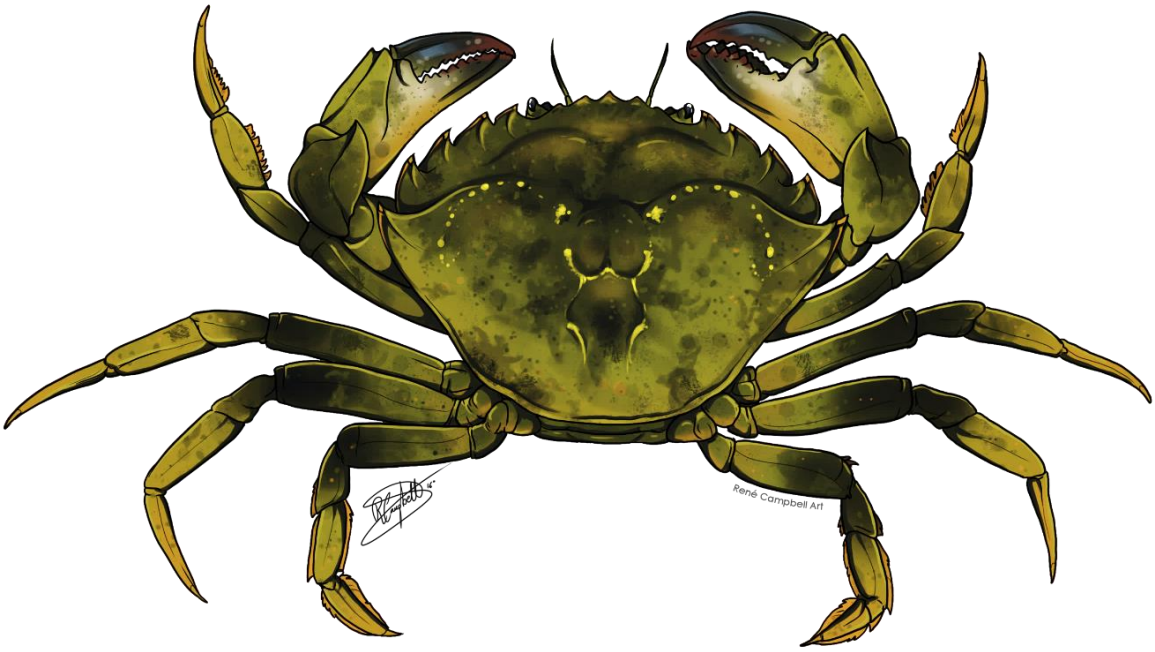
Morphological variation, reproductive biology and genetic structure of an invasive marine crab, *Carcinus maenas*



by

René Teresa Campbell (BSc Honours)

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Global Invasion" – watercolour painting of *Carcinus maenas* by René Campbell

"A supposedly daring insight came up, disguised as a question: 'Dr. Cole, aren't humans the most invasive species of all?"

"I'm not unsympathetic to that line of thinking,' she answered, 'but even if it's true, we're also the only species in any position to do anything about it."

— Joe Pitkin, Analog Science Fiction and Fact, June 2012

Thesis cover image: Digital illustration of *Carcinus maenas* by René Campbell

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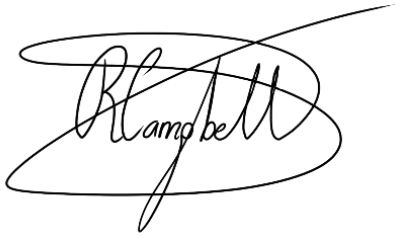
Thesis Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed

René Teresa Campbell



2nd October 2020

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Author contributions, permits and funding

The following specifies the author contributions for each thesis chapter

Chapter 1

Chapter writing, preparation of figures and tables: RC

Chapter editing and feedback: SD, MG and MD

Chapter 2

Experimental design and conception of the project: RC

Feedback and discussion of project ideas: SD, MG and MD

Chapter writing, preparation of figures and tables: RC

Fieldwork and data collection: RC

Laboratory analysis and DNA sequencing: RC

Statistical analysis: RC

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Chapter 3

Experimental design and conception of the project: RC

Feedback and discussion of project ideas: SD, MG and MD

Chapter writing, preparation of figures and tables: RC

Fieldwork and data collection: RC

Laboratory analysis and DNA sequencing: RC

Statistical analysis: RC

Chapter editing and feedback: SD, MG and MD

Chapter 4

Experimental design and conception of the project: RC

Feedback and discussion of project ideas: SD, MG and MD

Chapter writing, preparation of figures and tables: RC

Fieldwork and data collection: RC

Laboratory analysis, microscopy and histology: RC

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Chapter 5

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Chapter writing, preparation of figures and tables: RC

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Laboratory analysis and DNA sequencing: RC

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Chapter editing and feedback: SD, MG and MD

Chapter 6

Chapter writing, preparation of figures and tables: RC

Chapter editing and feedback: SD, MG and MD

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MD: Dr. Marty Deveney (Adjunct Supervisors, South Australian Research and Development Institute (SARDI) Aquatic Sciences)

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The handling of fish and cephalopod bycatch was conducted in accordance with the *Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition 2013* and the Flinders University Animal Welfare Committee (AWC) under the following Animal Ethics Approval: E430/16 (February 2016 – December 2019).

The collection of marine organisms for scientific research was conducted in accordance with the *Fisheries Management Act 2007: Section 115* under the following Ministerial Exemptions: ME9902877 (September 2016 – September 2017); ME9902954 (September 2017 – September 2018); ME9903005 (September 2018 – September 2019).

The collection, possession and/or control of a noxious species was conducted in accordance with the *Fisheries Management Act 2007: Section 78* under the following Noxious Species Permits: MP0050 (October 2016 – September 2017); MP0072 (September 2017 – September 2018); MP0102 (September 2018 – September 2019).

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Thesis Summary

Marine bioinvasions are becoming more frequent due to an increase in maritime activities especially with shipping transport carrying over 80% of the world trade. The European shore crab, *Carcinus maenas,* is one of the most widespread marine invasive species worldwide and can cause ecological and economic impacts on intertidal communities and shellfisheries. European shore crabs possess biological traits that increase their invasion success, such as high reproductive output, physiological tolerance and plasticity to local environment conditions, and varying genetic diversity. These traits allow new crab incursions to rapidly establish or help crabs expand their range. Research into *C. maenas* population biology and invasion ecology was limited for its invasive range in the southern hemisphere, including in southern Australia. This PhD project investigated *C. maenas* population biology and traits that may drive invasion success for this species throughout coastal habitats of Gulf St Vincent, South Australia. This thesis specifically addressed morphological variation, reproductive biology and genetic structure of South Australian *C. maenas*. Chapter 2 presents morphological and mitochondrial COI gene analyses to confirm the species of *Carcinus* present in South Australia. The study revealed that the species in South Australia is the Atlantic *C. maenas* and highlighted that *Carcinus* are accurately identified with molecular analysis. Chapter 3 used morphometrics and the COI gene to assess intraspecific variation of *C. maenas* across different habitats in Gulf St Vincent. Morphometric variation was observed across habitats as a possible indicator of phenotypic plasticity, while genetic homogeneity suggested *C. maenas* in South Australia comprise a single genetic population. Chapter 4 examined the ovary development, size at sexual maturity, fecundity and the reproductive period of female *C. maenas.* Females have early onset of maturity, produced an average of 200,000 eggs per brood, and spawn up to nine months of the year during cooler seasons. Chapter 5 presents next-generation sequencing of SNP markers (DArT-Seq) and the COI gene to contrast genetic diversity, genetic structure and demographic histories of South Australian *C. maenas* to other populations across its global range. South Australian *C. maenas* had high genetic diversity and were genetically distinct, while demographic history revealed Europe and southeast Australia as the most likely source populations. This research project identified biological characteristics such as morphological plasticity, high reproductive potential and diverse genetic structure that influence invasion success of *C. maenas* in South Australia. Understanding biology of this global invader will assist with research and management of marine invasive species.

Chapter 1. General Introduction

1.1 Overview of marine bioinvasions

Since the industrial revolution of the 1800's, anthropogenic activities have accelerated impacts on surrounding environments and biodiversity at an unprecedented level, leading to the Earth's sixth mass extinction event (Barnosky et al. 2011). The five most substantial causes of decreasing biodiversity are climate change, pollution, habitat loss, over-exploitation, and invasive species (Cafaro 2015). The number of invasive species has grown exponentially in the past 200 years due to increased global trade, novel trade routes, and increased demand for exotic species (Williams et al. 2013). Many successful invasive species are introduced intentionally as food or game (i.e. agriculture, hunting, fisheries), non-food species (i.e. exotic gardening, aquaria), or accidentally through transportation and development (i.e. shipping, infrastructure, accidental releases) (Lockwood et al. 2013).

The definition of an "invasive" species has been convoluted, with terminology changing depending on which stage a species is at during the invasion process among other factors (Colautti and MacIsaac 2004; Blackburn et al. 2011). Throughout this thesis, I use the term "invasive" for non-native species that have established self-sustaining populations outside of their native geographic range, have been introduced via anthropogenic vectors, are widespread and/or dominant, and have demonstrated negative impacts on the surrounding biodiversity, environment or human-used resources and industries (Craig 2010; Blackburn et al. 2011). The term "introduced" is used for non-native species that were transported by anthropogenic vectors and have established, and are characterised as either "localised but dominant" or "widespread but rare" in the introduced range. The term "non-established" is used for incidental findings or single records of species that have not yet formed a self-sustaining population. Native species range expansions occur when species have shifted outside their native range through unassisted dispersal, even when responding indirectly to anthropogenic pressures (i.e. climate change) (Colautti and MacIsaac 2004; Sorte et al. 2010; Gilroy et al. 2016). Although range shifts are a growing area of research in invasion ecology (see Sorte et al. 2010), this thesis is focused on invasive species that were introduced directly by anthropogenic vectors.

Since the development of boats and ships, humans have transported marine invasive species across the world for thousands of years (Hulme 2009). The annual rate of invasive species records has increased in the last 200 years due to globalisation of trade and economic growth (Hulme 2009). It has been predicted that maritime traffic will increase globally at a rate of 240 – 1,209% by the year 2050, with a three to 20-fold increase in marine bioinvasion risk (Sardain et al. 2019). Despite this increase, marine bioinvasion research has only emerged relatively recently since the 1960s, with 56.1% of current invasive species research focused on terrestrial ecosystems, 24.1% on freshwater ecosystems and 19.8% on marine ecosystems (Ojaveer et al. 2018; Geraldi et al. 2019). Marine bioinvasions are becoming more frequent due to an increase in maritime activities and human-mediated vectors such as global shipping and the aquarium trade (Hulme 2009).

Vectors are defined as physical transfer mechanisms responsible for the introduction and consequent spread of marine invasive species from one region to another (Geburzi and McCarthy 2018). Most marine vectors are attributed to shipping, including ballast water, biofouling and hull boring, fouled sea chests and intake pipes, and dry/semi-dry ballast (Bax et al. 2003; Seebens et al. 2013). Other vectors include deliberate introductions of exotic species for mariculture, the aquarium trade, offshore construction facilities and accidental/unknown introductions (Bax et al. 2003). Vector type is also associated with the diversity and number of marine invasive species that are transported. Williams et al. (2013) researched which vector types were responsible for the introduction of marine invasive taxa in California. For both single and multi-vector species, biofouling, ballast and aquaculture were the most responsible vectors of introduction. Crustaceans and molluscs were the only taxa that were introduced across all vector categories and contributed a large proportion of species found from each vector (Williams et al. 2013).

Once a non-native species has established a self-sustaining population, there is a risk that the species may have detrimental effects in the introduced range. Marine invasive species may threaten native biodiversity through competition, predation or pathogen spread, can modify habitat structure, and have the potential to impact on anthropogenic resources such as maritime infrastructure (e.g. through clogging of pipes and fouling of artificial substrates), tourism and fisheries (Bax et al. 2003; Lovell et al. 2006). Ecosystem services that may be impacted from marine bioinvasions include: human food resources (fisheries, aquaculture etc.); water storage; biotic materials/biofuels; quality of water and air; climate and biological

regulation; coastal protection; ocean nourishment; life cycle maintenance; symbolic/aesthetic values; and recreation/tourism (Katsanevakis et al. 2014). Associated changes to these ecosystem services and the monitoring, management and prevention efforts for invasive species can have severe economic repercussions.

Lovell et al. (2006) detailed that total economic damages and control costs for "harmful aquatic invasive species" in 2005 were USD $128 billion annually in the United States alone. In Australia, the cost of ballast water management for marine bioinvasions is estimated at AUD $36.2 million per year, eradication attempts between AUD $5–$20 million per year, and "living with invasives" between AUD $4 million and $1 billion per incursion event (Arthur et al. 2015). "Living with invasives" was defined as accepting the presence of an invasive species, its management costs and any potential impacts that it could cause in Australia. These impacts would include any non-market impacts (i.e. impacts to the environment or social amenity), loss of production to marine industries (i.e. impacts to aquaculture), and any additional management costs associated with mitigating the invasive species (Arthur et al. 2015).

1.2 Population biology of invasive marine crabs (Decapoda: Brachyura)

Crustaceans are some of the most successful taxonomic groups of aquatic invasive species worldwide (Hänfling et al. 2011). Invasive crustaceans have the highest number of species, geographic extent, ecological impact and invasion potential compared to all other marine invasive taxa such as molluscs, algae and fish (Molnar et al. 2008). Marine decapods (crabs, shrimps, lobsters and allies) comprise most of diversity of the known introduced and invasive marine crustaceans globally (Rilov et al. 2009). A comprehensive review on invasive marine crustaceans by Galil et al. (2011) identified 16 marine and estuarine crustacean taxa (Class and Orders) that were transportable by human-mediated vectors and corridors. Crustacean families of concern for invasion include crayfish (Cambaridae, Parastacidae), shrimp (Mysidae, Penaeidae), amphipods (Gammaridae), barnacles (Balanidae), isopods (Idoteidae, Sphaeromatidae), waterfleas (Cercopagididae, Daphniidae), sand fleas (Talitridae), anomuran crabs (Porcellanidae, Lithodidae) and brachyuran crabs (~31 families) (Galil et al. 2011). Out of all invasive crustaceans, brachyuran crabs were the most numerous in terms of species numbers, with approximately 96 species (31 families) recorded as introduced (Galil et al. 2011; McLay 2015; Swart et al. 2018; World Register of Marine Species "WoRMS" 2020). Of these 96 species, ~58 were invasive, with most consisting of swimming crabs (Family Portunidae) and rocky shore crabs (Family Grapsidae) (Fig. 1.1).

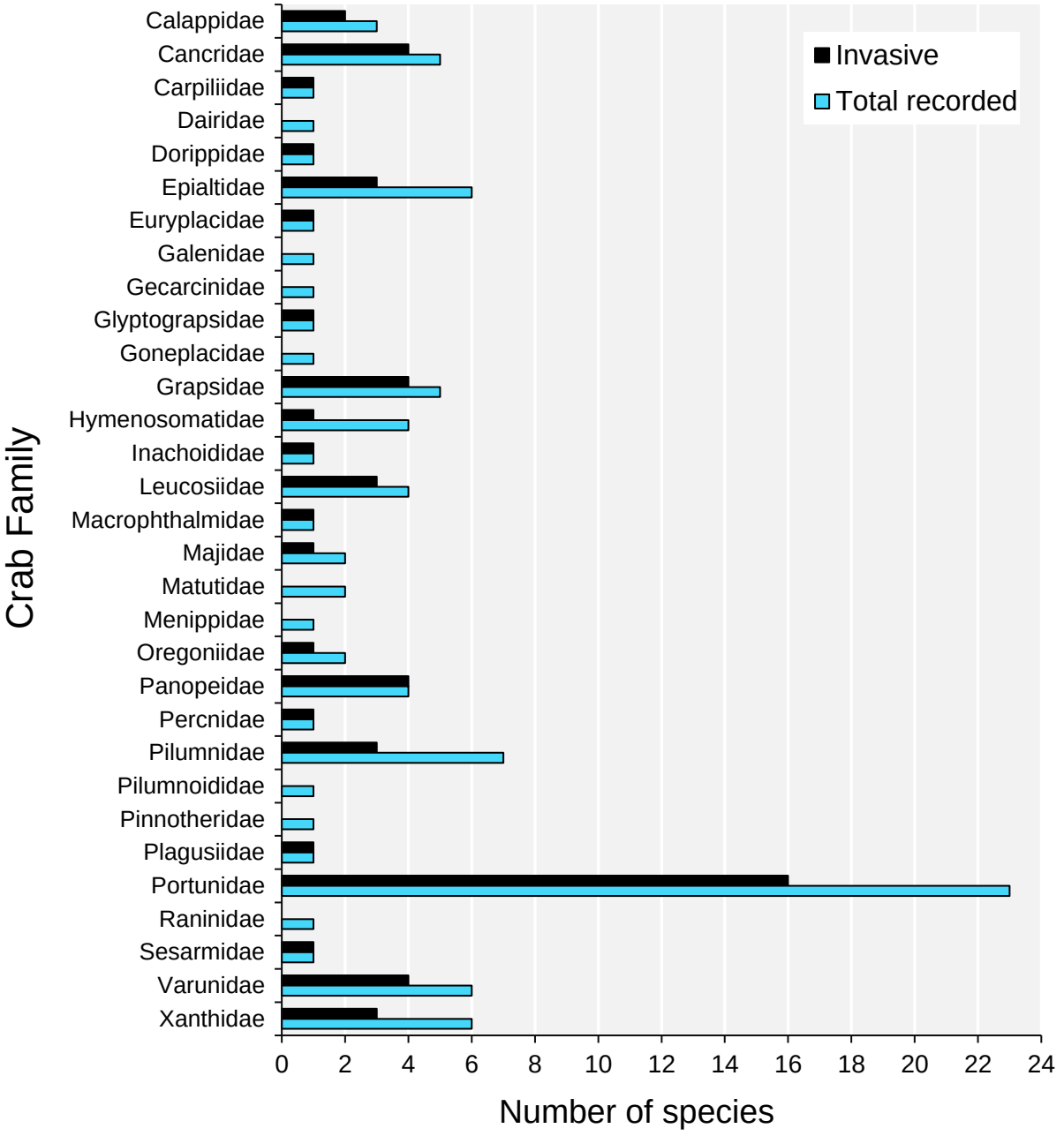

Figure 1.1 Number of globally invasive and introduced marine crab species recorded in each Family. "Total recorded" shows combined invasive and introduced species records in that Family. Data adapted and revised from Galil et al. (2011), McLay (2015) and Swart et al. (2018). Records and classifications were checked with the World Register of Introduced Marine Species (WRiMS - http://www.marinespecies.org/introduced/) and the Information System on Aquatic and Non-Indigenous and Cryptogenic Species (AquaNIS - http://www.corpi.ku.lt/ databases/index.php/aquanis/).

The main vectors that transport invasive marine crabs are ship ballast water (~20 species), biofouling assemblages (~17 species), and transfer with shellfish for aquaculture (~7 species) (Galil et al. 2011; McLay 2015). Construction of anthropogenic corridors (such as the

Suez Canal that connects the Mediterranean Sea and the Red Sea) were also responsible for introductions of ~15 crab species (Galil et al. 2011). Historically, invasive crabs were likely transported from fouling communities and the dry and semi-dry ballast of wooden ships (Bax et al. 2003). Many invasive marine crab introductions in modern day have been traced back to ballast water of commercial vessels, where zoea or megalopae larval stages were released during ballast water exchange at ports and harbours (Williams et al. 2013). The most prolific and widespread of the invasive marine crabs were likely transported this way, with an estimated hundreds of millions of tonnes of larvae-containing ballast water exchanged per year (McLay 2015). Invasive crabs are also transported via ship biofouling, where crabs shelter in the fouling communities encrusted on ship hulls, propellers, rudders, bow thrusters, sea chests and other vessel surfaces (Frey et al. 2014). Cuesta et al. (2016) identified biofouling as an important vector responsible for several crab introductions into Spain and remarked that biofouling is often overlooked compared to ballast water.

Impacts from invasive crabs are of concern to ecologists, conservationists, natural resource and biosecurity managers, industry stakeholders (i.e. aquaculture, fisheries, ballast water management) and the general public (Williams and Grosholz 2008). Invasive marine crabs have been documented to have numerous negative impacts on native habitats, communities or resources. These impacts can be direct through trophic interactions, competition or pathogen transmission, or indirect through habitat modification, and can result in various ecological or socio-economic consequences (Byers et al. 2010; Hänfling et al. 2011). In North America, invasive crustaceans (including crabs and other decapods) demonstrated various impact types: 10% competed with native species; 10% demonstrated economic impact; 5% had predatory impacts (including herbivory); 5% altered habitat; 5% provided a food/prey resource; 3% exhibited parasitism on host populations; and 3% had effects on threatened/endangered species (Ruiz et al. 2011).

Examples of invasive crab impacts include the Asian shore crab, *Hemigrapsus sanguineus*, which has widespread competition with native species in its introduced range, such as taking over burrows built by native fiddler crabs in Connecticut, USA (Wallentinus and Nyberg 2007; Epifanio 2013). The invasive Atlantic blue crab, *Callinectes sapidus*, competes with native brachyurans and has broad impacts on benthic communities in Greece (Mancinelli et al. 2017). The Chinese mitten crab, *Eriocheir sinensis*, can cause notable damage to riverbanks by burrowing and consuming netted fish and cutting nets, which resulted in

extensive efforts to mitigate the species in rivers, canals and associated facilities in Europe (Klaoudatos and Kapiris 2014). It is difficult to estimate economic costs of invasive marine crab (or even crustacean) impacts, with most estimates based around the most prolific crustacean species. Commercial fisheries losses attributed to invasive Chinese mitten crab are estimated between €73.4–€84.7 million since 1912 (Vilà et al. 2009).

Population biology plays an important role in understanding invasive species through life-history research, demographic structure, genetics, ecology and evolution (Sakai et al. 2001; Allendorf and Lundquist 2003). Populations of invasive species are often phenotypically, demographically, ecologically and genetically variable, and so there are different capacities for an invasive species to establish and impact on a native community (Simberloff 2003). Only a small percentage of species introduced into a new area successfully establish and have the potential to become invasive. General biological traits have been identified across marine invasive taxa that have successfully established, with many of these traits related to reproductive strategies, planktonic larval stages, opportunistic prey preferences, competitive behaviour, physiological tolerance to water conditions, genetic variation and phenotypic plasticity (Geburzi and McCarthy 2018). Key research areas in invasion ecology and population biology include the abundance and distribution of the invasive population, reproductive growth and demographic structure, habitat use and competition with native populations, gene flow and genetic adaptation, differentiation within and between populations, and evolutionary changes (Sakai et al. 2001; Rilov et al. 2009).

According to a review by Weis (2010), certain behaviours exhibited by invasive crustaceans are relevant for their success, including: 1) predation - outcompeting or eating native species; 2) predator avoidance – more efficient at avoiding predators than native species; 3) habitat modification – invasive species alter the environment or displace natives from their habitat; 4) movement – invader may be able to disperse more rapidly or over greater distances; 5) plasticity – able to change its phenotype in an altered environment; and 6) reproduction – frequency and abundance of spawning is greater than in native species. Swart et al. (2018) conducted biological trait analysis to investigate traits associated with invasion success of 56 predatory marine invasive crab species, nearly half of which were portunids (22 species). Traits that were analysed included: size, longevity, adult mobility, migratory behaviour, larval development, fecundity, generation time, range size and substrate preference. In contrast to Weis (2010), Swart et al. (2018) found no interaction between traits associated with successful

establishment of crabs, which was attributed to the lack of biological knowledge for many invasive taxa and the context-dependent nature of most bioinvasion studies.

Assessing the biological traits of invasive marine crabs assists with understanding the ecological processes that underpin their invasion success. Deudero et al. (2005) assessed the habitat preference, density, population structure and distribution of the invasive grapsid crab, *Percnon gibbesi*, in the Mediterranean Sea. Findings by Deudero et al. (2005) were useful for determining the future expansion of this invasive species, but highlighted that the complex nature of invasion ecology requires expansive research into many traits of a population such as reproduction. Kraemer (2019) observed that the population density of invasive Asian shore crabs, *Hemigrapsus sanguineus*, in Long Island Sound, USA, decreased by ~40% between years 2005–2017 and that the loss of larger female crabs may reduce reproductive population output for invasive *H. sanguineus.* The invasive Chinese mitten crab, *Eriocheir sinensis* (both an estuarine and freshwater species) in San Francisco Bay was found to have cyclical population dynamics, rapid establishment and population growth, and high reproductive rates that would make control of this species as a single population difficult (Rudnick et al. 2003).

Population research assists with prevention and early detection regimes in regions where invasive crabs have not yet been introduced (Geburzi and McCarthy 2018). Additionally, population biology research is a crucial component in understanding whether an invasive species' population could be feasibly controlled and where to focus management strategies for already-established species (Simberloff 2003). The most widespread invasive crabs from the genus *Carcinus* have a global distribution outside of their native European range (Leignel et al. 2014). This genus is ideal for understanding patterns and processes of marine bioinvasions due to attaining a global distribution that resulted from multiple introduction events (Young and Elliott 2019). Comparing multiple, spatially independent invasions of *Carcinus* in different coastal communities worldwide allows ecologists to identify biological traits associated with their widespread invasion success (Grosholz and Ruiz 1996). Findings help scientists and managers to predict which invasion characteristics allow the same species to establish in different biotic and abiotic contexts across their introduced range (Grosholz and Ruiz 1996).

1.3 Natural history and ecology of *Carcinus*

The European and Mediterranean shore crabs, or green crabs, of the genus *Carcinus* Leach, 1814 (Brachyura: Portunidae: Carcininae) are recognised as some of the most prolific marine invasive species worldwide (Leignel et al. 2014). Two species of *Carcinus* are currently described: the Atlantic *Carcinus maenas* (Linnaeus, 1758) and the Mediterranean *C. aestuarii* Nardo, 1847, both of which are morphologically similar and have high invasive potential (Behrens Yamada and Hauck 2001; Darling et al. 2008; Leignel et al. 2014). Both species are distinguishable from other portunid crabs by a fan-shaped carapace, five antero-lateral teeth on either side of the eyes, three undulations on the rostrum between the eyes, and no distinct swimming paddles on the last pair of legs (Fig. 1.2A). The crabs are also called "green crabs" due to their common dark green colouration, although other colour and pattern morphs are observed, predominantly in juveniles, which may aid in camouflage during early life stages (Todd et al. 2006; Stevens et al. 2014; Nokelainen et al. 2018).

Sexual dimorphism is also present in *Carcinus*: the width, length and convexity of the carapace and chelae are distinct between males and females. Females also have a wider, more convex and darker pleon to accommodate gonadal tissue and egg-carrying and are often slightly smaller than males (maximum carapace width in males = 100 mm; maximum carapace width in females = 80 mm; Fig. 1.2B and 1.2C) (Crothers 1967; Leignel et al. 2014). The ventral side of the carapace for both species is generally bright-green or yellow freshly after moult, and transitions to orange or red indicating a longer inter-moult period (Reid et al. 1997). The life span of *Carcinus* in their native range is approximately 5-7 years, where crabs will moult ~18 times over their life span with four moulting stages occurring during the zoea and megalopa larval stages (Crothers 1967; Young and Elliott 2019).

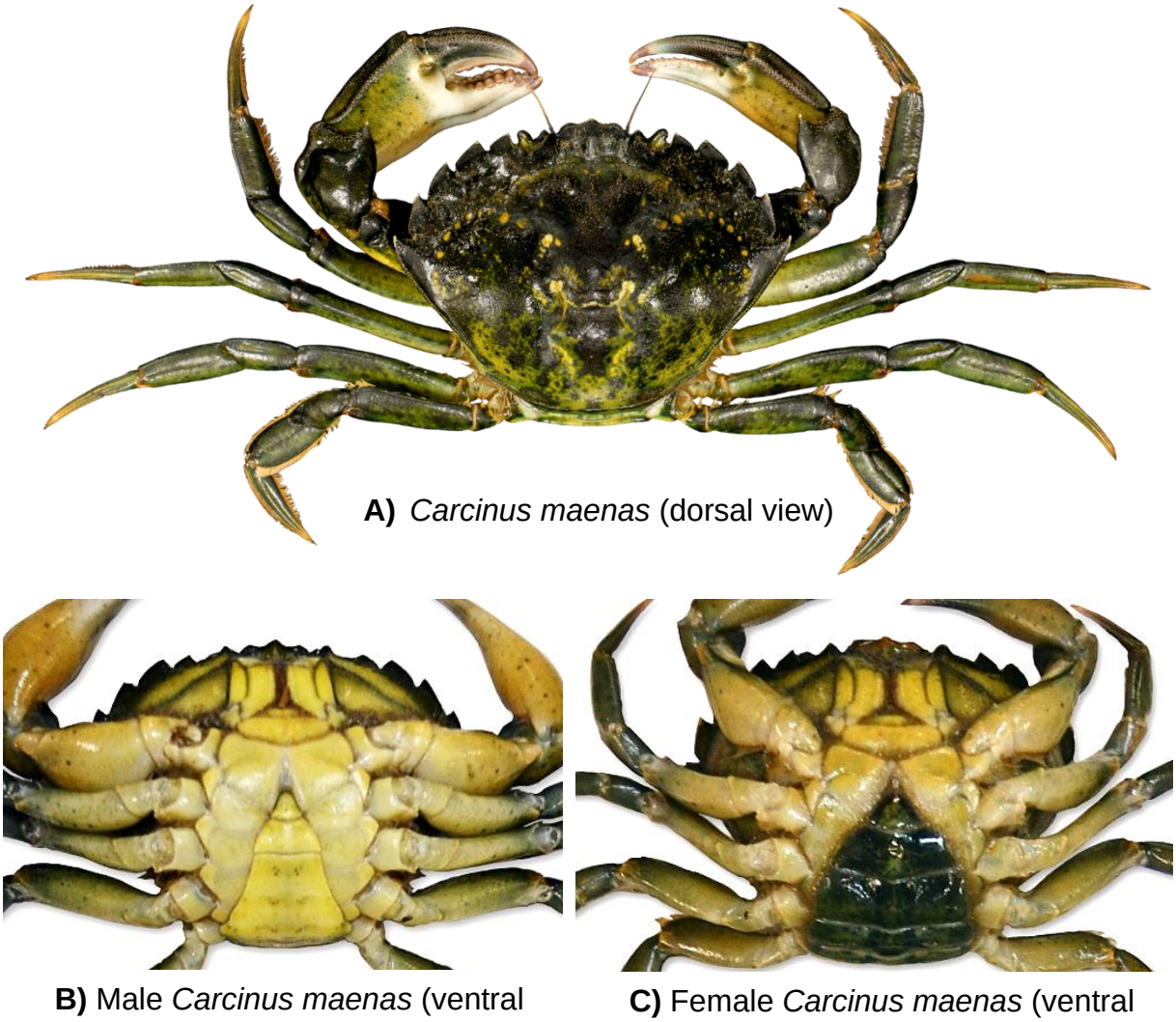

Figure 1.2 Photographs depicting specimens of *Carcinus maenas* collected from South Australia. A) dorsal view (male); B) male ventral view; and C) female ventral view.

The accepted taxonomic separation of *Carcinus* into two species has only occurred relatively recently. *Carcinus maenas* was first named by Carl Linnaeus in 1758 in *Systema Naturae* as "*Cancer maenas*"; the genus *Carcinus* was erected in 1814 by William Elford Leach where *C. maenas* became the monotypic species for this genus (Clark et al. 2001). Specimens of *Carcinus* from the Mediterranean Sea were observed to have varying morphological features compared to *Carcinus* from the Atlantic by Demeusy and Veillet (1953) and Zariquiey Álvarez (1957). Holthuis and Gottlieb (1958) resurrected the name *Carcinus mediterraneus* (Czerniavsky, 1884) for the Mediterranean form. Later, Manning and Holthius (1981) discovered a senior synonym for the Mediterranean form, *Carcinus aestuarii* Nardo, 1847, which is now the established name for this species.

The separation of *C. aestuarii* from *C. maenas* was based on morphological differences found between Atlantic and Mediterranean *Carcinus* specimens, most notably the male pleopod shape, presence or absence of setae on the cheliped carpus, carapace ratios, number and length of antenna segments, among other features (Demeusy and Veillet 1953; Zariquiey Alvarez 1968; Clark et al. 2001). Morphometric ratios have also been described for each of the two species, with *C. maenas* having a wider, thinner carapace compared to *C. aestuarii* (Behrens Yamada and Hauck 2001; Clark et al. 2001). Clark et al. (2001) found that morphology alone is not enough to assign *Carcinus* to species level and that most specimens were identified based on locality. Despite the morphological variation, genetic analysis using the mitochondrial COI gene and 16S rRNA have provided strong evidence for separation of the two *Carcinus* species (Geller et al. 1997; Roman and Palumbi 2004; Darling et al. 2008; Darling 2011a).

The native distribution of *C. maenas* extends along the European and North African coastlines, including the Baltic Sea to the east, northern Africa to the south, and Iceland and Central Norway to the north (Roman and Palumbi 2004; Darling et al. 2008; Fig. 1.3). *Carcinus aestuarii* are native to the Mediterranean Sea, Black Sea and Sea of Azov, although there is some minor geographic overlap between *Carcinus* at the Strait of Gibraltar (Darling et al. 2008; Deli et al. 2015). Outside of their native geographic range, *C. maenas* and hybrids have invaded five major regions worldwide: The Pacific and Atlantic coasts of North America; the coast of Argentina, South America; the cape of South Africa; southeastern Australia; and some parts of Japan (Carlton and Cohen 2003; Thresher et al. 2003; Baeta et al. 2005; Vinuesa 2007; Darling et al. 2008; Garside and Bishop 2014; Leignel et al. 2014; Young and Elliott 2019). For this reason, *C. maenas* is now regarded as the most widespread intertidal crab in the world, aided by the fact that both *Carcinus* species have likely been transported outside of Europe over ~200 years (Cohen et al. 1995; Carlton and Cohen 2003).

Aronson et al. (2014) stated that *C. maenas* has the potential to even become invasive in Antarctica, especially with increasing sea temperatures recorded in the western Antarctic Peninsula. The invasive range of *C. aestuarii* is more limited to Japan and South Africa, where hybridisation was confirmed between the two species in Japan, and potential hybridisation in South Africa (Geller et al. 1997; Carlton and Cohen 2003; Darling et al. 2008; Mabin 2018). There are no known established populations of invasive *C. aestuarii* outside the range of introduced *C. maenas*, therefore most research to-date has been focused primarily on *C.*

maenas. Further single records of *Carcinus* have been confirmed around the world, especially in tropical equatorial zones, but established populations have not occurred (Carlton and Cohen 2003; Young and Elliott 2019; Fig. 1.3).

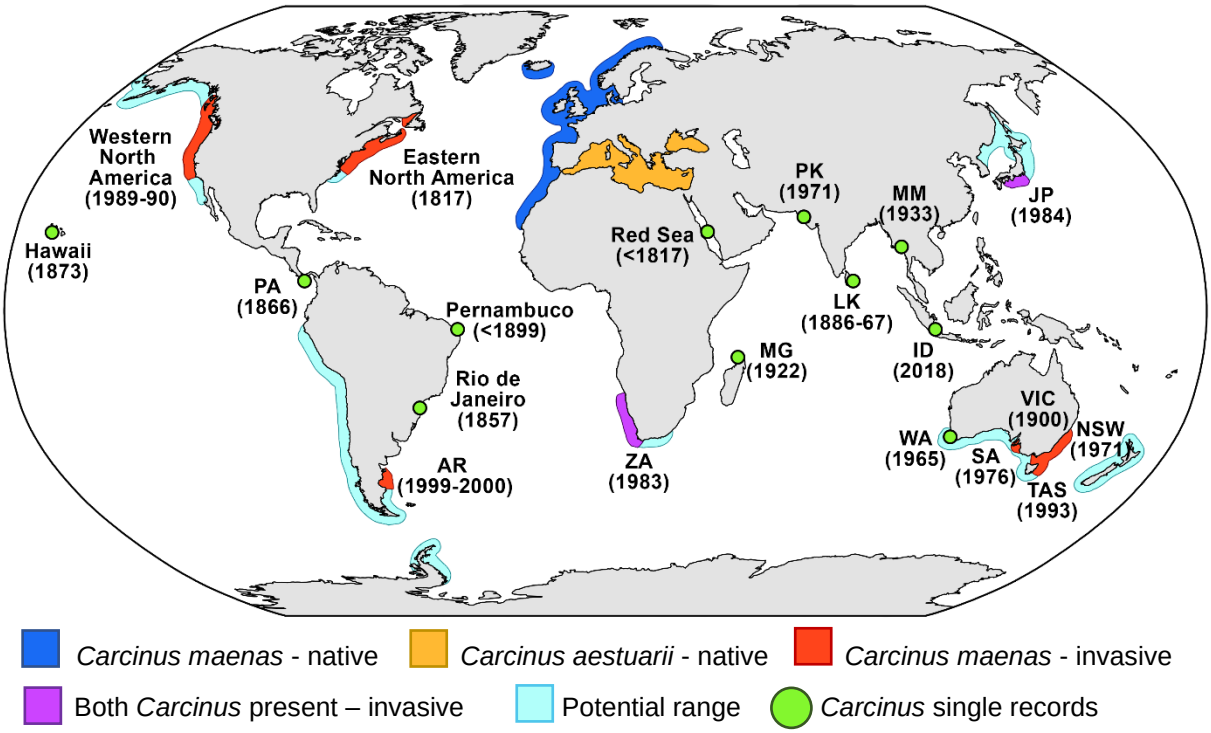

Figure 1.3 Distribution map for the native and invasive ranges of *Carcinus maenas*, *C. aestuarii* and complexes of the two species denoted by the different colour shading. Potential ranges of the genus are based on predictions by Carlton and Cohen (2003). Green dots represent non-established sightings of *Carcinus*, while dates of first sightings outside the native range are displayed between parentheses. Country codes are as follows: PA = Panama; AR = Argentina; ZA = South Africa; MG = Madagascar; LK = Sri Lanka; PK = Pakistan; MM = Myanmar; JP = Japan; ID = Indonesia. State codes for Australia are as follows: VIC = Victoria; NSW = New South Wales; WA = Western Australia; SA = South Australia; and TAS = Tasmania. Map adapted and modified from Carlton and Cohen (2003), Compton et al. 2010; Howard et al. (2018) and Young and Elliott (2019).

An evaluation by Carlton and Cohen (2003) suggested that global patterns of *Carcinus* dispersal were episodic, with three major episodes identified: 1) in a period around 1800; 2) in the 1850s – 70s; and 3) in the 1980s – 90s. Genetic analyses for both species have demonstrated that populations for most of the invaded range have separate COI haplotypes due to multiple introduction events (Darling et al. 2008; Darling 2011b). At least nine major vectors have been identified for *Carcinus* dispersal: 1) ship fouling/boring assemblages; 2) solid ballast; 3) fouled seawater plumbing and sea chests; 4) drilling platforms; 5) ballast water; 6) algae transported with fishery products; 7) accidental releases of education/research specimens; 8) private fishery

releases; 9) and transfer through aquaculture practices such as mussel seed (Carlton and Cohen 2003; Darbyson et al. 2009; Best et al. 2014). Crab larvae are also transported in ship ballast water, and once ballast has been released, ocean currents influence the distance and direction of dispersal before settlement (See and Feist 2010).

Both species are commonly observed in wave-protected rocky shores and estuaries in both intertidal and subtidal zones and may be found in up to 60 m depth (Leignel et al. 2014). They can colonise other habitats, such as saltmarsh, mangroves, rocky substrates, intertidal reefs, seagrass meadows, mudflats, woody debris, oyster reefs and anthropogenic habitats such as harbours (Almeida et al. 2008; Amaral et al. 2008; Garside and Bishop 2014). Open, rocky shorelines and sandy beaches exposed to higher wave energy are some of the few habitats *Carcinus* does not establish in, as they lack vertical tenacity and limb structure to grip onto rocky substrates adequately (Hampton and Griffiths 2007). Both juvenile and adult *Carcinus* prefer habitats with structural complexity that provide refuge areas, or alternatively soft and muddy sediments where they can conceal themselves via burrowing (Young and Elliott 2019).

Studies have demonstrated that *C. maenas* can survive a wide range of water temperatures, salinities and oxygen levels because they are eurythermal, euryhaline and hypoxia tolerant (Leignel et al. 2014; Young and Elliott 2019). Experiments on short-term temperature exposures showed that *C. maenas* tolerate water temperatures between 0–33°C before reaching their critical thermal maximum (CTMax) at 37°C (Kelley et al. 2011; Madeira et al. 2012; Tepolt and Somero 2014). For cooler water temperatures, invasive *C. maenas* were able to survive temperatures at or below 5°C in the laboratory for 18 weeks (Kelley et al. 2013). Crabs appear unable to breed when water temperatures exceed 26°C, and eggs are unlikely to develop past 18°C (Crothers 1967). Minimum temperatures required for growth and feeding usually occur around 7–10°C, therefore intertidal crab migrations are observed during seasonal winter and summer shifts globally (Naylor 1962; Young and Elliott 2019). Water temperature is likely the main driver that limits *Carcinus* spread beyond subtropical latitudes and appears to prevent crabs from establishing in tropical, equatorial regions and polar regions; however, *Carcinus* are more than capable of establishing in sub-polar and temperate latitudes (Carlton and Cohen 2003; Compton et al. 2010).

At high salinity, *C. maenas* is an osmotic conformer but shifts to an osmotic regulator at low salinity. Crabs are rarely found in salinities above 31% but can tolerate short-term exposure to high salinity of up to 54% and can survive salinities as low as 4–10% (Crothers

1967; McGaw and Naylor 1992; Jillette et al. 2011). *Carcinus* are tolerant to hypoxic conditions if suitable moisture is available. Experiments have shown *C. maenas* can survive out of water for at least ten days, and in low-oxygen environments (such as when burrowed in anoxic sediment during tidal changes) they can reverse the flow of air they breathe (Crothers 1967). While most physiological and biochemical responses to varying environmental stressors have been conducted on *C. maenas*, similar findings have also been demonstrated for *C. aestuarii* (Matozzo et al. 2011; Qyli and Aliko 2017; Qyli et al. 2020). The ability for *Carcinus* to tolerate such a wide variety of habitat types and environmental conditions are likely major contributors influencing their invasion success, especially in heavily modified habitats such as harbours and under future climate change conditions (Bessa et al. 2010; Behrens Yamada et al. 2015).

1.4 Ecological and commercial impacts of *Carcinus*

Only three crustacean species are considered to be in the world's top 100 worst invasive species by the Invasive Species Specialist Group (ISSG) and International Union for Conservation of Nature (IUCN): the fishhook waterflea, *Cercopagis pengoi* (Cercopagididae), the Chinese mitten crab, *Eriocheir sinensis* (Varunidae), and the European shore crab, *Carcinus maenas* (Portunidae) (Lowe et al. 2000). The invasion of *C. maenas* has had prominent impacts on a variety of marine species, and *C. maenas* was the first marine organism to be described as an "aquatic nuisance species" by the Aquatic Nuisance Species Task Force in 1988 (Leignel et al. 2014). In Australia, the Marine Pest Sectoral Committee updated a list of priority marine invasive species ("pests") and highlighted three established species which required national management to reduce their spread and impact. The three marine pests of national significance in Australia are the Japanese kelp (*Undaria pinnatifida*), the northern Pacific seastar (*Asterias amurensis*), and the European shore crab (*Carcinus maenas*), of which numerous environmental and commercial impacts were described (Marine Pest Sectoral Committee 2018).

In their native range, *Carcinus* are opportunistic benthic predators that feed primarily on bivalves such as cockles, oysters, clams and mytilid mussels, in addition to gastropods, annelids, other crustaceans and detritus (Scherer and Reise 1981; Mascaró and Seed 2001; Pickering and Quijón 2011). *Carcinus maenas* have been known to reduce populations in commercial species such as scallops (*Argopecten irradians*), soft-shelled clams (*Mya arenaria*), and blue mussels (*Mytilus edulis*) (MacPhail et al. 1955; Whitlow et al. 2003; Quinn

et al. 2012). *Carcinus maenas* can also influence benthic community composition through extensive predation, including mussel beds and oyster reefs which constitute important habitat engineers (Buschbaum et al. 2009). Smaller or thin-shelled bivalve prey are preferred due to higher profitability for the crab and lower damage risk to chelae, where extensive feeding on juvenile bivalves may reduce bivalve recruitment and abundance (Burch and Seed 2000; Walton et al. 2002; Pickering and Quijón 2011; Campbell et al. 2019).

Burrowing and feeding pits made by *C. maenas* in sediments and seagrass have altered community composition of associated benthic macrofauna (Griffen and Byers 2009; Lutz-Collins et al. 2016). In their invasive range in Newfoundland, Canada, *C. maenas* burrowing damaged rhizomes and plant shoots of native eelgrass meadows (*Zostera marina*), which resulted in a 50–100% decline in eelgrass cover at sites with established *C. maenas* compared to sites without crabs (Matheson et al. 2016). Finally, *C. maenas* can out-compete native and commercially important decapod species (e.g. *Cancer magister*, *Portunus armatus*) or may pose risks by exposing decapods and humans to parasites and disease (McDonald et al. 2001; Tanner 2007). Research on ecological impacts caused by invasive *C. aestuarii* and associated *Carcinus* hybrids are more limited, however they likely play similar ecological roles. In Japan, stomach content analysis of invasive *Carcinus* hybrids showed that crabs are omnivorous and demonstrate prey preference for invasive mussels (Doi et al. 2011).

Carcinus maenas have caused ecological impacts on coastal communities and socio-economic impacts on shellfisheries throughout their range (especially in North America). Predictions by Lafferty and Kuris (1996) and Lee and Gordon (2006) estimated that potential losses caused by *C. maenas* predation on commercial fishery species (clams, oysters, mussels and crabs) in the USA may be as high as USD $44 million per year. Other estimated annual losses to bivalve and crustacean shellfisheries attributed to *C. maenas* predation range between USD $22.6 million and USD $109 million (Colautti et al. 2006; Lovell et al. 2006). Grosholz et al. (2011) modelled the potential economic impact of invasive *C. maenas* to shellfisheries on the West Coast of the United States using a combination of ecological and economic models. In contrast to previous estimates, their findings suggested that past and present economic impacts are likely to be minor, but losses could increase to $0.09 million per year if crab densities increased or if they expanded their range into Alaska. In fact, some regions are considering utilising and managing invasive *C. maenas* by turning the species into a fishery resource. The Department of Fisheries and Oceans in Canada explored the potential of a

commercial fishery for invasive *C. maenas*, but the minimum impact of the species and the cost-benefit of the commercial fishery needed to be determined (St-Hilaire et al. 2016). Mach et al. (2014) estimated that invasive *C. maenas* of high density in Puget Sound, Washington, could consume between 0.15–4.46 million kg/year of shellfish worth between USD $1.03–$23.8 million, and cause a total economic loss of 2.8–64%.

1.5 Tools for assessing the invasion biology of *Carcinus*

The complex nature of marine bioinvasions has presented methodological challenges, including difficulties with distinguishing invasives from cryptogenic/cosmopolitan species, determining source populations and invasion routes, development of reliable detection tools, quantifying invasion success or impact, or predicting incursion events (Ojaveer et al. 2014; Ojaveer et al. 2018). Since the 1960s, marine bioinvasion studies have relied on historical records, museum collections, field surveys and taxonomy to determine the presence, spread and status of marine invasive species (Kamenova et al. 2017; Cardeccia et al. 2018; Ojaveer et al. 2018). The first proper attempts to define measures of ecological impact in marine invasive species using either field or controlled laboratory experiments did not occur until the 1990s (Ruiz et al 1999). Technological advancements in current marine bioinvasion research have assisted with surveillance and detection tools (genetics and genomics) and increased modelling and analysis capabilities (i.e. machine learning, bioinformatics, online databases) that are now routinely incorporated into marine bioinvasion management, policy and biosecurity (Ojaveer et al. 2014; Chan and Briski 2017; Kamenova et al. 2017). A review by Rato et al. (2021) highlighted the importance of integrating multiple methods and study fields (i.e. genetics, "omics", ecology, ecophysiology/ecotoxicology, behaviour and population dynamics) to better understand the invasion potential and fitness of marine crabs during global change.

The types of methods used for marine bioinvasion research will depend on the ecological questions and taxa being studied, in addition to costs, management priorities, effort and feasibility (Dana et al. 2013). As *C. maenas* is one of the most prominent ecotoxicological models for marine bioinvasion research, this species is ideal for comparing methodologies between studies or for developing new diagnostic tools (Leignel et al. 2014). Some aspects of invasive *Carcinus* biology are assessed with methods that have remained relatively unchanged over decades: for example, assessments of *C. maenas* reproduction used size of sexual maturity, fecundity counts and histological gonad staging to determine reproductive output and seasonality (Lyons et al. 2012; Best et al. 2017). Reproduction is a crucial phase that determines

if introduced species may persist and establish, and histological methods continue to be important for identifying reproductive biology of invasive species today (Carlton 1999; Cardeccia et al. 2018). Meanwhile, other methods of assessing biological traits (i.e. modelling predictions of dispersal mechanisms or assessing genetic structure and adaptation) are becoming more common due to technological advancements, reduced costs and changes to socio-economic priorities (Darling 2015; Chan and Briski 2017; Darling et al. 2017; Kamenova et al. 2017).

As molecular tools are advancing rapidly and becoming more accessible, genetic and genomic methods are now routinely used in marine bioinvasion research and management (Davey and Blaxter 2011). Genetic and genomic techniques can confirm species identification or hybridisation, identify introduction sources and/or invasion pathways, evaluate demographic history (i.e. expansion events or population bottlenecks) and assess population structure and connectivity (Fitzpatrick et al. 2012; Cristescu 2015; Sherman et al. 2016). Other molecular tools such as genomics and transcriptomics are now commonly used to identify genes that are under selection, which can indicate adaptation, acclimation or speciation of marine invasive species to novel environments (Rius et al. 2015; Viard et al. 2016; Oleksiak and Rajora 2020). Molecular tools such as environmental DNA ("eDNA"), metabarcoding and metagenomics are frequently used during surveillance to detect aquatic pests and diseases (Rius et al. 2015; Darling et al. 2017). Next-generation sequencing (NGS) methods are increasingly used to separate demographic and genetic processes from adaptive processes and provide greater reconstructions of invasion histories (Rius et al. 2015; Sherman et al. 2016). Despite the advancements in molecular technology, genetic and genomic markers contain different properties that determines their suitability for addressing the evolutionary ecology of marine invasive species.

The following properties of molecular markers vary and should be considered in all studies: inheritance, target genome, development time (if relevant markers have not been developed), cost, data comparison between studies, suitability for inferring evolutionary relationships, overall marker variability and of course, the specific question(s) being investigated (Davey and Blaxter 2011; Oleksiak and Rajora 2020). Genetic studies of marine invasive species initially used molecular markers that represented small fractions of the genome, such as allozymes or individual mitochondrial and nuclear genes (Ojaveer et al. 2018). Mitochondrial and microsatellite markers are still common in marine bioinvasion studies today,

however these markers are limited by their analytical power and differing properties. For example, mitochondrial markers are slow evolving, which makes them useful for reconstructing evolutionary pathways (Kamenova et al. 2017). Rapidly evolving markers such microsatellites have high resolution to detect recent demographic changes in a population (Kamenova et al. 2017). The recent development of next-generation sequencing tools has allowed for tens of thousands of single nucleotide polymorphisms (SNPs) to be detected and screened across the genome (Rius et al. 2015). However, sequencing hundreds of individuals is still expensive and the bioinformatic processing, filtering and analysis of SNP markers is demanding and time consuming.

A variety of molecular markers have been used to research the genetic structure and biological traits of invasive *Carcinus* (Table 1.1). Mitochondrial and nuclear microsatellite markers are the most common, however a smaller number of studies have used allozymes and genomic and transcriptomic SNPs. More molecular studies have been done on *C. maenas* compared to *C. aestuarii*, and few studies have compared both species to resolve taxonomy, evolutionary divergence, and differentiation between Atlantic and Mediterranean populations. For both species, most studies tended to use a combination of mitochondrial markers (COI or 16S) and nuclear microsatellite markers to assess hybridisation, genetic structure and connectivity, demographic events, source populations and phylogeography in native and/or invasive populations. Thousands of genomic and transcriptomic SNP loci have been characterised for *C. maenas* since 2010 to assess gene expression and selection, however SNP studies of *C. aestuarii* appear to be limited. Even with the high sequencing power of SNPs, genomic studies on invasive *C. maenas* often included mitochondrial and/or microsatellite markers. Ecology studies will often apply multiple markers to help reduce disparities in results that can occur due to different marker properties (Kirk and Freeland 2011; Oleksiak and Rajora 2020). Additionally, combined marker studies can address more ecological questions that may have otherwise been limited or misinterpreted when using only one molecular marker (Kirk and Freeland 2011).

Table 1.1 Summary table of common molecular markers used to assess both native and invasive populations of *C. maenas* and/or *C. aestuarii*. Dot points highlight the key genetic and/or genomic questions that were successfully addressed with each molecular marker.

Species	Markers used	Purpose of study	References
Carcinus maenas	Mitochondrial (COI and 16S)	• Confirmation of species-level identification and/or hybridisation • Population genetic structure across multiple spatial scales • Phylogeography and evolutionary history • Identification of cryptic invasions and shared haplotypes • Identity of thermal cline in haplotypes • Assessment of haplotype diversity and demographic events	Geller et al. 1997; Roman and Palumbi 2004; Roman 2006; Darling et al. 2008; Darling and Tepolt 2008; Darling 2011a; Marino et al. 2011; Darling et al. 2014; Pringle et al. 2011; Burden et al. 2014; Jeffery et al. 2017a; Jeffery et al. 2017b; Lehnert et al. 2018; Coyle et al. 2019; Cordone et al. 2020
	Allozymes	• Confirmation of species identity and genetic diversity • Used to assess genetic differentiation in the native range	Bulnheim and Bahns 1996; Brian et al. 2006
	Microsatellites	• Identity of source populations • Population genetic structure across multiple spatial scales • Assessment of population connectivity and demographic events • Phylogeography and evolutionary history • Confirmation of hybridisation and introgression • Detection of loci under putative selection	Tepolt et al. 2006; Darling et al. 2008; Pascoal et al. 2009; Tepolt et al. 2009; Domingues et al. 2010; Silva et al. 2010a; Darling 2011a; Domingues et al. 2011; Burden et al. 2014; Darling et al. 2014; Jeffery et al. 2017b; Lehnert et al. 2018
	SNPs (RAD-Seq, transcriptomics)	• Identity of source populations and reconstruction of invasion pathways • Assessment of population connectivity, seascape genomics and demographic events • Population genetic and genomic structure across multiple spatial scales	Tepolt and Palumbi 2015; Verbruggen et al. 2015; Jeffery et al. 2017a; Jeffery et al. 2017b; Jeffery et al. 2018; Lehnert et al. 2018; Tepolt and Palumbi 2020

		• Phylogeography and evolutionary history • Detection of loci under putative selection • Assessment of gene expression and adaptation to environmental variation • Confirmation of hybridisation and introgression	
Carcinus aestuarii	Mitochondrial (COI and 16S)	• Confirmation of species-level identification and/or hybridisation • Population genetic structure across multiple spatial scales • Phylogeography and evolutionary history • Identification of cryptic invasions and shared haplotypes • Assessment of haplotype diversity and demographic events	Geller et al. 1997; Roman and Palumbi 2004; Roman 2006; Darling et al. 2008; Darling and Tepolt 2008; Darling 2011a; Marino et al. 2011; Ragionieri and Schubart 2013; Deli et al. 2015; Deli et al. 2016a; Deli et al. 2018; Cordone et al. 2020
	Allozymes	• Confirmation of species identity and genetic diversity	Bulnheim and Bahns 1996
	Microsatellites	• Identity of source populations • Population genetic structure across multiple spatial scales • Assessment of population connectivity and demographic events • Phylogeography and evolutionary history • Confirmation of hybridisation and introgression	Darling et al. 2008; Marino et al. 2010; Darling 2011a; Deli et al. 2016a; Schiavina et al. 2014

1.6 Invasive *Carcinus* populations in southern Australia

Carcinus maenas was first recorded in Port Phillip Bay, Victoria, in the late 1800s, and was transported to Australia from Europe via the dry ballast of wooden vessels (Thresher et al. 2003; Ahyong 2005). Established populations of *C. maenas* are now found throughout the south-east coast of Australia in the States of Victoria (VIC), southern New South Wales (NSW) since 1971, South Australia (SA) since 1976, and Tasmania (TAS) since 1993 (Zeidler 1978; Thresher 2003; Dittmann et al. 2017). A single *C. maenas* specimen was recorded in the coastal marine areas of Fremantle, Western Australia (WA) in 1965, although there has been no recent evidence of establishment in this region (Wells et al. 2010). In South Australia, the first *Carcinus* specimen was recorded in Gulf St Vincent in 1976 and was suggested to be *C. maenas*, despite no genetic or morphological confirmation of species identity (Zeidler 1978).

Carlton and Cohen (2003) and Compton et al. (2010) reviewed environmental conditions that may be responsible for the thermogeographic expansion and limitation of *Carcinus* populations worldwide. It was observed that optimal summer sea temperatures between 13–16°C and winter sea temperatures between 5–9°C explained the general latitude limits of the genus. Based on these parameters, Compton et al. (2010) predicted that *C. maenas* populations in southeastern Australia will reach a northern limit south of Queensland, and in Jurien Bay should they establish in Western Australia. All of Tasmania, Australia's southernmost State, lies within *C. maenas*' potential range.

In South Australia, *Carcinus* are found throughout Gulf St Vincent in the Barker Inlet region and mangrove habitats north of Adelaide, along with the rocky shore habitats along Adelaide's southern metropolitan coastline (Dittmann et al. 2017; Fig. 1.4). The Port Adelaide River in Gulf St Vincent forms the main shipping harbour for Adelaide, where *Carcinus* were likely first introduced into South Australia (Zeidler 1978). The innermost channels and rivers near Port Adelaide include industrial ports, harbours, residential areas, and modified sandy beaches. Previously, *Carcinus* have also been sighted in northwestern Gulf St Vincent (Wiltshire et al. 2010; McArdle et al. 2012), however my preliminary survey in 2017 did not locate any *Carcinus* at these sites, suggesting they are no longer present or in low densities.

No current *Carcinus* populations were identified in Spencer Gulf during the 2017 surveys, despite potential suitable habitat being present such as mangroves, mudflats, shipping ports, and mussel beds (Fig. 1.4). Some small coastal townships in Spencer Gulf, such as Port

Pirie and Whyalla, have regular shipments of coal, iron ore and grains and regional shipping has the potential to bring in marine invasive species (Wiltshire et al. 2010). Coastal habitats in Spencer Gulf comprise inlets and mangrove forests similar to those found in Gulf St Vincent, which may also provide adequate habitat for *Carcinus* establishment in the future. Habitats found along South Australia's coastline include *Avicennia marina* mangrove forests, saltmarsh, seagrass meadows (mostly *Zostera* spp., *Posidonia* spp., and *Ruppia* spp.), soft sediments in enclosed estuaries, and rocky reefs and shores on the more southern tips of each peninsula (Bourman et al. 2016). Previous studies by Dittmann et al. (2017) have shown high variation in seasonal abundance and fine-scale habitat use by *Carcinus* in intertidal zones of Gulf St Vincent. Native bivalve species, such as habitat-forming mussels (*Xenostrobus inconstans*) and commercially important cockles (*Katelysia* spp.) are preyed upon by *Carcinus* in South Australian mangrove habitats (Campbell et al. 2019).

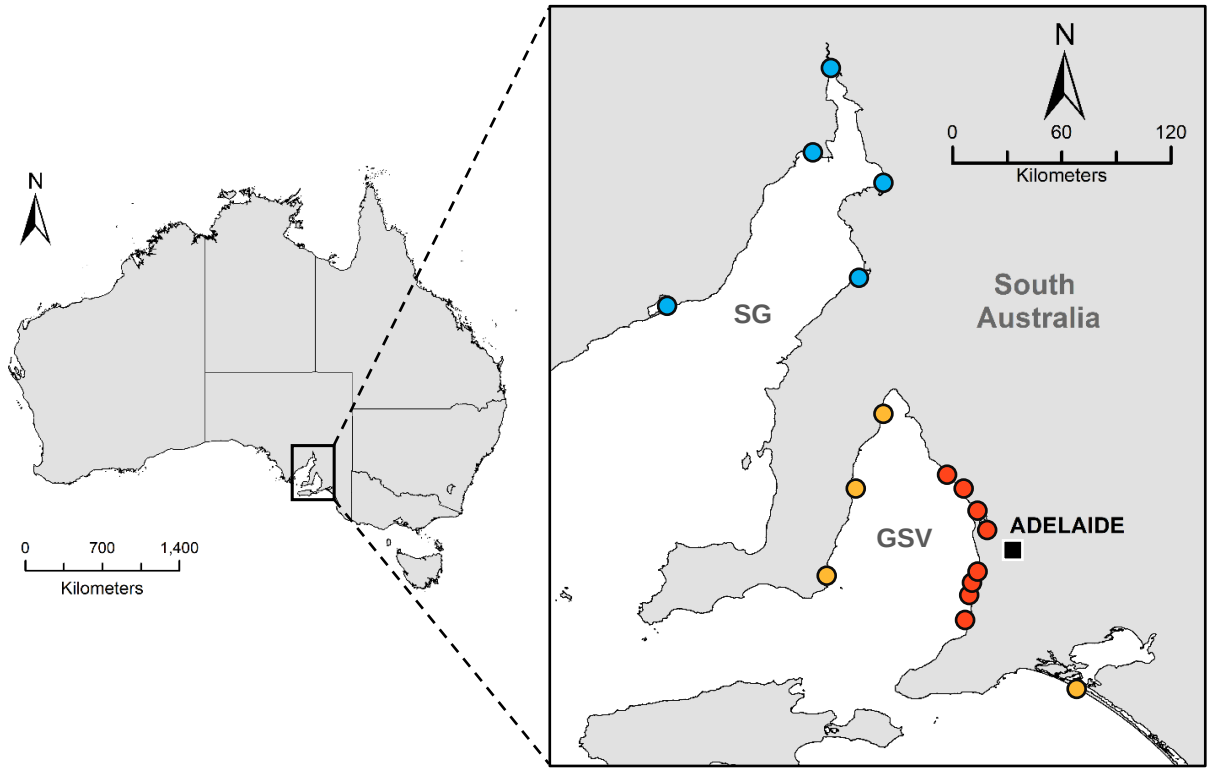

Figure 1.4 Map of Australia, with a focus on Gulf St Vincent (GSV) and Spencer Gulf (SG) in South Australia. Sites for the established population of *Carcinus* are shown by red circles, previous sightings of *Carcinus* in yellow circles, and potential range of *Carcinus* in blue circles. The capital city of Adelaide is represented by a black square. Grey shading indicates land.

Despite the global distribution of *Carcinus*, research into the ecology and population biology of this genus is limited across its southern hemisphere introduced range (Young and Elliottt 2019). *Carcinus maenas* is listed as one of the three marine priority pest species of national significance in Australia due to its documented impacts on coastal communities and shellfisheries (Grosholz et al. 2011; Leignel et al. 2014; Marine Pest Sectoral Committee 2018). As *Carcinus* populations in Australia are established it is unlikely that these crabs can ever be eradicated. Therefore, management efforts are focused on reducing spread of *Carcinus* to other viable habitats in southern Australia (Marine Pest Sectoral Committee 2020). Many of the temperate coastal habitats susceptible to *Carcinus* invasion in South Australia, such as mangrove forests in upper Spencer Gulf, host a variety of ecologically and commercially important benthic species that could be at-risk from *Carcinus* invasion. Understanding marine invasive species biology, ecology and life history is critical for fundamental emergency response and management strategies of marine pests such as *Carcinus*. Supporting marine pest research is therefore one of the key objectives in the Australian Marine Pest Plan 2018–2023 (Department of Agriculture and Water Resources 2018).

Research on the biological traits and life history of *Carcinus* in South Australia is limited and is one of the most understudied regions in *Carcinus*' global distribution (Young and Elliott 2019). The ~200-year introduction history of *Carcinus* spans multiple introduction events over five continents and varying coastal habitats (Carlton and Cohen 2003). As most introduced species will not establish, it is unlikely that *Carcinus* have attained a global distribution via chance alone. Rather, certain biological traits assisted *Carcinus* with overcoming ecological filters in the invasion process (Blackburn et al. 2011). Understanding aspects of *Carcinus*' population biology and ecology such as habitat-specific morphological variation, reproductive biology and genetic structure will highlight biological traits that underpin global invasion success (Geburzi and McCarthy 2018). This genus is ideal for investigating biological mechanisms that assist with colonisation under different biotic and abiotic conditions in marine ecosystems (Grosholz and Ruiz 1996).

1.7 Thesis aims

The overall aim of this PhD project was to assess aspects of invasive *Carcinus* population biology and ecology in South Australia. I specifically addressed species identity, morphological variation, reproductive biology and genetic structure of South Australian *Carcinus.* My PhD project consists of four major data chapters (excluding the general

introduction chapter [Ch. 1] and general discussion chapter [Ch. 6]) that expand understanding on biological traits that drive colonisation success of these global marine invaders. As research is limited for invasive *Carcinus* populations across the southern hemisphere, results will contribute new knowledge for *Carcinus* in temperate habitats of southern Australia. Research findings will be contrasted with *Carcinus* studies across their native and invasive distribution to identify biological traits that drive invasion success on a global scale. The broad aims of the four data chapters were:

Chapter 2: To confirm the species of *Carcinus* present (*C. maenas* and/or *C. aestuarii*) in South Australia using a combination of morphological characters, morphometric ratios and mtDNA COI gene sequencing. Carapace ratios and rostrum shape were variable, however mtDNA confirmed all specimens as *C. maenas*.

Chapter 3: To assess the morphological variation, genetic structuring and potential phenotypic plasticity of *Carcinus* across different coastal habitats in South Australia. A combination of linear and geometric morphometrics were used to assess morphological differences between habitats, while genetic variation was evaluated using an mtDNA haplotype network and analysis.

Chapter 4: To investigate the reproductive biology of female *Carcinus* in South Australia, with a focus on ovary development, size at sexual maturity, fecundity and reproductive period. This was achieved by using macroscopic and histological methods to assess ovary stage and egg counts and quantify reproductive biology.

Chapter 5: To identify genetic structure, genetic diversity and demographic history of *Carcinus* in South Australia and across their native and invasive global distribution. Both DArT-Seq SNP loci and mtDNA of *C. maenas* were assessed and compared over global spatial scales, while ABC analysis helped identify potential source populations.

1.8 Thesis structure

The following PhD thesis contains six chapters: a general introduction and general discussion chapter, and four manuscripts (as data chapters) that are currently in preparation to be submitted to scientific, peer-reviewed journals. The first chapter [Ch. 1] is a general introduction chapter that provides a background literature review to the topic and introduces the main knowledge gaps, aims and structure of this thesis.

The four data chapters [Ch. 2, Ch. 3, Ch.4 & Ch. 5] are written in manuscript format and consist of case studies carried out to answer specific hypotheses. Each data chapter is formatted consistently to match the layout, purpose and requirements of this thesis. Some repetition and overlap between data chapters is apparent as each data chapter was written as individual manuscripts to be submitted to international scientific journals.

Chapter 2 is a localised study that confirms the species identity of *Carcinus* in South Australia using morphological and molecular analyses and highlights appropriate methods to differentiate species of *Carcinus*. This chapter is currently in preparation for submission to *Transactions of the Royal Society of South Australia.*

Chapter 3 is a study that uses morphological and molecular analyses to assess variation of *Carcinus* across different coastal habitats in Gulf St Vincent, South Australia. This chapter is currently in preparation for submission to *Marine and Freshwater Research.*

Chapter 4 is a reproduction study that uses macroscopic and histological methods to address ovary development, size at sexual maturity, fecundity and reproductive period of female *Carcinus* in South Australia. This chapter is currently in preparation for submission to *Marine Biology.*

Chapter 5 is a large-scale study that assessed genetic structure and demographic history of South Australian, native and globally invasive *Carcinus* using next-generation sequencing and mitochondrial DNA analysis. This chapter is currently in preparation for submission to *Biological Invasions.*

The final chapter [Ch. 6] is a general discussion chapter that summarises the findings of the four data chapters, explores their implications in a broader context, highlights recommendations for further research, and concludes the thesis body of work. A single reference list for all six chapters is located at the end of the thesis along with separate appendices for each data chapter. Tables, figures and appendices are numbered specifically for each chapter in corresponding order throughout the thesis.

Chapter 2. Morphological and genetic evidence supports that invasive *Carcinus* Leach, 1814 in South Australia are *C. maenas* (Linnaeus, 1758)

ABSTRACT

Accurate identification of marine invasive species is important for understanding species distributions and expansions, invasion pathways and developing taxon-specific control methods. The shore crab genus *Carcinus* consists of two species: the Atlantic *Carcinus maenas*, and the Mediterranean *C. aestuarii*, which can be distinguished from each other with morphometric carapace ratios and genetic analysis. *Carcinus maenas* is globally invasive, whereas *C. aestuarii* is introduced to a limited distribution in Japan and South Africa. In temperate South Australia (which has a Mediterranean climate), invasive *Carcinus* have been referred to as *C. maenas* despite no detailed taxonomic analyses. The aim of this study was to confirm the identity of invasive *Carcinus* species in Gulf St Vincent, South Australia, using morphological characters, carapace ratios and mtDNA COI gene sequencing. The carapace ratios of 1,311 *Carcinus* and the COI sequences from 59 of these crabs were assessed. Carapace ratios were highly variable within and between sexes and indicated the likely presence of both *C. maenas* and *C. aestuarii* in South Australia. The COI sequencing of 59 crabs revealed ~100% sequence matches to *C. maenas* and diverged from *C. aestuarii* sequences on GenBank by 11.5% on average. Results supported that the South Australian population is *C. maenas*. The findings highlighted that carapace ratios alone do not distinguish *Carcinus* species due to intraspecific morphological variation and therefore genetic analysis is required to clearly differentiate *Carcinus* species during new incursions. Identification of *Carcinus* species assists in developing taxonomic and/or molecular diagnostic tools, and for assessing range expansions in the global distribution of this genus.

2.1 INTRODUCTION

Invasive species lists are important for bioinvasion management because they are commonly used in early detection activities such as port surveys and for prioritisation of risk assessments and mitigation (Hayes and Sliwa 2003; McGeoch et al. 2012). Being able to accurately identify or confirm the presence of an invasive species assists with early warning, prevention and control measures for bioinvasions. For example, taxonomic identification is required for accurate reconstructions of invasion history or for developing species-specific biological controls (Le Roux and Wieczorek 2009). These measures are dependent on knowing which species are present in a region and their invasion status elsewhere (Dias et al. 2017). Traditionally, morphological traits were used to describe and identify all species but can be ambiguous when intraspecific morphological variation is exhibited due to sexual dimorphism, ontogeny or phenotypic plasticity (Gianoli and Valladares 2012; Metri et al. 2017). Marine invasive species often experience rapid evolutionary change or hybridisation in novel environments; this results in genotypic variation (if through evolutionary processes) or phenotypic variation which adds further challenges to morphology-based species identifications (Whitney and Gabler 2008).

Incorrect invasive species identification occurs as a result of taxonomic uncertainty due to undescribed species characteristics or in cases where systematics is unresolved (McGeoch et al. 2012). Confirming the presence of an invasive or native species is difficult in closely-related species (i.e. within-genera) or for those that are cryptogenic or cosmopolitan (Hutchings 2018). Inability to identify species contributes to unreliable information on the presence, extent and population dynamics and may incorrectly categorise risk, impacts or indicate ineffective mitigation strategies for invasive species (McGeoch et al. 2012; Hutchings 2018). Loss of taxonomic expertise reduces confirmation of morphological identification, and it is now more common for species identification to proceed through a combination of morphological and molecular techniques (Hopkins and Freckleton 2002). Molecular techniques such as DNA barcoding/metabarcoding have since improved species discovery and identity by integrating morphological, physiological and ecological data (Darling and Blum 2007). Molecular-based identification of invasive species either serves as an alternative method to morphological identification, or may be used as additional “quality-control” to confirm initial morphological identity (Darling and Blum 2007).

