

**Studies investigating the outcome of a host-parasite interaction using
the stickleback-*Schistocephalus* model system**

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

Vicki Macnab

Department of Biology

University of Leicester

September 2011

Studies investigating the outcome of a host-parasite interaction using the stickleback-*Schistocephalus* model system

Vicki Macnab

This thesis firstly investigates the effect of the cestode parasite *Schistocephalus solidus* on the reproduction of its intermediate host, the three spined stickleback. The results indicated that the parasite reduced host reproduction and the physiological basis behind this was established. In males, parasite infection reduced the fishes ability to produce 11-ketotestosterone which resulted in reduced reproductive behaviour and sexual development. In infected females, vitellogenin production was reduced which prevented egg maturation. The adaptive nature of these changes seemed to be variable among different populations. This variation may result from genetic differences in the host or parasite population, co-evolutionary processes or reflect differences in the environmental conditions experienced by populations.

The second part of this thesis investigates the effect of two types of anthropogenic stressors – endocrine disrupting chemicals and temperature change as an outcome of global climate change – on disease progression in the stickleback-*Schistocephalus* system. The results indicated that the natural steroid 17β estradiol (E2) had a significant impact on disease progression in a sex and dose dependent manner, with males exposed to the high 100ngL^{-1} E2 treatment harbouring significantly larger parasites compared to females in this treatment and fish from the lower E2 and solvent control treatment. Elevated temperatures were also found to significantly increase parasite growth and reduce host growth. In addition, a behavioural study showed that fish harbouring infective parasites seek out high temperatures which could be adaptive on the part of the parasite as it would allow them to grow faster and potentially increase their reproductive output.

The clear outcome of this thesis is that anthropogenic stressors increase disease progression in the stickleback-*Schistocephalus* system and this is likely to lead to reduced host reproduction and increased parasite transmission and reproduction, potentially leading to the evolution of higher virulence in this host-parasite interaction.

Acknowledgements Page

Over the past four years many people have supported me and helped me along the way with my PhD.

Firstly, I can't thank Iain enough for all his help and support over the past few years. His positivity and enthusiasm for science and my project never failed to amaze me, and kept me going despite many setbacks!! I have learnt so much from my time at Leicester, and Iain's open and easy going approach has definitely brought out the best in me as a scientist.

I also need to say a big thanks to my supervisor at Cefas, Ioanna, for her help and support on my visits to Weymouth. Many other members of staff at Cefas have also helped me and I need to say a special thanks to Mathew Sanders, Marion Sebire and Alexander Scott for their help and patience whilst training me!

I would like to thank all my good friends that have supported me throughout my PhD, you have been great! To my St Leonards roomies Megan and Carolyn, you are the best! Megan, I know I definitely would not have got through my second year without you! You were always there to help, give advice and support no matter how busy you were!! And Carolyn, you were brilliant, always ready to listen, be it at work or the pub! And to the other uni girls I met along the way Sophie and Rachel, thank you for your friendship and support!

A big thanks to the past and present members of the Barber Lab! Tia, we have been through so much together in the past four years, its been great having someone to go through this with and I couldn't have chosen anyone better for the journey! Ceinwen, thank you for all your help during my experiments and your patience and kindness!

Next I would like to say a big thank you to my parents and brother, who have always been there for me and supported me whatever path I have taken. My parents have always managed to remind me that when I am stressed there is more to life than sticklebacks! They have always been ready to listen to my problems and give advice, regardless of whether they understand what I am talking about!! I would never have made it this far without the continued love and encouragement that I have received from them.

Kevin, I can't thank you enough, I am so lucky to have you. Your patience, love and support throughout my PhD has never wavered and I don't think you realise how much you have kept me going through this. You have kept me sane and reminded me of the important things in life!

Table of Contents

Abstract	I
Acknowledgements	II
Table of Contents	III
Table of Figures	IX
List of Tables	XI
List of Common Abbreviations	XII
Publication List	XIV
Collaborations	XIV

1. INTRODUCTION	1
1.1. The host-parasite interaction	2
1.1.1. The evolution of virulence in host-parasite interactions	2
1.1.2. Mechanisms of parasite avoidance and resistance	3
1.1.2.1. Behavioural resistance	4
1.1.2.2. Immunological mechanisms	6
1.1.3. Host behaviour changes associated with infection	7
1.1.4. Effects of parasites on host reproduction	9
1.2. Environmental stress influences host-parasite interactions	10
1.2.1. Interactions between stressors in perturbed environments	11
1.2.2. Endocrine disrupting chemicals	12
1.2.2.1. Estrogens in aquatic environments	14
1.2.2.2. Androgens in aquatic environments	15
1.2.2.3. Anti-androgens in aquatic environments	16
1.2.3. Elevated temperatures and global climate change	17
1.2.3.1. General effects on species and ecosystems	17
1.2.3.2. Temperature and host-parasite interactions	19
1.2.3.3. Temperature and disease in animals	21
1.3. The three spined stickleback, <i>Gasterosteus aculeatus</i>	22
1.3.1. Reproductive biology	23
1.3.1.1. Reproductive behaviour	23
1.3.1.2. Hormonal control of reproduction in males	26
1.3.1.3. Hormonal control of reproduction in females	28
1.3.2. Sticklebacks as models in ecotoxicology	29
1.4. The stickleback-<i>Schistocephalus</i> model host-parasite system	31
1.4.1. <i>Schistocephalus solidus</i> : life cycle and biology	31

1.4.2.	Interactions with stickleback hosts	33
1.5.	Aims and Objectives of the Thesis	35
2.	THE EFFECT OF <i>SCHISTOCEPHALUS SOLIDUS</i> INFECTION ON THE REPRODUCTIVE BEHAVIOUR AND ENDOCRINOLOGY OF MALE STICKLEBACKS	37
2.1.	Introduction	38
2.2.	Materials and methods	40
2.2.1.	Fish collection and husbandry	40
2.2.2.	Experimental procedure	41
2.2.3.	Post mortem analysis	42
2.2.4.	Quantification of kidney spiggin levels	43
2.2.5.	Quantification of 11-ketotestosterone (11KT) levels by radioimmunoassay (RIA)	43
2.2.6.	Statistical analysis	45
2.3.	Results	46
2.3.1.	Infection and nesting and courtship behaviour	46
2.3.2.	Infection and sexual development	47
2.3.3.	Relationships between 11-ketotestosterone (11KT) levels, reproductive behaviour and nuptial colouration	48
2.3.4.	Relationships between spiggin and reproductive behaviour	51
2.3.5.	Relationships between kidney index (Ik), 11-ketotestosterone (11KT) and spiggin	52
2.3.6.	Relationship between gonad index (I _G) and 11-ketotestosterone (11KT)	54
2.3.7.	Infection and body condition	54
2.3.8.	Relationships between fish mass (M-M _P) and 11-ketotestosterone (11KT)	57
2.3.9.	Body condition comparisons across both populations	57
2.4.	Discussion	59
2.4.1.	Are population differences in the condition of infected fish associated with differences in reproductive behaviour?	59
2.4.2.	How is the reproductive status of <i>S. solidus</i> infected male sticklebacks from the two populations linked to endocrinological phenotype?	62
2.4.3.	Adaptive or non adaptive reproductive disruption by parasites?	64
2.4.4.	Ideas for further work	66

3. THE EFFECT OF <i>SCHISTOCEPHALUS SOLIDUS</i> INFECTION ON THE REPRODUCTIVE CAPACITY OF WILD AND LABORATORY INFECTED STICKLEBACKS	68
3.1. Introduction	69
3.2. Materials and methods	71
3.2.1. Wild caught female fish study	71
3.2.2. Experimental infection study	71
3.2.3. Post mortem analysis (both studies)	74
3.2.4. Vitellogenin (VTG) analysis – ELISA (based on the OECD, 2011 test guidelines)	75
3.2.5. Quantification of kidney spiggin and 11KT levels	78
3.2.6. Statistical analysis	78
3.3. Results	79
3.3.1. Wild caught Carsington Reservoir females	79
3.3.2. Experimentally infected Carsington Reservoir females	82
3.3.3. Comparison of lab bred and wild caught Carsington Reservoir females	85
3.3.4. Experimentally infected Carsington Reservoir males	87
3.4. Discussion	89
3.4.1. Effects of <i>S.solidus</i> infection on the reproductive development of female sticklebacks	89
3.4.2. Effects of <i>S.solidus</i> infection on the body condition of female sticklebacks	91
3.4.3. Evolutionary significance of reduced reproductive capacity of <i>S. solidus</i> infected female sticklebacks	92
3.4.4. How does <i>S. solidus</i> exposure interfere with reproductive parameters in male sticklebacks?	95
3.4.5. Conclusions	97
4. THE EFFECT OF ENDOCRINE DISRUPTING CHEMICALS ON INFECTION SUSCEPTIBILITY AND DISEASE PROGRESSION	99
4.1. Introduction	100
4.1.1. Endocrine disruption in natural environments	100
4.1.2. Parasites and pollution	101
4.1.3. Aims of current study	104
4.2. Materials and Methods	105
4.2.1. Experimental design	105
4.2.2. Experimental infections of copepods and sticklebacks	106

4.2.3.	Post mortem analysis	107
4.2.4.	Vitellogenin (VTG) analysis	107
4.2.5.	Water sampling	108
4.2.6.	Molecular sex determination	109
4.2.7.	Statistical analysis	112
4.3.	Results	113
4.3.1.	Water analysis	113
4.3.2.	Effect of E2 treatment on infection susceptibility, condition and vitellogenin production of all fish	114
4.3.3.	Effect of E2 treatment on parasite size and relationships between parasite index and body condition measurements	118
4.4.	Discussion	121
4.4.1.	The impact of E2 treatment on parasite growth, condition and physiological measurements	121
4.4.2.	Possible mechanisms behind increased parasite size at high E2 levels in males	123
4.4.3.	Conclusions and future studies	130
5.	THE EFFECT OF TEMPERATURE ON DISEASE PROGRESSION IN THE STICKLEBACK-<i>SCHISTOCEPHALUS</i> SYSTEM	132
5.1.	Introduction	133
5.2.	Materials and methods	135
5.2.1.	Experimental infections	135
5.2.2.	Temperature treatments	136
5.2.3.	Experimental design	136
5.2.4.	Data collection	137
5.2.5.	Statistical analysis	138
5.3.	Results	138
5.3.1.	Effect of temperature on parasite establishment, growth and estimated adult fecundity	138
5.3.2.	Effect of temperature on fish growth and condition	140
5.4.	Discussion	144
5.4.1.	What underlying mechanisms might generate increased parasite growth at higher temperatures?	144
5.4.2.	Ecological consequences	149
5.4.3.	Conclusions	150

6. THE EFFECT OF <i>SCHISTOCEPHALUS SOLIDUS</i> INFECTION ON HOST THERMAL PREFERENCES IN STICKLEBACKS	152
6.1. Introduction	153
6.1.1. Host adaptive behavioural change	154
6.1.2. Parasite adaptive behavioural change	154
6.1.3. Altered host temperature preferences as adaptations to parasite infection	156
6.2. Materials and methods	158
6.2.1. Fish supply and husbandry (wild caught fish)	158
6.2.2. Fish supply and husbandry (experimentally infected fish)	158
6.2.3. Screening thermal preferences	159
6.2.4. Post mortem analysis	160
6.2.5. Behavioural trial analysis	160
6.2.6. Statistics and data analysis	161
6.3. Results	162
6.3.1. Trials using wild caught fish	162
6.3.2. Trials using laboratory fish	164
6.3.3. Comparisons between wild and lab trials: thermal preferences	168
6.4. Discussion	171
6.4.1. Behavioural change: side effects or adaptations of host / parasite	171
6.4.2. Potential mechanisms underlying thermal preferences in hosts harbouring non infective parasites (<50mg)	173
6.4.3. Potential mechanisms for thermal preferences in hosts harbouring infective parasites (>50mg)	175
6.4.4. Ecological consequences of parasite altered host thermal preferences	179
6.4.5. Ideas for further work	179
6.4.6. Conclusions	181
7. GENERAL DISCUSSION	182
7.1. Summary of main findings	183
7.1.1. Variation in the effects of infection on host reproduction in sticklebacks	183
7.1.2. Environmental effects on host – parasite interactions	186
7.1.2.1. The effects of EDCs on host – parasite interactions	186
7.1.2.2. The effects of temperature on host – parasite interactions	187
7.1.2.3. Ideas for further work	189
7.2. Main conclusions	190

Table of Figures

Figure 1.1 The thermal performance curve for a given process (e.g. growth, reproduction) of an ectotherm.....	18
Figure 1.2 The thermal performance curve of a host and its parasite	20
Figure 1.3 The life cycle of <i>Schistocephalus solidus</i>	33
Figure 2.1 A binding curve illustrating the proportion of radio-labelled antigen that was bound to the antibody at known steroid concentrations.....	45
Figure 2.2 The effect of <i>S. solidus</i> infection on reproductive behaviour of sticklebacks from two study populations	47
Figure 2.3 The effect of <i>S. solidus</i> infection on sexual development of sticklebacks from two study populations	49
Figure 2.4 The relationship between 11-ketotestosterone (11KT) levels and measures of sexual development and reproductive behaviour in <i>S. solidus</i> infected and non infected sticklebacks from the two study populations.....	50
Figure 2.5 The relationship between spiggin levels and reproductive behaviour in <i>S. solidus</i> infected and non infected sticklebacks from the two study populations.....	51
Figure 2.6 Relationships between measurements of sexual development in <i>S. solidus</i> infected and non infected sticklebacks from the two study populations.....	53
Figure 2.7 The relationship between 11-ketotestosterone (11KT) and spiggin levels in all fish separated by <i>S. solidus</i> infection status	53
Figure 2.8 The relationship between 11-ketotestosterone (11KT) levels and gonad index (I_G) in all fish separated by <i>S. solidus</i> infection status	54
Figure 2.9 The effect of <i>S. solidus</i> infection on measurements of body condition in sticklebacks from the two study populations.....	56
Figure 2.10 Differences in the index of physiological condition in infected sticklebacks across the two study populations, measured using principle component analysis.....	58
Figure 3.1 The effect of <i>S. solidus</i> infection on sexual development in female wild caught sticklebacks from Carsington Reservoir	80
Figure 3.2 Relationships between vitellogenin levels (VTG) and measurements of sexual development in <i>S. solidus</i> infected and non infected female wild caught Carsington sticklebacks	81
Figure 3.3 Effect of <i>S. solidus</i> infection status on indices of body condition in female wild caught Carsington females.....	82
Figure 3.4 The effect of experimental <i>S. solidus</i> exposure status on measurements of sexual development in lab raised Carsington females.....	83
Figure 3.5 The effect of experimental <i>S. solidus</i> exposure status on indices of body condition in lab raised Carsington females	84
Figure 3.6 Relationships between measurements of sexual development and body condition in <i>S. solidus</i> infected and non infected wild and lab female Carsington sticklebacks	85
Figure 3.7 Relationships between parasite index and reproductive and body condition parameters in <i>S. solidus</i> infected wild and lab female sticklebacks.....	86
Figure 3.8 The effect of experimental <i>S. solidus</i> exposure status on measurements of sexual development in lab raised Carsington males.....	88
Figure 3.9 The effect of <i>S. solidus</i> exposure status on reproductive behaviour and physiology in non infected male lab raised sticklebacks	89
Figure 3.10 The effect of <i>S. solidus</i> parasite index on levels of 11-ketotestosterone (11KT) in different populations of male sticklebacks.....	95

