Argulus infections in fisheries: status, control, and future prospects

Rhi Hunt

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A thesis submitted to Cardiff University for the degree of Doctor of Philosophy (PhD) in the School of Biosciences

Declaration

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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Collaborations

The Natural Environment Research Council (NERC GW4+ Doctoral Training Partnership) funded this PhD with the UK Environment Agency as a CASE partner (Collaborative Awards in Science and Engineering).

Chapter 2: Conducted in collaboration with the Environment Agency Brampton fish lab (Chris Williams, Amy Reading, Yasmin Sik-Harris and Connor Harvey), questionnaire was composed with input from Rhi Hunt and Jo Cable after which the Environment Agency handled delivery of the survey and collection of answers.

Chapter 3: Alex Titimeaua provided technical assistance during collection of the parasite growth data.

Chapter 4: Dr. Amy Ellison collaborated on this study and was involved in experimental design and analysis. Alex Titimeaua and Danielle Hillberg provided technical assistance during collection of circadian behaviour data.

Chapter 5: Dr. Amy Ellison collaborated on this study and was involved in experimental design, data collection and analysis. The Genome Hub at Cardiff University provided advice and conducted the library prep and sequencing.

Chapter 6: Conducted in collaboration with the Environment Agency Brampton fish lab (Chris Williams, Amy Reading, Yasmin Sik-Harris and Connor Harvey) with data collected by the Environment Agency.

Chapter 7: *Argulus* spp. samples were collected and provided by the Environment Agency Brampton fish lab (Chris Williams, Amy Reading, Yasmin Sik-Harris and Connor Harvey). Dr Peter Olson from the Natural History Museum assisted in obtaining CT scans of *Argulus* samples with Brett Clark preforming the scans. Dr. Amy Ellison offered advice regarding molecular work.

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"We understand the lights above the Arby's. We understand so much. But the sky behind those lights, mostly void, partially stars, that sky reminds us: We don't understand even more."

– Joseph Fink

Thesis Abstract

Among species farmed for consumption and trade, parasite epidemics are common due to the intense, often stressful conditions required to maximise production. For aquatic culture, parasite infections are often uncontrollable due to a lack of information and research on both parasites and hosts. Fish lice, ectoparasitic crustaceans, cause severe economic loss to industry each year through physical damage to hosts and subsequent secondary infections. Marine sea lice are notorious in salmon farming and have been the subject of intense research over past decades. Conversely, freshwater lice (Genus *Argulus*) have been relatively ignored. Here, the problems arising from *Argulus* spp. infections were investigated with the aim of reducing the economic and health impacts of these parasites. A survey of trout fisheries to ascertain the current state of UK *Argulus* spp. populations highlighted numerous variables associated with problematic infections and exposed a deficit in effective *Argulus* spp. control measures. Infection dynamics were then assessed with temperature, host species and infection density all playing a key role in *Argulus* spp. life histories and generation time. New and novel monitoring and control techniques were evaluated to help tackle infections. Light-baited traps and timed chemical dosage based on circadian rhythms have potential, while egg-laying substrate traps removed millions of *Argulus* spp. eggs from fisheries to reduce the next generation population size. Finally, a new and invading non-native *Argulus* species was discovered in UK fisheries and described here morphologically and genetically to facilitate future monitoring of this parasite and improve species identification. *Argulus* spp. will continue to negatively impact fisheries and aquaculture into the future, however the data and information provided here will help develop integrated management systems to improve fish health worldwide.

Chapter 1

General Introduction

1.1 Aquaculture and Fisheries

Fish are the most diverse group of vertebrates and are fundamental to life on earth. Approximately 71% of the earth's surface is covered by water (96.5% saltwater, 3.5% freshwater; Gleick 1993; Eakins and Sharman 2010) with 34,800 species of fish currently described (Froese and Pauly 2022). Fish span the trophic levels of food webs, regulating species interactions, population dynamics and nutrient availability/transfer across spatial boundaries (Holmlund and Hammer 1999). Fish also impact key environmental processes including sedimentation and bioturbation through foraging/burrowing behaviours and the carbon cycle via modulation of carbon fixers (Schindler *et al.* 1997; Adámek and Maršálek 2013).

Fish play a critical role in the economy primarily through food trade but also for activities, such as ornamental pet husbandry and recreational fishing (Tidwell and Allan 2001; Monticini 2010; Arlinghaus *et al.* 2015). Fish are an important source of protein globally and the most efficient meat produce available (salmon 2.6 times more efficient than poultry; Gjedrem *et al.* 2012), with global fish consumption growing at a higher rate than all other animal derived protein (FAO 2020). The ornamental pet trade and recreational fishing both provide important social and well-being aspects (Langfield and James 2009; McManus *et al.* 2011), with industry crucial in supporting rural economies (Moreau and Coomes 2007; Monticini 2010; Curtis *et al.* 2017). While all of these industries and fishery types are discussed throughout the thesis, food aquaculture and managed angling fisheries are the main focus throughout.

Food fish are collected via two main methodologies: wild-caught (fishing) and aquaculture. Production from marine and inland fishing has remained relatively stable over the past 20 years, with significant concerns globally regarding overfishing (Cunningham 2005, FAO 2020). Aquaculture involves raising fish in containment (fish farming) and can be conducted either in the natural environment using cages and pens positioned in water bodies (marine or freshwater), or in terrestrial/indoor constructions comprising of static or recirculating tank systems. Cage and pen systems are subject to natural environmental conditions (daily/seasonal patterns and climate change impacts), while indoor systems can be fully controlled with parameters often

set to maximise production (longer light periods, optimum temperatures, etc.). Compared to fishing, aquaculture has seen exponential growth with a 25% increase in production over the past 20 years and is considered a sustainable alternative to fishing and the future of fish production (FAO 2020). An estimated 59.51 million people worldwide in 2018 were involved in fisheries and aquaculture, with global production totalling 178.5 million tonnes of fish and other aquatic organisms for an estimated total first sale value of £295.6 billion (FAO 2020). Finfish dominate the market, accounting for 85% of total marine capture production and 66% of aquaculture production (FAO 2020). Anchoveta (*Engraulis ringens*) were the dominant species collected via marine fish capture, with grass carp (*Ctenopharyngodon idellus*) the most common species produced via aquaculture (FAO 2020). Asia is the highest producer, with China responsible for 35% of global fish production in 2018 (FAO 2020). In the UK for 2020, £3.2 billion in fish and related products were imported (most common species tuna) with £1.6 billion exported (most common species Atlantic salmon; National Statistics 2021; Uberoi *et al.* 2021). The highest weight of fish and fish-related products were imported from China (70.9 kt; National Statistics 2021), with the highest value of fish goods imported from Iceland (£298 million; Uberoi *et al.* 2021). Approximately 11,000 fishers were employed in 2020, with 5,986 full-time equivalent jobs aboard fishing vessels and 17,988 full-time equivalent jobs in associated fish processing (National Statistics 2021; Uberoi *et al.* 2021).

Although a substantially smaller business, ornamental fish are one of the most popular pets globally with over 2 billion live ornamental fish transported worldwide every year (Monticini 2010). The majority of ornamental fish sold are freshwater (90% of fish traded, ~2000 species) with most species raised and bred in aquaculture systems (as used by the food sector). This is contrasted by marine species which remain largely wild-caught (Monticini 2010; King 2019). In the UK ornamental fish are broadly categorised into tropical freshwater, cold freshwater and marine, with the most common tropical freshwater species sold belonging to the tetra group (25%), goldfish dominating cold freshwater sales (89%) and damselfish leading marine fish sales (20%; OATA 2021). Approximately 2000 pet shops across the UK sell ornamental fish with the industry worth £400 million per annum (King 2019; OATA 2021). Due to the nature of ornamental trade involving transport of live fish (compared to processed fish within the food trade), biosecurity is a constant issue with imports facilitating the spread of invasive species (Gippet and Bertelsmeier 2021). This is only compounded by a lack of identification and information regarding fish species and associated organisms (Smith *et al.* 2008).

Angling fisheries operate differently to food and ornamental fish aquaculture, with fish in natural settings caught by customers for recreational purposes. Angling can be conducted in completely wild/natural areas (e.g. rivers, ocean) or in managed water bodies (usually lakes) which are purposefully curated for fishing. Management of angling fisheries varies greatly between sites and can involve removal/addition of plants and animals (including stocking of target fish species for angling), structure building (e.g. pontoons, fish refuges), silt/algae control and supplemental fish feeding (IFM 2021). Anglers generate approximately £38.7 billion annually from retail sales in the USA (Southwick Associates 2020) with marine fishing alone accountable for £5 billion annually in expenditures across Europe (Hyder *et al.* 2018). Total economic value of marine fishing in Europe is estimated at £8.9 billion, with 100,000 jobs supported by the industry (Hyder *et al.* 2017). In the UK, anglers generated £1.7 billion in economic benefits with 835,000 freshwater anglers in 2019 (Environment Agency 2020a; Marsh 2021). Freshwaters are the main source of fishing licence sales with 97% of UK rod licence sales in 2018/2019 for non-migratory trout and coarse fish, a total value of £21.3 million (Environment Agency 2020a). Around 5000 - 6000 jobs in the UK are sustained by trout fisheries alone (Taylor *et al.* 2005a; Taylor *et al.* 2005b), with angling for introduced rainbow trout more popular than native brown trout (1.6 million vs 1.1 million days fished in 2015 respectively; Salado and Vencovska 2018). Angling fisheries are also linked to aquaculture, with many fisheries purchasing fish produced in culture for stocking (Taylor *et al.* 2006; Arlinghaus and Cooke 2009).

1.2 Problematic Aquatic Parasites

Aquaculture and cage systems are the future of food and pet fish production, with industry moving towards these approaches over wild caught fish (Diana 2009). These systems facilitate intensification of production, however such increase comes at a cost to fish welfare (Gabriel and Akinrotimi 2011). Increases in fish density, changes to natural light regimes and lack of environmental enrichment all lead to a rise in fish stress (Montero *et al.* 1999; Owen *et al.* 2010; Zhang *et al.* 2021). This, alongside a tendency for recirculating systems and monoculture (which aid spread of infection, Stewart 1991; Yanong 2003) translates to an increase in parasites and associated disease (Bondad-Reantaso *et al.* 2005). While angling fisheries are less intensive environments compared to aquaculture, fish are still exposed to multiple stressors and can be stocked to unnatural levels, creating an ideal environment for infections (Lewin *et al.* 2006). The process of angling (i.e. catching fish with rod/hook and use of a landing net) can

also stress and physically damage fish (Margenau 2007; Danylchuk *et al.* 2014; Lizée *et al.* 2018), facilitating infection (*Saprolegnia* spp. infection post netting, see Barthel *et al.* 2003).

Management of parasites and disease in aquatic systems is lacking compared to terrestrial species (Burka *et al.* 1997), with diagnosis and treatment difficult due to challenges in screening organisms and identifying pathogens (Li *et al.* 2002; Mitchell and Tully 2016). There is often a lag phase of one year between a major mortality event and pathogen identification/management (FAO 2020). Treatment is also limited, with many chemical options banned as a result of environmental toxicity and impact (Costello *et al.* 2001). As such, many parasites have persisted in aquatic systems and remain a constant problem. Estimation of global economic loss from parasites is difficult due to a lack of reporting, however calculations by Shinn *et al.* (2015) show an extreme range in losses, ranging from tens to millions of pounds per infection event. Some of the largest losses include £0.7 - £1.3 million from monogenean *Neobenedenia* spp. (marine/brackish) with 10 - 40% stock mortalities (Lopez *et al.* 2002; Ogawa *et al.* 2006; Shinn *et al.* 2015), £1.6 million from scuticociliate *Uronema nigricans* (marine, cause of swimmers disease) with only 4% mortalities but high value of fish (Deveney *et al.* 2005; Shinn *et al.* 2015) and £5.1 - £12.8 million from dinoflagellate *Amyloodinium ocellatum* (marine/brackish) with 20 - 50% mortalities and 100% parasite prevalence during peak months (Kuperman and Matey 1999; Shinn *et al.* 2015). Within the UK, annual losses for marine and freshwater aquaculture from parasites have been estimated at 5.8 - 16.5% of total production value (although this is noted to likely be an underestimate) with loss largely attributed to both amoebic gill disease impacts (caused by *Neoparamoeba perurans*) and management of sea lice infections (both marine, Shinn *et al.* 2015).

Ectoparasitic fish lice are perhaps the most notorious parasite in the fish trade, with infections a source of severe economic loss despite over a century of study (Wilson 1902; Costello 2009). There are two main types of fish lice: marine sea lice (primarily Genera *Lepeophtheirus* and *Caligus*) and freshwater lice (Genus *Argulus*). Sea lice mainly impact aquaculture production of food fish and have been the focus of the majority of studies due to the high value of marine fish species. Freshwater *Argulus* spp. infections, however, cause problems across aquaculture of food/ornamental fish and within angling fisheries (Mirzaei and Khovand 2015; Aalberg *et al.* 2016; Taylor *et al.* 2005b). Global costs of sea lice have been estimated at £260 million per year, with price of management and control the highest contributor to overall costs (Costello 2009). Mixed infection of *Caligus* spp. lice and blood fluke *Cardicola forsteri* resulted in losses

of £1 million over 12 weeks in ranched tuna (*Thunnus maccoyii*), with peak lice numbers of >265 lice per fish (Hayward *et al.* 2010; Shinn *et al.* 2015). Fewer estimates are available for losses from *Argulus* spp. infections due to the lack of research but in India (where *Argulus* spp. are the most common parasite in freshwater fish farms, Mishra *et al.* 2017) and Bangladesh, costs were calculated to be £293 - £313 per hectare of carp culture per year (Monir *et al.* 2015; Sahoo *et al.* 2013a). Carp farming in India utilises 383,000 hectares of land (Kunguma Kannika *et al.* 2019), which gives an estimated loss of £112 million per year using the costings from Sahoo *et al.* (2013a). This is an overestimate as this land use value includes all aspects of carp farming and thus does not translate exactly to hectares of carp culture, but it gives an indication of the value lost from *Argulus* spp. infections.

This thesis focuses on *Argulus* spp. and as such this genus alone is described below, however many concepts applied to freshwater lice can translate to marine lice or other aquatic parasites, with comments on this in Chapter 8.

1.3 Review of *Argulus* spp.

Argulus spp. (Crustacea: Branchiura) are found globally with 158 species currently described (Walter and Boxshall 2021), although due to the scarcity of genetic data it is likely that some species are synonymous with each other, and others have yet to be described. General anatomy can be observed in figure 1.1, males and females are easily distinguished via the presence/absence of testes, the presence of leg claspers in males, and the presence of eggs in gravid females. There are three established species of *Argulus* in the UK, native *A. foliaceus* and *A. coregoni* and the invasive *A. japonicus* (figure 1.2). *A. foliaceus* is the most common species, found throughout the temperate regions of the world (figure 1.3), as such this species is the focus throughout this thesis. *A. japonicus* is native to Asia but has been introduced worldwide (Rushton-Mellor 1992), with *A. coregoni* more common in Scandinavia (Møller 2006; Hakalahti *et al.* 2008).

Diagnosis of *Argulus* spp. is mainly achieved through visual observation of adults attached to hosts, although observation of free-swimming lice in the water may also occur (Møller 2006; Steckler and Yanong 2012). Consideration needs to be taken when capturing fish for examination as rod capture is biased towards healthy fish and *Argulus* spp. can display an aggregated distribution within host populations, thus use of netting methods (e.g. seine fishing) may be more appropriate (Bandilla *et al.* 2005; Taylor *et al.* 2009a). Identification of species

is achieved through morphological examination and comparison to published keys. *A. coregoni* can be identified from *A. foliaceus* and *A. japonicus* by its large size (up to 12 mm for *A. coregoni* versus 4 - 8 mm *A. foliaceus* and *A. japonicus*, Rushton-Mellor and Boxshall 1994; Yildiz and Kumandas 2002; Taylor *et al.* 2005a) and pointed abdominal lobe shape (Fryer 1982). Distinguishing between *A. foliaceus* and *A. japonicus* is more difficult as the lobe shape difference is less defined (rounded for *A. foliaceus* versus slightly pointed for *A. japonicus*), thus consideration of male breeding appendages on the swimming legs via microscope examination is recommended (Fryer 1982). A clear overview of the morphological differences between the established UK species (and a newly invading *Argulus* species) is available in Chapter 7.

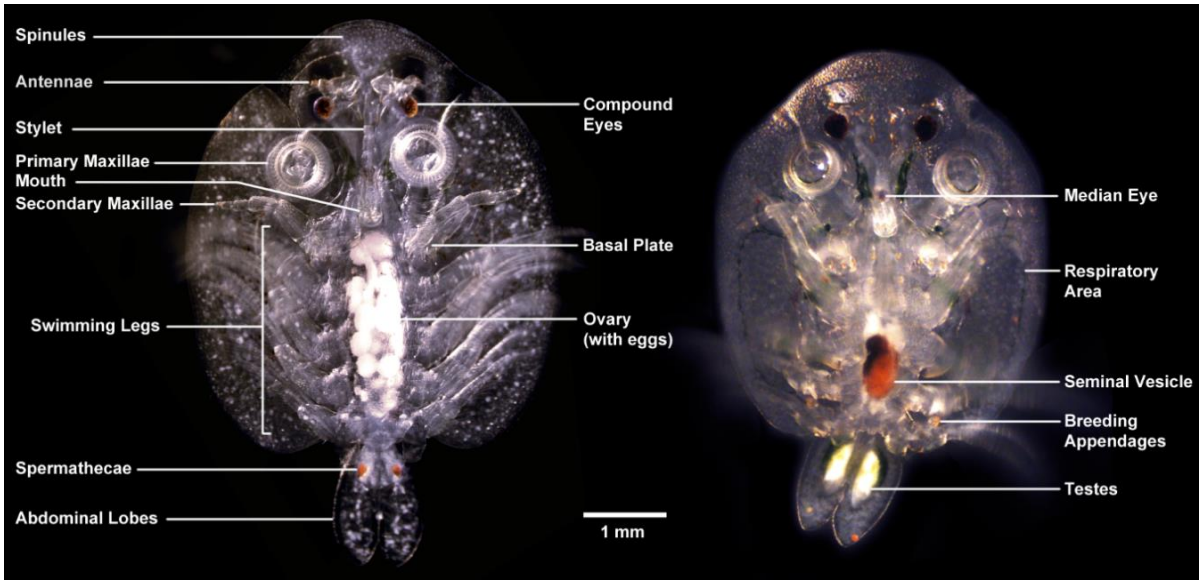

Figure 1.1 Labelled general anatomy of *Argulus* spp. female (left) and male (right). Images taken of live *Argulus foliaceus* using a dissecting microscope at x10.

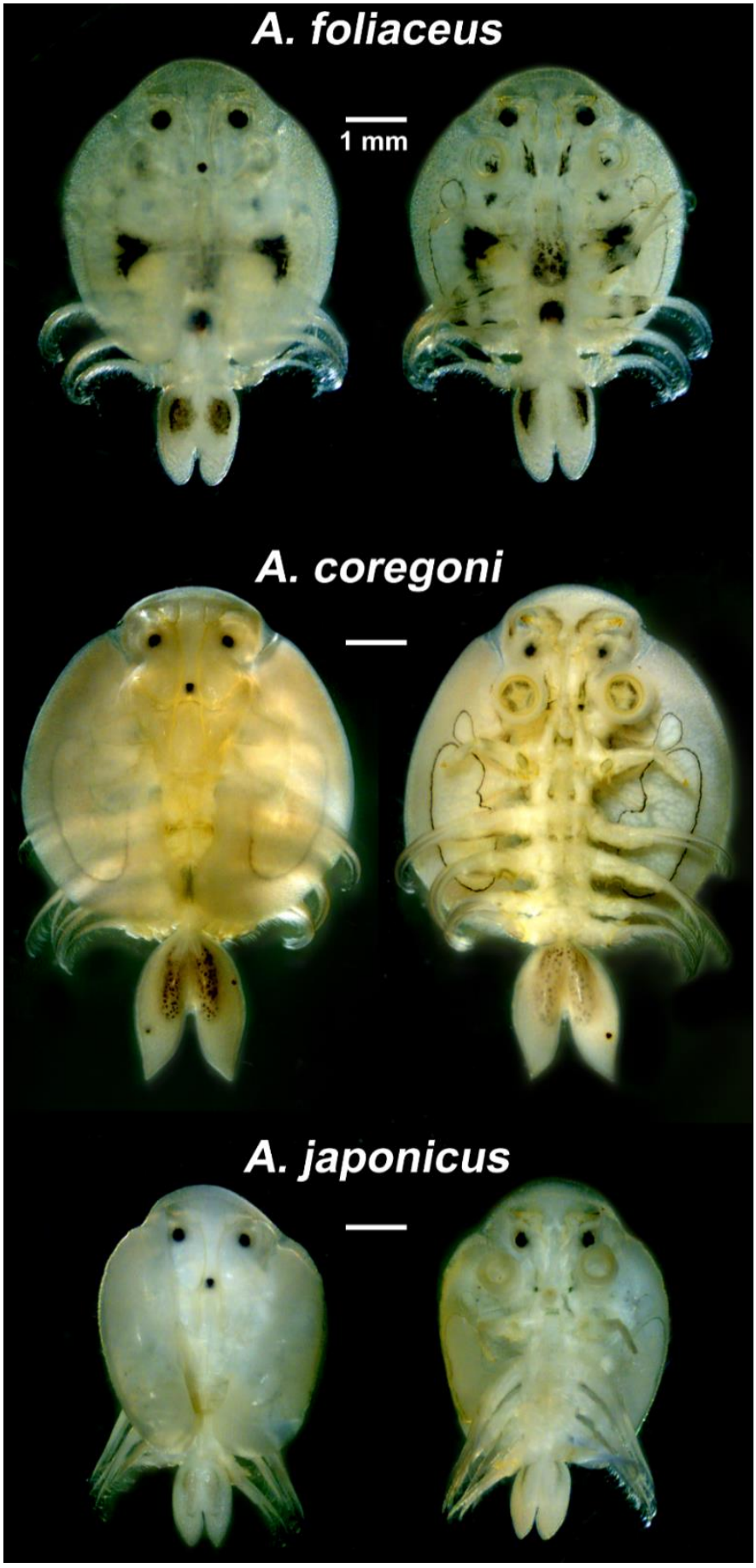

Figure 1.2 Dorsal and ventral views of male specimens of all three established UK *Argulus* species. Images taken of unstained specimens preserved in >90% ethanol at x10 using a dissecting microscope. All scale bars equal 1 mm.

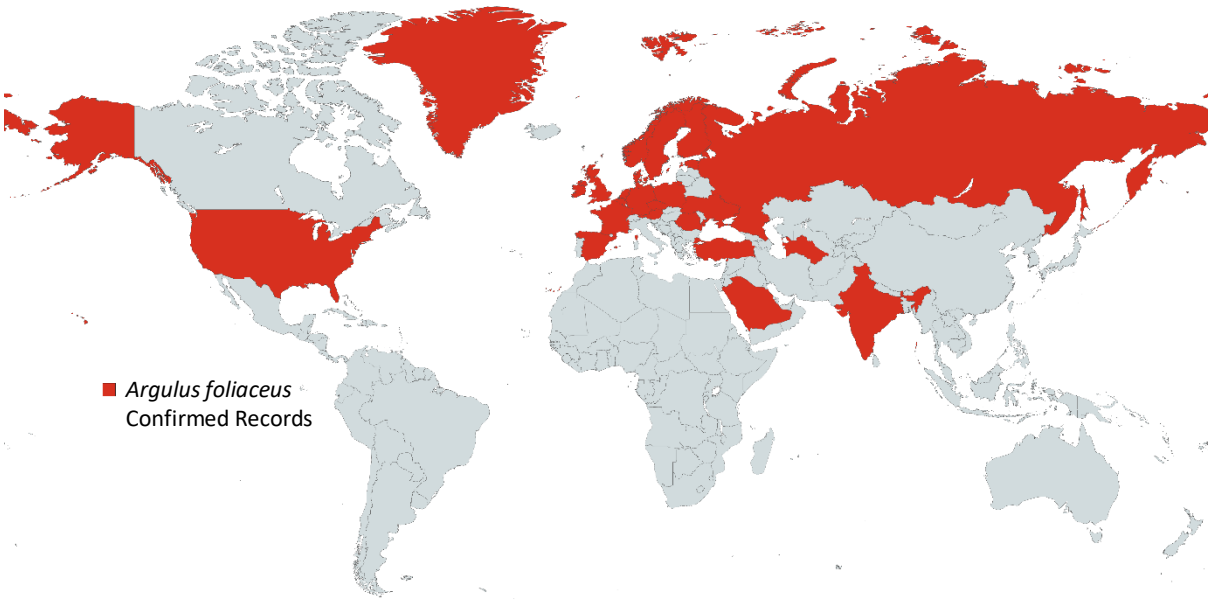

Figure 1.3 Worldwide distribution of *Argulus foliaceus*, countries coloured in red have confirmed records from https://www.gbif.org/ and literature. Crated using https://mapchart.net/.

Argulus spp. have a direct life cycle, requiring only fish (or for some *Argulus* species, amphibians) as a host for survival (figure 1.4; Walker *et al.* 2004). Despite this, *Argulus* spp. may switch hosts throughout their life in search of new hosts or mating opportunities (Bandilla *et al.* 2008). Mating is instigated by the male, may occur on or off host and lasts from 30 - 180 min (*A. japonicus*; see Avenant-Oldewage and Everts 2010). The male attaches to the dorsal carapace of the female and extrudes a spermatophore from the fourth swimming leg, which transfers sperm to the female spermathecae via spermathecal spines (Avenant-Oldewage and Swanepoel 1993; Avenant-Oldewage and Everts 2010). Gravid females then leave their host to lay eggs on a suitable hard substrate. Natural mortality or predation can occur during laying; however females may survive, return to a host, and lay multiple clutches of eggs over their lifetime (Hakalahti *et al.* 2004a).

For egg laying, *Argulus* spp. have shown a preference for dark coloured plastic or wood (Gault *et al.* 2002; Hakalahti *et al.* 2004b) although eggs have been recorded on a wide range of substrates including dead fish, snail shells, crayfish, plants, stones, and boats (Sahoo *et al.* 2013b; Taylor *et al.* 2005a). Eggs are laid in strings (figure 1.5) with the number of individual eggs in each string varying greatly between clutches, individuals, and species (throughout this study the number of eggs per string ranged from 4 - 256 for *A. foliaceus* cultures). Eggs are

attached to surfaces using a gelatinous cement (Hoffman 1977), strings can be collected off substrates using a sharp implement such as a scalpel with eggs remaining viable after removal (Stewart *et al.* 2017). Development of eggs is significantly affected by temperature and illumination (Shimura and Egusa 1980; Bai 1981; Hakalahti *et al.* 2004a; Sahoo *et al.* 2013b; Chapter 3). Metanauplii stage juveniles hatch from eggs and are immediately infective (figure 1.5), juveniles transition through several developmental stages to maturity dependent on species. *A. foliaceus* development consists of 9 - 11 juvenile stages (Rushton-Mellor and Boxshall 1994), *A. japonicus* 7 stages (Tokioka 1936) and *A. coregoni* 9 stages (Shimura 1981).

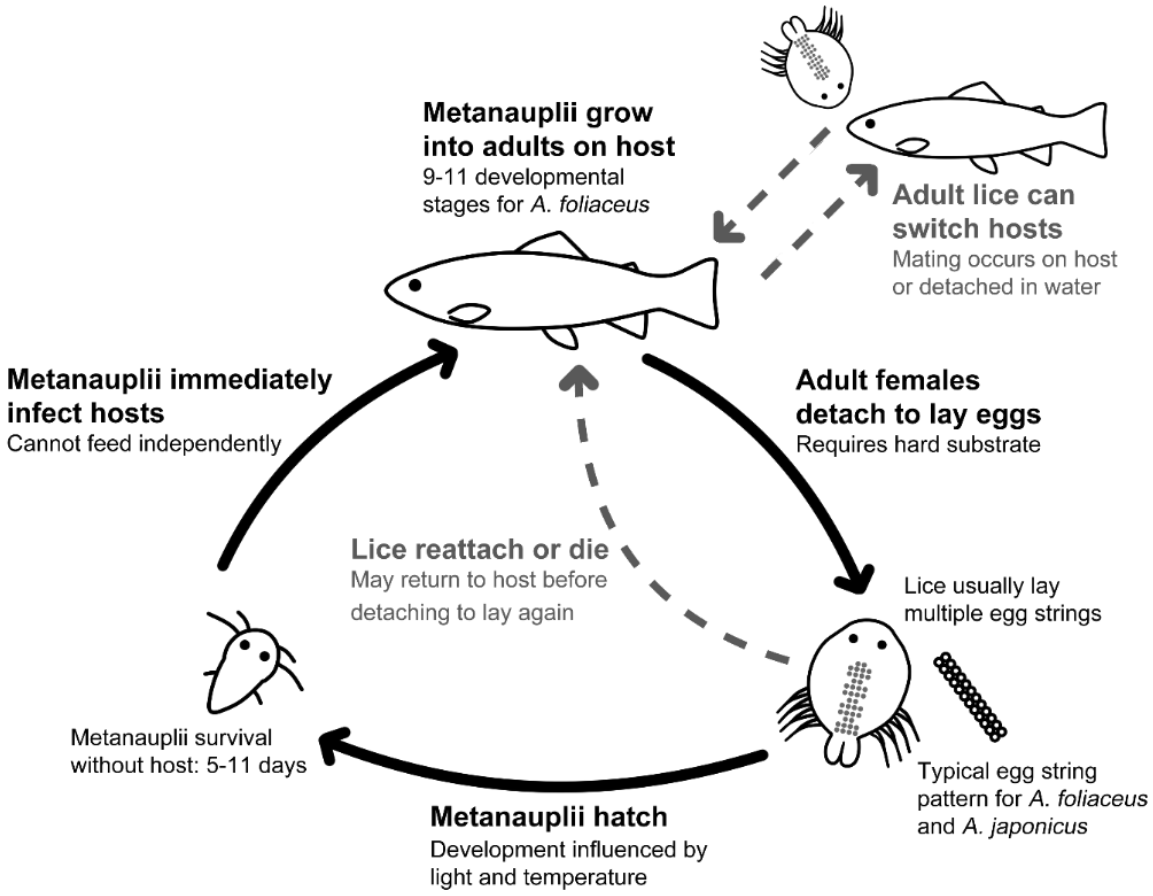

Figure 1.4 Simplified diagram of *Argulus* spp. general life cycle. Adult females can also attach to new hosts following egg laying. Created with reference to Rushton-Mellor and Boxshall 1994; Walker *et al.* 2004; Walker *et al.* 2011a.

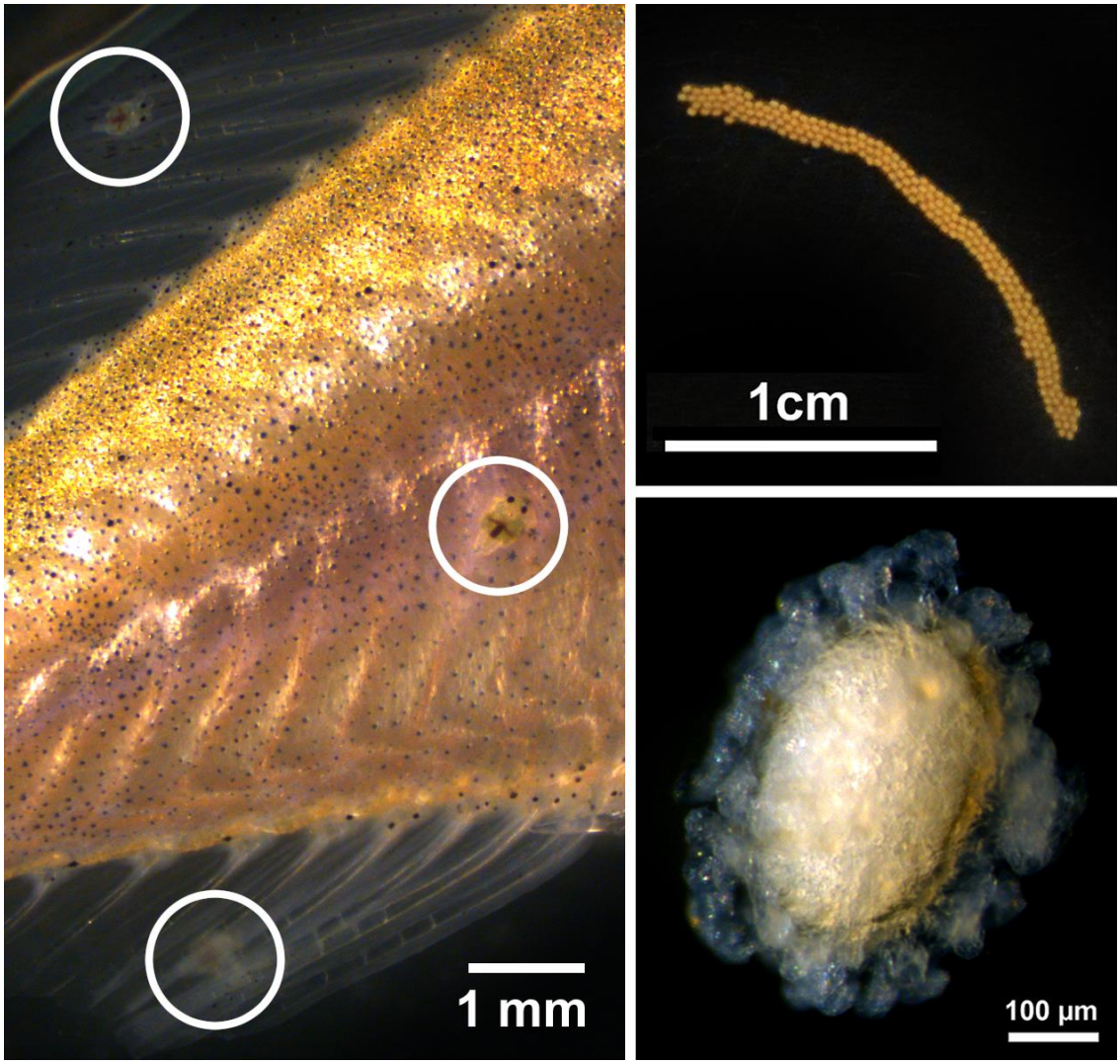

Figure 1.5 Newly hatched *Argulus foliaceus* metanuaplii on three-spined stickleback *Gasterosteus aculeatus* host (left), highlighted with white circles. *A. foliaceus* egg string (top right) and individual egg surrounded in gelatinous cement (bottom right). All images taken using a dissecting microscope.

Argulus spp. populations are seasonal when subject to natural environmental conditions (Shimura 1983; Hakalahti *et al.* 2004b; Harrison *et al.* 2006), with egg laying and development halted below 10 °C (Shafir and van As 1986; Mikheev *et al.* 2001). In UK fisheries, overwintered eggs hatch in spring, with peaks in population over summer and die off over winter (Gault *et al.* 2002; Harrison *et al.* 2006; Taylor *et al.* 2009b). *Argulus* spp. can be easily cultured in the lab where stable temperatures and access to mates allows constant and consistent reproduction (Stewart *et al.* 2017), although survival tends to be poor due to predation by the host. For *A. bengalensis* a maximum survival of 80 days was recorded in culture (Guha *et al.* 2013), while here a maximum survival of 278 days was recorded for *A. foliaceus* in culture.

Host specificity varies across species, *A. foliaceus* and *A. japonicus* are both considered generalists while *A. coregoni* shows a preference for salmonids (Pasternak *et al.* 2004; Walker *et al.* 2004; Bandilla *et al.* 2008). Transmission is linked to vision - *Argulus* spp. possess a pair of circular, darkly pigmented compound eyes and a singular median, naupliar eye (which likely only detects variation in light intensity; Mikheev *et al.* 1998; Meyer-Rochow *et al.* 2001; Walker *et al.* 2004). Pigment granules in the compound eyes differ in light/dark conditions, and *Argulus* spp. show an extremely strong positive phototaxis response across their life cycle (Meyer-Rochow *et al.* 2001; Bandilla *et al.* 2007; Yoshizawa and Nogami 2008). Mechanical and chemical cues also play a role in host location, but seemingly to a lesser degree or over a shorter distance (Bandilla *et al.* 2007).

Adult *Argulus* spp. attach to hosts primarily through their modified primary maxillae which function like suction discs and can move independently, allowing movement across the host (figure 1.6). Additional "hook-like" secondary maxilla and various spines and spinules then aid attachment (Walker *et al.* 2004). Adult *Argulus* spp. consume host blood; the mouth rasps at the host skin with the stylet injecting secretions. Enzymes from the mouth and stylet play a role in digestion, haemorrhaging and anti-coagulation (Walker *et al.* 2004; Walker *et al.* 2011b; AmbuAli *et al.* 2021). Metanauplii feed primarily on epithelial cells/mucous due to the morphology of their mouth parts preventing consumption of blood (Tam and Avenant-Oldewage 2006).

Infection causes an inflammatory and innate immune response (Forlenza *et al.* 2008; Saurabh *et al.* 2010; Saurabh and Sahoo 2010), with associated host condition loss, reduction in appetite, reduced growth rate and even mortality. Fish also become lethargic and prone to secondary infections due to skin damage caused by louse attachment (Hoffman 1977). Spring viraemia of carp (*Rhabdovirus carpio*) has been shown to be transmitted to uninfected carp via attachment of *A. foliaceus* from infected hosts, although multiplication of the virus does not occur within the louse making them a mechanical vector only (Ahne 1985). Fish may also display energetically costly flashing or jumping behaviours during the early stages of infection (Taylor *et al.* 2005a). It is due to these effects that *Argulus* spp. are problematic within aquaculture and angling fisheries (figure 1.7).

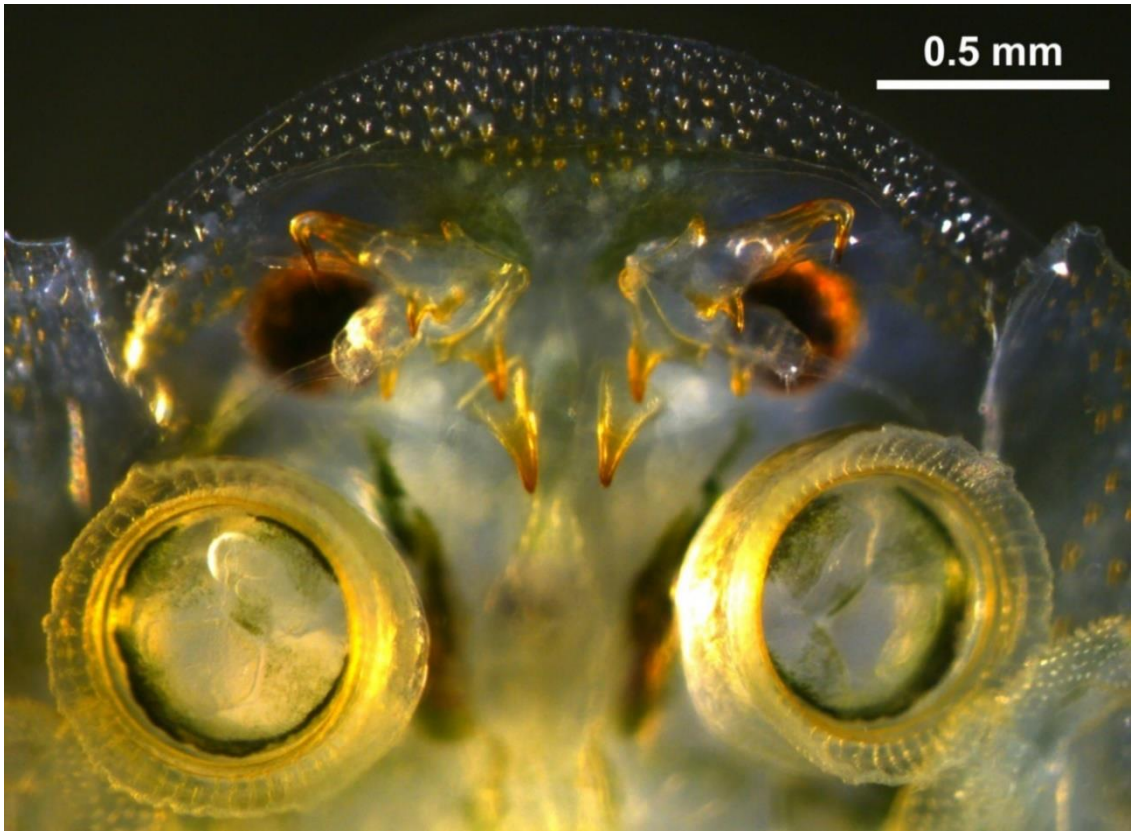

Figure 1.6 Primary maxillae suction discs and various spines and spinules on the carapace and antennae which aid attachment of *Argulus* spp. to their hosts. Image of the ventral side of *Argulus foliaceus*, taken using a dissecting microscope.

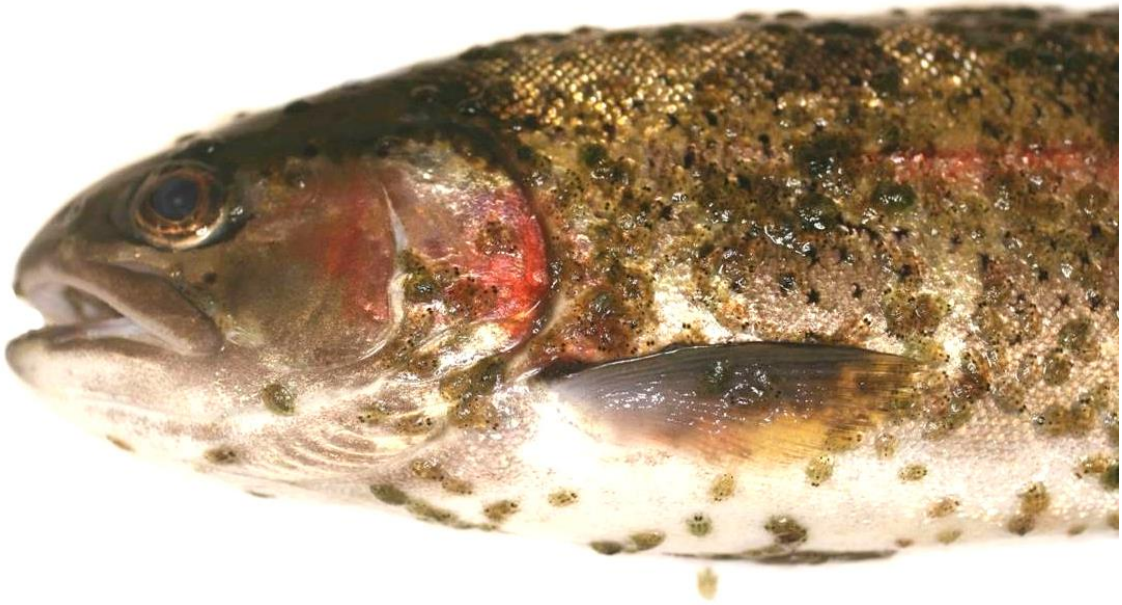

Figure 1.7 Heavily infected rainbow trout *Oncorhynchus mykiss* from a UK recreational fishery infected with *Argulus* spp. *Argulus* adults cover much of the external surface of the fish. Photograph taken by the Environment Agency.

Treatment of *Argulus* spp. infections is mainly achieved through chemical application, using many of the same products used for marine sea lice (Grave *et al.* 2004; Hakalahti *et al.* 2008). Outbreaks in the UK have been facilitated by the limited number of legal chemical treatments (Ernst *et al.* 2001); the organophosphate Diptrex 80 was the most common treatment before it was banned, although some fisheries still admit to its use due to a lack of alternatives (Taylor *et al.* 2005b). SLICE® (an avermectin based treatment) is available but only under veterinary cascade guidelines and fish can only be stocked 500-degree days post treatment, making it unfeasible for many farmers. Chemical treatments can also lead to drug resistance (Aaen *et al.* 2015), as such interest in chemical-free control methods is growing. Management strategies can help prevent infection (McPherson *et al.* 2012), however information is still lacking to fully inform decision making. Draining and desiccation of lakes (followed by a fallow period to ensure surviving lice have no access to hosts) used to be a popular technique, however the manual labour associated and inability to run fisheries during treatment has resulted in decreased use (Taylor *et al.* 2005b). Egg laying boards which aim to collect laid eggs and then remove them to reduce *Argulus* spp. numbers are a new method which have been investigated in a few studies but require further testing (Gault *et al.* 2002; Harrison *et al.* 2006; Taylor *et al.* 2009a).

1.4 Thesis Aims, Objectives and Layout

Due to a lack of research into freshwater lice and rising concerns from UK fisheries regarding infections, this thesis aims to address some of the key issues arising from *Argulus* spp. populations. The UK Environment Agency is a partner in this study, they are a government body tasked with protecting and improving the environment within their jurisdiction of England (Wales previously included, managed by Natural Resources Wales as of 2013). All data chapters within this thesis are written to be self-contained. Chapter 1 introduces the study system and gives a review of the biology of *Argulus* spp. with a focus on the UK established species. The current state of fisheries in the UK is assessed in Chapter 2 via a questionnaire-based study to ascertain the problems encountered by recreational fisheries associated with *Argulus* spp. infections. The future of *Argulus* spp. infections is then investigated in Chapter 3 which determined the impact of temperature and host on *Argulus* spp. life history traits to understand how parasite populations could change and react to shifts in environment or management. Novel control methods are evaluated in Chapters 4, 5 and 6 due to the limited control methods available to fisheries, using lab and field-based experiments. Chapters 4 and 5 explore the potential for light-based control with the topic of circadian rhythms introduced

within these chapters. Chapter 6 adds to previous investigations into egg laying substrate with field observations spanning multiple fisheries and years to assess the viability of this method. An emerging threat to UK fisheries in the form of a non-native *Argulus* species introduction is then described in Chapter 7 alongside appraisal of the genetic database currently available for *Argulus* spp. to aid biosecurity and improve *Argulus* spp. identification. Finally, Chapter 8 discusses the thesis as a whole with key points highlighted regarding *Argulus* spp. infection dynamics, the potential of new control techniques and the future of *Argulus* spp. infections worldwide.

1.5 Ethics Statement

All animal work was conducted under Home Office License PPL 303424. This work was approved by the Cardiff University's Animal Ethics Committee, followed ARRIVE guidelines and conformed to UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

Chapter 2

Argulus infections in UK trout fisheries: current trends revealed by questionnaire data

2.1 Abstract

Fishing is a traditional recreational hobby/sport and has remained a key component of society, providing social and economic benefits. In the UK angling fisheries offer employment and tourism, which are especially important in rural areas and small communities. Parasites are a constant threat to human and animal health, and conditions within fisheries often promote rapid spread of infections, ultimately resulting in monetary losses and closure. Fish lice are large ectoparasites that cause extensive mechanical damage to their hosts and facilitate secondary infections. Fish louse infections (*Argulus* spp.) in UK fisheries were last assessed in 2000, thus a new survey was launched to capture the current status of infections and problems faced by angling fisheries. In total 31.5% of fisheries surveyed faced problematic *Argulus* spp. infections, with high water temperature, stocking, presence of aerators and gravel pit substrates all associated with problematic infections. Economic threat from infections was largely influenced by fish and fishery impacts with fish health/capturability, angler satisfaction and fishery standing highlighted as the most significant factors, indicating that angler experience and approval is key to fisheries remaining economically viable. Infections were chronic (85% fisheries experiencing problems for 2 - 10+ years) with no significant impact from any treatment type on economic threat from infection, indicating that current control methods are inadequate. While changing stocking or careful selection of lakes for fisheries based on features could potentially help with infections, the complete lack of effect from all control methods emphasises the need for new and/or improved control methods against *Argulus* spp.

2.2 Introduction

Fish are a key economic resource and fundamental to life on earth. While fish are primarily caught for trade as a food resource (Tidwell and Allan 2001), recreational hobby and sport fishing remains an important industry both economically and socially (Ditton *et al.* 2002; Environment Agency 2018). The popularity of fishing varies greatly from country to country (for example, angling participation in Norway can reach up to 32% of the population) with a lack of documentation further obfuscating global estimation of anglers and industry output (Arlinghaus and Cooke 2009). Despite this, estimates suggest more than 10% population

participation across the industrialised world (Arlinghaus *et al.* 2015). Recreational fishing can negatively impact natural environments through modification and disturbance of habitats, selective removal of species and subsequent knock-on effects (Cooke and Cowx 2004; Lewin *et al.* 2006), however it can also compliment and support conservation efforts through economic contribution and sustainable use/incorporation into conservation plans (Cowx *et al.* 2010; Tufts *et al.* 2015).

In the UK, recreational fishing is key in supporting rural communities through fishery fees, commercial spending, and tourism, with trout fisheries alone providing 5000 - 6000 jobs (Taylor *et al.* 2005a,b). Fishing rod licence sales through the UK Environment Agency also drives protection of fish and improvement of fisheries, with 940,974 licences sold (totalling £20.9 million) in 2019/2020 (Environment Agency 2020b). Due to narrow profit margins, fisheries are often at risk with any persistent loss of stock, fishery performance or reputation threatening economic viability and potentially leading to closure. As such, the health status of fisheries is carefully monitored with the Environment Agency responding to and investigating cases of dying or dead fish. Incidents of known cause in 2019 - 2020 were mainly due to warm weather/algae (28%), pollution (21%), and fish disease (9%; Environment Agency 2020b). Aquatic parasites and disease remain a persistent problem in fisheries globally, with diagnosis and treatment lacking compared to that of terrestrial systems (Li *et al.* 2002). One of the most visible and persistent parasites in UK fisheries are ectoparasitic freshwater fish lice (Genus *Argulus*). Fish lice infections have been increasing in notoriety, with 29% of English and Welsh fisheries in 2000 experiencing problematic *Argulus* spp. infections (Taylor *et al.* 2006).

Argulus spp. are found throughout the UK although appear more commonly in the south of England and Wales with cases in Scotland scarce, potentially linked to the colder climate (Taylor *et al.* 2005a). Currently three species of *Argulus* are established in UK fisheries: native *Argulus foliaceus* and *A. coregoni* and the invasive *A. japonicus* (see Taylor *et al.* 2005a). While morphologically distinct with nuances in host preference, all three species impact fisheries though external damage to individual host fish. Wild populations are generally not impacted by *Argulus* spp. infections, however conditions within fisheries (high host density, potential monoculture, and anthropogenic stress; Lewin *et al.* 2006) can lead to problematic infections with fish succumbing to mortality from direct parasite damage and subsequent secondary infections (Ahne 1985; Taylor *et al.* 2005b).

Argulus spp. infections in still water trout fisheries were previously assessed 22 years ago via a questionnaire-based approach (Taylor *et al.* 2006). Problematic *Argulus* spp. infections were identified in 29% of fisheries surveyed. Infections were considered an economic threat as *Argulus* spp. reduced fish appeal and catchability, leading to a loss of anglers. Three main risk factors associated with problematic infections were identified: presence of algal blooms, low water clarity and slow fish stock turnover rate. With the last investigation conducted in 2000, the current status of *Argulus* spp. infections in UK fisheries is unknown. Here, in collaboration with the UK Environment Agency, an examination of *Argulus* spp. infections in UK fisheries was conducted to ascertain current status of infections and any change over the past 20 years.

