Anchors away: the susceptibility and response to infection between native and co-introduced fishes to the alien anchor worm *Lernaea cyprinacea*

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Murdoch University, Perth, Western Australia
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Mikayla McCredden

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Abstract
The introduction of alien fish species and their alien parasites pose one of the most important threats to freshwater fishes throughout the world. The south-west of Australia has a depauperate, although highly endemic freshwater fish fauna. Of the 200 native freshwater fish species in Australia 144 are exclusively confined to freshwater. In the extreme south-west there are only 11 native freshwater fish species and nine of these are endemic to the region. Six of the 11 freshwater fish species have restricted geographic ranges and four are listed as rare or likely to become extinct. In 2008, studies surveying the parasites of freshwater fishes in the South West Coast Drainage Division (SWCDD), reported the introduction of the alien parasite, *Lernaea cyprinacea*, into freshwater river systems in the region.

*Lernaea cyprinacea*, commonly known as anchor worm, is a parasitic copepod believed to have been brought in to Western Australia with the accidental release of its native host, *Carassius auratus* (goldfish). It is not native to Australia and, until recently, had only been reported in fish in eastern Australia. First reports of this parasite in the south-west identified it using morphological criteria from four native freshwater fishes: *Galaxias occidentalis* (western minnow), *Nannoperca vittata* (western pygmy perch), *Bostockia porosa* (nightfish) and *Tandanus bostocki* (freshwater cobbler).

The present study aimed to resolve the morphological uncertainty surrounding the taxonomy of the parasite using molecular techniques, specifically PCR and DNA sequencing, and to review the host range and geographic distribution of this invasive species within the south-west of Western Australia. A comparison of the infection success and pathogenicity of *L. cyprinacea* in a fish species, *Nannoperca vittata* (pygmy perch), that is endemic to the Southwestern Province Ichthyological, and that to the natural host, *Carassius auratus* (goldfish), is detailed.

*Lernaea cyprinacea* in south-western Australia had been morphologically identified in previous studies, but had not been identified using molecular tools. Parasite samples examined in this
study typed as *Lernaea cyprinacea* at the 28S ribosomal RNA (rRNA) locus. Sequences were identified using Finch TV Version 1.4.0 (Geospiza Research Team 2004-2006) and checked for identity using the nucleotide database, Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov).

The parasite appears to have increased its geographic range in the Southwestern Ichthyological Province; in 2008 it was reported in only one river (the Canning River), whereas in the present study it was found in another two rivers (the Murray River and Serpentine River). *Lernaea cyprinacea* was also found on two more host species, in addition to the four native hosts reported previously; *Galaxias occidentalis* (western minnow), *Nannoperca vittata* (western pigmy perch), *Bostockia porosa* (nightfish), *Tandanus bostocki* (freshwater cobbler), and now, *Pseudogobius olorum* (bluespot goby) and *Leptatherina wallacei* (western hardyhead). In the field, *L. cyprinacea* was more prevalent on native freshwater fish species than on the natural host *C. auratus*.

The difference in prevalence of *L. cyprinacea* on native fishes and *C. auratus* found in field studies may be due to differences in exposure to the parasite or to differences in susceptibility to infection. Laboratory experiments were used to compare the susceptibility to infection of native *N. vittata* and *C. auratus*. There was no difference found in the prevalence or intensity of infection on *N. vittata* or *C. auratus*, when they were exposed separately. In mixed communities however, a significantly greater proportion of *N. vittata* were infected compared to *C. auratus* (0.59 vs. 0.33), and the mean intensity of infection was also greater in *N. vittata* than in *C. auratus* (3.0 ± 0.3 vs. 2.2 ± 0.4).

*Nannoperca vittata* and *C. auratus* also exhibited significant differences in their behavioural reactions to infection, with putative defensive behaviours observed much more frequently in infected *C. auratus* than in infected *N. vittata*. Histologically, *C. auratus* had a greater pathological and inflammatory response to infection than *N. vittata*.
Due to the extensive and destructive effects of *C. auratus* on both native fishes and habitat, the control of *C. auratus* has become essential. Removal programs have been underway in Western Australia since 2005, however, we know very little about the effects of removal programs for *C. auratus* on the co-introduced parasite *L. cyprinacea*. In particular, it has been suggested that if goldfish are a less competent host species than native freshwater fish species, then removal may actually exacerbate the parasite problem by increasing prevalence of infection on native fishes.

This study provides no evidence that the removal of goldfish will exacerbate the problem of *L. cyprinacea* in river systems in south-western Australia. That being said, there is a need to expand this study to examine the comparative infectivity and pathogenicity of *L. cyprinacea* to other native fish species and, where possible, to monitor parasite infection rates in the field before and after goldfish control programs to ensure that there are no adverse effects from goldfish removal.
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Chapter 1
General Introduction

1.1 Threats to freshwater biodiversity

Freshwater ecosystems are extremely biodiverse. For example, although only 0.01% of the world’s total aquatic environments are freshwater habitats, approximately 40% of all fish species are restricted to these habitats (Allen, 1982, Nelson, 1994, Paxton and Eschmeyer, 1994). Freshwater fishes are considered to be represented by two groups, based on presumed habitats of their ancestral stocks. There are ~8,000 species that are believed to have originated in fresh water and are referred to as primary freshwater species. The secondary species, include ~1,500 species and are believed to have been evolved from marine ancestors (Allen, 1982).

Freshwater ecosystems are generally more threatened than terrestrial ecosystems (Dudgeon et al., 2006, Okamura and Feist, 2011). The various threats to freshwater ecosystems can be grouped into five interacting categories: overexploitation, water pollution, flow modification, destruction or degradation of habitat, and invasion by alien species (Figure 1.1) (Allan and Flecker, 1993, Naiman et al., 1995, Naiman and Turner, 2000, Jackson et al., 2001, Malmqvist and Rundle, 2002, Rahel, 2002, Postel and Richter, 2003, Revenga et al., 2005, Dudgeon et al., 2006).
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The effects of overexploitation are generally limited to vertebrates, whereas the other four categories have consequences that affect all freshwater biodiversity, from microbes to megafauna (Dudgeon et al., 2006). Pollution is a widespread problem and continues to be a major threat despite the considerable progress that has been made in reducing water pollution from domestic and industrial point sources (Colburn et al., 1996). Modifications in flow vary in severity and type, but tend to be more pronounced in areas with highly variable flow regimes (Dudgeon et al., 2006). There are a number of interacting factors that contribute to habitat degradation. These include direct impacts on the aquatic environment or indirect impacts resulting from changes within the drainage basin (Dudgeon et al., 2006). The widespread invasion and deliberate introduction of alien species contributes to the physical and chemical impacts of humans on freshwaters, partly because it is easier for alien species to successfully invade fresh waters that have already been degraded or modified by humans (Bunn and Arthington, 2002, Koehn, 2004, Beatty and Morgan, 2013).
Australian freshwater ecosystems, like those in other parts of the world, have suffered extensive habitat degradation, mostly due to human exploitation, and are now under increasing anthropogenic pressure (Morgan et al., 1998, Allen et al., 2002, Pollino et al., 2004), with some classic examples being the salinisation of south-western Australia (Rashnavadi et al., 2014), regulation of rivers (Olsen and Skitmore, 1991), and the invasion of the southern and eastern provinces by alien species, such as fishes (Howe et al., 1997, King et al., 1997, Gill et al., 1999, Morgan et al., 2004).

1.2 Southwestern Ichthyological Province

The Southwestern Province is one of 10 biogeographical provinces for freshwater fishes in Australia (Unmack, 2013, Morgan et al., 2014). This Province contains numerous lakes, flats and short coastal rivers that flow into the Indian Ocean and Southern Ocean south of the Arrowsmith River (near Dongara) to the east of Esperance (Figure 1.2) (Allen, 1989, Jaensch and Lane, 1993). The south-west region has a Mediterranean climate with warm, dry summers and cool, wet winters (Allen et al., 2002, Morgan et al., 2011), with rainfall highly seasonal, and mainly falling during winter and spring (Charles et al., 2010). Rivers in the region are characterised by large seasonal fluctuations in flow rates, with many ceasing to flow in the dry summer-autumn period (Allen, 1989, Jaensch and Lane, 1993). Over the past 160 years, since European settlement, the rivers of the south-west have changed greatly (Olsen and Skitmore, 1991). These changes have been, in part, due to direct changes to water flow (damming and water extraction), though they are more often a result from indirect changes in land use through agriculture, industry, forestry, mining and recreation (Olsen and Skitmore, 1991).

Wide scale clearing of native vegetation and reduced rainfall has also had a severe impact on the aquatic systems of Western Australia, causing secondary salinisation (Rashnavadi et al., 2014). As a consequence, only ~44% of flow in the largest 30 rivers in the region is now fresh (Mayer et al., 2005). In the Southwestern Province, secondary salinisation has also led to a change in the structure of freshwater fish assemblages, with many estuarine species now found inland and outside their historic range (Morgan et al., 1998, Morgan, 2003, Beatty et al., 2011).
1.3 Biodiversity of freshwater fishes in the Southwestern Province

Compared to other areas of the world of similar size, Australia has a depauperate, although highly endemic, freshwater fish fauna. Out of the 200 native species found in freshwater habitats in Australia, 144 are exclusively confined to freshwater and at least 30 are endemic (Allen, 1982, Paxton and Eschmeyer, 1994, Allen et al., 2002, Morgan et al., 2011, Unmack, 2013). This high level of endemism is due to Australia’s age, stability, isolation and aridity (Unmack, 2001, Allen et al., 2002, Morgan et al., 2011). Even though Western Australia shares a common Gondwanic history with south-eastern Australia, due to its ‘insular’ constitution, the freshwater fish fauna is surprisingly impoverished (Bunn and Davies, 1990, Allen et al., 2002, Morgan et al., 2011, Unmack, 2013). Other factors that have contributed to Western Australia’s diminished freshwater fish fauna include lack of extensive river systems, the physical barriers to aquatic migration created by desert and ocean bodies, and the impacts of weathering and

In the Southwestern Province, isolated from the rest of Australia by extensive arid zones, there are only 14 native fish species found in non-tidal freshwaters; 11 of which are primarily freshwater species and of these nine that are endemic to the region, giving the province the highest level of endemism in Australia (Morgan et al., 1998, Unmack, 2013, Morgan et al., 2014). The nine species that are endemic to the region are: *Tandanus bostocki* (freshwater cobbler); *Lepidogalaxias salamandroides* (salamanderfish); *Galaxias occidentalis* (western minnow); *Galaxiella nigrostriata* (black-stripe minnow); *Galaxiella munda* (western mud minnow); *Bostockia porosa* (nightfish); *Nannoperca vittata* (western pygmy perch); *Nannoperca pygmaea* (little pygmy perch) and *Nannatherina balstoni* (Balston’s pygmy perch) (Allen et al., 2002, Morgan et al., 2011, Morgan et al., 2014). There are also two other freshwater fish species that are found in, but are not restricted to, this region; *Galaxias maculatus* (spotted minnow) and *Galaxias truttaceus* (trout minnow) (Morgan et al., 1998, Morgan, 2003, Morgan and Beatty, 2006, Chapman et al., 2006). Four other native fish species are commonly found in this region, but are not restricted to freshwater; the estuarine *Leptatherina wallacei* (western hardyhead), *Pseudogobius olorum* (Swan River goby) and *Afurcagobius suppositus* (south-western goby), and the anadromous *Geotria australis* (pouched lamprey) (Morgan et al., 1998, Allen et al., 2002, Morgan et al., 2011, Morgan et al., 2014, Rashnavadi et al., 2014). Six of the 11 native species of fish found in the Southwestern Province have restricted geographic ranges and 4 are listed as rare or likely to become extinct (Table 1.1) (Morgan et al., 1998).
### Table 1.1. List of the native freshwater fish species of the Southwestern Ichthyological Province and their conservation status

<table>
<thead>
<tr>
<th>Family</th>
<th>Scientific Name</th>
<th>Common Name</th>
<th>Conservation Status</th>
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</thead>
<tbody>
<tr>
<td>Galaxiidae</td>
<td><em>Galaxiella nigrostriata</em></td>
<td>Black-stripe minnow</td>
<td>Lower Risk/Near Threatened (a)</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td><em>Galaxias occidentalis</em></td>
<td>Western minnow</td>
<td>---</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td><em>Galaxias truttaceus</em></td>
<td>Trout minnow</td>
<td>Critically Endangered (b)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td><em>Galaxias maculatus</em></td>
<td>Common jollytail</td>
<td>---</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td><em>Galaxiella munda</em></td>
<td>Western mud minnow</td>
<td>Lower Risk/Near Threatened (a) Vulnerable (c)</td>
<td>Yes</td>
</tr>
<tr>
<td>Lepidogalaxiidae</td>
<td><em>Lepidogalaxias salamandroides</em></td>
<td>Salamanderfish</td>
<td>Lower Risk/Near Threatened (a)</td>
<td>Yes</td>
</tr>
<tr>
<td>Percichthyidae</td>
<td><em>Bostockia porosa</em></td>
<td>Nightfish</td>
<td>---</td>
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<tr>
<td></td>
<td><em>Nannatherina balstoni</em></td>
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<tr>
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<td>Plotosidae</td>
<td><em>Tandanus bostocki</em></td>
<td>Freshwater cobbler</td>
<td>---</td>
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</table>


### 1.4 Invasive freshwater fishes

Invasive species are alien (exotic or non-native) organisms that have been introduced into an area outside of their natural range, established self-sustaining populations and spread beyond their initial point of introduction, with deleterious impacts on the environment, the economy, and human health (Figure 1.3) (Kolar and Lodge, 2001, Blackburn et al., 2011). Gallardo et al. (2016) reviewed published literature on invasive aquatic species throughout the world and
included a total of 67 invasive species (24 species of fish, 22 species of plants, 11 species of molluscs and seven species of crustaceans); three of the top ten most invasive species were fishes: *Cyprinus carpio* (common carp), *Agosia chrysogaster* (longfin dace) and *Oncorhynchus mykiss* (rainbow trout).

Alien fishes were first introduced into Australia through European settlers in the late 1800s (Allen et al., 2002). In the Southwestern Province of Western Australia, alien fishes have been co-introduced in three phases. In the first phase, species identified as a potential food or angling sources were released (Coy, 1979, Allen et al., 2002). These included *Salmo trutta* (brown trout), *Maccullochella peellii* (Murray cod), *Macquaria ambigu* (golden perch), *Bidyanus bidyanus* (silver perch), *Anguilla australis* (short-finned eels), *Cyprinus carpio* (common carp), *Tinca tinca* (tench), *Perca fluviatilis* (redfin perch) and *Oncorhynchus mykiss* (rainbow trout) (Coy, 1979).

In the second phase, species were released for aquaculture and as biological control agents (Coy, 1979). Most notably, this included the release of *Gambusia holbrooki* (eastern gambusia),
in the 1920s, to control mosquito populations (Allen et al., 2002). *Gambusia holbrooki* is now firmly established throughout much of the south-west and southern Pilbara (Morgan et al., 2004).

The third phase included ornamental fishes that have escaped or been set free (Coy, 1979). This has been occurring for at least the last four decades and has resulted in a number of self-sustaining populations of small ornamental fish, including *Carassius auratus* (goldfish), *Cyprinus carpio* (koi carp), *Oreochromis mossambicus* (Mozambique mouthbrooder or tilapia), and more recently *Xiphophorus hellerii* (swordtails), *Poecilia reticulata* (guppies), and *Phalloceros caudimaculatus* (one-spot livebearers) (Allen et al., 2002, Morgan et al., 2004, Beatty and Morgan, 2013).

Although many species of introduced fish have not been able to adapt to the environment of south-western Australia, particularly the seasonal nature of stream flow (Allen et al., 2002), other species have been able to establish self-sustaining populations and spread from their initial point of introduction (i.e. become invasive). Many factors have contributed to the establishment of self-sustaining populations of alien fishes, including fish behaviour (Molony et al., 2004), suitable water temperatures (Russell et al., 2003, Pusey and Arthington, 2003), suitable habitat for spawning (Pollino et al., 2004), minimal resource competition (Russell et al., 2003), abundant food supply (Morgan et al., 2002, Pusey and Arthington, 2003) and changes in river flow (Pollino et al., 2004).

Currently there are around 38 species of self-sustaining alien fishes nationwide in Australia’s fresh waters (Allen et al., 2002, Morgan et al., 2004, Lintermans, 2009). In the Southwestern Province there are currently 13 self-sustaining, invasive alien fishes (Table 1.2), several of which are the most widely introduced freshwater fishes globally, including *Carassius auratus* (goldfish), *G. holbrooki*, *O. mykiss* and *S. trutta* (Morgan et al., 1998, Allen et al., 2002, Morgan et al., 2004, Morgan and Beatty, 2007, Morgan et al., 2011, Beatty and Morgan, 2013).
Table 1.2. List of the self-sustaining, invasive fish species of the Southwestern Ichthyological Province and their geographical origin

<table>
<thead>
<tr>
<th>Family</th>
<th>Scientific Name</th>
<th>Common Name</th>
<th>Geographical Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cichlidae</td>
<td><em>Geophagus brasiliensis</em></td>
<td>Pearl cichlid</td>
<td>South America</td>
</tr>
<tr>
<td>Cyprinidae</td>
<td><em>Carassius auratus</em></td>
<td>Goldfish</td>
<td>Asia</td>
</tr>
<tr>
<td></td>
<td><em>Cyprinus carpio</em></td>
<td>European carp</td>
<td>Europe and Asia</td>
</tr>
<tr>
<td></td>
<td><em>Puntius conchonius</em></td>
<td>Rosy barb</td>
<td>Asia</td>
</tr>
<tr>
<td>Percidae</td>
<td><em>Perca fluviatilis</em></td>
<td>Redfin perch</td>
<td>Europe and Asia</td>
</tr>
<tr>
<td>Percichthydae</td>
<td><em>Macquaria ambigua</em></td>
<td>Golden perch</td>
<td>Australia (eastern)</td>
</tr>
<tr>
<td>Poeciliidae</td>
<td><em>Gambusia holbrooki</em></td>
<td>Mosquitofish</td>
<td>North America</td>
</tr>
<tr>
<td></td>
<td><em>Phalloceros caudimaculatus</em></td>
<td>Leopardfish</td>
<td>South America</td>
</tr>
<tr>
<td></td>
<td><em>Xiphophorus hellerii</em></td>
<td>Playfish or Green swordtail</td>
<td>North and Central America</td>
</tr>
<tr>
<td>Salmonidae</td>
<td><em>Salmo trutta</em></td>
<td>Brown trout</td>
<td>Europe</td>
</tr>
<tr>
<td></td>
<td><em>Oncorhynchus mykiss</em></td>
<td>Rainbow trout</td>
<td>North America</td>
</tr>
<tr>
<td>Terapontidae</td>
<td><em>Bidyanus bidyanus</em></td>
<td>Silver perch</td>
<td>Australia (eastern)</td>
</tr>
<tr>
<td></td>
<td><em>Leiopotherapon unicolor</em></td>
<td>Spangled perch</td>
<td>Australian (north-western, northern, eastern)</td>
</tr>
</tbody>
</table>

Modified from Beatty and Morgan (2013)

1.5 Effects of invasive fish species

Invasive fishes constitute a major threat to aquatic biodiversity throughout the world (Rahel, 2002). Introduction of non-native species poses a significant threat to the integrity and functioning of an ecosystem, and is classified as the second most important cause of native biodiversity loss worldwide (Wilcove et al., 1998, Grosholz, 2002, Clavero and Garcia-Berthou, 2005, Molnar et al., 2008). Invasive species can have numerous negative effects on native fishes through predation, degradation of habitat and water quality, competition for food and other resources, aggressive interactions such as fin nipping, and introduction of exotic pathogens and parasites (Arthington, 1991, Arthington and McKenzie, 1997, Dove and Ernst, 1998, Morgan et al., 2004).