Figure 4.1	The experimental design where sticklebacks were exposed to chemical E2 treatments before undergoing experimental <i>Schistocephalus solidus</i> infections	106
Figure 4.2	Molecular sex determination gel showing female sticklebacks producing a single band of approximately 300bp and males producing two products of 270bp and 300bp.....	112
Figure 4.3	The E2 steroid concentrations achieved in tanks across the three treatments over the course of the exposure.....	114
Figure 4.4	The effect of E2 treatment and fish sex on measurements of body condition in sticklebacks exposed to <i>S. solidus</i> infection	116
Figure 4.5	The effect of E2 treatment and fish sex on <i>S. solidus</i> parasite load.....	119
Figure 4.6	Relationships between parasite index and measurements of body condition in experimentally <i>S. solidus</i> infected sticklebacks from different E2 treatments.....	120
Figure 4.7	Relationships between parasite load and vitellogenin (VTG) levels in experimentally <i>S. solidus</i> infected sticklebacks from the different E2 treatments.....	121
Figure 4.8	The potential mechanisms involved in increased parasite growth in male fish in the high E2 group.....	123
Figure 5.1	The effect of thermal regime on disease progression in sticklebacks.....	140
Figure 5.2	The effect of temperature regime on growth in sticklebacks.....	141
Figure 5.3	The effect of exposure status and rearing temperature on indices of stickleback condition.....	142
Figure 5.4	The potential mechanisms behind increased parasite growth at elevated temperatures	146
Figure 6.1	The experimental set up used to test thermal preferences of sticklebacks.....	160
Figure 6.2	The proportion of time that <i>S. solidus</i> infected and non infected wild fish spent in the three thermal regimes	162
Figure 6.3	The effect of <i>S. solidus</i> infection status on wild stickleback thermal preferences..	163
Figure 6.4	The thermal preferences of wild caught sticklebacks that differ in their <i>S. solidus</i> infection status	164
Figure 6.5	The proportion of time that <i>S. solidus</i> exposed and non exposed lab fish spent in the three thermal regimes over the 5 tested time periods.....	166
Figure 6.6	The effect of experimental <i>S. solidus</i> exposure status on thermal preferences of lab sticklebacks over the 5 tested time points	167
Figure 6.7	The effect of experimental <i>S. solidus</i> exposure status on thermal preferences of sticklebacks at the final tested time point	167
Figure 6.8	The thermal preferences of laboratory bred three spined sticklebacks that differ in their <i>S. solidus</i> infection status.....	168
Figure 6.9	The thermal preferences of wild caught and laboratory bred three spined sticklebacks that differ in their <i>S. solidus</i> infection status	168
Figure 6.10	The relationship between thermal preferences (Σ deg) and disease progression in laboratory infected fish	170
Figure 6.11	The potential mechanisms associated with temperature preferences at different parasite sizes in wild caught fish.....	175

List of Tables

Table 2-1 Correlation matrix showing correlations between indicators of body condition and health status among infected fish grouped together from the two study populations	58
Table 2-2 Results of principal component analyses on various measures of body condition in male sticklebacks infected with <i>S. solidus</i>	58
Table 4-1 Results of two way ANOVAs performed to investigate the impact of infection status, E2 treatment and fish sex on measurements of body condition	117
Table 4-2 Results of two way ANOVAs performed to investigate the impact of infection status and fish sex on vitellogenin (VTG) production within a single E2 treatment group	117
Table 4-3 Result of two way ANOVA (I_p) and ANCOVA (M_p) performed to investigate the impact of E2 treatment and fish sex on <i>S. solidus</i> parasite load	118
Table 5-1 Results of two way ANOVAs investigating the impact of temperature treatment (15oC / 20oC) and exposure status (non exposed / exposed non infected / infected) on growth and the effect of family and temperature treatment on growth (rG).	141
Table 5-2 Results of two way ANOVAs investigating the impact of temperature treatment (15oC / 20oC) and exposure status (non exposed / exposed non infected / infected) on indices of condition.....	143
Table 6-1 Result of two way ANOVA performed to investigate the impact of fish sex and infection class on thermal preferences (Σdeg) of sticklebacks	163

List of Common Abbreviations

Σ_{deg} :	Temperature preference of fish
11KT:	11- ketotestosterone
A:	Dorsal profile area
E2:	17 β estradiol
EDC:	Endocrine disrupting chemical
F:	Egg output from parasite
G:	Specific growth rate
I _G :	Gonad index
I _H :	Hepatosomatic index
I _K :	Kidney index
I _P :	Parasite index
I _R :	Redness index
I _S :	Spleen index
K:	Body condition factor
KL:	Kendoon Loch population
L _B :	Length of blood sample in microhaematocrit tube (for PCV calculation)
L _R :	Length of red blood cells in microhaematocrit tube (for PCV calculation)
L _S :	Standard length of fish
M:	Mass of fish (including parasite mass)
M ₀ :	Mass at start of experiment (day 0)
M _G :	Mass of gonads
M _K :	Mass of kidney
M _L :	Mass of liver
M _P :	Mass of parasite
M _S :	Mass of spleen
M-M _P :	Mass of fish excluding parasite mass

PCV:	Packed cell volume of blood sample
$rA^{1/2}$:	Index of body swelling in <i>S. solidus</i> infected fish
rG:	The residual values from the relationship between M_0 and G
$rL_S M_P$:	The residual values from the relationship between L_S and M_P
t:	Time (number of days)
VP:	Victoria Park population
VTG:	Vitellogenin

Publication List

Chapter 2:

Macnab, V., Scott, A., Katsiadaki, I. & Barber, I. (2011). Variation in the reproductive potential of *Schistocephalus* infected male sticklebacks is associated with 11-ketotestosterone titre. *Hormones and Behaviour*. **60 (4)**, 371-379.

Macnab, V., Katsiadaki, I. & Barber, I. (2009). Reproductive potential of *Schistocephalus solidus* infected male three-spined stickleback *Gasterosteus aculeatus* from two UK populations. *Journal of Fish Biology*. **75 (8)**, 2095-2107.

Chapter 5 and 6:

In review: Macnab, V. & Barber, I. Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. *Global Change Biology*.

Collaborations

All work was carried out under the home office project license number: PPL 80/2327 (held by Dr. Iain Barber) and the personal license number: PIL 80/11486 under the supervision of Dr. Iain Barber (Leicester).

Chapter 2: The spiggin ELISA was carried out solely by our collaborators at the Centre for Environment, Fisheries and Aquaculture Science (Cefas). The 11KT radioimmunoassay was carried out by the candidate with supervision and help from Dr. Alexander Scott (Cefas). The rest of the work was carried out solely by the candidate at Leicester University.

Chapter 3: The spiggin ELISA was carried out solely by our collaborators at Cefas. The VTG ELISA and the 11KT radioimmunoassay were carried out by the candidate with supervision and help from Dr. Marion Sebire (Cefas). The rest of the work was carried out solely by the candidate at Leicester University.

Chapter 4: The running of the chemical exposure experiment and the collection of water samples was carried out by the candidate alongside Ceinwen Tilley (Leicester Uni). The VTG ELISA was carried out by the candidate with supervision and help from Dr. Ioanna Katsiadaki (Cefas). The steroid radioimmunoassay was carried out by the candidate with supervision and help from Dr. Alexander Scott (Cefas). The molecular sex determination work carried out by the candidate with supervision and help from Ceinwen Tilley (Leicester Uni). The rest of the work was carried out solely by the candidate at Leicester University.

Chapter 5: The work in this chapter was carried out solely by the candidate at Leicester University.

Chapter 6: The work in this chapter was carried out solely by the candidate at Leicester University.

1. Introduction



1.1. The host-parasite interaction

1.1.1. The evolution of virulence in host-parasite interactions

A parasite lives in symbiosis with another organism - its host - and has a detrimental effect on the fitness of this host (Clayton and Moore, 1997). The term “virulence” describes the degree to which host fitness is reduced by the parasite (Ebert and Herre, 1996, Poulin, 2007). Traditionally it was believed that parasites initially are highly virulent to their host but over evolutionary time the parasites become adapted to exploit their host in a manner that causes minimal harm leading to a reduction in virulence (Anderson and May, 1982, Minchella, 1985, Poulin, 2007). This belief was based on the idea that overly virulent parasites would reduce host numbers and lead to their extinction. However there is an increasing recognition that this is not always the case; evolution acts on parasites to maximise reproductive success under a given set of conditions, regardless of host mortality (Poulin, 2007). A trade-off typically exists between parasite reproduction and host survival. For example, if parasites exploit their hosts heavily, leading to high reproductive rates (high virulence) this will increase host mortality and reduce the survival time of the parasite (Lafferty and Kuris, 2009). The outcome of any particular host-parasite interaction will depend on how virulence is related to the transmission of the parasite (Anderson and May, 1982). For example, vector transmission may generate selection for higher virulence in vertebrate hosts because transmission relies on a healthy vector rather than healthy hosts. In contrast, where transmission is direct and depends on hosts being healthy and active, lower virulence may be selected for (Poulin, 2007). The reproductive rate of a parasite depends on parasite transmission and virulence, the death rate of uninfected hosts, host population size and host recovery (Anderson and May, 1982). As these factors can vary and show different inter-relationships, different levels of virulence can evolve in

isolated host-parasite populations (Ebert and Herre, 1996). An optimal virulence level may exist for a particular host-parasite system that maximises the parasites lifetime reproductive rate, but this may not be species wide as it will also depend on the environmental conditions that the system exists under, as these will also impact transmission (Poulin, 2007).

1.1.2. Mechanisms of parasite avoidance and resistance

Virulence is not determined solely by the parasite, but is also influenced by the extent to which the host responds to parasite infection. As host fitness is usually reduced as a result of parasite infection, there should be strong selection for the evolution of resistance mechanisms (Minchella, 1985). However, in many situations resistance to parasites has not evolved (Schmid-Hempel, 2003). Minchella (1985) lists four points that the evolution of host resistance will depend on: 1) the presence of resistance genes in the host population; 2) the harm that the parasite causes to the host; 3) the prevalence of the parasite and the likelihood that the host will come into contact with it i.e. the risk of infection and 4) the cost of resistance. Webster and Woolhouse (1999) demonstrated the evolutionary costs of resistance to *Schistosoma mansoni* in lines of snails (*Biomphalaria glabrata*). In comparison with susceptible lines, resistant snails had lower fecundity, producing less offspring, in the absence of parasites. This was not due to investment in resistance removing energy from reproduction, as no differences existed in egg production and other fecundity measures between susceptible and resistant lines. The authors propose that this reduced fertility may result from the absence of some internal component that is involved in both the development of embryos and parasites i.e. a pleiotropic effect, such as the component being used by the developing parasite and thus being lost in resistant lines. There are also costs of resistance at an individual level, as the activation of an immune response will take

energy away from other processes (Schmid-Hempel, 2003). In order for host resistance to evolve, its fitness cost must be less than the fitness costs associated with parasite infection (Minchella, 1985).

Even when host resistance does evolve, it is unlikely to lead to the extinction of the parasite. Genetic recombination in sexually-reproducing parasites increases the likelihood that some parasite offspring will evolve the capacity to cope with the host adaptations (Minchella, 1985). Likewise the exchange of genetic material during sexual reproduction in hosts will lead to offspring which differ genetically from their parents, with some individuals possessing traits that will allow them to combat parasite infection. Recently Morran et al. (2011) illustrated this principle in asexually reproducing nematodes (*Caenorhabditis elegans*) that went extinct when faced with a coevolving bacterial pathogen (*Serratia marcescens*) in the lab, whilst in wild populations, faced with a coevolving parasite, there was selection and maintenance for high levels of outcrossing. Therefore genetic recombination is expected to create parasites that differ in their infectivity to the host, and hosts that differ in their resistance and susceptibility to infection.

1.1.2.1. Behavioural resistance

Hosts can also resist parasites using behavioural mechanisms. Hosts that show behavioural defences against parasites will be favoured by natural selection if they produce more viable offspring (Hart, 1997). Consequently, there are several behaviours in animals that are associated with avoiding, reducing contact with or removing parasites. For such behaviours to evolve, Hart (1994) describes two criteria that must be met. Firstly, the parasites in question must have a negative effect on the fitness of the host and secondly the behaviour must remove or reduce the effect of the parasite. Behavioural defences are often costly; for example, the grooming of animals to remove

ectoparasites reduces the time that is spent surveying for predators (Mooring and Hart, 1995).

The avoidance of ectoparasites can be achieved by simply moving away from them, for example the great tit (*Parus major*) avoids nesting in boxes that are infested with the hen flea *Ceratophyllus gallinae* (Christe et al., 1994). The joining of a group could also dilute the effects of parasites (in a similar way to predators); however groups may also increase the transmission of parasites (Moore, 2002). Poulin and Fitzgerald (1989) found that three spined sticklebacks (*Gasterosteus aculeatus*) and black spotted sticklebacks (*Gasterosteus wheatlandi*) that were part of larger shoals had fewer attacks by the ectoparasite *Argulus canadensis*. Fish that were exposed to parasites also formed larger shoals than fish that were parasite-free. Some birds use plant material in their nest structure that repels parasites (Hart, 1997). For example, the European starling *Sturnus vulgaris*, incorporates plant material in to nests that reduce the hatching of arthropod eggs (louse *Menacanthus* sp and adult fowl mites *Ornithonyssus sylviarum*) and limit bacterial growth (Clark and Mason, 1985). Endoparasitic infections can be avoided by selective feeding that does not include intermediate hosts of the parasite (Moore, 2002). However, there may be perceptual or other limitations on parasite avoidance, as in some cases hosts seem unable to avoid eating infected prey. Wedekind and Milinski (1996) demonstrated that three spined sticklebacks did not avoid consuming copepods harbouring infective procercooids of the cestode parasite *Schistocephalus solidus*. In fact, sticklebacks fed on infected copepods more often, as they were more visible as they had higher activity rates but poorer escape responses. Another mechanism of avoidance of parasites is via mate selection whereby parasitized mates are avoided either because they could transmit infections directly or transmit parasites (or susceptibility) vertically to offspring (Hamilton and Zuk, 1982, Moore,

2002). Often sexually selected traits are reduced following parasite infections, which would allow mates to determine the “health” of the potential mate (Zahavi, 1975, Moore, 2002).

1.1.2.2. Immunological mechanisms

Host adaptations to prevent or reduce parasite success have resulted in the wide array of immune responses. There are two types of immune response: innate immunity and adaptive immunity (Kurtz et al., 2004). The innate non specific response can combat a wide range of pathogens and does not require previous exposure to such pathogens, whilst adaptive immunity produces a specific response to a pathogen, such as the production of antibodies (Janeway, 2005). The specific immune response in vertebrates relies on proteins of the major histocompatibility complex (MHC). During an immune response, MHC proteins will bind to foreign peptides activating T lymphocytes leading to the stimulation of B lymphocytes that will produce specific antibodies for the antigen (Kurtz et al., 2004, Janeway, 2005) . The MHC is highly polymorphic producing MHC class I and class II molecules. Paterson et al. (1998) illustrated that in a population of Soay sheep (*Ovis aries*) the presence of certain MHC alleles was associated with survival rates and parasite resistance. Reusch et al. (2001) illustrated that gravid female sticklebacks chose to mate with males with high numbers of MHC class IIB alleles. Therefore it appears that variation in the number and type of MHC molecules due to the heterozygosity at MHC loci (>200 alleles for some MHC loci in humans) and differential expression can determine the range of antigens that can be recognised (Kurtz et al., 2004). Parasites appear to have an important role in maintaining the high polymorphism of the MHC (Paterson et al., 1998, Reusch et al., 2001).

1.1.3. Host behaviour changes associated with infection

If a parasite manages to get past the first line of behavioural defence and the second – the immune response – then it will establish, and changes in the behaviour of infected hosts are often recorded. A behavioural change following infection could be adaptive for the host or the parasite or alternatively can occur as an “evolutionary neutral” side-effect of infection (Robb and Reid, 1996). Poulin (1995) states four criteria that should be used in determining whether a behavioural change is adaptive for host or parasite. Firstly, the behaviour should show a degree of complexity, as simple changes are more likely to be side effects. Secondly, behavioural changes should show a “purposiveness of design”. Thirdly, if changes have evolved independently in different host lineages they are more likely to be adaptive. And finally, behavioural changes should have an associated fitness advantage, for the host or parasite. In the case of the host this could be a reduction in the success of the parasite, whilst in the parasite it could be an increase in its transmission potential.

As described in section 1.1.2.1. host behavioural resistance will only evolve if the parasite has a negative effect on host fitness and the behaviour minimises this effect (Hart, 1994); this is also true of behavioural adaptations following infection. Examples include behavioural fevers and chills, whereby hosts alter their body temperature by selecting ambient temperatures that reduce parasite survival (Moore, 2002) and self medication (also known as “zoopharmacognosy”), in which animals show positive selection for specific non-nutritional dietary components to rid themselves of parasites (Moore, 2002, Huffman, 2010). One example of this is geophagy, in which animals eat soil or soil-like substances such as clay, which are proposed to have a medicinal purpose. Mahaney et al. (1996) illustrated that earth from termite mounds eaten by chimpanzees (*Pan troglodytes*) was composed of substances similar to those used in a

pharmaceutical for diarrhoea treatment. Though there were small sample sizes, the chimpanzees that showed this behaviour were observed to suffer from diarrhoea and parasite infections. It can sometimes be difficult to determine which party benefits from some changes, for example behavioural changes that enhance host survival may also be advantageous to parasites as it increases their survival time in the host (Moore, 2002).

