Black spot disease in freshwater fishes of south-western Australia: identification of the parasite, host range and potential as a bioindicator for water quality

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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.

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Abstract

The salinisation of freshwater ecosystems by anthropogenic influences is recognised as one of the largest threats to the highly endemic freshwater fish fauna of south-western Australia. There has also been some recent evidence that secondary salinisation is affecting the parasite community of freshwater fishes. A parasitic trematode, which causes black spot disease in the musculature of native fishes, has previously been tentatively identified as *Diplostomum galaxiae*, a species first described from a galaxiid fish (*Galaxias auratus)* in Tasmania. The aims of the current study were to confirm this specific identification using genetic analyses; investigate the host and geographic range of the parasite in south-western Australia; and determine whether the parasite could be a suitable bioindicator of secondary salinisation.

Encysted metacercariae were extracted from preserved fishes in the collection of the Freshwater Fish Group & Fish Health Unit, Murdoch University, and from a specimen of *Galaxias truttaceus* from Tasmania, and a section of the 18S rRNA gene sequenced. All parasites from south-western Australia and Tasmania were genetically identical, but did not group with other species in the *Diplostomum* genus in phylogenetic analyses. Instead, the Australian parasite aligned more closely to a clade containing *Posthodiplostomum* spp., and several genera from the Strigeidae family. Reclassification of the parasite causing black spot disease in south-western Australian freshwater fishes is thus recommended, following more extensive comparisons with the parasite throughout Australia. In the interim, the parasite has been designated with the temporary name Dip01.

Historical collections of fishes from the West Australian Museum and from the Freshwater Fish Group & Fish Health Unit, Murdoch University were assessed, to collate records of Dip01 metacercariae. The parasite appears to preferentially infect *Galaxias maculatus* and *Galaxias occidentalis* over other species; although it was also found in two percichthyids (a single *Nannatherina balstoni* and several *Bostockia porosa* from a single catchment) and has been reported previously from two estuarine fishes (*Leptatherina wallacei* and *Pseudogobius olorum*) that have colonised secondarily salinised rivers of the region. The overall prevalence of Dip01 in *G. maculatus* was 11.7% (95% CI 9.3-14.4%), and in *G. occidentalis* was 6.1% (4.8-7.7%). The mean intensity of infection in *G. maculatus* was 3.5 (2.0-7.3) parasites/infected fish, while the mean intensity in *G. occidentalis* was 7.8 (5.9-10.3) parasites per infected fish. The geographic range of Dip01 closely matches that of the preferred hosts, *G. maculatus* and *G. occidentalis.*

Prevalence and intensity data were compared against historical environmental data from the Water Information Reporting database of the Department of Water and Environmental Regulation, where available. There was a significant inverse relationship between parasite prevalence and salinity, with Dip01 only occurring in habitats with a conductivity of less than 1,000 μS/cm. Based on these data, the parasite appears to be a useful bioindicator of salinity, as it is restricted to low salinity waters, even though its preferred galaxiid hosts are tolerant of a wide range of salinities.

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1. Introduction

1.1 Parasites and parasitism

Parasitism occurs when an organism benefits from its host species, with the host gaining no benefit and usually being harmed in the process (Granroth-Wilding *et al*., 2015). Parasites account for more than half of all animal species, yet they are often an underappreciated component of a functioning ecosystem (Marcogliese, 2004). Parasitism is present in every ecosystem on earth, from the stomach and intestines of blue whales (de Vos *et al*., 2018) to phytoplankton in freshwater lakes (Lepere *et al*., 2008). Poulin (1999) states it is probably safe to say that for each species in a community of free-living animals, there is at least one species of parasite.

Parasites are a widely diverse ecological group, and modern molecular techniques are continually identifying new, cryptic species. Brief examples across a wide variety of taxonomic groups include new species of dipteran parasitoid flies in South America (Smith *et al*., 2006), new rhizocephalan barnacle species in Alaska (Noever *et al*., 2016), new trematode species in Europe (Georgieva *et al*., 2013), and new species of freshwater nematomorphs in North America (Hanelt *et al*., 2015). Despite this, parasite diversity is almost certainly underestimated and, as more species are being described, the question of "how many more are out there" is often debated. Poulin (2014) argues that this is not a useful question and taxonomic effort should be put into describing new species rather than pondering their potential existence.

The current rate of species extinction is as much as 100 times the normal, background rate through geological time (Pimm *et al*., 2014). It has been argued that coextinctions of parasites and mutualists are the most common form of species loss (Dunn *et al*., 2009), although coextinction events have been poorly documented (Strona, 2015). Despite the ubiquity of parasitic organisms, and their vulnerability to extinctions, it is only in the last two decades that the importance of parasites in natural ecosystems has been appreciated and that parasites have seriously been considered as conservation targets (Thompson *et al*., 2018).

1.2 Importance of parasites in ecosystems

Parasites play a key role in ecosystems and can influence their hosts in many ways. Parasites may reduce host fitness through direct effects on morbidity (e.g. ticks feeding on the blood of their mammalian host; Jones et al., 2015), mortality (e.g. the myxozoan endoparasite, *Tetracapsuloides bryosalmonae* in salmonid fish; Carraro *et al*., 2018) or fertility (e.g. the trematode *Schistosoma mansoni* in their snail host *Bomphalaria glabrata*; Webster and Woolhouse, 1999). Mathematical models have shown that parasites may provide density dependent regulation of their host populations through effects on both host mortality rates and host fecundity rates (Anderson and May, 1992; McCallum and Dobson, 1995), and these theoretical predictions have been confirmed empirically, for example in populations of red grouse (*Lagopus lagopus scotius*) infected by the nematode *Trichostrongylus tenuis* in northern England (Hudson *et al.,* 1998).

Parasites can also have indirect effects on the fitness of their hosts by mediating the outcome of competitive or predatory interactions with other free-living species (Granroth-Wilding *et al*., 2015). For example, the acanthocephalan, *Pomphorhynchus laevis*, reduces the immune competence of one of its intermediate hosts, a native amphipod, *Gammarus pulex*, as well as exerting a stronger negative influence on this species compared to that of the invasive amphipod species *Gammarus roeselii* and therefore promoting the competitive success of the invader in the Ouche River in France (Cornet *et al*. 2010). Similarly, the trematode *Microphallus* sp., found in in Michigan and northern Wisconsin, USA, alters the boldness of crayfish when a predator is present, making them less wary, and less likely to seek shelter, both of which increase predation risk; these behavioural changes differ in different crayfish species, meaning that the parasite can alter crayfish population dynamics (Reisinger *et al*., 2015).

These parasitic influences on their hosts can have cascading effects through an ecosystem, influencing trophic interactions, inter-community dynamics and biodiversity, especially when the parasite is utilising a keystone species (Hudson *et al*., 2006). Recent studies have suggested that parasites are vital components of food webs, increasing complexity and connectedness among species (Lafferty *et al*., 2006; Dunne *et al*., 2013). Parasites may also dominate food webs in terms of biomass and energy flow. This effect is proportional to their host specificity; generalist parasites (parasites that can infect more than one species of host), especially those with a complex life cycle, such as

trematodes, have a larger effect on food webs than specialists (parasites with a limited range of hosts). Preston *et al*. (2013), for example, found that trematode biomass equalled, or was greater than, the most abundant free-living insect orders in pond ecosystems; when combined with the amount of cercariae that are released from snail intermediate hosts, trematodes have the potential to exceed the energy contributions of all free-living taxa.

As a consequence of their vital role in ecosystem functioning, parasites may be important drivers of biodiversity. One such example is the aquatic microsporidian *Cougourdella* sp., and its effect on a species of caddisfly, *Glossosoma nigrior*. The *Cougourdella* sp. parasitises its host to a point where other invertebrate species increase in abundance, due to an increased availability of unutilised resources for these parasite-free species (Kohler & Wiley, 1997). Similarly, the intertidal snail *Littorina littorea* consumes up to 40% less macroalgae when infected with trematode parasites; as a result, there is a greater abundance of resources available for other species to utilise in the intertidal habitat (Wood *et al*., 2007).

1.3 Parasites as bioindicators

In recent years, a growing number of studies have suggested that parasites may be useful in environmental impact studies as indicators of environmental stress (Gilbert & Avenant-Oldewage, 2017). Parasite responses to environmental stresses may be complex, however. Rates of parasite infection may increase if anthropogenic impacts on the environment enhance the survival of infective parasite stages, increase the contact between infective stages and their intermediate or definitive hosts, or increase the susceptibility of the host to

infective stages of the parasite. It is also possible, however, for rates of parasite infection to decrease if environmental impacts reduce the survival of infective parasite stages or limit the contact between infective stages and their hosts (Pietrock & Marcogliese, 2003; Williams & Mackenzie, 2003; Mahmud *et al*., 2017).

Due to this sensitivity to natural and anthropogenic impacts on an ecosystem the distribution, abundance and physiological status of parasites can be useful indicators of environmental health (Sures, 2001; Flores-Lopes & Thomaz, 2011; Lafferty, 2012). For example, Khan & Billiard (2007) found that *Cryptocotyle lingua,* a digenean ectoparasite, was found to have a higher prevalence in polluted marine systems when its typical host, the winter flounder (*Pleuronectes americanus*), was stressed from pollutants such as runoff from nearby paper mills. By contrast, Pech *et al*. (2009) found that chemical pollution in coastal lagoons had a negative effect on the helminth parasites of the checkered puffer *Spheroides testudineus* sp. and Lacerda *et al*. (2017) showed that organic pollution had a negative effect on a range of fish parasites.

Parasites can also accumulate environmental contaminants at a higher rate than their hosts, with the sensitive species acting as excellent early warning systems (Malek *et al*., 2007). For example, nematodes of the genus *Contracaecum* accumulate metals more rapidly than host tissues and the larval stages can have between two to fifty times the concentration of heavy metals such as nickel and lead as their hosts (Leite *et al*., 2017). Other examples include cestodes in sharks being used as bioindicators of lead or cadmium levels (Malek *et al*.,

2007) and an intestinal acanthocephalan *Acanthocephalus lucii* in European perch as an indicator for lead (Jankovská *et al*., 2011).

Lafferty (1997), Blanar *et al*., (2009) and Vidal-Martínez *et al*., (2009) reviewed the responses of a range of parasite taxa to a range of environmental impacts, mostly in aquatic ecosystems. Two important points arose from these reviews. First, simple measures of parasite community structure, such as species richness, are unlikely to be useful indicators of specific environmental stresses, because different taxonomic groups can respond in completely different ways to the same environmental change. Second, no particular taxonomic group of parasites is likely to provide a universal indicator of environmental stress, because most taxonomic groups respond differently to different environmental changes. Nevertheless, there do seem to be some consistent responses of particular taxa to particular environmental effects, for example ciliates usually increase in number with increased effluent from a range of industrial processes, while acanthocephalans and digeneans decrease in number with increased heavy metal pollution (Lafferty, 1997). This may reflect a more general trend in the response of parasites to environmental stresses; populations of parasites with simple, direct life cycles often increase in size because of a compromised immune response by their host, whereas populations of parasites with more complex, indirect life cycles often decrease in size because the environmental change adversely affects free-living stages or intermediate host populations (Marcogliese, 2005). There are many exceptions to this general rule, however, and, as stressed by Marcogliese (2005), it is essential to understand the biology and transmission dynamics of any parasite species to be used as an

environmental indicator before patterns of population change can be interpreted correctly.

What are the advantages of parasites as environmental indicators? This depends on what we expect environmental indicators to tell us. Indicator taxa have been defined as those "whose characteristics, such as presence or absence, population density, dispersion or reproductive success are used as an index of attributes too difficult, inconvenient or expensive to measure" (Landres *et al.,* 1988). Parasites might be particularly useful in reflecting the effect of environmental stresses on ecosystem function, because of their key role in community food webs. That is, because the transmission pathways of parasites are often embedded in food webs (Thompson *et al.,* 2005), they can be extremely sensitive to environmental changes acting at any trophic level in the community. Before we can assess just how useful parasites are as functional indicators of environmental impacts, however, further theoretical and empirical studies are needed. From a theoretical perspective, we need models of parasite population dynamics that will provide quantitative predictions of how parasite population numbers will react to different environmental stresses. Building these models will require more empirical data on how these stresses affect parasites and their hosts. This in turn will require a combination of controlled laboratory experiments and properly designed and replicated field studies (Lafferty, 1997; Marcogliese, 2005).

Once these models and base knowledge of a parasites life cycle and population is established, it will then be possible for further studies as suggested by Vidal-Martinez & Wunderlich (2017), to determine parasite response to field and

laboratory effects of environmental degradation, assess bioaccumulation, and with an interdisciplinary approach utilise different types of biomarkers from both host and parasite populations to create long term data sets for both polluted and reference areas.

