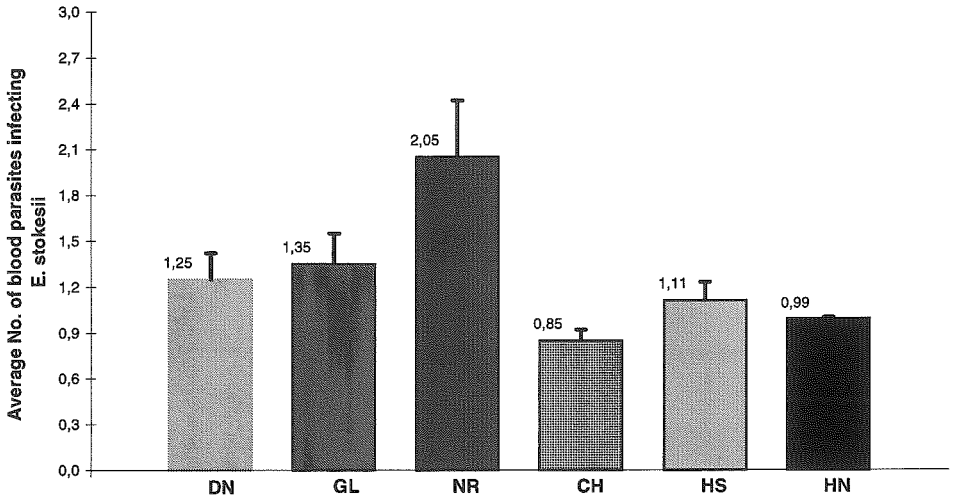

Figure 145. The means of the average number of blood parasite species infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges; the data were pooled per seasons investigated (1995-1998).

Table 104. ANOVA of the means of the average number of blood parasite species infecting *E. stokesii* comparing between the six study sites.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.685	5	0.537	14.432	**0.000**
Within Groups	0.447	12	0.037		
Total	3.132	17			

Table 105. Post-hoc (Tukey) analysis of the means of the average number of blood parasites infecting *E. stokesii* comparing individually between the six study sites.

Study site		Study site	Mean difference	SE	Sig.
DN	&	GL	-0.098	0.158	0.987
DN	**&**	**NR**	**-0.803**	**0.158**	**0.003**
DN	&	CH	0.398	0.158	0.191
DN	&	HS	0.142	0.158	0.940
DN	&	HN	0.255	0.158	0.603
GL	**&**	**NR**	**-0.705**	**0.158**	**0.008**
GL	&	CH	0.496	0.158	0.071
GL	&	HS	0.239	0.158	0.659
GL	&	HN	0.353	0.158	0.289
NR	**&**	**CH**	**1.201**	**0.158**	**0.000**
NR	**&**	**HS**	**0.944**	**0.158**	**0.001**
NR	**&**	**HN**	**1.057**	**0.158**	**0.000**
CH	&	HS	-0.256	0.158	0.597
CH	&	HN	-0.143	0.158	0.937
HS	&	HN	0.113	0.158	0.976

14.3.3.4 Prevalence of blood parasites within weight classes of *E. stokesii*

Figure 146 shows *E. stokesii* from all six studied populations structured by their body mass at the time of capture[14]. These data were not pooled by lizard individuals, thus repeatedly captured *E. stokesii* were included as often as they were captured over the three years.

The clustering of the populations by 30g weight classes most likely reflected the age composition of the lizard populations[15]. The relatively high frequency of *E. stokesii* in the first weight cluster reflected the numerous occurrence of newborns frequently caught during the late summer and autumn. The decreasing numbers of the following three weight classes reflected the high mortality juvenile lizards suffered during adolescence. *E. stokesii* within the five weight classes from 120g to 270g comprised the largest part of the population. These weight classes probably represent an amalgamation of several age classes. Body mass of the lizards rarely exceeded 300g. Also plotted on that figure was the average prevalence of the three blood parasite species that occurred on all of the sites over the entire three year period examined. In this analysis, *Schellackia* sp. was the most prevalent blood parasite throughout all weight groups of *E. stokesii*. Its prevalence steadily increased with each weight group. More than 60% of the lizards were infected in the weight class of 60g-90g, and this increased to over 80% in the heaviest weight groups. A decline of the prevalence in the heaviest weight class must be evaluated with caution due to the relatively small sample size of that class. Similar to *Schellackia* sp., the prevalence of *P. mackerrasae* and *Hem. mariae* increased with progressive weight groups, but always a much smaller part of the population was infected on average.

Figure 147 displays the weight class analysis for *E. stokesii* from study site CH. It was noteworthy that the number of lizards in the first weight group was lower compared to the other sites. During the long term investigation of the behaviour of *E. stokesii* on CH, a sudden lack of reproducing females was noticed from 1996 onwards (G. Duffield, pers. comm.). The reasons for this phenomenon remain unclear. *Schellackia* sp. was by far the most prevalent blood parasite at this site in all weight classes. The prevalence of *Schellackia* sp. steeply increased within the first few weight groups, and from the second weight class (30g-60g) onwards more than 80% of the lizards were infected. In most of the following

[14] The data of the third field season of the site CH were not included (see 14.2.3).

[15] It was assumed that the body mass of *E. stokesii* was a function of its age, although the indices of the body mass in relation to its age have not been established for this species in the field.

weight groups more than 90% of all *E. stokesii* were infected with *Schellackia* sp.. *Hem. mariae* and *P. mackerrasae* infected a small part of the population, with their highest average prevalence in weight classes of the medium sized adult lizards.

The *E. stokesii* populations at the study sites HS (Figure 148) and HN (Figure 149) were found to have rather similar patterns of blood parasite prevalences throughout their weight classes. Parallel to the population at the site CH, *Schellackia* sp. was the most prevalent blood parasite species that steadily increased its prevalence within each successive body mass group until more than 80% of a weight group was infected. In contrast to the population at the site CH, the prevalence of *Schellackia* sp. increased less steeply in the lizard population from the sites HS and HN, and the blood parasites *Hem. mariae* and *P. mackerrasae* occurred more frequently at those sites. The highest prevalences of *Hem. mariae* and *P. mackerrasae* were found within the adult weight classes, but the even the lowest weight classes of *E. stokesii* were found to be infected while no clear prevalence peaks were discernible. *P. circularis* was only found infecting a few medium sized adult *E. stokesii* at site HN

The prevalence patterns of the parasite species infecting *E. stokesii* from the sites GL and NR (Figure 150 and Figure 151) were very different from the ones found at the sites CH, HS and HN. *Schellackia* sp. was not the most dominant species, and in particular in the four weight classes from 30 g to 150 g, *P. mackerrasae* was the most prevalent blood parasite. All other blood parasite species showed a tendency of higher average prevalences with the increasing weight classes, but no clear peaks were discernible. Even the lowest weight classes were found to be infected with all of the parasite species, except *P. circularis*. *Hepatozoon* sp. occurred only in *E. stokesii* at the site NR.

In the last step of the analysis of the prevalence of each blood parasite species, the data were examined for a possible gender bias. No significant differences of the prevalence of any of the five blood parasites occurred among males and females in any of the six study sites.

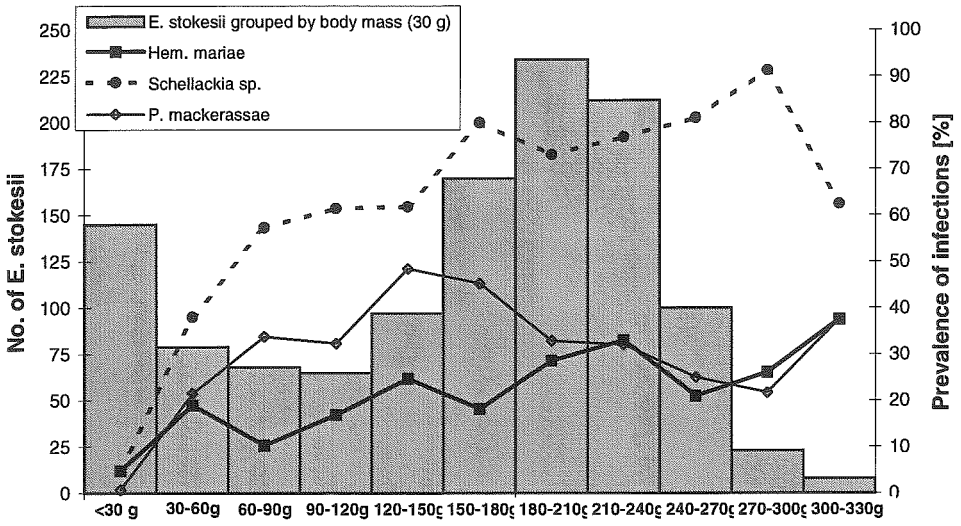

Figure 146. ***E. stokesii*** **from all six populations combined structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster.**

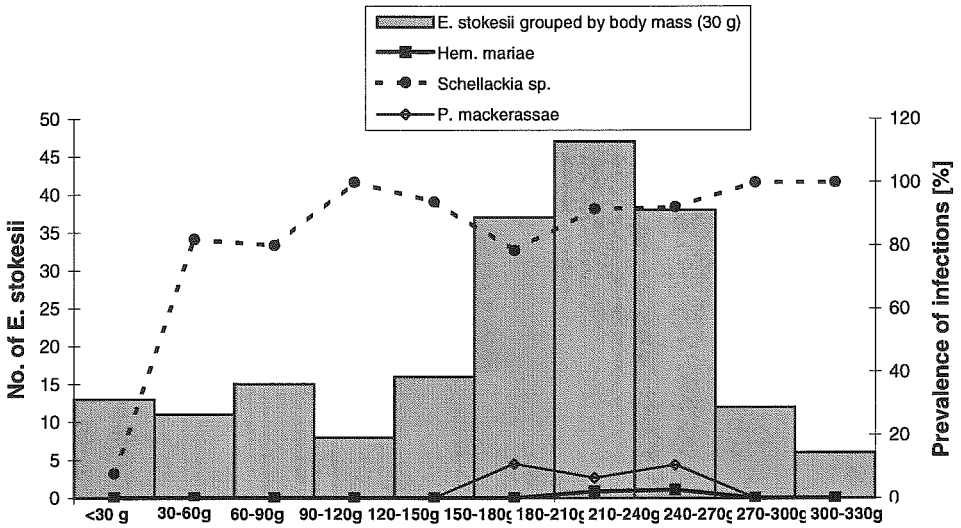

Figure 147. ***E. stokesii*** **from the study site CH structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster (data only from the first two field seasons).**

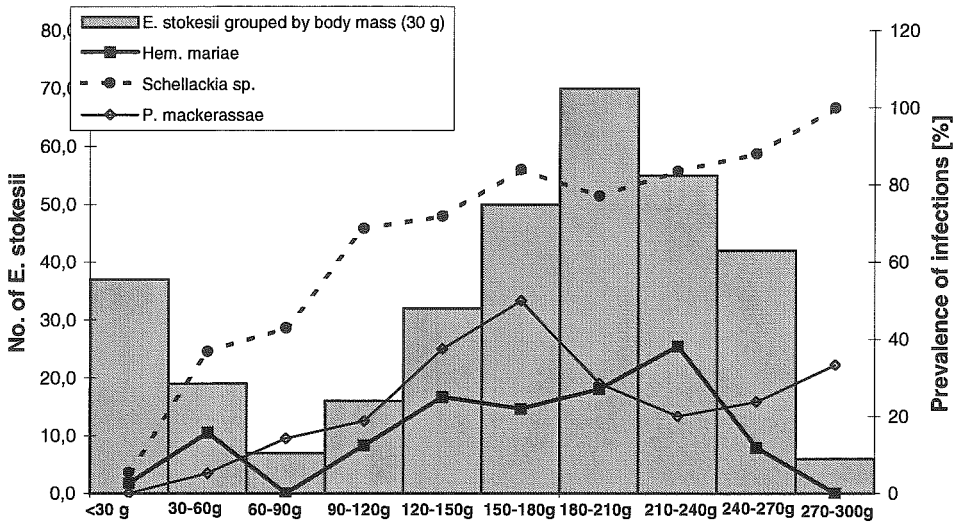

Figure 148. ***E. stokesii*** **from the study site HS structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster.**

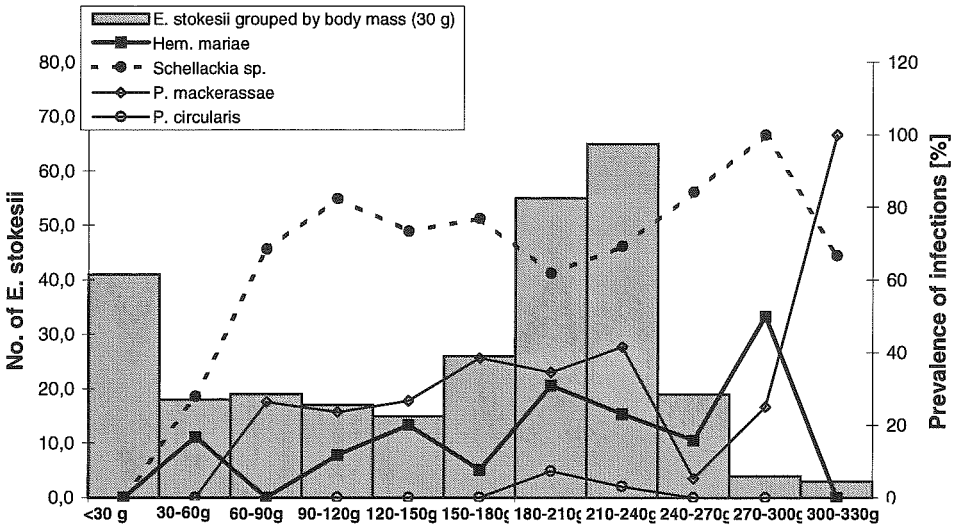

Figure 149. ***E. stokesii*** **from the study site HN structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster.**

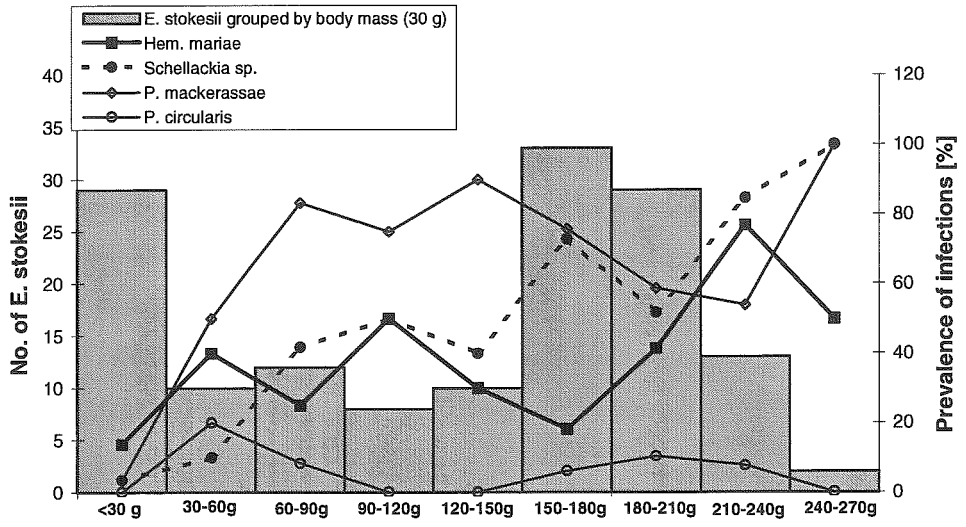

Figure 150. ***E. stokesii*** **from the study site GL structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster.**

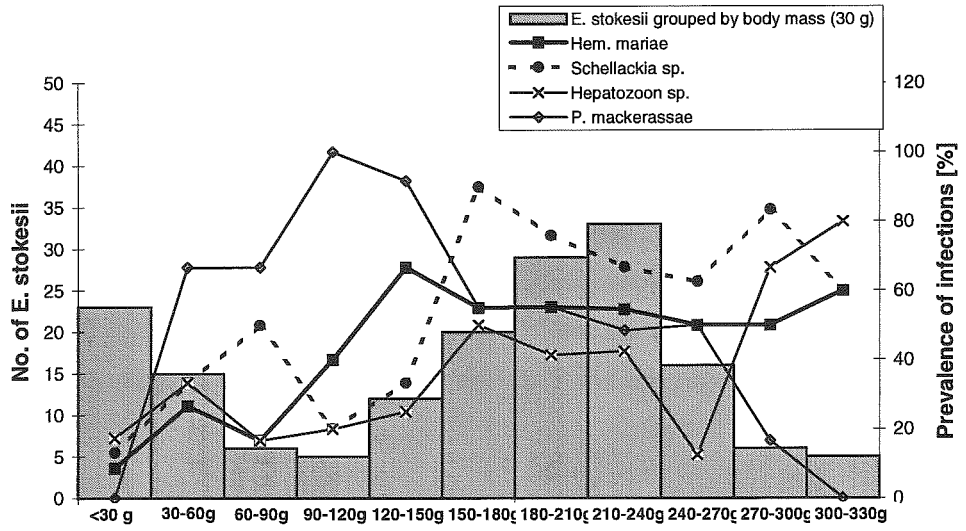

Figure 151. ***E. stokesii*** **from the study site NR structured by body mass; the mean prevalence of the most common blood parasites was plotted for each weight cluster.**

14.3.4 Average parasitemia of blood parasites at six study sites

The following analysis explores the differences of the average parasitemia of the five blood parasite species from the six populations over three consecutive seasons. For a better understanding of the data presented it is important to consider that parasitemia describes the number of parasites in an estimate of 10^4 erythrocytes (see 5.2.1.5) of an infected lizard individual, and the averages of the parasitemia for a given blood parasite species can therefore only be summarised for the lizards that are infected with that blood parasite species. This value is an estimate of how intensely infected host were infected. The mean per season was calculated from the average parasitemia in each of the three seasons, which is listed in detail for each parasite species except *P. circularis* in the Tables 107-110.