Decapod crustaceans represent some of the most widespread of all marine invasive taxa but can be misidentified with native species or other undescribed introduced species (Molnar et al. 2008; Brockerhoff and McLay et al. 2011). For example, the introduced Asian brush-clawed crab found in Germany in 1993 was originally identified as *Hemigrapsus penicillatus* (Markert et al. 2014). A sibling species of *H. penicillatus* was described in its native range in Japan: *Hemigrapsus takanoi* (Asakura and Watanabe 2005). The species of *Hemigrapsus* present in Germany and Japan was debated due to morphological inconsistencies suggesting that *H. takanoi* was a synonym of *H. penicillatus* (Sakai 2007; Asakura et al. 2008). Markert et al. (2014) assessed native and invasive *Hemigrapsus* using genetics and morphological characteristics and confirmed that the invasive species in Germany was *H. takanoi* and not *H. penicillatus* as first described. Similar species confirmations have occurred for other invasive crabs. Paddle crabs detected in New Zealand were suspected to be either *Charybdis japonica* or *C. miles* introduced from the Indo-Pacific region (Webber 2001). Morphological and genetic assessments confirmed the specimens as *C. japonica*, the first record of *C. japonica* outside of its native range (Smith et al. 2003). Confirmation of *C. japonica* helped locate source populations based on vessel movements from the native range (i.e. Hong Kong, Japan, Malaysia and Taiwan) into the Port of Auckland (Smith et al. 2003).

The genus *Carcinus* (Decapoda: Portunidae) consists of two sister species; *C. maenas* (Linnaeus, 1758) and *C. aestuarii* Nardo, 1847 which are commonly referred to as the European shore/green crab and Mediterranean shore/green crab, respectively. *Carcinus maenas* is native to the Atlantic coastline of Europe and North Africa and the Baltic Sea, while *C. aestuarii* is native to the Mediterranean Sea (Carlton and Cohen 2003). *Carcinus maenas* has now been introduced to every non-polar continent on Earth and is classified in the "world's top 100 worst invasive species" by the Invasive Species Specialist Group (ISSG) (Lowe et al. 2000; Leignel et al. 2014). The introduced range of *C. aestuarii* is more limited, with an established population in Japan where it can hybridise with introduced *C. maenas* (Darling 2011a). *Carcinus aestuarii* has also been detected in South Africa among a population of introduced *C. maenas*, suggesting that *C. aestuarii* is invasive within the same range as *C. maenas* (Geller et al. 1997; Darling et al. 2008). A recent metabarcoding study in Argentina detected COI gene sequences of both *C. maenas* and *C. aestuarii* and indicated that both species are present in this region (Cordone et al. 2020). Both species are omnivorous and can modify native species' abundances via predation and competition. Impacts on native species and fisheries have been documented for *C. maenas* worldwide; however, the impacts of *C. aestuarii*

are less understood given its introductions are more recent and less widespread (Doi et al. 2009; Leignel et al. 2014).

The taxonomy of *Carcinus* has been disputed which has caused historical inconsistencies in descriptions of distinguishing characteristics for the species (Bulnheim and Bahns 1996; Leignel et al. 2014). It is possible that both species were transported out of Europe over centuries, which probably contributed to difficulties in identifying *Carcinus* specimens and distributions (Cohen et al. 1995). Despite this confusion, morphological characters are described that can differentiate Atlantic and Mediterranean shore crabs and provided evidence that *C. maenas* and *C. aestuarii* are separate species (Cohen et al. 1995; Behrens Yamada and Hauck 2001; Clark et al. 2001; Table 2.1). Behrens Yamada and Hauck (2001) and Clark et al. (2001) stated that the carapace width to carapace length ratio (CW:CL) was the most reliable morphological indicator delimiting the two *Carcinus* species. Moderate morphological indicators included the carapace width to carapace height/depth ratio (CW:CH), the shape of the three lobes on the rostrum ("rostrum shape"), the shape of the posterior-lateral carapace margin, and the shape of the male copulatory pleopods (Behrens Yamada and Hauck 2001; Clark et al. 2001). Other characteristics such as the length of the walking legs and angle of anterior lateral teeth have been reported, however, these characters were variable and considered poor indicators to delimit *Carcinus* species (Zariquiey Alvarez 1968; Almaça 1972; Rice and Ingle 1975; Behrens Yamada and Hauck 2001).

Both Behrens Yamada and Hauck (2001) and Clark et al. (2001) assessed *Carcinus* using the same morphological criteria but had contrasting findings. Behrens Yamada and Hauck (2001) stated that while the two *Carcinus* species look similar, carapace ratios, rostrum shape and male pleopods can distinguish species in the field. However, Clark et al. (2001) said that morphological differences alone cannot unequivocally assign crabs as either *C. maenas* or *C. aestuarii* and recommended further confirmation through genetic sequencing. Genetic studies have supported the separation of *C. maenas* and *C. aestuarii* but have not been used to confirm morphological characters. Bulnheim and Bahns (1996) rejected a species-level separation between Atlantic and Mediterranean forms of *Carcinus* using allozyme electrophoresis, suggesting that a Mediterranean subspecies of *C. maenas* was present. Later studies using 16S rRNA sequences supported taxonomic species separation of *Carcinus* with a 2.5% level of divergence, while the mitochondrial COI gene displayed a 10.2–11% divergence, supporting the use of these markers for species confirmation (Geller et al. 1997;

Roman and Palumbi 2004; Darling et al. 2008; Darling 2011a). Nuclear microsatellites and mtDNA have revealed interspecific hybridisation between *C. maenas* and *C. aestuarii* in Japan (Darling 2011a). Genetic sequencing has been useful for confirming the identity of either *C. maenas* or *C. aestuarii* even though morphological similarities exist between both species.

Table 2.1 Key morphological features used for distinguishing between *C. maenas* and *C. aestuarii.* Adapted from Behrens Yamada and Hauck (2001) and Clark et al. (2001).

Morphological character	*Carcinus maenas*	*Carcinus aestuarii*	Indication of species differentiation
Carapace width to length ratio (CW:CL) for crabs ≥ 20 mm	Wider carapace 1.29 – 1.36	Narrower carapace 1.22 – 1.27	Reliable indicator
Carapace width to height/depth ratio (CW:CH)	Thinner carapace 2.32 – 2.5	Deeper carapace 2.19 – 2.26	Moderate indicator: females tend to have deeper carapaces
Shape of three lobes in the rostrum	Three distinct "bumps", margin is scalloped	Flatter "bumps" not as distinct, frontal area protrudes beyond eyes	Moderate indicator: frontal margin may be chipped or blunted
Posterior-lateral margin of carapace	Straight or convex	Concave	Moderate indicator: some overlap may be present, sexually dimorphic
Male pleopods (copulatory appendages)	Crescent-shaped, touch at the bend	Parallel and straight, don't touch at the bend	Moderate indicator, often requires microscopy

In southeast Australia, invasive *C. maenas* occur in Victoria, New South Wales and Tasmania, where its identity has been confirmed by genetic studies (Geller et al. 1997; Darling et al. 2008; Burden et al. 2014). In South Australia, Zeidler (1978) discovered introduced *Carcinus* in 1976, where it was referred to as *C. maenas*. Despite having a well-established population in Gulf St Vincent, South Australia, no detailed morphological or genetic taxonomic analyses have assessed which species of *Carcinus* occurs in this region. Differentiating *C. maenas* and *C. aestuarii* is important as these species can hybridise in the introduced range (Darling 2011). The hybridisation-invasion hypothesis suggests that hybrids may have enhanced invasiveness due to novel phenotypic and/or genotypic traits that hybrids can possess (Jeffery et al. 2017b). The species present will determine whether it gets identified through species-specific, genus-specific or universal molecular tests and directs the genetic protocols used for molecular surveillance and for assessing source populations (Le Roux and Wieczorek

2009). In this study, I aim to confirm the identity of *Carcinus* in Gulf St Vincent, South Australia, by using 1) morphometric carapace ratios and morphological characters; and 2) mitochondrial COI gene sequencing. Confirmation of invasive *Carcinus* in South Australia will assist with sourcing *C. maenas* and/or *C. aestuarii* specimens from other native and invasive ranges for reconstructing demographic histories and identifying source populations into South Australia. Additionally, comparing morphological and genetic analyses for species confirmation will highlight which methods are most useful for rapidly identifying *Carcinus* in new incursions.

2.2 MATERIALS AND METHODS

2.2.1 Sampling sites and crab collection

Carcinus was sampled along the metropolitan and regional coastlines of Gulf St Vincent, South Australia, a large, marine-hypersaline inverse estuary with mixed tides of total 2–3 m range (Fig. 2.1). Salinity in Gulf St Vincent ranges from 32–42, while mean sea-surface temperature is around 22°C in austral summer and 13°C in austral winter (Bye and Kämpf 2008). The sampling sites encompass the known established range of *Carcinus* which extends approximately ~70 km along the Gulf St Vincent coastline. Seven sampling sites in various coastal habitats were selected where monitoring had detected *Carcinus* (see Dittmann et al. 2017). The two northern sites, Middle Beach and Port Gawler, comprise dense mangrove (*Avicennia marina*) forests, seagrass (*Zostera* spp.) beds, saltmarsh and mudflats. Two sites were assessed in Port Adelaide; the Port (Adelaide) River and Old Port Reach, which comprise industrial ports, harbours, residential areas and modified mangrove beaches. The final three sites assessed were Hallett Cove, the Onkaparinga River estuary and Aldinga Beach. These sites consist of sandy beaches, rocky shorelines, limestone cliffs and temperate intertidal reefs that are exposed to larger swells and wave activity (Bourman et al. 2016).

To encompass morphological variability and maximise the number of *Carcinus* assessed, specimens captured by Dittmann et al. (2017) were included in the analyses, along with additional crabs collected in 2018 and 2019 for Chapter 3 and Chapter 4 (Appendix Table A2.1). Depending on the site assessed, either baited traps or timed searches were used to collect *Carcinus.* Opera-house traps (67 x 48 cm, 2 cm mesh; 7.8 cm diameter entrance rings) were deployed in the upper and lower intertidal at the following sites: Port Gawler, Middle Beach, Port River, Old Port Reach, and the Onkaparinga estuary. Traps were baited with a

commercially available sardine (*Sardinops sagax*), set at low tide and left overnight before retrieval the next day. Upon retrieval, traps were assessed and all *Carcinus* caught were deposited into labelled bags. Any live or dead fish caught as accidental bycatch were identified, counted and released into the water at the point of capture (Flinders University Animal Welfare Approval E430/16). Timed searches (30 minutes) were used to collect *Carcinus* by hand at Aldinga, Hallett Cove and Onkaparinga sites. All *Carcinus* were killed by freezing at -20°C and stored frozen until assessment.

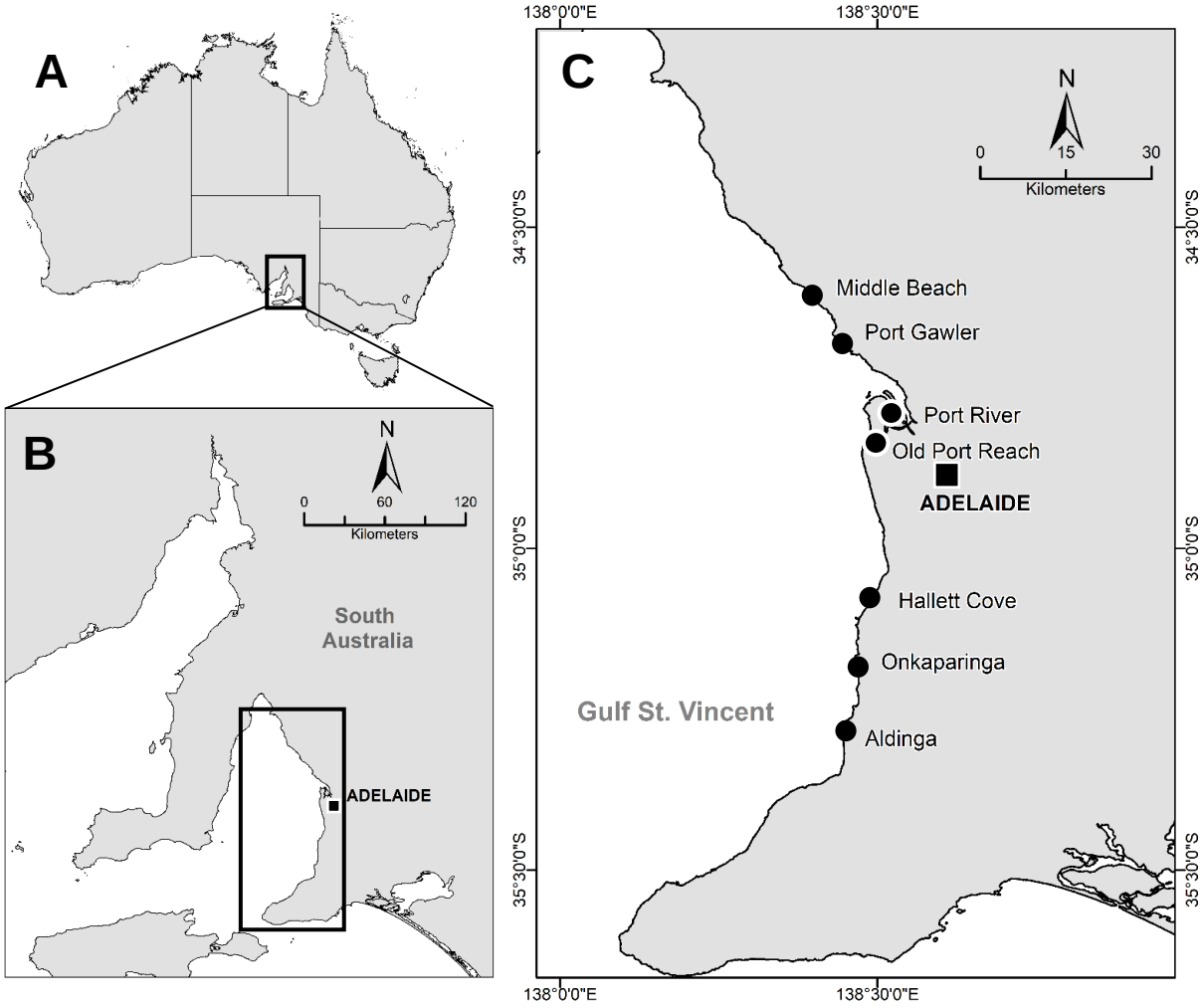

Figure 2.1 Map of sites where *Carcinus* was surveyed between 2013–2019. A) Australia; B) a sub-section of South Australia; and C) sampling sites on the Adelaide metropolitan coastline in Gulf St Vincent, South Australia. Sampling sites shown by black circles, the capital of South Australia, Adelaide, is shown by a black square. Grey shading indicates land.

2.2.2 Morphometrics and morphological characteristics

The sex of *Carcinus* collected between 2013–2019 was determined by the shape, structure and colour of the ventral pleon. Males have a narrow pleon that is the same colour as

the ventral surface, and females have a broad, convex pleon that is darker. *Carcinus* that could not be correctly sexed via the pleon (i.e. <20–30 mm carapace width) were considered morphologically immature and excluded from analysis. Frozen crabs were thawed and blotted dry, and the date, body condition and site of collection were recorded. The wet weight (g) of each crab was measured using electronic scales (A&D Weighing Pty Ltd, Adelaide, Australia) to the nearest 0.01 g.

Each crab was measured for linear morphometric dimensions across the carapace based on Behrens Yamada and Hauck (2001) and Clark et al. (2001). For carapace ratios, morphometric measurements of the carapace were made to the nearest 0.1 mm using digital Vernier callipers (Kincrome Australia Pty Ltd, Victoria, Australia). The carapace dimensions measured were carapace width (CW), carapace length (CL) and carapace height (CH) (Fig. 2.2). To reduce influence of size on crab morphology, these measurements were converted to the following carapace ratios after allometric relationships were examined: CW:CL (carapace width divided by length), and CW:CH (carapace width divided by height) (Clark et al. 2001). Another morphological feature that is a moderate indicator of species identification is the shape of three lobes on the rostrum (Behrens Yamada and Hauck 2001). Rostrum shape of each crab was noted as distinct, slightly blunted or blunted. The carapace ratios and morphological characters were compared to data collected by Behrens Yamada and Hauck (2001) and Clark et al. (2001) to indicate the identity of South Australian *Carcinus*.

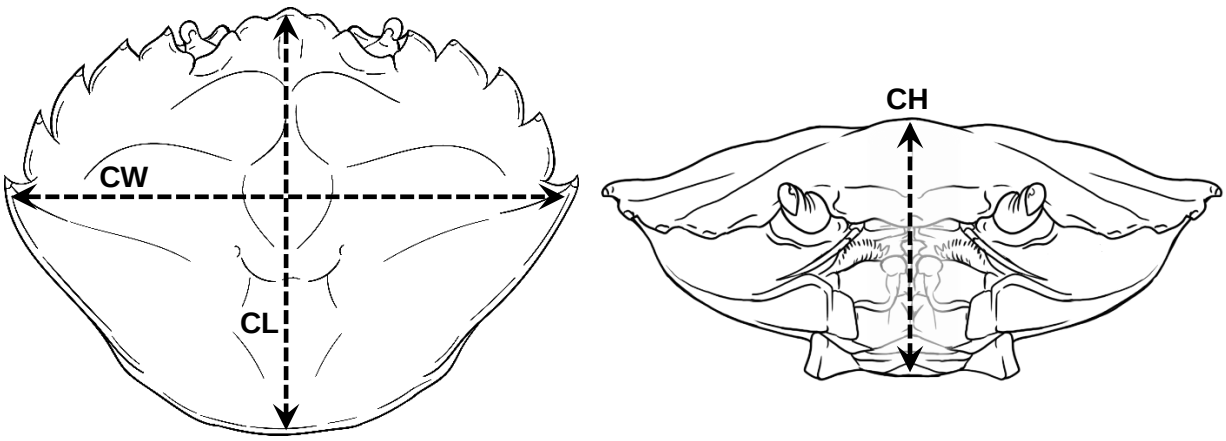

Figure 2.2 Measurement locations used for carapace ratios of *Carcinus* showing A) the dorsal view; and B) the anterior view of the carapace. CW = carapace width; CL = carapace length; CH = carapace height (sometimes referred to as carapace depth in other studies).

2.2.3 Molecular identification

DNA extraction

After morphometric measurement, total genomic DNA was extracted from 128 *Carcinus* samples. Crabs used for sequencing were selected across sampling sites and seasons to encompass variability in the system. The 128 individuals represented male and female crabs with CW:CL and CW:CH ratios that matched identities for *C. maenas* or *C. aestuarii* where possible, however *C. aestuarii* ratios were rarer (Appendix Table A2.2). One or two pereopods (walking legs) were removed from each crab after morphometric measurement and stored at -20°C until extraction. DNA was extracted from muscle tissue of frozen pereopods from each crab by overnight proteinase K digestion (20 mg/ml) at 55°C using the Gentra Puregene Tissue Kit (Gentra Systems Inc., Minneapolis, USA) following manufacturer's instructions. Purified DNA solutions were resuspended in 30–50 µl TE buffer and stored at 4°C. DNA concentration of all samples was checked with a Quantus Fluorometer (Promega Corporation, Wisconsin, USA) following the manufacturer's protocol.

DNA amplification and sequencing

Cytochrome *c* oxidase subunit I (COI) amplification was first tested with the following three primer pairs: universal primers HCO2198 (5' – TAAACTTCAGGGTGACCAAAAAATCA – 3') and LCO1490 (5' – GGTCAACAAATCATAAAGATATTGG – 3') (Folmer et al. 1994); degenerate primers COIF-PR115 (5' – TCWACNAAYCAYAARGAYATTGG – 3') and COIR-PR114 (5' – ACYTCNGGRTGNCCRAARARYCA – 3') (Folmer et al. 1994); and *Carcinus maenas*-specific primers M1745 (5' – GCTTGAGCTGGCATAGTAGG – 3') and M1746 (5' – GAATGAGGTGTTTAGATTTCG – 3') (Roman and Palumbi 2004). A subset of eight *Carcinus* samples were selected from the study to trial PCR parameters and primer optimisation. After each PCR optimisation test, 1.5% agarose gel electrophoresis was used to inspect the quality and strength of the resulting DNA bands. Two annealing temperatures (50°C and 55°C) and varying reagent concentrations were tested across the primer pairs (Appendix Table A2.3).

While the *C. maenas* specific primers showed the strongest DNA bands, degenerate primers are capable of sequencing both *C. maenas* and *C. aestuarii* and were selected for the current study (Darling et al. 2008; Darling and Tepolt 2008; Darling 2011a). A level of

degeneracy in the Folmer primers also maintains taxonomic utility in metazoan invertebrates (Geller et al. 2013; Mioduchowska et al. 2018). The universal Folmer primers were the least optimal primer pair to use after testing. In metazoan invertebrates (including decapod crustaceans) several studies have noted that Folmer's universal primers produced faint PCR products or failed to amplify due to sequence mismatches (Geller et al. 2013; Lobo et al. 2013; Mantelatto et al. 2016; Mioduchowska et al. 2018). Darling and Tepolt (2008) have shown that Folmer's degenerate primers can discriminate between *C. maenas* and *C. aestuarii* but noted that mismatches occur due to the high number of COI haplotypes found within the genus. No amplification was observed in negative controls using any of the primer pairs.

PCR amplifications using the degenerate primers were performed in a total volume of 24 µl containing ~1–50 ng/µl of extracted DNA, 1X MRT buffer (1x Immolase buffer, 2mM $MgCl_2$, 0.8 Mm total DNTP's, 0.5x BSA; Hayden et al. 2008; Garland et al. 2010), 0.5 µM of each primer, 1 mM $MgCl_2$ and 0.5 units Immolase (Bioline, NSW, Australia). PCR cycling conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 s, 50°C for 60 s and 72°C for 60 s, with a final extension of 72°C for 15 mins and 25°C for 2 mins (Appendix. Table A2.3). Negative controls were included in each round of PCRs. The quality of all PCR products was checked using 1.5% agarose gel electrophoresis, and any samples that did not display bands were excluded from PCR vacuum clean-up and sequencing.

From the 128 DNA extractions, 88 of these samples had successful PCR amplification viable for Sanger sequencing. Unsuccessful PCR amplification may have been due to poor performance of the degenerate primers, PCR inhibition caused by invertebrate pigments and chitin (Palmer 2008), or degraded tissue resulting from multiple freeze/thaw cycles and extended storage times (Bitencourt et al. 2007). Modifications to the DNA extraction and purification methods such as preserving tissue in ethanol, adding β-mercaptoethanol to lysis buffer (Bitencourt et al. 2007) or using RNase A solution to remove RNA contaminants (Gentra Puregene Handbook 2014) may be recommended in future work. Amplified PCR products were filtered with a $MultiScreen_{384}$-PCR plate vacuum cleanup (Millipore Australia Pty Ltd, NSW, Australia). PCR products were sequenced directly in both forward and reverse directions using the same degenerate primers used in the initial PCR. Samples were then sent for Sanger sequencing at the Australian Genome Research Facility (AGRF) in Adelaide, Australia.

2.2.4 Data analysis

Relationship between carapace dimensions

Morphometric carapace measurements and ratios were calculated for *Carcinus* collected across all sites and seasons in Gulf St Vincent, South Australia, between 2013–2019. All linear carapace measurements were first converted to averages (mean ± SD) to summarise sizes of male and female crabs used in analysis. To test if there was a relationship between the carapace measurements and to explore the effect of size on shape in the dataset (Baur and Leuenberger 2011), allometric regression analyses were first performed on all carapace dimensions. A positive relationship between carapace dimensions supports the use of these dimensions as carapace ratios and can therefore be used as species indicators regardless of crab size. Maximum carapace width (CW) was considered the independent variable and index of body size, as CW is less susceptible to variation from maturation and sexual dimorphism in most brachyuran crabs (Clark et al. 2001; Deli et al. 2014). The allometric relationship for CW (independent variable) was compared to CL and CH (dependent variables) for males and females separately to account for sexual dimorphism. The allometric growth equation used was: $Y = aX^b$, where a is the intercept, X is the independent variable (CW), and b is the allometric growth coefficient. Univariate ANOVA was used to test for a positive relationship between the independent variable and the dependent variables.

Carapace ratios and morphology

Carapace ratios were calculated by dividing carapace width by carapace length (CW:CL) and dividing carapace width by carapace height (CW:CH). Chi-square cross-tabulations were tested on male and female frequencies versus morphometric identity for CW:CL and CW:CH separately. A univariate nonparametric Mann-Whitney U test was used to test differences between CW:CL and CW:CH irrespective of sex or morphometric identity. The CW:CL and CW:CH ratios of males and females were individually tested against rostrum shape (factor with three levels) using univariate one-way PERMANOVA; distinct rostrum shapes were indicative of *C. maenas*, slightly blunted rostrums were undefined, and blunted rostrums were indicative of *C. aestuarii* (Behrens Yamada and Hauck 2001). Any crabs that had chipped rostrums were removed during tests on rostrum shape. All statistical analyses had a significance value at $\alpha = 0.05$, while post-hoc Mann-Whitney pairwise tests had sequential Bonferroni correction applied at the 5% level ($\alpha = 0.05$) to correct for multiple tests (Rice

1989). Statistical analyses were carried out in the software ORIGIN PRO version 2020 (OriginLab Corporation, Northampton, MA, USA) and PAleontological STatistics "PAST" version 4.01 (Hammer et al. 2001).

Genetic analysis

Mitochondrial COI sequence chromatograms were edited and proofread by eye, followed by multiple alignment using the software package Geneious Prime version 2020.1.1 (Biomatters Ltd, Auckland, New Zealand). Sequences that had poor quality reads, possibly due to sequencing error or primer mismatch were excluded from further sequence analysis. Of the 88 samples sent for Sanger sequencing, only 59 sequences were of high enough quality to be used in analysis. Sex, CW:CL, CW:CH and rostrum shape of the crabs used in the genetic analysis were evaluated to identify if morphological characters used to differentiate *Carcinus* species were supported by DNA sequences. Sequences were compared to the National Center for Biotechnology Information (NCBI) sequence database GenBank using the basic local alignment tool MegaBlast following Mantelatto et al. (2017). I compared the sequences with the resulting Blast sequences, including percent similarity and the GenBank distance tree to determine species.

A representative selection of thirteen *C. maenas* and five *C. aestuarii* COI haplotype sequences from multiple native and introduced localities and studies were retrieved from GenBank and aligned with my 59 South Australian *Carcinus* sequences in Geneious. The number and geographic range of representative haplotype samples selected will be affected by ascertainment bias, which can under or overestimate population parameters due to non-randomness of sample selection (Phillips et al. 2019). All sequences were trimmed to the shortest sequence length, which resulted in a 367 bp gap-less and unambiguously aligned sequence. Mean Kimura 2-parameter (K2P) genetic distances within and between South Australian *Carcinus*, global *C. maenas*, Mediterranean *C. aestuarii*, and outgroups were calculated with 1,000 bootstrap replications in MEGA version 6.0 (Tamura et al. 2013). Haplotype information such as the number of haplotypes, the number of parsimony informative and polymorphic sites, nucleotide diversity (π) and haplotype diversity (Hd) for all COI sequences were obtained using the software DnaSP version 6.12.03 (Rozas et al. 2017).

A neighbour-joining (NJ) tree of the COI sequences was constructed in Geneious using the Hasegawa–Kishono–Yano (HKY) genetic distance model with 1,000 bootstrap replications

(Felsenstein 1985). Proportions less than 70% were omitted from the tree. *Portunus armatus* (GenBank Accession: MN184695) was used as an outgroup to root the NJ tree, while *Nectocarcinus bennetti* (GenBank Accession: HQ944638) and *Ovalipes australiensis* (GenBank Accession: MN184694) were used as additional outgroup comparisons. Sample details for all sequences used in this study, including GenBank sequences, can be viewed in Appendix Table A2.4.

2.3 RESULTS

2.3.1 Relationships between carapace dimensions

A total of 1,311 *Carcinus* were analysed in this study, which consisted of 682 males and 629 females. Male crabs were larger than females on average with male carapace dimensions exceeding females across CW, CL and CH. A summary of the minimum, maximum and mean carapace sizes for all dimensions can be viewed in Appendix Table A2.5. Allometric relationships between CW and both CL and CH showed a strong, positive correlation in male and female *Carcinus*. The CW – CL regressions displayed a positive and significant relationship for male crabs (adjust. $R^2 = 0.993$, ANOVA $p < 0.001$; Appendix Fig. A2.1A) and female crabs (adjust. $R^2 = 0.988$, ANOVA $p < 0.001$; Appendix Fig. A2.1B). The CW – CH regressions were weaker than CW – CL but displayed a similar positive relationship for male crabs (adjust. $R^2 = 0.984$, ANOVA $p < 0.001$; Appendix Fig. A2.1A) and female crabs (adjust. $R^2 = 0.971$, ANOVA $p < 0.001$; Appendix Fig. A2.1B). The relationship between the carapace measurements supported their use in morphometric ratios for species delimitation.

2.3.2 Carapace ratios and morphology

The overall CW:CL ratio for males in this study ranged between 1.24 – 1.44 (mean = 1.34 ± 0.03), while for females the overall CW:CL ratio ranged between 1.18 – 1.40 (mean = 1.31 ± 0.03) (Fig. 2.3A). There was a significant association between the frequency of males and females and morphometric identity for CW:CL ratio ($X^2 = 55.03$, df = 2, $p < 0.001$). The CW:CH ratio for males ranged between 2.05 – 2.66 (mean = 2.39 ± 0.08), while for females the CW:CH ratio ranged between 2.0–2.57 (mean = 2.32 ± 0.09) (Fig. 2.3B). There was a significant association between the frequency of males and females and morphometric identity for CW:CH ratio ($X^2 = 138.93$, df = 2, $p < 0.001$). Irrespective of sex or morphometric identity, there was a significant difference between CW:CL and CW:CH values (Mann-Whitney U test, $U = 0$, $p < 0.001$).

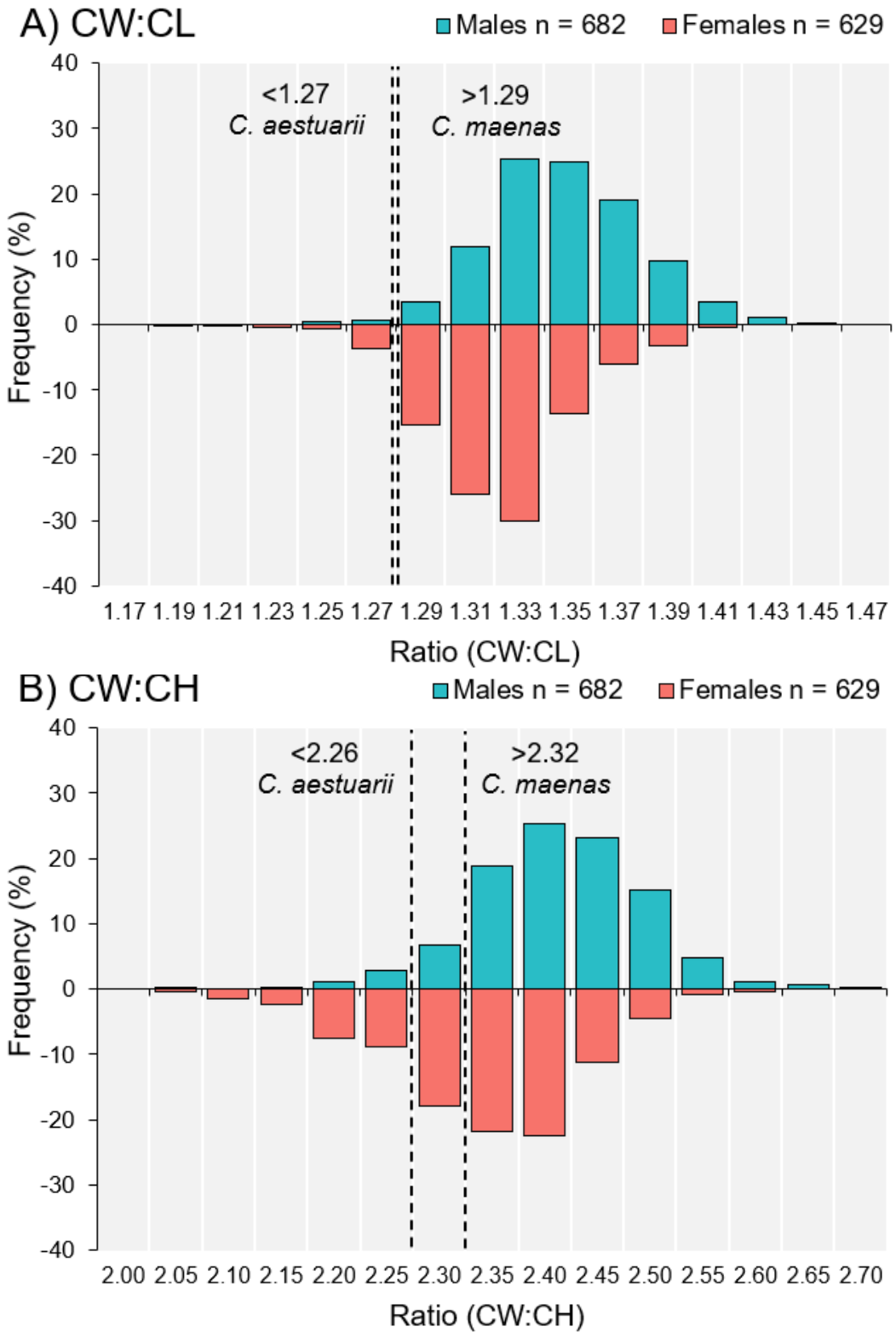

Figure 2.3 Size frequency distributions of two carapace ratios in male (n = 682) and female (n = 629) *Carcinus* collected from all sites and seasons in Gulf St Vincent, South Australia, between 2013–2019. A) CW:CL ratio and B) CW:CH ratio. Vertical dashed lines indicate undefined ratios that could be indicative of either species, where crabs that fall to the left of the dashed line are indicative of *C. aestuarii*, and crabs to the right of the dashed line are indicative of *C. maenas*. Please note difference in values on the x-axis.

For rostrum shape, 17 crabs had chipped rostrums and were excluded from analysis, resulting in 1,294 *Carcinus* used in rostrum comparisons. “Distinct” rostrum shapes indicative of *C. maenas* were most abundant (male n = 419, female n = 332), followed by “slightly blunted” rostrums (male n = 149, female n = 163) and finally “blunted” rostrums, which are indicative of *C. aestuarii* (male n = 108, female n = 123). In males, there was a significant difference between rostrum shape and CW:CL (PERMANOVA, $F = 9.258$, $p_{(perm)} < 0.001$), but not between rostrum shape and CW:CH (PERMANOVA, $F = 2.553$, $p_{(perm)} = 0.078$). Pairwise tests indicated a significant interaction between distinct and blunt rostrums based on CW:CL ratios in males (sequential Bonferroni, $p < 0.001$). In females, there was no significant difference between rostrum shape and CW:CL (PERMANOVA, $F = 0.902$, $p_{(perm)} = 0.4$) and CW:CH (PERMANOVA, $F = 0.179$, $p_{(perm)} = 0.83$).

Using the criteria of Behrens Yamada and Hauck (2001) and Clark et al. (2001), 1,196 specimens were identified as *C. maenas* based on CW:CL morphology and 54 specimens were identified as *C. aestuarii* (Fig. 2.4). There were 61 specimens that had an undefined CW:CL ratio between the two species. A total of 194 specimens were identified as *C. aestuarii* based on CW:CH morphology, while 961 specimens had a CW:CH ratio that matched *C. maenas* and 156 specimens were undefined. For rostrum shape, 751 crabs had distinct rostrums indicative of *C. maenas*, 312 crabs had slightly blunted rostrums, and 231 crabs had blunted rostrums indicative of *C. aestuarii*. Chipped rostrums were identified in 17 individuals. There were more females with CW:CL, CW:CH and rostrum shapes that matched with *C. aestuarii* and undefined species compared to males.

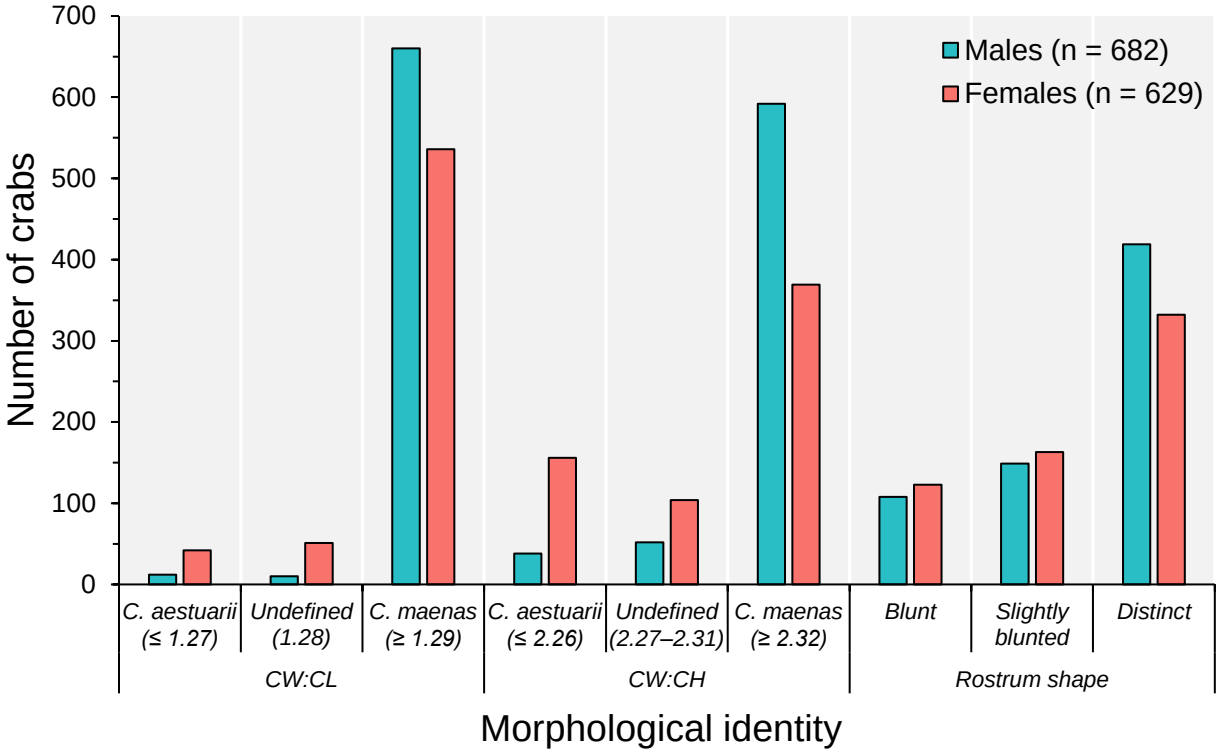

Figure 2.4 Total number of South Australian crabs with CW:CL ratio, CW:CH ratio or rostrum shape morphology indicative of *Carcinus* species identity. Teal bars indicate males, pink bars indicate females.

Combinations of morphological indicators (CW:CL, CW:CH and/or rostrum shape) showed that most specimens were undefined (Fig. 2.5). When using all three morphological indicators combined (CW:CL, CW:CH and rostrum shape), most specimens were undefined or indicative of *C. maenas*, while only eight individuals were classed as *C. aestuarii*. Similar patterns were seen for both ratio indicators combined (CW:CL and CW:CH) with most specimens classified as undefined or as *C. maenas*. When morphological indicators included one ratio combined with rostrum shape (CW:CL and rostrum shape, or CW:CH and rostrum shape), nearly all crabs were classified as undefined. Rostrum shape is a highly variable indicator that will incorrectly classify *Carcinus* even when combined with carapace ratios. As less crabs were classified as undefined using both carapace ratios, these morphological indicators are more reliable. Species identity varied between sexes when using different morphological indicator combinations.

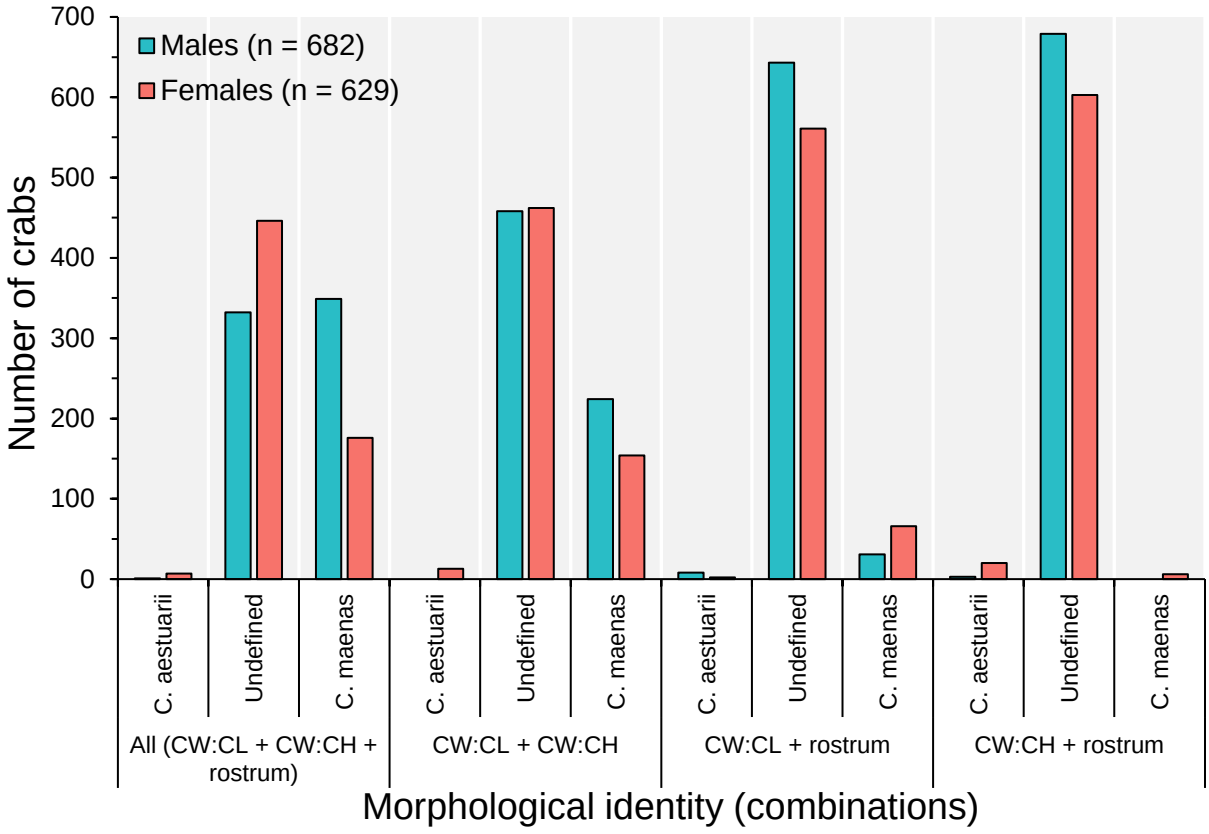

Figure 2.5 Total number of South Australian crabs with combinations of CW:CL ratio, CW:CH ratio and rostrum shape morphology indicative of *Carcinus* species identity. Teal bars indicate males, pink bars indicate females.

2.3.3 Genetic analyses and species identity

Haplotype summary and genetic distance

A fragment of 367 base pairs (bp) of mtDNA COI gene was successfully aligned and compared with 59 *Carcinus* samples from Gulf St Vincent, South Australia, 13 *C. maenas* samples from global native and invasive ranges, five *C. aestuarii* samples from the Mediterranean Sea, and three outgroup Portunid species (*N. bennetti*, *O. australiensis* and *P. armatus*; Table 2.2). The number of observed haplotypes was similar across groups, with a maximum of five unique haplotypes observed. Haplotype diversity (Hd) was highest for *C. aestuarii* and outgroup Portunid species and was lowest for South Australian *Carcinus* and *C. maenas* groups (0.693–0.705). The number of polymorphic sites in South Australian *Carcinus* and *C. maenas* was similar with five and seven sites observed respectively, while *C. aestuarii* had 23 polymorphic sites. Nucleotide diversity (π) was higher in *C. aestuarii* (0.03) than both South Australian *Carcinus* and *C. maenas* (0.003–0.004).

Table 2.2 Haplotype summary of *Carcinus* and GenBank sequences assessed in this study, based on a 367 bp region of the mtDNA COI gene. Codes = sample codes shown in the phylogenetic tree (Fig. 2.6); N = number of individuals sequenced; h = number of haplotypes; S = number of polymorphic sites; Hd = haplotype diversity; π = nucleotide diversity. Sample details for all individual sequences, including GenBank Accession numbers, can be viewed in Appendix Table A2.4

Species	Codes	Locality	N	h	S	Hd (± SD)	π
Carcinus	H, M, R	Gulf St Vincent, South Australia	59	5	5	0.693 ± 0.04	0.003
C. maenas	C	USA, CAN, southeast Australia, UK, Sweden, Norway, Germany, Iceland	13	5	7	0.705 ± 0.12	0.004
C. aestuarii	A	Italy, Turkey, Tunisia, Mediterranean Sea/Portugal	5	5	23	1 ± 0.13	0.03
P. armatus *N. bennetti* *O. australiensis*	N, O, P	Western Australia, New Zealand	3	3	100	1 ± 0.28	0.196
Total		All regions	80	16	131	0.756 ± 0.04	0.03

A total of 81 parsimony informative sites were detected for the 80 COI sequences across all crab species. There were four parsimony informative sites across South Australian *Carcinus* sequences, two parsimony informative sites across *C. maenas*, seven parsimony informative sites across *C. aestuarii*, and no parsimony informative sites in the outgroup species. Mean K2P genetic distances between the four taxa groups showed an average 0.4% divergence between South Australian *Carcinus* and *C. maenas* (Table 2.3). Both *C. maenas* and South Australian *Carcinus* had higher average divergence with *C. aestuarii* at 11.3–11.5%. All South Australian *Carcinus* sequences had highest average divergence with the outgroup species at ~25%. Mean K2P genetic distances (± SE) within the four taxa groups was: South Australian *Carcinus* = 0.003 ± 0.001; *C. maenas* = 0.004 ± 0.002; *C. aestuarii* = 0.031 ± 0.006; and outgroups = 0.229 ± 0.022.

Table 2.3 Mean pairwise genetic distances between the four taxa groups. Analysis based on 80 COI gene sequences (367 bp) and calculated with the Kimura 2-parameter (K2P) model with 1,000 bootstrap replications. Genetic distance values are shown below the diagonal while between group percentages are shown above the diagonal.

	Carcinus (SA)	*C. maenas*	*C. aestuarii*	Outgroups
Carcinus (SA)		0.4%	11.5%	25.2%
C. maenas	0.004		11.3%	25.3%
C. aestuarii	0.115	0.113		25%
Outgroups	0.252	0.253	0.25	

Species identification

All 59 South Australian *Carcinus* sequences in this study obtained between 99.2–100% identical matches to *Carcinus maenas* when compared to COI sequences on the GenBank database via the Megablast algorithm. Review of the GenBank distance tree for South Australian samples showed that sequences were placed on the same clade with all other GenBank sequences identified as *C. maenas*, but also had a close match to one *Liocarcinus pusillus* COI sequence (658 bp) on GenBank (Accession number: MG935274). On further inspection this sample matched closely only with *C. maenas* and no other *Liocarcinus* spp. This suggested that this *L. pusillus* sequence was a misidentification or named incorrectly by the authors and this match was disregarded. The neighbour-joining tree of the 80 COI gene sequences showed two major clades, with one belonging to *C. maenas* and the other belonging to *C. aestuarii* (Fig. 2.6). All 59 South Australian *Carcinus* sequences nested within the *C. maenas* clade. The GenBank identity matches, the mean genetic distances between taxa and the neighbour-joining tree provide strong support that South Australian *Carcinus* are *Carcinus maenas*. All South Australian *C. maenas* haplotype sequences were deposited into GenBank (Accession Numbers MT748791 – MT748849; Appendix Table A2.4).

Morphology of sequenced crabs

The 59 sequenced *C. maenas* from South Australia consisted of 37 males and 22 females. The CW of sequenced males ranged from 29.26 – 83.73 mm (mean CW = 57.32 ± 14.92), while for females the CW ranged from 42.47 – 72.15 mm (mean CW = 56.34 ± 9.15). Most sequenced crabs had a CW:CL and CW:CH ratio that matched *C. maenas* (n = 57 and n = 41, respectively; Table 2.4). The number of sequenced crabs with CW:CL ratios that matched

C. aestuarii was low but was much higher for CW:CH ratios. Distinct rostrum shapes were most abundant in sequenced crabs (n = 45), followed by slightly blunted rostrum shape (n = 13), and one individual with a chipped rostrum (n = 1). There were no significant differences between the CW:CL or the CW:CH ratios of sequenced crabs when compared with shape of the rostrum (CW:CL, PERMANOVA, $F = 2.19$, $p = 0.14$; CW:CH, PERMANOVA, $F = 0.002$, $p = 0.96$). The number of crab sequences obtained from each site in Gulf St Vincent were as follows: Middle Beach = 9; Port Gawler = 8; Port Adelaide River = 11; Old Port Reach = 8; Hallett Cove = 1; Onkaparinga River = 10; and Aldinga = 12.

Table 2.4 Morphological indicators of male and female *C. maenas* sequenced from Gulf St Vincent, South Australia (total crab n = 59). Ratio values used to indicate species of *Carcinus* based on Behrens Yamada and Hauck (2001) and Clark et al. (2001).

Morphological indicators			
CW:CL ratio			
Species indicator	*C. aestuarii* ≤ 1.27	Undefined 1.28	*C. maenas* ≥ 1.29
Female *N*	2	0	20
Male *N*	0	0	37
CW:CH ratio			
Species indicator	*C. aestuarii* ≤ 2.26	Undefined 2.27–2.31	*C. maenas* ≥ 2.32
Female *N*	11	2	9
Male *N*	2	3	32
Rostrum shape			
Species indicator	*C. aestuarii* Blunted	Undefined Slightly blunted	*C. maenas* Distinct
Female *N*	0 (1 chipped)	6	15
Male *N*	0	7	30

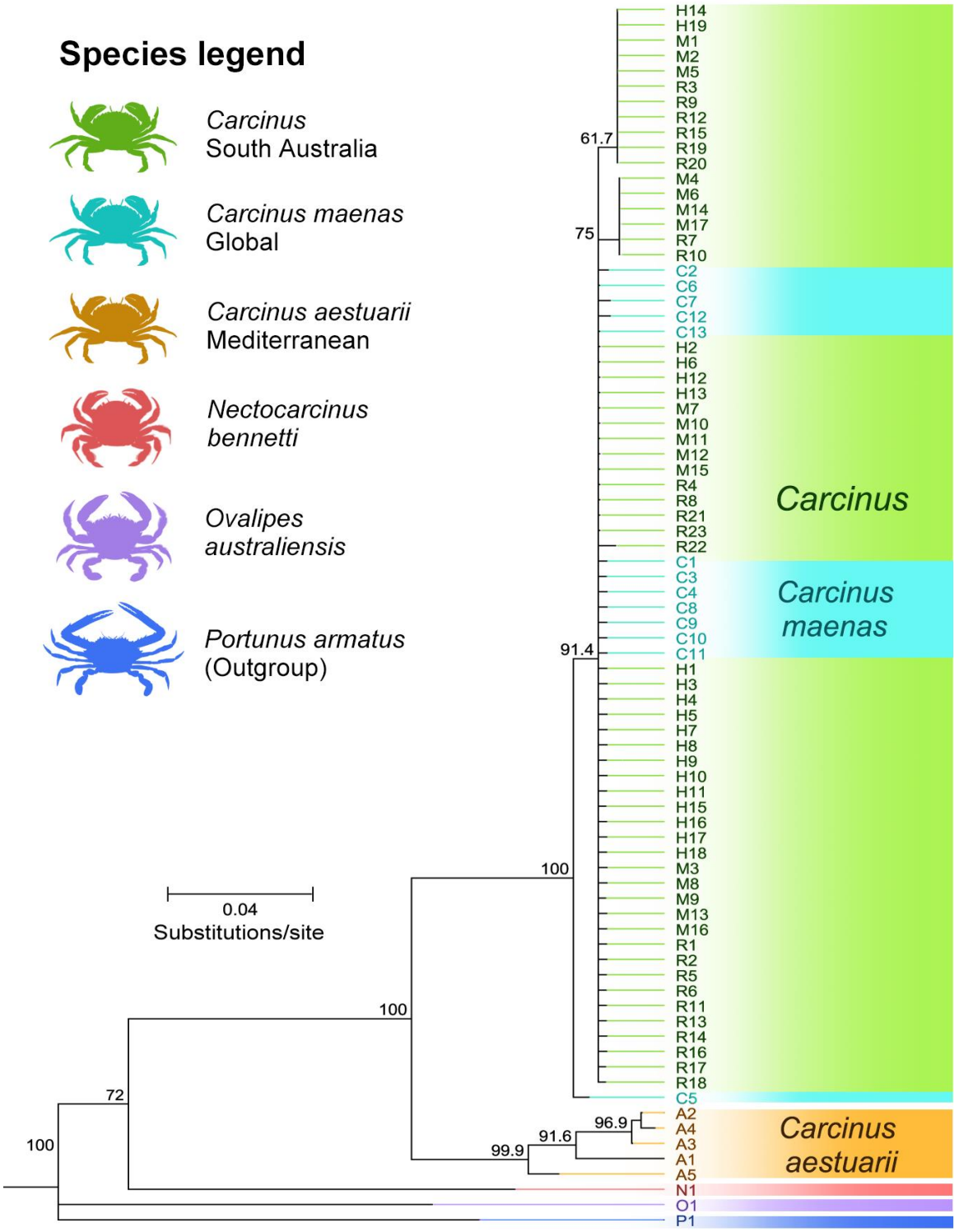

Figure 2.6 Neighbour-joining tree based on partial mtDNA COI sequences (367 bp) of *Carcinus* and closely related species obtained from this study and GenBank. The 59 South Australian *Carcinus* COI samples are shown in green. GenBank sequences are indicated by the corresponding colour key. Numbers above the branches indicate bootstrap proportions calculated from 1,000 replicates, with proportions less than 70% omitted from the tree. The distances were computed using the HKY genetic distance model with the number of base substitutions per site as the units. Information for all COI sequences including sample ID codes can be found in Appendix Table A2.4.

2.4 DISCUSSION

In temperate South Australia, invasive *Carcinus* had been referred to as Atlantic *C. maenas* without confirmation from morphological or genetic research. This study assessed the identity of invasive *Carcinus* in Gulf St Vincent, South Australia, using morphological carapace ratios (which can differentiate *Carcinus* species) and genetic analysis of the mtDNA COI gene. Despite morphological carapace ratios suggesting that both *C. maenas* and *C. aestuarii* are present in South Australia, genetic analysis confirmed the identity of South Australian *Carcinus* as *C. maenas.* When compared with genetic results, the best morphological indicator of *Carcinus* species was the CW:CL ratio. The CW:CH ratio and rostrum shape were highly variable due to sexual dimorphism and environmental factors and are thus unreliable discriminators of species in the *Carcinus* genus. The inclusion of a sister species (*C. aestuarii*) in *Carcinus* has had a contentious taxonomic history due to a lack of consistent and reliable morphological characters between species (Behrens Yamada and Hauck 2001; Clark et al. 2001; Leignel et al. 2014). This study rejected Behrens Yamada and Hauck's (2001) findings that *Carcinus* are distinguishable in the field based only on morphology and supported findings by Clark et al. (2001) that morphology alone cannot identify *Carcinus.*

2.4.1 Morphological indicators of *Carcinus*

Taxon-specific morphometric ratios and other morphological characters have been used to distinguish many species of decapod crustaceans, but can be affected by variation from sexual dimorphism, hybridisation and intraspecific variation (Macleod 2017). For example, Fazhan et al. (2020) measured 24 morphometric dimensions and assessed morphological characters to reveal identities of four *Scylla* species (mud crabs). Using discriminant function analysis, they determined which morphometric ratios differentiated *Scylla* spp., including variation attributable to sexual dimorphism. Fazhan et al. (2020) suggested that *Scylla* spp. with intermediate morphological characteristics could be hybrids, and that molecular work was needed to confirm these observations. In the crabs *Crytograpsus affinis* and *C. altimanis*, variability of morphometric ratios resulting from life history stages and phenotypic variation made it difficult to identify species without genetic sequencing (Spivak and Schubart 2003). Cartaxana (2015) found that morphometric ratios and diagnostic characters were highly variable in prawns of the genus *Palaemonetes*. Morphometric analysis alone could not unequivocally assign prawn specimens as *P. longirostris* or *P. garciacidi*, however 16S and COI gene sequencing provided evidence that the species are *P. longirostris* (Cartaxana 2015).

Comparisons of CW:CL and CW:CH ratios from this study to other studies showed overlap with both species of *Carcinus* across sexes and native and invasive localities (Table 2.5). As *C. maenas* displays sexual dimorphism in carapace shape (Ledesma et al. 2010), it is not surprising that female crabs in this study had carapace ratios that matched *C. aestuarii* more than males. The carapace of females is higher/deeper and narrower than males, which would affect the CW:CL and CW:CH ratios and increase morphological variation. Behrens Yamada and Hauck (2001) stated that sexual dimorphism needs to be considered when assessing carapace ratios of *Carcinus*. The CW:CH ratio is also more variable than CW:CL because carapace height is harder to measure and standardise than carapace width (Behrens Yamada and Hauck 2001). Intraspecific variation in carapace size and shape has been measured in both *C. maenas* and *C. aestuarii* and contributes to overlap in ratios. Brian et al. (2006) observed intraspecific morphological variation within male and female *C. maenas* in the UK which could be the result of phenotypic plasticity. *Carcinus aestuarii* has a smaller CW:CL ratio on average than *C. maenas*, but *C. aestuarii* can reach CW:CL ratios above 1.29 (Koçak et al. 2011) and as high as 1.49 (Aydin 2013), which is well within the ratios of *C. maenas* (Table 2.5).

In this study, rostrum shape was variable across all specimens. Cohen et al. (1995) and Behrens Yamada and Hauck (2001) described the rostrum of *C. aestuarii* as protruding beyond the eyes and is flatter/blunted, with 'bumps' not as distinct as the rostrum of *C. maenas.* Rostrum shape is often taxon-specific and a key identifying feature in many crustaceans (Poore and Ahyong 2004). However, rostrum shape is an unreliable morphological indicator as it can be blunted from environmental factors or change shape throughout moulting periods (Mazancourt et al. 2017). A morphological character not assessed in this study was the shape of the male copulatory pleopods ("gonopods"). Male pleopod shape and size is a standard character for taxonomic description of some brachyuran crabs (Plagge et al. 2016). *Carcinus maenas* has strongly curved pleopods while *C. aestuarii* has straight, parallel pleopods (Zariquiey Alvarez 1968; Behrens Yamada and Hauck 2001). Clark et al. (2001) used scanning electron microscopy and observed no notable differences in male pleopod shape between Atlantic *C. maenas* and Mediterranean *C. aestuarii* specimens. In other species such as *Liocarcinus corrugatus*, male pleopods were similar between Asian and European populations and was also considered an unreliable indicator of species differentiation (Plagge et al. 2016).

Table 2.5 Summary and comparison of CW:CL and CW:CH ratios measured for *Carcinus maenas* and *C. aestuarii* from other studies. Results from this study are highlighted in bold at the bottom of the table. Dashes (-) represent information that was not recorded or was not located in a study. Table expanded from Behrens Yamada and Hauck (2001). CH is referred to as CD in most of these studies.

Species	Location	Status	Sex	*N*	CW:CL	CW:CH	Study
Carcinus maenas	Iberian Peninsula	Native	Male	25	1.32 – 1.35	-	Zariquiey Alvarez 1968
	Iberian Peninsula	Native	Female	9	1.29 – 1.31	-	Zariquiey Alvarez 1968
	Iberian Peninsula	Native	-	-	1.27 – 1.35	2.27 – 2.57	Almaça 1972
	Plymouth, UK	Native	Female	2	1.29 – 1.32	-	Rice and Ingle 1975
	UK, Netherlands and Morocco	Native	Male	3	1.30 - 1.32	-	Behrens Yamada and Hauck 2001
	Oregon, USA	Invasive	Male	6	1.31 – 1.38	-	Behrens Yamada and Hauck 2001
	Atlantic coastline	Native	-	1301	1.25 - 1.36	2.22 - 2.60	Clark et al. 2001
	California, USA	Invasive	-	38	1.28 – 1.36	2.45 – 2.58	Clark et al. 2001
	Gulf St Vincent, South Australia	**Invasive**	**Male**	**682**	**1.23 - 1.43**	**2.0 - 2.66**	**This study**
		Invasive	**Female**	**629**	**1.18 - 1.40**	**2.0 - 2.57**	
Carcinus aestuarii	Iberian Peninsula	Native	Male	42	1.24 – 1.26	-	Zariquiey Alvarez 1968
	Iberian Peninsula	Native	Female	35	1.22 – 1.26	-	Zariquiey Alvarez 1968
	Iberian Peninsula	Native	-	-	1.24 – 1.27	2.25 – 2.31	Almaça 1972
	Tunis, Tunisia	Native	Female	1	1.25	-	Rice and Ingle 1975
	Tokyo Bay, Japan	Invasive	Male	6	1.25 – 1.31	2.13 – 2.45	Furota et al. 1999
	Tokyo Bay, Japan	Invasive	Female	2	1.24 – 1.25	-	Furota et al. 1999
	Sicily and Tunisia	Native	Male	5	1.25 - 1.29	-	Behrens Yamada and Hauck 2001
	Mediterranean Sea	Native	-	398	1.21 - 1.29	2.12 - 2.32	Clark et al. 2001
	Homa Lagoon, Turkey	Native	Male	608	1.18 - 1.31	-	Koçak et al. 2011
	Homa Lagoon, Turkey	Native	Female	559	1.18 - 1.28	-	Koçak et al. 2011
	Black Sea, Turkey	Native	Male	285	1.24 – 1.45	-	Aydin 2013
	Black Sea, Turkey	Native	Female	279	0.76 – 1.49	-	Aydin 2013

2.4.2 Genetic confirmation of South Australian *C. maenas*

Confirmation and delimitation of marine invasive species is beneficial for understanding introduction histories, identification of source populations and mapping the extent of the invasive range (Negri et al. 2018). Although some crabs in this study had carapace ratios that matched with *C. aestuarii*, analysis of the COI gene confirmed all species of *C. maenas.* The level of divergence between South Australian *C. maenas* and Mediterranean *C. aestuarii* was around ~11.5% and matches divergence between these two species in other studies (10.2–11%; Roman and Palumbi 2004; Darling 2011a). Genetic analysis has confirmed species identities of other invasive crabs when morphological discrepancies had occurred, such as in the Asian paddle crab *Charybdis japonica* (Smith et al. 2003) and the Asian brush-clawed crab *Hemigrapsus takanoi* (Markert et al. 2014). Clark et al. (2001) noted that since morphology alone does not support species separation of *Carcinus*, genetic analysis is useful for verifying crab identity, especially during new introductions. Unless a source population is known prior, specimens of *Carcinus* found in newly introduced areas or range expansions are likely to only be accurately identified through DNA sequencing (Carlton and Cohen 2003). Rapid confirmation of *Carcinus* is important for identifying possible source populations for reconstructing invasion pathways (e.g. Mediterranean Sea, Japan and South Africa sources in the case of *C. aestuarii*).

2.4.3 Study limitations and recommendations

This study was not intended to be a taxonomic review nor a phylogenetic reconstruction of *Carcinus*. Rather, I used morphological and genetic analysis to highlight discrepancies between methods used to confirm species identity within the *Carcinus* genus in South Australia. In order to classify taxon-specific morphological characters between *C. maenas* and *C. aestuarii*, a taxonomic re-assessment may be warranted. It is important to assess large sample sizes of male and female crabs to factor in variation caused by sexual dimorphism (Ledesma et al. 2010). Crab specimens should be assessed from native and invasive ranges and various crab sizes should also be included to address ontogenetic morphological changes (Duarte et al. 2014; Stevens et al. 2014). Unfortunately, no type specimens of *C. maenas* and *C. aestuarii* could be obtained from the native ranges to provide a morphological comparison with our South Australian samples.

Due to overlap of morphometric ratios and variability in morphological characters in *Carcinus*, specimens found during new incursions should be identified by molecular methods.

In cases where time or funding is limited, however, morphological identification may be adequate. If the specimens found during incursions are male, the CW:CL ratio should be a reliable indicator of species. If the specimen is female, caution is needed when using carapace ratios for species identification (including CW:CL) due to sexual dimorphism and morphological overlap.

The mtDNA COI gene can discriminate *C. maenas,* and Geller et al. (1997), Roman and Palumbi (2004), Darling et al. (2008) and Darling (2011a) successfully distinguished the two *Carcinus* species using COI and 16S sequences. Rubinoff and Holland (2005) argue that mtDNA markers can be problematic for taxonomic resolution especially in closely related sister taxa where introgression and hybridisation occurs (which is evident in *Carcinus;* Darling 2011a). The sole use of mtDNA without confirmation from nuclear molecular markers or morphological characters can lead to phylogenetic misinterpretation (Rubinoff and Holland 2005). South Australian *C. maenas* show similar mtDNA divergence to *C. maenas* from the USA (Darling 2011a), identified by mtDNA and nuclear microsatellites. While nuclear SNPs could be used to further assess hybridisation in South Australian *C. maenas,* further nDNA sequences would also have to be available for native *C. aestuarii*.

In this study, 59 of 128 crabs were successfully sequenced. The sequencing success did not appear to be influenced by date of collection/preservation, the date of DNA extraction, crab sex or size, sample site/habitat, carapace ratio, muscle tissue colour or amount, or DNA concentration. All reagents and primers used were fresh and multiple PCR conditions were tested (Table A2.3). Disparity in extractions versus sequences could be due to many factors, ranging from crustacean pigments and other contaminants (Palmer 2008), degraded tissue from freeze/thaw cycles (Bitencourt et al. 2007), dehydration of PCR products, user error, and primer mismatch.

Due to the poor performance of Folmer's universal primers, I suggest using taxon-specific primers for molecular identification such as the those developed for *C. maenas* (Roman and Palumbi 2004). While Folmer's degenerate primers have been used for *C. aestuarii* in previous studies, it may be of research interest to develop *C. aestuarii*-specific primers. This would help reduce primer mismatch and assist with detections, especially as *C. aestuarii* samples from the native population are limited compared to *C. maenas* (Darling and Tepolt 2008). The divergence in COI haplotypes in *Carcinus* make it difficult to identify conserved regions large enough for designing genus-specific PCR primers (Darling and Tepolt 2008).

This study confirmed the identity of *Carcinus* in South Australia as the globally invasive *C. maenas* using COI gene sequencing. Morphological variation within the *Carcinus* genus occurs due to intraspecific phenotypic variation, ontogenetic changes, sexual dimorphism, and in some cases, hybridisation. A taxonomic re-classification of morphological characters used to differentiate between *C. maenas* and *C. aestuarii* may be necessary. As *Carcinus* is globally widespread and can hybridise in its introduced range, rapid identification of the species using DNA sequencing should be undertaken during new incursions. Genetic confirmation of species identity will also be warranted if species-specific molecular tools are used during surveillance. Obtaining *C. maenas* specimens across its native and introduced range will help with assessing source populations and demographic history of this invasive species in temperate South Australia.

Chapter 3. Morphometric variation and genetic homogeneity of invasive *Carcinus maenas* across coastal habitats in South Australia

ABSTRACT

Population structure of marine invasive species may drive local adaptation or phenotypically plastic responses to local environments in the introduced range. The invasive European shore crab, *Carcinus maenas*, has established in various coastal habitats of Gulf St Vincent, South Australia. Morphological variation and genetic structure of *C. maenas* among habitats was investigated using linear and geometric morphometrics and mitochondrial COI gene sequencing. Both linear and geometric morphometrics showed significant differences in morphological size and shape among habitats. Carapace shape was wider in crabs from mangrove habitats compared to narrower carapaces in crabs from harbours and rocky shores. Geometric morphometrics were more informative for exploring visual shape differences than linear morphometrics by using shape wireframes and transformation grids, however the ecological relevance of carapace shape differences between habitat types was unclear. COI gene sequencing showed that crabs from all three habitats had similar haplotypes and there was no evidence of population structuring. Genetic homogeneity across the Gulf St Vincent population of *C. maenas* is likely to be the result of gene flow due to recruitment of larvae and/or migration of adults between habitats. Lack of genetic structure could also be the result of population bottlenecks or each habitat having a common source population. Morphometric differences and genetic homogeneity of *C. maenas* across habitats probably indicates phenotypically plastic responses to local environments, which contributes to invasion success by increasing survival under different abiotic and biotic conditions.

3.1 INTRODUCTION

Most introduced species do not successfully establish and become widespread in their introduced range (Crooks and Rilov 2009). Life-history strategies and biological traits such as high reproductive output, genetic adaptability and phenotypic plasticity are identified as indicators of invasion success that can increase chances of colonisation (Geburzi and McCarthy 2018). Successful marine invasive species often modify these traits (i.e. their physiology, behaviour, biochemistry, life-history and/or morphology) within a lifetime as a response to environmental cues (Smith 2009). Variation in these traits can be driven by genetic or epigenetic bases, but also by plastic responses to the environment for a single genotype (Pérez et al. 2006; Padilla and Savedo 2013; Eirin-Lopez and Putnam 2019).

Phenotypic plasticity is traditionally characterised as the ability for multiple phenotypes to arise from a single genotype and is induced under different environmental conditions (Gianoli and Valladares 2012). Altered gene expression and development pathways (i.e. genetic variation, natural selection and epigenetics) also play a key role in phenotypic variation, however the cause-effect interaction between phenotypic plasticity and adaptive evolution is still debated (Ghalambor et al. 2007; Padilla and Savedo 2013; Fox et al. 2019). To successfully colonise a novel environment, many introduced species may need to change phenotypically for populations to establish and grow under unfamiliar abiotic and biotic surroundings (Smith 2009; Padilla and Savedo 2013; Epstein and Smale 2018; Fox et al. 2019). The resulting phenotypic traits may vary in the introduced population, especially in heterogeneous environments which can increase fitness and survival (Ghalambor et al. 2007).

As marine invasive species will experience different environmental conditions in their introduced range (i.e. variations in water quality, habitat types, and biotic interactions), any subsequent genetic and phenotypic variation within the species can lead to population structure (Schmid and Guillaume 2017). Phenotypic responses to local environmental conditions can cause changes in allometry and ontogenetic development, which can result in morphological variation (Gianoli and Valladares 2012; Metri et al. 2017). In marine invertebrates, 40% of phenotypic plasticity studies examined morphological variability (Padilla and Savedo 2013). Morphological changes can facilitate invasive species survival, such as increased shell thickness to reduce predation risk in the invasive mud snail *Potamopygrus antipodarum* in New Zealand (Kistner and Dybdahl 2013). In Argentina the invasive golden mussel *Limnoperna fortunei* displayed morphological variation in shell shape (i.e. elongation of the shell) and gill

structure (i.e. gill cilia length and density) in response to high total suspended sediments (Paolucci et al. 2014). In Maine, USA, the European shore crab *Carcinus maenas*, displayed biogeographic differences in chelae (i.e. larger claw sizes and crushing force) as a response to differences in gastropod prey armour (Smith 2004). Morphological variation is, therefore, a phenotypic response in invasive species that may facilitate persistence in novel environments or under varying selection pressures.

Morphometrics is the analysis of size and shape and can quantify morphological variation (Rohlf 1990; Zelditch et al. 2012). Linear (or traditional) morphometrics are distance-based measures of morphological characters (e.g. length, width, and depth) and are used to measure size but contain limited shape information (Rohlf 1990). Linear measurements can be converted to ratios (i.e. of body proportions) to standardise size and provide some shape information, but ratios cannot characterise the entire shape of an organism (Baur and Leuenberger 2011; Cooke and Terhune 2015; MacLeod 2017). Geometric morphometrics using x and y coordinates (and z coordinates for 3D data) called 'landmarks', use positional information to analyse shape differences and overcome the limitations of linear morphometrics (Cooke and Terhune 2015). Centroid sizes within landmark data can separate size and shape information and are an increasingly used method for quantifying morphological variation (Parsons et al. 2003; Maderbacher et al. 2008). Linear and geometric morphometrics are useful for identifying phenotypic variation in marine invasive species and can also investigate spatially driven morphological differences (Cardini 2020).

Morphometrics are increasingly combined with molecular analyses to address questions regarding taxonomic classification, phenotypic plasticity or adaption, and structure within and between populations (Klingenberg 2002; Cǎndek and Kuntner 2015). If genetic morphological plasticity exists (i.e. the genotype – environment interaction is significant) then it is possible that morphological variation is the result of selection (Smith 2009). Population genetic structure at local geographic scales can also be attributed to multiple introduction events or geographic barriers between habitats (Cabezas et al. 2014). For example, populations of the invasive bivalve *Corbicula fluminea* have morphometric and genetic differences between two Portuguese estuaries; this could be the result of phenotypic plasticity or genotypic adaptation from different environmental and ecological conditions, or through different source populations and invasion routes for the two populations (Sousa et al. 2007). To consider the underlying mechanisms of morphological variation and genetic structuring, both

morphometrics and genes should be examined to obtain a more holistic view of any population differentiation (Silva et al. 2010b).