There are many examples of the extensive and dramatic effects of introduced alien species on indigenous species of freshwater fishes within Australia. *Gambusia holbrooki*, for example, has been associated with damaged caudal fins of native fishes, predation on juvenile native fishes...
and competition for food, causing growth retardation and suppressed reproductive activity of native fishes (Howe et al., 1997, Gill et al., 1999, Morgan et al., 2004). In eastern Australia, C. carpio is now widespread and has created major concerns with regards to the effects of the species on water quality by causing and increasing the frequency of algal blooms (King et al., 1997). Trout are also known to be associated with the decline of native fishes and amphibians in eastern Australia, and implicated in Western Australia, due to predation and competition for food and space (Crowl et al., 1992, Arthington and McKenzie, 1997, Lowe et al., 2000, Koehn and MacKenzie, 2004, Morgan et al., 2004, McDowall, 2006, Tay et al., 2007, Garcia De Leaniz et al., 2010). Feral populations of C. auratus have now been reported throughout Australia (McKay, 1984, Koehn and MacKenzie, 2004), and have been shown to be detrimental to both native freshwater flora and fauna (Morgan et al., 2004, Morgan and Beatty, 2007).

1.6 Carassius auratus as an invasive species

Carassius auratus is one of the world’s oldest domesticated fishes, arguably one of the most popular pets, and is also one of the most widely introduced freshwater fish species globally (McKay, 1984, Koehn and MacKenzie, 2004). Feral populations of C. auratus have been reported in almost every state of Australia and throughout much of the world (Fuller et al., 1999, Gido and Brown, 1999, Skelton, 2001). Within Western Australia, C. auratus appears to be most successful in modified or degraded waters and is generally restricted to the south-western corner, in close proximity to major populated areas (Morgan et al., 2004). The species is a particular problem in the Vasse River system, where removal programs have been in operation since 2005 (Morgan and Beatty, 2007).

Carassius auratus has the potential to prey on the eggs, larvae and adults of native fish species (Morgan and Beatty, 2007). The species also competes with native fishes for food and space and, as they grow to a much larger size than most native fish species, they are able to avoid piscivorous predation (Morgan and Beatty, 2007). Carassius auratus is a generalist/herbivore and so it can increase water turbidity and deplete aquatic vegetation through its benthic feeding habits (Richardson et al., 1995). This reduction of vegetation is believed to reduce both the
habitat and spawning sites for native fishes (Morgan and Beatty, 2007). In addition, *C. auratus* has been associated with an increase in blue-green algal blooms in rivers throughout the world (Kolmakov and Gladyshev, 2003, Morgan and Beatty, 2007). Kolmakov and Gladyshev (2003) found that significant growth of *Mycrocystis aeruginosa* (cynobacteria) was stimulated when passed through the intestines of the *C. auratus*. There was also an increase in growth of other cynobacteria, such as *Anabaena flos-aquae* and *Planktothrix agardhii*, compared to the controls. *Carassius auratus* has been associated with the introduction of parasites into South Africa and Australia (Fletcher and Whittington, 1998, Mouton et al., 2001, Hassan, 2008).

### 1.7 Parasites and invasive species

Parasites may play a key role in mediating the impacts of biological invasions at any of the three phases of introduction, establishment or spread. Parasites have the ability to directly affect endemic and exotic species or indirectly interfere with the interactions between exotic and endemic species, through the processes of parasite loss, spill-back, sinking and spillover (Prenter et al., 2004, Hassan, 2008, Peeler and Feist, 2011).

#### 1.7.1 Parasite loss

Alien species of plants and animals have been reported to host fewer parasites than related native species (Torchin et al., 2002, Torchin et al., 2003, Lymbery et al., 2010). Alien species generally originate from a founder population and so they may not carry the complete range of parasites found in the source location (Torchin et al., 2002, Torchin et al., 2003). There is also the risk of early extinction for those few parasites that do make it to the new environment due to inadequate environmental conditions or lack of specific intermediate hosts to complete their life cycle (Torchin et al., 2002, Torchin et al., 2003). Ewen et al. (2012) found that avian malaria parasites (*Plasmodium* spp.) that have successfully invaded New Zealand are more prevalent in their native range than related species of *Plasmodium* that have not invaded, and Torchin et al. (2003) reported similar findings across a range of host and parasite taxa. This may argue in favour of the importance of arrival with the host, as a higher prevalence means a greater probability of being present in host founders (Ewen et al., 2012), but a higher prevalence may also indicate a greater transmission efficiency and therefore a greater ability to persist in the
new environment. Distinguishing between these two processes is not usually possible because data on host and parasite founding populations are lacking. MacLeod et al. (2010) used a host/parasite system for which such data were available (i.e., chewing lice on introduced birds in New Zealand) and found that failure to persist in the new environment was a much more important source of loss of parasite species than was failure to arrive with their hosts in the new environment.

Parasite loss from introduced alien species has the potential to alter the new ecosystem by promoting demographic success and competitive ability of the alien over the native species (Torchin et al., 2002, Torchin et al., 2003, Prenter et al., 2004). In Norway, for example, the monogenean parasite Gyrodactylus salaris (gill fluke) switched from its original host (the Baltic strain of Atlantic salmon) to the Atlantic strain, which has no innate immunity (Peeler and Feist, 2011). Through the movement of fish for stocking and farming, G. salaris spread to over 40 rivers and caused declines of over 90% in wild populations (Peeler and Feist, 2011). This massive decline in salmon was presumably the result of reduced parasite load in the introduced Baltic strain, facilitating its improved competitive ability and leading to its demographic dominance over the endemic strain (Torchin et al., 2003).

1.7.2 Spill-back and dilution
Spill-back occurs when native parasites use alien species as a competent host, causing the disease to be sustained and magnified, and eventually spilled back to the natural host (Poulin et al., 2011, Thrush et al., 2011). In Lake Chichancanab, Mexico, Oreochromis spp. (African cichlid fish) were accidentally introduced, following damage to aquaculture facilities caused by hurricane Gilbert, resulting in a rapid increase in cichlid abundance. This population increase caused the dramatic decline of five native species of fish and the extinction of a sixth native fish species (Strecker, 2006). As cichlids are detritivore-planktivores, the decline of the native fishes was not caused by predation (Poulin et al., 2011). Both native and introduced cichlids act as an intermediate host for endemic trematodes, which complete their life cycle in piscivorous birds (Poulin et al., 2011). The larger size, greater abundance and greater use of open water habitats caused heavy predation of these exotic fishes by the definitive hosts, leading to an increased
trematode population and increased prevalence of the parasite in native hosts (Poulin et al., 2011).

Dilution occurs when endemic parasites use an alien species as a host, even though it may not be compatible, causing a reduction in disease risk for the native host (Poulin et al., 2011, Okamura and Feist, 2011). In New Zealand, the introduction of *Salmo trutta* (European brown trout) created a dilution effect for some native parasites, as it was a less competent host than native fishes (Dix, 1968, Poulin et al., 2011). A negative relationship was found between intensity of infection and index of local trout abundance in two native fishes, *Gobiomorphus breviceps* (upland bully) and *Galaxias anomalus* (roundhead galaxias) (Kelly et al., 2009). In other words, trematode infections in native fishes were less severe in sites where trout species are abundant (Poulin et al., 2011).

1.7.3 Spillover
If alien hosts introduce new parasites, then these may be transmitted to native hosts, leading to the emergence of new disease in the natives (known as spillover or pathogen pollution (Daszak et al., 2000, Taraschewski, 2006)). To threaten native hosts in a new locality, alien parasites must overcome the same barriers to introduction, establishment and spread as free-living aliens and, in addition, they must be able to switch from alien to native hosts. Lymbery et al. (2014) proposed using the terminology of ‘co-introduced’ for those parasites which have entered a new area outside of their native range with an alien host, and ‘co-invader’ for those parasites which have been co-introduced and then switched to native hosts (Figure 1.4).
Parasites may occasionally be introduced into a new location without their host(s). For example, eggs and juveniles of the swimbladder nematode *Anguillicola crassus*, a parasite of *Anguilla japonica* (Japanese eel), were introduced by aquaculture transport vehicles into the United Kingdom, where they have successfully parasitised native *Anguilla anguilla* (European eels) (Kirk, 2003). However, most invasive parasites involve co-introduction with their alien host species. A recent literature survey identified 98 examples of co-introductions of alien hosts and parasites, globally, across a wide range of taxa (Lymbery et al., 2014). The most common co-introduced parasites found in published studies were helminths (making up almost 49% of the total), arthropods (17%), and protozoans (14%). Fishes were by far the most common alien hosts in published studies making up 55% of the total; with 81% of fish hosts being either...
freshwater or diadromous. This may reflect a taxonomic bias in studies, but is also likely due to the propensity for freshwater ecosystems to be particularly affected by invasive fishes (García-Berthou, 2007, Johnson and Paull, 2011).

Figure 1.5. (a) Relative proportions of taxa represented in 98 examples of co-introduced parasites: prokaryotes (viruses and bacteria); protozoans; helminths (platyhelminths, nematodes and acanthocephalans); arthropods (crustaceans, arachnids); and a miscellaneous group including fungi, myxozoans, annelids, molluscs and pentasomids. (b) Relative proportions of alien hosts represented in 98 examples of co-introductions: molluscs; arthropods; fishes; mammals; and other vertebrates (amphibians, reptiles and birds). (c) Number of co-introduced parasite species with direct and indirect life cycles which have switched (black bars) or not switched (white bars) from alien to native host species (Lymbery et al., 2014).
It is usually considered that the establishment of parasites in a new environment is much more likely to occur in those species with simple, direct life cycles (i.e. vertical transmission or horizontal transmission without the need for intermediate hosts (Dobson and May, 1986, Bauer, 1991, Torchin and Mitchell, 2004)). There have been no empirical tests, however, of this hypothesis, because of the difficulty in obtaining data on parasite founding populations prior to establishment. In the 98 examples of parasite co-introductions documented by Lymbery et al. (2014), 64% of parasites had a direct life cycle and 36% had an indirect life cycle. This suggests that parasites with a direct life cycle might establish more readily in a new environment, but it is not a proper test of the hypothesis because no data were available on parasite co-introductions that failed to establish.

Parasites co-introduced with their hosts may spread geographically in their new range with their original, introduced host, without switching to native hosts. In the review by Lymbery et al. (2014), 78% of the 98 examples showed that co-introduced parasites were recorded in native hosts (i.e. became co-invaders), although this is likely to be an overestimate of the real incidence of host-switching, as null studies are less likely to be reported (Arnqvist and Wooster, 1995). For example, co-introductions without host-switching were found in monogenean parasites of the invasive *Lepomis gibbosus* (pumpkinseed fish) in the Danube River (Ondrackova et al., 2012), the lungworm *Rhabdias pseudosphaerocephala* of *Rhinella marina* (cane toads) in Australia (Pizzatto et al., 2012) and trematode *Haematoloechus longiplexus* in *Lithobates catesbeianus* (American bullfrogs) on Vancouver Island (Novak and Goater, 2013).

There is no evidence from published studies of an effect of life cycle on host switching. Of the 98 co-introduced parasites documented by Lymbery et al. (2014) 76% of parasites with a direct life cycle, and 80% of parasites with an indirect life cycle successfully switched to native hosts. This does not represent a strong test of the influence of life cycle on the propensity of introduced parasites to switch hosts, as it does not control for phylogeny or many of the other factors (e.g. host specificity and the similarity for host fauna and environmental conditions between source and recipient localities (Bauer, 1991, Kennedy, 1993)) which can influence the
propensity for host switching to occur. Nevertheless, it appears that not only are many parasites with complex, indirect life cycles able to be co-introduced and establish readily in a new environment, they are also no less likely to infect native hosts and become co-invasive than are parasites with direct life cycles.

1.8 Virulence of introduced parasites to native hosts

It has been suggested that parasites that switch from introduced species to native host species will have a greater pathogenic effect in the native hosts, where there is no coevolutionary history (naïve host syndrome (Mastitsky et al., 2010) or novel weapon hypothesis (Fassbinder-Orth et al., 2013)). Coevolution of parasite and host is often viewed as a contest between host resistance (ability to prevent infection) or tolerance (ability to limit damage caused by infection (Best et al., 2008, Svensson and Råberg, 2010)), and parasite virulence (parasite-induced reduction in host fitness; (Combes, 2001)). The naïve host theory states that parasites and hosts with long coevolutionary history will be co-adapted. Therefore, when an alien parasite is introduced into a new area and infects a naïve host that lacks coevolved resistance or tolerance, the naïve host will suffer serious disease (Allison, 1982, Mastitsky et al., 2010, Fassbinder-Orth et al., 2013).

The naïve host theory appears to be implied in many discussions of the impacts of co-invading parasites on native host (Daszak et al., 2000, Britton et al., 2011, Peeler et al., 2011, Peeler and Feist, 2011, Hatcher et al., 2012). There is, however, no evidence to suggest that the consequences of infection would be more severe in an immunologically naïve host species, than in a host species that has coevolved with the parasite (Lymbery et al., 2014). Parasites are expected to be ahead in the coevolutionary race as they generally have larger population sizes and shorter generation times than their hosts, making them locally adapted (i.e. having a greater mean fitness in local host populations than in foreign host populations (Kaltz and Shykoff, 1998). However, the fitness of the parasite can be enhanced by either a decrease or increase in virulence, depending on the circumstances of transmission (May and Anderson, 1983, Ebert and Herre, 1996). There is also the potential for an unknown level of virulence if the new host is not
closely related, phylogenetically, to the coevolved host, as virulence expressed in an unusual host will not necessarily relate to parasite fitness (Ebert, 1995).

Despite limited theoretical support for the naïve host theory, co-invading parasites may exhibit greater virulence to new, native hosts than to the alien hosts with which they were introduced, simply by chance. The probability of introduced hosts surviving the translocation process is likely to be inversely related to the virulence of any parasites they carry into their new range, because most introductions involve a few individuals being transported over geographic barriers or escaping from captivity (Blackburn et al., 2011). As a consequence, parasites with lower virulence in their natural host will be much more likely to be co-introduced (Strauss et al., 2012). If virulence of the parasite differs between the coevolved alien host and the new, native host, it is therefore more likely to be in the direction of increased virulence in the new host (Lymbery et al., 2014).

When a new, virulent parasite is introduced and spread there can be catastrophic effects on native host populations. Theoretical and empirical studies have both demonstrated that, through effects on host mortality and fecundity rates, parasites can provide density dependant regulation of their host population (Anderson and May, 1992, McCallum and Dobson, 1995, Hudson et al., 1998). On the International Union for Conservation of Nature list of the world’s worst invasive species, infectious disease is the main driver behind the impact of invasion in almost 25% of cases (Hatcher et al., 2012). In many instances, these diseases are caused by co-introduced parasites that have switched from alien to native hosts. For example, crayfish plague, caused by the fungus *Aphanomyces astaci*, has caused dramatic population declines in freshwater crayfish species throughout the world (Holdich and Reeve, 1991, Söderhäll and Cerenius, 1999, Evans and Edgerton, 2002). The parasite is largely asymptomatic in its natural North American freshwater crayfish hosts, but when spread with these hosts (or with ballast water or fish vectors) to new localities, has proved to be virulent in many European, Asian and Australian crayfish species (Holdich and Reeve, 1991, Söderhäll and Cerenius, 1999, Evans and Edgerton, 2002).
1.9 Control of invasive species and co-invading parasites

Invasive species are recognised as a major threat to biodiversity and much effort is extended in their control (Hauser and McCarthy, 2009, Britton et al., 2011, Sharp et al., 2011). The intended outcome of such control programs is the recovery of native species or ecosystems, but control of invasive species may have unintended consequences that prevent this outcome being realised (Bergstrom et al., 2009, Walsh et al., 2012). The effect of control programs on co-invading parasites has rarely been considered, but should be included in risk assessments prior to management interventions to control invasive species, because both invasive hosts and their co-invading parasites may fundamentally alter ecosystem function (Roy and Lawson Handley, 2012, Amundsen et al., 2013).

The relative competencies of native and alien hosts to transmit infections of co-invading parasites will determine whether the alien acts as a sink, to dilute the effects of the parasite, or a reservoir, to amplify the effects of the parasite on native hosts. In standard models of microparasite population dynamics, transmission rate is inversely related to virulence (Anderson and May, 1992), so the expectation would be that if introduced parasites are usually more virulent in native hosts, then alien hosts will act as reservoirs of infection. This seems to have occurred, for example, with avian malaria in Hawaii, the squirrel poxvirus in the UK and crayfish plague throughout Europe, where the natural, alien hosts increased transmission to native hosts (Dunn et al., 2009, Hatcher et al., 2012). The extent to which these cases can be generalised, however, is unclear. The expected inverse relationship between virulence and transmission rate arises from a simple mass action model of transmission, where transmission rate depends on the numbers (or densities) of infected and susceptible hosts, and increasing virulence removes infected hosts from the population (McCallum et al., 2001). In reality, the transmission process is likely to be much more complicated, particularly for parasites with complex life cycles, and there is limited theoretical or empirical support for a general trade-off between virulence and transmission rate (Ebert and Bull, 2003).
Alien hosts, therefore, may not always act as amplifying reservoirs, even when the parasite is less virulent in them than in native hosts. This has practical implications for the control of invasive alien species, when those species are associated with a co-invading parasite. If invasive aliens are more competent hosts than native species for a co-invading parasite, then control of the alien will reduce the infection pressure on native hosts. If, however, invasive aliens are less competent hosts, then control of the alien may inadvertently amplify infection of native hosts, with potentially devastating consequences on the native host population, particularly if other reservoirs are available. There are, unfortunately, very few empirical data on the relative competencies of different hosts for the transmission of any multi-host parasites (Haydon et al., 2002), let alone for alien and native hosts in transmitting co-invading parasites.

1.10 Introduced parasites in the Southwestern Province
There is very little known of the disease status of wild populations of native and exotic fishes in the Southwestern Province. A recent study by Lymbery et al. (2010) was the first comprehensive survey of parasites of freshwater fishes in the region. Forty-four putative species of parasites were found on native fishes, with most of these appearing to be native parasites that have not been previously described. This study also identified two introduced parasite species, *Lernaea cyprinacea* and *Ligula intestinalis* (Morgan, 2003, Marina et al., 2008, Lymbery et al., 2010).

1.11 *Lernaea cyprinacea* and Lernaeosis
There are currently 113 species of cyclopoid copepods classified in the family Lernaeidae (anchor worms), mostly known from females which are highly modified and parasitic on freshwater fishes (Ho, 1998). *Lamproglena* Nordmann and *Lernaea* Linnaeus are the two largest genera and make up a majority of the Lernaeidae family, accounting for more than two-thirds of the species (81/113). Of these two genera, *Lernaea* is both more diverse and widely distributed than *Lamproglen* (Ho, 1998).
Lernaeosis is a disease of freshwater fishes caused by parasitic copepods of the family Lernaeidae (Shariff et al., 1986, Lester and Hayward, 2006). The most common causative agent of lernaeosis is *Lernaea cyprinacea* (Hoffman, 1970, Ho, 1998, Lester and Hayward, 2006). *Lernaea cyprinacea* is not host specific and has a wide host range (Shariff et al., 1986). This species of parasite has been found in more than 45 species of cyprinids, fishes belonging to other orders and occasionally in tadpoles and amphibians (Tidd and Shields, 1963, Lester and Hayward, 2006). Its preferred hosts include the cyprinid species *C. carpio* (common carp), *C. auratus* (common goldfish) and *C. carassius* (crucian carp), though the parasite has been identified in over 100 fish species from 16 different orders (Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006).