14.3.4.1 Parasitemia-profile of blood parasites at six study sites

Figure 152 displays the mean of the average parasitemia in *E. stokesii* infected with *Hem. mariae* for the six study sites. The parasitemia was very different between the six sites investigated, ranging from 0.7 (site CH) to 105.7 infected blood cells (site DN). Not surprisingly, the site CH, which had the lowest average prevalence for *Hem. mariae,* also had the lowest parasitemia. Moreover, the lizards from the sites GL (43.5) and HS (35.1) had a medium-level parasitemia when compared to the other sites, which corresponded to the mean of the average prevalence found for those lizards. However, the remaining parasitemia values from the other three sites did not correlate as well with their respective prevalences. In fact, a reciprocal relation between the parasitemia and their prevalence was observable. No statistically significant correlation was found between the mean of the average prevalence and the mean of the average parasitemia of *Hem. mariae* in *E. stokesii* at the six sites (Table 106).

The average parasitemia of *Hem. mariae* in the infected lizards at the site DN was the highest among the studied populations (105.7; Figure 152), although the average prevalence was average when compared to the other sites. In contrast, the average parasitemia at the site NR was relatively low (32.3), although the average prevalence was the highest among the studied populations. At the site HN, lizards infected with *Hem. mariae* had the second lowest prevalence among the studied populations, but their parasitemia was the second highest (59.2) showing an extremely high variation.

Due to the lack of infections with *Hepatozoon* sp. apart from the *E. stokesii* at the site NR, no comparative data of the parasitemia were available (Figure 154). At the site NR, the mean of the average parasitemia was 38.4 in the lizard population infected with *Hepatozoon* sp (Table 108).

The mean of the average parasitemia of the *E. stokesii* infected with *Schellackia* sp. was extremely low and was very similar between the six sites, ranging from 7.1 at the site GL to 8.6 at the site CH (Figure 155). No statistically significant correlation was found between the mean of the average prevalence and the mean of the average parasitemia of *Schellackia sp.* from *E. stokesii* at the six sites (Table 106).

As mentioned before, the prevalence and parasitemia of *P. circularis* was only counted for the last season. For this index of the parasitemia, the data were not separated into the two *Plasmodium* species, because in order to create comparable data they had to be pooled over the three seasons. A more detailed analysis of the parasitemia of *P. mackerrasae* and *P. circularis* will be given later. Compared to the three haemogregarine species, the mean of the average parasitemia of *Plasmodium* spp. was extremely high, exceeding those of for example *Schellackia* sp. by the factor 1000 (Table 110; Figure 156). The parasitemia of *Plasmodium spp.* at the six study sites corresponded well with the mean of the average prevalences of *P. mackerrasae* found at each site. A statistically significant correlation was detected between the mean of the average prevalence and the mean of the average parasitemia of *Plasmodium* spp. at the six study sites (Table 106). The lowest average parasitemia occurred in the *E. stokesii* population at site CH (Figure 156). The average parasitemia at the site HS and HN was relatively low when compared to the other sites. The lizards with the highest average parasitemia were caught at the sites DN, GL and NR.

Table 106. Correlation between the mean of the average prevalence and the mean of the average parasitemia of three blood parasite species infecting *E. stokesii* at six sites in the Flinders Ranges.

Hem. mariae	Pearson Correlation	0.264
	Sig. (2-tailed)	0.614
Schellackia sp.	Pearson Correlation	0.777
	Sig. (2-tailed)	0.069
P. mackerrasae	Pearson Correlation	0.880
	Sig. (2-tailed)	**0.021**

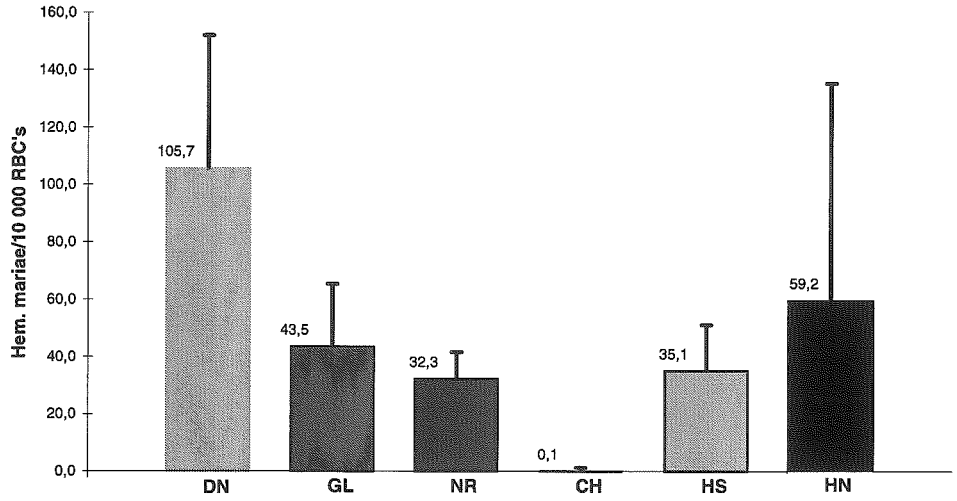

Figure 152. The averages of the mean parasitemia of *E. stokesii* infected with *Hem. mariae*; data of the three seasons investigated (1995-1998) were pooled (see also Table 107).

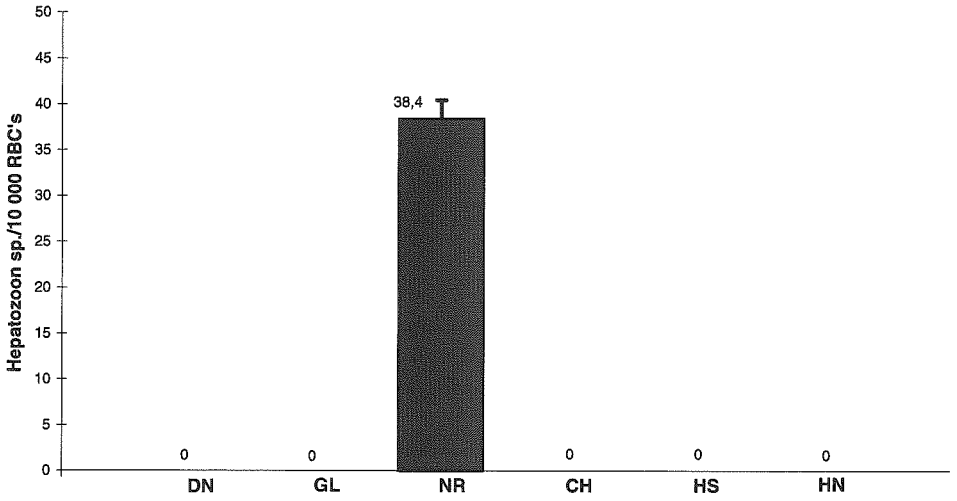

Figure 153. The averages of the mean parasitemia of *E. stokesii* infected with *Hepatozoon sp.*; data of the three seasons investigated (1995-1998) were pooled (see also Table 108).

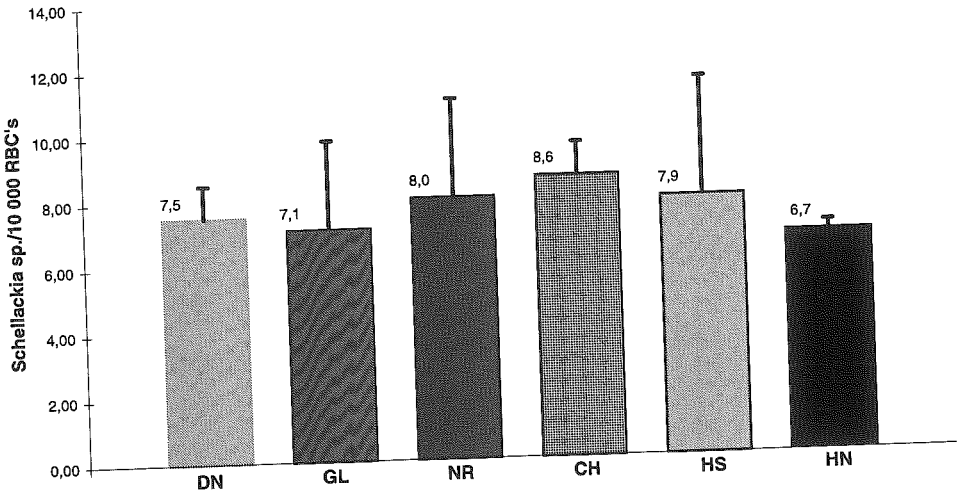

Figure 154. The averages of the mean parasitemia of *E. stokesii* infected with *Schellackia sp.*; data of the three seasons investigated (1995-1998) were pooled (see also Table 109).

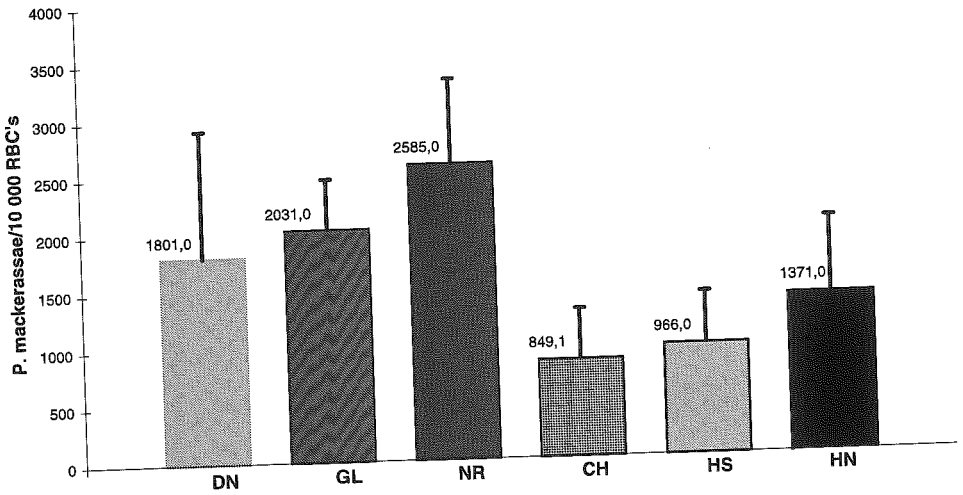

Figure 155. The averages of the mean parasitemia of *E. stokesii* infected with *Plasmodium spp.*; data of the three seasons investigated (1995-1998) were pooled (see also Table 110).

Table 107. Mean parasitemia of *Hem. mariae* infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges.

Site	1995/96				1996/97				1997/98				Average of the means of the three seasons	
	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	$\bar{X}$	SD
DN	44	15	97.7	80.4	16	3	64.0	53.0	12	2	155.5	12.0	105.7	46.3
GL	47	11	35.6	26.1	45	14	26.7	50.0	82	24	68.1	87.6	43.5	21.8
NR	51	19	42.3	35.4	46	15	23.9	26.4	101	56	30.6	62.0	32.3	9.3
CH	90	0			159	2	2.0	0.0	220	0			0.7	1.2
HS	91	15	27.3	13.9	107	21	24.4	70.6	174	38	53.5	180.0	35.1	16.0
HN	67	4	5.3	5.9	109	20	26.2	43.9	140	26	146.2	261.8	59.2	76.1

Table 108. Mean parasitemia of *Hepatozoon* sp. infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges.

Site	1995/96				1996/97				1997/98				Average of the means of the three seasons	
	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	$\bar{X}$	SD
DN	44	0			16	0			12	0				
GL	47	0			45	0			82	0				
NR	51	21	61.0	176.0	46	12	23.3	23.1	101	38	30.8	41.4	38.4	20.0
CH	90	0			159	0			220	0				
HS	91	0			107	0			174	0				
HN	67	0			109	0			140	0				

Table 109. Mean parasitemia of *Schellackia* sp. infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges.

Site	1995/96				1996/97				1997/98				Average of the means of the three seasons	
	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	$\bar{X}$	SD
DN	44	13	7.2	9.4	16	7	6.7	5.6	12	6	8.7	6.7	7.5	1.0
GL	47	27	5.2	5.0	45	15	10.3	9.8	82	36	5.9	5.2	7.1	2.7
NR	51	36	6.3	7.0	46	29	6.3	7.6	101	47	11.5	29.5	8.0	3.0
CH	90	65	9.8	13.4	159	141	7.9	13.4	220	168	8.2	15.5	8.6	1.0
HS	91	79	11.8	17.1	107	73	7.1	7.9	174	108	4.7	4.9	7.9	3.6
HN	67	45	6.8	11.1	109	70	7.0	7.2	140	69	6.3	8.3	6.7	0.3

Table 110. Mean parasitemia of *Plasmodium spp.* infecting *E. stokesii* from the six regularly monitored study sites in the Flinders Ranges.