Decapod crustaceans are among the most widespread marine invasive taxa, with phenotypic plasticity and maintenance of high genetic variation identified as contributors to their invasion success (Molnar et al. 2008; Hänfling et al. 2011; Geburzi and McCarthy 2018). Many crustaceans display plastic responses to their environment through changes in the morphology of the carapace and chelae (Anastasiadou and Leonardos 2008; Idaszkin et al. 2013; Hegele-Drywa et al. 2014; Bagheri et al. 2020). Farré et al. (2020) discovered variations in carapace morphology between native and invasive portunid crabs. The European shore crab, *Carcinus maenas*, is recognised as one of the most marine invasive species globally (Leignel et al. 2014). Native to the European North-Atlantic coastline, *C. maenas* has now been introduced to every continent apart from Antarctica (Carlton and Cohen 2003; Leignel et al. 2014). Across its native and invasive range, *C. maenas* is found in a wide variety of coastal habitats such as intertidal mudflats, estuaries, harbours, seagrass meadows, rocky shores, and oyster reefs (Hampton and Griffiths 2007; Young and Elliott 2019). Morphological and genetic variability has been observed in native and invasive *C. maenas* populations (Brian et al. 2006; Silva et al. 2010a). *Carcinus maenas* can adjust its phenotype to suit local environment pressures in the introduced range; for example, *C. maenas* that feed on hard-shelled prey developed stronger closing forces and longer sarcomeres than crabs feeding on soft-shelled prey (Edgell and Hollander 2011).

Populations of *C. maenas* in their invasive range in the southern hemisphere (Patagonia, South Africa and southern Australia) are poorly studied (Young and Elliott 2019). *Carcinus maenas* in southern Australia are the only populations occurring in mangrove (*Avicennia marina*) habitats (Morrisey et al. 2010; Garside et al. 2014; Garside and Bishop 2014). In South Australia, the *C. maenas* populations spans ~70 km along the Gulf St Vincent coastline (Dittmann et al. 2017), where it inhabits numerous habitat types, ranging from *A. marina* forests, *Zostera* spp. seagrass meadows, mudflats, ports and marinas, saltmarshes, estuaries, and rocky intertidal reefs. Sandy beaches and exposed shorelines along the southern coastline appear to be devoid of *C. maenas*, however they have established in all other coastal habitats (Wiltshire et al. 2010; Dittmann et al. 2017).

There is limited knowledge on the morphological variation and genetic structuring of invasive *C. maenas* in South Australia, and whether any population differences can be

attributed to habitat type is unknown. The aim of this study was to investigate morphological variation and genetic structure of invasive *C. maenas* across coastal habitats in Gulf St Vincent, South Australia. To achieve this aim, linear and geometric morphometrics were used to assess morphological variation, while mitochondrial DNA (mtDNA) analysis of the cytochrome *c* oxidase subunit I (COI) gene was used to assess genetic structuring. *Carcinus maenas* were sampled from mangrove, harbour, and rocky shore environments (hereafter referred to as habitats). I hypothesised that: 1) morphological variation in *C. maenas* would be associated with different habitat types in Gulf St Vincent; and 2) that distinct genetic structure between habitats would reflect genotypic variation, while a lack of genetic structure between habitats would reflect phenotypic variation. Identifying morphological variation and genetic structure across habitats may be evidence of phenotypic plasticity or genetic adaptations to different environmental conditions in *C. maenas* introduced range.

3.2 MATERIALS AND METHODS

3.2.1 Sampling sites and crab collection

Carcinus maenas were sampled during investigations from 2013–2019 along the metropolitan and regional coastlines of Gulf St Vincent, South Australia. Crabs were obtained from the following projects and field surveys to encompass morphological variability in the system: seasonal surveys between 2013–2017 (Dittmann et al. 2017), monthly sampling in 2018 (see Chapter 4), and one sampling event in 2019. Gulf St Vincent is a large inverse estuary with mixed tides of 2–3 m total range (Fig. 3.1). Average salinity ranges from 32–42, and mean sea-surface temperature is approximately 22°C and 13°C in the austral summer and winter respectively (Bye and Kämpf 2008). Sampling sites span approximately ~70 km of the Gulf St Vincent coastline, and comprised three distinct habitats. The mangrove habitat consists of dense mangrove (*Avicennia marina*) forests, seagrass (*Zostera* spp.) beds, saltmarsh, estuaries and mudflats. Two mangrove sites were sampled: Middle Beach and Port Gawler. The harbour habitat was at the margin of channels of the Inner Harbor of Port Adelaide at the Port River and Old Port Reach. The rocky shore habitats were characterised by rocky shorelines combined with intertidal reef platforms and estuaries. The two rocky shore sites were the Onkaparinga River estuary and Aldinga Beach, but some samples were also collected at Hallett Cove Beach. All sample sites were within the intertidal zone and clustered together in respective habitats i.e. each rocky shore site was closer to other rocky shore sites than to the mangrove and harbour sites (Fig. 3.1). Comparisons of sea surface temperature (°C), salinity (ppt) and dissolved

oxygen (mg/L) for the mangrove and harbour sites between 2013–2019 are shown in Table 3.1. Due to time constraints, water quality parameters were not recorded at the rocky shore sites.

Table 3.1 Summary of water quality parameters across four sites and two habitats between 2013–2019. SS Temp = sea surface temperature; DO = dissolved oxygen. Water quality parameters were not recorded at rocky shore sites due to time constraints. Minimum (Min), maximum (Max), mean and standard deviation (± SD) values are displayed. Site codes: Middle Beach (MB); Port Gawler (PG); Port Adelaide River (PAR); Old Port Reach (OPR).

		SS Temp (°C)			Salinity (ppt)			DO (mg/L)		
Habitat	Site	Min	Max	Mean (±SD)	Min	Max	Mean (±SD)	Min	Max	Mean (±SD)
Mangrove	MB	7.4	27.9	17.7 ± 5.4	28.8	45	39.7 ± 3.8	0.57	14.4	6.6 ± 3.0
Mangrove	PG	6.3	28.7	18.3 ± 5.6	28.8	52	41.5 ± 4.4	0.64	25.7	8.0 ± 4.7
Harbour	PAR	13.3	27.2	19.2 ± 4.2	28.9	44	37.9 ± 4.0	0.77	15.9	6.2 ± 2.9
Harbour	OPR	11.8	29.5	18.8 ± 4.7	31.4	42	37.8 ± 3.2	0.96	15.8	6.3 ± 3.0

Sampling effort varied at each site due to differences in survey frequency each year, but most sites were assessed seasonally (i.e. minimum four sampling events per site each year). *Carcinus maenas* were captured using either baited opera house traps or hand-collected during 30-minute timed searches (Table 3.2). On average, five baited opera house-style traps (67 x 48 cm, 2 cm mesh; 7.8 cm diameter entrance rings) were deployed at both sites in the mangrove habitat, both sites in the harbour habitat, and the Onkaparinga River site in the rocky shore habitat. However, at some sites (i.e. Port Gawler and Middle Beach) trapping effort was higher during some years due to multiple surveys being conducted at one time. Traps were baited with a commercially available sardine (*Sardinops sagax*), set at low tide (<0.6 m sea level) and left overnight before retrieval. Four timed searches were done at all rocky shore sites during each sampling event to collect *C. maenas* by hand at low tide. All *C. maenas* collected with either method were transported on wet-ice back to Flinders University, South Australia. Crabs were killed by freezing at -20°C and stored frozen until analysed.

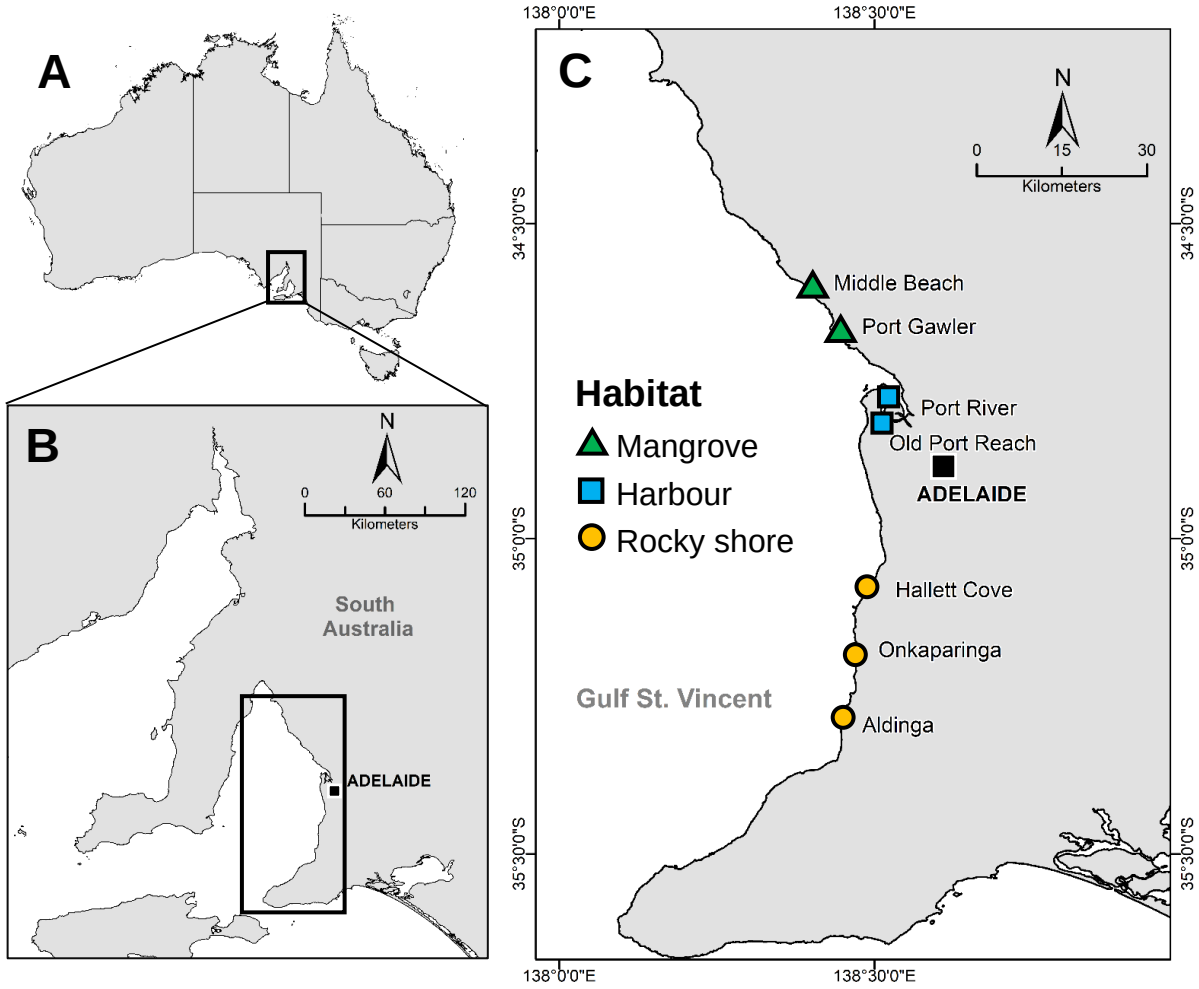

Figure 3.1 Map of sites where *C. maenas* was surveyed between 2013–2019. A) Australia; B) a sub-section of South Australia; and C) sampling sites on the Adelaide metropolitan coastline in Gulf St Vincent, South Australia. Sites in each habitat are denoted by symbol shape and colour. The capital of South Australia, Adelaide, is shown by a black square. Grey shading indicates land.

Crab samples were quality control checked. The 2013–2019 Gulf St Vincent dataset encompassed 1,364 crabs prior to quality control. Quality control excluded crabs so that the final data set comprised crabs with distinguishable sex based on the pleon shape (male or female); crabs larger than >30 mm CW to reduce ontogenetic variation; crabs with no missing or regenerating chelae; and crabs with no damaged pleons, chelae or rostrums, as these affect measurements. After quality control, a total of 1,011 *C. maenas* were used for linear morphometric measurements (Table 3.2). A subset of 150 crabs comprising 95 males and 55 females across sites and habitats were selected for geometric morphometrics in addition to linear morphometrics based on the following criteria: no damage to the carapace; no epibionts obstructing the carapace; adult sizes only (i.e. >30 mm CW; Table 3.2). Another subset of 59

crabs comprising 37 males and 22 females across sites and habitats were selected for molecular analysis in addition to linear morphometrics based on the following criteria: good tissue quality for DNA extraction; adult sizes only (i.e. >30 mm CW; Table 3.2). All crabs used in geometric morphometrics and DNA sequencing encompassed multiple sites, habitats and sexes as evenly as possible to represent morphological and genetic variability in the system but was also dependent on specimen availability and tissue quality.

Table 3.2 Summary of 1,011 *C. maenas* sampled from each habitat, site, assessment method (linear and geometric morphometrics, COI gene sequencing) and sex (M = male; F = female). After quality control, all crabs were used in linear morphometric measurements and are highlighted in bold to represent the total number of crabs collected across habitats and sites. A subset of crabs were used for either geometric morphometrics or COI gene sequencing (in addition to linear morphometrics) and are therefore not included in total counts. Site codes: Middle Beach (MB); Port Gawler (PG); Port Adelaide River (PAR); Old Port Reach (OPR); Hallett Cove (HC); Onkaparinga (ONK); Aldinga (ALD).

Habitat	Site	GPS coordinates	Collection methods	Sampling period	*N* crabs used per assessment method						Total
					Linear		Geometric*		COI*		
					M	**F**	M	F	M	F	
Mangrove	MB	S34°36.650' E138°24.700'	Trapping	July 2014 – Nov 2018	**99**	**82**	15	7	5	4	181
Mangrove	PG	S34°39.172' E138°26.334'	Trapping	May 2013 – Nov 2018	**277**	**203**	17	18	4	4	480
Harbour	PAR	S34°48.796' E138°30.716'	Trapping	July 2014 – July 2018	**43**	**61**	15	15	4	7	104
Harbour	OPR	S34°50.964' E138°29.817'	Trapping	Sep 2014 – Nov 2018	**33**	**27**	16	0	5	3	60
Rocky shore	HC	S35°04.847' E138°29.714'	Timed searches	Feb 2017 – Oct 2017	**2**	**4**	1	0	1	0	6
Rocky shore	ONK	S35°09.797' E138°28.283'	Trapping & timed searches	Oct 2015 – July 2019	**35**	**89**	19	15	9	1	124
Rocky shore	ALD	S35°16.332' E138°26.609'	Timed searches	Dec 2015 – Oct 2018	**34**	**22**	12	0	9	3	56
				Total	525	486	95	55	37	22	1,011

3.2.2 Morphometric measurements

Linear morphometrics

Each frozen *C. maenas* specimen was thawed and blotted dry, and the sex of the crab, the date, site of collection, and body condition were recorded. The sex of crabs was determined by the shape and structure of the pleon; narrow pleons in males and wide, convex pleons in females. Crabs with indeterminate pleons (usually <30 mm carapace width) were categorised as immature. The wet weight (g) of each crab was measured using electronic scales (A&D Weighing Pty Ltd, Adelaide, Australia) to the nearest 0.01 g. Twelve linear dimensions based on Clark et al. (2001), Brian et al. (2006) and Silva et al. (2009) were measured from each crab using digital Vernier callipers (Kincrome Australia Pty Ltd, Victoria, Australia) to the nearest 0.1 mm (Fig. 3.2). The linear measurements (including abbreviations) were: carapace width (CW); carapace length (CL); carapace height (CH); optical groove width (OGW); length of right chela (RCL); height of right chela (RCH); length of propodus of right chela (RPL); length of left chela (LCL); height of left chela (LCH); length of propodus of left chela (LPL); pleon width (PW); and pleon length (PL) (Fig. 3.2). A detailed description of the linear dimensions can be seen in Appendix Table A3.1. These linear dimensions were selected for *C. maenas* as they displayed intraspecific morphological variation across different habitat types and geographic distances between native Atlantic and introduced Pacific populations (Clark et al. 2001), and across sites in the UK (Brian et al. 2006) and Portugal (Silva et al. 2009). Systematic error was controlled by using the same brand and type of calibrated digital calipers, cross-checking measurements, and all measurements were made by the same operator.

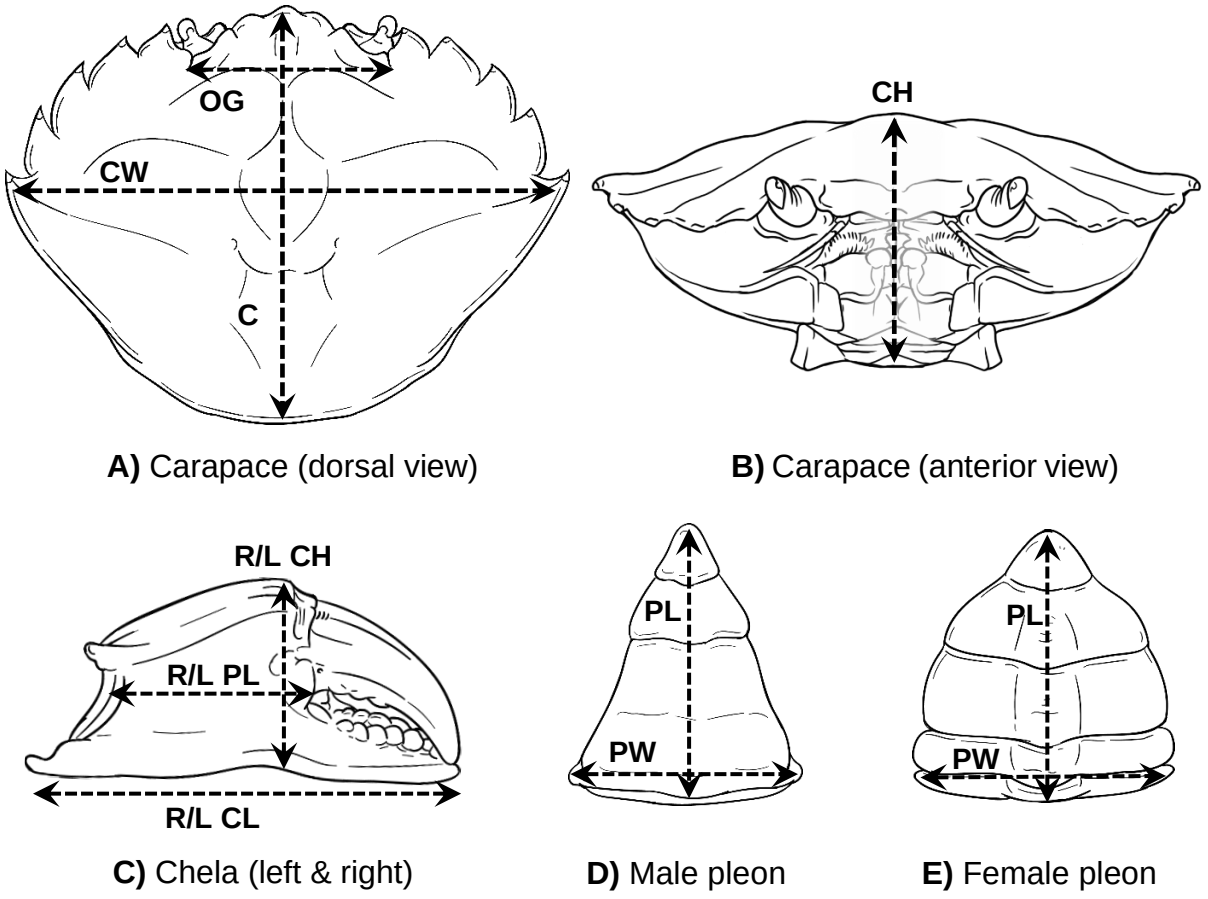

Figure 3.2 Locations of linear morphometric measurements taken from *C. maenas* showing A) the carapace (dorsal view); B) the carapace (anterior view); C) left and right chelae; D) male pleon; and E) female pleon. Measurements based on Clark et al. (2001). A detailed description of the linear dimensions and abbreviations can be seen in Appendix Table A3.1.

Geometric morphometrics

Cardini et al. (2015) determined that 15–20 specimens are required to achieve accuracy in geometric morphometrics. Geometric morphometrics was performed on 150 crabs (95 males and 55 females; see Table 3.2) across sites and habitats to assess visual variations in carapace shape. These crabs were measured, the limbs and epibionts were removed and the carapace was oriented for photography. Geometric morphometrics requires careful consideration of the image equipment and set-up used, including the presentation of samples and post-processing to reduce measurement error (Muir et al. 2012; Cardini 2016; Collins and Gazley 2017). A Nikon D610 DSLR full-frame camera was used, shooting in RAW format and manual mode with an AF-S Nikkor 50 mm F/1.8G lens (maximum reproduction ratio 0.15x). The camera was mounted onto a horizontal boom arm connected to a tripod to ensure that the lens was parallel to the sample. Test photographs were taken to find the desired settings and kept

consistent over consecutive photographing sessions. Carapace samples were each placed in the centre of a gridded sorting tray with a ruler for scale and lens distortion correction applied. Pleons were removed from female crabs to reduce their convexity and prevent specimens from rotating forward, which can affect landmark placement. No zoom was applied in any of the photographs and all crabs were photographed consistently regardless of crab size. Each photograph was checked by eye before specimens were discarded. The full camera settings used can be seen in Appendix Table A3.2.

Photographs for each specimen were edited to remove lens distortion in Adobe Photoshop CC 2019. First, the lens profile was specified in Photoshop (which matched the camera model and lens used for the photographs) so that automatic lens distortion was applied evenly across all photographs. After automatic lens distortion correction, each photograph was checked manually by eye and additional corrections were applied using the adaptive wide-angle filter with automatic correction. Image file data were constructed in tpsUtil v1.76 (Rohlf 2015), and images were digitised in tpsDig v2 (Rohlf 2015), including setting the scale for each image and the placement of landmarks. Fourteen landmarks that are the best representation of morphological differences for *C. maenas* (see Silva et al. 2009; Spani and Scalici 2018) were placed on the left-side of the carapace (Fig. 3.3). Landmarks were placed only on the left side to avoid duplication of equivalent landmarks in symmetrical structures, to avoid bias from differential carapace shape (Rufino et al. 2006; Zelditch et al. 2012) and for comparison purposes with Silva et al. (2009). A detailed description of the landmark positions is shown in Appendix Table A3.1. Digital post-editing processes and settings are in Appendix Table A3.2.

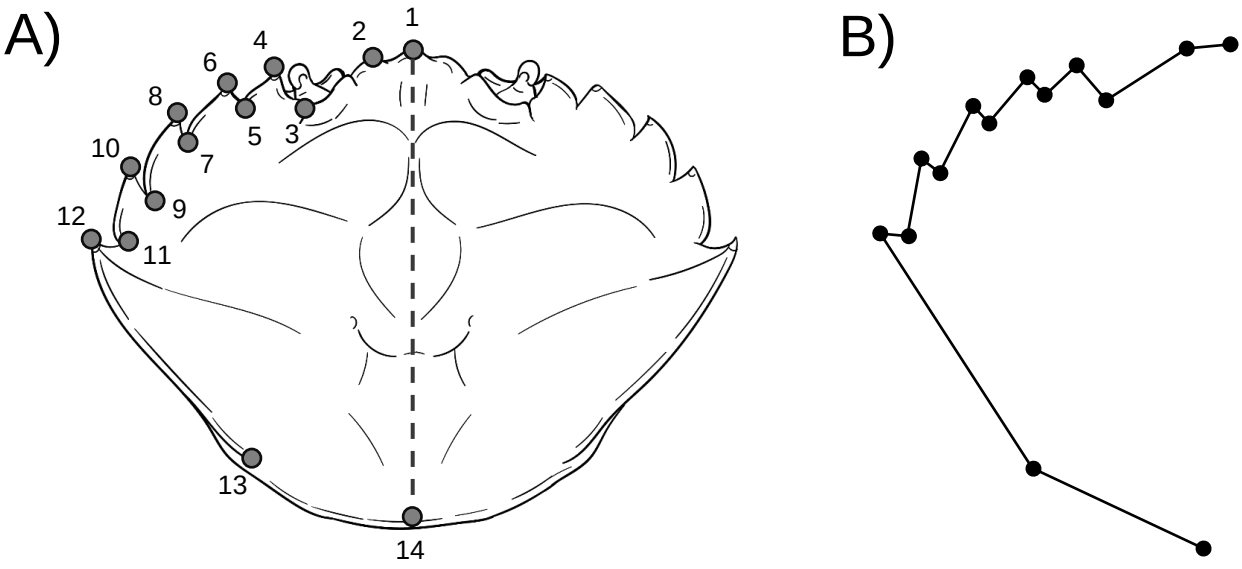

Figure 3.3 Landmark positions used for geometric morphometric analysis of *C. maenas* showing A) 14 digital landmark positions for the left side of the carapace; and B) illustration of a resulting wireframe that represents carapace shape. Landmark positions based on Silva et al. (2009). A detailed description of the landmarks can be seen in Appendix Table A3.1.

3.2.3 Molecular methods

DNA extraction

DNA was extracted from 59 frozen crab specimens used in Chapter 2 (37 males and 22 females) across sites to assess possible habitat-specific genetic structuring. After linear measurements, one or two pereopods (walking legs) were removed from each crab and stored in a -20°C freezer until extraction. Genomic DNA was extracted from muscle removed from frozen pereopods using the Gentra Puregene Tissue Kit (Gentra Systems, Minneapolis, USA) following the manufacturer's protocols after overnight proteinase K digestion (20 mg/ml) at 55°C. Purified DNA was resuspended in 30-50 µl TE buffer and stored at 4°C. DNA concentration of all samples was assessed using a Quantus Fluorometer (Promega Corporation, Wisconsin, USA) following the manufacturer's protocol.

DNA amplification and sequencing

As per Chapter 2, cytochrome *c* oxidase subunit I (COI) was amplified with the degenerate primers COIF-PR115 (5' – TCWACNAAYCAYAARGAYATTGG – 3') and COIR-PR114 (5' – ACYTCNGGRTGNCCRAARARYCA – 3') (Folmer et al. 1994). The PCR amplification was performed in 24 µl volumes containing ~1–50 ng/µl of extracted DNA,

with a final concentration of the following: 1X MRT buffer (1x Immolase buffer, 2mM $MgCl_2$, 0.8 Mm total DNTP's, 0.5x BSA; Hayden et al. 2008; Garland et al. 2010), 0.5 µM of the forward and reverse primer, 1 mM $MgCl_2$ and 0.5 units Immolase (Bioline, NSW, Australia). PCR cycling conditions were: initial denaturation at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 s, 50°C for 60 s and 72°C for 60 s, with a final extension of 72°C for 15 mins and 25°C for 2 mins. Amplification was checked using electrophoresis on a 1.5% agarose gel. Successfully amplified PCR products were filtered with a $MultiScreen_{384}$-PCR plate vacuum cleanup (Millipore Australia Pty Ltd, NSW, Australia). PCR products were Sanger sequenced directly in forward and reverse directions, using the same degenerate primers used in the initial PCR, at the Australian Genome Research Facility (AGRF) in Adelaide, Australia.

3.2.4 Data analysis

Morphometric analysis

All linear carapace measurements were first converted to averages (mean ± SD) to summarise sizes of male and female crabs used in analysis. Linear measurements were tested for normality using a Shapiro-Wilk Test and Levene's Test was used to assess homogeneity of variance. The linear measurements did not meet the assumptions of normality (Shapiro-Wilk $p < 0.05$) and homogeneity of variance (Levene's $p < 0.05$), so a log_{10}-transformation was applied to the linear measurements. Log_{10}-transformations are commonly used in morphometrics to linearise the relationships between different sized variables, and to meet assumptions of normality and homoscedasticity (Marcus 1990; Rohlf 1990). Twelve ratios were also constructed between specific untransformed linear measurements. Ratios help to standardise size, provide shape information, and reduce the effects of unspecified age and allometry of the crabs (Baur and Leuenberger 2011; Afkhami et al. 2016). Morphometric ratios for *C. maenas* were based on Clark et al. (2001) and Silva et al. (2009) and were CW/CL, CW/CH, OGW/CL, CW/OGW, PW/PL, PL/CW, CW/RCL, CW/LCL, RCL/RCH, LCL/LCH, RPL/RCH, and LPL/LCH (ratio abbreviations are described in Appendix Table A3.1).

Linear measurements and ratios were analysed separately using multivariate non-parametric permutational analysis of variance (PERMANOVA) with Euclidian distance and 9,999 permutations. As multiple measurement variables were being analysed at the same time (twelve linear measurement variables and twelve ratio variables), multivariate PERMANOVA was used. Sex (two levels) and habitat (three levels) were assigned as fixed factors. As

sampling effort varied at specific sites and surveys (i.e. some sites were sampled more frequently or had greater trapping effort) site was excluded as a factor and sies were pooled into their respective habitats. The inconsistent frequency and duration of sampling surveys between years prevents adequate assessment of temporal variation, and so sampling time was also excluded as a factor. Pairwise multivariate PERMANOVA tests were carried out to evaluate differences between all pair levels of each factor. All pairwise comparisons had sequential Bonferroni correction applied at the 5% level ($\alpha = 0.05$) to correct for multiple tests (Rice 1989). Multivariate principal components analysis (PCA) and canonical variate analysis (CVA) biplots were constructed to visualise differences in linear morphometric measurements and ratios within and between factors. As the statistical tests and multivariate ordinations showed distinct separation between males and females, sexual dimorphism was confirmed. Data for males and females were re-analysed separately to remove the effect of sex on morphological variation. Linear morphometric analyses were carried out in the software PAleontological STatistics "PAST" version 4.01 (Hammer et al. 2001).

150 *C. maenas* carapace shapes were analysed using geometric morphometrics. Differences between Procrustes distances and Euclidean distances in the tangent space were correlated using the software tpsSmall version 1.34 (Rohlf 2009). Procrustes distances are the square-root of the summed squared distance between homologous landmarks in two superimposed configurations at centroid size and is an absolute measure of shape deviation (Rohlf 2000; Cooke and Terhune 2015). The tpsSmall software determines if the variation in the shape data is small enough to permit multivariate statistical analyses. Combined and sex separated data had strong correlations between the Procrustes and Euclidean distances ($r = 0.99$ in all cases), suggesting that shape differences were sufficiently small to allow the use of multivariate analyses. Size (centroid size) and shape variables (Procrustes shape coordinates) were computed for the carapace landmark coordinates using generalised Procrustes superimposition in MorphoJ version 1.07a (Klingenberg 2011). Procrustes superimposition removes non-shape effects of scale, position, rotation and translation, and provides an 'average shape' by aligning and superimposing all landmark configurations (Rohlf and Slice 1990; Zelditch et al. 2012). Procrustes coordinates were used to generate a covariance matrix, followed by PCA to explore differences among individuals.

When comparing levels of factors where group membership was assumed to be known *a priori* (i.e. habitat), a CVA was used to explore differences among group means. The PCA

and CVA geometric morphometric data revealed clear separation by sex, so analyses were repeated separately for males and females to remove the effects of sexual dimorphism (Fruciano et al. 2016). Multivariate Procrustes ANOVA was performed for centroid size and shape of carapace landmarks to quantify relative amounts of variation in shape. Mahalanobis and Procrustes distances were calculated for each pair of habitat groups using the pooled within-group covariance matrix and 10,000 permutations. To correct for multiple tests, sequential Bonferroni was applied at the 5% level ($\alpha = 0.05$) on the pairwise Mahalanobis and Procrustes distances. Changes in carapace shape were visualised using deformation grids and shape wireframes that were obtained from MorphoJ and edited in Adobe Illustrator CC.

Molecular analyses

Mitochondrial COI sequence chromatograms were edited and proofread by eye, followed by multiple alignment using Geneious Prime version 2020.1.1 (Biomatters Ltd., Auckland, New Zealand). Any sequences with poor quality reads were excluded from further analysis. All COI sequences were trimmed to match the length of the shortest sequence, which provided 634 bp gapless and unambiguously aligned sequences. All COI sequences were then compared to the National Center for Biotechnology Information (NCBI) sequence database GenBank using the basic local alignment tool MegaBlast. Sequences were compared with the resulting Blast sequences, including percent similarity, to confirm that all sequences used in this study were *C. maenas*. All the resulting *Carcinus maenas* sequences from South Australia were deposited into GenBank. The COI sequences were sorted by their respective habitat and analysed using an unrooted neighbour-joining tree with the Tamura-Nei Model in Geneious. The resulting tree and aligned consensus sequences were used to generate an unrooted mtDNA haplotype network using Haplotype Viewer (Ewing 2010).

Nucleotide diversity (π) and haplotype diversity (Hd) for COI sequences were determined using DnaSP version 6.12.03 (Rozas et al. 2017). FASTA file formats were converted to Arlequin files via the online converter DNAcollapser (http://users-birc.au.dk/palle/php/fabox/dnacollapser.php) and the number of haplotypes were identified. Population genetic structure was examined using pairwise analysis via the fixation index (F_{ST}) with 10,000 permutations and an analysis of molecular variance (AMOVA) with 10,000 permutations using the software ARLEQUIN version 3.5.2 (Excoffier and Lischer 2010). Samples were grouped by habitat and partitioning of genetic variance was tested within and between groups. Decimal GPS coordinates that best represented each habitat were converted

to a geographic distance matrix in kilometers, and then log-transformed using Geographic Distance Matrix Generator (https://biodiversityinformatics.amnh.org/open_source/gdmg/index.php; Ersts 2020). To identify potential effects of isolation by distance (IBD) across the three habitats, a linearised pairwise F_{ST} matrix ($F_{ST}/1 - F_{ST}$) was correlated against the log-transformed geographic distance matrix using the Mantel Test with 10,000 permutations in ARLEQUIN. The presence of historic population bottlenecks or population expansions were assessed with neutrality tests Tajima's *D* (Tajima 1989) and Fu's F_s statistics (Fu 1997) in ARLEQUIN. These neutrality tests were conducted to detect if polymorphisms could be explained by genetic drift or other selective events for changes in population size. Statistical analyses had a significance value of $\alpha = 0.05$.

3.3 RESULTS

3.3.1 Morphometric analysis

Linear morphometrics

1,011 *C. maenas* were analysed by linear morphometrics after quality control checks were applied (total male n = 525; total female n = 486). Of this, 661 measured crabs were from the mangrove habitat, 164 crabs from the harbour habitat, and 186 crabs from the rocky shore habitat. All linear morphometric measurements (untransformed mean ± SD) were larger on average for male than female crabs across all habitats, except for pleon length and width, which were larger for females (Table 3.3). The carapace width (CW) of males ranged from 30.12–89.25 mm (mean CW = 66.12 ± 14.24), while CW of females ranged from 30.52–74.55 mm (mean CW = 54.25 ± 10.45). Crabs from the mangrove habitat were the largest across all morphometric dimensions, followed by the harbour habitat and then the rocky shore habitat.

Table 3.3 Summary of twelve linear morphometric measurements (mean ± SD) of each male and female *C. maenas*. All crabs were collected from three habitats in Gulf St Vincent, between 2013–2019. Measurements are untransformed with all units in mm.

Linear morphometric measurements (mean ± SD)	Male *C. maenas*				Female *C. maenas*			
	Mangrove (n = 376)	Harbour (n = 76)	Rocky shore (n = 73)	All habitats (n = 525)	Mangrove (n = 285)	Harbour (n = 88)	Rocky shore (n = 113)	All habitats (n = 486)
Carapace width	71.4 ± 1.0	56.0 ± 13.0	49.4 ± 11.5	66.1 ± 14.2	59.2 ± 8.4	48.8 ± 8.8	46.1 ± 9.0	54.3 ± 10.5
Carapace length	53.0 ± 8.0	42.0 ± 9.6	37.5 ± 8.7	45.4 ± 10.4	45.1 ± 6.5	37.1 ± 6.7	35.2 ± 6.9	41.3 ± 8.0
Carapace height	30.1 ± 4.8	23.3 ± 5.4	20.5 ± 5.0	27.8 ± 6.2	26.0 ± 4.0	20.8 ± 3.9	19.4 ± 3.9	23.5 ± 4.9
Optical groove-width	24.6 ± 3.5	19.5 ± 4.0	17.7 ± 3.8	22.9 ± 4.5	21.3 ± 2.8	17.6 ± 2.9	16.8 ± 3.0	19.6 ± 3.6
Right chela length	43.0 ± 8.5	32.3 ± 9.6	28.0 ± 8.8	39.4 ± 10.5	29.6 ± 4.8	23.8 ± 4.5	22.1 ± 4.9	26.8 ± 5.8
Right chela height	22.6 ± 5.3	15.9 ± 5.4	13.6 ± 5.0	20.3 ± 6.4	14.4 ± 2.8	11.1 ± 2.5	10.0 ± 2.5	12.8 ± 3.3
Right propodus length	29.6 ± 6.3	22.0 ± 6.8	18.9 ± 6.2	27.0 ± 7.6	19.5 ± 3.3	15.6 ± 3.0	14.3 ± 3.3	17.6 ± 4.0
Left chelae length	41.0 ± 8.3	31.0 ± 9.2	26.8 ± 7.9	37.6 ± 10.0	28.3 ± 4.5	22.8 ± 4.4	21.5 ± 4.7	25.7 ± 5.5
Left chela height	18.5 ± 4.5	13.6 ± 4.5	11.7 ± 3.9	16.8 ± 5.2	12.3 ± 2.4	9.6 ± 2.0	9.2 ± 2.3	11.1 ± 2.8
Left propodus length	27.2 ± 5.9	20.4 ± 6.3	17.6 ± 5.3	24.9 ± 7.0	18.1 ± 3.0	14.6 ± 2.9	13.7 ± 3.2	16.5 ± 3.6
Pleon width	23.1 ± 3.5	18.5 ± 4.3	16.4 ± 3.9	21.5 ± 4.5	23.7 ± 3.8	19.4 ± 4.0	18.2 ± 4.2	21.6 ± 4.7
Pleon length	25.0 ± 3.7	20.2 ± 4.5	17.8 ± 4.1	23.3 ± 4.8	25.8 ± 4.3	21.0 ± 4.2	19.7 ± 4.3	23.5 ± 5.1

Morphological variation was observed across twelve log_{10}-transformed linear measurements and thirteen ratios in both sexes across habitats (Fig. 3.4). For linear measurements of male crabs, canonical variate 1 (CV1) explained 94.5% of the linear measurement variation and was driven mostly by crabs from the mangrove habitat, while canonical variate 2 (CV2) explained 5.5% of the variation and was driven by crabs from the rocky shore (Fig. 3.4A). For ratios of male crabs, CV1 explained 91.7% of variation and was driven by crabs from the rocky shore, while CV2 explained 8.3% of variation and was driven by crabs from the mangroves (Fig. 3.4B). For linear measurements of female crabs, CV1 explained 96.9% of variation and was driven by crabs from the mangrove habitat, while CV2 explained 3.1% variation and was driven by crabs from both the harbour and rocky shore (Fig. 3.4C). For ratios of female crabs, CV1 explained 96.4% of variation and was driven by crabs from the harbour and rocky shore, while CV2 explained 3.6% variation and was driven by crabs from mangrove habitat (Fig. 3.4D). Corresponding CVA loading scores for linear measurements and ratios for male and female crabs are shown in Appendix Table A3.3.

While the CVA plots showed overlap in linear measurements and ratios across habitats, significant morphological differences were observed in all cases (PERMANOVA, $p < 0.005$; Table 3.4). Pairwise tests displayed significant differences for male and female *C. maenas* from the mangrove and harbour habitats (sequential Bonferroni $p < 0.005$), and the mangrove and rocky shore habitats (sequential Bonferroni $p < 0.005$). Differences were lesser but significant for males and females between the harbour and rocky shore habitats (sequential Bonferroni $p < 0.05$). The test outcomes supported the CVA plots which showed that there is less morphological variation in crabs between harbour and rocky shore habitats compared to the mangrove habitat.

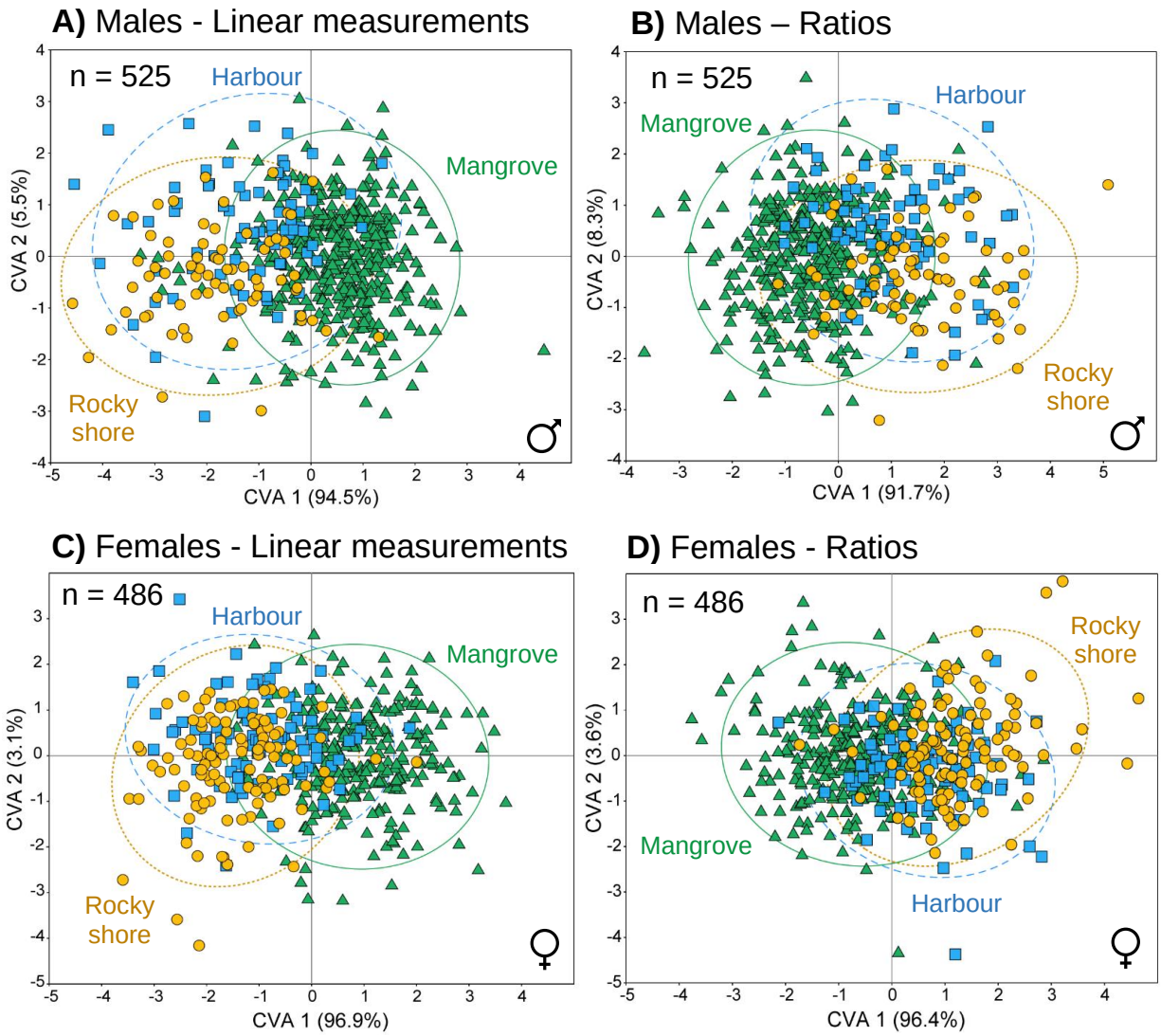

Figure 3.4 Canonical variate analysis (CVA) biplots of twelve linear measurements and thirteen ratios for *C. maenas* across three habitats. A) Linear measurements (log_{10}-transformed) of male crabs; B) ratios of male crabs; C) Linear measurements (log_{10}-transformed) of female crabs; and D) ratios of female crabs. Mangrove habitats = green triangles and solid ellipses; harbour habitat = blue squares and dashed ellipses; rocky shore habitat = yellow circles and dotted ellipses. Ellipses represent 95% confidence intervals. Note differences in axis values.

Table 3.4 One-way PERMANOVA and sequential Bonferroni-corrected pairwise test results of linear measurements (top) and ratios (bottom) for male and female *C. maenas* between three habitats (single factor). SS = sum of squares. Tests based on Euclidian distance measures with 9,999 permutations. Tests were done separately for each sex. Significant pairwise *p*-values after sequential Bonferroni correction are highlighted in bold (correction threshold: α = 0.05).

Linear measurements

Males ♂ (n = 525)				Females ♀ (n = 486)			
PERMANOVA	SS	*F*	*p*	PERMANOVA	SS	*F*	*p*
Habitats	102.2	119.1	**<0.001**	Habitats	60.49	109.4	**<0.001**
Pairwise tests (sequential Bonferroni)				Pairwise tests (sequential Bonferroni)			
Mangrove – Harbour		96.12	**<0.001**	Mangrove – Harbour		95.8	**<0.001**
Harbour – Rocky shore		7.859	**0.005**	Harbour – Rocky shore		5.5	**0.017**
Rocky shore – Mangrove		199.5	**<0.001**	Rocky shore – Mangrove		186.5	**<0.001**

Ratios

Males ♂ (n = 525)				Females ♀ (n = 486)			
PERMANOVA	SS	*F*	*p*	PERMANOVA	SS	*F*	*p*
Habitats	89.81	24.01	**<0.001**	Habitats	61.52	21.91	**<0.001**
Pairwise tests (sequential Bonferroni)				Pairwise tests (sequential Bonferroni)			
Mangrove – Harbour		19	**<0.001**	Mangrove – Harbour		14.6	**<0.001**
Harbour – Rocky shore		2.688	**0.041**	Harbour – Rocky shore		4.79	**0.001**
Rocky shore – Mangrove		35.86	**<0.001**	Rocky shore – Mangrove		34.9	**<0.001**

Geometric morphometrics

Morphological variation was observed for geometric carapace shape landmarks in both sexes across habitats (Fig. 3.5). The variation in carapace shape was visualised with CVA, in which the CVA morphospace displayed less overlap between all groups attributed to habitat compared to linear morphometrics. Male shape variation on CV1 showed that the carapace is narrower, with lateral teeth that point further outwards and are positioned more posteriorly down the carapace (Fig. 3.5A). Male shape variation on CV2 was associated with a wider carapace, and lateral teeth that were oriented more anteriorly on the carapace margin and point further inwards. For males, CV1 explained 76.2% of the variation and is driven mostly by crabs from the rocky shore, while CV2 explained 23.8% of the variation and is attributed to crabs from the mangroves. Female shape variation on CV1 displayed a carapace shape that is characterised by outward-pointing lateral teeth that are oriented more posteriorly (Fig. 3.5B).

Female shape variation on CV2 was characterised by lateral teeth that point slightly inwards and are positioned more anteriorly. For females, CV1 explained 67.6% of shape variation and was driven by harbour and rocky shore specimens, while CV2 explained 32.4% of shape variation and was attributed to the mangroves.

Procrustes ANOVA revealed significant differences in both centroid size and shape of male and female crabs between habitats ($p < 0.005$; Table 3.5). Crabs measured from the mangrove habitat displayed more morphological variation than crabs from the harbour and rocky shore habitats, which matched similar patterns observed in the linear morphometric CVAs. Pairwise Mahalanobis and Procrustes distances showed significant differences between the mangrove habitat compared to both the harbour and rocky shore habitats in all instances (sequential Bonferroni $p < 0.001$; Table 3.5). Carapace shape was more similar in male and female crabs between the harbour and rocky shore habitats. There were no significant differences in pairwise Procrustes distances for female crabs between the harbour and rocky shore habitat (sequential Bonferroni $p > 0.05$). Corresponding CVA loadings scores for the geometric morphometric analyses can be seen in Appendix Table A3.4.

Table 3.5 Geometric morphometric statistical analyses for male and female *C. maenas* carapace shapes between three habitats (single factor). Table displays Procrustes ANOVA (centroid size and Procrustes shape) and pairwise Mahalanobis and Procrustes distances with 10,000 permutations. SS = sum of squares. Tests were done separately for each sex. Significant pairwise *p*-values after sequential Bonferroni correction are highlighted in bold (correction threshold: $\alpha = 0.05$).

Males ♂ (n = 95)				Females ♀ (n = 55)			
Centroid size	SS	*F*	*p*	Centroid size	SS	*F*	*p*
Habitat	64.46	31.1	**<0.001**	Habitat	38.02	34.5	**<0.001**
Procrustes Shape	SS	*F*	*p*	Procrustes Shape	SS	*F*	*p*
Habitat	0.011	8.34	**<0.001**	Habitat	0.005	4.3	**<0.001**
Pairwise Mahalanobis	Distance		*p*	Pairwise Mahalanobis	Distance		*p*
Mangrove – Harbour	3.043		**<0.001**	Mangrove – Harbour	4.001		**<0.001**
Harbour – Rocky shore	1.833		**0.01**	Harbour – Rocky shore	3.317		**<0.001**
Rocky shore – Mangrove	2.718		**<0.001**	Rocky shore – Mangrove	3.380		**<0.001**
Pairwise Procrustes	Distance		*p*	Pairwise Procrustes	Distance		*p*
Mangrove – Harbour	0.020		**<0.001**	Mangrove – Harbour	0.020		**<0.001**
Harbour – Rocky shore	0.009		**0.03**	Harbour – Rocky shore	0.012		0.062
Rocky shore – Mangrove	0.023		**<0.001**	Rocky shore – Mangrove	0.017		**<0.001**

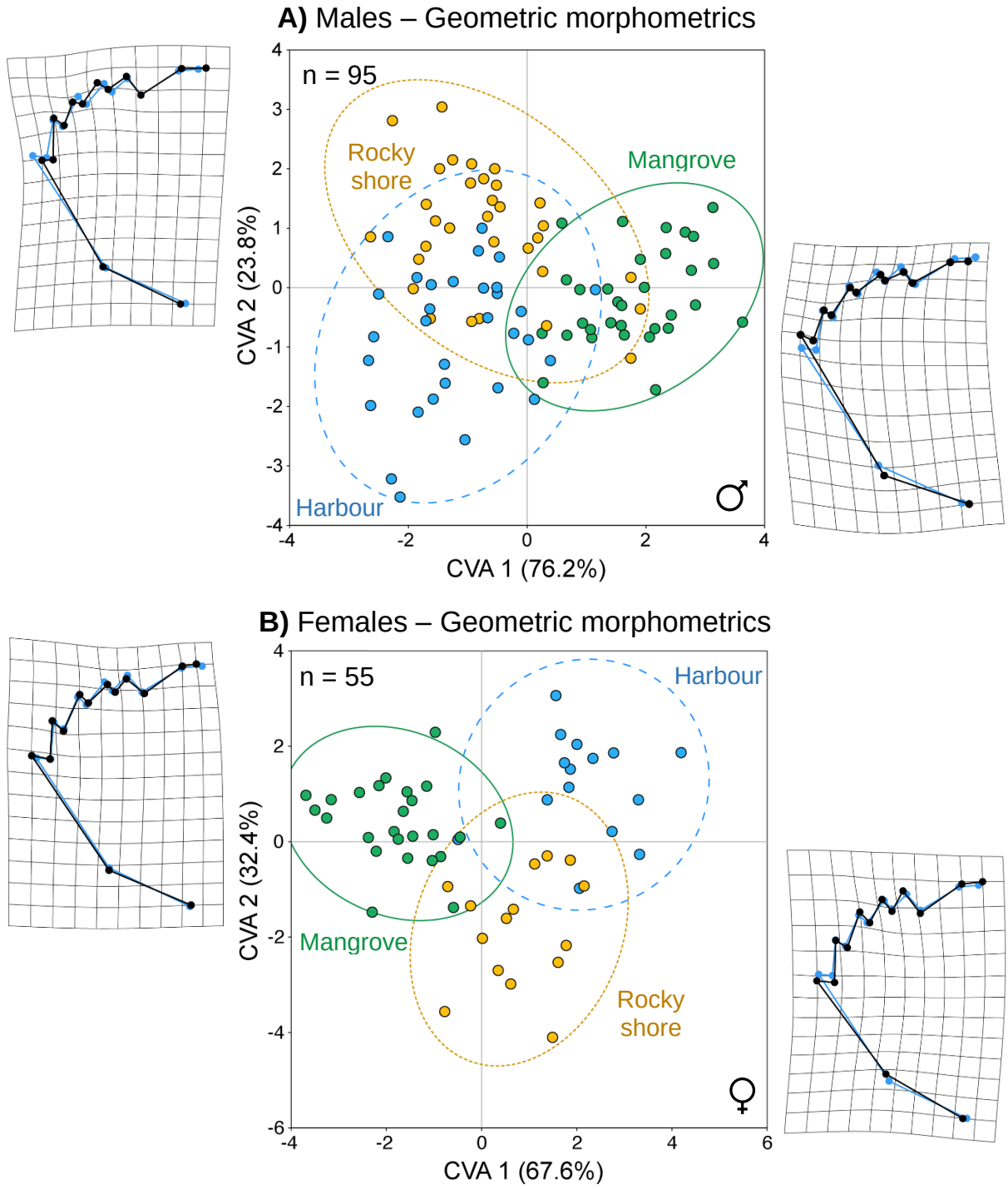

Figure 3.5 Canonical Variate Analysis (CVA) of carapace geometric landmark configurations for A) male; and B) female *C. maenas* specimens from three habitats. Ellipses represent 95% confidence. Shape wireframes in the upper left and bottom right corners display extreme shape variation along their respective canonical variates. Blue wireframes represent the average shape in each CV, while black wireframes represent deviation from the mean shape.

3.3.2 Molecular analysis

A 634 base pair (bp) fragment of mtDNA COI gene was amplified and sequenced from 59 *C. maenas* from Gulf St Vincent. The 634 bp region was sequenced from a ~1.07pg/ C=1.24 genome of *C. maenas* (Bonnivard et al. 2009). Of the 59 sequences, 17 sequences were from the mangrove habitat, 19 from the harbour habitat, and 23 from the rocky shore habitat. The overall nucleotide composition was C – 20%; T – 26.2%; A – 36.09%; and G – 17.71% (Appendix Table A3.5). All 59 sequences had between 99.2 – 100% identical matches to *C. maenas* when compared to COI sequences on the GenBank database via Blast (see Chapter 2 for specifics on *Carcinus* identity).

Of the 634 nucleotide sites, 10 were variable and 9 of these variable sites were parsimony informative. Six unique haplotypes were identified across the three habitats sampled in Gulf St Vincent, South Australia (Table 3.6; Fig. 3.6). Haplotypes were not very divergent from each other, with a maximum of three bp difference between haplotypes. Four haplotypes were shared across all three habitats, one haplotype (M4) was found in the mangrove and rocky shore habitat, and one haplotype (R22) appeared to be specific to one individual from the rocky shore habitat. Most haplotypes were separated by one bp difference, whereas M4 had two and H2 had three bp differences.

The most common haplotype was H1 (Table 3.6), and no distinct clades were apparent from the haplotype data. Haplotype diversity (Hd) was 0.818 for the South Australian population overall while Hd within habitats ranged from 0.632 in the harbour habitat up to ~0.816 in the mangrove and rocky shore habitats. Nucleotide diversity (π) was 0.0043 for the overall population and was lowest at 0.0036 in the harbour habitat and highest at 0.005 in the mangrove habitat (Table 3.6). High haplotype diversity (Hd > 0.5) but low nucleotide diversity ($\pi < 0.5$) suggests rapid population growth from a small population, assuming there was enough time for recovery of haplotype variation via mutation, but not enough time for accumulation of large sequence changes. Haplotype sequences were deposited in GenBank (Accession Numbers MT748791 – MT748849). Corresponding Accession Numbers for each sample are in Appendix Table A3.6.

Table 3.6 Haplotype summary, genetic diversity indices and neutrality tests based on a 634 bp region of the COI gene sequenced from *C. maenas* in each habitat. N = number of individuals sequenced; h = number of haplotypes; S = number of polymorphic sites; Hd = haplotype diversity; π = nucleotide diversity. *P*-values for Tajima's *D* and Fu's *F*s are displayed within parentheses.

Habitat	N	h	S	Hd (± SD)	π	Tajima's *D*	Fu's *F*s
Mangrove	17	5	9	0.816 ± 0.05	0.005	0.915 (*p* = 0.84)	1.902 (*p* = 0.83)
Harbour	19	4	7	0.632 ± 0.10	0.004	0.522 (*p* = 0.73)	2.157 (*p* = 0.87)
Rocky shore	23	6	10	0.814 ± 0.00	0.004	-0.007 (*p* = 0.54)	0.869 (*p* = 0.70)
Total	59	6	10	0.818 ± 0.03	0.004	0.802 (*p* = 0.70)	2.755 (*p* = 0.80)

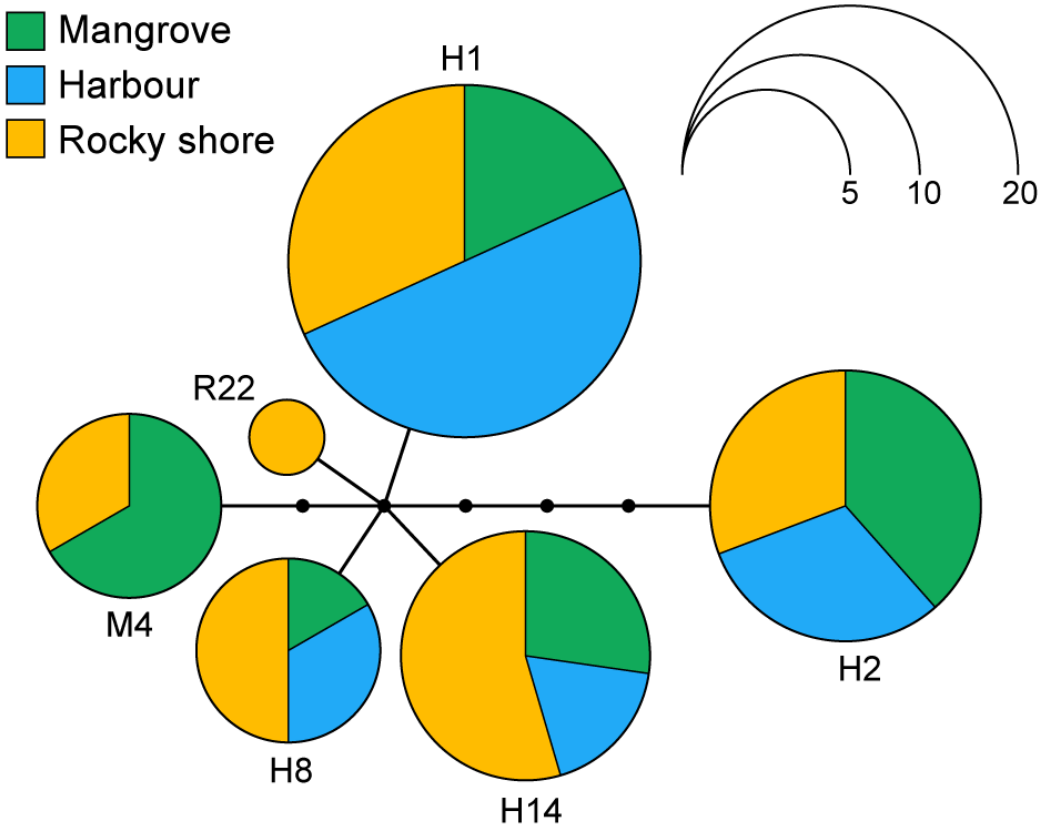

Figure 3.6 An unrooted mtDNA haplotype network of the COI gene observed in 59 individuals of *C. maenas* sampled from three habitats in Gulf St Vincent, South Australia. Each circle represents one unique haplotype (labelled next to the circle), and circle diameter represents mtDNA haplotype frequency. Colour represents the relative frequency of each mtDNA haplotype from the mangrove habitat (green), harbour habitat (blue) and rocky shore habitat (yellow) respectively. Length of lines connecting haplotypes denote the distance of relatedness, while small black circles represent mutational steps.

Neutrality tests (Tajima's *D* and Fu's *F*s) were both high for the population overall and for each habitat, however all were non-significant ($p > 0.05$) (Table 3.6). Tajima's *D* was positive overall, suggesting that there has been a decrease in population size and/or balancing selection has been reached. Fu's *F*s statistic also had positive values across all habitats, indicating a possible deficiency of alleles that could be the result of a recent population bottleneck. The Tajima's *D* value was negative in the rocky shore habitat (-0.0073) and the Fu's *F*s was also lower in this habitat (0.8685) when compared to the mangrove and harbour habitat. The Mantel test showed a negative and non-significant (R^2 = -0.96; p = 1.00) correlation between geographic distance and genetic distance (F_{ST}). The AMOVA indicated that the habitats accounted for 0.73% of total molecular variance, however variation was higher among individuals within habitats at 99.27% (Table 3.7). The global F_{ST} value for the population was low overall (0.007) and no significant variation was found among habitats ($p > 0.05$). Pairwise F_{ST} values were similar across interactions between the three habitats and there were no significant differences for the pairwise interactions between groups ($p > 0.05$) (Table 3.7).

Table 3.7 AMOVA test results (10,000 permutations) and F_{ST} values for COI gene sequences in *C. maenas* across three habitats. SS = sum of squares; df = degrees of freedom.

Source of variation	df	SS	Variance components	Percentage of variation (%)
Among populations (Va)	2	3.154	0.01	0.73
Within populations (Vb)	56	77.287	1.38	99.27
Total	58	80.441	1.39	100
F_{ST} = 0.007				
Fixation index (F_{ST}) significant tests (1023 permutations)				
Va and F_{ST}, p = 0.324				

Pairwise F_{ST} tests	F_{ST}	p
Mangrove - Harbour	0.0372	0.170
Harbour – Rocky shore	-0.0106	0.478
Rocky shore - Mangrove	0.0004	0.379

3.4 DISCUSSION

This study used a combination of morphometrics and molecular data to examine population structure of invasive *C. maenas* across habitats in Gulf St Vincent, South Australia. *Carcinus maenas* exhibited morphological variation across the three habitat types; crabs from the mangrove habitats were larger overall and had a wider carapace with lateral teeth that point further inwards than crabs from the harbour and rocky shore. There was no significant genetic structuring of *C. maenas* across habitat types, indicating that morphological differences may not be genetically driven. Morphometric variation and genetic homogeneity across habitats in Gulf St Vincent suggested that invasive *C. maenas* in South Australia are a single, self-sustaining population that can display phenotypic responses to local environment conditions.

3.4.1 Morphometric variation across habitats

Morphometric variation of *C. maenas* was most apparent in the mangrove habitat with less variation in the harbour and rocky shore habitats. The three habitats span ~70 km distance between the southernmost and northernmost sites, but they differ in vegetation, predator and prey diversity, substrate, tide/wave exposure and human activity (Bye and Kämpf 2008; Benkendoff et al. 2008). It is possible that morphometric differences in *C. maenas* between these habitats could be a response to the local environment conditions, driven by variations in their ontogenetic development (i.e. resource allocation during growth) and life history (Deli et al. 2017; Casties and Briski 2019). Brachyuran crabs display ecomorphological differences between habitats such as mangrove forests, sandy beaches and rocky shores (Marochi and Masunari 2016). For example, in the San José Gulf, Patagonia, geometric morphometric differences were displayed in the crab *Cyrtograpsus angulatus* from a coastal saltmarsh and rocky shore habitat separated by 3 km of sandy beach (Idaszkin et al. 2013). The mean carapace shape of *C. angulatus* from the rocky shore habitat was slender and lengthened compared to crabs from the saltmarsh, which may be due to phenotypic variation across habitats (Idaszkin et al. 2013).

The CVA loading scores of $\log_{10}$-transformed linear measurements and ratios showed that chelae dimensions and pleon ratios were main drivers of morphological variation in *C. maenas* between habitats. Morphological variation of chelae in marine crabs are driven mainly by feeding habits, defence and burrowing behaviour (Baldridge and Smith 2008; Parvizi et al. 2017; Vermeiren et al. 2020). For example, Silva et al. (2017) found that *Eriphia verrucosa*

and *Pachygrapsus marmoratus* had larger, worn-out chelae in rocky shore habitats where they consumed more hard-shelled prey compared to conspecifics from sandy shores. While *C. maenas* in Gulf St Vincent mangrove habitat feed on hard-shelled bivalves (Campbell et al. 2019), their diet and predatory behaviour in the harbour and rocky shore habitats are unknown. The chelae of male crabs were larger on average and had more pronounced shape variation which may be advantageous in male-male competition and mating behaviour (Lee and Seed 1992; Marochi et al. 2018). Additionally, thermogeographic variation in body size is observed in *C. maenas* in North America (Kelley et al. 2015), while *C. maenas* in southeast Australia preferred shaded patches in *A. marina* mangrove forests compared to bare areas (Garside and Bishop 2014). The largest male and female *C. maenas* were observed in the mangrove habitat in South Australia and it is possible that sexual dimorphism, temperature and habitat refugia influence size and morphology at these mangrove sites (Ledesma et al. 2010; Alencar et al. 2014; Williner et al. 2014).

Carcinus maenas displayed significant differences in their carapace shape across habitats. Unlike linear morphometrics and ratios, geometric morphometrics revealed variations in carapace shape through visual deformation grids and shape wireframes. In the mangrove habitats, *C. maenas* had a wider carapace with lateral teeth oriented more anteriorly and pointing further inwards in both sexes. In the harbour and rocky shore habitats, the carapace was narrower and had posteriorly oriented lateral teeth that pointed further outwards. In their native range, *C. maenas* displayed morphometric differences in their carapace and chelae among populations extending ~750 km along the Portuguese coastline (Silva et al. 2009). Southern Portuguese populations were characterised by concave carapace shapes, chelae with shorter pollexes and flattened posterior sections of the chelae compared to northern populations (Silva et al. 2009). At a smaller study site in Portugal ranging 2 km, *C. maenas* had thicker carapaces in the saltmarsh site and thinner carapaces in the estuary mouth site (Souza et al. 2011).

While *C. maenas* showed morphological variation throughout environmental clines and habitat types over varying spatial scales, the ecological significance of carapace shape changes is poorly understood (Miner et al. 2005; Smith 2009). Adaptive phenotypic variation is observed in native *C. maenas* across habitat types through variation in carapace colour and pattern (Todd et al. 2006; Stevens et al. 2014; Nokelainen et al. 2018). Juvenile *C. maenas* tend to have diverse colour and pattern variation that provides camouflage among different habitat

substrates which significantly reduced predation risk (Stevens et al. 2014). Phenotypic colouration has been observed for *C. maenas* in South Australia, with crabs displaying mottled patterns and greater colouration in the rocky shore compared to plain and dark crabs from the harbour and mangroves (R. Campbell, pers. obs.).

The adaptive significance of carapace shape variation in *C. maenas* is not as clear as phenotypic colouration. For *C. maenas* in South Australia, perhaps narrower carapaces are more advantageous in the rocky shore and harbour regions where reef, rocks and man-made structures are the main source of refuge for crabs? In Argentina, the freshwater crabs *Aegla neuguensis* and *A. riolimayana* showed habitat-specific variation in rostrum length and carapace shape between rivers and lakes (Giri and Loy 2008). Reductions in rostrum length may be a possible phenotypic response to living among rocky or sandy substrates in rivers, while carapace shape differences may assist with streamlining or burrowing in river and lake habitats (Giri and Loy 2008). The carapace shape of the introduced blue crab, *Callinectes sapidus*, was notably different to multiple native crab species, including other portunoids, in the Mediterranean Sea (Farré et al. 2020). Morphological traits of *C. sapidus* could contribute to invasion success through the following: a) over-development of the carapace anterior margin indicates larger gill chambers and higher respiratory efficiency; b) lengthened lateral spines act as defence against predators in the introduced range; and c) a wider carapace allows females to carry a greater volume of eggs and increase fecundity (Farré et al. 2020). Phenotypic variation in South Australian *C. maenas* is likely occurring across habitats as a plastic response to different selective pressures (i.e. resource availability, water quality, ecological interactions) which alter ontogenetic development and therefore body shape (Marochi et al. 2018).

3.4.2 Genetic homogeneity across habitats

Analysis of the mtDNA COI gene revealed no evidence of genetic structure in *C. maenas* across habitats in South Australia. The genetic homogeneity of *C. maenas* in the South Australian population matches other studies of this species in its native range (Brian et al. 2006; Silva et al. 2010a) and invasive range (Burden et al. 2014). In southwest England, Silva et al. (2010b) found no evidence of genetic structure in *C. maenas* using eight microsatellite loci even though crabs showed high morphometric variability between sites. Similar patterns were observed in *C. maenas* using other genetic markers; on the east and west coasts of the UK, allozymes also revealed genetic homogeneity despite morphometric variation among populations (Brian et al. 2006). In southeast Australia, COI gene sequences showed no genetic

structure in invasive *C. maenas* from estuaries in New South Wales and Victoria (Burden et al. 2014).

The genetic homogeneity of *C. maenas* in South Australia is likely due to mixing of individuals across habitats, either through larval dispersal, migration of adults or human-mediated translocations. If a small number of adult crabs from multiple generations migrate to different areas (i.e. within and between nearby embayments) and mate at random, this will likely provide enough genetic mixing to homogenise parts of the population (Chaplin et al. 2001). In Gulf St Vincent, South Australia, there is high population connectivity of *C. maenas* across habitats which can be due to limited restrictions to gene flow and substantial population mixing (Lowe and Allendorf 2010). Silva et al. (2010a) identified that *C. maenas* were separated by distances of 15–432 km between sites along the southwest English coast; the long larval mode and dispersal radius of *C. maenas* likely contributed to larval flow between distant sites and homogenised the population (Silva et al. 2010a).

In southeast Australian estuaries, genetic homogeneity of *C. maenas* was demonstrated across large spatial scales of over 400 km coastline from New South Wales to Victoria (Burden et al. 2014). However, the Tasmanian population was genetically distinct from mainland Australia which may have been attributed to ocean currents in the Bass Strait acting as a barrier to gene flow (Burden et al. 2014; Aguilar et al. 2019). Seascape dynamics, such as the intermittent closing of estuaries along the southeast Australian coastline, may alter intertidal migrations and mixing of adult *C. maenas* (Garside et al. 2014). While adult *C. maenas* move intertidally and between embayments to forage, their dispersal potential is limited by their mobility; most adult *C. maenas* in Australia have a dispersal radius of 1–15 km per generation (Thresher et al. 2003).

Genetic mixing of South Australian *C. maenas* is likely due to larval dispersal than adult dispersal. *Carcinus maenas* has an extended larval period (50 days development with ~60 km dispersal distance; Silva et al. 2010a) which promotes mixing, and in turn removes genetic structure and randomises distributions in the population (Weersing and Toonen 2009). In Gulf St Vincent, South Australia, allozyme, mtDNA and microsatellite analysis have revealed genetic homogeneity in other native marine species such as snapper (*Chrysophrys auratus*; Fowler 2016), western king prawns (*Penaeus Melicertus latisulcatus*; Beckmann and Hooper 2016a), and blue swimmer crabs (*Portunus armatus;* Chaplin et al. 2001). Despite these marine species occupying heterogenous habitats over diverse coastlines in Gulf St Vincent, genetic

homogeneity is maintained over larger spatial scales due to high mobility (snapper), larval dispersal and/or random mating of adults. Few studies have assessed genetic heterogeneity or homogeneity in invasive marine species within Gulf St Vincent. However, some crabs with larval spawning do display genetic heterogeneity at localised spatial scales elsewhere (<100 km), such as the marbled crab *Pachygrapsus marmoratus* in Italy, which may be the result of local larval retention, variations in reproductive success and *en masse* larval dispersion (Iannucci et al. 2020).

As no genetic structuring of *C. maenas* was evident throughout Gulf St Vincent (i.e. no genotype – environment interaction), the observed morphometric variation across habitats may be evidence of phenotypic plasticity (Smith 2009). Significant morphometric variation but genetic homogeneity over habitats and spatial scales were found in other crab species. In the European stone crab, *Xantho poressa,* the COI gene identified genetic homogeneity across sites in Europe, although morphometric and colour variation was detected (Reuschel and Schubart 2007). The semiterrestrial crab, *Armases aungustipes*, has significant morphometric differences in carapace and chelae shape across populations that extended ~4,000 km along the Brazilian coastline; however, COI gene analysis showed genetic homogeneity despite the large latitudinal distribution (Marochi et al. 2017).

There is still debate as to whether genes for plasticity are targeted by selection, or if plasticity is a by-product of selection on traits in different environments (Smith 2009; Ghalambor et al. 2007; Fox et al. 2019; Westneat et al. 2019). Marochi et al. (2017) suggested that incongruences between morphological variation and genetic structure may not be the result of phenotypic plasticity but could be due to incomplete lineage sorting or very recent, ongoing genetic divergence. Epigenetic mechanisms also underpin phenotypic plasticity (e.g. DNA methylation); this influences an organism's response to its environment even when populations are genetically homogeneous (Clark et al. 2018). Invasion dynamics and the invasion process itself also impact mitochondrial haplotype diversity, such as genetic bottlenecks, founder effects and drift (Geller et al. 2010).