There are several fascinating examples of behavioural changes in hosts that are proposed to increase parasite transmission. One of the most well known examples is the effect of the lancet fluke *Dicrocoelium dentriticum* on the behaviour of its ant intermediate host. Herbivorous mammals are the definitive host of this parasite and ants are not typical prey. At night, infected ants climb to the tops of grass stems and remain there overnight in a move that is expected to increase transmission of the parasite to grass eating animals (Adamo, 2002, Moore, 2002). However due to the complexity of this cycle, experimental evidence demonstrating adaptive manipulation is difficult to gather. This behaviour contrasts strongly with “normal” ant behaviour and is clearly advantageous to the parasite. Thus it is difficult to imagine that they could be side effects of infection; but studies in controlled experimental set ups are needed to provide more firm evidence.

Another example where parasite behavioural manipulation seems to be operating is in insect hosts infected by hairworms (Nematomorpha). Crickets (*Nemobius sylvestris*) infected with the hairworm *Paragordius tricuspidatus* are more likely to enter the water than non infected individuals. On attaining adulthood, worms need to enter an aquatic environment to survive (Thomas et al., 2002a). This behaviour is associated with host death by drowning and as it clearly has a fitness advantage for the parasite, and a “purposiveness” of design this example is often referred to as parasite manipulation (Thomas et al., 2002a, Biron et al., 2005). It is also important to

note that many behavioural changes that are presumed to be advantageous to the parasite in terms of increasing transmission success i.e. increased activity, inhabiting open areas can also increase the temperature of the host i.e. behavioural fever (Moore, 2002). This indicates again the difficulties in understanding whether an adaptation is host- or parasite-mediated.

1.1.4. Effects of parasites on host reproduction

Another change often observed following parasite infection is reduced host reproduction. Parasites utilise nutrients from their host in order to facilitate their own growth and development, and this has the potential to reduce host survival (Hurd, 2001). This will not be advantageous to parasites that rely on host survival for transmission, thus mechanisms that provide energy without reducing host longevity would prove to be advantageous (Ebert and Herre, 1996). One way of doing this is by exploiting host reproductive resources, as reproduction is an energetically expensive process in most species (Poulin, 2007). By reducing host reproduction the parasite can free up energy in a way that does not reduce host survival and may even prolong life or allow hosts to increase in size. Reduced reproduction has been shown to exist in several species following infection (Hurd, 2001, Poulin, 2007). Parasites can directly do this by feeding on the host gonads (Sitja-Bobadilla, 2009) or via more indirect routes such as nutrient theft and endocrine disruption (Trubiroha et al., 2010).

The evolutionary explanations for parasite-reduced host reproductive development are often debated (Poulin, 2007) and typically fall into three broad categories. Firstly, parasites may be capable of manipulating resource allocation to prevent reproduction and release energy for parasite growth or to prolong the survival of their host thus increasing their transmission potential (Ebert et al., 2004, Heins et al., 2004). For example Webb and Hurd (1999) illustrated that the rat tapeworm

Hymenolepis diminuta produces a molecule that reduces synthesis of vitellogenin (VTG) in the fat body of female *Tenebrio molitor* beetles. On the other hand reduced investment in reproduction could be a host adaptation if it reduces the effect of the parasite on the host, for example if the host uses the energy available to increase in size and outlive the parasite (gigantism) (Minchella, 1985, Major et al., 1997). In the *H. diminuta* example, it appears that female hosts also actively reduce reproduction. Haemolymph from infected females can reduce VTG levels in the ovaries of non infected females, whereas haemolymph taken from infected males does not have this effect, suggesting that the substance responsible for reduced VTG production is a host derived molecule produced only in females. Third, reduced host reproduction might benefit neither parasite nor host, and arise as a simple ‘side effect’ of infection (Major et al., 1997, Webb and Hurd, 1999, Hurd, 2001), for example as a result of up-regulated host immune defences (Moret and Schmid-Hempel, 2000) or nutrient theft (Heins et al., 2010a).

1.2. Environmental stress influences host-parasite interactions

The environments inhabited by parasites and their hosts are changing rapidly as a result of global change associated with anthropogenic activities (Poulin, 2007) and these changes are dramatically altering the conditions under which hosts and parasites interact. These alterations could have long term evolutionary effects, for example, if the transmission success of a parasite is increased as a result of the new conditions then this could lead to the selection for higher virulence (Poulin, 2007), since high virulence is typically associated with high transmission (Anderson and May, 1982). Poulin (2007) states that climate change and pollution are the most significant anthropogenic alterations facing natural environments.

1.2.1. Interactions between stressors in perturbed environments

It is increasingly recognised that animals in natural environments are being exposed to a suite of anthropogenic stressors alongside natural stressors such as parasites, predation and competition (Christensen et al., 2006, Coors and De Meester, 2008). Multiple stressors can interact with unpredictable consequences for animal health (Marcogliese et al., 2010). One possibility is that they act **additively**, to produce an outcome equal to the effects of each single stressor, when they are added together. Five estrogenic chemicals when applied alone led to the production of the female egg yolk protein precursor VTG in male fathead minnows, *Pimephales promelas*; and when applied together in a mixture, produced additive effects on the production of VTG (Brian et al., 2005). Alternatively, multiple stressors can have **synergistic** effects, whereby one stressor enhances the effect of another, producing an effect that is bigger than expected (Marcogliese et al., 2010). For example, trematode infections and pesticide exposure have synergistic effects on the proportion of limb deformities observed in wood frogs *Rana sylvatica* (Kiesecker, 2002). Stressors can also act **antagonistically**, such that the effect produced is less than expected. For example, thermal stress reduced cadmium toxicity to juvenile channel catfish *Ictalurus punctatus*, which showed higher survival rates (Perschbacher, 2005, Holmstrup et al., 2010). This was proposed to be due to mechanisms that combat cadmium toxicity being reduced at lower temperatures (i.e. enzyme activity).

Therefore it is clear that studies are required to investigate the effects of different types of multiple stressors. One of the most important types of natural stressors that animals experience are parasite infections. Parasites are ubiquitous in nature, however they are often not considered in studies testing the effects of anthropogenic stress on organisms which was highlighted by Poulin (1992) and

Marcogliese et al. (2010). In this thesis the effects of two types of anthropogenic stressors on a host-parasite interaction have been studied: endocrine disrupting chemical contaminants and temperature change as an outcome of global climate change. The following paragraphs will describe each of these stressors and the problems that are associated with them.

1.2.2. Endocrine disrupting chemicals

A wide range of synthetic chemicals are presently available in an attempt to meet the growing demands of an ever expanding human population (Sumpter, 2009) and these chemicals reach the environment through a variety of routes including air, soil and water (Greytak et al., 2010). These chemicals pose a great risk to living systems once they enter their environment by accumulating in the food chain and interfering with the physiological, immunological and endocrine systems of animals. For example, the anti-inflammatory pharmaceutical drug diclofenac was shown to be involved in severe population declines of the Oriental white backed vulture, *Gyps bengalensis*, in Pakistan, between the years 2000 and 2003 (Oaks et al., 2004). Dead vultures collected at this time displayed renal failure and visceral gout, and residues of diclofenac were discovered in the birds. The association between renal failure and diclofenac residues was highly significant and experimental studies confirmed its renal toxicity. The drug had been given to hoofed animals and consumed by the vultures when they fed on dead livestock.

The aquatic environment is particularly at threat due to the fact that most environmental chemicals eventually reach water bodies as runoff into rivers or atmospheric deposition (Sumpter, 2005). In addition, human populations rely heavily on water for normal day to day tasks and industrial activity, requiring large volumes of water to be recycled (Sumpter, 2009). Sewage treatment plants (STPs) are now utilised

to “clean” used water before it is re-circulated. However with new chemicals continuously appearing, the extent to which STPs can remove them is often unknown, or varies remarkably depending on the processes used at each site (Combalbert and Hernandez-Raquet, 2010). Estimates of the number of anthropogenic chemicals used range from 30 000 to 100 000 (Boxall et al., 2004, Matthiessen and Johnson, 2007, Sumpter, 2009). It is likely that a large majority of these chemicals will arrive at STPs due to the widespread use of water, and very little is known about the amount of these that leave STPs in effluent. In the UK effluent can contribute up to 50% of the water in a river, and this becomes higher when there are low levels of rainfall to dilute it (Routledge et al., 1998). It is well known that many “unnatural” chemicals arrive in rivers due to effluent discharges from STPs (Sumpter, 2009).

A group of chemicals causing environmental concern have been defined as endocrine disrupting chemicals (EDCs) (Colborn et al., 1993). These chemicals interfere with the endocrine system of organisms leading to changes in developmental and reproductive processes (Combalbert and Hernandez-Raquet, 2010). The chemicals can act **agonistically** mimicking hormones, by binding to steroid receptors and causing a response, or **antagonistically**, by binding to a receptor and blocking a response (Matthiessen and Johnson, 2007). Alternatively they can interfere with hormone synthesis and / or metabolism e.g. with hepatic enzymes that are involved in the metabolism of steroids (Guillette and Gunderson, 2001, Sumpter, 2005). The effects of EDCs can be difficult to detect, as although larval or embryonic stages may be sensitive to such chemicals, their effect is often not evident until the animal reaches sexual maturity (Matthiessen and Johnson, 2007). EDCs have important implications for human health. For example, the synthetic estrogen, diethylstilbestrol (DES), was given to pregnant females during the 1940-70s to prevent miscarriages (Soto and

Sonnenschein, 2010). Daughters of mothers who took the drug often had problems with their reproductive organs, immune systems and during pregnancy, and they also had a higher risk of developing cancers associated with reproductive organs (Colborn et al., 1993). More recently, increases in male reproductive problems are being noted and EDCs have been suggested to be involved (Toppari et al., 1996, Skakkebaek, 2004), whilst EDCs such as bisphenol A have also been shown to possess carcinogenic properties (Soto and Sonnenschein, 2010). I will now provide an overview of the three main classes of EDCs that can cause problems in aquatic ecosystems.

1.2.2.1. *Estrogens in aquatic environments*

This group of chemicals has received the most attention and their effects dominate the scientific literature (Jobling et al., 1998, Sumpter, 2005). The feminisation of male wildlife led to the discovery that environmental estrogens existed, and in the United Kingdom this was illustrated by the feminisation of male fish in rivers receiving STP effluent (Purdom et al., 1994). In the USA, the ability of many contaminants to have estrogenic effects on alligators (*Alligator mississippiensis*) raised concern (Guillette et al., 2000). Known endocrine disruptors with estrogenic activity include the natural steroids estrone (E1), 17 β estradiol (E2) and estriol (E3) and synthetic estrogens used in medical drugs, including 17 α ethynylestradiol (EE2) which is the active component in the female contraceptive pill. In addition, man-made chemicals with estrogenic activity known as xenoestrogens (Sumpter, 2005) are present in the environment and include components used in the production of pesticides, plasticisers such as bisphenol A (BPA) and polychlorinated biphenyls (PCBs) (Combalbert and Hernandez-Raquet, 2010). Estrogens produced by plants also exist and are known as phytoestrogens (e.g. the isoflavines, Combalbert and Hernandez-Raquet, 2010).

The persistence of estrogens in treated STP effluent is one of the main reasons for the availability of estrogens in the wild. In living organisms the site of catabolism of estrogens is the liver; here, they are conjugated to sulphate, glucoronide or sulfoglucoronide forms. This increases the solubility of estrogens and allows them to be eliminated in urine. A proportion of estrogens are also excreted in faeces as their free unconjugated forms (Combalbert and Hernandez-Raquet, 2010). Conjugated estrogens have low estrogenic activity compared to the unconjugated free forms. However, intestinal and fecal microorganisms can break down conjugated estrogens into their free forms, and this process also occurs at STPs (Combalbert and Hernandez-Raquet, 2010). The removal of estrogens at STPs depends on the types of treatments in place. Plants equipped with only primary treatments display low hormone removal rates (<14%), whilst secondary treatment (biological or trickling filters) provides mean removal rates of 70% for E2 and 30% for E1. The highest removal rates are achieved using the activated sludge process, with removal rates of 85% for E2 and E3 and >60% for EE2 and E1 (Combalbert and Hernandez-Raquet, 2010). Another source of estrogens is from animal waste; as they also excrete hormones in their faeces and urine. These estrogens will often end up in water due to runoff into rivers / lakes (Combalbert and Hernandez-Raquet, 2010).

1.2.2.2. *Androgens in aquatic environments*

Androgenic chemicals appear to be environmentally available, and also persist in the effluent from STPs. Effluents taken from five STPs with only primary treatment were shown to have elevated levels of androgenic activity. The effluent from one of these sites was studied further to reveal that six natural steroids were responsible for the majority of the androgenic activity (Thomas et al., 2002b). Effluents from pulp and paper mill factories appear to be particularly androgenic. The masculinisation of female

mosquitofish (*Gambusia affinis holbrooki*) was shown to occur at sites downstream of a paper mill in Florida (Parks et al., 2001). This masculinisation can be observed in the anal fin of the fish, which is a trait controlled by androgens. In affected females the anal fin has a larger number of segments and is elongated, resembling a gonopodium - a structure possessed only by male fish, which use it for insemination (Howell et al., 1980, Parks et al., 2001).

Synthetic androgens also exist, and have the potential to cause problems for wildlife. Trenbolone acetate (TBA) is an anabolic steroid fed to cattle in the USA and Canada to promote growth. Internally, TBA is hydrolysed into the active androgen 17 β trenbolone, which is excreted by the animal. 17 β trenbolone appears to be an androgen receptor agonist, binding to the receptor and causing a reaction (Wilson et al., 2002). It is proposed that this chemical will reach water bodies that are located near TBA-fed livestock, potentially causing the masculinisation of wildlife (Wilson et al., 2002, Seki et al., 2006). Another synthetic chemical displaying androgenic activity is perchlorate, which is used in the USA in a range of household and industrial products and in rocket propellant. Recently, testing has revealed its widespread presence in the environment, including its existence in water, milk and vegetables (Bernhardt et al., 2006). Female sticklebacks that were raised through to maturity in perchlorate were masculinised, leading to functional hermaphroditism, whereas exposed males displayed a dose dependent enlargement of their testes (Bernhardt et al., 2006).

1.2.2.3. *Anti-androgens in aquatic environments*

Recently, anti-androgenic chemicals have also been found to exist in the environment. These chemicals are antagonists of the androgen receptor, binding to it and preventing activation (Kelce and Wilson, 1997, Katsiadaki et al., 2006). Anti-androgens have a “demasculinising” effect and examples include some pesticides, fungicides and

plasticizers (Sumpter, 2005, Eustache et al., 2009). The fungicide vincozlin is used on fruit and vegetables and is a known contaminant of the human diet (Eustache et al., 2009) with one study showing that over 80% of a population had been exposed to readable levels of vincozlin and similar pesticides. Lifelong exposure to the estrogen genistein, and vincozlin or to higher doses of vincozlin alone in male rats resulted in abnormalities of the reproductive tract and reduced fertility including changes in sperm production and quality (Eustache et al., 2009).

1.2.3. Elevated temperatures and global climate change

Global climate change is occurring as a result of increased anthropogenic production of greenhouse gases. These gases, mainly CO₂, methane and nitrous oxide, trap thermal energy in the lower atmosphere leading to a warming effect (McMichael et al., 2003, McMichael et al., 2006). Climate scientists now predict that these activities will lead to long term climatic changes worldwide and that a rise in world temperature is occurring at present as a result (IPCC, 2001, McMichael et al., 2003). Models predict that a temperature rise of 3-5°C over the next 100 years will occur in temperate regions (IPCC, 2007); such a change is rapid in terms of natural climatic changes (McMichael et al., 2003). Other changes will occur due to these temperature rises, including a rise in sea levels, changes in precipitation levels and patterns, and an increased frequency of extreme weather events such as floods, droughts and heat waves (Harvell et al., 2002, Patz et al., 2005, McMichael et al., 2006). Changes will not be evenly spread and higher latitudes will experience faster rates of warming (Woodward et al., 2010).