1.4. Threats to freshwater environments

Freshwater environments cover a large swathe of geomorphic settings; from large connected systems like rivers, to isolated areas such as lakes. Freshwater ecosystems cover less than 1% of the earth's surface yet support approximately 10% of global biodiversity, with estimates that they contain more than 126,000 species of plants and animals (Balian *et al*., 2008), including around 33% of all vertebrates (Strayer & Dudgeon, 2010). Freshwater ecosystems are critical habitats for many species both aquatic and terrestrial (Bedford, *et al.,* 2001), and they are highly affected by anthropogenic activities, primarily because of the tendency for human development on or near the water, such as agriculture or ports. These conflicting land uses mean that freshwater ecosystems are more threatened than either marine or terrestrial ecosystems (Brinson & Malvárez, 2002; Strayer & Dudgeon, 2010).

There are many threats facing freshwater ecosystems and these can be broadly categorised as habitat degradation or alteration, introduced species, pollution, climate change and salinisation (Vörösmarty *et al*., 2010), all of these threats can exacerbate or potentially dampen the effects of the others, while still causing harm in their own way.

Habitat degradation or alteration

Habitat change can have major effects on ecosystem structure, with a dramatic change in available habitat and potential flow on-effects such as food resources. Some examples of habitat alteration are removal of surrounding vegetation, siltation in a water body, changing flow regimes, damming, abstraction, and shoreline modification (Vörösmarty *et al*., 2010). For example, mass deforestation through China has caused rivers to experience high levels of siltation, and the removal of accompanying wetlands has led to a large number of flood events (Zhao *et al*., 2006). The basins are either reduced or incapable of preventing flooding, with the reclamation of wetlands for agricultural use resulting in a dramatic decline of the species richness of fish, waterfowl and vascular plants (Fang *et al*., 2006).

Alteration of physical conditions such as damming or obstructing a water body, altering its path or by abstracting it is seen as a major global threat to freshwater diversity (Vörösmarty *et al*., 2010). While this is altering the habitat by removal or change of water as discussed above it originates from a single source, i.e. the obstruction, change in path or its abstraction point and has different effects to other habitat changes. Some examples of ecoregions threatened by damming or abstraction are the Murray-Darling basin in eastern Australia, watercourses in West Korea, and the Middle and Lower Indus basin (Poff *et al*., 2007).

The presence of adjacent agricultural changes can also alter the ecosystem. Livestock can alter the riparian zone though grazing or wallowing, destabilise riverbanks and increase the nutrient levels of streams and rivers through their

excrement. These activities can have far-reaching consequences. For example, Saunders & Fausch (2018), in a study in the USA, found that as intensity of grazing by cattle increased on riparian vegetation, less terrestrial invertebrates were in the diet of the trout.

Introduced species

Introduced species can pose a major threat to freshwater ecosystems, leading to biotic homogenisation as native species are replaced by widespread alien species (Simberloff, 2011). This is of particular concern to freshwater fishes in many parts of the world, with introduced fishes being regarded as the prime threat to native freshwater fish in the Mediterranean (Hermoso, *et al*., 2011). A global assessment of Mediterranean climate-based freshwater habitats found that fish assemblages in these habitats historically had few species in common, but are now more homogenised due to introduced species (Marr *et al*., 2010). A topical example of the threat posed by introduced species is European carp, *Cyprinus carpio*, which has proliferated globally and is now one of the most widely distributed fish in the world (Vilizzi *et al*., 2015). Since the introduction of carp into Australia, the species has spread throughout waterways in south-eastern Australia, inhabiting one million km^2 (Koehn, 2004). The degradation of habitat can also benefit invasive species adapted to harsher conditions such as *Geophagus brasiliensis* in Western Australia, as it is more saline tolerant and has a different diet to natives (Beatty *et al.,* 2013). Parasites may play a key role in mediating the impacts of introduced species. Introduced hosts often have fewer parasite species and a lower prevalence of parasites than native hosts, which may provide them with a competitive advantage (Torchin & Mitchell, 2004).

Once introduction has occurred, parasite transmission may occur from native hosts to introduced hosts, leading to an increase in infection of natives if the introduced hosts amplify transmission (Kelly *et al*., 2009) or a decrease in infection of native species if introduced hosts reduce transmission (Paterson *et al*., 2011). If introduced hosts carry new parasites (coinvaders), then these may be transmitted to native hosts, leading to the emergence of new disease in the natives (Lymbery *et al*., 2010; 2014).

Pollution

Anthropogenic pollution is a major stressor in riverine health (Vörösmarty *et al*., 2010), with multiple contaminants falling under this category, such as; light, thermal, chemical and physical pollutants. Light pollution is caused by anthropogenic light in otherwise natural darkness, with the potential to disrupt circadian rhythms of fish and alter abundances, due to behavioural changes such as shoaling and presence of higher order predators in the presence of unnatural light (Becker *et al*., 2013; Brüning *et al*., 2015). Thermal pollution is caused by changes to the natural temperature of the water, for example by discharge from power stations, and has the potential to affect spawning and development in aquatic species (Graham *et al*., 2016). Thermal pollution can also alter benthic cover and fish species richness (Teixeira *et al*., 2009). Chemical pollution can be lethal to organisms, with some such as the zebrafish (*Danio rerio*) being used for toxicology analysis (Dubińska-Magiera *et al*., 2016). Chemical pollutants can also alter parasite pathogenicity, making understanding their interactions with the environment crucial (Nakayama *et al*., 2017). Physical pollution such as plastics, especially micro plastics, are harmful to

aquatic fauna, with potential for intestinal blockages, behavioural changes and changes in lipid metabolism (Jovanović, 2017). Pollution has been found to have a greater effect on fish that are also suffering water stress, in particular affecting fish in a Mediterranean climate (Karaouzas *et al*., 2018).

Climate change

Changes to climate are readily apparent at a global scale, with changes to rainfall, temperature and migratory patterns; with Mediterranean climates such as south-western Australia, predicted to become drier and warmer (Beatty *et al*., 2014; Hallett *et al*., 2018). In south-western Australia rainfall has decreased by 16%, and stream discharge by 50% since the 1970s (Silberstien *et al*., 2012). The change to a drier climate will affect each fish populations differently, with potential threats such as habitat fragmentation, degradation and pollution all likely to be exacerbated by climate change (Pratchett *et al*., 2011). The drier climate has, and will continue to, decrease freshwater flows, which reduces habitat and may lead to increased salinity levels (Beatty *et al*., 2014).

Salinisation

Secondary salinisation is the increase of dissolved salts in the water, and is a growing global threat to freshwater ecosystems (Cañedo-Argüelles *et al*., 2013) with approximately 10% of the earth's surface suffering from increased salinity (Perri *et al*., 2018). Anthropogenic or secondary salinisation occurs when a saline groundwater table is raised and brought into contact with surface water either by the percolation of excess irrigation water (irrigation salinity) or by rainfall, as a result of the clearing of perennial vegetation (dryland salinity), or when urban sources of salts, such as de-icing salt, are washed into waterways

(Pannell & Ewing, 2006). In Western Australia, secondary salinisation of waterways is principally a consequence of dryland salinity, caused by the removal of native, deep-rooted, perennial vegetation for agricultural purposes, typically being replaced with short rooted annual crop species (Delaney *et al*., 2016).

An increase of salinity in waterways as a consequence of extensive land clearing is a particularly important threat to freshwater fish, their parasites and other freshwater organisms in south-western Australia (Beatty *et* al., 2011; Rashnavadi *et al*., 2014). With the increase in salinity levels, it is very likely that less tolerant species will be severely adversely affected, with potential for severe physiological effects, and even death (Kefford *et al*., 2016). While the salt-tolerant taxa may not exhibit any physiological or behavioural changes due to the salinity at any given level, it may still be indirectly affecting their habitat. This could be by the increase in salinity altering the geochemical properties of the waterway or by affecting the other biotic components of the ecosystem such as competitors, predators, prey, or parasites (Kefford *et al*., 2004).

1.5 Freshwater fishes of south-western Australia

Globally there are nearly 30,000 species of fish, of which approximately 13,000 are obligatory freshwater species (Lévêque *et al*., 2008). This means that freshwater fishes comprise around 25% of all vertebrate life, and 13-15% of all freshwater life (Lévêque *et al*., 2005). These 13,000 exclusively freshwater species live in approximately 1% of the earth's surface, as opposed to the roughly 70% of the surface for marine species. Around half of freshwater fish species are found in the Neotropical (4,035 species) and Afrotropical (2,938

species) regions, while the Australian region only has 261 species, of which 102 are endemic (Lévêque *et al*., 2008).

Freshwater fish species are facing the same primary threats as all freshwater organisms (see 1.4). While appropriate inventories of freshwater fish species are incomplete, hundreds of freshwater fish species are at risk of extinction (Lévêque *et al*., 2008). Freshwater fishes, especially those with shorter life cycles or specialised habitats are highly susceptible to severe population declines in the event of habitat loss (Wedderburn *et al*., 2014) or changes in environmental factors (Beatty *et al*., 2014; Leigh *et al*., 2015). The requirements of a sustainable freshwater habitat and therefore the conservation management of freshwater fish species is often a very complex and large-scale process, involving more than just the body of water; beginning in the catchment and ending at the discharge point, and covering all the potential affecting factors between. The prerequisites of this large-scale management are rarely met (Lévêque *et al*., 2008).

Australia has a unique and diverse assemblage of freshwater fishes, with 10 ichthyological regions in the country, five of which are within Western Australia (Unmack, 2013). There are 102 species of native fish in Western Australia, of which 66 are obligate freshwater species (Morgan *et al*., 2014a). South-western Australia has a depauperate, but highly endemic, freshwater fish fauna, with 11 described species, of which 9 are found nowhere else in the world (Rashnavadi *et al*., 2014) (Table 1.1). The South-western Province is dominated by species which have ancient lineages and are either unique to the region or have

historical ties that have since been severed due to the formation and uplift of the Nullarbor Plain.

Ecosystems in south-western Australia have undergone fundamental changes due to salinisation (Morgan *et al*., 2003), with the freshwater fish species of the region experiencing downstream range contractions due to secondary salinisation of waterways (Beatty *et al*., 2014). As a consequence, six of the 11 native freshwater fish species in south-western Australia are either listed, or in the process of being listed, as threatened or near threatened (Table 1.1).

Table 1.1 Native freshwater fishes of south-western Australia and their conservation status. DBCA – Western Australian Department of Biodiversity, Conservation and Attractions; EPBC – Environmental Protection and Biodiversity Conservation; IUCN – International Union for the Conservation of Nature. Information from Morgan *et al*., (2014a).

Species	**Common name**	**Conservation status**	**Endemism**
Tandanus bostocki	Freshwater cobbler	Not listed	Endemic
Lepidogalaxias salamandroides	Salamanderfish	Near threatened (IUCN)	Endemic
Galaxias maculatus	Common jollytail	Not listed	Non-endemic
Galaxias occidentalis	Western minnow	Not listed	Endemic
Galaxias truttaceus	Trout minnow	Endangered (DBCA) Critically endangered (EPBC Act 1999)	Non-endemic
Galaxiella munda	Western mud minnow	Lower risk/near threatened (IUCN)	Endemic
Galaxiella nigrostriata	Black-stripe minnow	Endangered (EPBC Act 1999)	Endemic
Bostockia porosa	Nightfish	Not listed	Endemic
Nannatherina balstoni	Balstons pygmy perch	Vulnerable (EPBC Act 1999/DBCA)	Endemic
Nannoperca pygmaea	Little pygmy perch	Not listed but extremely rare	Endemic
Nannoperca vittata	Western Pygmy perch	Not listed	Endemic

Many of the fishes inhabiting south-western Australia are very habitat-restricted during the typical Mediterranean hot and dry summers, and rely on small refuge water bodies to survive (Morgan *et al*., 2014a). These refuge habitats are at very high risk of evaporation and evapoconcentration due to their typically small size and fragmented nature (Robson *et al*., 2013). Climate change is predicted to dramatically decrease the number, size and connectivity

1.6. Trematode parasites of freshwater fishes in south-western Australia

The parasites of freshwater fishes in Australia, and particularly in south-western Australia have been poorly studied. Lymbery *et al*., (2010), in the first general survey of the parasite fauna, found 44 different morphospecies, most of which had not been previously described. Only two species, both introduced, could be definitively identified to species level from published descriptions; the tapeworm *Ligula intestinalis* and the copepod crustacean *Lernaea cyprinacea*. A third species was tentatively identified as the trematode *Diplostomum galaxiae*.

Diplostomum galaxiae is a strigeoid trematode, which was first described in 1983 by Smith & Hickman parasitising *Galaxias auratus* in Tasmania. The fish is an intermediate host, with encysted metacercariae visible as black spots ("black spot disease") in the musculature of the body and head, this was the first description of black spot disease in Australia. Adult flukes were obtained from domestic ducklings, *Anas platyrhynchos*, that had been fed cysts, and these were identical in morphological appearance to flukes obtained naturally from white-faced heron, *Egretta* (=*Ardea*) *novaehollandiae*.