2.3 Methods

Questionnaire design and approach

Between 2018 - 2019, the UK Environment Agency sent a questionnaire to 1000 still water trout fisheries across England to establish the extent of *Argulus* spp. infections and identify factors that might be associated with problematic infections. The questionnaire included two main sections, one on fishery information (water specifications, 11 questions) and the other on management (fish stock and associated practices, 11 questions), followed by a third section for fisheries with *Argulus* problems to ascertain the impact of infection (12 questions). The questionnaire consisted mostly of closed questions, with two open questions on other health problems and a general comments box to capture any additional information not covered elsewhere in the questionnaire. A copy of the blank questionnaire is available at: figshare.com/s/69a6ec7dc93ad9a2d98d

Statistical analysis

A total of 240 responses were received from still water trout fisheries (24% response rate). Three main topics were examined during analyses: (i) the current status of UK still water trout fisheries, (ii) differences between fisheries with and without problematic *Argulus* spp. infections, and (iii) variables that influenced the perceived economic threat of *Argulus* spp. infections.

Any fisheries not fished and/or currently stocked were removed, leaving 225 fisheries for further analysis. For statistical analyses, factors with <5 observations were not included due to lack of statistical strength. Analyses were conducted using R statistical software (v4.0.2; R Core Team 2017) with the level of significance in all tests taken as $p<0.05$. R package "ggplot2"

was additionally used to visualise data (Wickham 2009). Models were refined through stepwise deletion of insignificant terms and Akaike information criterion comparisons, with visual examination of model plots to check standardised residuals for normal distribution and homogeneity of variance (Crawley 2007).

To examine overall trends in still water fisheries, two proportion z-tests were used to ascertain differences in water parameters (type and source) and stocking times (spring, summer, autumn, winter) across all fisheries. Modelling and proportion tests were then conducted for each section of the questionnaire to determine which parameters were more common in fisheries with problematic *Argulus* spp. infections vs fisheries without problems, alongside which variables contributed to the economic threat posed by *Argulus* spp. infections. All models (generalised linear model; GisedLM) used a binomial family and a logit link function. Two GisedLMs were used to analyse the fishery information section. The first model examined if water parameters (maximum depth/temperature and clarity) had an impact on *Argulus* spp. infection status. The second determined if water parameters had an influence on the economic threat of *Argulus* spp. infections. Two proportion z-tests were then conducted to examine differences in water type (gravel pit, chalk, clay lined, reservoir) and source (spring fed, river/stream, static, land run off) between fisheries with problematic vs non-problematic infections and fisheries with perceived economic threat vs no perceived threat from infections.

For the management section another two GisedLMs were used. The first model examined if number of fish stocked, management type (catch and release, put and take, combination of both) and other health problems had an impact on *Argulus* spp. infection status. Catch and release management involves fish that are caught being released back into the fishery (generally resulting in low stock turnover), while put and take involves putting fish into the fishery that can then be taken by anglers to keep (generally resulting in high stock turnover). The second model determined if number of fish stocked, management type and length of *Argulus* problem had an influence on the economic threat of *Argulus* spp. infections. Two proportion z-tests were then conducted to examine differences in fishery features (dam wall, aerator, buoys, fishing pontoons, reed beds) and stocking time (spring, summer, autumn, winter) between fisheries with problematic vs non-problematic infections and fisheries with perceived economic threat vs no perceived threat from infections. Further two proportion z-tests were conducted for the *Argulus* spp. infection information section to ascertain the influence of *Argulus* spp. management/treatment (fishery closure, trickle stocking, SLICE® application,

destocking, culling affected fish, egg traps), fish symptoms (look unsightly, lose condition, harder to catch, mortality, lethargic; assessed by fishery owners considering fish appetite, scale/weight loss and behaviour) and fishery impacts (reduced catches, less anglers, reputation loss, reduced satisfaction) on the economic threat of *Argulus* spp. infections.

2.4 Results

All fisheries

The questionnaire responses provided insight into current practices in still water trout fisheries across England. There were an average of two water bodies per site (range 1 - 25) covering an average size of 45.7 acres (range 0.25 - 3100 acres) with average maximum depth of 5.4 m (range 0.9 - 45.7 m). Clay lined waters were the most common (54%, all comparisons $\chi^2(1) = 41.84 - 91.34$, $p<0.001$, two proportion z-tests), followed by reservoir (21%) which was significantly more common than gravel pit and chalk lined ($\chi^2(1) = 5.57$, $p = 0.02$ and $\chi^2(1) = 12.25$, $p<0.001$ respectively, two proportion z-tests; figure 2.1). Spring fed and river/stream were the primary water sources (41% and 37% respectively), with both significantly more common than static, land run off and pumped (all $\chi^2(1) = 28.02 - 106.90$, $p<0.001$, two proportion z-tests; figure 2.1). Fishery management was split between put and take, catch and release, and both combined (41%, 32% and 27% respectively; with put and take significantly more common than both combined, $\chi^2(1) = 7.16$, $p = 0.007$, two proportion z-test) with an average of 2100 total fish (range 0 - 80000 total fish) equal to 204 fish per acre (range 0 - 2400 fish per acre) stocked annually. The majority of stocking occurred in spring ($\chi^2(1) = 49.05 - 127.45$, $p<0.001$ for all comparisons, two proportion z-tests) with lowest stocking during winter ($\chi^2(1) = 17.06 - 127.45$, $p<0.001$ for all comparisons, two proportion z-tests; figure 2.1). While this study was focused on *Argulus* spp. infections, other health concerns noted by fishery owners included gill maggots (4 fisheries) and puffy skin disease (6 fisheries), alongside fungus, eye flukes (digenean larvae) and other skin conditions. These additional concerns did not correlate with *Argulus* spp. infections.

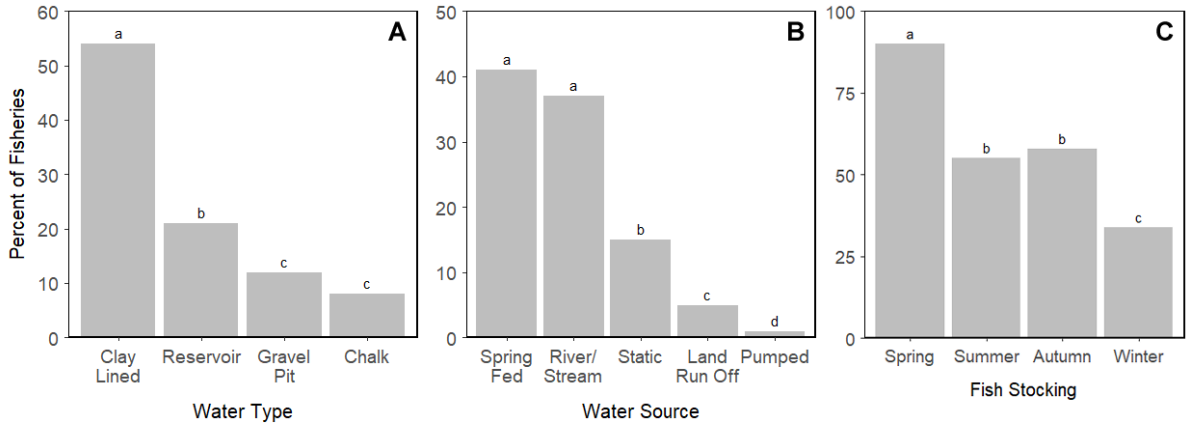

Figure 2.1 Questionnaire responses from all still water trout fisheries across England showing frequency of **A**: different water types, **B**: different water sources and **C**: seasonal fish stocking practices. Bars that do not share letters are significantly different from each other.

Problematic Argulus infections

In total, 31.5% of fisheries had problematic *Argulus* spp. infections (minor to severe). Of these fisheries, a further 35% stated that *Argulus* spp. were a threat to their economic viability/sustainability while 21% stated that the impact was unknown. Most fisheries had experienced *Argulus* problems for >2 years (85%), with problems spanning 2 - 5 years the most common (42%; significantly more common than 1 - 2 or 5 - 10 years, $\chi^2(1) = 9.67$ and $p = 0.002$ for both, two proportion z-tests). Almost a third of fisheries had experienced problems with *Argulus* spp. for >10 years (28%; figure 2.2), and infections were reported to be stable (51%; significantly more common than improving: $\chi^2(1) = 9.67$, $p = 0.002$ or worsening: $\chi^2(1) = 7.84$, $p = 0.005$, two proportion z-tests). Fisheries with problematic *Argulus* spp. infections experienced significantly higher maximum temperatures than fisheries without problematic infections (average 21.5 °C vs 18.9 °C, likelihood ratio test (LRT) value = 15.632, $p<0.001$, GisedLM). Infections were most problematic in July and August, with summer infections significantly more problematic than all other seasons ($\chi^2(1) = 83.41$ - 207.57, $p = 0.001$ for all, two proportion z-tests; figure 2.2).

Fisheries with problematic infections stocked more fish than fisheries without problematic infections, both annually (250 versus 159 fish per acre) and across the summer, autumn, and winter ($\chi^2(1) = 6.96$ - 18.61, $p = 0.008$ - <0.001, two proportion z-tests; figures 2.3 and 2.4). High stocking annually and in autumn also correlated with increased economic threat from *Argulus* spp. infections (LRT = 4.27, $p = 0.04$, GisedLM and $\chi^2(1) = 7.72$, $p = 0.005$, two proportion z-test; figure 2.5). Gravel pit water types were significantly more common in

fisheries with problematic infections ($\chi^2(1) = 8.61$, $p = 0.003$, two proportion z-test), with clay lined significantly more common in fisheries with non-problematic infections ($\chi^2(1) = 5.36$, $p = 0.02$, two proportion z-test; figures 2.3 and 2.4). Perceived economic threat was lower in fisheries with a river/stream water source ($\chi^2(1) = 4.94$, $p = 0.03$, two proportion z-test; figure 2.5), with no significant influence from water depth/clarity, fishery features or management type. Aerators were significantly more common in fisheries with problematic infections, while dam walls were more common in fisheries with non-problematic infections ($\chi^2(1) = 8.14/8.15$ respectively, $p = 0.004$ for both, two proportion z-tests; figures 2.3 and 2.4). Fishery infection status was not influenced by water depth/clarity/source or management type.

Fish under 5lb were most susceptible with rainbow trout the most commonly infected species (75%, likely a reflection of species stocking practices) although brown trout, blue trout, tiger trout, carp, pike, and perch were also reported susceptible. The main impacts on fish were loss of condition (more common than mortality and harder for anglers to catch, $\chi^2(1) = 5.53$, $p = 0.02$ and $\chi^2(1) = 7.63$, $p = 0.006$ respectively, two proportion z-test), unsightly appearance (more common than harder to catch, $\chi^2(1) = 5.31$, $p = 0.02$, two proportion z-test) and lethargy (figure 2.2). Harder to catch and lethargy were associated with a significant increase in perceived economic threat ($\chi^2(1) = 13.64$, $p<0.001$ and $\chi^2(1) = 6.56$, $p = 0.01$ respectively, two proportion z-test; figure 2.5). Reduced angler catches and satisfaction, less anglers and reputation loss all increased perceived economic threat ($\chi^2(1) = 4.88 - 18.75$, $p<0.001$ for all except reduced satisfaction where $p = 0.03$, two proportion z-tests; figure 2.5). Fishery closure also increased perceived economic threat of *Argulus* spp. infections (LRT = 12.13, $p<0.001$, GisedLM), with 24% of fisheries closing for 1 - 9 months.

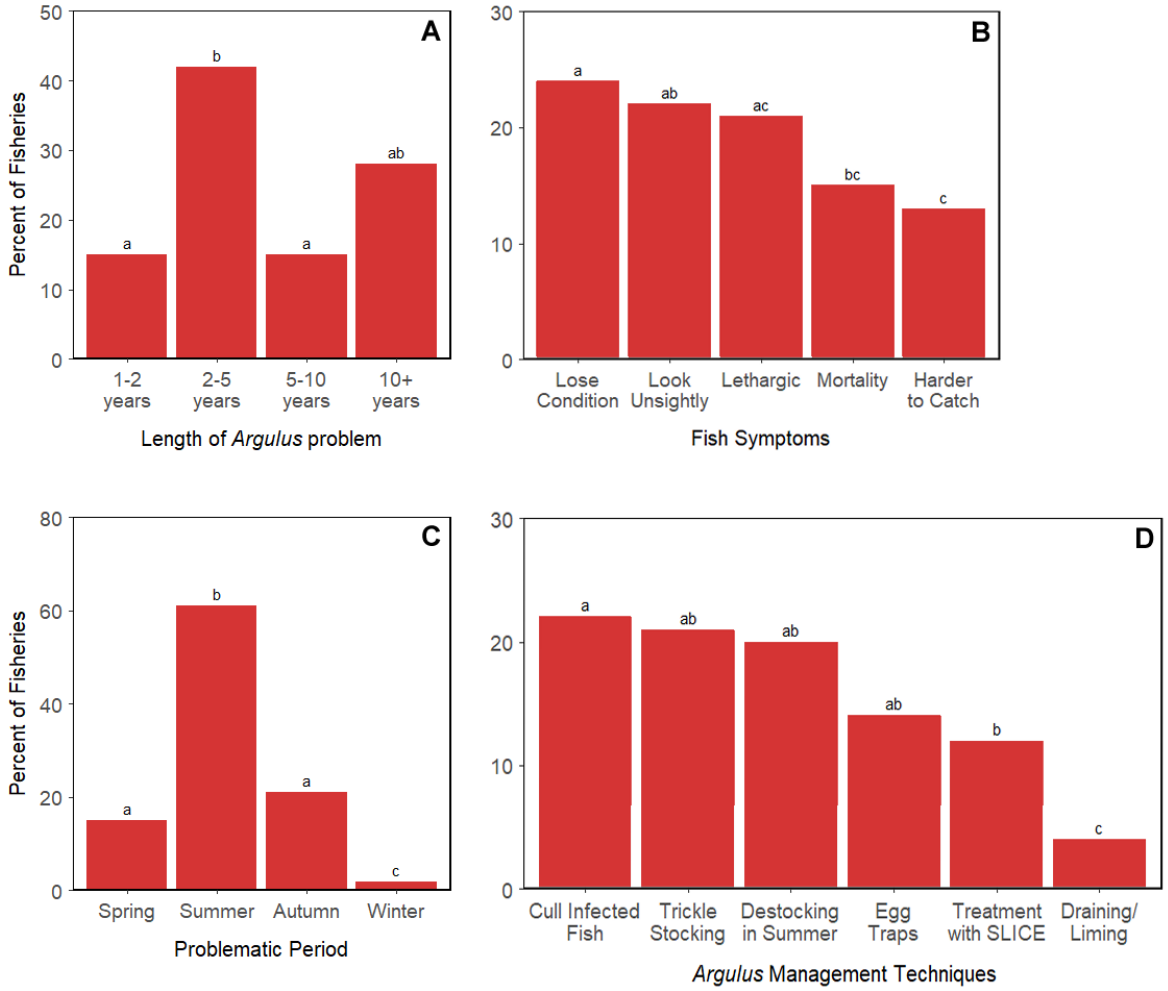

Figure 2.2 Questionnaire responses from still water trout fisheries across England with problematic *Argulus* spp. infections, showing **A**: duration of *Argulus* spp. infections experienced by fisheries, **B**: main symptoms experienced by fish, **C**: problematic infection period by season and **D**: management techniques employed to counter *Argulus* spp. infections. Bars that do not share letters are significantly different from each other.

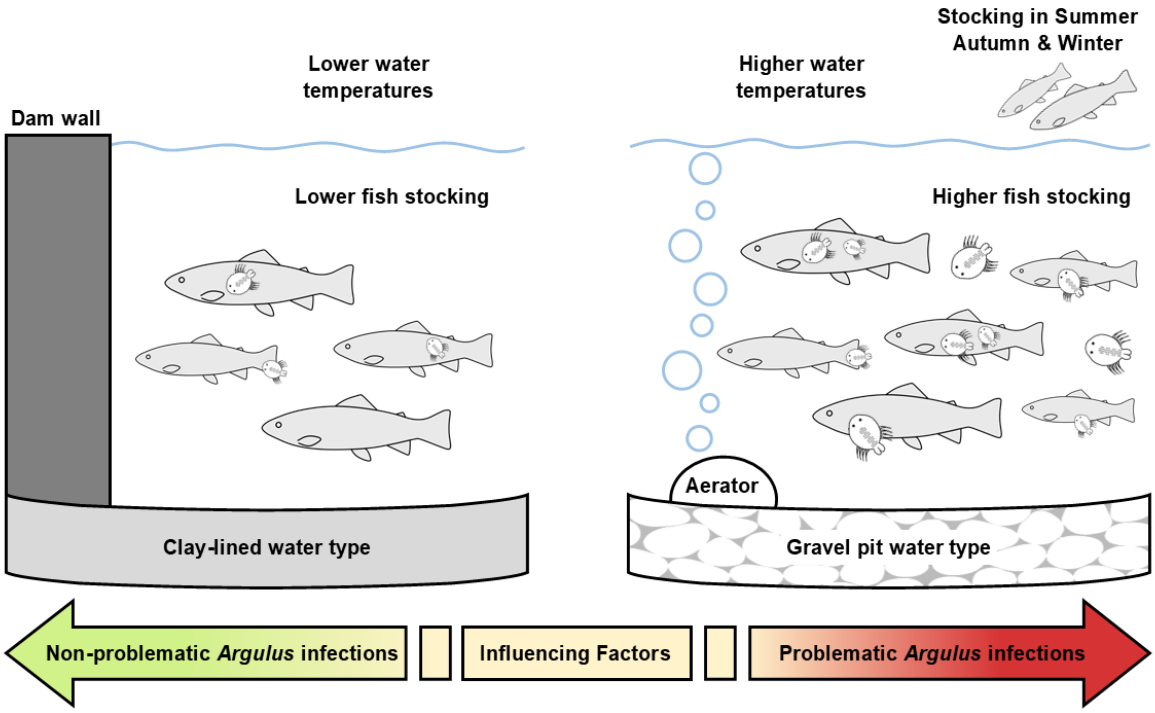

Figure 2.3 Factors significantly influencing ($p<0.05$) status of *Argulus* spp. infections in UK still water trout fisheries.

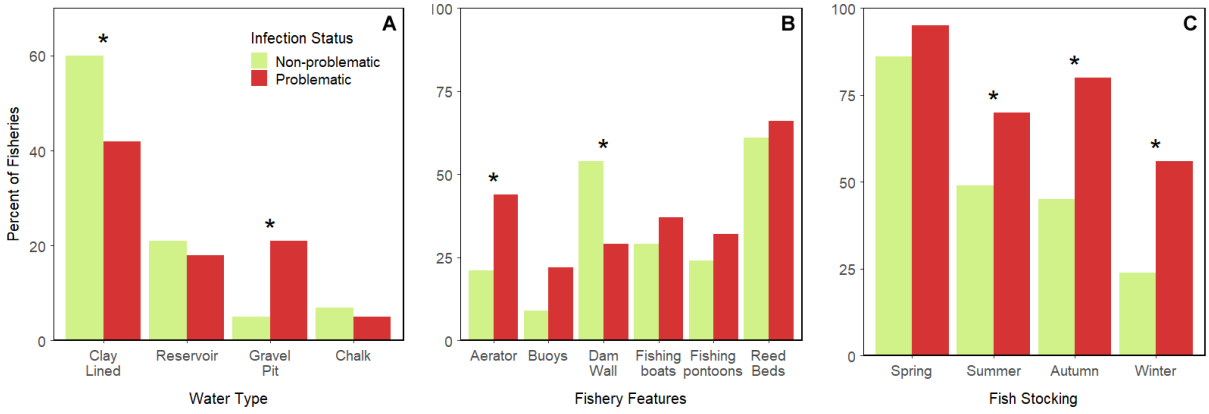

Figure 2.4 Questionnaire responses from still water trout fisheries across England showing comparisons between fisheries with problematic *Argulus* spp. infections and fisheries with non-problematic infections. **A**: Water types present in fisheries, **B**: Features present in fisheries **C**: Fish stocking periods. Legend in figure A also applies to figures B and C, significant differences between fisheries are indicated with an asterisk (*) symbol.

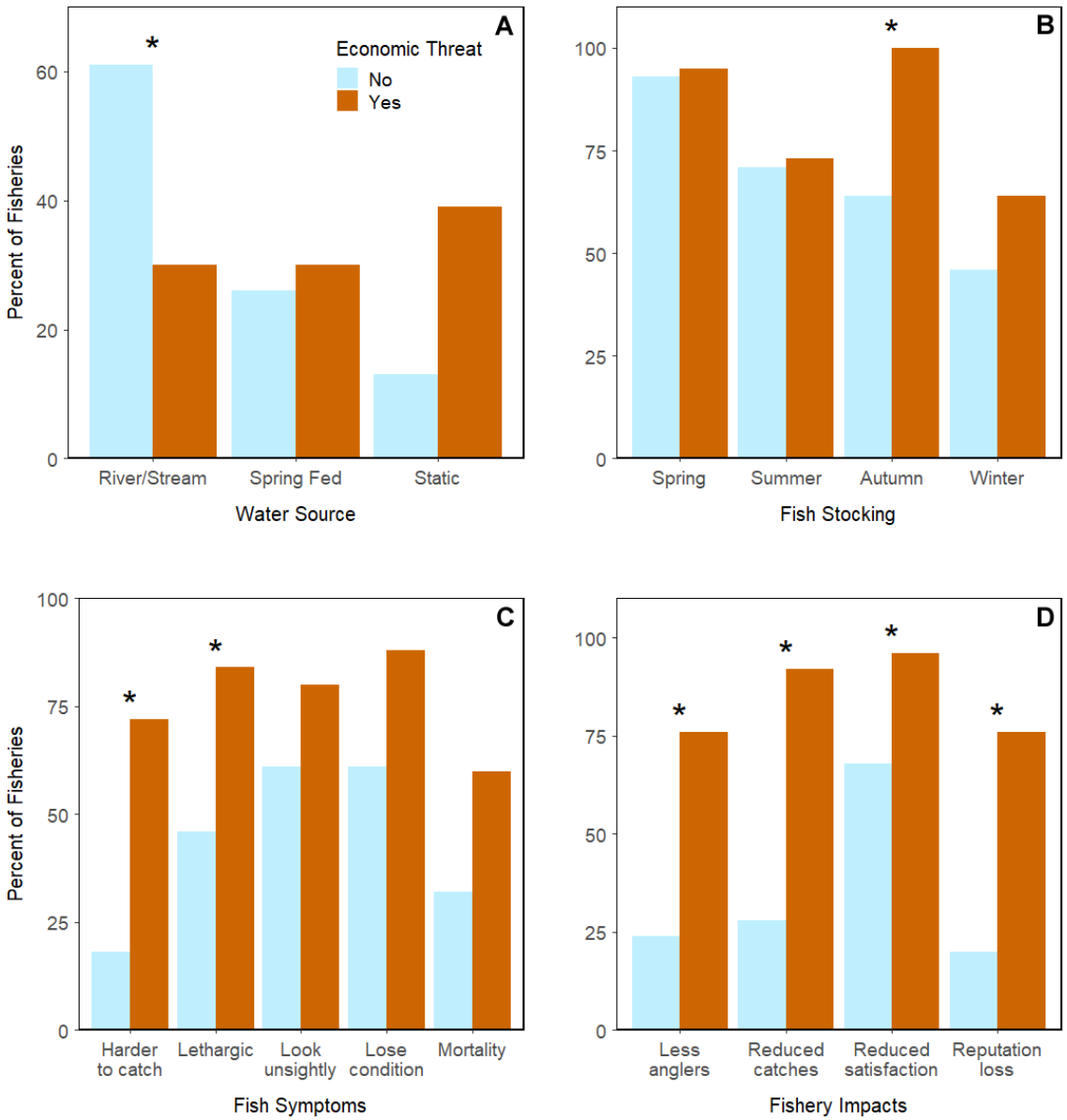

Figure 2.5 Questionnaire responses from still water trout fisheries across England with problematic *Argulus* spp. infections showing comparisons between fisheries that perceive infection to be an economic threat versus fisheries that do not. **A**: Primary water source of fisheries, **B**: Fish stocking periods, **C**: Fish symptoms from *Argulus* spp. infection and **D**: Fishery impacts from *Argulus* spp. infection. Legend in figure A also applies to figures B, C, and D, significant differences between fisheries are indicated with an asterisk (*) symbol.

Trickle stocking, destocking in summer and culling affected fish were the most common *Argulus* spp. management techniques (20 - 22% of fisheries employed culling, significantly more common than SLICE® treatment, $\chi^2(1) = 4.10$, $p = 0.04$, two proportion z-test) followed

by egg laying traps and treatment with SLICE® (14% and 12% respectively; figure 2.2). Drain and lime was the least common technique ($\chi^2(1) = 4.43 - 17.05$, $p = 0.04$ to <0.001 for all comparisons, two proportion z-tests; figure 2.2). Other techniques used by only 1 - 2 fisheries (and could therefore not be statistically assessed) include low stocking density, not stocking during hot weather, destocking over winter, removal of weeds/egg laying material and regular feeding. Economic threat of *Argulus* spp. was not impacted by treatment use.

2.5 Discussion

Still water fisheries across England are diverse in their structure and management. Natural features dominate with half of fisheries possessing clay lined waters and a majority fed via spring or river/streams. Put and take was the most popular management style, although catch and release and a combination of both were also frequently used. The average stocking rate of 204 fish per acre was within recommended levels of stocking for mature gravel pit fisheries (assuming an average stock fish weight of 5lb; Giles and Agency 2000) and above levels previously considered to result in low captures (<121 fish per acre; Giles *et al.* 2004, Templeton 1995).

Under managed fishery environments *Argulus* spp. infections can thrive and here almost a third of fisheries reported problematic *Argulus* spp. infections, comparable to levels reported in 2000 (Taylor *et al.* 2006). The stability of these problematic infections suggests that over the last 20 years fisheries have not changed sufficiently to impact these parasite populations, thus the problem remains. Temperature was a key factor with higher overall maximum water temperatures correlating with problematic *Argulus* spp. infections. The most common UK species, *A. foliaceus*, matures more quickly at higher temperatures (Chapter 3) with egg laying/hatching halted below 10 °C (Shafir and van As 1986; Mikheev *et al.* 2001). Additionally, salmonids as a cold water species suffer under high temperatures and become more susceptible to infections (Rebl *et al.* 2020). Thus, *Argulus* spp. infections are highly seasonal (Shimura 1983; Hakalahti *et al.* 2004b; Harrison *et al.* 2006); UK infections are typically highest over summer (Gault *et al.* 2002; Taylor *et al.* 2009a) and here July and August were the most problematic months (typically the hottest months in the UK).

Aerators are structures placed in water bodies to increase oxygen levels and reduce stratification of the water column. They come in a wide range of shapes and types (including floating or submerged). One of the most common types involves a submerged pipe with holes

- air is pumped into the pipe and as bubbles escape through the holes they rise through the water column/disturb the water's surface, promoting oxygen exchange. Here, aerators were more common in fisheries with problematic infections, however aerator type was not disclosed thus it is unknown if specific technologies or methods of aeration correlate with problematic infections. Despite this, aerators (regardless of type) are typically installed for two main reasons: to improve fish health and prevent algal blooms. Algal blooms are a previously identified risk factor for problematic *Argulus* spp. infections (although in this questionnaire only one fishery noted presence of algae), thus increased use of aerators here in fisheries with problematic infections may be a result of fisheries addressing this known risk factor (Taylor *et al.* 2006; Liu *et al.* 2015; El-Sheekh *et al.* 2019). Alternatively, fisheries with problematic infections may be installing aerators to improve health of sick fish following a loss of condition from *Argulus* spp. infections. There is no known impact of aerators on *Argulus* spp., although the additional oxygen could also potentially influence parasite population growth.

Water clarity was previously found to increase risk of problematic *Argulus* spp. infections, however here we found no correlation regarding clarity and infection status. This may simply be due to our larger sample size improving representation of fisheries (240 fisheries here vs 77 in Taylor *et al.* 2006). Alternatively, aerators can improve water clarity (Sheng *et al.* 2013) and here were more common in fisheries with problematic infections (where we would expect lower clarity based on Taylor *et al.* 2006). A river/stream source was associated with a lack of economic threat from infections; this source can provide cool water inflow resulting in cooler overall water temperatures. Additionally, the presence of running water could supress parasite population growth as *A. foliaceus*, the most common UK species, are not usually found in areas with running water (Mikheev *et al.* 2015).

Physical fishery parameters also influenced *Argulus* spp. infections: fisheries with problematic infections were more likely to have gravel pit water types, while fisheries with non-problematic infections commonly had clay lined waters and dam walls. These water types may be linked to reproductive output, as female *Argulus* require suitable hard surfaces (such as stones) for egg laying which may have higher availability in gravel pit waters compared to waters lined with soft substrate such as clay (Sahoo *et al.* 2013b). Dam walls should also provide an area for egg laying yet were more common in non-problematic fisheries. This may simply be due to dam wall presence being linked to clay-lined water types (presence was most common in reservoir and clay-lined waters, with 25 fisheries of each type possessing a dam wall). Alternatively,

dam walls increase trapping and build-up of sediment which can prevent *Argulus* spp. egg laying (Taylor *et al.* 2009a; Sahoo *et al.* 2013b; Kondolf *et al.* 2014).

Problematic *Argulus* spp. infections and economic threat were also linked to higher fish stocking, and additional stocking in summer, autumn, and winter. While increased stocking could simply be a consequence of angling activity and/or fish loss requiring replacement, stocking itself can impact infection levels. Fish stocking is a biosecurity risk - each stocking event has the potential to introduce new *Argulus* species or strains into the fishery (Giles *et al.* 2004), including invasive non-native species of parasites. Higher host density increases the parasite carrying capacity of a population and improves infection/transmission success (Pasternak *et al.* 2000), although a fast stock turnover can reduce infection levels by removing and preventing establishment of parasites (McPherson *et al.* 2012; Taylor *et al.* 2005b). Low stock turnover is a known risk factor for problematic *Argulus* spp. infections as fish (and therefore attached parasites) are not removed (Taylor *et al.* 2006). We were unable to calculate stock turnover in this study as it requires acquisition of catch logs from each fishery, however high stocking can contribute to low stock turnover if angler catch rate does not equal or exceed fish stock rate. As stocking influences *Argulus* spp. populations, strategic application of stocking techniques can aid management of infections, and here they were the most common *Argulus* spp. management technique employed by fisheries.

Overall, control of *Argulus* spp. infections is inefficient as 85% of fisheries with problematic infections have tackled them for 2+ years, with 28% experiencing infections for 10+ years. This combined with infections remaining stable for most fisheries suggests that these problematic *Argulus* spp. infections are chronic, likely compounded by a lack of appropriate control options. The main treatments used by the fisheries surveyed were stocking related (Trickle stocking, destocking in summer and culling affected fish) but application of such techniques varies between fisheries and can be difficult to apply effectively (McPherson *et al.* 2012). Egg laying substrate is a newer technique employed by 14% of fisheries, this involves installing substrate suitable for *Argulus* spp. egg laying, which is then removed to reduce parasite reproductive output. This technique shows promise, but further testing is required to ascertain its viability as a control method (Gault *et al.* 2002; Chapter 6). Chemical control is one of the most effective methods to remove *Argulus* spp. from fisheries (Hakalahti *et al.* 2004c; Hakalahti *et al.* 2008) and 12% of fisheries used SLICE®, an avermectin based treatment. In the UK, this is the only legal chemical treatment against *Argulus* spp., it is only

available under veterinary guidelines and stock cannot be fished for 500-degree days post application, which limits its use. Additionally, drug resistance remains a rising threat in fish lice (Aaen *et al.* 2015). Draining/liming was a popular management technique in the past, but here only 5 fisheries total had employed this technique. This decrease in use likely stems from fisheries being unusable during treatment (impacting income) with parasite eradication not guaranteed. Despite the wide range of control techniques employed here, none of them impacted the perceived economic threat of *Argulus* spp. to fisheries.

Argulus spp. infections impact economic viability directly through fish mortality (requiring replacement of fish) and purchase/application of treatment, alongside indirectly though lowering angler satisfaction and the standing of the fishery (through host symptoms including lethargy and harder for anglers to catch). These indirect impacts increased the perceived economic threat from infections, likely due to fish symptoms being more common when infections are problematic, alongside a subsequent reduction in customers. This suggests that over the past 20 years, the factors driving the economic threat from *Argulus* spp. infections have not changed as Taylor *et al.* (2006) also found angler satisfaction to dictate economic loss. Fishery closure also significantly correlated with perceived economic threat as closure prevents acquisition of profits. Closed seasons over winter may also contribute to parasite success as stocking is often conducted in early spring prior to reopening, resulting in a large host population for newly hatched *Argulus* spp. from over-wintered eggs (Taylor *et al.* 2005b).

While the current study focused on trout fisheries (the fishery type with traditionally the highest number of *Argulus* problems), more recently the number of reports from carp fisheries regarding problems and mortalities from *Argulus* spp. infections has increased (personal observations C. Williams). Historically, *Argulus* spp. in Europe have caused problems in carp fisheries with the parasite often referred to as a carp louse (Herter 1927; Taylor *et al.* 2005a). This recent rise in problems in UK carp fisheries is of great concern, as these fisheries generally stock large, expensive prize fish that are under catch and release management, with no turnover of fish stock. Loss of fish can therefore be devastating for these fisheries. As such, considerations should be made into the future regarding infections in carp fisheries alongside trout fisheries within the UK.

Chapter 3

Life in the fast lane: temperature, parasite density and host species impact survival and growth of the fish ectoparasite *Argulus foliaceus*

3.1 Abstract

With expanding human populations, the food sector has faced constant pressure to sustainably expand and meet global production demands. In aquaculture this frequently manifests in an animal welfare crisis, with fish increasingly farmed under high production, high stress conditions. These intense environments can result in fish stocks having a high susceptibility to infection, with parasites and associated disease one of the main factors limiting industry growth. Prediction of infection dynamics is key to preventative treatment and mitigation. Considering the climatic and technology driven changes facing aquaculture, an understanding of how parasites react across a spectrum of conditions is required. Here we assessed the impact of temperature, infection density and host species on the life history traits of *Argulus foliaceus*, a common palearctic fish louse, representative of a parasite group problematic in freshwater aquaculture and fisheries worldwide. Temperature significantly affected development, growth, and survival; parasites hatched and developed faster at higher temperatures, but also experienced shorter lifespans when maintained off the host. At high temperatures, these parasites will likely experience a short generation time as their life history traits are completed more rapidly. *A. foliaceus* additionally grew faster on host species encountered in their native range (versus a novel host species) and at lower infection densities. Ultimately such results contribute to prediction of population dynamics, aiding development of effective control to improve animal welfare and reduce industry loss.

3.2 Introduction

Aquaculture has global economic benefits, providing food security and supplying stock for sport fishing and the ornamental pet trade (FAO 2020). As fisheries intensify to meet global demands, animals are subject to an increasing number of stressors (Wedemeyer 1997; Wood 2001; Conte 2004; Lewin *et al.* 2006). Such conditions facilitate and amplify parasite transmission and disease outbreaks, with infections arguably the most important factor limiting sustainable industry expansion (Granada *et al.* 2016). Management of parasites and disease in fish is grossly lacking compared to mammalian species (Burka *et al.* 1997) with diagnosis and treatment difficult to accomplish. This is exacerbated by a lack of information regarding

parasite behaviours, life cycles and life-history traits, coupled with high diversity in fishery cultures and structure (Li *et al.* 2002).

Fish farm infrastructure ranges from near natural systems to highly controlled artificial environments. Despite this diversity, all farms can experience temperature shifts due to climate change and/or increased use of technology (Jiang 2010). Temperature is crucial in farming, significantly influencing animal physiology and wellbeing. Associated parasites and disease are equally affected by temperature, which can lead to drastic shifts in infection dynamics. For parasites, high ambient temperatures typically lead to a short generation time as life history traits are completed more quickly; however, each trait can respond differently to temperature leading to trade-offs (e.g. Gophen 1976; Andersen and Buchmann 1998; Soleng *et al.* 1998; Sahoo *et al.* 2013b). Examination of a suite of life history traits is therefore required to understand how temperature impacts overall parasite population dynamics. This is critical if we are to predict infection dynamics and develop more effective management practices.

One of the most conspicuous parasite groups plaguing fisheries are ectoparasitic fish lice, relatively large (compared to most fish parasites) crustaceans responsible for widespread damage in both marine and freshwater systems (Hakalahti *et al.* 2008; Costello 2009). Elevated temperatures are linked to outbreaks (Shimura 1983; Hakalahti *et al.* 2004a; Harrison *et al.* 2006), with modelling of marine sea lice showing a higher epidemic potential at higher temperatures (Groner *et al.* 2014). Freshwater lice (Genus *Argulus*) are particularly dependent on spring warming to induce hatching of overwintered eggs, which kick-start early population growth (Mikheev *et al.* 2001). As such, wild fisheries are predicted to encounter *Argulus* spp. earlier in the year and for prolonged periods under climate change scenarios, while in aquaculture systems maintained above 10 °C *Argulus* spp. can reproduce continuously (Hakalahti *et al.* 2006; Taylor *et al.* 2009b; Stewart *et al.* 2017). Freshwater lice are also a rising problem in UK angling fisheries; only one legal chemical treatment is currently available (SLICE®, typically used against sea lice) with resistance a concern (Hakalahti *et al.* 2004c; Taylor *et al.* 2005b). Management strategies focusing on stocking practices can help reduce infection depending on application (McPherson *et al.* 2012), while control methods such as egg-laying traps lack testing (Taylor *et al.* 2005b). To improve current application of management, an understanding of how *Argulus* spp. population dynamics in fisheries change under differing environmental conditions is needed.

Here, we examined the impact of temperature on one of the most common freshwater fish lice, *A. foliaceus* (Radkhah 2017), before infection, during establishment and post-infection. Specifically, we aimed to identify changes in parasite hatching, growth, and survival on and off the host. The impact of infection density and host species on parasite growth was also considered due to the variety of hosts across farming systems and tendency of *Argulus* spp. to aggregate on the host. Temperature also has the potential to alter both of these factors by influencing host-parasite optima.

3.3 Methods

Host origins and maintenance

Permission was obtained from local authorities prior to fish collection. Three-spined sticklebacks (*Gasterosteus aculeatus*) were collected via hand netting from Roath Brook, Cardiff (ST 18897 78541) on 19/04/18 and 20/06/18, with ornamental guppies (*Poecilia reticulata*) purchased from a wholesaler on 29/06/18. Upon arrival at Cardiff University, all fish were lightly anaesthetised with 0.02% MS222 (tricaine methanesulfonate) and screened for ectoparasites using a dissection microscope with fibre optic illumination. Both species were infected with *Gyrodactylus* spp.; guppies were treated with Levamisole according to Schelkle *et al.* (2009) while for sticklebacks gyrodactylids were removed manually with watchmaker's forceps due to the low prevalence. No *Argulus* spp. were found on either fish species. All fish were acclimatised in a laboratory setting on a 12h light:12h dark cycle, fed daily and maintained in stock tanks at a density <1 fish/L for 2 weeks prior to experimental use. Sticklebacks were maintained at 14 ± 0.5 °C and fed *Tubifex* bloodworm, while guppies were kept at 24 ± 0.5 °C and fed Aquarian® tropical fish flakes. Prior to experimental use, all fish were screened clear of ectoparasites three times (Schelkle *et al.* 2009) and measured for standard and fork length (using callipers accurate to 0.1 mm). Throughout all experiments, location of *A. foliaceus* on the host was recorded to examine parasite movement.

Parasite cultures and infection

A. foliaceus were obtained from laboratory cultures, maintained using three-spined stickleback hosts (see Stewart *et al.* 2017). Two *A. foliaceus* populations were used in this study, lab population (cultured 4 years in lab, origins detailed in Stewart *et al.* 2017) and wild population (cultured 1 year in lab) originating from *A. foliaceus* adults (identified morphologically according to Fryer 1982) obtained from Rudd (*Scardinius erythrophthalmus*) from a fishery in Surrey on the 19/10/17. To hatch *A. foliaceus* for experimental use, eggs laid in culture were

removed from storage at 7 °C (development ceases <10 °C; Shafir and van As 1986; Mikheev *et al.* 2001) and gradually acclimated to incubation temperature (14 or 24 °C; experiment dependent) by placing them at ambient air temperature to allow gradual warming of the water (10 h to reach 24 °C from 7 °C, 4 h to reach 14 °C). Temperatures were maintained using thermostatically controlled rooms; average temperature = 24 °C ± 0.52 SD and 14 °C ± 0.55 SD. Eggs were checked daily and given weekly water changes until hatching. No significant differences were found between lab and wild population *A. foliaceus* regarding incubation time, hatching success/period and survival on/off the host (data not shown). As such, the most prolific culture was used at the time of each experiment (lab population for hatching and survival experiments, wild population for parasite growth experiments).

Infections were performed by placing a single fish into 100 ml water and introducing parasites (single louse for all experiments except parasite density which used 3 to 5 individuals) via a pipette. In all cases, *Argulus* naturally attached to the fish within 10 min of exposure. For the hatching and survival experiments, time to infect was recorded for each parasite, however, no significance was found between temperature treatments (shock, gradual or no temperature change), with host length or with parasite position post infection. To measure *A. foliaceus* on the host, infected fish were anesthetised using 0.02 % MS222 and placed under a dissecting microscope in a glass dish with 200 ml of dechlorinated water. Images were taken of attached *A. foliaceus* with fish laying flat on their sides, at x10 magnification using a Lumenera Infinity 1 camera with Infinity Capture software version 6.5.4. *A. foliaceus* were measured from the rostral tip of their carapace to caudal end of the abdominal lobes using ImageJ version 1.51j8 (Schneider *et al.* 2012). To measure *A. foliaceus* off the host, parasites were placed onto a slide using a pipette, restrained by reducing their pool of water to a minimal amount, and then imaged as mentioned above. All images were calibrated for measurements using a 1/100 mm micrometre scale.

Temperature impact on parasite hatching and survival

To determine the effect of temperature on *A. foliaceus* hatching success and survival, three temperature treatments were investigated: gradual temperature change (eggs incubated at 24 °C with newly hatched parasites gradually cooled to 14 °C over 24 h), shock temperature change (eggs incubated at 24 °C with newly hatched parasites introduced to 14 °C water 24 h post hatching without acclimation) and finally no temperature change (eggs incubated and parasites maintained thereafter at 14 °C). For hatching success trials, three separate groups of eggs were

incubated per temperature (14 °C N = 132 eggs total, 24 °C N = 476 eggs) with daily checks and weekly water changes.

For survival on the host, individual sticklebacks (average standard length = 40 mm ± 0.45) were infected with five individuals of *A. foliaceus* (all from the same temperature treatment) 24 h post-hatching and placed into 1 L tanks at 14 °C (standard stickleback infection level; Stewart *et al.* 2017, N = 15 fish and N = 75 parasites per treatment). Any *A. foliaceus* lost during infection (N = 19 total) were presumed eaten and replaced (Bandilla *et al.* 2008). *A. foliaceus* survival was monitored on infected sticklebacks daily for 7 days and then weekly until 21 days post-infection.

Parasite survival off the host was assessed by placing newly hatched *A. foliaceus* into 50 ml dechlorinated water at 14 °C (N = 30 - 65 parasites per treatment). Here an additional temperature treatment was tested with parasites hatched and maintained at 24 °C (N = 37 parasites). *A. foliaceus* were monitored daily using a dissecting microscope with the number alive, moribund, and dead recorded. Consistently, one day prior to death parasites were moribund - as this displayed the same trend as survival, it is not reported further.

Temperature, parasite density and host species impact on A. foliaceus growth

To ascertain the impact of temperature on *A. foliaceus* growth, sticklebacks acclimatised to 14, 19 and 24 °C (1 week acclimation period) were infected with a single *A. foliaceus* metanauplius measured prior to infection (day 0, length = 0.618 mm ± 0.049 SD; N = 15 fish and parasites per temperature). To investigate any additional impact of host species and infection density on *A. foliaceus* growth, sticklebacks and ornamental guppies were selected as two extremes. Sticklebacks are a temperate, natural host found in most waterbodies across the UK, versus guppies, a tropical fish and one of the most popular pet species with reports of *A. foliaceus* infection in aquaculture/pet trade (Walker *et al.* 2007; Momeni Shahraki *et al.* 2014; Maceda-Veiga *et al.* 2016). For experimental work sticklebacks and guppies acclimatised to 19 °C were infected with one individual of *A. foliaceus* per 7.4 mm of host standard length (based on maximum non-lethal infection density of 5 parasites per stickleback: Stewart *et al.* 2017; N = 10 fish per host species, N = 3 - 5 parasites per fish). Post-infection, all fish were maintained individually in 1 L tanks with water changes every 48 h to maintain water quality. One day post-infection (day 1) and subsequently every 48 h for two weeks, *A. foliaceus* were measured

on the host with their position noted. After 2 weeks, all *A. foliaceus* were removed from fish, sexed, and re-measured off the host to give final parasite length.

Statistical analysis

All statistical analyses were conducted in R statistical software v3.4.3 (R Core Team 2017) using the following packages: "ggplot2" to visualise the data (Wickham 2009), "survival" to run survival analyses (Therneau and Grambsch 2000) and "lme4" to run generalised linear mixed models (Bates *et al.* 2014). Models were refined through stepwise deletion of insignificant terms and Akaike information criterion comparisons, with visual examination of model plots to check standardised residuals for normal distribution and homogeneity of variance. In all mixed models fish ID was included as a random factor to account for pseudo-replication, and in all tests the level of significance was taken as $p<0.05$.

To examine the survival of *A. foliaceus* on stickleback hosts, a generalised linear mixed model (GisedLMM) with Poisson family and square root link function was used with number of days post infection, temperature treatment (gradual, shock or none), host standard length and an interaction between day and treatment as dependent variables. Survival analysis was used to determine the effect of temperature treatment and time on *A. foliaceus* survival off the host. Hatching success of eggs was compared across treatments using a Chi-squared test.

A GisedLMM with gaussian family and log link function was used to assess the impact of parasite sex, host standard length, host species, days post-infection, temperature and an interaction between day and temperature on *A. foliaceus* length. To analyse the impact of parasite density and host species on *A. foliaceus* length, two general linear mixed models (GLMM) with gaussian family and identity link function were used to examine the effect of days post-infection and host standard length, alongside either infection density and an interaction between infection density/day, or host species with an interaction between host species/day.

Additionally, for the growth experiments, a GisedLMM with binomial family and logit link function was used to assess whether parasite location (on the body of the host instead of the fins, yes/no) was affected by temperature, infection density, host species and time. To examine overall movement of *A. foliaceus* on hosts, a GisedLMM with binomial family and logit link function compared whether a parasite moved (yes/no) to temperature, host species, infection

density, days post-infection and host length. Two proportion z-tests were also used to compare the number of parasites on the body of the fish versus the fins across five parasite size groups: 0.40 - 0.79, 0.80 - 1.19, 1.20 - 1.59, 1.60 - 1.99 and 2.00 - 2.39 mm. These size ranges were based on *A. foliaceus* developmental stages (see Rushton-Mellor and Boxshall 1994), parasites larger than 2.4 mm length were not statistically assessed due to small sample size.

3.4 Results

Temperature impact on parasite hatching and survival

At 24 °C, *A. foliaceus* eggs hatched after an average incubation period of 27 days (range 19 - 39 days) while at 14 °C eggs hatched after 67 days (range 60 - 75 days). Hatching success of eggs across temperature treatment ranged from 57.7% to 63.7%, and success did not differ between eggs incubated at 24 vs 14 °C.

A. foliaceus maintained at 24 °C off the host had significantly lower survival than parasites maintained at 14 °C ($\chi^2(3) = 54.10$, $p<0.001$, survival analysis; figure 3.1). When examining *A. foliaceus* survival on stickleback hosts at 14 °C, survival significantly decreased over time ($F_{1, 420} = 67.02$, $p<0.001$, GisedLMM), with just under 50% survival 21 days post-infection (figure 3.1). Host length did not significantly impact parasite survival. Survival of *A. foliaceus* on and off stickleback hosts was not impacted by incubation temperature (24 or 14 °C) or temperature treatment post-hatching (gradual, shock or no thermal change).

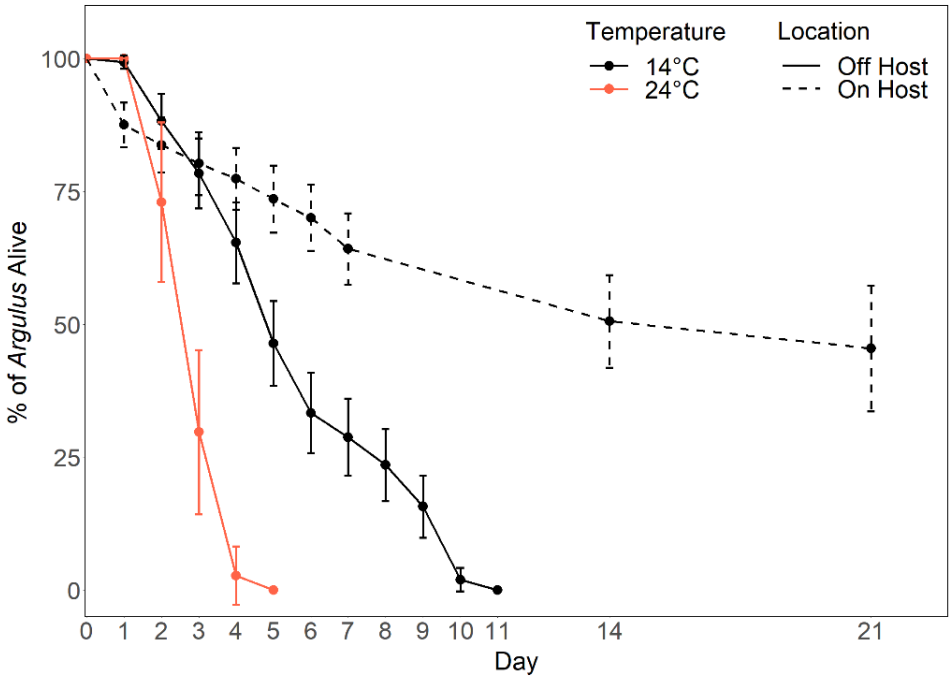

Figure 3.1 *Argulus foliaceus* survival off the host at 14 or 24 °C and on host (three-spined stickleback; *Gasterosteus aculeatus*) at 14 °C. For on host survival, all fish began with five metanauplii. Error bars represent 95% confidence intervals.

Temperature, parasite density and host species impact on A. foliaceus growth

A. foliaceus length increased with temperature and over time ($F_{2,\ 360}$ = 104.96, p<0.001, GisedLMM; figure 3.2). At 14 days post-infection, *A. foliaceus* length averaged 2.5 mm at 24 °C, 1.9 mm at 19 °C and 1.1 mm at 14 °C. Parasite growth was significantly slower at high parasite density compared to low parasite density (figure 3.2; $F_{1,\ 195}$ = 34.15, p<0.001, GLMM). Parasite length was also affected by host species over time ($F_{1,\ 156}$ = 11.69, p<0.001, GLMM): when infected with multiple parasites, but at an equivalent density, sticklebacks had larger *A. foliaceus* than guppies. In all tests, host length and parasite sex did not significantly impact *A. foliaceus* growth.