Site	1995/96				1996/97				1997/98				Average of the means of the three seasons	
	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	No. of bled *E. stokesii*	No. of infected *E. stokesii*	$\bar{X}$	SD	$\bar{X}$	SD
DN	44	15	1380	2212	16	9	3061	2508	12	6	960	1160	1801	1112
GL	47	27	2357	2861	45	24	1536	2433	82	39	2210	2498	2035	437
NR	51	28	3296	2798	46	18	1823	2331	101	47	2636	2260	2585	738
CH	90	5	1309	1004	159	8	426	339	220	7	811	1121	849	442
HS	91	20	477	569	107	34	1355	1722	174	33	1065	1806	966	447
HN	67	16	1884	2096	109	22	1623	2497	140	40	605	731	1371	675

14.3.4.2 Changes of the average parasitemia within annual seasons

For this examination, the average parasitemia of infected *E. stokesii* was plotted for each of the 20 field excursions over the entire three-year period of the study. Each blood parasite species was analysed separately, but the data from all six sites had to be pooled in order to obtain a tolerable sample size. Over the three years studied, *Hem. mariae* did not show a clear seasonal pattern in its average parasitemia of the infected lizards (Figure 156). Small peaks or troughs of the parasitemia were observed throughout the years, and although some values could give the impression of a biologically meaningful curve, no entrainment to any season of the year was detectable. The variance at each sample time was too high to allow any trends to be detected. The sample size of infected lizards during the third season was considerably larger than that of the previous seasons. During the last season a pattern occurred showing an increasing average parasitemia with a higher variation during spring and summer, and a decrease in autumn that might hint a seasonal pattern. However, further studies with larger sample sizes are needed to confirm this result. The few lizards infected with *Hepatozoon* sp. did not show any seasonal pattern of the average parasitemia (Figure 157), and the sample size was too low to allow any interpretation of the data. Similar to *Hem. mariae* and *Hepatozoon* sp., the analysis of the average parasitemia of *Schellackia* sp. on the field trips did not show any consistent patterns, although the sample size was relatively large all through the three years (Figure 158). The average parasitemia of *Plasmodium* spp. showed a strong association with the seasons of the year (Figure 159). In all three years investigated, the average parasitemia and its variation rose during spring, and it steadily fell during summer and autumn. Relatively low parasitemias were found during the winter months, with the exception of the last field trip in April 1998, where a relatively high average parasitemia was recorded. The average parasitemia of *P. circularis* from the season 1997/98 did not show any meaningful pattern (Figure 160). Due to the relatively low prevalence of *P. circularis* at the six study sites (Table 44), it was likely that even the recounting of the previous two seasons would probably not deliver a large enough sample size to investigate its patterns of parasitemia.

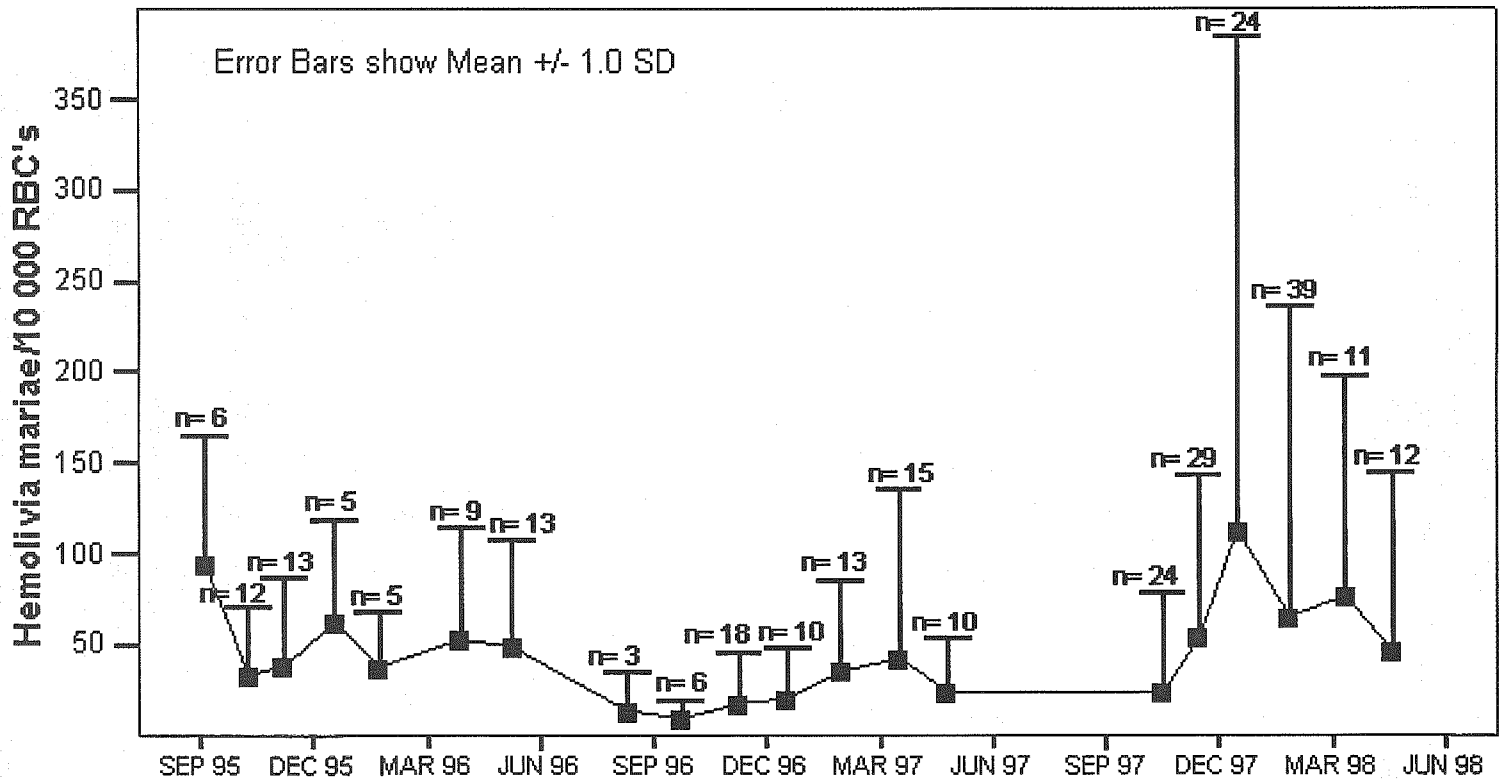

Figure 156. The average parasitemias of captured *E. stokesii* infected with *Hem. mariae* combining all data from the six regularly monitored study sites in the Flinders Ranges over the entire three year period of this study (1995-1998).

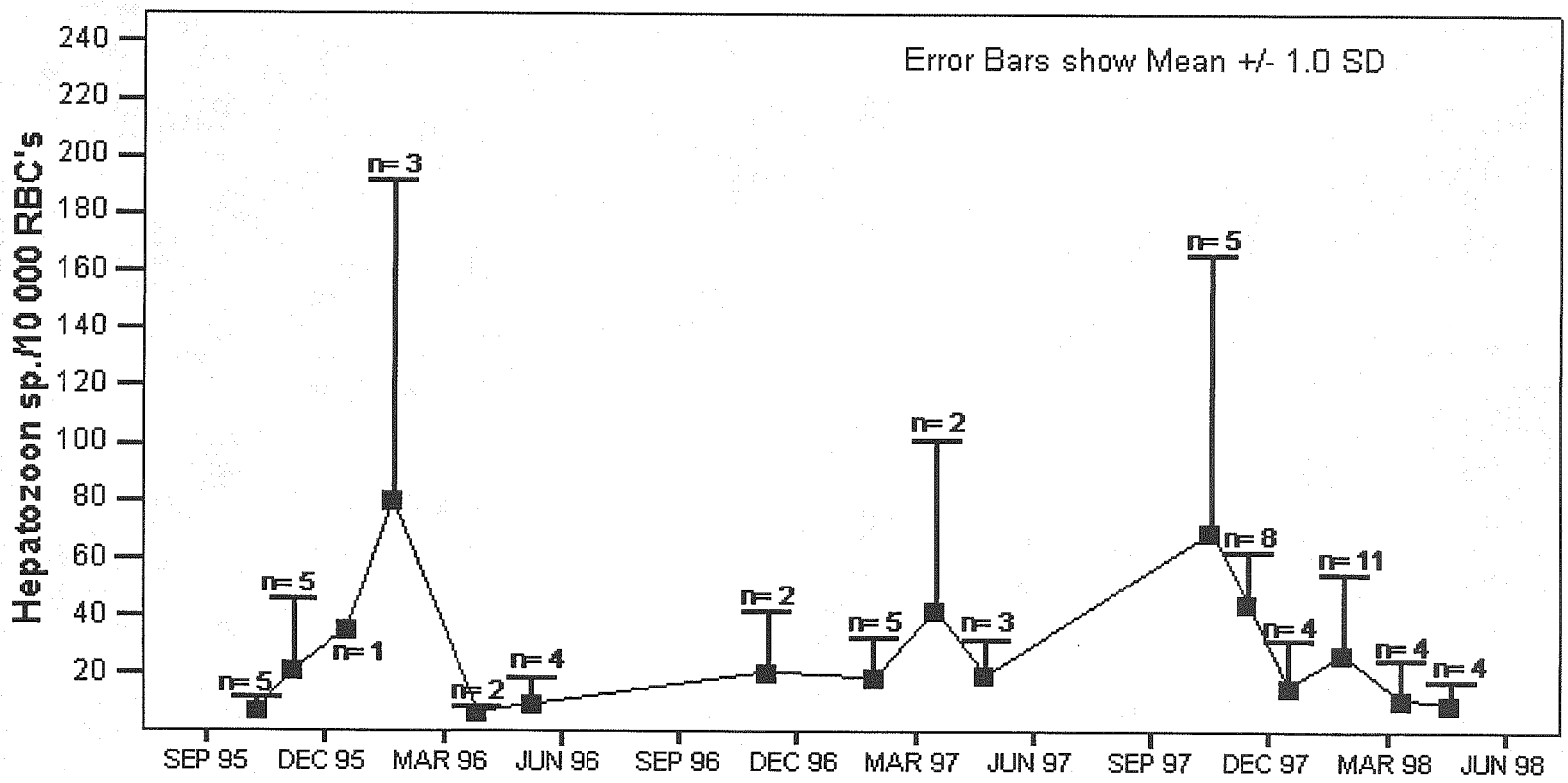

Figure 157. The average parasitemias of captured *E. stokesii* infected with *Hepatozoon* sp. combining all data from the six regularly monitored study sites in the Flinders Ranges over the entire three year period of this study (1995-1998).

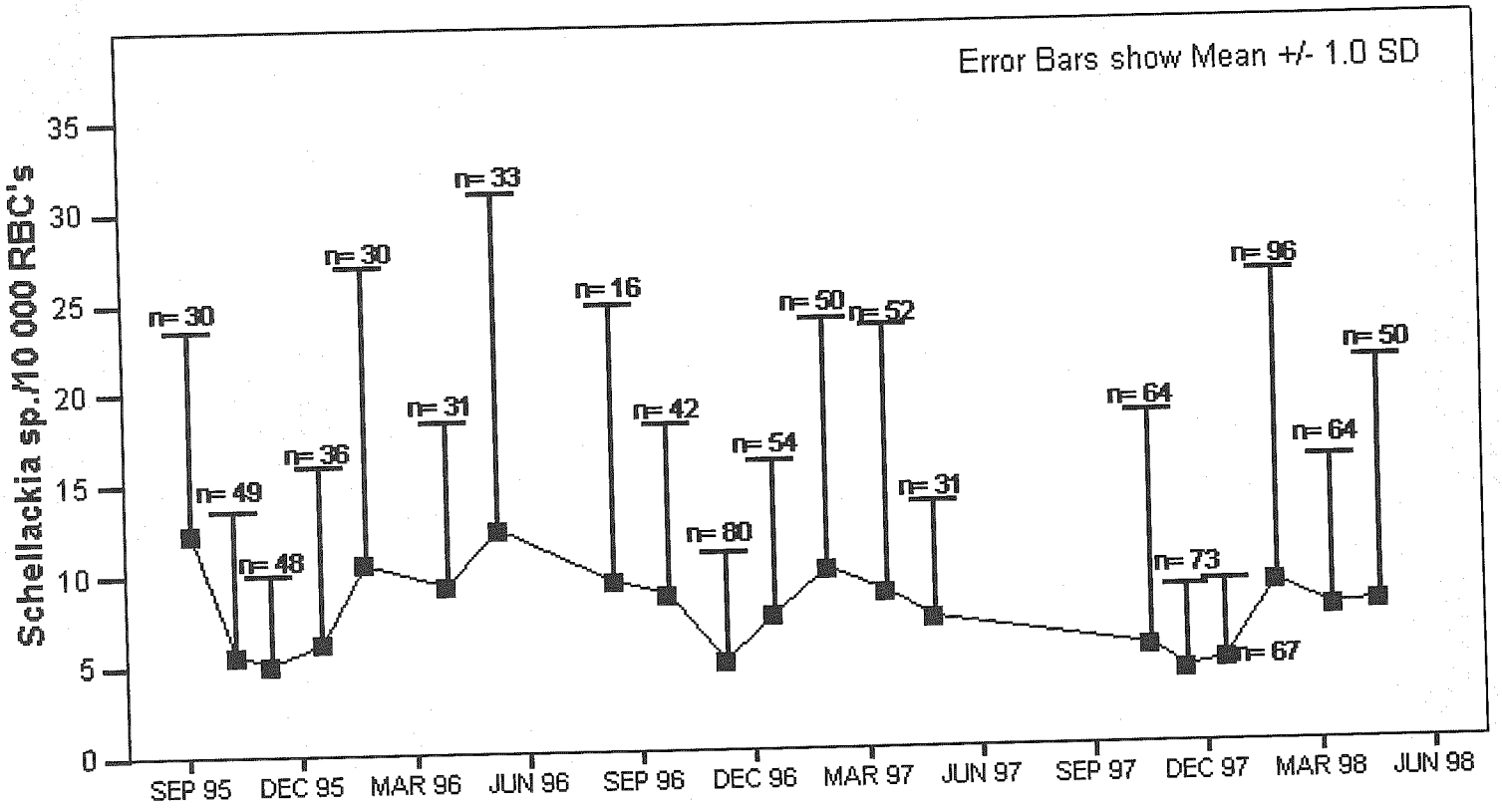

Figure 158. The average parasitemias of captured *E. stokesii* infected with *Schellackia sp.* combining all data from the six regularly monitored study sites in the Flinders Ranges over the entire three year period of this study (1995-1998).

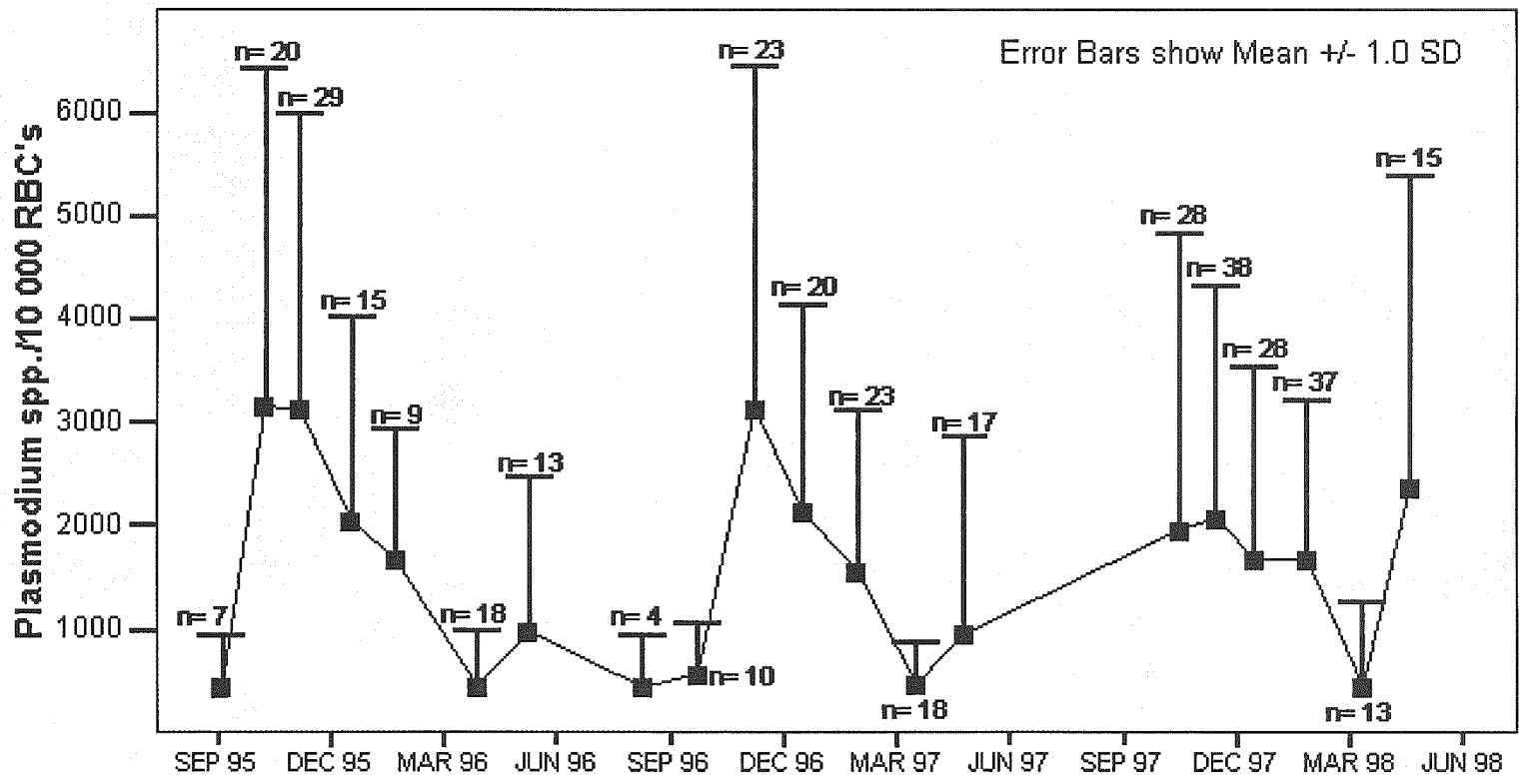

Figure 159. The average parasitemias of captured *E. stokesii* infected with *Plasmodium* spp. combining all data from the six regularly monitored study sites in the Flinders Ranges over the entire three year period of this study (1995-1998).