Members of the Superfamily Diplostomoidea, which includes *Diplostomum* spp., typically have a three host life cycle (Blasco-Costa & Locke, 2017; see Figure 1.1) definitive hosts are piscivorous birds, mammals or reptiles. Eggs are shed into the water with the host's faeces and hatch into free-swimming miracidia, which infect the first intermediate host, a gastropod mollusc. Following three generations of asexual reproduction, cercariae emerge from the gastropod and then locate and penetrate the second intermediate host, typically a freshwater fish or amphibian. The life cycle is completed when the second intermediate host is predated by the definitive host.

Chapman *et al*. (2006) assessed the helminth parasitism of *Galaxias maculatus* in several water bodies in south-western Australia and found a black spot causing trematode, which was tentatively identified as *Diplostomum galaxiae.* The study found black spot infections in Moates Lake, which is a freshwater system, but infection was not found in two saline systems nearby, although the same fish species and potential bird definitive hosts were present, which suggests that the parasite may be susceptible to salinity and therefore an indicator of secondary salinisation in freshwater ecosystems in south-western Australia. Similarly, Rashnavadi *et al*. (2014) in a survey of the salinised Blackwood River in south-western Australia, found *Diplostomum* sp. infecting fish in the lower salinity sections of the river, and not in the upper secondarily salinised section of the catchment.

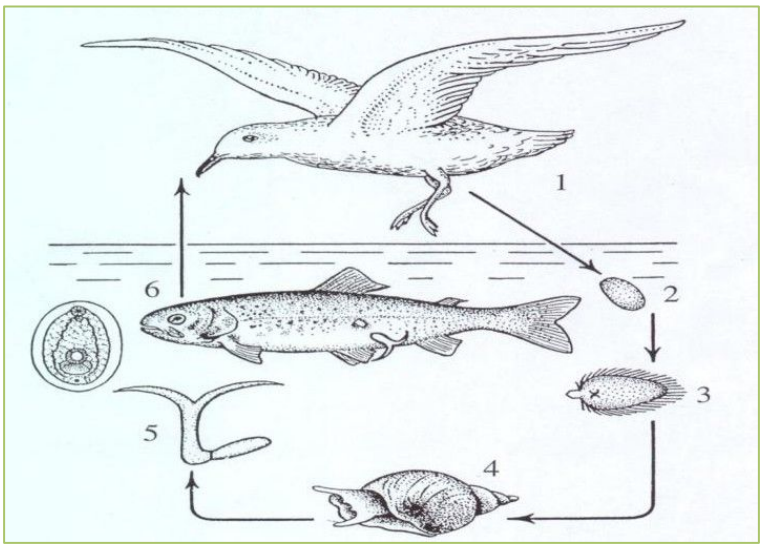

Figure 1.1. The generalised life cycle of a diplostomoid trematode. Parasites breed sexually in the gut of piscivorous birds (1), eggs are released in the host's faeces (2), these eggs hatch into free-swimming miracidia (3). Miracidia infect snails (4), the parasite asexually reproduces in the snail host and produces cercariae (5). The cercariae penetrate the fish and develop into metacercariae (6). When a piscivorous bird eats the infected fish, the parasite completes its life cycle. (Modified from Hakalahti et al., 2006).

1.7 Taxonomy of diplostomoid trematodes

Members of the trematode superfamily Diplostomoidea are characterised by possession of a holdfast organ posterior to the ventral sucker. There are over 250 described species, in 88 genera, in the superfamily (Blasco-Costa & Locke, 2017). Diplostomoid parasites are most frequently encountered in the second intermediate host, freshwater fishes. Species identification of diplostomoid metacercariae is complicated by the fact that the larval stages bear no morphological similarity to the adult parasite, and themselves have limited morphological features that would allow for identification (Locke *et al*., 2010). In recent years, the use of molecular techniques vastly improved the prospects of species identification from metacercariae, and has also led to a dramatic increase in the recognised species diversity in the superfamily, as a large

number of new, cryptic species have been described (e.g. Brabec *et al*., 2015; Kudlai *et al*., 2017; Soldánová *et al*., 2017).

The phylogeny of the Diplostomoidea has traditionally been based on definitive host occurrence and morphological features of the adult parasite, such as structure and shape of the holdfast and arrangement of the reproductive organs (Blasco-Costa & Locke, 2017). This has led to a variety of different taxonomic schemes, depending on the relative importance attached to different characters. Unfortunately, molecular studies have to date not been able to resolve these difficulties, with different studies often producing very different phylogenetic topologies, depending on the sequences analysed and the species included in the analysis (Blasco-Costa & Locke, 2017). The higher-level taxonomy of the superfamily is in need of thorough revision.

1.8. Aims and hypothesis

The overall aim of this study is to determine the suitability of the black spot trematode, provisionally identified as *Diplostomum galaxiae*, as a bioindicator of secondary salinisation of freshwater ecosystems in south-western Australia. The specific aims and hypotheses of the study are:

- Use molecular techniques to determine the species of parasite responsible for black spot infection in fishes in south-western Australia. I hypothesise that the parasite is *Diplostomum galaxiae*, originally described in fish from Tasmania.

- Determine the geographic distribution and range of fish hosts used by the parasite. I hypothesise that the parasite will be found more

frequently in species of Galaxiidae, the family of the type host, than in species from other families, and that the geographic range of the parasite will mirror that of its preferred host(s).

- Relate parasite prevalence and intensity of infection to water salinity, to determine whether the parasite has potential as a bioindicator of secondary salinisation. I hypothesise that there will be a negative correlation between water salinity and parasite prevalence and / or intensity.

2. Methods

2.1 Species identification

Metacercariae were collected from preserved (100% ethanol) specimens of *Galaxias maculatus* (one sample, one location) and *Galaxias occidentalis* (five samples, four locations) from Western Australia, and from *Galaxias truttaceus* specimens (two samples, one location) from Tasmania. Samples were limited to those available in storage at the Freshwater Fish Group & Fish Health Unit at Murdoch University. Cysts were excised from the host using a scalpel blade, pooled from each individual and placed into an Eppendorf tube for extraction. Genomic DNA was extracted using the DNeasy blood and tissue kit in accordance with the Qiagen handbook (Qiagen, Hilden, Germany). A negative control was included in the extraction process. DNA was stored at -20°C until required.

Samples were initially screened for amplification of the 18S rRNA gene using the generic primer set 18S9F and 18S637R (Table 2.1; Moszczynska et al, 2009) under the following conditions: pre- amplification step of 94°C for 2 min; followed by 40 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min; and a final extension step at 72°C for 10 min. The reaction was performed in a 25µl volume consisting of 12.5µl 2 x GoTaq PCR master mix (Promega, Alexandria NSW, Australia), 2µl of each primer, 7µl water and 1.5µl DNA template. A negative control was included in the PCR to control for contamination. Samples that did not amplify using the primers in in the first PCR run (PCR 1) were amplified using primers 01Diplo18SF and 01Diplo18SR (PCR 2) or 02Diplo18SF and 02Diplo18SR (PCR 3) (Table 2.1). The thermal conditions for PCR 2 and

PCR 3 were as previously described, except the annealing temperature for both reactions was 53°C instead of 50°C, and the extension time was 2 min instead of 1 min. All reactions were carried out in a PT100 thermocycler (MJ-Research), and PCR products were run on a 2% agarose gel stained with SYBR Safe (Invitrogen, USA) and visualised under LED light. Bands were cut out using an in- house filter tip purification method as described by Yang *et al*., (2013).

Table 2.1: Primer sets used for amplification of 18S rRNA gene in samples of metacercariae from freshwater fishes from Western Australia and Tasmania.

PCR run	**Primers**	**Reference**
PCR 1	18S9F 5'-TGATCCTGCCAGTAGCATATGCTTG-3'	Moszczynska *et al.*, 2009
	18S637R 5'-TACGCTATTGGAGCTGGAGTTACCG- 3'	Moszczynska *et al.*, 2009
PCR 2	01Diplo18SF 5' -TACCTTAAAACGGTGAAACC- 3'	This study
	01Diplo18SR 5' -CGACCCAGATCCAACTACGA- 3'	This study
PCR 3	01Diplo18SF 5'-AAACGGTGAAACCGCGAATG-3'	This study
	01Diplo18SR 5'- CCTTGGCAAATGCTTTCGCTGT-3'	This study

Purified PCR products were sequenced in both directions using the ABI Prism Dye Terminator Cycle Sequencing Kit (Applied Biosystems, California) according to the manufacturer's instructions. The chromatograms were edited in Geneious (Kearse *et al.*, 2012) and a consensus sequence (Dip01) was generated from the products of all three primer pairs using MUSCLE (Edgar, 2004). Phylogenetic analyses were carried out by generating a 964bp alignment (MUSCLE Edgar, 2004) containing Dip01 and 27 references downloaded from GenBank. A GTR + G + I model was selected using jModelTest (Posada, 2008) to construct a phylogenetic tree using Bayesian analysis. Posterior probabilities

were generated using Mr Bayes v. 3.1.2 (10,000,000 generations, sampling frequency of 1000, burn- in 3000).

2.2 Host preference and geographic range

Fish specimens from the collections housed at both the Freshwater Fish Group & Fish Health Unit (FFGFHU) at Murdoch University and the wet store of the Ichthyology Section of the Western Australian Museum (WAM) were examined for black spot infection by non-invasive visual assessment. Between both collections the fish specimens assessed ranged from being collected in 1936 to 2018. All WAM specimens were limited to non-invasive visual assessment. All *Galaxias maculatus* (n= 651) and *G. occidentalis* (n= 1081) samples from both collections were inspected first, because the parasite had previously been found in these species (AJ Lymbery, pers. comm.). Black spots under the skin were confirmed as encysted metacercariae by examination with a dissecting microscope; metacercarial cysts are composed of a thin, transparent inner layer of parasite origin and a thick, partially opaque outer layer of host origin Smith & Hickman, (1983). If infected *G. maculatus* and *G. occidentalis* were found at any location, all other fish species at that location within the collections were also examined (n= 5,452). Unless the same water body had multiple collection events it is assumed that all fish were collected from a singular site per collection.

Geographic origins of infected fish were mapped using GPS coordinates, where they were provided in the collections, or from site descriptions, where GPS coordinates were not provided. For each fish species at each location, and for each fish species over all locations, prevalence was recorded as the proportion

of infected hosts, with 95% confidence intervals (CI) calculated assuming a binomial distribution, and intensity of infection as the mean number of metacercariae per infected host, with 95% confidence intervals calculated by bootstrapping, using the software QPweb (Reiczigel *et al*., 2013). Differences in prevalence among fish species were assessed using a χ^2 test, or a Fisher exact test if only two species were present at a location. Differences in intensity of infection among fish species were assessed by a non-parametric Kruskal Wallis test.

2.3 Correlation with environmental factors

To assess the environmental conditions of the fish collection sites the Water Information Reporting (WIR) database of the Department of Water and Environmental Regulation was used. Gauging station data from the WIR were selected for use based on proximity to the latitude and longitude of the fish collection localities, and availability in a five year period immediately prior to fish collection. The only environmental variables for which sufficient data were available for analysis were water temperature (°C), conductivity (µS/cm) and pH.

As the two species of fish most commonly infected were *G. maculatus* and *G. occidentalis*, with no significant difference in parasite prevalence or mean intensity of infection (see Results), I pooled parasite data for these two species to examine correlations of parasite prevalence and intensity per site with mean water temperature, conductivity and pH. Data were not available for all environmental variables for the same localities, meaning that sample sizes were too small for multivariate analyses. Instead, separate univariate analyses were

conducted for each environmental variable, using non-parametric Spearman's rank correlation.

3. Results

3.1 Species identification

Black spot metacercariae were found on four host species: *Nannatherina balstoni* (Balston's pygmy perch), *Galaxias maculatus* (common jollytail), *Galaxias occidentalis* (western minnow) and *Bostockia porosa* (nightfish) (Figure 3.1).

Figure 3.1. The four species with black spot disease from the WAM collection, A: *Nannatherina balstoni* (Balston's pygmy perch), B: *Galaxias maculatus*, C: *Galaxias occidentalis*, and D: *Bostockia porosa* (cysts circled).

Sequence data were obtained for metacercariae from three fish specimens; *G. maculatus* from the Goodga River, Western Australia, *G. occidentalis* from the Serpentine River, Western Australia, and *G. truttaceous* from Tasmania. All three sequences were identical, with no matches from GenBank.