Considering *A. foliaceus* averaged 0.618 mm length at birth, adulthood (4.7 mm; taken from Rushton-Mellor and Boxshall 1994; Taylor *et al.* 2009b) would take 124 days at 14 °C, 45 days at 19 °C and 30 days at 24 °C at low parasite density on stickleback hosts (assuming a linear growth pattern). For the higher infection density tests at 19 °C, *A. foliaceus* would take 50 days to reach adulthood on sticklebacks and 55 days on guppies. These values are however an estimate as *Argulus* species display diverse growth profiles (Rushton-Mellor and Boxshall 1994; Pasternak *et al.* 2004), especially under natural, wild conditions (Taylor *et al.* 2009b).

Position and movement of A. foliaceus on hosts

Significantly more *A. foliaceus* were found on the fins of hosts versus the body as temperature and time spent on host increased ($F_{1, 822} = 4.60$, p = 0.04 and $F_{1, 823} = 6.86$, p = 0.008 respectively, GisedLMM). Parasite density and host species did not affect parasite position on host. *A. foliaceus* movement frequency was higher at high infection density and temperature ($F_{1, 446} = 50.80$, p<0.001 and $F_{1, 445} = 24.89$, p<0.001 respectively, GisedLMM), but was not affected by host species, time spent on host or host length. *A. foliaceus* position was also significantly influenced by parasite size; 70% of *A. foliaceus* 2.0 - 2.4 mm in length were located on the host's body, versus 47% of newly hatched parasites 0.4 - 0.8 mm length ($\chi^2(1) = 4.36$, p = 0.04, two proportion z-test).

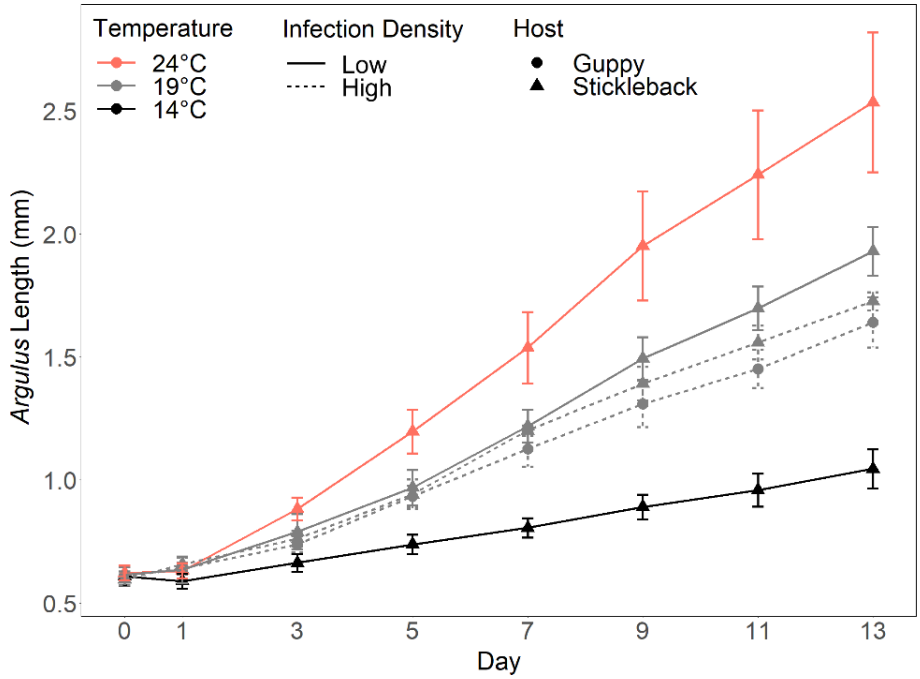

Figure 3.2 Impact of temperature, infection density and host species on *Argulus foliaceus* growth. Three-spined sticklebacks (*Gasterosteus aculeatus*) were infected with one individual *A. foliaceus* metanauplii at 14 °C, 19 °C and 24 °C (infection density = low). Additional guppies (*Poecilia reticulata*) and sticklebacks at 19 °C were infected with one *A. foliaceus* metanauplii per 7.4 mm of host standard length (creating a starting infection number of 3 - 5 parasites per fish, infection density = high). Error bars represent 95% confidence intervals.

3.5 Discussion

Parasite generation time is intrinsically linked to environmental variables and here, *A. foliaceus* responded positively to increasing temperature with faster incubation and growth. However, life span off the host was reduced at higher temperatures, potentially impacting infection success. *A. foliaceus* also demonstrated a high resistance to sudden temperature changes, with a 10 °C temperature shock having no impact on parasite survival on or off the host. Infection density and host species also affected parasite growth, variables which could alter infection dynamic predictions especially as they can change drastically across temperature and farming system.

Both egg incubation and *A. foliaceus* growth on stickleback hosts were significantly faster at 24 °C compared to 14 °C, suggesting *A. foliaceus* at higher temperatures would experience a shorter generation time as birth and development occur more rapidly. While fast life history traits potentially allow parasites to rapidly exploit hosts, they may also limit infection depending on host density; for example in entomopathogenic nematodes, prolific availability of hosts benefits parasites with fast infection rates while limited host availability favours parasites with slower rates (Crossan *et al.* 2007). Nematodes with fast infection rates also had a corresponding trade off in fecundity and survival (Crossan *et al.* 2007), comparable to this study where *A. foliaceus* at 24 °C experienced fast incubation and growth, but also a significant reduction in survival off the host. High host densities (such as those encountered in aquaculture systems) could override this trade off, negating any impact of reduced survival at high temperatures. For angling fisheries however, replacement stocking to maintain low fish densities has been previously predicted to decrease parasite populations (McPherson *et al.* 2012). In this case parasite survival could potentially influence overall parasite population success, as survival at 14 °C was double compared to 24 °C allowing parasites at low temperatures more opportunities for infection. This low temperature survival was also double the observed survival in a previous report by Walker *et al.* (2011a) examining 1 day old *A. foliaceus* at 15 °C. This difference potentially arises from inter-population variation, the 1 °C difference and/or the inclusion of aerators by Walker *et al.* (2011a), which could cause higher parasite activity and subsequent increased metabolic cost/shorter lifespan.

A. foliaceus were maintained at constant temperature under laboratory conditions, however in heterogenous environments the parasite could maximise survival by moving to thermally optimal areas. *A. foliaceus* have a thermal preference of 28 - 30 °C (Herter 1927), although

shadows present in the experimental setup may have affected preference results (Lagerspetz and Vainio 2006). This suggests *A. foliaceus* do not select cooler temperatures (such as 14 °C) to increase off host survival. Regardless, the ability of any parasite to select preferential microclimates should be considered when examining infections in fisheries and aquaculture.

Another key factor contributing to the success of *A. foliaceus* is their broad host range; they are found on practically all fish species within their natural habitat, alongside successfully infecting novel, unnatural hosts including ornamentals such as goldfish, koi, and guppies (Walker *et al.* 2007; Momeni Shahraki *et al.* 2014; Mirzaei and Khovand 2015). Despite a difference in growth rate, *A. foliaceus* successfully infected and survived on both natural, native stickleback hosts and novel guppy hosts. Possessing a broad host range allows parasites to exploit a wider host pool, providing an advantage over specialist parasites in systems with mixed host species (such as wild/angling fisheries). In comparison, monoculture aquaculture systems will likely give rise to specialist parasites as they outcompete generalists. Regardless of system, host availability should be considered when assessing problematic infections as both specialist (Pasternak *et al.* 2004) and generalist (shown here) *Argulus* species show differential growth across host species.

Argulus spp. typically display an aggregated distribution within host populations (Bandilla *et al.* 2005; Walker *et al.* 2008). Here higher *A. foliaceus* density on the host resulted in lower parasite growth rate, as such parasite populations could display reduced growth as aggregation increases. Regarding parasite position on host, parasites increasingly moved from the host fins to the body as time progressed and at higher temperatures. *A. foliaceus* moved to the body when they reached 2.0 - 2.4 mm length (7^{th} developmental stage, at which point the mouth-tube is fully developed; Rushton-Mellor and Boxshall 1994), indicating the start of blood feeding. Juveniles feed only on mucous/skin cells, whereas the blood feeding adults cause greater host damage with the potential for secondary infections (Bower-Shore 1940; Bandilla *et al.* 2006; Walker *et al.* 2011b). Prioritising identification and treatment of *A. foliaceus* infections when parasites are <2 mm length (pre-blood feeding) would improve control by addressing infections before they cause significant damage, as currently (in UK angling fisheries) infections are primarily tackled once adults are present in high numbers.

Understanding the dynamics of parasites across their life cycle is critical for predicting changes to infection. *Argulus* spp. populations have been previously modelled to examine the impact of

control methods and predict population changes/generation time (Fenton *et al.* 2006; Taylor *et al.* 2009b; McPherson *et al.* 2012; Kumar *et al.* 2017). Of these studies, Taylor *et al.* (2009b) used *A. foliaceus* length-frequency data to estimate egg incubation, hatching period and maturation rate of different cohorts within an angling fishery. The authors calculated a maturation time of 34 days at 24 °C, comparable to our estimate of 30 days with the difference likely caused by variables such as host species and infection density (as shown here), and/or our use of a linear growth assumption for estimation. However, Taylor *et al.* (2009b) also observed an increase in hatching period with decreasing temperature (not observed here) suggesting that this trait may be governed by different seasonal variables such as light period (Bai 1981). Incorporating this new empirical data into existing and future models should therefore help verify outputs and improve modelling predictions. This in turn will help farmers select appropriate control methods and conditions to give the best trade-off for host growth and parasite restriction, reducing the impact of parasites on industry.

Chapter 4

Shining a light on parasite behaviour: daily patterns of *Argulus* fish lice

4.1 Abstract

Parasites display a wide range of behaviours that are frequently overlooked in favour of host responses. Understanding these behaviours can improve parasite control through more precise application or development of new behaviour-based strategies. In aquaculture fish lice are an ongoing problem, infections reduce fishery production and control options are limited. Fish lice are distinct in their ability to survive and swim off hosts, allowing transmission to multiple fish hosts across their lifespan. Here we assessed off host behaviour of *A. foliaceus* (a freshwater fish louse) and observed a diurnal rhythmical pattern in behaviour. This pattern was lost when lice were exposed to constant darkness, indicating that the behaviour is not endogenously driven. Males were consistently active in light with reduced activity in darkness. In contrast, females were active during light and dark phases with peak activity at the start of dark periods. *A. foliaceus* was also strongly attracted to a light stimulus, preferring white and blue coloured light to green or red. Light is a strong driver of fish louse activity and could be used to trap parasites. Aquaculture light regimes could also be altered to reduce parasite attraction and activity.

4.2 Introduction

Parasites are a fundamental component of ecosystems; practically all known species carry parasites and food webs can be dominated by their presence (Marcogliese and Cone 1997; Poulin and Morand 2000; Lafferty *et al.* 2006; Dobson *et al.* 2008). In addition to their ecological importance, parasitic infections play a critical role in the global health of humans and both domesticated and wild species. Conflict between humans and parasites drives development and use of control strategies to prevent and reduce the health and socio-economic impacts of infection. Understanding behaviour can aid development and employment of control strategies, but research tends to focus on host rather than parasite behaviours (Barnard 1990; Sukhdeo and Chappell 1994; Lewis *et al.* 2002). This is despite the fact that parasites have developed a wide range of complex behaviours to facilitate transmission, infection, reproduction, and survival (Rea and Irwin 1994; Sukhdeo and Chappell 1994; Lewis *et al.* 2002). Behaviours involved in host finding are of particular interest regarding development of control strategies to interrupt and prevent infection. Many parasites have adopted 'active' host

finding behaviours to locate suitable hosts, whereby a parasite responds to environmental and/or host signals (Rea and Irwin 1994). Parasites utilise a range of stimuli (such as chemical, thermal, mechanical, and visual), often in combination to locate hosts and assess their suitability (Van Leerdam *et al.* 1985; Ashton *et al.* 1999; Bailey *et al.* 2006; Mordue (Luntz) and Birkett 2009).

Organisms can temporally synchronise to their environment by detecting and responding to external cues, resulting in biological rhythms of physiology and behaviour (Vitaterna *et al.* 2001; Bell-Pedersen *et al.* 2005). Light/dark cycles are the dominant cue for a majority of organisms, however for parasites both environmental and host cues influence rhythmicity (Bell-Pedersen *et al.* 2005; Reece *et al.* 2017). By synchronising with hosts, parasites can increase their survival. During dispersal and transmission, rhythms allow parasites to maximise infection success by optimizing presence of infective stages with host availability (Sukhdeo and Chappell 1994; Bogéa *et al.* 1996). In addition, infection success and parasite survival can be influenced by fluctuations (daily and/or seasonal) in host immune responses (Martinez-Bakker and Helm 2015; Kiessling *et al.* 2017; Carvalho Cabral *et al.* 2019). Identification of cues used by parasites and the rhythms they exhibit could help reduce infection and transmission risks. For example, by avoiding/preventing access to known infection locations (e.g. top of the water column for marine sea lice) during peak parasite presence or deploying control measures at such times to maximise capture.

The environmental and/or host cues utilised by parasites can differ between life stage or sexes. While this can reduce the efficacy of broad control applications and induce bias, it can also be used for highly targeted control. Sex-specific control schemes have been employed to successfully reduce parasite populations or their vectors. Females can be targeted to reduce the next generation by directly removing reproducers, while male targeting uses sterilisation and release techniques to lower population fecundity (Epsky *et al.* 1999; Alphey *et al.* 2010). Discrete sexes can be caught using sex-specific behaviours such as pheromone or food-based attraction (Epsky *et al.* 1999). These sex-specific behaviours likely lead to sex-specific rhythms, which could also be exploited to further promote control success. Sexual differences in parasite rhythms have yet to be explored (Sikkel *et al.* 2009).

Fish lice are ectoparasitic crustaceans, problematic worldwide in fisheries. Control options are limited, with reduction in chemical applications due to environmental concerns and rising drug

resistance (Costello 2009; Taylor *et al.* 2005a). Recent developments in control of marine sea lice capitalises on louse behaviour: lice frequent the top of the water column, consequently fish are held at >10 m below sea surface to reduce infection. For freshwater lice (Genus *Argulus*) however, control options remain insufficient with some farmers turning to illegal options (Taylor *et al.* 2005b). Thus, there is a need to explore alternative, behaviour-based control methods. *Argulus* spp. are unusual in that they retain the ability to free swim throughout their life cycle with host switching frequent, especially among male parasites as they seek female partners (Bandilla *et al.* 2008). No studies have tested for the presence of endogenous rhythms in *Argulus* spp. (or any other aquatic ectoparasitic crustacean), although a diurnal pattern is present in the strength of their positive phototaxis response (Yoshizawa and Nogami 2008). *Argulus* spp. also react to light/dark changes with differing activity, however this has not been observed over a circadian period or between sexes (Mikheev *et al.* 1999).

Here we examine host seeking behaviour of a globally problematic fish ectoparasite over a diurnal period, testing for the presence of endogenous cues in males and females. Strength of light attraction and wavelength specific preferences are also assessed to aid control development.

4.3 Materials and methods

Parasite and host maintenance

Argulus spp. used in this study were collected from Risca Canal (Newport, UK; grid reference: ST 24344 90686) on 06/06/18 and 07/08/19 by hand netting naturally infected three-spined stickleback *Gasterosteus aculeatus.* Parasites were removed from fish in the field by lifting the host fish out of water using a net for a 10 s period; upon re-submersion into a container of freshwater the parasite detached and was collected using a wide-bore pipette. *Argulus* spp. were transported to the laboratory off host in sealed containers of dechlorinated water. Once in the Cardiff aquarium, parasites were morphologically identified as *A. foliaceus* (according to Fryer 1982) and maintained in male/female pairs on three-spined sticklebacks collected from Roath Brook, Cardiff (ST 18897 78541; an *Argulus* spp. naïve population). Fish were infected by placing the parasites into fish holding tanks (9 L) containing an individual stickleback to allow natural parasite attachment. All fish and parasites were maintained under a 12 h light:12 h dark cycle, with fish fed daily with *Tubifex* bloodworm. *A. foliaceus* were acclimated to laboratory conditions on their hosts for 1 week prior to experimentation. Parasites were not re-used within or across experiments. For the circadian rhythm experiment both male and non-gravid female

parasites were used. For the light attraction/colour preference experiments only male *A. foliaceus* were used due to higher availability of male parasites versus non-gravid females (as female parasites continuously produce eggs after mating and egg bearing females exhibit egg laying behaviour when off host).

A. foliaceus were removed from sticklebacks for use in experiments using the same collection method as described above. All *A. foliaceus* were checked visually for damage before use and measured from the rostral edge of the carapace to the anterior end of the abdominal lobes using a dissecting microscope at x10 magnification with a Lumenera Infinity 1 camera and Infinity Capture software version 6.5.4.

Circadian rhythm of parasite swimming activity off host

To understand how *A. foliaceus* behave off host/during transmission over a circadian period, individual adult male and non-gravid female *A. foliaceus* (males: N = 22, average size = 3.93 mm ± 0.23 SD, females: N = 18, average size = 4.43 mm ± 0.44 SD,) were placed into glass petri dishes (10 cm diameter) filled with 50 ml dechlorinated water. The water level in the petri dishes was sufficient to allow full horizontal movement, while minimizing vertical motion for behavioural tracking. Additionally, the sides of each dish were covered with white fabric to reduce reflections and prevent visual disturbance (Mikheev *et al.* 1998). Parasites were then subject to 12 h light:12 h dark (LD; average 1000 lux) for 48h, after which they were removed from the setup, given one day of recovery on stickleback hosts (to allow feeding/prevent starvation) before returning to the setup for another 48 h under total darkness (DD). The order of light condition (12:12 LD vs DD) could not be randomised as the total darkness regime would disrupt any entrained circadian rhythm, altering any tests post exposure. The setup was completely reset between trials and light condition tests. Parasite behaviour was recorded during the 48 h exposures via 24 h infrared CCTV cameras (Sentient Pro HDA DVR 8 Channel CCTV, Maplin). Every 4 h (zeitgeber time = ZT, ZT0 = 7 am, ZT4 = 11 am, ZT8 = 3 pm, ZT12 = 7 pm, ZT16 = 11 pm, ZT20 = 3 am; lights on at ZT0 and off at ZT12) the total distance covered by the parasite and subsequent average swimming speed was calculated over a 2 min period using ImageJ version 1.51j8 (Schneider *et al.* 2012) to prepare video files for analysis and Kinovea version 0.8.27 (Ganni *et al.* 2018) to track parasite movement. Proportion of time spent swimming was obtained from Kinovea by calculating the time spent swimming at >1 mm/s (approximately ¼ body length). Patterns of parasite activity were then assessed for a 24 h period and between 12:12 LD/DD trials to determine activity and entrainment of rhythm.

Argulus light attraction in the presence of fish hosts

The attraction of *A. foliaceus* to a light source versus a live fish host was assessed using two different behavioural assays: fish vs light trials in which adult male *A. foliaceus* were given the choice of either a white light or a stickleback in darkness over a 24 h period (N = 20 parasites, average size = 4.12 mm ± 0.31 SD; figure 4.1A), and lit fish vs dark fish trials offering the choice of a stickleback with a white light or a stickleback in darkness (N = 18 parasites, average size = 4.14 mm ± 0.35 SD; figure 4.1B) over a 2 h period. Arenas comprised of glass tank filled to 10 cm water depth, split into three identical sized sections (left, middle and right) using a 1 cm aperture mesh to allow free movement of parasites while restricting fish movement (figure 4.1A and B). Stimuli were placed into the left and right thirds, with two *A. foliaceus* restrained under a glass dish in the middle third for 30 min to allow acclimation. After acclimation, the lice were released and monitored via infrared CCTV cameras. All light stimuli used a waterproof LED white light (average 50 lux at a distance of 7 cm), while all stimuli in darkness contained the same type of LED white light but turned off to ensure each section had the same structure. The positions of the stimuli were swapped in between trials to avoid any potential side bias. For the lit fish vs dark fish trials, all host pairs were size matched.

Argulus light colour preference

To investigate whether certain wavelengths of light are more attractive to *A. foliaceus*, adult males (N = 20, average size = 4.08 mm ± 0.33 SD) were placed individually into the centre of a 2.5 L opaque white square arena (14 x 14 cm) filled with 1 L water (5 cm water depth). The arena was split into four equal quarters, with four waterproof lights (3 x 3 x 2 cm, LED with RGB colour) placed into the arena and positioned flush inside each corner (figure 4.1C). Lights were randomly assigned to emit either red (635 - 700 nm), green (520 - 560 nm), blue (450 - 490 nm), or white (emits all wavelengths, 450 - 700 nm) light, with brightness controlled so each light individually generated an average 50 lux (lux meter positioned 7 cm away from light). There was no visual overlap in the colours emitted from each light, and initial testing found that parasites did not swim erratically or behave in any other abnormal manner in the experimental arena (following previous observations in the lab and by Mikheev *et al.* 1998). The inclusion of an acclimation period in initial testing also had no impact on parasite behaviour, thus parasites were observed immediately after introduction to the arena and did not experience any time break between replicate recordings. After being introduced to the centre of the arena, parasites were monitored for 2 min with their time at each colour recorded.

Location at a colour was classified as the parasite being present anywhere in the quarter containing the light (with more than half of the parasites body present in the quarter for when the parasite crossed between sections). Parasites were observed live, with the observer stationed next to the arena looking down into the tank. Room lights were turned off so the only light source during experimentation came from the lights in the arena - this provided enough light to observe parasite movement while preventing casting of shadows into the arena from the observer. Individual parasites were tested 3 times consecutively with average time spent in each light corner calculated. The arena was reset and light position randomised between each replicate. Parasites did not linger or remain stationary on boundary lines between quarters during observations.

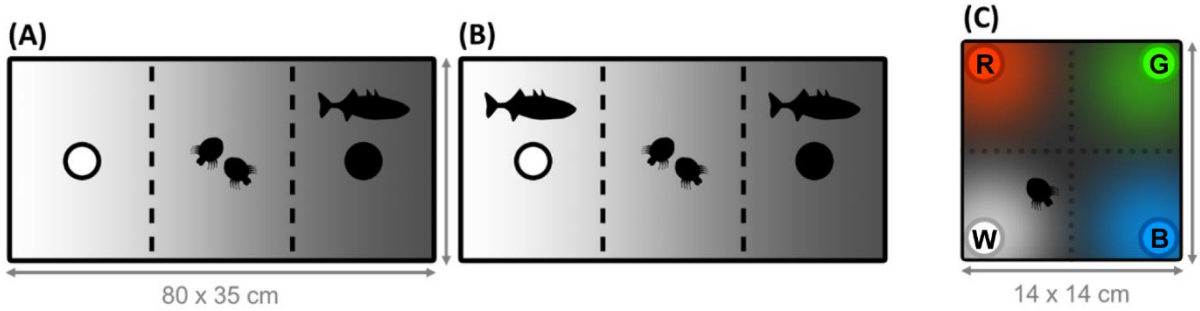

Figure 4.1 Plan view of experimental arenas for *Argulus foliaceus* **A/B**: light vs fish host preference and **C**: light colour preference trials. In each arena circles represent LED light sources. Arena A gives a choice of white light vs a three-spined stickleback (*Gasterosteus aculeatus*) host with a turned off light, B gives a choice of a white light + stickleback vs a turned off light + stickleback. In A/B, dashed lines represent 1cm aperture mesh which allows the parasites to swim through while blocking fish movement. In C dotted lines indicate the total area of each coloured corner for behavioural recording, R = red light, G = green, W = white and B = blue (coloured light placement was changed/randomised for each trial).

Statistical analysis

All statistical analyses were conducted using R statistical software (v3.6.2; R Core Team 2017) with the level of significance in all tests taken as $p<0.05$. Models were refined through stepwise deletion of insignificant terms and Akaike information criterion comparisons, with visual examination of model plots to check standardised residuals for normal distribution and homogeneity of variance (Crawley 2007). The following packages were used for analyses: "ggplot2" to visualise data (Wickham 2009), "lme4" to run general linear mixed models (Bates

et al. 2014), "emmeans" for post-hoc analyses (Searle *et al.* 1980), "RAIN" and "MetaCycle" to determine circadian rhythmicity (Thaben and Westermark 2014; Wu *et al.* 2016) and "circacompare" to compare rhythms (Parsons *et al.* 2020). For all rhythm analysis the time period being examined was set to 24 h.

To detect rhythmicity, RAIN was used due to its capability in detecting and accounting for asymmetrical patterns (Thaben and Westermark 2014) alongside MetaCycle due to its inclusion of multiple methods for rhythm evaluation (Wu *et al.* 2016). The test "rainresult" was used to examine patterns across parasite sex and light condition by examining phase and peak shape. The phase of a rhythm refers to the time point at which a peak occurs, with peak shape the time (in this case hours) between a peak and the next trough. Comparison of rhythms between different conditions were then investigated using circacompare to assess MESOR (Midline Estimating Statistic of Rhythm), amplitude and phase across rhythms. MESOR is a mean value adjusted for circadian rhythms, amplitude refers to "a measure of half the extent of predictable variation within a cycle" (Cornelissen 2014; Otsuka *et al.* 2016). A general linear mixed model (GLMM) with gaussian family and identity link function was then conducted using only the 12:12 LD data to compare activity at each ZT time point by examining *A. foliaceus* activity against ZT time, parasite sex and length with an interaction between ZT time/parasite sex. This GLMM was then repeated using the DD trials only. All GLMMs used parasite ID as a random factor to account for pseudoreplication. To determine *A. foliaceus* colour preference, a general linear model (GLM) with gaussian family and identity link function was used to compare swimming activity (average over 3 trials) against light colour and parasite length. Across all tests and trials, parasite length had no significant impact and is thus not reported further.

4.4 Results

Circadian rhythm of parasite swimming activity off host

A strong diurnal pattern in off host swimming activity was observed for both male and female *A. foliaceus* when maintained under 12:12 LD conditions (RAIN $p<0.001$ for both males and females, MetaCycle $p<0.001/0.004$ for males/females respectively; figure 4.2); however, under total darkness (DD) this diurnal rhythm was lost, suggesting this pattern is stimulated by light and not endogenously driven. Under 12:12 LD, male parasites had different phase to females ($p = 0.02$, male phase = 5.69 h post ZT0, female = 8.56 h, circacompare), but there was no difference in MESOR or amplitude (figure 4.3).

Under 12:12 LD, overall average swimming speed of *A. foliaceus* did not differ among sexes (0.77 and 0.83 cm/s for males and females respectively). However, when directly comparing ZT timepoints females had a significantly higher swim speed at ZT12 (7pm when the lights turn off; t-ratio = 2.92, df = 127, p = 0.004, GLMM; figure 4.2). Under DD, females had marginally significant higher overall activity than males (0.86 cm/s for females, 0.62 cm/s for males; likelihood ratio test value = 3.86, p = 0.04, GLMM). When examining the proportion of time spent swimming, no patterns were observed except for females under DD which showed a peak at ZT0/20 and drop at ZT8/12 (Rain p = 0.005, MetaCycle p = 0.04; figure 4.4).

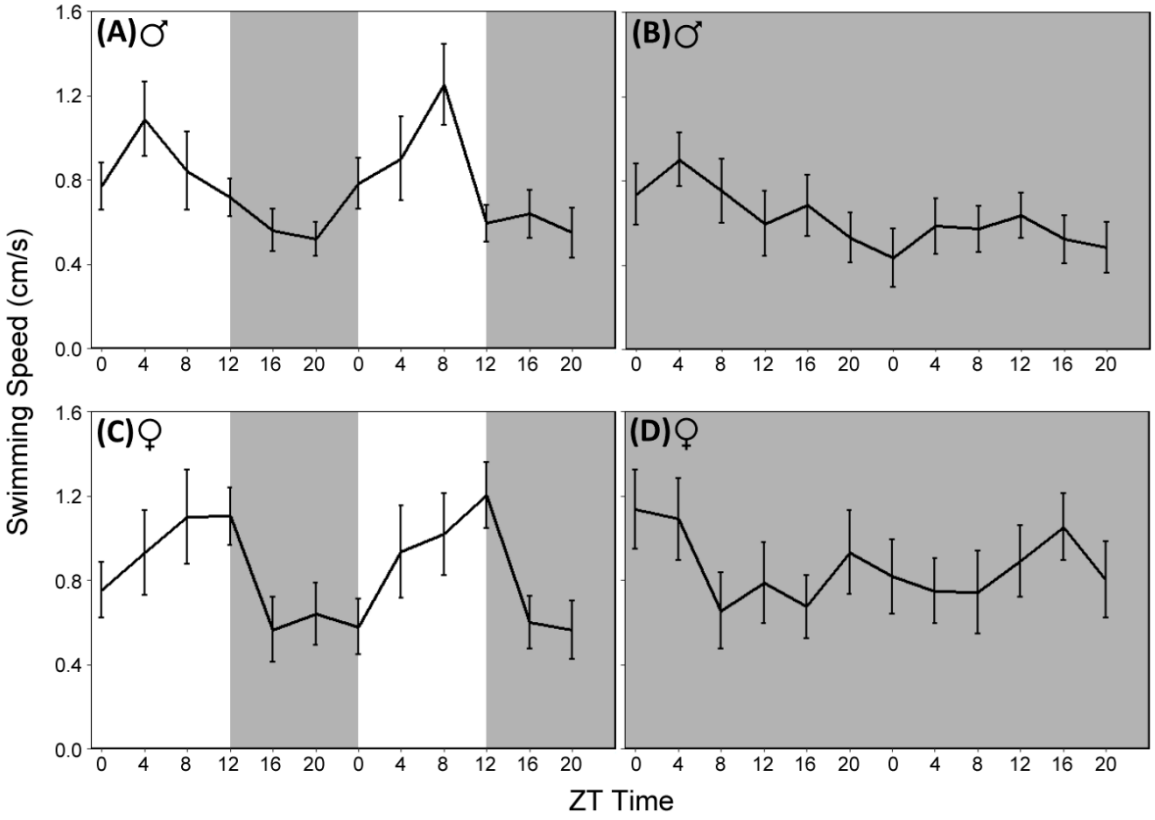

Figure 4.2 Average swimming speed of *Argulus foliaceus* off host over a 48 h period under two different light conditions. **A**: Male *A. foliaceus* under 12h light:12h dark. **B**: Male *A. foliaceus* under total darkness. **C**: Female *A. foliaceus* under 12h light:12h dark. **D**: Female *A. foliaceus* under total darkness. White backgrounds indicate periods of light, dark grey backgrounds indicate periods of darkness. ZT0 = 7am, ZT12 = 7pm.

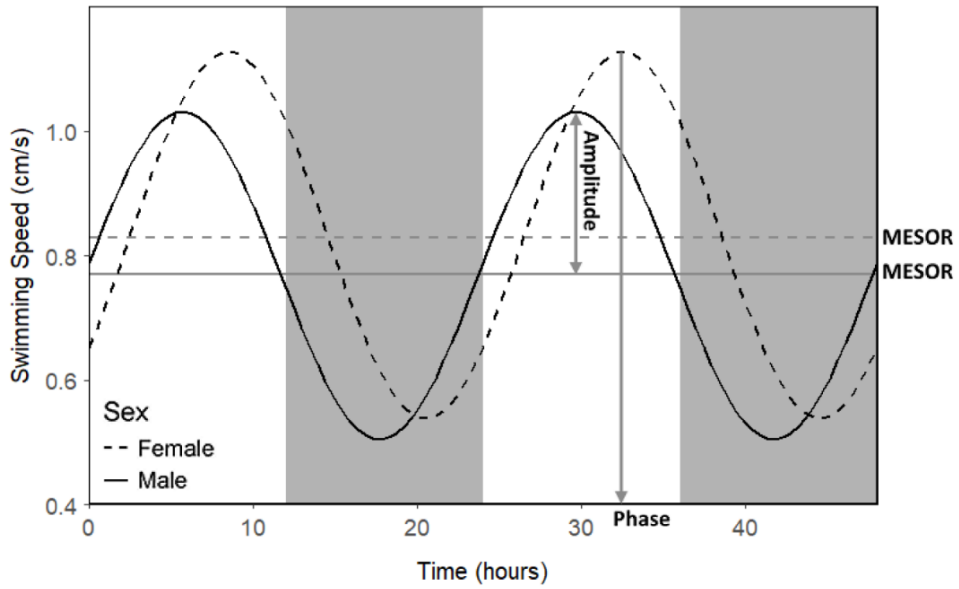

Figure 4.3 Circacompare output plot of male and female *Argulus foliaceus* swimming speed over a 12h light:12h dark 48 h period. Lights turn on/off at 0/12 and 24/36.

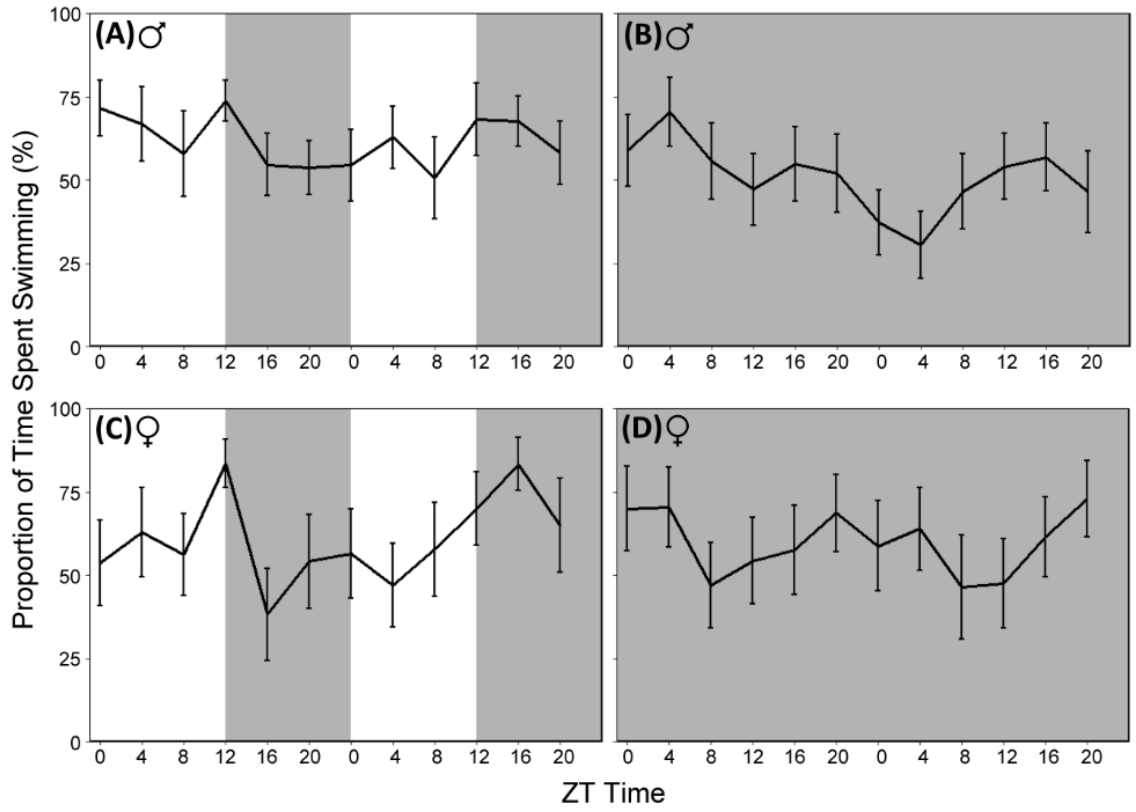

Figure 4.4 Proportion of time *Argulus foliaceus* spent swimming off host over a 48 h period under differing light conditions. **A**: Male *A. foliaceus* under 12h light:12h dark. **B**: Male *A. foliaceus* under total darkness. **C**: Female *A. foliaceus* under 12h light:12h dark. **D**: Female *A. foliaceus* under total darkness. White backgrounds indicate periods of light, dark grey backgrounds indicate periods of darkness. ZT0 = 7am, ZT12 = 7pm.

Argulus light attraction in the presence of fish hosts

When assessing preference between a light stimulus or a fish host, the average time taken for lice to first enter the light section was 59 s. After 24 h, 85% of parasites were located at the light stimulus and the remaining 15% had been consumed by the fish host (time to consumption ranged from 11 s - 378 s). No fish became infected during these trials.

For trials assessing preference between a fish host with or without a light source turned on, 100% of parasites moved to the section containing a fish host with a light on. After 2 h, 17% of these parasites had been eaten by the fish, 22% infected the fish and 61% remained swimming around this section.

Argulus light colour preference

A. foliaceus significantly preferred white and blue coloured light over green or red (all comparisons t-value = 3.818 - 5.689, $p<0.001$, except white vs green in which t-value = 2.214, $p = 0.03$, GLM), with preference for blue light over white close to significance ($p = 0.05$; figure 4.5).

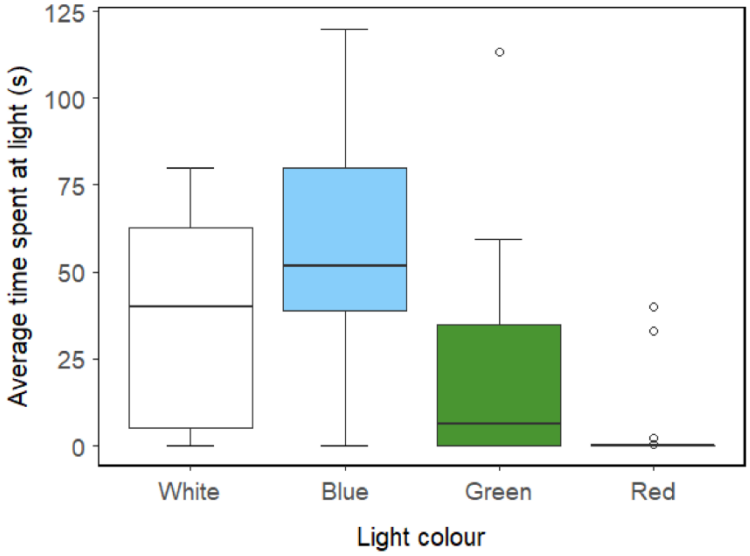

Figure 4.5 Light preference of male *Argulus foliaceus* off the host. Average time spent by free-swimming *A. foliaceus* in the vicinity of different coloured lights over a 2 minute period. White light wavelength = 450 - 700 nm, blue = 450 - 490 nm, green = 520 - 560 nm, red = 635 - 700 nm.

4.5 Discussion

During dispersal, hosts provide a spatially patchy environment in which parasites need to anticipate host availability (Skelton *et al.* 2015). As such, parasites must develop strategies to increase host-parasite contact and facilitate infection and transmission. In many parasites this

involves host-seeking behaviours and synchronisation with their hosts. For fish lice, hosts are located by free-swimming parasites responding to host and environmental cues, with light their dominant stimulus (Bandilla *et al.* 2007). While previous studies have recorded variations in fish lice behaviour over diurnal periods (Yoshizawa and Nogami 2008; Heuch *et al.* 2011), none have determined if these rhythms are endogenously driven. Here *A. foliaceus* off host activity followed a diurnal, non-endogenous, circadian pattern as the distinct behavioural rhythm under light/dark conditions was lost under total darkness. There was also a sexual difference in off host behaviour with male and female rhythms offset by approximately 4 h. When examining light attraction *A. foliaceus* consistently displayed a strong attraction to light over combined host cues (in the form of a live host) and preferred shorter wavelengths of light.

Argulus spp. display sexually dimorphic host switching behaviour with males frequently leaving their hosts to find mates while non-gravid females remain on host (Bandilla *et al.* 2008). This dimorphism continues in off host behaviour. As shown previously by (Mikheev *et al.* 1999), female *A. foliaceus* had highest activity when the lights turned off and low activity when lights turned on. Examining activity over a circadian period however indicates that this is not sustained, 4+ hours after lights turn off female parasite activity drops, and inversely 4+ hours after lights turn on female activity increases. Males do not follow the same pattern with activity consistently higher during light periods and lower during dark periods. The continued high average speed of females when lights turn off (versus a drop in activity for males) could be related to their host switching behaviours: females are not predisposed to spending time off host, and thus may not react as quickly as males to light changes. Alternatively, the lights used in this study (and in Mikheev *et al.* 1999) turned on/off immediately and could be simulating a passing shadow (a trigger of fish lice activity, Bohn 1910; Poulin *et al.* 1990). Females could react stronger than males to potential host cues (due to a higher tendency for females to remain on the host) resulting in high activity when lights turn off. The distinct and strong diurnal rhythm observed when using average swimming speed measurements was not observed when using measurements that only record time spent active. Average swim speed is more comprehensive accounting for variation in activity, while time spent active (i.e. a simple proportion of time moving or not) cannot discern these nuances and would lead to assumption of arrhythmic behaviour. This highlights the importance of selecting the correct activity measure when assessing rhythmical patterns in behaviour.

Light is an integral component of aquaculture systems, with differing light wavelengths, intensity and photo periods used to manipulate fish growth and maturation (Boeuf and Le Bail 1999; Oppedal *et al.* 1999; Villamizar *et al.* 2011). The subsequent impact of these altered light regimes on both fish behaviour and health is now being considered. Recent studies have also found parasitic infection can alter host circadian gene expression, further complicating the relationship between parasites, hosts and the rhythms they both follow (Ellison *et al.* 2018; 2020). Considering the positive phototactic response of fish lice, aquaculture lights could attract lice to cages and facilitate infection (Trippel 2010; Stewart *et al.* 2013). In this study male *A. foliaceus* were more active under light versus dark, suggesting lit cages would not only attract lice but also increase their activity which could lead to higher infection success. Shifting the wavelength of light used in aquaculture systems could potentially allow retention of fish manipulation, while limiting the impact on pathogenic organisms. For example, when inhibiting *Salmo salar* sexual maturation to increase production, green and red light treatments used less energy versus white light treatments (Leclercq *et al.* 2011). Additionally, *Oncorhynchus mykiss* raised under red light showed improved growth compared to fish raised under blue or white light (Karakatsouli *et al.* 2008). Red light was the least attractive light colour to *A. foliaceus* (and *A. japonicus*: see Yoshizawa and Nogami 2008), therefore cages lit with red light could attract less parasites to those lit with shorter wavelengths. This may only be beneficial in outdoor systems where wild parasites enter containers/cages to infect fish, versus enclosed systems where parasites may be trapped in with the fish.

In addition to altering the light regimes in aquaculture to reduce parasite attraction and infection, light could be used to purposefully attract parasites into traps. Light traps have successfully captured sea lice in both the lab and field (where, in comparison, plankton tows captured none) and were suggested as a monitoring tool (Novales Flamarique *et al.* 2009). Unlike sea lice which show differing reaction strength to light across their life stages, *Argulus* spp. appear to be consistent in their light attraction from hatching to adulthood (Bai 1981; Novales Flamarique *et al.* 2000; Bandilla *et al.* 2007; Novales Flamarique *et al.* 2009). Additionally, freshwater habitats used for aquaculture are often small, enclosed areas (e.g. rearing ponds and raceways, recreational fishing lakes and reservoirs) relative to marine systems, potentially increasing the chance of *Argulus* spp. to encounter traps. Therefore, light traps could be more effective and a feasible management tool for freshwater fisheries and aquaculture. Our findings suggest that over relatively short distances lice are strongly attracted to light, therefore future studies should

examine the attraction distance of light coupled with trials in freshwater aquaculture systems to determine the efficacy of light traps in controlling lice infections.

Parasite behaviour can be complex and diverse with host cues, external stimulus and diurnal rhythms all affecting parasite activity. When developing control strategies, understanding behaviour allows more effective application (i.e. during parasite emergence) and offers the potential for identifying new targets for control. Sexual differences are also critical to consider, as differing behaviour could lead to one sex avoiding control application. By understanding and manipulating parasites, the impact of infection on global health and economics can be reduced. Parasite behaviour is therefore an important component of management and should be considered for all problematic infections.

Chapter 5

Daily patterns in parasite processes: diel variation of fish louse transcriptomes

5.1 Abstract

Parasites, like all other organisms, time themselves to environmental cues using a molecular clock to generate and maintain circadian rhythms. Chronotherapeutic (timed treatment) techniques based on such rhythms offer great potential for improving control of chronic, problematic parasites. Fish lice are a key disease threat in aquaculture, with current control insufficient. Assessing the rhythmicity of fish lice transcriptomes offers not only insight into the viability of chronotherapy, but the opportunity to identify new drug targets. Here, for the first time in any crustacean parasite, diel changes in gene expression are examined, revealing that approximately half of the *Argulus foliaceus* annotated transcriptome displays significant daily rhythmicity. We identified rhythmically expressed putative clock genes, including core *Clock/Cycle* and *Period/Timeless* pairs, alongside rhythms in feeding-associated genes and processes involving immune response, as well as fish louse drug targets. A substantial number of gene pathways showed peak expression in hours immediately preceding onset of light, potentially in anticipation of peak host anti-parasite responses or in preparation for increased feeding activity. Genes related to immune haemocyte activity and chitin development were more highly expressed 4 h post light onset, although inflammatory gene expression was highest during dark periods. Our study provides an important resource for application of chronotherapy in fish lice; timed application could increase efficacy and/or reduce dose requirement, improving the current landscape of drug resistance and fish health while reducing the economic cost of infection.

5.2 Introduction

Temporal rhythms are a core element of life on earth, with most environmental cues cycling over tidal (12.4 h), daily (24 h), lunar (monthly) and/or seasonal scales (Helm and Stevenson 2014; Neumann 2014). Of these rhythmic cues, the most dominant is the daily change in light (Vitaterna *et al.* 2001). These highly predictable light cycles - which in turn drive diel variation in other factors such as temperature and food availability - have led to the evolution of endogenous circadian rhythms, wherein environmental cues entrain feedback loops to regulate patterns in activity and biological processes (Vitaterna *et al.* 2001; Bell-Pedersen *et al.* 2005).

Organisms often face entrainment from multiple oscillators, with symbionts experiencing an additional set of cues originating from host rhythms (Rijo-Ferreira *et al.* 2017a). Despite the impact of parasites on human and animal health, parasite rhythms are severely understudied compared to free-living organisms with most investigations into parasite-host systems focusing on the host. Parasites and their associated hosts are intertwined in an arms race; to fully understand one you must consider the other, thus investigating parasite rhythms is key to improving global health (Martinez-Bakker and Helm 2015).

Parasite rhythm studies focus on transmission and reproductive behaviours as these are key for infection dynamics (Martinez-Bakker and Helm 2015). These include temporal peaks in malaria asexual reproduction (with the exact mechanism behind this still unconfirmed; Mideo *et al.* 2013), rhythmical discharge of infective stages including eggs, oocysts and cercariae, which typically synchronise with host behaviours to maximise transmission (Hawking 1975; Bogéa *et al.* 1996; Lu *et al.* 2009; Martinez-Bakker and Helm 2015) and timed behavioural manipulation of hosts to promote transmission to a secondary host (Trail 1980; de Bekker *et al.* 2014). Genetic studies of parasite circadian rhythms have primarily involved identification of putative 'clock' genes required to generate rhythms (Hevia *et al.* 2015; Sun *et al.* 2018; Rawlinson *et al.* 2021). More recently whole transcriptome rhythmicity has been assessed in a few parasitic species (fungi, de Bekker *et al.* 2017; protists *Plasmodium* spp., Smith *et al.* 2020 and trypanosomes, Rijo-Ferreira *et al.* 2017b). These studies reveal substantial portions of parasite gene repertoires may be expressed rhythmically over 24 h periods, even in endoparasites not directly exposed to light cycles. Like free-living organisms, parasites likely need to anticipate daily variations in risks and rewards such as host immune activity and nutrient availability (Rouzine and McKenzie 2003; O'Donnell *et al.* 2011; Prior *et al.* 2018).

Research into circadian rhythms of host immune systems and their pathogens has led to the development of chronotherapeutic control strategies - timed application of treatments to improve efficacy and/or reduce dosage (Smolensky and Peppas 2007). Synchronisation to patient rhythms such as cell-cycle stage or blood pressure is forefront (Hermida *et al.* 2003; Altinok *et al.* 2007), with treatments timed to exploit parasite rhythms under investigation. Experimental trials with malaria show promise (Cambie *et al.* 1991; Owolabi *et al.* 2021) and the potential application of timed treatment has been documented for other parasite species (Honorio-Franca *et al.* 2013; Davis *et al.* 2018). Despite the rapidly developing chronotherapeutic revolution in human medicine, the potential for harnessing chronobiology

to understand the dynamics of both captive and wild animal health remains largely unexplored (Smolensky and D'alonzo 1993; Reinberg and Smolensky 2012; McKenna *et al.* 2018).

Integrated chronotherapeutic strategies could be key for improving parasite control. Ectoparasitic fish lice - large crustaceans that attach to fish skin and cause extensive mechanical damage (Møller 2006) - have plagued farmers since the conception of aquaculture. Light appears an important external cue for both marine and freshwater species, influencing activity (Novales Flamarique *et al.* 2000; Bandilla *et al.* 2007; Yoshizawa and Nogami 2008; Novales Flamarique *et al.* 2009; Chapter 4), transmission (Mikheev *et al.* 1999) and reproduction (Shimura and Egusa 1980; Bai 1981; Harrison *et al.* 2007). Until recently, chemical treatments have been the main control method in fish farms (Grave *et al.* 2004). However, rising drug resistance, stress to fish and environmental concern over their use means treatments need to be used more sparingly while remaining effective (Aaen *et al.* 2015). Considering the strong evidence of daily rhythmic phenotypes in fish lice, and the urgent need to improve louse control in aquaculture, profiling of fish lice at the transcriptional level over daily cycles is a vital first step towards a deeper chronobiological understanding of these economically devastating parasites and the development of novel mitigation strategies.

Here we examined transcriptome rhythmicity of *A. foliaceus*, the most common freshwater fish louse in the UK (also found throughout temperate areas of Europe, Central Asia, and North America). *A. foliaceus* infections are a chronic problem in UK fisheries with treatment limited compared to marine systems due to environmental concerns. Despite taxonomic differences, *Argulus* spp. also act as a sea louse model due to their comparable functionality and impact on hosts. *Argulus* spp. *de novo* transcriptomes have been generated previously to mine for specific therapeutic targets including neuropeptides, hemocyanin and feeding-associated proteins (Sahoo *et al.* 2013b; Christie 2014; Pinnow *et al.* 2016; AmbuAli *et al.* 2021). This study is the first to investigate diel changes in gene expression and identify putative clock genes in a crustacean parasite, alongside rhythmical key processes relating to infection and potential drug targets.

5.3 Methods

Animal husbandry

Juvenile triploid female rainbow trout (*Oncorhynchus mykiss*) were acquired from Bibury Trout Farm in January 2019 and maintained at Cardiff University at 14 °C in a recirculating

aquaculture system. Fish were clear of external parasites, established by lightly anesthetising fish with 0.02% MS222 (tricaine methanesulfonate) and examining them under a dissection microscope with fibre optic illumination. Throughout the study, fish were maintained under a 12h light:12h dark light cycle and fed daily with commercially available trout food (Nutraparr, Skretting, UK) *ad libitum.*

Parasite culture

A. foliaceus eggs were collected by capturing adult females (morphologically identified according to Fryer, 1982) from Risca Canal (Newport, UK; grid reference: ST 24344 90686) by hand netting naturally infected three-spined stickleback *Gasterosteus aculeatus*. Lice were removed from fish in the field by lifting the host fish out of water using a net for a 10 s period; upon re-submersion into a container of freshwater the parasite detached and was collected using a wide-bore pipette. Lice were transported to the lab off host in sealed containers of dechlorinated water. Once in the Cardiff aquarium, eggs laid by female parasites were collected and stored at <10 °C to prevent incubation (Shafir and van As 1986; Mikheev *et al.* 2001). To hatch *A. foliaceus* metanauplii, eggs were warmed to 24 °C (over approximately 8 h by placing eggs at ambient temperature) and maintained at 24 °C for 20 days with daily monitoring until hatching. Due to the high mortality of lice during the first week of infection (Chapter 3) and to ensure lice would be large enough to harvest sufficient RNA, *A. foliaceus* were cultured on rainbow trout (average 10 cm standard length, SL) prior to experimentation. Rainbow trout were infected with 50 metanauplii each (within 24 h of hatching) by placing the fish into a small receptacle of water and introducing the parasite via a pipette, allowing for natural attachment of the parasite to the host. Fish were monitored daily for 1 week at 18 °C, after which all *A. foliaceus* were removed from hosts.