Carcinus maenas population structure fluctuates across its globally invasive range, including levels of genetic diversity, expansion rates, bottlenecks and abundances (Thresher et al. 2003; Darling et al. 2008). At intercontinental scales, *C. maenas* displayed genetic structuring over continents when assessed with mtDNA and microsatellite markers (Darling et al. 2008). The mtDNA neutrality tests were non-significant but *C. maenas* in South Australia

may have experienced a reduction in population size due to a genetic bottleneck resulting from founder effects. Genetic bottlenecks can purge an introduced population of less common haplotypes associated with the source population (Geller et al. 2010). Further analysis of population genetic structure, gene flow and comparisons of native and invasive ranges would help to detect underlying mechanisms driving genetic homogeneity in South Australian *C. maenas* (Karl et al. 2012).

3.4.3 Study limitations and ecological implications

Removing juvenile crabs (<30 mm CW) from the analysis prevented ontogenetic variation from confounding the morphometric results (Zelditch et al. 2012). However, as Cardini (2020) noted, using adults only in geometric morphometric studies will bias results due to ontogeny. As crabs allocate resources to their biomass differently throughout their development (Metri et al. 2017), assessing ontogenetic changes in morphology may help to further identify variation and provide data about its likely drivers (Cardini and Elton 2007; Cardini 2020). Morphology of crabs varied across habitats based on linear morphometric measurements in this study, however linear morphometrics do not contain the shape information necessary to explore habitat-specific variation in detail (Parsons et al. 2003). Habitat-specific size variation may be further influenced by sampling methods (i.e. trapping versus timed searches) in addition to local environment conditions. This may have confounded the size of crabs collected across sites because juvenile crabs are less likely to enter traps when larger, antagonistic adults are present, or due to mesh sizes and selectivity of traps preventing crabs of certain sizes from entering (Smith et al. 2004; Bellchambers and de Lestang 2005).

This study is also limited in its genetic interpretation, because only mtDNA sequences were used. mtDNA markers cannot produce *a priori* higher estimates of gene flow than hypervariable nuclear markers such as microsatellites (Karl et al. 2012). Fratini et al. (2016) identified genetic homogeneity in mtDNA COI in the marbled crab, *Pachygrapsus marmoratus*, across the Mediterranean Sea. Genetic heterogeneity was, however, also observed in Mediterranean *P. marmoratus* populations when analysed with polymorphic nuclear microsatellite markers by Deli et al. (2016b). Mitochondrial markers may therefore underestimate population structure in relation to biogeography and so nuclear markers should also be used (Deli et al. 2016b; Fratini et al. 2016). Assessing gene flow to investigate genetic homogeneity in South Australian *C. maenas* requires further study with nuclear markers such as SNPs and is explored further in Chapter 5.

Morphological variation and genetic homogeneity were displayed in invasive *C. maenas* across habitat types in this study. Genetic homogeneity throughout the South Australian population suggests that local adaptation is unlikely, extremely subtle or operates on different parts of the genome (Brian et al. 2006; Hoban et al. 2016). Comparing South Australian mtDNA haplotypes with native and other invasive populations of *C. maenas* may help to identify source populations through shared haplotypes (Darling et al. 2008). Comparing genotypes from the native and introduced range is vital for understanding the origin of plasticity in invasive species (Drown et al. 2010). Nuclear genetic markers such as single-nucleotide polymorphisms (SNPs) in addition to mtDNA may provide a more robust understanding of genetic structuring, and also provides the potential to identify adaptive processes in South Australian *C. maenas* (i.e. loci under selection). It is unclear what adaptive benefit, if any, the carapace shape variations observed provide to *C. maenas* in different habitats. Environmental (i.e. water temperature, substrate type) or ecological (i.e. prey availability, predation pressure) factors across habitats in Gulf St Vincent are likely to affect life history and survival of *C. maenas.*

Chapter 4. Early sexual maturity, high fecundity and a prolonged spawning period in invasive female *Carcinus maenas*

ABSTRACT

Reproductive traits such as high fecundity, prolonged reproduction, and early onset of sexual maturity contribute to the invasion success of marine invasive species. The European shore crab, *Carcinus maenas*, is a global marine invader with high fecundity. Research on European shore crab reproduction is limited to its northern hemisphere distribution, while reproduction in its southern hemisphere invasive range has not been evaluated. This study investigated the reproductive biology of female *C. maenas* from an invasive population in Gulf St Vincent, South Australia. Crabs were collected monthly between March – November in 2018, and ovarian indices and histological staging was determined. Histological analyses of the ovaries showed more large females with mature or spent ovaries than smaller females. 50% of females reached sexual maturity by ~51 mm carapace width, but the smallest egg-bearing female was 24 mm wide, suggesting early onset of sexual maturity occurs in some individuals. Average fecundity was estimated for a larger sample set of ovigerous females collected during seasonal monitoring from 2012 – 2018. Females produced an average 210,000 eggs per clutch, and fecundity was positively correlated with crab size. The reproductive period was identified, with high proportions of sexually mature and ovigerous females found in cooler months in winter/early spring compared to autumn/late spring. *Carcinus maenas* has high reproductive output and fecundity, size at maturity and seasonal spawning similar to studies in the northern hemisphere. Understanding the reproductive biology of *C. maenas* in their southern Australian range expands our understanding of life-history strategies that contribute to the invasion success of this species.

4.1 INTRODUCTION

Marine invasive species are of increasing global concern due to their potential negative impacts on marine ecosystems through predation, competition, pathogen spread and habitat modification (Bax et al. 2003; Williams et al. 2013). Whether introduced species can establish self-sustaining populations in their introduced range is influenced by their reproductive biology and life history strategies (Sakai et al. 2001). Most individuals in the initial colonising population are unlikely to survive, and successful colonisation often relies on repeated supply of propagules or a reproducing population that is large enough to persist (Johnston et al. 2009; Hänfling et al. 2011). Invasive species often have similar traits that increase chances of survival throughout the invasion process, such as high fecundity, early onset of sexual maturity and spawning plasticity to suit local environment conditions (Geburzi and McCarthy 2018). Investigating reproductive biology helps to identify marine invasive species with higher potential for establishment or range expansion (Blackburn et al. 2011).

Overcoming barriers to reproduction is a major step in the establishment stage of the invasion process (Blackburn et al. 2011). Reproductive traits which are characteristic of *r*-selected breeding strategies are indicators of invasion success: early onset of sexual maturity; short generation times; short embryonic development; long reproductive periods; multiple spawning events; fast growth; high fecundity; high nutrient allocation towards reproductive investment; and optimal timing of spawning events to increase offspring survival (Hänfling et al. 2011; Zeng et al. 2014; Geburzi and McCarthy 2018; Griffen 2018). These reproductive traits are documented to contribute to successful invasion by marine taxa, including ascidians, comb jellyfish, bryozoans, polychaete worms, bivalves, echinoderms, fish, gastropods, crustaceans and macroalgae (Nyberg and Wallentinus 2005; Harding et al. 2008; Hänfling et al. 2011; Gardner et al. 2015; Zhan et al. 2015; McFarland et al. 2016; Micael et al. 2016; Agüera and Byrne 2018; Jaspers et al. 2018; Lee et al. 2018). The mode of sexual reproduction (i.e. asexual or sexual depending on species), the ability to store sperm for prolonged periods, and mating/brooding behaviours contribute to marine bioinvasion success (Ramirez Llodra 2002; Weis 2010; Hänfling et al. 2011). Asexual reproduction (which is commonly observed in marine algae and some invertebrates) may allow a single founding individual or fragments to produce an entire population (Havel et al. 2015). Meanwhile, sexual reproduction increases genetic variance and novel trait combinations during establishment, especially if multiple introductions have occurred from various source populations (Facon et al. 2008).

Marine crustaceans are some of the most diverse and widespread marine invasive taxa worldwide (Brockerhoff and McLay 2011; Swart et al. 2018). Brachyuran crabs reproduce sexually, therefore males and females need to be proximal to each other to mate, but female crabs can store sperm, which provides opportunity for reproduction at low population densities during early colonisation (Anderson and Epifanio 2010; Hänfling et al. 2011; Farias et al. 2017). Invasive crabs may have competitive advantages over native crabs by reaching sexual maturity earlier, having longer reproductive periods and seasonal spawning events, or having higher fecundity than similar-sized and/or closely related species (Hänfling et al. 2011). Such reproductive traits have been seen in the invasive Asian shore crab, *Hemigrapsus sanguineus* (Griffen and Riley 2015; Brousseau and McSweeney 2016); the Asian paddle crab, *Charybdis japonica* (Wong and Sewell 2015); and the blue crab, *Callinectes sapidus* (Türeli et al. 2018). In rare cases, some invasive crabs do not have higher reproductive output as seen in introduced *Charybdis hellerii* in the Caribbean Sea (Bolaños et al. 2012), however, most invasive crabs exhibit high reproductive output which has contributed to their invasion success.

The European shore crab (or green crab), *Carcinus maenas* is one of the most successful marine invasive species worldwide and can negatively impact shell fisheries and intertidal habitats through predation and competition (Leignel et al. 2014). European shore crabs are iteroparous and have multiple and variable reproductive cycles over the course of their 5-7-year lifespan (Crothers 1967; Crothers 1968; Baeta et al. 2005). They exhibit species-specific reproductive patterns in response to varying environmental conditions (e.g. water temperature) that are likely to assist with establishment in their global introduced range (Anger et al. 1998; DiBacco and Therriault 2015). *Carcinus maenas* mate at water temperatures between 3–26°C (Young and Elliott 2019), while development of eggs is limited to temperatures below 18°C (Behrens Yamada 2001). Crabs can mate multiple times per year and male *C. maenas* produce sperm throughout the year regardless of season, therefore seasonality of spawning events is determined by female reproductive status (Van der Meeren 1994; Lyons et al. 2012). The seasonality and frequency of spawning events for *C. maenas* varies geographically. In their native range in Ireland, *C. maenas* have a biannual spawning season (Lyons et al. 2012), while in Portugal they spawn throughout the year (Baeta et al. 2005). In their invasive range in the Canada, *C. maenas* displayed one annual spawning event (Best et al. 2017).

A part of *C. maenas* invasion success is linked to the high fecundity of adults and the long developmental stages of their planktonic larvae (Moksnes et al. 2014; DiBacco and Therriault 2015). It usually takes *C. maenas* 1–2 years to reach sexual maturity in both their

native and invasive range, with maturity occurring anywhere between 20–50 mm CW (Young and Elliott 2019). The number of eggs produced by *C. maenas* varies by location and crab size. In its invasive range in Canada, fecundity of *C. maenas* ranges between 4,700 – 400,000 eggs per clutch (Audet et al. 2008; Griffen 2014). In their native range, *C. maenas* produces up to 370,000 eggs per clutch (Leignel et al. 2014). Although *C. maenas* are well-studied in their invasive range, little research has been done on their reproductive biology, especially in their invasive range in the southern hemisphere including Argentina, South Africa, and southeastern Australia (Young and Elliott 2019). As successful marine bioinvasions will depend on biotic and abiotic factors in the recipient community (i.e. ecological interactions, environmental conditions) it is important to consider population differences and similarities across *C. maenas*' global distribution (Thomsen et al. 2011). If reproductive traits are similar across globally invasive populations of *C. maenas*, then these traits may be indicative of the limits of *C. maenas*' reproductive plasticity (Morris 2014).

Population assessments of invasive *C. maenas* in South Australia showed the presence ovigerous females in the austral winter–spring (May–October) (Dittmann et al. 2017). This study assessed reproductive traits of *C. maenas* including fecundity, size at sexual maturity and seasonal ovary development. I aimed to investigate the reproductive biology of invasive female *C. maenas* in South Australia using egg counts and macroscopic and histological ovarian assessments. I hypothesised that: 1) larger females have higher reproductive output (gonad indices, mature gonad stages, fecundity) compared to smaller crabs; 2) females have an early size at sexual maturity similar to *C. maenas* from other invasive ranges; and 3) the number and frequency of mature and gravid females increases in cooler months and will be reflected in a female-biased sex ratio. This is the first study to assess the reproductive biology of *C. maenas* in its invasive range in the southern hemisphere. This study was designed to identify when crabs have spawned and at what size they become sexually mature, which can provide insight into recruitment and dispersal events that sustain introduced populations.

4.2 MATERIALS AND METHODS

4.2.1 Sampling sites and events

Carcinus maenas were sampled in coastal habitats of Gulf St Vincent, South Australia, using baited traps and timed search surveys (Fig. 4.1). Sample sites were in low-energy coastline with mixed tides and a 2–3 m spring tidal range (Bye and Kämpf 2008). Baited traps

were deployed in mangrove habitats at Middle Beach and Port Gawler, in a harbour at Port Adelaide River and Old Port Reach, and in a rocky shore habitat at the Onkaparinga River. Timed search surveys were done only in the rocky shore region at two sites: the Onkaparinga River and Aldinga Beach due to presence of sanctuary zones and site accessibility. The mangrove habitat is comprised of dense mangrove (*Avicennia marina*) forests with saltmarsh and sandflats in the upper intertidal zones, and seagrass beds in the subtidal zones. Habitats in the harbour varied in composition; the Port Adelaide River has a large shipping channel in the deeper subtidal zone with industrial infrastructure, a wide sediment mudflat next to a boat ramp and marina, and sparse *A. marina* mangroves. This channel forms the main shipping port for Adelaide and is where *C. maenas* were likely introduced via shipping (Zeidler 1978; Dittmann et al. 2017). Old Port Reach has a small mangrove cove and mudflat/clay banks, with man-made rock walls next to high-density urban housing and a private marina. The rocky shore habitats consisted of rocky, temperate reef and shorelines (Bourman et al. 2016). The trapping and/or timed search methods were consistent with previous monitoring at the same sites and have effectively caught *C. maenas* in all cases (see Dittmann et al. 2017).

Sampling took place monthly between March and November 2018 (nine sampling events) and coincided with the new moon period and spring tides where possible. Monthly sampling targeted the periods in which female *C. maenas* are reproductively active and to identify potential major spawning events. All sampling occurred during daytime low tides (<0.6 m sea level) to gain access to the shoreline for placement of traps and timed searches. In March 2018, only the mangrove and harbour sites were assessed due to time restrictions preventing assessments at the rocky shore sites during that month. All sites were successfully assessed each month between April–November 2018. Dates of each specific sampling event are shown in Appendix Table A4.1.

Ovarian development was assessed monthly between March and November 2018. Monitoring from 2012–2017 showed no evidence of gravid females in summer (December–February) in Gulf St Vincent, and abundances of both male and female *C. maenas* were lower in summer each year (Dittmann et al. 2017). I therefore focused the sampling effort from early austral autumn to late spring (March–November) to maximise female crab catches to assess ovary development. For fecundity assessments, I analysed any gravid females collected during 2012–2018 in Gulf St Vincent from studies by Dittmann et al. (2017) and this study.

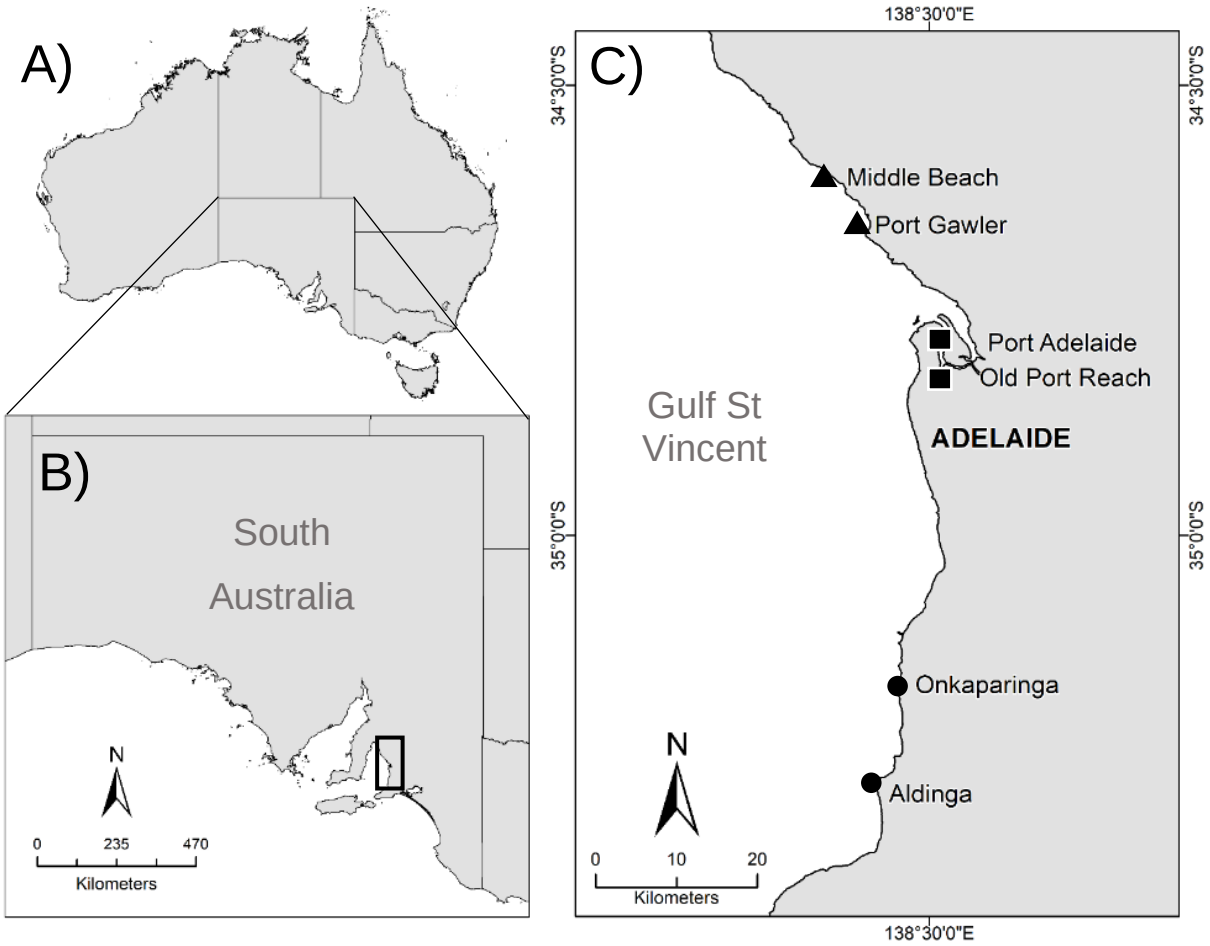

Figure 4.1 Map of A) Australia; B) South Australia; and C) Gulf St Vincent and the Adelaide metropolitan coastline with six sampling sites. Black tringles represent mangrove sites, black squares represent harbour sites, and black circles represent rocky shore sites. Dark grey shading indicates land.

4.2.2 Crab collection

Carcinus maenas were trapped with opera-house style traps (67 x 48 cm, opening diameter 7.8 cm, and 2 cm mesh size) each baited with one frozen sardine (*Sardinops sagax*). All baited traps were deployed in shallow water at low tide and secured to the sediment with wooden stakes. Five traps were deployed at each of the mangrove sites, the harbour sites, and at the Onkaparinga River rocky shore on all occasions. There was a minimum distance of 5 m between traps. Traps in mangrove and harbour sites were retrieved after approximately 24 hours in the following low tide. Overnight deployment of traps has higher likelihood of catching *C. maenas* due to their nocturnal feeding behaviour and intertidal migrations during the tidal cycle (Reid and Naylor 1989). Traps in the Onkaparinga River were deployed for approximately 3–4 hours to reduce theft because this site has high recreational activity. No

trapping occurred at Aldinga because this site is a Sanctuary Zone and therefore trapping is not permitted even with scientific collection permits.

On retrieval of traps, all *C. maenas* were collected into labelled sample bags and transported on ice to Flinders University for processing and assessment. Environmental parameters (i.e. water temperature, salinity, dissolved oxygen) were recorded at the mangrove and harbour sites during all sampling events using a handheld HANNA multi-parameter probe. Recorded sea surface temperatures across the mangrove and harbour sites ranged from 7.35°C in June to 26.43°C in March (mean SST: 17.55°C ± 5.14). Recorded salinity across the mangrove and harbour sites ranged from 34.5 in November to 46.33 in July (mean salinity: 41.06 ± 2.99). Environmental parameters were not recorded in rocky shore sites due to time constraints and restrictions with the tidal window.

The rocky shore sites were searched by two researchers per sampling event for 30 minutes per timed search (four timed searches per site). Timed searches were not possible at the mangrove and harbour sites due to habitat complexity, maritime traffic and site accessibility. Any *C. maenas* located during the timed searches were collected by hand and placed into labelled sample bags; no other organisms were removed during the search. At the Onkaparinga River site, where both trapping and timed search methods were used, not all sample bags specified the method used to collect crabs. When either trapping or time search methods could not be confirmed for crabs caught at the Onkaparinga River site, they were classified as "unspecified." Small crabs and gravid females can avoid baited traps or seek refuge to reduce predation or avoid antagonistic interactions with other crabs (Young et al. 2017; Young and Elliott 2019). Active methods such as hand collection can locate more immature or gravid females than trapping alone (Kent and McGuinness 2006). It should be noted that as different collection methods were used at different sites in this study, spatial bias is evident and will influence the number, size and maturity of females caught.

4.2.3 Laboratory procedures

All male *C. maenas* were killed via freezing at -20°C and preserved frozen. Males were counted and measured across the carapace width (CW) to evaluate sex ratios and size distributions. Gravid females with noticeable egg clutches were killed via freezing and kept frozen for later fecundity assessments. Live, non-gravid female *C. maenas* collected from each site were placed in aerated seawater aquaria and fed sardines until ovary analysis. Assessment

of female crabs occurred within one to five days after field work to avoid further ovary development. Only females were selected for maturity analyses because spermatogenesis does not follow obvious seasonal patterns, and because mature male crabs can inseminate females regardless of month sampled (Lyons et al. 2012; Best et al. 2017). Any crabs that could not be sexed because the pleons were immature (< ~25 mm CW) were excluded from this study.

For histology assessments, female crabs were removed from aquaria and anesthetised at -20°C for 10–30 minutes depending on crab size. Crab whole body wet weight was measured on electronic scales (A&D Weighing Pty Ltd, Adelaide, Australia) to the nearest 0.01 g. Carapace and pleon measurements (width, length) of female crabs were taken as per Chapter 3 using digital Vernier callipers (Kincrome Australia Pty Ltd, Victoria, Australia) to the nearest 0.1 mm. The presence of missing limbs (body condition), colouration, and epibionts were recorded for each individual. Each female crab was then killed by injection of Davidson's AFA Fixative into the hepatopancreas (protocols by The World Organisation of Animal Health – Manual of Diagnostic Tests for Aquatic Animals 2016). To ensure rapid death and effective fixation, the fixative was injected to an equivalent 10% of the crab's body weight, after which signs of life would rapidly cease (i.e. no reflexes to stimuli or movement after 5–10 seconds). All non-gravid female crabs used for ovary analysis were killed using Davidson's AFA Fixative instead of freezing; this avoided ice crystals from damaging cell and tissue structures which can affect sectioning and staining (Costa 2018). The top of the carapace was dissected from dead female crabs following Crothers (1968).

Macroscopic and histological descriptors were used to stage ovary development as per Lyons et al. (2012) and Best et al. (2017) (Table 4.1; Fig. 4.2; Fig. 4.3). Descriptions of each ovary stage were as follows: immature ovaries (Stage 0) were small and translucent and could not be adequately examined or separated from the hepatopancreas or digestive gland. Histology was trialled on suspected ovary tissue of an immature Stage 0 female which confirmed the presence of hepatopancreas, muscle and foregut cells and tissue only. Previtellogenic ovaries (Stage 1) were able to be identified macroscopically, and in most cases could be separated from the hepatopancreas. This stage consisted of a small white and/or cream ovary (Fig. 4.2A). Histological descriptors of Stage 1 ovaries showed oogonia found in "nests", rounded follicle cells and many small primary oocytes that stained blue and purple (Fig. 4.3A). Intermediate ovaries (Stage 2) were easily distinguished by a larger ovary that was creamy mustard yellow to pale orange in colour (Fig. 4.2B). Histological descriptors of Stage 2 ovaries showed larger

oocytes in various stages of vitellogenesis, appearance of yolk globules in the cytoplasm, and stained a deep purple/pink colour (Fig. 4.3B). Mature ovaries (Stage 3) were easiest to identify macroscopically due to their large size and bright orange to red colouration (Fig. 4.2C). Histology of Stage 3 ovaries showed very large oocytes with large amounts of cytoplasm filled with yolk globules, distinguishable nuclei, flattened follicle cells, and bright pink stains (Fig. 4.3C). Ovigerous females did not require ovary staging due to carrying obvious egg clutches in their pleon but were assessed for fecundity and maturity analysis (Fig. 4.2D). Resorbing ovaries (Stage 4) had a deflated ovary that ranged in colour from white, grey and brown with flecks of orange, but was sometimes difficult to separate from the hepatopancreas. Remnant hatched eggs were often still attached to the pleon in resorbing stages and used as an additional descriptor. Histological analysis of Stage 4 ovaries showed large nests of oogonia and primary follicles developing in the next cycle of oogenesis, while any mature oocytes that were not released were in phases of atresia and resorption (Fig. 4.3D) (Table 4.1).

In all cases where ovaries were distinguishable and large enough to be separated from other tissues and organs, the whole ovary was carefully removed with forceps and weighed on electronic scales. All immature ovaries (Stage 0) and some previtellogenic (Stage 1) and resorbing (Stage 4) ovaries were too small to be separated from the hepatopancreas/foregut and in this case, only the macroscopic stage of the ovary was recorded. A small section of each ovary was removed and placed into a histology cassette, then preserved in Davidson's AFA Fixative for a minimum of 48 hours to penetrate the tissue (10:1 fixative: tissue ratio). Ovary samples remained in fixative for a minimum of three days to several weeks. No noticeable effect of prolonged fixation occurred during sectioning and staining (i.e. brittleness). Specimens fixed in Davidson's AFA Fixative were then transferred to 70% ethanol until tissue dehydration.

4.2.4 Estimations of reproductive output

Ovary histology and microscopy

For histological analyses, all preserved ovaries were dehydrated following a graded ethanol series from 70–100%, then de-alcoholised in chloroform for approximately 12 hours. Ovaries were embedded in paraffin wax, and paraffin blocks were chilled and sectioned at approximately 7 µm thick using a Leica RM2135 rotary microtome. Some sections had to be cut slightly thinner or thicker (i.e. 4–9 µm) to accommodate varying tissue size. Sections were

dried at 37°C overnight prior to staining. All sections were stained with a routine Harris' haematoxylin and eosin (H&E) method following standard protocols at Flinders University Microscopy and mounted onto permanent glass slides using DPX mounting medium. Detailed descriptions of the histology and H&E staining methods can be seen in Appendix Fig. A4.1. An Olympus Brightfield BX53 microscope was used for the examination of the slides under various magnifications for exploring the tissue and cell structure.

Microscopic images were taken with an Olympus DP27 Microscope Colour Camera and Olympus cellSens Entry Software with manual white balance applied. Three images of different areas of the section of the ovary were taken from each female, and all images were taken consistently at 200x magnification. To ensure that images were adequate for analysis, thin black boundaries were manually drawn around the cytoplasm of intact egg cells (oocytes) using Adobe Photoshop CC and a Wacom drawing tablet. Outlined images were imported in FIJI (Image J) version 1.52s for calibration and scaling (Schindelin et al. 2012). The Cell Counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html) was used to count each type of oocyte (i.e. previtellogenic, intermediate, mature and/or resorbing) per image before a greyscale threshold was applied.

The wand tool in FIJI was used to select approximately five oocytes of each type in the image, and the surface area in μm^2 was calculated for each oocyte. Any oocytes at the edge of the image, obstructed by other cells, or not sectioned through the nucleus were not analysed. The presence of other cell types, such as oogonia and post-ovulatory follicles (POFs) were noted. Oogonia were detected across all ovarian stages (Stage 0 to Stage 4), however POFs were only ever detected in resorbing (post-spawning) stages (Stage 4). Due to the inconsistently shaped boundaries of POFs and small size of oogonia, these cells could not be measured accurately and so were marked only on presence. The cell counts and surface area measurements from the three images were pooled and averaged for each female. Histological stage of the whole ovary was based on the presence, number and frequency of the most advanced oocytes in the ovary section as per Lyons et al. 2012 and Best et al. 2017. The macroscopic and histological maturity stage of each ovary were classified as per Table 4.1, Figure 4.2 and Figure 4.3.

Table 4.1 Descriptions of ovary development stages for female *C. maenas* based on macroscopic and histological characteristics of ovaries. Macroscopic stages of the ovaries and eggs can be seen in Figure 4.2, while histological stages of the ovaries can be seen in Figure 4.3. Gravid females (which spawned between Stage 3 and Stage 4; Fig. 4.2D) did not require ovary examination. SA = surface area.

Development stage	Maturity class	Macroscopic characteristics	Histological characteristics	Min – max oocyte SA (μm^2)
Stage 0 Immature	Immature	Ovary is indistinguishable or hard to locate from the hepatopancreas.	No ovarian tissue/cells are identifiable.	N/A
Stage 1 Previtellogenic (Fig. 4.2A, 4.3A)	Immature	Thin, small ovary. Often translucent, white or cream in colour. Can be distinguished from the hepatopancreas but sometimes difficult to separate and measure.	Numerous, densely packed oogonia proliferating from the germination zone, often found in "nests." Some larger previtellogenic primary oocytes present with obvious nucleoli. Oocytes consisted mostly of nuclei and small amounts of cytoplasm. Round follicle cells. Stain colour is blue/purple.	116 – 5000 μm^2 (mean: 948 ± 712)
Stage 2 Intermediate (Fig. 4.2B, 4.3B)	Immature	Ovary is larger and easy to separate from hepatopancreas. Ovary colour ranges from pale-mustard yellow to pale orange.	Oogonia reduced to smaller clusters. Maturing oocytes increase in size and number, with darker nuclei and nucleoli visible. Lipid and yolk globules start to appear via vitellogenesis. Oocytes have increased amounts of cytoplasm. Follicle cells begin to flatten. Stain colour is purple/deep pink.	2379 – 16,673 μm^2 (mean: 7875 ± 3003)
Stage 3 Mature (Fig. 4.2C, 4.3C)	Mature	Ovary size is large and fills up body cavity. Easy to separate from the hepatopancreas. Colour ranges from bright orange to orange-red.	Oocytes have matured and are very large in volume. Nuclei can be seen in mature oocytes. Cytoplasm filled with yolk and is very large. Follicle cells completely flattened. Infrequent, small clusters of oogonia present throughout ovary. Stain colour is bright pink/red-pink.	8338 – 60,801 μm^2 (mean: 27,147 ± 9660)
Stage 4 Resorbing/spent (Fig. 4.3D)	Mature	Ovary is reduced in size, and ranges from orange/grey/white in colour. Flecks of orange oocytes may still be present in the ovary or oviducts. Hatched eggs often present in the pleon from spawning event.	Mature oocytes often in the process of reabsorption and reducing in size. Oogonia and primary oocytes reappear for the next cycle. Reduced size of cytoplasm. Atretic, post-ovulatory follicles present. Follicle cells are rounder. Stain colour is often blue/purple with sections of bright pink.	16,342 – 44,047 μm^2 (mean: 30,885 ± 10,667)

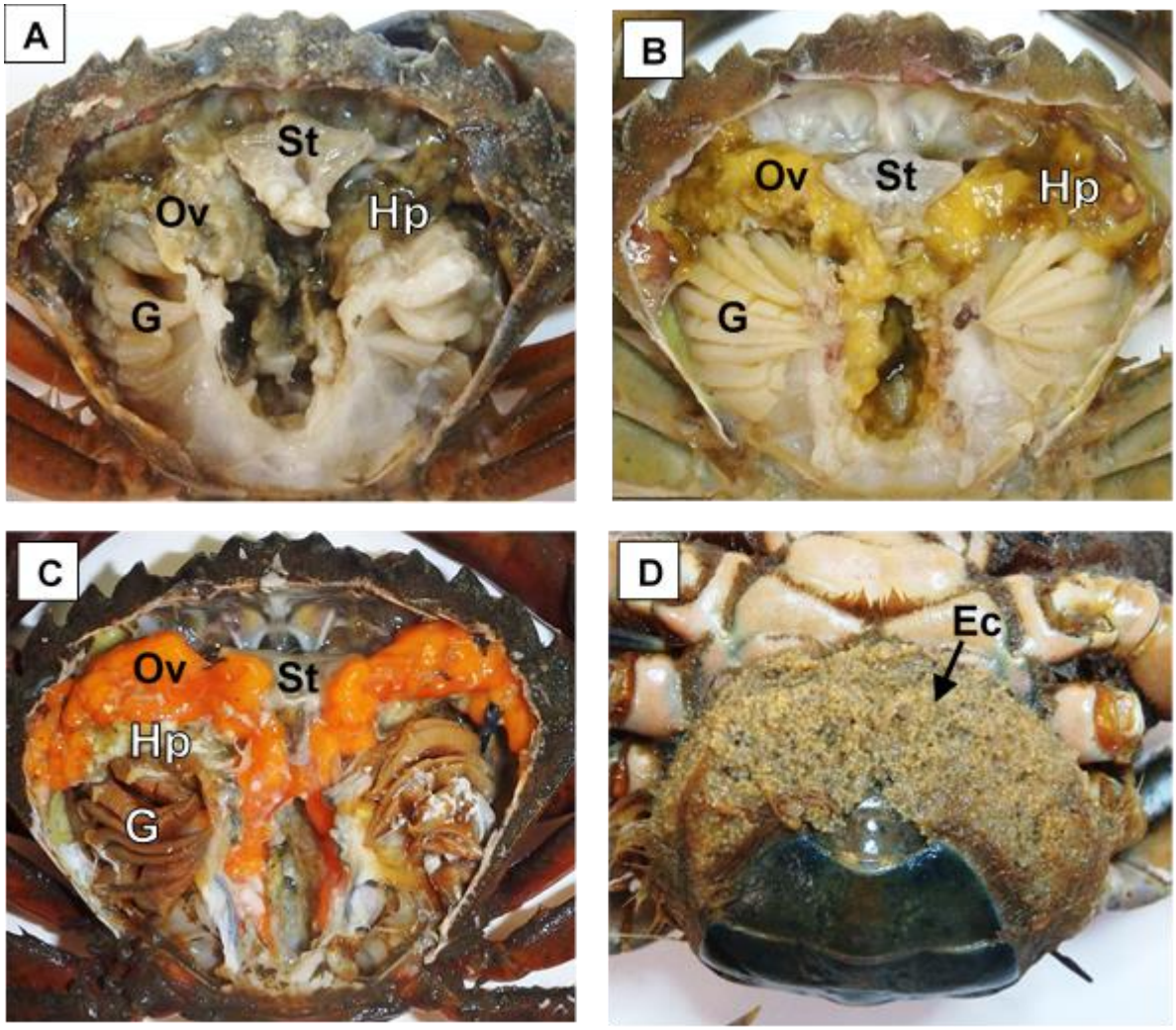

Figure 4.2 Macroscopic stages of ovarian and egg development in female *C. maenas.* A) Stage 1 – early development/previtellogenic; B) Stage 2 – intermediate; C) Stage 3 – mature; D) Gravid. Ov: ovaries; Hp: hepatopancreas; G: gills; St: stomach; Ec: egg clutch. Stage 0 (immature) and stage 4 (resorbing/spent) ovaries are not shown. The corresponding description of the macroscopic characteristics for each stage is shown in Table 4.1.

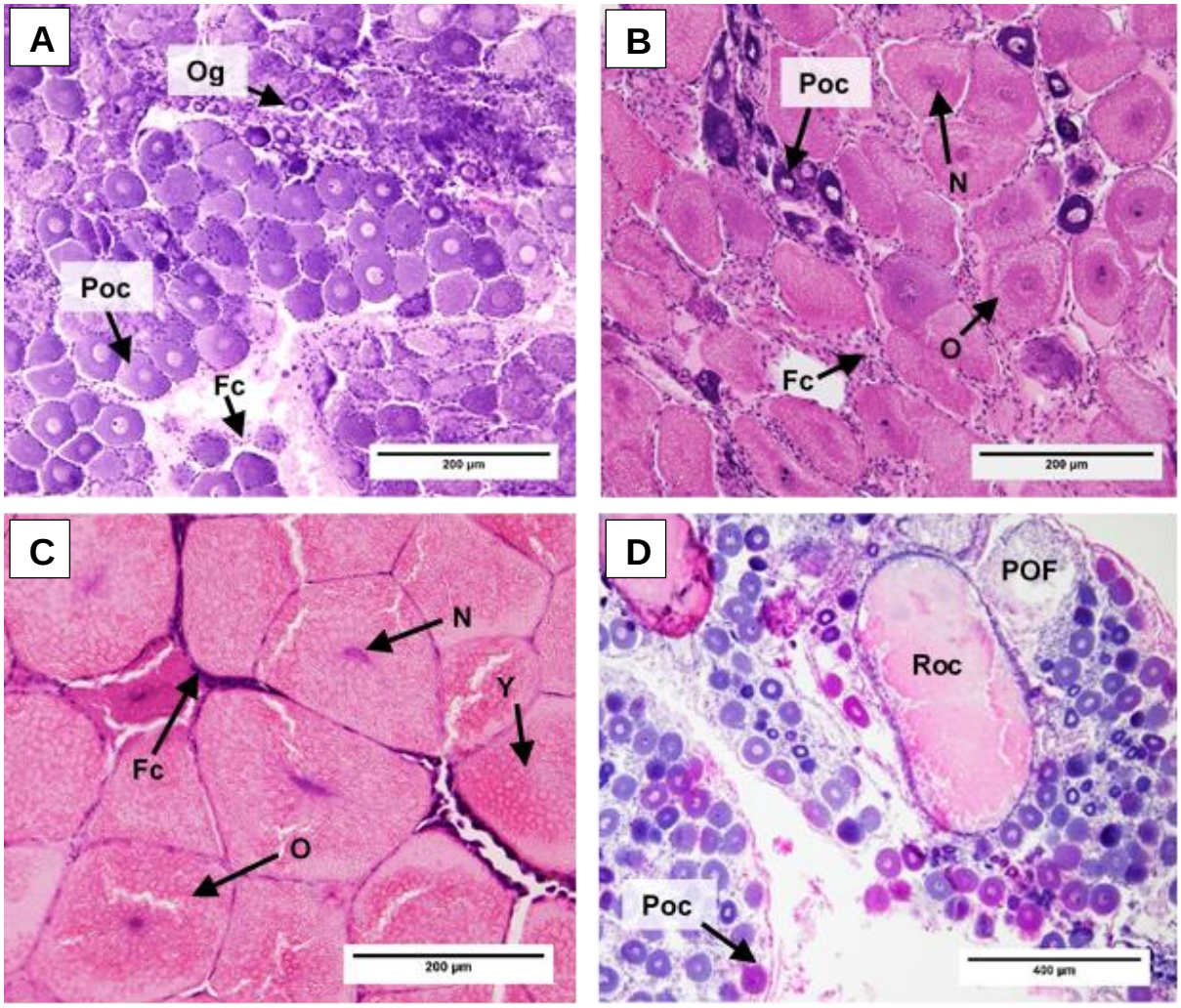

Figure 4.3 Histological stages of ovarian development in female *C. maenas* under 200x magnification (D is shown at 100x magnification). A) Stage 1 – early development/previtellogenic; B) Stage 2 – intermediate; C) Stage 3 – mature; D) Stage 4 – spent/resorbing. Og: oogonia; Poc: primary oocyte; Fc: follicle cell; Oc: mature oocyte; N: nucleus; POF: post-ovulatory follicle; Roc: resorbing oocytes; Yg: yolk globules. Stage 0 (immature) not shown as this stage could not be detected under the microscope. The corresponding description of the histological characteristics for each stage is shown in Table 4.1.

Fecundity estimates

Gravid females collected in 2018 were frozen at -20°C until fecundity analysis. Additional gravid females that had been collected and stored frozen from studies in 2012–2017 were also assessed in this study. Fecundity was defined as the estimated number of eggs per clutch produced by ovigerous females during the breeding season (Díaz et al. 1983; Ramirez Llodra 2002). All gravid females were rinsed and thawed, then weighed on electronic scales with the egg clutch attached. The entire egg clutch was then carefully separated from the pleopods and pleon with forceps, rinsed over a sieve and blotted dry. The egg clutch was weighed again separately, and the overall colouration of the clutch was noted. For each separated egg clutch, three similar sized sub-samples were removed from the clutch and preserved in 70% ethanol until egg counting. Each subsample was weighed to the nearest 0.01 g, placed into petri dishes and examined under a dissecting microscope. The number of eggs in each subsample were counted manually using an analogue hand tally calculator. The development stage of the fertilised eggs was described as either uneyed (no embryo present), eyed (embryo present), or hatched (Appendix Fig. A4.2). Five females had hatched egg clutches which could not be accurately counted and weighed and were removed from the fecundity analyses.

4.2.5 Data analysis

To assess the population dynamics of *C. maenas* in South Australia, raw abundance data for male and female *C. maenas* collected during the 2018 sampling period (March–November) were converted to monthly male:female sex ratios and pooled across sites. Deviations from the expected 1:1 (male:female) ratio for each month were analysed with a Chi-square cross-tabulation test (X^2: Sokal and Rohlf 1995). Chi-square cross-tabulations were tested on female size-frequency (CW) versus ovary stage, and for ovary stage frequency versus sampling month. Averages of crab size are expressed as mean ± SD. For seasonal reproduction, average monthly sea surface temperatures for Gulf St Vincent in 2018 were obtained from the Australian Ocean Data Network (AODN; https://portal.aodn.org.au/search) via the Integrated Marine Observing System (IMOS).

Normality and homogeneity of variance of the data were visually inspected using quantile-quantile (Q-Q) plots and histograms and any outliers present were examined. A Shapiro-Wilk Test was used to test normality of the data, while Levene's Test was used to test

for homogeneity of variance. As the data did not meet the assumptions of normality (Shapiro-Wilk $p < 0.05$) and homogeneity of variance (Levene's $p < 0.05$), multivariate non-parametric permutational analysis of variance (PERMANOVA) was used for statistical tests. All PERMANOVA tests were run with 9,999 permutations and Euclidean distance in the software PAleontological STatistics "PAST" version 4.01 (Hammer et al. 2001). All statistical analyses had a significance value at $\alpha = 0.05$, while all pairwise comparisons had sequential Bonferroni-correction applied at the 5% level ($\alpha = 0.05$) to correct for multiple tests (Rice 1989). Female crab measurements (i.e. wet weight, carapace width/length, and pleon width/length) were $\log_{10}$-transformed. All measurement variables were tested against development stage (factor with six levels) using multivariate one-way PERMANOVA and pairwise comparisons. Due to the different collection methods used across sampling sites and the low number of females collected at some sites, data from all sites were pooled and site was excluded as a factor in the analysis. Construction of all graphs were done using either ORIGIN PRO version 2018 (OriginLab Corporation, Northampton, MA, USA) or R version 3.6.3 (R Core Team 2020) and RStudio version 1.2.5042 (RStudio Team 2020).

Oogenesis and gonad indices

Univariate one-way PERMANOVA and pairwise comparisons were used to test and oocyte surface area (μm^2) against oocyte type (factor with four levels). The gonadosomatic index (GSI; adapted from Baklouti et al. 2013) and gonad-carapace width index (GCW; adapted from Best et al. 2017) were calculated for females with weighed ovaries as per the equations below. Univariate one-way PERMANOVA and pairwise comparisons were used to test GSI and GCW against ovary stage (factor with four levels). A univariate non-parametric Mann-Whitney U test was used to test differences between GSI and GCW irrespective of ovary stage.

Equation 1 (adapted from Baklouti et al. 2013):

$$GSI = \frac{Gonad\ wet\ weight\ (g)}{Total\ crab\ wet\ weight\ (g)}\ x\ 100$$

Equation 2 (adapted from Best et al. 2017):

$$GCW = \frac{Gonad\ wet\ weight\ (g)}{CW\ (mm)}\ x\ 100$$

Size at sexual maturity

To estimate gonad size at sexual maturity (L_{50}), females were classed as "immature" or "mature" based on development stage (ovary stages 0, 1 and 2 were immature, while ovary stages 3, 4 and gravid were mature). The corresponding development stage and maturity class used in the L_{50} analyses are shown in Table 1. A logistic function was fitted to the data in the R package '*sizeMat*' version 1.1.1 to estimate the size at which a randomly chosen specimen has a 50% change of being sexually mature (Torrejon-Magallanes 2020). In the regression analysis, *X* (Carapace width) was the explanatory variable, while *CL* (immature = 0; mature = 1) was the binomial response variable. The variables were fitted to a logistic function with the following form:

$$P_{CL} = \frac{1}{(1 + e^{-(beta_0 + beta_1 * X)})}$$

Where P_{CL} is the probability of an individual being mature at a determinate *X* size (CW), and $beta_0$ (intercept) and $beta_1$ (slope) are parameters estimated. The L_{50} was then calculated as:

$$L_{50} = \frac{-beta_0}{beta_1}$$

Fecundity estimates

Fecundity was estimated by extrapolating the egg counts of each subsample to the total weight of the egg clutch per crab using the following gravimetric equation (adapted from Bithy et al. 2012):

$$\mathrm{F_1} = \mathrm{nG/g}$$

Where F_1 = fecundity of the subsample; n is the number of eggs in the subsample; G is the total weight of the egg clutch; and g is the weight of the subsample. The resulting fecundity values for all three subsamples (F_1, F_2 and F_3) were then averaged to calculate the estimated mean fecundity (F) per gravid female from the equation below:

$$\mathrm{F} = \frac{\mathrm{F_1} + \mathrm{F_2} + \mathrm{F_3}}{3}$$

Linear regression was then used to analyse the relationship between carapace width (mm) and clutch weight (g) against estimated mean fecundity (F). The development stage of the fertilised eggs in the clutches (uneyed or eyed) were compared in the regressions using an analysis of covariance (ANCOVA). The ANCOVA was used to check if development stage of embryos during incubation could influence the relationship between fecundity and female size (Wehrtmann et al. 2010; Ahamed and Ohtomi 2011; Zimmermann et al. 2015; Fields et al. 2020). In the ANCOVA tests, the dependent variable was fecundity, the fixed factor was the egg stage, and the covariate was either carapace width or clutch weight.

4.3 RESULTS

4.3.1 Population dynamics of *C. maenas*

359 *C. maenas* were collected during 2018 comprising 199 males (55%) and 160 females (45%). Of the 359 crabs, baited traps caught 81 females and 116 males (Appendix Fig. A4.3). 46 females and 72 males were captured during timed searches. There were 33 females and 11 males collected from the Onkaparinga River with an unspecified method (Appendix Fig. A4.4). Most females were collected from the rocky shore habitat (n = 105), followed by the mangrove habitat (n = 34) and harbour habitat (n = 21). Similar patterns were observed for males with most collected from the rocky shore habitat (n = 116), followed by the mangrove habitat (n = 73) and harbour habitat (n = 10). The overall male to female sex ratio across sampling months for 2018 was 1: 0.80, which was different to the expected ratio of 1:1 (X^2 = 19.99, df = 8, $p < 0.05$), and displayed an overall male bias (Table 4.2). Monthly sex ratios showed a male bias across most months, apart from April (0.57: 1) and May (0.52: 1) where there was a female bias.

Table 4.2 Total male and female abundances and sex ratios for *C. maenas* collected monthly in 2018 from all sites in Gulf St Vincent. Sex ratios are displayed as male:female. Asterisks (*) denote months where the sex ratio was female biased.

Abundance	Mar 18	Apr 18	May 18	Jun 18	Jul 18	Aug 18	Sep 18	Oct 18	Nov 18
Females	2	21	25	12	19	11	3	40	27
Males	3	12	13	16	22	23	13	51	46
Sex ratio	1: 0.67	0.57: 1*	0.52: 1*	1: 0.75	1: 0.86	1: 0.48	1: 0.23	1: 0.78	1: 0.59
Total	**1: 0.80**								

4.3.2 Oogenesis and stages of ovarian development

The ovaries of 144 non-gravid female *C. maenas* collected monthly between March and November 2018 were staged using macroscopic methods (see Table 4.1 for staging). Of the 144 ovaries assessed macroscopically, I was able to histologically stage and measure oocytes from 78 of these ovaries (Table 4.3). For the macroscopic staging, immature ovaries (Stage 0) and mature ovaries (Stage 3) were the most abundant. Previtellogenic ovaries (Stage 1) and intermediate ovaries (Stage 2) were less abundant, while resorbing ovaries (Stage 4) were the least abundant overall. I was unable to identify any immature ovaries (Stage 0) or count oocytes in some previtellogenic ovaries (Stage 1) and resorbing ovaries (Stage 4) using histological assessments. Discrepancies between macroscopic and histological methods for immature and resorbing ovaries are likely caused by difficulty separating small and/or undeveloped ovarian tissue from the other organs. All ovaries that were intermediate (Stage 2) or mature (Stage 3) were successfully identified using both macroscopic and histological examination (Table 4.3).

Table 4.3 The number of ovary stages of non-gravid female *C. maenas* identified with macroscopic and histological methods. Please refer to Table 4.1, Fig. 4.2 and Fig. 4.3 for ovary stages.

Ovarian stage	Macroscopic staging *N*	Histological staging *N*
Stage 0	57	0
Stage 1	12	6
Stage 2	17	17
Stage 3	52	52
Stage 4	6	3
Total	144	78

Of the 78 ovaries examined histologically, 1,688 oocytes were identified, counted and measured. Oocyte surface area (μm^2) was significantly different across the four types of oocytes (PERMANOVA, $F = 1871$, $p_{(perm)} < 0.001$). Mature and resorbing oocytes had greater surface area and size variation than previtellogenic and intermediate oocytes (Fig. 4.4). Pairwise comparisons showed that interactions between all pairs of oocyte types were significant (sequential Bonferroni, $p < 0.005$) except between mature oocytes versus resorbing oocytes (sequential Bonferroni, $p = 0.251$). Mature and previtellogenic oocytes were the most abundant, while resorbing oocytes were the least abundant.

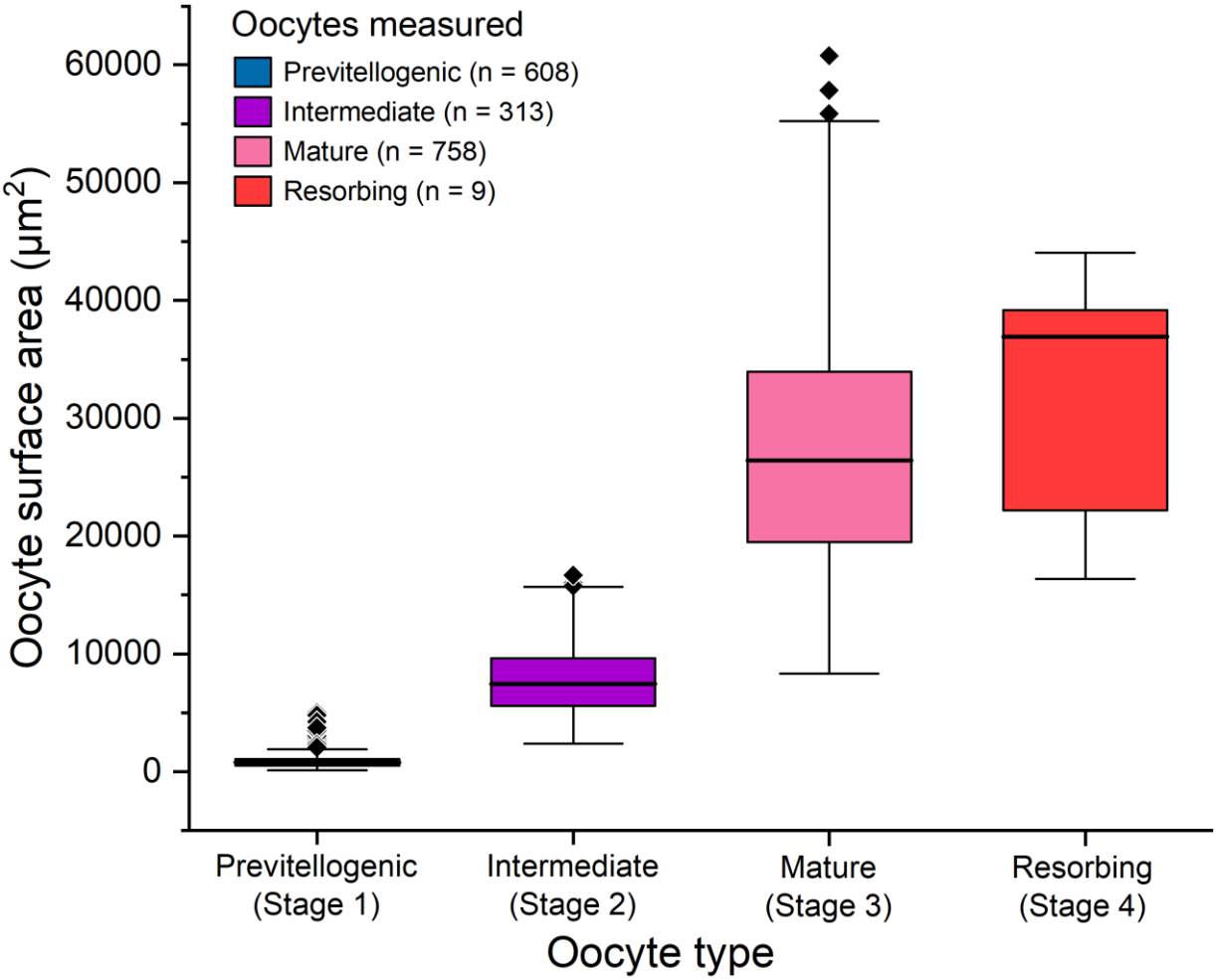

Figure 4.4 Oocyte surface area (μm^2) of four oocyte types from histological ovary examination of 78 *C. maenas* collected monthly between March–November 2018. Black circles represent outliers. Crabs with immature ovaries that were indistinguishable from the hepatopancreas were excluded.

The GSI of 78 females ranged between 0.12–14.58% with the largest GSI values found in intermediate, mature and resorbing ovary stages (Fig. 4.5). While GSI increased slightly with ovary stage, this increase was non-significant (PERMANOVA, $F = 2.174$, $p_{(perm)} = 0.09$). The GCW ranged between 0.04–18.21% with the largest GCW values found in mature and resorbing ovary stages (Fig. 4.5). There was a non-significant increase in GCW and ovary stage (PERMANOVA, $F = 2.338$, $p_{(perm)} = 0.08$). The minimum and maximum range of GCW values showed less variation than GSI, but the GCW index included more extreme outliers. There was a significant difference between GSI and GCW irrespective of ovary stage (Mann-Whitney U test, $U = 2215$, $p = 0.003$). Kumar et al. (2003) suggested that GSI becomes more biased with increasing body weight due to variations in mass (i.e. missing/regenerating limbs, epibionts). Meanwhile GCW uses carapace width rather than body weight, which is standardised and does not change as a function of ovary stage (Devlaming et al. 1982; Best et al. 2017).

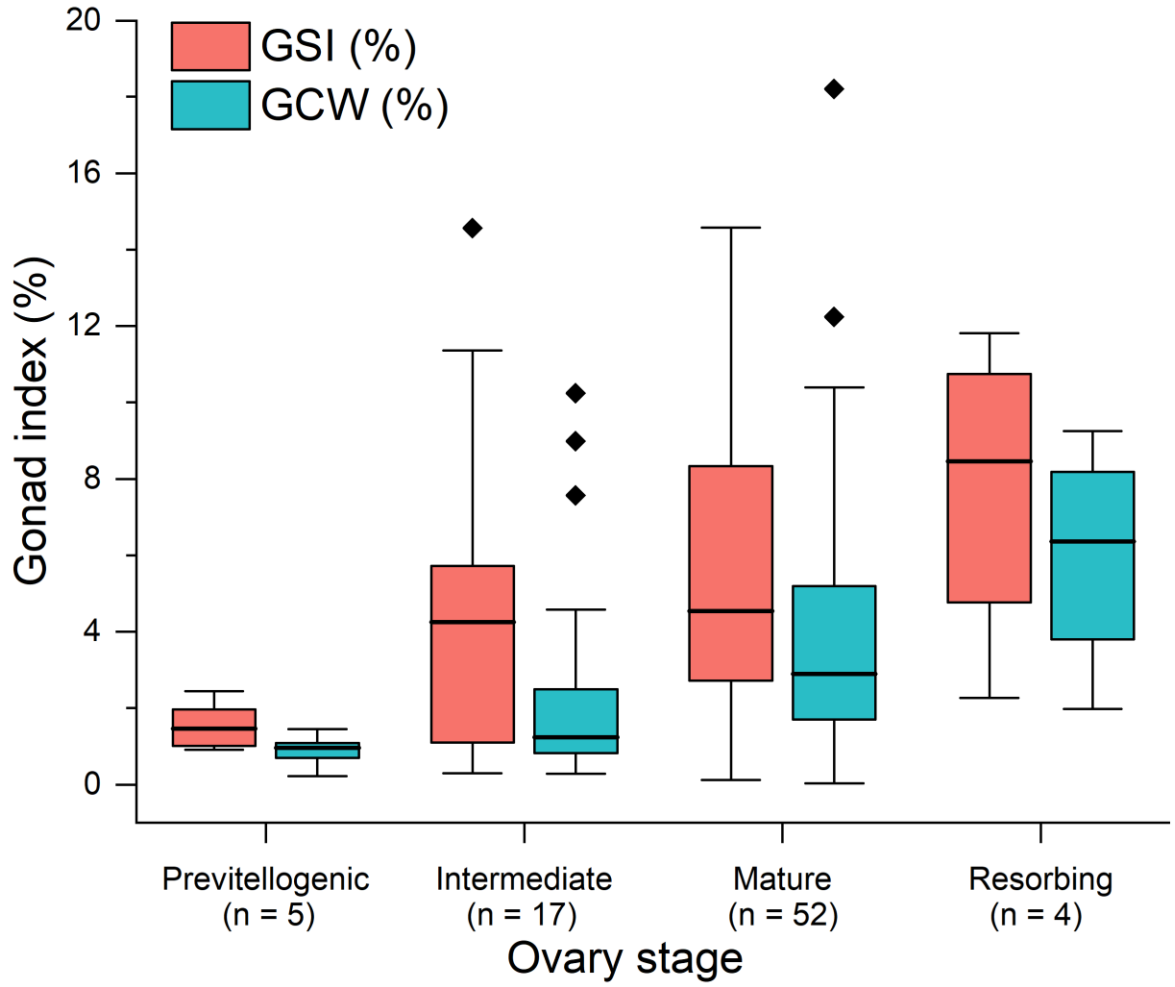

Figure 4.5 Boxplots displaying gonadosomatic index (GSI%, pink boxes) and gonad-carapace width index (GCW%, teal boxes) across four ovary stages in 78 female *C. maenas* collected monthly in 2018. Stage 1 = previtellogenic; Stage 2 = intermediate; Stage 3 = mature; Stage 4 = resorbing/spent. Black diamonds represent outliers, while black horizontal lines in each box represent the median.

4.3.3 Size at sexual maturity

As females matured, the mean wet weight (g), carapace width/length (mm), and pleon width/length (mm) increased (Table 4.4). All mean measurements decreased again for gravid females, whose sizes were more comparable to crabs with previtellogenic and intermediate ovaries (Stages 1 and 2) than to crabs with mature and resorbing ovaries (Stages 3 and 4). Females with Stage 3 and Stage 4 ovaries were the largest across all dimensions, while females with Stage 0 and Stage 1 ovaries were the smallest for all dimensions. There was a significant difference between the five $\log_{10}$-transformed measurements and development stage (PERMANOVA, F = 8.584, $p_{(perm)} < 0.001$). Pairwise comparisons (sequential Bonferroni corrected) showed a significant difference in measurements for Stage 0 vs. Stage 2 (p = 0.043), Stage 0 vs. Stage 3 (p = 0.002), Stage 0 vs. Stage 4 (p = 0.015), and Stage 1 vs. Stage 3 (p = 0.018). Pairwise comparisons for all other ovary stages were non-significant ($p > 0.05$).

Table 4.4 Summary table of measurements (mean ± SD) for each development stage assessed in 157 female *C. maenas* collected monthly in 2018.

Ovarian stage	*N*	Mean wet weight (g)	Mean carapace width (mm)	Mean carapace length (mm)	Mean pleon width (mm)	Mean pleon length (mm)
Stage 0	57	18.62 ± 12.7	42.57 ± 10.4	32.48 ± 7.9	16.67 ± 4.7	18.11 ± 4.9
Stage 1	12	20.3 ± 16.7	43.61 ± 11.2	33.16 ± 8.2	16.65 ± 5	18.22 ± 5.5
Stage 2	17	34.14 ± 22.2	52.33 ± 12.2	39.17 ± 8.6	20.63 ± 5.3	22.37 ± 5.5
Stage 3	52	38.02 ± 21.1	54.33 ± 11.1	41.10 ± 8.3	21.79 ± 4.7	23.72 ± 5.4
Stage 4	6	46.2 ± 14.2	60.12 ± 7	45.56 ± 5.9	24.19 ± 3.2	25.71 ± 3.4
Gravid	13	25.97 ± 18.4	47.12 ± 11.8	37.19 ± 8.8	19.37 ± 5.1	20.87 ± 5.5

157 female *C. maenas* (144 non-gravid, 13 gravid) sampled monthly in 2018 were analysed for gonadal size at sexual maturity. Gravid females were considered mature, despite not having gonads analysed histologically, and were included in gonad maturity (L_{50}) analysis. The fitted logistic equation predicted that 50% of females reached gonad maturity (L_{50}) at a size of 51.7 mm CW (R^2 value = 0.17; 95% CI: 46.2–58.5) (Fig. 4.6). The smallest female with immature ovaries measured 19.94 mm, while the largest immature female measured 69.69 mm CW (immature mean CW: 44.64 ± 11.43). The smallest female with mature ovaries measured 23.27 mm, while the largest mature female measured 74.55 mm CW (mature mean CW: 53.5 ± 11.36). The correlation between CW and sexual maturity was weak, which was probably due to females being capable of reaching sexual maturity across all size ranges.

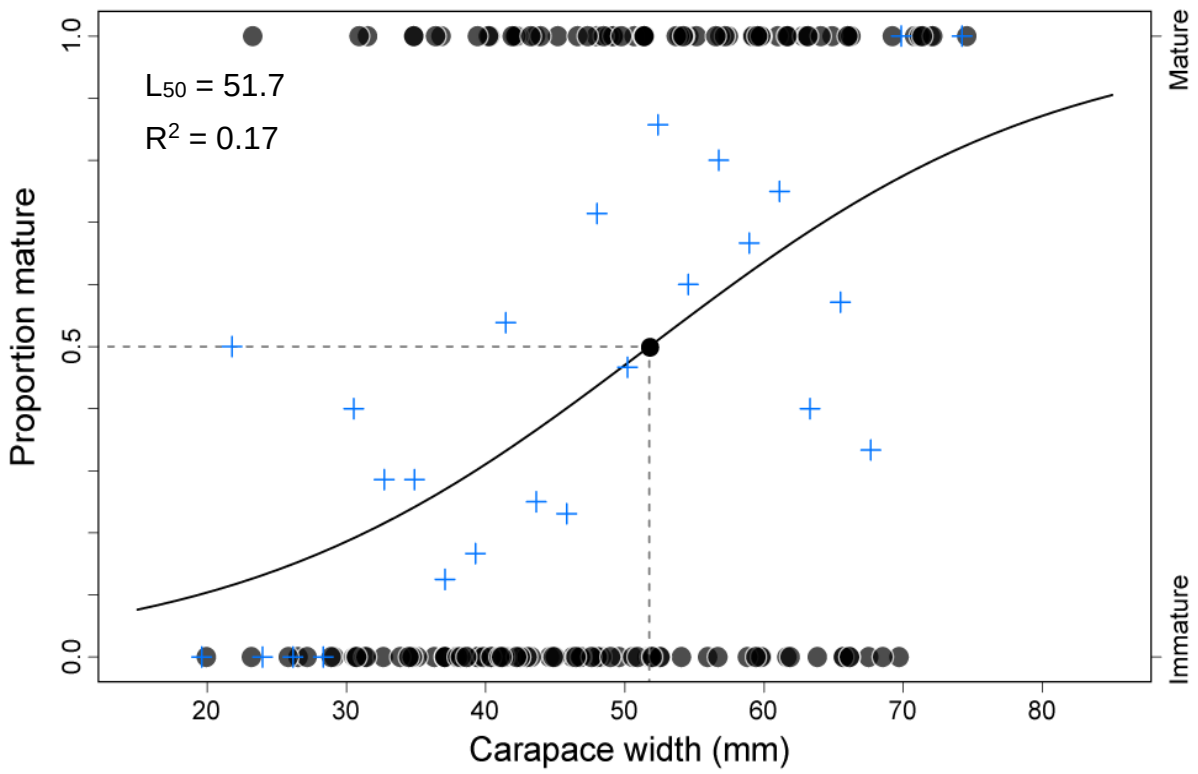

Figure 4.6 The relationship between carapace width (mm) and the proportion of female *C. maenas* reaching gonad maturity (0.0 = immature; 1.0 = mature). 157 females (144 non-gravid, 13 gravid) were assessed monthly in 2018 from Gulf St Vincent, South Australia. The fitted logistic regression is shown by a solid black line, the individual data are shown as black circles, and the proportion that are mature for several categories of the x-axis are shown by blue plus signs. Fifty percent of females reached gonad maturity (L_{50}) at 51.7 mm CW. Gravid females were considered mature and included in the L_{50} analysis. 95% confidence intervals for L_{50} were 46.2–58.5.

Immature and early ovary stages were more common in smaller female *C. maenas*, while mature ovaries and gravid females were more common in larger crabs (Fig. 4.7). Immature ovaries (Stage 0) had highest frequency between 20–60 mm CW, while previtellogenic ovaries (Stage 1) were frequent between 30–40 mm CW. Intermediate ovaries (Stage 2) were most frequent between 50–70 mm CW, while mature ovaries (Stage 3) were highly frequent between 40–80 mm CW. Resorbing ovaries (Stage 4) were found at large size classes between 50–70 mm CW. Gravid females were found across most size classes between 30–80 mm CW, but were more frequent at larger sizes (i.e. 50–60 mm CW). There was a significant association between ovary stage (including gravid) and CW for female *C. maenas* sampled in 2018 ($X^2 = 65.63$, df = 35, $p < 0.005$).

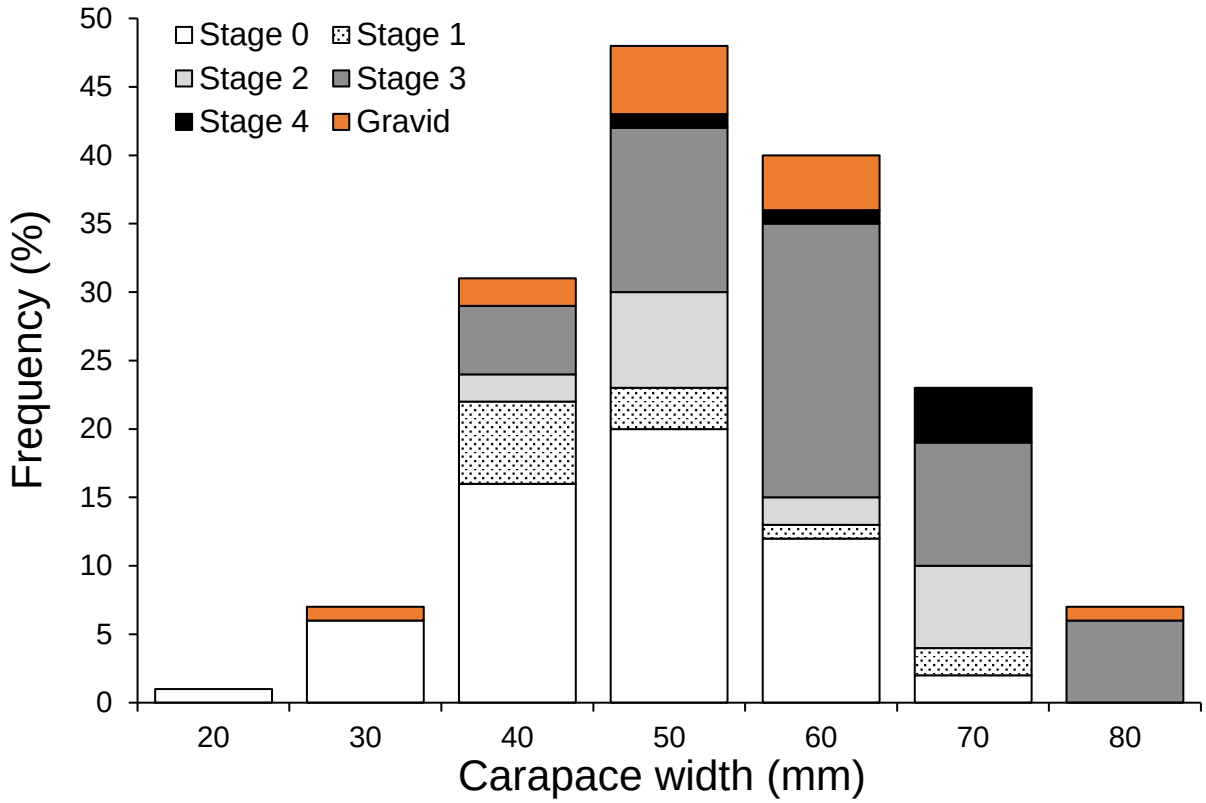

Figure 4.7 Proportion (frequency %) of each development stage in *C. maenas* and grouped according to carapace width (mm). Crabs were collected monthly from March–November 2018 across all sites in Gulf St Vincent, South Australia (total n = 157). Gravid females were considered mature and included in the size-frequency distribution.

4.3.4 Fecundity

40 gravid female crabs were collected between 2012–2018 across all sampling sites in Gulf St Vincent, 17 of which were from 2018. From all gravid females, 23 had uneyed egg clutches, 12 females had eyed egg clutches, and five females had hatched egg clutches. Any clutches that were classified as "hatched" could not be counted and weighed and were removed from fecundity analysis. After data were filtered, 35 gravid females with uneyed (65.7%) and eyed (34.3%) egg clutches were used in the fecundity analyses. The smallest gravid female from the 2012–2018 monitoring period was 23.27 mm, while the largest gravid female was 71.95 mm CW (mean gravid CW: 48.50 mm ± 9.9). Estimated fecundity (F) ranged between 29,048 eggs and 526,521 eggs (mean F: 210,847 ± 110,627). The wet weight of egg clutches ranged between 0.719 g and 11.15 g (mean egg clutch weight: 4.68 g ± 2.39). Linear regression analysis revealed a significant, positive correlation between estimated fecundity and CW for both egg stages combined (Adj. $R^2 = 0.64$, $F = 62.14$, df = 33, $p < 0.001$) (Fig. 4.8A). Similarly, estimated mean fecundity and clutch weight were positively correlated for both egg stages combined (Adj. $R^2 = 0.91$, $F = 350$, df = 33, $p < 0.001$) (Fig. 4.8B).