Phylogenetic analysis placed the parasite in a clade containing a number of species of *Posthodiplostomum* (Family Diplostomatidae), and several genera from the family Strigeidae (Figure 3.2). Sequence similarity with the other species in this clade varied between 93.8 and 97.7%, within the range of similarities found for other described species in the tree; on this basis, it will henceforth be referred to as a putative new species, Dip01. Dip01 was not closely related to other species of *Diplostomum* in the tree. Furthermore, the phylogenetic analysis suggested that both the genus *Diplostomum*, and the familes Diplostomatidae and Strigeidae are paraphyletic (Figure 3.2).

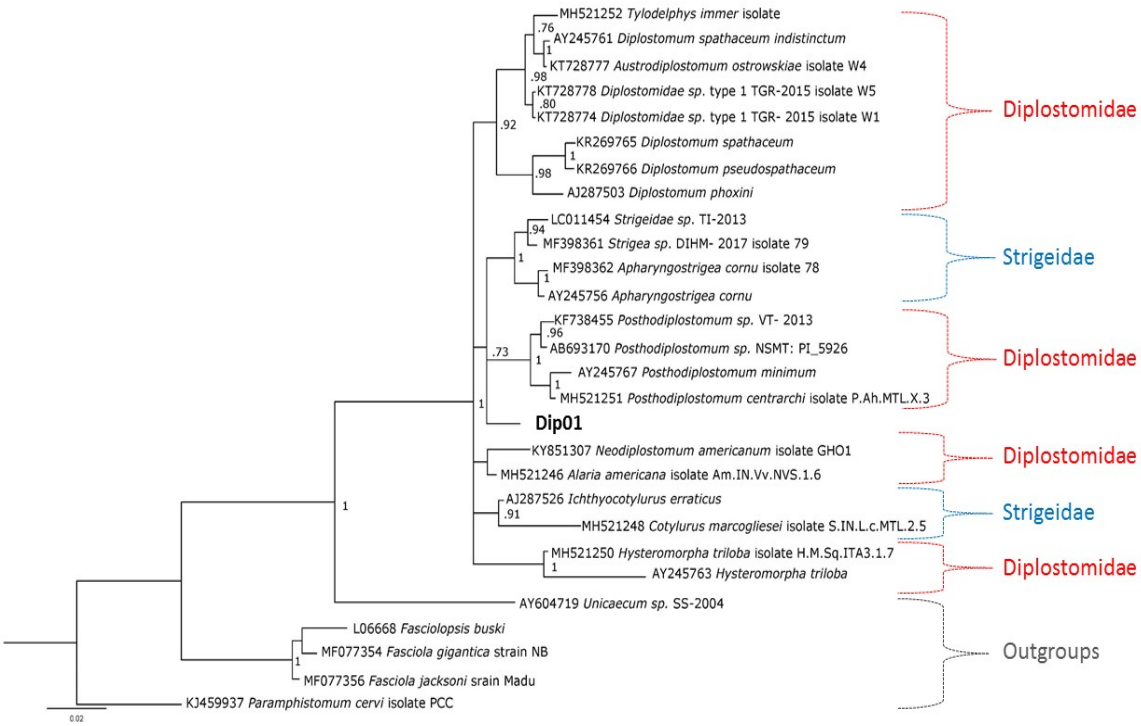

Figure 3.2. Phylogenetic analysis of 964 bp sequence of the 18S rRNA gene from black spot metacercariae found in this study (Dip01) and 27 reference sequences from GenBank (22 from the superfamily Diplostomoidea and five from other trematode superfamilies, used as outgroups). Tree constructed using Bayesian analysis. Numbers at nodes represent Bayesian posterior probabilities.

3.2 Host preference and geographic range

Nearly eight thousand fish specimens were assessed from the collections at the Murdoch University and the Western Australian Museum, with 7,135 specimens used for final analysis. Eleven of the species of fish assessed had at least one sample site with more than 10 fish present. Of the 56 systems assessed 17 had presence of at least one parasite. Table 3.1 shows for each species the sample size, number of sites examined and prevalence and intensity of the infection of Dip01 with 95% confidence intervals. Four species of the fishes examined were infected by the parasite Dip01, however *N. balstoni* had only one infected fish and was excluded from further statistical analysis due to insufficient samples size (n<10). The only other fish species infected were two *Galaxias* species, *G. maculatus* and *G. occidentalis* and *B. porosa*. *Galaxias maculatus* was the species most commonly infected, with an overall prevalence of 11.7%, while *G. occidentalis* had the highest intensity of infection, with an overall mean intensity of 7.8 parasites per infected fish. There were significant differences among fish species in overall prevalence ($\chi^2_2 = 6.5$, $p = 0.04$) and overall mean intensity of infection ($\chi^2_2 = 6.5$, $p = 0.04$). Dunn's multiple comparison test indicated that the prevalence of infection in *G. maculatus* was significantly greater than in *B. porosa* (Figure 3.3), while the intensity of infection in *G. maculatus* and *G. occidentalis* was significantly greater than in *B. porosa* (Figure 3.4).

Table 3.1. Fish species surveyed with sites containing at least 10 individuals, and prevalence and intensity of infection of Dip01, with 95% confidence intervals (CI). Overall prevalence and intensity were calculated from all fish examined, regardless of site; while mean site prevalence and intensity were calculated as averages of prevalences and intensities at each site.

Fish species	Common name	Number at sites	Number of sites examined	Overall prevalence (±95% CI)	Mean site prevalence (±95% CI)	Overall intensity (±95% CI)	Mean site intensity (±95% CI)
Galaxias maculatus	Common jollytail	651	18	0.117 (0.093-0.144)	0.212 (0.105-0.318)	3.5 (2.0-7.3)	8.3 (1-18.8)
Galaxias occidentalis	Western minnow	1081	34	0.061 (0.048-0.077)	0.348 (0.132-0.563)	7.8 (5.9-10.3)	4.5 (1.4-7.7)
Bostockia porosa	Nightfish	674	19	0.030 (0.019-0.046)	0.353 (0-0.840)	1.4 (1.2-1.9)	1.2 (1-1.7)
Nannatherina balstoni	Balston's pygmy perch	85 [87]	4 [5]	0.012 (0.001-0.063)	-	-	-
Geotria australis	Pouched lamprey	25	1	0	0	0	0
Galaxias truttaceus	Trout minnow	10	1	0	0	0	0
Galaxiella munda	Western mud minnow	1305	15	0	0	0	0
Galaxiella nigrostriata	Black-stripe minnow	2266	18	0	0	0	0
Nannoperca vittata	Western pygmy perch	1037	24	0	0	0	0
Pseudogobius olorom	Blue-spot goby	19	1	0	0	0	0
Gambusia holbrooki	Mosquitofish	29	2	0	0	0	0

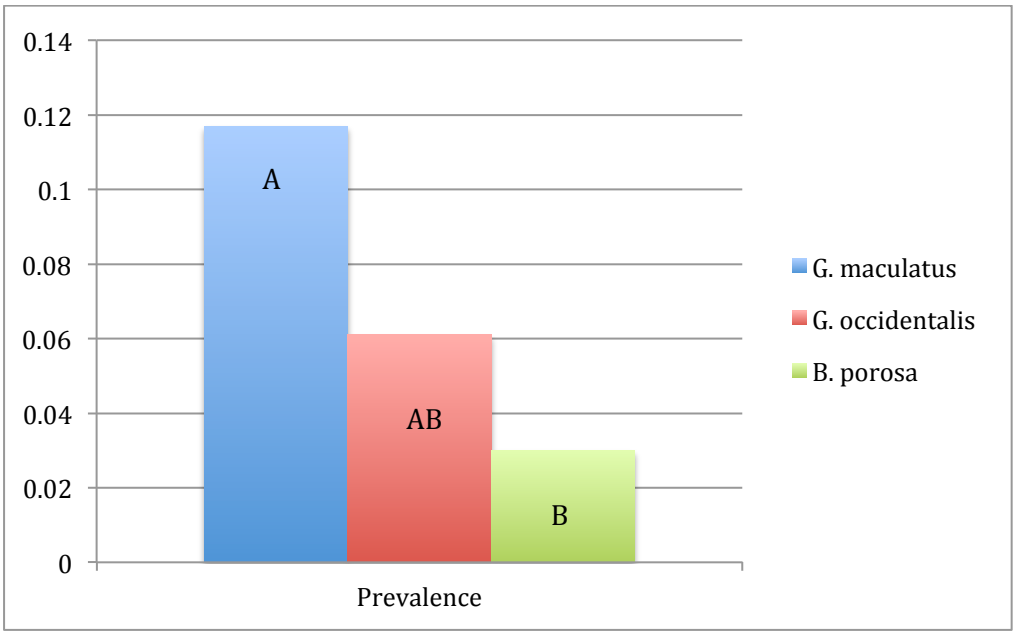

Figure 3.3. Overall prevalence of infection with Dip01 on different fish hosts. Bars with the same letter not significant difference found ($p > 0.05$).

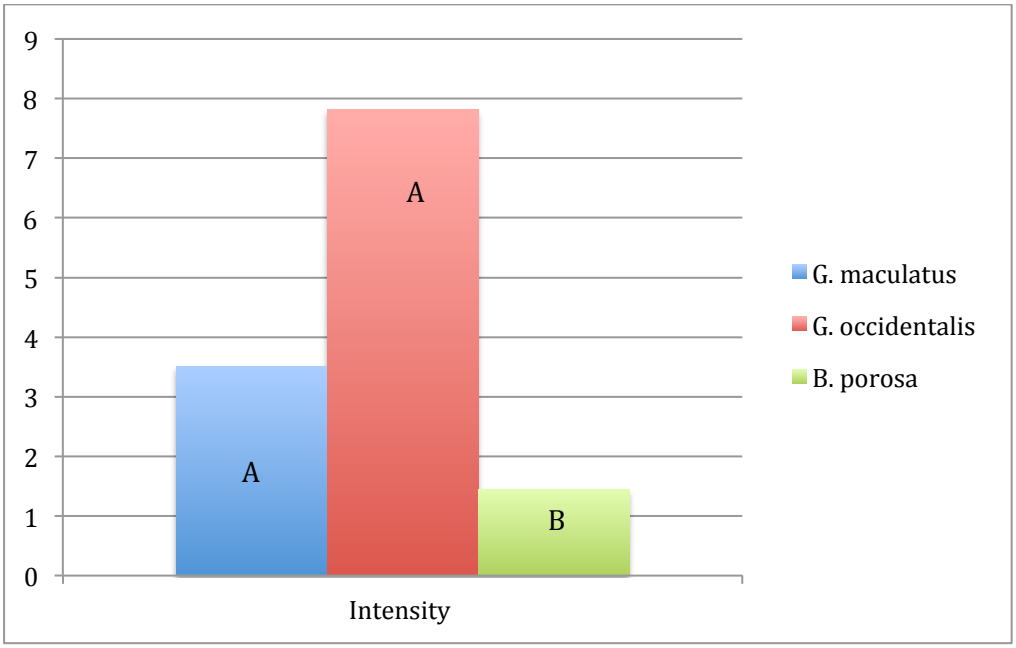

Figure 3.4. Overall mean intensity of infection with Dip01 on different fish hosts. Bars with the same letter not significantly difference ($p > 0.05$).

Table 3.2 shows the prevalence and mean intensity of infection for all sites where the parasite was present at any point in time (see also Appendix 1 for further details). The highest prevalence was found on *G. occidentalis* in the Serpentine River (94% infected), while the greatest mean intensity of infection

was on *G. maculatus* in Cape Le Grand (19.2 parasites per infected fish). Only two sites where the parasite was present had more than one species of fish host infected, with two tributaries of the Hill River, Mundina Creek and Coomaloo Creek, both housing infected *G. occidentalis* and *B. porosa*. These two sites are also the only two where *B. porosa* was infected.

The parasite causing black spot disease was identified in samples from 14 rivers/creeks, two dams, and two lakes across south-western Australia, including the Hill River's Tributaries Mundina and Coomaloo Creek, through to Cape Le Grande in the south-east of the state (Figure 3.5).

Table 3.2. Infected fish species, and prevalence and mean intensity of infection of Dip01, with 95% confidence intervals (CI) by site. Where more than one species of fish was infected, prevalence's and mean intensities are calculated over all fishes.

