7. Surface features and attachment mechanism of the diplectanid monogenean, *Lepidotrema bidyana* Murray, 1931

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7.1 Abstract

Lepidotrema bidyana (Monogenea: Diplectanidae) is a common problematic parasite on silver perch *Bidyanus bidyanus* farms. The effect of *L. bidyana* on silver perch remains poorly understood and infections are therefore often left unmanaged on farms. Specimens of *L. bidyana* were examined by scanning electron microscopy (SEM) and histopathology was conducted on gills infected with *L. bidyana*. When adult *L. bidyana* attach to the host gills the hamuli penetrate the gill epithelium, the haptoral margin stretches and the hooklets penetrate and grip the gill epithelium, then the haptor contracts, pushing the accessory spines into the gill epithelium at the base of the interlamellar space and finally the intrinsic body musculature causes the body to expand dorso-ventrally, pressing the rodlet rows of the squamodisc against the adjacent lamellae wall, further maintaining attachment through friction. Attachment causes lateral displacement of the secondary lamellae with associated epithelial distortion and punctures of the epithelium, which perforate blood vessels. Juvenile parasites attach at the base of the interlamellar space by gripping the epithelium with the hooklets, with the majority of the body of the worm in the space between the secondary lamellae. High intensities of *L. bidyana* are associated with white outgrowths on the distal half of gill filaments, which histology demonstrated were round-to-oval shaped granular basophilic cysts within the epithelial cells, consistent with epitheliocystis. It is likely that *L. bidyana* infections facilitate secondary infections, including bacteria, characterised as epitheliocystis.

7.2 Introduction

The Diplectanidae includes monogeneans that typically infect the gills of freshwater and marine perciform teleosts around the world (Oliver, 1993; Desdevises et al., 2001; Domingues and Boeger, 2008) and are characterised by possessing a haptor with two pairs of large lateral hamuli and three bars, 14 small hooklets and a dorsal and/or ventral squamodisc (Bychowsky, 1957; Desdevises et al., 2001; Domingues and Boeger, 2008; Sánchez-García et al., 2011). Diplectanids can be problematic in aquaculture (Katharios et al., 2006; Dezfuli et al., 2007). *Lepidotrema bidyana* is recorded only from the gills of silver perch, *Bidyanus bidyanus* (Mitchell), an Australian endemic freshwater fish, which has potential for aquaculture (Rowland, 2009). *Lepidotrema bidyana* feeds on host mucus and epithelial cells and possesses two pairs of dorsal and ventral hamuli, a dorsal and a ventral squamodisc and one row of nine spines convergent behind each squamodisc (Murray, 1931; Young, 1969). The current recommended treatment for *L. bidyana* is formalin (FOR) (Rowland et al., 2006; Read et al., 2007) and praziquantel (PZQ) has shown potential as an alternative (Forwood et al., 2013) but due to a lack of knowledge about the host impact, *L. bidyana* is often left unmanaged in aquaculture (M. Landos. pers. comm.).

Gill dwelling monogeneans have evolved diverse attachment strategies and haptor structures (Bychowsky, 1957; Kearn, 1994; Sánchez-García et al., 2011). Different attachment mechanisms used by different parasites cause variable structural damage to host gills. Diplectanids such as *Diplectanum aequans* cause major structural damage and marked pathological changes in the gills (Dezfuli et al., 2007; Sánchez-García et al., 2011), while *Lamellodiscus* spp. cause minor structural damage to the gills and appear to have little associated pathology (Katharios et al., 2006; Sánchez-García et al., 2011). In high intensity infections, however, *Lamellodiscus* spp. can

decrease host growth (Katharios et al., 2006). Pathological changes associated with *Lepidotrema* spp. infections are not described, but stereomicroscope observations of gill tissue indicate that *L. bidyana* caused epithelial hyperplasia and the formation of white out-growths (Rowland et al., 2006).

Here we describe the surface features of *L. bidyana*, the structures that facilitate attachment, the mode of attachment and the resultant pathology, to better inform management decisions.

7.3 Materials and methods

7.3.1 Source of parasites

Lepidotrema bidyana were obtained from infected silver perch sourced from Pioneer Fish Farm (Gloucester, NSW) and maintained in a 2 000 L fibre glass recirculation tank at Flinders University, South Australia. Upon arrival five fish were examined to confirm the presence of the parasite following Forwood et al. (2012) and infection was maintained by cohabitation of infected fish (Hirazawa et al., 2004). Twenty fish were randomly sampled from the source population and euthanized with an overdose (a 40 mL / 1000 L bath) of Aqui-S® (Aqui-S NZ, Lower Hutt, New Zealand), the gill baskets were removed, separated and viewed under a dissection microscope. Mean intensity of *L. bidyana* was 346 ± SD 249 (54 – 1476). Parasites were removed for individual study or attached to pieces of gill.