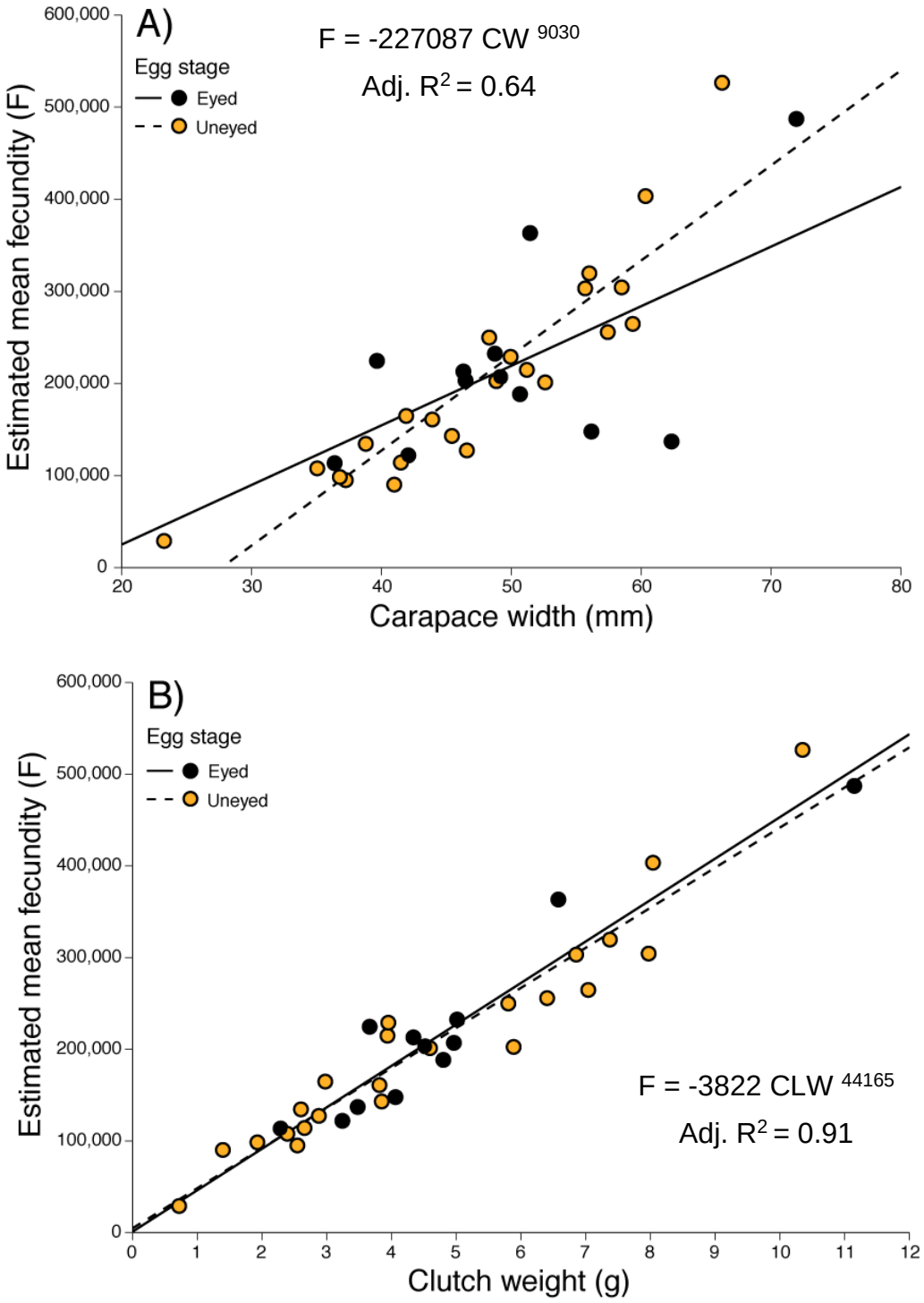

Figure 4.8 Linear regression between A): estimated fecundity and carapace width (mm); and B) estimated fecundity and clutch weight (g) for gravid female *C. maenas* sampled between 2012–2018 (gravid n = 35). Eyed egg stages are represented by solid points and a solid line, while uneyed egg stages are represented by yellow points and a dashed line. Note differences in the x-axis scale. Power regressions are applied to gravid females for both egg stages combined. F = fecundity; CW = carapace width; CLW = clutch weight.

The ANCOVA detected an effect of CW on fecundity (F = 55.22, df = 1, $p < 0.001$) but not for egg stage on fecundity (F = 2.17, df = 1, p = 0.15). There was no significant interaction between CW, egg stage and fecundity (ANCOVA F = 2.47, df = 1, p = 0.12). For clutch weight, the ANCOVA detected an effect of clutch weight on fecundity (F = 229.67, df = 1, $p < 0.001$) but not for egg stage on fecundity (F = 0.017, df = 1, p = 0.89). There was no significant interaction between clutch weight, egg stage and fecundity (ANCOVA F = 0.079, df = 1, p = 0.78). Fecundity increased with CW and clutch weight of gravid females, however egg stage did not have a significant effect on fecundity.

4.3.5 Reproductive period

Over the 2018 monthly sampling period, a seasonal pattern of gonad maturation was evident (Fig. 4.9). The frequency of mature ovaries increased with decreasing sea surface temperature (SST°C) and seasonal change from austral autumn to winter and spring. There was a higher frequency of gravid and resorbing stages in May–August, followed by a secondary, smaller rise in these stages in October–November. The proportion of immature, previtellogenic and intermediate ovary stages decreased during cooler winter/early spring months, before increasing again in late spring. The abundance of females collected in March and September 2018 was low across all sites compared to the other months. There was a significant association between the frequency of gonad stages (including gravid) and sampling month in 2018 (X^2 = 86.15, df = 40, $p < 0.001$).

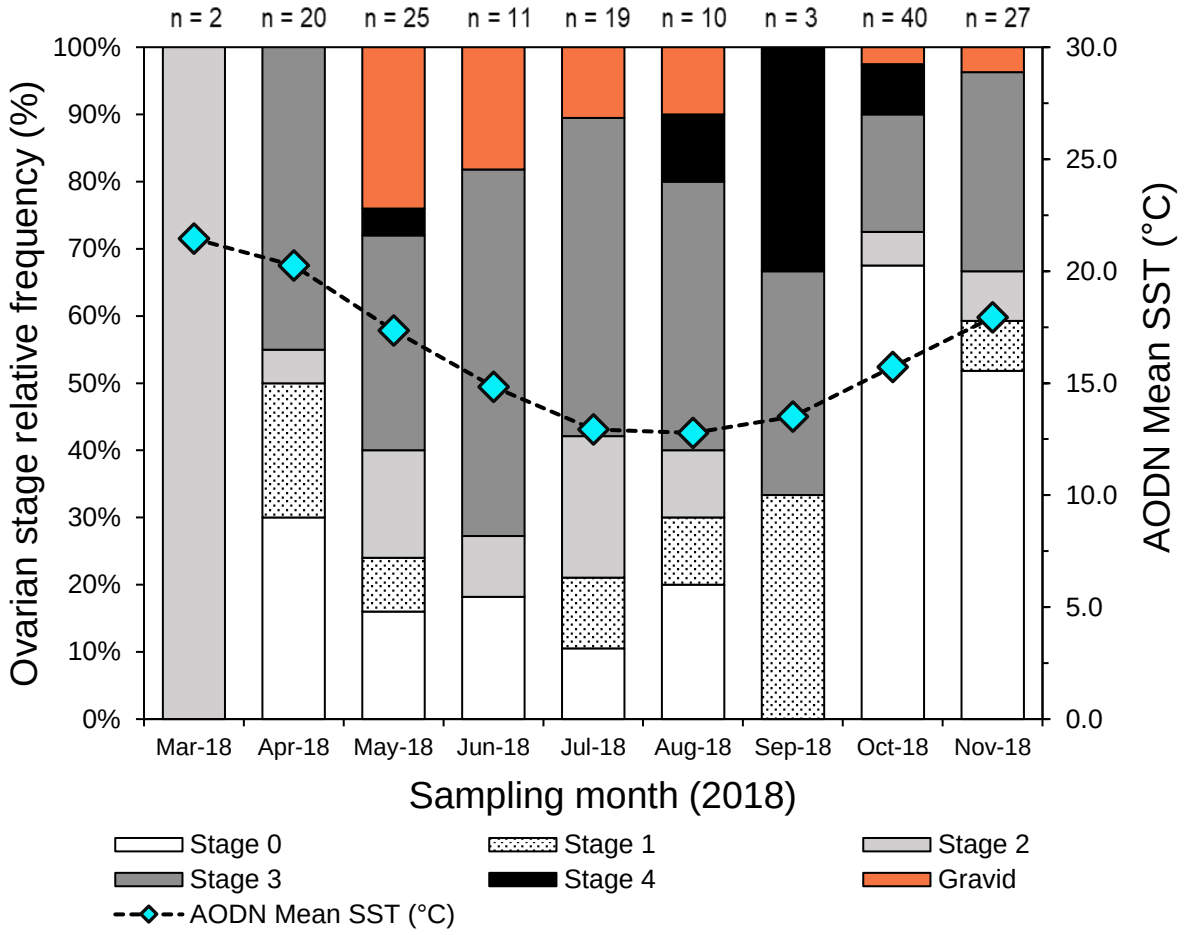

Figure 4.9 Stacked histogram displaying relative frequencies (%) of each ovarian stage in female *C. maenas* (including gravid females). Crabs were collected monthly from March–November 2018 across all sites in Gulf St Vincent, South Australia (total n = 157). The number of females collected per month is displayed at the top of each bar. Monthly mean sea surface temperature (°C) for Gulf St. Vincent in 2018 is shown as blue diamonds. SST data for Gulf St Vincent was obtained from the Australian Ocean Data Network (AODN: https://portal.aodn.org.au/search).

4.4 DISCUSSION

Reproduction is an essential process for the establishment and spread of marine invasive species during colonisation (Blackburn et al. 2011). The reproductive biology of invasive female *Carcinus maenas* in South Australia was investigated in this study through analysis of ovarian development, fecundity, size at sexual maturity and the reproductive period. Larger female *C. maenas* had higher reproductive output than smaller crabs including greater gonad indices, frequency of mature gonads at larger sizes, and higher fecundity at larger sizes. Invasive *C. maenas* in South Australia displayed onset of sexual maturity between 23–50 mm CW which is similar to *C. maenas* in the northern hemisphere (Young and Elliott 2019). *Carcinus maenas* in South Australia displayed a prolonged spawning period; they reproduce from early austral autumn to late spring (approximately nine months). Ovary maturation and egg production increased as sea surface temperatures decreased in Gulf St Vincent in 2018, with gravid females observed at water temperatures below ~18°C. Female *C. maenas* can spawn multiple times throughout their 5–7 year lifespan, and as often as three times per year (Crothers 1967; Crothers 1968; Baeta et al. 2005). Prolonged spawning periods allow females to increase their reproductive output during favourable conditions over consecutive seasons. Reproductive strategies of *C. maenas* can assist with invasion success by increasing offspring survival, establishment, and population stability (Best et al. 2017).

4.4.1 Size at sexual maturity and fecundity

The size at sexual maturity is a crucial factor in determining the reproductive output of crabs and the growth rate of the population (Ramirez Llodra 2002; Waiho et al. 2017). In this study, size at gonadal sexual maturity was 51.7 mm CW, at which 50% of females in the sampled population were mature. This matches records of female *C. maenas* in Ireland, where size of gonadal sexual maturity was 49.96 mm CW (Table 5; Lyons et al. 2012). Onset of sexual maturity varies between and within species, and resource availability and environmental selective pressures can affect sexual maturation (Ramirez Llodra 2002). In some brachyuran crabs, including *C. maenas*, physiological maturity precedes morphological maturity, and gonad staging is therefore important for accurately determining sexual maturation (Lyons et al. 2012). In this study, the smallest female to reach sexual maturity was 23.27 mm CW. Female *C. maenas* in their North American invasive range reach sexual maturity between 22–45.8 mm CW, while in their native range they can reach sexual maturity at ~20 mm CW (Table 4.5; Young and Elliott 2019).

Table 4.5 Size at sexual maturity for female *C. maenas* determined from three maturity classifications: morphometric maturity, physiological gonad maturity, and functional (gravid) maturity. CW_{50} represents the size at which 50% of females were considered sexually mature, CW_{max} and CW_{min} are the maximum and minimum mature size. Results from this current study are highlighted in bold. Table adapted from Waiho et al. (2017).

Maturity	CW_{50} (mm)	CW_{max} (mm)	CW_{min} (mm)	Maturity indicator(s)	N	Location	Year	Native/introduced	Reference
Morphometric maturity	-	41.44	28.66	CW/pleon width	1063	St. Lawrence, Canada	2000 - 2001	Introduced	Audet et a. 2008
Physiological gonad maturity	49.96	59.99	38.6	Gonad stages 3, 4 & 5	74	Southwest Ireland	2006 - 2008	Native	Lyons et al. 2012
	-	-	37	Gonad stages, 3, 4 & 5	152	Newfoundland, Canada	2012	Introduced	Best et al. 2017
	51.7	**74.55**	**30.91**	**Gonad stages 3, 4 & gravid**	**157**	**Gulf St. Vincent, South Australia**	**2018**	**Introduced**	**This study**
Functional maturity (gravid)	-	55	37	Ovigerous		Newfoundland, Canada	2008 - 2010	Introduced	Best et al. 2017
	-	-	38.18	Ovigerous	33	St. Lawrence, Canada	2000 - 2001	Introduced	Audet et al. 2008
	-	64	29	Ovigerous		Mondego Estuary, Portugal	2003 - 2004	Native	Baeta et al. 2005
	-	**71.95**	**23.27**	**Ovigerous**	**40**	**Gulf St. Vincent, South Australia**	**2012 - 2018**	**Introduced**	**This study**

Invasive *C. maenas* in South Australia have high fecundity and produced ~210,000 eggs per clutch. Fecundity was correlated to crab size; females as small as 23 mm CW produced 29,048 eggs, while females of 65–70 mm CW produced 526,521 eggs in a clutch. In their native European range, *C. maenas* of ~46 mm CW produced ~185,000–200,000 eggs (Young and Elliott 2019). In St. Lawrence, Canada, invasive *C. maenas* have an estimated fecundity of 140,000–200,000 eggs, but large females could spawn between 300,000–400,000 eggs (Audet et al. 2008). In New Hampshire, USA, *C. maenas* produced between 4,700–166,000 eggs in a clutch (based on dry weights, 34–49 mm CW) (Griffen 2014). The closely-related *Carcinus aestuarii* has an estimated fecundity between 6,000–129,969 eggs for individuals ranging between 16–40 mm CW (Özbek et al. 2012). The high fecundity of invasive crabs increases the proportion of offspring that will survive in each clutch, and therefore assists with colonisation and range expansion as fecundity drives per capita population growth rates (Griffen 2016; Geburzi and McCarthy 2018). Other invasive crabs also display high fecundity for their size, such as the shore crab, *Hemigrapsus takanoi*, which produces ~50,000 eggs per clutch at 20 mm CW (Gothland et al. 2014). The Asian paddle crab, *Charybdis japonica*, produces 94,000–1,786,000 eggs per clutch at sizes between 46–100 mm CW (average of 255,000 eggs at 60 mm CW; Kolpakov and Kolpakov 2011). In the Chinese mitten crab, *Eriocheir sinensis*, females produced 141,000 – 686,000 eggs per clutch at sizes between 46–80 mm CW (Przemysław and Marcello 2013).

Higher fecundity of *C. maenas* compared to native crab species may reduce competition with native crab recruits as indicated by Garside et al. (2015) for *C. maenas* in southeast Australia. Interspecific competition from native crabs in the recipient community is a potential biotic factor that can reduce establishment and spread of marine invasive species (Geburzi and McCarthy 2018). To compare with native portunid crabs of South Australia, the commercially important blue swimmer crab (*Portunus armatus*) in southern Australia has an estimated fecundity between 463,000–1,781,000 eggs (105–134 mm CW; Kumar et al. 2003; Johnson et al. 2010; Beckmann and Hooper 2016b). The greater fecundity of native *P. armatus* compared to *C. maenas* is expected given their larger size at sexual maturity (~70 mm CW). In the related rough rock crab (*Nectocarcinus integrifrons*) that is native to Gulf St Vincent, fecundity ranges between 6,940–56,430 eggs (55.1–54.15 mm CW), with an average production of 28,252 eggs per clutch (Hackett 2006). *Nectocarcinus integrifrons* are related to *C. maenas* (previously placed in Family Carcinidae, but now tentatively placed in Geryonidae or Ovalipidae) and are similar in size (maximum CW of ~80 mm). They lack distinct paddles on the hind legs and

occupy sheltered reef and seagrass habitats in the subtidal zone (Edgar 2012). Despite these similarities, *C. maenas* in this study produced three to four times as many eggs as similar-sized *N. integrifrons* which may provide *C. maenas* with a competitive advantage (Hackett 2006).

4.4.2 Reproductive period of *C. maenas* in South Australia

The ability to tolerate and reproduce in varying environmental conditions is important for invasive species to establish, especially in dynamic environments such as intertidal habitats (Hänfling et al. 2011). *Carcinus maenas* in South Australia has a prolonged spawning period as females reproduced for nine months of the year spanning multiple seasons. This study assessed only seasonal gonad development for nine months of 2018, however, and thus is not a comprehensive representation of *C. maenas* reproduction over longer temporal scales. When environmental conditions become unfavourable, invasive marine crabs can alter their spawning times by storing sperm or increasing energetic reserves for embryo development (Grosholz and Ruiz 2003; Rey et al. 2017). Reproduction in *C. maenas* is influenced by food availability, salinity, and water temperature, with observations that mating will not occur above 26°C, and that eggs do not develop above 18°C (Young and Elliott 2019). Monitoring of *C. maenas* in Gulf St Vincent between 2012–2017 showed no evidence of ovigerous females during austral summer when water temperatures exceeded ~18°C (Dittmann et al. 2017). While no ovigerous females have been observed in South Australian populations during summer, it is possible that females can mate during this time and store sperm until conditions become favourable for spawning in cooler months. Histological analyses of resorbing ovaries showed primary oocytes were developing after a spawning event; this suggests that individual females are capable of spawning multiple times during this nine-month period and will undergo oogenesis during summer months.

The seasonal reproduction of *C. maenas* varies across its native and invasive range. In Ireland, *C. maenas* have two major spawning events comprising a primary winter cycle and a secondary summer cycle, where oogenesis began as sea temperatures reached 13–14°C (Lyons et al. 2012). In temperate estuaries in Portugal, *C. maenas* spawn year-round in no obvious seasonal pattern (Baeta et al. 2005). In their Canadian range, *C. maenas* have one major spawning event and an annual reproduction cycle that corresponded to water temperature between 6–18°C (Best et al. 2017). The variation in seasonal reproduction for *C. maenas* globally shows that this species can alter reproductive strategies to suit the local environment. In New South Wales, southeastern Australia, Garside et al. (2015) observed that invasive *C.*

maenas juvenile recruitment peaked in later winter and spring, while the recruitment of many native crabs peaked in autumn. The asynchrony in the reproductive cycles of invasive *C. maenas* and native crabs is most likely due to optimum water temperatures, but may provide a secondary benefit for *C. maenas* recruits by reducing interspecific competition with native crab recruits (Garside et al. 2015). Crabs are poikilothermic and will need to adapt to changes in water temperature and seasonal shifts under climate change (Azra et al. 2020). Environmental changes will affect metabolism, development, reproduction and population dynamics of invasive *C. maenas* and recipient communities in South Australia.

4.4.3 Study limitations and implications for management

Immature crabs avoid entering traps if larger mature crabs are present to avoid antagonistic interactions (Kent and McGuinness 2006; Young et al. 2017; Young and Elliott 2019). Baited traps may therefore be biased towards capturing large sexually mature crabs, which may overestimate the proportion of mature crabs in each size class and underestimate the mean size at maturity (Smith et al. 2004; Johnson et al. 2010). While not possible at all sites due to accessibility, habitat complexity and tidal restrictions, using an active method such as timed searches helped to obtain smaller, immature crabs and gravid females which may avoid traps or seek shelter. This has been demonstrated in the Japanese mitten crab, *Eriocheir japonica*, where hand nets caught a wider range of crab sizes than crab pots and were more representative of the crabs' reproductive ecology and demography (Kobayashi and Vazquez-Archdale 2016). The combination of trapping and timed searches at the Onkaparinga site was effective at capturing a wide range of gravid and non-gravid female sizes. The size at sexual maturity is comparable to other studies (Table 4.5), and the methods used in this study are the same as used by Dittmann et al. (2017). Fyke nets or otter trawls may reduce trap selectivity and bias (Duncombe and Therriault 2017; Young et al. 2017) but these methods are difficult to deploy in habitats such as busy shipping channels and mangroves, are subjected to local fishing regulations, and have increased risk of bycatch.

In this study, the number of ovaries staged with macroscopic and histological methods differed for females with immature and resorbing ovaries. Histological methods are used to determine crab reproduction and stock biomass in commercial fisheries and aquaculture (Quinitio et al. 2007; Ravi et al. 2013; Ghazali et al. 2017; Sun et al. 2018; Aaqillah-Amr et al. 2018). Accurate macroscopic identification of tissues and organs can be difficult without examining histological structure. In contrast, histological methods are time consuming and

expensive. Histological validation is important because macroscopic observation of gonads reveals limited information (i.e. size and colour) on reproductive patterns of seasonally reproducing species (Grant and Tyler 1983). Using both methods was a good way of classifying the macroscopic and histological stages of *C. maenas* ovarian development.

Reproductive success is a major factor that determines whether introduced species establish and spread (Geburzi and McCarthy 2018). Control mechanisms should account for a variety of factors, including seasonal reproduction of the invasive species and optimising collection methods prior to spawning events. The high fecundity, long spawning period and early onset of sexual maturity of invasive *C. maenas* highlights the importance of understanding this species' reproductive biology in South Australia. While *C. maenas* is established in Gulf St Vincent, there is potential for range expansion to other coastal areas of South Australia with larval dispersal and propagules transported by shipping. If physical removal of *C. maenas* in southern Australia is attempted during new incursions, it should be done prior to spawning to reduce chances of establishment. Summer water temperatures (December – February) in temperate coastlines of southern Australia are too warm to effectively locate and capture *C. maenas*, therefore trapping and surveying crabs between March and April would be ideal. From May onwards, abundance of gravid and sexually mature females is higher, and therefore any larvae released during this time are likely to settle by mid-winter (~50 days larval duration; Silva et al. 2006).

Carcinus maenas is a globally invasive marine species and can have negative impacts on marine habitats, native species and commercial fisheries. The reproductive biology of *C. maenas* had not been assessed in its southern hemisphere invasive range despite its wide distribution. This study has provided data to understand the frequency or period of spawning events and reproductive potential (i.e. fecundity, size at sexual maturity) of this successful marine invader. Using macroscopic and histological methods, this study showed that invasive *C. maenas* in South Australia are highly fecund (average 210,000 eggs per clutch), have early onset of sexual maturity (23–51 mm CW), and have a prolonged spawning period that spans nine months of the year. High reproductive output and prolonged spawning periodicity of *C. maenas* in South Australia helps identify reproductive strategies that increase chances of establishment or spread.

Chapter 5. Genetic diversity, distinct population structure and demographic history of *Carcinus maenas* in South Australia

ABSTRACT

The distribution and abundance of marine invasive species have increased rapidly worldwide largely due to industrial shipping and the maritime trade. Genetic tools are useful for investigating aspects of marine bioinvasions including population genetic structure, genetic diversity and introduction pathways, which influence ecological and evolutionary success of colonising species. The European shore crab, *Carcinus maenas*, is a highly successful marine invasive species that has global distribution. *Carcinus maenas* is established in South Australia but it is unclear how this population is related to the native and globally invasive ranges of this species so this study was undertaken to determine the genetic diversity, population structure, and demographic history of invasive *C. maenas* in South Australia. A genome complexity-reduction method (DArT-Seq™) generated 3,509 single nucleotide polymorphism (SNP) loci for *C. maenas*. SNP loci and mtDNA COI gene sequences from other studies were used to contrast populations of *C. maenas* that spanned its native and invasive distributions. The South Australian population had genetic diversity that was higher than in the native range. Significant genetic structure revealed differentiation in the South Australia population that is a result of isolation by distance or other invasion processes. Admixture of SNP loci, shared mtDNA haplotypes and demographic histories suggested that South Australian *C. maenas* may have originated from multiple introductions of European and invasive Australian populations. Propagule pressure from multiple sources help invasive species overcome founder effects which contributes to the genetic diversity and genetic structure of South Australian *C. maenas.* Genetic reconstructions of invasion history are a useful tool for identifying sources of this globally invasive species and can assist in management of relevant invasion pathways if *C. maenas* expands its range.

5.1 INTRODUCTION

The distribution, frequency and potential impacts of marine invasive species have increased substantially due to shipping and the maritime trade, habitat modification, fisheries and aquaculture, the aquarium trade, and marine infrastructure (Williams et al. 2013). An estimated ~10,000 different species are transported between vast biogeographic regions at any one time via ship ballast tanks and biofouling communities on vessel surfaces (Bax et al. 2003). Many marine invasive species can go undetected until populations are well-established due to the complexity of the marine environment (Ruiz et al. 2011; Richardson et al. 2016). The 'open' nature of marine environments and unique life-histories of marine taxa, along with globally connected shipping pathways, make marine ecosystems ideal for understanding invasion ecology (Rius et al. 2015). Recent advances of genetic and genomic tools have further improved our understanding of marine bioinvasion processes and assisted with confirming species taxonomy, identifying introduction sources and invasion pathways, and assessing genetic diversity that may assist with the colonisation process (Lawson Handley et al. 2011; Fitzpatrick et al. 2012; Cristescu 2015; Sherman et al. 2016; McCartney et al. 2019; Oleksiak and Rajora 2020).

Marine invasive species are often characterised by high genetic diversity as a result of repeated introductions and propagule pressure which can overcome founder effects (i.e. limited genetic diversity due to small numbers of founding individuals) in colonising populations (Roman and Darling 2007; Rius et al. 2015; Chiesa et al. 2019). The founder effect has been reduced in modern marine bioinvasions compared to historical introductions simply due to sheer increase in maritime shipping and associated vectors repeatedly increasing propagule pressure (Roman 2006). Invasive species with low genetic diversity may be the result of a single-source introduction that lacks sufficient propagule load from diverse sources or hybridisation or due to inbreeding in the founder population (Roman and Darling 2007; Viard et al. 2016). Many marine invasive species display increased genetic diversity over time through multiple introduction events and human-mediated gene flow (Roman and Darling 2007; Viard et al. 2016). Genetic structure research of marine invasive species is useful for deducing gene flow, levels of diversity, inbreeding, connectivity, effective population size, selective processes and demographic events (Geller et al. 2010; Chan and Briski 2017). Understanding these population processes helps identify ecological or evolutionary factors that may be predictors of invasion success. Decapod crustaceans are some of the most successful

marine invasive species due to their life history strategies (i.e. reproduction, dispersal, adaptation), ecological roles and distribution, and are candidate organisms for understanding invasion processes on genetic structure (Stillman et al. 2008; Hänfling et al. 2011).

European shore crabs (*Carcinus maenas*) are native to the northeast Atlantic coastline but have been introduced to every continent apart from Antarctica (Leignel et al. 2014; Young and Elliott 2019). Investigations of *C. maenas* population genetic structure have largely used mitochondrial and nuclear microsatellite markers (Roman and Palumbi 2004; Darling et al. 2008; Tepolt et al. 2009; Domingues et al. 2010; Burden et al. 2014; Mabin 2018). Darling et al. (2008) undertook one of the most comprehensive assessments on the distribution of native and introduced populations of *Carcinus* spp. (*C. maenas* and the sister species *C. aestuarii*). Using the mtDNA COI gene and nuclear microsatellites, Darling et al. (2008) assessed genetic diversity, admixture, and global population genetic structure of *Carcinus*. Population genetic and phylogeographic analysis of *C. maenas* supported observations of multiple, episodic introduction events throughout its global distribution (Carlton and Cohen 2003; Darling et al. 2008). Genetic diversity of invasive *C. maenas* populations varies by region: populations derived from secondary introductions that occurred ~100 years ago (i.e. western North America, Tasmania, Argentina) displayed significantly lower levels of genetic diversity compared to native populations (Roman 2006; Darling et al. 2008). However, populations of *C. maenas* from southeast Australia, South Africa and Nova Scotia have intermediate or higher levels of genetic diversity than in the native range (Darling et al. 2008). Assessments of invasion pathways and genetic structure of *C. maenas* in the southern hemisphere, however, are limited compared to their northern hemisphere range (Young and Elliott 2019).

Published studies on genome-wide discovery and genotyping of single-nucleotide polymorphisms (SNPs) using next-generation sequencing (NGS) technology have increased for invasive species since 2010 (McCartney et al. 2019). SNPs obtained from restriction-site associated DNA sequencing (RAD-seq) can dramatically increase the power to detect hybridisation, fine-scale spatial structure and loci under selection (Davey and Blaxter 2011). This has been observed in invasive *C. maenas* in Nova Scotia and New Brunswick using the mtDNA COI gene and RAD-seq SNPs. While the COI gene identified population structure and isolation by distance equally to SNPs, SNPs detected finer genetic differences between northern and southern groups in *C. maenas* (Jeffery et al. 2017a). The use of NGS methods for *C. maenas* is largely limited to RAD-seq and transcriptome analysis of native populations in

Europe and invasive populations in North America (Tepolt and Palumbi 2015; Jeffery et al. 2017a; Jeffery et al. 2017b; Jeffery et al. 2018; Lehnert et al. 2018; Tepolt and Palumbi 2020). Outside of these biogeographic regions, population genetic structure of *C. maenas* is limited to a few studies (Burden et al. 2014; Mabin 2018) and assessments of *C. maenas* genetic structure in the southern hemisphere using SNPs have not yet been undertaken (Young and Elliott 2019). It is important to note that different molecular markers have different properties (see Chapter 1) and can show contrasting results (Kirk and Freeland 2011; Oleksiak and Rajora 2020). Using a combination of molecular markers, such as mitochondrial and nuclear markers, will help reduce disparities when comparing results to other genetic studies on *C. maenas* (see Kirk and Freeland 2011).

In southeast Australia, *C. maenas* is invasive in four States: Victoria (VIC), New South Wales (NSW), Tasmania (TAS) and South Australia (SA). *Carcinus maenas* was first introduced into Port Phillip Bay, VIC, in the late 1800s via the dry ballast of European wooden vessels (Thresher et al. 2003). After establishment, the crabs spread widely throughout Port Phillip Bay and along the southeast coasts of Victoria. The crab was first reported in southern New South Wales in 1971, in South Australia in 1976, and in Tasmania in 1993 (Thresher et al. 2003; Ahyong et al. 2005). Australian populations of *C. maenas* exhibit marked genetic differences compared to the European native range, which likely occurred due to founder effects, genetic drift and isolation from Europe over ~120 years (Darling et al. 2008). Secondary introduction of *C. maenas* from mainland Australia (VIC and NSW) into Tasmania probably occurred through human-mediated shipping between States (Carlton and Cohen 2003; Darling et al. 2008). Oceanographic currents between South Australia and southeast Australia form a biogeographic barrier which prevents larval dispersal, while changes in sea surface temperature, habitat type and biological interactions contribute to geographic isolation of South Australian *C. maenas* (Thresher et al. 2003; Aguilar et al. 2019). International shipping ports along the southeast Australian coastline receive regular arrivals from Asia, North America and Europe (Burden et al. 2014), enabling multiple introductions and admixture of *C. maenas* populations which markedly decreased after ballast water controls were implemented in 2001. However, Carlton and Cohen (2003), Darling et al. (2008) and Burden et al. (2014) did not assess the South Australian population of *C. maenas* and invasion dynamics and structure of this population are unknown.

The history of European colonisation is important for understanding invasion pathways and genetic structure of *C. maenas* in South Australia. The South Australian population may have arisen from source populations in southeast Australia and/or introductions from other native or invasive ranges. The South Australian population is geographically isolated from the southeast Australian populations which may affect admixture rates and alter genetic differentiation of South Australian *C. maenas*. This study aimed to characterise the genetic structure and infer the likely introduction history of invasive South Australian *C. maenas*. This will be achieved by using SNPs and the mtDNA COI gene to compare with native and globally invasive *C. maenas* populations. The specific questions addressed were: 1) do South Australian populations of invasive *C. maenas* have similar genetic structure and diversity to invasive or native populations?; 2) do SNP markers reveal similar patterns in global genetic structure of *C. maenas* as studies that used other genetic markers?; and 3) what are the most likely demographic scenarios for the introduction history of *C. maenas* into South Australia? Genetic comparisons will help identify genetic diversity, population differentiation and potential invasion pathways of *C. maenas* into South Australia for the first time.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection and genomic DNA extraction

Specimens of *Carcinus maenas* were obtained from various regions across their native and invasive global distribution. South Australian *C. maenas* samples used in Chapter 2 and Chapter 3 were also used for SNP and mtDNA COI analyses. South Australian specimens were collected using either baited opera house traps or timed searches, and DNA was extracted from frozen (-20°C) pereopod muscle tissue as detailed in Chapter 2 and Chapter 3. All *C. maenas* specimens obtained from outside South Australia were collected and preserved from research collaborators, where combinations of hand collection, trapping or already-preserved samples were used. Interstate samples were obtained from the Australian States of New South Wales, Victoria and Tasmania, and internationally from Sweden, Portugal, South Africa, and the east and west coasts of North America (Nova Scotia, Maine, Washington and British Columbia) (Table 5.1; Fig. 5.1). Samples represented a wide distribution of *C. maenas*' known range except the native range in Iceland and the invasive ranges in Argentina and Japan as my attempts to obtain samples from these regions were unsuccessful. Samples were sourced from the most likely sources of historic and current shipping pathways to South Australia (i.e. southeast Australia and Europe, especially the United Kingdom and northern Europe) (State

Library of South Australia 2020: https://guides.slsa.sa.gov.au/immigration). Interstate and international samples were preserved in vials of 70% EtOH to meet Australian aviation requirements (Special Provision A180) and Biosecurity Import Conditions. Each sample consisted of 1–2 preserved pereopods removed from each crab (shell cracked open where possible to allow ethanol penetration) or pereopod muscle tissue only. All imported samples were transferred into fresh vials of 70% EtOH once received and kept refrigerated until DNA extraction.

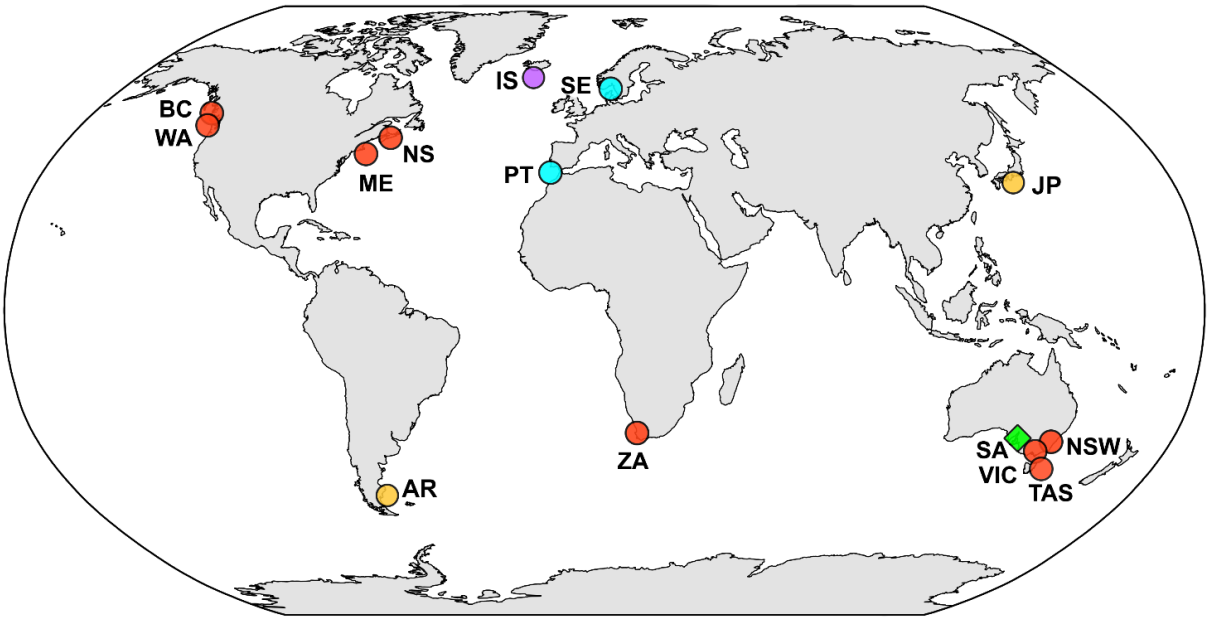

Figure 5.1 Sampling localities of *C. maenas* analysed in this study. Blue circles indicate sampled native range, red circles indicate sampled invasive range, and the green diamond indicates the sampled invasive range in South Australia. The purple circle represents the unsampled native range (IS = Iceland) and yellow circles represent the unsampled invasive range (AR = Argentina; JP = Japan). Corresponding region codes, sample numbers, collection methods and GPS coordinates of samples used in this study are in Table 5.1.

Following the manufacturer's protocols, total genomic DNA was extracted from frozen and ethanol-preserved muscle tissue using the Gentra Puregene Tissue Kit (Gentra Systems Inc., Minneapolis, USA) after overnight proteinase K digestion (20 mg/ml) at 55°C. Purified DNA solutions were resuspended in 30-50 µl TE buffer and stored at 4°C. DNA concentration of all purified samples were checked with a Quantus Fluorometer (Promega Corporation, Wisconsin, USA) following the manufacturer's protocol. Samples with high DNA concentrations (i.e. ~100 ng/µl) were diluted with TE buffer to between ~10 and 50 ng/µl. Samples that had low gDNA concentration (≤ ~5 ng/µl) due to tissue degradation or poor preservation were excluded from further analyses. A 'mock incubation' test was performed on

a subset of 38 samples haphazardly selected from all geographic locations. The mock incubation protocol helps to detect presence of buffer-activated nucleases (usually Mg dependent) with no restriction enzymes used. The mock test was performed by incubating 1 µl of gDNA per sample in Restriction Enzyme buffer at 37°C for one hour, then resolving samples on a 0.8% TAE agarose gel. Good quality gDNA showed a high molecular weight band on the gel, which was apparent for nearly all tested *C. maenas* samples. For each sample, 20 µl of aqueous gDNA solution was loaded into fully skirted 96-well PCR plates, with two empty wells in each plate acting as controls. Duplicate gDNA samples were also included within and between plates as an internal quality control check. A total of 188 *C. maenas* gDNA samples, including 17 duplicate samples, were submitted to Diversity Arrays Technology Pty. Ltd. (DArT P/L: Canberra, Australia) for DArT-Seq^{TM} genotyping.

Table 5.1 Sample details of *C. maenas* obtained from native and invasive geographic locations. *N* is the number of samples obtained. Bold text highlights samples from South Australia. For detailed sample information of each individual see Appendix Table A5.1.

Code	*N*	Collection method(s)	Preservation	Collection locality	Geographic region	GPS coordinates	Status
SE	11	Hand collection	70% EtOH	Kristineberg, Fiskebäckskil, Sweden	Europe, North Sea	58.252437 11.465743	Native
PT	12	Hand collection	70% EtOH	Esteiro das Charradas, Faro, Portugal	Portugal, Atlantic northeast	37.00900 -7.989200	Native
ZA	11	Baited traps	Frozen, then 70% EtOH	Table Bay Harbour, South Africa	South Africa, South Atlantic	-33.920555 18.4425000	Invasive
NS	10	Unspecified	70% EtOH	St Catherines River, Nova Scotia, CAN	East Canada, Atlantic northwest	43.841564 -64.835988	Invasive
ME	10	Unspecified	70% EtOH	Biddeford Pool, Maine, USA	East USA, Atlantic northwest	43.442017 -70.341453	Invasive
WA	15	Fukui/Minnow traps, Hand collection	70% EtOH	Puget Sound, Washington, USA	West USA, Pacific northeast	48.121126 -122.66170	Invasive
BC	30	Unspecified	70% EtOH	Vancouver Island, British Columbia, CAN	West Canada, Pacific northeast	48.38823 -123.6338	Invasive
NSW	15	Within oyster baskets	70% EtOH	Wagonga Inlet, New South Wales	Southeast Australia	-36.218273 150.110624	Invasive
VIC	2	Hand collection	70% EtOH	Port Phillip Bay, Victoria	Southeast Australia	-37.860548 144.864450	Invasive
TAS	7	Hand collection	95% EtOH, then 70% EtOH	Beauty Bay & Cornelian Bay, Tasmania	Southeast Australia	-42.857267 147.324281	Invasive
SA	**66**	**Baited traps, hand collection**	**Frozen (-20 °C)**	**Gulf St. Vincent, South Australia**	**Southern Australia**	**-34.812701 138.512220**	**Invasive**

5.2.2 DArT-Seq™ 1.0 genotyping

Library preparation

Genotyping of *C. maenas* was achieved with DArT-Seq™ 1.0 proprietary next-generation sequencing technology (Kilian et al. 2012). DArT-Seq™ represents a combination of DArT complexity reduction methods and recent application of this concept on NGS platforms (Sansaloni et al. 2011; Kilian et al. 2012). DArT-Seq™ is a restriction enzyme-based genome complexity reduction method that tests for polymorphism in tens of thousands of genomic loci and is optimised for each organism. The *PstI* and *HpaII* method was used for *C. maenas*. DNA samples were processed in digestion and ligation reactions similarly to Kilian et al. (2012), but a single *PstI*-compatible adapter was replaced with two different adapters corresponding to two different restriction enzyme overhangs. The Illumina flowcell attachment sequence was included with the *PstI* adapter, while the reverse adapter contained the flowcell attachment region and *HpaII* overhang sequence. Mixed fragments of *PstI-HpaII* were amplified in 30 rounds of PCR using the following reaction conditions: initial denaturation for 1 min (94°C), followed by 30 cycles of denaturation for 20 sec (94°C), annealing for 30 sec (58°C), extension for 45 sec (72°C), and a final extension for 7 min (72°C). Equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to a c-Bot (Illumina, San Diego, USA) bridge PCR, followed by sequencing on a single lane of an Illumina HiSeq2500. The sequencing was run for 77 cycles.

SNP calling and quality control

Sequences generated from each lane were processed with in-house proprietary DArT analytical pipelines. In the primary pipeline, FASTQ files were processed to filter out poor quality sequences, with more stringent selection criteria applied to the barcode region compared to the rest of the sequence. This ensured that specific sequences carried in the 'barcode split' step were reliable. Filtering was performed on the raw sequences using the following parameters in the software PHRED: minimum pass score of 30 and 75% for the barcode region, and minimum pass score of 10 and 50% for the whole read. Approximately 1,410,000 sequences per sample were identified and used in marker calling. Identical sequences were collapsed into FastQC files, which were 'groomed' using DArT `P/L's proprietary algorithm. Grooming corrects a low-quality base from singleton tags into a correct base using collapsed tags with multiple membership as the template.

The resulting FastQC files were used in a secondary pipeline for proprietary DArT SNP and SilicoDArT calling algorithms 'DArTSoft14.' Selection criteria added to the algorithm consisted of analysing ~1,000 controlled cross-populations. Mendelian distribution of alleles in these populations was also tested. Multiple samples were processed from DNA as technical replicates with high quality, low error rate markers used as main selection criteria. The call quality was assured by high average read depth per locus (average across all markers was 20 reads/locus). Only two *C. maenas* samples failed the DArT sequencing pipeline, however the duplicate of one of these samples was sequenced successfully and included in downstream analyses. This resulted in 186 individuals (including 16 duplicates) in the final DArT dataset. No loci were called in the controls.

Duplicate samples were used to identify differences in the number of loci called within and between plates. Of the 17 duplicates received by DArT, 16 duplicate samples were assessed (eight samples within plates and eight samples between plates) as one duplicate pair failed the sequencing pipeline and was removed. The total number and percentage of missing loci between each pair of duplicates was compared, with the sample retaining the highest number of loci kept for data analyses. Average between-run reproducibility was 94.2% (total missing loci between duplicates between plates = 18,274 loci ± 3,088) and was calculated when there was a call for the same locus in duplicates on different plates. Average within-run reproducibility was higher at 97.45% (total missing loci between duplicates within plates = 9,884 loci ± 1,223) and was calculated when there was a call for the same locus in duplicates on the same plate. After duplicates were removed, the resulting DArT dataset consisted of 170 individuals. All samples were organised into nine pre-defined geographic populations designated by sampling locality at large spatial scales (i.e. State/country-wide level). Samples from Victoria, New South Wales and Tasmania were pooled into a "southeast Australia" geographic population for convenience.

Secondary SNP filtering

Secondary filtering of SNP data obtained from DArT P/L was performed in R version 3.6.3 (R Core Team 2020; www.r-project.org) and RStudio version 1.2.5042 (RStudio Team 2020) using the package '*DartR*' version 1.3.5 (Gruber et al. 2018). This package was developed specifically so that DArT SNP and SilicoDArT data generated by DArT P/L can be loaded into R effectively for downstream analyses. Filtering SNP datasets is important to detect, minimise and/or remove errors that may have occurred due to laboratory work, library

preparation or bioinformatic processing (O'Leary et al. 2018). Inadequate filtering protocols can create artefacts that may be incorrectly interpreted as indicators of population structure or loci under selection (O'Leary et al. 2018). Filtering steps by locus and individual were done across the whole dataset following dartR recommendations. After each step, the changes in loci within populations and for the whole dataset were visualised to check the number of loci retained. Hardy-Weinberg Equilibrium (HWE), linkage disequilibrium and relatedness, however, were filtered by population to prevent population-specific bias from affecting population structure. The resulting 'unfiltered' DArT dataset consisted of 170 individuals, nine populations and 28,850 binary SNPs. *Carcinus maenas* SNPs were filtered in *DartR* using sequential steps shown in Table 5.2. Loci retention was checked after each filtering step both within populations and across the whole dataset.

Table 5.2 Filtering steps applied to the DArT-Seq™ dataset of *C. maenas*, showing the number of loci, number of individuals and % missing data remaining after each filtering step. Stringent filtering was performed in the R package '*DartR*' version 1.3.5 (Gruber et al. 2018). Filtering steps were done across the whole dataset unless otherwise specified (i.e. within population filtering).

Filtering step applied for *C. maenas* SNP data	*N* SNP loci	*N* individuals	Missing data %
DArT-Seq™ dataset received from DArT P/L	28,850	170	17.99
Remove loci that represent secondary SNPs	20,852	170	17.56
Remove loci that were not represented in all technical replicates	16,610	170	18.33
Remove all monomorphic loci	16,557	170	18.32
Remove by loci call rate <95%	6,660	170	2.45
Remove by individual call rate <95%	6,597	157	0.49
Remove by minor allele frequency (MAF) <1%	3,954	157	0.47
Remove by HWE deviation (Bonferroni-corrected $p < 0.05$) within each population	3,954	157	0.47
Remove by pairwise Hamming distance <10%	3,876	157	0.47
Remove by min. read depth < 5X and max. read depth of 80X	3,841	157	0.47
Remove by linkage disequilibrium ($r^2 > 0.2$, $p < 0.05$) within each population	3,512	157	0.47
Remove loci by individual relatedness within each population ($r > 0.5$)	3,512	156	0.47
Remove loci under putative selection (FDR < 10%)	3,509	156	0.47

Hardy-Weinberg Equilibrium, linkage disequilibrium, relatedness and outlier loci

As shown in Table 5.2, deviation from Hardy-Weinberg Equilibrium (HWE) within each of the nine geographic populations was tested in *DartR.* All pairwise HWE comparisons had Bonferroni-correction applied at the 5% level ($\alpha = 0.05$) to correct for multiple tests as HWE is tested across multiple genomic loci (Rice 1989; Sethuraman et al. 2019). Since no loci deviated from HWE in any population, linkage disequilibrium (LD) was tested to detect the non-independence of alleles at different loci. The SNP dataset was separated for each geographic population and imported into the software PLINK version 1.9 (Chang et al. 2015; www.cog-genomics.org/plink/1.9/). LD was calculated as the squared pairwise correlation coefficient (r^2) with a significance value of $\alpha = 0.05$. All loci that were linked (i.e. LD threshold higher than $r^2 > 0.2$, $p < 0.05$) were removed to reduce LD bias before recombing the dataset in R for further filtering.

High levels of relatedness and inbreeding can alter population structure and so relatedness between individuals of *C. maenas* was estimated in the software COANCESTRY version 1.0.1.9 (Wang 2011). Relatedness coefficients using sequence data is computed by obtaining allele frequencies in the population from which two individuals are sampled, comparing genotypes of the two individuals, and then constructing a relatedness estimate from these comparisons (Ackerman et al. 2017). Factoring in varying sample sizes of each geographic population, the relatedness estimates were first calculated for the whole dataset with all populations combined, then for each population separately (Wang 2017). Five relatedness estimators were used for each *C. maenas* geographic population: Queller and Goodnight (1989), Li et al. (1993), Ritland (1996), Lynch and Ritland (1999) and Wang (2002). As no reference individuals were defined, the Dyadic and Triadic estimators were not used in the current study. All relatedness estimates were calculated with 95% confidence intervals and 10,000 bootstrap permutations. Following Domingues et al. (2011), relatedness of *C. maenas* was defined as the following: unrelated individuals $r = 0.00$; half-sibs $r = 0.25$; full-sibs, parent-offspring $r = 0.50$; monozygotic twins, duplicated samples $r = \sim 1$. Pairwise relatedness estimates > 0.50 were considered highly related; individuals were removed from the dataset if any one of the estimators exceeded > 0.50 in a dyad.

Loci under putative selection can bias population genetic structure and were identified using F_{ST} outlier analyses in BAYESCAN version 2.1 (Foll and Gaggiotti 2008) and the R package '*OutFLANK*' version 0.2 (Whitlock and Lotterhos 2015). BAYESCAN implements

Bayesian methods to assess differences in allele frequencies that can identify loci under selection. The current SNP dataset was analysed in BAYESCAN using the following default parameters: a sample size of 5,000 and a thinning interval of 10, 20 pilot runs (5,000 pilot run length), and an additional burn-in of 50,000 (100,000 total iterations). The neutral model had 10 prior odds and a false discovery rate (FDR) of 10%. Results of BAYESCAN were exported into R for exploration and plotting. The same dataset was independently analysed in *OutFLANK*. Extreme F_{ST} values were removed to estimate the null F_{ST} distribution by selecting a left trim faction of 0.14 and a right trim fraction of 0.69. I defined F_{ST} outlier loci as having a q-value threshold < 0.1 (FDR < 10%). The threshold for minimum expected heterozygosity was < 0.1, where loci below this threshold were removed. Outlier loci detected with either method were removed from the SNP dataset; these loci may be explored in future research to investigate selection. The final filtered dataset therefore had no loci deviating from HWE, no loci in LD, no highly related pairs of individuals, and no putative loci under selection from both the BAYESCAN and *OutFLANK* method combined.

5.2.3 Data analysis

Summary statistics and genetic diversity

Summary statistics and genetic diversity estimates were calculated on the filtered, putatively neutral SNP dataset globally and for each *C. maenas* geographic population. Mean observed heterozygosity (H_O) and mean unbiased expected heterozygosity (uH_E; also known as Nei's gene diversity, *D*) was calculated overall and for each population using the R package '*hierfstat*' version 0.04–22 (Goudet 2004). Unbiased expected heterozygosity can be applied to any sample size, including a small number of individuals, if many loci are used (Nei 1978). Using unbiased heterozygosity results in larger populations having smaller differences between biased and unbiased heterozygosity values (Pagnotta 2018). The inbreeding coefficient (F_{IS}) was estimated for each population using the function 'divBasic' in the R package '*diveRsity*' version 1.9.9 (Keenan et al. 2013). Bias-corrected confidence intervals (95%) were generated for population-specific F_{IS} estimates with 1,000 bootstrap iterations. The total number of alleles observed (N_A) and the percentage of missing SNP data for each population and overall were calculated using the R package '*adegenet*' version 2.1.3 (Jombart 2008; Jombart and Collins 2015).

Mean allelic richness (A_R) was calculated across all loci in each population using the R package '*PopGenReport*' version 3.0.4 (Adamack and Gruber 2014). Allelic richness is a

simple measure of genetic diversity, which is defined as the average number of alleles per locus in a population (Kalinowski 2004). Allelic richness was calculated using the rarefaction method, which accounts for variation in sample size across populations (even when alleles and private alleles may be absent in some populations) and 'rarefacts' these alleles over populations and samples (Kalinowski 2004; Foulley and Ollivier 2006; Szpiech et al. 2008). The number of unique private alleles in each population (N_P) were counted once, regardless of dosage, using the R package '*poppr*' version 2.8.5 (Kamvar et al. 2014).

The SNP dataset was converted using PGDSPIDER version 2.1.1.5 (Lischer and Excoffier 2012) into other data formats for further analyses. To explore divergence between geographic populations, pairwise F_{ST} values between pairs of each population were generated in the program ARLEQUIN version 3.5.2.2 (Excoffier and Lischer 2010). Significance of pairwise F_{ST} values was calculated with 10,000 permutations and samples were grouped by the nine pre-defined populations. A hierarchical analysis of molecular variance (AMOVA) was calculated with 10,000 permutations in ARLEQUIN to assess genetic variation at different levels of sampling (i.e. within populations, among populations within groups, and among groups). Separate AMOVA analyses were run with different combinations of population groupings for genetic cluster observations.

Genetic clusters and population structure

Isolation-By-Distance (IBD) using the Mantel test between F_{ST} genetic distance and geographic distance matrices for nine geographic populations was calculated with the R package *DartR*. The null hypothesis of the Mantel test assumes no relationship between genetic and geographic distance. GPS coordinates for each population were expressed in latitude-longitude decimal degrees under Google Earth Mercator projection. The IBD analysis is carried out using log (Euclidean distance) of geographic coordinates in metres against between-population pairwise $F_{ST}/1\text{-}F_{ST}$. The Mantel test for IBD was run with 999 permutations under the Pearson's product-moment correlation. All statistical analyses had a significance value of $\alpha = 0.05$.

Identification of genetic clusters and population structure was first explored using principal coordinates analysis (PCoA) and discriminant analysis of principal components (DAPC) on the filtered dataset. The PCoA was used to visualise genetically similar groups of *C. maenas* for nine pre-defined geographic populations and was carried out using the "gl.pcoa"

function in *DartR*. The PCoA uses SNP data for individuals in each population and runs a Gower ordination with Euclidean distance. Individuals classified by population were visualised on a PCoA bivariate plot with ellipses representing 95% confidence intervals, while the proportion of variation was explained by the two axes.

The DAPC required prior groups to be defined, which were identified using the *K*-means clustering algorithm implemented in *adegenet*. The algorithm first transforms the data using principal components analysis (PCA), and then runs a discriminant analysis (DA) depending on the number of principal components retained (Jombart et al. 2010). The function "find.clusters" was used to identify *K* in *adegenet*, which is the number of genetically distinct groups present within the dataset with prior group membership defined by population locality (Jombart and Collins 2015). The most likely *K* values were determined through a Bayesian information criterion (BIC) score that was generated for each sequential *K* scenario. The lowest BIC score was selected as this corresponds to the optimal number of clusters (Jombart and Collins 2015). Five principal components (PCs) and two discriminant functions were retained for the DAPC, as these were the optimum amount required to explain the variation in the dataset without sacrificing information (Jombart and Collins 2015). The DAPC results were visualised using *adegenet* by constructing a scatterplot of the optimum *K*-means clusters with 95% inertia ellipses and eigenvalues, and an individual density plot of the clusters of the first discriminant function.

As PCoA and DAPC can assign individuals to populations but cannot detect admixture, it is important to use multiple methods to address population structure (Jombart et al. 2009; Ibrahim et al. 2020). After assessing genetic clusters with PCoA and DAPC, the Bayesian clustering software STRUCTURE version 2.3.4 (Pritchard et al. 2000) was used to further investigate population genetic structure and admixture. STRUCTURE characterises population clusters using multilocus allele frequencies and assigns all individuals to genetic clusters even without prior spatial population information (Pritchard et al. 2000). The filtered dataset was analysed in STRUCTURE and tested for a range of *K* between 1 to 10. Five independent replicates were run for each value of *K*. Each model had a burn-in period of 50,000 iterations followed by 100,000 Markov Chain Monte Carlo (MCMC) repetitions. All STRUCTURE simulations were run using the admixture model of ancestry with correlated allele frequencies across populations.

The STRUCTURE analysis for the whole *C. maenas* dataset was run independently twice; the first analysis was run without prior location information included, while the second analysis included the prior location information (Locprior model). The Locprior model uses sampling locations (i.e. geographic populations) as prior information to assist clustering in cases where the signal of structure is relatively weak (Pritchard et al. 2000). Results from the Locprior model showed no differences in population structure and was excluded from the current study. To decrease computing time, all STRUCTURE analyses were run in parallel by deploying '*StrAuto*' version 1.0 (Chhatre and Emerson 2018) on a local high-performance computing cluster.

STRUCTURE results were imported into STRUCTURE SELECTOR (Li and Liu 2018), a web-based software used to detect and visualise the optimal numbers of clusters (*K*) using multiple methods. This software is similar to the commonly used STRUCTURE HARVESTER software (Earl and vonHoldt 2012), which uses post-hoc analyses such as the Evanno ΔK (DeltaK) method (Evanno et al. 2005) and the log probability method ln Pr($X \mid K$) to identify the optimal *K* (Pritchard et al. 2000). However, issues can arise when using these methods, such as the inability of ΔK to determine $K = 1$ or to recognise weak population structure, difficulty in interpreting *K* when uneven sampling has occurred across populations, or difficulty interpreting *K* when hierarchical population structure is evident (i.e. subpopulations within populations) (Gilbert 2016; Janes et al. 2017). STRUCTURE SELECTOR uses both methods, but also uses new estimators (MedMedK, MedMeanK, MaxMedK, and MaxMeanK) to help select the optimal *K* for the mean and median of the individual membership coefficients. I used MedMedK and MedMeanK for my dataset with a default threshold of 0.5. Once the optimal *K* was selected, results were imported into CLUMPAK (Kopelman et al. 2015), a web-based software used to merge runs of each *K* and build the final admixture barplots.

Hierarchical population structure was analysed as results from STRUCTURE SELECTOR and CLUMPAK suggested that subpopulations may be evident based on the identified clusters. Putative subpopulations were formed using the proportion of individual membership assigned to each cluster for the optimum *K*. Subpopulations were converted into separate datasets using the *DartR* package in R, and any monomorphic loci were removed from each dataset. The STRUCTURE analyses were run independently on each subpopulation dataset with the same parameters as above, except the range of *K* tested varied to match the

number of populations in each dataset. STRUCTURE results for each subpopulation were then imported into STRUCTURE SELECTOR and CLUMPAK as above for hierarchical results.

Mitochondrial DNA analysis

Since samples of *C. maenas* could not be obtained from geographic regions in Iceland, Argentina, and Japan, the global distribution of *C. maenas* was explored further using the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. COI gene sequences were also used to expand on population structure in Victoria and New South Wales as samples sizes using SNP loci were not adequate in these populations. The 59 South Australian *C. maenas* COI gene sequences that were extracted and analysed for Chapter 2 and Chapter 3 were included in this study (GenBank Accession Numbers MT748791 – MT748849). Haplotype sequences representing the global native and invasive range of *C. maenas* was obtained from Darling et al. (2008) (n crabs sequenced = 653; GenBank Accession Numbers FJ159008 – FJ159085) and Burden et al. (2014) (n crabs sequenced = 57; GenBank Accession Numbers KF709201 – KF709206). No *Carcinus aestuarii* or *Carcinus* hybrid sequences were included.

COI gene sequences were imported into the software package Geneious Prime version 2020.1.1 (Biomatters Ltd, Auckland, New Zealand). Sequences were checked for ambiguities by eye, aligned and then trimmed to the shortest sequence length, resulting in a 367 bp gap-less and unambiguously aligned sequence. An unrooted neighbour-joining tree of all sequences was constructed using the Tamura-Nei Model in Geneious. The NJ tree and aligned sequences were imported into Haplotype Viewer (Ewing 2010) to generate an unrooted mtDNA haplotype network which was colour-coded by common haplotype assignments across geographic regions.

To determine haplotype frequencies, the mtDNA sequence alignment in FASTA format was uploaded to the online converter DNAcollapser (http://users-birc.au.dk/palle/php/fabox/dnacollapser.php). Resulting haplotype frequencies and groupings are shown in Appendix Table A5.7. The number of individuals sampled per haplotype was obtained from Darling et al. (2008) and Burden et al. (2014) and grouped into geographic regions following Darling et al. (2008): Off-shelf Europe (Iceland and Faroe Islands); northern Europe (east of the UK to Norway); western Europe (west of the UK to Portugal); Nova Scotia; eastern North America; western North America; Japan; Argentina; South Africa; mainland Australia (Victoria and New South Wales); Tasmania; and South Australia. Haplotype

frequencies across geographic regions were visualised as pie charts. Unique haplotypes (also referred to as private haplotypes) were defined as two or more individuals having the same haplotype restricted to one geographic population. Singleton haplotypes (i.e. only one haplotype from one individual observed in any population) were removed from the pie chart visualisations. Haplotype frequency values for each geographic region are displayed in Appendix Table A5.8.

The number of unique haplotypes, number of polymorphic and parsimony-informative sites, nucleotide diversity (π) and haplotype diversity (Hd) of COI gene sequences in each geographic region were obtained using the software DnaSP version 6.12.03 (Rozas et al. 2017). Pairwise F_{ST} between geographic populations were calculated with 10,000 permutations in ARLEQUIN. Population genetic structure results between mtDNA COI gene sequences and DArT-Seq^{TM} SNP loci were visually compared to address similarities or discrepancies in the findings.

Identifying introduction history of C. maenas into South Australia

To infer potential introduction histories of *C. maenas* into South Australia, Approximate Bayesian Computation (ABC) methods were used in the program DIYABC version 2.1.0 (Cornuet et al. 2014). The ABC approach generates simulated datasets, then selects simulated datasets that are closest to the observed dataset, and then finally estimates posterior distributions of parameters through regression (Cornuet et al. 2014). This method is useful for reconstruction of demographic histories and multiple evolutionary scenarios, which are described as a succession of "events" and "inter-event periods" that have occurred back in time. Several scenarios are constructed, with each scenario including a historical model that describes how the populations are connected to a common ancestor and how allelic states change along their genealogical trees (Cornuet et al. 2014).

To construct these scenarios, prior knowledge about the biology and natural history of the species is required, which may include historical records, demographic information, or population genetic structure. As the population genetic structure and introduction history of *C. maenas* have not been studied for South Australia, I constructed my scenario models based on the following knowledge of the species: 1) I identified major introduction events and invasion pathways of *C. maenas* globally and into southeast Australia as inferred from historical records (Carlton and Cohen 2003; Thresher et al. 2003); 2) I investigated additional genetic structure

using global mitochondrial haplotypes obtained from Darling et al. (2008) and Burden et al. (2014); and 3) I used the population genetic structure results in this current study to identify which populations had greater admixture and similarity with the South Australian population. I then constructed the import file for DIYABC manually in R.

Analysis 1 was run on the sampled genetic dataset of 3,509 loci, 156 individuals and four major geographic populations based on effective population sizes (Ne): N1) the native European range that included Sweden and Portugal; N2) the invasive global range that included all North American populations and the South African population; N3) the southeast Australian population; and N4) the South Australian population. Analysis 2 was run separately on the same sampled dataset from Analysis 1, but an unsampled "ghost" population was included in the simulations. That is, unsampled genetic data that was not obtained from geographic populations (i.e. Iceland, Argentina, Japan, NSW/VIC or elsewhere) were modelled in the demographic scenarios (Estoup and Guillemaud 2010; Lawson Handley et al. 2011). All scenarios were set as uniform distributions and had the European population as the common ancestor. I indicated that genetic bottlenecks (db) occurred after all founder events resulting from divergence or admixture in each population. Reduced effective population sizes after a founder event followed Mabin (2018). The default DIYABC values for admixture rates were used (i.e. 0.001–0.999) (Table 5.3). Six scenarios were modelled in both the sampled dataset (Analysis 1; Fig. 5.2) and in the unsampled ghost dataset (Analysis 2; Fig. 5.3).

Time of introduction events (t) was inferred from known historical records of *C. maenas* and was measured in generations; t1 was the introduction of *C. maenas* into South Australia; t2 was the introduction into southeast Australia; t3 was the first introductions outside of Europe; and t4 was any unsampled introduction event that could have occurred outside of Europe (Analysis 2 only). Generation time for *C. maenas* is suggested to be one year (see Behrens Yamada 2001 and Mabin 2018). The following fixed conditions for time were set: t4 > t3, t2 and t1; t3 > t2 and t1; t2 > t1; and r2 > r1. The Hudson simulation algorithm for SNP markers was used in both datasets, which is the equivalent to applying a default minor allele frequency (Cornuet et al. 2014). Custom mutation models cannot be calculated for SNP data in DIYABC as SNP loci are bi-allelic; therefore, DIYABC assumes that a single mutation occurs during a population(s) gene tree (Cornuet et al. 2014). Parameter values across all scenarios were drawn until conditions were fulfilled. All 3,509 SNP loci in both datasets were used in the ABC analysis and started from the first locus. Summary statistics calculated were

the mean genic diversity across loci, the mean F_{ST} distances between samples, the mean Nei's distances between samples, and the mean admixture rates using maximum likelihood. Analysis for both datasets was run with 1 x 10^5 simulations, which is the minimum number of simulations required by DIYABC and significantly reduced computation time.

Table 5.3 Demographic model parameters and prior distributions of two DIYABC analyses for sampled and unsampled *C. maenas* datasets. Analysis 1 (sampled dataset) only used samples from this study with SNP data. Analysis 2 (unsampled dataset) included an unsampled "ghost" population in the simulations. Each analysis consisted of six different scenarios and all parameters were set as uniform distributions. Both analyses were run with 10^5 simulations per scenario. Units for effective population size (N_e) and reduced effective population size (Nb) are the number of individuals, while time of introduction events (t) and genetic bottleneck events (db) are measured in generations (years). Admixture rates (ra) are defaults set by DIYABC.

Analysis 1 – sampled dataset (3,509 loci, 156 individuals with no "ghost" population)		
Parameter	Description	Priors (min – max)
N1	Effective population size for native European range	100 – 10,000
N2	Effective population size for globally invasive range	100 – 10,000
N3	Effective population size for southeastern Australian population	100 – 10,000
N4	Effective population size for South Australian population	100 – 10,000
t1	Introduction of the South Australian population	10 – 180
t2	Introduction of the southeastern Australian population	10 – 230
t3	Collective introductions for all globally invasive populations	10 – 520
db	Length of genetic bottleneck event	5 – 50
ra	Admixture rate (default)	0.001 – 0.999
N2b	Reduced size of globally invasive populations (post-db)	5 – 1,000
N3b	Reduced size of southeastern Australian population (post-db)	5 – 1,000
N4b	Reduced size of South Australian population (post-db)	5 – 1,000
Analysis 2 – ghost dataset (3,509 loci, 156 individuals unsampled "ghost" population)		
Parameter	Description	Priors (min – max)
N1	Effective population size for native European range	100 – 10,000
N2	Effective population size for globally invasive range (sampled)	100 – 10,000
N3	Effective population size for southeastern Australian population	100 – 10,000
N4	Effective population size for South Australian population	100 – 10,000
G5	Effective population size of an unsampled ghost population	100 – 10,000
t1	Introduction of the South Australian population	10 – 180
t2	Introduction of the southeastern Australian population	10 – 230
t3	Introductions for all globally invasive populations (sampled)	10 – 500
t4	Possible introduction of unsampled ghost population	10 – 520
db	Length of genetic bottleneck event	5 – 50
ra	Admixture rate (default)	0.001 – 0.999
Gpb	Reduced size of unsampled ghost population (post-db)	10 – 1,000
N2b	Reduced size of globally invasive populations (post-db)	10 – 1,000
N3b	Reduced size of southeastern Australian population (post-db)	10 – 1,000
N4b	Reduced size of South Australian population (post-db)	10 – 1,000

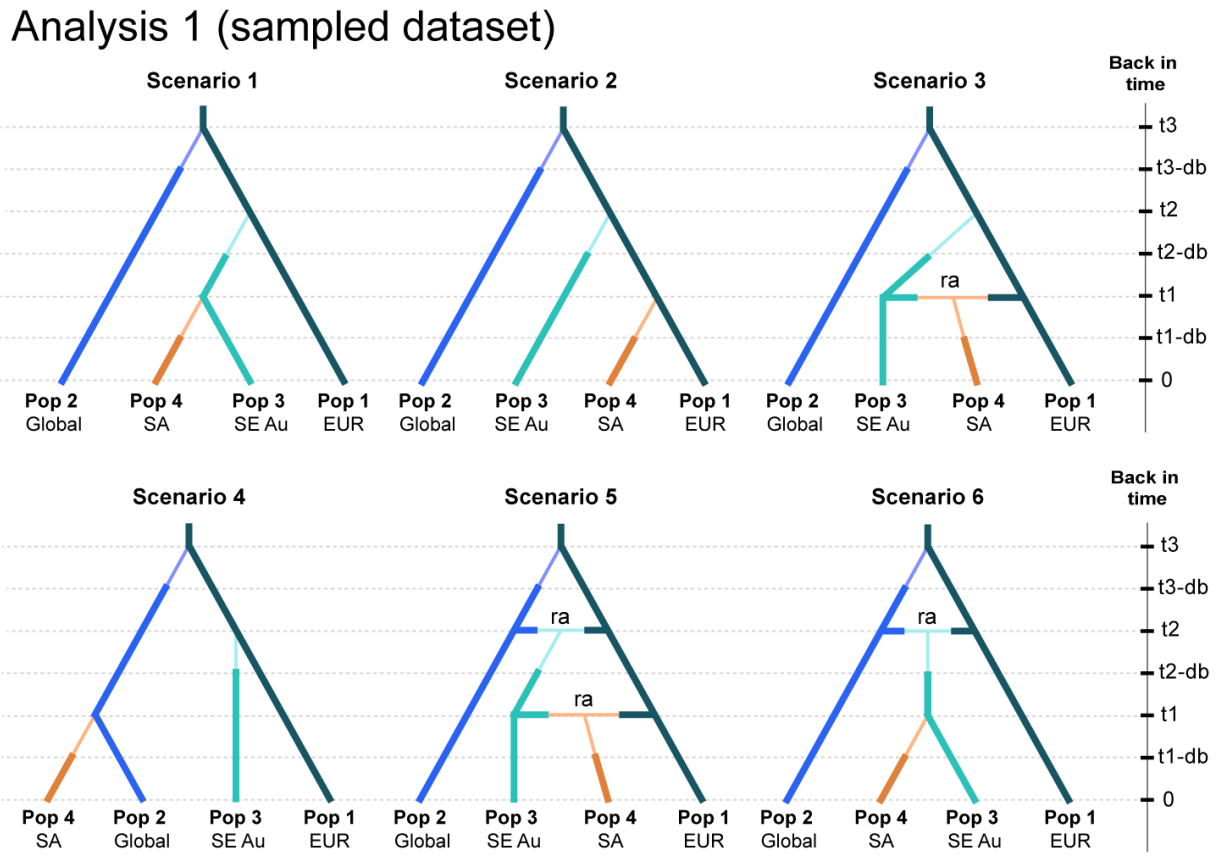

Figure 5.2 Potential scenarios for *C. maenas* introduction into South Australia as illustrated by DIYABC. Analysis 1 consists of sampled populations only (i.e. no unsampled ghost populations were included in analysis). Analysis is for 3,509 loci, 156 individuals and four population groups across six scenarios: native European range (Pop 1), global invasive range (Pop 2), southeast Australia (Pop 3) and South Australia (Pop 4). Thin branches in the tree represent bottleneck events (db), while "T" junctions represent admixture events (ra). Please note that time is not to scale.

Models for each scenario in Analysis 1 were as follows (Fig. 5.2): Scenario 1) *C. maenas* in South Australia were a secondary introduction from southeast Australia, which was a direct introduction from Europe; Scenario 2) *C. maenas* in South Australia and southeast Australia were both independent introductions from Europe; Scenario 3) South Australian *C. maenas* were the result of an admixture event between southeast Australian and European introductions; Scenario 4) South Australian *C. maenas* were introduced directly from Europe, while southeast Australian *C. maenas* were introduced from other global introductions; Scenario 5) South Australian *C. maenas* were the result of multiple introductions and admixture events between Europe, globally invasive and southeast Australian populations; Scenario 6) South Australian *C. maenas* were introduced from southeast Australia, which was the result of an admixture event between European and global introductions.

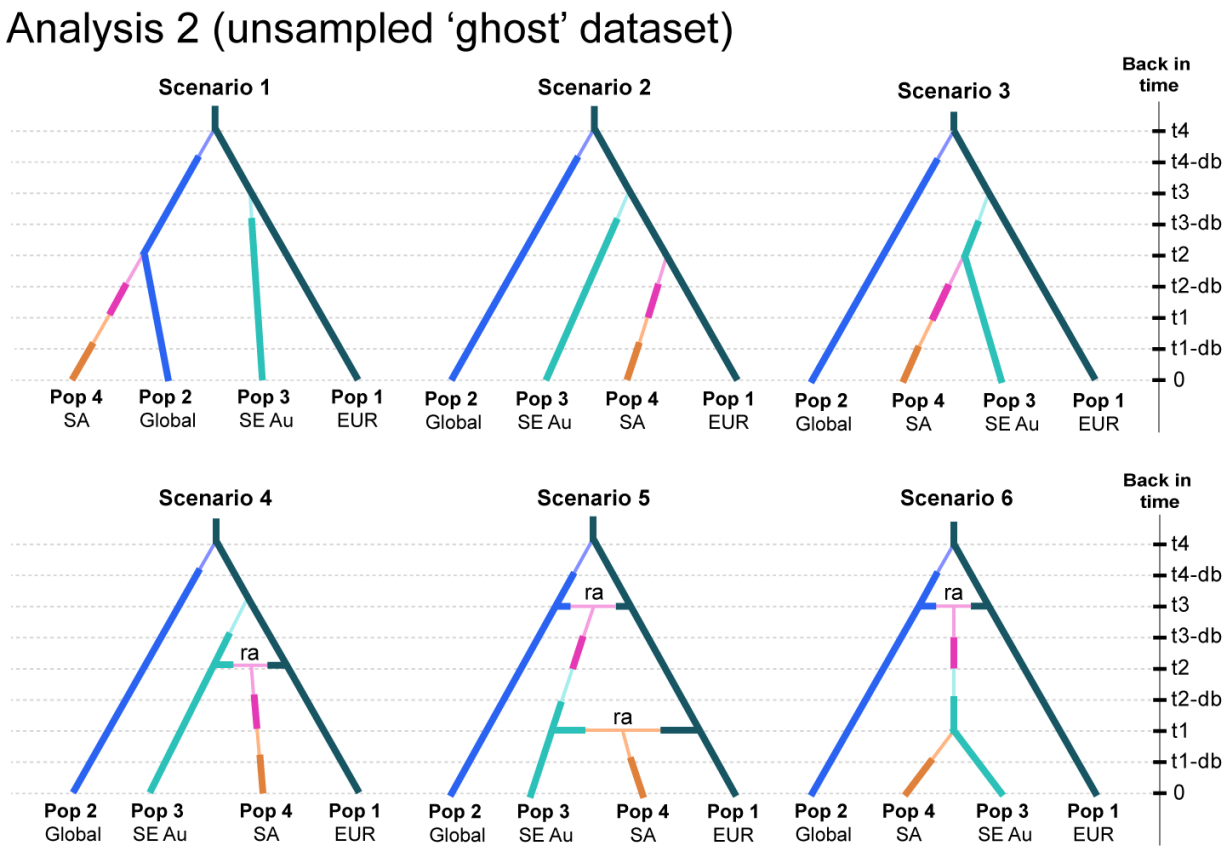

Figure 5.3 Potential scenarios for *C. maenas* introduction into South Australia as illustrated by DIYABC. Analysis 2 consists of both sampled populations and a simulated unsampled 'ghost' population. The ghost population is represented by pink branches in each tree. Analysis is for 3,509 loci, 156 individuals, an unsampled ghost population, and four sampled population groups across six scenarios: native European range (Pop 1), global invasive range (Pop 2), southeast Australia (Pop 3) and South Australia (Pop 4). Thin branches in the tree represent bottleneck events (db), while "T" junctions represent admixture events (ra). Please note that time is not to scale.