1.2.3.1. *General effects on species and ecosystems*

The effect of temperature on species will depend on their thermal tolerance and how close they are presently to their physiological limit (Deutsch et al., 2008). For example, in European diving beetles (*Deronectes* spp.), species with the highest upper

temperature tolerance (UTT) also had the highest acclimatory ability (Calosi et al., 2008). They therefore predicted that species that have a low UTT are most at risk from warming. In the case of the European diving beetles, these species also had the smallest geographical range; putting them further at risk from warming. Ectotherms are particularly at risk from climate change, as their physiological processes are influenced by the environmental temperature that they are experiencing (Harvell et al., 2002). This is in contrast to endotherms, which possess thermoregulatory equipment that allows them to maintain their body at a constant temperature (Deutsch et al., 2008). The ability for ectotherms to perform a particular function, such as reproduction or growth, over a range of different temperatures is shown by a thermal performance curve (Huey and Stevenson, 1979, Deutsch et al., 2008) (Figure 1.1.). For any given process there will be a minimum and a maximum temperature at which it can function, and an optimal temperature at which the process functions best (Huey and Stevenson, 1979, Deutsch et al., 2008) (Figure 1.1.). Deutsch et al. (2008) illustrated that insects living close to their optimal temperature in the tropics were likely to be most severely affected by warming as a result of their sensitivity to temperature. Whilst species existing at higher latitudes could have their fitness enhanced from warming, as their optimal temperature was higher than what they were currently experiencing.

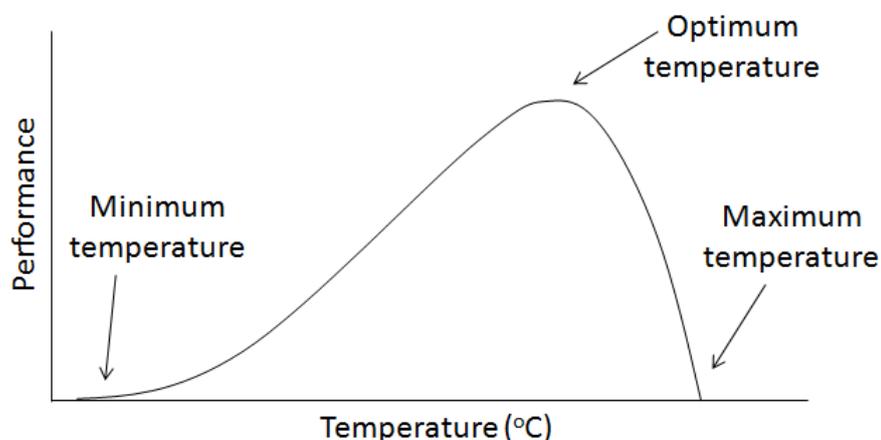


Figure 1.1 The thermal performance curve for a given process (e.g. growth, reproduction) of an ectotherm (Redrawn from Huey and Stevenson, 1979).

1.2.3.2. *Temperature and host-parasite interactions*

Temperature can substantially affect the interaction between a host and its parasite (Thomas and Blanford, 2003). Firstly, it can alter the extent to which the host and parasite come into contact with one another (Harvell et al., 2002, Purse et al., 2005) and secondly, once a host is parasitized it will influence the outcome of this host-parasite interaction. The effect that temperature has on a host-parasite interaction depends on the thermal performance curves of both the parasite and their host (Thomas and Blanford, 2003). If their curves overlap then both groups will perform similarly across a range of temperatures and thus the interaction will remain unaffected. However, if their curves show a degree of separation then temperature can affect the host-parasite interaction. For example, in Figure 1.2., high temperatures increased parasite performance, whilst host performance is reduced, resulting in increased virulence. Conversely at low temperatures host performance is increased and parasite performance is reduced, thus reducing virulence in the same host-parasite interaction. The effects of temperature on parasite virulence may not be linear; for example, high and low temperatures could increase virulence with intermediate temperatures reducing it (Thomas and Blanford, 2003). Environmental warming has been implicated in species extinctions due to the provision of optimal growth conditions for pathogens e.g. the chytrid fungus *Batrachochytrium dendrobatidis* has led to mass extinctions of frog species in the tropics (Pounds et al., 2006).

Environmental temperatures will strongly affect ectotherm-parasite interactions (Harvell et al., 2002, Lazzaro and Little, 2009). **Thermoregulators** are ectotherms that can use behavioural mechanisms to regulate their body temperature by moving to the desired temperature regime. Environmental temperatures will still have an impact here, as they will provide the given set of temperature regimes that can be selected. In these

scenarios there is potential for the host or parasite to use temperature as a “weapon” against the opposite partner. **Thermoconformers** are incapable of controlling their body temperature via behavioural mechanisms and thus tend to track the temperature of the environment. Here, the effect of the environmental temperature on the host will be more straightforward and neither the host nor parasite will be able to use temperature to their advantage (Thomas and Blanford, 2003).

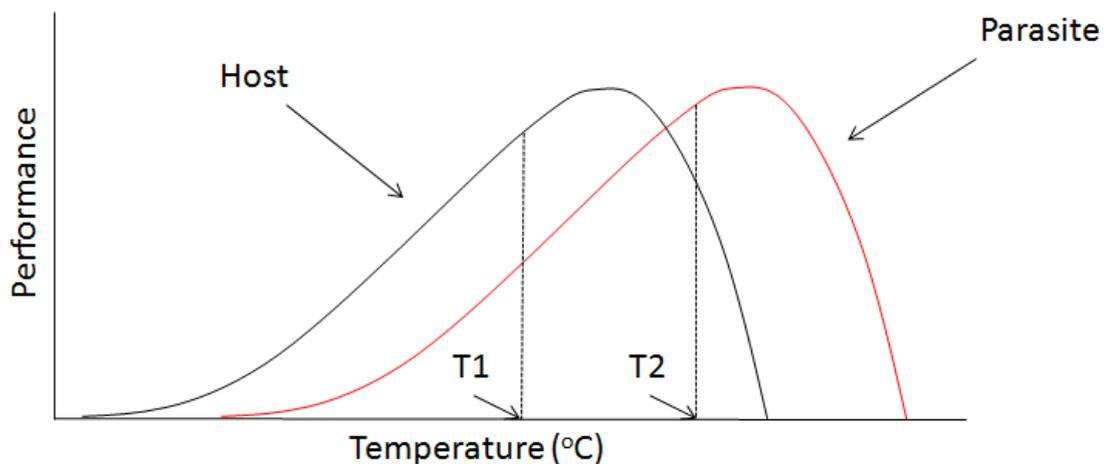


Figure 1.2 The thermal performance curve of a host and its parasite (redrawn from Thomas and Blanford, 2003). At temperature 1 (T1) the host outperforms the parasite, whilst at temperature 2 (T2) the parasite outperforms the host.

As described in section 1.1. parasites and hosts are typically engaged in a long term co-evolutionary “arms race” whereby one party develops an adaptation to outwit the other, causing the other party to develop a counteradaptation. Frequency-dependent selection is thought to maintain this relationship; for example if a host develops an adaptation to reduce the success of the parasite this would spread in a population giving them a fitness advantage (Thomas and Blanford, 2003). This relationship is often described as the “Red Queen” effect and is a “race” against one another to stay in the same place (Blanford et al., 2003). Many studies focus on the genetic variation of host resistance and parasite virulence when investigating co-evolution. However, the environment in which the host and parasite exist can also influence the parasite and host

“race” (Blanford et al., 2003). In the field, it has been harder to illustrate examples of co-evolution, and this may be due to the role of environmental factors (Blanford et al., 2003, Thomas and Blanford, 2003). Temperature had a significant effect on the susceptibility of the pea aphid *Acyrtosiphon pisum* to the fungal pathogen, *Erynia neoaphidis* (Ferrari et al., 2001, Blanford et al., 2003). Temperature changed the ranking of the resistance of the pea aphid clones, for example, one clone that had previously been shown to be resistant to the pathogen became susceptible under a different temperature regime. The authors state that environmental temperature, which can show large fluctuations, may keep the effect of frequency-dependent selection in a wild population at zero. Under different temperature regimes, selection therefore may favour different phenotypes.

1.2.3.3. *Temperature and disease in animals*

One major likely consequence of changing thermal regimes is an increased importance of infectious disease (Pounds et al., 2006), as a result of range expansion of hosts and vectors, increased susceptibility of thermally-stressed hosts and altered seasonal survival of infective stages (Purse et al., 2005). Harvell et al. (2002) state three ways in which climate change could interact with host-pathogen interactions. First, it could lead to increases in pathogen growth, transmission and reproduction. Secondly, it could change susceptibility of hosts to pathogen. Third, as climate change is predicted to increase winter temperatures more than summer temperatures, pathogen survival may increase because cool winter temperatures often limit pathogen survival.

Vector borne diseases are likely to increase as a result of climate change, due to expansion of the range at which vectors can survive (Harvell et al., 2002). For example, the emergence of bluetongue disease in Europe in 1998 was proposed to be due to the expansion of the range of its vectors, biting midges (*Culicoides* spp.), as a result of

increases in temperature and precipitation (Purse et al., 2005). Diseases transmitted by vectors are often limited by the failure of a pathogen to complete development before their vector dies (Harvell et al., 2002). Increased temperatures could therefore lead to faster development of parasites, allowing for the spread of disease. For example, increased temperatures shortened the developmental time of the malaria parasite *Plasmodium mexicanum* within its vector, the sandfly *Lutzomyia vexator* (Fialho and Schall, 1995). Climate change is likely to have a significant effect on parasites with complex life cycles, with free living stages and ectothermic hosts most likely to be affected (Harvell et al., 2002). There is also the potential for opposing effects of temperature change on different parts of the life cycle.

Studer et al. (2010) illustrated the potential for temperature to interfere with host-parasite interactions in the trematode *Maritrema novaezealandensis* and its two intermediate hosts; a snail (*Zeacumantus subcarinatus*) and an amphipod (*Paracalliope novizealandiae*). An increase in temperature (from <20°C to 25°C) led to higher rates of cercarial production from the snail host, and increased their infectivity to amphipods. High temperatures increased parasite development in the amphipod host, however high temperatures also had negative effects on cercarial and amphipod survival, illustrating the complexities in determining the overall effect of temperature on a parasite life cycles.

1.3. The three spined stickleback, *Gasterosteus aculeatus*

In this research the model species that has been used is the three spined stickleback fish, *Gasterosteus aculeatus*. These small teleosts are members of the family Gasterosteidae and are widely distributed across the northern hemisphere (Wootton, 1976, Wootton, 1984). Three spined sticklebacks originated as a marine species with freshwater populations becoming established 8000-10,000 years ago as glacial ice sheets melted

creating freshwater habitats (Bell and Foster, 1994). Since then, freshwater populations have undergone rapid adaptive radiation as they became specialised to their environment. Freshwater populations are highly diverse in terms of morphology and behaviour, making them ideal for use in evolutionary ecology studies (Barber and Nettleship, 2010). Many freshwater populations that are geographically separated show similar phenotypic traits, suggesting that repeated convergent evolution has occurred and that these traits are adaptive for that particular environment (Rundle et al., 2000, Katsiadaki et al., 2007).

Sticklebacks are now commonly used as a model species in a range of different fields including evolutionary ecology, reproductive biology, parasitology, ecotoxicology and molecular genetics. They are a popular model species as they are easily bred by natural or IVF methods (Barber and Arnott, 2000) and their small size make them ideal for housing in large numbers in freshwater aquariums (Barber and Nettleship, 2010). They are widespread across the UK and can be collected in large numbers as they are not considered threatened (Katsiadaki, 2007). They are also a robust species that cope well under laboratory conditions. Importantly, their genome has now been fully sequenced which makes them ideal for molecular genetics studies (Kingsley, 2003).

1.3.1. Reproductive biology

1.3.1.1. *Reproductive behaviour*

The reproductive behaviour of three spined sticklebacks is complex and males exhibit a number of reproductive behaviours and secondary sexual characteristics, which has led to them becoming a popular model species in fish reproductive studies (Katsiadaki et al., 2007). The reproductive season in the stickleback begins in spring as the fish move from deeper water into their shallow water breeding grounds, and the males take up

their territories (Wootton, 1976). The reproductive season extends approximately from late April to July (Östlund-Nilsson, 2007), coinciding with the time period when there is food available of appropriate size and sufficient quantity to sustain the young sticklebacks (Wootton, 1984). The male establishes nuptial colouration which consists of a red throat and fore belly, and the iris develop a bright blue colour, whilst the patterns on the females body become darker (Wootton, 1976). The male stickleback begins to build a nest on a sandy substrate by using filamentous material, transported to the location with his mouth and compacted together to form a tunnel, where the female stickleback can deposit eggs (Van Iersel, 1953). When the male creeps through the tunnel, this signals that the nest is now finished and the male is ready for courting (Wootton, 1976). The nesting materials are held together by a glycoprotein “glue” named spiggin, which is produced in the kidney and whose synthesis is under androgenic hormonal control (Jakobsson et al., 1999).

The male’s nest has been proposed as a sexual signal that females use to assess mate quality. A study by Östlund-Nilsson (2001) on the fifteen spined stickleback, *Spinachia spinachia*, showed that females preferred to mate with males whose nests had more glue in them. The amount of glue present was found to correlate with food intake, and thus could be an “honest” signal of mate quality. A later study by Little et al. (2008) illustrated that the glue secretion produced by male three spined sticklebacks had antimicrobial properties, reducing bacterial and fungal growth and increasing the survival of eggs. It was not determined whether the protein spiggin was responsible for this, or whether another peptide released in the glue was. Therefore, choosing to mate with males with more glue in their nest may increase the chance of producing healthy fry. The nuptial colouration that develops in the male is also a secondary sexual characteristic thought to signal male condition. Colouration is obtained through

carotenoids in their diet (Wedekind et al., 1998) and it has been shown to correlate with a number of factors influencing male fitness (Milinski and Bakker, 1990). Females have been shown to prefer males with brighter nuptial colouration and thus it is thought to signal the males condition (Milinski and Bakker, 1990). Barber et al. (2001) illustrated that maternal half-siblings generated from males with bright colouration were more resistant to parasite infection with *Schistocephalus solidus* compared to those produced from males with duller colouration.

Courtship begins when a gravid female comes into contact with a male, who in response to this stimulus performs a zigzag dance and proceeds to bite various locations of the female's body. He will then return to his territory whilst continuing to exhibit his zigzag display, and insert his head into the nest, often showing fanning behaviour or "sweeps" of the nest (Östlund-Nilsson, 2007). If the female is receptive to courtship she will track the male and assume a head-up pose. Spawning occurs when the female enters the nest, and the male usually continues to bite her tail whilst she deposits her eggs. Immediately following the spawning, the male enters the nest and fertilises the eggs. The male then proceeds to chase the female away (Wootton, 1976). The male is unable to mate with another female during a refractory period following spawning, during which he flattens the eggs into a layer, and reconstructs his nest to repair any damage by the female (Wootton, 1976). The male is capable of mating again approximately an hour after fertilisation, and if successful in courting, clutches will be deposited on the preceding clutch. The courtship phase can occur up to about seven times, after which the male will terminate courtship and switch to the paternal phase (Wootton, 1976).

During the paternal stage, the male is not sexually active, becoming duller in colour and instead participating in parental duties (Östlund-Nilsson, 2007). Parental

care by males has been shown to be energetically expensive (Smith and Wootton, 1999), with males participating in the fanning of their nest to oxygenate eggs as insufficient ventilation will lead to the eggs dying (Wootton, 1976). Fanning behaviour involves the males positioning themselves with their snout facing downwards towards the nest, and using their pectoral fins to produce water currents over the nest. The male also removes dead eggs and those infected with fungus, and repositions eggs that have become displaced from the nest (Wootton, 1976). He will provide protection for his eggs, chasing off potential predators (Östlund-Nilsson, 2007) and when the eggs become metabolically active, males will change the construction of their nest probably to increase ventilation (Wootton, 1984). The eggs take approximately 5-10 d to hatch depending on ambient water temperature (Östlund-Nilsson, 2007).