*Lernaea cyprinacea* is not native to Australia but has been recorded in a number of native fish species in New South Wales and Victoria in eastern Australia, including *Maccullochella peellii* peellii (Murray cod), *Maccullochella macquariensis* (trout cod), *Macquaria ambigua* (golden perch), *Macquaria australasica* (Macquarie perch), *Bidyanus bidyanus* (silver perch), *Tandanus tandanus* (freshwater catfish), *Galaxias olidus* (mountain galaxias), *Prototroctes maranae* (Australian grayling) and *Gadopsis marmoratus* (river blackfish) (Ashburner, 1978, Hall, 1983, Bond, 2004). More recently there has been a report of *L. cyprinacea* in the Canning River in Western Australia (Marina et al., 2008). This is the first time that the parasite has been reported in Western Australia, being found on four native freshwater species: *Galaxias occidentalis* (western minnow), *Nannoperca vittata* (western pygmy perch), *Bostockia porosa* (nightfish) and *Tandanus bostocki* (freshwater cobbler).

*Lernaea cyprinacea* was most likely introduced into Western Australia accidentally through the release or escape of infected aquarium fishes into natural waterways (Marina et al., 2008). It is presumed that the parasite was brought in with cyprinid hosts such as *C. auratus* and *C. carpio* (Marina et al., 2008). Morgan et al. (2004) found that many streams, irrigation drains and lakes in the Perth vicinity contain *C. auratus* and *C. carpio*. These species, particularly, *C. auratus,
are also found in a number of natural waterways between the Moore and Vasse Rivers on the Swan Coastal Plain (Morgan et al., 2004, Beatty and Morgan, 2013).

1.11.1 Life cycle
The life cycle of *L. cyprinacea* is quite complex and has nine main stages, including three free-living naupliar stages, five copepodid stages, and one adult stage (Figure 1.6) (Grabda, 1963). Each of these stages are marked by a moult (Grabda, 1963, Shields, 1978). It is the copepodid and adult stages that are parasitic (Grabda, 1963). The copepodid larvae are usually localised on the gills and body surface of the host, where they mature and mate (Grabda, 1963, Shields, 1978, Berry et al., 1991, Lester and Hayward, 2006). Copepodids are also known to feed on the gill tissue of the fish hosts (Goodwin, 1999). Copepodids have low host specificity and a relatively loose connection with their fish host, meaning that they are able to move freely from host to host (Grabda, 1963, Shields, 1978). Goodwin (1999) observed that the copepodids were attached to gill filaments but would occasionally detach and move to new sites. Once the males and females have mated on the fish host, the males die and the females metamorphose (this is where the female undergoes significant morphological changes) (Grabda, 1963). In the sedentary phase, marked by the metamorphosed adult female, the female permanently attaches to the host by inserting its anterior body into the host tissue, becoming an egg producing organism (Grabda, 1963, Nagasawa et al., 2007). The eggs hatch into free living naupliar larvae, which moult into infective copepodids after about four days and attach to the gill of a fish host. After a week or so copepodids moult to adults, depending on the temperature, with optimal development occurring at 28-36°C and little development occurring below 20°C (Shields and Tidd, 1968, Lester and Hayward, 2006).
**Figure 1.6.** The life cycle of *Lernaea cyprinacea* L. on the host *Carassius auratus* (L.) (re-drawn from Shields (1978)).

### 1.11.2 Clinical signs and pathology

The attachment of *L. cyprinacea* can have a number of serious pathogenic consequences for the fish host. The pathogenicity of *L. cyprinacea* is determined by the two parasitic stages: the copepodids and the metamorphosised adult female.

Haemorrhaging has been associated with the attachment of the copepodids to the fish host. Shields (1978) used the haemorrhaging as a quick visual sign of infection, finding the most extensive damage around the fin areas. An infection of copepodids on the gill of the fish host typically causes respiratory distress and sluggishness (Kabata, 1979). Damage caused by copepodids is particularly seen around the attachment site on the gills, this includes epithelial hyperplasia, displacement and erosion of lamellae, telangiectasis, and congestion or hemorrhage in the filament central sinus (Goodwin, 1999). This disruption and necrosis of gill epithelium
can result in fish death (Khalifa and Post, 1976). It has also been suggested that the copepods of *Lernaea* may open routes for secondary infections (Woo and Shariff, 1990).

The adult female stage of *L. cyprinacea* is often found on the tail and body of the fish host (Shields and Tidd, 1974). The principal pathogenic effects of the disease lernaeosis are associated with the metamorphosised female’s attachment and feeding behaviour on tissue debris and erythrocytes (Kabata, 1985). This causes chronic exhaustion of the energy reserves of the host (Kabata, 1985), as well as weight loss, stunted growth and reduced reproductive performance (Kabata, 1985, Khan et al., 2003). The attachment of adult females is often accompanied by haemorrhages and muscle necrosis (Khalifa and Post, 1976, Berry et al., 1991, Lester and Hayward, 2006). Bond (2004) found that there was also reduced swimming ability and high mortality rates (usually associated with epithelial destruction and secondary wound infection). Host fins are damaged or destroyed while scales are lost resulting in circular ulcers (Kabata, 1985). The anchor apparatus normally triggers an intense inflammatory response at the attachment site, which may be encapsulated by a thick fibrotic layer (Khalifa and Post, 1976, Kabata, 1985, Berry et al., 1991, Lester and Hayward, 2006). Those fishes that survive infections are generally left with large scars (Marina et al., 2008). The pathological effects of adult female parasites are often found to be greater on smaller fishes because the attachment organ penetrates more deeply into the body of the fish causing damage to the internal organs (Khalifa and Post, 1976, Lester and Hayward, 2006).

### 1.11.3 Detection and characterisation

*Lernaea cyprinacea* is primarily a freshwater species (Shields and Sperber, 1974, Kabata, 1979). It is a thermophilic parasite and more prevalent during the warmer months in temperate climates, preferring water temperatures between 25-32°C (Shields and Tidd, 1968, Bulow et al., 1979, Lester and Hayward, 2006). The species tends to favour environments that provide suitable conditions for attachment and so are more commonly found in lentic ecosystems and slow flowing waters (Haley and Winn, 1959, Demaree, 1967, Al-Hamed and Hermiz, 1973, Bulow et al., 1979, Medeiros and Maltchik, 1999).
When referring to *L. cyprinacea*, the common name ‘anchor worm’ describes the anchor-like processes (holdfast) which the adult female uses to attach itself to the host (Noga, 2000). The arms of the holdfast penetrate the host’s body allowing the parasite’s head to firmly anchor as the rest of the body floats freely in the water (Grabda, 1963). This anchorage allows the copepod to stay firmly attached to the fish body so that it cannot be easily washed away (Grabda, 1963). The hold is so strong that mechanical removal of the parasite with forceps results in the death of the copepod as the head remains in the body of the fish (Noga, 2000).

There are a number of morphological features that are used to distinguish between species in the family Lernaeidae, including the cephalothorax, metasomal somites, egg sac, antenna, exopods and endopods of legs, maxilla and maxilliped (Ho, 1998). In particular, the shape of the holdfast of the metamorphosed female is unique to each species and is a fundamental taxonomic character to identify *L. cyprinacea* Figure 1.7 (Harding, 1950, Fryer, 1961). *Lernaea cyprinacea* is identified by the anchoring apparatus developing from outgrowths posterior to the parasites head with two pairs of cylindrical structures (arms) (Grabda, 1963). The dorsal pair is larger than the ventral pair and divides into two branches at its base (T or Y shaped dorsal ramified pair) (Grabda, 1963, Kabata, 1979, Kabata, 1985). Unlike the dorsal pair, the ventral pair is not ramified but is willowy (Kabata, 1985). Although the female holdfasts are often used in species identification, there is evidence of morphological plasticity which causes complications with morphological identification (Kabata, 1979, Kabata, 1982, Lester and Hayward, 2006). This means that definitive confirmation of species identity will requires molecular characterisation.
Figure 1.7. Lateral view of an adult metamorphosed female of *Lernaea cyprinacea* (Demaree, 1967). List of abbreviations: ab = abdomen; al = anal laminae; c = cephalothorax; es = egg sac; h = head; pp = pregenital prominence; sl = swimming legs; tl = total length; tr = trunk
1.12 Thesis aims and objectives

Without knowing the effects of *Lernaea cyprinacea* on native freshwater fishes we are unable to gain a full understanding of its current impact on our fauna or know how far spread it has become. The more information we have the greater our ability to help preserve our native freshwater ecosystems.

The overarching aim of this study was to determine the geographic range, prevalence and pathogenicity of the introduced parasite *Lernaea cyprinacea* on native freshwater fishes in south-western Australia. More specifically, the study aimed to investigate the following hypotheses:

1. Using molecular characterisation will confirm the presence of *L. cyprinacea* in Western Australia.
2. There will be an increase in the geographical distribution and host range of *L. cyprinacea* in the Southwestern Province since it was first recorded.
3. The native freshwater fish, *N. vittata*, will show greater levels of susceptibility to *L. cyprinacea* than its presumed ancestral host, *C. auratus*.
4. There will be physiological and behavioural differences among fish species, which may contribute to differences in infectivity, when exposed to the parasite.
5. Native freshwater fishes will have a greater level of pathogenicity to the parasite when compared to *C. auratus*. 
Chapter 2
Distribution of *Lernaea cyprinacea* in south-western Australia

2.1 Introduction
Alien species are recognised as invasive when they establish self-sustaining populations in a locality outside of their natural range and spread beyond their point of introduction (Vitousek et al., 1997, Sakai et al., 2001). Human population growth, increasing transport capacity and economic globalisation have accelerated the rate of introductions of alien species throughout the world (Vitousek et al., 1997, Sakai et al., 2001). Not only are invasive species now recognised as a major cause of biodiversity loss, they have also been associated with changes in ecosystem functioning, resulting in biotic homogenisation as native species are replaced by widespread alien species (Pimentel, 2002, Rahel, 2002, Simberloff, 2011). Invasive species are able to affect native species directly (e.g., competition or predation) and indirectly (e.g., altering habitat or changing disease dynamics).

Introduced alien hosts often have fewer parasite species and a lower prevalence of parasites than native hosts, which may provide them with a competitive advantage (enemy release; Mitchell and Power, 2003, Torchin et al., 2003). Once introduced, parasite transmission may occur from native hosts to alien hosts, leading to either an increase in infection of native species if alien species amplify transmission (spillover; Daszak et al. 2000, Kelly et al., 2009) or a decrease in infection of native species if alien species reduce transmission (dilution; Keesing et al., 2006, Poulin et al., 2011). If alien hosts introduce new parasites, then these may be transmitted to native hosts, leading to the emergence of new disease in the native species (spillover or pathogen pollution; Daszak et al., 2000, Taraschewski, 2006).

Co-invading parasites are increasingly being recognised as important causes of disease emergence, often producing high morbidity and mortality in native hosts (Smith and Carpenter,
2006, Taraschewski, 2006, Peeler et al., 2011). Freshwater ecosystems are particularly impacted by invasive species and co-invading parasites.

As mentioned previously, Southwestern Ichthyological Province of Australia has a depauperate, although highly endemic, freshwater fish fauna, with nine of the 11 species of native freshwater fish endemic to the region (Lymbery et al., 2010). Since 1970, there has been a 63% increase in alien fish introductions to the Southwestern Province, with 13 alien fish species having established self-sustaining populations (Beatty and Morgan, 2013). Most alien fish introductions in the last 10 years have been aquarium species (Beatty and Morgan, 2013).

Recent studies on the parasites of freshwater fishes in south-western Australia have reported the first cases of *Lernaea cyprinacea* on native fishes (Marina et al., 2008, Basile, 2011). *Lernaea cyprinacea* is a generalist parasite with a wide host range (Shariff et al., 1986). Although its preferred hosts include cyprinid species (such as *Cyprinus carpio* (common carp) and *Carassius auratus* (goldfish)), it has been identified in over 100 fish species from 16 orders (Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006), and reported from Africa, Asia, Europe, North America and Australia (Hoffman, 1999, Durham et al., 2002). Marina et al. (2008) reported *L. cyprinacea* on four native freshwater species; *Galaxias occidentalis* (western minnow), *Nannoperca vittata* (western pygmy perch), *Bostockia porosa* (nightfish) and *Tandanus bostocki* (freshwater cobbler) in the Canning River, which runs through the Western Australian capital city, Perth.

The conclusions drawn by Marina et al. (2008) about the presence and geographic extent of *L. cyprinacea* in south-western Australia were subject to two caveats. First, their species identification relied on morphological criteria, which may be compromised by the considerable morphological plasticity of species of *Lernaea* (Kabata, 1979, Lester and Hayward, 2006). Second, although they examined fishes from 12 river systems spanning the range of the Southwestern Province, they did not sample other rivers in the vicinity of the Perth metropolitan area. The aim of the current study was to use molecular techniques to confirm the
species identity of the parasite and to more precisely map the geographic and host range in south-western Australia.

2.2 Methods
2.2.1 Sampling
Fishes were sampled from a number of localities in 22 different river systems in the Southwestern Ichthyological Province: Moore, Preston, Abba, Sabina, Ludlow, Vasse, Margaret, Blackwood, Donnelly, Warren, Gardner, Shannon, Deep, Styx, Kent, Denmark, Hay, King, Kalgan, Canning, Serpentine and Murray River. All rivers were sampled in 2011, except for the Serpentine River, which was sampled in both 2011 and 2013, during the months of summer.

All fish sampled were caught with the approval of the Department of Parks and Wildlife (Permit number: SF009875)

All rivers were sampled with two-winged fyke nets (75 x 105 cm mouth opening; 55 x 400 cm wing; 500 cm long pocket with two funnels; 0.2 cm mesh size), with the nets facing both upstream and downstream to catch all migrating fishes (Figure 2.1). To ensure the capture of nocturnal species, the nets were set mid-afternoon and retrieved early the next morning.

Collected fishes were identified to species and measured for total length (TL) on site. All fishes were then examined for *Lernaea* spp. by visually inspecting on their body surface and were considered positive if the adult female parasite was still attached to the host. Native fishes that were not found to be infected (lacked the presence of an adult female) were immediately released, whereas all infected fishes and all alien fishes (infected and uninfected) were transported to the laboratory in aerated containers. Fishes were then euthanised with an overdose of anaesthetic (Aqui-S; 30mL/L) and fixed in 10% buffered formalin. Infection was confirmed by visual inspection using a dissecting microscope and the numbers of parasites and location on the host (fins, head, body or tail) recorded.
2.2.2 Molecular characterisation

For samples that had been preserved in formalin, a minimum of 3 phosphate-buffered saline (PBS) washes were conducted before DNA extraction, using tissue from ~8-10 adult *Lernaea* sp. (per sample) with a PowerSoil DNA Isolation Kit (Mo Bio, California, USA), as per the manufacturer’s instructions. DNA was stored in a freezer at -20°C until required.

2.2.2.1 PCR Using 18S and 28S rDNA Primers

Using non-specific primers, a standard PCR protocol was used to amplify a product of ~744bp at both the 18S and 28S rRNA loci (Song et al., 2008). The PCR was conducted in a final volume of 25µl and consisted of a final concentration of 200ng genomic DNA, 0.2µM of the forward and reverse primers (18SF (5′–AAGGTGTGMCCCTATCAACT–3′) and 18SR (5′–TTACTTCCCTCTAAACGCTC–3′), 28SF (5′–ACAACTGTGTGCCCTTAG–3′) and 28SR (5′–TGGTCCGTGTTCAGACG–3′)), 50µM of deoxynucleotide triphosphates (dNTPs) (Fisher Biotec), 1xPCR buffer (with 2 mM MgCl₂) (Fisher Biotec), 2.5U of ExTaq DNA polymerase (Fisher Biotec) and 2µl of template DNA. Ultra-pure PCR water was added to a final volume of 25µl. PCR conditions consisted of: an initial cycle of 94°C for 5 minutes,
followed by 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 1 minute, a final extension at 72°C for 5 minutes and a hold of 14°C.

A positive control and negative control were used in each PCR reaction. The positive control consisted of 2µl of *L. cyprinacea*, and the negative control used no template DNA. Final PCR products were run on a 1% (w/v) agarose gel (Invitrogen, New Zealand) in a TAE buffer (containing 40mM Tris-HCl; 20mM EDTA; pH 7.0) stained with SYBER® safe DNA gel stain (10,000 concentration in DMSO. Invitrogen Molecular probes®, Eugene, Oregon, USA). A 100 bp molecular weight ladder was used (Axygen, Fisher Biotech, Australia) and DNA was visualized using UVP dual-density transillumination (positive samples were identified through a single band on the gel).

**2.2.2.2 Sequencing**
Positive amplicons were cut from the gel using disposable scalpel blades and were placed into 1.5ml eppendorf tubes. Samples not sequenced immediately were frozen at -20°C. Using an Ultra Clean 15 DNA Purification kit (Geneworks), a high salt solution provided by the kit was added to the samples (enough to cover the gel). These samples were then placed on heating bricks at ~56°C until the gel melted. Solutions were resuspended and 5µl of ultrabind, containing silica to bind the DNA, was added to each of the samples, which were then mixed by inverting the tubes several times and left at room temperature for 5 minutes. The samples were then centrifuged for 5 seconds at 14000xg to pellet the DNA/silica mixture and the supernatant discarded. Ultrawash (400µl) was added and each tube, vortexed and centrifuged at 14000xg for 5 seconds and the supernatant was once again discarded. To remove any excess moisture, samples were vacuum-dried using a rotorvac for 5-10minutes. Ultrapure water (10-20µl) was added to each sample to elute the DNA from the silica and incubated at 56°C for 5 minutes. Once again the samples were centrifuged at 14000xg for 1 minute and the supernatant containing the DNA was transferred into a 0.6µl tube. DNA concentration for each sample was tested using a NanoDrop.
PCR products were sequenced using a Big Dye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). The sequencing reactions contained 3.2 pmol of primer, 2 µl of Big Dye version 3.1 (Applied Biosystems), 1.5 µl of 5 x sequence buffer (Applied Biosystems), 2.5-5 µl of template (depending on the DNA concentration) and ultrapure PCR water added to 10µl. Cycle sequencing was conducted using an initial heating of 96°C for 2 minutes and then 25 cycles of 96°C for 10 seconds, 55°C for 5 seconds, 60°C for 4 minutes and a final hold of 72°C for 7 minutes.

Cycle sequencing products were placed into 0.6 mL eppendorf tubes and precipitated by adding 1 µl of 125 mM EDTA (disodium salt), 1 µl of 3 mM sodium acetate pH 5.2 and 25 µl of 100% ethanol. The solution was mixed and left for at least 20 minutes at room temperature to precipitate the DNA. Samples were then spun at 14000xg for 30 minutes, the supernatant was discarded, and the pellet rinsed by adding 125 µl of 75% ethanol. The samples were once again spun at 14000xg for 5 minutes. The supernatant was discarded and the samples placed in the speed vac for 5-10 minutes. Finally the samples were air dried in the dark for 15 minutes.

2.2.2.3 Species identification and phylogenetic analysis
Sequences were viewed using Finch TV Version 1.4.0 (Geospiza Research Team 2004-2006) and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Distance, Parsimony and Maximum Liklihood (ML) trees were constructed using MEGA version 7 (Tamura et al., 2011). Bootstrap support for branching was based on 1000 replications and checked for identity using the nucleotide database, Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov).