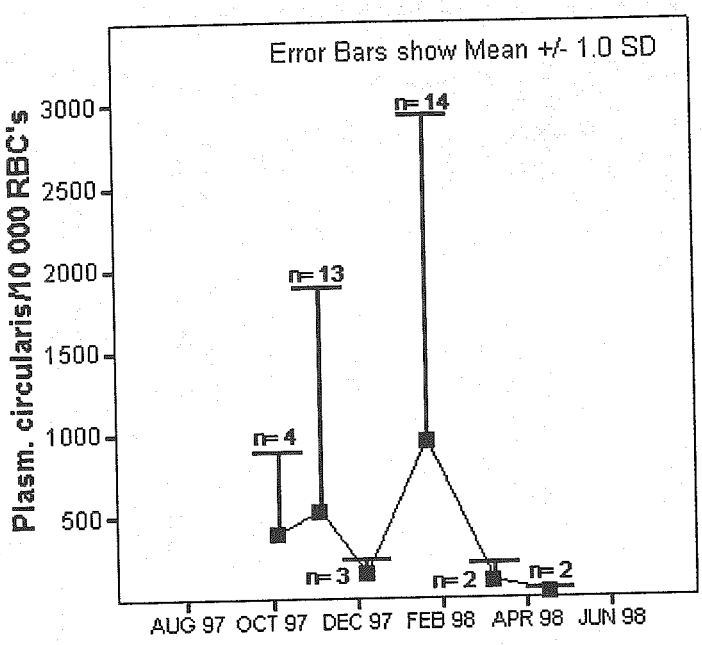

Figure 160. The average parasitemias of *E. stokesii* infected with *P. circularis* combining all data from the six regularly monitored study sites in the Flinders Ranges over the entire three year period of this study (1995-1998).

14.3.5 Occurrence of ectoparasites of *E. stokesii* at six study sites

The number and the prevalence of ectoparasites of *E. stokesii* was evaluated during the examination of the lizards at the field station in Hawker. The only ectoparasites found attached to the lizard during the entire three-year study were ticks. The numbers of phlebotomine sand flies observed at the field sites engorging on *E. stokesii* were listed in Table 39. The following analysis examines the spatial pattern of the of tick instars infesting *E. stokesii*. The only adult ticks detected at the six regularly monitored study sites belonged to the species *Amb. limbatum*. Thus it was assumed that all larvae and nymphs found belonged to that species as well.

Larvae and nymphs were by far the most frequently found tick instars infesting *E. stokesii* (Table 111). The site with the maximum number of ticks found attached to *E. stokesii* was HN (79), followed by GL (64), NR (55) and HS (42). Twelve ticks were found infesting *E. stokesii* at site DN and no tick was detected at site CH. The average number of ticks attached per captured host showed that the *E. stokesii* at site GL had the highest average number of attached ticks (0.438, Table 111), followed by the lizards at site NR (0.324), HN (0.280), DN (0.182) and HS (0.126).

Over the three years investigated, the average number of captured *E. stokesii* found to be parasitised by ticks was highest at site GL (Table 112, 0.164), followed by the *E. stokesii* of sites NR (0.153), DN (0.106), HN (0.092) and HS (0.063). No ticks were found at site CH. The order of sites in this analysis of the lizard infestation resembled that of the average number of ticks attached (Table 111), although sites DN and HN swapped places.

The final step of the analysis examined how many lizard individuals were found to be infested over the three year study. Each *E. stokesii* was only counted once and it was considered as infested when a tick has been found attached to it once at any time during the field survey. The data show that the highest proportion of infested *E. stokesii* hosts was found at site NR (0.566; Table 113), closely followed by the lizards at site GL (0.526). Markedly lower numbers of lizard individuals were infested with ticks at site HN (0.319). The average number of infested lizard individuals was lowest at the sites DN (0.286) and HS (0.233).

In summary, the lizards at sites GL and NR had on average the highest number of ticks attached among the six study sites. Considering each capture event singly, *E. stokesii* from GL and NR were also most frequently infested with ticks. Furthermore, each lizard individual at those two sites was on average more frequently infested with ticks that the lizard individuals at the other four sites when the data were pooled for each lizard individual over three years. Although the *E. stokesii* at site GL were found to have on average more ticks attached than those from site NR, the number of infected *E. stokesii* individuals at GL was slightly lower than at site NR, indicating that ticks infested a larger part of the lizard population at site NR. The levels of tick infestation of *E. stokesii* at the sites HN were similar to those at the sites DN and HS, although the *E. stokesii* at site HS constantly had the lowest levels of infestation on average. The *E. stokesii* at site CH were not infested with ticks.

Table 111. The number of tick instars that were found attached to *E. stokesii* during the routine examinations from September 1995-April 1998.

Site	No. of *E. stokesii* examined	Number of ticks found attached to *E. stokesii*														
		Larvae			Nymphs			Males (*Amb. limbatum*)			Females (*Amb. limbatum*)			Sum of tick instars		
		Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD
DN	66	1	0.015	0.123	10	0.152	0.533	0			1	0.015	0.123	12	0.182	0.579
GL	146	33	0.226	1.119	31	0.212	0.815	0			0			64	0.438	1.409
NR	170	12	0.071	0.319	43	0.253	1.443	0			0			55	0.324	1.548
CH	203	0			0			0			0			0		
HS	334	21	0.063	0.584	21	0.063	0.336	0			0			42	0.126	0.741
HN	282	16	0.057	0.544	61	0.216	1.033	2	0.007	0.084	0			79	0.280	1.158

Table 112. The number of *E. stokesii* that were found with tick instars attached during the routine examinations from September 1995-April 1998.

Site	No. of *E. stokesii* examined	Number of *E. stokesii* found with ticks attached														
		Larvae			Nymphs			Males (*Amb. limbatum*)			Females (*Amb. limbatum*)			Sum of tick instars		
		Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD	Σ	$\bar{X}$	SD
DN	66	1	0.015	0.123	6	0.091	0.290	0			1	0.015	0.123	7	0.106	0.310
GL	146	8	0.055	0.228	17	0.116	0.322	0			0			24	0.164	0.372
NR	170	9	0.053	0.225	20	0.118	0.323	0			0			26	0.153	0.361
CH	203	0			0			0			0			0		
HS	334	10	0.030	0.171	15	0.045	0.207	0			0			21	0.063	0.243
HN	282	4	0.014	0.118	20	0.045	0.257	2	0.007	0.084	0			26	0.092	0.290

Table 113. The average number of lizard individuals that were found to be infested with ticks during the routine examinations from September 1995-April 1998.

Site	No. of *E. stokesii* examined	Number of ticks found attached to *E. stokesii*									
		Larvae		Nymphs		Males (*Amb. limbatum*)		Females (*Amb. limbatum*)		Sum of tick instars	
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
DN	24	0.042	0.204	0.125	0.338	0.167	0.381	0	0	0.286	0.713
GL	54	0.148	0.359	0.296	0.461	0.296	0.461	0	0	0.526	0.856
NR	60	0.150	0.360	0.300	0.462	0.300	0.462	0	0	0.566	0.926
CH	88	0	0	0	0	0	0	0	0	0	0
HS	152	0.066	0.249	0.086	0.281	0.086	0.281	0	0	0.233	0.672
HN	78	0.051	0.222	0.154	0.363	0.154	0.363	0.026	0.159	0.319	0.691

14.4 Discussion

South Australian populations of the gidgee skink, *E. stokesii*, were found to be frequently infected with a variety of blood parasites. Six coccidian blood parasite species were identified during this study and the presence of a micro-filaria was recorded. Depending on the species of blood parasite, *E. stokesii* often were infected with extremely high parasitemias and mixed infections consisting of five different blood parasites were recorded. The spatial distribution of the blood parasite species on a larger geographical scale appeared random. It was noteworthy that except for *T. rugosa,* no blood infections were found in most of the other lizard species captured, although most lizards from species other than *E. stokesii* were caught within the same habitat as *E. stokesii. T. rugosa* was only diagnosed with *Hem. mariae*, and a possible infection with *P. mackerrasae* remains to be proven.

The reasons for this exceptional accumulation of blood parasite species in *E. stokesii* remain unclear. Three possible explanations will be briefly discussed in the following. It can be speculated that *E. stokesii* is more susceptible to infections with blood parasites. In this hypothesis, intrinsic factors like the lack of efficiency in their immune response could lead to relatively long phases of chronic infections. In case of *Plasmodium* it could lead to frequent relapses of the infection. These chronically infected lizards could infect new vectors for long periods of time and therefore more new lizard hosts of a population would become infected. This theory was supported by the finding that *T. rugosa* from the same habitat and with the same tick species attached as *E. stokesii* were less likely to be infected with *Hem. mariae*, and their parasitemia was generally lower that in *E. stokesii*, implying that on average *T. rugosa* lost their infection with *Hem. mariae* faster than *E. stokesii.* However, the original transmission experiments with *Hem. mariae* and *T. rugosa* (Smallridge, 1998) and the transmission experiments with *Hem. mariae* and *E. stokesii* within this study showed no marked differences between the duration of the chronic phases of the blood infection between the two lizard species when *E. stokesii* were single infected with *Hem. mariae.*

The second hypothesis explains the high prevalences of blood parasites in *E. stokesii* as a result of interactions of the different blood parasite species. This theory is based on the finding that in transmission experiments in this study a very long prepatent phase was observed when *E. stokesii* was infected with *Hem. mariae* and *Hem. biplicata* (see chapter seven). The following chronic phase of experimentally induced mixed blood infections of *E. stokesii* with *Hem. mariae* and *Hem. biplicata* was observable for more than 18 month, and a

relatively high parasitemia was maintained during this time. In contrast, *E. stokesii* infected with *Hem. mariae* alone went through a relatively short chronic phase of infection. The duration of the chronic phase of those infections could not be determined, because this study was terminated before the blood infections ceased, but the parasitemias at the end of this study were already very low. *E. stokesii* with mixed infections caught in the field would often show extremely high parasitemias and chronic infections with several species of blood parasites were maintained in some cases for over three years, while single infected lizards would usually lose their infection within month. The cause or the nature of the proposed interactions of blood parasite species remain to be elucidated, but the suggestion is that the presence of one parasite weakens the host resistance to another parasite.

In the third hypothesis, *E. stokesii* were more prone to blood parasite infections due to their behavioural peculiarities. In this explanation, factors like the refuge in the rock crevice, their tendency to occur in groups, the ingestion of ticks and the ingestion of their placenta would enhance the chance of parasite transmission. The sheltered rock crevices probably not only protect *E. stokesii* from its predators, but the relatively benign microclimate inside those crevices also protects the vectors of *Hem. mariae* and *Hem. biplicata*, the ticks *Amb. limbatum* and *Amb. vikirri*, from the harsh climatic conditions outside. In addition, the haematophagous sand fly, *Australophlebotomus dycii*, develops inside the rock crevices. At some sites, the dipteran parasitises *E. stokesii* in large numbers during late summer and autumn (see Table 39), and its larvae probably live on the faeces of *E. stokesii* and overwinter inside those crevices. A vector role of *A. dycii* could not be proven in this study, but the fact that a dipteran so adapted to the wet tropics manages to survive inside those crevices in a semi-arid habitat sheds a light on the quality of the crevices as a microclimatic safe haven, ideal for the development and shelter of many species of arthropod vectors. The apparent grouping behaviour of the lizards in combination with their very narrow home range (G. Duffield, pers. comm.) and their habit of returning into the same crevices makes *E. stokesii* an ideal host for many ectoparasites and the diseases they transmit. Furthermore the grouping of *E. stokesii* most likely leads to a quick spread of ectoparasites with otherwise limited motile abilities like ticks and the micro-organisms they transmit.

It was remarkable that the prevalences of the different blood parasite species had large differences among most study sites, but they remained very consistent over the years within the six populations studied in detail. Each of the study sites maintained a characteristic pattern, described as the prevalence profile of the blood parasite species. In general, three types of prevalence profiles were discernible, and those types corresponded well with differences in the vegetational structure of the sites and the vector species found. The first type, represented by the site CH, had a low blood parasite species diversity, with mainly *Schellackia* sp. infecting the lizards. Ectoparasites at this site were extremely rare. No ticks were found in the three year study, and no sand flies were detected. The vegetation was rather lush when compared to the other sites. Most likely the lack of vector species caused the relatively low prevalences of most blood parasites at this site. The high prevalence of *Schellackia* sp. could be explained by the frequent occurrence of transmissions from mother to offspring, which will be discussed in more detail later on. Possibly the good vegetational cover provided ample food for the lizards, so that infections by the ingestion of ticks were extremely rare. Why vector species were so rare at this site remains a mystery.

The second type of a prevalence-profile was represented by sites HS and HN. At both sites, the blood parasite species diversity was relatively low and *Schellackia* sp. was the most prevalent species. Ticks and sandflies were found occasionally (see Table 39 and Tables 111-113), and the vegetational cover was good but not as excellent as at the site CH. Not surprisingly, in this case the number of infections increased with the presence of vector species.

The third category of a prevalence-profile was represented by the sites DN, GL and NR. Most *E. stokesii* at those sites had mixed infections with rather high average parasitemias. Ticks and sandflies were found regularly (see Table 39 and Tables 111-113). The vegetational cover was scarce. The high prevalences of the various blood parasites at those sites were probably due to the high abundance of vectors. The relatively high parasitemias are possibly due to frequent re-infections and super-infections. Moreover, under those circumstances of harsh environmental conditions and sparse vegetational cover the chances for vector species to become a more regular part of the diet of *E. stokesii* and thus to transmit eg. *Hem. mariae* more frequently might be enhanced as well.