1.3.1.2. *Hormonal control of reproduction in males*

The major androgen produced by the male gonad is 11-ketoandrostenedione (11KA) and this can be converted to 11-ketotestosterone (11KT) extra-testicularly (Mayer et al., 2004). It has been illustrated that there is a positive correlation between plasma levels of 11KT and the display of courtship behaviours in the male stickleback (Páll et al., 2002a). Páll et al.(2002a) investigated levels of 11KT in males over the nesting cycle, showing a peak in plasma levels of 11KT that coincides with the breeding season, where plasma levels in courting males can be as high as 400ngml^{-1} . However as males move into the paternal phase, plasma levels of 11KT decline drastically to as low as 10ngml^{-1} . Experimental castration of adult males before they enter the nesting cycle results in the complete inhibition of nesting and courtship, whilst subsequent supplementation with 11KA (which is converted into 11KT in the fish) re-establishes both behaviours (Borg, 1987, Mayer and Páll, 2007). In contrast, castration of males already in the process of nesting resulted in courtship behaviour continuing (Páll et al.,

2002b). Thus it is proposed that these androgens are important in instigating the nesting cycle, but their significance declines once they are participating in these behaviours. The reduced levels of 11KT during the paternal phase suggests their role in this stage is small (Mayer and Páll, 2007). This was confirmed in a study by Páll et al. (2002b) in which the nest fanning behaviour of males that had spawned and had subsequently been either: (i) castrated (ii) castrated and given 11KA implants or (iii) sham operated was quantified. No differences in the fanning behaviour of these groups indicated that these behaviours appear to occur irrespective of androgen levels.

In the male stickleback the kidney undergoes substantial hypertrophy during the breeding season and produces the glycoprotein spiggin, a nest building glue (Borg et al., 1993, Jakobsson et al., 1999). Spiggin synthesis is under the control of androgens (notably 11KT); its production can be induced in female sticklebacks after administration of androgens (Matthiessen et al., 2002) and inhibited in breeding males after administration of anti-androgenic chemicals (Sebire et al., 2008, Sebire et al., 2009). The male stickleback becomes ready for reproduction at the beginning of the breeding season in terms of secondary sexual characteristics displayed: nuptial colouration, nest building, kidney hypertrophy and spiggin production (Guderley, 1994).

Gonadotropic hormones (GTHs) are thought to have a considerable role in the reproduction of male three spined sticklebacks (Guderley, 1994). The stickleback has two GTHs that are proposed to have a role in breeding: luteinising hormone (LH) and follicle stimulating hormone (FSH). They are composed of two subunits; a common α -subunit and a unique β subunit (Borg, 2007). The seasonal cycle of the expression of the β -subunits of these two hormones has been studied by Hellqvist et al. (2006). In males, LH- β expression peaked in May and FSH- β expression peaked in January.

Hellqvist et al. (2006) found that spermatogenesis activity occurred predominantly in July to October, when levels of both GTHs were low, along with low levels of androgens. The authors state that the results found in this study are agreeable with other investigations on sticklebacks, but they contrast with other fish, where spermatogenesis is stimulated in part by GTHs promoting androgen production in the testes. Spermatogenesis may still be under GTH control; it could be controlled by low levels of GTHs or it could be stimulated by another GTH that does not promote androgen production (Guderley, 1994).

1.3.1.3. *Hormonal control of reproduction in females*

The female's predominant function is to produce eggs and deposit them in a male's nest (Wootton, 1976). However, there are many processes involved in reaching this endpoint. Initially the germ cells divide to produce oocytes, and these are present in the ovaries at different stages of development in the lead up to spawning, giving the female the capability of multiple spawnings (Guderley, 1994). Oocyte maturation begins in the late summer; however vitellogenesis ("yolking-up") of the egg does not occur till spring time the following year (Wootton, 1976). The yolk precursor, vitellogenin (VTG), is a product of the liver and its production is under estrogenic control (Wootton, 1984). Coinciding with oocyte maturation is the increase in ovary weight, which is highest just prior to spawning (Hellqvist et al., 2006). The female may spawn up to 15 clutches during one breeding season and initiation of spawning is influenced by photoperiod, temperature and food availability (Wootton, 1984). Usually, maturation of oocytes is initiated by long day lengths at high temperatures (Guderley, 1994).

The expression of the β -subunits of LH and FSH have been studied in females as well as males (Hellqvist et al., 2006). Similarly to males, it was found that LH- β expression peaked in May closely mirroring the gonadosomatic index (GSI), whilst

FSH- β expression peaked earlier in January and remained low during the breeding season. GTH activity generally leads to estrogen production in vertebrates, and estrogens in turn stimulate vitellogenesis. In the stickleback, treatment with estrogens does increase vitellogenin concentrations and incorporation of the yolk protein into the oocytes (Ollevier and Covens, 1983). Thus, the patterns of GTH- β expression seems to be similar to other teleosts (Borg, 2007).

1.3.2. Sticklebacks as models in ecotoxicology

Recently the stickleback has become an important species in ecotoxicological studies, particularly in studies investigating endocrine disruption. As a recently-emerging pollutant, model species for toxicity testing of endocrine disruptors are still being developed. Much focus has been placed on the fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), sheephead minnow (*Cyprinodon variegates*), medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Katsiadaki et al., 2007). But these species are not native in Europe, which makes it impossible for lab studies and field studies in this region to be compared. The three spined stickleback has been shown to be suitable for studies of endocrine disruption (Katsiadaki et al., 2002b). It has four important advantages over other species; it is native to northern Europe, it occupies both freshwater and marine environments, it has genetic sex markers (Griffiths et al., 2000), and it has a quantifiable estrogen, androgen and anti-androgen endpoint giving it superiority over other model species that only have estrogen endpoints (Katsiadaki et al., 2002b). The exposure of male sticklebacks to estrogenic chemicals results in the production of VTG, whilst the exposure of female sticklebacks to androgens results in the production of spiggin, both of which can be quantified using an ELISA (Katsiadaki et al., 2002a, Katsiadaki et al., 2002b, Hahlbeck et al., 2004). Using these procedures, a number of androgens have been shown to induce spiggin induction in females including

17 α methyltestosterone and 5 α dihydrotestosterone (Katsiadaki et al., 2002a). The masculinising effect of pulp mill effluent has also been shown to significantly induce spiggin production in female sticklebacks (Katsiadaki et al., 2002a, Katsiadaki et al., 2002b). A test has now been developed by Katsiadaki et al. (2006) to test for anti-androgenic activity in the stickleback by adapting the androgen bioassay whereby the female stickleback is subjected to a moderate concentration of a model androgen, resulting in spiggin production. Alongside this, the hypothesised anti-androgen is administered at a range of concentrations. Thus the anti-androgenic activity of the chemical can be measured and Katsiadaki et al.(2007) describes the test as “robust and reproducible”. However, there are also disadvantages associated with using the stickleback as an ecotoxicological model, such as they do not breed all year round, making it difficult to conduct several studies on eggs and fry. In addition, they are sometimes criticised for not being sensitive to pollution as they are quite a robust species (Katsiadaki et al., 2007).

Several studies have now utilised the stickleback as a model species to investigate chemicals as potential endocrine disruptors. The anti-androgenic activity of flutamide, vinclozolin, linuron and fenitrothion has been shown using the stickleback (Katsiadaki et al., 2006, Sebire et al., 2008, Sebire et al., 2009). In addition, the effects of synthetic estrogens on stickleback reproduction have been investigated. A study by Maunder et al.(2007a) exposed groups of sticklebacks to two concentrations of ethinyl estradiol (EE2); 27.7ngL⁻¹ and 1.75ngL⁻¹ for four weeks following hatching before rearing them in an unpolluted environment. Adult males that were exposed to the higher concentration of EE2 as juveniles exhibited the intersex condition; conversely application of a lower concentration of EE2 did not produce gonadal abnormalities in males. Nesting ability was observed when the males became mature and it was found

that at both concentrations significantly fewer nests were built compared to the control group. Thus it is highlighted that even when gonadal abnormalities do not occur, reproduction can be affected in more subtle ways. Another important reason for using the stickleback as a model species is because they show a range of observable reproductive behaviours that can also be used as endpoints in endocrine disruption studies (Katsiadaki, 2007). Studies have illustrated that exposure to estrogenic chemicals reduces aggressive behaviour (Bell, 2001), increases growth and risky behaviour (Bell, 2004), influences nesting behaviour (Brian et al., 2006), increases the time that it takes for a male to build a nest and reduces paternal care (Wibe et al., 2002).

1.4. The stickleback-*Schistocephalus* model host-parasite system

Parasites are ubiquitous on and in fish, and sticklebacks can become infected with a wide range of parasites. This, alongside the ease at which sticklebacks can be maintained in the laboratory makes them ideal for studying host-parasite interactions from an ecological and evolutionary perspective (Barber, 2007). Sticklebacks have therefore been used in several studies that investigate different areas of parasitology including the MHC complex (Reusch et al., 2001, Wegner et al., 2003, Eizaguirre et al., 2011), sexual selection (Milinski and Bakker, 1990, Barber et al., 2001) and the immunocompetence handicap hypothesis (Kurtz et al., 2007). During this research the cestode parasite *Schistocephalus solidus* was used as a model parasite species.

1.4.1. *Schistocephalus solidus*: life cycle and biology

The cestode *Schistocephalus solidus* has a three host life cycle (Smyth, 1946, Barber and Scharsack, 2010). Parasite eggs hatch in the water releasing free swimming coracidia, and after feeding on these, cyclopoid copepods, the 1st intermediate host, become infected. If infection develops in this host a proceroid will begin to grow in the haemocoel of the copepod. When the proceroid is infective it develops a cercomer, and

sticklebacks feeding on these copepods can now become infected (Smyth, 1969). Once consumed, the infective proceroid sheds its outer layer including its cercomer in the stomach exposing the underlying tegument and microtriches before moving through the intestinal wall to reach the body cavity, where development takes place (Hammerschmidt and Kurtz, 2007). The stickleback is the only obligate host in the parasite's life cycle (Smyth, 1946, Barber and Scharsack, 2010). In this 2nd intermediate host the parasite is referred to as a plerocercoid and here it undergoes most of its development, attaining an advanced stage of sexual development known as "progenesis" (Smyth, 1994, Dörücü et al., 2007).

On reaching approximately 50mg in size the parasite becomes infective to its definitive host, which can be any endothermic vertebrate; however ecological trophic interactions mean that this tends to be a piscivorous bird (Tierney and Crompton, 1992, Barber and Scharsack, 2010). Fish harboring infective parasites show reduced antipredator behaviour that could increase predation susceptibility and may potentially arise from adaptive parasite manipulation (Milinski, 1985, Tierney et al., 1993, Barber et al., 2004). Once in the intestine of its definitive host the parasite begins reproduction within 48h, producing eggs that are released in the birds faeces (Barber and Scharsack, 2010). If these eggs reach a water body, the life cycle is completed. The parasite is hermaphroditic and can produce eggs sexually either by selfing (if only one worm present in the avian intestine) or by outcrossing with another worm (in multiple infections) (Barber and Scharsack, 2010). Outcrossed offspring have been shown to have higher fitness in terms of egg hatchability and infection success (Milinski, 2006). Smyth (1954) developed an *in vitro* method for the culture of plerocercoids dissected from infected sticklebacks, allowing the collection of parasite eggs that can then be incubated and hatched to produce coracidia that can be used to infect copepods.

Copepods exposed to infective coracidia can then be screened under the microscope to identify their infection status before being fed to sticklebacks.

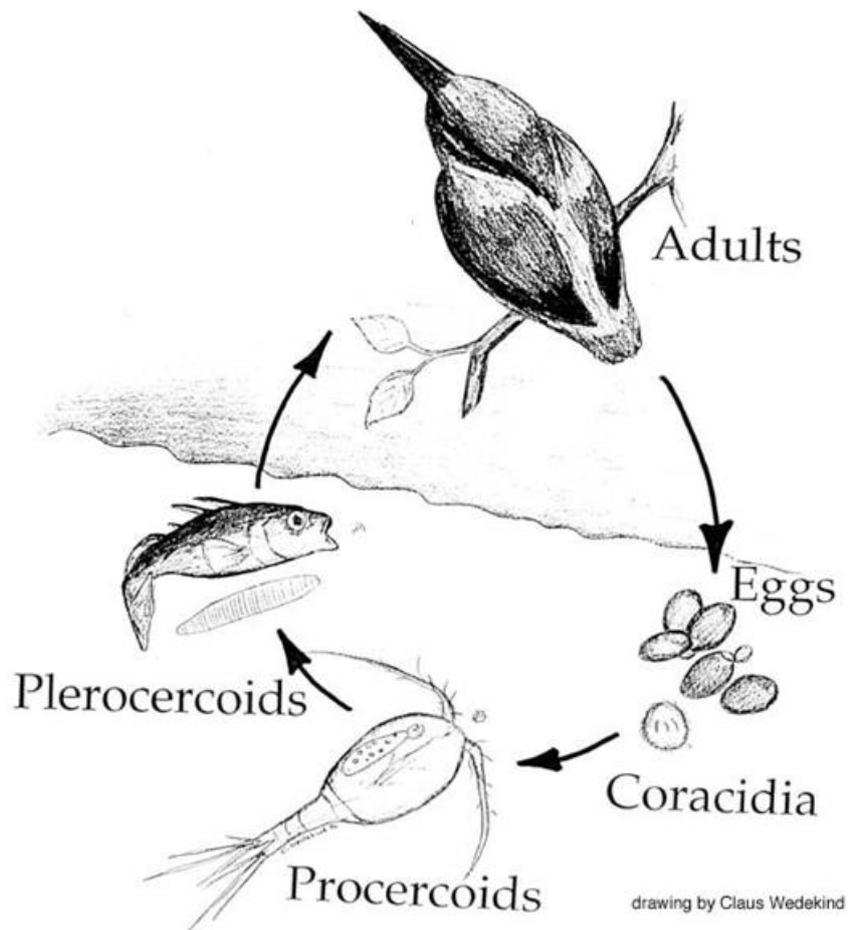


Figure 1.3 The life cycle of *Schistocephalus solidus*

1.4.2. Interactions with stickleback hosts

Sticklebacks can avoid becoming infected by *S. solidus* in three main ways. Firstly, they can employ behavioural defenses as discussed in section 1.1.2.1. However, in terms of the copepod-stickleback interaction this seems to be limited as sticklebacks do not avoid feeding on infected copepods (Wedekind and Milinski, 1996). The second defense mechanism is physical barriers and in the stickleback system is represented by the aggressive environment of the stomach; this defense mechanism appears to be most

effective in removing parasites (Hammerschmidt and Kurtz, 2007). Hammerschmidt and Kurtz (2007) illustrated in an experimental infection study that 50-75% of the parasites failed to pass through the intestinal wall. On reaching the body cavity it seems that the parasite can only be cleared by the host early on infection (Scharsack et al., 2007, Barber and Scharsack, 2010).

Finally, hosts can attempt to target parasites by activating immune responses against them (Barber and Scharsack, 2010). A study by Scharsack et al. (2007) investigated the immune response of experimentally infected sticklebacks. They showed that the respiratory burst reaction was not activated early on in infection, but was upregulated once the parasite became infective to its next host (ie. when it reached 50mg in size; Tierney and Crompton, 1992). The activation of monocytes from the head kidney was demonstrated early on in infection, but in a cyclical manner whereby monocytes were upregulated at 7 days post exposure (dpe), dropping at 17dpe before peaking again at 27dpe and then gradually falling as infection proceeded (Scharsack et al., 2007). This suggests that the parasite is capable of manipulating the host's immune response in order to avoid detection (Barber and Scharsack, 2010). Further evidence for this is provided by a study by Scharsack et al. (2004), in which monocytic leukocytes taken at 45dpe from the head kidney of *S. solidus* infected sticklebacks did not react to *S. solidus* antigens exposed to them *in vitro*, but did respond to non-specific antigens. Adaptive immunity takes 2-3 weeks to develop in fish and is dependent on temperature (Rijkers et al., 1980); it does not appear to be heavily involved in the clearance of *S. solidus* infections (Scharsack et al., 2007, Barber and Scharsack, 2010). However, adaptive immunity has been shown to be involved in disease progression. Kurtz et al. (2004) illustrated that *S. solidus* grew faster in fish that had a low and high number of

MHC class IIB molecules compared to fish that possessed an intermediate number of molecules.

1.5. Aims and Objectives of the Thesis

Anthropogenic stressors pose a significant threat to natural ecosystems worldwide, but it is unclear how these stressors will interact with natural stressors such as parasite infections, to affect wildlife. The overall aim of this research was to investigate the effect of anthropogenic stressors on parasite infection in the stickleback, using the cestode parasite *S. solidus* as an example of a natural stressor. However, before investigating the effects of multiple stressors, it was important to first identify the effect of *S. solidus* on the host stickleback reproductive phenotype. This is dealt with in the first section of the thesis. The main objectives of Chapters 2 and 3 were to firstly identify the physiological basis for reduced reproduction and investigate population differences, and secondly to investigate the adaptive nature (or otherwise) of reduced host reproduction to determine whether it resulted from host and / or parasite adaptations. A third objective was to investigate the effects of experimental *S. solidus* infection on host reproductive phenotypes, in comparison to those observed in the wild.