2.2.3 Data analysis
Parasite data were expressed as prevalences (proportion of infected hosts) and intensities of infection (number of attached parasites per infected host). Ninety five percent confidence intervals were calculated for prevalences, assuming a binomial distribution, and for mean intensities, from 2,000 bootstrap replications using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Within each river, prevalences were compared among fish species using chi-square tests, and mean intensities were compared among fish species using a non-parametric
Kruskall-Wallis test. For the Serpentine River, where fish were sampled in 2011 and 2013, prevalences were compared between times for each fish species using a Fisher exact test and intensities were compared using a non-parametric Wilcoxin signed rank test. The relationship between fish length and parasite infection, pooled over rivers, was tested using generalised linear models, with infection status (present or absent, modelled as a binomial distribution with a logit link function) and intensity (modelled as a Poisson distribution with a log link function), as response variables and fish total length nested within species as a predictor variable. Differences among fish species in site of infection, pooled over rivers, were compared by chi-square tests. All statistical comparisons were performed using JMP v10 (SAS Institute Inc., 2009).

2.3 Results
2.3.1 Species identification
Of the 22 samples taken from fish hosts and tested at the 18S and 28S locus, due to time constraints and issues with sequencing, only three tested positive for *Lernaea cyprinacea* and were sequenced at the 28S locus. All parasite isolates had an identical DNA sequence and a maximum identity match of 99% to *Lernaea cyprinacea* isolate LCM 28S ribosomal RNA gene, partial sequence (GenBank accession number: DQ107548), from an isolate identified in a study of freshwater parasitic copepods in China (their sequences ranging from 686 to 696 bp in length) (Song et al., 2008). Phylogenetic analysis using distance, parsimony and ML methods produced identical trees (data not shown) and showed that the three sequences from the present study grouped within the *L. cyprinacea* clade of available sequences from GenBank at this locus (Fig. 2.2).
Figure 2.2. Phylogenetic tree of *L. cyprinacea* sequences generated during this study at the 18S/28S locus inferred using distance analysis. Bootstrap values (>50% for 1,000 replicates) are indicated at the nodes.

The three sequences generated as part of the present study have been submitted to GenBank under the accession number KY346866-KY346868.

### 2.3.2 Distribution of infection among rivers and fish species

Fishes infected with attached adult *L. cyprinacea* were found in only three of the 22 rivers sampled; the Canning River, Serpentine River and Murray River (Figure 2.3). A total of 3,540 fishes belonging to 17 different species (14 native and 3 alien) were sampled from these rivers. Two hundred and seventy seven of the fishes sampled (7.8%) were found to be infected (the adult female of the parasite was visible on the fish host), and of these, 258 (93%) of these were native fish (six species), while all three alien fish species were also found to have infections.
Figure 2.3. Sampling sites in the Southwestern Ichthyological Province, Western Australia. Rivers negative for *Lernaea cyprinacea* (red dots). Rivers positive for *L. cyprinacea* (blue dots). Sites within positive rivers identified as having *L. cyprinacea* (green dots).

Infections with adult *L. cyprinacea* were found on six native fish species; *Pseudogobius olorum* (bluespot goby), *Nannoperca vittata* (western pygmy perch), *Tandanus bostocki* (freshwater cobbler), *Bostockia porosa* (nightfish), *Leptatherina wallacei* (western hardyhead) and *Galaxius occidentalis* (western minnow). Infections were also found on the alien *Phalloceros caudimaculatus* (leopard fish), *Carassius auratus* (goldfish) and *Gambusia holbrooki* (eastern mosquitofish) (Figure 2.4).
Figure 2.4. Percentage of fishes infected with *L. cyprinacea* belonging to different species.

Prevalences and intensities of infection for each fish species found in each river are shown in Table 2.1. Within each river, there were significant differences among fish species in prevalence (Canning River $\chi^2_6 = 106.45$, $P < 0.0001$; Murray River $\chi^2_5 = 303.76$, $P < 0.0001$; Serpentine River 2011 $\chi^2_6 = 106.33$, $P < 0.0001$; Serpentine River 2013 $\chi^2_6 = 154.24$, $P < 0.0001$), but not in intensity, except for the Serpentine River in 2013 (Canning River $\chi^2_6 = 7.42$, $P = 0.28$; Murray River $\chi^2_1 = 0.28$, $P = 0.59$; Serpentine River in 2011 $\chi^2_4 = 6.45$, $P = 0.17$; Serpentine River in 2013 $\chi^2_5 = 18.56$, $P = 0.002$).

For the Serpentine River, where fishes were sampled in 2011 and 2013, there were no consistent differences in rates of infection over time. Prevalences were significantly greater in 2013 for *C. auratus* (Fisher exact test, $P < 0.0001$) and *T. bostocki* ($P = 0.02$), but significantly greater in 2011 for *N. vittata* ($P = 0.005$). Intensities of infection differed significantly only for *T. bostocki* ($z = 2.32$, $P = 0.02$), with intensity being greater in 2013.
Table 2.1. Prevalences (proportion of infected fish) and mean intensities of infection of *Lernaea cyprinacea* of fish species in the Canning, Murray and Serpentine Rivers. 95% confidence intervals in parentheses. N = total number of fish sampled.

<table>
<thead>
<tr>
<th>River</th>
<th>Fish Species</th>
<th>N</th>
<th>Prevalence</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canning</td>
<td>Native</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. porosa</em></td>
<td>54</td>
<td>0.11 (0.05-0.23)</td>
<td>2.2 (1.0-3.0)</td>
</tr>
<tr>
<td></td>
<td><em>G. occidentalis</em></td>
<td>116</td>
<td>0.17 (0.11-0.25)</td>
<td>1.3 (1.1-1.4)</td>
</tr>
<tr>
<td></td>
<td><em>N. vittata</em></td>
<td>269</td>
<td>0.07 (0.05-0.11)</td>
<td>1.2 (1.0-1.4)</td>
</tr>
<tr>
<td></td>
<td><em>P. olorum</em></td>
<td>25</td>
<td>0.08 (0.01-0.26)</td>
<td>1.5 (1.0-2.0)</td>
</tr>
<tr>
<td></td>
<td><em>C. auratus</em></td>
<td>33</td>
<td>0.12 (0.04-0.28)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>G. holbrooki</em></td>
<td>367</td>
<td>0.003 (0-0.02)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>P. caudimaculatus</em></td>
<td>507</td>
<td>0.002 (0-0.01)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alien</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>C. auratus</em></td>
<td>33</td>
<td>0.12 (0.04-0.28)</td>
<td>1</td>
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<td></td>
<td><em>G. holbrooki</em></td>
<td>367</td>
<td>0.003 (0-0.02)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>P. caudimaculatus</em></td>
<td>507</td>
<td>0.002 (0-0.01)</td>
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<tr>
<td>Murray</td>
<td>Native</td>
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<td></td>
<td></td>
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<td><em>G. occidentalis</em></td>
<td>127</td>
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<tr>
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<td><em>L. wallacei</em></td>
<td>361</td>
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<td><em>N. vittata</em></td>
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<td>0</td>
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<td><em>T. bostocki</em></td>
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<td>0.32 (0.27-0.39)</td>
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<td>147</td>
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<td>0</td>
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<td></td>
<td>Alien</td>
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<td><em>G. holbrooki</em></td>
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<td><em>B. porosa</em></td>
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<td>0.33 (0.14-0.60)</td>
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<td><em>G. occidentalis</em></td>
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<td>0.11 (0.06-0.19)</td>
<td>1.1 (1.0-1.3)</td>
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<td><em>N. vittata</em></td>
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<td>0.55 (0.32-0.75)</td>
<td>2.2 (1.4-3.1)</td>
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<td><em>P. olorum</em></td>
<td>28</td>
<td>0.14 (0.05-0.32)</td>
<td>1.2 (1.0-1.5)</td>
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<td><em>T. bostocki</em></td>
<td>59</td>
<td>0.51 (0.38-0.64)</td>
<td>1.6 (1.3-2.0)</td>
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<tr>
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<td>0</td>
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<td></td>
<td><em>G. holbrooki</em></td>
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<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>Alien</td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>C. auratus</em></td>
<td>42</td>
<td>0.26 (0.27-0.39)</td>
<td>1.3 (1.2-1.4)</td>
</tr>
<tr>
<td></td>
<td><em>G. holbrooki</em></td>
<td>167</td>
<td>0.006 (0-0.04)</td>
<td>1</td>
</tr>
</tbody>
</table>

The risk of infection was significantly influenced by fish size over all species ($\chi^2 = 371.37$, P < 0.0001), with larger fishes more likely to be infected, but there was no effect of fish size on intensity of infection ($\chi^2 = 4.93$, P = 0.76).

### 2.3.3 Predilection sites for attachment

The relative frequency of attachment sites were compared among those fish species where at least five infected fish were found (*T. bostocki*, *B. porosa*, *G. occidentalis*, *N. vittata* and *C. auratus*). Of the 210 parasites found on these fishes, 80.4% were located on the fins, 13.4% on the body, 1.9% on the tail and 4.3% on the head. Fish species had a significant effect on the
attachment site of *L. cyprinacea* ($\chi^2 = 83.36$, df = 64, P = 0.05) with predilection for the fins and the body in *T. bostocki, B. porosa, G. occidentalis* and *N. vittata*, and for the fins, tail and body in *C. auratus* (Figure 2.5).

![Pie charts showing attachment site preferences of Lernaea cyprinacea on different fish species.](image)

**Figure 2.5.** Percentage of *Lernaea cyprinacea* attached at different body sites on (a) *Tandanus bostocki*, (b) *Bostockia porosa*, (c) *Galaxias occidentalis*, (d) *Nannoperca vittata*, (e) *Carassius auratus*. 
2.4 Discussion
2.4.1 Species identification

DNA sequencing at the 28S rRNA loci confirmed that *L. cyprinacea* is present in the Southwestern Ichthyological Province. In previous studies, species identification was based only on morphology (Marina et al., 2008). The metamorphosed females are known to have holdfasts unique to their species, and often this is used as a fundamental characteristic to aid in identification of *Lernaea* species (Harding, 1950, Fryer, 1961). However, studies have cast some doubt on the reliability of the holdfasts in species identification (Kabata, 1979, Kabata, 1982, Lester and Hayward, 2006), therefore both molecular and morphological identification is required in species confirmation.

Although *L. cyprinacea* was positively identified, given the limited number of samples sequenced, we cannot be sure that *L. cyprinacea* is the only species of *Lernaea* present in south-western Australia. Nevertheless, the limited morphological variation observed among isolates from this and previous studies in south-western Australia (Marina et al., 2008; Basile, 2001) suggest that the occurrence of multiple species is unlikely. The three positive samples were collected from the Serpentine and Canning Rivers, all identified on *C. auratus*.

*Lernaea cyprinacea* has been identified as an invasive species across many continents, including Africa, Europe, North America and Australia (Hall, 1983, Hoffman, 1970, Kennedy, 1993, Robinson and Avenant-Oldewage, 1996, Durham et al., 2002, Lester and Hayward, 2006, Marina et al., 2008, Innal and Avenant-Oldewage, 2012, Koyun et al., 2015). It has been suggested that the parasite originated from Asia and then spread to different parts of the world, from movement of aquarium species, although we do not know this for certain (Robinson and Avenant-Oldewage, 1996, Innal and Avenant-Oldewage, 2012, Acosta et al., 2013). Currently, Australia’s quarantine policies are based on the risk analysis guidelines that were established under the World Trade Organisation’s Sanitary and Phytosanitary (SPS) Agreement. However, a review of Australia’s ornamental fish importation has suggested that these guidelines do not incite and acceptable level of protection (Whittington and Chong, 2007). Therefore, the inability
to construct meaningful risk analysis for ornamental fish importation leaves Australia at risk of additional exotic disease incursion.

An attempt to use these *L. cyprinacea* sequences to find a possible origin for its introduction into Western Australia, using the National Centre for Biotechnology Information (NCBI) database, was performed by comparing these sequences with known *L. cyprinacea* sequences from other regions, but unfortunately the origins of the species could not be determined in this study.

Information regarding the genomic sequence of *Lernaea cyprinacea* is still very limited, with a study by Pallavi et al. (2015), being the first to comprehensively examine *L. cyprinacea*. Molecular characterisation of *L. cyprinacea* has been principally focused on the partial sequences of 18S and 28S rDNA (Song et al., 2008, Stavrescu-Bedivan et al., 2014). Due to the limited number of studies in this area it is difficult to say whether or not molecular characterisation is, in itself, a reliable tool for species identification. However, the strength and reliability in species identification comes when both morphology and molecular characterisation are used together.

### 2.4.2 Distribution

This study has extended the known geographic range of *L. cyprinacea* in south-western Australia. The initial study by Marina et al. (2008) sampled fishes from 11 rivers within the Southwestern Province (including the Moore, Canning, Murray, Harvey, Harris, Vasse, Blackwood, Warren, Kalgan, Goodga and Pallinup Rivers) and found that *L. cyprinacea* was limited to the Canning River. In the present study, the parasite was identified on fishes in the Canning River as well as both the Murray River and Serpentine River. This is the first time *L. cyprinacea* has been reported in the Murray River and Serpentine River.

The reason for the introduction of *L. cyprinacea* into the south-west of Australia has yet to be established, although Marina et al. (2008) suggested that the initial infection started with the release or escape of infected ornamental fishes such as *C. auratus*. In Australia, there are around
38 species of self-sustaining exotic freshwater fishes nationwide, with 16 identified in Western Australia and at least 13 found in the Southwestern Ichthyological Province, including *C. auratus* (Department of Fisheries, 2002). *Carassius auratus* has been found in many streams, irrigation drains and lakes in the Perth vicinity, as well as a number of natural waterways between the Moore and Vasse Rivers on the Swan Coastal Plain (Morgan et al., 2004). They are a particular problem in the Vasse River System where removal programs have been in operation since 2005 (Morgan and Beatty, 2007).

As the Canning and Serpentine Rivers are connected by Birriga Drain, infected fishes may have used this as a corridor to move from one river to the other. The most likely explanation for the appearance of the parasite in the Murray River is a separate introduction of infected fishes, as a number of alien species have been recorded in the river (Morgan et al., 2004). There is also the possibility that infected fishes may have used the Peel-Harvey Estuary to move from the Serpentine River to the Murray River. The parasite was identified on both *C. auratus* and *Pseudogobius olorum* (bluespot goby) in Peel Drain, part of the Serpentine River. Of these two fishes, *P. olorum* would be more likely to migrate between rivers using the Peel-Harvey Estuary. Gobiidae are among some of the most abundant taxa in temperate estuaries and lagoons, often dominating these spaces numerically, or at least contributing substantially to estuarine ichthyofaunal assemblages (Potter and Hyndes, 1999, Whitfield, 1999). Although *L. cyprinacea* is relatively salt-sensitive (Lester and Hayward, 2006, Idris and Amba, 2011), studies have shown that the parasite can survive in up to 10-15 ppt of salt for a short period of time (Idris and Amba, 2011). The Peel-Harvey Estuary is generally high in salinity but has been known to fall as low as 25 ppt (Lukatelich and McComb, 1986). Given the possibility that *L. cyprinacea* could survive a short exposure to higher levels of salt, it is still possible that infected *P. olorum* would be able to cross the small section of the estuary from the mouth of the Serpentine River into the Murray River.

Currently, *L. cyprinacea* has not been found in any other rivers in the Southwestern Ichthyological Province. This does not necessarily mean that the parasite is absent from these
rivers. Most rivers in recent times have been sampled at only a limited number of sites and fishes have only been examined for attached adult female parasites, so it is possible that *L. cyprinacea* is more widespread, but at low prevalence or with a patchy distribution within infected river systems. Furthermore, the apparent increase in distributional range (from one river to three rivers) since the parasite was first reported in 2008 suggests that further spread is very likely to occur.

Finding a preference for parasite attachment on the fins is not uncommon and has previously been observed in other studies (e.g. Shields and Tidd, 1974, Bulow et al., 1979, Goodwin, 1999, Marina et al., 2008). It has been suggested that these attachment sites provide greater protection against being dislodged by currents (Medeiros and Maltchik, 1999). The scales/lack of scales of a fish may also determine parasite attachment (Dalu et al., 2012).

### 2.4.3 Host range
*Lernaea cyprinacea* has a wide host range (Shariff et al., 1986) and has been identified in over 100 species of fish from 16 different orders (Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006). In the present study, as well as documenting an increased geographic range of *L. cyprinacea*, additional host species were identified. *Lernaea cyprinacea* infections were found on six native and three alien fish species. These included all the species previously found to be infected by Marina et al. (2008), with the addition of *L. wallacei* and *P. olorum*, which are native estuarine species often found in salinised river systems in Western Australia (Morgan et al., 2014).

At all the sites in which *L. cyprinacea* infestation was found, the parasite was more prevalent in native freshwater species than in alien fishes, including the presumed ancestral host *C. auratus*. There may be a number of reasons for the differences in prevalence found between native and alien fishes: intrinsic parasite features, such as a simple life cycle and low host-specificity, could help facilitate host-switching; environmental requirements or behaviours could enhance the frequency of contact between the parasite and native hosts, causing increased host-parasite
encounters; and differences in host susceptibility may account for a greater attachment rate of the parasite to native hosts.

2.4.3.1  Host switching
Host-switching occurs when an alien fish species is translocated to a new geographical region carrying with it parasites from the source; here the introduced parasite transfers to native fish species (Taraschewski, 2006). Host-switching is more likely to occur with an introduced parasite that has both a simple, direct life cycle and broad host range (Peeler and Feist, 2011, Poulin et al., 2011, Thrush et al., 2011). The transmission of the infective stages of *L. cyprinacea* occurs by environmental contact, not via an intermediate host, and the parasitic copepodid and adult stages are characterised by low host specificity (Lester and Hayward, 2006). Therefore, *L. cyprinacea* satisfies both requirements for a high probability of host-switching (Grabda, 1963, Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006). In particular, the copepodid stages have a loose connection with the host, where they may simply cling to the surface of the host body for a time and then swim away to seek another host (Grabda, 1963, Shields, 1978). Although the metamorphosed adult female anchors more permanently to the host, it has still been isolated from more than 100 fish species (Lester and Hayward, 2006). This ease of parasite attachment to a new host species may be due to the morphological plasticity of the anchoring apparatus (Harding, 1950, Fryer, 1961, Kabata, 1979).

2.4.3.2  Host-parasite contact
Differences in parasite prevalence between native and alien fish species may be determined by differences in host-parasite contact frequencies. For example, as most native fishes undertake regular migration for spawning and feeding (Pen and Potter, 1990, Pen and Potter, 1991, Morgan et al., 1998, Beatty and Morgan, 2010, Morgan et al., 2011), this may increase the chances of encountering *L. cyprinacea*. The habitat requirements of fishes may also influence host-parasite contact frequency. In the present study, *T. bostocki*, the native freshwater cobbler, was found to have the highest prevalence of *L. cyprinacea*. This is a benthic species of fish, preferring lentic and slow flowing waters (Pen and Potter, 1990, Morgan et al., 1998, Morgan et al., 2011). As still and slow flowing waters are abiotic factors that help in the development and
attachment of \( L. \ cyprinacea \), and the infective copepodid stage is negatively phototactic (Shields, 1978), the habitat choice of \( T. \ bostocki \) may aid in the frequency of parasite encounters (Demaree, 1967, Bulow et al., 1979). A study of \( L. \ cyprinacea \) by Marcogliese (1991), found the parasite to be more prevalent on detritivorous fish species than planktivorous fish species, presumably because of increased contact rates.