Location	# Times sampled	Infected Species present	# Fish	Prevalence	Intensity
Angove River	4	*G. maculatus*	190	0.311 (0.25-0.38)	N/A
Cape Le Grand	2	*G. maculatus*	128	0.1 (0.057-0.17)	15.7 (8.8-34.4)
Gairdner River	1	*G. maculatus*	14	0.071 (0.004-0.320)	1
Pallinup River	5	*G. maculatus*	192	0.016 (0.004-0.046)	1
Serpentine River (lower gauging station)	1	*G. occidentalis*	18	0.94 (0.73-1)	4.94 (3.9-6.1)
Hill River (Coomaloo creek)	1	*G. occidentalis* *B. porosa*	16 30	0.74 (0.59-0.85)	10.1 (7.01-13.9)
Millyeannup Brook	3	*G. occidentalis*	84	0.18 (0.11-0.28)	4.27 (2-10.7)
Lake Jasper	1	*G. occidentalis*	25	0.120 (0.034-0.303)	2.0 (1-2.7)
Blackwood River (Bridgetown)	1	*G. occidentalis*	19	0.105 (0.019-0.316)	10.0 (1-10.0)
Donnelley River (Pemberton)	1	*G. occidentalis*	20	0.1 (0.018-0.32)	2.5 (2-2.5)
Marbellup Brook	1	*G. occidentalis*	10	0.1 (0.005-0.447)	1
Blackwood River (upper catchment)	1	*G. occidentalis*	24	0.083 (0.015-0.267)	1.5 (1-1.5)
Margaret River	1	*G. occidentalis*	30	0.067 (0.0120-0.213)	1.5 (1-1.5)
Hill River (Mundina Creek)	1	*G. occidentalis* *B. porosa*	9 20	0.28 (0.14-0.47)	1.9 (1.12-2.4)

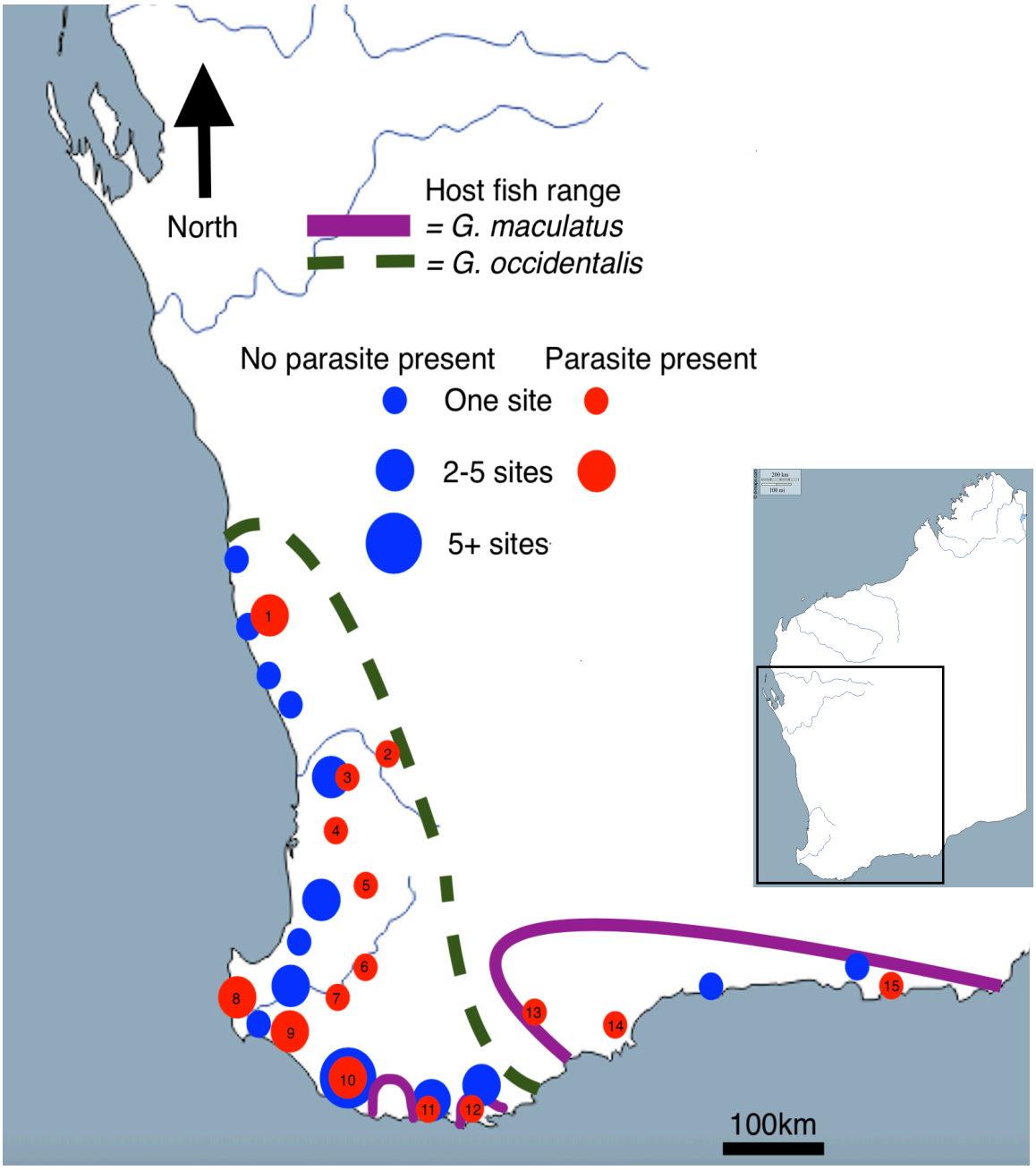

Figure 3.5. Sites from which fishes were examined for the presence of Dip01, with size of the dot proportional to the number of sites in that area, and the colour indicating presence (red) or absence (blue) of the parasite. Also shown are the ranges of the two principal host species. Note the isolated, disjunct population of *G. maculatus* (Morgan *et al.*, 2006). Sites with the parasite present are as follows; 1: Hill River Tributaries, Mundina and Coomaloo Creek, 2: Cunderdin, 3: Bickley Reservoir, 4: Serpentine Dam, 5: Boddington Weir, 6: Blackwood River, 7: Bridgetown, 8: Margaret River, Boronup, 9: Milyeannup brook, Lake Jasper, 10: Lake Maringup, Pemberton, 11: Angove River, 12: Marbellup Brook, 13: Pallinup River, 14: Gairdner River, 15: Cape Le Grande. (Map adapted from d-maps.com).

3.3 Correlation with environmental factors

Parasite prevalence at a site was significantly inversely correlated with conductivity (rho = -0.55, p = 0.01) (Figure 3.6). Both *G. occidentalis* and *B. porosa* were not present in sites with conductivity greater than 10,000uS/cm, while *G. maculatus* was found at sites exceeding 30,000 μS/cm. However, no fish samples were infected with Dip01 at sites with mean conductivity >1,000 μS/cm. The mean conductivity of sites where the parasite was found on any host species was 503.4 ± 2519.4 μS/cm, compared to a mean of 9150.9 ± 2295.2 μS/cm for sites where the parasite was not found on any host species (Welch t = 2.7, p = 0.02).

No significant relationships were found between parasite prevalence and either pH (Figure 3.7; rho = -0.16, p = 0.59) or temperature (Figure 3.8; rho = 0.47, p = 0.17).

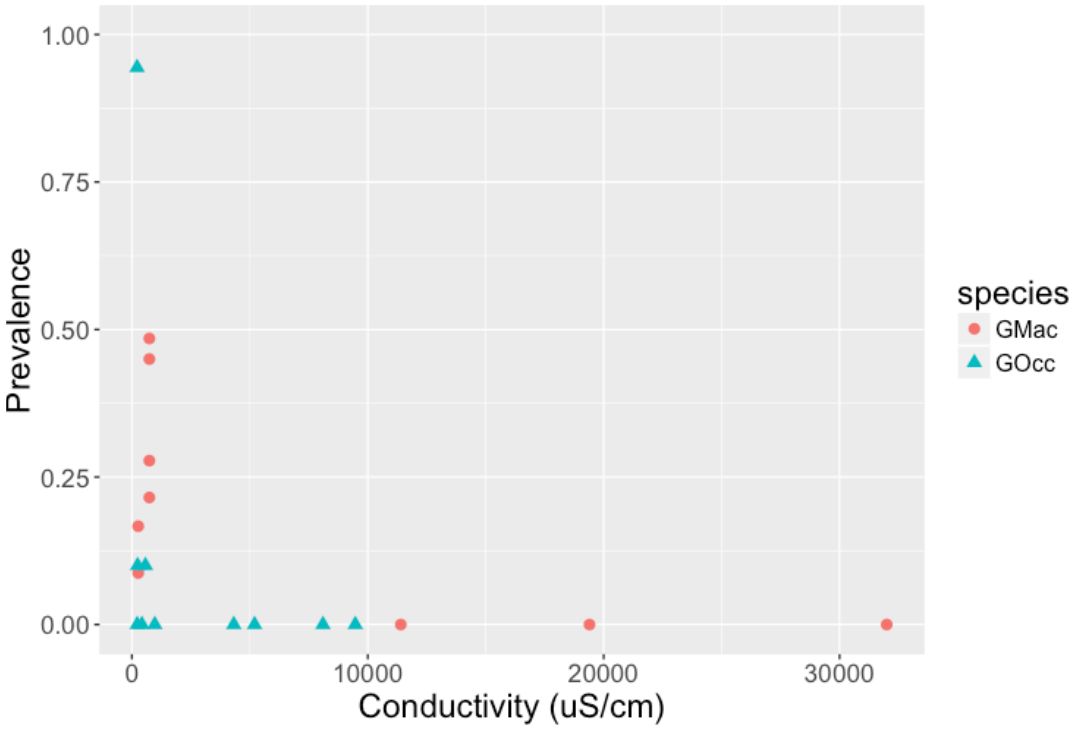

Figure 3.6. Prevalence of the parasite Dip01 in *Galaxias* spp. compared to the conductivity (µS/cm) of the sample sites.

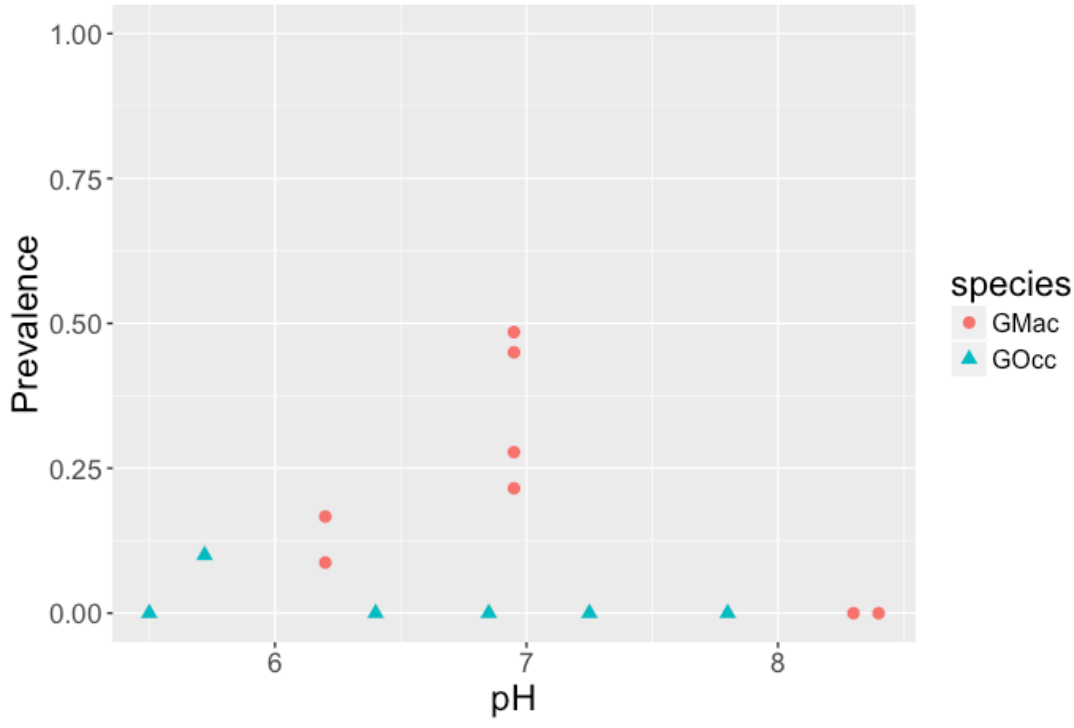

Figure 3.7. Prevalence of the parasite Dip01 in *Galaxias* spp. compared to the pH of the sample sites.