The finding that the sites with the lowest vector activity had the highest prevalence of *Schellackia* sp. (CH, HS and HN) supported the theory of a transmission of *Schellackia* sp. from mother to offspring. *Schellackia* sp. infected the lizards at the site CH when they were still very young, and the appearance and the prevalence of the parasite in the youngest part of the population resembled the patterns found for the spontaneous infections in *E. stokesii* born and raised in the ACU. Similar to the newborns kept in the ACU, those field lizards were probably infected by the ingestion of the placenta (see chapter three). However, if *Schellackia* sp. was transmitted from mother to offspring, it remains unclear why the prevalence of *Schellackia* sp. infecting the *E. stokesii* at the sites DN, GL and NR was not as high as at the other sites. Possibly the parasite did actually infect a smaller part of the population at the sites DN, GL and NR. Another possibility is, that in the presence of other blood parasite species, the extremely low parasitemia of *Schellackia* sp. was reduced even further, so that it slipped under the detectable threshold. The latter explanation seems most plausible in the presence of *Plasmodium* species, because eg. *P. mackerrasae* undergoes a massive proliferation during which it infects often more than 90% of blood cells and subsequently probably caused an anaemia in lizards by the destruction of erythrocytes. The *E. stokesii* at the sites DN, GL and NR were most frequently infected with *P. mackerrasae*, and the prevalence especially in the younger part of the population was very high. Whether or not in those cases *Plasmodium* spp. could have been also transmitted by the ingestion of the placenta remains to be investigated.

From all parasites investigated, only *P. mackerrasae* showed a pattern of parasitemia that varied over the season. It was possible that the observed spring peak of parasitemia was not caused by recently acquired infections, but either by recurring chronic infections or by infections acquired during the last season, that stayed dormant until the new spring. Successful experimental transmissions are sorely needed to elucidate those speculations.

Possibly the pattern of a peak parasitemia in spring reflected an adaptation to a vector species. Although a development of *P. mackerrasae* in *A. dycii* could not be demonstrated, it was more than likely that *A. dycii* played a role in the transmission of *P. mackerrasae*. The transmission of lizard malaria by phlebotomine sand flies has been reported before (Ayala & Lee, 1970), and apart from *A. dycii* no other dipteran vector was observed in this study. However, the peak parasitemia of *P. mackerrasae* occurred in spring, but *A. dycii* was most abundant in autumn. As explained in the following, a vector of a *Plasmodium* species does not

inevitably need to be most abundant during the time of peak parasitemia as well. In the life cycle of *Plasmodium*, the gametocytes are the stages that must be imbibed by a vector, because only they can mate and accomplish further development. From the evaluations of blood smears it became apparent that the mature sexual stages of *P. mackerrasae* and *P. circularis* were most frequently observed in autumn. Furthermore it is important to bear in mind that for a successful transmission by an dipteran vector, *Plasmodium* has to develop from the sexual stage in the lizard blood into the infective sporozoite in the salivary glands before the vector feeds again. In eg. a phlebotomine sand fly, *Plasmodium* has to develop into sporozoites fast, because many sand fly species feed only twice and those events are often only days apart. As described by Fialho *et al.* (Fialho & Schall, 1995), *Plasmodium* species might have only a narrow window of opportunity where the outside temperature conditions are sufficiently high to develop into the infective stage fast enough before the vector feeds again.

The finding of three additional parasite species in the faeces of *E. stokesii* and a microfilaria in the blood (see Figure 7, p. 31) underlined the fact that for some reason, *E. stokesii* was hosting a diverse community of parasitic species. In this study, *E. stokesii* was found to be an excellent target organism for the study of blood parasites. It appears likely that further studies on other populations of the skink will reveal new parasite species, and because *E. stokesii* occurs throughout the entire southern half of the Australian continent, such a survey would be very promising for the study of for example the life cycle, the ultrastructure, the molecular genetics, the demographic relationships and the parasitic biodiversity of micro-parasites.

15. GLOSSARY

Gamogony. Sexual phase of reproduction in the life cycle of certain protozoa, leading to the formation of gametocytes or gametes.

Merogony. Also called schizogony. Formation of merozoites by multiple fission.

Mixed infection. The infection with more than one species of pathogenic microorganism.

Parasitemia. The number of infected blood cells in a vertebrate host individual (usually expressed as infected cells in 10^4 blood cells or as a percentage)

Prevalence. The number of infected host organisms in regard to the entire population (usually expressed in %).

Schizogony. Asexual reproduction of a sporozoan by multiple fission in which merozoites are produced.

Semaphoront. Any stage in the life cycle of one species

Sporogony. Formation of sporocysts and sporozoites in members of the phylum Apicomplexa. Multiple fission of a zygote.

Thin-film. A monolayer of blood cells on a glass slide.

Xenodiagnosis. The diagnosis of infectious diseases in vertebrates by the examination of vector competent invertebrates that were allowed to feed on an infected vertebrate host.

16. APPENDIX

TWO MALARIA PARASITES (APICOMPLEXA: PLASMODIIDAE) OF THE AUSTRALIAN SKINK EGERNIA STOKESII.

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ABSTRACT: The Australian skink Egernia stokesii is parasitized uncommonly by Plasmodium circularis n. sp. and by P. mackerrasae. Plasmodium circularis is distinguished from all other plasmodiids by immature schizonts that encircle host cell nuclei, forming an unbroken ring from apparent fusion of the attenuated ends. Mature schizonts contract into halteridial or dumbbell-shaped forms 15.6 X 4.3 µm, LW 66.2 μm^2, with 19-52 nuclei. Rounded or oval gametocytes are 9.0 X 7.3 µm, LW 66.9 μm^2, and L/W 1.24. Gametocyte LW is 2.63 X host erythrocyte nucleus size, and 1.79 X uninfected erythrocyte nuclei. Plasmodium mackerrasae occurs in high prevalence and often massive parasitemia in E. stokesii. Schizonts, often oblong, elongate or oval, are 5.1 X 3.7 µm, LW 19.8 μm^2, with 7.2 merozoites. Immature gametocytes, elongate with terminal nucleus, may produce multiple infections of 6 or more parasites. Mature gametocytes, usually rounded, are 5.8 X 4.6 µm, LW 26.7 μm^2, and L/W 1.29. Gametocyte size is 0.98 X host erythrocyte nucleus size, and 1.03 X uninfected erythrocyte nuclei. Phanerozoites, in endothelium or connective tissue of most organs, may appear in large numbers in circulating blood as seemingly intact bodies of regular form, similar to or larger than phanerozoites seen in sections. Previously

unreported phenomena for hemosporidian parasites include extremely large, highly irregular EE-schizonts, in circulating blood, perhaps torn from endothelial lining of blood vessels and sinuses, and a visible flooding of free merozoites into the blood stream.

A three year study of the hemoparasites of the Australian skink Egernia stokesii found a diverse infracommunity of at least six apicomplexan species within this host. Two Plasmodium species were found, 1 of which is undescribed, 2 species of Hemolivia, a Schellackia species, and an Hepatozoon species. Plasmodium mackerrasae Telford 1979, originally described from scanty material, was most common. It is re-described here from abundant material, along with taxonomic description of the second species.

MATERIALS AND METHODS

Thin blood smears, made by puncture of the caudal vein and air-dried, were fixed in absolute methanol and stained by Giemsa at pH 7.0 for 20 min. Slides were screened at 400 X, and parasites measured by ocular micrometer and photographed at 1000X under oil immersion. Data were transformed (log X+1) and compared with known species and among samples by 1-way ANOVA. Statistical significance was $P < 0.05$. Taxonomic characters employed were those of Telford (1988). Hapantotype, a slide previously designated as syntype (Telford, 1979) and parahapantotype slides were deposited in the Queensland Museum, with other parahapantotype slides reserved for deposition with the Telford collection. Unsuccessful experimental infections of captive-born E. stokesii were attempted by IP inoculation of blood from the caudal vein; experimental infection of Egernia whitei resulted from IP inoculation of blood from clipped toes of Egernia cunninghami (Telford, 1979).

DESCRIPTION

Plasmodium circularis n. sp.

(Figs. 1-48)

Asexual stages: Smallest trophozoites rounded (Figs. 1, 2), 3.0-3.5 X 2.5-3.0 µm, becoming elongate at 6.0-7.0 X 2.5-3.0 µm (Figs. 3, 4) with no vacuole, a prominent nucleus, and dark pigment in 1-2 granules. Binucleate schizonts (Figs. 1, 5) more variably shaped, at 6-7.5 X 3-5 µm, with nuclei often terminal. At second nuclear division (Figs. 6-8), elongate schizonts with 3 nuclei up to 11-15 X 1.5-3.5 µm. Tetranucleate schizonts 7 X 3 to 15 X 3.5 µm (Figs. 9-11). At third nuclear division (Figs. 12, 13), dimensions greatly increased and shapes bizarre with 4-8 nuclei. From third nuclear division, all schizonts elongate (Figs. 2, 14-21) and curving around host cell nucleus. Before segmentation, erythrocyte nuclei usually completely encircled (84%) by still immature schizonts (Figs. 22-24), with apparent fusion of their elongated ends into unbroken cytoplasm (Figs. 25, 26). Ring formed by schizonts 32.3±3.3 µm in circumference (26-38 µm, N=12), with greatest width tapering from 2.5-6 µm to 2-2.5 µm at minimum. Nuclei in ring-shaped schizonts 23.3±2.7 (21-28, N=12). At segmentation schizonts contracted into elongate forms with broadened ends (Figs. 27-37), most often halteridial around host cell nucleus (48%). A few lentiform schizonts polar in position (12%), more elongate or dumbbell-shaped parasites lateropolar (24%) or lateral (16%) to erythrocyte nucleus. Few (12%) of apparently mature schizonts with cytoplasmic division beginning (Figs. 38-40); definitive segmenters not found. Occasional maturing schizonts broken into two portions (Figs. 32, 40). Apparently mature schizonts 15.6±4.9 X 4.3±0.7 µm (7.5-26.0 X 3.0-6.0 µm, N=25), with LW 66.2±20.6 μm^2 (33.7-108), and 19-52 nuclei (26.2). Schizont size relative to infected erythrocyte nuclei 2.85±0.92 (1.5-4.6), and to normal erythrocyte nuclei 2.52±0.78 (1.3-4.1). Host cells of asexual stages erythrocytes, but neither cells nor their nuclei distorted, and nucleus rarely distorted (4%). Erythrocytes host to immature

schizonts enlarged in width (F=13.334, P <0.01, d/f=1) and LW (F=5.406, P <0.05, d/f=1) but their length not different from uninfected cells. Infected erythrocyte nuclei significantly shorter in length (F=37.385, P <0.001, d/f=1), but with greater width (F=6.753, P <0.025, d/f=1) and LW (F=61.743, P <0.001, d/f=1). Erythrocytes infected by mature schizonts enlarged in length (F=6.121, P <0.025, d/f=1), width (F=13.188, P <0.001, d/f=1), and LW (F=14.151, P <0.001, d/f=1). Although length of infected erythrocyte nuclei greater (F=12.236, P <0.01, d/f=1), width not different from that of uninfected cells, and LW less (F=5.883, P <0.025, d/f=1).

Sexual stages: Immature gametocytes oval, 5 X 4.5 to 8 X 6 µm with band-shaped nucleus 2-3 µm in width, often subterminal in position. Mature gametocytes (Figs. 41-48) 9.0±1.3 X 7.3±1.0 µm (7-12 X 3.6-11 µm, N=27), with LW 66.9±18.3 μm^2 (48-132) and L/W 1.24±0.15 (1.0-1.5). Gametocyte size relative to infected erythrocyte nucleus size 2.63 (1.7-4.7), and to normal erythrocyte nuclei 1.79 (1.3-3.5). Gametocytes usually polar (48%), but commonly lateral (30%) or lateropolar (22%) as well to host cell nucleus. Few small, black discrete pigment granules, scattered. All erythrocytic, usually distorting host cell (78%) and displacing its nucleus (93%), commonly distorting nucleus (30%). Although length (F=4.165, P <0.05) and width (F=4.924, p<0.05) of host erythrocytes greater, no significant effect upon LW. Infected erythrocyte nuclei with lower values of length (F=21.047, P <0.001, d/f=1), width (F=12.174, P <0.001, d/f=1) and LW (F=31.136, P <0.001, d/f=1) than nuclei of uninfected cells. Gametocytes sexually dimorphic in width (F=5.261, P <0.05, d/f=1), macrogametocytes (Figs. 41-44) broader (7.7±1.1, N=16) than microgametocytes (Figs. 45-48, 6.8±0.6, N=11) with greater LW (F=5.020, P <0.05, d/f=1), 72.7±21.0 vs. 58.4±8.6. Length and L/W ratio not different significantly, macrogametocyte length 9.4±1.5 and L/W 1.23±0.17, in comparison to microgametocytes, 8.5±0.7 and 1.26±0.13, respectively. Nuclei of macrogametocytes 4-8 X 1.5-3.5

µm, usually marginal; chromatin diffuse in microgametocytes, and nucleoli not observed.

Taxonomic summary

Type host: Egernia stokesii (Dumeril) (Sauria: Scincidae)

Other hosts: None known.

Type locality: South Australia, Neuroodla (31°50'S, 138°08'W).

Additional localities: South Australia: Hawker (31°53'S, 138° 25'W); The Four Mile (31°50'S, 138°30"W); Quorn (32°15'S, 138° 05'W); Drakes Nob (32°05'S, 138°09'W); Island Lagoon (31°42'S, 136° 68'W); Chace Range (31°45'S, 138°35'W).

Site of infection: erythrocytes

Prevalence: 7% at type locality by examination of blood films

Type specimens: Hapantotype deposited in Queensland Museum, No. G462650 and parahapantotype, Queensland Museum No. G462651; other parahapantotypes retained for deposition with the Telford collection.

Etymology: The specific name refers to the characteristic immature schizonts that encircle the host cell nucleus.

Remarks

Plasmodium circularis is unique among all Plasmodium species yet described by the formation of immature schizonts that completely encircle the host cell nucleus (Figs. 25, 26), with apparent fusion of opposite tips of the cytoplasm into an unbroken ring. This character alone eliminates the need for detailed comparison with other described species. The commonly seen halteridial or dumbbell-shaped, apparently mature schizonts (Figs. 27-31, 33-39) are somewhat similar in appearance to the larger schizonts of Plasmodium (Sauramoeba) heischi (Garnham and Telford, 1984), described from the East African skink Mabuya striata. Gametocytes of P. heischi, however, are elongate and spindle-shaped,

and considerably larger than those of P. circularis. Gametocyte size relative to that of uninfected erythrocyte nuclei (1.8) and merozoite number (19-52) are consistent with characteristics of the subgenus Lacertamoeba (Telford, 1988), but relative schizont size (2.5) slightly exceeds that found in this subgenus (0.5-2.0 times erythrocyte nucleus LW). Segmenters are evidently briefly present in circulating blood, and both maturing schizonts and gametocytes appear to be seasonal in their occurrence, present most commonly in the Australian spring from September to December.

Three other Plasmodium species have been described from Australian lizards. Plasmodium australis Garnham was originally reported as P. giganteum (Mackerras, 1961,) classified later as a subspecies, P. giganteum australis (Garnham, 1966; Ayala, 1978; Telford, 1984), and as a distinct species (Telford, 1988). It is a parasite of the agamid lizard Amphibolurus barbatus in northern Queensland, and has large schizonts that can nearly fill the host erythrocyte, and elongate to bulky gametocytes. The range in merozoite numbers (22-49) and schizont size, estimated from illustrations (Mackerras, 1961) to be 14 X 7 and 11 X 9 µm, or LW of 98-99 μm^2, is similar to that of P. circularis. Gametocyte shape, however, is elongate or bulky, and estimated dimensions of 2 macrogametocytes (13 X 5 and 12 X 9 µm, or LW of 65-108 μm^2) compared with 1 microgametocyte (10 X 5 µm, LW 50 μm^2) suggest sexual dimorphism in size, typical of the subgenus Sauramoeba (Telford, 1988). Plasmodium egerniae, Mackerras from Egernia m. major of southern Queensland (Mackerras, 1961), produces very large schizonts (estimated size 14 X 11 µm, or LW of 162 μm^2) that can fill the host cell with 40-50 merozoites, and huge, elongate to bulky gametocytes that also fill the host erythrocyte. Measurements estimated from photographs (Mackerras, 1961) indicate dimensions of 18 X 9 and 14 X 11 µm (LW 162 and 154 μm^2) for 2 macrogametocytes, and 15 X 8 µm (LW 120 μm^2) for 1 microgametocyte. Both schizont and gametocyte sizes greatly

exceed those of P. circularis, although the numbers of merozoites apparently overlap. Neither schizonts nor gametocytes of P. circularis fill the host cell.