The second section of this thesis (Chapters 4 – 6) examines the effects of anthropogenic stressors (endocrine disrupting chemical contaminants and elevated environmental temperature, as an outcome of global climate change) on infection susceptibility and disease progression. A second aim was to determine the combined effects of parasite infections and anthropogenic stress on host condition. A final objective, addressed in Chapter 6, was to study the effect of parasite infection on the thermal preference behaviour of *Schistocephalus* infected sticklebacks.

By identifying the effects of anthropogenic stress on disease progression and parasite infection on host reproduction, the likely effects that anthropogenic stress will have on host reproduction can be inferred.

2. The effect of *Schistocephalus solidus* infection on the reproductive behaviour and endocrinology of male sticklebacks



2.1. Introduction

Three spined sticklebacks (*Gasterosteus aculeatus*) infected with plerocercoids of the pseudophyllidean cestode *Schistocephalus solidus* often show reduced sexual development, and the results of a number of influential studies suggest that infected fish are unlikely to engage in reproduction (Arme and Owen, 1967, Pennycuick, 1971, McPhail and Peacock, 1983, Tierney et al., 1996). There is increasing recognition, however, that the effects of *S. solidus* infections on host reproduction may be more variable, and that the classical model of the parasite as an absolute castrator of sticklebacks may not be universally applicable. For example, *S. solidus*-infected females in a number of Alaskan populations are known to develop mature gonads, engage in reproductive behaviour and apparently are capable of spawning despite heavy plerocercoid loads (Heins and Baker, 2003, Schultz et al., 2006, Heins and Baker, 2008). There is also evidence that the effects of *S. solidus* infection on male testicular development are not consistent across infected populations (Heins and Baker, 2008). Since successful reproduction by males requires a complex suite of reproductive behaviours (Wootton, 1976) as well as gonad development, understanding the effects of infection on male reproductive potential is not readily established from post mortem analysis of field samples. Any effects of *S. solidus* infection on these behavioural traits could severely limit reproductive potential, even in the absence of effects on gonad development.

Male sticklebacks show a range of reproductive behaviours including nest-building and courtship, which are under the control of the 11-ketoandrogens, especially 11-ketotestosterone (Borg, 1994, Borg, 2007). Male fish produce a nest building glue, spiggin which is also under the control of androgens that exert their effect on the kidney, which becomes hypertrophied during this process (Borg et al., 1993, Jakobsson

et al., 1999). Males also provide the parental care for embryos and newly hatched fry in this species, participating in nest fanning to ensure the eggs constantly receive a supply of O₂ (Páll et al., 2002b). Thus, reproduction is an energetically demanding process for male sticklebacks (Frischknecht, 1993, Smith and Wootton, 1999) and as a result shortly after completing reproduction most male fish die. Parasites such as *S. solidus*, which compete with their host for energy reserves, could therefore benefit by castrating hosts at an early stage, possibly through endocrine disruption. As 11KT is responsible for reproductive development and initiating reproductive behaviour in males, as well as being energetically expensive (Ros et al., 2004), 11KT synthesis could be a potential target of endocrine disrupting parasites.

The evolutionary explanations for parasite-reduced host reproductive development are often debated and typically fall into three broad categories (Poulin, 2007). First, reduced reproductive effort may be an adaptive host response to infection if it mitigates the effects of parasites; for example, long-lived hosts may forego a reproductive attempt whilst infected in order to maximise their lifetime fitness (Major et al., 1997). Second, parasites might benefit by reducing host sexual development if this maximises their own fitness; for example, non-reproductive hosts may be capable of harbouring more, or larger, parasites, live longer, or contain energy that is more readily accessible (Ebert et al., 2004). Third, reduced host reproduction might benefit neither parasite nor host, and arise as a simple ‘side effect’ of infection (Major et al., 1997, Webb and Hurd, 1999, Hurd, 2001), for example as a result of up-regulated host immune defences (Moret and Schmid-Hempel, 2000). Infection-associated reductions in host reproductive development may therefore benefit parasites, hosts or neither party, and its adaptive function often remains unclear (Lafferty and Kuris, 1999).

In this study we investigated the reduced reproductive capacity of *Schistocephalus*-infected male sticklebacks in two UK populations. We related behavioural and physiological endpoints of male sexual development to circulating levels of 11KT in infected and non-infected male sticklebacks from these populations. We address three main questions: (1) Do population differences exist in the ability of *S. solidus*-infected male *G. aculeatus* to engage in reproductive behaviour, and is this related to body condition indicators in the fish? (2) How is the reproductive status of *S. solidus* infected male sticklebacks from the two populations linked to endocrinological phenotype? (3) To what extent do the data support hypotheses of adaptive and non-adaptive host reproductive disruption by parasites?

2.2. Materials and methods

2.2.1. Fish collection and husbandry

Adult sticklebacks were collected by trapping from two sites with endemic *S. solidus* infection: Victoria Park pond (VP), Leicester (N52°37'16'', W1°07'07'') and Kendoon Loch (KL), Dumfriesshire (N55°11'12'', W4°10'54''). Differences in latitude, elevation and water body size have consequences for the observed timing of breeding season at the two sites (V. Macnab, pers. obs.). To ensure that fish were caught immediately prior to the natural breeding season, VP fish were collected in late February, and KL fish in early May 2008. Fish were returned to the laboratory, where they were held in mixed-sex stock tanks under identical conditions, on a filtered, re-circulating freshwater system and fed daily, *ad libitum* to excess with frozen bloodworms, *Chironomus* sp. larvae. Temperature was maintained at 16.5°C, range $\pm 1.5^\circ\text{C}$ throughout the study, and a photoperiod of 16L: 8D was implemented.

2.2.2. Experimental procedure

Nest building trials began with fish from VP on the 20 March 2008 and with fish from KL on 13 June 2008. Male sticklebacks ($n_{VP} = 21$; $n_{KL} = 19$), recognizable by the development of blue iris colour and red throat colouration, were transferred from stock tanks to individual 14L glass nesting aquaria (36×20×20 cm), each fitted with a sponge airlift biofilter, a plastic plant and a gravel substratum covered in a layer of washed sand. Based on their dorsal profile (Barber and Svensson, 2003) and sexual ornamentation, an equivalent number of putative parasitized and non-parasitized males were selected for the study. Because infected male fish often lack red throat colouration, the presence of the blue iris colour was used as the main selection criteria. On the day after transfer to individual nesting tanks, males were provided with two hundred 70 mm long black polyester threads as nesting material and opaque dividers were placed between adjacent aquaria to prevent visual contact between males. Gravid females were presented to males for a period of 10 min, three times each week. During nesting trials, fish from VP experienced a measured ration of 8–10% body mass d^{-1} , whereas those from KL were fed daily to excess *ad libitum* with frozen bloodworms, *Chironomus* sp. larvae. Despite these differences, there was a minimal effect on the amount of food received by fish from the two populations; this was especially true for infected fish, for which ration was calculated using total mass (i.e. including parasite mass), and uneaten food was regularly removed from the tanks of infected fish from both populations. From this, it can be assumed that the slight differences in the feeding regime did not affect the performance of infected fish in the study. Nesting trials lasted for 35d, during which each fish was scored regularly (at least once a week) for nest development (0–3) and courtship level (0–5) (Rushbrook and Barber, 2006). The maximum nesting and courtship scores attained by each fish during the reproductive

behaviour study were used in the analysis. Fish attaining a nest score of 2 or above (i.e. having a visible nest structure with threads glued into a pit; Rushbrook and Barber, 2006) are subsequently referred to as 'nesters'.

2.2.3. Post mortem analysis

On completion of the nesting trials, fish were killed by exposure to an overdose of Benzocaine anaesthetic (Stock solution: 10g prepared in 1L of 70% EtOH). Individual fish were blotted, measured (standard length, L_S , to 0.1 mm) and weighed (wet mass, M , to 0.001 g). Blood samples, collected in heparinised microhaematocrit capillary tubes following caudal severance, were centrifuged at 11,800 rpm for 5 min (13,000g). The packed cell volume (PCV; the proportion of the blood that red blood cells account for) was taken by measuring the length of the red blood cells in the tube (L_R) and the length of the whole blood sample (L_B). The PCV was then calculated using the equation $PCV=100(L_R/L_B)$. The plasma was then stored at -20°C for future determination of 11KT titres (see below). Systematic dissection then allowed the wet blotted mass of the kidney (M_K), gonads (M_G), liver (M_L) and spleen (M_S) to be recorded (to 0.0001 g). The total mass of plerocercoids recovered from infected fish was quantified (M_P , to 0.001g) and allowed the calculation of parasite index: $I_P = M_P/(M-M_P)$, where $(M-M_P)$ is the weight of infected fish excluding parasite mass. Body condition factor (K) was calculated using the equation: $K= [(M -M_P)L_S^{-3}] \times 10^5$ (Pennycuick, 1971). Hepatosomatic (I_H), spleen (I_S), kidney (I_K) and gonad (I_G) indices were calculated using the formula $I_x = 100[M_x(M -M_P)^{-1}]$, where x is the organ considered. Digital photographs of the throat of each fish, taken under standardized light conditions, were analysed using Adobe Photoshop 5.5 and a redness index (I_R) calculated for each fish (Frischknecht, 1993, Barber et al., 2001).

2.2.4. Quantification of kidney spiggin levels

Kidney spiggin protein was measured using a well-established and validated enzyme linked immunosorbent assay (ELISA) as described previously (Katsiadaki et al., 2002a, Allen et al., 2008) and was undertaken by the Cefas collaborator. Briefly, the kidney of each fish was excised and digested in a strong urea buffer before analysis using a competitive ELISA (explained in more detail in chapter 3 Section 3.2.4., with respect to vitellogenin analysis) with polyclonal anti-spiggin serum. In this assay kidney spiggin levels of up to 200 units/g of body weight are considered as background levels indicating the absence of kidney hypertrophy. An actively breeding male typically has spiggin levels exceeding 100,000 units / g of body weight (Rushbrook and Barber, 2006).

2.2.5. Quantification of 11-ketotestosterone (11KT) levels by radioimmunoassay (RIA)

Thawed plasma samples were centrifuged at 13,000 rpm (1600g) for 60s. 2µl of each sample was added to 30µl of deionised water in a 1.5ml Eppendorf tube, recentrifuged (13,000 rpm; 1600g; 60s) and heated for 15min at 80°C. 220µl of RIA buffer (0.5M phosphate buffer containing 0.2% bovine serum albumen, 0.8% sodium chloride, 0.03% EDTA and 0.01% sodium azide) was added to each sample and the tube vortexed briefly. Nine 11KT standards of known concentrations were added to the assay (ranging from 1-250pg/100µl). The assay also had three controls: a “blank”, containing the radio-labelled antigen (but no antibody added); a “maximum”, containing the antibody and radio-labelled antigen separated by charcoal (which removes any unbound antigen) and thus provides a maximum binding capacity of the antibody (i.e. there is no “cold antigen” present); a “total”, which contained the antibody and radio-labelled antigen but

with no charcoal added this provides a measurement of the total amount of radioactive material present.

To all of the sample tubes (and the controls) 100µl of buffer mix was added, containing buffer, radio-labelled antigen and antibody that bound to it in the absence of a radioinert steroid (note: blanks only had radio-labelled antigen added, no antibody). The buffer mix was produced using a radioactive antigen (here, radio-labelled 11KT) and a known amount of antibody for this antigen. When this mixture was added to the plasma samples, it introduced an unknown amount of “cold” antigen (our sample). This “cold” sample antigen competed with the labelled antigen for binding sites with the antibody. As the concentration of the “cold” antigen increased more of this bound to the antibody displacing the radio-labelled antigen. This reduces the ratio of bound labelled antigen to unbound labelled antigen. The tubes were then left in the fridge overnight to equilibrate.

The following day 1ml of charcoal (0.5M phosphate buffer containing 0.1% gelatine, 0.1% dextran and 0.5% charcoal) was added to all tubes, except the “total” control. The tubes were then centrifuged at 2500rpm (1000g) for 12 min, which resulted in a charcoal pellet containing any unbound antigen, radio labelled or cold, and a supernatant. The supernatant (containing radio-labelled and cold antigen bound to the antibody) was then decanted into scintillation vials, 10ml of scintillation fluid was added, and the tubes were shaken then loaded into the scintillation counter. The radioactivity of the bound mixture was then measured using a scintillation counter (Scott et al., 1980). Higher measurements mean that more of the radio-labelled antigen was bound to the antibody and so indicated lower amount of steroid in the blood sample.

The standards and controls were used to plot a binding curve (Figure 2.1.) where the amount of unlabelled antigen can be measured. The maximum on the graph was calculated by working out the average radioactive counts from the two “maximum” samples and then dividing by the average “total” radioactive counts. This gives the maximum % binding capacity of the antibody (how much of the antigen that the antibody is capable of binding to). The standards were the average of the two radioactive counts divided by the average “total”, indicating how much radio-labelled antigen was bound to the antibody at a known steroid concentration. This was then used to work out the given concentration of the “cold” antigen in our samples using the standard curve. The binding curve had a detection threshold of 1ng.ml^{-1} . In our analysis, samples with 11KT concentrations below the detection threshold were allocated the concentration of 1ng.ml^{-1} ; any effects therefore err on the conservative side.

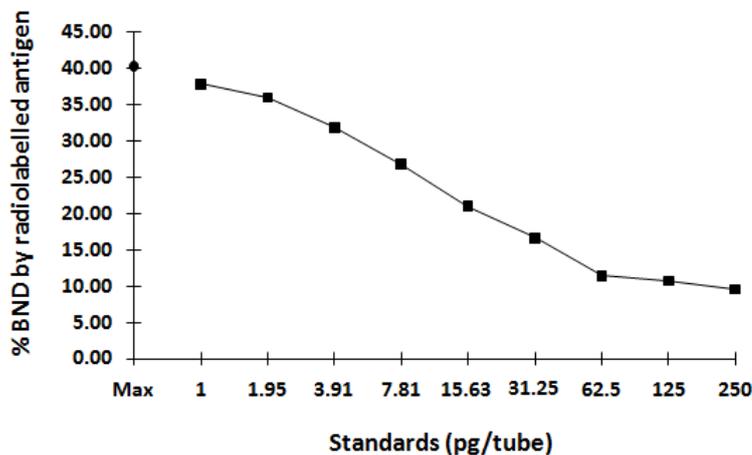


Figure 2.1 A binding curve illustrating the proportion of radio-labelled antigen that was bound to the antibody at known steroid concentrations.

2.2.6. Statistical analysis

Proportional data (K , I_R , I_H , I_S , I_G , I_K , I_P) were arcsine square root transformed and normality was tested using the Kolmogorov-Smirnov statistic. Non parametric data

were normality transformed using logarithm, square root or inverse transformation or it was reflected and then logarithm transformed; if normality was still not achieved non parametric statistical tests were used.

The effect of infection status (infected / non infected) on all the measured variables was tested using t tests or Mann Whitney U tests. Correlations between variables were tested using Pearson's or Spearman's correlation tests. A principal component analysis (PCA), incorporating all quantified traits of condition ($M-M_P$, L_S , M_S , M_L , PCV), was undertaken to generate a single axis of physiological condition among infected fish from both populations. The first emerging axis (PC1) was then used to test for differences between populations in terms of host physiology.

On dissection, only three of the fish from KL were found to be non-infected, precluding the statistical comparison of infection classes in this population. The statistical analysis of KL fish was therefore confined to examining relationships between variables and I_P among infected fish only.

2.3. Results

2.3.1. Infection and nesting and courtship behaviour

Victoria Park population (VP)

Among VP fish, those harbouring *S. solidus* infections showed significantly lower maximum nesting ($W_{8,13}=38.5$, $P < 0.001$) and courtship scores ($W_{8,13}=41$, $P < 0.001$) than non infected conspecifics, with no infected fish attaining a nest development score of >1 or a courtship score of >3 (Figure 2.2.). Neither score correlated with parasite index (I_P) among infected fish from VP ($n=8$; nesting: $r_s=0.394$, $P=0.334$; courtship: $r_s=-0.577$, $P=0.134$; Figure 2.2.).