Models for each scenario in Analysis 2 were as follows (Fig. 5.3): Scenario 1) South Australian *C. maenas* were introduced directly from Europe, and southeast Australian *C. maenas* were introduced from an unsampled global population; Scenario 2) *C. maenas* in South Australia were introduced from an unsampled European population, while southeast Australia was introduced directly from Europe; Scenario 3) *C. maenas* in South Australia were a secondary introduction from an unsampled population in southeast Australia, which was a direct introduction from Europe; Scenario 4) South Australian *C. maenas* were the result of an unsampled admixture event between southeast Australian and European introductions; Scenario 5) South Australian *C. maenas* were the result of multiple unsampled introductions and admixture events between Europe, globally invasive and southeast Australian populations; Scenario 6) South Australian *C. maenas* were introduced from southeast Australia, which was

the result of an unsampled admixture event between European and global introductions.

After completion of the simulated datasets, DIYABC's statistical analysis tools were used to compare differences between the scenarios for both Analysis 1 and Analysis 2 separately. First, scenarios and prior distributions were pre-evaluated using principal components analysis (PCA) to identify if the models were on target. Posterior distributions of parameters for all scenarios were estimated using 1% of the simulated dataset and logistic regression transformation. Model checks were used to evaluate how well the scenario priors of parameters fit the data. Models for each scenario were checked with applied logistic regression transformation and PCA. The number of selected data in each model check was approximately 5% of the total number of simulated data. To determine which scenarios were most likely, all scenarios were compared with each other using 1% of the simulated dataset, 10 local logistic regressions, and a direct estimate value of 500. Summary statistics were transformed with linear discriminant analysis for the scenario comparisons. This produced a direct estimate plot and a logistic regression plot that displayed the most likely demographic scenarios for Analysis 1 and Analysis 2. Finally, the confidence in scenario choice was calculated with numerous scenario-parameter combinations using global posterior-based error. Linear discriminant analysis and logistic regression was applied with 500 simulated datasets ("pods") and a direct estimate of 500 for both analyses.

5.3 RESULTS

5.3.1 SNP quality control and filtering

The DArT-Seq^{TM} pipeline delivered a final dataset consisting of 28,850 SNPs across 170 individuals of *C. maenas* (hereinafter referred to as the unfiltered dataset). The unfiltered dataset had 17.99% missing data, an average call rate per locus of 0.82 and a missing rate of 0.18, an average read depth of 14.97, and 63 monomorphic loci. The resulting filtered dataset consisted of 3,509 neutral SNPs across 156 individuals. All samples from VIC and most samples from NSW did not pass the sequencing and filtering pipelines due to poor DNA quality from degraded tissue samples. The filtered dataset had 0.47% missing data, an average call rate per locus of 0.995, a missing rate of zero, an average read depth of 26.2, and zero monomorphic loci. The composition of base frequencies in the filtered dataset were A = 24.3%, G = 26.6%, T = 23.78% and C = 25.33%.

No significant deviations from Hardy-Weinberg Equilibrium (HWE) were observed within any population (Bonferroni $p < 0.05$). A total of 329 loci in LD ($r^2 > 0.2$, $p < 0.05$) were identified within populations and all linked loci were removed from the dataset. Relatedness estimates for all pairs of individuals in each population was low overall (i.e. mean relatedness for any pair of individuals in each population did not exceed 0.005; Appendix Table A5.2). However, one dyad of individuals from Portugal had abnormally high relatedness > 0.9 for all estimates, which may be attributed to monozygotic twins or more likely mis-handled samples through contamination, switched identities or sample duplication (Blouin 2003). Both individuals in this dyad had similar low relatedness values with all other individuals in the Portuguese population. Due to suspected duplication of samples in this dyad, only one individual was subsequently removed from the dataset.

Finally, BAYESCAN identified 69 outlier loci with a false discovery rate of 10%. From the 69 outlier loci detected, 60 were potentially under diversifying selection, while nine loci were under balancing selection. Of the 3,512 DArT loci scanned, a total of 60 loci were under putative diversifying selection, 1,765 loci were under balancing selection, and 1,688 loci were under neutral selection (Appendix Fig. A5.1). *OutFLANK* detected fewer outlier loci in the same SNP dataset, with only three outlier loci identified (Appendix Fig. A5.2). The three outlier loci detected by *OutFLANK* were also detected by BAYESCAN and were thus removed during the DArT filtering pipeline and will be explored in future investigations. The remaining outlier loci detected by BAYESCAN were retained, as BAYESCAN has shown high false-positive rates in outlier detection, while *OutFLANK* is more conservative (Storfer et al. 2018).

5.3.2 Summary statistics and genetic diversity

Carcinus maenas from South Australia had the highest genetic diversity measures of all geographic populations in the native and invasive ranges (Table 5.4). South Australia had the highest mean observed and unbiased expected heterozygosity (0.148 and 0.158, respectively), while southeast Australia had the lowest mean observed and unbiased expected heterozygosity (0.12 and 0.131, respectively). The inbreeding coefficient (F_{IS}) was low overall for the whole dataset (0.067), with South Australia and South Africa having the highest average inbreeding coefficients (0.053 and 0.052 respectively). The lowest average F_{IS} values were observed in Sweden and Maine (-0.013 for both). South Australia had the highest mean allelic richness after rarefaction (1.364), number of observed alleles (6,269), and number of private alleles (251). Southeast Australia had the lowest mean allelic richness (1.272) and number of

observed alleles (4,765). Zero private alleles were detected in Maine and Washington USA and only one private allele was detected in Sweden.

Pairwise F_{ST} values ranged from 0.008 (British Columbia vs. Washington) to 0.174 (British Columbia vs. southeast Australia) (Table 5.5). All pairwise F_{ST} values were significantly different between all population pairs ($p < 0.001$), expect for British Columbia vs. Washington ($F_{ST} = 0.008$; $p > 0.05$). Hierarchical analysis of molecular variance (AMOVA) showed the largest degree of variation occurred within populations, accounting for ~87% of variation overall in each K grouping (Table 5.6). At $K = 2$, the first group consisted of Australian, European, South African and Nova Scotia populations, while the second group consisted of the remaining North American populations. As K groups increased to $K = 5$, South Australia, southeast Australia and Nova Scotia populations subsequently formed their own groups. Variation among populations within K groups ranged from 6.77% at $K = 2$ and decreased to 2.63% at $K = 5$. Variation among groups ranged from 7.37% at $K = 2$ and increased to 9.51% at $K = 5$. All hierarchical AMOVA comparisons were significantly different ($p < 0.05$).

Table 5.4 Genetic diversity estimates of *C. maenas* from nine geographic populations for 3,509 DArT-Seq™ loci. *N*, number of individuals; uH_E, mean unbiased expected heterozygosity; H_O, mean observed heterozygosity; F_{IS}, inbreeding coefficient; A_R, mean allelic richness after rarefaction; N_A, total number of alleles observed per locus per population; N_p, number of unique private alleles observed per population. Parentheses indicate the following: standard deviation (± SD), bias corrected 95% confidence intervals (95% CI) and percentage of missing data per population (%). Highest values for each of these measures are highlighted in bold.

Population	*N*	uH_E (± SD)	H_O (± SD)	F_{IS} (95% CI)	A_R (± SD)	N_A	N_P	Missing data (%)
Sweden	10	0.146 (± 0.17)	0.14 (± 0.18)	-0.013 (-0.1 – 0.04)	1.327 (± 0.35)	5,432	1	0.30
Portugal	11	0.142 (± 0.17)	0.135 (± 0.18)	0.0 (-0.06 – 0.04)	1.319 (± 0.35)	5,450	6	0.30
South Africa	11	0.148 (± 0.17)	0.133 (± 0.17)	0.052 (0.0 – 0.09)	1.333 (± 0.35)	5,509	21	0.54
Nova Scotia	10	0.155 (± 0.18)	0.145 (± 0.18)	0.012 (-0.06 – 0.05)	1.344 (± 0.36)	5,446	20	0.46
Maine USA	10	0.152 (± 0.18)	0.146 (± 0.19)	-0.013 (-0.08 – 0.03)	1.335 (± 0.37)	5,369	0	0.25
Washington USA	12	0.15 (± 0.18)	0.141 (± 0.19)	0.016 (-0.32 – 0.03)	1.328 (± 0.37)	5,318	0	0.35
British Columbia	29	0.147 (± 0.18)	0.14 (± 0.18)	0.03 (0.01 – 0.05)	1.328 (± 0.36)	5,566	7	0.29
Southeast Australia	6	0.131 (± 0.20)	0.12 (± 0.2)	0.0 (-0.18 – 0.07)	1.272 (± 0.39)	4,765	7	0.55
South Australia	**57**	**0.158** (± 0.16)	**0.148** (± 0.16)	**0.053** (0.04 – 0.07)	**1.364** (± 0.33)	**6,269**	**251**	**0.67**
All populations	156	0.148 (± 0.18)	0.139 (± 0.18)	0.067	1.328 (± 0.36)	7,018	313	0.47

Table 5.5 Pairwise F_{ST} values (below diagonal) calculated between pairs of *C. maenas* populations for 3,509 putatively neutral SNPs. Corresponding *p*-values are shown above the diagonal, with significant values ($p < 0.05$) between pairs highlighted in bold. Non-significant values are marked with an asterisk (*). F_{ST} values were calculated with 10,000 bootstrap permutations.

	Sweden (n = 10)	Portugal (n = 11)	South Africa (n = 11)	Nova Scotia (n = 10)	Maine (n = 10)	Washington (n = 12)	British Columbia (n = 29)	Southeast Australia (n = 6)	South Australia (n = 57)
Sweden		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Portugal	0.058		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
South Africa	0.032	0.040		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Nova Scotia	0.040	0.091	0.061		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Maine USA	0.091	0.114	0.086	0.085		**<0.001**	**<0.001**	**<0.001**	**<0.001**
Washington USA	0.121	0.121	0.111	0.116	0.024		0.062*	**<0.001**	**<0.001**
British Columbia	0.125	0.122	0.115	0.126	0.038	0.008		**<0.001**	**<0.001**
Southeast Australia	0.135	0.148	0.125	0.151	0.148	0.170	0.174		**<0.001**
South Australia	0.095	0.076	0.079	0.124	0.135	0.145	0.147	0.118	

Table 5.6 Hierarchical analysis of molecular variance (AMOVA) of nine *C. maenas* geographic populations and various genetic clusters calculated with 10,000 permutations. Analysis for 3,509 SNPs and 156 individuals. Genetic clusters (*K*) infer the number of groups and were derived from population structure results. Groups of populations indicated in the parentheses. Significance values are shown in bold ($p < 0.05$); df = degrees of freedom; SS = sum of squares. Population abbreviations: South Australia (SA); southeast Australia (SE Au); Sweden (SE); Portugal (PT); South Africa (ZA); Nova Scotia (NS); Maine (ME); Washington (WA); British Columbia (BC).

Genetic cluster	Population groupings	Source of variation	df	SS	Variance components	Percentage of variation	Sig.
K = 2	(SA, SE Au, SE, PT, ZA, NS) + (ME, WA, BC)	Among groups	1	4429.5	22.53	7.37	**0.01**
		Among pops within groups	7	5944.49	20.69	6.77	**<0.001**
		Within populations	303	89,906.5	262.48	85.86	**<0.001**
K = 3	(SA) + (SE Au, SE, PT, ZA, NS) + (ME, WA, BC)	Among groups	2	6860.25	22.62	7.56	**0.003**
		Among pops within groups	6	3513.72	14.3	4.78	**<0.001**
		Within populations	303	79,532.56	262.48	87.67	**<0.001**
K = 4	(SA) + (SE Au) + (SE, PT, ZA, NS) + (ME, WA, BC)	Among groups	3	7823.54	26.84	8.9	**<0.001**
		Among pops within groups	5	2550.44	10.11	3.38	**<0.001**
		Within populations	303	79,532.56	262.48	87.66	**<0.001**
K = 5	(SA) + (SE Au) + (SE, PT, ZA) + (NS) + (ME, WA, BC)	Among groups	4	8523	28.4	9.51	**<0.001**
		Among pops within groups	4	1850.93	7.84	2.63	**<0.001**
		Within populations	303	79,532.56	262.48	87.86	**<0.001**

5.3.3 Global population genetic structure

Isolation-by-distance (IBD) showed a strong and significant correlation between genetic distance and geographic distance for the nine geographic populations (Mantel r = 0.561, p = 0.001; Fig. 5.4A and 5.4B). The IBD scatterplot supported spatial genetic structure in the global range of *C. maenas* and IBD may be driving population genetic structure.

The PCoA revealed three clusters for the overall *C. maenas* dataset (Fig. 5.5). The first grouping comprised South Australia, the second grouping comprised southeast Australia, the European native range (Sweden and Portugal), South Africa, and Nova Scotia, and the third grouping comprised North America (Maine, Washington and British Columbia). The first PCoA axis explained 9.9% of variation, while the second axis explained 4.2% of variation, accounting for 14.1% of variation overall and relatively weak clustering. Further explanation of variation is shown in the scree plot in Appendix Fig. A5.2. Individuals from all North American populations (including Nova Scotia) and southeast Australia had higher variation within populations as shown by wider spread of points. Two individuals from Nova Scotia appeared to be divergent from their respective clusters.

The DAPC also identified three clusters (K = 3) in the scatterplot (Fig. 5.6A) and density plot (Fig. 5.6B). The Bayesian Information Criterion (BIC) value for K = 3 was 787.28 and provided the most support for the clustering compared to other K values (Appendix Fig. A5.4). The BIC values ranged between 799.8 at K = 1 to 806 at K = 10. The DAPC clusters showed the exact same population group assignments seen in the PCoA; Group 1 consisted of the European native range, southeast Australia, Nova Scotia, and South Africa; Group 2 consisted of the North American populations (Maine, Washington, and British Columbia); and Group 3 consisted of South Australia only (Fig. 5.6A). Several individuals appeared as possible outliers, most noticeably from Group 1. No overlap occurred for any of the DAPC clusters shown in the two-dimensional plots; however, the first discriminant function showed less separation between Group 1 and Group 2 (Fig. 5.6B).

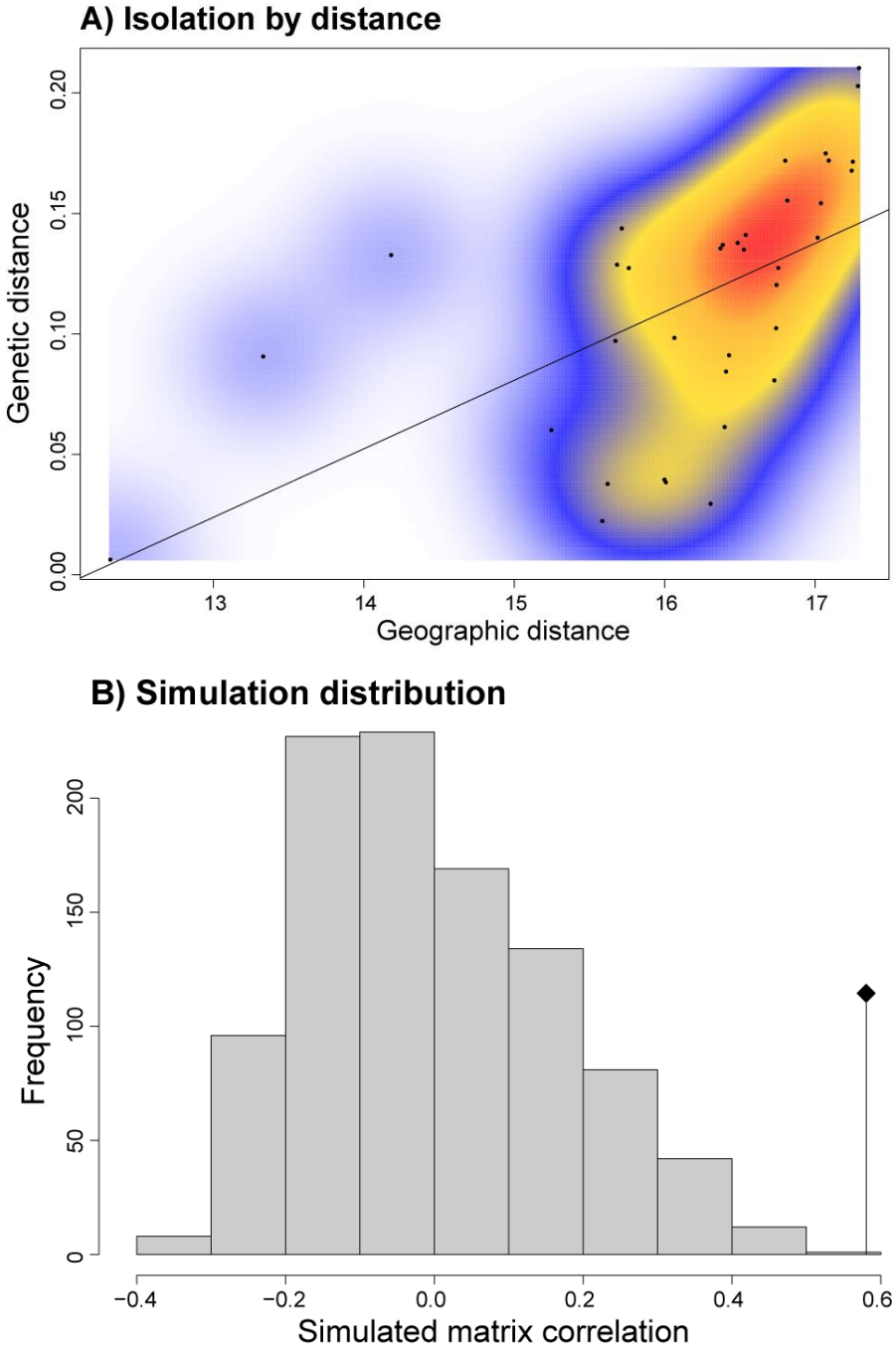

Figure 5.4 Isolation by distance plots of *C. maenas* for 3,509 SNPs, 156 individuals and nine geographic populations (Mantel r = 0.58, p = 0.001). A) Density plot of genetic versus geographic pairwise IBD distances; warmer colours indicate higher density, while the solid diagonal line is the regression between genetic and geographic distance. B) Histogram of 10,000 Pearson correlations between pairwise F_{ST} genetic distance and geographic distance matrices. The original correlation value between distance matrices is represented as a black line and diamond. Significant spatial structure is inferred when the original value is positioned out of the reference distribution. The corresponding genetic and geographic distance matrices can be viewed in Appendix Table A5.3.

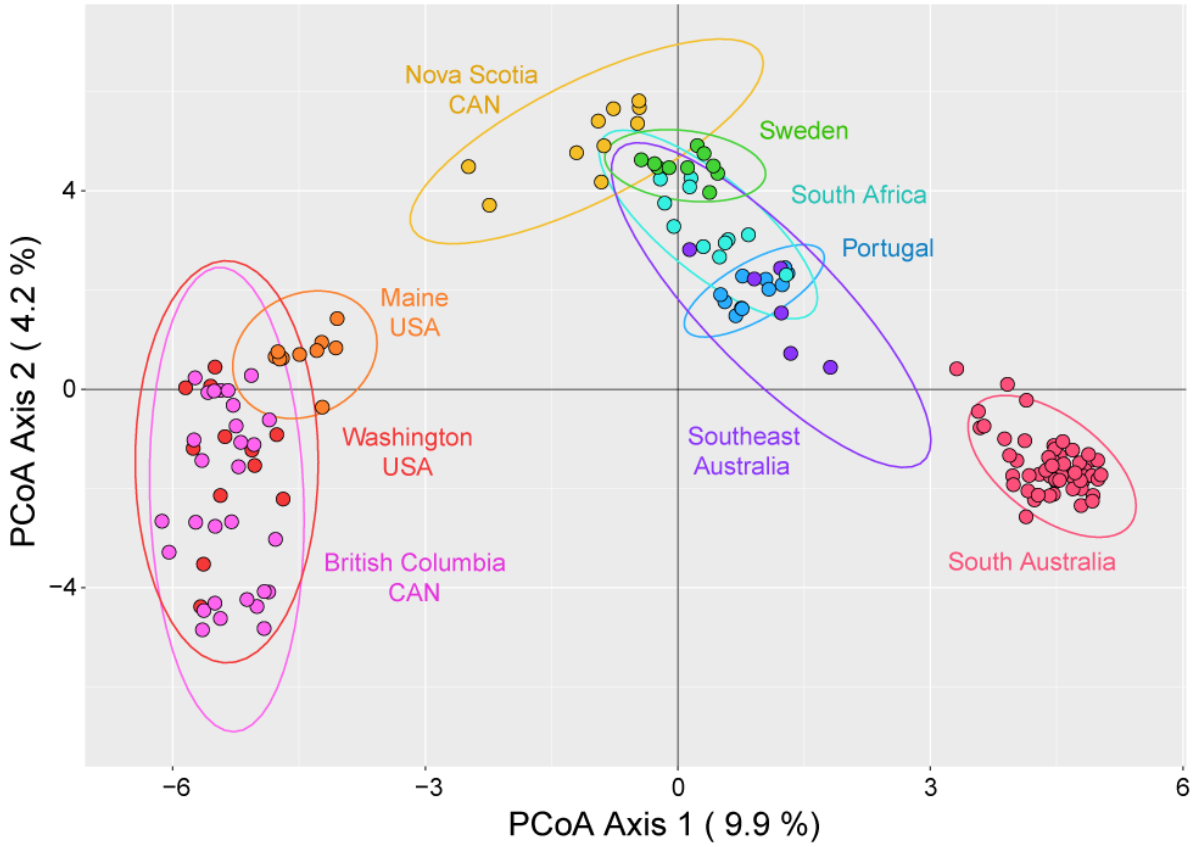

Figure 5.5 Principal coordinate analysis (PCoA) for nine global populations of *C. maenas* for 3,509 SNPs, 156 individuals and nine geographic populations. Individuals are colour-coordinated by geographic population, and ellipses represent 95% confidence. The percent variation for the PCoA is explained by each axis. The corresponding scree plot of PCoA eigenvalues can be seen in Appendix Fig. A5.3.

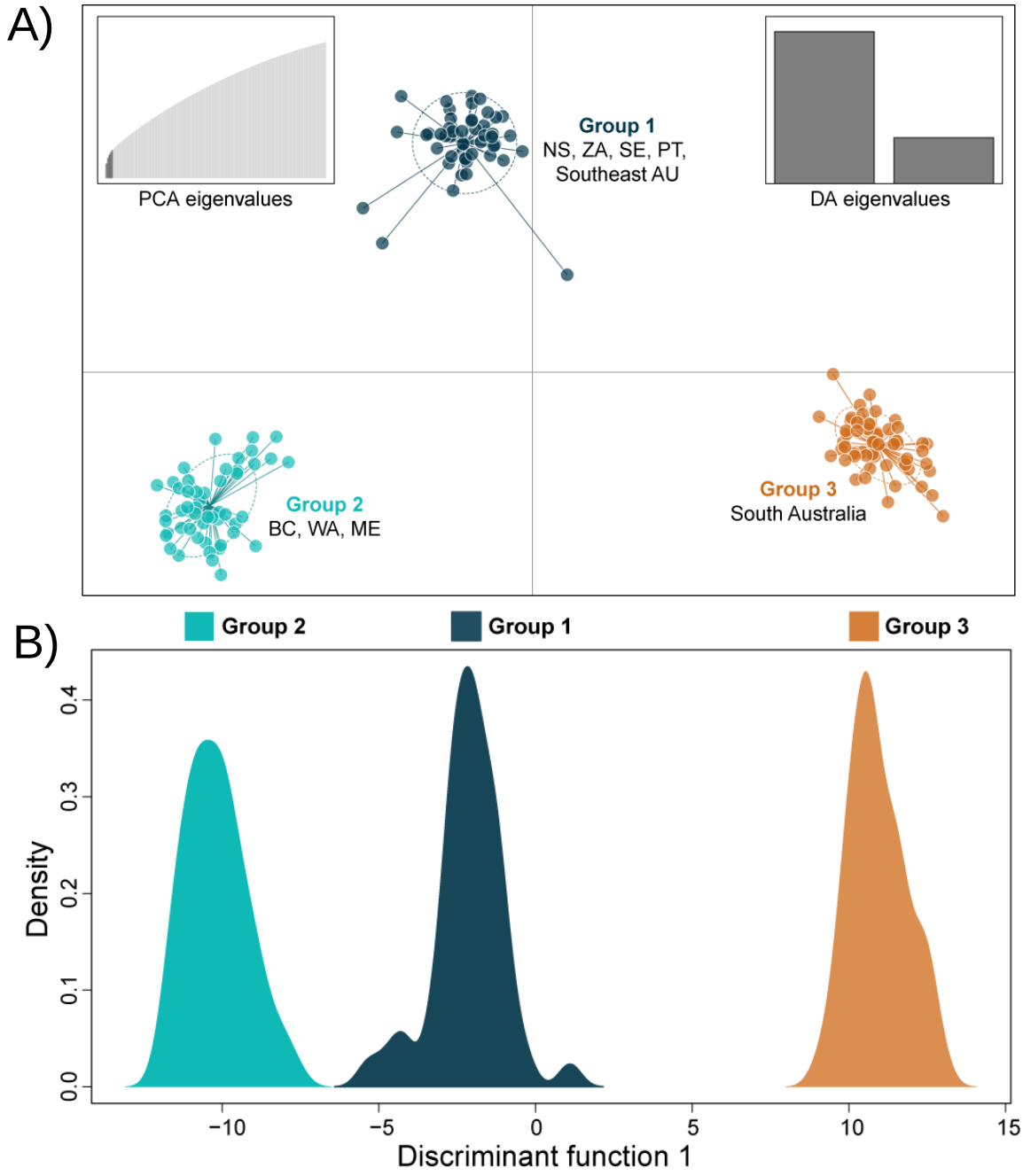

Figure 5.6 Discriminant Analysis of Principal Components (DAPC) with *K*-means clustering of *C. maenas* for 3,509 SNPs, 156 individuals, and nine geographic populations. A) DAPC scatterplot, where each dot represents an individual, coloured groups represent each *K*-means cluster and associated populations with 95% inertia ellipses, and insets show the retained PCA and DA eigenvalues. B) Individual density plot against the first discriminant function resulting from the DAPC. Corresponding BIC values are in Appendix Fig. A5.4.

Bayesian STRUCTURE analysis was broadly compatible with the membership of different genetic clusters shown by the PCoA and DAPC (Fig. 5.7). For the overall dataset of 3,509 SNPs, the ΔK method suggested that $K = 2$, while the mean LnP(K), MedMed K and MedMean K methods all suggested that $K = 5$ (Appendix Fig. A5.5; Appendix Table A5.4). Patterns of admixture and possible substructure could only be observed by exploring multiple K values. At $K = 2$, the South Australian population and the North American populations (Maine, Washington, and British Columbia) showed distinct clustering. The southeast Australian population, Europe, South Africa and Nova Scotia appeared to have intermediate admixture between the two clusters at $K = 2$ but were grouped with the South Australian cluster. At $K = 3$, clustering showed that southeast Australia, Europe, South Africa, and Nova Scotia formed a third cluster that matched the PCoA and DAPC results. At $K = 4$, the southeast Australian population formed a fourth genetic cluster with the one individual from NSW in southeast Australia showing different admixture compared to the five samples from Tasmania. At $K = 5$, Nova Scotia formed a fifth genetic cluster, and admixture across the populations became more apparent. South Australia had minor admixture with southeast Australia and Europe, and Nova Scotia had minor admixture with Maine.

Hierarchical population structure (initial separation by $K = 2$) demonstrated that the North American populations (excluding Nova Scotia) were distinctly separated from the rest of the global distribution (Fig. 5.7). Hierarchical structure analysis identified separation between South Australia and southeast Australia, Europe, South Africa and Nova Scotia at $K = 2$. At $K = 4$, southeast Australia and Nova Scotia formed separate genetic clusters, while South Africa, Portugal and Sweden were clustered together. For this hierarchical structure, $K = 4$ was most likely (Appendix Table A5.5). Within the North American populations, substructure affirmed $K = 2$ (Appendix Table A5.6). The STRUCTURE results supported an optimum $K = 5$ for the overall dataset with major clusters as follows: South Australia (1); southeast Australia (2); Europe + South Africa (3); Nova Scotia (4); and North America (5). The ΔK method did not adequately reflect the optimum K for this dataset.

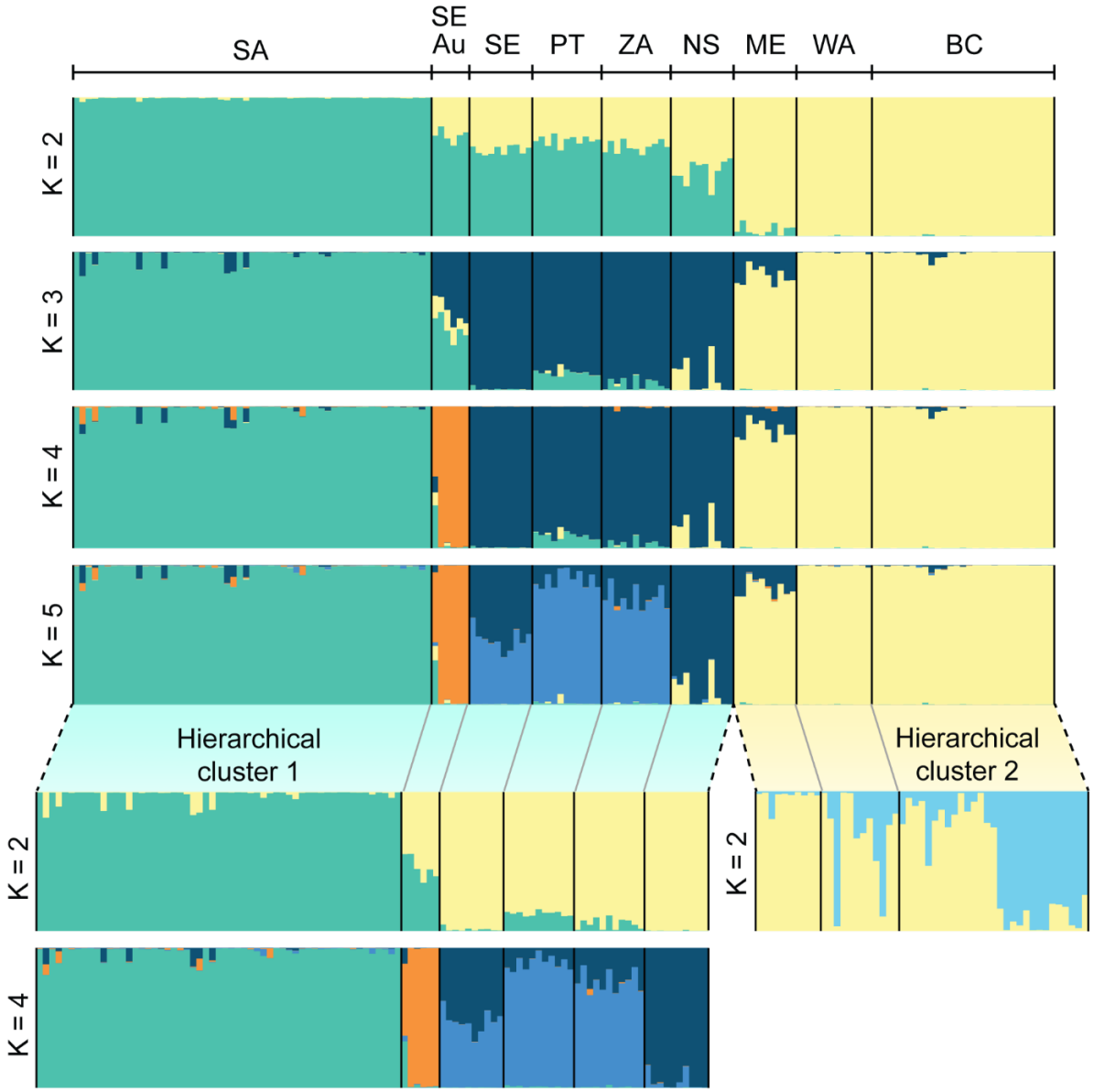

Figure 5.7 Population structure analysis for nine geographic populations of *C. maenas* for 3,509 putatively neutral SNPs. Admixture barplots using Bayesian clustering analysis from STRUCTURE and generated in CLUMPAK. Each vertical-coloured line represents one individual (156 individuals total), while vertical black lines separate each geographic population. Results show 5 replicate runs for $K = 2$ to $K = 5$. Optimal K values can be viewed in Appendix Fig. A5.5 and Appendix Table A5.4. Hierarchical population structure separates two major genetic clusters, with cluster 1 showing $K = 2$ and $K = 4$ as the most likely indicators of substructure (Appendix Table A5.5), and cluster 2 showing $K = 2$ as the most likely indicator of substructure (Appendix Table A5.6). Population abbreviations: South Australia (SA); southeast Australia (SE Au); Sweden (SE); Portugal (PT); South Africa (ZA); Nova Scotia (NS); Maine (ME); Washington (WA); British Columbia (BC).

A fragment of 367 base pairs (bp) of mtDNA COI gene sequence was successfully compared for *C. maenas* across its global distribution. A total of 62 unique mtDNA haplotypes were identified from the global distribution of *C. maenas* (total number of individuals sequenced = 769; South Australia crab n = 59; Darling et al. 2008 crab n = 653; Burden et al. 2014 crab n = 57) (Table 5.7). Of the 367 nucleotide sites, 46 were polymorphic and 31 were parsimony informative. Haplotype diversity (Hd) for *C. maenas* globally was 0.741 and nucleotide diversity (π) was 0.004. Genetic diversity for western North America, Argentina and Japan was zero as only one unique haplotype was recovered in these populations. South Australia had higher genetic diversity (Hd = 0.693; π = 0.003) than Tasmania (Hd = 0.185; π = 0.001), but both were lower than mainland Australia (Hd = 0.832; π = 0.004). Haplotype diversity was higher in mainland Australia (Victoria and New South Wales) than populations in all native and invasive ranges. South Australia's haplotype diversity was comparable to South Africa and Nova Scotia and contrasted with DArT SNP loci results where South Australia had the highest diversity compared to all native and invasive populations.

Table 5.7 Haplotype summary and diversity of a 367 bp region of the COI gene sequenced from *C. maenas* global geographic populations. N = number of individuals sequenced; h = number of unique haplotypes; S = number of polymorphic sites; pS = number of parsimony-informative sites; Hd = haplotype diversity; π = nucleotide diversity. South Australia is highlighted in bold. All other sequences were obtained from Darling et al. (2008) and Burden et al. (2014).

Population	N	h	S	pS	Hd (± SD)	π
Off-shelf Europe	38	3	7	4	0.383 ± 0.081	0.004
Northern Europe	135	28	24	11	0.794 ± 0.03	0.004
Western Europe	126	36	31	11	0.813 ± 0.028	0.004
Nova Scotia	20	4	3	3	0.721 ± 0.051	0.004
Eastern North America	71	2	1	0	0.028 ± 0.027	<0.001
Western North America	74	1	0	0	0.0	0.0
Argentina	15	1	0	0	0.0	0.0
South Africa	49	12	12	6	0.787 ± 0.046	0.004
Japan	55	1	0	0	0.0	0.0
Mainland Australia	86	7	6	5	0.832 ± 0.013	0.004
Tasmania	41	3	3	3	0.185 ± 0.079	0.001
South Australia	**59**	**5**	**5**	**4**	**0.693 ± 0.041**	**0.003**
Total	769	62	46	31	0.741 ± 0.016	0.004

The network of 62 unique haplotypes across *C. maenas* geographic populations showed that the most common haplotypes had a global distribution (represented by the letter E) and occurred at high frequencies (Fig. 5.8). A high abundance of single haplotypes were observed from European regions. Some single haplotypes were found from other regions, most notably Nova Scotia (N1), South Africa (Z1) and the Faroe Islands and Iceland (F1, F2 and I1). The overall haplotype network had a radiating pattern that showed a dominant clade and a secondary clade. Mutational base-pair steps between haplotypes was low, and European haplotypes were most divergent overall. South Australian haplotypes (letter S) were often shared with European and southeast Australian haplotypes (represented by the letter E and letter B).

Haplotype frequencies on the number of sampled individuals (excluding singleton haplotypes) revealed how haplotypes were shared across geographic regions (Fig. 5.9). Following results by Darling et al. (2008), the most common haplotype (grey-blue) was found in all populations apart from off-shore Europe (Iceland and Faroe Islands) and Tasmania. This common haplotype was the only haplotype found in west and east North America (except an individual from Connecticut, USA), Argentina and Japan. Nova Scotia, South Africa, mainland Australia and South Australia shared multiple haplotypes with the northern and western European populations. Tasmania and off-shore European populations had the most unique haplotype frequencies. South Australia had six individuals of a unique haplotype that was not observed in any other geographic region and shared more haplotypes with Europe and mainland Australia than it did with Tasmania.

Pairwise F_{ST} values indicated that haplotypes between most geographic regions were significantly different and supported population structure (Table 5.8). South Australia was significantly different to all other populations which supports its differentiation as seen in SNP loci ($p < 0.05$). There were no differences between northern and western Europe with each other ($p > 0.05$). No differences were found between Argentina, Japan, western North America and eastern North America pairs; this is not surprising given the low number of unique haplotypes in these four populations ($p > 0.05$). Tasmania and off-shore European populations were the most significantly different in all pairwise comparisons ($p < 0.001$).

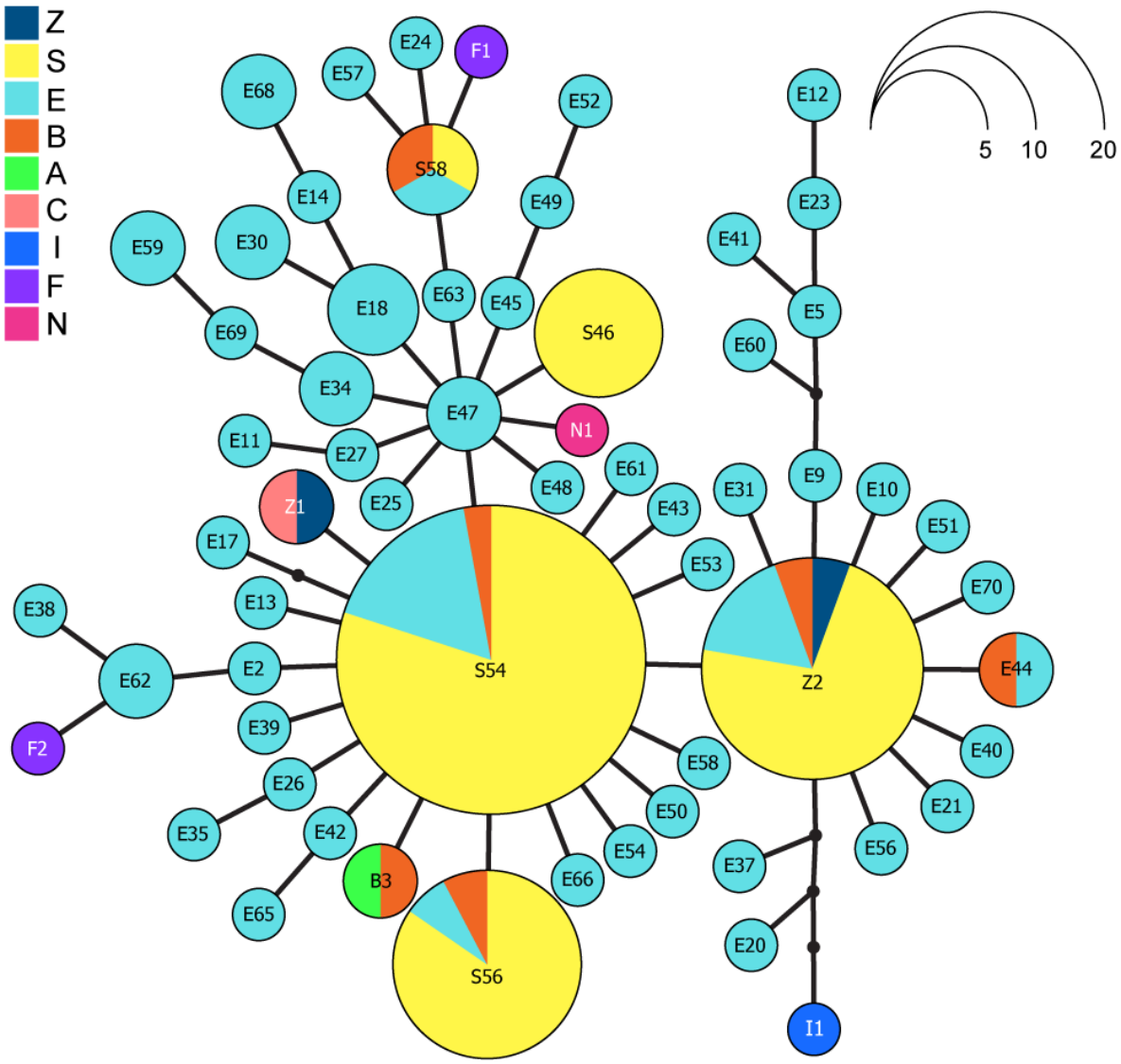

Figure 5.8 An unrooted mtDNA haplotype network of the COI gene observed in global populations of *C. maenas* for this study, Darling et al. (2008) and Burden et al. (2014) (total haplotype n = 62). Each circle represents one unique haplotype (labelled in the circle), and circle diameter is the mtDNA haplotype frequency. Colour represents the relative frequency of each mtDNA haplotype from the following regions: Z (South Africa); S (South Australia); E (Europe); B (southeast Australia, Burden et al. 2014); A (southeast Australia, Darling et al. 2008); C (Connecticut, USA); I (Iceland); F (Faroe Islands); N (Nova Scotia). Unique haplotype frequency information is seen in Appendix Table A5.7 and has been modified from Darling et al. (2008) and Burden et al. (2014).

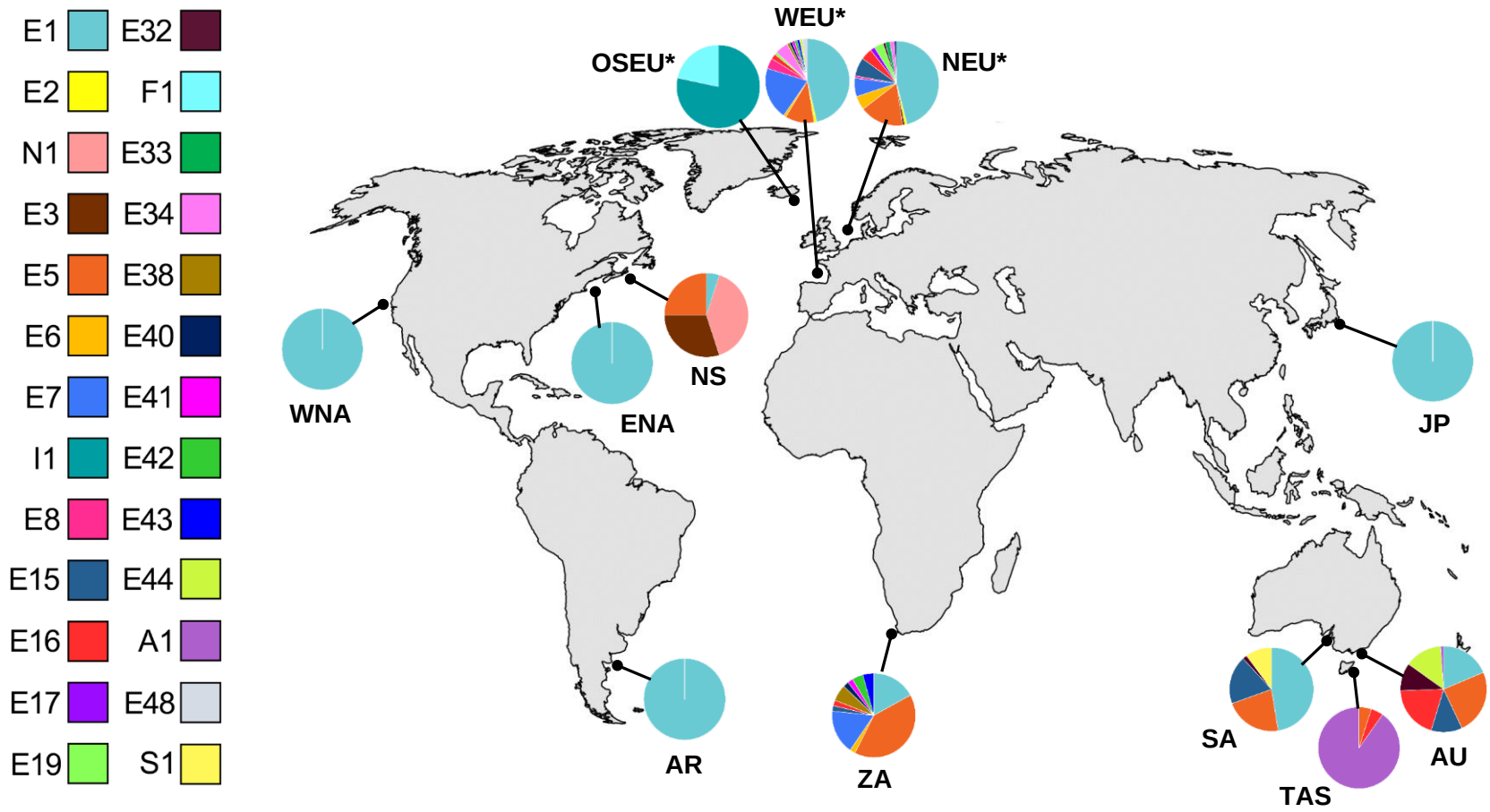

Figure 5.9 Global distribution of COI haplotypes of *C. maenas* from this study, Darling et al. (2008) and Burden et al. (2014). Each pie chart shows proportion of haplotype groups in each geographic region. Region codes are as specified: OSEU (Off-shelf Europe); WEA (western Europe); NEU (northern Europe); NS (Nova Scotia); ENA (eastern North America); WNA (western North America); AR (Argentina); ZA (South Africa); JP (Japan); TAS (Tasmania, Australia); AU (Victoria and New South Wales, Australia); and SA (South Australia). Asterisks (*) denote native European range. Individual haplotype frequencies across geographic regions are denoted by colours in the legend (haplotype details are shown in Appendix Table A5.8). Singleton haplotypes have been excluded.

Table 5.8 Pairwise F_{ST} values (below diagonal) calculated between pairs of *C. maenas* populations for a 367 bp region of the COI gene sequenced from global *C. maenas* populations (n sequences = 769). Corresponding *p*-values are shown above the diagonal, with significant values ($p < 0.05$) between pairs highlighted in bold. South Australian sequences are from Chapter 2 and Chapter 3, while remaining sequences were obtained from Darling et al. (2008) and Burden et al. (2014). Non-significant values are marked with an asterisk (*). F_{ST} values were calculated with 10,000 bootstrap permutations. Population abbreviations: Off-shelf Europe (OSEU); northern Europe (NEU); western Europe (WEU); Nova Scotia (NS); eastern North America (ENA); western North America (WEA); Argentina (AR); South Africa (ZA); Japan (JP); mainland Australia (AU); Tasmania (TAS); South Australia (SA).

	OSEU (n = 38)	NEU (n = 135)	WEU (n = 126)	NS (n = 20)	ENA (n = 71)	WNA (n = 74)	AR (n = 15)	ZA (n = 49)	JP (n = 55)	AU (n = 86)	TAS (n = 41)	SA (n = 59)
OSEU		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NEU	0.675		0.4*	**<0.001**	**<0.001**	**<0.001**	**<0.05**	**<0.01**	**<0.001**	**<0.001**	**<0.001**	**<0.005**
WEU	0.663	-0.0001		**<0.001**	**<0.001**	**<0.001**	**<0.01**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
NS	0.691	0.216	0.190		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ENA	0.864	0.121	0.134	0.736		0.49*	0.99*	**<0.001**	0.99*	**<0.001**	**<0.001**	**<0.001**
WNA	0.871	0.124	0.137	0.756	0.0005		0.99*	**<0.001**	0.99*	**<0.001**	**<0.001**	**<0.001**
AR	0.756	0.066	0.076	0.516	-0.032	0.0		**<0.001**	0.99*	**<0.001**	**<0.001**	**<0.05**
ZA	0.649	0.038	0.039	0.195	0.344	0.357	0.200		**<0.001**	**<0.05**	**<0.001**	**<0.001**
JP	0.848	0.111	0.123	0.710	-0.003	0.0	0.0	0.316		**<0.001**	**<0.001**	**<0.001**
AU	0.660	0.074	0.081	0.218	0.333	0.340	0.0	0.020	0.310		**<0.001**	**<0.001**
TAS	0.809	0.410	0.406	0.668	0.817	0.836	0.705	0.500	0.809	0.482		**<0.001**
SA	0.723	0.029	0.051	0.352	0.162	0.170	0.073	0.096	0.146	0.110	0.513	

5.3.4 Reconstructions of invasion history

The pre-evaluation of priors for the six demographic scenarios in each analysis was successfully visualised with PCA. For both DIYABC analyses, the observed dataset (yellow circle) fell within the simulated data for each scenario (coloured circles), suggesting a good fit between the simulated and observed datasets (Appendix Fig. A5.6A and A5.6B).

In Analysis 1 (sampled dataset), scenario comparisons using the direct estimate method showed that scenarios 2, 3 and 5 were most likely, however posterior probability was relatively low overall (< 0.35) (Fig. 5.10A). The logistic regression method showed that scenario 5 was most likely, with a very high posterior probability of 1.0 (Fig. 5.10B). All other scenarios had a posterior probability of zero using the logistic regression method. DIYABC analysis of the sampled dataset provides evidence that scenario 5 is the most likely, but scenario 2 and 3 should also be considered. Scenario 5 indicates that the South Australia population possibly resulted from multiple source introductions (i.e. southeast Australia, Europe, and possibly other invasive ranges) and admixture events. Scenario 2 indicates that South Australia resulted from a direct European introduction, while Scenario 3 supports admixture between southeast Australia and Europe in the resulting South Australian population. Confidence intervals for all scenario values are found in Appendix Table A5.9.

In Analysis 2 (unsampled dataset), scenario comparisons using the direct estimate method suggested scenarios 4 and 5 were most likely, but probability was low (< 0.4) (Fig. 5.11A). The logistic regression method suggested that scenario 5 was most likely, with a high probability around 0.9 (Fig. 5.11B). The DIYABC analysis supports that the South Australia population resulted from multiple introductions and admixture events from southeast Australia, Europe, the sampled invasive range and a potential ghost population. Both Analysis 1 and Analysis 2 did not support scenarios where South Australia resulted from a direct introduction (i.e. without admixture) from any of the native and invasive populations, including ghost populations. Multiple introductions from various source locations have contributed to admixture and genetic structure in South Australian *C. maenas.* Confidence intervals for all scenario values are found in Appendix Table A5.10.

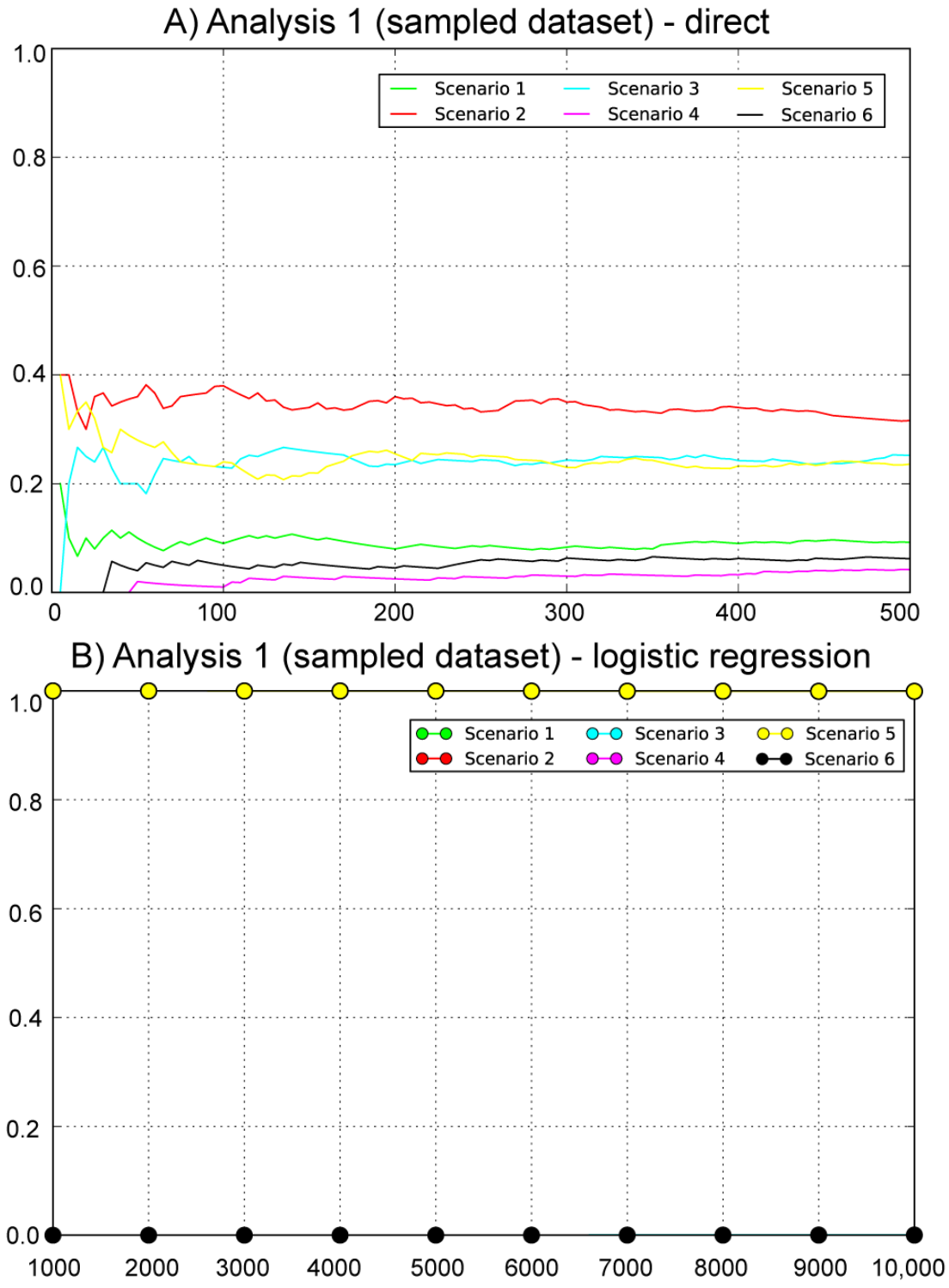

Figure 5.10 Comparison of the posterior probabilities for six modelled scenarios in the sampled dataset. A) a model comparison of the six scenarios using the direct estimate method, with scenario 2, 3 and 5 being the most likely. B) a model comparison of the six scenarios using the logistic regression method, with scenario 5 being the most likely (all other scenarios were zero). Y-axis = posterior probability; x-axis = number of simulated datasets that are closest to the observed data.

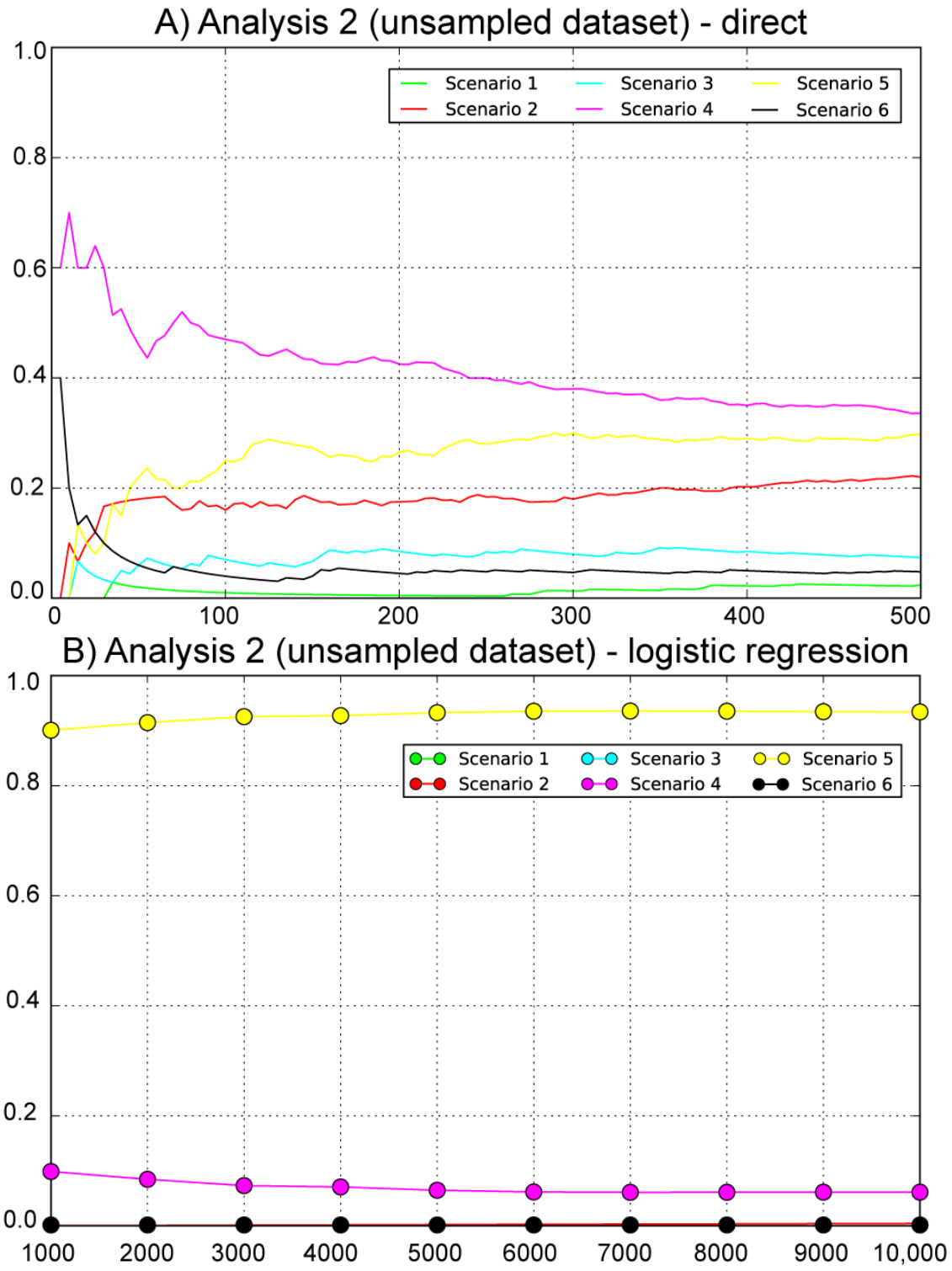

Figure 5.11 Comparison of the posterior probabilities for six modelled scenarios in the unsampled ghost dataset. A) a model comparison of the six scenarios using the direct estimate method, with scenario 4 and 5 being the most likely. B) a model comparison of the six scenarios using the logistic regression method, with scenario 5 being the most likely. Y-axis = posterior probability; x-axis = the number of simulated datasets that are closest to the observed data.

5.4 DISCUSSION

Genetic diversity of marine invasive species is an important underlying factor that assists with overcoming founder effects during establishment and identification of invasion pathways and source populations for management (Lawson Handley et al. 2011; Cristescu 2015; Darling et al. 2017). I analysed the genetic structure of *C. maenas* in South Australia using DArT-Seq^{TM} SNP loci and mtDNA COI gene sequencing. 3,509 SNP loci revealed high genetic diversity in the South Australian population, while both SNP loci and mtDNA supported significant population differentiation in South Australian *C. maenas*. The DIYABC analysis, Bayesian admixture plots, and shared mtDNA haplotypes support that Australian *C. maenas* populations originate from multiple introductions and admixture events from Europe, Tasmania and southeast Australia.

5.4.1 Genetic diversity of *Carcinus maenas*

Genetic analysis of SNPs revealed slightly higher genetic diversity in the South Australian population of *C. maenas* than other populations analysed in this study. Rarefaction allelic richness (which accounted for variation in sample size) showed marked genetic diversity in the South Australian population, with over 250 private alleles observed. Observed and unbiased expected heterozygosity in South Australian *C. maenas* was slightly higher than other populations. Genetic diversity determines an invader's ability to adapt to new or changing environments, and is estimated through measurements such as heterozygosity, proportion of polymorphic loci, and allelic richness (Lawson Handley et al. 2011). Differences in genetic diversity between populations result from evolutionary and demographic processes, such as genetic bottleneck events, differences in effective population size, and restrictions to accumulation of genetic variation (Geburzi and McCarthy 2018).

After an introduction event, many invasive populations will experience reductions in genetic diversity due to bottleneck events impacted on a small number of founding individuals in the colonisation process (Viard et al. 2016; Geburzi and McCarthy 2018). Multiple introductions from genetically diverse sources can help marine invasive species overcome founder effects and create populations that display similar or higher genetic diversity than observed in the native range (Roman and Darling 2007; Chan and Briski 2017). On average, *C. maenas* displayed greater genetic diversity in introduced populations than native populations when analysed with mtDNA and microsatellite markers (Darling et al. 2008).

SNPs revealed that the lowest genetic diversity was found in southeast Australia (comprising mostly Tasmanian samples), low genetic diversity in Maine, Washington and British Columbia, high genetic diversity in South Africa and Nova Scotia, and highest genetic diversity in South Australia. This is consistent with Darling et al. (2008), who found that western North America, eastern USA, Argentina and Tasmania had reduced genetic diversity for all measures, followed by intermediate genetic diversity in mainland Australia (Victoria and New South Wales), and high genetic diversity in South Africa and Nova Scotia. Rius et al. (2015) observed that when high marine invasive species have high genetic diversity it is the result of introductions from either genetically diverse or disparate sources. The ~200-year introduction history, multiple vectors (i.e. dry ballast and ballast water, biofouling assemblages) and source populations responsible for *C. maenas'* global distribution have contributed to genetic variation across populations (Darling et al. 2008).

Genetic diversity is important for overcoming founder effects during colonisation, but higher genetic diversity does not automatically translate to greater impacts of established invasive populations (Thresher et al. 2003; Darling et al. 2008; Rius et al. 2015). Introduced populations of *C. maenas* with high genetic diversity have the lowest rates of population expansion and studies are needed to documented impact (Thresher et al. 2003; Darling et al. 2008). The estimated rate of *C. maenas* population expansion in genetically diverse populations is 1.7 km year^{-1} in South Australia (and similar for mainland Australia), 1.9 km year^{-1} in South Africa, and 8.7 km year^{-1} in northeast America near Nova Scotia (Thresher et al. 2003). In introduced *C. maenas* populations with the lowest genetic diversity (i.e. California, USA to British Columbia, Canada), the rate of expansion was estimated to be 200 km year^{-1} (Thresher et al. 2003). Rates of expansion in Japan and Argentina are unknown. While multiple introductions from genetically diverse sources have contributed to high genetic diversity in South Australian *C. maenas*, rates of expansion in South Australia have been slow and documented impacts in this region are limited (Walton et al. 2002; Campbell et al. 2019).

5.4.2 Global patterns of genetic structure

The South Australian population of *C. maenas* was differentiated from other global populations by significant IBD, ordination and clustering (PCoA, DAPC and Bayesian STRUCTURE), pairwise F_{ST} values and mtDNA haplotype differences. Population genetic structure of species is shaped by the dispersal abilities of adults and their larvae, geographical barriers to gene flow (including ocean currents and thermoclines), and human-mediated vectors

(Hellberg 2009). Most of the dispersal potential of *C. maenas* is attributed to their pelagic larval stage, which have a dispersal distance of approximately 60 km during its long larval phase (~50 days) and is driven by local oceanographic conditions such as currents (Silva et al. 2010a). In South Africa, IBD was significant in several marine species that exhibited pelagic larval dispersal distances <10 km per generation (Wright et al. 2015).

Geographic distances and larval dispersal are not the only drivers behind gene flow, or lack thereof, in marine populations. In Nova Scotia, invasive *C. maenas* populations showed a stronger isolation-by-environment (IBE) relationship than IBD, indicating that environmental conditions such as sea surface temperature and sea ice have greater constraints on gene flow than geographic distances among populations (Jeffery et al. 2018). *Carcinus maenas* displays rapid adaptation in their invasive Northwest Atlantic and native European ranges, driven by temperature and mediated by a putative genomic island of divergence that varied with cold tolerance (Tepolt and Palumbi 2020). The environmental drivers for genetic adaptation of *C. maenas* in South Australia are currently unknown. As the global thermogeographic range of *C. maenas* is expected to shift due to climate change, the genetic basis of adaptation is a priority for marine bioinvasion management (Compton et al. 2010; Lehnert et al. 2018).

The PCoA and DAPC results in this study clustered southeast Australia with Europe, however Bayesian STRUCTURE analysis separated southeast Australia at $K = 4$ and $K = 5$. The mtDNA haplotype network supported differences between Tasmania, mainland Australia and South Australia. Darling et al. (2008) revealed that mainland Australia (with Tasmania) formed its own genetic cluster with Argentina. South Australia is a genetically distinct population from mainland Australia and Tasmania. The distinct population structure of South Australian *C. maenas* is most likely due to geographical isolation from other *C. maenas* populations in southeast Australia. Thresher et al. (2003) and Darling et al. (2008) indicated that Tasmanian *C. maenas* were introduced from mainland Australia, either through interstate shipping, aquaculture transport or larval dispersal. Thresher et al. (2003) noted that the East Australian Current "EAC" (which flows southward from Queensland through to Tasmania) may have caused southward transport of *C. maenas* larvae from Port Phillip Bay, Victoria.

South Australia is connected to northwestern Tasmania through the seasonal Zeehan and South Australian Currents that flow eastward. Aguilar et al. (2019) noted however that the Southeast Australian Biogeographic Barrier (SEABB) makes larval connectivity between South Australia and southeast Australia unlikely for most marine species. Burden et al. (2014)

found higher levels of gene flow for *C. maenas* among estuaries situated on mainland Australia compared to gene flow between Tasmanian and mainland Australian populations. Even with the EAC, Tasmania is separated from mainland Australia by the Bass Strait which is 240 km wide and makes dispersal difficult (Burden et al. 2014). Other factors such as seasonal sea surface temperatures, substrate type and biotic interactions also limit *C. maenas* connectivity between South Australia and southeast Australia (Thresher et al. 2003; Garside et al. 2014). The most feasible explanation of connectivity between South Australia and southeast Australia *C. maenas* is through human-mediated vectors. Since *C. maenas* was introduced to South Australia in the 1970s, it has likely accrued genetic changes over time which have contributed to its differentiation (Bilton et al. 2002; Hellberg 2009).

Population pairwise F_{ST} values from SNPs and mtDNA supported that there is significant genetic structure across global *C. maenas* populations. The AMOVA results showed that most genetic variation occurred within populations. The number of genetic clusters in this study varied depending on method used (PCoA, DAPC or Bayesian STRUCTURE analyses). When factoring in admixture, five major genetic clusters were identified: Europe and South Africa (1); southeast Australia (2); South Australia (3); Nova Scotia (4), and North America (5). South Australia consistently formed its own genetic cluster regardless of method and had one unique haplotype not found anywhere else (Darling et al. 2008; Burden et al. 2014). The South African population consistently clustered with native European populations for all analyses. Darling et al. (2008) found that the South African population clustered with native *C. maenas,* but also showed similarities with native *C. aestuarii* populations in the Mediterranean Sea. Further work by Mabin (2018) used mtDNA and microsatellites to confirm the presence of admixture and possible hybridisation between *C. maenas* and *C. aestuarii* in South Africa.

I found that Nova Scotian *C. maenas* are significantly different to those from Maine, USA, and even formed its own genetic cluster at $K = 4$. This result is supported by mtDNA haplotype network analysis by Darling et al. (2008); Nova Scotia formed its own separate cluster when analysed with mtDNA, but clustered with native European populations when using microsatellite loci. There is evidence that the *C. maenas* population in Nova Scotia resulted from a secondary introduction to the east coast of North America, and SNP loci have identified a genetic cline between the Nova Scotia and Maine populations (Darling et al. 2008; Jeffery et al. 2017a, 2017b; Jeffery et al. 2018). While F_{ST} values are different between Maine

and the west coast of North America (British Columbia and Washington), all three populations clustered together consistently in this study. Hierarchical *K* clustering found separation between the two sample sites in British Columbia: half of the samples in this study were from Sooke Basin, the other half from Barkley Sound. Limited genetic studies have indicated that *C. maenas* in Sooke Basin are isolated and genetically distinct from other populations in the Salish Sea (Drinkwin et al. 2019).

5.4.3 Source populations of South Australian *C. maenas*

Results from DIYABC analysis on SNP loci showed that introduction of *C. maenas* to South Australia was the result of admixture events between multiple source populations and was consistent with genetic clustering and admixture demonstrated by the PCoA, DAPC and Bayesian STRUCTURE methods. The most likely demographic scenario indicated admixture between individuals from native European, globally invasive and southeast Australian populations, even when unsampled "ghost" populations were factored into the model. There was little support for scenarios where South Australian *C. maenas* were introduced directly from another globally invasive population, or through direct introductions from Europe or southeast Australia in the absence of admixture. The Bayesian STRUCTURE results also showed some admixture between South Australia, Europe and southeast Australia.

Similar admixture events and demographic histories have been revealed in ABC analyses in other invasive species. Mabin (2018) used DIYABC on microsatellite loci to show that invasive *C. maenas* in South Africa were derived from admixture of individuals from native and globally invasive ranges. In the globally invasive ascidian (*Microcosmus squamiger*) DIYABC analysis on microsatellite loci showed evidence for non-independent introduction processes and admixture in this widespread marine species (Rius et al. 2012). DIYABC analysis of 1,653 genotype-by-sequencing (GBS) SNPs in the invasive vase tunicate (*Ciona intestinalis*) indicated that northwest Atlantic populations resulted from admixture between Sweden and the English Channel (Hudson et al. 2020). ABC analysis is therefore useful for reconstructing introduction histories using different genetic markers in marine organisms.

Mitochondrial haplotypes from Darling et al. (2008) and Burden et al. (2014) were successfully contrasted with South Australian mtDNA sequences to expand on putative source populations. The mtDNA haplotypes covered the known global distribution of *C. maenas*,

including populations where SNP loci were not sampled in this study (i.e. off-shelf Europe (Iceland and Faroe Islands), Japan, Argentina, Victoria and New South Wales). South Australia shared most haplotypes with Europe and a subset of haplotypes with southeast Australia, most of which were from mainland Australia and a small proportion from Tasmania. Thresher et al. (2003) and Darling et al. (2008) concluded that the Tasmanian population is a secondary introduction resulting from mainland Australia (known as an invasive "bridgehead" introduction; Estoup and Guillemaud 2010). While Victoria is the most likely source of the Tasmanian population due to connectedness by shipping and ocean currents, it was speculated that South Australian *C. maenas* could have also contributed to the introduction into Tasmania and not the other way around (Thresher et al. 2003). South Australia and mainland Australia shared more haplotypes with each other than they did with Tasmania; this provided further evidence that DIYABC unsampled "ghost" populations and admixture in the South Australian population have come from Victoria and New South Wales. Haplotype analysis provided additional evidence that South Australia *C. maenas* are descended from multiple introductions from the southeast coast of Australia (Victoria and New South Wales) and Europe.