TAXONOMIC REDESCRIPTION

Plasmodium mackerrasae Telford 1979

(Figs. 49-80)

Asexual stages: Trophozoites 1.5 X 0.8 to 2-3 X 1.0-1.5 µm, elongate, triangular or rounded, without vacuole (Figs. 49-52, 65-67, 74, 75,77-79). Nucleus usually terminal, sometimes in middle. Pigment visible early, well before nuclear division begins. Binucleate schizonts (Figs. 51, 52, 74, 75, 77-80) 3 X 2 to 4 X 2.5 µm, LW averaging 7.6 μm_2. Immature tetranucleate schizonts (Figs. 52, 53, 66-68) 4-5 X 2.5-4 µm, averaging 15 μm^2. Mature or nearly mature schizonts (Figs. 54, 55, 65-67, 69-73) 5.1$\pm$1.1 X 3.7$\pm$1.1 µm (3-7 X 2.5-6.0 µm, N=25). Schizont size (LW) 19.8$\pm$9.3 μm^2 (7.5-42 μm^2). Merozoite number 4 to 14 (7.2$\pm$2.8, N=25), arranged variably, most commonly in oblong (32%) or elongate schizont (28%), rarely as rosette (4%), fan (8%), cruciform (8%), oval (12%), or in ill-defined manner. All erythrocytic, but never with distortion of the host cell or nucleus, nor displaced nucleus. Schizont size relative to infected erythrocyte nucleus 0.75$\pm$0.35 (0.29-1.75) and to normal erythrocyte nuclei 0.75$\pm$0.36 (0.28-1.62). Schizonts usually polar to the host cell nucleus (84%), seldom lateropolar (12%), or lateral (4%) in position. Erythrocytes parasitized by single schizont only no different in any dimension from normal cells. When 1-4 additional parasites present within cells, erythrocyte length ($F=11.206$, $P<0.01$, d/f=1), width ($F=62.699$, $P<0.001$, d/f=1), and LW ($F=52.960$, $P<0.001$, d/f=1) significantly enlarged, but nuclear dimensions no different from uninfected cells.

Sexual stages: Immature gametocytes 5-7 X 1.5-2.5 µm, elongate (Figs. 56-59), with a terminal nucleus, and a small cluster of pigment granules,

often producing multiple infections in host cells of 6 or more parasites. Sexually distinctive staining present in elongate gametocytes as small as 6 X 2.5 µm (Fig. 59). Mature gametocytes (Figs. 60-64, 68, 74-80) usually rounded, 5.8±0.9 X 4.6±0.7 µm (3.5-9.0 X 3.0-6.0 µm, N=75), with LW 26.7±6.6 μm^2 (10.5-42.0) and L/W 1.29±0.25 (1.0-2.3). Gametocyte size relative to infected erythrocyte nucleus size 0.98±0.26 (0.4-1.6), and to normal erythrocyte nuclei 1.03±0.25 (0.4-1.6). Pigment dispersed in one to several small dark granules. Vacuoles rarely present. Gametocytes predominantly polar (83%), seldom occupying lateral (4%) or lateropolar (13%) positions in the host cell. All erythrocytic, never distorting host cell, rarely displacing its nucleus (1%), or distorting it (4%). Gametocytes from intense active infections not sexually dimorphic in size or shape. Nuclei marginal in macrogametocytes (Figs. 60, 61, 74-77), usually lateral, sometimes polar to long axis of gametocyte, and usually diffuse in microgametocytes (Figs. 62-64, 78-80), imparting pink tint to the cell, but occasionally visible as indistinct mass, either polar or lateral. Nucleoli not noted. Gametocytes from infection in chronic phase, lacking schizonts but with numerous immature gametocytes, 6.1±0.9 X 4.6±0.5 µm (5.0-9.0 X 3.5-6.0 µm, N=25), with LW 27.8±4.5 μm^2 (20-36) and L/W 1.35±0.3 (1.0-2.3). Many gametocytes irregular in shape, from almost rectangular to slightly lobate, in contrast to more regular rounded or oval shapes in active infections. Chronic phase gametocytes with different mean dimensions of length and LW by sex. Macrogametocytes significantly greater in length (6.5±1.0 µm, F=5.116, P=0.034, d/f=1) and LW (29.9±3.9 μm^2, F=6.132, P=0.021, d/f=1) than microgametocytes (5.8±0.7 µm, 25.9±4.3 μm^2). Host erythrocytes and their nuclei, in intense infection where gametocytes were smaller in all dimensions and thus newly mature, no different in dimensions from uninfected cells except in LW (F=4.508, P=0.041, d/f=1). Each infected cell with 1-5 (3.0±1.3) additional parasites. In 2 other infections with larger gametocytes, 1 chronic with 1-3 (1.6±0.8) parasites per cell, and 1 active, intense

infection with 0-4 (2.1±0.9) additional parasites present per cell, erythrocyte lengths (F=17.515, P <0.001, d/f=1 and F=17.926, P <0.001, d/f=1, respectively), widths (F=5.622, P=0.237, d/f=1 and F=41.443, P <0.001, d/f=1, respectively), and LW (F=19.135, P <0.01, d/f=1 and F=55.553, P <0.001, d/f=1, respectively) significantly increased. Only width of their nuclei greater (F=5.492, P=0.025, d/f=1 and F=5.537, P=0.025, d/f=1) than that of uninfected cells.

Taxonomic summary

Host: Egernia stokesii (Duméril)

Other hosts: Egernia cunninghami (type host), E. striolata, E. whitei (experimental host).

Type locality: Southern Queensland, no other data.

Additional localities: South Australia, at any location where the host species occurs between 31°42'S and 32°15'S.

Site of infection: erythrocytes and connective tissue or endothelium in circulatory, respiratory, digestive, excretory, and muscular systems.

Prevalence: 5-54% by examination of blood films

Type specimens: Syntype from Telford collection deposited as hapantotype at Queensland Museum No. G462652; parasyntypes deposited as parahapantotypes at Queensland Museum No.s G462653, G462654, G462655, and G462656; other parasyntypes at Museum National d'Histoire Naturelle, Paris, in Garnham collection, and retained for deposition with the Telford collection.

Etymology: The specific name recognizes the contribution of M. Josephine Mackerras to Australian parasitology.

Remarks

Gametocytes of Plasmodium mackerrasae were compared with samples from 3 other Egernia species (Table I). Comparisons of gametocyte dimensions from E. stokesii 16 and 620 with those from E. whitei found

significant differences in length (F=23.715, P <0.001, d/f=1), LW (F=14.438, P <0.001, d/f=1) and L/W (F=8.753, P <0.01, d/f=1,) (Table I). Only gametocyte width in E. stokesii 620 did not differ from that of E. whitei gametocytes; width was significantly different (F=4.527, P=0.038, d/f=1) in E. stokesii 16. In a comparison of schizont characters (length, width, LW, nuclei) between samples from E. whitei and E. stokesii 16, only the mean number of nuclei differed significantly (F=24.389, P <0.001, d/f=1).

Three types of EE-schizonts were found. Occasionally thrombocytes were infected in E. stokesii with extremely high parasitemias. Those in fixed tissues were usually intensely stained (Figs. 81-89), with dimensions of 12-26 X 8-16 µm (17.2±4.2 X 10.7±2.7, N=19), varying in shape from round at 13 X 13 µm to elongate at 26 X 8 µm, with most ovoid. They were most common in heart (intertrabecular sites and pericardium) and often in clusters in blood vessel endothelium and connective tissue (Figs. 81, 82, 85, 87, 88) but occurred variably in the 4 lizards examined histologically in endothelium of pulmonary capillaries, and in connective tissue of kidney (Fig. 83), ovary, testis, striated muscle (Fig. 89), colon (Figs. 86, 88), urinary bladder (Fig. 84), and even fat body. Parasites seen in liver were in endothelium of blood vessels or sinuses, while most of those found in small intestine appeared to lie within the lamina propria. None were present in the splenic pulp, or connective tissue of the stomach and gall bladder.

Other schizonts, all extracellular, often of great size and highly variable form were found on a slide of circulating blood (Figs. 90-104). Some were oval to elongate in shape, 23-53 X 12-35 µm. Minimum numbers of nuclei from 5 schizonts were 55 in a schizont 23 X 15 µm; 71 in one 16 X 12 µm; 132 in a schizont 33 X 12 µm; 160 at 53 X 15 µm; and >300 in a schizont 44 X 35. There were many schizonts of irregular form, usually large (Figs. 102-104), sometimes occupying most of the microscopic field at 1,000X. Some were elongate and slender, others arachniform, with

jagged cytoplasmic processes. No attempt was made to count nuclei in these schizonts, but their numbers certainly extended into the upper hundreds or low thousands. Some of the very large schizonts were apparently releasing merozoites into the blood stream. On several occasions, blood smears showed a flood of free merozoites among the erythrocytes (Figs. 105, 106), without presence of the large, irregular schizonts. Relapses occurred among captive lizards from 1-20 months (8.7 months) post-capture, with parasitemias varying from 5-85%.

DISCUSSION

Plasmodium mackerrasae was originally isolated into E. whitei from a chronic infection in E. cunninghami (Telford, 1979), in which only 3 gametocytes and a few immature gametocytes were found. The few gametocytes available from the type host did not differ in any dimension from those in the chronic infection, comprised only of mature gametocytes, from E. striolata. However, comparisons of the 3 samples from E. stokesii among themselves found comparable significant differences in mean gametocyte width and LW, and in 2 of 3 comparisons of length with differences found among the other three hosts. Only gametocyte L/W did not differ significantly among the 3 samples. Given the overlap in ranges of all gametocyte characters from the 4 host species and in schizont characters from 2 hosts, it is concluded that a single species is present, P. mackerrasae. Mean differences in dimensions by themselves among samples from congeneric hosts probably are insufficient for taxonomic distinction if the characters used show substantial overlap in ranges. Phase of infection and host immunity might influence dimensional mean values. As with the type material, the morphometric characteristics of P. mackerrasae in E. stokesii are consistent with those of the subgenus Lacertamoeba Telford 1988.

The designation of a parasite from the gecko Phyllodactylus marmoratus as a separate genus Billbraya australis Paperna and Landau (Paperna

often producin[illegible] ...rd, 1994) on the basis that none of
Sexually dis[illegible] ...ing it from Plasmodium was
6 X 2.5 [illegible] ...Plasmodium mexicanum. Its
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LW 2[illegible] ...australis is preoccupied. It
size[illegible] ...6-11.2 µm) than P. circularis, but
tr[illegible] ...ped, merozoite numbers being limited to
...its observed. The most distinctive character
...ultiple infections of 5-12 immature gametocytes in
...ythrocytes. However, 5-8 immature gametocytes in multiple
...ion were commonly seen in the P. mackerrasae infections from E. stokesii. The study of additional infections is sorely needed.

Allocation to Plasmodium species of exoerythrocytic (EE) schizonts observed (Figs. 81-104) cannot be made with certainty, in view of the presence of mixed infections in some lizards. Plasmodium mackerrasae was present in every lizard positive for malarial parasites, often at very high parasitemia levels, and very likely produced the EE-schizonts found in tissues (Figs. 81-89) and circulating blood (Figs. 90-104). Forms similar to those described for Plasmodium sasai (Telford, 1989, 1994, 1996, 1998) and Plasmodium mexicanum (Thompson and Huff, 1944) were present in fixed tissues, and presumably represent phanerozoites (Figs. 81-89). On slides of circulating blood, compact schizonts with a regular shape (Figs. 90-92, 99, 100) could have been phanerozoites of similar origin to those described above. Some more irregular schizonts (Figs. 93-98, 101) were similar in appearance to two extracellular schizont fragments reported in infections of Saurocytozoon tupinambi in its natural host (Lainson et al., 1974; Telford, 1978). Those very large schizonts, with highly variable form (Figs. 102-104), often elongate or jagged in appearance with irregular cytoplasmic projections, must have formed within the endothelial surfaces of blood vessels or sinuses. No similar descriptions are available in the literature of the Plasmodiidae, or indeed,

of the Haemosporidia. Smaller masses, formed within cerebral capillaries in lizards naturally or experimentally infected by P. mexicanum have been reported (Ayala, 1970; Telford, 1984), but it was suggested (Telford, 1984) that they might be pathologic, rather than a normal stage in the life cycle. The presence of merozoites flooding the blood stream (Figs. 105, 106) perhaps resulted from a synchronous rupture of intracellular phanerozoites. This massive flooding of the circulatory system with merozoites, not previously reported for plasmodiid parasites, possibly contributed to the high prevalence of erythrocytes multiply infected by P. mackerrasae, a characteristic of most infections examined during this study.

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and Landau, 1990) was criticized (Telford, 1994) on the basis that none of the characters supposedly distinguishing it from Plasmodium was exclusive, inasmuch as all are present in Plasmodium mexicanum. Its redescription as a Plasmodium species, using a different specific name was recommended because the specific name australis is preoccupied. It has larger gametocytes (9.8-19.2 X 4.8-11.2 µm) than P. circularis, but mature schizonts were not described, merozoite numbers being limited to 6-8 in the larger schizonts observed. The most distinctive character appears to be multiple infections of 5-12 immature gametocytes in individual erythrocytes. However, 5-8 immature gametocytes in multiple infection were commonly seen in the P. mackerrasae infections from E. stokesii. The study of additional infections is sorely needed.

Allocation to Plasmodium species of exoerythrocytic (EE) schizonts observed (Figs. 81-104) cannot be made with certainty, in view of the presence of mixed infections in some lizards. Plasmodium mackerrasae was present in every lizard positive for malarial parasites, often at very high parasitemia levels, and very likely produced the EE-schizonts found in tissues (Figs. 81-89) and circulating blood (Figs. 90-104). Forms similar to those described for Plasmodium sasai (Telford, 1989, 1994, 1996, 1998) and Plasmodium mexicanum (Thompson and Huff, 1944) were present in fixed tissues, and presumably represent phanerozoites (Figs. 81-89). On slides of circulating blood, compact schizonts with a regular shape (Figs. 90-92, 99, 100) could have been phanerozoites of similar origin to those described above. Some more irregular schizonts (Figs. 93-98, 101) were similar in appearance to two extracellular schizont fragments reported in infections of Saurocytozoon tupinambi in its natural host (Lainson et al., 1974; Telford, 1978). Those very large schizonts, with highly variable form (Figs. 102-104), often elongate or jagged in appearance with irregular cytoplasmic projections, must have formed within the endothelial surfaces of blood vessels or sinuses. No similar descriptions are available in the literature of the Plasmodiidae, or indeed,