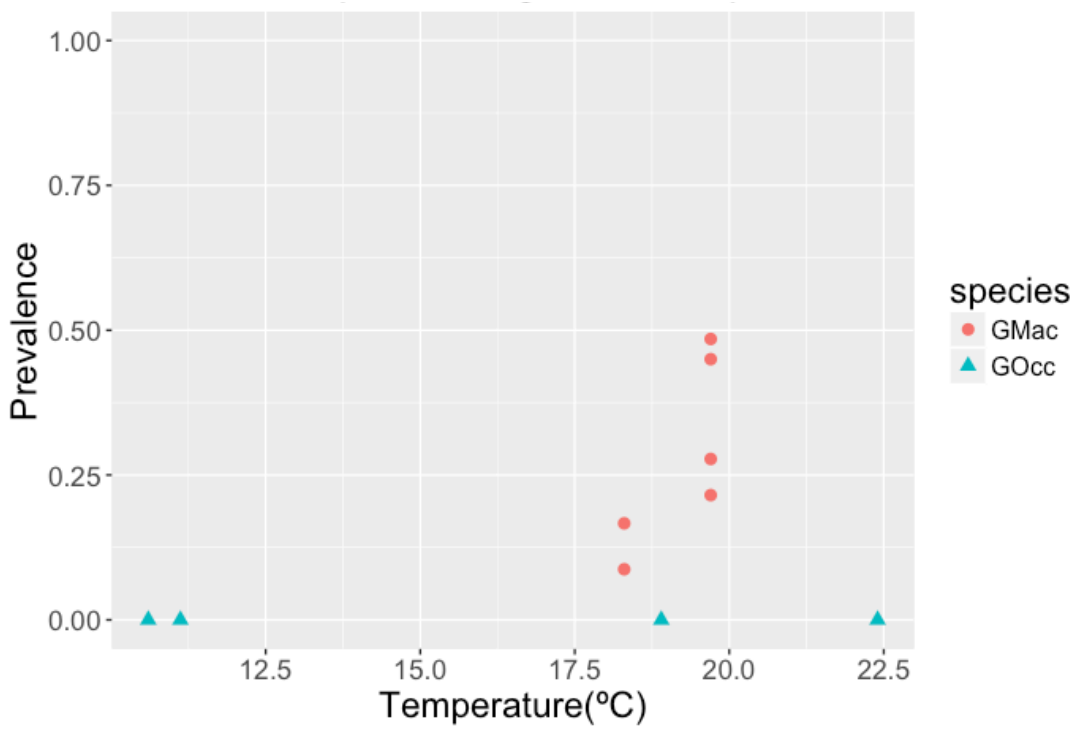

Figure 3.8. Prevalence of the parasite Dip01 in *Galaxias* spp. compared to the temperature (°C) of the sample sites.

Although there was a trend for intensity of infection with Dip01 to be inversely related to conductivity, this was not significant (Figure 3.9; rho = -0.602, p = 0.09), and there was no significant relationship between intensity and pH (Figure 3.10; rho = -0.52, p = 0.23). There was, a significant inverse relationship between intensity and temperature (Figure 3.11; rho = -0.98, p = 0.0006).

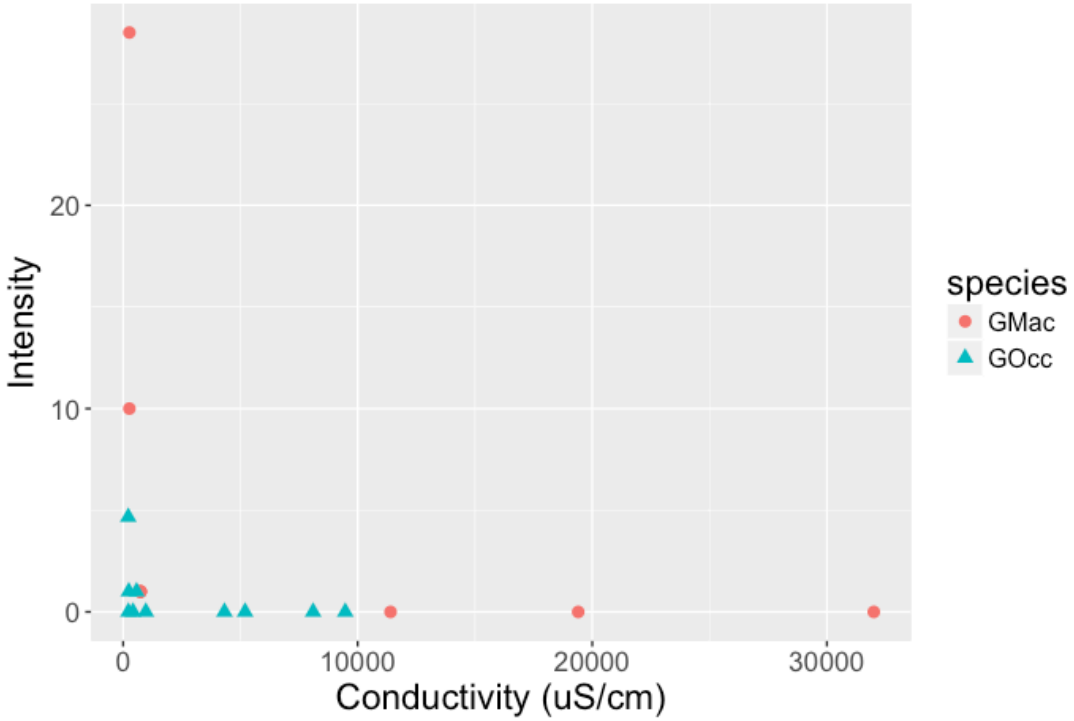

Figure 3.9. Intensity of the parasite Dip01 in *Galaxias* spp. compared to the conductivity (uS/cm) of the sample sites.

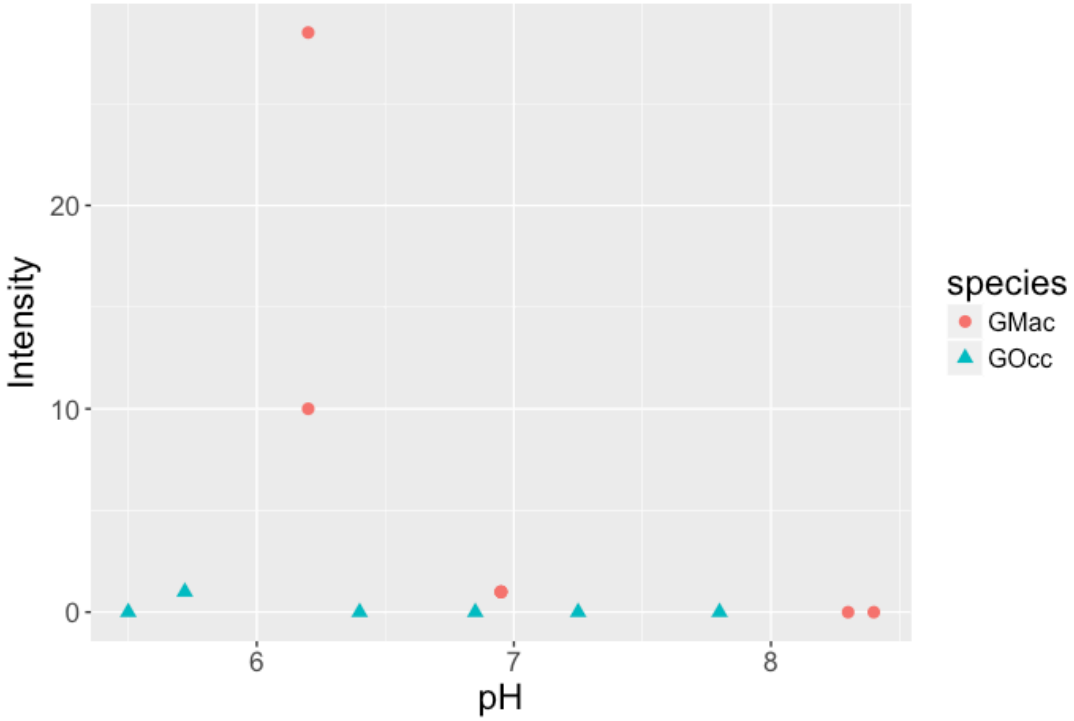

Figure 3.10. Intensity of the parasite Dip01 in *Galaxias* spp. compared to the pH of the sample sites.

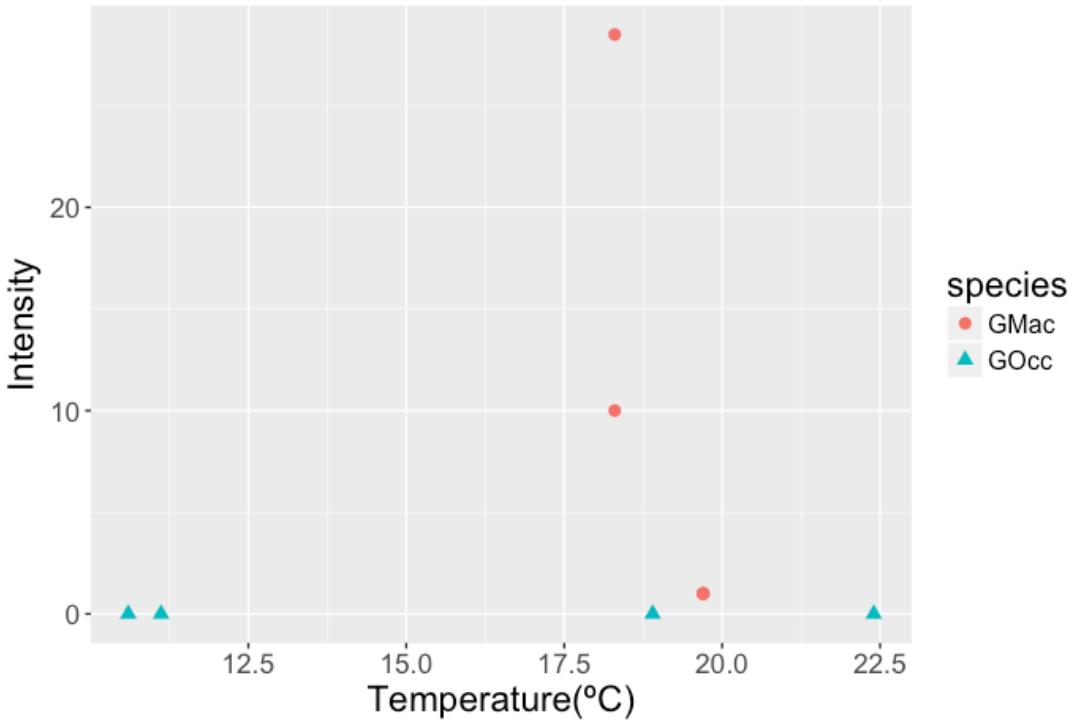

Figure 3.11. Intensity of the parasite Dip01 in *Galaxias* spp. compared to the temperature (°C) of the sample sites.

4. Discussion

Primary findings

This is the first comprehensive study of the parasite causing black spot disease in freshwater fishes in south-western Australia. There were three major findings from this study. First, genetic analysis revealed that while parasite specimens from south-western Australia and Tasmania were genetically identical in rDNA sequence, the parasite (here provisionally named as Dip01) was not closely related to other species of *Diplostomum,* and hence the current nomenclature (*Diplostomum galaxiae*) requires revision. Second, Dip01 appears to preferentially use *Galaxias maculatus* and *Galaxias occidentalis* as second stage intermediate hosts, and the geographic range of the parasite closely matches that of the preferred host species. Third, the prevalence of Dip01 appears to be inversely related to salinity, with the parasite never found in water with conductivity above 1,000 μ S/cm.

4.1 Species identification

A 964bp region of the rRNA gene was used to determine species identity of the black spot parasite. 18S rRNA gene sequences are commonly used in species-level taxonomic studies, and have been recommended for species delimitation in the Diplostomoidea (Moszczynska *et al*., 2009; Brabec *et al*., 2015). Two parasite specimens from south-western Australia, from the Serpentine River and the Goodga River (approximately 400 km apart) were identical in rRNA gene sequence. Although I was not able to obtain samples from the type specimens of *Diplostomum galaxiae*, originally described from *Galaxias auratus* in Lake Crescent, Tasmania (Smith & Hickman, 1983), the Western Australian

specimens were also identical to a specimen of the parasite from *Galaxias truttaceus* in Tasmania. This suggests that the same species of parasite is the causative agent of black spot disease in freshwater fishes throughout southern Australia, although confirmation of this will require much more extensive sampling of host species and geographic areas.

Phylogenetic analysis did not support the current nomenclature of the black spot parasite as *Diplostomum galaxiae*. In fact, the parasite was most closely related to a clade containing species of *Posthodiplostomum*, and members of the Strigeidae family (*Strigea* spp. and *Apharyngostrigea* sp.). Species in all of these genera utilise snails and fish as intermediate hosts and piscivorous birds as definitive hosts (Ondračková *et al*., 2004; Schleppe & Goater, 2004). *Galaxias maculatus* has been found to be infected with a species of *Posthodiplostomum* in South America (Ritossa *et al*., 2013), although the *Posthodiplostomum* sp. in this case was encysted in the abdominal cavity of the fish, rather than the muscle. *Posthodiplostomum minimum*, from white sands pupfish (*Cyprinodon tularosa*) in the USA, has been found to negatively affect fish growth (Stockwell *et al*., 2011). To date, there have been no studies of the potential pathogenic effects of black spot infections in Australian freshwater fishes.