Sample collection

Argulus naïve rainbow trout (SL = 60.1 ± 4.94 mm, range 47.5 - 69.0 mm, N = 49) were infected using the same method as above with 7 individuals of *A. foliaceus* per fish (collected from the culture, average parasite length = 0.88 mm ± 0.10 SD, range = 0.73 - 1.04 mm). Fish were held individually in 4 L tanks at 14 °C, with cleaning every 48 h and feeding at 10 am every day. After 1 week, 7 fish were sampled every 4 h over a 24 h period (starting at 7 am and finishing with a sample at 7 am the next day; zeitgeber time = ZT, ZT0 = 7 am, ZT4 = 11 am, ZT8 = 3 pm, ZT12 = 7 pm, ZT16 = 11 pm, ZT20 = 3 am; lights on at ZT0 and off at ZT12). As two samples were collected for ZT0 (the first at the start of the recording period and the

second at the end to create a full loop of sampling), when referring to the ZT0 timepoints separately the starting ZT0 is labelled ZT0A with the ending ZT0 labelled ZT0B. During sampling all *A. foliaceus* on an individual host were collected using sterilised fine tweezers, taking care to only touch the lice and not the fish skin. All lice from an individual fish were pooled together into RNAlater to form one sample; the number of *A. foliaceus* collected from each fish ranged from 4 - 7 lice.

Transcriptome sequencing

Total RNA was extracted from samples using the QIAGEN RNeasy® Mini Kit and quantified using Qubit High-Sensitivity RNA assays (Qubit® 3.0 Fluorometer, ThermoFisher Scientific: RNA concentration = 245 ± 63 ng/µl, range 88 - 352 ng/µl). RNA sequencing was performed in the School of Biosciences Genome Hub, Cardiff University. Quality control of the RNA samples was confirmed by TapeStation (TapeStation Analysis Software 3.2) with a minimum RIN score of 10.0. Out of 49 samples, 48 were used in sequencing; 13 for ZT0 and 7 each for ZT4, 8, 12, 16 and 20. Libraries were generated according to manufacturer's instructions (Illumina, San Diego, CA) using an Illumina NeoPrep Library Workstation. Prior to equimolar pooling, library quality was quantified and assessed using Agilent TapeStation. The library pool was run on an Illumina NextSeq500 Sequencer (2 × 75 PE) to achieve a minimum of 16 million read pairs per sample. Raw reads are available at NCBI BioProject ID: PRJNA764202 with metadata at figshare.com/s/3aa6cedc8ec1b4e6c754.

De novo assembly

Reads were filtered and trimmed using Trimmomatic version 0.38 to perform the following: cut 15 bases from the start of the read and cut bases off the start/end if they were below Q3, cut adapter/other Illumina-specific sequences from the read, trim anywhere within each read where a 4 bp window dropped below Q15 and drop any trimmed reads below 36 bp long. Fastqc version 0.11.7 was used before and after trimming as part of quality assessment. Prior to assembly, putative contaminant host reads were removed using BBsplit (Bushnell 2014) and published rainbow trout genome (Berthelot *et al.* 2014). *De novo* assembly was performed on the filtered trimmed reads using Trinity version 2.6.6 and the default parameters. Assembly quality was assessed by examining the ExN50 generated by the trinity report and applying BUSCO (Benchmarking universal single-copy orthologs) using BUSCO version 4.1.4 with the eukaryota_odb10 (2020-09-10) BUSCO set (Simão *et al.* 2015). To further ensure no host or other contaminant sequences were included, all contigs were screened using DIAMOND

(Buchfink *et al.* 2015) blastx searches against the NCBI nr database, retaining only those with an arthropod as the top hit (51,755 contigs). Annotation including gene ontology (GO) assignment was performed using the Trinotate automated pipeline (Bryant *et al.* 2017). Annotation output is available at figshare.com/s/de1515267957f9376a12.

Gene expression analyses

Filtered and trimmed reads were mapped against the *de novo* assembly using Trinity scripts (salmon abundance estimation method) to generate raw and normalised (TMM) count matrices at gene level. Raw counts were imported into R (v4.0.3; R Core Team 2020) and differential expression tests were performed using DESeq2 version 1.30.1 (Love *et al.* 2014) to compare expression between all time points. Rhythmically expressed genes were determined using eJTK (Hutchison *et al.* 2015) to examine normalised counts data, with circacompare version 0.1.1 (Parsons *et al.* 2020) used to plot rhythm graphs. For both DESeq2 and eJTK, false discovery rate (FDR) corrected p-values of less than 0.05 were considered for significant differential expression and rhythmicity. Principal component analysis (PCA) plots were generated in R using logged raw counts. GO functional enrichment tests were carried out using topGO version 2.42.0 (R) to detect significantly overrepresented biological processes of groups of rhythmically expressed genes with ViSEAGO version 1.4.0 used to visualize GO enrichment at each time point. GO functional enrichment tests were performed for all rhythmic genes and for genes with significantly different expression between ZT0A and ZT0B (determined via DESeq2). Putative clock genes were identified via manual searching with BLAST using model *Drosophila melanogaster* clock genes. The web-based tool Galaxy was also used to perform phylogenetically-informed annotation via a tree-based approach to look for light-interacting genes (Blankenberg *et al.* 2010; Speiser *et al.* 2014). Genes were putatively assigned as feeding-related by selecting protein sequences listed in AmbuAli *et al.* (2020) and NCBI entries which matched to proteins listed in AmbuAli *et al.* (2021) to manually search for hits against the transcriptome using BLAST.

5.4 Results

De Novo Assembly

Raw reads generated per sample ranged from 7.8 - 10.7 million reads, with a total of 168,593 transcripts generated for whole tissue samples of *A. foliaceus.* Out of 51,755 total putative arthropod genes, 10,290 were annotated. Percent GC was 41.05 with an average contig length of 1212.12 based on all transcript contigs and 676.19 based on longest isoform per gene. The

transcriptome assembly was considered good, with an N50 value of 2420 and an E90N50 value of 2125. BUSCO results reported 98.4% completeness.

Transcriptome rhythmicity

Analyses using eJTK found 4906 genes significantly rhythmic out of a total of 10,290 annotated genes (47.7%; output available at figshare.com/s/5fcbc7f613dc835015d3). Rhythmicity totals varied by phase, with 14% of genes peaking at phase 0, 19% at phase 4, 18% at phase 8, 9% at phase 12, 15% at phase 16 and 25% at phase 20 (figure 5.1). The genes with highest significance in rhythmicity included the core clock gene *Timeless* and various genes relating to molecular processes and feeding activity. Results from DESeq2 analyses did not show high numbers of significant genes when examining differences between two timepoints (output available at figshare.com/s/1da183599fce58d60946). However, when examining all significant genes across all timepoint comparisons (raw p-value with cut-off at <0.01) 96.6% of gene IDs matched to those found significant in eJTK analyses.

There is little clustering of individuals by time point when examining all rhythmic genes with a PCA plot (figure 5.1). When filtering the data to genes with $p<0.01$ rhythmicity according to eJTK analyses (453 genes), some clustering can be observed with three groups corresponding to ZT0A/4, ZT8/12/16 and ZT20/0B. When examining just the clock genes (which are known to have a 24 h period of rhythmicity), ZT0A and ZT0B overlap more compared to the $p<0.01$ plot. Investigation into differences in functional profiles between ZT0A and ZT0B revealed that the most significant differences were related to metabolic processes (involving amino sugars; GO:1901071, GO:0006040 and GO:0006022, carbohydrate derivatives; GO:1901135 and organonitrogen compounds; GO:1901564) and chitin/cuticle development (GO:0006030, GO:0042335 and GO:0040003).

Functional profiles displayed distinct blocks of activity and changes over a 24 h period (figure 5.2, output available at figshare.com/s/b609ebdde0d10047fced). Metabolic processes and localisation displayed most activity at ZT20, with high enrichment at ZT4 (one hour after host feeding) relating to amino sugar metabolic process (GO:0006040), glucosamine-containing compound metabolic process (GO:1901071) and chitin metabolic process (GO:0006030). Nucleobase-containing compound metabolic processes at ZT20 were related to RNA processing. Development at ZT20 and ZT0 involved reproductive development and neurogenesis, while cuticle development peaked at ZT4 (GO:0040003 and GO:0042335).

Immune system processes were particularly apparent at ZT4 (e.g. haemocyte activation/degranulation: GO:0043303, GO:0045576), and included a Toll-like receptor (TRINITY_DN15356; TLR2-like) and lectins (e.g. TRINITY_DN3872, lectin-1-like). Response to stress was enriched at ZT12, including regulation of acute inflammatory response (GO:0002673 and GO:0002526). Transport function peaks occurred across the 24 h period, with regulation of transport focused at ZT20.

Clock genes

In total 10 rhythmic putative clock genes were identified in *A. foliaceus* (table 5.1). Clock genes peaked in all phases except for ZT12. *Cycle* peaked from ZT20 - ZT0 when lights turned on with expression lowest just after at ZT4 - ZT8, inverse of *Clock* which peaked from ZT4 - ZT12 and was lowest from ZT20 - ZT0 (figure 5.3). Both *Period* and *Timeless* followed the same pattern with expression low during the light period and highest at ZT16 in the dark period. *Timeless* (TRINITY_DN37479_c0_g1) was also the second most significant rhythmic gene according to eJTK analyses.

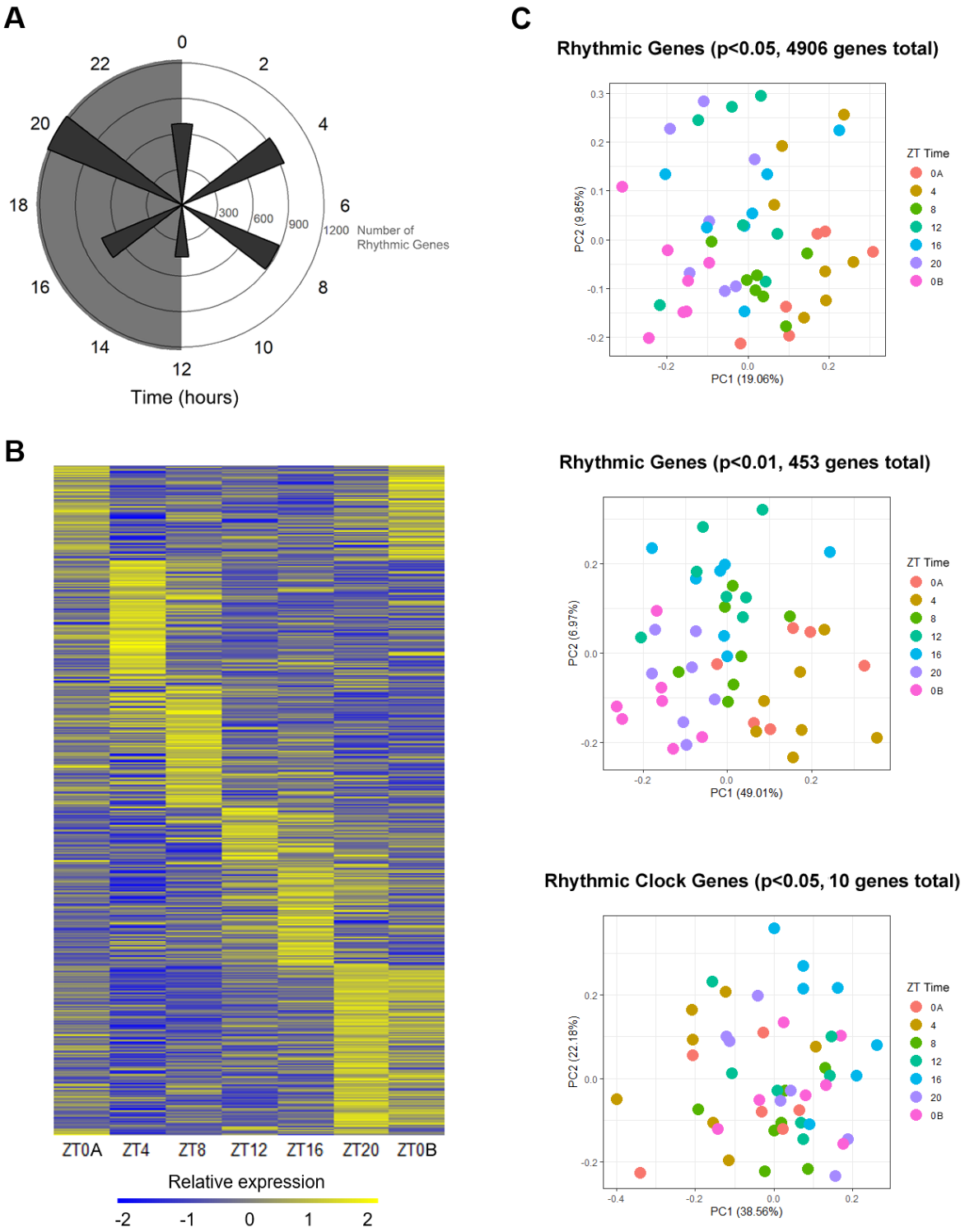

Figure 5.1 *Argulus foliaceus* rhythmic transcriptome assessment. **A**: Number of significantly rhythmic genes at each timepoint, white background = light period, grey background = dark period. **B**: Heatmap showing rhythmic gene expression over a 24 h period, ZT0 = 7am/light on, ZT12 = 7pm/light off. **C**: Principal component analysis plots of rhythmic gene expression.

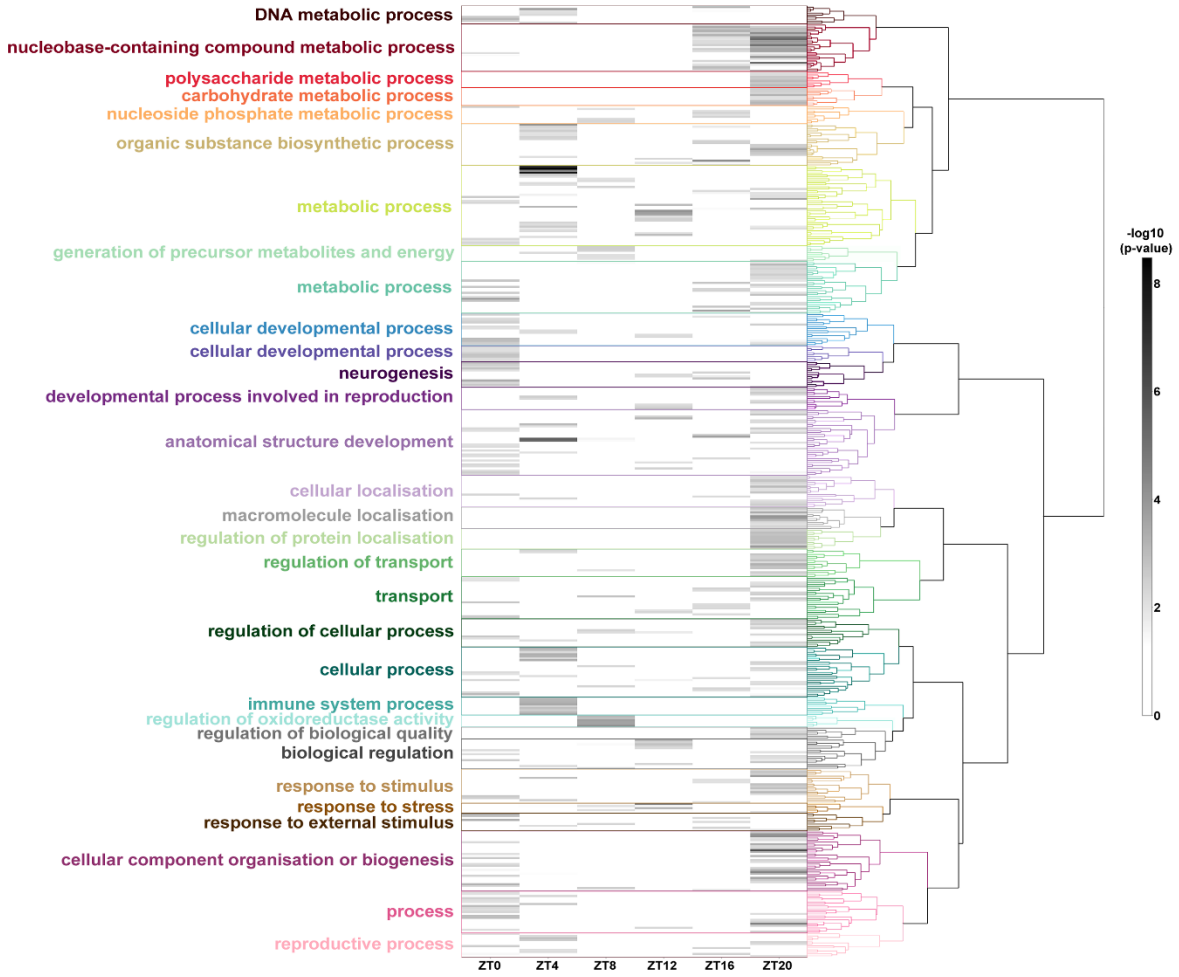

Figure 5.2 Clustering heatmap for comparison of enriched gene ontology term functional profiles across time, generated from *Argulus foliaceus* transcriptome. Dendrogram is based on Wang's semantic similarity distance and *ward.D2* aggregation criterion, heatmap displays -log10 (p-value) from functional enrichment tests for each timepoint. ZT0 = 7am, lights turn on, ZT12 = 7pm, lights turn off.

Feeding-related genes

A total of 42 significantly rhythmic genes were found associated with feeding enzymes (figure 5.4, table 5.2). Overall, the majority of these genes peaked at ZT20 (4 h before lights on; 31%) with few genes peaking at ZT12 (lights off; 4.8%). Anti-haemostatic proteins were highly expressed at ZT4 and ZT20 (21.9% and 31.3%) as were digestion/degradation proteins (ZT4 = 28.6%, ZT20 = 35.7%), while anti-inflammatory proteins peaked mostly at ZT20 (37.5%) followed by ZT4 and ZT8 (both 25%). Other specific proteins of note include: hemocyanin which was highest at ZT16 and lowest at ZT4, ferritin which was also lowly expressed at ZT4 but peaked at ZT12, and immune system protein adenosine deaminase, which displayed a linear decrease in expression with highest at ZT0 and lowest at ZT20.

Table 5.1 Summary of rhythmic clock gene hits from *Argulus foliaceus* transcriptome using Galaxy phylogenetically-informed annotation. Phase and rhythmicity p-values are from eJTK analyses.

Gene	Trinity ID	Hits (isoforms)	Phase	Rhythmicity (p-value)
Cryptochrome 2	TRINITY_DN10110_c0_g2	1	0	0.015803
Pigment Dispersing Hormone	TRINITY_DN5985_c0_g1	2	0	0.027781
Clock	TRINITY_DN3505_c0_g1	3	4	0.026201
Slowpoke	TRINITY_DN31_c0_g1	3	4	0.006378
Vrille	TRINITY_DN6730_c0_g1	2	8	0.031003
Period	TRINITY_DN7846_c0_g1	2	16	0.008084
Timeless	TRINITY_DN37479_c0_g1	3	16	7.89E-05
Cycle	TRINITY_DN1637_c0_g1	5	20	0.017545
Lark	TRINITY_DN8177_c0_g1	4	20	0.014336
Tango	TRINITY_DN1637_c0_g1	4	20	0.017545

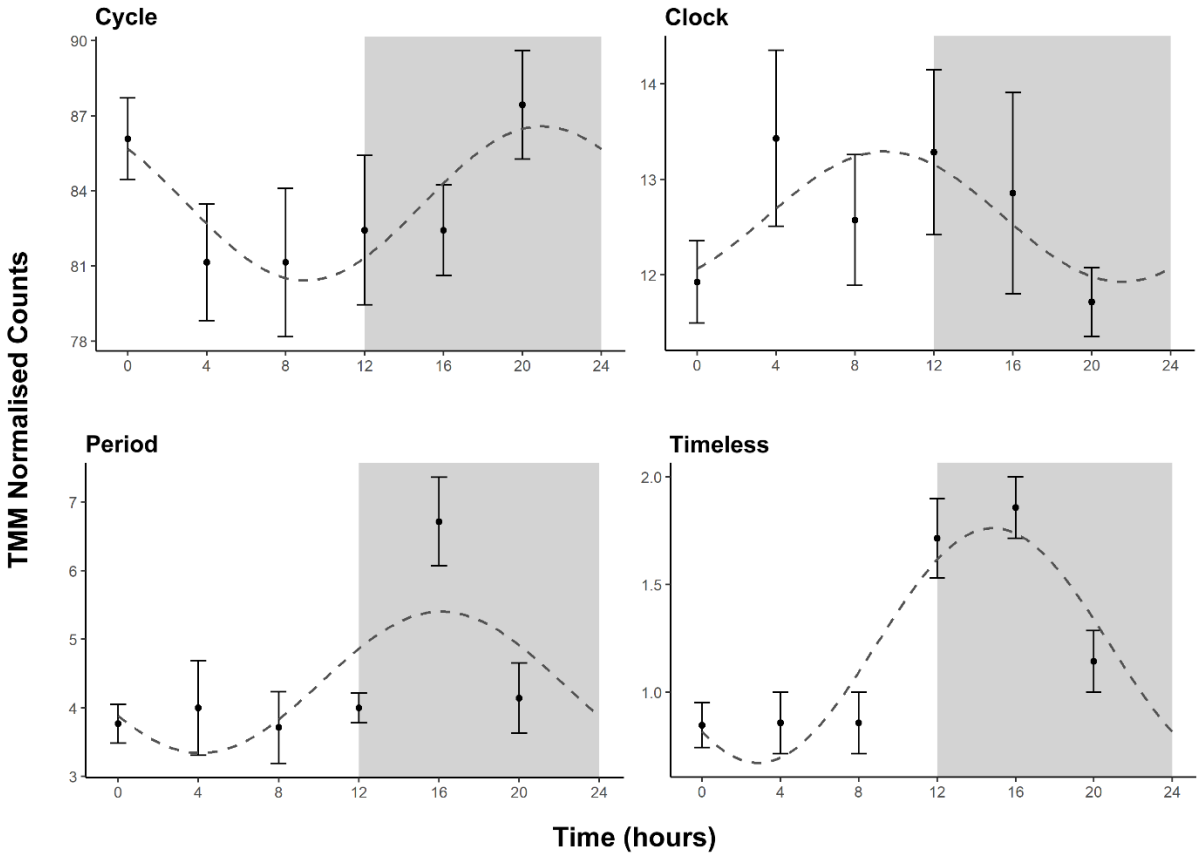

Figure 5.3 Rhythmic plots of putative clock gene expression in *Argulus foliaceus*. Error bars represent standard error, dotted line indicates rhythmic pattern generated by circacompare package in R, white background = light period, grey background = dark period.

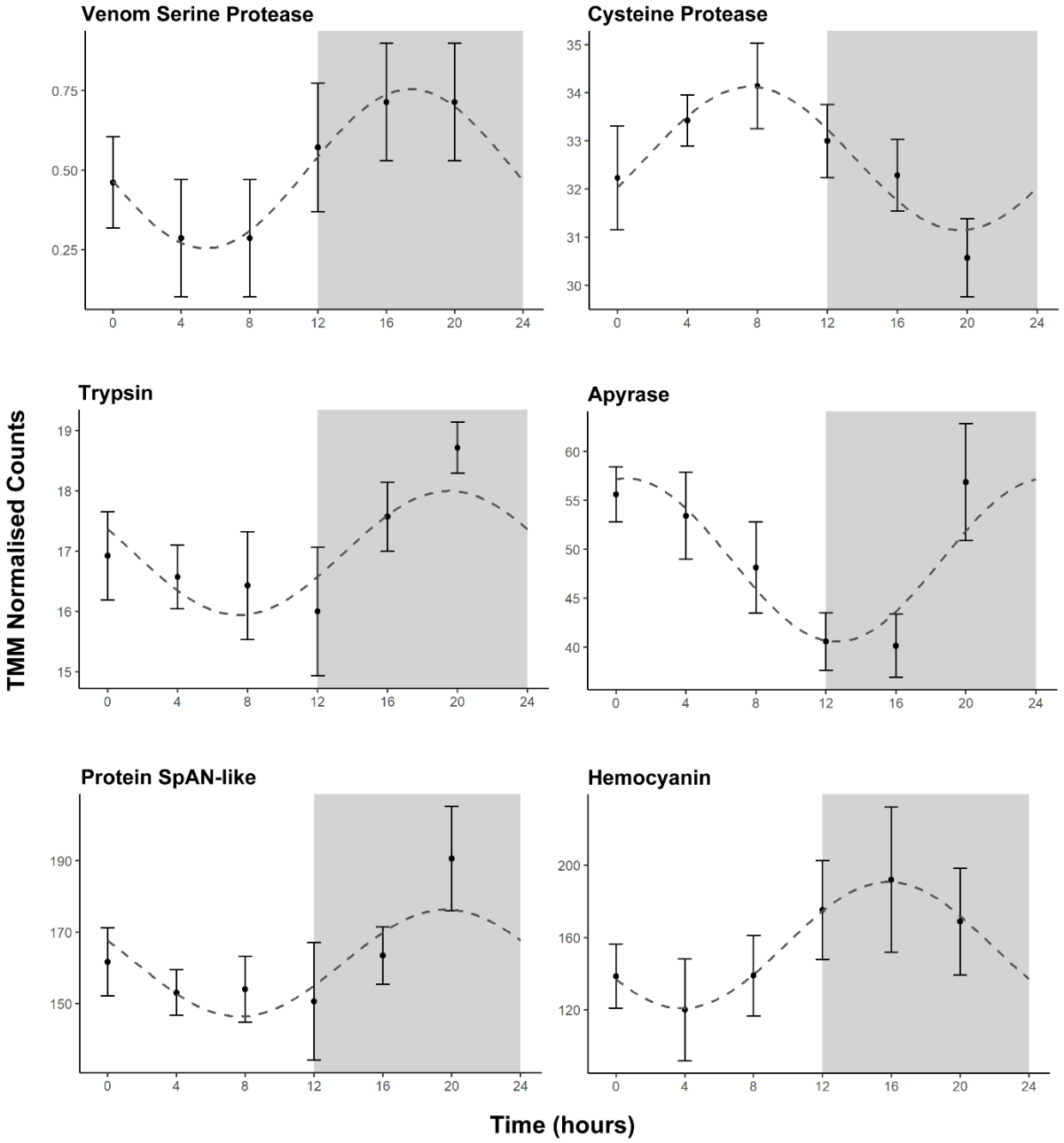

Figure 5.4 Rhythmic plots of *Argulus foliaceus* gene expression associated with feeding proteins. Venom serine protease, Cysteine protease and Trypsin produce anti-coagulant activity, Apyrase is an anti-coagulant and anti-inflammatory, Protein SpAN-like is related to immunity and Hemocyanin is involved in respiration and protein storage. Error bars represent standard error, dotted line indicates rhythmic pattern generated by circacompare package in R, white background = light period, grey background = dark period.

Table 5.2 Significantly rhythmic genes associated with feeding proteins identified from *Argulus foliaceus.* Protein selection for transcriptome searches and functional profiles were obtained from AmbuAli *et al.* (2020 & 2021). Phase and rhythmicity p-values are from eJTK analyses.

Protein class/domain	**Protein function(s)**	**Protein hit(s)**	**Trinity ID**	**Phase**	**Rhythmicity (p-value)**
Trypsin/Serine protease	Digestion and anti-haemostatic	Trypsin-1, Transmembrane protease serine 9	TRINITY_ DN13101_c0_g1	0	0.043473
		Trypsin	TRINITY_ DN2823_c0_g1	4	0.034037
		Serine protease1/2	TRINITY_ DN14783_c0_g1	4	0.033285
		Trypsin-1, Transmembrane protease serine 9, Transmembrane protease serine 9-like	TRINITY_ DN6711_c0_g1	4	0.017913
		Serine protease 29	TRINITY_ DN3732_c0_g1	8	0.027292
		Serine protease 29	TRINITY_ DN4009_c0_g2	8	0.014336
		Trypsin	TRINITY_ DN13135_c0_g1	20	0.016171
		Trypsin, Transmembrane protease serine 9	TRINITY_ DN11746_c0_g1	20	0.014842
		Trypsin-1, Serine protease1/2	TRINITY_ DN2412_c0_g1	20	0.006697
Protease	Anti-haemostatic	Metalloprotease	TRINITY_ DN7888_c0_g1	4	0.013433
	Anti-inflammatory, haemoglobin digestion	Cysteine protease	TRINITY_ DN5223_c0_g1	4	0.033285
		Cysteine protease	TRINITY_ DN5390_c0_g1	4	0.013672
		Cysteine protease	TRINITY_ DN5981_c0_g1	8	0.010528
	Anti-coagulant	Cathepsin-L	TRINITY_ DN21402_c0_g1	0	0.018496
		Venom serine protease	TRINITY_ DN7084_c0_g1	8	0.019468
		Venom serine protease, Trypsin-1	TRINITY_ DN7433_c0_g1	12	0.039253
		Cathepsin-L	TRINITY_ DN35513_c0_g1	12	0.049166
		Venom serine protease	TRINITY_ DN19000_c0_g2	16	0.022241
		Venom serine protease	TRINITY_ DN22100_c0_g2	16	0.034037
		Venom serine protease	TRINITY_ DN19000_c0_g1	20	0.041951
		Venom serine protease	TRINITY_ DN39641_c0_g1	20	0.025455

		Cathepsin-L	TRINITY_ DN4145_c0_g1	20	0.031003
Protease inhibitor	Anti-coagulant, anti-haemostatic, platelet aggregation, anti-complement activation, modulate host immune response, regulation of host inflammation	Serpin B6-like, Leukocyte elastase inhibitor-like	TRINITY_ DN6560_c0_g1	4	0.02882
		Alaserpin, Serpin B6-like, Leukocyte elastase inhibitor-like, Serine protease inhibitor	TRINITY_ DN1680_c0_g1	8	0.025455
		Serpin B6-like	TRINITY_ DN2191_c0_g1	8	0.014744
		Alaserpin, Serpin B6-like	TRINITY_ DN7534_c0_g1	20	0.016274
Diphospho-hydrolase	Anti-pain, anti-inflammatory, anti-haemostatic, platelet aggregation inhibitor	Apyrase	TRINITY_ DN534_c1_g1	16	0.015003
		Apyrase	TRINITY_ DN305_c0_g1	20	0.019976
		Apyrase	TRINITY_ DN104_c0_g1	20	0.007141
Phospholipase	Hydrolyses phospholipids (deactivates platelet-activating factor)	Phospholipase A2	TRINITY_ DN7380_c0_g1	4	0.018966
		Phospholipase A3	TRINITY_ DN1187_c0_g1	16	0.017091
Serine protease inhibitor (serpin)	Anti-coagulant	Thrombin inhibitor	TRINITY_ DN431_c0_g1	16	0.028035
		Thrombin inhibitor	TRINITY_ DN3019_c0_g1	20	0.008969
Astacin	Immunity including antifungal activity, food digestion, host penetration and immune evasion or activation	Protein SpAN-like	TRINITY_ DN1331_c0_g1	20	0.036208
		Protein SpAN-like	TRINITY_ DN5781_c0_g1	20	0.039778
Peptidase M14	Proteolytic-enzyme	Mast cell carboxypeptidase A	TRINITY_ DN4543_c0_g1	0	0.02232
Fasciclin	Mediate cell adhesion	Beta-ig-h3 fasciclin	TRINITY_ DN6864_c0_g1	0	0.015474
Purine metabolism enzyme	Vasodilator and anti-platelet	Adenosine deaminase	TRINITY_ DN11760_c0_g1	0	0.014388
Hemocyanin	Respiratory, protein storage	Hemocyanin subunit type 1 precursor, Hemocyanin A chain	TRINITY_ DN913_c0_g1	16	0.036492
Glycoprotein	Iron storage and transport	Ferritin	TRINITY_ DN10052_c0_g1	16	0.016171
Metalloenzyme	Degrades plasminogen	Enolase	TRINITY_ DN6653_c0_g1	16	0.043473
Vault protein Inter-alpha-Trypsin	Proteinase inhibitor	Inter-alpha-trypsin inhibitor heavy chain H4-like isoform X2	TRINITY_ DN9469_c0_g2	20	0.005655

5.5 Discussion

Daily rhythms are ubiquitous to life and diel variation in wide-ranging physiological processes in both hosts and their parasites may be pivotal to infection outcomes. Investigation of parasite rhythmicity at the transcriptional level provides an opportunity to examine and understand which processes are temporally coordinated. Here, almost half of annotated genes in the fish louse *A. foliaceus* had significant diel variation under light/dark cycles, indicating a high level of transcriptome rhythmicity. These included genes related to key processes such as feeding enzyme activity, cuticle development and immune responses. Our study highlights the magnitude of rhythmicity that can be encountered in parasite transcriptomes and the potential for chronotherapy applications considering the patterns seen in key processes and drug targets.

Few other studies have examined parasite transcriptional rhythmicity, and for those that have, the results vary significantly across taxa. For example, trypanosomes and parasitic fungi appear to express a relatively small proportion of their gene repertoire rhythmically (*Trypanosoma brucei* ~15% of genes; Rijo-Ferreira *et al.* 2017b, *Ophiocordyceps kimflemingiae* ~5%; de Bekker *et al.* 2017). In contrast, the blood stage of *Plasmodium falciparum* exhibits rhythmicity in up to 93% of the transcriptome (Smith *et al.* 2020). The current study is the first to examine diel gene variation in a parasitic crustacean despite their importance in aquaculture and fisheries as primary infections and vectors of secondary diseases. In non-parasitic crustaceans, however, 68.2% of copepod *Calanus finmarchicus* genes were rhythmic (Payton *et al.* 2021) while in Antarctic krill *Euphausia superba* only 27% of genes were deemed rhythmic (of which, 2.7% were clock controlled; Biscontin *et al.* 2019). Considering the importance of light in *Argulus* spp. off host behaviour and host searching (Chapter 4; Mikheev *et al.* 1999; Yoshizawa & Nogami 2008), it is perhaps unsurprising that almost half of the annotated transcriptome displayed significant light-based rhythmicity.

Generation and regulation of circadian rhythms is reliant on transcription-translation feedback loops of "clock" genes. These molecular clocks are found from bacteria to mammals, although the constitution of core clock genes varies across taxa (Rijo-Ferreira and Takahashi 2019; Rijo-Ferreira *et al.* 2017a). Previously for crustaceans up to 15 rhythmic circadian clock genes have been identified (Bernatowicz *et al.* 2016; Biscontin *et al.* 2019; Payton *et al.* 2021). Here, 10 putative clock genes were identified as having diel cycles in the *A. foliaceus* transcriptome: *Cycle, Pigment Dispersing Hormone, Timeless, Lark, Period, Slowpoke, Clock, Tango, Vrille* and *Cryptochrome 2* (*Cry2*). The presence of *Cry2* but lack of *Cryptochrome 1* (*Cry1*) suggests

A. foliaceus clock genes resemble those of sand hopper *Talitrus saltator* and isopod *Eurydice pulchra* (see Zhang *et al.* 2013; O'Grady *et al.* 2016), rather than *Drosophila melanogaster* or copepod *Calanus finmarchicus*, which possess only *Cry1* (Yuan *et al.* 2007; Payton *et al.* 2021), or the cladoceran *Daphnia pulex*, which possess both (Tilden *et al.* 2011). Clock genes are responsible for prolonged occurrence of rhythmic activity in the absence of zeitgeber cues (i.e. 24 h light or darkness), known as endogenous rhythms. While specific endogenous activity cannot be identified in this study, the presence of a suite of clock genes indicates the possibility. Endogenous rhythms have not yet been assessed in any parasitic crustaceans, although their presence has been confirmed in other parasites (monogeneans, parasitoid wasps and trypanosomes; Kearn 1973; Bertossa *et al.* 2010; Rijo-Ferreira *et al.* 2017b).

When examining clustering of rhythmic genes using PCA plots there were no distinct clusters for all genes, however grouping could be observed when the gene list was condensed to genes with stronger significance in rhythmicity ($p<0.01$). The *A. foliaceus* used in this study were hatched from wild caught females and thus are genetically diverse compared to a lab culture, contributing to the lack of clear clustering. Despite an overall distinct 24 h rhythmical pattern in gene expression, there were some differences in expression between the start and end point of sampling (ZT0). Some of these differences may be due to disturbance of the fish and therefore the attached parasites over the sampling period. While fish and attached parasites were maintained in isolated tanks, individuals still experienced disruption in the form of researcher presence every 4 h during sample collection. Differences could also be driven by genes with rhythmic periods shorter or longer than 24 h, as when examining the PCA plot of clock genes (which are known to have 24 h periods), the two ZT0 timepoints overlapped greatly (compared to the PCA plot of $p<0.01$ rhythmic genes). Metabolic processes and chitin/cuticle development were the main functional profiles different between the start/end ZT0 timepoints. Metabolic processes have varying cycles (Tu and McKnight 2006) and while we observed 24 h rhythmicity here in some chitin/cuticle development genes, growth is a complex process compounded by our use of actively growing juvenile *A. foliaceus*. As such, it is unlikely all chitin-based/growth related genes follow a 24 h pattern.

Rhythmic transcriptomes produce rhythmic behaviours, the occurrence of which can potentially be predicted from transcriptome diel variation. Adult *Argulus* spp. blood feed and as such express proteins to facilitate this process (Gresty *et al.* 1993; Walker *et al.* 2011b; AmbuAli *et al.* 2020), although it is currently unknown if feeding occurs at specific times. As

Argulus spp. feeding causes extensive external damage to their host, knowing when feeding occurs is important for understanding host vulnerability. Despite the *A. foliaceus* used in this study being too young to blood feed, genes were identified relating to blood feeding activity, indicating expression of these proteins is not adult restricted and could facilitate juvenile feeding on mucous and skin cells. The genes identified here included trypsins, proteases and protease inhibitors, which have been found previously in *A. foliaceus* and other blood feeding parasites (Francischetti *et al.* 2009; Robinson *et al.* 2009; Tirloni *et al.* 2015; Xavier *et al.* 2019; AmbuAli *et al.* 2020). Further confirmation of these proteins through functional assays could aid development of compounds to mitigate parasite feeding impact or improve host defence against feeding. Numerous putative feeding genes also had significant rhythms suggesting temporal patterns to feeding, with their expression generally highest at ZT20, 4h before lights on. This may be due to *A. foliaceus* feeding at this time, or in preparation of host protective immune response (inflammation) which triggers at onset of the light period (Montero *et al.* 2019; Ellison *et al.* 2021).

Chronotherapies - timed treatments based on host-parasite immune rhythms and targeting/disruption of key processes - hold great potential for parasite control. Here *A. foliaceus* rhythmic immune expression (including functional enrichment related to haemocyte degranulation, lectin, and Toll-like receptor genes; Theopold *et al.* 2004; Viswambari Devi *et al.* 2010; Lin and Söderhäll 2011; Watthanasurorot *et al.* 2011; Grigorian and Hartenstein 2013) was predominantly highest at ZT4 (4 h after lights on). Mounting an immune response requires a high energetic cost, as such it is beneficial for organisms to time expression to when it is most required (Demas *et al.* 1997). Response to stress peaked at ZT12 when lights turned off, potentially in preparation of host adaptive responses and wound repair which increase in activity during dark periods (Ellison *et al.* 2021). Intriguingly, genes related to crustacean parasite drug targets also showed daily rhythmicity. For example, acetylcholinesterase genes (a target of organophosphate treatments for sea lice, *Argulus* spp. and other parasites) peak mainly at/around ZT0 (lights turn on). Timed treatment could therefore improve efficacy, especially considering rising resistance concerns (Aaen *et al.* 2015; Agusti-Ridaura *et al.* 2018). This has been proven previously for glyphosate treatment of weeds where application time had a higher impact than dosage on control (Mulugeta and Boerboom 2000). Chitinases are the only drug group currently not facing resistance (Aaen *et al.* 2015; Macken *et al.* 2015); they control fish lice by targeting development (Eichner *et al.* 2015). Chitinases are ineffective against adult sea lice as moulting stops upon reaching adulthood, conversely *Argulus* spp.

continue to moult even as adults making them ideal targets. Here *A. foliaceus* chitin development genes were highly rhythmic in their expression with activity at ZT4 (4 h after lights on), comparable to chitin synthesis in krill which occurred 2 - 9 h post lights on (Biscontin *et al.* 2019). Due to the presence of some chitin development genes when examining differences between the start/end ZT0 time points, it is advised that further investigation into chitin development rhythmicity over a longer time period is conducted prior to testing of timed chitinase treatment to confirm period length of growth-related rhythmicity.

Parasites are often overlooked with regard to examining rhythmical processes in favour of their hosts, despite the potential in exploiting parasite rhythms to improve control. Here, gene expression related to numerous key processes including immune responses, chitin development and feeding were found to be significantly rhythmic in their expression over day/night cycles. While further functional assays to confirm gene function are required, this work provides a baseline for rhythmical patterns within louse gene expression and showcases the potential for chronotherapy. Going forward this will aid reduction of drug use in aquaculture without compromising impact, improving fish welfare, and mitigating economic loss.

Chapter 6

Towards integrated fisheries management: low cost and efficient self-assessment monitoring and control tool for louse infections

6.1 Abstract

Fish lice are one of the largest economic costs to fisheries, responsible for fish condition loss, mortality, and decrease in fishery performance. Despite decades of research fish lice remain problematic, with monitoring limited to host observations and chemotherapeutant control linked to drug resistance. Freshwater systems face even greater threats than marine systems, with environmental concerns and practical/legal constraints preventing standard chemical control. There is interest in employing integrated management strategies combining techniques to prevent, monitor and reduce infections, but appropriate tools and application are lacking. Thus, there is a need to develop new and alternative methods for tackling fish lice infections. Here, novel substrates exploiting the egg laying behaviour of freshwater lice (*Argulus* spp.) are assessed over successive years in still water trout fisheries with varying management styles. Eggs of *Argulus foliaceus* were collected from substrates without disrupting fishery function, providing estimations of population size and seasonal variation in reproduction. *A. foliaceus* mostly displayed two annual peaks in reproduction, likely tied to two separate generations. Removal of eggs from substrates also led to a reduction in eggs collected the following year, suggesting substrates may function as a control method to reduce louse population. As egg substrates function alongside other management methods, they are ideal for integrated strategies offering a low cost, simplistic method of parasite monitoring and egg removal.

6.2 Introduction

Monitoring and control of parasites differs across habitats, but generally fewer options are available for aquatic systems compared to terrestrial ecosystems (Burka *et al.* 1997; Li *et al.* 2002; OIE 2009). This is due to difficulties in accessing habitats for sampling, a lack of research impeding identification, and increased environmental concerns regarding unfocused treatment applications (Bain and Stevenson 1999; Diaz *et al.* 2004; Kamel *et al.* 2021). As such, many parasites persist in aquaculture with ectoparasitic fish lice one of the most notorious. Problematic infections of fish lice are rampant in both freshwater fisheries (Genus *Argulus*) and marine aquaculture (Genera *Lepeophtheirus* and *Caligus*). Fish lice attach to hosts and feed on skin, mucous, and blood, causing extensive mechanical damage to external host tissues

(Gresty *et al.* 1993; Walker *et al.* 2004; Walker *et al.* 2011; AmbuAli *et al.* 2020). This results in host behavioural changes of restlessness and aggravated movements followed by listlessness (Walker *et al.* 2004; Taylor *et al.* 2005b; Forlenza *et al.* 2008). Secondary infections are also common as host susceptibility is increased and other pathogens gain direct access to tissue (Shimura *et al.* 1983; Ahne 1985; Singhal *et al.* 1990; Nylund *et al.* 1993; Shameena *et al.* 2021). Direct losses are incurred from fish mortality with indirect losses from reduced fish condition. Fish louse infections have remained a source of severe economic loss over the last century (Wilson 1902; Costello 2009), thus new and improved monitoring and control methodologies are required.

Monitoring of fish lice is mainly achieved via fish captures followed by lice counts on host. This methodology is subject to error due to the aggregated distribution of lice in host populations, with fish subjected to stress during capture and examination (affecting fish health; Murray 2002; Bandilla *et al.* 2005; Cox *et al.* 2017). For sea lice, light-baited traps can be deployed to collect lice independent of fish captures (Novales Flamarique *et al.* 2009), with drones that sense lice on hosts (and subsequently treat them using lasers) being tested in Norwegian and Scottish fish farms (Dumiak 2017). For freshwater *Argulus* spp. no alternative methods are currently available to monitor lice, however collection of eggs is possible by deploying removable egg laying substrates. This is due to a key difference in life cycle between marine sea lice and freshwater *Argulus* spp. - sea lice carry eggs on their bodies in strings which hatch *in situ* while *Argulus* spp. must leave their host and lay eggs onto a suitable surface. Collection of eggs allows an estimation of parasite population size and dynamics through examination of reproductive output (Gault *et al.* 2002; Taylor *et al.* 2009a). Previous implementation of this method resulted in significantly higher estimates of breeding female lice population compared to fish captures (up to 37,956 individual female lice vs 15,875). The estimate from egg laying substrates was considered more accurate as heavily infected hosts were missed during fish captures (Gault *et al.* 2002).

Fish lice infections are usually treated and controlled using drug-based methods, with pyrethrins and pyrethroids, organophosphorous compounds, chitin-synthesis inhibitors and avermectins all used to treat sea lice infections (Grave *et al.* 2004; Aaen *et al.* 2015). *Argulus* spp. infections can be treated using many of the same products (Grave *et al.* 2004; Hakalahti *et al.* 2008); however application can often be difficult due to practicality, legality, and efficacy issues, such that there are no legal chemical treatments available for *Argulus* spp. within the

UK. Rising drug resistance is also a concern and has led to development and implementation of alternative control methods (Aaen *et al.* 2015). For *Argulus* spp., these include draining/fallowing of lakes (usually accompanied by liming), stocking/management methods to increase stock turnover and culling of infected fish (Hakalahti *et al.* 2008; Taylor *et al.* 2005b). Draining/fallowing is often used as a last resort since this method is highly intensive, has significant environmental impact and despite general public perception, does not guarantee eradication (Hakalahti *et al.* 2004; Taylor *et al.* 2006; Rico *et al.* 2012). Management techniques (stocking/culling) can help reduce the impact of infection, but due to the unique features of each fishery they can be difficult to apply effectively (Taylor *et al.* 2005a, b). As an alternative, egg laying substates used to collect eggs for monitoring may also work as a control method - destruction of eggs laid on substates could reduce parasite population size by decreasing the next generation of parasites. In 1999, Gault *et al.* (2002) removed 228,000 egg strings from a UK trout fishery, with egg captures the following year drastically lowered (only 1566 strings collected).

Considering the potential for egg laying substrates in both monitoring and controlling *Argulus* spp. infections, further testing should be conducted to ascertain feasibility and efficacy across a range of fisheries with varying infection levels and management. Egg laying substates work by offering a preferable egg laying location for adult female lice (hard, dark surfaces most preferable; Hakalahti *et al.* 2004b; Sahoo *et al.* 2013b) to reduce laying elsewhere in the fishery and maximise egg collection. *Argulus* spp. can free swim throughout their life cycle and gravid females must leave their host to find a suitable substrate on which to lay their eggs (Walker *et al.* 2004). Eggs are attached to surfaces using a gelatinous cement (Hoffman 1977) and laid in "strings" which can contain tens to hundreds of eggs each (inter- and intra-specific variability; Hakalahti *et al.* 2004). Females can lay multiple strings in succession and although mortality is possible during/after laying, they can survive, return to host, and become gravid again (Hakalahti *et al.* 2004). As substrates passively capture eggs, they do not impede fishery use and can be implemented alongside other measure such as stocking techniques, making them viable for use in integrated management systems. Previous substrate designs have shown promise, but are labour intensive, not installation/user friendly and may interfere with angler activity or other water users (e.g. boating; Taylor *et al.* 2005b).

Having identified freshwater coarse fisheries with a known *Argulus* problem, five fisheries each with a different management strategy trialled novel egg laying substrates over successive

years. Ultimately, such data can inform installation of egg laying substrates as a monitoring tool and component of an integrated louse management strategy.

6.3 Methods

Site selection

Five still water trout fisheries in mid to southern England (figure 6.1) experiencing problematic *Argulus* spp. infections installed novel egg laying substrates in 2017-2018. Management level of each fishery was defined by the Environment Agency following assessment of each site regarding monitoring, fish stocking, and maintenance protocols. Baseline management levels need to be defined when assessing trap efficacy as management can affect trap implementation, monitoring, and use. Each of the five fisheries were sampled for 1 to 4 years (see table 6.1). In Fishery A, drain and lime treatment was previously applied however it was unsuccessful leading to the fishery seeking alternative control measures, installing traps 1 year after treatment (Wijeyaratne and Gunawardene 1988). Maximum air temperature was obtained for each sampling date for each fishery using the HadUK-Grid gridded dataset at 1 km resolution (Hollis *et al.* 2019). Air temperature was taken for consistency as water temperature was not available for all dates/fisheries. All five fisheries were host to *A. foliaceus* infections (the most common UK species) identified via morphological examination (Fryer 1982).

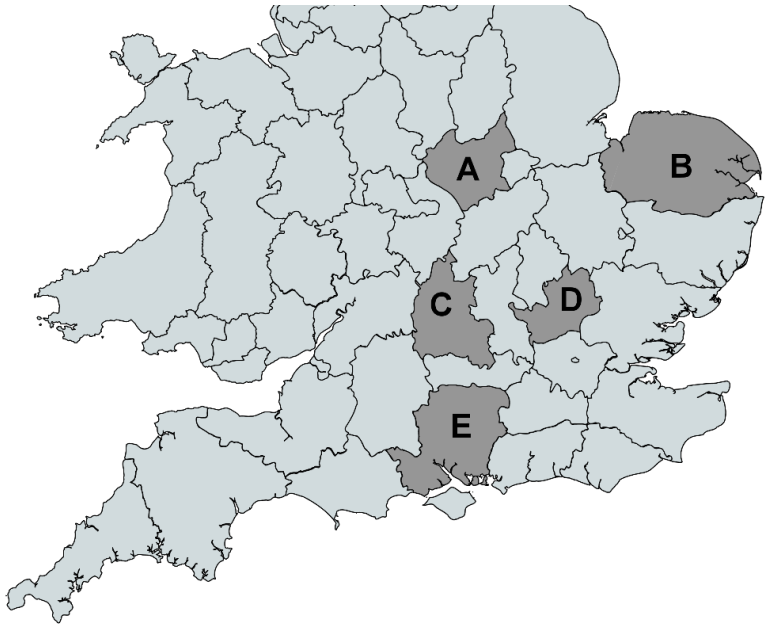

Figure 6.1

Fishery sites in England with known *Argulus foliaceus* infections sampled during this study, designated by county. A = Leicestershire, Fishery A. B = Norfolk, Fishery B. Fishery. C = Oxfordshire, Fishery C. D = Hertfordshire, Fishery D. E = Hampshire, Fishery E. Map created using mapchart.net

Trap design, installation, and egg string recording

Plastic drainage pipes (1m length x 110mm diameter) were positioned in the lakes vertically as egg laying substrates, with a black colour selected due to *Argulus* spp. preferring dark coloured substrates for egg deposition (Hakalahti *et al.* 2004b). These pipe substrates were fitted over agricultural wood steaks (fence posts, 1.8 m length x 75 mm diameter) that had been driven until stable into the bed at the perimeter of each water body (figure 6.2). The substrates were submerged to a depth of 1 m to allow easy access by fisheries using waders without need of a boat. Additionally, *A. foliaceus* preferentially lay eggs withing the top 1m of the water column (Harrison *et al.* 2007). At biweekly intervals, the substrates were either raised above water using a peg and chain method (figure 6.2) or by lifted off the posts by hand to allow counting of eggs followed by desiccation (100% egg mortality after 48 h drying; Hakalahti *et al.* 2008) and mechanical removal of eggs from the substrate. This style of egg laying structure ensures angler's casts are not impeded, a concern with previously used structures that float or hang in the water body (Taylor *et al.* 2005b). Substrate material cost was as follows: wood steaks £3.42 each (Jacksons-Fencing, 2018), 1m*110mm black soil piping £5.98 each (Screwfix, 2018) and peg and chain £0.35 each (Homebase, 2018) resulting in a total cost of £9.75 per substrate. The number of substrates installed per site varied from 10 - 40 (table 6.1). For Fishery C in 2017 and 2020 when egg string numbers were high, maximum numbers were recorded as >300 egg strings, and counts were taken as 300 for analyses.