7.3.2 Scanning electron microscope and histology processing

For scanning electron microscopy *L. bidyana* were fixed in 2% glutaraldehyde in 0.025 M sodium cacodylate buffer, ten free and ten anchored to the gill filament. Samples were dehydrated in a graded ethanol series, immersed in 1:1 dry analytical grade ethanol and hexamethyldisilazane (HMDS) for 10 mins, transferred to 100%

HMDS for 20 min and allowed to air dry for 12 h. Dried specimens were mounted on aluminium stubs using conductive carbon tape, and examined and photographed using a scanning electron microscope (SEM) (FEI Phenom). For the histology study, ten left anterior gill arches were removed from freshly euthanased silver perch and immediately fixed in 10% neutral buffered formalin (NBF). The gills were processed and embedded in paraffin, sectioned at 5 μm, stained with haematoxylin and eosin (H & E), observed using a compound microscope and photographed (Nikon)

7.4 Results

7.4.1 Surface features of *L. bidyana*

Both the dorsal and ventral hamuli have the distal part of the hamulus extending from the tissue but the distal part of the dorsal hamuli are not obviously hooked in SEM images (Fig 7.1A-D). Dorsal and ventral squamodiscs are similar in size and structure each containing 12 rows of anteriorly directed rodlets, the anterior eight rows are rounded distally, whereas the posterior four rows have a distal point. At the base of each squamodisc nine spines with a distal hook extend posteriorly from the squamodisc and have an overlying membrane (Fig 7.1B - D). Each squamodisc is a complete, cupped structure. The body of the worm is covered in layered, anteriorly directed, distally rounded scales from the base of the haptor, which become less prominent anteriorly (Fig 7.1A-D).