Kendoon Loch population (KL)

The small number of non infected KL fish meant that behavioural scores of infected and non infected fish could not be compared statistically. Both behaviours, however, were more variable among infected fish from KL, spanning the full range of available scores. Furthermore, among infected fish from KL, I_p correlated negatively with both maximum nest score ($r_s = -0.611$, $n=16$, $P=0.012$) and maximum courtship score ($r_s = -0.523$, $n=16$, $P=0.038$) (Figure 2.2.).

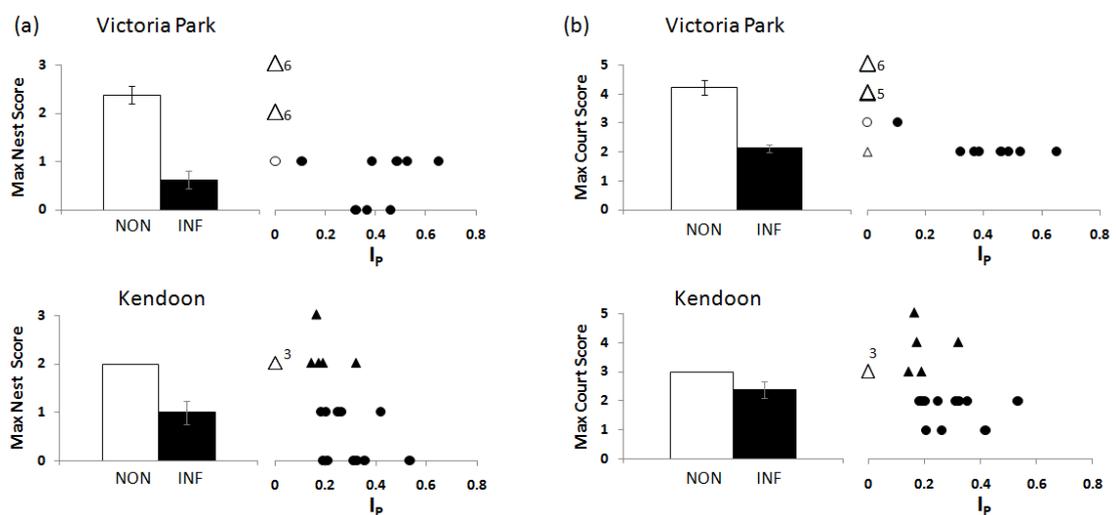


Figure 2.2 The effect of *S. solidus* infection on reproductive behaviour of sticklebacks from two study populations. Histograms of the maximum (a) nesting score and (b) courtship score attained by non infected (open bars) and *Schistocephalus* infected (filled bars) male fish. Bar heights are means \pm s.e. Scatter plots show the relationship between the parasite index (I_p) and the maximum scores achieved among individual fish from each population separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles).

2.3.2. Infection and sexual development

Victoria Park Population (VP)

Infection status had a significant effect on indices of sexual development in VP fish.

Infected fish had significantly reduced I_R , I_G , and I_{KS} (I_R : $t_{19} = -5.8$, $P < 0.001$; I_G : $t_{19} = -2.3$, $P = 0.035$; I_{KS} : $t_{19} = -10.2$, $P < 0.001$; Figure 2.3.a.b.d). The levels of 11-ketotestosterone (11KT) and spiggin were significantly reduced in infected fish (11KT: $W_{8,13} = 36$, $P < 0.001$; spiggin: $W_{8,13} = 36$, $P < 0.001$; Figure 2.3.c.e). Among infected VP fish there

were no correlations between parasite index (I_P) and sexual development measurements (n=8; I_R : $r_s = -0.429$, $P = 0.289$; 11KT: $r_s = 0.245$, $P = 0.558$; I_G : $r = 0.103$, $P = 0.809$; I_K : $r = -0.578$, $P = 0.134$; spiggin: $r = 0.006$, $P = 0.989$; Figure 2.3.).

Kendoon Loch Population (KL)

Again, due to the small sample size of non infected fish from KL, the effect of infection status on sexual development measurements could not be compared statistically.

Typically measurements were more variable in KL compared to VP and spanned the full range of values. Among infected KL fish, significant negative correlations existed between I_P and I_R , I_K , 11KT and spiggin levels (n=16; I_R : $r = -0.597$, $P = 0.015$; I_K : $r = -0.746$, $P = 0.001$; 11KT: $r = -0.618$, $P = 0.011$; spiggin: $r = -0.592$, $P = 0.016$; Figure 2.3.a.c.d.e). Similar to the VP population no correlation existed between I_P and I_G ($r = -0.189$, n=16, $P = 0.483$; Figure 2.3.b).

2.3.3. Relationships between 11-ketotestosterone (11KT) levels, reproductive behaviour and nuptial colouration

An individual male's 11KT level correlated significantly with both his maximum nesting and courtship scores in each population (VP, n=21: Nesting $r_s = 0.844$, $P < 0.001$; Courtship $r_s = 0.843$, $P < 0.001$; KL, n=19: Nesting $r_s = 0.781$, $P < 0.001$; Courtship $r_s = 0.626$, $P = 0.004$; Figure 2.4.a.b). When considering infected males only, there was no correlation between 11KT levels and either nesting or courtship scores in VP males (n=8; Nesting $r_s = 0.194$, $P = 0.646$; Courtship $r_s = -0.283$, $P = 0.496$; Figure 2.4.a.b).

However, among infected KL males, 11KT correlated positively with nesting score ($r_s = 0.705$, n=16, $P = 0.002$; Figure 2.4.a) but not quite significantly with courtship score ($r_s = 0.484$, n=16, $P = 0.058$; Figure 2.4.b).

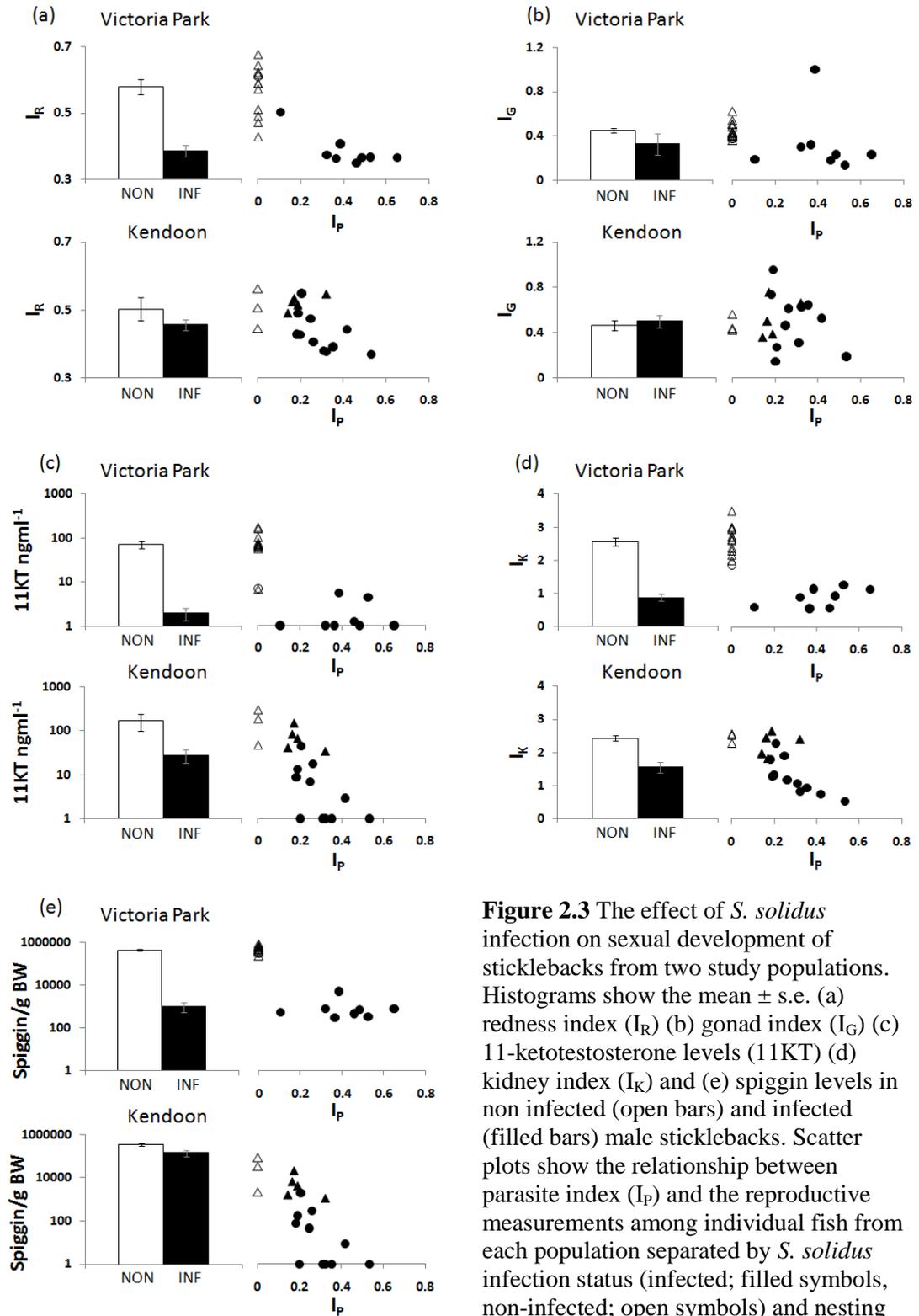


Figure 2.3 The effect of *S. solidus* infection on sexual development of sticklebacks from two study populations. Histograms show the mean \pm s.e. (a) redness index (I_R) (b) gonad index (I_G) (c) 11-ketotestosterone levels (11KT) (d) kidney index (I_K) and (e) spiggin levels in non infected (open bars) and infected (filled bars) male sticklebacks. Scatter plots show the relationship between parasite index (I_P) and the reproductive measurements among individual fish from each population separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles).

A significant positive correlation existed between I_R and 11KT levels in VP fish ($r_s=0.682$, $n=21$, $P=0.001$; Figure 2.4.c) and KL fish ($r=0.826$, $n=19$, $P<0.001$; Figure 2.4.c). This correlation did not exist among infected fish from VP ($r_s=0.109$, $n=8$, $P=0.797$), but remained in KL fish ($r=0.880$, $n=16$, $P<0.001$).

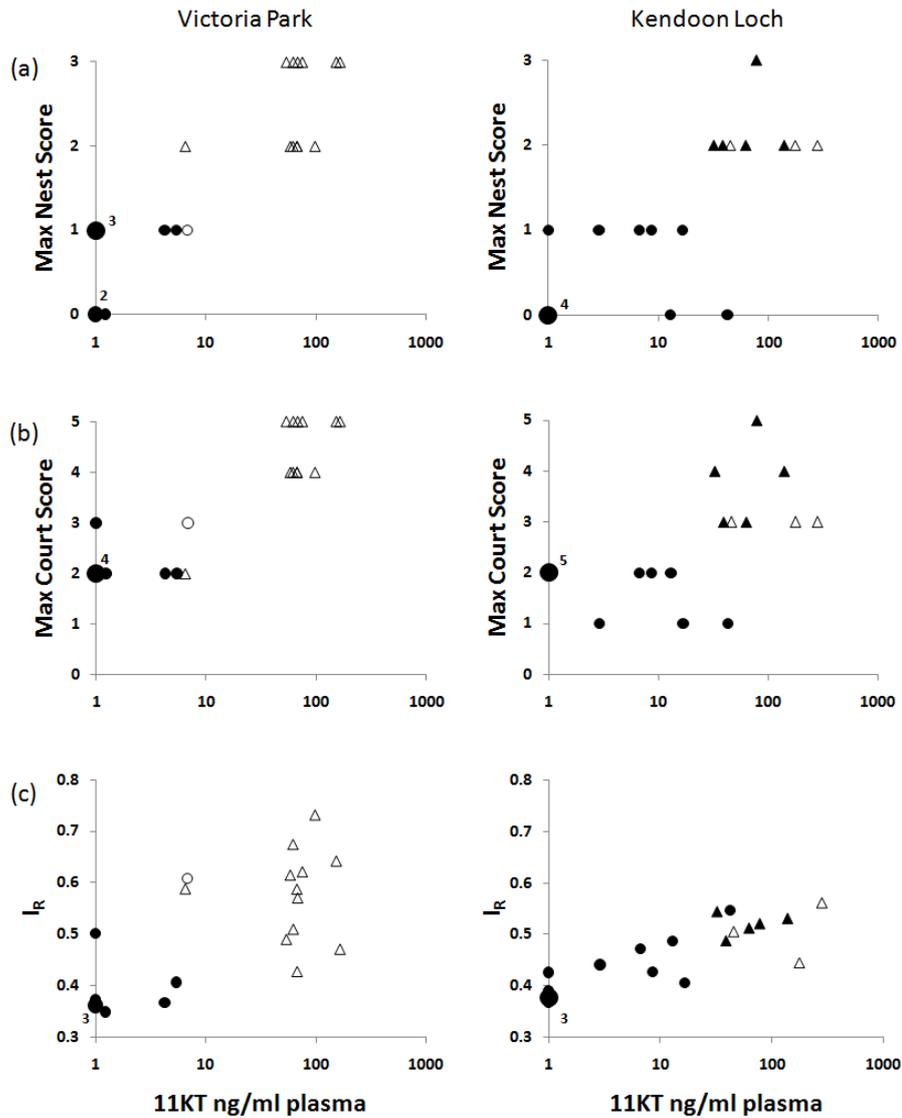


Figure 2.4 The relationship between 11-ketotestosterone (11KT) levels and measures of sexual development and reproductive behaviour in *S. solidus* infected and non infected sticklebacks from the two study populations. The relationship between 11KT levels and (a) the maximum nesting (b) the maximum courtship score and (c) redness index (I_R) achieved by male sticklebacks separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non nesters; circles) in the Victoria Park population and the Kendoon Loch population.

2.3.4. Relationships between spiggin and reproductive behaviour

An individual male's spiggin level correlated significantly with both his maximum nesting and courtship scores in each population (VP, n=21: nesting $r_s = 0.820$, $P < 0.001$; courtship $r_s = 0.764$, $P < 0.001$; KL, n=19: nesting $r_s = 0.614$, $P = 0.005$; courtship $r_s = 0.458$, $P = 0.048$; Figure 2.5.). Amongst infected males from VP, no relationship existed between reproductive behaviour and spiggin levels (n=8; Nesting $r_s = 0.282$, $P = 0.499$; Courtship $r_s = -0.082$, $P = 0.846$; Fig 2.5.a.b). Amongst KL infected males, spiggin levels correlated positively with maximum nesting score ($r_s = 0.569$, n=16, $P = 0.021$; Figure 2.5.a), but was not related to courtship behaviour ($r_s = 0.382$, n=16, $P = 0.144$; Figure 2.5.b).