Blasco-Costa & Locke (2017) concluded that the higher systematics of the Diplostomoidea is in need of molecular evaluation and thorough revision. The current study, although limited in scope, reinforces that view. Phylogenetic analysis of reference 18SrRNA gene sequences from GenBank did not find a monophyletic origin for either the Diplostomatidae or Strigeidae. Furthermore,

the genus *Diplostomum* appeared to be paraphyletic, with some species of the genus being more closely related to a species of *Austrodiplostomum.*

4.2 Host preference and geographic range

Dip01, the parasite causing black spot disease, was present with more than one infected host in three species, two galaxiids *G. maculatus* and *G. occidentalis,* and the percichthyid *B. porosa*, and was found on one occasion in the percichthyid *N. balstoni*. It is worth noting that the one record from *N. balstoni,* was from the Boranup area (Turner Brook), and has since been lost from the system (Morgan *et al.,* 2014b). Rashnavadi *et al.* (2014) also reported black spot disease on the atherinid *Leptatherina wallacei* at a prevalence of 0.03, and on a single goby (*Pseudogobius olorum*) in low salinity waters of the Blackwood River.

There was a clear host preference in the current study, with the parasite having a higher prevalence and intensity of infection in the two *Galaxias* species sampled than in *B. porosa* samples. Dip01 was not found on the galaxiids *Galaxiella nigrostriata or Galaxiella munda*, despite a very large number of specimens being examined. One other galaxiid species, *G. truttaceus*, was also assessed, but only 22 specimens were present in the collections, with all sites having 10 or fewer individuals. No black spot infections were found on these fish.

No significant differences were found in either prevalence or intensity of infection between *G. maculatus* and *G. occidentalis*. *Galaxias maculatus* had the highest intensity of infection, with one specimen having 80 metacercariae, with the highest infection intensity in *G. occidentalis* being 28, and in *B. porosa* being

four on one individual. The greatest prevalences were found in *G. occidentalis* in two tributaries of the Hill River, Mundina Creek (prevalence = 100%) and Coomaloo Creek (prevalence = 67%). These were also the only sites where *B. porosa* was infected. It is possible that the abundance of infective cercariae was so great in this area that non-preferred hosts, such as *B. porosa,* were infected due to a spill back effect, a phenomenon whereby transmission to all hosts is increased when one host species amplifies the parasite population (Ondračková *et al*., 2015).

The only other site where the parasite was found and which had multiple fish species present was the Serpentine River. This site also had the second highest prevalence of the parasite, with 94% of *G. occidentalis* present infected. The other fish species present, the invasive mosquitofish, *Gambusia affinis* did not have any infection of the parasite, unlike *B. porosa* from the Hill River tributaries samples. Rashnavadi *et al.* (2014) also reported *G. affinis* to be free of black spot disease in their study of fish in the Blackwood River. This may indicate that *G. affinis* is not a suitable host for Dip01. Invasive species, if they are not competent hosts for native parasite species, can cause a dilution effect where they reduce the overall parasite load in an ecosystem (Gendron & Marcogliese, 2016).

Although trematode metacercariae have traditionally been regarded as host generalists, molecular studies, particularly in the Diplostomatidae, are now showing that they often have a narrow range of potential second intermediate hosts, some to only a single species (Blasco-Costa & Locke, 2017). Cercariae have shown to have the ability to preferentially choose the more susceptible

hosts when presented with multiple potential host species (Sears et al., 2012). The host preference of Dip01 for *G. maculatus* and *G. occidentalis* could be due to a number of (not mutually exclusive) reasons, such as overlapping habitat preferences, ease of penetration through the skin of the host or the feeding preference of the definitive hosts *Galaxias maculatus* and *G. occidentalis* are primarily insectivorous and spend most of their time in the water column (Pen & Potter, 1991; Becker & Laurenson, 2007). This may make them particularly susceptible to infection by free-swimming cercariae, although it does not explain why other pelagic species, such as *Nannoperca vittata* or *Gambusia holbrooki* were not infected.

The skin of a fish is the first stage of defense against parasite infection (Micallef *et al*., 2012), and as *G. maculatus* and *G. occidentalis* are both scaleless, their lack of scales could allow for easier access for trematode cercariae to burrow under the skin and encyst. This does not explain why *G. munda* or *G. nigrostriata* were not infected, as they are also scaleless, but that could be due to other factors, such as that these fish are almost exclusively restricted to specific habitats (e.g. ephemeral environments for *G. nigrostriata* or headwater streams for *G. munda*) (Morgan *et al*., 1998; Ogston *et al*., 2016). In the case of *G. nigrostriata*, this species also undergoes an annual aestivation, which is unlikely to support metacercariae. The plotosid catfish *Tandanus bostocki* is also scaleless but was not investigated in this study, Hassan (2008) reported four putative trematode species from fish in the Blackwood River, but did not detect '*Diplostomum galaxiae*'. The fish that Hassan (2008) examined were from the salinised main channel of the river, which may not be conducive to the survival of

metacercariae (see below and also Rashnavadi *et al.*, 2010). Other populations of *T. bostocki* in low salinity catchments should be examined in the future to determine if it is a suitable host for Dip01.

As some trematodes are able to selectively infect intermediate hosts, it is possible that the parasite Dip01 has adapted to infect fish that are part of the diet of piscivorous bird hosts. Gwiazda & Amirowicz (2006) found that grey herons (*Ardea cinera*) spent most of their time hunting in areas with higher turbidity, lower fish abundance but larger fish body size, with fish larger than the median prey length constituting 51.9% of the total diet. The three species that were found to be infected in the current study, *G. maculatus, G. occidentalis* and *B. porosa,* all reach 15-19 cm, which is substantially larger than many of the other freshwater fish species found in south-western Australia.

Dip01 was found throughout the ranges of its preferred second intermediate host species, *G. maculatus* and *G. occidentalis,* which span the entire Southwestern Province, although these two species are rarely encountered in sympatry (Morgan *et al*., 2006). While the parasite Dip01 was found over a wide geographic range in the Southwestern Province, from Cape Le Grand in the south-east of state, to the Hill River north of Perth, further exploration into the range is required within the Pilbara Province and also into eastern Australia.

The distribution of Dip01 would almost certainly be critically linked to the presence of the first intermediate host, and the terminal host just as much as the fish secondary intermediate hosts. Most trematodes can only infect one mollusc genus, potentially even one species (Lockyer *et al*., 2004). While not assessed in

this study, specificity for the first intermediate host has the potential to dramatically limit the distribution of the parasite, although trematode parasites can influence host distributions to cater for their transmission (Curtis, 2007). The distribution and habitat preference of the bird definitive host would also affect parasite distribution, as it would govern the dispersal and recruitment of the larval stages of a trematode into the ecosystems containing the intermediate host populations (Hechinger & Lafferty, 2005). This would exert some control on the parasite's population, and would potentially explain the distribution of the parasite, as without additional recruitment into a system it would not be able to survive, especially as the two main intermediate fish host species *G. maculatus* and *G. occidentalis,* usually do not live longer than a few years (Pen & Potter, 1991; Chapman *et al*., 2006b).

4.3 Correlation with environmental factors

The prevalence of the black spot parasite Dip01 was significantly inversely related to conductivity, and there was also a non-significant inverse relationship between conductivity and intensity of infection. The parasite was not found in any sites with mean conductivity greater than 1,000 μS/cm, and a significant difference was found between conductivity where the parasite was present and where it was absent, indicating that salinity limits the distribution of the parasite. The two main second stage intermediate host species, *G. maculatus* and *G. occidentalis*, are relatively salt-tolerant, with both able to tolerate salinities of at least 15 ppt (10,00-20,000 μS/cm, depending on water temperature) (see Morgan *et al*., 2003, 2006; Beatty *et al*., 2011). Furthermore, the parasite was only found on other halo-tolerant species (e.g. the typically estuarine *L. wallacei*

and *P. olorum*) in low salinity sections of the Blackwood River, being absent from secondarily salinised habitats in the catchment (Rashnavadi 2010; Rashnavadi *et al*. 2014). These authors also reported a prevalence of 0.143 for black spot disease in *G. occidentalis* in fresh tributaries, compared to salinised sections of the Blackwood River, and also found a significantly higher prevalence during winter compared to summer. Salinity is therefore likely to be affecting the parasite directly or affecting the first intermediate or definitive hosts.

Salinity in freshwater exudes physiological stress on freshwater organisms if they are not adapted to it and is one of, if not the most important, environmental stressors for freshwater ecosystems, with an overall trend of reduced biodiversity, alteration of community structure and altered ecosystem processes in high salinity systems (Kefford *et al*., 2016). Salinity could adversely affect parasite cercariae when they are transitioning from the snail to fish intermediate host. Activity of cercariae of the trematode *Maritrema novaezealandensis*, for example, is adversely affected by increasing salinity (Studer & Poulin, 2013). Pinder *et al*. (2005) surveyed 230 wetlands in Western Australia, and found that invertebrate diversity decreased as salinity increased from 4.1gL^{-1}, and with halophillic species excluded, from 2.6g l^{-1}. This is supported by the consensus that freshwater snails are usually adversely affected by salinity, typically with the exception of invasive species (Kašovská *et al*., 2014). An increase in salinity can also change the macrophyte populations in a river or lake (Davis *et al*., 2003), and this could alter available food and habitat

for the mollusc first stage host, which would then limit the habitats the parasite could infect.

A review of water bird conservation by Ma *et al*. (2010) details how high salinity can negatively impact birds through dehydration, degrading feathers and reducing waterproofing of feathers, however salinity itself doesn't affect waterbirds as much as it does its chosen food source. Herbivorous waterbirds tend to occupy water bodies with the highest quality vegetation and depending on the environment, that could be in either fresh or salinised systems, and the same is true for piscivorous birds (Ma *et al*. 2010). This indicates that it is likely salinity affects the snail first stage intermediate host of Dip01 more than it affects the definitive bird host, however further analysis will be required to test this assumption.

There was a significant inverse relationship between intensity of infection and water temperature, although biological interpretation of this relationship is constrained by the fact that temperature readings were available from only six sites where the parasite was present. A positive relationship, rather than a negative relationship, might be expected between temperature and infection intensity because warmer water would promote more algae and freshwater plants for the snail intermediate hosts, with temperature being the third highest response variable for algal growth after phosphorous/carbon levels and strong illumination (Wang *et al*., 2016). Rashnavadi (2010) found prevalence to be significantly higher on *G. occidentalis* during summer than winter, whereas Chapman *et al*. (2006), for *G. maculatus* found prevalence to range from 1.72% in summer, to 26.01 and 21.49% in autumn and winter, respectively, which is

similar to 24% prevalence on *G. occidentalis* in autumn in Turner Brook (Morgan *et al.,* 2013). Some herons such as the grey heron (*Ardea cinerea*) have also been shown to have an increase in population numbers in conjunction with warmer winter temperatures (Fasola *et al*., 2010). Without knowing the identity of the first intermediate or definitive hosts for Dip01, however, it is difficult to speculate on how water temperature might affect the parasite.

4.4 Limitations and recommendations for future research

Although molecular analysis of Dip01 suggests that the parasite causing black spot disease is the same species throughout Australia, this is based on only three samples for which 18S rRNA gene sequence data could be obtained. A much more comprehensive study is required, examining parasite specimens from a wider range of sites in Western Australia and eastern Australia, and also obtaining sequences from other gene regions, such as the mitochondrial cytochrome c oxidase I gene (Locke *et al*., 2010). In addition, in the absence of DNA data from the original type specimen of *Diplostomum galaxiae*, it will be necessary to obtain adult parasites, either from naturally infected definitive host or from artificial infections of birds, to compare adult worm morphology with the original description. If the morphology of adult Dip01 is similar to the type specimen of *D. galaxiae*, this will provide justification for a revised nomenclature, removing the species from the genus *Diplostomum*. Morphological analysis of the cercariae and metacercariae would also be required for a complete species description.

One of the biggest limitations of the current study was the use of historical collections and the corresponding database records for environmental data,

rather than field sampling of fishes and environmental variables. While this enabled a much larger range of fish specimens to be examined and therefore provided robust data for parasite prevalence, intensity of infection and host preference, it meant other hosts in the life cycle (molluscs and birds) were not assessed from the sites where fish were sampled, and not every site could be used for all of the analysis, potentially missing out on confirmation of environmental parameters affecting the parasite.

Future studies should build on the findings from this research, by sampling contemporary fish populations to confirm that Dip01 does show a preference for the two galaxiid species, *G. maculatus* and *G. occidentalis*. More detailed collection of water quality and other environmental parameters would enable a multivariate analysis of the species distribution, to see whether it is indeed limited by salinity. *Galaxias maculatus*, which has a wide environmental tolerance, would be an ideal host to sample for such a study.

Fieldwork should also be conducted to determine the lifecycle of Dip01. The entire life cycle needs to be assessed, with infection rates and in the first intermediate hosts (probably snails) and the definitive hosts (piscivorous birds) determined. The bird and snail hosts could have a larger influence on the distribution of the parasite and its sensitivity to environmental parameters, such as salinity. The impact of environmental variables on cercarial shedding from snails should also be assessed as this could explain patterns of distribution and host preference of the parasite.