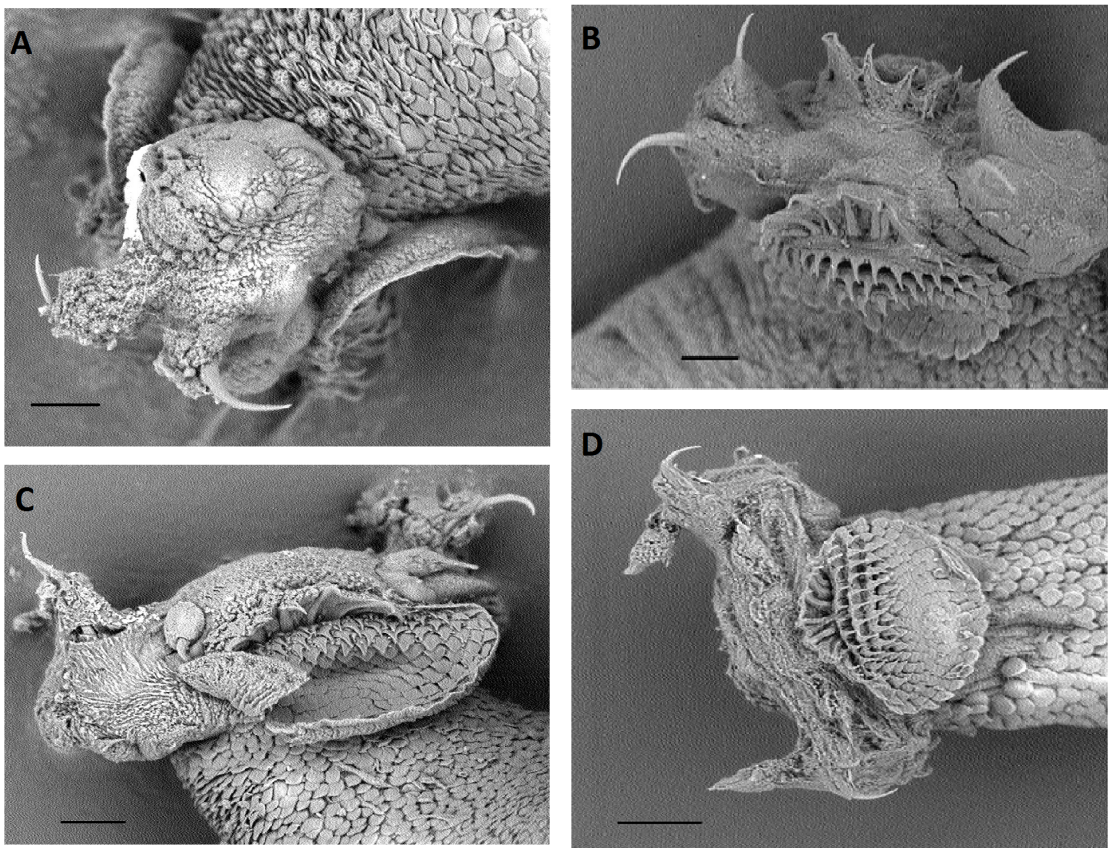

Figure 7.1: SEM images of detached *Lepidotrema bidyana*: Lateral view (A); top view (B); dorsal view (C); and ventral view (D) of the haptor of *L. bidyana*. Scale bars: A, B and C = 12.5 µm; D = 17.5 µm.

7.4.2 Attachment by *L. bidyana*

Lepidotrema bidyana adults attach to the gills by placing the haptor between an adjacent pair of secondary lamellae (Fig 7.2A). The hamuli curve laterally and penetrate the epithelium then the haptor contracts, which push the accessory spines into the gill epithelium at the base of the interlamellar space (Fig 7.2B and C). The intrinsic body microstructure causes the body to expand dorso-ventrally, pressing the rodlet rows of the squamodisc against the adjacent lamellae wall, further maintaining attachment through friction and suction (Fig 7.2B and D). The edges of the haptor extend and the hooklets penetrate the walls of the adjacent secondary lamellae, holding the edges of the haptor to the surface. The combined effect of the hamuli penetrating the basement membrane and the accessory spines and hooklets attaching

to the epithelium secures the worm in place (Fig 7.3A and B). Juvenile worms attach at the base of the interlamellar space by gripping the epithelium with the hooklets (Fig 7.2E). The majority of the body of attached juvenile worms remained in the interlamellar space and the hamuli of juvenile worms did not appear to function in attachment.