Egg and invertebrate identification

Fishery owners were shown how to morphologically recognise *Argulus* spp. egg strings on substrates by the Environment Agency for recording. For Fishery B, presence of other invertebrates and eggs attached to the substrates was also recorded. As such, samples were collected by the Environment Agency and sent to Cardiff University for identification using molecular analysis. Invertebrate species were identified by extracting DNA from eggs collected by the fishery using a QIAGEN DNeasy Blood & Tissue Kit. Kit instructions were followed with exception of eggs fragmented before digestion using a sterile eppendorf pestle with subsequent overnight digestion of samples. DNA was then amplified using general invertebrate primers described in Folmer *et al.* (1994), with a total PCR mixture volume of 30 µl containing 3 µl DNA, 1 µl of each primer (forward and reverse; 10 µM concentration), 15 µl of QIAGEN 2x Multiplex PCR Master Mix (including HotStarTaq DNA Polymerase: 5 units/µl concentration), and 10 µl of nuclease-free water. A PCR was then conducted with the following conditions: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 30

s, annealing at 49 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min (Khan *et al.* 2016). Following amplification samples were sent to Eurofins Genomics for sequencing and genus confirmed via BLAST search.

Environmental variables

For Fishery A, B, C and D, an acoustic mapping survey was performed using a Lowrance Elite Ti7 echosounder and processed using BioBase, with lake bottom substrate hardness categorised into 5 groups (soft, lower medium, medium, upper medium, hard) beneath each egg laying substrate. At Fishery E, oxygen and water clarity were recorded at each egg count during 2017. O_2 (ppm) was recorded using an electronic meter and turbidity (cm) was measured using the Secchi disk method. At Fishery E, egg laying substrate specific variables were also collected, namely: surrounding silt/gravel (percent coverage over a 1 m radius from substrate), angle of slope (if present), distance from bank and presence of cold water (spring), root structures, wooden structures, and submerged plastic (such as standpipes). For Fishery B, aquatic macrophyte presence (submerged weed and emergent reed) around each egg laying substrate was recorded.

Figure 6.2 Substrates in use at Fishery E. Egg laying substrate raised above water level for inspection (left), peg and chain holding the pipe in place on the stake is visible at the water surface. *Argulus foliaceus* eggs covering a substrate (right). Photographs by Conor Harvey, 2018.

Table 6.1 Summary of fishery characteristics of still water trout fisheries across England using *Argulus* egg substrates. BioBase = topography analysis conducted to assess substrate hardness across the fishery.

Variable	**Fishery A**	**Fishery B**	**Fishery C**	**Fishery D**	**Fishery E**
Lake surface area (m^2)	5,994	10,0370	8,7801	9,918	6,405
Management level	Low	High	High	Low	Medium
Date substrates installed	09/06/2017	17/04/2018	19/05/2017	05/05/2017	21/06/2017
Number of substrates	12	40	28	10	20
Egg laying substrate surface area (m^2)	2.82	9.4	7.05	2.35	4.7
Egg laying substrate to Lake ratio (%)	0.05	0.01	0.01	0.02	0.07
Data collected	2017 - 2018	2018	2017 - 2020	2017 - 2018	2017 - 2018
BioBase	Yes	Yes	Yes	Yes	No

Statistical analysis

All statistical analyses were conducted using R statistical software (v4.0.3; R Core Team 2020) with the level of significance in all tests taken as $p<0.05$. Package "mgcv" (version 1.8-31) was used to construct general additive models (GAM), "lme4" to run general linear mixed models (Bates *et al.* 2014), "leaflet" used to create maps and "ggplot2" used to visualise data (Wickham 2009). All models were refined through stepwise deletion of insignificant terms and comparisons of residual plots, Akaike information criterion, and for GAMs k-index.

For all fisheries, impact of date and egg laying substrate ID on number of egg strings recorded was examined using a negative binomial GAM with log link function. All models used a thin plate regression spline to smooth the date variable, except for Fishery B data which used an adaptive smoother instead. For Fisheries A, C, D and E, the impact of sample year on egg string collection was also examined. For Fishery B egg laying substrate aquatic macrophyte presence and attachment of other invertebrates were included to assess their potential impact on *Argulus* egg strings. Changes in individual substrate use over time were examined for each fishery and sample year using a Chi-squared test with simulated p-values (based on 2000 replicates) due to low counts (<5 observations). Dates containing zero egg string counts were removed

(recordings at the start/end of the year when it was too cold for laying, thus all substrates had 0 captures) alongside substrate IDs with no observations.

Post hoc analysis with "emmeans" (Searle *et al.* 1980) was conducted on each model to examine contrasts between substrates (and for Fishery C, sample year). Additional data collected at Fishery E during 2017 allowed examination of whether egg laying substrate specific variables (surrounding silt/gravel, angle of slope, distance from bank and presence of cold water, weeds, root structures, wooden structures, and submerged plastic) affected total *Argulus* egg strings collected using a negative binomial generalised linear model (GisedLM) with log link function. Dynamics of other invertebrates was assessed at Fishery B by comparing invertebrate presence on substrates (yes/no) to date, substrate ID and weed and reed presence using binomial GisedLMs with logit link function for snails, leeches, and red mite eggs, and probit link function for snail eggs and caddis fly larvae.

A gamma GisedLM with inverse link function was used to assess the impact of substrate hardness and bank direction on percent of *Argulus* egg strings captured at each fishery. A general linear model (GLM) with gaussian family and identity link function was used to examine overall impact of maximum air temperature on percent of *Argulus* egg strings collected.

6.4 Results

In total 238,817 egg strings were removed from fisheries in this study, equivalent to over 12.4 million eggs (assuming average number of eggs per string = 52, calculated from Cardiff University *A. foliaceus* lab cultures). Following the calculation used in Gault *et al.* (2002) wherein female *A. foliaceus* lay an average of 6 strings over their lifetime (Pasternak *et al.*, 2000) adult female population size was calculated for all fisheries, with the lowest population size 40 individuals for Fishery D in year 2 and the highest 11,337 individuals for Fishery E in year 1 (figure 6.3).

For all fisheries with 2+ years of observation (Fisheries A, C, D and E), significantly less eggs were collected in year 2 compared to year 1 ($p<0.001$ for all, A: $\chi^2(1) = 69.44$; D: $\chi^2(1) = 49.73$; E: $\chi^2(1) = 43.32$, GAM), indicating a reduction in *A. foliaceus* population post trap implementation. In fisheries A and D, a decrease of 86 and 75% respectively was observed, while fisheries C and E experienced a lower decrease of 43 and 36% respectively (table 6.2).

This difference may be linked to *Argulus* population size, as fisheries A and D had smaller populations compared to fisheries C and E (28 - 70 times higher number of egg strings collected in fisheries C and E). For Fishery C, year 1 had the highest egg captures across 4 years of collecting, with the lowest counts in year 3 (year 1 comparisons: t-ratio = 8.061 - 12.120, $p<0.001$ for all, year 3 comparisons: t-ratio = 6.191 - 12.120, $p<0.001$ for all, GAM). There was no significant difference in the number of egg strings captured in year 2 vs year 4 (table 6.2).

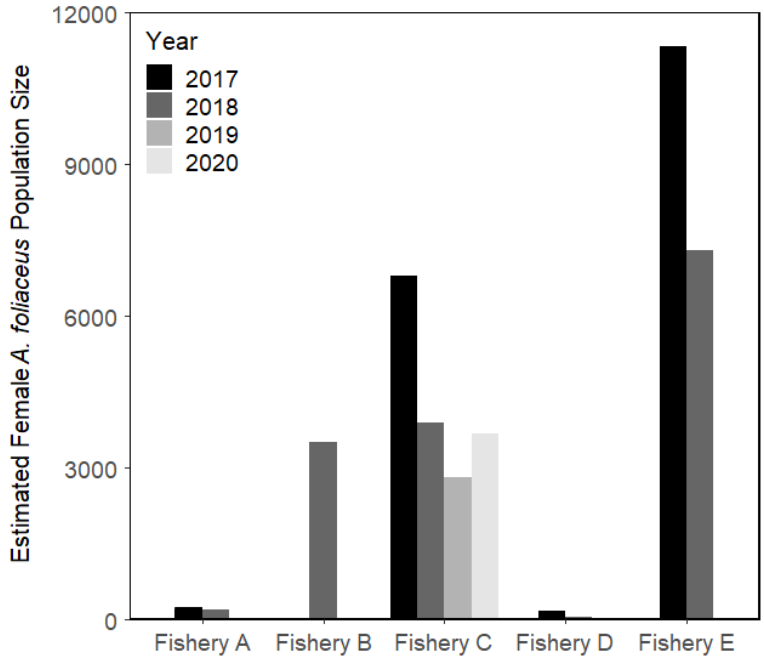

Figure 6.3 Estimated population size (total number of individuals) of reproducing adult female *Argulus foliaceus* within still water trout fisheries, based on total number of egg strings collected (assuming 6 strings laid by females on average, table 6.2).

Table 6.2 Summary of data collected from still water trout fisheries across England using *Argulus* egg substrates.

Data	**Fishery A**	**Fishery B**	**Fishery C**	**Fishery D**	**Fishery E**
Number of egg string peaks/year	1 - 2	2	1 - 2	1	2
Total strings collected	1641	21098	103019	1207	111852
Strings collected in Year 1 (2017)	1442	NA	40730	967	68021
Strings collected in Year 2 (2018)	199	21098	23370	240	43831
Strings collected in Year 3 (2019)	NA	NA	16922	NA	NA
Strings collected in Year 4 (2020)	NA	NA	21997	NA	NA
Decrease in strings (Year 1 - Year 2)	86%	NA	43%	75%	36%

For each fishery, the number of *Argulus* egg strings laid on the substrates showed a significant non-linear pattern over time (figure 6.4), with one or two peaks per year ($p<0.001$ for all, Fishery A: $\chi^2 = 333$, edf = 13.57; B: $\chi^2 = 1233$, edf = 10.64; C: $\chi^2 = 4829$, edf = 36.42; D; $\chi^2 = 447.6$, edf = 13.30; E: $\chi^2 = 1750$, edf =16.85, GAM). For Fishery A two peaks occurred in year 1, however for year 2 the data ended in July after one peak thus it is unknown if further peaks occurred. Fishery B had only one year of sampling in which two distinct peaks occurred. Fisheries D and E consistently had one or two peaks per year respectively, while Fishery C showed variation across 4 years of sampling. For fisheries with two clear annual peaks, the trough between peaks occurred between July - August (except for Fishery E in year 1 with a later trough from August - September). These egg laying troughs did not correlate with temperature, but across all fisheries increasing temperature lead to significant increases in *Argulus* egg strings (t-value = 4.609, df = 130, $p<0.001$, GLM).

Individual substrates within a fishery significantly varied in their captures between each other (figure 6.5, $p<0.001$ for all, Fishery A: $\chi^2(11) = 110.94$; B: $\chi^2(39) = 474.499$; C: $\chi^2(28) = 612.56$; D: $\chi^2(9) = 139.00$; E: $\chi^2(19) = 154.70$, GAM), over time ($p<0.001$ for all fisheries/comparisons, Chi-squared tests, figure 6.6), and between sample year ($p<0.001$ for all fisheries/comparisons, except for Fishery A where $p = 0.002$, Chi-squared tests).

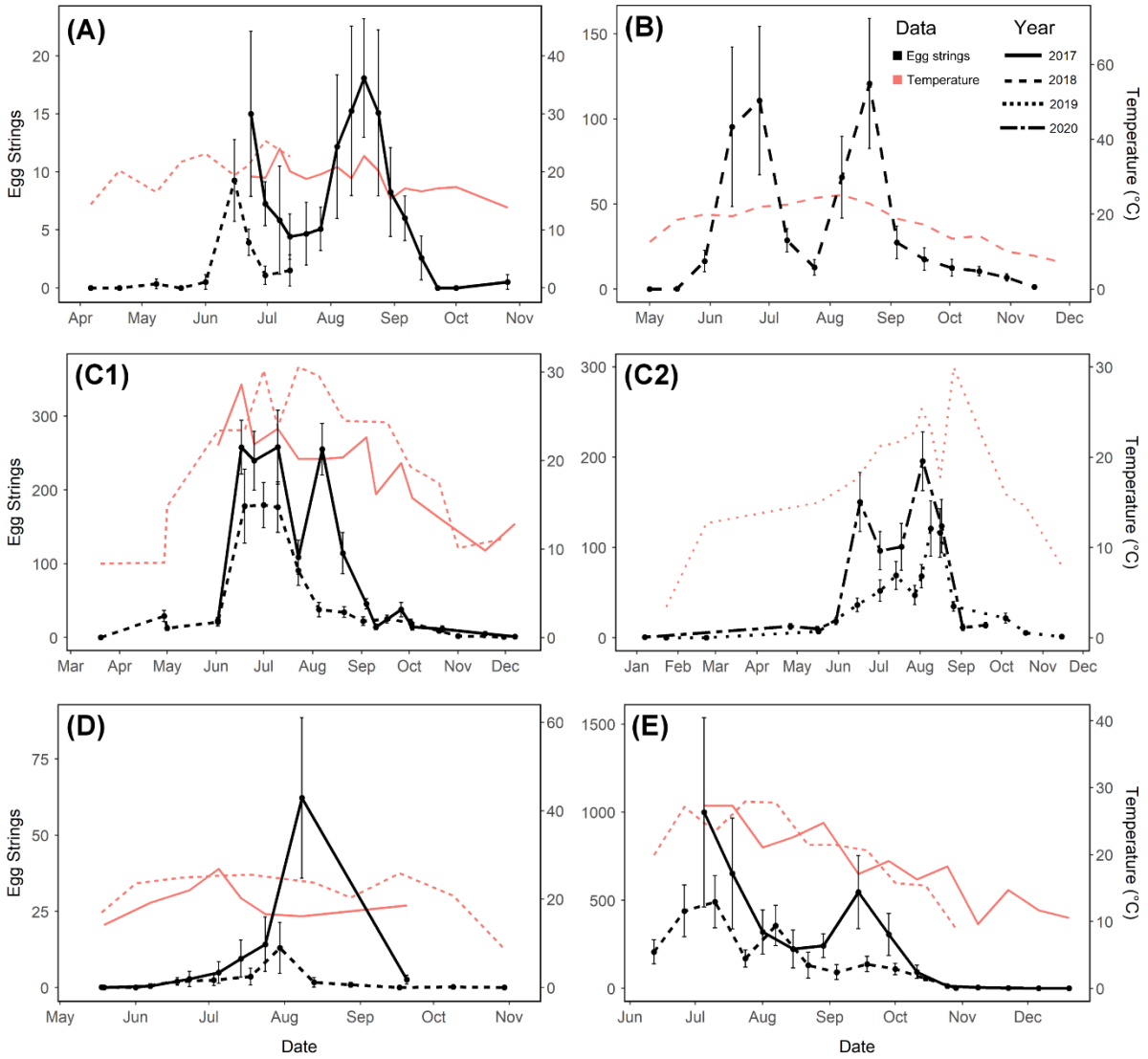

Figure 6.4 Average number of *Argulus foliaceus* egg strings collected in each still water trout fishery over a year. Sampling years 1 - 4 = 2017 - 2020. Letter IDs of graphs refer to the fisheries as referenced in text with Fishery C split into 2 graphs to improve visualisation of data (C1 = 2017 and 2018, C2 = 2019 and 2020). Legend in graph B applies to all plots. Temperature is maximum air temperature, for C2 data is not yet available for 2020. Error bars represent 95% confidence intervals.

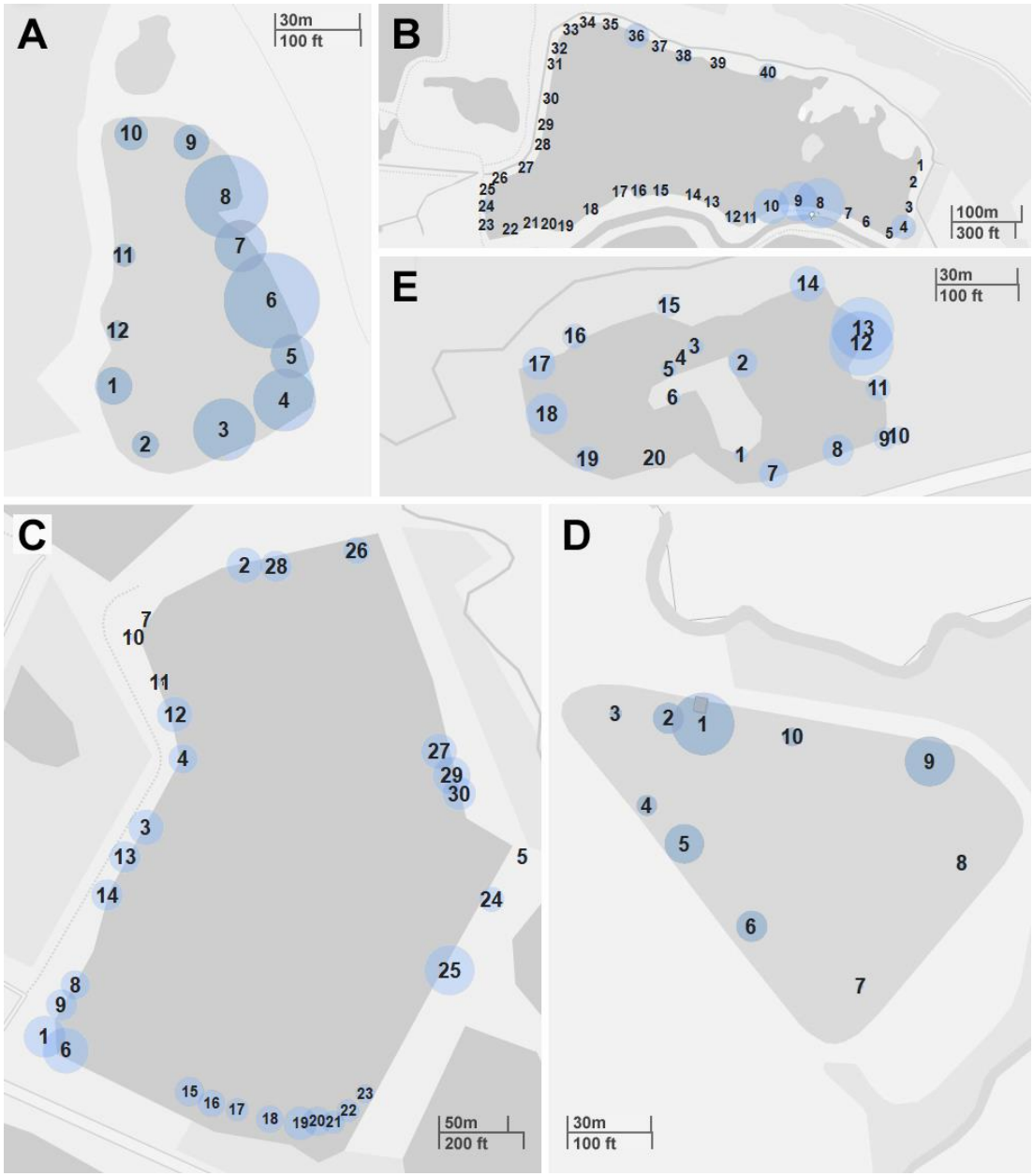

Figure 6.5 Map of each fishery showing percentage of *Argulus foliaceus* egg strings captured (indicated by blue circle size) for each identified egg laying substrate over the entire sampling period. A = 2017 - 2018, B = 2018, C = 2017 - 2020, D = 2017 - 2018 and E = 2017 - 2018. Letter IDs of graphs refer to the fisheries as referenced in text. Maps created with the leaflet R package using map tiles CartoDB positron (maps B, C and E) and Esri World Topographic Map (maps A and D).

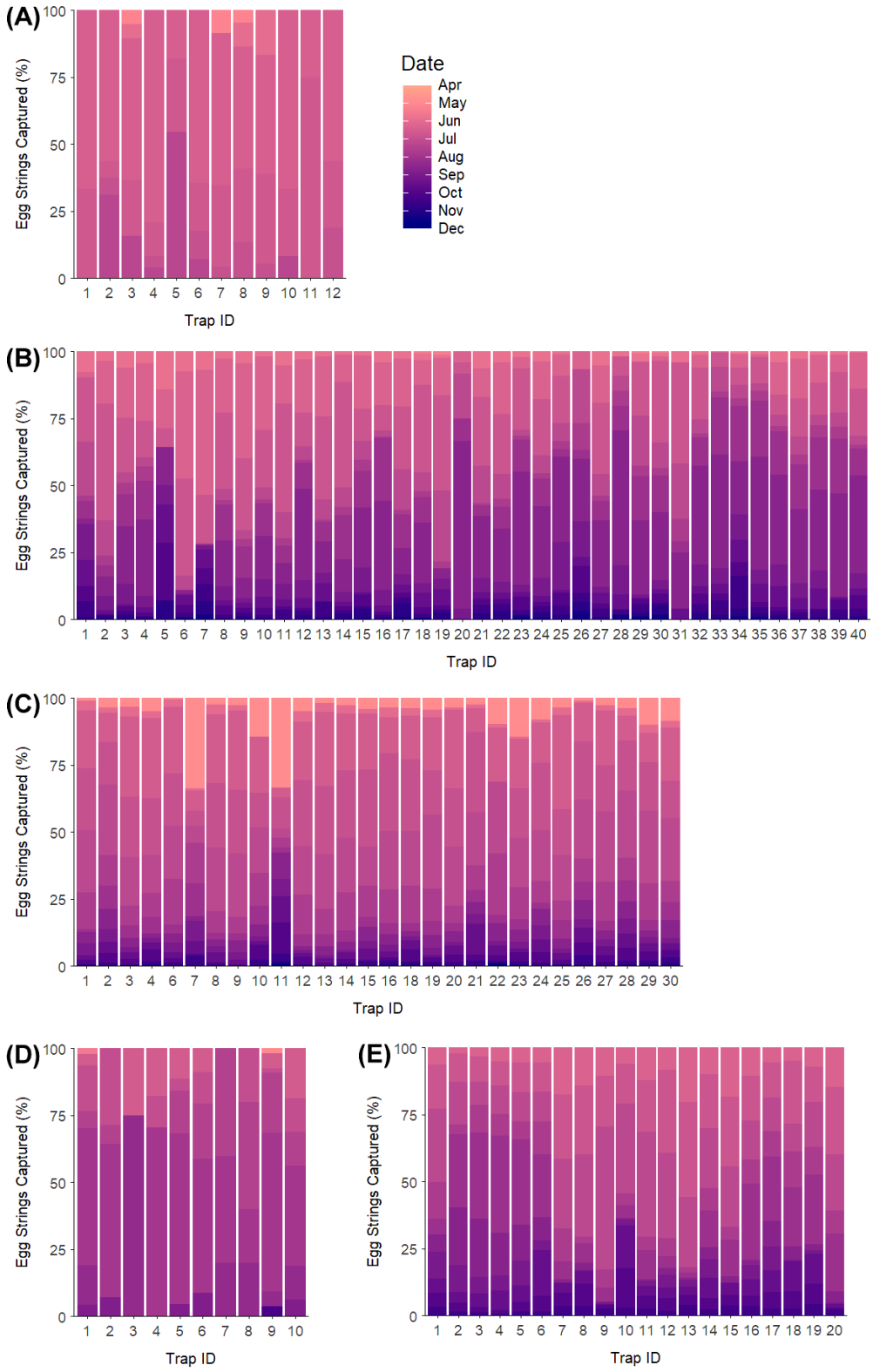

Figure 6.6 Stacked percent bar chart showing change in *Argulus foliaceus* egg captures across time from individual substrates used within still water trout fisheries. Letter IDs of graphs refer to the fisheries as referenced in text. For all fisheries data displayed is from 2018. Legend applies to all graphs, substrates with lighter/peach bars collected more eggs early in the year, while substrates with darker bars collected more eggs later in the year.

The environment around substrates influenced the number of *Argulus* egg strings laid. In Fishery B, a lack of reeds and weeds around substrates resulted in significantly higher egg captures (reed: $\chi^2(1) = 10.45$, $p = 0.001$, weed: $\chi^2(1) = 3.94$, $p = 0.04$, GAM; although for weeds the result was close to non-significance). In Fishery E, weeds did not impact egg captures, nor did roots, slope angle, distance from bank or presence of plastic/wood structures. Silt and gravel presence however had a significant impact, with silt around substrates negatively impacting egg captures and gravel positively impacting egg captures (z-value = -3.14, p = 0.002, GisedLM). For all fisheries with BioBase analyses, substrate hardness beneath egg laying substrates and bank direction had no impact on egg string collection.

For fishery B, five other invertebrates were identified on egg laying substrates alongside *Argulus* eggs: red mite eggs; *Limnesia* and *Eylais* spp., caddis fly larvae, snails, snail eggs and leeches; *Erpobdella* and *Helobdella* spp.). Presence of these invertebrates on substrates did not impact *A. foliaceus* egg captures. These invertebrates were also significantly influenced by date (red mite eggs: likelihood ratio test (LRT) value/p = 8.41/0.004, caddis fly larvae: LRT/p = 45.23/<0.001, snails: LRT/p = 5.33/0.02, snail eggs: LRT/p = 133.85/<0.001, leeches: LRT/p = 10.47/<0.001, GisedLM). Red mite eggs were positively affected by presence of weeds and reeds (LRT = 11.59 and 21.24 respectively, $p<0.001$ for both, GisedLM) and differed significantly with substrate ID (LRT = 55.20, p = 0.04, GisedLM). Caddis fly larvae presence was also positively impacted by reed presence (LRT = 10.85, $p<0.001$, GisedLM), while snails were positively affected by weed presence (LRT = 10.99, $p<0.001$, GisedLM).

6.5 Discussion

The management, monitoring and control of lice in freshwater systems remains challenging, despite efforts spanning the last 2 decades. Here, installation of egg laying substrates across five fisheries with known problems associated with *A. foliaceus* infection provided a cheap and efficient means of assessing parasite reproductive output in real time. One year after installation of the substrates, the *A. foliaceus* populations were reduced as evidenced by fewer collected egg strings. Our data, together with willingness of fishery managers to install these newly designed substrates, suggests that they will provide a useful integrated management tool across a range of management conditions.

A. foliaceus egg laying varies spatially and temporally; here displaying strong seasonal variation in fecundity, in line with previous studies (table 6.3). Temperature is the primary driver of *Argulus* spp. egg laying (Hakalahti *et al.* 2006; Harrison *et al.* 2006) and dictates the length of the reproductive season (May - October for these UK fisheries). Between June and September, two annual peaks in egg laying occurred in 4 out of 5 fisheries (despite consistent suitable egg laying temperatures throughout this summer period), likely arising from two separate generations of *A. foliaceus.* This is supported by previous observations of bi-annual *A. foliaceus* generations in UK fisheries (Gault *et al.* 2002; Taylor *et al.* 2009a).

Table 6.3 *Argulus foliaceus* dynamics in fisheries as recorded in literature.

Data	Location	Sample dates	Host / substrate	Results	Reference
Louse number	Lithuania	Jun - Aug	Peled	Linear increase in louse numbers	Žiliukienė *et al.* 2012
		May - Aug	Pike, Peled, Pike-Perch, Vimba, Bream	Two peaks	
	Turkey	May - Jan	Bleak	One large peak	Koyun 2011
		May - Jan	Crucian and Golden Carp	Two small peaks	
	Finland	Feb - Nov	Perch, Roach and Brown Trout	Two peaks: first larval and second adults. Adult female numbers varied June-August.	Pasternak *et al.* 2000
	UK	Jun - Dec, Jan - May	Rainbow Trout	Two peaks in one fishery, single peak in three fisheries	Taylor *et al.* 2009a
Egg laying	UK	Jun - Dec, Jan - May	Grey foam-PVC boards	One peak in three fisheries, indistinct/potentially two peaks in two fisheries	Taylor *et al.* 2009a
	Northern Ireland	Apr - Nov	Corrugated polypropylene boards	One peak in site 1 (only two timepoints available for site 2)	Harrison *et al.* 2006
	Northern Ireland	Apr - Oct	Corrugated polypropylene boards	One peak in 1999 (high egg numbers), three peaks in 2000 (low egg numbers)	Gault *et al.* 2002

If peaks in egg laying are tied to individual generations, then the trough between the first and second peak is likely caused by lice mortality. This is because *Argulus* spp. continually produce

eggs and can have multiple clutches assuming appropriate mate availability and temperatures (Stewart *et al.* 2017). *Argulus* spp. have an estimated lifespan of up to 1 year (wild populations, Shafir and Oldewage 1992; laboratory cultures at 14 °C, personal observations), as such overwintered adults would be reaching the end of their life expectancy during the first peak. *A. foliaceus* can also die from predation upon leaving their hosts (necessary to lay eggs, Kearn 2004; Bandilla *et al.* 2008) and natural mortality during/post laying (Hakalahti *et al.* 2004a). Additionally for these UK based fisheries, the hottest months result in higher fish mortalities, altering availability of hosts and potentially impacting louse populations. Taylor *et al.* (2009b) also noted that the presence of overwintering adults could impact subsequent numbers of generations - it is possible that Fishery D, the only fishery here with a single peak each year, lacked overwintered adults and hence had no egg laying in June despite appropriate temperatures. This could have been driven by the management approaches implemented in this fishery (one large stocking, low stock turnover and high availability of unmanaged alternative egg laying substrates). Regardless of the number of generations a fishery experiences, understanding the dynamics of an individual fishery is key for fishery owners to manage louse infections through proactive application of control methods.

Egg laying substrates show potential as a control method with egg captures significantly reduced in all fisheries in subsequent years following substrate installation and use. Such substrates will not remove all eggs in a fishery as female lice will lay their eggs on a wide variety of substrates (Sahoo *et al.* 2013b). Thus, a small population will always subsist, hatched from eggs laid elsewhere in the fishery. Where we were able to collect 4 years of data and assess longer-term substrate usage, egg captures almost halved in the second year compared to the first year (from 40,730 to 23,370 egg strings collected, 43% drop). In subsequent years however the number of egg strings collected remained stable, likely due to this subsisting population. It should also be noted that egg collection in 2020 (year 4) could have been affected by the worldwide Covid-19 pandemic, by restricting fishery owner ability to monitor substrates. Few control treatments are 100% effective; even the comprehensive and expensive lime and drain treatment does not guarantee eradication (Taylor *et al.* 2005). Egg laying substrate installation was significantly cheaper and more sustainable than application of chemicals or draining/fallowing of lakes and allowed fisheries to remain operational throughout. The aim of this approach is to reduce lice numbers below problem levels rather than complete eradication. For this, an understanding of how many substrates need to be deployed when implemented

alongside other management strategies is required to prevent economic loss and improve the efficacy of *Argulus* spp. monitoring and control (Hakalahti *et al.* 2008; McPherson *et al.* 2012).

The impact of egg laying substrates is dependent on their attractiveness to both lice and fish as *Argulus* spp. are dependent on their host for food and likely move around a fishery on their fish hosts, using their free-swimming capabilities over shorter distances to switch hosts or lay eggs. Substrate positioning is therefore key to ensure suitable environmental parameters; silt prevents *Argulus* spp. from laying (Taylor *et al.* 2009a; Sahoo *et al.* 2013b; current study) thus choosing silt free areas should increase substrate use. The impact of adjacent plants such as weeds and reeds varies between fisheries and may depend on overall fishery composition or fish behaviour. Non-target invertebrates, including red mites, leeches, snails, and caddis fly larvae, were also found on the egg laying substrates. While they did not affect substrate use by lice, all of these organisms contribute to the ecosystem of a fishery (Macadam and Stockan 2015; Collier *et al.* 2016), thus care needs to be taken regarding the removal of these organisms alongside target *Argulus* spp. eggs. Presence of these other organisms on substrates did allow for observation of their seasonality, meaning these substrates could be used to monitor other organisms that utilise hard surfaces in their life cycle.

There is a growing need for sustainable (non-invasive, non-chemical) tools that can be used in integrated management systems to reduce and control pests. To be viable in an integrated system, new methods or tools need to provide a service and be suitable alongside other treatment options. Genetic diagnostics (e.g. assessment of environmental DNA for species presence/prevalence) offer great potential for quick and comprehensive infection identification and monitoring, but still face limitations in the form of accuracy and development of systems for widespread use (Harper *et al.* 2019). Here egg-laying substrates were quick and simple to install at all sites, extremely cost-effective (<£10 per trap), non-polluting and did not hinder fishery use, with maintenance required only during the louse reproductive season. These substrates offer great potential as a monitoring tool: fisheries can actively see the extent of their *Argulus* problem and how it changes across the year, allowing proactive adjustments to stocking or other management interventions. There is also potential for the substrates to be useful as a control method in their own right, as egg captures were significantly lower in subsequent years post substrate implementation. Stocking practices can continue with the substrates in place, allowing development of integrated management strategies in angling fisheries without reliance on draining/fallowing or chemical application. For farms or other

systems where chemical application is available, use of substrates may be particularly effective alongside treatments that only target the lice themselves and have no impact on eggs (such as the organophosphate Diptrex 80; Taylor *et al.* 2005b).

Despite the promising results from this study, successful utilisation of these substrates requires appropriate management. Substrates must be monitored regularly with good estimates of egg captures to provide the necessary resolution for examination of population changes. If monitoring (and subsequent egg removal) is stopped, eggs substrates could instead promote *Argulus* populations though provision of appropriate egg laying surfaces. Optimisation of this approach will likely take several years of use within a fishery post implementation; thus egg laying substrates are an investment to be used alongside other management strategies. While optimisation still requires further research, egg laying substrates are a viable option for *Argulus* spp. management and could become a low-cost, standard tool for integrated parasite management strategies.

Chapter 7

An updated guide to the Branchiura in Britain: The threat of *Argulus mongolianus*

7.1 Abstract

Parasitic invasions have previously been responsible for widespread impacts to human/animal health and subsequent economic loss. Introduction of parasites is facilitated by difficulties in identifying infections, with screening of aquatic organisms complicated by challenges in handling, contamination of travel water/substrate and a lack of information compared to terrestrial organisms. Recent mortalities within a UK carp fishery led to the discovery of an unknown species of *Argulus*, identified morphologically as *A. mongolianus*. A key outlining a single female sample was the only descriptive text previously available for *A. mongolianus*, with only one other relevant reference citing mass mortalities of carp in Russia. Here, we depict male and female *A. mongolianus* and morphologically compare this species to the three established UK *Argulus* species to create a guide allowing future ease of identification. To compliment the morphological assessment, 3D models were constructed via micro-CT scanning to create a manipulatable model of male and female *A. mongolianus* and male *A. japonicus*. The online genetic database for *Argulus* spp. was also assessed to ascertain relationships between species and updated to include new cytochrome c oxidase subunit 1 (COI) sequences for *A. mongolianus*, *A. japonicus* and *A. coregoni*. This resource will help to identify *A. mongolianus* and ascertain its spread throughout the UK, alongside improving future genetic investigations and species identification of *Argulus* spp. This in turn will help reduce the ongoing introduction of *Argulus* spp. worldwide.

7.2 Introduction

Introduction of invasive non-native species can lead to disruption of ecosystems with severe economic loss, negative impacts to human and/or animal health and even species extinction (Andersen *et al.* 2004; Clavero and García-Berthou 2005; Charles and Dukes 2007; Bradshaw *et al.* 2016). Introduction and establishment of parasitic species can be dangerous due to native hosts having no prior experience of the evolutionary arms race with the novel parasite (Kirk 2003; Mastitsky *et al.* 2010; Lymbery *et al.* 2014). While free-living organisms must surpass numerous challenges to establish themselves outside their native range (Hulme *et al.* 2008), parasites face additional barriers due to their dependency on hosts for survival. Introduction

and establishment of parasites in areas outside their natural range thus requires not only appropriate environmental factors, but viable host populations (Lymbery *et al.* 2014; Dunn and Hatcher 2015).

Parasite invasions mainly occur through import or invasion of infected host species (Mack *et al.* 2000; Juhásová *et al.* 2016), although accidental importation without the host is possible for eggs/larvae and free-living stages (Chapman *et al.* 2012; Lymbery *et al.* 2014). The global trade of live organisms for hobby and leisure is now considered one of the main invasion routes, with an extensive variety of species transported (estimated 24% of all terrestrial bird, mammal, amphibian, and reptile species traded; Scheffers *et al.* 2019) in a multibillion-dollar industry (Bush *et al.* 2014; Sinclair *et al.* 2021). Correct identification of species is key to ensuring legal trade, improving biosecurity, and minimising invasion risks (Collins *et al.* 2013). This is an ongoing issue; for 2000 - 2005 US imports, only 3.8% of aquatic organism shipments were identified to family level or below, with widespread use of broad taxonomic designations (Smith *et al.* 2008). For parasites, recognition and identification is even more difficult (and in many cases impossible without clinical investigation) due to their size, life cycle and lack of research compared to that of free-living organisms (Besansky *et al.* 2003; Tavares *et al.* 2011).

Identification of aquatic parasites faces further complications due to difficulties in handling and examining aquatic organisms; removal from water can be extremely stressful or even damaging to the organism, and for fish anaesthetic is often required for accurate evaluation (Li *et al.* 2002; Mitchell and Tully 2016). Additionally, the water used to transport aquatic organisms and soil of aquatic plants may harbour parasites that can spread to other tanks if placed into a recirculating system, or be introduced via spillage and disposal (Liltved and Hansen 1990; Rodgers *et al.* 2011; Assis *et al.* 2014). Because of this, numerous problematic aquatic parasites have been introduced worldwide including *Aphanomyces astaci* (cause of crayfish plague with severe mortalities in European crayfish species; Svoboda *et al.* 2017), *Anguillicoloides crassus* (eel swimbladder parasite, causes mortalities which threaten European eel populations/cultures; Lefebvre *et al.* 2012), *Myxobolus cerebralis* (cause of whirling disease, affects wild and cultured salmonids; Hoffman 1990) and *Sphaerothecum destruens* (intracellular parasite responsible for extinction of fish populations; Andreou *et al.* 2012). Introduction of the monogenean *Gyrodactylus salaris* to Norway resulted in mass mortalities of wild Atlantic salmon (>95% fish infected in some areas), as such infected rivers were treated by eradicating all fish hosts within the system and restocking with uninfected

individuals (Johnsen and Jenser 1991; Sandodden 2018). Discovery and identification of aquatic parasites outside their native range is therefore paramount to minimising health impacts and reducing economic cost through monitoring, recognition of invasion routes and prevention of further spread.

In 2019, mortalities within a UK fishery led to the discovery of an unknown species of parasitic fish louse belonging to the genus *Argulus*. *Argulus* spp. are ectoparasitic fish lice that cause direct damage to host fish skin and tissue through attachment and feeding, alongside vectoring/facilitating secondary diseases (Ahne 1985; Walker *et al.* 2011b; AmbuAli *et al.* 2020). There are currently three established *Argulus* spp. in the UK: native *A. coregoni* and *A. foliaceus* and the introduced *A. japonicus*. Of these *A. foliaceus* is the most common and *A. japonicus* the least due to its recent introduction (first records in 1990; Rushton-Mellor 1992); although difficulties in distinguishing it from *A. foliaceus* may have led to fewer records due to miss-identification (Rushton-Mellor and Boxshall 1994; Taylor *et al.* 2005a,b). For genetic species identification, DNA barcoding systems wherein reference libraries are constructed for standardised gene regions, are considered a fast, accurate and cost-effective tool (Hubert and Hanner 2015). Cytochrome c oxidase subunit I (COI) genetic marker is considered to be the primary animal barcode sequence (Hebert *et al.* 2003; Ratnasingham and Hebert 2007), and most of the genetic data available for *Argulus* spp. uses this marker. The sudden appearance of an unidentified *Argulus* species in the UK provided the basis for the current study, which in turn led to a review of the existing data on morphological distinction of UK *Argulus* species through updating the key produced by Fryer (1986) and an assessment of the genetic data available for the genus.

7.3 Methods

Collection and specimen preparation

In 2019 the UK Environment Agency was alerted to fish mortalities within an undisclosed UK carp fishery. Upon investigation, *Argulus* species were recovered primarily on/in the gills, mouth, and head of the fish - host locations not commonly frequented by the three known UK *Argulus* species (figure 7.1). Lice were collected and placed into ethanol >70% for morphological assessment and >90% for molecular identification. Further infections have since been confirmed in multiple fisheries on common carp and their hybrids, roach, and bream.

Morphological examination

Fewer males than females of the unknown *Argulus* species were found on hosts, as such a total of 14 females and 5 males were measured for morphological analyses. Using ImageJ version 1.51j8 (Schneider *et al.* 2012), measurements were taken for total length (from rostral tip of carapace to caudal end of abdominal lobes), carapace length (from rostral tip of carapace to end of carapace), carapace width, width and height of carapace protrusion, abdominal lobe length (taken vertically from point of lobe), abdominal lobe width, abdominal lobe sinus length, distance between eyes, eye diameter and first maxillae (suction disc) diameter. All widths and diameters were taken at the widest point. All measurements from images were calibrated using a 1/100 mm micrometre scale.

Figure 7.1 Live female *Argulus mongolianus* on host common carp (*Cyprinus carpio*) mouth (left) and gill (right). Note the "shovel" like carapace protrusion, visible to the naked eye. Photographs taken by the Environment Agency.

Images were taken of lice samples without staining at x10 - x80 magnification using a Lumenera Infinity 1 camera mounted on a dissecting microscope with Infinity Capture software version 6.5.4. Additional images were taken of wet mounted samples in Lactophenol at x100 using a compound microscope. *A. japonicus* (male) and unknown *Argulus* species (male and female) samples were selected for 3D modelling via micro-CT scanning at the Natural History Museum (London). Samples were stained for 1 week using 1% iodine or 0.3%

PTA (phosphotungstic acid) and held in place within a 1.5 ml Eppendorf tube during scanning. Only male *A. japonicus* were used as a comparison due to time/budget limitations; males are more useful than females in *Argulus* species determination due to the species-specific male mating apparatus morphology. Scanning was performed using the using the Carl Zeiss Xradia Versa 520 high resolution micro-CT scanner with the following parameters: 40.0 kV, 75.0 µA and 3.0 W for all samples, 3201 projections and 5.0 s exposure time for male *A. japonicus* and male unknown sample, 1601 projections and 7.0 s exposure time for female unknown sample. Voxel size (three-dimensional pixel) was 0.0036, 0.0076 and 0.0085 mm for male *A. japonicus,* male unknown sample and female unknown sample respectively. Avizo Software version 2019.1 (Westenberger 2008) was used to render the models, remove the Eppendorf mount, and visualise the final models. Drishti version 2.7 was then used to generate virtual dissections of the female sample (Limaye 2012).

Molecular analyses

Individual specimens of unknown *Argulus* species (N = 2), *A. japonicus* (N = 6), *A. coregoni* (N = 7) *and A. foliaceus* (N = 5) from UK fisheries were first examined under a dissecting microscope in >90% ethanol to confirm sample quality prior to DNA extraction. DNA was extracted from whole tissue samples macerated with a pestle using the QIAGEN DNeasy Blood & Tissue Kit following manufacturer's instructions. Cytochrome c oxidase subunit I (COI) gene region was selected for analysis as there are more publicly available sequences for this region (table 7.1), reflecting its status as the primary animal barcode sequence (Hebert *et al.* 2003; Ratnasingham and Hebert 2007). For all PCR reactions a total volume of 30 µl PCR mixture was used containing 3 µl DNA, 1 µl of each primer (forward and reverse; 10 µM concentration), 15 µl of QIAGEN 2x Multiplex PCR Master Mix (including HotStarTaq DNA Polymerase: 5 units/µl concentration), and 10 µl of nuclease-free water. A 710 bp fragment of DNA was amplified using "Universal" COI invertebrate primers LCO1490: 5´-GGTCAACAAATCATAAAGATATTGG-3´ and HCO2198: 5´-TAAACTTCAGGGTGACCAAAAAATCA-3´ (Folmer *et al.* 1994) with the following conditions: initial denaturation at 95 °C for 15 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min (Khan *et al.* 2016). Following PCR, 5 µl of each PCR product was combined with 5 µl of bromophenol blue (6x) and analysed on a 1% agarose gel (100 ml) prepared with 2 µl of SYBR Green I and electrophoresed at 90 V for 1 h. The gel was then examined using a GelDoc-It Imaging System (UVP). Following visual confirmation of PCR amplicon, samples were sent

to Eurofins Genomics for sequencing (10 µl nuclease-free water, 5 µl PCR product, 2 µl primer at 10 µM concentration, forward or reverse). All chromatograms generated were assessed to ensure good sequence quality (defined as clear single peaks for each base), with F/R sequences paired and ends trimmed using BioEdit version 7.2.5 (Hall 1999). In total, forward and reverse sequences were generated from two unknown *Argulus* species, four *A. japonicus* and six *A. coregoni* specimens. Sequences are available on GenBank under accession numbers OL841700 - OL841711.

Table 7.1 Diversity of *Argulus* spp. sequences currently available online. For cytochrome c oxidase subunit 1 (COI), sequences of *A. siamensis, A. foliaceus, A. japonicus* and *A. indicus* were not included due to poor sequence quality (see main text).

Region sequenced	**No. of identified species**	**Identified species**	**No. of unknown species**	**Unknown species ID**
COI	6	*A. funduli* *A. appendiculosus* *A. stizostethii* *A. catostomi* *A. longicaudatus* *A. americanus*	14	BACAZ-034 BACZP380-15 Arg.G.Fh.LWT.13.1 Ar.Ext.Mc.SEM.2.1 Arg.Lg.G.SEM.3.1 Arg.Ca.Ext.CHU.1.1 Arg.Ca.Ext.CHU.1.2 A.Ca.Ext.CHU.4.1 ECTCR162-14 ECTCR093-14 CH756-G04 CH759-D05 BIOUG19282-D03 CH727-G02
18S	8	*A. bengalensis* *A. siamensis* *A. rhipidiophorus* *A. foliaceus* *A. japonicus* *A. coregoni* *A. monodi* *A. nobilis*	3	*Argulus* sp. WBUAFS/A1 *Argulus* sp. Yu-2006 *Argulus* sp. UTOM3
28S	3	*A. foliaceus* *A. japonicus* *A. rhipidiophorus*	4	*Argulus* sp. Yu-2006 *Argulus* sp. OC-2001 *Argulus* sp. JMM-2003 *Argulus* sp. KS-2017
NAD1	1	*A. japonicus*	0	NA
ND4	1	*A. japonicus*	0	NA
PPO	1	*A. foliaceus*	0	NA
COMPLETE GENOME	1	*A. americanus*	0	NA

All *Argulus* spp. COI sequences available online from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and BOLD (Barcode of life data system v4; https://boldsystems.org/) were collected together to create a custom database for analyses, with *Dolops bidentata* (Branchiura) and *Lepeophtheirus salmonis* (Copepoda) included as outgroups (GenBank accession numbers MT582371.1 and MG936209.1 respectively). Sequences <300 bp were excluded. All sequences were trimmed to an equal length resulting in a total length of 499 bp for all sequences. The exception to this was unknown *Argulus* species BACZP380-15; this sequence was only 403 bp however removal did not alter results, as such it was included in the final analyses to maximise database diversity. Prior to trimming sequences ranged from 499 to 658 bp, trimming did not alter tree clustering. BLAST searches were conducted using Genome Workbench version 3.7.1 (Kuznetsov and Bollin 2021), with a word size of 11 and expect value of 0.05 (Wheeler and Bhagwat 2007). Clustal X version 2.1 (Larkin *et al.* 2007) was used to align sequences with MEGA version 10.2.4 (Kumar *et al.* 2018) then used to generate a Maximum-Likelihood Tree with 1000 bootstrap iterations, visualise the tree and calculate pairwise and mean p-distance (proportion of nucleotide sites that are different) between sequences using default parameters. Neighbour-Joining and Minimum-Evolution trees were also created using the default parameters in MEGA and showed the same structure/clustering as the Maximum-Likelihood Tree. During analyses, sequences of *A. siamensis, A. foliaceus, A. japonicus* and *A. indicus* from Khan *et al.* (2016) were excluded as they all clustered together and did not group with any other sequences, suggesting cross contamination. *A. japonicus* sequences from Wadeh *et al.* (2010) were also excluded as they clustered separately from other *A. japonicus* and were only 1 - 7 mutations different from the *A. americanus* sequence, suggesting miss-identification or contamination. After removing these sequences, the database totalled 39 samples: 37 *Argulus* spp. sequences (9 known species plus unknown species) and 2 outgroup species samples. All publicly available sequences used are listed in table 7.2.

Identification

Morphological identification was achieved through published species descriptions/keys and comparison to museum samples. During this, 45 species of *Argulus* from the Natural History Museum (London) collections were assessed, of which none matched the unknown *Argulus* samples. Molecular analyses could not identify the species due to a lack of genetic information for *Argulus* spp., however examination of COI indicated that the species sampled was

genetically distinct from the previous *Argulus* spp. sequences available. According to a single key describing a female sample only (Tokioka 1939), the species was identified as *A. mongolianus.* No prior molecular data existed for *A. mongolianus* and internet searching revealed very little information on the species with only two mentions in published literature, one of which being the key (Tokioka 1939; Shedko *et al.* 2018).

Table 7.2 Cytochrome c oxidase subunit 1 (COI) *Argulus* spp. sequences obtained from BOLD (Barcode of life data system v4; https://boldsystems.org/). GenBank (https://www.ncbi.nlm.nih.gov/genbank/) IDs are also available for *A. americanus* (AY456187.1) and Unknown Species BACAZ-034 (MG449868.1). Sequences of *A. siamensis, A. foliaceus, A. japonicus* and *A. indicus* were not included due to predetermined issues with the sequences (see main text).