It is unlikely that the South Australian population was introduced from off-shelf Europe due to its genetic distinctiveness, no shared haplotypes with any other population and cold climate (Roman and Palumbi 2004; Darling et al. 2008). Japan is also an improbable source population for South Australia. The Japanese *Carcinus* population is a hybridised species complex of *C. maenas* and *C. aestuarii* (see Carlton and Cohen 2003; Darling 2011a), however there is no evidence that *C. aestuarii* occurs in South Australia (see Chapter 2). Argentina has an interesting invasive population of *C. maenas*; Darling et al. (2008) found that Australian and Argentine populations consistently clustered together using microsatellite loci. While South Australian mtDNA haplotypes were significantly different than Argentinian haplotypes, the significance value was less ($p < 0.05$) than all other population comparisons ($p < 0.001$). Argentina is an unlikely source population for South Australian *C. maenas* because *C. maenas* was first recorded in Argentina in 2003 although it may have been present since 1999 (Hidalgo et al. 2005), over 25 years after *C. maenas* was discovered in South Australia. Darling et al. (2008) suspected that *C. maenas* from mainland Australia were introduced to Argentina via a secondary bridgehead introduction but this requires further investigation.

5.4.4 Study limitations and implications

Comparisons of diversity of different genetic markers (i.e. mtDNA, microsatellites and

genomic SNPs) should be viewed with caution. Genetic diversity from mtDNA contrasted the DArT SNP loci results for the South Australian population in this study. The mtDNA diversity of South Australian *C. maenas* was lower (Hd = 0.693; π = 0.003) than the native ranges from Darling et al. (2008) (Hd = 0.794–0.813; π = 0.004); this contrasted DArT SNPs where South Australia had higher genetic diversity than the native range. Differences in genetic diversity between DArT SNPs and mtDNA is due to the number and type of markers being compared. DArT-Seq^{TM} SNPs have shown fine-scale structure in organisms where mitochondrial markers revealed little structure and diversity. For example, in the Galapagos shark (*Carcharhinus galapagensis*), 8,103 neutral DArT SNPs revealed fine-scale population structure, while the mtDNA Control Region (CR) showed no significant population structure (Pazmiño et al. 2017). Genetic diversity in the invasive vase tunicate (*C. intestinalis*) showed inconsistent results between mtDNA and microsatellite loci (Zhan et al. 2012). Differences in effective population size and changes in genetic drift between mitochondrial and nuclear markers may be responsible for marker discrepancies (Zhan et al. 2012). Inferences between nuclear markers and mtDNA also differ due to stochastic factors affecting mtDNA evolution, while SNPs that use thousands of genome-wide markers detect diversity and structure not found using mtDNA (Pazmiño et al. 2017).

Genetic diversity inferred from DArT SNP loci contradicted Mabin (2018), who used mtDNA sequences and microsatellite loci to assess genetic structure of invasive *Carcinus* populations. Mabin (2018) included samples from Gulf St Vincent, South Australia, and is the only other study to include South Australian *C. maenas* in genetic diversity and structure assessments. Mabin (2018) analysed eight microsatellite loci from 30 *C. maenas* and detected one private allele, lower allelic richness and lower heterozygosity in the South Australian population than the native range. Disparity between this study and Mabin (2018) is due to different genetic markers used: the discriminatory power of ~100 neutral SNPs is roughly equivalent to 10-20 microsatellites (Helyar et al. 2011). Differences in sample size (30 individuals by Mabin 2018 and 57 individuals in this study) may have contributed to variation in genetic diversity estimates (Bashalkhanov et al. 2009).

Uneven sample sizes are also known to effect genetic cluster results generated in the program of STRUCTURE, especially when isolation by distance is present (Puechmaille 2016). In this study, sample size of *C. maenas* varied across geographic populations (six individuals in southeast Australia up to 57 individuals in South Australia) which may have

altered inferences of hierarchical structure or estimates of genetic clusters. Suggestions to overcome this issue include using both Bayesian and non-Bayesian clustering approaches (i.e. DAPC and STRUCTURE; as was done in this study). A combination of estimators for selecting the optimal *K* should also be used rather than Δ*K* alone, such as those implemented by STRUCTURE SELECTOR, which was also undertaken in this study (Puechmaille 2016). Finally, subsampling populations to even out sample size will assist with STRUCTURE results and interpretation (Meirmans 2019). I explored a subsampled dataset (i.e. 20 individuals randomly selected from South Australia and British Columbia, all other population sample sizes the same) using the same analysis for the original filtered dataset. The DAPC and Bayesian STRUCTURE subsample analysis did not significantly change the genetic cluster results. The separation of South Australia from southeast Australia was less, but still clustered into its own group using all methods including optimal *K* and DAPC BIC values (Appendix Table A5.11).

Genome scanning (BAYESCAN, *OutFLANK*) showed a discrepancy in the detection of outlier loci under putative selection in *C. maenas.* While both methods had a false discovery rate (FDR) of 10%, BAYESCAN detected 69 outlier loci while *OutFLANK* only detected three outlier loci. As both genome scan methods detected the same three outlier loci, these loci were removed during filtering to prevent possible selection from biasing genetic structure. Differences in genome scan results using DArT-Seq™ was found in another marine study; BAYESCAN detected 28 outlier loci while *OutFLANK* detected three outlier loci in the grey reef shark (*Carcharhinus amblyrhynchos*) and was attributed to higher false-positives in BAYESCAN (Momigliano et al. 2017). While selection in *C. maenas* is an important aspect of genetic structure and could explain some patterns observed in this study, it was not the primary research aim and only a small portion of the genome is associated with climatic or environmental adaptation (Meirmans 2015). Genome scans using genome-complexity reduction markers (i.e. DArT-Seq™) sample small portions of the genome at random; for this reason, loci associated with environmental/climatic adaptation may be missed, resulting in a small number of outlier loci detections (Meirmans 2015).

A difficulty in reconstructing invasion pathways is the need to assess historical specimens that were collected in early phases of introduction. Specimens of *C. maenas* analysed in this study were collected recently (i.e. most within the last few years, and up to 12 years for the NSW samples). *Carcinus maenas* have been recorded in Australia for >100 years

(Carlton and Cohen 2003; Thresher et al. 2003) and genetic changes have likely occurred over generations that may cloud the ability to accurately identify source populations. Estoup and Guillemaud (2010) identified limitations with using ABC methods due to stochastic events that strongly effect genetic composition of analysed samples: 1) genetic drift effects genetic composition of the source populations; 2) only part of genetic variability is sampled during introductions; 3) genetic bottlenecks occur after a few generations; 4) mutational events occur throughout all stages in the populations' history; and 5) limited sample sizes or small number of genetic loci may not accurately characterise the population. Such stochastic events make it difficult to infer "true" introduction histories, however ABC methods aim to select the "best" scenario among limited sets of scenarios (Fraimout et al. 2017). Despite these limitations, ABC methods can adequately reconstruct invasion scenarios if the suspected source population shows genetic differentiation, and if historical records exist for baseline comparisons, which was the case for *C. maenas*.

Genetic tools are becoming cheaper and are desirable methods for surveillance and management of marine bioinvasions (Bott et al. 2010; Viard et al. 2016). Genetics and genomics can identify marine invasive organisms using metabarcoding and eDNA, characterise gene expression and loci under selection that determine adaptive qualities of invaders, and reconstruct invasion histories to determine source populations (Lawson Handley et al. 2011). While ABC methods are complex and time consuming, they are a powerful tool for identifying biogeography that can factor in unsampled populations and expand on historical records and observations (Rius et al. 2012; Cristescu 2015). Detection of source populations is crucial for heightening vigilance for monitoring and managing vector pathways (Estoup and Guillemaud 2010; Darling 2015). If *C. maenas* expands its range further in southern Australia or elsewhere, genetic methods such as DIYABC analysis can rapidly identify source populations.

This study identified genetic structure in invasive *C. maenas* in South Australia. Multiple introductions from genetically diverse sources have contributed to higher genetic diversity in South Australian *C. maenas.* Genetic diversity is an important trait of an invader that helps founding individuals persist through founder effects such as genetic bottlenecks and increases establishment. The South Australian population was genetically distinct from the global distribution of *C. maenas*. Geographic isolation between South Australian *C. maenas* and southeast Australia has contributed to genetic differentiation of the South Australian

population. DIYABC analyses, Bayesian admixture and shared haplotypes revealed demographic history of South Australian *C. maenas*. Multiple introductions and admixture events, mostly from Europe and southeast Australia (including Victoria and New South Wales), were the most likely source populations for the South Australian population. Diverse genetic structure has assisted with successful establishment of *Carcinus maenas* across different environments throughout their global distribution. Methods such as DIYABC analysis and assessments of admixture are a useful management tool for locating source populations and developing prevention strategies during new incursions.

Chapter 6. General Discussion

The European shore crab, *Carcinus maenas*, is one of the world's worst marine invasive species (Leignel et al. 2014), but when I began my studies, little attention had been given to the population biology and invasion ecology of *C. maenas* in its introduced range in the southern hemisphere. This thesis provided new knowledge on aspects of the population biology that may contribute to the invasion success of *C. maenas* in coastal habitats of South Australia. I examined morphological variation, reproductive biology and genetic structure of *C. maenas* using morphometrics, histology, DNA sequencing and multivariate analyses. Here, I summarise the main findings of the four data chapters and review how these findings assist with our understanding on the ecology and population biology of invasive *C. maenas*. I then expand on the biological factors that contribute to successful invasions and discuss the implications of the work for management and future research.

6.1 Overview of main results

6.1.1 Chapter 2 – Species confirmation of *C. maenas*

My results confirmed that South Australian *Carcinus* are *C. maenas.* While the assessment of morphometric carapace ratios and rostrum shapes suggested that both Atlantic *C. maenas* and Mediterranean *C. aestuarii* are found in Gulf St Vincent, South Australia, analysis of mtDNA revealed ~100% matches with *C. maenas* on GenBank. There was an average 11.5% divergence between South Australian *C. maenas* and Mediterranean *C. aestuarii* GenBank sequences. Morphologically, the CW:CL ratio had been applied as a reliable morphological indicator differentiating species of *Carcinus* (see Behrens Yamada and Hauck 2001; Clark et al. 2001) and the ratio for 91% of South Australian specimens matched *C. maenas.* The CW:CH ratio and rostrum shapes were variable, with 73% and 57% of crabs matching *C. maenas* descriptors using these two indicators. My study supports Clark et al. (2001) who suggested morphology alone is inadequate for differentiating *Carcinus* spp. Sexual dimorphism, intraspecific variation and environmental variables can all affect morphology of *Carcinus*, and a taxonomic re-assessment of both species may be warranted. During new incursions or range expansions, *Carcinus* can be rapidly and accurately identified by molecular approaches (Bott et al. 2015).

6.1.2 Chapter 3 – Morphological variation and genetic homogeneity

Habitat-specific morphological variation in *C. maenas* across mangrove, harbour and rocky shore habitats spanning ~70 km in Gulf St Vincent, South Australia were identified using linear and geometric morphometrics. Linear morphometrics of 1,011 crabs showed that chelae dimensions are responsible for variation in males while pleon dimensions are responsible for variation in females between habitats. Landmark coordinate geometric morphometrics facilitated accurate visualisation of morphological variation. Analysis of 150 crabs using geometric morphometrics illustrated finer morphological differences in carapace shape than linear morphometrics, with crabs from mangroves having wider carapaces than crabs from the harbour and rocky shore. Morphological variation in crabs may be the result of differential growth and development, feeding habits and burrowing behaviour (Baldridge and Smith 2008; Silva et al. 2009; Parvizi et al. 2017). Analyses of the mtDNA COI gene of 59 crabs revealed no significant genetic structuring between habitat types. Six mtDNA haplotypes were identified within the South Australian population and no clear haplotype network was displayed. Intraspecific morphological variation in South Australian *C. maenas* may be the result of phenotypic plasticity and/or ontogenetic changes during development, while genetic homogeneity is likely the result of mixing between larvae and adults throughout the population (Brian et al. 2006; Silva et al. 2010a). The ecological relevance of these shape changes across habitat types is unknown but the capacity of *C. maenas* to modify morphology to local environment conditions may influence invasion success.

6.1.3 Chapter 4 – Reproductive biology of female *C. maenas*

My findings support that invasive *C. maenas* display early onset of sexual maturity, high fecundity and s prolonged spawning period in South Australia. Ovarian development and maturity of 157 female *C. maenas* collected in 2018 were analysed with macroscopic and histological methods. Females reach sexual maturity at as little as 23 mm carapace width, while 50% of the sampled population reached maturity by 50 mm carapace width. The size at sexual maturity is similar to that observed in *C. maenas* across its native and invasive ranges (Baeta et al. 2005; Audet et al. 2008; Lyons et al. 2012; Best et al. 2017; Young and Elliott 2019). Fecundity of females averaged ~200,000 eggs per clutch. Small females < 25 mm carapace width produced ~29,000 eggs per clutch, while females > 65 mm produced up to ~500,000 eggs per clutch. Monthly ovarian assessments from March to November 2018 revealed a prolonged spawning period in female *C. maenas*. Female

C. maenas spawn for nine months of the year in South Australia during periods when temperatures facilitate egg development. As average sea surface temperatures decreased below 18°C, the prevalence of mature, gravid and resorbing females increased. In New South Wales, *C. maenas* reproduces more in winter and spring while native crabs spawn in summer and autumn (Garside et al. 2015) which may reduce larval and juvenile interspecific competition. In South Australia, the prolonged spawning period of *C. maenas* may increase larval density and dispersal during favourable conditions, while high fecundity and early onset of sexual maturity assists with maintaining population density and expanding the range of invasive populations.

6.1.4 Chapter 5 – Genetic structure and demographic history

Genetic analyses revealed higher diversity and more unique private alleles in the South Australian population compared to native and other invasive ranges. 3,509 SNPs were analysed in 156 *C. maenas* individuals spanning nine global geographic populations. The South Australian population formed its own separate genetic cluster when data were analysed using PCoA, DAPC and Bayesian clustering methods. Genetic clusters matched other studies on *C. maenas* global population structure (Darling et al. 2008; Burden et al. 2014). Bayesian structure plots indicated that South Australia and Tasmania form distinct populations that were admixed with each other and with Europe. Additional analyses of the mtDNA COI gene sequences in Chapters 2 and 3 compared my data with global structure studies by Darling et al. (2008) and Burden et al. (2014) and confirmed that some haplotypes are shared between South Australia, mainland Australia, Tasmania and Europe. South Australian *C. maenas* also had one globally unique mtDNA haplotype. Significant isolation by distance was observed which may drive the genetic structure of the South Australian *C. maenas* population. DIYABC analysis of demographic scenarios with and without modelled ghost populations supported that the South Australian population was introduced from multiple sources admixed with individuals introduced from Europe and Eastern Australia (Estoup and Guillemaud 2010; Cristescu 2015). Multiple introductions from varying source localities can help invasive species overcome founder effects by increasing genetic diversity in invasive populations and may be one reason why *C. maenas* has a global distribution (Roman and Darling 2007; Viard et al. 2016).

6.2 Invasion success of *Carcinus maenas* in South Australia

Introduced species must survive and reproduce under the biotic and abiotic conditions faced in unfamiliar and challenging environments to establish and spread (Colautti and MacIsaac 2004). Only a small proportion of introduced species establish and fewer become invasive; supported by life history and ecological traits that predict invasion success (Geburzi and McCarthy 2018). Many of these traits include reproduction such as high fecundity or multiple spawning events, moderate-to-high genetic diversity to overcome founder effects, phenotypic plasticity and physiological tolerance to varying biotic and abiotic conditions, mobility, behaviour, and ecological interactions such as competition with native species for food and refugia (Fig. 1; Geburzi and McCarthy 2018; Swart et al. 2018). Success is not only determined by the traits of the invasive species, but also abiotic conditions in the introduced range and traits of the recipient community (Havel et al. 2015; Rato et al. 2021). Global change will further drive aquatic environments to become more hostile or receptive to marine invasive species (Occhipinti-Ambrogi and Galil 2010).

Carcinus maenas, is a highly successful marine invader. Their success is underpinned by multiple biological traits which have allowed them to pass through ecological filters multiple times over the course of their ~200-year introduction history (Leignel et al. 2014). European shore crabs have established in sub-polar and temperate latitudes where they were exposed to a wide range of biotic and abiotic pressures during colonisation (Carlton and Cohen 2003). Ecological interactions with recipient communities, habitat availability, and environmental conditions vary throughout *C. maenas'* global distribution and have probably contributed to fluctuations in *C. maenas* populations across its range (Darling et al. 2008). The conditions experienced by *C. maenas* in one population often differ to conditions experienced by *C. maenas* in other populations; for example, conditions experienced by *C. maenas* in South Australia are different to those experienced in Nova Scotia, and so biological traits will likely differ between these regions (Darling 2011b). *Carcinus maenas* is a generalist/opportunistic species which has contributed to its establishment across a global spatial scale and varying coastal environments (Brian et al. 2006; Edgell and Hollander 2011; Casties and Briski 2019). Coyle et al. (2019) discovered cold-adapted mitochondrial haplotypes in invasive *C. maenas* in the USA, and highlighted that haplotype influences ecological phenotypes and invasion outcomes across its distribution.

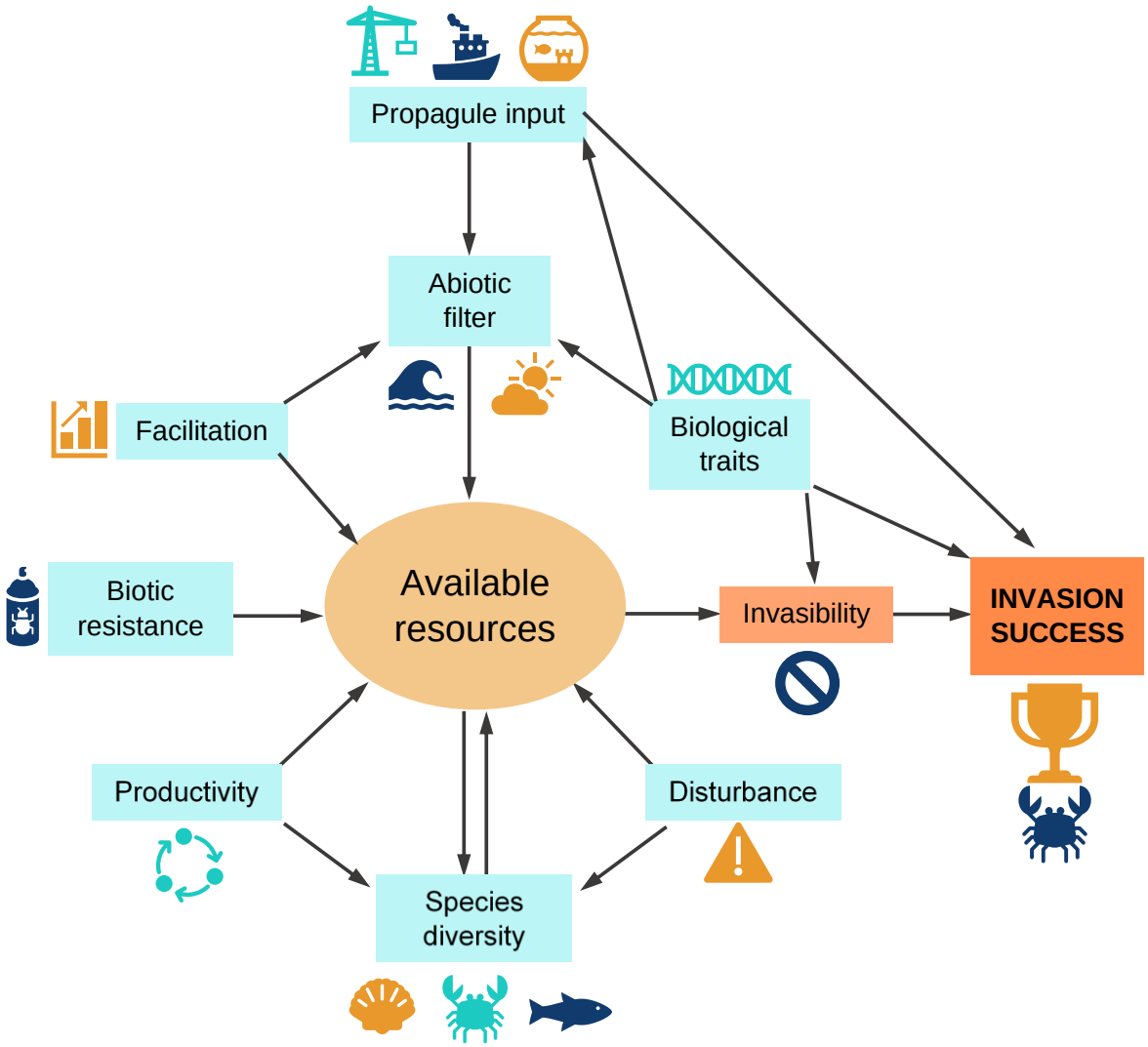

Figure 6.1 Conceptual diagram of factors known to affect invasions of introduced marine species. Vectors supply propagules and propagule pressure is influenced by reproductive traits of the introduced species. When species survive local environmental conditions (abiotic filter), the ability of that species to establish is affected by multiple interacting factors such as biological traits (i.e. physiology, genetics), species interactions, resistance and disturbance. Diagram based on Geburzi and McCarthy (2018).

Phenotypic plasticity is a key trait that contributes to invasion success (Geburzi and McCarthy 2018). The ability of an invader to change its phenotype to suit local environmental conditions help it to overcome biotic and abiotic stressors in new environments or during climate change (Ghalambor et al. 2007; Gianoli and Valladares 2012; Fox et al. 2019). The morphological variation of *C. maenas* I discovered in Gulf St Vincent may be the result of phenotypic plasticity as a response to local environment conditions. Substrate type, predation pressure and prey availability, tide/wave exposure and anthropogenic impacts differ across

these coastal habitats, and *C. maenas* may therefore experience different ecological pressures across its South Australian population (Hampton and Griffiths 2007; Baldridge and Smith 2008; Garside and Bishop 2014). Genetic homogeneity in South Australian *C. maenas* is the result of mixing between larvae during dispersal or migration of adults, or perhaps through multiple introductions coming from genetically similar sources (Weersing and Toonen 2009; Lowe and Allendorf 2010).

The exact causes and ecological relevance of morphological variation in *C. maenas* is unknown because genotypic and phenotypic relationships are difficult to separate (Brian et al. 2006). An aspect not studied in this project was colour polymorphism in *C. maenas*; in its native range, *C. maenas* display carapace colour and pattern variation depending on habitat type and crab size which reduces predation risk (Todd et al. 2006; Stevens et al. 2014; Nokelainen et al. 2018). In South Australia, *C. maenas* display colour and pattern variations across habitat type, with higher proportions of mottled and spotted crabs found in the rocky shore than plain crabs, which are more common in the mangroves and harbour (R. Campbell, unpublished observations.). Observations of consistent differences in colour phenotypes between habitats provides further evidence that the morphology of *C. maenas* is influenced by its surroundings.

Reproduction is another key trait that determines the success of invasive species and is crucial in the establishment process (Blackburn et al. 2011; Geburzi and McCarthy 2019). In sexually reproducing organisms, males and females need to be introduced into the same area to mate or gravid females must be transported for reproduction to occur (Ramirez Llodra 2002; Zeng et al. 2014). Reproduction with successful recruitment increases population density and the likelihood that the population will become self-sustaining (Anderson and Epifanio 2010; Hänfling et al. 2011). My findings support that European shore crabs show early onset of sexual maturity, high fecundity and extended reproduction in their invasive range (Baeta et al. 2005; Lyons et al. 2012; Best et al. 2017). I assessed reproduction of *C. maenas* for the first time in the southern hemisphere. In South Australia, *C. maenas* spawn over an extended nine-month period between the austral autumn and spring when water temperature is less than 18°C. This temperature facilitates successful egg and larval development and is advantageous for *C. maenas* growth and survival (Young and Elliott 2019).

The population genetics of marine invasive species is a useful tool for identifying genetic diversity, genetic structure and admixture, gene flow, hybridisation, gene expression

and neutral and selective processes (Lawson Handley et al. 2011). My study showed that South Australian *C. maenas* have strong genetic structure; SNP loci identified higher genetic diversity than occurs in the native range, strong differentiation of the South Australian population, and adxmiture of European and southeast Australian populations which indicates that the South Australian population is descended from individuals from multiple source populations. While high genetic diversity assists with overcoming founder effects during establishent, genetic diversity alone does not determine the population dyanmics of the invader (i.e. expansion; Roman and Darling 2007). For example, *C. maenas* populations with moderate-to-high genetic diversity have some of the lowest rates of population expansion (i.e, South Africa, Nova Scotia, southeast Australia), while populations in North America with low genetic diversity have the highest rates of expansion and greatest commercial and ecological impact (Thresher et al. 2003; Darling et al. 2008).

6.3 Implications for management and future research recommendations

Up to 90% of the global ocean surface has been impacted by human activity through combinations of climate change, overexploitation of natural resources, pollution, habitat modification and reduction, biodiversity loss and invasive species (Pyšek et al. 2020). Marine invasions are increasing globally and cause negative environmental and socio-economic impacts (Anton et al. 2019; Sardain et al. 2019). The sheer scale of marine invasive species' distributions, the open nature of the marine environment, and the lack of marine bioinvasion research and prioritisation compared to terrestrial bioinvasions makes implementing management strategies difficult in marine systems (Ojaveer et al. 2018; Geraldi et al. 2019). Giakoumi et al. (2019) showed that most management actions for marine bioinvasions are not ideal due to poor effectiveness, feasibility, acceptability, impacts on native species and cost. Management of marine invasive species becomes more difficult and costly as species progress through the invasion process (i.e. introduction, establishment, spread and impact) (Hulme 2009).

In Australia, three established marine invasive species are of national significance: Japanese kelp (*Undaria pinnatifida*), the northern Pacific seastar (*Asterias amurensis*) and the European shore crab (*Carcinus maenas*) (Marine Pest Sectoral Committee 2018). *Carcinus maenas* was listed due to its negative impacts on bivalve and seagrass communities and fisheries overseas (Grosholz et al. 2011; Leignel et al. 2014; Mach and Chan 2014). *Carcinus maenas* will not be eradicated from established areas, therefore management is

focused on prevention of *C. maenas* spread via human-mediated transport (ship biofouling, ballast water and aquaculture). This is prioritised to prevent establishment of this species in Western Australia and from spreading further to at-risk coastal habitats in southern and southeast Australia (Marine Pest Sectoral Committee 2020).

Research on *C. maenas* biology and ecology in southern Australia is warranted, as the Australian rapid response manual for *Carcinus maenas* (see Marine Pest Sectoral Committee 2020) highlights that understanding pest biology, ecology and life history is fundamental for an effective emergency response. Marine bioinvasion research assists with threat evaluations of marine pests, highlights feasibility of management responses, and helps with the development of effective methods for awareness, surveillance, containment, eradication and control (Marine Pest Sectoral Committee 2020). One of the key objectives in the Australian Marine Pest Plan 2018 – 2023 (Department of Agriculture and Water Resources 2018) is to support marine pest research and development to improve understanding on the biology of marine pests.

While this research was not focused on management strategies for invasive *C. maenas* in southern Australia, my findings may assist with identifying management strategies for this species. Chapter 2 supported findings by Clark et al. (2001) that morphology alone cannot differentiate between *C. maenas* and *C. aestuarii* and refuted that morphology can identify *Carcinus* spp. (see Behrens Yamada and Hauck 2001). While *Carcinus* is easily distinguishable from other crab genera, intraspecific variation and morphological overlap between *Carcinus* species makes them difficult to identify in the field. Carapace ratios and other morphological features mis-classify *Carcinus* species and a taxonomic reassessment of the genus may be valuable, especially because hybridisation has been observed (Geller et al. 1997; Darling 2011a; Jeffery et al. 2017; Mabin 2018; Cordone et al. 2020). Unless a clearly identified pathway of *Carcinus* can be located during new incursions, genetics is the most accurate method for confirming identity of *Carcinus* spp. Species confirmation is essential in marine bioinvasion management for determining the presence, extent and population dynamics of the marine invader, which is used to help categorise risk assessments and locate source populations (McGeoch et al. 2012; Hutchings 2018). Rapid responses to marine bioinvasions often start with confirming species identity and invaders are identified immediately to prevent species dispersal or mismanagement (Locke and Hanson 2009; Viard et al. 2016; Ma et al. 2019).

Colour polymorphism has been observed in *C. maenas* in the South Australian population, and assessments of carapace colour and pattern variation could add further evidence to support phenotypic plasticity between habitats (Todd et al. 2006; Stevens et al. 2014; Nokelainen et al. 2018). In addition to phenotypic variation, genetic adaptation could be examined using epigenetic, transcriptomic and loci under selection approaches, which could describe how invasive *C. maenas* overcome barriers to establish in novel environments and adapt to unfamiliar marine ecosystems (Tepolt and Palumbi 2015; Ardura et al. 2017; Eirin-Lopez and Putnam 2019; Fox et al. 2019; Angers et al. 2020). Understanding adaptation and tolerance of *C. maenas* is important because thermogeographic modelling predicts range expansion of this species under global climate change scenarios, including in southern Australia (Compton et al. 2010).

With further development the loci detected in Chapter 5 could be used to identify correlations with environmental clines in southern Australia, which have been demonstrated in invasive *C. maenas* in northeast America (Jeffery et al. 2018; Coyle et al. 2019). Comparisons of mitochondrial haplotypes and thermal tolerance may also reveal differences in the southeast Australian population, such as a cold-adapted haplotype in Tasmania (Coyle et al. 2019). Temperature and habitat type are important factors that determine where *C. maenas* can establish and expand their range, and therefore managers should prioritise habitats and environmental conditions that match thermal optimum limits and substrates of *C. maenas* (see Hampton and Griffiths 2007; Iacarella et al. 2015). Understanding phenotypic plasticity, genetic adaptation and environmental matching would help model changes in invasive *C. maenas* population dynamics and distributions in current and future scenarios (Keller et al. 2011; Iacarella et al. 2015).

Reproductive output and life history strategies are crucial factors that determine if introduced species can form self-sustaining populations (Ramirez Llodra 2002; Geburzi and McCarthy 2018). When introduced species start reproducing, population density increases and eradication becomes unlikely (Havel et al. 2015; Locke and Hanson 2019). My study showed that *C. maenas* has a prolonged spawning period lasting ~nine months in Gulf St Vincent and that timing of management strategies such as removal should account for seasonal reproduction. *Carcinus maenas* trapping and surveys should be focused prior to spawning events that occur in March onwards, as higher abundances of gravid and sexually mature females in winter-spring will increase larval density and dispersal. Fecundity estimates and

histology are also vital for understanding the reproductive output in invasive species populations and for predicting the timing and severity of new incursions (Lee et al. 2018).

Finally, genetic analysis of SNP loci assisted with identifying population genetic structure of *C. maenas* and reconstructed demographic history in South Australia. Genetic tools are increasingly used by resource managers and end-users as historical records or observations do not provide enough information to identify source populations of introduced species (Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Cristescu 2015; Darling et al. 2017). ABC analysis is useful for identifying source locations during early introductions (i.e. if *C. maenas* are sighted in Western Australia then rapidly identifying the source is essential). ABC analysis can confirm source populations for regions where the introduction history and source population are debated (i.e. Argentine *C. maenas*; Darling et al. 2008). While ABC analysis is time-consuming and complex (especially for genomic datasets), it is a powerful tool for understanding source populations when paired with biological information and historical records of a marine invader (Bourne et al. 2020). Determining demographic history early in the invasion process can promote heightened vigilance for monitoring and management against the identified source populations (Estoup and Guillemaud 2010). Should *C. maenas* expand its range in southern Australia, ABC analysis, genetic admixture and shared mtDNA DNA sequences can be used to rapidly locate source populations and develop intervention strategies.

6.4 Conclusion

Carcinus maenas has a global invasive distribution and is the most widespread intertidal crab in the world (Leignel et al. 2014). Although *C. maenas* is well studied in the northern hemisphere, the population biology and ecology of *C. maenas* in Australia were poorly described (Thresher et al. 2003; Young and Elliott 2019) prior to the commencement of my study. Invasive *C. maenas* have been established in southeast Australia for over a century; while they have not spread as rapidly as invasive *C. maenas* populations in North America, *C. maenas* has documented predatory impacts on native and commercially important bivalves in Australia which warrants management (Walton et al. 2002; Campbell et al. 2019). *Carcinus maenas* is unlikely to be eradicated anywhere in its established range, thus management efforts are directed at asset protection and preventing spread (i.e. containment) to at-risk coastal habitats such as Spencer Gulf, South Australia, and Fremantle, Western Australia (Marine Pest Sectoral Committee 2018). Management of marine invasive species benefits from research in their biology, physiology and ecology.

This thesis revealed aspects of invasive *C. maenas* population biology and identified morphological variation, high reproductive output and significant genetic structure in the South Australian population using multiple approaches. The findings are relevant not only to South Australia (i.e. should *C. maenas* continue to expand its range further along Gulf St Vincent or into Spencer Gulf), but also provide updated ecological understanding of this species in the southern hemisphere invasive range where researched is limited. As one of the three priority established marine pests in Australia, research on *C. maenas* is important for informing management decisions at State and Commonwealth Government levels and supports objectives for marine pest research and development as specified in the Australian Marine Pest Plan 2018 – 2023 (Department of Agriculture and Water Resources 2018). Marine habitats face rapid changes in the 21st century such as environmental shifts resulting from climate change, natural resource depletion and additional habitat modifications (Occhipinti-Ambrogi and Galil 2010; Ojaveer et al. 2018). Biological traits underpinning the success of globally distributed marine invaders such as *C. maenas* will determine rates of expansion, impacts on recipient communities, and management strategies of marine bioinvasions as significant changes continue to affect marine ecosystems worldwide.

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APPENDIX – CHAPTER 2

Table A2.1 Summary of sampling events to collect 1,311 *Carcinus* used for morphometric carapace ratios and rostrum shape. Habitats, sampling sites (with GPS coordinates), collection methods and sampling periods are displayed. The total number of crabs collected from each site and used in carapace ratio analysis is also shown. Asterisks (*) denote sampling sites that were only assessed once and are not displayed in the study map (Fig. 2.1). Crabs collected from 2013–2017 are from Dittmann et al. (2017), while crabs caught in 2018 and 2019 are from Chapter 3 and Chapter 4, respectively.

Habitat	Sampling site	GPS coordinates	Collection method used	Sampling period	N crabs
Mangrove	Middle Beach	S34°36.650' E138°24.700'	Trapping	July 2014 to Nov 2018	230
Mangrove	Port Gawler	S34°39.172' E138°26.334'	Trapping	May 2013 to Nov 2018	570
Harbour	Port Adelaide River	S34°48.796' E138°30.716'	Trapping	July 2014 to July 2018	141
Harbour	Old Port Reach	S34°50.964' E138°29.817'	Trapping	Sep 2014 to Nov 2018	93
Rocky shore	Hallett Cove	S35°04.847' E138°29.714'	Timed searches	Feb 2017 to Oct 2017	9
Rocky shore	Onkaparinga	S35°09.797' E138°28.283'	Trapping and timed searches	Oct 2015 to July 2019	166
Rocky shore	O'Sullivan Beach*	S35°07.164' E138°28.081'	Trapping	October 2018 (one event)	1
Rocky shore	Aldinga	S35°16.332' E138°26.609'	Timed searches	Dec 2015 to Oct 2018	101

Table A2.2 Carapace ratio summary of 128 male and female *Carcinus* used in DNA extractions. The number of crabs with CW:CL and CW:CH ratios that matched identities of *C. aestuarii*, *C. maenas* or undefined species is shown. The CW:CL and CW:CH ratios were obtained once from each of the 128 crabs. 59 of these 128 crabs were successfully sequenced, and their carapace ratios can be viewed in Table 2.4.

	CW:CL			CW:CH		
Identity	*C. aestuarii*	Undefined	*C. maenas*	*C. aestuarii*	Undefined	*C. maenas*
Ratio	≤ 1.27	1.28	≥ 1.29	≤ 2.26	2.27 – 2.31	≥ 2.32
Male *N*	1	0	78	5	6	68
Female *N*	5	5	39	18	6	25
Total *N*	**128**					

Table A2.3 Optimisation of PCR cycling parameters and reagent concentrations tested for three mtDNA COI primer pairs. Optimum conditions resulting from the tests of degenerate and *C. maenas* specific primers are highlighted in bold with asterisks (**) and yellow shading.

Primer pair	Test	PCR cycling parameters for primer optimisation	Reagents (µl per sample reaction)	Reference
HCO2198 and LCO1490 ("Universal")	1	94°C 5 min; (94°C 30 sec, 50°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min	Water (17.9 µl); MRT 5X buffer (5 µl); F-primer (0.5 µl); R-primer (0.5 µl); DNA template (1 µl)	Folmer et al. 1994
	2	94°C 5 min; (94°C 30 sec, 50°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min	Water (15.8 µl); MRT 5X buffer (5 µl); $MgCl_2$ 50mM (0.5 µl); F-primer (1.25 µl); R-primer (1.25 µl); Immolase 5u/µl (0.2 µl); DNA template (1 µl)	
	3	94°C 5 min; (94°C 30 sec, 55°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min		
COIF-PR115 and COIR-PR114 ("Degenerate")	1	94°C 5 min; (94°C 30 sec, 50°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min	Water (17.9 µl); MRT 5X buffer (5 µl); F-primer (0.5 µl); R-primer (0.5 µl); DNA template (1 µl)	Folmer et al. 1994
	2**	94°C 5 min; (94°C 30 sec, 50°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min	Water (15.8 µl); MRT 5X buffer (5 µl); $MgCl_2$ 50mM (0.5 µl); F-primer (1.25 µl); R-primer (1.25 µl); Immolase 5u/µl (0.2 µl); DNA template (1 µl)	
	3	94°C 5 min; (94°C 30 sec, 55°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min		
M1745 and M1746 ("*Carcinus maenas* specific")	1	94°C 5 min; (94°C 30 sec, 50°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min	Water (15.8 µl); MRT 5X buffer (5 µl); $MgCl_2$ 50mM (0.5 µl); F-primer (1.25 µl); R-primer (1.25 µl); Immolase 5u/µl (0.2 µl); DNA template (1 µl)	Roman and Palumbi 2004
	2**	94°C 5 min; (94°C 30 sec, 55°C 60 sec, 72°C 1 min) x 35 cycles; 72°C 15 min; 25°C 2 min		

Table A2.4 Sample details of crab mtDNA COI gene sequences (367 bp) analysed in this study, showing sample ID, species identity, locality, COI length before trim, and GenBank Accession Number. Sample IDs correspond to the phylogenetic tree shown in Fig. 2.6.

Sample ID	Species	Sample locality	COI length (before trim)	GenBank Accession Number
H1	*Carcinus*	Gulf St Vincent, SA	634	MT748791
H2	*Carcinus*	Gulf St Vincent, SA	634	MT748792
H3	*Carcinus*	Gulf St Vincent, SA	634	MT748793
H4	*Carcinus*	Gulf St Vincent, SA	634	MT748794
H5	*Carcinus*	Gulf St Vincent, SA	634	MT748795
H6	*Carcinus*	Gulf St Vincent, SA	634	MT748796
H7	*Carcinus*	Gulf St Vincent, SA	634	MT748797
H8	*Carcinus*	Gulf St Vincent, SA	634	MT748798
H9	*Carcinus*	Gulf St Vincent, SA	634	MT748799
H10	*Carcinus*	Gulf St Vincent, SA	634	MT748800
H11	*Carcinus*	Gulf St Vincent, SA	634	MT748801
H12	*Carcinus*	Gulf St Vincent, SA	634	MT748802
H13	*Carcinus*	Gulf St Vincent, SA	634	MT748803
H14	*Carcinus*	Gulf St Vincent, SA	634	MT748804
H15	*Carcinus*	Gulf St Vincent, SA	634	MT748805
H16	*Carcinus*	Gulf St Vincent, SA	634	MT748806
H17	*Carcinus*	Gulf St Vincent, SA	634	MT748807
H18	*Carcinus*	Gulf St Vincent, SA	634	MT748808
H19	*Carcinus*	Gulf St Vincent, SA	634	MT748809
M1	*Carcinus*	Gulf St Vincent, SA	634	MT748810
M2	*Carcinus*	Gulf St Vincent, SA	634	MT748811
M3	*Carcinus*	Gulf St Vincent, SA	634	MT748812
M4	*Carcinus*	Gulf St Vincent, SA	634	MT748813
M5	*Carcinus*	Gulf St Vincent, SA	634	MT748814
M6	*Carcinus*	Gulf St Vincent, SA	634	MT748815
M7	*Carcinus*	Gulf St Vincent, SA	634	MT748816
M8	*Carcinus*	Gulf St Vincent, SA	634	MT748817
M9	*Carcinus*	Gulf St Vincent, SA	634	MT748818
M10	*Carcinus*	Gulf St Vincent, SA	634	MT748819
M11	*Carcinus*	Gulf St Vincent, SA	634	MT748820
M12	*Carcinus*	Gulf St Vincent, SA	634	MT748821
M13	*Carcinus*	Gulf St Vincent, SA	634	MT748822
M14	*Carcinus*	Gulf St Vincent, SA	634	MT748823

M15	*Carcinus*	Gulf St Vincent, SA	634	MT748824
M16	*Carcinus*	Gulf St Vincent, SA	634	MT748825
M17	*Carcinus*	Gulf St Vincent, SA	634	MT748826
R1	*Carcinus*	Gulf St Vincent, SA	634	MT748827
R2	*Carcinus*	Gulf St Vincent, SA	634	MT748828
R3	*Carcinus*	Gulf St Vincent, SA	634	MT748829
R4	*Carcinus*	Gulf St Vincent, SA	634	MT748830
R5	*Carcinus*	Gulf St Vincent, SA	634	MT748831
R6	*Carcinus*	Gulf St Vincent, SA	634	MT748832
R7	*Carcinus*	Gulf St Vincent, SA	634	MT748833
R8	*Carcinus*	Gulf St Vincent, SA	634	MT748834
R9	*Carcinus*	Gulf St Vincent, SA	634	MT748835
R10	*Carcinus*	Gulf St Vincent, SA	634	MT748836
R11	*Carcinus*	Gulf St Vincent, SA	634	MT748837
R12	*Carcinus*	Gulf St Vincent, SA	634	MT748838
R13	*Carcinus*	Gulf St Vincent, SA	634	MT748839
R14	*Carcinus*	Gulf St Vincent, SA	634	MT748840
R15	*Carcinus*	Gulf St Vincent, SA	634	MT748841
R16	*Carcinus*	Gulf St Vincent, SA	634	MT748842
R17	*Carcinus*	Gulf St Vincent, SA	634	MT748843
R18	*Carcinus*	Gulf St Vincent, SA	634	MT748844
R19	*Carcinus*	Gulf St Vincent, SA	634	MT748845
R20	*Carcinus*	Gulf St Vincent, SA	634	MT748846
R21	*Carcinus*	Gulf St Vincent, SA	634	MT748847
R22	*Carcinus*	Gulf St Vincent, SA	634	MT748848
R23	*Carcinus*	Gulf St Vincent, SA	634	MT748849
C1	*Carcinus maenas*	Hooksiel, Germany	658	KT209504
C2	*Carcinus maenas*	Southeast Australia	403	KF709206
C3	*Carcinus maenas*	Southeast Australia	403	KF709201
C4	*Carcinus maenas*	United Kingdom	580	JQ306003
C5	*Carcinus maenas*	Seltjarnarnes, Iceland	502	AY616441
C6	*Carcinus maenas*	Bremerhaven, Germany	502	AY616439
C7	*Carcinus maenas*	Mongstadt, Norway	502	AY616438
C8	*Carcinus maenas*	Oeresund, Sweden	658	MG935203
C9	*Carcinus maenas*	English Channel, UK	654	MH931378
C10	*Carcinus maenas*	New Brunswick, Canada	507	MK634801
C11	*Carcinus maenas*	Maine USA	507	MK634890
C12	*Carcinus maenas*	Nova Scotia, Canada	507	MK634899
C13	*Carcinus maenas*	Canada, Newfoundland	658	MN184686

A1	*Carcinus aestuarii*	Turkey	654	KC789147
A2	*Carcinus aestuarii*	Sicily, Italy	619	JQ305890
A3	*Carcinus aestuarii*	Italy	480	JN990065
A4	*Carcinus aestuarii*	Mediterranean Sea/Portugal	546	HF952777
A5	*Carcinus aestuarii*	Tunisia	587	MG798808
N1	*Nectocarcinus bennetti*	New Zealand	658	HQ944638
O1	*Ovalipes australiensis*	Western Australia	658	MN184694
P1	*Portunus armatus*	Western Australia	658	MN184695

Table A2.5 Summary of linear carapace measurements for all male and female *Carcinus* collected from all sites and seasons in Gulf St Vincent, South Australia, between 2013–2019. Minimum (Min), maximum (Max), mean and standard deviation (± SD) values are displayed.

Carapace measurements (mm)	Males (n = 682)				Females (n = 629)			
	Min	Max	Mean	± SD	Min	Max	Mean	± SD
Carapace width (CW)	12.9	91.94	63.74	16.6	16.58	76.29	53.36	11.7
Carapace length (CL)	10.31	68.16	47.54	12.1	13.21	60.2	40.7	8.9
Carapace height (CH)	5.4	39.99	26.8	7.2	6.82	34.24	23.13	5.5

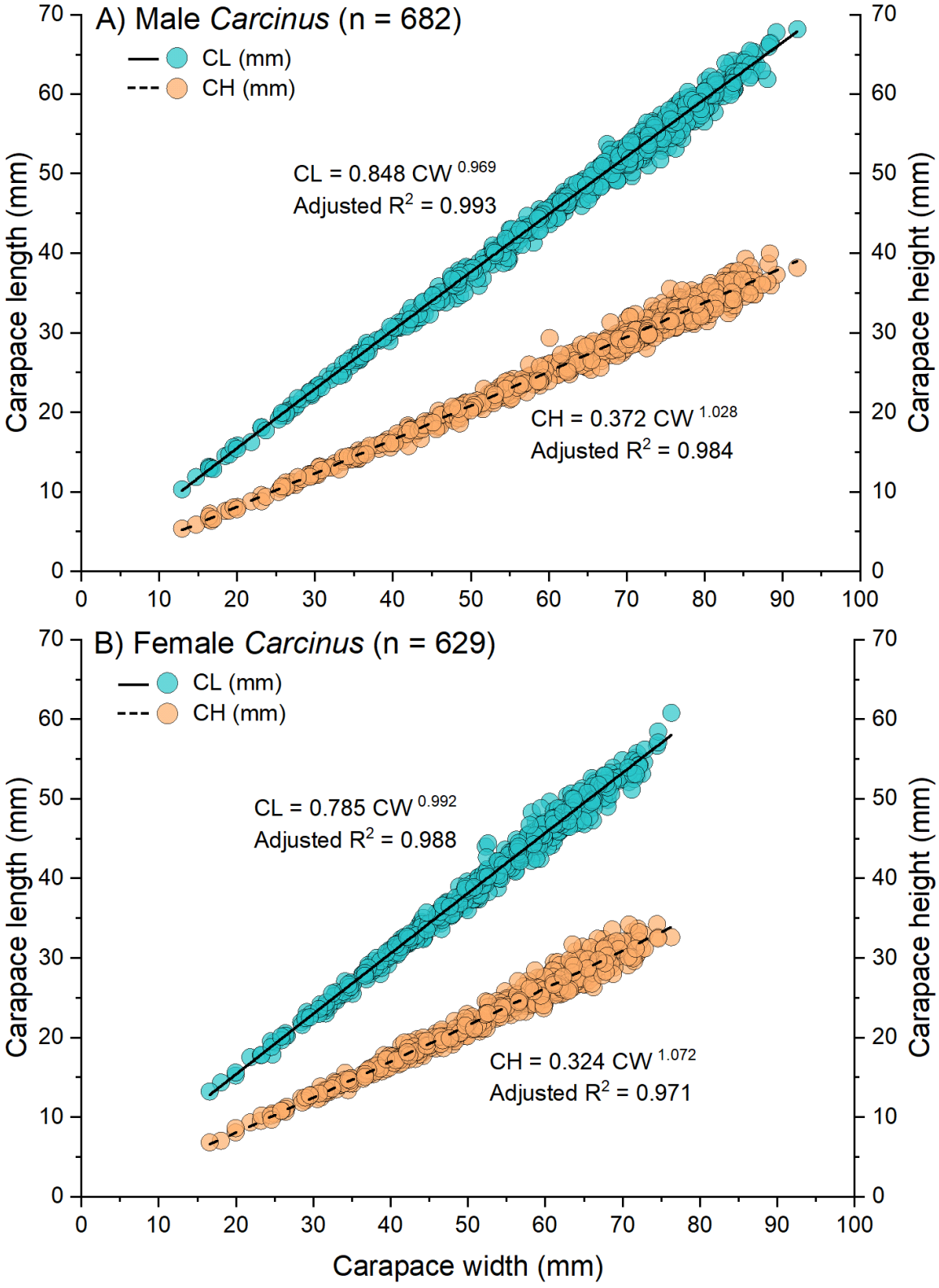

Figure A2.1 Allometric relationships for A) male *Carcinus*; and B) female *Carcinus* between carapace width (CW) and carapace length (CL; teal points, solid line) and carapace height (CH; orange points, dashed line). Lines display allometric fit with associated power functions.

APPENDIX – CHAPTER 3

Table A3.1 Descriptions of linear morphometric measurements and geometric landmark positions for *Carcinus maenas*. Associated linear dimensions can be seen in Fig. 3.2, while landmark positions can be seen in Fig. 3.3.

Measurement abbreviation	Description of linear dimensions measured with Vernier calipers
CW	Maximum carapace width (tips of both 5th antero-lateral teeth)
CL	Carapace length (outermost tip of rostrum to centre of posterior margin)
CH	Carapace height (most convex points on the top and bottom of carapace)
OGW	Optical groove notch width
CHL	Length of chelae (for both left and right chelae)
CHH	Height of chela (for both left and right chelae)
CHP	Length of propodus (excluding Pollex – for both left and right chelae)
PW	Width of penultimate pleomere 'pleon' (male and female)
PL	Pleon length including telson (male and female)
Landmark position	**Description of landmarks on dorsal surface of the carapace (left side only)**
1-2	Extreme tips of the middle and the left protrusions of the rostrum
3-4	Optical groove notch to tip of 1st antero-lateral tooth
5-6	Anterior notch and tip of the 2nd antero-lateral tooth
7-8	Anterior notch and tip of the 3rd antero-lateral tooth
9-10	Anterior notch and tip of the 4th antero-lateral tooth
11-12	Anterior notch and tip of the 5th antero-lateral tooth
13	Maximum curvature of posterior carapace margin
14	Centre of posterior margin on the carapace

Table A3.2 Digital camera settings and digital post-processing for photographing *Carcinus maenas* specimens and editing images used for landmark placement and geometric morphometric analysis. Example image of carapace photograph with distortion corrections applied shown underneath the table.

Camera settings and shooting mode		Digital editing process & software	
Camera type	Nikon D610 DSLR	Adaptive wide angle	Photoshop
Megapixels	24.3 megapixels	Lens correction	Photoshop
ISO	100	Image conversion to TPS	tpsUtil
Image format	RAW + JPEG	Landmark placement	tpsDig
Shooting mode	Manual	Image scaling/calibration	tpsDig
Shutter speed	4 seconds		
Focal length	50 mm		
Aperture	F/16		
Lens used	AF-S Nikkor 50 mm F/1.8G		
Resolution	300 dpi/1080 p		
Zoom/distance	None		
White balance	Manual, 5000 K		
Distortion control	Auto-straighten		
Shutter press	Hand-held remote		
Flash	Off		

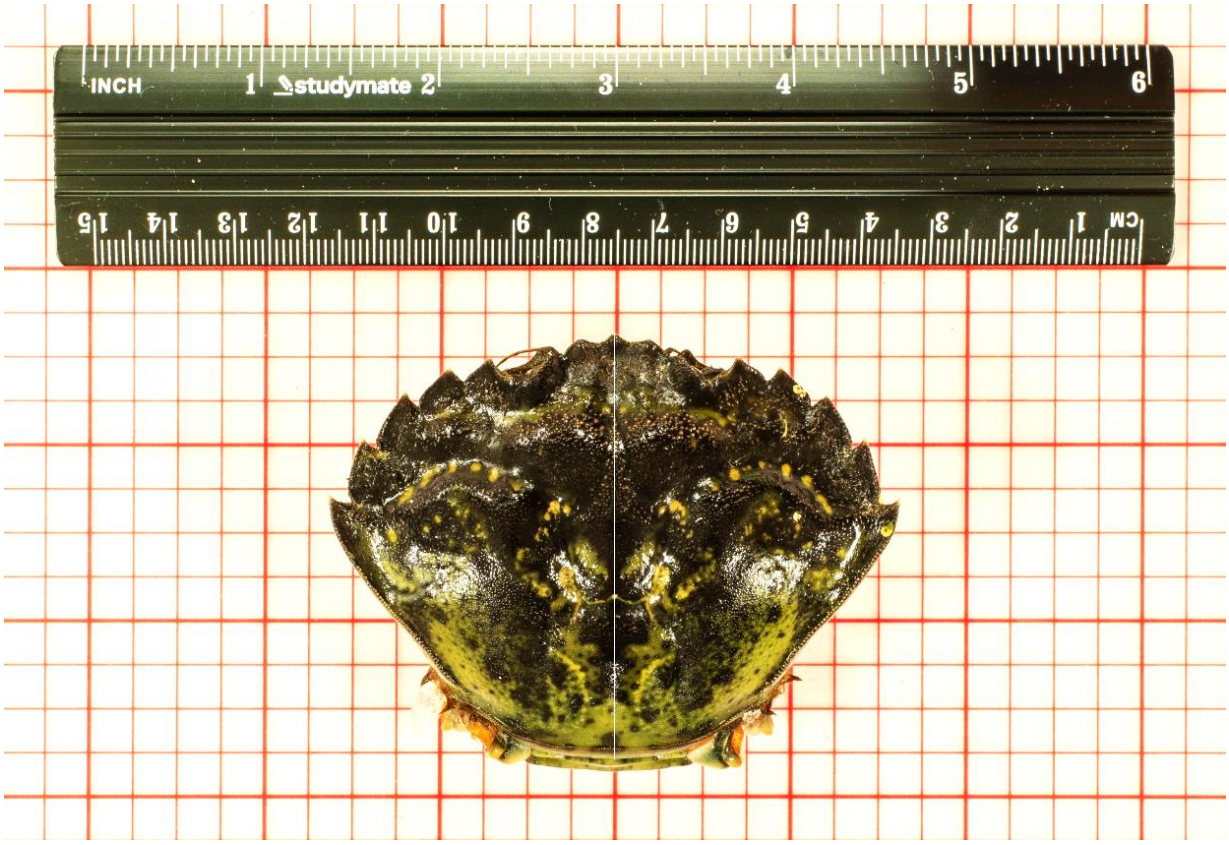

Table A3.3 Canonical Variate Analysis (CVA) loading scores for twelve linear measurements (log_{10}-transformed) and thirteen ratios measured from all male and female *Carcinus maenas* in Gulf St Vincent, South Australia. Corresponding CVA plots can be seen in Fig. 3.4.

Linear measurements				
	Males ♂		Females ♀	
Dimension	Axis 1	Axis 2	Axis 1	Axis 2
CW	33.04	48.24	1.57	16.10
CL	-25.80	-49.03	-26.29	-37.20
CH	20.83	-2.03	40.39	14.01
OGW	20.92	-57.56	20.36	-57.95
RCL	-22.54	14.42	-9.66	6.47
RCH	15.66	-5.04	15.57	-3.69
RPL	-7.62	-6.89	-5.05	18.45
LCL	-5.46	9.50	0.61	-4.53
LCH	7.39	-3.62	5.00	-5.02
LPL	-6.29	-8.35	2.53	16.13
PW	-7.70	-3.10	-20.59	24.40
PL	-9.50	63.36	-12.26	2.52
-	-	-	-	-

Ratios				
	Males ♂		Females ♀	
Ratio	Axis 1	Axis 2	Axis 1	Axis 2
CW/CL	-18.70	-27.49	-13.92	-8.62
CW/CH	6.10	0.29	9.30	-0.51
OGW/CL	-10.70	34.39	-2.20	-43.31
CW/OGW	-2.96	13.75	-2.29	-12.88
PW/PL	-10.60	-46.64	2.16	-34.49
PW/CL	29.60	95.08	3.89	44.82
PL/CW	1.31	-48.04	5.07	-64.96
CW/RCL	-1.49	-1.06	1.01	4.26
CW/LCL	1.32	0.51	3.30	-1.06
RCL/RCH	2.65	3.12	3.47	1.44
LCL/LCH	2.23	2.25	2.05	1.42
RPL/RCH	2.84	-2.77	1.64	-7.73
LPL/LCH	0.73	-2.22	-0.16	-6.48

Table A3.4 Canonical Variate Analysis (CVA) loading scores for fourteen geometric landmark coordinates from all male and female *C. maenas* in Gulf St Vincent, South Australia. Corresponding CVA plots can be seen in Fig. 3.5.

	Males ♂		Females ♀	
Landmark	Axis 1 (x)	Axis 2 (y)	Axis 1 (x)	Axis 2 (y)
1	0.28	0.19	0.28	0.20
2	0.22	0.18	0.22	0.19
3	0.10	0.11	0.10	0.12
4	0.06	0.16	0.05	0.17
5	0.01	0.12	0.01	0.12
6	-0.01	0.14	-0.02	0.15
7	-0.07	0.08	-0.07	0.08
8	-0.09	0.10	-0.10	0.10
9	-0.14	0.01	-0.14	0.00
10	-0.17	0.03	-0.17	0.02
11	-0.19	-0.09	-0.18	-0.09
12	-0.23	-0.08	-0.22	-0.09
13	-0.01	-0.43	0.00	-0.42
14	0.24	-0.54	0.24	-0.54

Table A3.5 Sequence variation of mtDNA COI gene among *C. maenas* sequenced from three habitats in Gulf St Vincent, South Australia (total crab n = 59). Numbers indicate the nucleotide positions in the first 634 bp of mtDNA COI region; dots represent identical nucleotides when compared with the H1 sequence. The number of crabs observed in each haplotype is indicated in parentheses.

Haplotype	**Nucleotide position**										**Frequency (%)**
	25	28	76	130	292	298	319	454	541	625	
H1 (22)	G	C	T	C	A	G	A	A	G	A	0.37 ± 0.06
H2 (13)	A	T	.	.	.	A	.	.	A	.	0.22 ± 0.05
H8 (6)	.	.	C	.	.	.	.	.	.	.	0.1 ± 0.04
H14 (11)	.	.	.	.	.	.	.	G	.	.	0.19 ± 0.05
M4 (6)	.	.	.	A	G	.	.	.	.	.	0.1 ± 0.04
R22 (1)	.	.	.	.	.	.	G	.	.	G	0.02 ± 0.02

Table A3.6 Sample details of *C. maenas* mtDNA COI sequences used this study, including habitat, mtDNA haplotype and GenBank Accession Number. N/A = no information provided. Linear morphometric measurements were taken from all sequenced specimens.

Sample ID	Sample site	Habitat	Sex	Date of sample collection	Date of DNA extraction	Undiluted gDNA concentration (ng/µm)	mtDNA haplotype	GenBank Accession Number
H1_503	Old Port Reach	Harbour	F	N/A	20/06/2017	1.1	H1	MT748791
H2_504	Old Port Reach	Harbour	M	N/A	20/06/2017	63	H2	MT748792
H3_513	Old Port Reach	Harbour	F	20/06/2017	20/06/2017	66	H1	MT748793
H4_531	Port Adelaide River	Harbour	M	28/03/2017	22/06/2017	0.141	H1	MT748794
H5_564	Port Adelaide River	Harbour	F	4/11/2016	22/06/2017	0.756	H1	MT748795
H6_605	Port Adelaide River	Harbour	F	27/06/2017	17/07/2017	109	H2	MT748796
H7_610	Old Port Reach	Harbour	M	27/06/2017	17/07/2017	0.819	H1	MT748797
H8_616	Port Adelaide River	Harbour	M	27/06/2017	17/07/2017	5.8	H8	MT748798
H9_632	Port Adelaide River	Harbour	F	27/06/2017	20/07/2017	0.28	H1	MT748799
H10_633	Port Adelaide River	Harbour	M	27/06/2017	20/07/2017	2.02	H1	MT748800
H11_636	Port Adelaide River	Harbour	F	27/06/2017	20/07/2017	0.308	H1	MT748801
H12_847	Port Adelaide River	Harbour	F	26/10/2017	22/03/2018	211	H2	MT748802
H13_969	Old Port Reach	Harbour	M	18/04/2018	7/08/2018	12	H2	MT748803
H14_1132	Old Port Reach	Harbour	F	28/09/2018	11/12/2018	51	H14	MT748804
H15_1218	Old Port Reach	Harbour	M	28/09/2018	9/06/2019	0.579	H1	MT748805
H16_1219	Old Port Reach	Harbour	M	12/10/2018	9/06/2019	1.45	H1	MT748806
H17_1227	Port Adelaide River	Harbour	F	16/06/2015	9/06/2019	1.47	H1	MT748807
H18_1229	Port Adelaide River	Harbour	F	16/06/2015	9/06/2019	0.127	H8	MT748808

H19_1234	Port Adelaide River	Harbour	M	16/06/2015	9/06/2019	15	H14	MT748809
M1_492	Middle Beach	Mangrove	M	4/11/2016	20/06/2017	66	H14	MT748810
M2_501	Port Gawler	Mangrove	M	4/11/2016	20/06/2017	24	H14	MT748811
M3_606	Port Gawler	Mangrove	M	27/06/2017	17/07/2017	153	H8	MT748812
M4_660	Port Gawler	Mangrove	F	8/07/2016	24/07/2017	5.6	M4	MT748813
M5_844	Middle Beach	Mangrove	F	27/06/2017	22/03/2018	140	H14	MT748814
M6_846	Port Gawler	Mangrove	F	2/07/2015	22/03/2018	195	M4	MT748815
M7_967	Port Gawler	Mangrove	M	18/05/2018	7/08/2018	8.1	H2	MT748816
M8_976	Port Gawler	Mangrove	F	27/06/2017	8/08/2018	2.68	H1	MT748817
M9_977	Middle Beach	Mangrove	F	27/06/2017	8/08/2018	0.867	H1	MT748818
M10_1099	Port Gawler	Mangrove	M	18/04/2018	11/12/2018	1.73	H2	MT748819
M11_1102	Middle Beach	Mangrove	M	13/07/2018	11/12/2018	1.6	H2	MT748820
M12_1110	Middle Beach	Mangrove	M	18/05/2018	11/12/2018	0.378	H2	MT748821
M13_1122	Middle Beach	Mangrove	M	13/07/2018	11/12/2018	Lower than blank	H1	MT748822
M14_1133	Port Gawler	Mangrove	F	28/09/2018	11/12/2018	74	M4	MT748823
M15_1134	Middle Beach	Mangrove	F	28/09/2018	11/12/2018	31	H2	MT748824
M16_1135	Middle Beach	Mangrove	F	28/09/2018	11/12/2018	37	H1	MT748825
M17_1245	Middle Beach	Mangrove	M	9/11/2018	9/06/2019	16	M4	MT748826
R1_470	Aldinga	Rocky shore	F	16/11/2016	19/06/2017	0.873	H1	MT748827
R2_471	Aldinga	Rocky shore	M	16/11/2016	19/06/2017	0.843	H1	MT748828
R3_530	Aldinga	Rocky shore	M	2/02/2017	22/06/2017	0.484	H14	MT748829
R4_571	Hallett Cove	Rocky shore	M	31/03/2017	23/06/2017	0.579	H2	MT748830
R5_845	Onkaparinga	Rocky shore	F	6/10/2017	22/03/2018	218	H8	MT748831

R6_973	Onkaparinga	Rocky shore	M	16/07/2018	7/08/2018	17	H1	MT748832
R7_1097	Onkaparinga	Rocky shore	M	15/08/2018	11/12/2018	0.161	M4	MT748833
R8_1098	Aldinga	Rocky shore	M	16/05/2018	11/12/2018	1.59	H2	MT748834
R9_1100	Aldinga	Rocky shore	M	16/07/2018	11/12/2018	2	H14	MT748835
R10_1107	Onkaparinga	Rocky shore	M	15/08/2018	11/12/2018	15	M4	MT748836
R11_1113	Onkaparinga	Rocky shore	M	15/08/2018	11/12/2018	11	H1	MT748837
R12_1114	Onkaparinga	Rocky shore	M	15/08/2018	11/12/2018	0.188	H14	MT748838
R13_1117	Onkaparinga	Rocky shore	M	15/08/2018	11/12/2018	9	H1	MT748839
R14_1120	Aldinga	Rocky shore	M	15/08/2018	11/12/2018	0.321	H1	MT748840
R15_1127	Onkaparinga	Rocky shore	M	19/04/2018	11/12/2018	5	H14	MT748841
R16_1128	Aldinga	Rocky shore	M	19/04/2018	11/12/2018	1	H1	MT748842
R17_1136	Aldinga	Rocky shore	F	1/10/2018	11/12/2018	38	H8	MT748843
R18_1137	Aldinga	Rocky shore	F	1/10/2018	11/12/2018	23	H8	MT748844
R19_1216	Aldinga	Rocky shore	M	15/10/2018	9/06/2019	54	H14	MT748845
R20_1217	Aldinga	Rocky shore	M	15/10/2018	9/06/2019	15	H14	MT748846
R21_1222	Onkaparinga	Rocky shore	M	5/10/2018	9/06/2019	12	H2	MT748847
R22_1242	Aldinga	Rocky shore	M	23/10/2017	9/06/2019	0.418	R22	MT748848
R23_1246	Onkaparinga	Rocky shore	M	7/11/2018	9/06/2019	0.887	H2	MT748849

APPENDIX – CHAPTER 4

Table A4.1 Sampling dates for monthly reproductive assessments of female *C. maenas* at six sampling sites using baited traps and timed search methods. Sampling occurred monthly between March and November 2018. Moon phases (○ = full moon, ● = new moon). Note that no timed searches occurred in March 2018.

Season	Month (2018)	Baited traps	Timed searches	Moon phase
Autumn	March	1st & 2nd	N/A	○
Autumn	April	17th & 18th	19th	●
Autumn	May	17th & 18th	16th	●
Winter	June	27th & 28th	18th	○ ●
Winter	July	12th & 13th	16th	●
Winter	August	13th & 14th	15th	●
Spring	September	27th & 28th	1st October	○
Spring	October	11th & 12th	15th	●
Spring	November	8th & 9th	7th	●

Figure A4.1 Flowchart showing the histology and H&E staining process for *C. maenas* ovary samples. Adapted and modified slightly from the standard Flinders University Microscopy method.

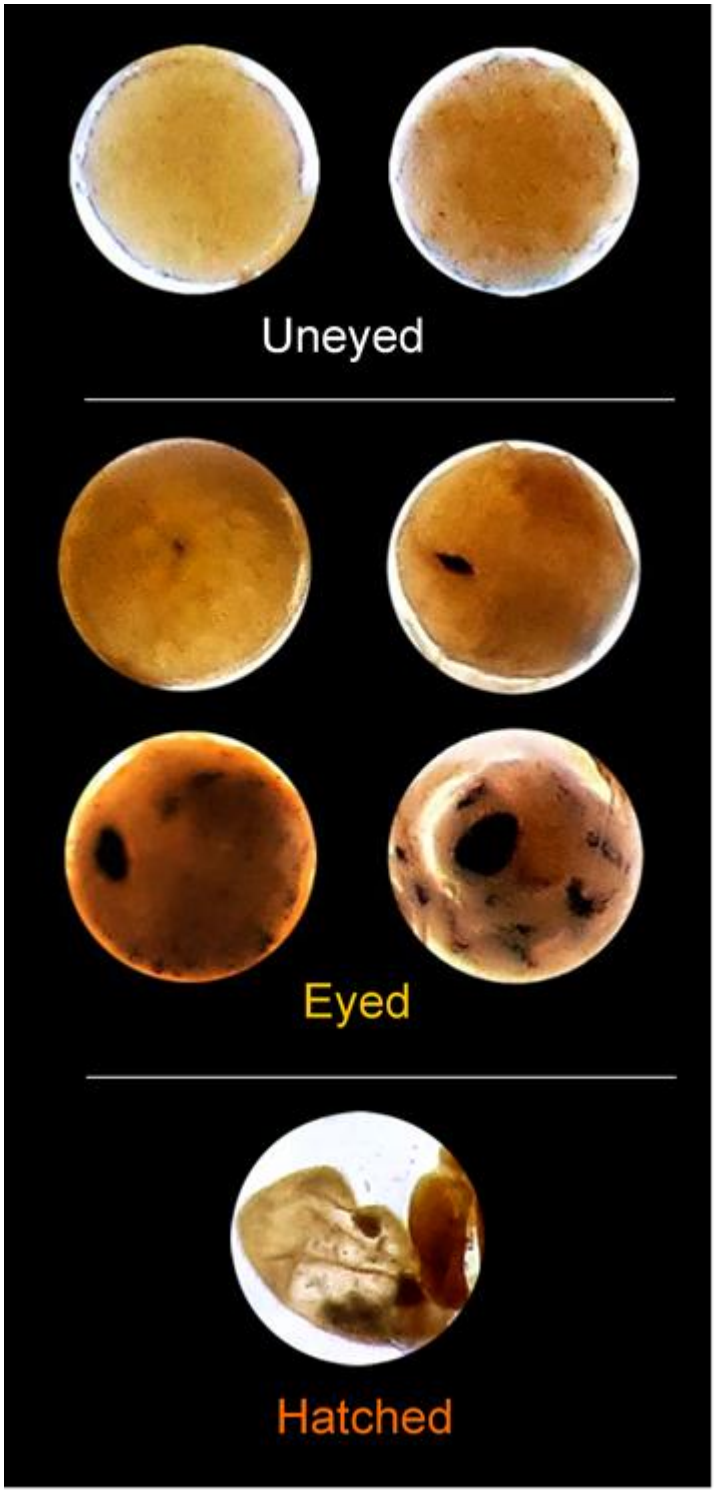

Figure A4.2 Development stages of fertilised eggs in gravid *C. maenas* clutches. Uneyed stages (yolk only) and eyed stages (developing embryo) were noted in fecundity estimates. Hatched stages were noted but not included in fecundity analysis.

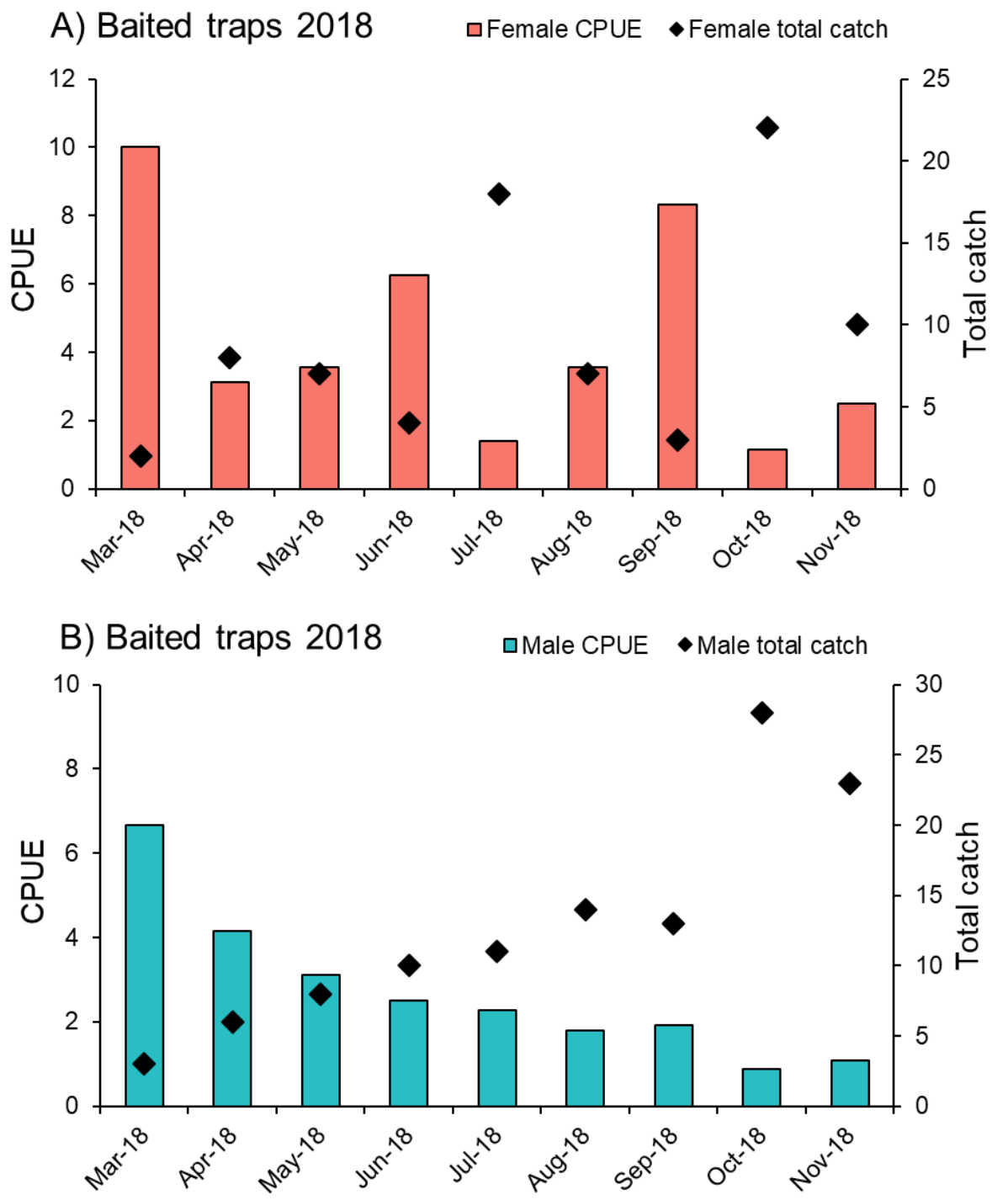

Figure A4.3 Baited trap CPUE (black diamonds) and total catch (coloured bars) of *C. maenas* caught during the 2018 sampling period in Gulf St. Vincent, South Australia. A) Female *C. maenas*; B) Male *C. maenas.* Please note the difference in y-axes.