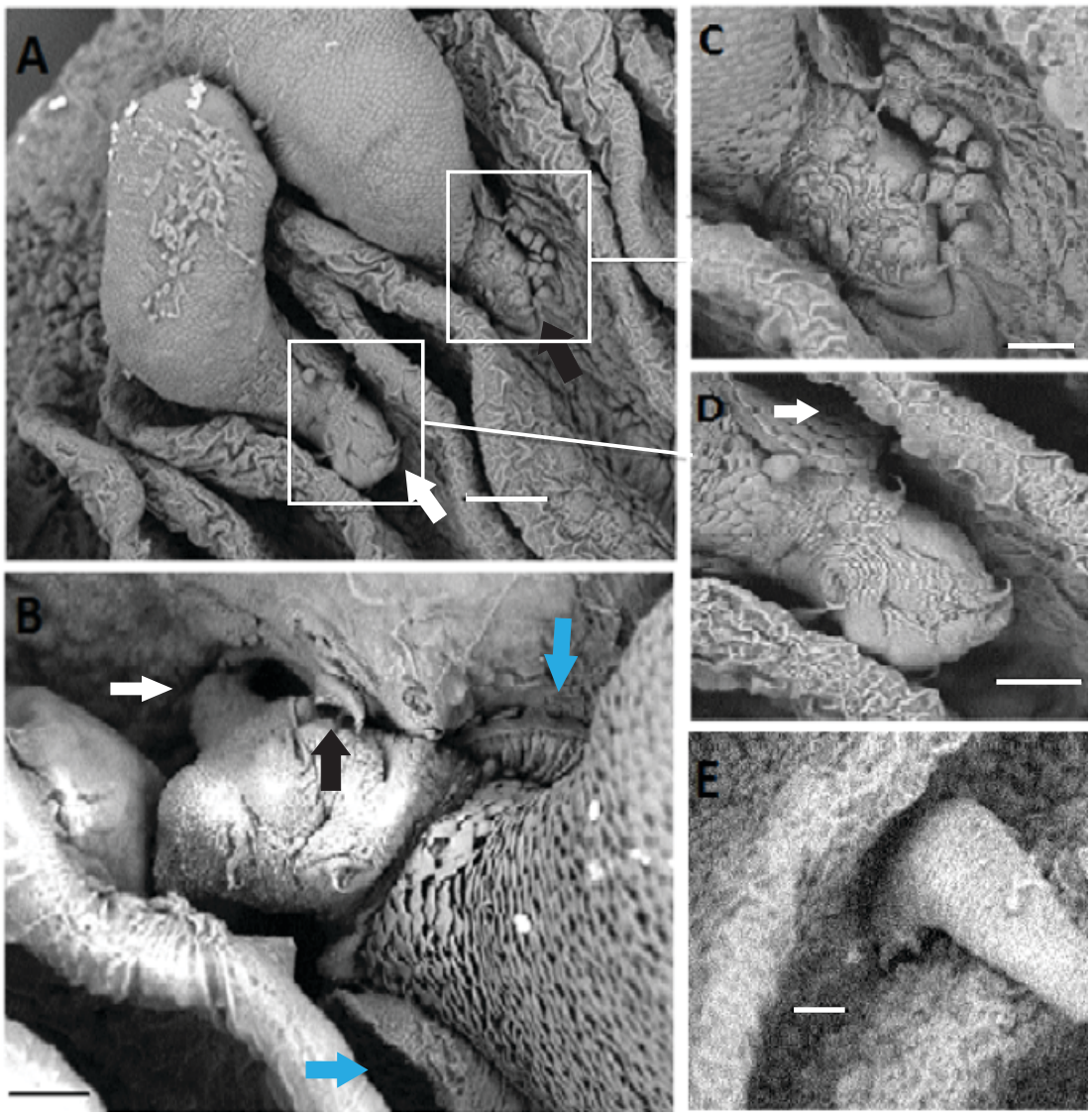

Figure 7.2: SEM pictures of attached *Lepidotrema bidyana* from *Bidyanus bidyanus*: Two *L. bidyana*, one fully attached with the haptor penetrating the space between the two seconday lamellae (black arrow) and one semi-attached inbetween the seconday lamellae (white arrow) (A); *L. bidyana* attached to gill fillament, with haptor penetrating the gill epithilum (white arrow), marginal hooks penetrating and tearing the gill epithilium (black arrow) and dorsal and ventral squamodiscs (blue arrows) (B); detail of the hamuli penetrating the gill epithelium (C); detail of the dorsal squamatodiscs aiding in

attachment (white arrow) (D); Juvenile *L. bidyana* using hooklets to form an attachment in-between the secondary lamellae (E). Scale bars: A = 25 µm, B, C, D and E = 10 µm,.

Attachment caused up to 36 punctures in the gill epithelium (Fig 7.3C). The worm pushes the secondary lamellae laterally (Fig 7.3B). Distortion and displacement of the epithelium and perforation of blood vessels was observed (Fig 7.3A and B). Round-to-oval granular basophilic cysts were observed, consistent with epitheliocystis (Fig 7.4). Tissue changes associated with attachment appear minor.