Species	**Online ID**
A. funduli	ECTCR119-14
A. appendiculosus	429A-01_COIR
A. appendiculosus	429B_COI
A. stizostethii	507G_COI
A. americanus	AY456187
A. catostomi	ZOOPS_0119
A. catostomi	ZOOPS_0458
A. catostomi	ZOOPS_0459
A. catostomi	ZOOPS_0455
A. catostomi	ZOOPS_0456
A. catostomi	ZOOPS_0457
A. longicaudatus	ZOOPS_0349
A. longicaudatus	ZOOPS_0350
Unknown Species	BACAZ-034
Unknown Species	BACZP380-15
Unknown Species	Arg.G.Fh.LWT.13.1
Unknown Species	Ar.Ext.Mc.SEM.2.1
Unknown Species	Arg.Lg.G.SEM.3.1
Unknown Species	Arg.Ca.Ext.CHU.1.1
Unknown Species	Arg.Ca.Ext.CHU.1.2
Unknown Species	A.Ca.Ext.CHU.4.1
Unknown Species	ECTCR162-14
Unknown Species	ECTCR093-14
Unknown Species	CH756-G04
Unknown Species	CH759-D05
Unknown Species	BIOUG19282-D03
Unknown Species	CH727-G02

7.4 Results

Morphological examination

Detailed measurements for *A. mongolianus* are given in table 7.3. Females were on average just over 1 mm larger than males, with carapace on average 67% of total parasite length. Pigmentation is present on dorsal side of female and male carapace and dorsal side of male abdominal lobes, framing the testes (figure 7.2). Compared to other *Argulus* species, *A. mongolianus* has a rounded circular carapace (especially in large females) versus the typical "shield" like shape. The main distinctive characteristics for morphological identification include the "shovel" like carapace protrusion, lack of ventral carapace spinules above the antennae, large secondary maxillae basal plate fully covered in spinules (strawberry in shape) and morphology of mating apparatus on 2nd, 3^{rd,} and 4th pairs of male swimming legs. The "shovel" like protrusion is a unique characteristic not reported in any other documented *Argulus* species. It is likely related to the lack of ventral carapace spinules above the antennae, as this is also unique among known *Argulus* species. The protrusion is more distinct in females than males, with an "m" shaped groove visible in females (comparisons made between males and females of comparable sizes; see figures 7.3 and 7.4).

Table 7.3 *Argulus mongolianus* measurements of 14 female and 5 male samples, which had been preserved in >70% ethanol. All measurements are in mm.

Measurement	**FEMALE**			**MALE**		
	Average	Standard Deviation (±)	Range	**Average**	Standard Deviation (±)	Range
Total length	**6.79**	1.91	3.84 - 10.22	**4.58**	0.73	3.56 - 5.67
Carapace length	**4.70**	1.42	2.66 - 7.48	**2.98**	0.47	2.32 - 3.59
Carapace width	**4.51**	1.45	2.67 - 7.63	**2.68**	0.44	2.14 - 3.28
Carapace protrusion width	**0.88**	0.13	0.6 - 1.13	**0.72**	0.12	0.54 - 0.87
Carapace protrusion height	**0.20**	0.06	0.08 - 0.28	**0.18**	0.04	0.13 - 0.21
Abdominal lobe length	**1.91**	0.42	1.16 - 2.54	**1.34**	0.23	1.01 - 1.71
Abdominal lobe width	**1.90**	0.65	0.98 - 2.91	**0.98**	0.15	0.76 - 1.23
Abdominal lobe sinus length	**1.41**	0.36	0.7 - 2	**0.81**	0.18	0.52 - 1.09
Distance between eyes	**0.79**	0.18	0.58 - 1.2	**0.57**	0.09	0.43 - 0.7
Eye diameter left	**0.20**	0.04	0.11 - 0.29	**0.18**	0.01	0.16 - 0.2

Eye diameter right	**0.20**	0.04	0.11 - 0.28	**0.17**	0.02	0.14 - 0.19
First maxillary diameter left	**1.07**	0.26	0.65 - 1.57	**0.57**	0.06	0.52 - 0.69
First maxillary diameter right	**1.04**	0.26	0.66 - 1.54	**0.58**	0.06	0.49 - 0.68

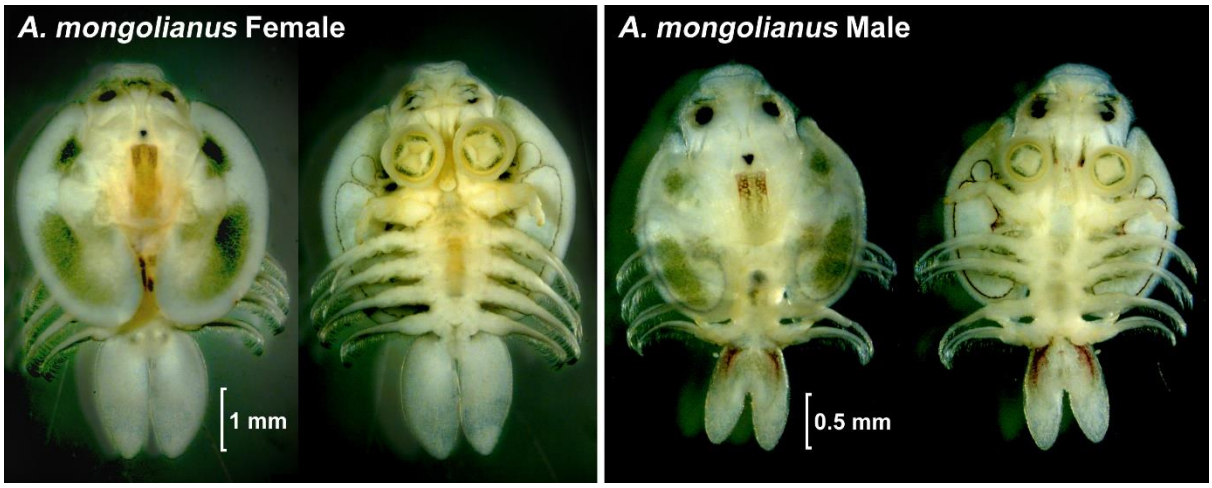

Figure 7.2 Dorsal and ventral views of whole *Argulus mongolianus* samples preserved in >70% ethanol. Images taken of unstained samples at x10 for female and x20 for male using a dissecting microscope.

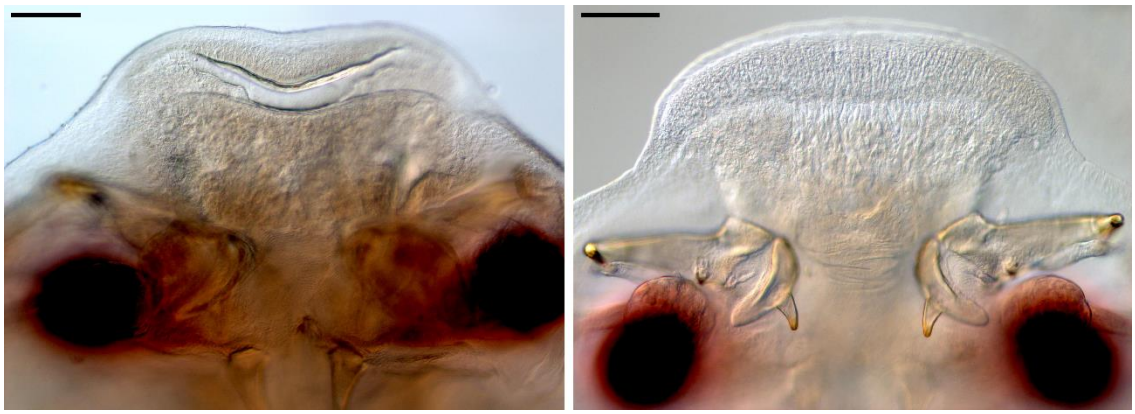

Figure 7.3 Comparison of carapace protrusion in female (left) and male (right) *Argulus mongolianus.* Scale bars equal 100 µm, images were taken of lactophenol wet mount samples at x100 using a compound microscope by the Environment Agency.

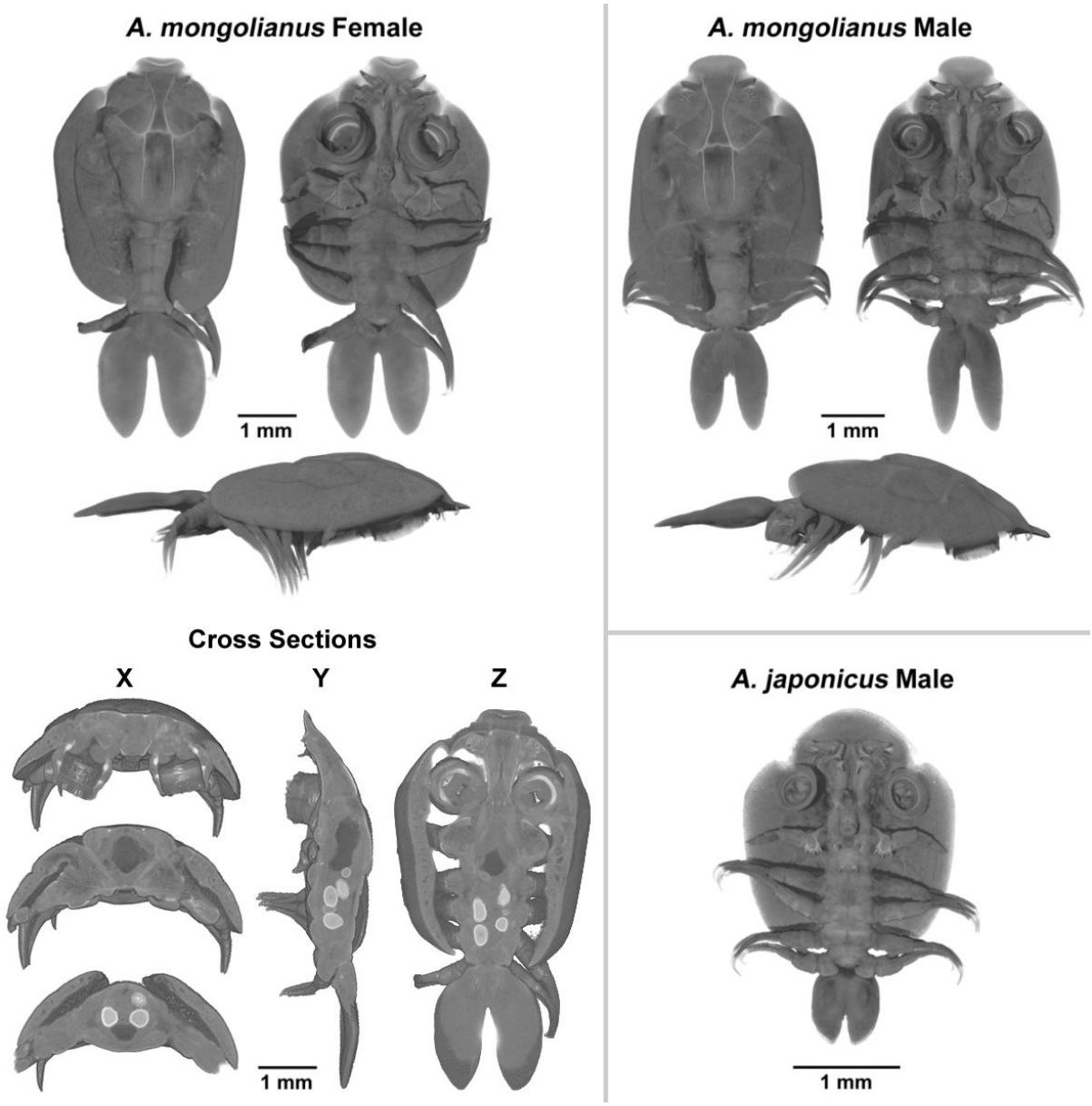

Figure 7.4 3D models of *Argulus* spp. created using micro-CT scanning. For *A. mongolianus* female X axis cross sections were taken: top image ~30% in from rostral tip of carapace (halfway through the first maxillae/suction discs), middle image 50% through thorax, bottom image ~75% in from rostral tip of carapace. Y axis and Z axis cross sections both taken at 50% through thorax for their respective planes. White/light-coloured ovoid shapes in cross sections are eggs *in-situ* in the ovary. Images are displayed on a white background for clarity, carapace protrusion and margins are transparent while dorsal ridges, primary maxillae rings and eggs (light/white coloured areas) are areas of dense tissue. Models are available online as image stacks (.tiff), *A. mongolianus* female: figshare.com/s/fd46a0f9b7db57fa735a,
A. mongolianus male: figshare.com/s/f5aae6725a2650affb95,
A. japonicus male: figshare.com/s/7e8d5baa39222b9ea5a8.

Updated outline drawings based on Fryer (1986) highlight differences between the established UK *Argulus* species (*A. coregoni*, *A. foliaceus* and *A. japonicus*) and the newly invading *A. mongolianus* (figure 7.5). Species identification requires examination of morphological characteristics; however, host niche can be indicative of taxon. *A. coregoni*, *A. foliaceus* and *A. japonicus* are typically found along the head, body and fins of the fish (although extreme infections of *A. foliaceus* can result in colonisation of the mouth too; personal observations C. Williams) while *A. mongolianus* is mainly found on the head and on/in the gills and mouth. *A. coregoni* is reported to prefer salmonid species (Mikheev *et al.* 2007), *A. foliaceus* and *A. japonicus* are broad generalists found on all species (Walker *et al.* 2004; Kearn 2004), *A. mongolianus* has mainly been reported on carp, although roach and bream are also susceptible. The main morphological distinctions between the established UK species and *A. mongolianus* are listed in table 7.4 with supporting photographs and morphological comparisons in figures 7.6, 7.7 and 7.8.

Table 7.4 Prominent morphological characteristics for identification of *Argulus mongolianus*, *A. coregoni*, *A. foliaceus* and *A. japonicus*. A microscope is required to observe most features.

Anatomical structure	***A. coregoni***	***A. foliaceus***	***A. japonicus***	***A. mongolianus***
Carapace protrusion	None	None	None	Yes, "shovel" like protrusion
Spinules on ventral side of carapace above antennae (refer to figure 7.6)	Yes	Yes	Yes	No
Basal plate of secondary maxillae (refer to figure 7.6)	Small oval patch of spinules	Oval patch of spinules	Oval patch of spinules	Fully covered in spinules
Abdominal lobe sinus length (refer to figure 7.7)	Approximately half of lobe length	Less than half of lobe length	Approximately half of lobe length	Almost ¾ of total lobe length
Abdominal lobe shape (refer to figure 7.7)	Pointed tips	Extremely rounded tips	Slightly pointed tips	Rounded tips
Abdominal lobe margins (refer to figure 7.6)	Smooth margins	Denticulate margins	Denticulate margins	Denticulate margins
Male 2nd swimming leg underside of coxa (refer to figure 7.8)	Two protruding, rounded knob-like bumps, one at each end of coxa	Flat with slightly curved pointed protrusion near thorax	Curved with small bump at each end of coxa	Flat with small bump near thorax
Male 3rd swimming leg topside of coxa (refer to figure 7.8)	Small bumps towards leg bristles	Smooth/no protrusions	Smooth/no protrusions	Protruding, rounded knob-like bump towards leg bristles
Male 4th swimming leg topside of coxa (refer to figure 7.8)	Dual pointed clasper	Rounded clasper	Sharply pointed clasper	Dual pointed clasper

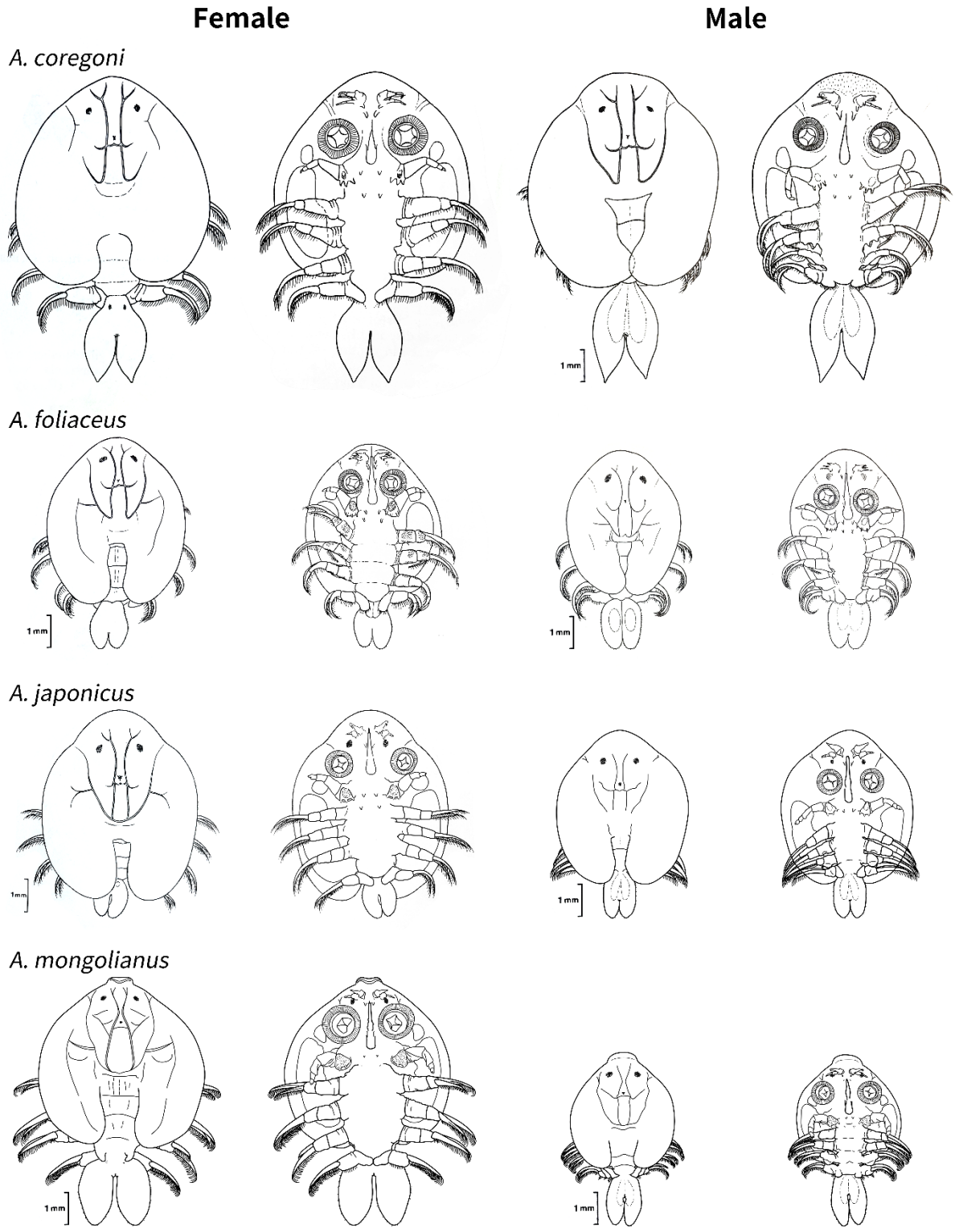

Figure 7.5 Updated key of *Argulus* species in the UK. *A. coregoni* and *A. foliaceus* from Fryer 1986. *A. japonicus* dorsal female from Fryer 1986, ventral female and male images from author. *A. mongolianus* by author. Female *A. coregoni* did not have any scale or measurements and thus were sized to match male *A. coregoni*. The 1 mm scale bar applies to both dorsal and ventral images, all *Argulus* images with scale are in proportion to each other.

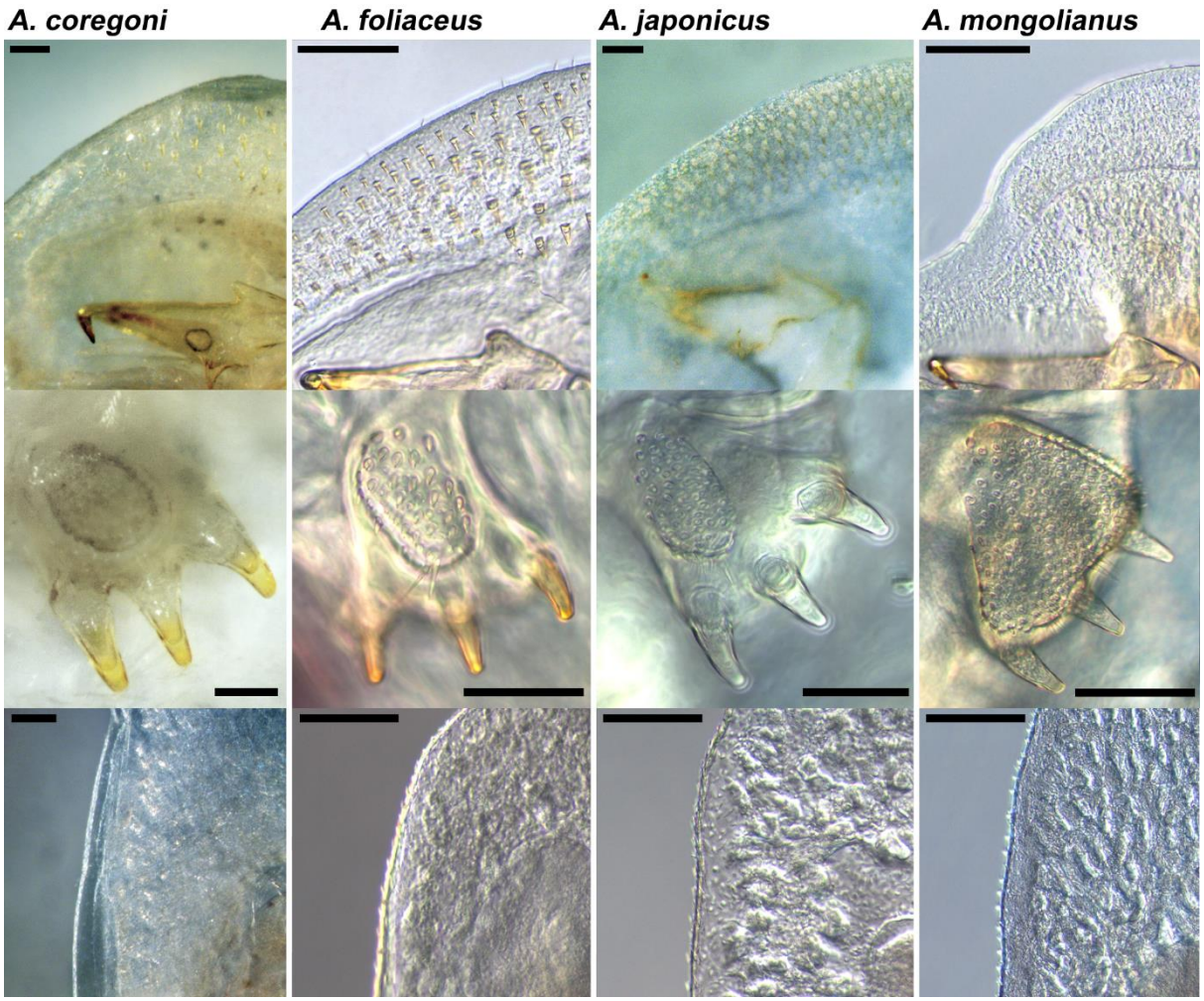

Figure 7.6 Comparisons of key morphological differences between *Argulus mongolianus* and the three established UK *Argulus* species: *A. coregoni*, *A. foliaceus* and *A. japonicus*. Top row shows presence of spinules above antennae for the established UK species which are not present for *A. mongolianus*. Middle row shows variation in secondary maxillae basal plate with *A. mongolianus* fully covered in spinules versus a small patch of spinules for the established UK species. bottom row shows smooth margins of abdominal lobes for *A. coregoni* with denticulate margins for the other species. All scale bars equal 100 µm, images taken of unstained samples at x60 - x80 using a dissecting microscope and lactophenol wet mount samples at x100 using a compound microscope.

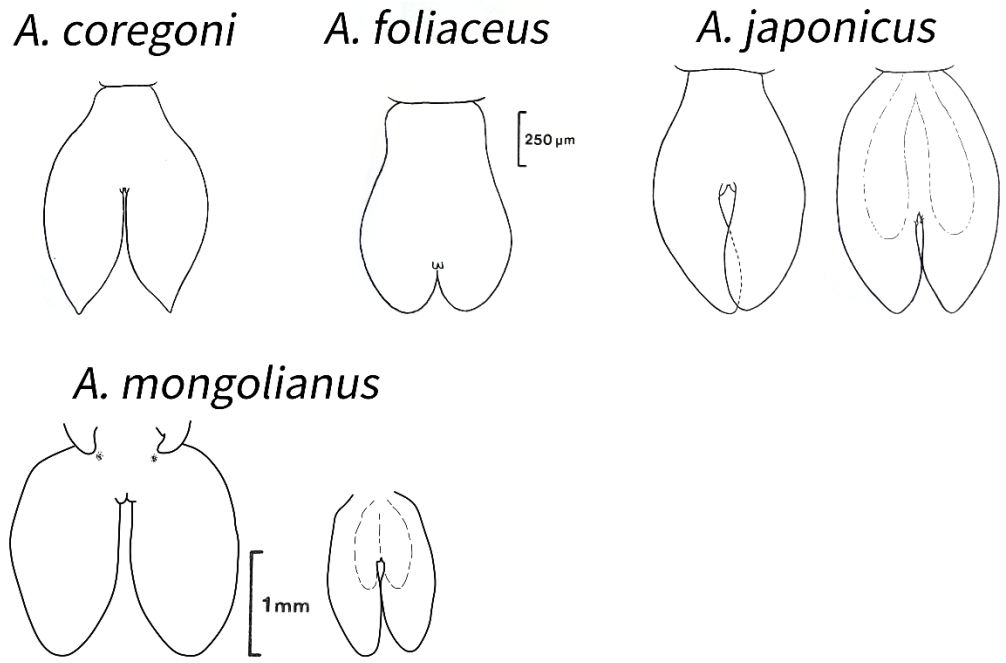

Figure 7.7 Abdominal lobe shape of *Argulus foliaceus* female, *A. coregoni* female, *A. japonicus* female (left) and male (right), *A. mongolianus* female (left) and male (right). Lobe tip shape and sinus length (the slit separating the lobes) can be used in species identification. First row images are from Fryer (1986), no scale bar provided for *A. coregoni* and *A. japonicus*. *A. mongolianus* 1 mm scale applies to both female and male images.

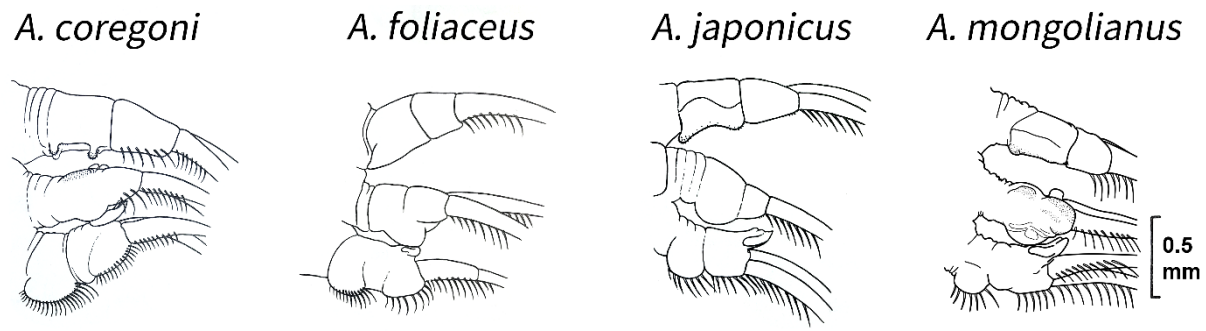

Figure 7.8 Male breeding appendages on 2nd, 3rd and 4th swimming legs of *Argulus coregoni, A. foliaceus, A. japonicus* and *A. mongolianus.* Shape and position of protrusions on coxa (base of appendage) for all three legs should be considered when identifying species. First three images are from Fryer (1986), no scale bar provided.

Molecular analyses

Based on the 499 bp COI sequence alignment, the 9 known *Argulus* species clustered separately from each other with 5 distinct clusters of unknown *Argulus* species observed (figure 7.9). Across all *Argulus* spp. sequences there was a mean p-distance of 0.20, with intraspecies variation ranging from 0.00 - 0.04. The mean p-distance between *Argulus* spp. and the outgroup containing *Dolops bidentata* and *Lepeophtheirus salmonis* was 0.34. The *A. mongolianus* sequences generated here from two specimens matched exactly (with one sequence 10 bp longer prior to trimming). *A. mongolianus* was closest in relation to *A. coregoni* (82.9 - 83.1% identity, 97.2% coverage), followed by *A. japonicus* (81.2 - 82.2% identity, 99.6% coverage). The *A. japonicus* sequences generated also clustered with unknown *Argulus* spp. sequences CHU 1.1, 1.2 and 4.1 (samples collected from Chenhu Lake, Hubei, China; native range of *A. japonicus*) with 96.6 - 98.4% identity and 100% coverage. As such, these unknown sequences from China are now identified as *A. japonicus.* Also, the unknown *Argulus* species samples ECTCR162-14 and ECTCR093-14 on BOLD were collected at the same place/time as *A. funduli* sample ECTCR119-14. These unknown species sequences matched 98.4 - 99.8% identity with 100% coverage to *A. funduli* and thus can be identified as this species.

A. foliaceus is one of the most common and studied *Argulus* spp. Despite this only two COI sequences were labelled as such on GenBank and BOLD (Khan *et al.* 2016) but were likely mis-identified (see methods above). During this study we were unable to obtain a COI sequence for *A. foliaceus* using the LCO1490/HCO2198 primers (Folmer *et al.* 1994). The only sequence we generated from an *A. foliaceus* sample matched to *Skrjabillanus tincae,* a fish nematode that uses *A. foliaceus* as an intermediate host (Molnár and Székely 1998). This, combined with Andres *et al.* (2019) being unable to obtain a COI sequence for *A. flavescens*, *A. americanus* and *A. bicolor* (the only *A. americanus* COI available is from whole genome sequencing, *A. flavescens* and *A. bicolor* have no COI sequences available online) indicates the standard "universal" COI primer set are unsuitable for all *Argulus* species, and for *A. foliaceus* there is at least one mutation in the primer binding region. As such, the Environment Agency is currently developing new COI primers for *Argulus* species identification.

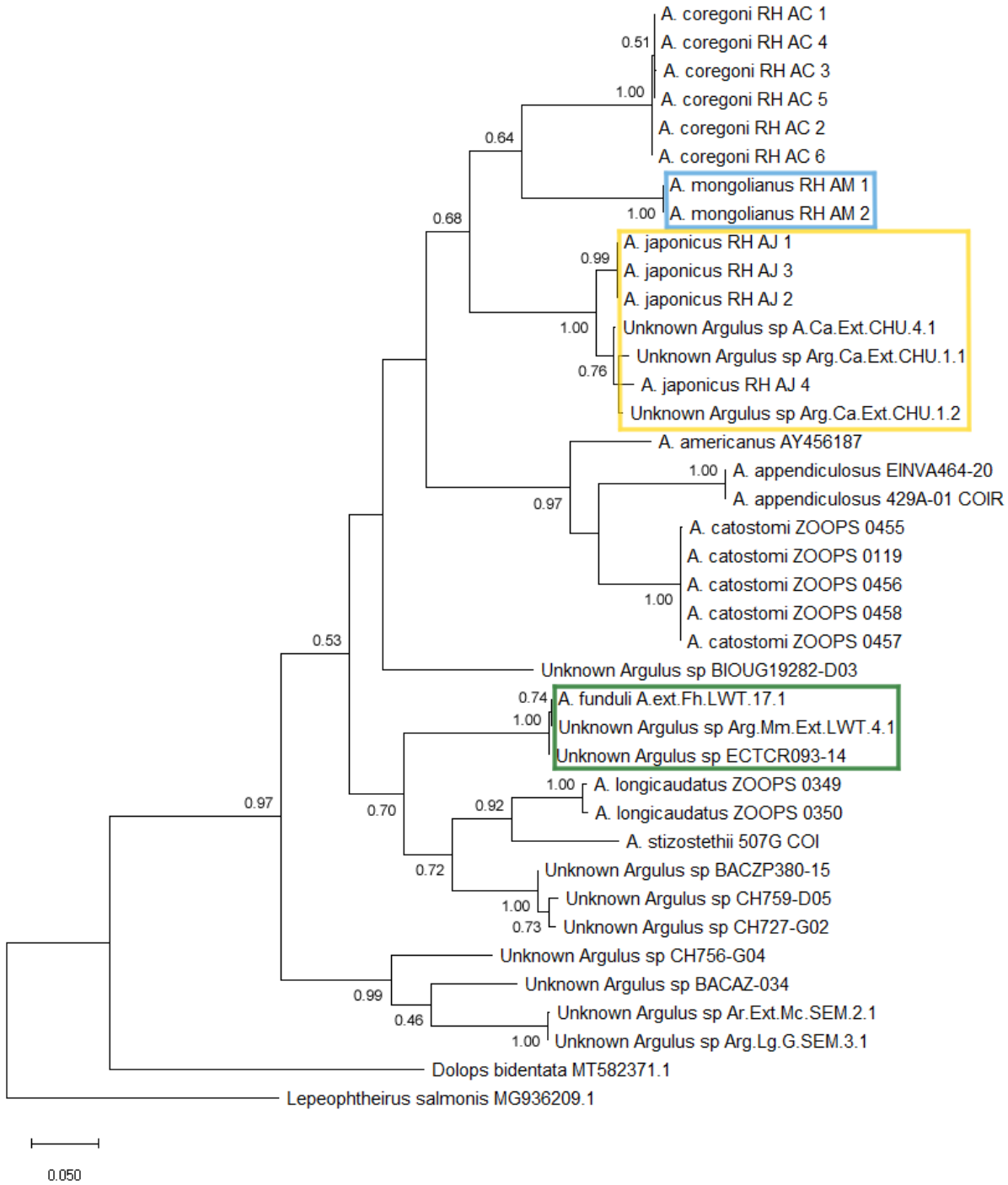

Figure 7.9 Maximum-Likelihood Phylogenetic Tree of *Argulus* mtDNA Cytochrome Oxidase I gene (all 499 bp except BACZP380-15 which equalled 403 bp) with bootstrap ratios. All species with "RH" in their name ID were generated in the study, other sequences were obtained from BOLD (Barcode of life data system v4; https://boldsystems.org/, see table 7.2). *A mongolianus* are highlighted with a light blue box, *A. japonicus* cluster with unknown species sequences is highlighted with a yellow box, *A. funduli* cluster with unknown species sequences is highlighted with a dark green box. Scale bar indicates branch length.

7.5 Discussion

Identification of organisms is reliant on robust morphological and/or molecular data, for which information on both is lacking for many species within the genus *Argulus*. Here, we were able to identify the unknown *Argulus* discovered in UK as *A. mongolianus* via a single key documenting only a female sample due to the unique morphological characteristics of this species. We have thus improved morphological identification of *A. mongolianus* by documenting male and female characteristics, alongside their comparisons to the established UK species. The molecular database for *Argulus* has also been expanded with COI sequences generated for *A. mongolianus, A. coregoni* and *A. japonicus.*

Anatomically, *A. mongolianus* is comparable to the established UK species with a few key distinct traits that enable morphological identification. The full spinule cover of the secondary maxillae basal plate is easily distinguished from the ovoid patch of spinules seen in all the established UK species. The male mating appendages on the 2nd, 3rd and 4th swimming legs are considered species specific, likely a mechanism to prevent or reduce inter-specific reproduction (Reproductive Isolation; Masly 2012). The rostral carapace protrusion of *A. mongolianus* appears to be unique among known *Argulus* species, accompanied by a lack of spinules on the ventral carapace above the antennae. Other *Argulus* species recorded in the oral cavity and gills do not possess a similar feature (Wilson 1902; Roberts 1957; Wang 1961; Shimura *et al.* 1983), suggesting that the protrusion does not play a role in attachment to this area, especially considering the lack of spinules which normally aid attachment to host. The protrusion also bears a "m" shaped groove in females which is not seen in males, suggesting a sex-specific role. The groove could aid copulation or egg laying (female specific behaviours), however no other *Argulus* species possess female specific mating or egg laying features. Sexually dimorphic attachment capabilities have been recorded previously for beetles attributed to a trade off in males to enhance attachment to smooth surfaces such as female elytra, versus females which only need to attach to rough plant surfaces (Voigt *et al.* 2008). Further examination of the protrusion and its tissue composition is needed to ascertain its function, although the micro-CT scanning model does not show any areas of high tissue density in the protrusion, compared to the high-density rings of tissue observed in the primary maxillae suction discs and dorsal carapace ridges.

Creation of 3D models via micro-CT scanning can enhance species description by providing a digital dataset that can be manipulated and examined (Stoev *et al.* 2013; Staab *et al.* 2018).

This method of creating a 3D model also enables "virtual dissections" without damaging material, especially important for valuable samples (Simonsen and Kitching 2014). Here, these virtual dissections allowed visualisation of eggs within the ovary of the female sample alongside examination of internal structures. The models also help highlight anatomical features such as the difference in carapace protrusion between males and females and the shape of dorsal ridges which can prove difficult to photograph. These models cannot replace traditional imaging - despite being high-resolution small details can still be lost (such as the spinules on underside of carapace/secondary maxillae basal plate) due to micro movements in the sample during scanning creating blur. Instead, they provide a manipulatable view of the organism that complements traditional microscope imaging.

Genetically, *A. mongolianus* matched closest to *A. coregoni and A. japonicus.* Both of these species are found in Asia, with *A. japonicus* native (now introduced worldwide; Rushton-Mellor 1992) and *A. coregoni* a European species, reported in Japan from 1938 (Hoshina 1950; Nagasawa and Yuasa 2019). All other known species in the COI genetic database are native to the USA (Wilson 1902; Wilson 1944; Cressey 1972). While COI can be used in *Argulus* species identification, the inability to amplify COI for all *Argulus* species using the "universal" LCO1490/HCO2198 primers leaves the current methodology unsuitable for further investigation. It has been previously noted for other organism groups that the Folmer *et al.* (1994) primers are not truly universal and as such alternative primer pairs have been developed for other taxa (Lohman *et al.* 2009; Hamsher *et al.* 2011). Development of *Argulus* specific COI primers should therefore be priority for expanding the genetic database further. In addition, shorter COI sequences (<300 bp) have been used successfully for species identification of museum samples (Shokralla *et al.* 2011; Coetzer and Grobler 2018) and thus could be applied to expand the *Argulus* genetic database using samples (such as from a museum) that may not successfully sequence using the "universal" LCO1490/HCO2198 primers or an edited *Argulus* specific primer pair due to the length of the barcode.

One issue that could arise from developing new COI primers for *Argulus* spp. identification is the potential for hybridisation. Hybridisation has not yet been reported within the Genus *Argulus* - mixed species infections are uncommon with a previous investigation showing dominance by one species (mixed species population: 93.5% *A. japonicus*, 6.5% *A. siamensis*; Sahoo *et al.* 2012). However, the Environment Agency has collected *Argulus* spp. samples from a UK fishery which possess a mix of morphological characteristics, matching both *A.*

foliaceus and *A. japonicus*. It is currently unknown if these samples are hybrids and genetic investigation is in progress. If hybridisation is possible, then additional development of mitochondrial DNA (mtDNA) markers would be favourable as mtDNA is maternally inherited (Cronin *et al.* 1991). Previously mtDNA has been used to examine natural hybridisation in decapods and copepods (Imai and Takeda 2005; Parent *et al.* 2012). Further, mtDNA can be used in population genetic analyses, an area of interest to the Environment Agency considering the isolated nature of *Argulus* spp. populations within contained lake systems.

A. mongolianus has not been previously recorded in the UK or Europe, with the only literature regarding this species (other than the key) reporting over 90% cultured carp mortalities in Russia (Shedko *et al.* 2018). It is likely *A. mongolianus* was introduced to the UK from European carp imports. As parasite discovery, identification and recording can be difficult and/or not perceived as important, many species are transported across the globe as contaminants. This poses a major biosecurity risk - considering the high mortalities seen from the parasite in Russia and the mortalities recorded here in the UK that led to its discovery, this parasite could become a major problem for UK carp fisheries should it spread. *Argulus* spp. are usually conspicuous due to their large size and position on the host body, although introductions to the UK still occur with parasites hidden within locations such as the folds of fish fins (personal observations UK Pet Shops). As *A. mongolianus* frequents the gill cavity and mouth of the fish, it may be significantly more difficult to diagnose compared to other *Argulus* species, hence identification only occurring once mortalities have occurred. Molecular diagnosis using techniques such as eDNA (Trujillo-González *et al.* 2019, 2020) could circumvent the need to handle hosts and observe parasites, improving identification and biosecurity. Better screening of aquatic organisms and subsequent identification of parasites is required to reduce and prevent the continued introduction of parasites to areas outside their range. The description of *A. mongolianus* and comparison to UK *Argulus* species generated here will aid morphological identification upon parasite discovery. Genetic data will aid further progression in genetic identification and diagnosis of *Argulus* species infections.

Chapter 8

General Discussion

8.1 Overview

This thesis sought to improve our understanding of freshwater fish lice, a common and often devastating group of parasites that have been largely neglected in favour of research into their marine counterparts. While marine fisheries remain dominant in the global market (Tidwell and Allan 2001; FAO 2020), freshwater fisheries are a core component (Mohan Dey *et al.* 2005; Monticini 2010; Rath 2018; Sinclair *et al.* 2021) with severe economic loss endured worldwide from *Argulus* spp. infections (Taylor *et al.* 2006; Sahoo *et al.* 2012; Sahoo *et al.* 2013a). Three main areas were explored within this thesis: (1) current status of UK angling fisheries and problems associated with *Argulus* spp. infections, established in Chapters 2 and 3, (2) options for improved monitoring and control explored throughout Chapters 4, 5 and 6, and (3) how *Argulus* spp. infections might change into the future with shifts in climate and host factors in Chapter 3 and identification of an invading non-native *Argulus* spp. recently discovered in UK fisheries in Chapter 7.

While this thesis largely focused on angling fisheries and infections within the UK, aquaculture systems worldwide also benefit from the information generated regarding parasite identification (Chapter 7), infection dynamics (Chapter 3) and control (Chapters 4 and 5). The egg laying substrate control method within Chapter 6 was built for and deployed in angling fisheries, but this control method could easily be adjusted for cage-based systems situated within freshwater bodies. For indoor recirculating systems, an understanding of how and where *Argulus* spp. lay their eggs can improve monitoring and targeting of treatments.

8.2 Current State of Fisheries

One of the biggest hurdles in addressing *Argulus* spp. infections is communication with fisheries, highlighted in Chapters 2 and 7 with only 24% of fisheries contacted responding to the survey and the successful invasion of *A. mongolianus* to UK waters (with no documentation of the parasite in Europe despite it likely arising from European imports). It can be difficult to obtain information from industry regarding parasitic infection, especially when such information could lead to a lowering of reputation and associated economic loss should it become public (Chapter 6). Survey response rates vary dramatically (range 3 - 92.2%; Baruch

and Holtom 2008) and although only a quarter of fisheries responded, this was more than triple the number of fisheries surveyed in 2000 (240 fisheries here versus 77 in Taylor *et al.* 2006) and thus gives an improved basis to ascertain the current status of UK fisheries.

Management (fish stocking amount/timing) and temperature contributed to fisheries facing problematic infections, supporting previous literature on risk factors for *Argulus* spp. infections in fisheries (Shimura 1983; Gault *et al.* 2002; Hakalahti *et al.* 2004b; Harrison *et al.* 2006; Taylor *et al.* 2006; Taylor *et al.* 2009a). Further assessment of temperature in Chapter 3 showcased how high temperatures lead to faster life history traits and short generation time, reflected in the seasonal pattern of *Argulus* spp. infections in the UK with peak problematic periods during summer and the potential for multiple parasite cohorts (Chapters 3 and 6). Fishery features also impacted infections, and this data could be used to "profile" and identify water bodies that are at higher risk of problematic infections. Perhaps the most alarming finding was that current control methods are not sufficient in handling problematic *Argulus* spp. infections, resulting in fish and angler impacts that led to economic loss. Because of this, new options for monitoring and control were examined over Chapters 4, 5 and 6.

8.3 Progress with Monitoring and Control

The monitoring and control methods explored in this thesis aimed to reduce the reliance on/application of chemical treatments in fisheries, through examination of new monitoring/non-chemical control techniques (Chapters 4 and 6) and investigating the potential for timed treatment to improve efficacy of chemical application (Chapter 5). Exploration into chemical attraction of *Argulus* spp. has also begun (Appendix 1), with the aim of identifying host chemical cues to allow artificial replication and testing as another form of bait within louse traps.

Light is a common tool in aquaculture and fisheries, used to increase fish food intake/growth, manipulate fish behaviour/maturation/spawning, and for ease of maintenance (Boeuf and Le Bail 1999; Oppedal *et al.* 1999; Juell *et al.* 2003; Karakatsouli *et al.* 2008; Villamizar *et al.* 2011). Light is also a strong attractant of *Argulus* spp. and is an ideal bait for trap-based methods as it is non-consumable and customisable (intensity and wavelength). As a monitoring tool, light traps should be effective as previous tests for marine sea lice were successful (Novales Flamarique *et al.* 2009) and *Argulus* spp. have been shown to have stronger light attraction than their marine counterparts (MacKinnon 1993; Mikheev *et al.* 1999; Genna *et al.*

2005; Bandilla *et al.* 2007; Mordue (Luntz) and Birkett 2009). For control, a "push-pull" methodology (Cook *et al.* 2007; Khan *et al.* 2011) could be applied wherein lights on fish cages/in fisheries are turned off or changed to red to stop louse attraction (push) and traps with white or blue light are deployed to attract and trap lice (pull). With the data from this thesis, light traps can be designed and tested within fisheries to ascertain viability based on capture rate (including non-target captures), cost, and ease of use. The circadian rhythms that arise from light cycles can also be exploited to improve efficacy of chemical control through timed dosage based on both parasite and host rhythms. Here, we found the *A. foliaceus* transcriptome to be highly rhythmic indicating chronotherapeutic techniques could be effective. For forthcoming tests of timed dosage, the data produced here can be used to determine suitable initial time-points for examination. The transcriptome data also offers the potential for mining of new drug targets. This is key considering the rise in drug resistance within fish lice (Hakalahti *et al.* 2008; Aaen *et al.* 2015) and could also result in formulation of new chemical treatments with higher efficacy and/or lower environmental impact compared to current options.

Another "trap" based tool employing egg laying substrate was examined in UK fisheries to determine the viability of this technique as previous investigations were inconclusive. The structures used here were constructed to ensure angler casts would be unaffected and deployment/use both fast and cost-effective, concerns that had not been achieved with previous designs (Taylor *et al.* 2005b; personal observations C. Williams). The traps were extremely effective as a monitoring tool, providing easy, real-time observation of louse populations without interfering with fish hosts. For one fishery, they showcased how treatment of drain/lime (both time consuming and costly due to fishery closure) was not 100% effective as eggs were recovered from the substrate traps after treatment. The traps also proved to be a viable control method as they removed large volumes of *Argulus* spp. eggs; in total 238,817 egg strings were removed from fisheries in this study, equivalent to over 12.4 million eggs (assuming average number of eggs per string = 52, calculated from lab cultures). This led to an overall decrease in eggs collected (and therefore adult breeding population) the following year.

Another factor of monitoring and control briefly touched upon in Chapter 3 is preventive/early treatment of infections (as blood feeding appears to commence from >2 mm parasite size). *Argulus* spp. infections are commonly treated only once parasite populations are large enough to cause sufficient damage to hosts/economic loss. Early diagnosis of *Argulus* spp. infections can be challenging as juvenile lice are small in size (~0.6 mm at hatching) and cryptic (not

species specific), with difficulties in thoroughly examining hosts without laboratory equipment. Light traps could offer an early warning sign of infections as *Argulus* spp. are attracted to light from hatching, although determination of juvenile *Argulus* spp. compared to other invertebrates may be impossible without microscope examination. The egg laying substrates employed in Chapter 6 were able to capture eggs even at low adult population levels (both after treatment and as early as late April, before problematic summer peaks), therefore application of treatment upon discovery of eggs when populations are "non-problematic" could prevent large population spikes and subsequent problems later in the year.

The control methods examined here can also be extrapolated and applied to other parasite species. Light traps can be used to capture any species that display light-attracted behaviour, with the potential for light-baited traps previously investigated for juvenile monogenean *Neobenedenia girellae* (see Skilton *et al.* 2020). The same chemical treatments are often used for both *Argulus* spp. and marine sea lice (Hakalahti *et al.* 2008; Mayer *et al.* 2013), thus the circadian rhythms of drug targets identified in Chapter 5 may also apply to sea lice. The concept of chronotherapeutic techniques can be applied to all parasites and here almost half of the *A. foliaceus* transcriptome was rhythmic indicating other crustacean parasites could display this level of rhythmicity. As with the light traps, egg laying substrates can be employed for any organism that preferentially attaches itself or eggs to a substrate. In Japan, mesh is often removed/changed within cage aquaculture due to entanglement and colonisation by monogenean eggs (*Neobenedenia* spp., see Ogawa *et al.* 2006; Lowell 2012; *Heterobothrium okamotoi*, see Ogawa and Yokoyama 1998) and this has been suggested as a form of control.

Fishery managers hope for a "silver bullet" treatment that will fix all problems with parasitic infections in one fell swoop. The reality is that for *Argulus* spp., populations are extremely difficult to eradicate once established and reducing/maintaining pathogen populations to low, manageable levels ("non-problematic") is the goal. Towards this goal combination treatments used in parallel or sequentially are key, with all the monitoring and control methods examined in this thesis compatible with each other and other techniques. The difficulty lies in appropriate selection and application of these techniques, with physical and managerial diversity of fisheries complicating matters. Using the data generated in this thesis, the Environment Agency aims to develop a risk-based predictive model wherein fisheries can input their parameters regarding features, management, and monitoring/control to ascertain their risk of facing problematic *Argulus* spp. infections and receive recommendations to reduce their risk.

8.4 The Future of *Argulus* spp. Infections

Almost two decades ago, problematic *Argulus* spp. infections were noted in 29% of UK trout fisheries (Taylor *et al.* 2006). Here, 31.5% of trout fisheries surveyed experienced problematic infections, an increase of 2.5%. As the criteria for a "problematic" infection is subjective (and likely changes for each fishery due to their diverse structure and management), it is difficult to ascertain the accuracy of this increase. The Environment Agency, however, has noted a rise in enquires regarding *Argulus* spp. infections over the last decade, with carp farms with no previous history of *Argulus* infestations now also facing problems. The recent discovery of *A. mongolianus* may be linked to this increase, as the three established *Argulus* species mainly cause problems within trout farms. Following on from the results of Chapter 2, an investigation into carp farms with the Environment Agency will be conducted to ascertain why carp farms are now facing problematic infections.

Models have also been previously developed to predict changes within *Argulus* spp. populations (Taylor *et al.* 2009b; McPherson *et al.* 2012); the data generated in Chapters 3 and 6 will improve accuracy of predictions and aid development of new models. Host species and infection density have not been previously considered when modelling, yet they both play a role in the dynamics of population growth and thus should be considered in future models. Both specialist (*A. coregoni*; see Pasternak *et al.* 2004) and generalist (*A. foliaceus*; Chapter 3) *Argulus* spp. growth is impacted by host selection. Previous investigations by Bandilla *et al.* (2005) found that rainbow trout (*Oncorhynchus mykiss*) showed no protective acquired resistance to *Argulus* spp. infection, however initial investigations in Appendix 2 suggests that three-spined sticklebacks (*Gasterosteus aculeatus*) do as re-infection led to significantly lower survival of *A. foliaceus*. Currently, selection of species for stocking in fisheries and aquaculture is based on public demand, maximising natural resources (via polyculture; Welcomme and Bartley 1998), species hardiness and culture knowledge (Minchin and Rosenthal 2002; Arlinghaus *et al.* 2014). For farmers facing problems from *Argulus* spp. infections, consideration of species cultured/stocked (and for angling/cage-based fisheries, presence of other host species in water body) could therefore aid control.