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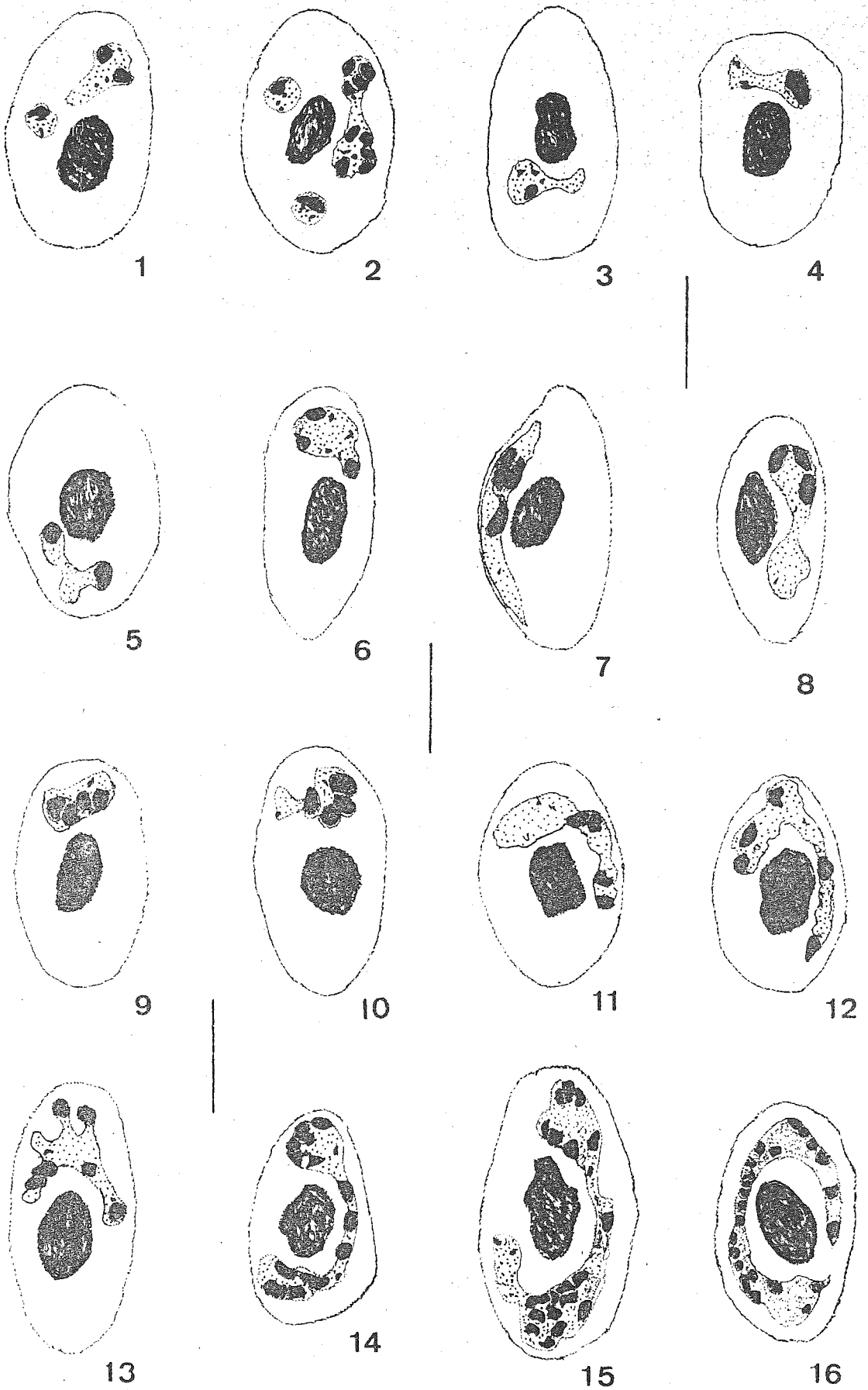
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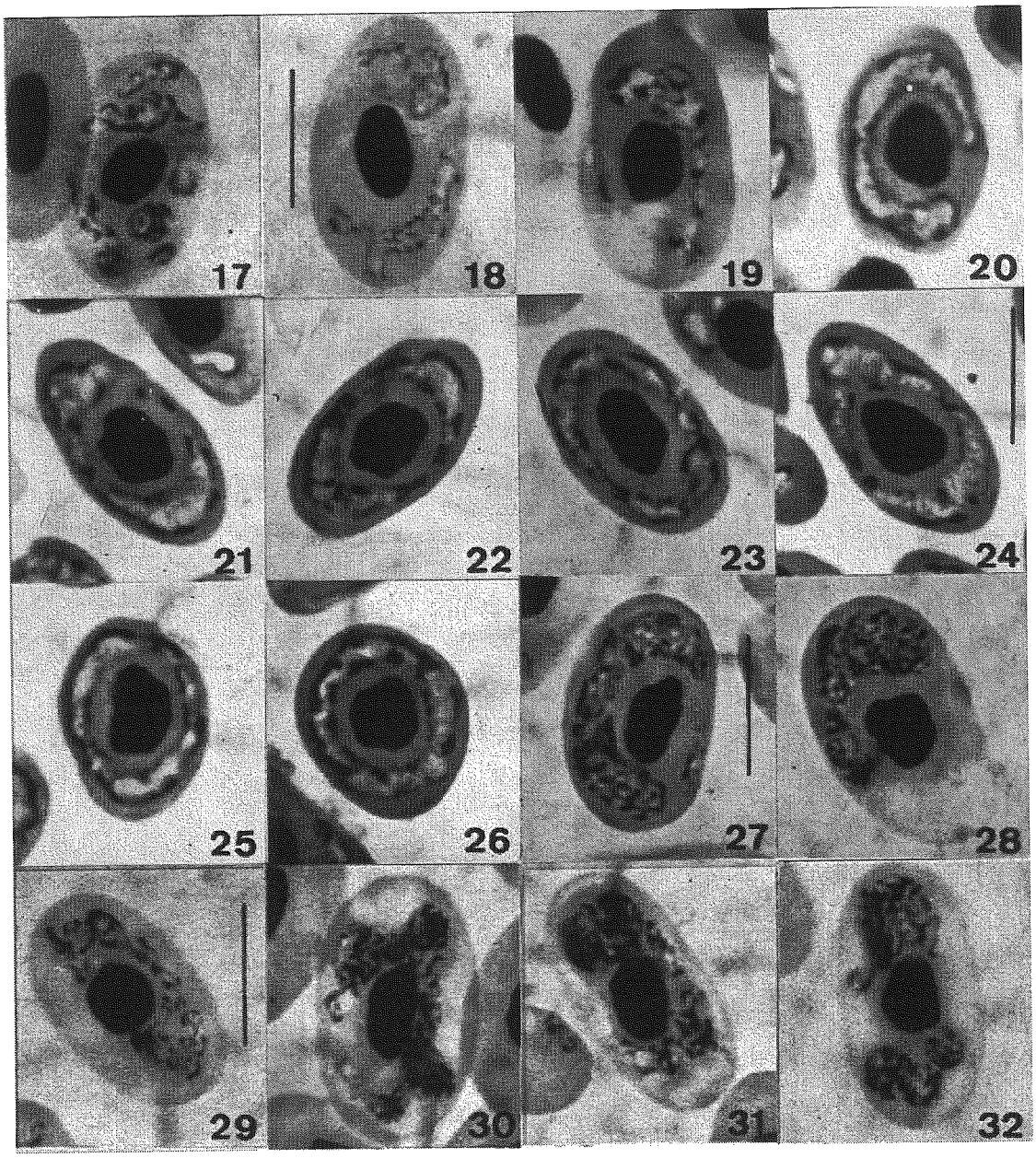
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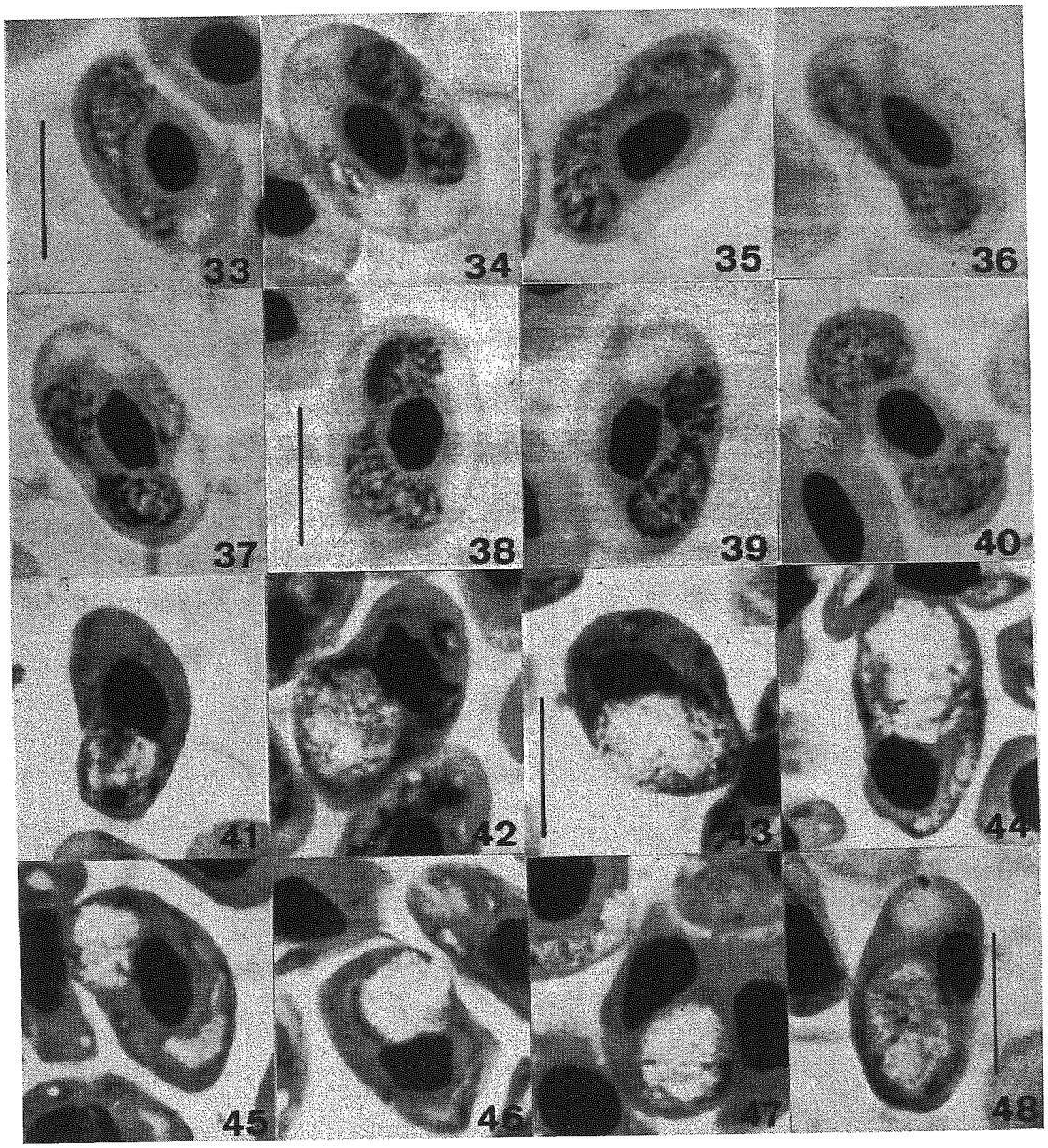
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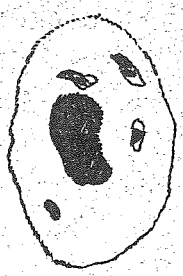
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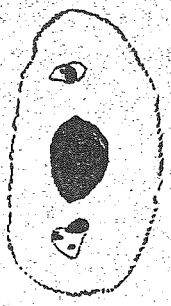
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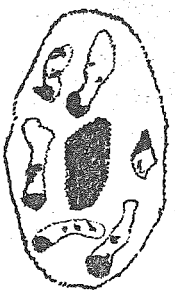
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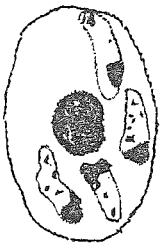
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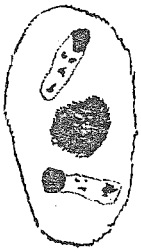
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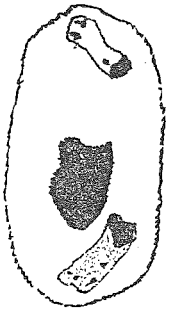
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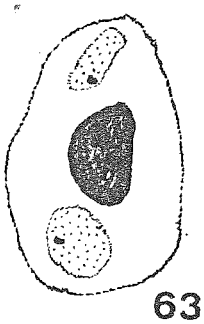
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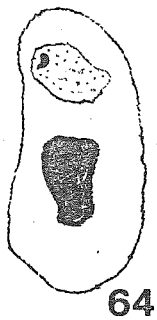
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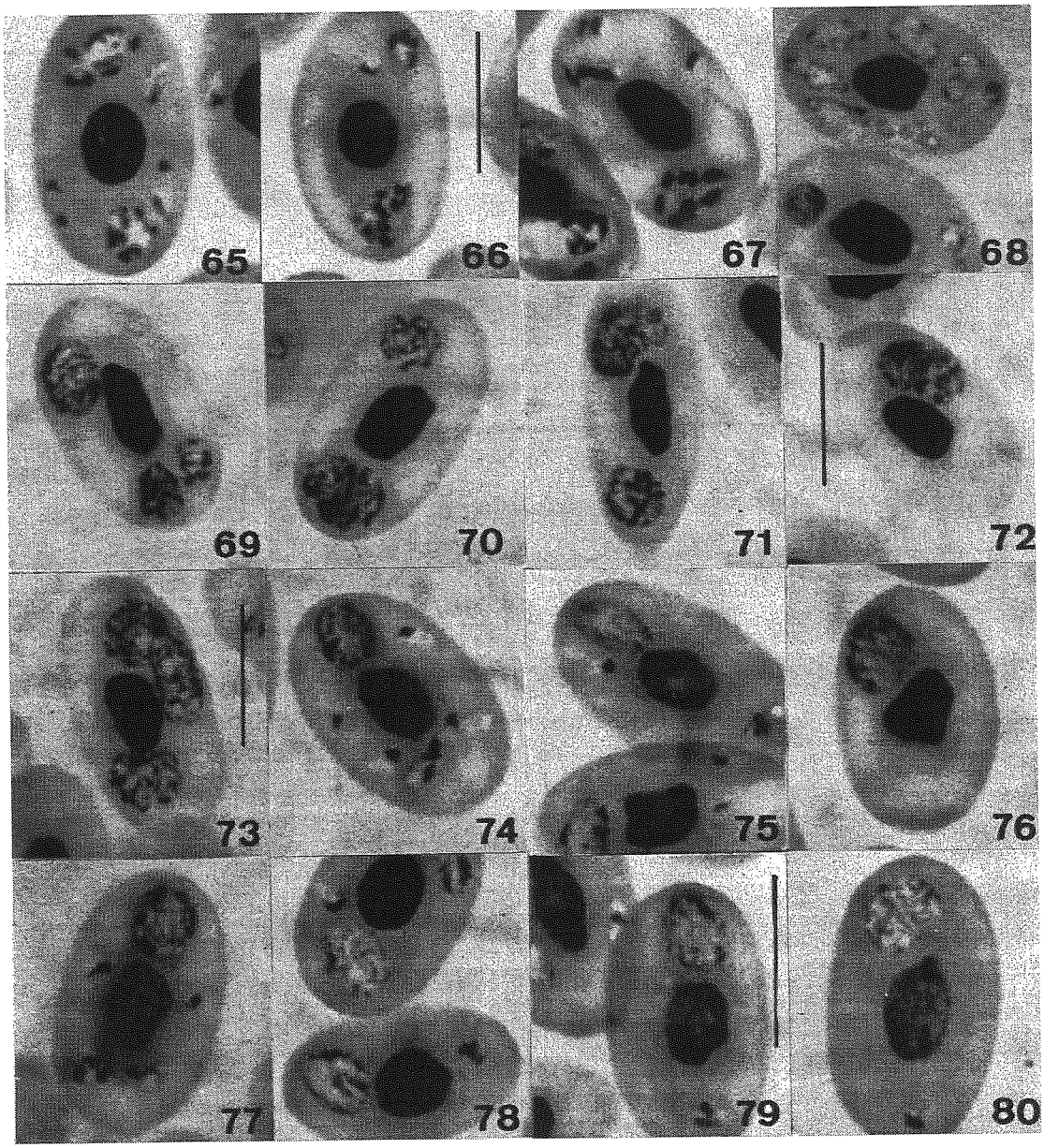
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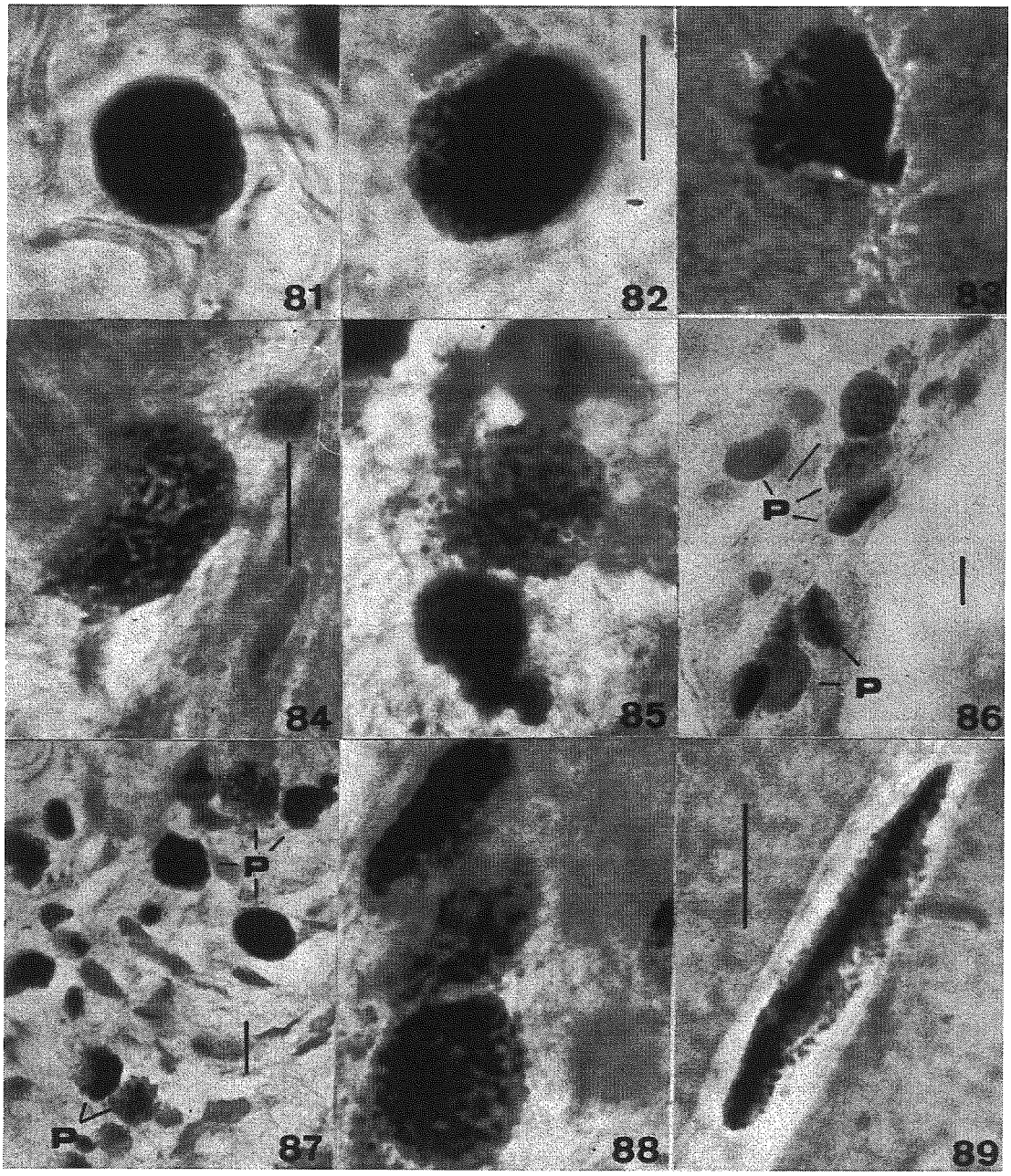
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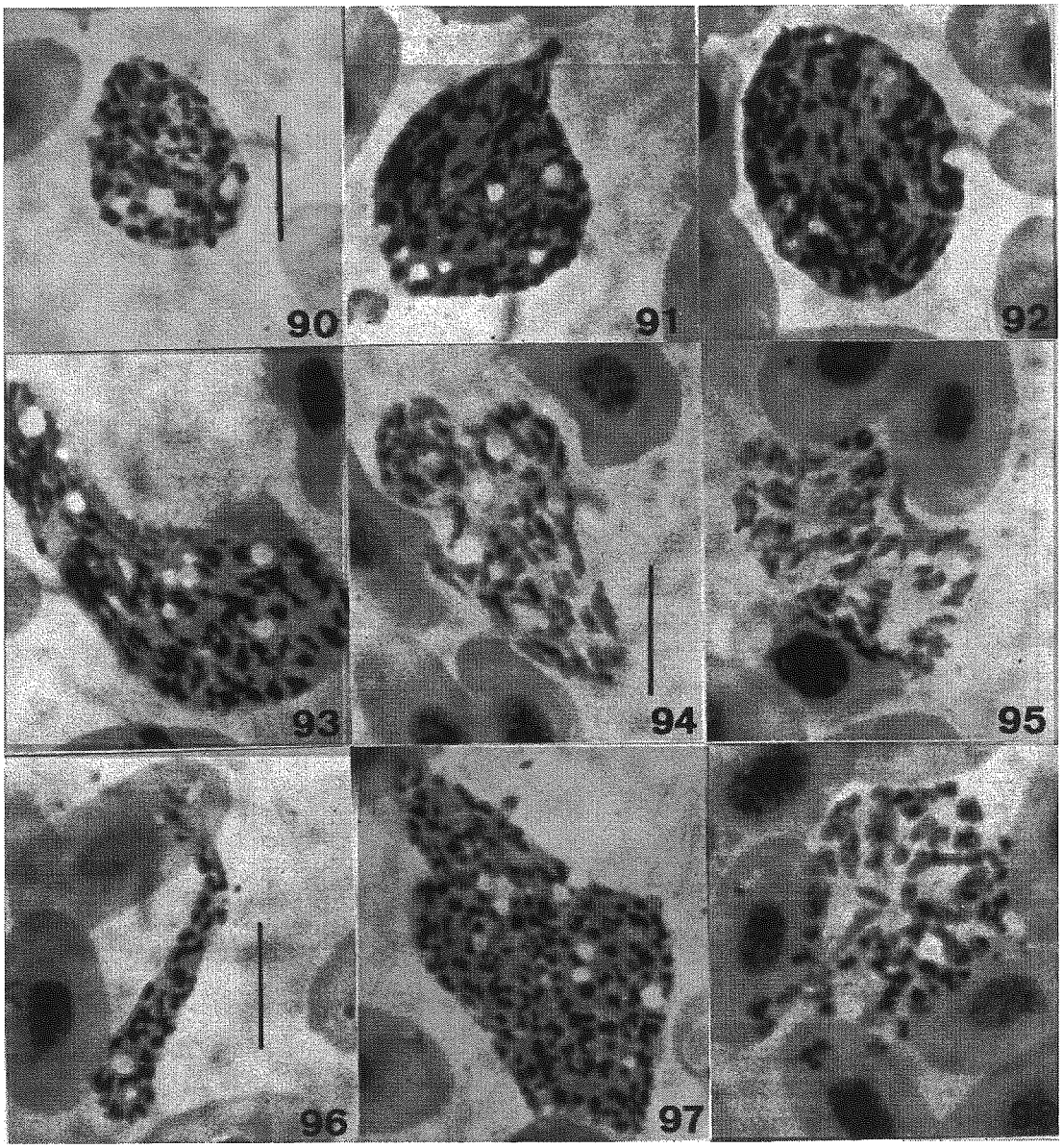
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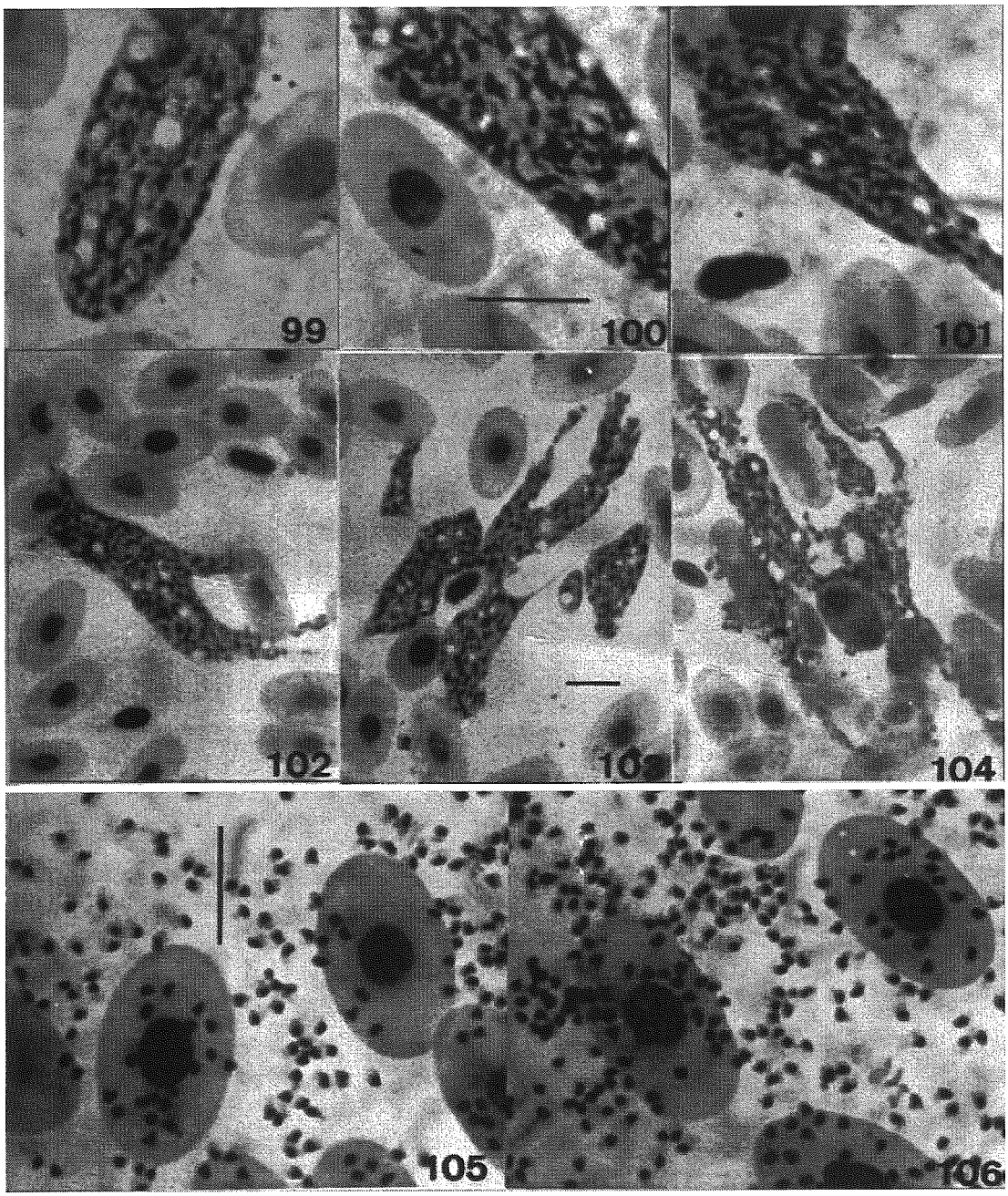
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Legends to Figures