As well as being influenced by fish life cycle and habitat preference, contact rates may also reflect host size. A positive relationship between likelihood of infection and fish length within species was identified, and this relationship may also extend to different species. \( Tandanus bostocki \) is by far the largest native freshwater fish species in south-western Australia, reaching a total body length of up to 500mm (Morgan et al., 1998). Other studies have also examined the relationship between host size and \( L. \ cyprinacea \) infections, but have had conflicting results. Marcogliese (1991) found no correlation between fish host size and infection in a lake in North Carolina, USA, whereas Pe´rez-Bote (2010) identified a significant relationship between \( Barbus comizo \) size and \( L. \ cyprinacea \) infection in the Guadiana River in Spain. Pe´rez-Bote (2000) found a positive, but not significant, relationship between host size and fish lengths for \( Barbus sclateri, Squalius alburnoides \) and \( Chondrostoma willkommii \) in the Guadiana River. Similar results were reported by Gutiérrez-Galindo and Lacasa-Millán (2005) in a community of cyprinids from the Llobregat River in northeastern Spain. Adams (1984) noted an increase in copepod abundance with host size, however, Amin et al. (1973), found that it was usually the smaller fishes of the species studied that were more heavily infected.

2.4.3.3 Host susceptibility
Differences in rate of parasite attachment due to host morphology or immune responsiveness could be a factor in determining the differences in infection levels among fish species and particularly between native and alien fish species. Although many native freshwater fish species, such as \( T. \ bostocki \) and \( G. \ occidentalis \), are scaleless, this is unlikely to explain the higher rates of \( L. \ cyprinacea \) infection seen on them than on scaled alien fishes. Meyer (1966) suggested that it was scaled, rather than scaleless, teleosts that were more likely to be susceptible to \( L. \ cyprinacea \) infections. A histopathological study by Hemaprasanth et al. (2011)
found that the head of *L. cyprinacea* penetrated the host tissue at an angle between overlapping scales, suggesting the possibility of greater anchoring and protection for the parasite. Therefore, it appears more likely that differences in host behaviour, skin bio-chemistry or immunological mechanisms may explain differences in host susceptibility to parasite attachment. The physiochemical characteristics of the skin mucus, or other related mechanisms such as a localised immune response or defensive behavioural reactions, may act as a physical barrier to copepodids, although not necessarily to the anchoring apparatus of the adult female (Hemaprasanth et al., 2011). However, due to the mobility of the copepodids (Grabda, 1963, Shields, 1978), they are likely to abandon a less susceptible host species for one that provides better attachment, given a choice of different fish species as hosts.

### 2.4.4 Conclusions

Using a combination of molecular work with previous morphological identification (from Marina et al. (2008)), it can now be definitively reported that *Lernaea cyprinacea* has been introduced and identified on native freshwater fishes in the south-west of Australia. This study also confirms that this parasite appears to have a greater affinity for native freshwater fishes than its native host, *C. auratus*, and other exotic fish species. The greater prevalence on native freshwater fishes may be due to a greater rate of exposure to the parasite and/or to a greater infectivity of the parasite on these species. Disentangling these causes is the topic of the next chapter.
Chapter 3
Are native fish at higher risk than alien fish to alien parasites?

3.1 Introduction

Invasive species are considered the second most important cause of biodiversity loss throughout the world, posing significant threats to the integrity and functioning of ecosystems (Wilcove et al., 1998, Grosholz, 2002, Clavero and Garcia-Berthou, 2005, Molnar et al., 2008). Freshwater ecosystems are particularly threatened by invasive species, as they are likely to successfully invade fresh waters that have already been altered or degraded by humans, as well as contribute to the physical and chemical impacts of humans on fresh waters (Bunn and Arthington, 2002, Koehn, 2004).

One of the biggest threats associated with the introduction of an invasive species is the introduction of co-invading parasites and pathogens. Co-invaders are parasites that have been co-introduced with an alien species to a new location, outside of their natural range, and spread to new native hosts (Lymbery et al., 2014). It has been suggested that parasites which switch from introduced host species to native host species will have greater infectivity and pathogenicity in native hosts, with which they have no co-evolutionary history (e.g. naïve host syndrome - (Mastitsky et al., 2010); novel weapon hypothesis - (Fassbinder-Orth et al., 2013)). The naïve host theory proposes that parasites and hosts with a long co-evolutionary history will be co-adapted; alien parasites that are introduced into a new area meet naïve hosts that lack co-evolved resistance (the ability to prevent infection) or tolerance (the ability to limit the detrimental effects of infection), therefore they suffer greater infection rates and more serious disease (Allison, 1982, Mastitsky et al., 2010, Fassbinder-Orth et al., 2013, Lymbery et al., 2014).

*Lernaea cyprinacea* is a copepod crustacean parasite known to have serious pathogenic effects on cultured freshwater fishes. The adults, in particular, cause high rates of mortality in young
fish because of their relatively large size and mode of attachment and feeding, and they may also cause secondary infections by transmitting viruses and bacteria (Woo and Shariff, 1990). *Lernaea cyprinacea* is not host specific and has a wide host range, being identified in over 100 fish species from 16 different orders (Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006), as well as occasionally in amphibians (Tidd and Shields, 1963, Lester and Hayward, 2006). Cyprinids, such as *Carassius auratus* (goldfish) and *Cyprinus carpio* (koi carp), appear to be the ancestral hosts (Ho, 1998, Barson et al., 2008). *Carassius auratus* and *C. carpio* are among the most invasive freshwater fishes in the world (McKay, 1984, Fuller et al., 1999, Gido and Brown, 1999, Skelton, 2001, Koehn and MacKenzie, 2004). *Lernaea cyprinacea* appears to have been co-introduced with cyprinid hosts in many different countries (Hoffman, 1970, Marcogliese, 1991, Robinson et al., 1998, Marina et al., 2008).

*Lernaea cyprinacea* is not native to Australia, but has been recorded in a number of native fishes in New South Wales and Victoria in eastern Australia (Ashburner, 1978, Hall, 1983, Callinan, 1988, Rowland and Ingram, 1991, Dove, 2000, Bond, 2004), and also in a number of rivers in Western Australia (Marina et al., 2008). In the field, *L. cyprinacea* appears to be more prevalent on native freshwater fishes than on *C. auratus* (with which the parasite was presumably introduced) or other alien fish species (Marina et al., 2008) also see Chapter 2). This may be a consequence of greater rates of exposure of native fishes to infective stages of the parasite and/or greater infectivity of the parasite to native fishes. Although other studies have also found differences among fish species in the prevalence and intensity of infections with *L. cyprinacea* (Adams, 1984, Marcogliese, 1991, Robinson et al., 1998, Thilakaratne et al., 2003, Choudhury et al., 2004, Gutiérrez-Galindo and Lacasa-Millán, 2005, Barson et al., 2008, Mancini et al., 2008, Kuperberg et al., 2009, Tasawar et al., 2009, Dalu et al., 2012, Stavrescu-Bedivan et al., 2014, Tavares-Dias et al., 2015), the reason for these differences has rarely been investigated.

The aim of the current study is to compare the infectivity of *L. cyprinacea* to a native freshwater fish species, *Nannoperca vittata* (western pygmy perch) and to *C. auratus*, under controlled
laboratory conditions. By eliminating variation in exposure rate of the parasite, any differences among host species in prevalence or intensity of infection should be due to differences in infectivity. It is hypothesised that, when both fish species are exposed to the parasite, *N. vittata* will be more likely to be infected and to have a greater intensity of infection.

### 3.2 Methods

#### 3.2.1 Experimental fishes

All fishes were purchased from commercial suppliers who had no history of infection with *L. cyprinacea* (i.e. no previous reports of visible signs of infection). Fish (a total of 225 *N. vittata* and 214 *C. auratus*) were transported to a secure laboratory at the Fish Health Unit, Murdoch University, and maintained in 1,000 L tanks with a recirculating, aerated water supply, and fortnightly 25% water exchanges. Once in the laboratory all *N. vittata* were kept in one tank and all *C. auratus* were together in a second, separate, tank. Ammonia, nitrite and pH levels were monitored weekly and fishes fed once daily to satiety (Aqua One Goldfish Flakes for *C. auratus* and New Life Spectrum Grow for *N. vittata*). All fishes were acclimatised and quarantined in the laboratory for at least seven days before being used in experiments. Fish were also examined under a dissection microscope to confirm that there was no exposure to *L. cyprinacea* prior to the commencement of the experiments. Fish were not given any treatment preceding the start of the experiment.

Prior to use in experiments, fishes (no more than 50 of each species at a time) were moved to six 50 L tanks in an air conditioned laboratory and acclimatised gradually to a water temperature of 24°C, which was maintained for all experiments. The 50 L tanks had a static water supply, with aeration through a sponge filter and the same feeding and monitoring regime as in the maintenance tanks (Figure 3.1).
Figure 3.1. 50L aerated and heated tanks used for infection experiments.

3.2.2 Laboratory culture of *Lernaea cyprinacea*
To establish the life cycle of the parasite in the laboratory, infected wild fishes were captured in the field and transported to the laboratory (see Chapter 2), where they were placed into a 500 L tank with a static water supply and aeration through sponge filters, and a standard feeding and monitoring regime. Water temperature was maintained at 26°C, which is regarded as optimal for completion of the parasite life cycle (Shields, 1978). Uninfected *C. auratus* were added to the tank (20-30 fish at a time, depending on fish size) to act as a host to maintain the life cycle (Figure 3.2). Fish that did not become infected or those showing adverse signs of infection (poor swimming performance, no feeding, abnormal behaviour) were removed from the culture tank and replaced with new fish.
3.2.3 Experimental design

Infection experiments were conducted in 12 identical 50 L aquarium tanks, each aerated through a sponge air filter and maintained at a constant temperature of 24°C. Water quality was monitored weekly and 25% water exchanges undertaken at least once a fortnight. All fish were fed once daily to satiety. Prior to the commencement of the experiment, each tank was seeded with *L. cyprinacea* by placing two infected *C. auratus* (adult female parasites visible on the fish), each containing 3-5 parasites, in each tank for 5 days (this ensured that there would be enough time for the egg sacs to develop and hatch into the free living infective copepodid stage (Shields, 1978)). Once the tanks had been seeded, no further water exchanges were undertaken until the experiment ended.

After the seeder fish were removed, each tank was stocked with 8-10 experimental fishes, either all *C. auratus*, all *N. vittata*, or an equal mixture of each species. Prior to stocking, fishes were anaesthetised with AQUI-S (0.1 mL/L), measured for total length and examined under a
dissecting microscope to ensure they were free from *L. cyprinacea*. Fishes in each tank were observed daily over a 14 day period, after which all fishes were removed, anaesthetised, measured for total length, examined under a dissecting microscope and the number and location of attached adult parasites recorded (Figure 3.3). Infected fishes, identified by the presence of an adult female (copepods were not recorded), were euthanised in an ice slurry and then preserved in 10% formalin for histological examination. Any fishes which died prior to the end of the experiment were preserved and examined for infection, but were not replaced. Fishes which showed signs of distress during the experiment (obvious skin lesions, lethargy, no feeding activity for two consecutive days), were removed, euthanised and examined for infection and recorded as mortalities for data analyses. Data were collected from 214 *C. auratus* (108 in single species groups and 106 in mixed species groups) and 225 *N. vittata* (121 in single species groups and 104 in mixed species groups).

All experiments were conducted with approval from Murdoch University Animal Ethics committee (permit numbers R2448/11).

![Figure 3.3. Heavily infected *Carassius auratus* with adult *Lernaea cyprinacea* (circles).](image)
3.2.4 Data analysis
Infection prevalence (proportion of infected hosts) and mean intensity of infection (number of parasites per infected host) were calculated for each fish species (C. auratus or N. vittata) in each type of tank community structure (single species or mixed species). Ninety five percent confidence intervals were calculated for prevalences, assuming a binomial distribution, and for mean intensities, from 2,000 bootstrap replications using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000).

Infection and mortality data were analysed using JMP v10 (SAS Institute Inc., 2009). Generalised linear models (GLMs), with either infection status (infected or uninfected), intensity of infection and mortality status (alive or dead) as response variables and fish species (C. auratus or N. vittata), tank community structure (single species or mixed species) and interaction of fish species x community structure as predictor variables. Fish total length and number of fishes per tank were also initially included as predictor variables in all models. With mortality as a response variable, the full model would not converge and these covaraites were removed. Neither fish total length nor number of fishes per tank were significantly related to mortality rate in univariate logistic regression analyses. A binomial distribution was assumed for infection status and mortality, with logit link functions, and a Poisson distribution was assumed for intensity, with a log link function. For each type of community structure (single species or mixed species), prevalences and mortality rates were compared between fish species using Fisher exact tests and intensities were compared using a non-parametric Wilcoxon signed-rank test. For each fish species, the relationship between mortality rate and intensity of infection (pooled over community structure) was investigated using logistic regression.

3.3 Results
3.3.1 Prevalence and intensity of infection
Results from the GLM analyses are shown in Tables 3.1 and 3.2. Although no main effects were significant, there was a significant interaction of fish species and community structure on both infection status (P = 0.02) and intensity of infection (P = 0.005).
Table 3.1. Effect tests from GLM analysis of predictor variables for prevalence of infection with *Lernaea cyprinacea*. Significant effects shown in bold.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>2.73</td>
<td>0.10</td>
</tr>
<tr>
<td>Community structure</td>
<td>0.23</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Species x community structure</strong></td>
<td><strong>5.09</strong></td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td>Fish length</td>
<td>0.27</td>
<td>0.60</td>
</tr>
<tr>
<td>Number of fishes per tank</td>
<td>2.83</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 3.2. Effect tests from GLM analysis of predictor variables for intensity of infection with *Lernaea cyprinacea*. Significant effects shown in bold.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>3.00</td>
<td>0.08</td>
</tr>
<tr>
<td>Community structure</td>
<td>3.62</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Species x community structure</strong></td>
<td><strong>7.76</strong></td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>Fish length</td>
<td>3.59</td>
<td>0.06</td>
</tr>
<tr>
<td>Number of fishes per tank</td>
<td>1.56</td>
<td>0.21</td>
</tr>
</tbody>
</table>

These significant interactions arise because in single species communities there is little difference between *N. vittata* and *C. auratus* in the proportion of fish infected (0.52 and 0.47, respectively; Fisher exact test, $P = 0.50$) and *N. vittata* have a slightly (non-significantly) lower intensity of infection (2.0 ± 0.3 compared 2.6 ± 0.3, $z = 0.97$, $P = 0.33$). In mixed species communities, however, *N. vittata* has a significantly greater infection rate than *C. auratus* (0.59 compared to 0.33; Fisher exact test, $P = 0.0003$) and a greater (although not quite significantly greater) intensity of infection (3.0 ± 0.3 compared to 2.2 ± 0.4, $z = 1.74$, $P = 0.08$) (Figure 3.4).
3.3.2 Mortality rate

Mortality rate differed among fish species (P < 0.0001), but was not affected by community structure or the interaction of community structure and species (Table 3.3). *Nannoperca vittata* had a significantly greater mortality rate than *C. auratus* in both single species communities (34.8% compared to 2.5% mortality, Fisher exact test, P < 0.0001) and mixed species communities (40.9% versus 0% mortality, Fisher exact test, P < 0.0001). Mortality rate was too low in *C. auratus* to test for an effect of intensity of infection, but in *N. vittata*, mortality was positively related to intensity ($\chi^2 = 18.51$, P < 0.0001). The mean intensity of infection in fish that died was 3.9 (95% CI 2.6 – 5.1), compared to 2.2 (95% CI 1.6 - 2.9) in fish that survived.

Table 3.3. Effect tests from GLM analysis of predictor variables for mortality during infection with *Lernaea cyprinacea*. Significant effects shown in bold.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>$\chi^2$</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>62.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Community structure</td>
<td>1.39</td>
<td>0.24</td>
</tr>
<tr>
<td>Species x community structure</td>
<td>2.08</td>
<td>0.15</td>
</tr>
</tbody>
</table>
3.4 Discussion
Field surveys in south-western Australia have found consistent differences among fish species in the prevalence of the introduced parasite *L. cyprinacea*, with the parasite more prevalent on native freshwater fishes than on *C. auratus* or other alien fish species (Marina et al. (2008); Chapter 2). This may be due to differences in the rate of exposure of different fish species to the parasite, or to differences in infectivity of the parasite to different fish species. In this study, infection experiments in the laboratory were used to minimise differences in exposure rate. Aquaria were seeded with infective copepodids, and *C. auratus*, or native *N. vittata*, were introduced, either separately or in mixed communities. The results strongly suggest that *L. cyprinacea* differs in its infectivity to these two fish species. When fishes were exposed in single species groups, there were no differences in prevalence or intensity of infection with attached adult females. However, when fishes were exposed in mixed species communities, *N. vittata* were infected more frequently and had a greater intensity of infection. Furthermore, *N. vittata* were more likely to die than *C. auratus* in these infection experiments, and the risk of mortality was positively related to the intensity of infection. It should be noted that these infection experiments monitored only the likelihood and consequences of the attachment of adult parasites and give no information on differences in the infectivity or pathogenicity of the copepodid stage to the fish hosts.

The development of immunity is not uncommon in *C. auratus*. A study by Kadhim (2009) showed that *C. auratus* were able to develop acquired immunity after prolonged exposure to *L. cyprinacea*. Unfortunately due to the limited number of studies focusing on native freshwater fishes it is hard to know whether or not *N. vittata* are able to develop immunity towards external parasites. In this experiment no immunity was detected as fish exposure was not prolonged enough. It is also important to note that while no controls were used in this experiment, unexposed fish were maintained in the laboratory at the same time, with no recorded mortalities. While these cannot be used as proper controls, the data provided strongly suggests that mortalities were due to infection, and a significant relationship was identified between risk of mortality and number of parasites per host.
A number of other studies have reported differences among fish species in the prevalence and/or intensity of infection with *L. cyprinacea*, both in the wild (Gutiérrez-Galindo and Lacasa-Millán, 2005) and in aquaculture systems (Bauer et al., 1962, Shariff et al., 1986, Babey and Berry, 1989, Goodwin, 1999, Hemaprasanth et al., 2011). As far as can be ascertained, no previous studies have used experimental infections to separate differences in infectivity from differences in the rate of exposure to the parasite.

There may be a number of proximate reasons for the differences in infectivity and pathogenicity of *L. cyprinacea* to *C. auratus* and *N. vittata*. Fish hosts may respond to parasite infections with behavioural and immunological defences (Zaccone et al., 2009). Behavioural defences may involve the avoidance of infective parasite stages or adaptations to reduce parasite loads, such as physical removal of ectoparasites and ingestion of anti-parasitic compounds (Barber et al., 2000). Immunological defences in fishes include both innate and adaptive immune systems (Magnadottir, 2010). There is some evidence of differences among fish species in their immunological responses to infection with *L. cyprinacea* (Shields and Goode, 1978, Shariff et al., 1986), but no previous studies have investigated differences in behavioural responses.