Temperature increases can lead to intensification of infections, as salmonid fish hosts experience higher susceptibility and *Argulus* spp. display shorter generation times allowing additional cohorts within a year. As temperatures across the globe rise with climate change, an increase in problematic *Argulus* spp. infections is expected assuming no intervention. Control

of water temperature within angling fisheries is unachievable, as such fishery owners are at the mercy of the climate. While indoor recirculating aquaculture allows for control of temperature (mitigating climate change), fish face worsening conditions as the industry expands and intensifies, with cage-based aquaculture enduring both intensification and climate stressors. Fisheries therefore stand on a precipice of change; infections will likely worsen in the foreseeable future without improvement to fish stress and development of efficacious *Argulus* spp. control strategies.

Currently three species of *Argulus* are established within the UK, however there is a constant threat of introduction of new, non-native species due to trade of live fish and lack of parasite screening/identification. The Environment Agency have previously encountered *A. americanus* in the UK on lungfish from aquaria (personal observations C. Williams), and here we complete the description of *A. mongolianus* following its discovery in UK fisheries. Considering the source/invasion route of *A. mongolianus* remains unknown and its presence confirmed in multiple fisheries, spread across the UK is possible. Fisheries with known infections are being monitored to prevent parasite transfer to new waters, however imports from abroad remain a major risk. With the enhanced description and complimentary genetic data generated in this thesis, it is hoped that more *A. mongolianus* infections will be identified on into the future, aiding understanding of this species' movement from its native range of Asia. Genetic data could also prove a valuable tool for diagnosis of *Argulus* spp. infections; eDNA testing of water samples is a rapidly developing area with interest in its use in screening for infection due to the non-invasive methodology and ability to detect low levels of infection (e.g. Huver *et al.* 2015; Trujillo-González *et al.* 2019; Spikmans *et al.* 2020; Trujillo-González *et al.* 2020). Detection of *Argulus* spp. currently requires host examination and while adults can be easily observed with the naked eye, low infections, colonisation of gills/mouth and infection with small juveniles can make recognition challenging. While eDNA techniques still face hurdles regarding accurate detection (Harper *et al.* 2019; Beng and Corlett 2020; Sieber *et al.* 2020), their integration into parasite screening and monitoring as methodology improves will be key to tackling infections and preventing pathogen spread.

Although this thesis focused on aquaculture and fisheries as these industries typically face high parasite burdens (with subsequent economic loss), wild fish are also subject to parasitic infections. Parasites are a key part of ecosystems/food webs, with infections usually a consistent, non-lethal presence. However, stress (such as from pollution events), "spillover"

(wherein parasites introduced by aquaculture infect wild fish) or novel invading parasites can cause parasite prevalence to dramatically increase (Bouwmeester *et al.* 2021). Transmission of sea lice from farmed to wild salmon has been observed previously (Krkošek *et al.* 2006), thus spillover of *Argulus* spp. from freshwater fisheries and aquaculture is likely possible. Additionally, as *Argulus* spp. continue to spread across the globe, invading non-native *Argulus* spp. may disrupt wild fish populations. Loss of wild biodiversity and/or populations can have devastating affects through impacts on food security and other industries (especially those reliant on tourism). As for fisheries/aquaculture, *Argulus* spp. infections in wild fish/habitats can be addressed using the techniques and methods proposed in this thesis. Light traps could easily be implemented in any water body to monitor *Argulus* spp. presence, with eDNA techniques also viable (bolstered by the improved *Argulus* spp. genetic database created here). Egg laying substrates can also be applied to wild lakes, with testing required for canal/river-based habitats. Monitoring of *Argulus* spp. (and other parasites) in wild ecosystems is critically important to ensure fish populations, and thus the wider community, remain healthy (Timi and Poulin 2020). This is especially important considering the global threat posed by climate change and its potential impact on parasite infections.

In conclusion, this thesis has greatly expanded the current knowledge base for *Argulus* spp. Current problems faced by UK trout fisheries have been documented with calculated differences in *A. foliaceus* life histories across different temperatures, infection densities, and hosts. The survey highlighted how current control methods are failing farmers, with infections chronic and not improved by treatment application. A suite of new and novel monitoring and control techniques were also assessed, with endogenous rhythms and circadian gene expression examined for the first time for any crustacean parasite. All the control methods explored here show promise, with the light-based methods pending field testing and egg substrates an ongoing investigation with the Environment Agency offering it as an option to interested fisheries. An updated description for newly invading *A. mongolianus* was also produced, with additions to the *Argulus* spp. genetic database.

Parasite infections will likely increase and intensify on into the future with changes to industrialisation/climatic stressors and invasion of non-native species. Chapter 3 highlighted how temperature resulted in shorter generation times for *Argulus* spp., a trait seen in other parasites (Andersen and Buchmann 1998; Hakalahti *et al.* 2006; Studer *et al.* 2010; Macnab and Barber 2012; Groner *et al.* 2014) thus many infections will worsen with global warming.

Monitoring of infections is key to proactive tackling of problems; the survey produced in Chapter 2 was centred around *Argulus* spp. infections, but the concept can be altered and applied to assess any parasite and/or associated disease faced by industry. Identification of parasite infections faces many challenges, however genetic diagnosis offers promise of basic and often non-invasive diagnosis with accuracy improving as technology and methodology develop. Control is an ongoing arms race between humanity and parasites. Numerous non-chemical control options are now being explored with a reduction in chemical application to mitigate environmental impacts and rising drug resistance. With continued improvements to communication, screening, monitoring and treatment (the groundwork for which is provided across this study), *Argulus* spp. and infections from other parasitic species can be successfully tackled to reduce economic loss and improve fish health globally.

References

Aaen, S.M., Helgesen, K.O., Bakke, M.J., Kaur, K. and Horsberg, T.E. 2015. Drug resistance in sea lice: a threat to salmonid aquaculture. *Trends in Parasitology* 31(2), pp. 72–81.

Aalberg, K. *et al.* 2016. A study of fish lice (*Argulus* sp.) infection in freshwater food fish. *Folia Veterinaria* 60(3), pp. 54–59.

Agusti-Ridaura, C. *et al.* 2018. *Caligus rogercresseyi* acetylcholinesterase types and variants: a potential marker for organophosphate resistance. *Parasites & Vectors* 11(1), p. 570.

Ahne, W. 1985. *Argulus foliaceus* L. and *Piscicola geometra* L. as mechanical vectors of spring viraemia of carp virus (SVCV). *Journal of Fish Diseases* 8(2), pp. 241–242.

Alphey, L., Benedict, M., Bellini, R., Clark, G.G., Dame, D.A., Service, M.W. and Dobson, S.L. 2010. Sterile-insect methods for control of mosquito-borne diseases: an analysis. *Vector Borne and Zoonotic Diseases* 10(3), pp. 295–311.

Altinok, A., Lévi, F. and Goldbeter, A. 2007. A cell cycle automaton model for probing circadian patterns of anticancer drug delivery. *Advanced Drug Delivery Reviews* 59(9), pp. 1036–1053.

AmbuAli, A., Monaghan, S.J., McLean, K., Inglis, N.F., Bekaert, M., Wehner, S. and Bron, J.E. 2020. Identification of proteins from the secretory/excretory products (SEPs) of the branchiuran ectoparasite *Argulus foliaceus* (Linnaeus, 1758) reveals unique secreted proteins amongst haematophagous ecdysozoa. *Parasites & Vectors* 13(1), p. 88.

AmbuAli, A., Taggart, J.B., Bekaert, M., Wehner, S., Monaghan, S.J. and Bron, J.E. 2021. Identification of transcripts relating to host-parasite interactions and feeding-associated activities through de novo transcriptome sequencing of branchiuran fish lice, *Argulus foliaceus* (Linnaeus, 1758) and *Argulus coregoni* Thorell, 1865. PREPRINT (Version 2) available at Research Square [https://doi.org/10.21203/rs.3.rs-285509/v2].

Andersen, M.C., Adams, H., Hope, B. and Powell, M. 2004. Risk assessment for invasive species. *Risk Analysis* 24(4), pp. 787–793.

Andersen, P.S. and Buchmann, K. 1998. Temperature dependent population growth of *Gyrodactylus derjavini* on rainbow trout, *Oncorhynchus mykiss*. *Journal of Helminthology* 72(1), pp. 9–14.

Andreou, D., Arkush, K.D., Guégan, J.-F. and Gozlan, R.E. 2012. Introduced pathogens and native freshwater biodiversity: a case study of *Sphaerothecum destruens*. *PLOS ONE* 7(5), p. e36998.

Andres, M.J., Higgs, J.M., Grammer, P.O. and Peterson, M.S. 2019. *Argulus* from the Pascagoula River, MS, USA, with an emphasis on those of the threatened Gulf sturgeon, *Acipenser oxyrinchus desotoi*. *Diversity* 11(12), p. 232.

Arlinghaus, R., Beardmore, B., Riepe, C., Meyerhoff, J. and Pagel, T. 2014. Species-specific preferences of German recreational anglers for freshwater fishing experiences, with emphasis on the intrinsic utilities of fish stocking and wild fishes. *Journal of Fish Biology* 85(6), pp. 1843–1867.

Arlinghaus, R. and Cooke, S.J. 2009. Recreational fisheries: socioeconomic importance, conservation issues and management challenges. In: Dickson, B., Hutton J., and Adams, W.M. eds. *Recreational Hunting, Conservation and Rural Livelihoods*. Oxford, UK: Wiley-Blackwell, pp. 39–58.

Arlinghaus, R., Tillner, R. and Bork, M. 2015. Explaining participation rates in recreational fishing across industrialised countries. *Fisheries Management and Ecology* 22(1), pp. 45–55.

Ashton, F.T., Li, J. and Schad, G.A. 1999. Chemo- and thermosensory neurons: structure and function in animal parasitic nematodes. *Veterinary Parasitology* 84(3), pp. 297–316.

Assis, D.A.S. de, Cavalcante, S.S. and Brito, M.F.G. de 2014. Aquarium trade as a potential disseminator of non-native invertebrates in Northeastern Brazil. *Neotropical Biology and Conservation* 9(2), pp. 115–119.

Avenant-Oldewage, A. and Everts, L. 2010. *Argulus japonicus*: Sperm transfer by means of a spermatophore on *Carassius auratus* (L). *Experimental Parasitology* 126(2), pp. 232–238.

Avenant-Oldewage, A. and Swanepoel, J.H. 1993. The male reproductive system and mechanism of sperm transfer in *Argulus japonicus* (Crustacea: Branchiura). *Journal of Morphology* 215(1), pp. 51–63.

Bai, A.S. 1981. Photic effects on embryonation and phototactic responses by the larvae of *Argulus siamensis*. *Proceedings: Animal Sciences* 90(5), pp. 513–517.

Bailey, R.J. *et al*. 2006. The role of semiochemicals in host location and non-host avoidance by salmon louse (*Lepeophtheirus salmonis*) copepodids. *Canadian Journal of Fisheries and Aquatic Sciences* 63(2), pp. 448–456.

Bandilla, M., Hakalahti, T., Hudson, P.J. and Valtonen, E.T. 2005. Aggregation of *Argulus coregoni* (Crustacea: Branchiura) on rainbow trout (*Oncorhynchus mykiss*): a consequence of host susceptibility or exposure? *Parasitology* 130(2), pp. 169–176.

Bandilla, M., Hakalahti-Sirén, T. and Valtonen, E.T. 2007. Experimental evidence for a hierarchy of mate- and host-induced cues in a fish ectoparasite, *Argulus coregoni* (Crustacea: Branchiura). *International Journal for Parasitology* 37(12), pp. 1343–1349.

Bandilla, M., Hakalahti-Sirén, T. and Valtonen, E.T. 2008. Patterns of host switching in the fish ectoparasite *Argulus coregoni*. *Behavioral Ecology and Sociobiology* 62(6), pp. 975–982.

Bandilla, M., Valtonen, E.T., Suomalainen, L.-R., Aphalo, P.J. and Hakalahti, T. 2006. A link between ectoparasite infection and susceptibility to bacterial disease in rainbow trout. *International Journal for Parasitology* 36(9), pp. 987–991.

Barnard, C.F. 1990. *Parasitism and Host Behaviour*. London: CRC Press, p. 393.

Barthel, B.L., Cooke, S.J., Suski, C.D. and Philipp, D.P. 2003. Effects of landing net mesh type on injury and mortality in a freshwater recreational fishery. *Fisheries Research* 63(2), pp. 275–282.

Baruch, Y. and Holtom, B.C. 2008. Survey response rate levels and trends in organizational research. *Human Relations* 61(8), pp. 1139–1160.

Bates, D., Mächler, M., Bolker, B. and Walker, S. 2014. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67(1), 1–48.

de Bekker, C., Merrow, M. and Hughes, D.P. 2014. From behavior to mechanisms: an integrative approach to the manipulation by a parasitic fungus (*Ophiocordyceps unilateralis* s.l.) of its host ants (*Camponotus* spp.). *Integrative and Comparative Biology* 54(2), pp. 166–176.

de Bekker, C., Will, I., Hughes, D.P., Brachmann, A. and Merrow, M. 2017. Daily rhythms and enrichment patterns in the transcriptome of the behavior-manipulating parasite *Ophiocordyceps kimflemingiae*. *PLOS ONE* 12(11), p. e0187170.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L. and Zoran, M.J. 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nature Reviews Genetics* 6(7), pp. 544–556.

Beng, K.C. and Corlett, R.T. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation* 29(7), pp. 2089–2121.

Bernatowicz, P.P., Kotwica-Rolinska, J., Joachimiak, E., Sikora, A., Polanska, M.A., Pijanowska, J. and Bębas, P. 2016. Temporal expression of the clock genes in the water flea *Daphnia pulex* (Crustacea: Cladocera). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 325(4), pp. 233–254.

Berthelot, C. *et al.* 2014. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nature Communications* 5, p. 3657.

Bertossa, R.C., van Dijk, J., Beersma, D.G. and Beukeboom, L.W. 2010. Circadian rhythms of adult emergence and activity but not eclosion in males of the parasitic wasp *Nasonia vitripennis*. *Journal of Insect Physiology* 56(7), pp. 805–812.

Besansky, N.J., Severson, D.W. and Ferdig, M.T. 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology* 19(12), pp. 545–546.

Biscontin, A. *et al.* 2019. Analysis of the circadian transcriptome of the Antarctic krill *Euphausia superba*. *Scientific Reports* 9(1), p. 13894.

Blankenberg, D. *et al.* 2010. Galaxy: a web-based genome analysis tool for experimentalists. *Current Protocols in Molecular Biology* 89(1), pp.1-21.

Boeuf, G. and Le Bail, P.-Y. 1999. Does light have an influence on fish growth? *Aquaculture* 177(1), pp. 129–152.

Bogéa, T., Favre, T.C., Rotenberg, Lúc., Silva, H.S. and Pieri, O.S. 1996. Circadian pattern of cercarial emergence in *Schistosoma Mansoni* (Platyhelminthes: Digenea) from isolated *Biomphalaria Glabrata*. *Chronobiology International* 13(2), pp. 93–101.

Bohn, G. 1910. Sur les réactions comparées de deux parasites des poissons vis-à-vis de la lumière. *Compte-Rendu de l'Association Française Pour l'Avancement des Sciences* 38, pp. 726–729.

Bondad-Reantaso, M.G. *et al.* 2005. Disease and health management in Asian aquaculture. *Veterinary Parasitology* 132(3), pp. 249–272.

Bouwmeester, M.M., Goedknegt, M.A., Poulin, R. and Thieltges, D.W. 2021. Collateral diseases: aquaculture impacts on wildlife infections. *Journal of Applied Ecology* 58(3), pp. 453–464.

Bower-Shore, C. 1940. An investigation of the common fish louse, *Argulus foliaceus* (Linn.). *Parasitology* 32(4), pp. 361–371.

Bradshaw, C.J.A. *et al.* 2016. Massive yet grossly underestimated global costs of invasive insects. *Nature Communications* 7(1), p. 12986.

Brooks, K.M. 2009. Considerations in developing an integrated pest management programme for control of sea lice on farmed salmon in Pacific Canada. *Journal of Fish Diseases* 32(1), pp. 59–73.

Bryant, D.M. *et al.* 2017. A tissue-mapped axolotl de novo transcriptome enables identification of limb regeneration factors. *Cell reports* 18(3), pp. 762–776.

Buchfink, B., Xie, C. and Huson, D.H. 2015. Fast and sensitive protein alignment using DIAMOND. *Nature Methods* 12(1), pp. 59–60.

Burka, J.F., Hammell, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J. and Speare, D.J. 1997. Drugs in salmonid aquaculture – A review. *Journal of Veterinary Pharmacology and Therapeutics* 20(5), pp. 333–349.

Bush, E.R., Baker, S.E. and Macdonald, D.W. 2014. Global trade in exotic pets 2006–2012. *Conservation Biology* 28(3), pp. 663–676.

Bushnell, B. 2014. *BBMap: A Fast, Accurate, Splice-Aware Aligner*. Berkeley, USA: Lawrence Berkeley National Lab. Available at: https://www.osti.gov/servlets/purl/1241166.

Cambie, G., Caillard, V., Beauté-Lafitte, A., Ginsburg, H., Chabaud, A. and Landau, I. 1991. Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy. *Annales de Parasitologie Humaine et Comparée* 66(1), pp. 14–21.

Carvalho Cabral, P., Olivier, M. and Cermakian, N. 2019. The Complex Interplay of Parasites, Their Hosts, and Circadian Clocks. *Frontiers in Cellular and Infection Microbiology* 9, p. 425.

Chapman, J.W., Dumbauld, B.R., Itani, G. and Markham, J.C. 2012. An introduced Asian parasite threatens northeastern Pacific estuarine ecosystems. *Biological Invasions* 14(6), pp. 1221–1236.

Charles, H. and Dukes, J.S. 2007. Impacts of invasive species on ecosystem services. In: Nentwig, W. ed. *Biological Invasions*. Berlin, Germany: Springer, pp. 217–237.

Christie, A.E. 2014. Peptide discovery in the ectoparasitic crustacean *Argulus siamensis*: identification of the first neuropeptides from a member of the Branchiura. *General and Comparative Endocrinology* 204, pp. 114–125.

Clavero, M. and García-Berthou, E. 2005. Invasive species are a leading cause of animal extinctions. *Trends in Ecology & Evolution* 20(3), p. 110.

Coetzer, W. and Grobler, J. 2018. Identifying *Rhabdomys* Museum specimens following taxonomic changes: use of short COI sequences. *Vertebrate Zoology* 68, pp. 191–197.

Collier, K.J., Probert, P.K. and Jeffries, M. 2016. Conservation of aquatic invertebrates: concerns, challenges and conundrums. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26(5), pp. 817–837.

Collins, R.A., Armstrong, K.F., Holyoake, A.J. and Keeling, S. 2013. Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. *Biological Invasions* 15(6), pp. 1209–1215.

Conte, F.S. 2004. Stress and the welfare of cultured fish. *Applied Animal Behaviour Science* 86(3), pp. 205–223.

Cook, S.M., Khan, Z.R. and Pickett, J.A. 2007. The use of push-pull strategies in integrated pest management. *Annual Review of Entomology* 52(1), pp. 375–400.

Cooke, S.J. and Cowx, I.G. 2004. The role of recreational fishing in global fish crises. *BioScience* 54(9), pp. 857–859.

Cornelissen, G. 2014. Cosinor-based rhythmometry. *Theoretical Biology and Medical Modelling* 11(1), p. 16.

Costello, M.J., Grant, A., Davies, I.M., Cecchini, S., Papoutsoglou, S., Quigley, D. and Saroglia, M. 2001. The control of chemicals used in aquaculture in Europe. *Journal of Applied Ichthyology* 17(4), pp. 173–180.

Costello, M.J. 2009. The global economic cost of sea lice to the salmonid farming industry. *Journal of Fish Diseases* 32(1), pp. 115–118.

Cowx, I.G., Arlinghaus, R. and Cooke, S.J. 2010. Harmonizing recreational fisheries and conservation objectives for aquatic biodiversity in inland waters. *Journal of Fish Biology* 76(9), pp. 2194–2215.

Cox, R., Groner, M.L., Todd, C.D., Gettinby, G., Patanasatienkul, T. and Revie, C.W. 2017. Mate limitation in sea lice infesting wild salmon hosts: the influence of parasite sex ratio and aggregation. *Ecosphere* 8(12), p. e02040.

Crawley, M.J. 2007. *The R Book*. West Sussex: John Wiley & Sons Ltd, p. 1078.

Cressey, R.F. 1972. *The Genus Argulus (Crustacea: Branchiura) of the United States*. Washington D.C.: U.S. Environmental Protection Agency, p. 14.

Cronin, M.A., Palmisciano, D.A., Vyse, E.R. and Cameron, D.G. 1991. Mitochondrial DNA in wildlife forensic science: species identification of tissues. *Wildlife Society Bulletin* 19(1), pp. 94–105.

Crossan, J., Paterson, S. and Fenton, A. 2007. Host availability and the evolution of parasite life-history strategies. *Evolution* 61(3), pp. 675–684.

Cunningham, L. 2005. Assessing the contribution of aquaculture to food security: a survey of methodologies. FAO Fisheries Circular. No. 1010. pp. 1–25.

Curtis, J., Breen, B., O'Reilly, P. and O'Donoghue, C. 2017. The economic contribution of a recreational fishery in a remote rural economy. *Water Resources and Rural Development* 10, pp. 14–21.

Danylchuk, A.J., Suski, C.D., Mandelman, J.W., Murchie, K.J., Haak, C.R., Brooks, A.M.L. and Cooke, S.J. 2014. Hooking injury, physiological status and short-term mortality of juvenile lemon sharks (*Negaprion bevirostris*) following catch-and-release recreational angling. *Conservation Physiology* 2(1), p. cot036.

Davis, E.L. *et al.* 2018. Seasonally timed treatment programs for *Ascaris lumbricoides* to increase impact—an investigation using mathematical models. *PLOS Neglected Tropical Diseases* 12(1), p. e0006195.

Demas, G.E., Chefer, V., Talan, M.I. and Nelson, R.J. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 273(5), pp. R1631–R1637.

Deveney, M.R., Bayly, T.J., Johnston, C.J. and Nowak, B.F. 2005. A parasite survey of farmed Southern bluefin tuna, *Thunnus maccoyii* (Castelnau). *Journal of Fish Diseases* 28(5), pp. 279–284.

Diana, J.S. 2009. Aquaculture production and biodiversity conservation. *BioScience* 59(1), pp. 27–38.

Ditton, R.B., Holland, S.M. and Anderson, D.K. 2002. Recreational fishing as tourism. *Fisheries* 27(3), pp. 17–24.

Dobson, A., Lafferty, K.D., Kuris, A.M., Hechinger, R.F. and Jetz, W. 2008. Homage to Linnaeus: How many parasites? How many hosts? *Proceedings of the National Academy of Sciences of the United States of America* 105(Suppl 1), pp. 11482–11489.

Dunn, A.M. and Hatcher, M.J. 2015. Parasites and biological invasions: parallels, interactions, and control. *Trends in Parasitology* 31(5), pp. 189–199.

Eakins, B.W. and Sharman G.F. 2010. Volumes of the World's Oceans from ETOPO1, NOAA National Geophysical Data Center, Boulder, CO.

Eichner, C., Harasimczuk, E., Nilsen, F., Grotmol, S. and Dalvin, S. 2015. Molecular characterisation and functional analysis of LsChi2, a chitinase found in the salmon louse (*Lepeophtheirus salmonis salmonis*, Krøyer 1838). *Experimental Parasitology* 151–152, pp. 39–48.

Ellison, A.R., Uren Webster, T.M., Rey, O., Garcia de Leaniz, C., Consuegra, S., Orozco-terWengel, P. and Cable, J. 2018. Transcriptomic response to parasite infection in Nile tilapia (*Oreochromis niloticus*) depends on rearing density. *BMC Genomics* 19(1), p. 723.

Ellison, A.R., Uren Webster, T.M., Rodriguez-Barreto, D., de Leaniz, C.G., Consuegra, S., Orozco-terWengel, P. and Cable, J. 2020. Comparative transcriptomics reveal conserved impacts of rearing density on immune response of two important aquaculture species. *Fish & Shellfish Immunology* 104, pp. 192–201.

Ellison, A.R. et al. 2021. Circadian dynamics of the teleost skin immune-microbiome interface. *Microbiome* 9(1), p. 222.

El-Sheekh, M.M., Abdeldaim, M.M., Gharib, S.M. and El-Ksassas, H. 2019. Green technology applications for algal bloom control. In: Ravishankar, G.A., and Ambati, R.R. eds. *Handbook of Algal Technologies and Phytochemicals*. London, UK: CRC Press, pp. 13–19

Environment Agency 2018. *Annual fisheries report 2016 to 2017*. Bristol, UK: Environment Agency, p. 94.

Environment Agency 2020a. Annual summary of rod licence sales. Available at: https://data.gov.uk/dataset/2b303513-bc81-4bef-880f-8a587db9b3a1/annual-summary-of-rod-licence-sales [Accessed: 17 October 2021].

Environment Agency 2020b. Fisheries annual report 2019 to 2020. Available at: https://www.gov.uk/government/publications/fisheries-annual-report-2019-to-2020/fisheries-annual-report-2019-to-2020 [Accessed: 17 October 2021].

Epsky, N.D. *et al.* 1999. Field evaluation of female-targeted trapping systems for *Ceratitis capitata* (Diptera: Tephritidae) in seven countries. *Journal of Economic Entomology* 92(1), pp. 156–164.

Ernst, W., Jackman, P., Doe, K., Page, F., Julien, G., MacKay, K. and Sutherland, T. 2001. Dispersion and toxicity to non-target aquatic organisms of pesticides used to treat sea lice on salmon in net pen enclosures. *Marine Pollution Bulletin* 42(6), pp. 432–443.

FAO 2020. *The State of World Fisheries and Aquaculture 2020: Sustainability in action*. Rome, Italy: Food and Agriculture Organization, p. 244.

Fenton, A., Hakalahti, T., Bandilla, M. and Valtonen, E.T. 2006. The impact of variable hatching rates on parasite control: a model of an aquatic ectoparasite in a Finnish fish farm. *Journal of Applied Ecology* 43(4), pp. 660–668.

Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3(5), pp. 294–299.

Forlenza, M., Walker, P.D., de Vries, B.J., Wendelaar Bonga, S.E. and Wiegertjes, G.F. 2008. Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura). *Fish & Shellfish Immunology* 25(1), pp. 76–83.

Francischetti, I.M.B., Sá-Nunes, A., Mans, B.J., Santos, I.M. and Ribeiro, J.M.C. 2009. The role of saliva in tick feeding. *Frontiers in bioscience: a journal and virtual library* 14, pp. 2051–2088.

Froese, R. and Pauly, D. 2022. FishBase. World Wide Web electronic publication. www.fishbase.org, version (02/2022).

Fryer, G. 1982. *The parasitic Copepoda and Branchiura of British freshwater fishes: A handbook and key*. Ambleside, UK: Freshwater Biological Association, p. 87.

Gabriel, U. and Akinrotimi, O. 2011. Management of stress in fish for sustainable aquaculture development. *Researcher* 3, pp. 28–38.

Galarowicz, T. and Cochran, P.A. 1991. Response by the parasitic crustacean *Argulus japonicus* to host chemical cues. *Journal of Freshwater Ecology* 6(4), pp. 455–456.

Ganni, S., Botden, S.M.B.I., Chmarra, M., Goossens, R.H.M. and Jakimowicz, J.J. 2018. A software-based tool for video motion tracking in the surgical skills assessment landscape. *Surgical Endoscopy* 32(6), pp. 2994–2999.

Gault, N.F.S., Kllpatrick, D.J. and Stewart, M.T. 2002. Biological control of the fish louse in a rainbow trout fishery. *Journal of Fish Biology* 60(1), pp. 226–237.

Genna, R.L., Mordue, W., Pike, A.W. and Mordue (Luntz), A.J. 2005. Light intensity, salinity, and host velocity influence presettlement intensity and distribution on hosts by copepodids of sea lice, *Lepeophtheirus salmonis*. *Canadian Journal of Fisheries and Aquatic Sciences* 62(12), pp. 2675–2682.

George, J.E., Pound, J.M. and Davey, R.B. 2004. Chemical control of ticks on cattle and the resistance of these parasites to acaricides. *Parasitology* 129(S1), pp. S353–S366.

Gippet, J.M.W. and Bertelsmeier, C. 2021. Invasiveness is linked to greater commercial success in the global pet trade. *Proceedings of the National Academy of Sciences* 118(14), p. e2016337118

Gjedrem, T., Robinson, N. and Rye, M. 2012. The importance of selective breeding in aquaculture to meet future demands for animal protein: a review. *Aquaculture* 350–353, pp. 117–129.

Gleick, P.H., 1993. *Water in crisis: a guide to the world's fresh water resources*. New York: Oxford University Press, p.13.

Gophen, M. 1976. Temperature effect on lifespan, metabolism, and development time of *Mesocyclops leuckarti* (Claus). *Oecologia* 25(3), pp. 271–277.

Granada, L., Sousa, N., Lopes, S. and Lemos, M.F.L. 2016. Is integrated multitrophic aquaculture the solution to the sectors' major challenges? – a review. *Reviews in Aquaculture* 8(3), pp. 283–300.

Grave, K., Horsberg, T., Lunestad, B. and Litleskare, I. 2004. Consumption of drugs for sea lice infestations in Norwegian fish farms: methods for assessment of treatment patterns and treatment rate. *Diseases of Aquatic Organisms* 60, pp. 123–131.

Gresty, K.A., Boxshall, G.A. and Nagasawa, K. 1993. The fine structure and function of the cephalic appendages of the branchiuran parasite, *Argulus japonicus* Thiele. *Philosophical Transactions of the Royal Society B* 339(1287), pp. 119–135.

Grigorian, M. and Hartenstein, V. 2013. Hematopoiesis and hematopoietic organs in arthropods. *Development genes and evolution* 223, pp. 103–115

Groner, M.L., Gettinby, G., Stormoen, M., Revie, C.W. and Cox, R. 2014. Modelling the impact of temperature-induced life history plasticity and mate limitation on the epidemic potential of a marine ectoparasite. *PLOS ONE* 9(2), p. e88465.

Guha, A., Aditya, G. and Saha, S.K. 2013. Survivorship and fecundity of *Argulus bengalensis* (Crustacea; Branchiura) under laboratory conditions. *Invertebrate Reproduction & Development* 57(4), pp. 301–308.

Hakalahti, T., Häkkinen, H. and Valtonen, E.T. 2004a. Ectoparasitic *Argulus coregoni* (Crustacea: Branchiura) hedge their bets – studies on egg hatching dynamics. *Oikos* 107(2), pp. 295–302.

Hakalahti, T., Pasternak, A.F. and Valtonen, E.T. 2004b. Seasonal dynamics of egg laying and egg-laying strategy of the ectoparasite *Argulus coregoni* (Crustacea: Branchiura). *Parasitology* 128(Pt 6), pp. 655–660.

Hakalahti, T., Lankinen, Y. and Valtonen, E.T. 2004c. Efficacy of emamectin benzoate in the control of *Argulus coregoni* (Crustacea: Branchiura) on rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* 60(3), pp. 197–204.

Hakalahti, T., Karvonen, A. and Valtonen, E.T. 2006. Climate warming and disease risks in temperate regions – *Argulus coregoni* and *Diplostomum spathaceum* as case studies. *Journal of Helminthology* 80(2), pp. 93–98.

Hakalahti, T., Mikheev, V.N. and Valtonen, E.T. 2008. Control of freshwater fish louse *Argulus coregoni*: a step towards an integrated management strategy. *Diseases of Aquatic Organisms* 82(1), pp. 67–77.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series* 41, pp. 95–98.

Hamsher, S.E., Evans, K.M., Mann, D.G., Poulíčková, A. and Saunders, G.W. 2011. Barcoding diatoms: exploring alternatives to COI-5P. *Protist* 162(3), pp. 405–422.

Harper, L.R. *et al.* 2019. Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. *Hydrobiologia* 826(1), pp. 25–41.

Harrison, A., Gault, N. and Dick, J. 2006. Seasonal and vertical patterns of egg-laying by the freshwater fish louse *Argulus foliaceus* (Crustacea: Branchiura). *Diseases of Aquatic Organisms* 68, pp. 167–173.

Harrison, A.J., Gault, N.F.S. and Dick, J.T.A. 2007. Diel variation in egg-laying by the freshwater fish louse *Argulus foliaceus* (Crustacea: Branchiura). *Diseases of Aquatic Organisms* 78(2), pp. 169–172.

Hawking, F. 1975. Circadian and other rhythms of parasites. *Advances in Parasitology* 13, pp. 123–182.

Hayward, C.J., Ellis, D., Foote, D., Wilkinson, R.J., Crosbie, P.B.B., Bott, N.J. and Nowak, B.F. 2010. Concurrent epizootic hyperinfections of sea lice (predominantly *Caligus chiastos*) and blood flukes (*Cardicola forsteri*) in ranched Southern Bluefin tuna. *Veterinary Parasitology* 173(1), pp. 107–115.

Hebert, P.D.N., Cywinska, A., Ball, S.L. and deWaard, J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 270(1512), pp. 313–321.

Helm, B. and Stevenson, T.J. 2014. Circannual rhythms: history, present challenges, future Directions. In: Numata, H. and Helm, B. eds. *Annual, Lunar, and Tidal Clocks: Patterns and Mechanisms of Nature's Enigmatic Rhythms*. Tokyo, Japan: Springer, pp. 203–225.

Hermida, R.C., Ayala, D.E. and Iglesias, M. 2003. Administration time–dependent influence of aspirin on blood pressure in pregnant women. *Hypertension* 41(3), pp. 651–656.

Herter, K. 1927. Reizphysiologische Untersuchungen an der Karpfenlaus (*Argulus foliaceus* L.). *Zeitschrift für vergleichende Physiologie* 5(2), pp. 283–370.

Heuch, P.A., Parsons, A. and Boxaspen, K. 2011. Diel vertical migration: A possible host-finding mechanism in salmon louse (*Lepeophtheirus salmonis*) copepodids? *Canadian Journal of Fisheries and Aquatic Sciences* 52(4), pp. 681–689.

Hevia, M.A., Canessa, P., Müller-Esparza, H. and Larrondo, L.F. 2015. A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 112(28), pp. 8744–8749.

Hoffman, G.L. 1977. *Argulus, a Branchiuran parasite of freshwater fishes*. Lincoln, USA: U.S. Fish and Wildlife Service, p. 9.

Hoffman, G.L. 1990. *Myxobolus cerebralis*, a worldwide cause of salmonid whirling disease. *Journal of Aquatic Animal Health* 2(1), pp. 30–37.

Honorio-Franca, A.C., Lanes, P.K.D., Ribeiro, E.B. and Franca, E.L. 2013. Chronopharmacology for anthelmintic: immune and modified release of drugs prospectus. *Science International* 1(2), pp. 22–28.

Hoshina, T. 1950. Über eine *Argulus*-art im salmonidenteiche. 日本水産学会誌 16(6), pp. 239–243.

Hoy, M.A. 1998. Myths, models and mitigation of resistance to pesticides. *Philosophical Transactions of the Royal Society B: Biological Sciences* 353(1376), pp. 1787–1795.

Hubert, N. and Hanner, R. 2015. DNA Barcoding, species delineation and taxonomy: a historical perspective. *DNA Barcodes* 3, pp. 44–58.

Hulme, P.E. *et al.* 2008. Grasping at the routes of biological invasions: a framework for integrating pathways into policy. *Journal of Applied Ecology* 45(2), pp. 403–414.

Hutchison, A.L. *et al.* 2015. Improved statistical methods enable greater sensitivity in rhythm detection for genome-wide data. *PLOS Computational Biology* 11(3), p. e1004094.

Huver, J.R., Koprivnikar, J., Johnson, P.T.J. and Whyard, S. 2015. Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. *Ecological Applications* 25(4), pp. 991–1002.

Hyder, K. *et al.* 2018. Recreational sea fishing in Europe in a global context—participation rates, fishing effort, expenditure, and implications for monitoring and assessment. *Fish and Fisheries* 19(2), pp. 225–243.

Hyder, K., Radford, Prellezo, Weltersbach, Lewin, Zarauz and Ferter 2017. *Research for PECH Committee - Marine recreational and semi-subsistence fishing - its value and its impact on fish stocks*.

Brussels, Belgium: European Parliament, Policy Department for Structural and Cohesion Policies, p.134.

IFM 2021. *Fisheries Management During the Coronavirus Pandemic*. Hull, UK: Institute of Fisheries Management, p. 1.

Imai, H. and Takeda, M. 2005. A natural hybrid mud crab (Decapoda, Portunidae) from Japan. *Journal of Crustacean Biology* 25(4), pp. 620–624.

Jiang, S. 2010. Aquaculture, capture fisheries, and wild fish stocks. *Resource and Energy Economics* 32(1), pp. 65–77.

Johnsen, B.O. and Jenser, A.J. 1991. The *Gyrodactylus* story in Norway. *Aquaculture* 98(1), pp. 289–302.

Juell, J.-E., Oppedal, F., Boxaspen, K. and Taranger, G.L. 2003. Submerged light increases swimming depth and reduces fish density of Atlantic salmon *Salmo salar* L. in production cages. *Aquaculture Research* 34(6), pp. 469–478.

Juhásová, L. *et al.* 2016. Population structure and dispersal routes of an invasive parasite, *Fascioloides magna*, in North America and Europe. *Parasites & Vectors* 9(1), p. 547.

Karakatsouli, N., Papoutsoglou, S.E., Panopoulos, G., Papoutsoglou, E.S., Chadio, S. and Kalogiannis, D. 2008. Effects of light spectrum on growth and stress response of rainbow trout *Oncorhynchus mykiss* reared under recirculating system conditions. *Aquacultural Engineering* 38(1), pp. 36–42.

Kearn, G.C. 1973. An endogenous circadian hatching rhythm in the monogenean skin parasite *Entobdella soleae* , and its relationship to the activity rhythm of the host (*Solea solea*). *Parasitology* 66(1), pp. 101–122.

Kearn, G.C. ed. 2004. The common fish louse – *Argulus*. In: *Leeches, Lice and Lampreys: A Natural History of Skin and Gill Parasites of Fishes*. Dordrecht, Netherlands: Springer, pp. 237–264.

Khan, K.F., Sanker, G. and Kumar, C.P. 2016. Linking eggs and adults of *Argulus* spp. using mitochondrial DNA barcodes. *Mitochondrial DNA Part A* 27(6), pp. 3927–3931.

Khan, Z., Midega, C., Pittchar, J., Pickett, J. and Bruce, T. 2011. Push—pull technology: a conservation agriculture approach for integrated management of insect pests, weeds and soil health in Africa. *International Journal of Agricultural Sustainability* 9(1), pp. 162–170.

Kiessling, S., Dubeau-Laramée, G., Ohm, H., Labrecque, N., Olivier, M. and Cermakian, N. 2017. The circadian clock in immune cells controls the magnitude of *Leishmania* parasite infection. *Scientific Reports* 7(1), p. 10892.

King, T.A. 2019. Wild caught ornamental fish: a perspective from the UK ornamental aquatic industry on the sustainability of aquatic organisms and livelihoods. *Journal of Fish Biology* 94(6), pp. 925–936.

Kirk, R.S. 2003. The impact of *Anguillicola crassus* on European eels. *Fisheries Management and Ecology* 10(6), pp. 385–394.

Kondolf, G.M. *et al.* 2014. Sustainable sediment management in reservoirs and regulated rivers: experiences from five continents. *Earth's Future* 2(5), pp. 256–280.

Koyun, M. 2011. The effect of water temperature on *Argulus foliaceus* L. 1758 (Crustacea; Branchiura) on different fish species. *Notulae Scientia Biologicae* 3(2), pp. 16–19.

Krkošek, M., Lewis, M.A., Morton, A., Frazer, L.N. and Volpe, J.P. 2006. Epizootics of wild fish induced by farm fish. *Proceedings of the National Academy of Sciences* 103(42), pp. 15506–15510.

Kumar, S., Sathish Kumar, T., Vidya, R. and Pandey, P.K. 2017. A prospective of epidemiological intervention in investigation and management of argulosis in aquaculture. *Aquaculture International* 25(1), pp. 303–325.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35(6), pp. 1547–1549.

Kunguma Kannika, M., Vignesh, K., Revathi, K. and Senthil Kumar, V. 2019. An economic analysis of Indian major carp production in Cauvery Delta Zone, Tamil Nadu. *Infokara Research* 8, pp. 1335–1342.

Kuperman, B.I. and Matey, V.E. 1999. Massive infestation by *Amyloodinium ocellatum* (Dinoflagellida) of fish in a highly saline lake, Salton Sea, California, USA. *Diseases of Aquatic Organisms* 39(1), pp. 65–73.

Kuznetsov, A. and Bollin, C.J. 2021. NCBI genome workbench: desktop software for comparative genomics, visualization, and GenBank data submission. In: Katoh, K. ed. *Multiple Sequence Alignment: Methods and Protocols*. Methods in Molecular Biology. New York, USA: Springer, pp. 261–295.

Lafferty, K.D., Dobson, A.P. and Kuris, A.M. 2006. Parasites dominate food web links. *Proceedings of the National Academy of Sciences* 103(30), pp. 11211–11216.

Lagerspetz, K.Y.H. and Vainio, L.A. 2006. Thermal behaviour of crustaceans. *Biological Reviews* 81(2), pp. 237–258.

Langfield, J. and James, C. 2009. Fishy tales: experiences of the occupation of keeping fish as pets. *British Journal of Occupational Therapy* 72(8), pp. 349–356.

Larkin, M.A. *et al.* 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23(21), pp. 2947–2948.

Leclercq, E., Taylor, J., Sprague, M. and Migaud, H. 2011. The potential of alternative lighting-systems to suppress pre-harvest sexual maturation of 1+ Atlantic salmon (*Salmo salar*) post-smolts reared in commercial sea-cages. *Aquacultural Engineering* 44, pp. 35–47.

Lefebvre, F., Fazio, G. and Alain, C. 2012. *Anguillicoloides crassus*., pp. 310–326. In: Woo, P.T.K. and Buchmann, K. eds. *Fish Parasites: Pathobiology and Protection*. Oxfordshire, UK: CABI, pp. 310–326

Lewin, W.-C., Arlinghaus, R. and Mehner, T. 2006. Documented and Potential Biological Impacts of Recreational Fishing: Insights for Management and Conservation. *Reviews in Fisheries Science* 14(4), pp. 305–367.

Lewis, E.E., Campbell, J.F. and Sukhdeo, M.V.K. 2002. *The Behavioural Ecology of Parasites*. Oxfordshire, UK: CABI, p. 384.

Li, D., Fu, Z. and Duan, Y. 2002. Fish-Expert: a web-based expert system for fish disease diagnosis. *Expert Systems with Applications* 23(3), pp. 311–320.

Liltved, H. and Hansen, B.R. 1990. Screening as a method for removal of parasites from inlet water to fish farms. *Aquacultural Engineering* 9(3), pp. 209–215.

Limaye, A. 2012. Drishti: a volume exploration and presentation tool. In: Stock, S.R. ed. *Developments in X-Ray Tomography VIII*. Washington, USA, SPIE, pp. 191–199.

Lin, X. and Söderhäll, I. 2011. Crustacean hematopoiesis and the astakine cytokines. *Blood* 117(24), pp. 6417–6424.

Liu, C., Shao, S., Fan, C. and Shen, Q. 2015. Removal of volatile organic sulfur compounds by aeration during algae-induced black blooms in shallow lakes. *Journal of Water Supply: Research and Technology-Aqua* 65(2), pp. 172–180.

Lizée, T.W. *et al.* 2018. Influence of landing net mesh type on handling time and tissue damage of angled brook trout. *North American Journal of Fisheries Management* 38(1), pp. 76–83.

Lohman, D.J., Prawiradilaga, D.M. and Meier, R. 2009. Improved COI barcoding primers for Southeast Asian perching birds (Aves: Passeriformes). *Molecular Ecology Resources* 9(1), pp. 37–40.

Lopez, C., Rajan, P.R., Lin, J.H.-Y., Kuo, T.-Y. and Yang, H.-L. 2002. Disease outbreak in seafarmed Cobia (*Rachycentron canadum*) associated with *Vibrio* Spp., *Photobacterium damselae* ssp *piscicida*, monogenean and myxosporean parasites. *Bulletin of The European Association of Fish Pathologists* 22(3), pp. 206–211.

Love, M.I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15(12), p. 550.

Lowell, J.M.S. 2012. *Effect of Netting Materials on Fouling and Parasite Egg Loading on Offshore Net Pens in Hawaii*. Kailua-Kona, USA: Blue Ocean Mariculture, p. 5.

Lu, D.-B., Wang, T.-P., Rudge, J.W., Donnelly, C.A., Fang, G.-R. and Webster, J.P. 2009. Evolution in a multi-host parasite: chronobiological circadian rhythm and population genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host reservoirs by habitat. *International Journal for Parasitology* 39(14), pp. 1581–1588.

Lymbery, A.J., Morine, M., Kanani, H.G., Beatty, S.J. and Morgan, D.L. 2014. Co-invaders: the effects of alien parasites on native hosts. *International Journal for Parasitology: Parasites and Wildlife* 3(2), pp. 171–177.

Macadam, C.R. and Stockan, J.A. 2015. More than just fish food: ecosystem services provided by freshwater insects. *Ecological Entomology* 40(S1), pp. 113–123.

Maceda-Veiga, A., Domínguez-Domínguez, O., Escribano-Alacid, J. and Lyons, J. 2016. The aquarium hobby: can sinners become saints in freshwater fish conservation? *Fish and Fisheries* 17(3), pp. 860–874.

Mack, R.N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M. and Bazzaz, F.A. 2000. Biotic invasions: causes, epidemiology, global consequences, and control. *Ecological Applications* 10(3), pp. 689–710.

Macken, A., Lillicrap, A. and Langford, K. 2015. Benzoylurea pesticides used as veterinary medicines in aquaculture: risks and developmental effects on nontarget crustaceans. *Environmental Toxicology and Chemistry* 34(7), pp. 1533–1542.

MacKinnon, B.M. 1993. Response of the copepodid larvae of *Caligus elongatus* to light, and the ultrastructure of the eyes. *Canadian Journal of Fisheries and Aquatic Sciences* 50(4), pp. 793–799.

Macnab, V. and Barber, I. 2012. Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. *Global Change Biology* 18(5), pp. 1540–1548.

Marcogliese, D.J. and Cone, D.K. 1997. Food webs: a plea for parasites. *Trends in Ecology & Evolution* 12(8), pp. 320–325.

Margenau, T.L. 2007. Effects of angling with a single-hook and live bait on muskellunge survival. In: Diana, J. S. and Margenau, T. L. eds. *The Muskellunge Symposium: A Memorial Tribute to E.J. Crossman.* Developments in environmental biology of fishes 26. Dordrecht, Netherlands: Springer, pp. 155–162.

Marsh, D. 2021. *Natural Capital of Freshwater Fisheries in England*. Callington, UK: The Rivers Trust, p. 34.

Martinez-Bakker, M. and Helm, B. 2015. The influence of biological rhythms on host–parasite interactions. *Trends in Ecology & Evolution* 30(6), pp. 314–326.

Masly, J.P. 2012. 170 years of "Lock-and-Key": genital morphology and reproductive isolation. *International Journal of Evolutionary Biology* 2012, p. 247352.

Mastitsky, S.E., Karatayev, A.Y., Burlakova, L.E. and Molloy, D.P. 2010. Parasites of exotic species in invaded areas: does lower diversity mean lower epizootic impact? *Diversity and Distributions* 16(5), pp. 798–803.

Mayer, J., Hensel, P., Mejia-Fava, J., Brandão, J. and Divers, S. 2013. The use of lufenuron to treat fish lice (*Argulus* sp) in koi (*Cyprinus carpio*). *Journal of Exotic Pet Medicine* 22(1), pp. 65–69.

McKenna, H., van der Horst, G.T.J., Reiss, I. and Martin, D. 2018. Clinical chronobiology: a timely consideration in critical care medicine. *Critical Care* 22(1), p. 124.

McManus, A., Hunt, W., Storey, J. and White, J. 2011. *Identifying the health and well-being benefits of recreational fishing.* Perth, Australia: Centre of Excellence for Science, Seafood and Health, p. 64.

McPherson, N.J., Norman, R.A., Hoyle, A.S., Bron, J.E. and Taylor, N.G.H. 2012. Stocking methods and parasite-induced reductions in capture: Modelling *Argulus foliaceus* in trout fisheries. *Journal of Theoretical Biology* 312, pp. 22–33.

Meyer-Rochow, V.B., Au, D. and Keskinen, E. 2001. Photoreception in fishlice [Branchiura]: The eyes of *Argulus foliaceus* Linne, 1758 and *A. coregoni* Thorell, 1865. *Acta Parasitologica* 46(4), pp. 321–331.

Mideo, N., Reece, S.E., Smith, A.L. and Metcalf, C.J.E. 2013. The Cinderella syndrome: why do malaria-infected cells burst at midnight? *Trends in Parasitology* 29(1), pp. 10–16.

Mikheev, V.N., Mikheev, A.V., Pasternak, A.F. and Valtonen, E.T. 1999. Light-mediated host searching strategies in a fish ectoparasite, *Argulus foliaceus* L. (Crustacea: Branchiura). *Parasitology* 120 (Pt 4), pp. 409–416.

Mikheev, V.N., Pasternak, A.F. and Valtonen, E.T. 2007. Host specificity of *Argulus coregoni* (Crustacea: Branchiura) increases at maturation. *Parasitology* 134(12), pp. 1767–1774.

Mikheev, V.N., Pasternak, A.F. and Valtonen, E.T. 2015. Behavioural adaptations of argulid parasites (Crustacea: Branchiura) to major challenges in their life cycle. *Parasites & Vectors* 8(1), p. 394

Mikheev, V.N., Pasternak, A.F., Valtonen, E.T. and Lankinen, Y. 2001. Spatial distribution and hatching of overwintered eggs of a fish ectoparasite, *Argulus coregoni* (Crustacea: Branchiura). *Diseases of Aquatic Organisms* 46(2), pp. 123–128.

Mikheev, V.N., Valtonen, E.T. and Rintamäki-Kinnunen, P. 1998. Host searching in *Argulus foliaceus* L. (Crustacea: Branchiura): the role of vision and selectivity. *Parasitology* 116(5), pp. 425–430.

Minchin, D. and Rosenthal, H. 2002. Exotics for Stocking and Aquaculture, Making Correct Decisions. In: Leppäkoski, E., Gollasch, S., and Olenin, S. eds. *Invasive Aquatic Species of Europe. Distribution, Impacts and Management*. Dordrecht, Netherlands: Springer, pp. 206–216.

Mirzaei, M. and Khovand, H. 2015. Prevalence of *Argulus foliaceus* in ornamental fishes [goldfish (*Carassius auratus*) and Koi (*Cyprinus carpio*)] in Kerman, southeast of Iran. *Journal of Parasitic Diseases* 39(4), pp. 780–782.