The most suitable locations to sample for life cycle studies would be Cape Le Grande, Goodga River, Serpentine River and the Hill River's tributaries, Coomaloo and Mundina Creeks. These locations span the range of both *G. maculatus* and *G. occidentalis*, which are rarely sympatric, and the highest prevalence of the parasite, and the tributaries of the Hill River tributaries contained the only non-galaxiid species infected found in this study (excluding the one *N. balstoni* from Turner Brook). Without current environmental data it is difficult to say if the conditions are still potentially suitable for the parasite or its hosts, but they hold the highest potential for finding the parasite again.

4.5 Conclusions

The parasite causing black spot disease in freshwater fish in south-western Australia (provisionally labelled Dip01) appears to be the same species that is found in eastern Australia, but current classification in the genus *Diplostomum* is likely to be incorrect. Dip01 was most commonly associated with the two galaxiid species *G. maculatus* and *G. occidentalis* as intermediate hosts. The parasite was never found in sites with conductivity >1,000 μS/cm, but further testing is required to confirm this association and determine which life cycle stage(s)are adversely affected by salinity. If the parasite Dip01 is limited in its distribution by salinity, it would make a useful bioindicator. The cysts of Dip01 are visible to the naked eye and infected fish have been found across a wide geographic range. Parasite absence may therefore be useful in identifying catchments that are experiencing secondary salinisation.

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6. Appendices:

Appendix 1:

Table of every site sampled with n ≥ 10 fish present with 95% confidence intervals (CI). Note that there were only 9 *Galaxias occidentalis* at the Hill River Mundina Creek junction, however they were collected at the same time with *Bostockia porosa* and therefore was included.

Date	Location	Species	n	Mean prevalence (±95% CI)	Mean intensity (±95% CI)
13/12/06	Angove River	*G. maculatus*	33	0.485 (0.316-0.653)	N/A
13/12/06	Angove River	*G. maculatus*	20	0.450 (0.244-0.680)	N/A
13/12/06	Angove River	*G. maculatus*	72	0.278 (0.186-0.395)	N/A
13/12/06	Angove River	*G. maculatus*	65	0.215 (0.129-0.330)	N/A
N/A	Cape le Grande	*G. maculatus*	24	0.167 (0.059-0.372)	28.5 (5.0-64.2)
3/2/86	Pallinup River	*G. maculatus*	19	0.105 (0.019-0.316)	1
15/4/72	Cape le Grande	*G. maculatus*	104	0.087 (0.044-0.158)	10.0 (5.2-14.8)
4/4/81	Gairdner River	*G. maculatus*	14	0.071 (0.004-0.32)	1
12/7/59	Pallinup River	*G. maculatus*	18	0.056 (0.003-0.271)	1
30/3/81	Pallinup River	*G. maculatus*	14	0.000	0
3/2/86	Pallinup River	*G. maculatus*	116	0.000	0
22/11/76	Pallinup River	*G. maculatus*	25	0.000	0
30/10/85	Bandy Creek	*G. maculatus*	12	0.000	0
12/4/81	Bandy Creek	*G. maculatus*	49	0.000	0
16/1/86	Fitzgerald River	*G. maculatus*	25	0.000	0
1971	Fitzgerald River	*G. maculatus*	31	0.000	0
31/3/81	Bremer River	*G. maculatus*	10	0.000	0
26/1/59	Hill River junction of Coomallo Creek	*G. occidentalis*	16	1.000 (0.792-1.0)	19.81 (16.4-23.1)
9/12/10	Serpentine	*G. occidentalis*	18	0.944	4.9

	Dam LGS			(0.729-0.997)	(3.94-6.23)
6/10/76	Milyeannup Brook	*G. occidentalis*	10	0.800 (0.447-0.963)	7.0 (3-16.9)
24/1/59	Hill River junction of Mundina Creek	*G. occidentalis*	9	0.667 (0.323-0.902)	2.2 (1.3-2.7)
13/3/94	Lake Jasper	*G. occidentalis*	25	0.120 (0.034-0.303)	2.0 (1-2.7)
Dec-94	Blackwood River (Bridgetown)	*G. occidentalis*	19	0.105 (0.019-0.316)	10.0 (1-10.0)
16/3/36	Donnelly River (Pemberton)	*G. occidentalis*	20	0.100 (0.018-0.320)	2.5.0 (2.0-3.0)
13/9/06	Milyeannup Brook	*G. occidentalis*	10	0.100 (0.005-0.447)	1
21/11/76	Lower Marbellup Brook	*G. occidentalis*	10	0.100 (0.005-0.447)	1
30/1/86	Milyeannup Brook	*G. occidentalis*	64	0.094 (0.042-0.913)	1.20 (1-1.50)
13/5/08	Upper catchment Blackwood River	*G. occidentalis*	24	0.083 (0.015-0.267)	1.5 (1-1.5)
11/3/09	Brookfield Estate (Margaret River)	*G. occidentalis*	30	0.067 (0.012-0.213)	1.5 (1-1.5)
14/12/06	Phillips Creek	*G. occidentalis*	20	0.000	0
30/1/86	Shannon River	*G. occidentalis*	30	0.000	0
22/12/10	Shannon River	*G. occidentalis*	23	0.000	0
15/9/92	Shannon River	*G. occidentalis*	19	0.000	0
8/2/10	Hill River	*G. occidentalis*	11	0	0
18/3/86	Northcliffe	*G. occidentalis*	36	0	0
22/2/05	Napier Creek (Kalgan River)	*G. occidentalis*	30	0	0
5/9/58	Armadale (Canning River)	*G. occidentalis*	12	0	0
30/1/86	Luke Samuel	*G. occidentalis*	10	0	0
5/6/09	Below weir at Boddington	*G. occidentalis*	10	0	0
18/8/78	Arrowsmith River	*G. occidentalis*	53	0	0
1978	Capel River	*G. occidentalis*	14	0	0
6/6/09	Hay/Mitchell River	*G. occidentalis*	12	0	0

21/4/81	Tone River	*G. occidentalis*	12	0	0
10/1/86	Gardner River	*G. occidentalis*	21	0	0
28/3/81	Chelgiup Creek	*G. occidentalis*	14	0	0
27/3/81	Willyung creek	*G. occidentalis*	23	0	0
24/4/62	Scott River	*G. occidentalis*	22	0	0
7/4/65	Victoria Resevior (Canning River)	*G. occidentalis*	387	0	0
21/10/14	Hotham Fishway	*G. occidentalis*	12	0	0
18/3/86	Doggerup Lake	*G. occidentalis*	44	0	0
24/5/06	Canning River	*G. occidentalis*	11	0	0
1965	Hill River junction of Coomaloo Creek	*B. porosa*	30	0.6 (0.416-0.764)	1.5 (1.2-1.9)
1964	Hill River junction of Mundina Creek	*B. porosa*	20	0.1 (0.018-0.320)	1
18/2/10	Hill River	*B. porosa*	13	0	0
22/11/1977	Moore River	*B. porosa*	12	0	0
27/03/1981	King River	*B. porosa*	13	0	0
21/04/1981	Tone River	*B. porosa*	10	0	0
23/04/1981	Shannon River	*B. porosa*	11	0	0
29/04/1982	Isolated pool	*B. porosa*	14	0	0
09/01/1986	Boorara Brook	*B. porosa*	30	0	0
17/01/1986	Warren River	*B. porosa*	66	0	0
17/06/1986	Warren River	*B. porosa*	100	0	0
31/01/1985	Lake Samuel vicinity	*B. porosa*	15	0	0
31/01/1986	Shannon River	*B. porosa*	15	0	0
31/01/1986	Shannon River	*B. porosa*	19	0	0
01/02/1986	Windy Harbour Road	*B. porosa*	19	0	0
01/02/1986	Windy Harbour Road	*B. porosa*	29	0	0
01/02/1986	Windy Harbour Road	*B. porosa*	30	0	0
02/02/1986	Weld River	*B. porosa*	198	0	0
00/00/1978	Capel River	*B. porosa*	26	0	0
00/00/1958	Pemberton	*G. australis*	25	0	0
04/02/1986	Goodga Swamp	*G. truttaceus*	10	0	0
02/11/1976	Canebreak Rd	*G. Munda*	22	0	0
03/02/1986	Upper Marbellup	*G. Munda*	10	0	0
31/01/1986	34*143',	*G. Munda*	18	0	0

	116*029’				
06/01/1978	Shannon River	*G. Munda*	22	0	0
02/02/1978	Mt. Frankland	*G. Munda*	10	0	0
23/02/1986	Gardiner River	*G. Munda*	22	0	0
20/03/1977	Frankland River	*G. Munda*	40	0	0
03/02/1978	Paffy Inlet	*G. Munda*	10	0	0
02/02/1986	Inlet River	*G. Munda*	106	0	0
00/02/1978	Quinnup	*G. Munda*	34	0	0
15/02/1982	Northcliffe	*G. Munda*	61	0	0
02/02/1986	34*116’	*G. Munda*	94	0	0
31/01/1986	34*116’	*G. Munda*	127	0	0
29/04/1982	Shannon River	*G. Munda*	94	0	0
24/04/1962	Scott River	*G. Munda*	635	0	0
11/11/1988	Northcliffe	*G. Nigrostriata*	47	0	0
00/04/1989	Northcliffe	*G. Nigrostriata*	28	0	0
06/12/1988	Northcliffe	*G. Nigrostriata*	11	0	0
28/04/1982	Northcliffe	*G. Nigrostriata*	145	0	0
05/01/1989	Northcliffe	*G. Nigrostriata*	29	0	0
10/11/1988	Northcliffe	*G. Nigrostriata*	40	0	0
06/10/1988	Northcliffe	*G. Nigrostriata*	50	0	0
17/01/1986	Northcliffe	*G. Nigrostriata*	521	0	0
31/01/1985	33*116’	*G. Nigrostriata*	23	0	0
16/01/1986	33*116’	*G. Nigrostriata*	13	0	0
09/01/1986	34*116’	*G. Nigrostriata*	11	0	0
17/06/1986	34*116’	*G. Nigrostriata*	30	0	0
01/02/1986	34*116’	*G. Nigrostriata*	136	0	0
05/09/1988	34*116’	*G. Nigrostriata*	62	0	0
08/01/1986	34*116’	*G. Nigrostriata*	87	0	0
01/02/1986	34*116’	*G. Nigrostriata*	410	0	0
17/01/1986	34*116’	*G. Nigrostriata*	522	0	0
30/05/1964	Shannon River	*G. Nigrostriata*	89	0	0
21/11/1976	Upper Marbellup	*G. Nigrostriata*	12	0	0
23/9/05	Wilyabrup	*Nannatherina blastoni*	10	0	0
25/2/93	Deep R (Beard-moore)	*Nannatherina blastoni*	17	0	0
22/12/10	Fly brook	*Nannatherina blastoni*	20	0	0
22/2/86	Weld River	*Nannatherina blastoni*	23	0	0
31/1/86	Shannon River	*Nannatherina blastoni*	15	0	0
16/3/36	Pemberton	*N. Edelia*	100	0	0
20/2/64	Waychinicup River	*N. Edelia*	31	0	0

3/10/59	Shannon River	*N. Edelia*	14	0	0
27/3/81	King River	*N. Edelia*	30	0	0
21/4/81	Tone River	*N. Edelia*	10	0	0
9/1/86	Boorara Brook	*N. Edelia*	23	0	0
17/1/86	Northcliffe	*N. Edelia*	11	0	0
17/6/86	Warren River	*N. Edelia*	28	0	0
18/1/86	Warren River	*N. Edelia*	40	0	0
30/1/86	Milyeanup Brook	*N. Edelia*	29	0	0
31/1/86	Doggerup Creek	*N. Edelia*	11	0	0
31/1/86	Lake Samuel	*N. Edelia*	69	0	0
31/1/86	Shannon River	*N. Edelia*	45	0	0
31/1/86	Shannon River	*N. Edelia*	13	0	0
1/2/86	Northcliffe	*N. Edelia*	27	0	0
1/2/86	Mount Chudalup	*N. Edelia*	13	0	0
1/2/86	Mount Chudalup	*N. Edelia*	90	0	0
2/2/86	Shannon River	*N. Edelia*	98	0	0
2/2/86	Weld River	*N. Edelia*	224	0	0
3/2/86	Upper Marbellup Creek	*N. Edelia*	14	0	0
4/2/86	Goodga Swamp	*N. Edelia*	10	0	0
18/3/86	Doggerup Lake	*N. Edelia*	29	0	0
00/00/1978	Capel River	*N. Edelia*	68	0	0
7/6/09	Meerup River	*N. Edelia*	10	0	0
00/00/1964	Hill River	*P. olorom*	19	0	0
09/12/10	Serpentine Dam	*G. holbrooki*	19	0	0
23/11/10	Serpentine Dam	*G. holbrooki*	10	0	0