Also unclear are the ultimate (evolutionary) reasons for the greater infectivity and pathogenicity of *L. cyprinacea* to the new, native host, *N. vittata*, than the co-introduced host, *C. auratus*. The co-introduction of alien hosts and parasites does not appear to be common. Studies have shown that introduced alien species usually harbour significantly fewer parasites than native species (Mitchell and Power, 2003, Torchin et al., 2003). This could be due to the founding populations of aliens not carrying the complete range of parasites into the new location, or because the parasites are unable to complete their life cycles in the new environment (Torchin et al., 2003, MacLeod et al., 2010, Ewen et al., 2012). Despite this tendency for introduced alien species to harbor fewer parasites, inevitably there will be some parasites that are co-introduced.
Although the frequency of host switching may not be high, it has been suggested that a parasite that switches from an introduced host species to a native host species will have greater infectivity and pathogenic effects in native hosts, where there is no coevolutionary history (naïve host syndrome (Mastitsky et al., 2010, Fassbinder-Orth et al., 2013)). The results from this study, showing both a greater infectivity and greater pathogenicity of *L. cyprinacea* to the new host (*N. vittata*) than its presumed ancestral host (*C. auratus*) would appear to support the naïve host theory.

The theoretical basis of the naïve host theory, however, is not well established. Coadaptation is the evolution of reciprocal adaptations in two or more interacting species. More formally, coadaptation of two species can be defined as the evolution of adaptation(s) in one species in response to selection imposed by a second species, followed by the evolution of adaptation(s) in the second species in response to reciprocal selection imposed by the first species (Clayton et al., 1999). Coadaptation between parasites and hosts may be viewed in the context of the Red Queen Hypothesis (Van Valen, 1973), as an evolutionary arms race, with hosts constantly evolving new defence mechanisms against parasites, and parasites evolving new ways of overcoming host defences (Mode, 1958, Ehrlich and Raven, 1964).

Who is ahead in this coevolutionary arms race is determined by the relative slopes of the parasite and host selection gradients. The slope of the selection gradient is influenced by many factors, including the intensity of selection, the amount of genetic variation in the trait, the effective population size and the generation time of the population undergoing selection. Conventional wisdom has it that parasites will respond more rapidly because of a greater intensity of selection, larger population sizes and shorter generation times than their hosts (Kaltz and Shykoff, 1998, Combes, 2001). In theory, this should lead to local adaptation of parasites, whereby a parasite population has greater mean fitness on host populations with which it has co-evolved (Lively, 1996, Gandon and Van Zandt, 1998, Kaltz and Shykoff, 1998). It may therefore be expected that parasites which switch hosts would have lower fitness on (and to the extent that infectivity reflects parasite fitness, have lower infectivity to) the new host species.
Empirical studies have not universally supported this theory; while local adaptation has been found in some studies, other studies have found either no evidence for local adaptation or local maladaptation, which indicates that hosts, rather than parasites, are responding more rapidly to reciprocal selection (reviewed by Kaltz and Shykoff (1998)). Still other studies have detected a complex pattern of both local adaptation and local maladaptation at different geographic scales in the same parasite/host system (Hanks and Denno, 1994, Imhoof and Schmid-Hempel, 1998).

The reason for this diversity of empirical results lies in the complex oscillatory nature of parasite/host coadaptation. Simple, single locus population genetic models, in which parasite traits adapt to the most common host traits, generate time-lagged cycles in allele frequencies (Clarke, 1979, Hutson and Law, 1981, Nee, 1989). More realistic quantitative genetic models show that cycling is also possible for polygenic traits (Diekmann et al., 1995, Gavrilets, 1997). Clearly, if different populations of parasites and hosts are at different temporal phases of their oscillatory cycle, then different spatial snapshots of local adaptation will yield different results.

There is, therefore, no theoretical reason from a coevolutionary perspective why parasites should have consistently greater infectivity and pathogenic effects on naïve hosts. The reason why co-invading parasites often seem to be more pathogenic to native hosts than to the alien hosts with which they were introduced, as is the case with *L. cyprinacea* in south-western Australia, may simply be an accident of invasion. Parasites which are not highly pathogenic are more likely to be co-introduced and therefore any difference in virulence of the parasite between the co-evolved alien host and the new native host is more likely, simply by chance, to be in the direction of increased virulence in the new host (Lymbery et al., 2014). In any case, whatever the ultimate explanation of this difference in pathogenicity, it may have profound effects upon the population dynamics of native host species.

### 3.4.1 Conclusions

Previous studies in south-western Australia have found that *L. cyprinacea* is more prevalent on native freshwater fishes than its co-introduced host, *C. auratus*, or other alien species (Marina et al. (2008); Chapter 2). This difference in rate of infection may be due to either (or both)
differences in exposure rates of the fish species to the parasite, or differences in infectivity of
the parasite to the fish species. By using infection experiments under controlled conditions in
the laboratory, differences in exposure rate were minimised. In these infection experiments,
there was little difference between *N. vittata* and *C. auratus* in the prevalence or intensity of
infection with attached adult female parasites when fish were exposed in single-species groups,
but there was a considerable difference when fish were exposed in mixed-species groups, with
*N. vittata* having a significantly greater infection rate and intensity of infection. Furthermore,
the mortality rate of *N. vittata* was significantly greater than the mortality rate of *C. auratus*, and
was positively related to the intensity of infection. The most parsimonious explanation for these
results is that adult *L. cyprinacea* are able to attach to both species of fish, but exhibit a
preference for *N. vittata* over their presumed ancestral host, with a concomitant increase in
pathogenicity to the new host species. In the next chapter, the possible reasons for these
differences are investigated.
Chapter 4
Does the behaviour of naïve hosts change on exposure to *Lernaea cyprinacea*?

4.1 Introduction

The naïve host theory postulates that co-invading parasites that switch from the host species with which they were introduced to native hosts in the new locality, will have greater infectivity to, and pathogenic effects upon, their new hosts. This is because these new, naïve hosts lack the coevolved resistance or tolerance of their traditional hosts (Mastitsky et al., 2010, Fassbinder-Orth et al., 2013). Although the theoretical basis of the naïve host theory is questionable (see Chapter 3), a recent literature review found that in 85% of documented host switching of alien parasites from alien hosts to native hosts, pathogenic effects were more pronounced in the new, native host. Resistance refers to the ability of a host to avert a parasite infection, reduce the parasite burden or recover from infection, while tolerance is the ability of a host to limit the damage caused by a given parasite burden (Hayward et al., 2014). Most studies of host resistance and tolerance focus on immune responses, but there are also important non-immunological defence mechanisms, such as behaviours that prevent or combat infection (Parker et al., 2011).

There have been a variety of host-parasite systems in which behavioural changes in parasitised animals have been reported (Poulin, 1994). The magnitude of these changes in host behaviour vary greatly, ranging from small shifts in time on a particular activity, to strange and drastically new behaviours (Poulin, 1995). There are three possible explanations for parasite-induced changes in host behaviour. First, these changes in behaviour may be the inevitable side effects of infection which benefit neither parasite nor host (Barber and Wright, 2005). For example, the parasite *Diplostomum spathaceum* on invading the lens tissue of animals, including fishes, amphibians, reptiles, birds and mammals causes parasitic cataract disease (known also as diplostomatosis or eye fluke disease) (Palmieri et al., 1977, Chappell et al., 1994). Second, behavioural changes in an infected host may reflect parasite adaptations, increasing the
probability of successful transmission. This may occur through either direct mechanisms (where the parasites themselves, or their biochemical secretions, act directly on the host) or indirect physiological mechanisms (where a constraint is imposed on some other part of the host’s physiology). Lafferty and Morris (1996), for example, found that infection of California killifish (*Fundulus parvipinnis*, Cyprinodontidae) with *Euhaplorchis californiensis*, a brain-encysting trematode, increased the frequency of conspicuous behaviours performed by fish hosts, making them 30 times more likely to be eaten by herons and egrets (the parasite’s definitive host).

Finally, behavioural changes may be adaptations by the host to either prevent infection, rid themselves of the parasites or compensate for their effects (Table 4.1).

Immune responses are energetically costly (Parker et al., 2011), so to reduce demand on their immune systems, hosts are expected to evolve behavioural mechanisms that limit their contact with infective stages of parasites (Hart, 1990). For example, some fish have been show to avoid certain habitats that are associated with an infection risk (Poulin and Fitzgerald, 1989) and reject parasitised sexual partners (Kennedy et al., 1987, Milinski and Bakker, 1990, Rosenqvist and Johansson, 1995). Hosts that are already parasitised may perform a broad range of behaviours in an attempt to eliminate or remove parasites. These include self-medication (a complex behaviour where infected hosts seem to actively seek out substances that appear to have a negative effect on the parasite, but a positive effect on the host (Clayton and Wolfe, 1993)) and the physical removal of ectoparasites, for example in fishes by ‘flashing’ or rubbing against structural components of their environment, or by visiting ‘cleaning stations’ where parasites are actively removed by other organisms (Losey, 1987, Urawa, 1992, Poulin and Grutter, 1996).

If the parasite burden cannot be reduced, hosts may use behavioural mechanisms to compensate for the effects of the parasite. Changes in foraging behaviour and prey preference, for example, may increase food intake rate that, to some extent, compensates for the parasites nutritional demands (Milinski, 1990, Ranta, 1995).
Table 4.1. Previously recorded host defensive behaviours and their presumed benefits

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Presumed Benefit</th>
<th>Examples/References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avoidance or habitat shifting</td>
<td>Avoiding areas or conspecifics with parasites</td>
<td>• Sticklebacks (<em>Gasterosteus aculeatus</em>) avoid areas with the crustacean ectoparasite <em>Argulus canadensis</em> (Poulin and Fitzgerald, 1989)</td>
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<td></td>
<td></td>
<td>• Sticklebacks avoid infected fish displaying odd behaviours (Dugatkin et al., 1994)</td>
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<td>• Hippopotamus (<em>Hippopotamus amphibious</em>) habitat choice influenced by the presence of tabanids (Moore, 2002)</td>
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<td></td>
<td></td>
<td>• Reindeer (<em>Rangifer tarandus</em>) move to water to avoid flying insect attacks (Mehlhorn et al., 2008)</td>
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<td></td>
<td></td>
<td>Bats change their day roosting spots in response to severe ectoparasite attacks (Mehlhorn et al., 2008)</td>
</tr>
<tr>
<td>Temperature control</td>
<td>Moving to different areas to compensate for temperature changes caused by parasites, decreasing the affects</td>
<td>• Poikilothermic (ectothermic) hosts, such as reptiles, affected by endoparasites may raise their temperature by moving to warm, sunny microhabitats to improve their immune response function (Mehlhorn et al., 2008)</td>
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<tr>
<td></td>
<td></td>
<td>• In response to ‘behavioural fever’ some infected hosts may prefer environments with lower temperatures, slowing down parasite development (Mehlhorn et al., 2008)</td>
</tr>
<tr>
<td>Choice of movement</td>
<td>Small changes in normal movements can help decrease exposure to ectoparasites</td>
<td>• Red deer (<em>Cervus elaphus</em>) spend twice as much time lying down on days when they are heavily harassed by head flies (<em>Hydrotaea irritans</em>) (Moore, 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tadpoles of <em>Bufo</em> and <em>Rana</em> species can make explosive movements when they sense cercariae contacting their skin, thus preventing these ectoparasites from attaching to them (Mehlhorn et al., 2008)</td>
</tr>
<tr>
<td>Changes in diet/foraging areas</td>
<td>Changes in diet and foraging areas can help reduce parasite exposure</td>
<td>• Some species of waterfowl are known to alter their diet choice with the changing season (Lozano, 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• The feeding ranges of Mangabeys (<em>Cercocebus atys</em>) shift to avoid foraging in areas that may be contaminated by their own faeces (Freeland, 1980)</td>
</tr>
<tr>
<td>Self-medicating</td>
<td>Using substances to defend against parasites</td>
<td>• Hexabranchus sanguineus uses macrolides derived from sponges to defend against predators and fungi (Keman and Faulkner, 1987)</td>
</tr>
<tr>
<td>Paratite removal behaviours</td>
<td>Removal of parasite if infection cannot be avoided</td>
<td>• Some animals have specialized muscles for twitching skin and tails for swatting flies (Hart, 1997)</td>
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<tr>
<td></td>
<td></td>
<td>• Some birds and marsupials have comb-like claws (Mehlhorn et al., 2008)</td>
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<tr>
<td></td>
<td></td>
<td>• House mice (<em>Mus domesticus</em>) have specialised lower incisors (teeth) effective at combing away ectoparasites (Rammath, 2009)</td>
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<td></td>
<td>• Elephants can swat their backs with branches as a tool to repel flies (Hart and Hart, 1994)</td>
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<td></td>
<td></td>
<td>• Grooming performed by different animals aid in parasite removal (Dunbar, 1991, Hawlena et al., 2007)</td>
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<tr>
<td></td>
<td></td>
<td>• Some fish rub against structural components of their environment to dislodge ectoparasites (Urawa, 1992)</td>
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<tr>
<td></td>
<td></td>
<td>• Fish may visit ‘cleaning stations’ on coral reefs (Losey, 1987, Poulin and Grutter, 1996)</td>
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</table>

*Lernaea cyprinacea* is a cosmopolitan copepod that is a non-specific parasite of many freshwater fish species as well as amphibians and aquatic insects (Williams and Bunkley-Williams, 1996, Piasecki et al., 2004, Nagasawa et al., 2007, Kupferberg et al., 2009). Although the native range of the species appears to be within Asia, it is currently much more widely
distributed through co-invasion, primarily with teleost hosts (Oscoz et al., 2010). Species of *Lernaea* have a wide-spread impact on both ornamental, farmed and wild fishes all over the world, with *L. cyprinacea* being responsible for causing high mortality rates and serious economic losses among ornamental fishes due to haemorrhage and secondary infections (Woo, 2006), as well as impacting new, native hosts as their range expands through human intervention (Demaree, 1967, Robinson and Avenant-Oldewage, 1996, Hoffman, 1999, Allen et al., 2002, Piasecki et al., 2004, García-Berthou, 2007, Marina et al., 2008, Sánchez-Hernández, 2011). Not only can infections of *L. cyprinacea* directly harm fishes, but they can also result in disfigurement, making ornamental fish and fish grown for food unsuitable for sale, resulting in a high level of loss to the fishing industry (Thilakaratne et al., 2003, Boxshall, 2004, Piasecki et al., 2004, Kir, 2007, Dalu et al., 2012).

The history of fish mortalities caused by *L. cyprinacea* dates back to 1880, where lernaeosis almost wiped out an entire population of crucian carp (*Carassius carassius*) from one of the lakes of the Masurian Lake district (Kocylowski and Miaczy´nski, 1960). In North America, Goodwin (1999) reported that massive infections with *L. cyprinacea* caused major losses to three farms polyculturing *Hypophthalmichthys nobilis* (bighead carp) and *Ictalurus punctatus* (channel catfish). *Lernaea cyprinacea* was introduced into South America via the importation of *Cyprinus carpio* (common carp), during the beginning of the 20th century. Since then, there has been a rapid spread of the copepod, and it is now a common parasite infecting farmed species in Brazil and wild fish in all main drainage basins in the country (Piasecki et al., 2004). In Spain, *L. cyprinacea* is an exotic, invasive species, now well established in a number of rivers, and has recently been recorded in wild populations of non-migratory *Salmo trutta* (brown trout) in central Spain (Sánchez-Hernández, 2011).

There are nine main stages in the life cycle of *L. cyprinacea*, including three free-living naupliar stages, five copepodid stages and one adult stage (Grabda, 1963). The free-living naupli have been known to survive as long as 13 days without signs of further changes (Shields and Tidd, 1968), and a relatively loose connection to the fish host means that the copepodid larvae can
readily detach itself and infect a new site or a new host (Grabda, 1963, Shields, 1978, Goodwin, 1999). Once the males and females have mated on the fish host, the male dies and the females metamorphose (Grabda, 1963). Unlike the copepodids, the adult female parasite penetrates the fish host and becomes embedded, making it very difficult to remove (Bauer et al., 1962).

The pathogenicity of *L. cyprinacea* is determined by the copepodids and the metamorphosised adult female. For the copepodids, attachment is usually localised on the gills and body surface of the host (Grabda, 1963, Shields, 1978, Lester and Hayward, 2006). An infestation of copepodids on the gill of a host fish typically can lead to respiratory distress and sluggishness within the host (Kabata, 1979). Copepodids can also cause disruption and necrosis of gill epithelium, resulting in fish death (Khalifa and Post, 1976). Attached adult female stages are often found on the fins and body of the fish host (Shields and Tidd, 1974, Adams, 1984, Kabata, 1985, Medeiros and Maltchik, 1999, Stavrescu-Bedivan et al., 2014, Koyun et al., 2015), but may also infect the head, gills and cloaca (Goodwin, 1999, Medeiros and Maltchik, 1999, Acosta et al., 2013). The metamorphosed adult female’s attachment and feeding behaviour (on erythrocytes and tissue debris) is responsible for the most severe pathogenic effect of the disease lernaeosis (Kabata, 1985). Lernaeosis can cause chronic exhaustion of the energy reserves of the host (Kabata, 1985), as well as weight loss, stunted growth and reduced reproductive performance (Kabata, 1985, Khan et al., 2003). Haemorrhaging and muscle necrosis often result from the attachment of the adult female (Khalifa and Post, 1976, Berry et al., 1991, Lester and Hayward, 2006). Histopathologically, some of the more common changes associated with lernaeosis vary from acute inflammatory reactions to severe degenerative changes, and necrosis in the skin and underlying musculatures (Noor El-Deen et al., 2013).

A native teleost of south-western Australia, *Nannoperca vittata* (western pygmy perch) was found to more susceptible to experimental infections with *L. cyprinacea* than the presumed ancestral host, introduced *Carassius auratus* (goldfish) (Chapter 3). Furthermore, the greater susceptibility of *N. vittata* to infection resulted in a greater rate of mortality. In this chapter, I investigate whether this observed difference in susceptibility to infection can be explained by
behavioural differences between the host species and whether differences in mortality rates are reflected in histopathological differences between species. I hypothesise that *C. auratus*, but not *N. vittata*, will respond to infection by exhibiting defensive behaviours that either prevent attachment by adult female *L. cyprinacea*, or reduce the parasitic burden, with consequent reductions in pathogenic consequences of infection.

4.2 Methods

4.2.1 Experimental fishes and laboratory culture of *Lernaea cyprinacea*

Fishes were purchased and maintained, and laboratory cultures of *L. cyprinacea* were established and maintained as described in Chapter 2 (Sections 2.2.1 and 2.2.2).

4.2.2 Experimental design

Experiments were conducted in 12 identical 50 L aquariums, each aerated through a sponge air filter and maintained at a constant temperature of 24°C. Water quality was monitored weekly and 25% water exchanges were undertaken fortnightly. All fish were fed daily to satiety using Aqua One Goldfish Flakes for *C. auratus* and New Life Spectrum Grow Life for *N. vittata*. Prior to the commencement of the experiment, each tank was seeded with *L. cyprinacea* by placing two *C. auratus* with visible adult female parasites in each tank for between 5 and 8 days. No controls (unexposed) tanks were used in this experiment as infected and uninfected fish were compared in the same tanks.

After the seeder fish were removed, each tank was stocked with five *C. auratus* and five *N. vittata*. Prior to stocking, fish were anaesthetised with AQUI-S (0.1 mL/L), measured for total length (mm) and examined under a dissecting microscope to ensure they were free from *L. cyprinacea*. Behavioural data were collected daily from each tank during 5 minute observational periods, as described below. After 10 days, fish were removed, anaesthetised, measured for total length, examined under a dissecting stereomicroscope, and the number and location of attached adult parasites recorded (Figure 4.1). Infected fish were then euthanised in an ice slurry and then preserved in 10% formalin for histological examination.
4.2.3 Behavioural observations

The behavioural observations were based on continuous sampling (Lehner, 1979), with all behaviours of one fish species recorded over a five minute (minimum) observational period. This was done for both fish species and each tank was treated as a separate entity. A five minute period of acclimatisation preceded the behavioural observations, where the observer was standing still and close enough to the tanks to let the fish become accustomed to her presence.