Mishra, S.S. *et al.* 2017. Present status of fish disease management in freshwater aquaculture in India: state-of-the-art-review. *Journal of Aquaculture & Fisheries* 1, p. 3.

Mitchell, M. and Tully, T.N. 2016. *Current Therapy in Exotic Pet Practice*. Pennsylvania, USA: Saunders, Elsevier Health Sciences, p. 512.

Mohan Dey, M., Rab, M.A., Paraguas, F.J., Bhatta, R., Ferdous Alam, Md., Koeshendrajana, S. and Ahmed, M. 2005. Status and economics of freshwater aquaculture in selected countries of Asia. *Aquaculture Economics & Management* 9(1–2), pp. 11–37.

Mohney, B.K., Matz, T., LaMoreaux, J., Wilcox, D.S., Gimsing, A.L., Mayer, P. and Weidenhamer, J.D. 2009. In situ silicone tube microextraction: a new method for undisturbed sampling of root-exuded thiophenes from marigold (*Tagetes erecta* L.) in Soil. *Journal of Chemical Ecology* 35(11), p. 1279.

Molento, M.B. 2009. Parasite control in the age of drug resistance and changing agricultural practices. *Veterinary Parasitology* 163(3), pp. 229–234.

Møller, O. 2006. *Argulus foliaceus*. In: Woo, P.T.K. and Buchmann, K. eds. *Fish Parasites: Pathobiology and Protection*. Oxfordshire, UK: CABI, pp. 337–346.

Molnár, K. and Székely, C. 1998. Occurrence of skrjabillanid nematodes in fishes of Hungary and in the intermediate host, *Argulus foliaceus* L. *Acta Veterinaria Hungarica* 46(4), pp. 451–463.

Molyneux, D.H. 2006. Control of Human Parasitic Diseases: Context and Overview. In: Molyneux, D. H. ed. *Advances in Parasitology 61*. London, UK: Academic Press, pp. 1–45.

Momeni Shahraki, M., Asgari, M.R., Khamesipour, F. and Raissy, M. 2014. Prevalence of *Argulus foliaceus* and fungal infections in some ornamental fishes [discus (*Symphysodon discus*), dwarf gourami (*Trichogaster lalius*) and guppy (*Poecilia reticulata*)] in Isfahan City of Iran. *Kafkas Universitesi Veteriner Fakultesi Dergisi* 20(5), pp. 817–820

Monir, M., Bagum, Rahman, N., Ashaf-Ud-Doulah, M., Bhadra and Chakra Borty, S. 2015. Parasitic diseases and estimation of loss due to infestation of parasites in Indian major carp culture ponds in Bangladesh. *International Journal of Fisheries and Aquatic Studies* 2(5), pp. 118-122.

Montero, D., Izquierdo, M.S., Tort, L., Robaina, L. and Vergara, J.M. 1999. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiology and Biochemistry* 20(1), pp. 53–60.

Montero, R. *et al.* 2019. Dawn to dusk: diurnal rhythm of the immune response in rainbow trout (*Oncorhynchus Mykiss*). *Biology* 9(1), p. 8.

Monticini, P. 2010. *the ornamental fish trade. production and commerce of ornamental fish: technical-managerial and legislative aspects*. Rome, Italy: GLOBEFISH Research Programme, Food and Agriculture Organization, p. 134.

Mordue (Luntz), A.J. and Birkett, M.A. 2009. A review of host finding behaviour in the parasitic sea louse, *Lepeophtheirus salmonis* (Caligidae: Copepoda). *Journal of Fish Diseases* 32(1), pp. 3–13.

Moreau, M.-A. and Coomes, O.T. 2007. Aquarium fish exploitation in western Amazonia: conservation issues in Peru. *Environmental Conservation* 34(1), pp. 12–22.

Mulugeta, D. and Boerboom, C.M. 2000. Critical time of weed removal in glyphosate-resistant *Glycine max*. *Weed Science* 48(1), pp. 35–42.

Murray, A.G. 2002. Using observed load distributions with a simple model to analyse the epidemiology of sea lice (*Lepeophtheirus salmonis*) on sea trout (*Salmo trutta*). *Pest Management Science* 58(6), pp. 585–594.

Nagasawa, K. and Yuasa, A. 2019. The fish louse *Argulus coregoni* from *Oncorhynchus masou ishikawae* (Salmonidae) cultured in Shikoku, western Japan, with a list of occurrence records of *A. coregoni* from fishes reared in Japan. *Crustacean Research* 49, pp. 1–8.

National Statistics 2021. *UK Sea Fisheries Statistics 2020*. Newcastle, UK: Marine Management Organisation, p. 66.

Neumann, D. 2014. Timing in tidal, semilunar, and lunar rhythms. In: Numata, H. and Helm, B. eds. *Annual, Lunar, and Tidal Clocks: Patterns and Mechanisms of Nature's Enigmatic Rhythms*. Tokyo, Japan: Springer, pp. 3–24.

Novales Flamarique, I., Browman, H.I., Bélanger, M. and Boxaspen, K. 2000. Ontogenetic changes in visual sensitivity of the parasitic salmon louse *Lepeophtheirus salmonis*. *The Journal of Experimental Biology* 203(Pt 11), pp. 1649–1657.

Novales Flamarique, I., Gulbransen, C., Galbraith, M. and Stucchi, D. 2009. Monitoring and potential control of sea lice using an LED-based light trap. *Canadian Journal of Fisheries and Aquatic Sciences* 66(8), pp. 1371–1382.

Nylund, A., Wallace, C. and Hovland, T. 1993. The possible role of *Lepeophtheirus salmonis* (Kroyer) in the transmission of infectious salmon anaemia. In: Boxshall, G.A. and Defaye, D. eds. *Pathogens of wild and farmed fish: sea lice*. London, UK: Ellis Horwood, pp. 367–373

OATA 2021. *Fishing for facts: an introduction to the UK ornamental fish trade*. Wiltshire, UK: Ornamental Aquatic Trade Association, p. 33.

O'Donnell, A.J., Schneider, P., McWatters, H.G. and Reece, S.E. 2011. Fitness costs of disrupting circadian rhythms in malaria parasites. *Proceedings of the Royal Society B: Biological Sciences* 278(1717), pp. 2429–2436.

Ogawa, K., Miyamoto, J., Wang, H.-C., Lo, C.F. and Kou, G.-H. 2006. *Neobenedenia girellae* (Monogenea) infection of cultured Cobia *Rachycentron canadum* in Taiwan. *Fish Pathology* 41, pp. 51–56.

Ogawa, K. and Yokoyama, H. 1998. Parasitic diseases of cultured marine fish in Japan. *Fish Pathology* 33(4), pp. 303–309.

O'Grady, J.F., Hoelters, L.S., Swain, M.T. and Wilcockson, D.C. 2016. Identification and temporal expression of putative circadian clock transcripts in the amphipod crustacean *Talitrus saltator*. *PeerJ* 4, p. e2555.

OIE (International Office of Epizootics and Aquatic Animal Health Standards Commission) 2009. *Manual of diagnostic tests for aquatic animals*. Paris, France: Office International des épizooties.

Oppedal, F., Taranger, G.L., Juell, J.-E. and Hansen, T. 1999. Growth, osmoregulation and sexual maturation of underyearling Atlantic salmon smolt *Salmo salar* L. exposed to different intensities of continuous light in sea cages. *Aquaculture Research* 30(7), pp. 491–499.

Otsuka, K., Cornelissen, G. and Halberg, F. 2016. *Chronomics and Continuous Ambulatory Blood Pressure Monitoring: Vascular Chronomics: From 7-Day/24-Hour to Lifelong Monitoring*. Tokyo, Japan: Springer, p. 870.

Owen, M.A.G., Davies, S.J. and Sloman, K.A. 2010. Light colour influences the behaviour and stress physiology of captive tench (*Tinca tinca*). *Reviews in Fish Biology and Fisheries* 20(3), pp. 375–380.

Owolabi, A.T.Y., Reece, S.E. and Schneider, P. 2021. Daily rhythms of both host and parasite affect antimalarial drug efficacy. *Evolution, Medicine, and Public Health* 9(1), pp. 208–219.

Parent, G.J., Plourde, S. and Turgeon, J. 2012. Natural hybridization between *Calanus finmarchicus* and *C. glacialis* (Copepoda) in the Arctic and Northwest Atlantic. *Limnology and Oceanography* 57(4), pp. 1057–1066.

Parsons, R., Parsons, R., Garner, N., Oster, H. and Rawashdeh, O. 2020. CircaCompare: a method to estimate and statistically support differences in mesor, amplitude and phase, between circadian rhythms. *Bioinformatics* 36(4), pp. 1208–1212.

Pasternak, A., Mikheev, V. and Valtonen, E.T. 2004. Growth and development of *Argulus coregoni* (Crustacea: Branchiura) on salmonid and cyprinid hosts. *Diseases of Aquatic Organisms* 58(2–3), pp. 203–207.

Pasternak, A.F., Mikheev, V.N. and Valtonen, E.T. 2000. Life history characteristics of *Argulus foliaceus* L. (Crustacea: Branchiura) populations in Central Finland. *Annales Zoologici Fennici* 37(1), pp. 25–35.

Payton, L. *et al.* 2021. Widely rhythmic transcriptome in *Calanus finmarchicus* during the high Arctic summer solstice period. *iScience* 24(1), p. 101927

Pinnow, P., Fabrizius, A., Pick, C. and Burmester, T. 2016. Identification and characterisation of hemocyanin of the fish louse *Argulus* (Crustacea: Branchiura). *Journal of Comparative Physiology B* 186(2), pp. 161–168.

Poulin, R., Curtis, M.A. and Rau, M.E. 1990. Responses of the fish ectoparasite *Salmincola edwardsii* (Copepoda) to stimulation, and their implication for host-finding. *Parasitology* 100(3), pp. 417–421.

Poulin, R. and Morand, S. 2000. The diversity of parasites. *The Quarterly Review of Biology* 75(3), pp. 277–293.

Prior, K.F. *et al.* 2018. Timing of host feeding drives rhythms in parasite replication. *PLOS Pathogens* 14(2), p. e1006900.

R Core Team 2017. *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing. Available at: https://www.R-project.org/.

Radkhah, A.R. 2017. Introduction to some species of Argulus (Crustacea: Branchiura), parasitic infections in the freshwater fishes. *Journal of Applied Sciences and Environmental Management* 21(7), pp. 1268–1271.

Rath, R.K. 2018. *Freshwater Aquaculture, 3rd Ed.* Jodhpur, India: Scientific Publishers, p. 597.

Ratnasingham, S. and Hebert, P.D.N. 2007. BOLD: the barcode of life data system (http://www.barcodinglife.org). *Molecular Ecology Notes* 7(3), pp. 355–364.

Rawlinson, K.A. *et al.* 2021. Daily rhythms in gene expression of the human parasite *Schistosoma mansoni*. BMC Biology 19(1), p. 255.

Rea, J.G. and Irwin, S.W. 1994. The ecology of host-finding behaviour and parasite transmission: past and future perspectives. *Parasitology* 109 Suppl, pp. S31–39.

Rebl, A., Korytář, T., Borchel, A., Bochert, R., Strzelczyk, J.E., Goldammer, T. and Verleih, M. 2020. The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (*Oncorhynchus mykiss*). *Scientific Reports* 10(1), p. 14913.

Reece, S.E., Prior, K.F. and Mideo, N. 2017. The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. *Journal of Biological Rhythms* 32(6), pp. 516–533.

Reinberg, A. and Smolensky, M.H. 2012. *Biological Rhythms and Medicine: Cellular, Metabolic, Physiopathologic, and Pharmacologic Aspects*. New York, USA: Springer-Verlag, p. 305.

Rijo-Ferreira, F., Pinto-Neves, D., Barbosa-Morais, N.L., Takahashi, J.S. and Figueiredo, L.M. 2017b. *Trypanosoma brucei* metabolism is under circadian control. *Nature Microbiology* 2(6), pp. 1–10.

Rijo-Ferreira, F. and Takahashi, J.S. 2019. Genomics of circadian rhythms in health and disease. *Genome Medicine* 11(1), p. 82.

Rijo-Ferreira, F., Takahashi, J.S. and Figueiredo, L.M. 2017a. Circadian rhythms in parasites. *PLoS Pathogens* 13(10), p. e1006590.

Roberts, L.S. 1957. Parasites of the Carp, *Cyprinus carpio* L. in Lake Texoma, Oklahoma. *The Journal of Parasitology* 43(1), pp. 54–54.

Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P. and Ranganathan, S. 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. *Molecular & Cellular Proteomics: MCP* 8(8), pp. 1891–1907.

Rodgers, C., Mohan, C. and Peeler, E. 2011. The spread of pathogens through trade in aquatic animals and their products. *Revue Scientifique et Technique (International Office of Epizootics)* 30, pp. 241–56.

Rouzine, I.M. and McKenzie, F.E. 2003. Link between immune response and parasite synchronization in malaria. *Proceedings of the National Academy of Sciences* 100(6), pp. 3473–3478.

Rushton-Mellor, S.K. 1992. Discovery of the fish louse, *Argulus japonicus* Thiele (Crustacea: Branchiura), in Britain. *Aquaculture Research* 23(2), pp. 269–271.

Rushton-Mellor, S.K. and Boxshall, G.A. 1994. The developmental sequence of *Argulus foliaceus* (Crustacea: Branchiura). *Journal of Natural History* 28(4), pp. 763–785.

Sahoo, P.K., Hemaprasanth, Kar, B., Garnayak, S.K. and Mohanty, J. 2012. Mixed infection of *Argulus japonicus* and *Argulus siamensis* (Branchiura, Argulidae) in carps (Pisces, Cyprinidae): loss estimation and a comparative invasive pattern study. *Crustaceana* 85(12–13), pp. 1449–1462.

Sahoo, P.K. et al. 2013a. Estimation of loss due to argulosis in carp culture ponds in India. *Indian Journal of Fisheries* 60, pp. 99–102.

Sahoo, P.K., Mohanty, J., Hemaprasanth, Kar, B., Mohanty, B.R., Garnayak, S.K. and Jena, J.K. 2013b. Egg laying strategies and effect of temperature on egg development of *Argulus siamensis. Journal of Parasitic Diseases: Official Organ of the Indian Society for Parasitology* 37(2), pp. 158–162.

Salado, R. and Vencovska, J. 2018. *A survey of freshwater angling in England. Phase 1: angling activity, expenditure and economic impact*. Bristol, UK: Environment Agency, p. 129.

Sandodden, R. 2018. Eradication of *Gyrodactylus salaris* infested Atlantic salmon (*Salmo salar*) in the Rauma River, Norway, using rotenone. *Management of Biological Invasions* 9(10), pp. 67–77.

Saurabh, S. and Sahoo, P.K. 2010. Non-specific immune responses of the Indian major carp *Labeo rohita* Hamilton to infestation by the freshwater fish louse *Argulus siamensis* (Wilson). *Indian Journal of Fisheries* 57(2), pp. 45–53.

Saurabh, S., Sahoo, P.K., Mohanty, B.R., Mohanty, J., Jena, J.K., Mukherjee, S.C. and Sarangi, N. 2010. Modulation of the innate immune response of rohu *Labeo rohita* (Hamilton) by experimental freshwater lice *Argulus siamensis* (Wilson) infection. *Aquaculture Research* 41(9), pp. e326–e335.

Scheffers, B.R., Oliveira, B.F., Lamb, I. and Edwards, D.P. 2019. Global wildlife trade across the tree of life. *Science* 366(6461), pp. 71–76.

Schelkle, B., Shinn, A.P., Peeler, E. and Cable, J. 2009. Treatment of gyrodactylid infections in fish. *Diseases of Aquatic Organisms* 86(1), pp. 65–75.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9(7), pp. 671–675.

Searle, S.R., Speed, F.M. and Milliken, G.A. 1980. Population marginal means in the linear model: an alternative to least squares means. *The American Statistician* 34(4), pp. 216–221.

Shafir, A. and van As, J. 1986. Laying, development and hatching of eggs of the fish ectoparasite *Argulus japonicus* (Crustacea: Branchiura). *Journal of Zoology* 210, pp. 401–413.

Shafir, A. and Oldewage, W.H. 1992. Dynamics of a fish ectoparasite population: opportunistic parasitism in *Argulus japonicus* (Branchiura). *Crustaceana* 62(1), pp. 50–64.

Shameena, S.S. *et al.* 2021. Dose-dependent co-infection of *Argulus* sp. and *Aeromonas hydrophila* in goldfish (*Carassius auratus*) modulates innate immune response and antioxidative stress enzymes. *Fish & Shellfish Immunology* 114, pp. 199–206.

Shedko, M.B., Ermolenko, A.V. and Aseeva, N.L. 2018. Rare and poorly studied species of parasites of cyprinid fish from the Primorye Territory. *The 1st International Conference on North East Asia Biodiversity* Vladivostok, Russia, pp. 70–71.

Sheng, Y., Qu, Y., Ding, C., Sun, Q. and Mortimer, R.J.G. 2013. A combined application of different engineering and biological techniques to remediate a heavily polluted river. *Ecological Engineering* 57, pp. 1–7.

Shimura, S. 1981. The larval development of *Argulus coregoni* Thorell (Crustacea: Branchiura). *Journal of Natural History* 15(2), pp. 331–348.

Shimura, S. 1983. Seasonal occurrence, sex ratio and site preference of *Argulus coregoni* Thorell (Crustacea: Branchiura) parasitic on cultured freshwater salmonids in Japan. *Parasitology* 86(3), pp. 537–552.

Shimura, S. and Egusa, S. 1980. Some ecological notes on the egg deposition of *Argulus coregoni* Thorell (Crustacea, Branchiura). *Fish Pathology* 15(1), pp. 43–47.

Shimura, S., Inoue, K., Kudo, M. and Egvsa, S. 1983. Studies on effects of parasitism of *Argulus coregoni* (Crustacea: Branchiura) on furunculosis of *Oncorhynchus masou* (Salmonidae). *Fish Pathology* 18(1), pp. 37–40.

Shinn, A.P., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E.E. and Brooker, A.J. 2015. Economic costs of protistan and metazoan parasites to global mariculture. *Parasitology* 142(1), pp. 196–270.

Shokralla, S., Zhou, X., Janzen, D.H., Hallwachs, W., Landry, J.-F., Jacobus, L.M. and Hajibabaei, M. 2011. Pyrosequencing for mini-barcoding of fresh and old museum specimens. *PLOS ONE* 6(7), p. e21252.

Sieber, N., Hartikainen, H. and Vorburger, C. 2020. Validation of an eDNA-based method for the detection of wildlife pathogens in water. *Diseases of Aquatic Organisms* 141, pp. 171–184.

Sikkel, P.C., Ziemba, R.E., Sears, W.T. and Wheeler, J.C. 2009. Diel ontogenetic shift in parasitic activity in a gnathiid isopod on Caribbean coral reefs. *Coral Reefs* 28(2), pp. 489–495.

Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V. and Zdobnov, E.M. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31(19), pp. 3210–3212.

Simonsen, T.J. and Kitching, I.J. 2014. Virtual dissections through micro-CT scanning: a method for non-destructive genitalia 'dissections' of valuable Lepidoptera material. *Systematic Entomology* 39(3), pp. 606–618.

Sinclair, J.S., Stringham, O.C., Udell, B., Mandrak, N.E., Leung, B., Romagosa, C.M. and Lockwood, J.L. 2021. The international vertebrate pet trade network and insights from US imports of exotic pets. *BioScience* 71(9), pp. 977–990.

Singhal, R.N., Jeet, S. and Davies, R.W. 1990. The effects of argulosis-saprolehniasis on the growth and production of *Cyprinus carpoi*. *Hydrobiologia* 202(1), pp. 27–31.

Skelton, J., Creed, R.P. and Brown, B.L. 2015. A symbiont's dispersal strategy: condition-dependent dispersal underlies predictable variation in direct transmission among hosts. *Proceedings of the Royal Society B: Biological Sciences* 282(1819), p. 20152081.

Skilton, D.C., Saunders, R.J. and Hutson, K.S. 2020. Parasite attractants: identifying trap baits for parasite management in aquaculture. *Aquaculture* 516, p. 734557.

Smith, K.F., Behrens, M.D., Max, L.M. and Daszak, P. 2008. U.S. drowning in unidentified fishes: Scope, implications, and regulation of live fish import. *Conservation Letters* 1(2), pp. 103–109.

Smith, L.M. *et al.* 2020. An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*. *Science* 368(6492), pp. 754–759.

Smolensky, M.H. and D'alonzo, G.E. 1993. Medical chronobiology: concepts and applications. *American Review of Respiratory Disease* 147(6 Pt 2), pp. S2–S19.

Smolensky, M.H. and Peppas, N.A. 2007. Chronobiology, drug delivery, and chronotherapeutics. *Advanced Drug Delivery Reviews* 59(9), pp. 828–851.

Soleng, A., Jansen, P. and Bakke, T. 1998. Transmission of the monogenean *Gyrodactylus salaris*. *Parasitology International* 47, p. 319.

Southwick Associates 2020. *Sportfishing in America: A Reliable Economic Force*. Florida, USA: Southwick Associates, produced for the American Sportfishing Association, p. 18.

Speiser, D.I. *et al.* 2014. Using phylogenetically-informed annotation (PIA) to search for light-interacting genes in transcriptomes from non-model organisms. *BMC Bioinformatics* 15(1), p. 350.

Spikmans, F. *et al.* 2020. Impact of the invasive alien topmouth gudgeon (*Pseudorasbora parva*) and its associated parasite *Sphaerothecum destruens* on native fish species. *Biological Invasions* 22(2), pp. 587–601.

Staab, M., Garcia, F.H., Liu, C., Xu, Z.-H. and Economo, E.P. 2018. Systematics of the ant genus *Proceratium* Roger (Hymenoptera, Formicidae, Proceratiinae) in China – with descriptions of three new species based on micro-CT enhanced next-generation-morphology. *ZooKeys* (770), pp. 137–192.

Steckler, N. and Yanong, R.P.E. 2012. *Argulus (Fish Louse) Infections in Fish*. Florida, USA: University of Florida, Institute of Food and Agricultural Sciences, p. 4.

Stewart, A. *et al.* 2017. Hook, line and infection: a guide to culturing parasites, establishing infections and assessing immune responses in the three-spined stickleback. In: Rollinson, D. and Stothard, J. R. eds. *Advances in Parasitology 98*. London, UK: Academic Press, pp. 39–109.

Stewart, H., Nomura, M., Piercey, G.E., Dunham, A. and Lelliott, T.L. 2013. *Ecological effects of blue LED lights used in aquaculture*. Ontario, Canada: Fisheries and Oceans Canada, p. 4.

Stewart, J.E. 1991. Approaches to problems of disease in aquaculture. *ICES marine science symposia* 192, pp. 206–210.

Stoev, P. *et al.* 2013. *Eupolybothrus cavernicolus* Komerički & Stoev sp. n. (Chilopoda: Lithobiomorpha: Lithobiidae): the first eukaryotic species description combining transcriptomic, DNA barcoding and micro-CT imaging data. *Biodiversity Data Journal* 1, p. e1013.

Strong, L. and Wall, R. 1990. The chemical control of livestock parasites: problems and alternatives. *Parasitology Today* 6(9), pp. 291–296.

Studer, A., Thieltges, D.W. and Poulin, R. 2010. Parasites and global warming: net effects of temperature on an intertidal host–parasite system. *Marine Ecology Progress Series* 415, pp. 11–22.

Sukhdeo, M.V.K. and Chappell, L.H. 1994. *Parasites and Behaviour*. Cambridge, UK: Cambridge University Press, p. 151.

Sun, G. *et al.* 2018. Large-scale gene losses underlie the genome evolution of parasitic plant *Cuscuta australis*. *Nature Communications* 9(1), p. 2683.

Svoboda, J., Mrugała, A., Kozubíková-Balcarová, E. and Petrusek, A. 2017. Hosts and transmission of the crayfish plague pathogen *Aphanomyces astaci*: a review. *Journal of Fish Diseases* 40(1), pp. 127–140.

Tam, Q. and Avenant-Oldewage, A. 2006. The digestive system of larval *Argulus japonicus* (Branchiura). *Journal of Crustacean Biology* 26(4), pp. 447–454.

Tavares, R.G. *et al.* 2011. Molecular techniques for the study and diagnosis of parasite infection. *Journal of Venomous Animals and Toxins including Tropical Diseases* 17, pp. 239–248.

Taylor, N.G.H., Sommerville, C. and Wootten, R. 2006. The epidemiology of *Argulus* spp. (Crustacea: Branchiura) infections in stillwater trout fisheries. *Journal of Fish Diseases* 29(4), pp. 193–200.

Taylor, N.G.H., Sommerville, C. and Wootten, R. 2005a. *A review of Argulus spp. occurring in UK freshwaters*. Bristol, UK: Environment Agency., p. 30. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/290424/scho0705bjik-e-e.pdf.

Taylor, N.G.H., Sommerville, C. and Wootten, R. 2005b. *Argulus spp. infections in UK stillwater trout fisheries*. Bristol, UK: Environment Agency., p. 57. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/290430/scho0705bjil-e-e.pdf.

Taylor, N.G.H., Wootten, R. and Sommerville, C. 2009a. The influence of risk factors on the abundance, egg laying habits and impact of *Argulus foliaceus* in stillwater trout fisheries. *Journal of Fish Diseases* 32(6), pp. 509–519.

Taylor, N.G.H., Wootten, R. and Sommerville, C. 2009b. Using length-frequency data to elucidate the population dynamics of *Argulus foliaceus* (Crustacea: Branchiura). *Parasitology* 136(9), pp. 1023–1032.

Thaben, P.F. and Westermark, P.O. 2014. Detecting rhythms in time series with RAIN. *Journal of Biological Rhythms* 29(6), pp. 391–400.

Theopold, U., Schmidt, O., Söderhäll, K. and Dushay, M.S. 2004. Coagulation in arthropods: defence, wound closure and healing. *Trends in Immunology* 25(6), pp. 289–294.

Therneau, T.M. and Grambsch, P.M. 2000. *Modeling Survival Data: Extending the Cox Model*. New York, USA: Springer Science & Business Media, p. 350.

Tidwell, J.H. and Allan, G.L. 2001. Fish as food: aquaculture's contribution: ecological and economic impacts and contributions of fish farming and capture fisheries. *EMBO reports* 2(11), pp. 958–963.

Tilden, A.R., McCoole, M.D., Harmon, S.M., Baer, K.N. and Christie, A.E. 2011. Genomic identification of a putative circadian system in the cladoceran crustacean *Daphnia pulex*. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics* 6(3), pp. 282–309.

Timi, J.T. and Poulin, R. 2020. Why ignoring parasites in fish ecology is a mistake. *International Journal for Parasitology* 50(10), pp. 755–761.

Tirloni, L. *et al.* 2015. Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study. *Parasites & Vectors* 8(1), p. 338.

Tokioka, T. 1936. Larval development and metamorphosis of *Argulus japonicus*. *Memoirs of the college of Science Kyoto Imperial University, Series B* 12(1), pp. 93–114.

Tokioka, T. 1939. *Argulus* of Manchukuo. *Annotationes Zoologicae Japonenses* 18(1), pp. 42–45.

Trail, D.R.S. 1980. Behavioral interactions between parasites and hosts: host suicide and the evolution of complex life cycles. *The American Naturalist* 116(1), pp. 77–91.

Trippel, E.A. 2010. Pathway of effects of artificial light on non-target organisms at aquaculture sites in Canada. *DFO Canadian Science Advisory Secretariat* Research Document 2010/023, p. vi + 14.

Trujillo-González, A., Becker, J.A., Huerlimann, R., Saunders, R.J. and Hutson, K.S. 2020. Can environmental DNA be used for aquatic biosecurity in the aquarium fish trade? *Biological Invasions* 22(3), pp. 1011–1025.

Trujillo-González, A., Edmunds, R.C., Becker, J.A. and Hutson, K.S. 2019. Parasite detection in the ornamental fish trade using environmental DNA. *Scientific Reports* 9(1), p. 5173.

Tu, B.P. and McKnight, S.L. 2006. Metabolic cycles as an underlying basis of biological oscillations. *Nature Reviews Molecular Cell Biology* 7(9), pp. 696–701.

Tufts, B.L., Holden, J. and DeMille, M. 2015. Benefits arising from sustainable use of North America's fishery resources: economic and conservation impacts of recreational angling. *International Journal of Environmental Studies* 72(5), pp. 850–868.

Uberoi, E., Hutton, G., Ward, M. and Ares, E. 2021. *UK fisheries statistics* Number CBP 02788. London, UK: House of Commons, p. 42.

Van Leerdam, M.B., Smith, J.W. and Fuchs, T.W. 1985. Frass-mediated, host-finding behavior of *Cotesia flavipes*, a braconid parasite of *Diatraea saccharalis* (Lepidoptera: Pyralidae). *Annals of the Entomological Society of America* 78(5), pp. 647–650.

Villamizar, N., Blanco-Vives, B., Migaud, H., Davie, A., Carboni, S. and Sánchez-Vázquez, F.J. 2011. Effects of light during early larval development of some aquacultured teleosts: A review. *Aquaculture* 315(1), pp. 86–94.

Viswambari Devi, R., Basilrose, M.R. and Mercy, P.D. 2010. Prospect for lectins in arthropods. *Italian Journal of Zoology* 77(3), pp. 254–260.

Vitaterna, M.H., Takahashi, J.S. and Turek, F.W. 2001. Overview of circadian rhythms. *Alcohol Research and Health* 25(2), pp. 85–93.

Voigt, D., Schuppert, J.M., Dattinger, S. and Gorb, S.N. 2008. Sexual dimorphism in the attachment ability of the Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) to rough substrates. *Journal of Insect Physiology* 54(5), pp. 765–776.

Wadeh, H., Alsarakibi, M. and Li, G. 2010. Analysis of genetic variability within *Argulus japonicus* from representatives of Africa, Middle East, and Asia revealed by sequences of three mitochondrial DNA genes. *Parasitology Research* 107(3), pp. 547–553.

Walker, P.D., Flik, G. and Bonga, S.E.W. 2004. The biology of parasites from the genus *Argulus* and a review of the interactions with its host. *Symposia of the Society for Experimental Biology* (55), pp. 107–129.

Walker, P.D., Harris, J., van der Velde, G. and Bonga, S.E.W. 2007. Size matters: stickleback size and infection with *Argulus foliaceus* (L., 1758) (Branchiura, Arguloida). *Crustaceana* 80(11), pp. 1397–1401.

Walker, P.D., Harris, J.E., van der Velde, G. and Bonga, S.E.W. 2008. Differential host utilisation by different life history stages of the fish ectoparasite *Argulus foliaceus* (Crustacea: Branchiura). *Folia Parasitologica* 55(2), pp. 141–149.

Walker, P.D., Russon, I.J., Duijf, R., Velde, D., Van, G. and Bonga, S.E.W. 2011a. The off-host survival and viability of a native and non-native fish louse (*Argulus*, Crustacea: Branchiura). *Current Zoology* 57(6), pp. 828–835.

Walker, P.D., Russon, I.J., Haond, C., Van der Velde, G. and Wendelaar Bonga, S.E. 2011b. Feeding in adult *Argulus japonicus* thiele, 1900 (maxillopoda, Branchiura), an ectoparasite on fish. *Crustaceana* 84(3), pp. 307–318.

Walter, T.C. and Boxshall, G. 2021. World of Copepods database. *Argulus* Müller O.F., 1785. Available at: https://www.marinespecies.org/copepoda/aphia.php?p=taxdetails&id=104071 [Accessed: 10 December 2021].

Wang, K.-N. 1961. Notes on the ecology and life history of *Argulus* (Parasitic Copepoda) from the freshwater fishes of China. *Acta Zoologica Sinica* 14, pp. 154–170.

Watthanasurorot, A., Söderhäll, K., Jiravanichpaisal, P. and Söderhäll, I. 2011. An ancient cytokine, astakine, mediates circadian regulation of invertebrate hematopoiesis. *Cellular and Molecular Life Sciences* 68(2), pp. 315–323.

Wedemeyer, G.A. 1997. Effects of rearing conditions on the health and physiological quality of fish in intensive culture. *Fish Stress and Health in Aquaculture*, pp. 35–71.

Welcomme, R.L. and Bartley, D.M. 1998. Current approaches to the enhancement of fisheries. *Fisheries Management and Ecology* 5(5), pp. 351–382.

Westenberger, P. 2008. Avizo—Three-Dimensional Visualization Framework. In: Brady, S. R., Sinha, A. K., Gundersen, L. C. eds. *Geoinformatics 2008—Data to Knowledge*, pp. 13–14.

Wheeler, D. and Bhagwat, M. 2007. BLAST QuickStart: Example-Driven Web-Based BLAST Tutorial. In: Bergman, N.H. ed. *Comparative Genomics: Volume 2*. Totowa, USA: Humana Press, pp. 149–176.

Wickham, H. 2009. *ggplot2: Elegant Graphics for Data Analysis*. New York, USA: Springer-Verlag, p. 213.

Wilson, C.B. 1902. North American parasitic copepods of the family Argulidae, with a bibliography of the group and a systematic review of all known species. *Proceedings of the United States National Museum* 25(1302), pp. 635–742.

Wilson, C.B. 1944. Parasitic copepods in the United States National Museum. *Proceedings of the United States National Museum* 94(3177), pp. 529–582.

Wood, E. 2001. *Collection of Coral Reef Fish for Aquaria: Global Trade, Conservation Issues and Management Strategies*. Herefordshire, UK: Marine Conservation Society, p. 56.

Wu, G., Anafi, R.C., Hughes, M.E., Kornacker, K. and Hogenesch, J.B. 2016. MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics* 32(21), pp. 3351–3353.

Xavier, M.A. *et al.* 2019. Blood anticlotting activity of a *Rhipicephalus microplus* cathepsin L-like enzyme. *Biochimie* 163, pp. 12–20.

Yanong, R.P.E. 2003. *Fish Health Management Considerations in Recirculating Aquaculture Systems – Part 2: Pathogens*. Florida, USA: University of Florida, Institute of Food and Agricultural Sciences, p. 8.

Yildiz, K. and Kumandas, A. 2002. *Argulus foliaceus* infection in a goldfish (*Carassius auratus*). *Israel Journal of Veterinary Medicine* 57(3), pp. 118–120.

Yoshizawa, K. and Nogami, S. 2008. The first report of phototaxis of fish ectoparasite, *Argulus japonicus*. *Research in Veterinary Science* 85(1), pp. 128–130.

Yuan, Q., Metterville, D., Briscoe, A.D. and Reppert, S.M. 2007. Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Molecular Biology and Evolution* 24(4), pp. 948–955.

Zhang, L. *et al.* 2013. Dissociation of Circadian and circatidal timekeeping in the marine crustacean *Eurydice pulchra*. *Current Biology* 23(19), pp. 1863–1873.

Zhang, Z., Fu, Y., Guo, H. and Zhang, X. 2021. Effect of environmental enrichment on the stress response of juvenile black rockfish *Sebastes schlegelii*. *Aquaculture* 533, p. 736088.

Žiliukienė, V., Žiliukas, V. and Stankus, S. 2012. Infestation of *Argulus foliaceus* L. on fish fry reared in illuminated cages. *Veterinarija ir Zootechnika* 57(79), pp. 83–88.

Appendix 1

Chemical Attraction of *Argulus foliaceus*

A1.1 Aims

Argulus spp. have been previously observed to be attracted to host chemical cues, however characterisation of these cues has not yet been conducted. This study aimed to establish attraction of *Argulus foliaceus* to host cues and test success of chemical extraction techniques using a behavioural assay.

A1.2 Methods

Parasite and host collection

Three-spined stickleback *Gasterosteus aculeatus* naturally infected with *A. foliaceus* (morphologically identified according to Fryer 1982) were hand netted from Risca Canal (Newport, UK; grid reference: ST 24344 90686). *A. foliaceus* were removed from hosts in the field by removing fish from water for 10 s and re-submerging in a container of canal water following Chapter 3 and transported to the laboratory off host in sealed containers of dechlorinated water. Here, parasites were maintained as a culture (see Stewart *et al.* 2018) on three-spined sticklebacks collected from Roath Brook, Cardiff (ST 18897 78541; an *Argulus* spp. naïve population). Sticklebacks were fed daily with *Tubifex* bloodworm, and all fish and parasites were maintained under a 12 h light:12 h dark cycle. *A. foliaceus* were used in experiments after 1 week acclimatisation to laboratory conditions. Following removal from hosts for experimentation, *A. foliaceus* were measured from the rostral edge of the carapace to the anterior end of the abdominal lobes using a dissecting microscope at x10 magnification with a Lumenera Infinity 1 camera and Infinity Capture software version 6.5.4 (no visible damage to any of the removed parasites).

Stimulus origin

Fish conditioned water was created by placing a single *Argulus* naive stickleback (N = 3) into a glass tank containing 500 ml autoclaved dechlorinated water for 24 h. The three tanks of fish conditioned water were then mixed to create a homogenous stimulus solution for experimental use. Female gravid and non-gravid *Argulus* conditioned water was created by placing individual female *A. foliaceus* (gravid N = 5, non-gravid N = 5) into a glass dish with 20 ml autoclaved dechlorinated water for 24 h, after which the water was mixed for all gravid or non-

gravid lice respectively to again create a homogenous stimulus solution. Control water was created by placing 500 ml autoclaved dechlorinated water under the same conditions as fish conditioned water minus the fish for 24 h.

Conditioned water assays

Experimental design was based on methodology by Galarowicz (1991), in which response to a stimulus is determined by an increase in parasite activity. While cruder than the Y-maze assays performed by Bandilla *et al.* (2007) this method provided a quick and easy assay, although camera tracking was used, over the personal observation approach employed by Galarowicz (1991), for higher accuracy data collection. Adult male *A. foliaceus* (4.52 ± 1.15 SD) were removed from hosts and placed into a glass dish with 20ml autoclaved dechlorinated water for 24 h to increase behavioural response via starvation (Mikheev *et al.* 1999; Bandilla *et al.* 2007). After 24 h, each male was placed into a glass petri dish containing 50 ml autoclaved dechlorinated water (sides covered with white material to reduce behaviour altering reflections; Mikheev *et al.* 1998) and left to acclimatise for 30 min (based on Mikheev *et al.* 1999). After 30 min, 1ml of either fish, female gravid *Argulus*, female non-gravid *Argulus* or control water (N = 15 each) was introduced to the centre of each petri dish via a glass pipette. *A. foliaceus* activity was filmed for 1 min prior to and after introduction of stimuli, with difference in average speed calculated. Each louse was tested against each stimulus in a random order, with apparatus completely reset between trials (cleaned using ethanol and dried fully at 70 °C) to ensure removal of residue stimulus.

Chemical extraction

This methodology largely follows Mohney *et al.* (2009), using ethanol for extraction over methanol and slight adjustments to accommodate tubing placement in water instead of soil. Prior to extraction, polydimethylsiloxane (PDMS) tubing was cleaned by soaking in ethanol for 24 h, draining and then drying at 70 °C. For collection, tubing (1 m in length) was coiled into tanks of fish conditioned water (generated as above, N = 3), ensuring an even distribution of tubing throughout the tank with the ends left exposed approximately 2.5 cm above the water level. Tubing was left for 24 h, removed from the water and then placed between two pieces of sterile filter paper and blotted dry. To extract stimulus, one end of tubing was placed into a glass collection vial, with 500 µl of ethanol pipetted into the opposite end of the tubing using a Gilson pipette and filter tip placed into glass stretched pipette. The end containing the stretched pipette was raised to create a flow rate of 1 ml/min. After 500 µl of ethanol had passed

through the tubing into the collection tube, two 500 µl bolus of air (one after the other) were pumped into the tubing to remove any residual solvent. Extract was stored at -80 °C.

Extraction assays

To test whether the stimulus causing increased *A. foliaceus* activity had been extracted from fish conditioned water via the PDMS tubing method, behavioural assays using the extract were performed. As PDMS tubing extraction has a capture rate of ~40%, a x3 re-dilution of extract was used (~120% concentration, 30µl extract in 10ml autoclaved dechlorinated water). Extract control stimulus was created by diluting pure ethanol (using the same volume as used for extract re-dilution) with autoclaved dechlorinated water to ensure ethanol by itself did not impact *A. foliaceus* behaviour. Assays were then performed as above using the following stimuli: extract x3 (N = 20), extract control, fish and water control (N = 29 each).

Statistical analysis

All statistical analyses were conducted using R statistical software (v3.6.3; R Core Team 2017) with the level of significance in all tests taken as $p<0.05$ using package "ggplot2" to visualise the data (Wickham 2009). A generalised linear model (GisedLM) with binomial family and logit link function was used to examine whether an increase in activity (y/n) was influenced by stimulus type. A general linear model (GLM) with gaussian family and identity link function was then used to determine if the difference in average *A. foliaceus* speed post-stimulus application was significantly affected by stimulus type. Models were refined through stepwise deletion of insignificant terms and Akaike information criterion comparisons, with visual examination of model plots to check standardised residuals for normal distribution and homogeneity of variance (Crawley 2007).

A1.3 Results

When presented with a fish stimulus, *A. foliaceus* activity significantly increased when examined as a yes/no increase in activity post-application ($p = 0.006$, GisedLM; figure A1.1). However when only looking at the difference in average swim speed pre- and post-stimulus there was no significance (although fish stimulus is the treatment with the highest difference versus control, $p = 0.09$, GLM; figure A1.2). No other treatments showed a significant difference in activity compared to the control. These results suggest that the extraction method

employed here was not successful in extracting the chemical stimulus from fish that attracts *A. foliaceus.*

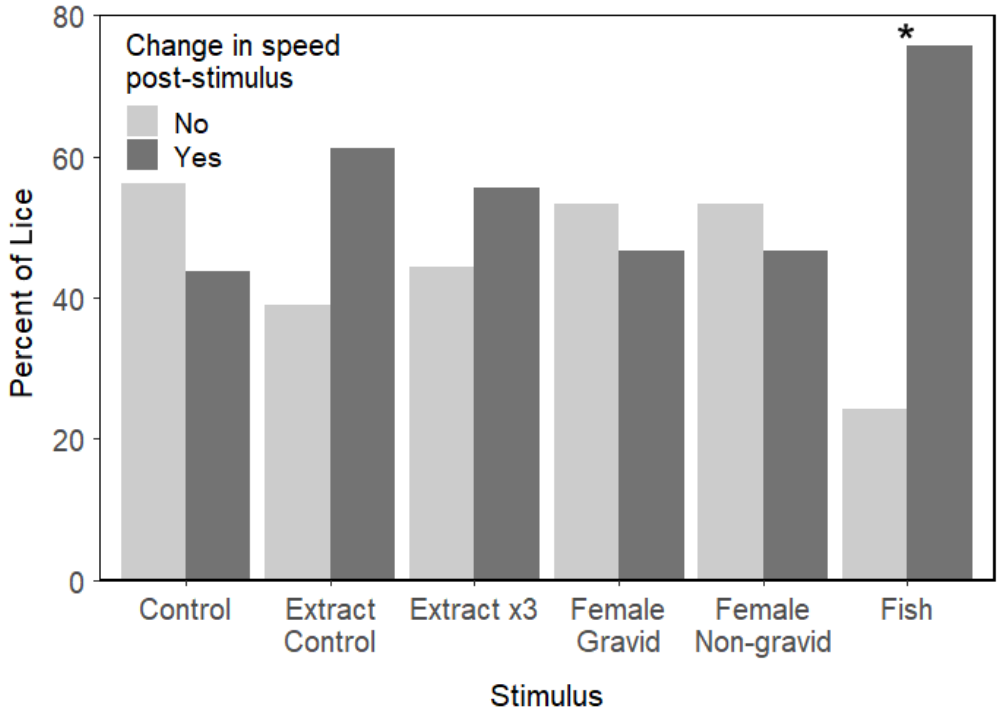

Figure A1.1 Change in *Argulus foliaceus* activity post-application of different stimuli. The "*" indicates significant difference between yes/no columns.

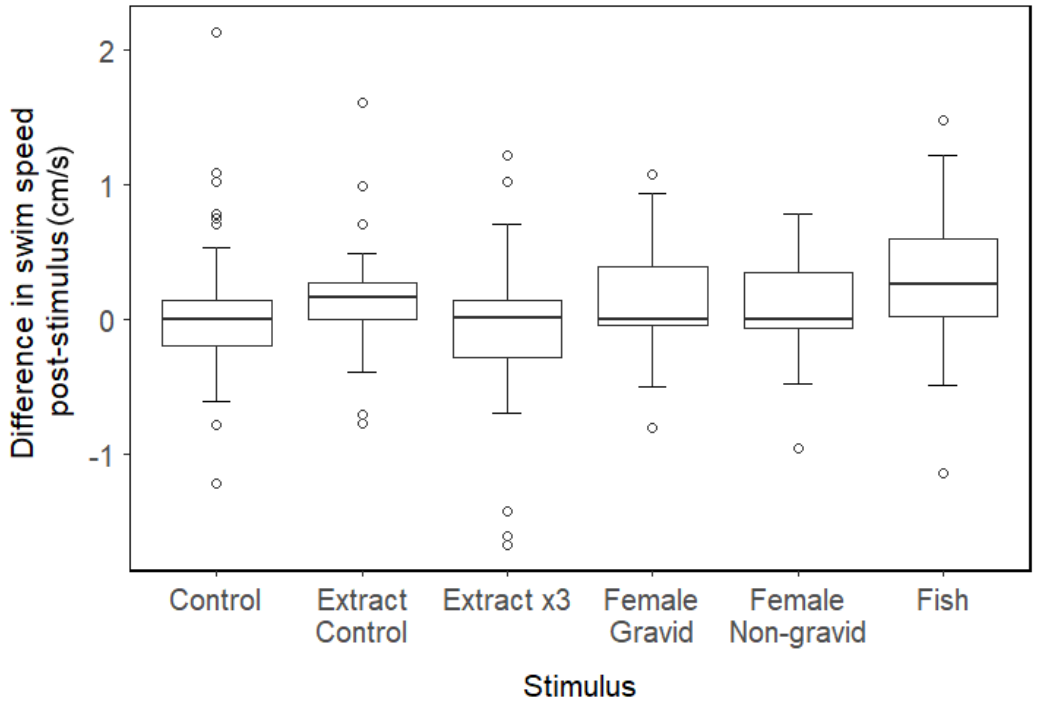

Figure A1.2 Change in average swim speed of *Argulus foliaceus* post-application of different stimuli.

Appendix 2

Argulus foliaceus infections in non-naïve fish

A2.1 Aims

This study investigated acquired resistance of three-spined stickleback *Gasterosteus aculeatus* to infection with *Argulus foliaceus* and determined what happens to lice lost during infection.

A2.2 Methods

Parasite survival

Adult three-spined sticklebacks *Gasterosteus aculeatus* (originating from Roath Brook, Cardiff, ST 18897 78541) maintained as an *A. foliaceus* culture (see Stewart *et al.* 2017) were isolated into 1 L tanks 1-2 weeks post natural loss of infection (N = 5). Each fish was re-infected with 5 *A. foliaceus* metanauplii (total N = 25) hatched from the laboratory culture and the position and presence of each parasite on the host recorded for two weeks. To ascertain whether missing lice had detached and died or been consumed by the host, water in the tanks was filtered everyday through a mesh of aperture <0.5 mm (tested prior to experimentation in lab to ensure capture of metanauplii) and examined for the presence of *A. foliaceus.* All waste produced by the fish was then investigated under a dissecting microscope for presence of *A. foliaceus.* Data regarding survival of *A. foliaceus* on naïve stickleback hosts was taken from Chapter 3 for use in analyses.

Statistical analysis

All statistical analyses were conducted using R statistical software (v3.6.3; R Core Team 2017) with the level of significance in all tests taken as $p<0.05$. Package "survival" was used to run survival analyses (Therneau and Grambsch 2000) with "ggplot2" used to visualise the data (Wickham 2009). Survival analysis was conducted to determine the impact of stickleback prior infection status (naïve or non-naïve) on *A. foliaceus* survival.

A2.3 Results

Over the two weeks there was a 92% reduction in infection for previously infected fish, a significantly greater decrease versus infections on *Argulus* naïve fish ($p<0.001$; figure A2.1). Only 8% of lost lice were recovered, found on the bottom of the tank. As the stickleback waste only showed evidence of bloodworm (fed daily to laboratory housed sticklebacks) through the

presence of undigested mouth parts, the remaining 84% of lice lost were deemed to have been consumed and completely digested.

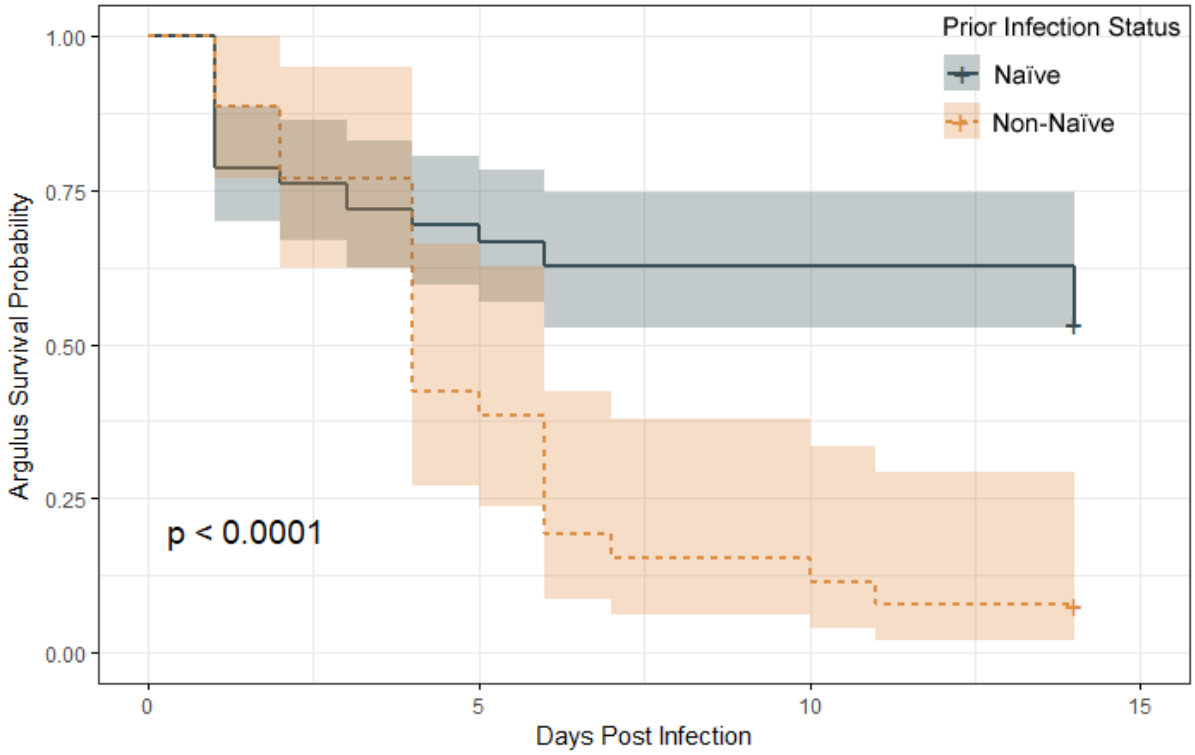

Figure A2.1 Survival probability of *Argulus foliaceus* on naïve and non-naïve three-spined stickleback *Gasterosteus aculeatus.*