Figures 1-16. Trophozoites and immature schizonts of Plasmodium circularis n. sp. from Egernia stokesii. **1-4** Trophozoites. **1,5** Binucleate schizonts. **6-11** Trinucleate and tetranucleate schizonts. **2,12-14** Schizonts at 4th nuclear division. **15,16** Schizonts at 5th nuclear division, curving around erythrocyte nucleus. Scale bar = 10 µm.

Figures 17-32. Schizonts of P. circularis and P. mackerrasae. **17** Three young schizonts of P. circularis and 3 asexual stages of P. mackerrasae (lower right quarter of erythrocyte) within same cell. **18,19** Young schizonts of P. circularis. **20-26** Immature schizonts of P. circularis incompletely (20-24) and completely (25 and 26) encircling erythrocyte nucleus. **27-31** Nearly mature schizonts of P. circularis. **32** Nearly mature, broken schizont. Scale bar = 10 µm.

Figures 33-48. Nearly mature schizonts and mature gametocytes of P. circularis. **33-37** Nearly mature schizonts. **38,39** Mature schizonts at onset of segmentation. **40** Segmenting schizont broken at midpoint. **41-44** Macrogametocytes. **45-48** Microgametocytes. Scale bar = 10 µm.

Figures 49-64. Plasmodium mackerrasae in E. stokesii. **49-52** Trophozoites. **51,52** Binucleate schizonts. **52,53** Tetranucleate schizonts. **54,55** Schizonts nearing maturity. **56-59** Immature gametocytes. **59,60** Nearly mature macrogametocytes. **60,61** Macrogametocytes. **62-64** Nearly mature and mature microgametocytes. Scale bar = 10 µm.

Figures 65-80. Plasmodium mackerrasae in E. stokesii. **65-67** Trophozoites and schizonts in multiply infected erythrocytes, with a hemococcidian at lower left. **68** Twelve asexual parasites in host cell (upper), macrogametocyte and young schizonts in lower cell. **69-72** Mature schizonts. **73** Three mature schizonts in erythrocyte. **74-77** Macrogametocytes, usually in multiple infection with trophozoites and young schizonts. **78-80** Microgametocytes in multiple infections with asexual stages.

Figures 81-89. Phanerozoites, probably of *P. mackerrasae*, in tissues of *E. stokesi*. **81,82** In endothelium or connective tissue of a major blood vessel. **84** In connective tissue of urinary bladder. **85** In connective tissue of blood vessel in heart. **86,87** clusters of phanerozoites (**P**) in colon wall and blood vessel of Heart. **88** In connective tissue of colon wall. **89** In connective tissue between striated muscle fibers. Scale bar = 10 µm.

Figures 90-98. Phanerozoites, probably of *P. mackerrasae*, in circulating blood of *E. stokesi*. Scale bar = 10 µm.

Figures 99-104. Phanerozoites, probably of *P. mackerrasae*, in circulating blood of *E. stokesi*. Scale bar = 10 µm.

Figures 105 and 106. Merozoites, probably of *P. mackerrasae*, flooding the circulating blood of *E. stokesi*. Scale bar = 10 µm.

TABLE 1. Comparison of *Plasmodium mackerrasae* gametocytes from *Egernia stokesii* with infections in two additional natural hosts and an experimental host.

Host type *Egernia* species	number measured	gametocyte length (μm)	width (μm)	LW (μm^2)	L/W
natural					
stokesii I	25	5.1±0.7 (3.5-6.5)	4.1±0.6 (3.0-5.5)	21.3±4.6 (10.5-33.0	1.26±0.24 (1.0-1.7)
stokesii II	25	6.2±0.7 5.0-8.0)	5.0±0.6 (4.0-6.0)	31.0±6.3 24.0-42.0)	1.25±0.18 (1.0-1.6)
stokesii III	25	6.1±0.9 (5.0-9.0)	4.6±0.5 (3.5-6.0)	27.8±4.5 (20.0-36.0)	1.35±0.29 (1.0-2.3)
striolata	25	6.5±0.9 (5.0-8.0)	5.6±1.0 (4.0-7.0)	37.0±10.7 (20.0-56.0)	1.17±0.12 (1.0-1.5)
cunninghami	3	6.3±0.6 (6.0-7.0)	6.0±1.0 (5.0-7.0)	38.3±9.7 (30.0-49.0)	1.07±0.12 (1.0-1.2)

TABLE 1. (continued)

Host type *Egernia* species	number measured	gametocyte length (µm)	width (µm)	LW (μm^2)	L/W
experimental					
whitei 11.08.72	40	5.4±0.6 (4.0-7.0)	4.8±0.5 (4.0_6.0)	26.3±4.8 (16.0-36.0)	1.13±0.12 (1.0-1.4)
whitei 16.08.72	28	5.3±0.7 (4.0-7.0)	4.6±0.7 (3.0-6.0)	24.6±6.2 (12.0-36.0)	1.16±0.15 (1.0-1.5)

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