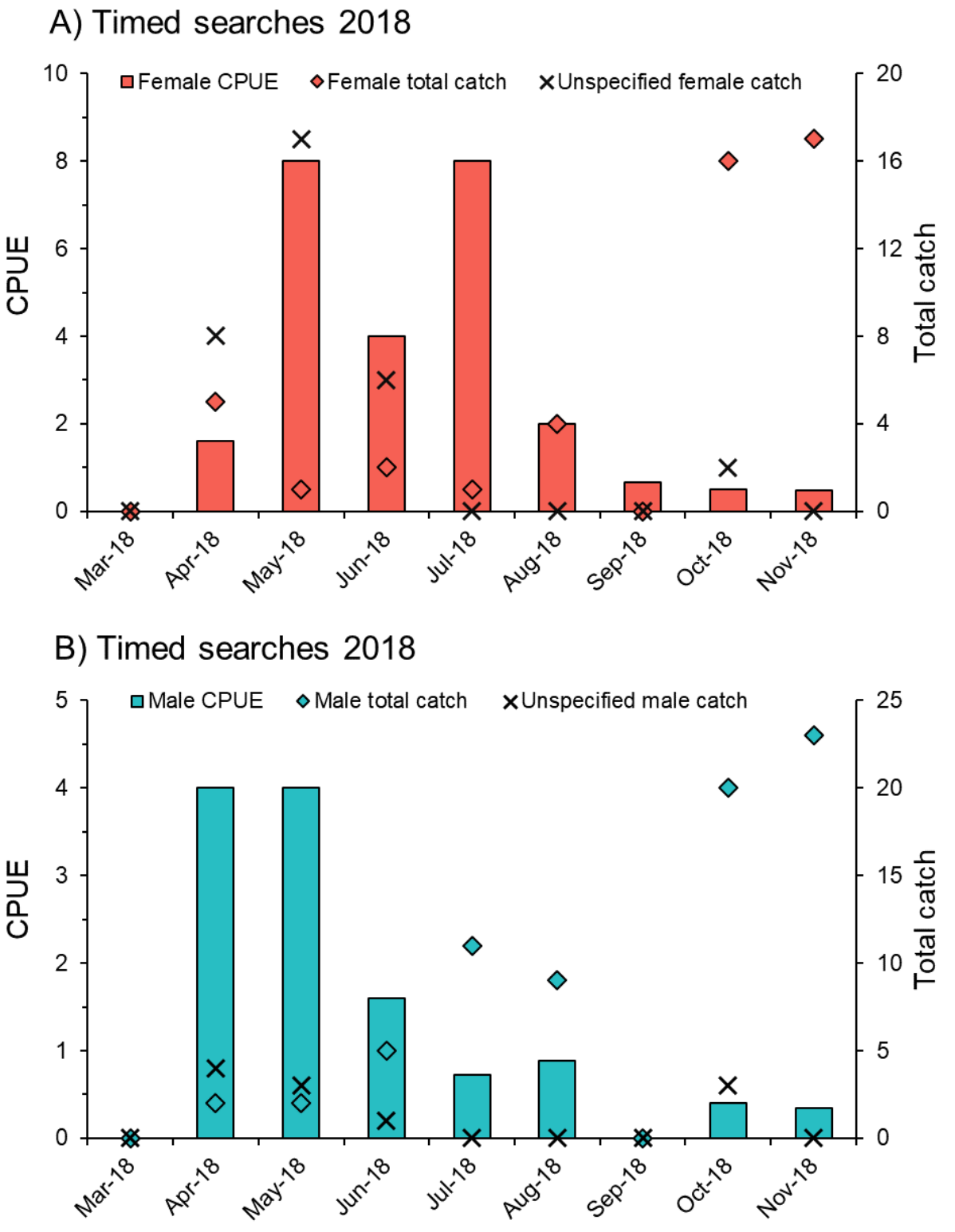

Figure A4.4 Timed search CPUE (coloured bars), total catch (diamonds) and unspecified catch (black crosses) of *C. maenas* collected during the 2018 sampling period in Gulf St Vincent, South Australia. A) Female *C.* maenas; B) Male *C. maenas.* Black crosses represent crabs that were collected with an unspecified method (either timed search or baited traps) at the Onkaparinga River site. Please note the difference in y-axes.

APPENDIX – CHAPTER 5

Table A5.1 *Carcinus maenas* sample details used for DArT-Seq™ sequencing as part of this study. Category 1 = samples that did not have high enough gDNA quantity and/or quality after extraction and excluded from sequencing entirely; Category 2 = samples were sent to DArT-Seq™ but did not pass the quality control pipeline; Category 3 = samples passed the DArT-Seq™ quality control pipeline, but did not pass the secondary filtering pipeline; Category 4 = samples successfully passed quality control and all filtering steps and were used for all data analyses. Duplicate samples not indicated in the table. N/A = no information provided for sex or carapace width.

Sample ID	Geographic location	Sample site	Sex	Carapace width (mm)	Date of sample collection	Date of DNA extraction	gDNA concentration (ng/µm)	Category
CMSAH1	South Australia	Port Adelaide River, Gulf St Vincent	F	59.6	4/11/2016	22/06/2017	18	3
CMSAH2	South Australia	Old Port Reach, Gulf St Vincent	M	58.06	27/06/2017	17/07/2017	9.2	4
CMSAH3	South Australia	Port Adelaide River, Gulf St Vincent	F	64.4	4/11/2016	20/06/2017	16	4
CMSAH4	South Australia	Port Adelaide River, Gulf St Vincent	F	44.2	26/10/2017	22/03/2018	23	4
CMSAH5	South Australia	Port Adelaide River, Gulf St Vincent	F	47.74	27/06/2017	17/07/2017	20	4
CMSAH6	South Australia	Old Port Reach, Gulf St Vincent	M	63.22	18/04/2018	7/08/2018	12	4
CMSAH7	South Australia	Old Port Reach, Gulf St Vincent	F	54.06	28/09/2018	11/12/2018	24	4
CMSAH8	South Australia	Port Adelaide River, Gulf St Vincent	M	46.79	27/06/2017	17/07/2017	5.8	3
CMSAH9	South Australia	Port Adelaide River, Gulf St Vincent	M	47.67	16/06/2015	9/06/2019	15	4
CMSAH10	South Australia	Old Port Reach, Gulf St Vincent	M	58.97	12/10/2018	9/06/2019	8.3	3
CMSAH11	South Australia	Old Port Reach, Gulf St Vincent	M	72.54	12/10/2018	9/06/2019	25	4
CMSAH12	South Australia	Old Port Reach, Gulf St Vincent	M	50.46	12/10/2018	15/06/2019	8.3	4
CMSAH13	South Australia	Port Adelaide River, Gulf St Vincent	F	51.92	16/06/2015	9/06/2019	16	4
CMSAH14	South Australia	Port Adelaide River, Gulf St Vincent	F	40.65	16/06/2015	9/06/2019	16	4
CMSAH15	South Australia	Port Adelaide River, Gulf St Vincent	M	57.49	18/04/2018	7/08/2018	44	4
CMSAH16	South Australia	Old Port Reach, Gulf St Vincent	M	56.11	13/07/2018	7/08/2018	26	4
CMSAM1	South Australia	Port Gawler, Gulf St Vincent	M	80.5	28/03/2017	19/06/2017	15	4
CMSAM2	South Australia	Port Gawler, Gulf St Vincent	M	80.21	4/11/2016	22/06/2017	37	4

CMSAM3	South Australia	Middle Beach, Gulf St Vincent	M	79.37	27/06/2017	20/07/2017	13	4
CMSAM4	South Australia	Port Gawler, Gulf St Vincent	F	63.59	21/06/2016	24/07/2017	18	3
CMSAM5	South Australia	Middle Beach, Gulf St Vincent	M	70.54	8/07/2016	24/07/2017	8.5	3
CMSAM6	South Australia	Port Gawler, Gulf St Vincent	M	79.98	27/06/2017	17/07/2017	21	4
CMSAM7	South Australia	Middle Beach, Gulf St Vincent	F	58.95	27/06/2017	22/03/2018	14	4
CMSAM8	South Australia	Port Gawler, Gulf St Vincent	M	70.25	18/05/2018	7/08/2018	8.1	4
CMSAM9	South Australia	Port Gawler, Gulf St Vincent	M	81.29	4/11/2016	20/06/2017	24	4
CMSAM10	South Australia	Port Gawler, Gulf St Vincent	F	63.43	2/07/2015	22/03/2018	29	4
CMSAM11	South Australia	Middle Beach, Gulf St Vincent	M	81.31	4/11/2016	20/06/2017	17	4
CMSAM12	South Australia	Middle Beach, Gulf St Vincent	F	59.21	28/09/2018	11/12/2018	37	4
CMSAM13	South Australia	Middle Beach, Gulf St Vincent	F	59.21	28/09/2018	11/12/2018	38	4
CMSAM14	South Australia	Middle Beach, Gulf St Vincent	F	72.11	28/09/2018	11/12/2018	31	4
CMSAM15	South Australia	Port Gawler, Gulf St Vincent	F	46.6	28/09/2018	11/12/2018	16	4
CMSAM16	South Australia	Port Gawler, Gulf St Vincent	M	80.86	9/11/2018	9/06/2019	49	4
CMSAM17	South Australia	Middle Beach, Gulf St Vincent	F	62.06	27/06/2017	17/07/2017	14	4
CMSAM18	South Australia	Middle Beach, Gulf St Vincent	M	83.73	9/11/2018	9/06/2019	16	4
CMSAM19	South Australia	Port Gawler, Gulf St Vincent	F	65.79	8/07/2016	24/07/2017	5.6	3
CMSAM20	South Australia	Middle Beach, Gulf St Vincent	M	71.87	21/06/2016	24/07/2017	48	3
CMSAM21	South Australia	Middle Beach, Gulf St Vincent	M	76.04	13/07/2018	7/08/2018	36	4
CMSAM22	South Australia	Middle Beach, Gulf St Vincent	M	74.31	18/04/2018	24/09/2018	12	4
CMSAM23	South Australia	Port Gawler, Gulf St Vincent	M	69.71	18/05/2018	24/09/2018	8.5	4
CMSAM24	South Australia	Port Gawler, Gulf St Vincent	M	75.67	8/07/2016	24/07/2017	11	4
CMSAM25	South Australia	Middle Beach, Gulf St Vincent	M	79.53	27/06/2017	17/07/2017	13	4
CMSAM26	South Australia	Middle Beach, Gulf St Vincent	M	75.09	4/11/2016	19/06/2017	17	4
CMSAM27	South Australia	Middle Beach, Gulf St Vincent	M	59.96	4/11/2016	22/06/2017	18	4
CMSAM28	South Australia	Port Gawler, Gulf St Vincent	M	75.88	8/07/2016	24/07/2017	21	4
CMSAM29	South Australia	Port Gawler, Gulf St Vincent	M	77.51	7/08/2015	19/06/2017	13	4
CMSAM30	South Australia	Middle Beach, Gulf St Vincent	M	62.67	13/07/2018	7/08/2018	26	4
CMSAM31	South Australia	Port Gawler, Gulf St Vincent	M	68.48	18/05/2018	7/08/2018	18	4
CMSAM32	South Australia	Middle Beach, Gulf St Vincent	F	69.97	27/06/2017	22/03/2018	18	4

CMSAM33	South Australia	Middle Beach, Gulf St Vincent	M	72.08	21/06/2016	24/07/2017	18	4
CMSAM34	South Australia	Middle Beach, Gulf St Vincent	M	74.67	13/07/2018	24/09/2018	7.4	4
CMSARS1	South Australia	Aldinga, Gulf St Vincent	M	55	16/11/2016	19/06/2017	20	4
CMSARS2	South Australia	Aldinga, Gulf St Vincent	F	49.73	23/10/2017	8/08/2018	15	4
CMSARS3	South Australia	Onkaparinga, Gulf St Vincent	F	61.02	6/10/2017	22/03/2018	52	4
CMSARS4	South Australia	Onkaparinga, Gulf St Vincent	M	54.8	16/07/2018	7/08/2018	17	4
CMSARS5	South Australia	Onkaparinga, Gulf St Vincent	M	42.21	15/08/2018	11/12/2018	9	4
CMSARS6	South Australia	Onkaparinga, Gulf St Vincent	M	59.49	19/04/2018	11/12/2018	5	4
CMSARS7	South Australia	Aldinga, Gulf St Vincent	F	42.47	1/10/2018	11/12/2018	23	4
CMSARS8	South Australia	Onkaparinga, Gulf St Vincent	M	52.87	15/08/2018	11/12/2018	11	4
CMSARS9	South Australia	Onkaparinga, Gulf St Vincent	M	43.26	15/08/2018	11/12/2018	15	4
CMSARS10	South Australia	Aldinga, Gulf St Vincent	M	41.538	15/10/2018	9/06/2019	15	3
CMSARS11	South Australia	Onkaparinga, Gulf St Vincent	M	70.02	5/10/2018	9/06/2019	12	3
CMSARS12	South Australia	Aldinga, Gulf St Vincent	M	67.91	15/10/2018	9/06/2019	17	4
CMSARS13	South Australia	O'Sullivans Beach, Gulf St Vincent	M	72.77	19/10/2018	15/06/2019	8	4
CMSARS14	South Australia	Aldinga, Gulf St Vincent	F	52.37	23/10/2017	8/08/2018	15	4
CMSARS15	South Australia	Onkaparinga, Gulf St Vincent	M	60.89	16/07/2018	7/08/2018	13	4
CMSARS16	South Australia	Onkaparinga, Gulf St Vincent	M	54.08	16/05/2018	24/09/2018	7.9	4
CMVIC1	Victoria	Jawbone Sanctuary, Port Phillip Bay	N/A	N/A	Jul-19	5/12/2019	5.4	3
CMVIC2	Victoria	Jawbone Sanctuary, Port Phillip Bay	N/A	N/A	Jul-19	5/12/2019	20	3
CMTAS1	Tasmania	Beauty Bay, Lindisfarne	M	51	26/06/2019	5/12/2019	49	4
CMTAS2	Tasmania	Beauty Bay, Lindisfarne	M	43	26/06/2019	5/12/2019	31	4
CMTAS3	Tasmania	Beauty Bay, Lindisfarne	M	40	26/06/2019	5/12/2019	48	4
CMTAS4	Tasmania	Beauty Bay, Lindisfarne	M	30	26/06/2019	5/12/2019	48	4
CMTAS5	Tasmania	Cornelian Bay	F	39	27/06/2019	5/12/2019	49	4
CMTAS6	Tasmania	Cornelian Bay	M	27	27/06/2019	5/12/2019	0.0885	1
CMTAS7	Tasmania	Cornelian Bay	M	20	27/06/2019	5/12/2019	0.333	1
CMNSW1	New South Wales	Clyde River	F	21.2	1/11/2008	5/12/2019	0.0418	1
CMNSW2	New South Wales	Clyde River	M	23.5	1/11/2008	5/12/2019	0.282	1
CMNSW3	New South Wales	Clyde River	F	22.0	1/11/2008	5/12/2019	0.126	1

CMNSW4	New South Wales	Clyde River	M	20.7	1/11/2008	5/12/2019	0.0317	1
CMNSW5	New South Wales	Clyde River	M	19.1	1/11/2008	5/12/2019	0.0545	1
CMNSW6	New South Wales	Pambula Lake	F	28.3	16/01/2008	5/12/2019	0.021	1
CMNSW7	New South Wales	Pambula Lake	F	19.3	16/01/2008	5/12/2019	0.0183	1
CMNSW8	New South Wales	Pambula Lake	M	24.2	12/02/2007	5/12/2019	0.01	1
CMNSW9	New South Wales	Pambula Lake	F	18.1	12/02/2007	5/12/2019	0.0075	1
CMNSW10	New South Wales	Pambula Lake	M	29.8	14/01/2008	5/12/2019	0.0175	1
CMNSW11	New South Wales	Wagonga Inlet	M	29.1	12/02/2013	5/12/2019	1.48	1
CMNSW12	New South Wales	Wagonga Inlet	F	26	12/02/2013	5/12/2019	0.546	1
CMNSW13	New South Wales	Wagonga Inlet	M	28.5	12/02/2013	5/12/2019	0.386	1
CMNSW14	New South Wales	Wagonga Inlet	F	28.4	12/02/2013	5/12/2019	10	4
CMNSW15	New South Wales	Wagonga Inlet	F	26.9	12/02/2013	5/12/2019	0.087	1
CMKR2	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	0.681	1
CMKR3	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	11	4
CMKR5	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	34	4
CMKR9	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	30	4
CMKR10	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	31	4
CMKR12	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	23	4
CMKR13	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	50	4
CMKR18	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	40	4
CMKR19	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	14	4
CMKR21	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	20	4
CMKR23	Sweden	Kristineberg, Fiskebäckskil	M	N/A	19/07/2019	5/12/2019	20	4
CMPA1	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	39	4
CMPA2	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	37	4
CMPA3	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	35	4
CMPA4	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	34	4
CMPA5	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	33	4
CMPA6	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	25	4
CMPA7	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	21	4

CMPA8	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	33	4
CMPA9	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	40	4
CMPA10	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	40	4
CMPA11	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	38	4
CMPA12	Portugal	Esteiro das Charradas, Faro	N/A	N/A	4/07/2019	13/2/2020	49	3
CMAFR1	South Africa	Table Bay harbour	M	72	16/07/2019	9/12/2020	42	4
CMAFR2	South Africa	Table Bay harbour	F	66	16/07/2019	9/12/2020	29	4
CMAFR3	South Africa	Table Bay harbour	M	76	16/07/2019	9/12/2020	43	4
CMAFR4	South Africa	Table Bay harbour	M	60	16/07/2019	9/12/2020	37	4
CMAFR5	South Africa	Table Bay harbour	M	74	16/07/2019	9/12/2020	39	4
CMAFR6	South Africa	Table Bay harbour	M	68	16/07/2019	9/12/2020	32	4
CMAFR7	South Africa	Table Bay harbour	F	42	16/07/2019	9/12/2020	12	4
CMAFR8	South Africa	Table Bay harbour	M	51	16/07/2019	9/12/2020	33	4
CMAFR9	South Africa	Table Bay harbour	F	51	16/07/2019	9/12/2020	28	4
CMAFR10	South Africa	Table Bay harbour	M	50	16/07/2019	9/12/2020	39	4
CMAFR11	South Africa	Table Bay harbour	F	57	16/07/2019	9/12/2020	22	4
CMNS1	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	27	4
CMNS2	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	13	4
CMNS3	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	20	4
CMNS4	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	11	4
CMNS5	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	20	4
CMNS6	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	24	4
CMNS7	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	40	4
CMNS8	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	10	4
CMNS9	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	18	4
CMNS10	Nova Scotia, CAN	St Catherines River	M	N/A	Sep-19	9/12/2019	28	4
CMMA1	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	49	4
CMMA2	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	22	4
CMMA3	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	19	4
CMMA4	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	44	4

CMMA5	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	29	4
CMMA6	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	36	4
CMMA7	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	24	4
CMMA8	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	22	4
CMMA9	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	21	4
CMMA10	Maine, USA	Biddeford Pool	F	N/A	Sep-19	9/12/2019	23	4
CMWA1	Washington, USA	Dungeness NWR, Puget Sound	F	47	17/05/2019	7/1/2020	15	3
CMWA2	Washington, USA	Dungeness NWR, Puget Sound	M	60	9/05/2019	7/1/2020	5.5	3
CMWA3	Washington, USA	Dungeness NWR, Puget Sound	M	82	17/05/2019	7/1/2020	35	4
CMWA4	Washington, USA	Dungeness NWR, Puget Sound	F	47	17/05/2019	7/1/2020	10	2
CMWA5	Washington, USA	Dungeness NWR, Puget Sound	M	52	17/05/2019	7/1/2020	9.8	4
CMWA6	Washington, USA	Chicken Coop Creek, Puget Sound	M	80	7/05/2019	7/1/2020	23	4
CMWA7	Washington, USA	Scow Bay, Puget Sound	M	62	21/09/2018	7/1/2020	37	4
CMWA8	Washington, USA	Jimmycomelately Creek, Puget Sound	F	52	15/08/2017	7/1/2020	43	4
CMWA9	Washington, USA	Kala Point Lagoon, Puget Sound	M	77	8/09/2018	7/1/2020	38	4
CMWA10	Washington, USA	Sequim Bay, Puget Sound	M	79.7	21/06/2019	7/1/2020	22	4
CMWA11	Washington, USA	Samish Bay, Puget Sound	M	73	3/01/2019	7/1/2020	9.4	4
CMWA12	Washington, USA	Samish Bay, Puget Sound	M	63	5/01/2019	7/1/2020	37	4
CMWA13	Washington, USA	Lagoon Point, Puget Sound	M	57.5	5/06/2018	7/1/2020	41	4
CMWA14	Washington, USA	Dungeness Landing, Puget Sound	M	55	15/06/2018	7/1/2020	37	4
CMWA15	Washington, USA	Westcott Bay, Puget Sound	M	61	26/06/2018	7/1/2020	42	4
CMS1BC1	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	17	4
CMS1BC2	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	18	4
CMS1BC3	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	11	4
CMS1BC4	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	19	4
CMS1BC5	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	19	4
CMS1BC6	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	25	4
CMS1BC7	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	15	4
CMS1BC8	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	39	4
CMS1BC9	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	28	4

CMS1BC10	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	19	4
CMS1BC11	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	27	4
CMS1BC12	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	40	4
CMS1BC13	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	45	4
CMS1BC14	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	25	4
CMS1BC15	British Columbia, CAN	Pipestem Inlet, Barkley Sound	N/A	N/A	Jul-19	12/12/2019	21	4
CMS2BC1	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	23	4
CMS2BC2	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	14	4
CMS2BC3	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	2.02	1
CMS2BC4	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	22	4
CMS2BC5	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	14	4
CMS2BC6	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	25	4
CMS2BC7	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	35	4
CMS2BC8	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	16	4
CMS2BC9	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	20	4
CMS2BC10	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	16	4
CMS2BC11	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	15	4
CMS2BC12	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	19	4
CMS2BC13	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	15	4
CMS2BC14	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	41	4
CMS2BC15	British Columbia, CAN	Hutchinson Cove, Sooke Basin	N/A	N/A	Jul-19	12/12/2019	15	4

Table A5.2 Summary table of five relatedness estimates with 95% confidence intervals for dyads of *C. maenas* in each population. *N* Dyads are the numbers or pairwise comparisons in each population. The number of individuals in Portugal are highlighted in bold with an asterisk as one individual had an extremely high relatedness ($r > 0.5$) and was subsequently removed from the dataset. The resulting number of individuals for Portugal are shown in parentheses. All relatedness estimates were based on 3,512 loci as per the filtering pipeline (Table 5.2).

Population	*N* Ind	*N* Dyads	Wang [95% CI]	LynchLi [95% CI]	LynchRd [95% CI]	Ritland [95% CI]	QuellerGt [95% CI]
South Australia	57	1596	-0.044 [-0.115 – 0.026]	-0.062 [-0.137 – 0.011]	-0.018 [-0.05 – 0.016]	-0.019 [-0.053 – 0.017]	-0.018 [-0.077 – 0.041]
Southeast Australia	6	15	-0.044 [-0.115 – 0.026]	-0.096 [-0.178 – -0.016]	-0.201 [-0.245 – -0.156]	-0.198 [-0.244 – -0.151]	-0.200 [-0.267 – -0.131]
Sweden	10	45	-0.002 [-0.067 – 0.062]	-0.041 [-0.114 – 0.03]	-0.111 [-0.145 – -0.077]	-0.109 [-0.144 – -0.073]	-0.114 [-0.178 – -0.049]
Portugal	**12*** (11)	66	-0.001 [-0.069 – 0.065]	-0.042 [-0.118 – 0.032]	-0.091 [-0.124 – -0.057]	-0.091 [-0.126 – -0.055]	-0.09 [-0.154 – -0.026]
South Africa	11	55	-0.046 [-0.115 – 0.022]	-0.103 [-0.182 – -0.026]	-0.101 [-0.134 – -0.067]	-0.104 [-0.140 – -0.067]	-0.101 [-0.163 – -0.038]
Nova Scotia	10	45	-0.019 [-0.085 – 0.045]	-0.067 [-0.141 – 0.005]	-0.112 [-0.146 – -0.076]	-0.112 [-0.148 – -0.075]	-0.111 [-0.174 – -0.049]
Maine	10	45	-0.002 [-0.066 – 0.062]	-0.040 [-0.112 – 0.03]	-0.111 [-0.147 – -0.075]	-0.109 [-0.146 – -0.072]	-0.112 [-0.175 – -0.049]
Washington	12	66	-0.023 [-0.091 – 0.043]	-0.061 [-0.136 – 0.012]	-0.091 [-0.129 – -0.053]	-0.092 [-0.131 – -0.052]	-0.092 [-0.154 – -0.029]
British Columbia	29	406	-0.026 [-0.097 – 0.044]	-0.047 [-0.122 – 0.026]	-0.036 [-0.073 – 0.003]	-0.036 [-0.075 – 0.002]	-0.035 [-0.097 – 0.026]

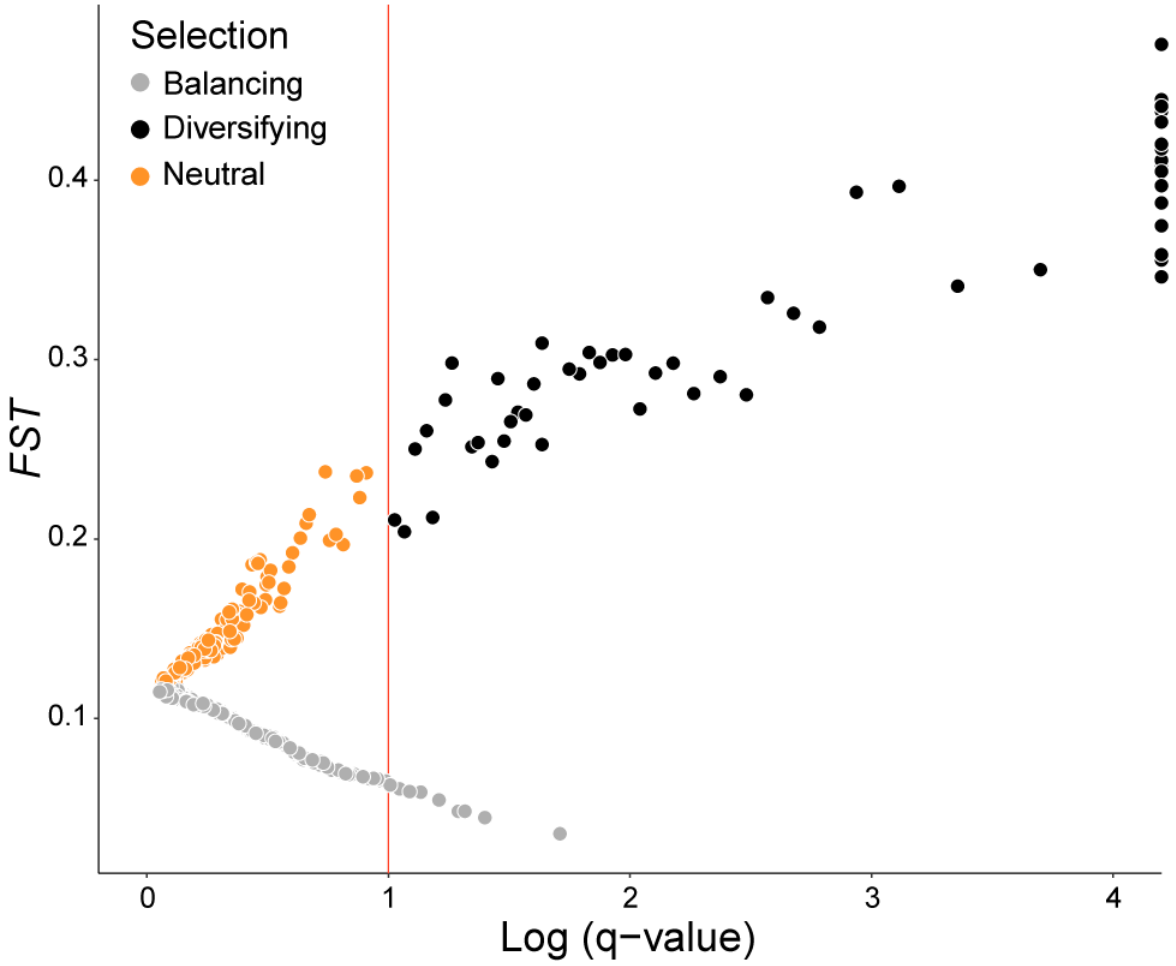

Figure A5.1 Separation of 69 outlier SNP loci based on BAYESCAN F_{ST} outlier detection in *C. maenas*. Black circles represent loci under putative diversifying selection (n = 60 loci), grey circles represent loci under balancing selection (n = 1,764 loci), and orange circles represent loci under neutral selection (n = 1,688 loci). The vertical red line indicates the q-value threshold (0.1), where circles that fall to the right of the threshold line are outlier loci. Of the 69 outlier loci detected, 60 were potentially under diversifying selection, and nine loci were under balancing selection. Genomic scan conducted on 3,512 SNPs, 156 individuals and nine geographic populations.

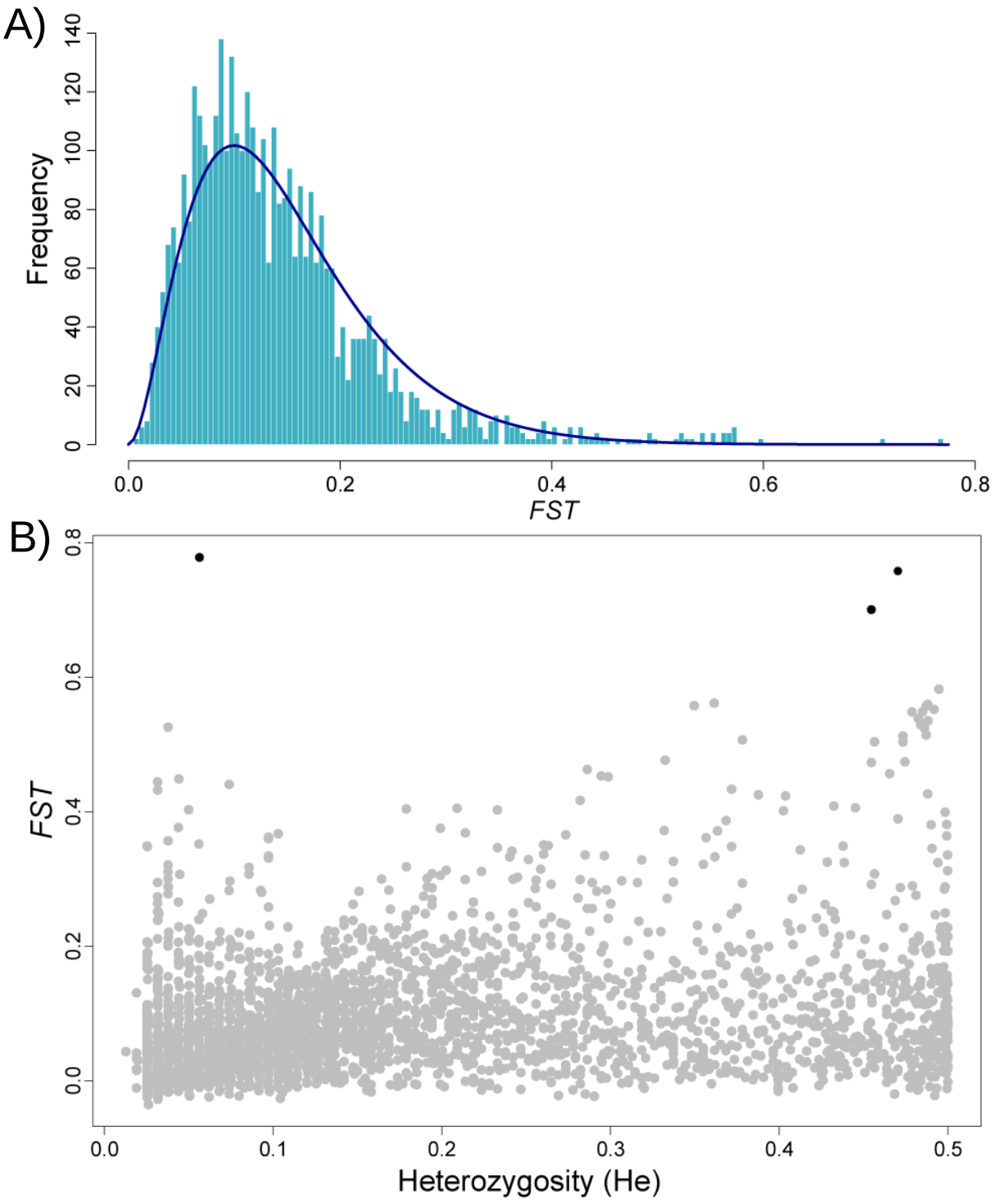

Figure A5.2 A) *OutFLANK* frequency distribution of per locus F_{ST} without sample size correction, with the solid line indicating the smoothed distribution of the null model based on left and right-trim fractions. B) Separation of putatively neutral and adaptive SNP loci based on *OutFLANK* F_{ST} outlier detection in *C. maenas*. The black circles indicate three outlier loci that were potentially under selection and removed from the dataset (q-value threshold = 0.1, minimum expected heterozygosity = 0.1). Genomic scan conducted on 3,512 SNPs, 156 individuals and nine geographic populations.

Table A5.3 Isolation by distance results of nine *C. maenas* populations displaying geographic pairwise distances (below diagonal) and genetic pairwise distances (above diagonal) for 3,509 SNPs, 156 individuals and nine geographic populations.

	South Australia (n = 57)	Southeast Australia (n = 6)	Sweden (n = 10)	Portugal (n = 11)	South Africa (n = 11)	Nova Scotia (n = 10)	Maine (n = 10)	Washington (n = 12)	British Columbia (n = 29)
South Australia		0.133	0.102	0.081	0.084	0.140	0.154	0.168	0.171
Southeast Australia	14.182		0.155	0.172	0.138	0.175	0.172	0.203	0.210
Sweden	16.741	16.816		0.060	0.029	0.039	0.098	0.135	0.141
Portugal	16.729	16.802	15.247		0.038	0.097	0.127	0.132	0.137
South Africa	16.408	16.486	16.306	16.008		0.061	0.091	0.120	0.127
Nova Scotia	17.017	17.071	15.999	15.673	16.399		0.091	0.129	0.144
Maine	17.039	17.091	16.065	15.762	16.428	13.331		0.022	0.038
Washington	17.244	17.286	16.527	16.371	16.744	15.683	15.586		0.006
British Columbia	17.251	17.293	16.539	16.387	16.756	15.715	15.621	12.314	

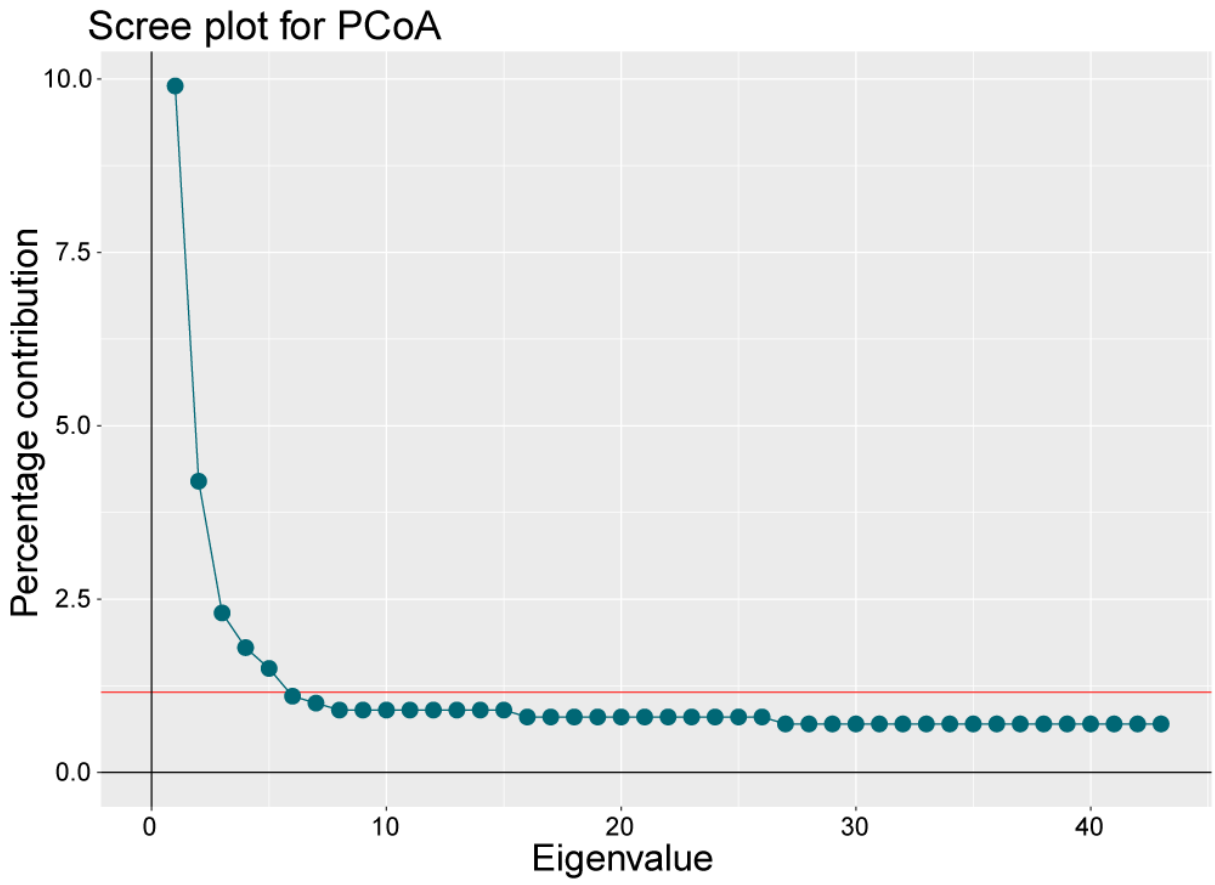

Figure A5.3 Scree plot of PCoA eigenvalues showing the percentage of variation explained by each axis.

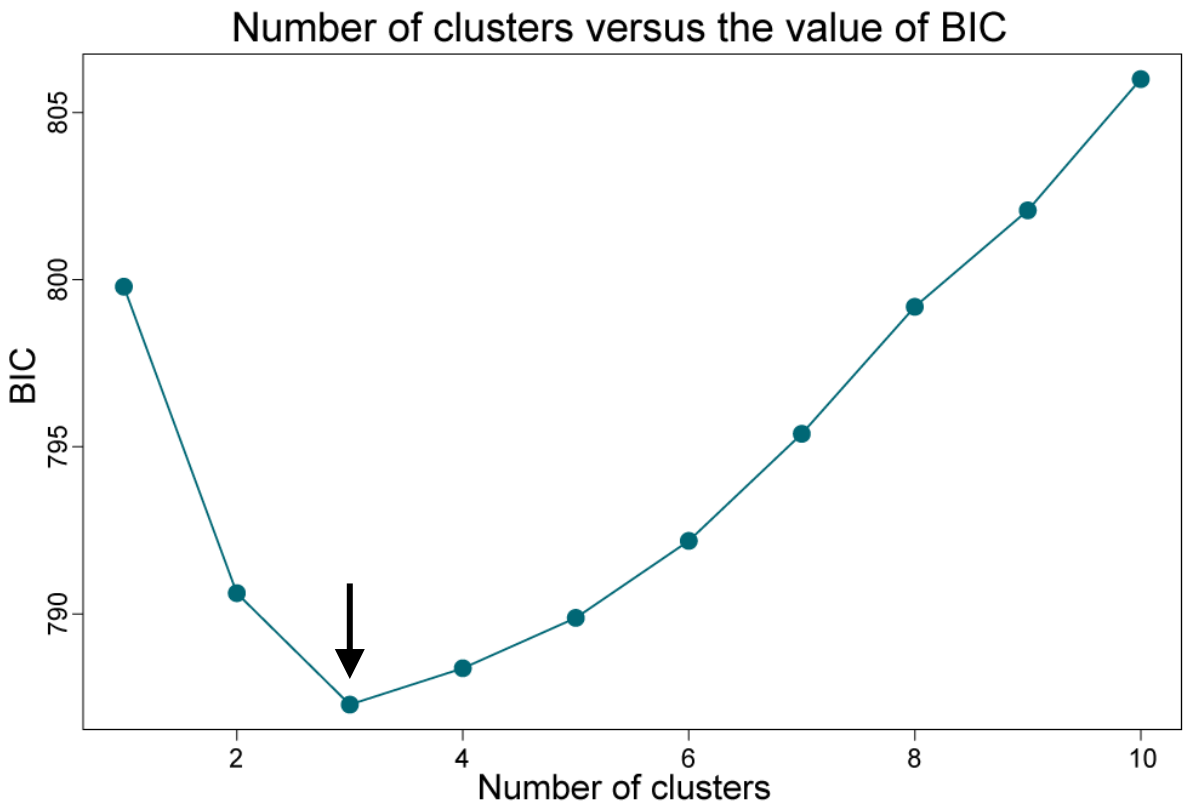

Figure A5.4 Bayesian Information Clustering (BIC) values resulting from the DAPC analysis of *C. maenas* populations. The optimum number of clusters (*K*) is inferred from the lowest BIC value that falls after an "elbow" in the curve of BIC values. The optimum $K = 3$ is highlighted by an arrow where the BIC value was lowest at 787.29.

Table A5.4 Results from STRUCTURE SELECTOR displaying the optimum *K* values for Bayesian STRUCTURE analysis. For the Evanno Δ*K* method, yellow highlights the value that corresponds to an optimum *K* = 2. For the mean LnP(K) method, blue highlights the value that corresponds to an optimum *K* = 5. For the MedMedK and MedMeanK methods, the optimum *K* = 5 are highlighted in green. Based on the whole filtered dataset of 3,509 SNPs, 156 individuals and nine populations. Corresponding optimal *K* plots can be seen in Appendix Fig. A5.5.

Loci	Ind	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
3,509	156	1	5	-310,138	3.48	—	—	—
3,509	156	2	5	-288,542	10.82	21,596	12,542	1159.5
3,509	156	3	5	-279,488	8.05	9053	6592	806.81
3,509	156	4	5	-276,927	29.94	2560	1669	55.77
3,509	156	5	5	-276,036	859.60	891.2	151,901	176.7
3,509	156	6	5	-427,046	277,394	-151,010	250,551	0.9
3,509	156	7	5	-327,505	83,790	99,541	223,093	2.66
3,509	156	8	5	-451,057	392,762	-123,552	165,147	0.42
3,509	156	9	5	-409,462	178,138	41,594	375,560	2.1
3,509	156	10	5	-743,428	560,250	-333,965	—	—

Loci	Ind	K (range)	MedMedK	MedMeanK	MaxMedK	MaxMeanK
3,509	156	1-10	**5**	**5**	6	6

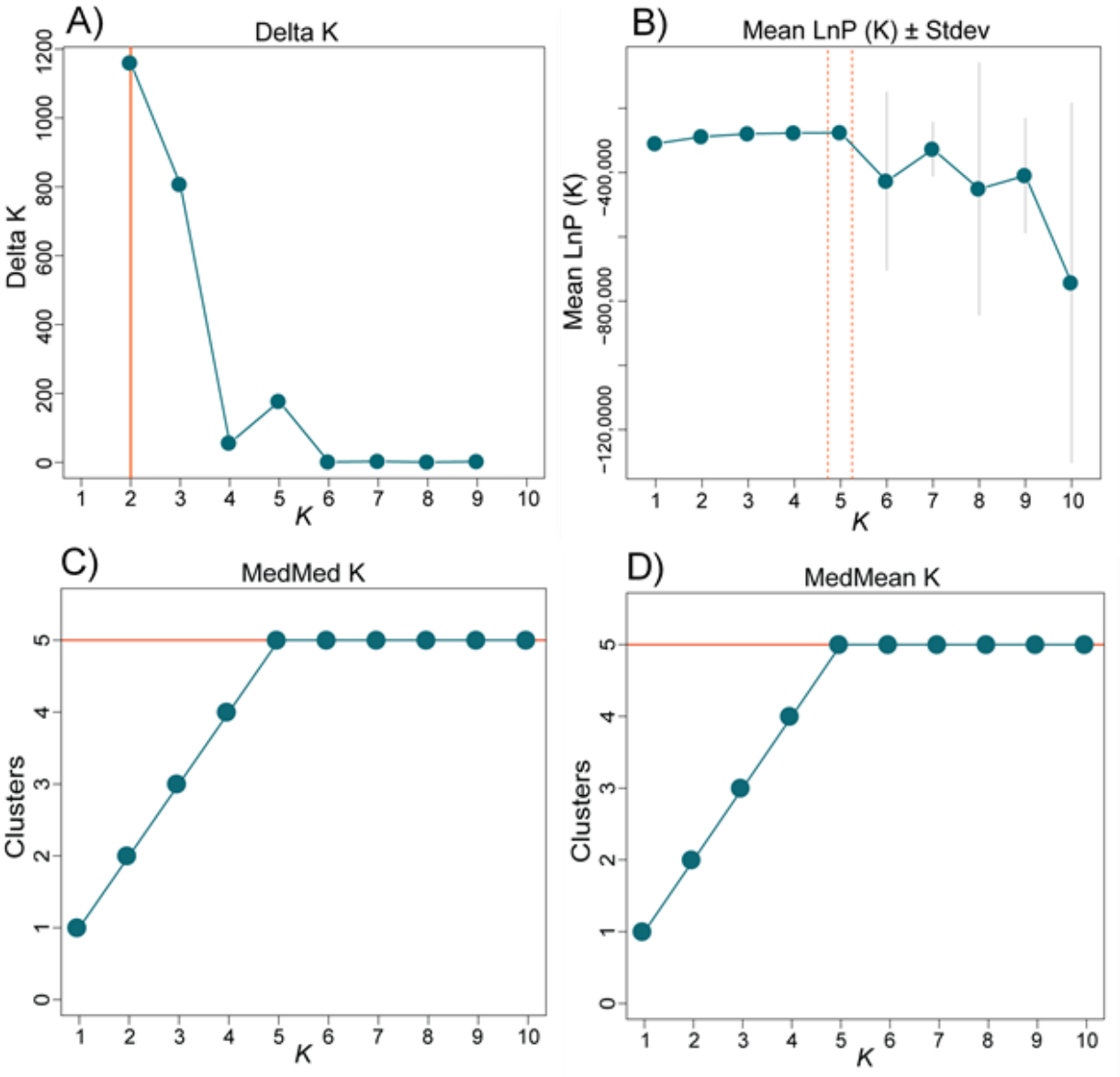

Figure A5.5 Results from STRUCTURE SELECTOR displaying the optimum *K* for Bayesian STRUCTURE analysis. A) the Evanno Δ*K* method showing *K* = 2; B) the mean LnP(K) method showing *K* = 5; C) the MedMedK method showing *K* = 5; and D) the MedMeanK method showing *K* = 5. The red lines indicate the optimum *K*. Based on the whole filtered dataset of 3,509 SNPs, 156 individuals and nine populations. Corresponding values can be seen in Appendix Table A5.4.

Table A5.5 Hierarchical structure of the first major genetic cluster resulting from the primary STRUCTURE analysis. Based on 3,389 loci, 105 individuals, and six populations (South Australia, southeast Australia, Sweden, Portugal, South Africa, and Nova Scotia). For the Evanno Δ*K* method, yellow highlights the value that corresponds to an optimum *K* = 2. For the mean LnP(K) method, blue highlights the value that corresponds to an optimum *K* = 4. For the MedMedK and MedMeanK methods, the optimum *K* = 4 are highlighted in green.

Loci	Ind	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
3,389	105	1	5	-203,107	4.36	—	—	—
3,389	105	2	5	-192,752	8.8	10,355	7789	885.9
3,389	105	3	5	-190,187	7.8	2565	1821	233.65
3,389	105	4	5	-189,443	770.13	743.92	28,528	37.04
3,389	105	5	5	-217,227	28,287	-27,784	32,058	1.13
3,389	105	6	5	-212,953	51,410	4274	—	—

Loci	Ind	K (range)	MedMedK	MedMeanK	MaxMedK	MaxMeanK
3,389	105	1-6	**4**	**4**	4	4

Table A5.6 Hierarchical structure of the second major genetic cluster resulting from the primary STRUCTURE analysis. Based on 2,347 loci, 51 individuals, and three populations (Maine, Washington and British Columbia). For the Evanno Δ*K* method, yellow highlights the value that corresponds to an optimum *K* = 2. For the mean LnP(K) method, blue highlights the value that corresponds to an optimum *K* = 2. For the MedMedK and MedMeanK methods, the optimum *K* = 2 are highlighted in green.

Loci	Ind	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2,347	51	1	5	-86,527	2.41	—	—	—
2,347	51	2	5	-83,863	13.8	2664	8330	603.84
2,347	51	3	5	-89,529	5114	-5666	—	—

Loci	Ind	K (range)	MedMedK	MedMeanK	MaxMedK	MaxMeanK
2,347	51	1-3	**2**	**2**	2	2

Table A5.7 Assignment of *C. maenas* COI gene sequences to unique haplotypes shown in the haplotype network in Fig. 5.8. The number of unique haplotypes are shown in the far left column, followed by the haplotype network label, the number of sequences per haplotype, the study reference, and the GenBank Accession Number for each sequence. Haplotypes with two or more sequences are separated by horizontal black lines in the table.

Haplotype number	Label	*N* sequences	Sequences belonging to haplotype	Reference	GenBank Accession No.
1	Z2	18	Z2	Darling et al. 2008	FJ159085
			S59	This study	MT748849
			S57	This study	MT748847
			S44	This study	MT748834
			S40	This study	MT748830
			S30	This study	MT748803
			S29	This study	MT748802
			S23	This study	MT748796
			S19	This study	MT748792
			S15	This study	MT748824
			S12	This study	MT748821
			S11	This study	MT748820
			S10	This study	MT748819
			S7	This study	MT748816
			E67	Darling et al. 2008	FJ159081
			E8	Darling et al. 2008	FJ159018
			E4	Darling et al. 2008	FJ159013
			B5	Burden et al. 2014	KF709205
2	Z1	2	Z1	Darling et al. 2008	FJ159019
			C1	Darling et al. 2008	FJ159009
3	S58	3	S58	This study	MT748848
			E32	Darling et al. 2008	FJ159044
			B4	Burden et al. 2014	KF709204
4	S56	13	S56	This study	MT748846
			S55	This study	MT748845
			S51	This study	MT748841
			S48	This study	MT748838
			S45	This study	MT748835
			S39	This study	MT748829
			S36	This study	MT748809
			S31	This study	MT748804
			S5	This study	MT748814
			S2	This study	MT748811
			S1	This study	MT748810
			E15	Darling et al. 2008	FJ159026
			B2	Burden et al. 2014	KF709202

5	S54	35	S54	This study	MT748844
			S53	This study	MT748843
			S52	This study	MT748842
			S50	This study	MT748840
			S49	This study	MT748839
			S47	This study	MT748837
			S42	This study	MT748832
			S41	This study	MT748831
			S38	This study	MT748828
			S37	This study	MT748827
			S35	This study	MT748808
			S34	This study	MT748807
			S33	This study	MT748806
			S32	This study	MT748805
			S28	This study	MT748801
			S27	This study	MT748800
			S26	This study	MT748799
			S25	This study	MT748798
			S24	This study	MT748797
			S22	This study	MT748795
			S21	This study	MT748794
			S20	This study	MT748793
			S18	This study	MT748791
			S16	This study	MT748825
			S13	This study	MT748822
			S9	This study	MT748818
			S8	This study	MT748817
			S3	This study	MT748812
			E64	Darling et al. 2008	FJ159078
			E55	Darling et al. 2008	FJ159069
			E46	Darling et al. 2008	FJ159060
			E33	Darling et al. 2008	FJ159045
			E28	Darling et al. 2008	FJ159040
			E1	Darling et al. 2008	FJ159008
			B1	Burden et al. 2014	KF709201
6	S46	6	S46	This study	MT748836
			S43	This study	MT748833
			S17	This study	MT748826
			S14	This study	MT748823
			S6	This study	MT748815
			S4	This study	MT748813
7	N1	1	N1	Darling et al. 2008	FJ159011
8	I1	1	I1	Darling et al. 2008	FJ159017
9	F2	1	F2	Darling et al. 2008	FJ159050
10	F1	1	F1	Darling et al. 2008	FJ159032

11	E70	1	E70	Darling et al. 2008	FJ159084
12	E69	1	E69	Darling et al. 2008	FJ159083
13	E68	2	E68	Darling et al. 2008	FJ159082
			E36	Darling et al. 2008	FJ159048
14	E66	1	E66	Darling et al. 2008	FJ159080
15	E65	1	E65	Darling et al. 2008	FJ159079
16	E63	1	E63	Darling et al. 2008	FJ159077
17	E62	2	E62	Darling et al. 2008	FJ159076
			E22	Darling et al. 2008	FJ159029
18	E61	1	E61	Darling et al. 2008	FJ159068
19	E60	1	E60	Darling et al. 2008	FJ159067
20	E59	2	E59	Darling et al. 2008	FJ159066
			E6	Darling et al. 2008	FJ159015
21	E58	1	E58	Darling et al. 2008	FJ159072
22	E57	1	E57	Darling et al. 2008	FJ159071
23	E56	1	E56	Darling et al. 2008	FJ159070
24	E54	1	E54	Darling et al. 2008	FJ159068
25	E53	1	E53	Darling et al. 2008	FJ159067
26	E52	1	E52	Darling et al. 2008	FJ159066
27	E51	1	E51	Darling et al. 2008	FJ159065
28	E50	1	E50	Darling et al. 2008	FJ159064
29	E49	1	E49	Darling et al. 2008	FJ159063
30	E48	1	E48	Darling et al. 2008	FJ159062
31	E47	2	E47	Darling et al. 2008	FJ159061
			E7	Darling et al. 2008	FJ159016
32	E45	1	E45	Darling et al. 2008	FJ159059
33	E44	2	E44	Darling et al. 2008	FJ159057
			B6	Darling et al. 2008	KF709206
34	E43	1	E43	Darling et al. 2008	FJ159056
35	E42	1	E42	Darling et al. 2008	FJ159055
36	E41	1	E41	Darling et al. 2008	FJ159054
37	E40	1	E40	Darling et al. 2008	FJ159053
38	E39	1	E39	Darling et al. 2008	FJ159052
39	E38	1	E38	Darling et al. 2008	FJ159051
40	E37	1	E37	Darling et al. 2008	FJ159049
41	E35	1	E35	Darling et al. 2008	FJ159047
42	E34	2	E34	Darling et al. 2008	FJ159046
			E19	Darling et al. 2008	FJ159030
43	E31	1	E31	Darling et al. 2008	FJ159043
44	E30	2	E30	Darling et al. 2008	FJ159042
			E29	Darling et al. 2008	FJ159041
45	E27	1	E27	Darling et al. 2008	FJ159039
46	E26	1	E26	Darling et al. 2008	FJ159038
47	E25	1	E25	Darling et al. 2008	FJ159037
48	E24	1	E24	Darling et al. 2008	FJ159036
49	E23	1	E23	Darling et al. 2008	FJ159035

50	E21	1	E21	Darling et al. 2008	FJ159033
51	E20	1	E20	Darling et al. 2008	FJ159031
52	E18	3	E18	Darling et al. 2008	FJ159029
			E16	Darling et al. 2008	FJ159027
			E3	Darling et al. 2008	FJ159012
53	E17	1	E17	Darling et al. 2008	FJ159028
54	E14	1	E14	Darling et al. 2008	FJ159025
55	E13	1	E13	Darling et al. 2008	FJ159024
56	E12	1	E12	Darling et al. 2008	FJ159023
57	E11	1	E11	Darling et al. 2008	FJ159022
58	E10	1	E10	Darling et al. 2008	FJ159021
59	E9	1	E9	Darling et al. 2008	FJ159020
60	E5	1	E5	Darling et al. 2008	FJ159014
61	E2	1	E2	Darling et al. 2008	FJ159010
62	B3	2	B3	Burden et al. 2014	KF709203
			A1	Darling et al. 2008	FJ159058

Table A5.8 mtDNA haplotype frequencies of *C. maenas* across global geographic regions. South Australian haplotypes are highlighted in yellow. All other haplotypes and frequencies were obtained from Darling et al. (2008) and Burden et al. (2014). Blank spaces indicate zeros. Corresponding haplotype sample codes and geographic regions are shown in Fig. 5.9. Singleton haplotypes were excluded.

Haplotype	OSEU	NEU	WEU	NS	ENA	WNA	ZA	JP	AR	AU	TAS	**SA**
E1		52	46	1	70	74	8	55	15	16		**28**
E2		1	1									
N1				8								
E3		1		6								
E4		19	11	5			19			21	2	**13**
E6		6	1				1					
E7		8	20				8					
I1	29											
E8		1	4									
E15		8					1			10		**11**
E16		5	2				1			17	2	
E17		2										
E19		4	1									
F1	8											
E32		1								9		**1**
E33		2										
E34		2	5									
E38			1				3					
E40		1	1				1					
E41			1				1					
E42			1				2					
E43			1				2					
E44			1							12		
A1										1	37	
E48			2									
S1												**6**

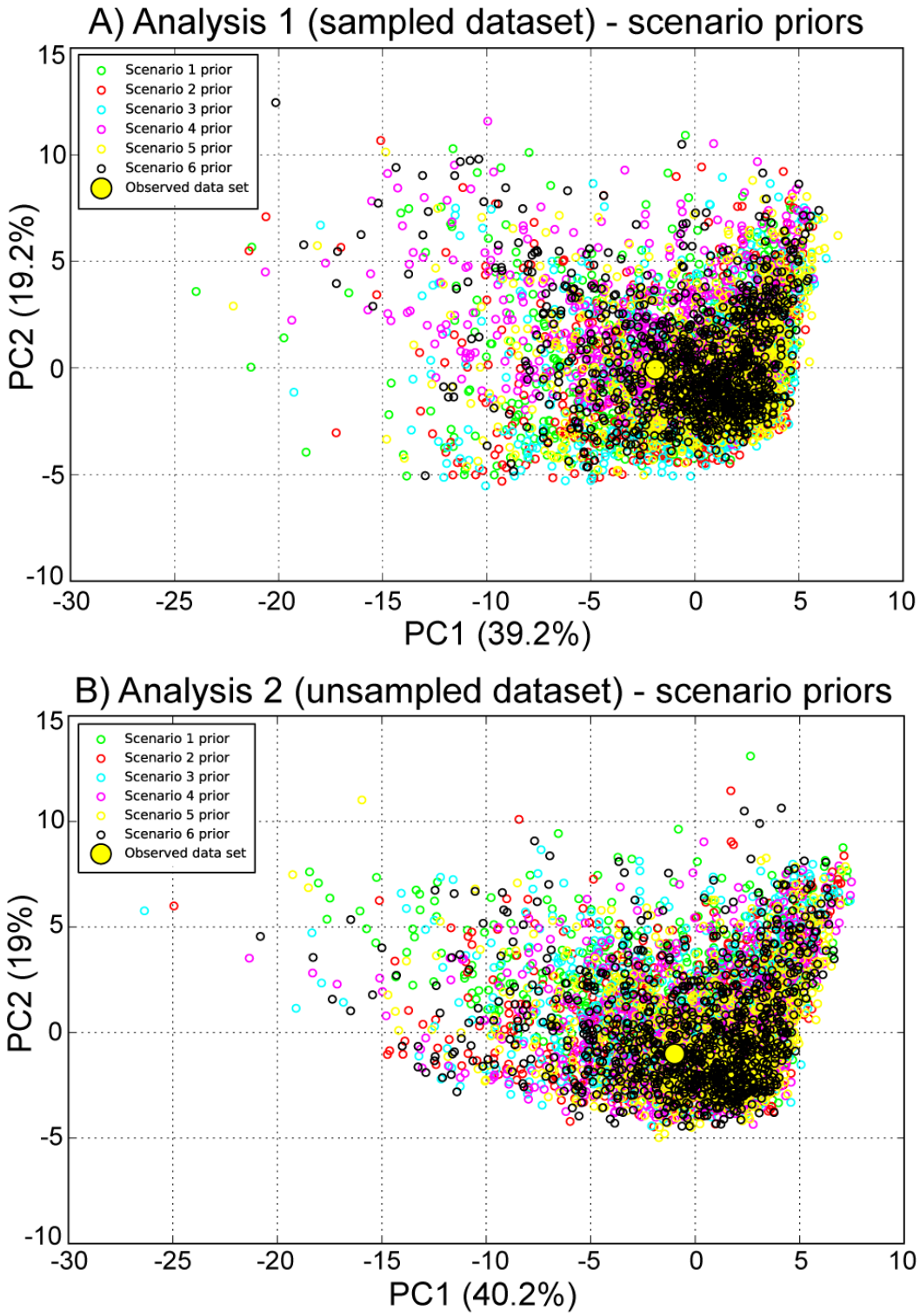

Figure A5.6 Principal components analysis (PCA) of pre-evaluation of scenario-prior parameters displaying similarity between the observed SNP dataset and the modelled scenarios for: A) the sampled dataset for all populations; and B) the unsampled dataset that included a ghost population. The large yellow/black circle represents the observed dataset, while the smaller, coloured circles represent the simulated datasets for the historical scenarios in each analysis.

Table A5.9 Analysis 1 (sampled dataset) scenario comparisons for the direct approach and the logistic approach of six modelled scenarios (Fig. 5.2). Values in bold indicate the most likely scenario choice for each approach. Brackets contain 95% confidence intervals.

Analysis 1 – sampled dataset (3,509 loci, 156 individuals with no "ghost" population)						
Direct approach						
Closest	Scenario 1	**Scenario 2**	Scenario 3	Scenario 4	Scenario 5	Scenario 6
50	0.100 [0.0 – 0.36]	**0.360** [0.0 – 0.78]	0.200 [0.0 – 0.55]	0.020 [0.0 – 0.14]	0.280 [0.0 – 0.67]	0.040 [0.0 – 0.21]
100	0.090 [0.0 – 0.34]	**0.380** [0.0 – 0.80]	0.230 [0.0 – 0.59]	0.010 [0.0 – 0.09]	0.240 [0.0 – 0.61]	0.050 [0.0 – 0.24]
150	0.100 [0.0 – 0.36]	**0.340** [0.0 – 0.75]	0.260 [0.0 – 0.64]	0.026 [0.0 – 0.16]	0.220 [0.0 – 0.58]	0.053 [0.0 – 0.25]
200	0.080 [0.0 – 0.31]	**0.360** [0.0 – 0.78]	0.235 [0.0 – 0.60]	0.025 [0.0 – 0.16]	0.255 [0.0 – 0.63]	0.045 [0.0 – 0.22]
250	0.084 [0.0 – 0.32]	**0.332** [0.0 – 0.74]	0.244 [0.0 – 0.62]	0.028 [0.0 – 0.17]	0.252 [0.0 – 0.63]	0.060 [0.0 – 0.26]
300	0.083 [0.0 – 0.32]	**0.350** [0.0 – 0.76]	0.243 [0.0 – 0.61]	0.030 [0.0 – 0.17]	0.230 [0.0 – 0.59]	0.063 [0.0 – 0.27]
350	0.080 [0.0 – 0.31]	**0.331** [0.0 – 0.74]	0.248 [0.0 – 0.62]	0.031 [0.0 – 0.18]	0.242 [0.0 – 0.61]	0.065 [0.0 – 0.28]
400	0.090 [0.0 – 0.34]	**0.340** [0.0 – 0.75]	0.242 [0.0 – 0.61]	0.032 [0.0 – 0.18]	0.232 [0.0 – 0.60]	0.062 [0.0 – 0.27]
450	0.095 [0.0 – 0.35]	**0.328** [0.0 – 0.74]	0.237 [0.0 – 0.61]	0.040 [0.0 – 0.21]	0.235 [0.0 – 0.60]	0.062 [0.0 – 0.27]
500	0.092 [0.0 – 0.34]	**0.316** [0.0 – 0.72]	0.252 [0.0 – 0.63]	0.042 [0.0 – 0.21]	0.236 [0.0 – 0.60]	0.062 [0.0 – 0.27]
Logistic approach						
N	Scenario 1	Scenario 2	Scenario 3	Scenario 4	**Scenario 5**	Scenario 6
1000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
2000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
3000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
4000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
5000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
6000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
7000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
8000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
9000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 1.0]
10,000	0.0 [0.0 – 0.95]	0.0 [0.0 – 0.95]	0.0 [0.0 – 0.95]	0.0 [0.0 – 0.95]	**0.999** [0.99 – 1.0]	0.0 [0.0 – 0.95]

Table A5.10 Analysis 2 (unsampled ghost dataset) scenario comparisons for the direct approach and the logistic approach of six modelled scenarios (Fig. 5.3). Values in bold indicate the most likely scenario choice for each approach. Brackets contain 95% confidence intervals.

Analysis 2 – unsampled dataset (3,509 loci, 156 individuals with "ghost" populations)						
Direct approach						
Closest	Scenario 1	Scenario 2	Scenario 3	**Scenario 4**	Scenario 5	Scenario 6
50	0.020 [0.0 – 0.14]	0.180 [0.0 – 0.51]	0.060 [0.0 – 0.26]	**0.460** [0.02 – 0.89]	0.220 [0.0 – 0.58]	0.060 [0.0 – 0.26]
100	0.010 [0.0 – 0.09]	0.160 [0.0 – 0.48]	0.070 [0.0 – 0.29]	**0.470** [0.03 – 0.90]	0.250 [0.0 – 0.62]	0.040 [0.0 – 0.21]
150	0.006 [0.0 – 0.07]	0.180 [0.0 – 0.51]	0.066 [0.0 – 0.28]	**0.433** [0.0 – 0.86]	0.273 [0.0 – 0.66]	0.040 [0.0 – 0.21]
200	0.005 [0.0 – 0.06]	0.175 [0.0 – 0.50]	0.085 [0.0 – 0.32]	**0.425** [0.0 – 0.85]	0.265 [0.0 – 0.65]	0.045 [0.0 – 0.22]
250	0.004 [0.0 – 0.05]	0.184 [0.0 – 0.52]	0.084 [0.0 – 0.32]	**0.400** [0.0 – 0.82]	0.280 [0.0 – 0.67]	0.048 [0.0 – 0.23]
300	0.013 [0.0 – 0.11]	0.180 [0.0 – 0.51]	0.080 [0.0 – 0.31]	**0.380** [0.0 – 0.80]	0.300 [0.0 – 0.70]	0.046 [0.0 – 0.23]
350	0.014 [0.0 – 0.11]	0.200 [0.0 – 0.55]	0.091 [0.0 – 0.34]	**0.360** [0.0 – 0.78]	0.288 [0.0 – 0.68]	0.045 [0.0 – 0.22]
400	0.022 [0.0 – 0.15]	0.202 [0.0 – 0.55]	0.085 [0.0 – 0.32]	**0.350** [0.0 – 0.76]	0.290 [0.0 – 0.68]	0.050 [0.0 – 0.24]
450	0.024 [0.0 – 0.15]	0.211 [0.0 – 0.56]	0.077 [0.0 – 0.31]	**0.351** [0.0 – 0.76]	0.288 [0.0 – 0.68]	0.046 [0.0 – 0.23]
500	0.024 [0.0 – 0.15]	0.220 [0.0 – 0.58]	0.074 [0.0 – 0.30]	**0.336** [0.0 – 0.75]	0.298 [0.0 – 0.69]	0.048 [0.0 – 0.23]
Logistic approach						
N	Scenario 1	Scenario 2	Scenario 3	Scenario 4	**Scenario 5**	Scenario 6
1000	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.0 [0.0 – 1.0]	0.098 [0.0 – 1.0]	**0.900** [0.76 – 1.0]	0.0 [0.0 – 1.0]
2000	0.0 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.084 [0.0 – 0.16]	**0.914** [0.83 – 0.99]	0.0 [0.0 – 0.0]
3000	0.0 [0.0 – 0.0]	0.001 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.073 [0.01 – 0.12]	**0.925** [0.86 – 0.98]	0.0 [0.0 – 0.0]
4000	0.0 [0.0 – 0.0]	0.001 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.070 [0.02 – 0.11]	**0.927** [0.88 – 0.97]	0.0 [0.0 – 0.0]
5000	0.0 [0.0 – 0.0]	0.002 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.064 [0.02 – 0.10]	**0.932** [0.89 – 0.97]	0.0 [0.0 – 0.0]
6000	0.0 [0.0 – 0.0]	0.002 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.061 [0.02 – 0.09]	**0.935** [0.90 – 0.96]	0.0 [0.0 – 0.0]
7000	0.0 [0.0 – 0.0]	0.003 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.060 [0.03 – 0.09]	**0.935** [0.90 – 0.96]	0.0 [0.0 – 0.0]
8000	0.0 [0.0 – 0.0]	0.003 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.061 [0.03 – 0.08]	**0.935** [0.90 – 0.96]	0.0 [0.0 – 0.0]
9000	0.0 [0.0 – 0.0]	0.004 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.061 [0.03 – 0.08]	**0.934** [0.90 – 0.96]	0.0 [0.0 – 0.0]
10,000	0.0 [0.0 – 0.0]	0.004 [0.0 – 0.0]	0.0 [0.0 – 0.0]	0.061 [0.03 – 0.08]	**0.934** [0.90 – 0.96]	0.0 [0.0 – 0.0]

Table A5.11 Results from STRUCTURE SELECTOR displaying the optimum *K* values for Bayesian STRUCTURE analysis of the subsampled dataset. Bayesian Information Clustering (BIC) *K* values from DAPC results also shown. The subsampled dataset had 3,498 loci, 110 individuals and nine geographic populations. The number of individuals analysed from South Australia and British Columbia was reduced to 20 each, while all other populations had the same number of individuals as the original dataset. For the Evanno Δ*K* method, yellow highlights the value that corresponds to an optimum *K* = 2. For the mean LnP(K) method, blue highlights the value that corresponds to an optimum *K* = 4. For the MedMedK and MedMeanK methods, the optimum *K* = 5 are highlighted in green. For the DAPC BIC method, pink highlights the value that corresponds to an optimum *K* = 3.

Loci	Ind	K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
3,498	110	1	5	-216,335	36.27	—	—	—
3,498	110	2	5	-203,185	6.33	13,150	7423	1172
3,498	110	3	5	-197,458	22.66	5727	3540	156.17
3,498	110	4	5	-195,271	433.46	2187	2321	5.35
3,498	110	5	5	-195,405	1086.78	-134.54	36,440	33.53
3,498	110	6	5	-231,980	37,179	-36,575	62,330	1.67
3,498	110	7	5	-206,225	26,577	25,755	47,460	1.78
3,498	110	8	5	-227,929	37,658	-21,704	285,158	7.57
3,498	110	9	5	-534,793	712,454	-306,863	536,391	0.75
3,498	110	10	5	-305,265	154,221	229,527	—	—

Loci	Ind	K (range)	MedMedK	MedMeanK	MaxMedK	MaxMeanK
3,498	110	1-10	**5**	**5**	5	5

DAPC BIC Values

K = 1	K = 2	K = 3	K = 4	K = 5	K = 6	K = 7	K = 8	K = 9	K = 10
560.40	555.34	554.72	555.86	557.16	558.98	562.00	564.43	567.40	570.65