All observations occurred at the same time each morning, increasing to twice a day at the first signs of infection (once in the morning and once in the afternoon). This behavioural observation protocol was determined from a previous experiment (Basile, 2011). Each tank was treated as a separate entity, and all observable behaviours were recorded for the fish in each tank. Fish were recorded as infected if an attached adult female parasite was visible, and uninfected if a parasite was not visible. Infected and uninfected status was confirmed at the end of the experiment, when fishes were examined under a dissecting stereomicroscope.
The behaviours recorded in the experiment had been previously defined in a preliminary trial, in which the behaviours of parasite-free fishes (*C. auratus* and *N. vittata*) were compared with the behaviours of fishes in tanks which contained infective stages of *L. cyprinacea* (Basile, 2011). A number of specific behaviour patterns, as described in Table 4.2, were observed only in fishes in the tanks which had been seeded with parasites and these are labelled as putative “defensive” behaviours. A range of other behaviours were observed in all tanks, and these are labelled collectively as “standard” behaviours (Table 4.2).

**Table 4.2. Definitions of “standard” and “defensive” behavioural patterns.**

<table>
<thead>
<tr>
<th>Behavioural type</th>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Normal swimming and grouping activity, mouthing and eating objects in water column or attached to tank sides or bottom, defaecating</td>
<td></td>
</tr>
<tr>
<td>Defensive</td>
<td>Gulping</td>
<td>Expansion of oral cavity, with water taken in and expelled through open gill arches</td>
</tr>
<tr>
<td></td>
<td>Jerking</td>
<td>Rapid forward propulsion for 1-3 seconds, performed while swimming</td>
</tr>
<tr>
<td></td>
<td>Scrapping</td>
<td>Brushing body against objects within the tank or the tank sides</td>
</tr>
<tr>
<td></td>
<td>Pecking</td>
<td>Directed mouthing action at conspecific</td>
</tr>
</tbody>
</table>

### 4.2.4 Analysis of behavioural observations

Each of the five behavioural patterns (standard, gulping, jerking, scraping and pecking) were recorded as present or absent for each fish in each tank during the 5 minute observational period on each day. Fish were not individually marked, so fish ID could not be included in analyses, but behaviours were recorded for all fish in each tank during each observation period, so each fish should contribute equally to the total number of behaviours recorded. The frequency of occurrence of each type of putative defensive behaviour was compared between uninfected *C.*
auratus and N. vittata, and between infected C. auratus and N. vittata, using Fisher exact tests. Time was not considered a factor in the analysis of this experiment as preliminary trials showed that the behavioural responses did not differ over the time period of observations. Information on parasite prevalence and mean intensity was also collected at the end of the experiment.

4.2.5 Pathology of infection
Following the behavioural observations, 47 C. auratus and 47 infected N. vittata were examined and the location of all attached L. cyprinacea recorded as on fins, body or head. Differences among fish species in site of infection were compared using a chi-square test.

Tissues around parasite infection sites in 30 fish each of both C. auratus and N. vittata were processed for paraffin sections and stained with haematoxylin and eosin. Prepared sections were examined under a light microscope and histopathological lesions were evaluated semi-quantitatively using methods modified from Schwaiger (2001). The severity of tissue lesions in each organ of each fish were ranked on a scale from 1 to 3, where grade 1 = no or minimal alterations from normal tissue; grade 2 = focal mild to moderate changes; grade 3 = extended, pathological alterations, involving tissue necrosis and inflammation. Histopathology scores were compared using a Mann-Whitney U test.

4.3 Results
4.3.1 Behavioural differences between species
The frequency of different behaviours in uninfected and infected fishes is shown in Table 4.3. Putative defensive behaviours were rarely seen in uninfected fish of either species, and there were no significant differences between species in the frequency of occurrence of any of these behaviours (Fisher exact tests: for gulping, P = 1.00; for jerking, P = 0.62; for scraping, P = 1.00; for pecking, P = 1.00). In infected fish however, there was a clear difference between species, with putative defensive behaviours observed much more frequently in C. auratus than in N. vittata (Fisher exact tests: for gulping, P = 0.0001; for jerking, P < 0.0001; for scraping, P = 0.06; for pecking, P = 0.01).
Table 4.3. Mean percentage occurrence (with SE in parentheses) of different behaviours in *Carassius auratus* and *Nannoperca vittata*, either uninfected or infected with *Lernaea cyprinacea*. Observations taken over four replicate tanks for each combination of species and infection status.

<table>
<thead>
<tr>
<th>Species and infection status</th>
<th><em>C. auratus</em></th>
<th><em>N. vittata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>Gulping</td>
<td>0</td>
<td>13.2 (3.1)</td>
</tr>
<tr>
<td>Jerking</td>
<td>6.0 (3.6)</td>
<td>31.6 (2.8)</td>
</tr>
<tr>
<td>Scraping</td>
<td>3.6 (3.6)</td>
<td>4.8 (3.0)</td>
</tr>
<tr>
<td>Pecking</td>
<td>1.9 (1.9)</td>
<td>14.8 (2.9)</td>
</tr>
<tr>
<td>Standard</td>
<td>87.3 (7.2)</td>
<td>35.6 (5.4)</td>
</tr>
</tbody>
</table>

4.3.2 Differences in pathology of infection between species

In both fish species, over half of all attached female parasites were on the fins (particularly the dorsal fins and caudal fin). More parasites were found on the body than on the head of *C. auratus*, which was in contrast to infections on *N. vittata*, where more parasites were found on the head when compared to the body (Figure 4.3), but these differences in attachment sites between species were not significant ($\chi^2 = 4.06, P = 0.13$). The total surface area (Figure 4.4) of the head and body were similar for both fish species (the head made up about ~15% for both fish species, and the body ~85%). Therefore the surface area of the head and body would not account for the variations seen in the preference of parasite attachment.

![Figure 4.2. Parasite attachment sites for a) Carassius auratus and b) Nannoperca vittata.](image)
Figure 4.3. Measurements of the total surface area of a fish

Histological examination of infection sites showed a much greater inflammatory response in *C. auratus* than in *N. vittata*. (Figures 4.5 - 4.7). This was confirmed by tissue lesion scores, which were significantly greater in *C. auratus* (mean score = 2.8, 95% CI = 2.7 - 3.0) than in *N. vittata* (mean score = 1.8, 95% CI = 1.5 – 2.1) (Mann-Whitney U test, z = 4.59, P < 0.0001).
Figure 4.4. a) *Nannoperca vittata* with adult *Lernaea cyprinacea* in muscle (arrow) with a minimal to mild inflammatory response (20x). b) *Carassius auratus* with adult *L. cyprinacea* in the gill (arrow) with a severe inflammatory response (20x). c) Close up of capsule surrounding adult *L. cyprinacea* in *N. vittata* (arrow) with minimal to mild inflammatory response (100x). d) Close up of severe inflammatory response of *C. auratus* to adult *L. cyprinacea*. Eosinophilic granulocytic cell (ECG) (circle). Macrophage (rectangle). Lymphocyte (arrow).

Figure 4.5. Adult *Lernaea cyprinacea* in *Nannoperca vittata* (circled), with view of pincers (white arrow). Inflammatory response (black arrows) (x20).
During histological examinations, some *N. vittata* were observed to have infections of microspordia while a myxozoan infection was seen in one of the *C. auratus*. Interestingly, the difference in inflammatory response to these infections mirrored the difference seen with *L. cyprinacea*; minimal response in *N. vittata* and a severe response in *C. auratus*.

Parasite prevalence was found to be the same for both fish species (0.67, 95% CI 0.60-0.75), whereas mean intensity was 2.6 (SE 0.4) for *C. auratus* and 2.0 (SE 0.2) for *N. vittata*.
4.4 Discussion
When the presumed ancestral host *C. auratus* and the naïve native host *N. vittata* were together exposed to *L. cyprinacea* under experimental conditions, there was a significantly greater rate of infection and parasite intensity on *N. vittata* (Chapter 3). Additionally and significantly, *N. vittata* suffered a greater rate of mortality than *C. auratus*. In this Chapter, I have established that differences between the species in the rate and intensity of infection may be due, at least in part, to different behavioural responses to infection; with infected *C. auratus* exhibiting a range of behavioural responses that were largely absent in *N. vittata*. Surprisingly, however, there was no evidence from histological studies of infection sites for a greater pathological response in *N. vittata* than in *C. auratus*. On the contrary, tissue necrosis and inflammatory response were significantly greater in *C. auratus* than in *N. vittata*.

4.4.1 Defensive behaviours
Because parasites exert major costs on their hosts, either directly through their pathogenic effects or indirectly through their stimulation of energetically expensive immune responses, natural selection is expected to favour hosts that utilise behavioural strategies to avoid infection, reduce the parasite burden or compensate for the effects of infection (Barber et al., 2000, Wisenden et al., 2009). In the present study there was limited capacity for hosts to avoid infection with *L. cyprinacea*, but the behaviours seen in infected fishes (principally *C. auratus*), such as gulping, jerking, scaping and pecking, may serve to prevent attachment of adult female parasites or dislodge those that have already attached.

Whether these behaviours are truly adaptive responses to parasitic infection is not known. Adaptive changes are typically defined by 4 criteria: complexity, purposiveness of design, convergence and fitness effects (Futuyma, 1986, Ridley, 1993, Poulin, 1995). The most important criterion is fitness effects; the demonstration that a trait leads to an increase in the survival or reproductive success of the bearer. As this is often difficult to demonstrate experimentally, adaptations are frequently inferred from their complexity, which requires there to be an organising principal (i.e. natural selection), purposiveness of design, which means that traits fit with their environment and perform their function too well to have arisen by chance, or
convergence, whereby similar traits may be seen in several different lineages and are therefore presumed to have arisen independently in response to selection. It is important to note that, in the absence of evidence for direct fitness benefits, these other criteria provide only circumstantial evidence that a particular trait represents an adaptation, and an adaptive explanation remains a hypothesis until it is experimentally tested.

There is no direct evidence that the presumed defensive behaviours observed in this study actually reduce parasite load or have direct fitness benefits for the host, but their complexity, purposiveness of design and (in some cases) convergence suggest that they may have evolved as defences against parasite attachment. Scraping (also reported as chafing or flashing), in which fishes with ectoparasitic infection scrape their body against a firm surface, has been recorded in a large number of fish species and is usually interpreted as an attempt to dislodge the parasite (Wyman and Walters-Wyman, 1985, Urawa, 1992, Barber et al., 2000, Wisenden et al., 2009). Jerking, which consists of rapid, burst swimming alternating with resting periods, has been previously reported in carp fingerlings exposed to copepodids of *L. cyprinacea* (Hemaprasanth et al., 2011) and also in tadpoles exposed to trematode cercariae (Thiemann and Wassersug, 2000) and has been hypothesised to interrupt attachment of infective stages (Wisenden et al., 2009). Gulping, in which water is taken in through the mouth and rapidly expelled through the gills, has been reported in other fish species in response to low oxygen concentration (Jones, 1952); and may therefore be a response to respiratory difficulties, perhaps due to the activity of copepodids on the gills. Pecking, in which fishes mouthed at the head and body of conspecifics, has not, to my knowledge, been previously reported. The behaviour did not appear to be an aggressive interaction and evoked no response in the fish that was pecked; it was similar to the substrate pecking behaviour which has been observed as a feeding response in *C. auratus* (Hara, 2006). In a number of instances the pecking appeared to be directed towards an attached parasite, but I was not able to record this accurately enough to determine whether it was more than a chance effect. The behaviour may be a case of allogrooming, analogous to the cleaning interactions in reef fishes. There is abundant evidence that cleaner species significantly reduce ectoparasite loads on reef fishes (Grutter, 1999, Cheney and Côté, 2001, Grutter et al., 2002).
and Sikkel et al. (2004) provided evidence that the presence of ectoparasites is the proximate cause of cleaning interactions.

If the behaviours seen in infected fishes are adaptive responses to parasite attachment, this may explain their greater occurrence in *C. auratus*, the presumed ancestral host of *L. cyprinacea*, than in *N. vittata*, which has no co-evolutionary history with the parasite. There are two possible explanations for the lack of behavioural responses to infection observed in *N. vittata*. First, the selection pressure from ectoparasites may not have been great enough for native fish species to develop (or retain) defensive adaptations. Behavioural defences are likely to have energetic costs, both directly and through reduced foraging activity (Wisenden et al., 2009), so will only evolve (or be retained) if these costs are exceeded by fitness benefits. The Southwestern Province not only has a relatively small number of freshwater fish species, it has a correspondingly depauperate parasite fauna. Lymbery et al. (2010) in a comprehensive survey of parasites of freshwater fishes in the region, found 44 morphospecies, with only 12 of these (including the introduced *L. cyprinacea*) being ectoparasites. Second, even if native ectoparasites have provided sufficient selection pressure for the development or retention of defensive behaviours, they may only occur in response to particular stimuli. Of the (presumed) native ectoparasites (five protozoan species, a myxozoan, two monogeans, two ergalisid copepods and a trematode), all, except the trematode metacercariae were found on the gills and none had a similar mode of attachment to *L. cyprinacea*.

At present, while the results from this study are very suggestive, it is not possible to say definitively that the behaviours observed in *C. auratus* are adaptations against *L. cyprinacea* or that their infrequent occurrence in *N. vittata* contributes to the greater infectivity of the parasite to this species. The ability to label host behaviours as defensive adaptations is not an easy task. Further experimentation is necessary to help differentiate between behavioural modifications that are actually adaptive and those that are just accidental by-products of infection. Coupling this with studies that examine the immune response of fish hosts will help provide a fuller picture of defensive adaptations associated with parasite infection.
Although steps were put in place to limit possible influences on the results, there is always the potential for limiting factors. This includes the use of different feed for each fish species, the lack of individual identification for fish, human presence during observations and the impact of light on fish and parasite. However, none of these factors are likely to have had any major effects on the results.

4.4.2 Pathological response

In contrast to findings from natural infection in the wild (Chapter 2), there was no significant difference between *C. auratus* and *N. vittata* in the attachment sites of *L. cyprinacea*. The majority of adult female *L. cyprinacea* were found on the fins (particularly the dorsal fins) of both fish species, with 61% found on the fins of *C. auratus* and 67% found on the fins of *N. vittata*. Localisation to the fins has previously been observed for this species in other studies (e.g. Shields and Tidd, 1974, Bulow et al., 1979, Goodwin, 1999, Marina et al., 2008), with Medeiros and Maltchik (1999) identifying that a greater proportion of parasites attached to the base of the fins of fishes during periods of higher water flow in an intermittent stream, and suggested that these attachment sites provided greater protection against being dislodged by currents. Attachment at these sites, however, may also provide protection against host defensive behaviours, such as scraping.

The scales/lack of scales of a fish may also determine parasite attachment. Some scale-less fish species, such as *Clarias gariepinus* (African sharptooth catfish), may produce hormones or secrete mucous that make attachment for the copepod unacceptable or create immunity in the fish host. Whereas the structure and arrangement of scale in some species, such as *Hydrocynus vittatus* (African tigerfish), might not allow for easy implantation of the parasite’s anchor as they are tightly packed (Dalu et al., 2012).

*Carassius auratus* was found to have significantly greater levels of pathogenicity and inflammation at parasite attachment sites, when compared with *N. vittata*. To a certain extent, the greater inflammatory response in *C. auratus* is not surprising; immune responses, including
inflammation, have previously been reported in *C. auratus* in response to the attachment of *L. cyprinacea* (Khalifa and Post, 1976, Shields and Goode, 1978, Shariff et al., 1986, Woo and Shariff, 1990, Kadhim, 2009). Preliminary studies in our laboratory have also identified significantly increased alternative complement pathway activity (ACH50; part of the innate immune system) in the mucous coating of *C. auratus* when exposed to *L. cyprinacea* (Kanani et al., 2014).

What was unexpected was the severity of the reaction in *C. auratus*, compared to that in *N. vittata*, considering the much greater likelihood of mortality in *N. vittata*. The lack of inflammation in response to parasite attachment in *N. vittata* may suggest that the parasite does not provoke an immune response in this host, which in turn might explain the greater infectivity (see Chapter 3). However, there was little evidence of tissue necrosis or other pathogenic effects which might be responsible for the greater morality rate of infected *N. vittata*.

It is possible that the increased parasite load may affect osmoregulation, accounting for the higher mortality rates seen in *N. vittata*. A review by Thorstad et al. (2015) of the effects of salmon lice (*Lepeophtheirus salmonis*) on wild sea trout (*Salmo trutta*), found that heavily infected fish were most affected by osmoregulatory disturbances, and moribund fish suffered from a complete osmoregulatory breakdown (Bjørn and Finstad, 1997). It has also been reported that trophonts cause physical changes to the epithelium through attachment and feeding (Lom and Lawler, 1973) to such an extent that osmoregulation is compromised (Noga and Levy, 2006). The mechanical attachment of *L. cyprinacea*, plus parasite load, may be enough to affect the osmoregulation of *N. vittata* in a similar fashion, causing the greater mortalities seen, when compared with *C. auratus*. However, this is speculative and requires further investigation before any answers can be determined.

### 4.4.3 Conclusions

In this study, *L. cyprinacea* was found to have a preference for parasitising the naïve native teleost host over the natural host. It was also demonstrated that there is a significantly greater rate of infection; parasite intensity and mortality rate in the naïve host compared to the natural
host (see Chapter 3). The differences in rate and intensity of infection between species were, in part, due to differences in behavioural response of host to parasite infection. *Carassius auratus* displayed a range of behavioural responses to infection, which were not observed in *N. vittata*. However, whether the behaviours observed in this study are adaptive against *L. cyprinacea* or that their infrequent occurrence in *N. vittata* contributes to the greater infectivity of the parasite to this species is yet to be determined. Further experimentation is necessary to help differentiate between behavioural modifications, adaptive behaviours and those that are just accidental by-products of infection. Combining this with studies examining the immune response of fish hosts (such as continuing in the attempts to use immunohistochemistry markers) will help to provide a more comprehensive picture of defensive adaptations associated with parasite infection.

Histological studies of the infection site of *L. cyprinacea* established that the natural host had a significantly greater pathogenic and inflammatory response than the naïve host. This was somewhat surprising, considering that there is a greater likelihood of mortality in the naïve host. This suggests that the parasite does not provoke an immune response in *N. vittata*, possibly providing an explanation for the greater infectivity observed (Chapter 3) but does not explain the higher mortality rate seen in the naïve host.
Chapter 5
General Discussion

5.1 Identification of *Lernaea cyprinacea* as a co-invading parasite

It is not always straightforward to determine whether a newly discovered parasite is alien or native to a region. For many parasites, morphology alone does not provide sufficient resolution for accurate species identification (Lymbery and Thompson, 2012). Marina et al. (2008) identified *Lernaea cyprinacea* in native freshwater fishes of the south-west of Australia based on morphological criteria. As the shape of the holdfast of the metamorphosised adult female is unique to each species of *Lernaea*, it has often been used as a fundamental tool in species identification (Harding, 1950, Fryer, 1961). However, evidence of morphological plasticity means that morphological identification by the female holdfasts is unreliable (Kabata, 1979, Kabata, 1982, Lester and Hayward, 2006). In the present study, the species identity of the parasite was confirmed by using DNA sequence analysis at the 18S and 28S loci.