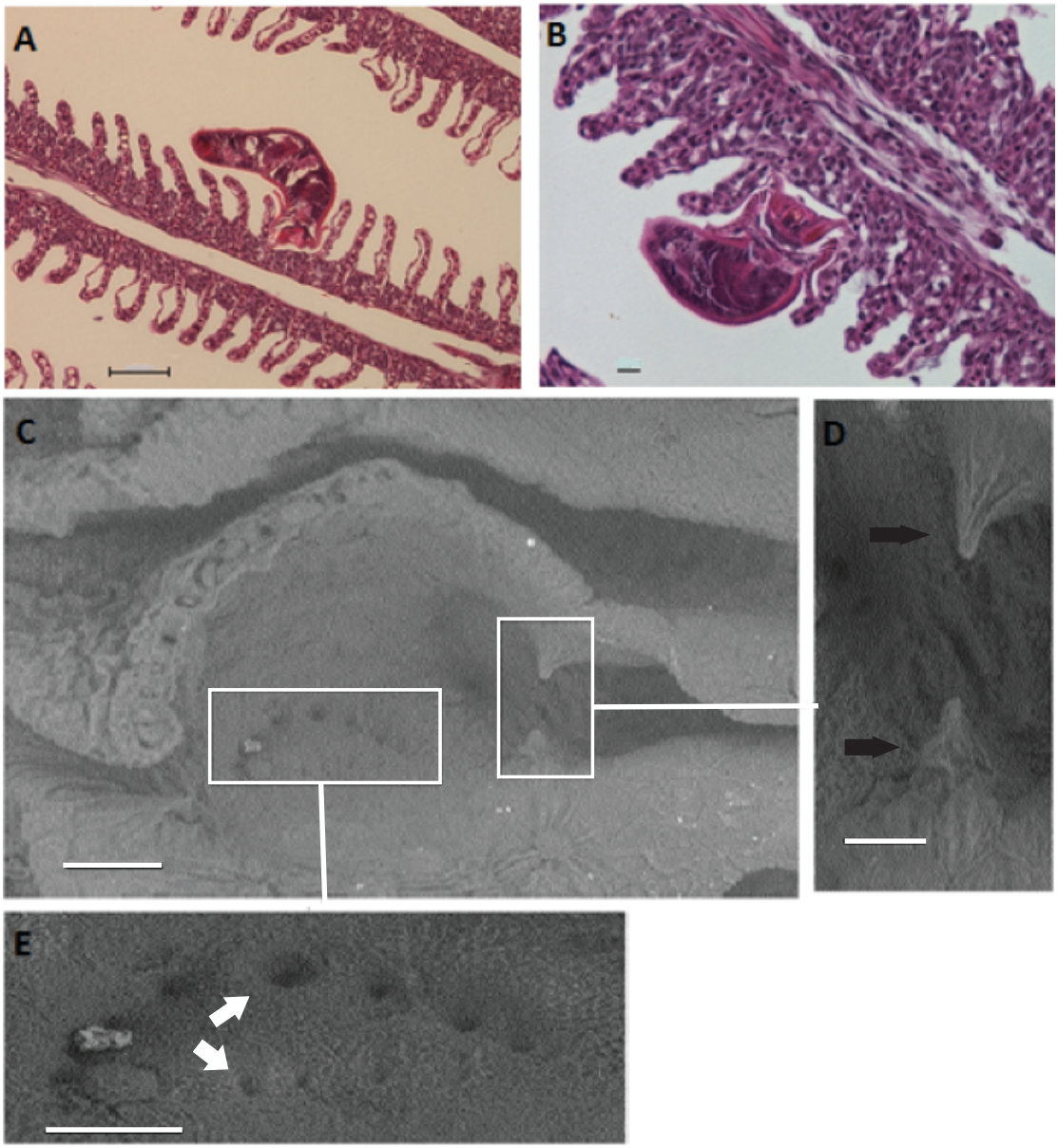

Figure 7.3: *Lepidotrema bidyana* from *Bidyanus bidyanus*. A and B, H-E sections: longitudinal section of *L. bidyana* attached to the gill filament (A); detail of the haptor penetrating the lamellae during attachment (B). C-E, SEM images: *L. bidyana* haptor impression on the primary and secondary lamellae, with marked depressions at the base of the interlamellae space (white arrows) and epithelial swelling on each side of the secondary lamellae (black arrows) (C); D and E, details of Fig C, epithelial perforations produced by the 9 accessory spines (E), epithelial swelling produced by the marginal hooks (D). Scale Bars: A = 100 µm; B = 10 µm; C = 15 µm; D-E = 7.5 µm.

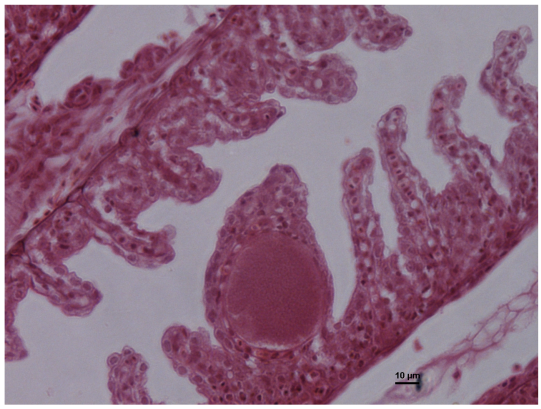

Figure 7.4: Epitheliocystis from *Bidyanus bidyanus*. H-E sections: longitudinal section of round-to-oval shaped granular basophilic cysts. Scale Bar = 10 µm.

7.5 Discussion

Diplectanids with different attachment mechanisms cause varying structural damage to the host. The hamuli of *Lepidotrema bidyana* created point penetrations of the host tissue and attachment induced moderate epithelial displacement and perforation of epithelia and blood vessels. The squamodiscs created friction against the secondary lamellae wall, but SEM showed that they could also probably independently create suction that decreases the likelihood of the worm being detached. *Diplectanum aequans* is a comparatively large (2 – 3 mm) diplectanid, compared to *L. bidyana* (1 mm), whose hamuli and squamodisc spines penetrate the host epithelium deeply and cause severe disruption of the epithelium (Dezfuli et al., 2007). *Diplectanum aequans* can induce a severe hyperplastic response with disruption and fusion of the secondary lamellae and marked inflammation (González-Lanza et al., 1991; Dezfuli et al., 2007; Sánchez-García et al., 2011). In high intensities, *D. aequans* causes host death (Dezfuli et al., 2007). *Lepidotrema bidyana* infections did not cause substantial epithelial disruption or an inflammatory response in this study. The pathology of *L. bidyana* is consistent with that of *Lamellodiscus* spp. in which penetration during attachment is also shallow and epithelial disruption is minor (Sánchez-García et al., 2011). Infections by *Lamellodiscus* spp. are associated with mild hyperplasia at the

base of the secondary lamellae and, rarely, hypertrophy of the gill epithelial cells (Katharios et al., 2006; Sánchez-García et al., 2011). *Lamellodiscus* spp. are not highly pathogenic and are not generally associated with host mortality (Athanassopoulou et al., 2009). Katharios et al. (2006) suggested that high intensity *Lamellodiscus* spp. infections reduce the area available for respiration causing hypoxia and numerous epithelial punctures, which lead to osmoregulatory dysfunction. This is consistent with our observations of heavy (> 1000 parasites per host) *L. bidyana* infections in aquaculture.

Rowland et al. (2007) stated that high intensity *L. bidyana* infections caused reduced appetite and growth, increased stress and facilitated secondary infections. Forwood et al. (2012) reported a strong correlation between higher *L. bidyana* intensities and silver perch condition, suggesting that high intensities of *L. bidyana* are associated with reduced host condition. High intensity *L. bidyana* infections could induce a stress response and decreased feed intake (Bonga, 1997). Quantifying the impact of *L. bidyana* intensity on the host would facilitate development of a 'trigger level' to inform when benefits of treatment outweigh costs and stress associated with treatment.

Monogenean infections facilitate secondary infections (Paperna, 1991). Epithelial disruptions may permit entry of microbial pathogens (Cusack and Cone, 1986; Buchmann and Bresciani, 1998). Attachment by *L. bidyana* caused up to 36 epithelial punctures (Fig 7.3), which could facilitate the entry of secondary pathogens. Histopathology revealed that the "white outgrowths" of Rowland et al. (2006) were likely epitheliocystis, which has been reported from silver perch (Frances et al., 1997). Co-infections with monogeneans and epitheliocystis are common (reviewed by Nowak and LaPatra, 2006) and caused ongoing chronic mortality in juvenile

seabream *Sparus aurata* (see Padros and Crespo, 1995). The relationship between *L. bidyana* infections and secondary pathogens including the oomycete *Saprolegnia parasitica*, which is problematic on silver perch farms (Lategan et al., 2004), warrants further investigation.

Bath treatments are often ineffective against small gill monogeneans (Chisholm and Whittington, 2002). Such worms may obtain protection from bath treatments by withdrawing their body into the interlamellar space, limiting exposure to the medicine and reducing efficacy (Thoney and Hargis, 1991). Praziquantel is emerging as the treatment of choice against monogeneans in aquaculture and is an effective bath treatment against adult *L. bidyana* (Forwood et al., 2013). Single PZQ treatments of up to 48 h, however, are ineffective against juvenile *L. bidyana* (see Forwood et al., 2013). This was also noted in monocotylid monogeneans infecting giant shovelnose rays, *Rhinobatos typus* and was attributed to post-oncomiracidia and juvenile worms avoiding exposure to the treatment by retracting deeply between the secondary lamellae (Chisholm and Whittington, 2002). Strategically timed repeat treatments are necessary for high efficacy against small gill dwelling monogeneans. While *L. bidyana* appears not to be highly pathogenic, monitoring and management in aquaculture systems is necessary and specific data on the lifecycle including egg-hatching time and maturity interval is required to facilitate strategic repeat bath treatments for more effective management.

7.5 Acknowledgments

We are grateful to Dr Matt Landos and to the Scifleet family who run the Pioneer Fish Farm in Gloucester, NSW for providing silver perch to work with. We also thank A. Blanch and M. Norman for technical assistance during various technical aspects of the project. This work was supported by internal funds from the School of Biological

Sciences, Flinders University and was done under approval from the Flinders University Animal Welfare Committee (Permit E354).