Even when species identity is confirmed, however, there may still be doubt over the origin of the parasite. Cryptogenic species, those that are not demonstrably alien or native, appear to be remarkably common in terrestrial, freshwater and marine ecosystems because human-mediated transport of organisms began long before taxonomic surveys and species monitoring programs (Carlton, 1996, Thomsen et al., 2010). *Lernaea cyprinacea* is now reported all over the world, including Africa, Asia, Europe, North America and Australia (Hoffman, 1999, Durham et al., 2002). Its preferred hosts include cyprinid species, but it has been identified on more than 100 fish species from 16 different orders (Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006). Although *L. cyprinacea* is not native to Australia, it has now been recorded in a number of native fish species in New South Wales and Victoria, in eastern Australia, and more recently, in Western Australia (Ashburner, 1978, Hall, 1983, Bond, 2004). Hall (1983) was one of the first to report *L. cyprinacea* in Australia, identifying it on *Prototroctes maraena* (Australian grayling) in the Tambo River in Victoria, eastern Australia.
Marina et al. (2008) first reported *L. cyprinacea* in the Canning River in Western Australia, and though reasons for the introduction of the parasite into Western Australia remain unknown, it has been suggested that co-introduction through the accidental release of cyprinid hosts, such as *Carassius auratus* and *Cyprinus carpio*, into natural waterways is the most likely cause (Marina et al., 2008). In Western Australia, *C. auratus* and *C. carpio* have been identified in many streams, irrigation drains and lakes in the Perth metropolitan area (Morgan et al., 2004). These species, in particular *C. auratus*, have also been found in a number of natural waterways between the Moore and Vasse Rivers on the Swan Coastal Plain (Morgan et al., 2004).

Although we know this parasite in native to Asia and is now widely distributed in the world (Wellborn and Lindsey, 1970, Kupferberg et al., 2009), its origin into Australia’s south-west is still unknown. Molecular genetic studies on isolates of *L. cyprinacea* may shed more light on the origin of the parasite, but, unfortunately, there is very little information publically available about the genetic variation of *L. cyprinacea* populations from different geographic regions. A recent study by Pallavi et al. (2015) was the first to gain any fundamental molecular knowledge of this parasite. This study focused on the genes associated with parasitism, examining the gene expression changes associated with parasitism of *L. cyprinacea* during the transit from the free living to parasitic stage, in an attempt to set a foundation for the development of novel interventions against *L. cyprinacea*.

As previously stated, there are a number of limitations with this study. Firstly, due to time constraints only three samples were able to be sequenced, therefore, although *L. cyprinacea* was positively identified, it cannot be said for certain that no other species of *Lernaea* was present. Secondly, as there have only been a few studies that have focused on molecular characterisation of *L. cyprinacea* there is not enough evidence to say, with confidence, that molecular characterisation, by itself, is a reliable identification tool.
5.2 Distribution and host range of *Lernaea cyprinacea* in Western Australia

Co-invading parasites have been defined as those which are co-introduced into a new locality with an alien host and then spread from their initial point of introduction and switch to native hosts (Lymbery et al., 2014). *Lernaea cyprinacea* was originally identified on four native and three alien fish species in Australia’s Southwestern Ichthyological Province (Marina et al., 2008). In this study both the distribution and host range of *L. cyprinacea* was reviewed and both had increased since 2008.

In the present study, *L. cyprinacea* was identified in both the Serpentine River and Murray River; in addition to the river reported in Marina et al. (2008) (the Canning River). This is the first time that this parasite has been reported in the Murray and Serpentine Rivers. A further two native fish species infected with *L. cyprinacea* were also identified, in addition to those identified by Marina et al. (2008): *Leptatherina wallacei* (western hardyhead) and *Pseudogobius olorum* (bluespot goby).

It is usually considered that the establishment of parasites in a new environment is much more likely to occur in those species with simple, direct life cycles (vertical transmission or horizontal transmission) without the need for intermediate hosts (Dobson and May, 1986, Bauer, 1991, Torchin and Mitchell, 2004). Dobson and May (1986), for example, suggest an order of magnitude difference in the establishment of directly transmitted parasites compared to those with an indirect life cycle. The life cycle of *L. cyprinacea* is direct, requiring only one host to complete all nine stages (Grabda, 1963, Shields, 1978). Of the nine stages in the life cycle, two are parasitic; the copepodid and adult stage (Grabda, 1963). Although *L. cyprinacea* only requires one host to complete its life cycle, a low host specificity and relatively loose attachment to the fish host by the copepodids means they are able to move freely from host to host (Grabda, 1963, Shields, 1978). It is only in the final stage where permanent attachment to the host occurs by metamorphosed adult female (Grabda, 1963, Nagasawa et al., 2007).
The lack of host specificity and wide host range of *L. cyprinacea* (Shariff et al., 1986), makes it an ideal candidate for host-switching (Grabda, 1963, Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006).

The main reason for the spread of *L. cyprinacea* throughout the world has been attributed to international trade of tropical fishes (Robinson and Avenant-Oldewage, 1996), and it is now identified on a large range of fish species, as well as tadpoles and amphibians, all over the world (Tidd and Shields, 1963, Bulow et al., 1979, Kabata, 1979, Shariff et al., 1986, Lester and Hayward, 2006, Nelson, 2006). It is not unusual for this parasite to have serious deleterious effects on freshwater fish hosts, and death can occur as a result of haemorrhaging and secondary bacterial infections (Oscoz et al., 2010).

This present study identified *L. cyprinacea* on a further two species of native freshwater fish in Australia’s south-west, since the initial report in 2008, which suggests that this parasite is now spreading. With its ever increasing host range, and known detrimental effects (including major fish losses), control of this parasite and prevention of further incursions become important in the conservation of native freshwater fishes. Future monitoring is important, not only in Western Australia, but all over the world as the invasiveness of *L. cyprinacea* makes it one of the major ectoparasites of freshwater fish worldwide (Pallavi et al., 2015). I recommend an ongoing monitoring program in the south-west of Western Australia to help contain further spread of *L. cyprinacea*. It may be possible to involve community catchment groups and freshwater angling societies in such a monitoring program, as the parasite (in it’s attached adult stage) is conspicuous and can be readily identified from photographic evidence.

5.3 Differences in infectivity and pathogenicity of *Lernaea cyprinacea* to alien and native host species

The naïve host hypothesis suggests that when a co-invading parasite switches from the host species with which they were introduced to native hosts in the new location, there will be
greater infectivity and pathogenic effects in the native hosts, as these hosts lack any coevolved resistance or tolerance (Allison, 1982; Mastitsky et al., 2010; Fassbinder-Orth et al., 2013). Resistance refers to the host’s ability to avert parasite infection, reduce the parasite burden or recover from infection, and tolerance is the ability for a host to limit the damage caused by a given parasite burden (Hayward et al., 2014). Although there is no strong theoretical basis to the naïve host hypothesis, Lymbery et al. (2014) found that of 16 published studies of co-introduced parasites that switched to native hosts and where good evidence of relative virulence of infection was available, virulence was greater in the new, native host in 85% of cases.

Field surveys found a consistently greater prevalence of *L. cyprinacea* on native freshwater fish species than on the parasite’s presumed ancestral host, *C. auratus*, or other alien species. This may be a consequence of greater exposure rates of native freshwater fishes to the infective stages of the parasite rather than greater infectivity of the parasite to native fishes. To separate these effects, the infectivity of *L. cyprinacea* to *Nannoperca vittata*, a native freshwater fish species, and *C. auratus* was compared, under controlled laboratory conditions. When fishes were exposed to infective stages of *L. cyprinacea* in single species groups, there was little difference between *N. vittata* and *C. auratus* in the rate or intensity of infection. However, in mixed species communities, *N. vittata* had a significantly greater infection rate than *C. auratus* and a greater (although not significant) intensity of infection. Furthermore, the mortality rate of *N. vittata* was greater than that of *C. auratus* in both single species and mixed species groups, and the risk of mortality was positively related to the intensity of infection.

On the face of it, these results support the predictions of the naïve host hypothesis. It is possible that the co-invading parasite may exhibit a greater virulence in native hosts than in the alien hosts with which they were introduced, simply by chance. The probability of an introduced host surviving the translocation process is likely to be inversely related to the virulence of any parasites they carry with them into the new range, because most introductions involve a few individuals being transported over geographic barriers or escaping from captivity (Blackburn et al., 2011). As a result, parasites with a lower virulence in their natural host are more likely to be
introduced (Strauss et al., 2012). If the virulence of the parasite differs between the coevolved alien host and the new, native host, it is more likely to be in the direction of increased virulence in the new host (Lymbery et al., 2014).

5.4 Behavioural differences between alien and native host species in response to infection

Differences in the infectivity of *L. cyprinacea* to *C. auratus* and *N. vittata* may result from differences in the behavioural and immunological defences of the fish hosts in response to parasite infections. Behavioural defences can range from avoidance of the infective parasite stages to reducing parasite load through adaptations, such as the physical removal of ectoparasites and ingestion of anti-parasitic compounds (Barber et al., 2000). Immunological defences in fishes include both the innate and adaptive immune systems (Magnadottir, 2010).

Host behaviours may explain, at least partially, differences in infectivity of *L. cyprinacea* to *N. vittata* and *C. auratus*. Infected *C. auratus* exhibited a range of behaviours that were largely absent from *N. vittata*. These behaviours included: gulping (where water is taken in quickly through the mouth and rapidly expelled through the gills); jerking (rapid bursts of swimming alternating with resting periods); scraping (where fishes with ectoparasitic infection scrape their body against a firm surface) and pecking (where fishes mouthed at the head and body of conspecifics). Although it is not possible to say with certainty that these behaviours were effective in reducing the rate or intensity of infection with *L. cyprinacea*, a number of lines of evidence support this conjecture.

These behaviours were much more common in fishes with *L. cyprinacea* infections, and although there is no direct evidence that they are defensive behaviours, their complexity, intent and convergence (identification in other fish species in response to parasitic infection) suggest they may have evolved as defences against parasite attachment. Nevertheless, the evidence to date is circumstantial and further experimentation is required to differentiate between behavioural modifications that are actually adaptive and those that are just accidental by-
products of infection. The behaviour of other native fish species, such as _Tandanus bostocki_ (freshwater cobbler), _Galaxias occidentalis_ (western minnow) and _Bostockia porosa_ (nightfish) should be examined, and compared with _C. auratus_ and other cyprinid species following infection with _L. cyprinacea_. Additionally, more direct studies are needed of the effectiveness of putative defensive behaviours in reducing parasite burdens and increasing the fitness (survival and/or reproductive success) of infected fishes. These should also be coupled with studies examining the immune response of fish hosts, to provide a more complete picture of the defensive adaptations associated with ectoparasite infections.

5.5 Differences in parasite pathogenicity to native and alien host species

The naïve host hypothesis predicts not only a greater infectivity of alien parasites to native hosts, but also greater pathogenicity. Pathological effects of parasitic infection arise partly from the action of the parasite in the destruction of host tissue and the production of toxins or other virulence factors, such as proteases, and partly from the physiological response of the host (Schmid-Hempel, 2011). The attachment of _L. cyprinacea_ can have serious pathogenic consequences for the fish host, including skin lesions (or ulcerations) and secondary bacterial infections (Shariff and Roberts, 1989). Histopathologically, the more common changes associated with parasite infection vary from acute inflammatory reactions to severe degenerative changes, and necrosis in the skin and underlying musculatures (Noor El-Deen et al., 2013).

In the present study, there was a significantly greater mortality rate in the native species _N. vittata_, than in the presumed ancestral host, _C. auratus_, following experimental infection with _L. cyprinacea_. Histopathological studies, however, were unable to shed light on the reason for this increased mortality rate. There was a much greater inflammatory response to infection in _C. auratus_ than in _N. vittata_. Although it is not entirely surprising to observe this reaction of _C. auratus_ to infection with _L. cyprinacea_, as previous studies have demonstrated an immune response by this host to the parasite (Khalifa and Post, 1976, Shields and Goode, 1978, Shariff et al., 1986, Woo and Shariff, 1990, Kadhim, 2009), the severity of the inflammatory response
was unexpected. This becomes even more striking when compared to *N. vittata*, especially considering the much greater mortality rate in *N. vittata*.

It is possible that the increased parasite load may affect osmoregulation, resulting in the higher mortality rates seen in *N. vittata*, even without obvious histopathological changes at the attachment site. This hypothesis should be investigated further through experiments that measure the plasma chloride levels in infected and uninfected fish hosts (Thorstad et al., 2015). Further immunological studies, such as the use of immunochemistry markers, will also be necessary to help us understand the differences seen in the immunological response of *N. vittata* and *C. auratus* to *L. cyprinacea*. It will also be important to expand the study to include other native freshwater fishes to see whether or not this lack of response is common to native fishes, or if it is a phenomenon only seen in *N. vittata*.

### 5.6 Possible impacts of *Lernaea cyprinacea* for the freshwater fishes of south-western Australia

There is an increasing recognition of the important role co-invading parasites play as a cause of disease emergence, often producing high morbidity and mortality rates in native hosts (Smith and Carpenter, 2006, Taraschewski, 2006, Peeler et al., 2011). Freshwater ecosystems are particularly impacted by invasive species and co-invading parasites, especially as there has been an increase in the rate of alien freshwater fish introductions throughout the world, doubling in the last 30 years (Gozlan, 2008, Gozlan et al., 2010). A review of the literature by Lymbery et al. (2014) found that, of 98 cases of co-introductions of alien hosts and parasites, 51% of alien hosts were fishes and, of these, 81% were freshwater or diadromous species.

The freshwater ecosystems of Australia have suffered extensive habitat degradation, mostly as a result of human exploitation, and are now under increasing anthropogenic pressure (Morgan et al., 1998, Allen et al., 2002, Pollino et al., 2004). Invasive fish species represent an important threat to native species in the Southwestern Ichthyological Province through predation, degradation of habitat and water quality, competition for food and other resources, aggressive
interactions such as fin nipping, and introduction of exotic pathogens and parasites (Beatty and Morgan, 2013).

The morbidity and mortality associated with infections of directly transmitted parasites tends to be density dependent, as most parasites are transmitted more readily in dense populations of their hosts. This might suggest, a priori, that parasitic disease is not likely to be a major problem for endangered animal species, with small or declining populations. However, for generalist parasites that can infect any number of host species, non-endangered hosts can act as a reservoir of infection for endangered hosts, even when the endangered species exists at low population densities (McCallum and Dobson, 1995, Holt et al., 2003). Because L. cyprinacea is a generalist parasite, it poses a potential threat to native fish species such as Nannatherina balstoni, Nannoperca pygmaea, Galaxiella nigrostriata, Lepidogalaxias salamandroides and Galaxias truttaceus, all of which have very restricted ranges (Morgan et al., 2014).

Since the first report of L. cyprinacea in south-western Australia, although the cause for its introduction has yet to be established, it has been suggested that the most likely route of initial infection was the release or escape of infected ornamental fishes, such as C. auratus (Marina et al., 2008). Self-sustaining populations of C. auratus have been reported in almost every state of Australia and throughout much of the world (Fuller et al., 1999, Gido and Brown, 1999, Skelton, 2001). Within Western Australia, C. auratus is most frequently found in modified or degraded waters and is generally restricted to the south-western corner, in close proximity to major populated areas (Morgan et al., 2004). The species is a particular problem in the Vasse River system, where removal programs have been in operation since 2005 (Morgan and Beatty, 2007).

Control programs for invasive alien species should consider the potential impacts on co-invading parasites, because the alien host may act either as a sink, to dilute the effects of the parasite, or as a reservoir, to amplify the effects of the parasite on native host species. Whether the alien host acts as a sink or a reservoir will depend on the relative competencies of native and alien hosts to transmit infections. Because L. cyprinacea is more infective to native fish species
than to *C. auratus*, it has been suggested that the removal of *C. auratus* could amplify parasitic infection on native species (A. J. Lymbery, pers. comm.). This is likely to be countered, however, by the greater mortality rate of infected native species (at least *N. vittata*) compared to *C. auratus*, which will increase transmission rates from *C. auratus* compared to those from native species.

The results from the present study, while not directly addressing this question, suggest that *C. auratus* is a more competent host than *N. vittata* and is therefore likely to amplify infections. Both the intensity of infection and the mortality rate of *N. vittata* were greater in the presence, than in the absence of *C. auratus*. There is therefore no evidence from this study that the removal of goldfish will exacerbate the problem of *L. cyprinacea* in river systems in southwestern Australia. There is a need, however, to expand this study to examine the comparative infectivity and pathogenicity of *L. cyprinacea* to other native fish species and, where possible, to monitor parasite infection rates in the field before and after goldfish control programs to ensure that there are no adverse effects from goldfish removal.

### 5.7 Options for control of *Lernaea cyprinacea* in the Southwestern Ichthyological Province

As *L. cyprinacea* is a generalist parasite, with a direct life cycle and considerable time spent as free-living stages, eradication of the parasite is not a viable control option. Any management actions which are undertaken should therefore be aimed at minimising the potential for spread of the parasite. At present, it appears that *L. cyprinacea* is confined to the Canning, Serpentine and Murray Rivers. Further spread is likely to be through the translocation of infected fishes from these rivers into rivers currently free of infection or the accidental or deliberate release of infected ornamental fishes into such rivers.

Taking this into account, the best way to reduce the threats of translocation and further release is through educational campaigns. Richter et al. (2015) conducted a survey of an environmental educational program that had been applied to a cohort of 542 students. This study found that
there was a significant increase in the environmental knowledge of students receiving environmental education compared to the controls. Even a year after the environmental education ended, those students who had used the environmental program still tested high in their environmental knowledge. Another study, by Cobo et al. (2010), looking at the trends in non-indigenous freshwater species records in the Iberian Peninsula, suggested that the reason for the reduction in vertebrate inflow may be attributed to educational programs that had recently been put into place.

Public awareness of the risks posed by invasive fish species is essential, and will be an important step in helping preserve freshwater ecosystems in Australia and around the world.

5.8 Conclusions

Lernaea cyprinacea is a generalist parasite, known to have severe detrimental effects on its fish host (Shariff et al., 1986, Oscoz et al., 2010). It is an invasive species that has now been identified on a wide range of fish species throughout the world, and is considered a major parasite of freshwater fishes (Wellborn and Lindsey, 1970, Kupferberg et al., 2009, Pallavi et al., 2015). Its recent introduction into Western Australia has already seen an increase in host range and distribution since the initial report in 2008 (Marina et al., 2008). Although L. cyprinacea has now been positively identified, the origin of infection has yet to be established. What is known is that this parasite has greater morbidity and mortality rates in native freshwater fishes (as demonstrated in Chapter 3) compared with C. auratus. However, despite this, histopathological studies have shown a greater response in C. auratus than N. vittata to L. cyprinacea infections. This study also suggests that C. auratus is a more competent host than N. vittata and is therefore likely to amplify infections. Both the intensity of infection and the mortality rate of N. vittata were greater in the presence, than in the absence of C. auratus. The behavioural observations of this study have shown that the complexity, intent and convergence of behaviours displayed by C. auratus but not N. vittata appear to be defensive behaviours, although this cannot be said with certainty.
What we do know is that *L. cyprinacea* needs to be controlled, and further infestations on native fishes need to be prevented. In order to do this, further research is necessary, extending the current study to include more freshwater fish species to gain a more complete understanding of how *L. cyprinacea* infections affect native fishes. Any information gained builds a foundation and enables us to have a full understanding of the impacts of this parasite on freshwater ecosystems. This foundation gives us the opportunity to develop effective public awareness campaigns, and, ultimately, aid in the control of *L. cyprinacea* worldwide.
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