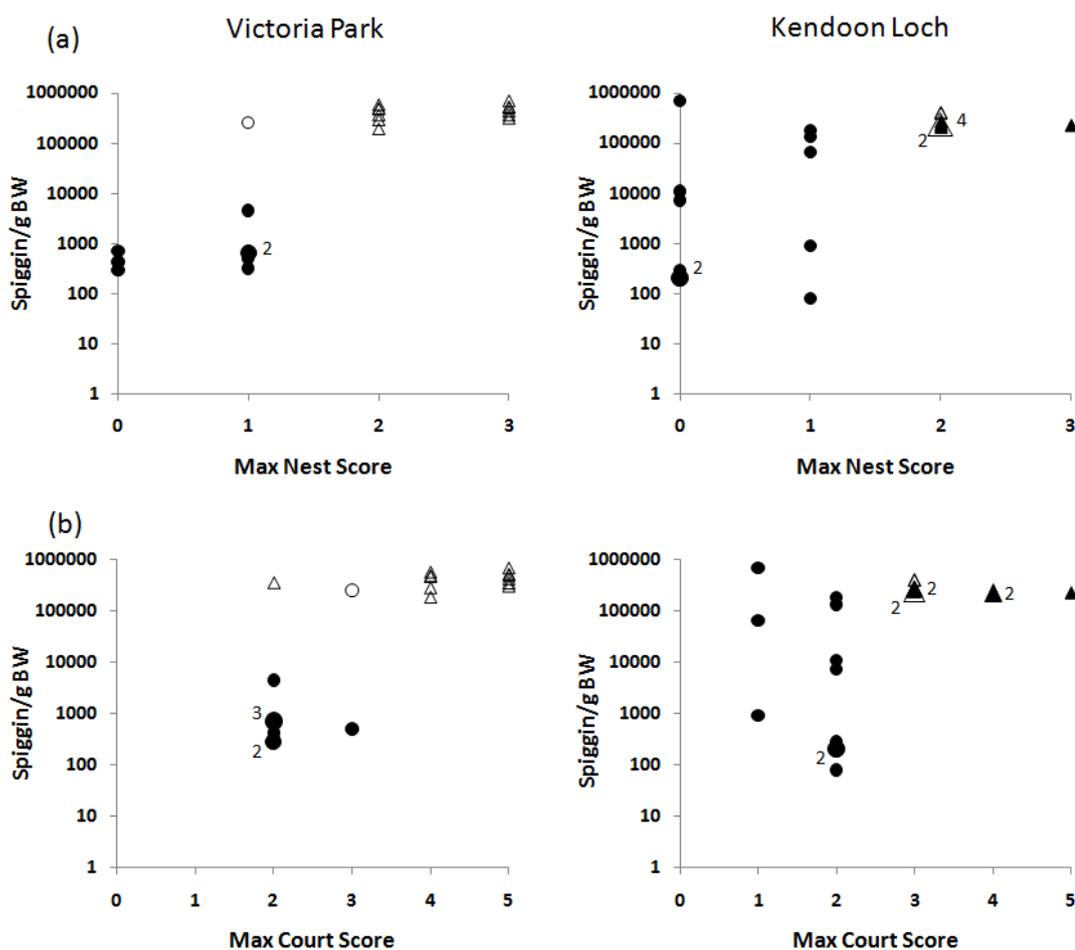


Figure 2.5 The relationship between spiggin levels and reproductive behaviour in *S. solidus* infected and non infected sticklebacks from the two study populations. The relationship between spiggin levels and the maximum (a) nesting and (b) courtship score achieved by male sticklebacks separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non nesters; circles).

2.3.5. Relationships between kidney index (I_K), 11-ketotestosterone (11KT) and spiggin

An individual male's 11KT levels correlated with I_K in both populations (VP; $r_s=0.773$, $n=21$, $P<0.001$; KL $r=0.845$, $n=19$, $P<0.001$; Figure 2.6.a); this relationship did not exist among infected fish from VP ($r_s=0.518$, $n=8$, $P=0.188$) but remained in infected fish from KL ($r=0.823$, $n=16$, $P<0.001$).

Amongst all fish in each population spiggin levels also correlated with I_K (VP; $r_s=0.874$, $n=21$, $p<0.001$; KL; $r=0.774$, $n=19$, $P<0.001$; Figure 2.6.b). The correlation did not exist among infected fish from VP ($r=-0.396$, $n=8$, $P=0.331$) but did among infected KL males ($r=0.864$, $n=16$, $P<0.001$).

A strong correlation existed between 11KT and spiggin titres among the global sample i.e. combining both populations ($r_s=0.785$, $n=40$, $P<0.001$; Figure 2.7.a.b).

11KT and spiggin titres correlated positively among infected KL males ($r=0.761$, $n=16$, $P=0.001$), but not among those from VP ($r_s=0.082$, $n=8$, $P=0.847$).

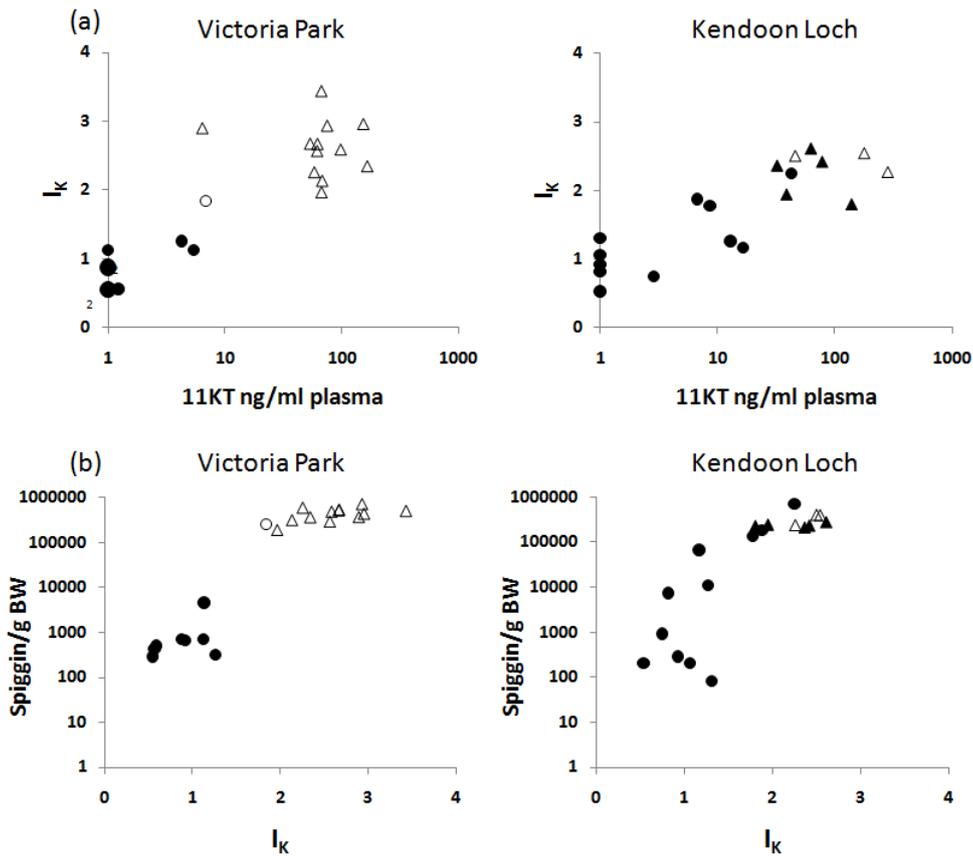


Figure 2.6 Relationships between measurements of sexual development in *S. solidus* infected and non infected sticklebacks from the two study populations. The relationship between (a) 11-ketotestosterone (11KT) levels and kidney index (I_K) and (b) I_K and spiggin levels in the Victoria Park and Kendoon Loch population. Fish are separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles).

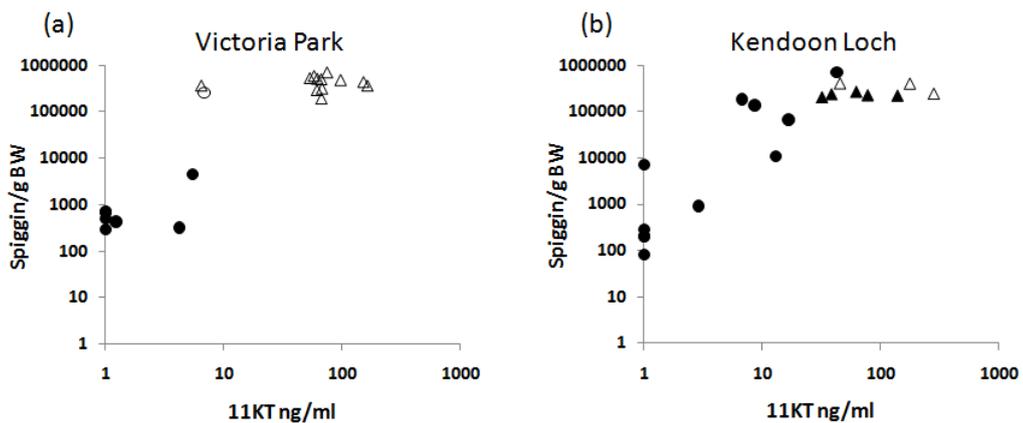


Figure 2.7 The relationship between 11-ketotestosterone (11KT) and spiggin levels in all fish separated by *S. solidus* infection status (infected; filled symbols, non infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles) in (a) the Victoria Park population and (b) Kendoon Loch population.

2.3.6. Relationship between gonad index (I_G) and 11-ketotestosterone (11KT)

Although there was a significant correlation between I_G and 11KT among the VP population ($r_s=0.699$, $n=21$, $P<0.001$) this disappeared when six unresponsive (i.e. 11KT titres <1.25 ng.ml⁻¹) infected males were removed ($r_s=0.325$, $n=15$, $P=0.237$; Figure 2.8.a). Therefore the correlation only existed due to the extreme low levels of 11KT in these six individuals, rather than a true correlation. A relationship did not exist between I_G and 11KT in the KL population ($r=0.144$, $n=19$, $P=0.556$; Fig 2.8.b). There was no significant correlation among infected fish from VP ($r_s=-0.109$, $n=8$, $P=0.797$) or KL ($r=0.224$, $n=16$, $P=0.405$).

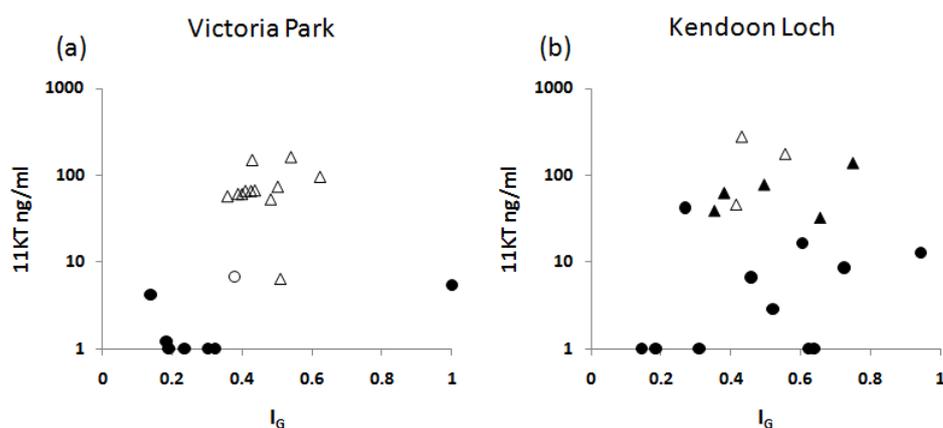


Figure 2.8 The relationship between 11-ketotestosterone (11KT) levels and gonad index (I_G) in all fish separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles) in (a) the Victoria Park population and (b) the Kendoon Loch population.

2.3.7. Infection and body condition

Victoria Park Population (VP)

Infected fish had significantly reduced body condition (K), packed cell volume (PCV) and fish mass ($M-M_P$) compared to non infected conspecifics (K: $t_{19}=-2.94$, $P=0.008$; PCV: $t_{19}=-4.4$, $P<0.001$; $M-M_P$: $t_{19}=-2.8$, $P=0.011$; Figure 2.9.b.d.e). The spleen index of infected fish was significantly enlarged ($t_{19}=3.5$, $P=0.002$; Figure 2.9.c) and no

differences existed between infected and non infected fish with respect to hepatosomatic index (I_H) or fish length (L_S) (I_H : $t_{19}=0.64$, $P=0.531$; L_S : $t_{19}=-1.9$, $P=0.067$; Figure 2.9.a.f.). There were no correlations between I_P and any of the measured variables in infected VP fish ($n=8$; I_H : $r=0.522$, $P=0.185$; K : $r=0.584$, $P=0.128$; I_S : $r=0.523$, $P=0.184$; PCV : $r=0.379$, $P=0.354$; $M-M_P$: $r=0.19$, $P=0.652$; L_S : $r=-0.139$, $P=0.742$; Figure 2.9.).

Kendoon Loch Population (KL)

Differences in condition could not be compared across infection classes in KL, due to a low number of non infected fish. However, significant negative correlations existed between I_P and K and $M-M_P$ ($n=16$; K : $r=-0.698$, $P=0.003$; $M-M_P$: $r=-0.547$, $P=0.028$; Fig 2.9.b.e). There was no relationship between I_P and the other condition variables ($n=16$; I_H : $r=-0.142$, $P=0.599$; PCV : $r=-0.353$, $P=0.18$; L_S : $r=-0.446$, $P=0.084$; I_S : $r=-0.115$, $n=16$, $P=0.672$; Fig 2.9.a.c.d.f).

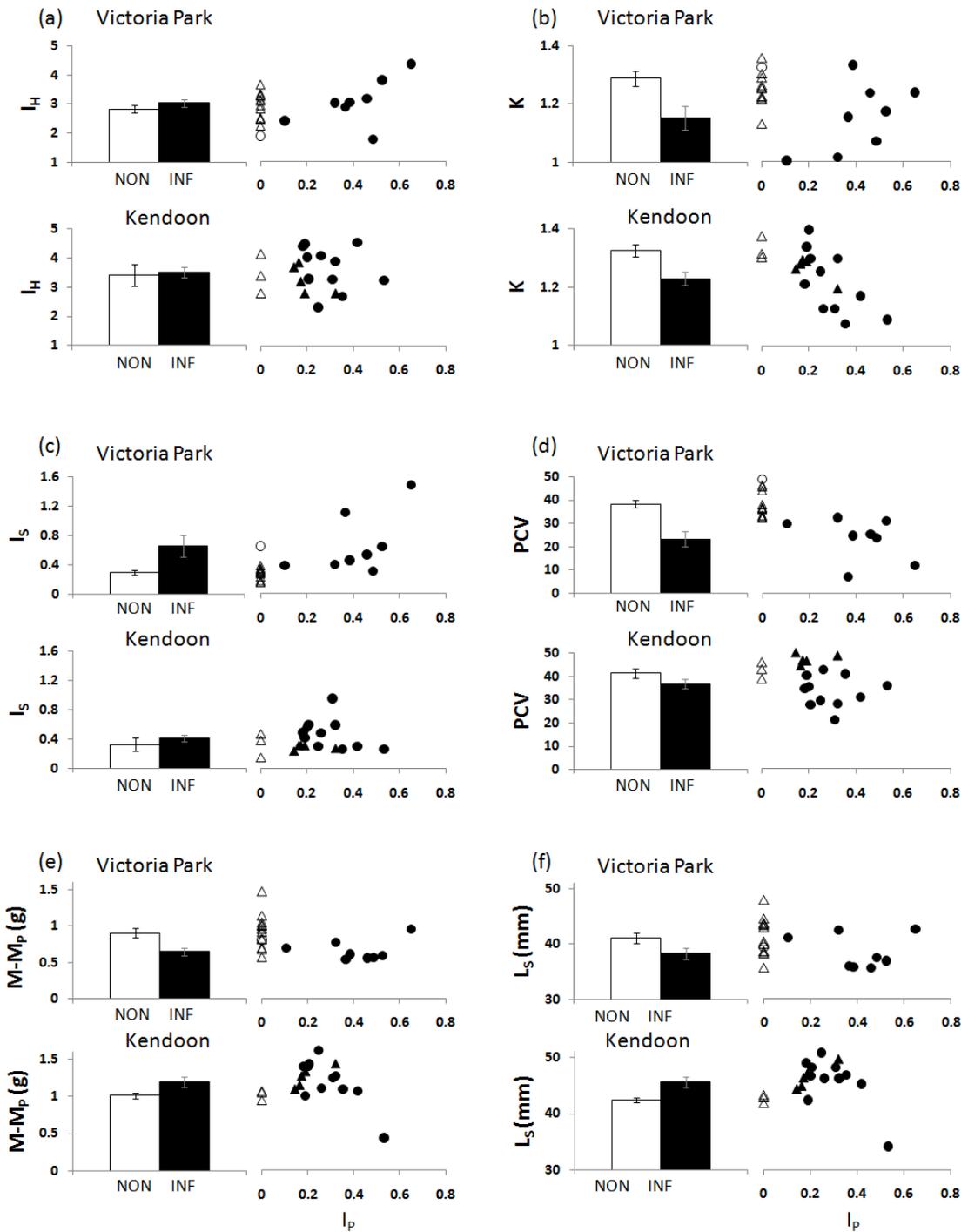


Figure 2.9 The effect of *S. solidus* infection on measurements of body condition in sticklebacks from the two study populations. Histograms show the mean \pm s.e. (a) hepatosomatic index (I_H) (b) body condition factor (K) (c) spleen index (I_S) (d) packed cell volume (PCV) (e) fish mass ($M-M_P$) and (f) fish length (L_S) in non infected (open bars) and infected (filled bars) male sticklebacks. Scatter plots show the relationship between parasite index (I_p) and the body condition measurements among individual fish from each population separated by *S. solidus* infection status (infected; filled symbols, non-infected; open symbols) and nesting status (nesters; triangles, non-nesters; circles).

2.3.8. Relationships between fish mass (M-M_P) and 11-ketotestosterone (11KT)

There was no relationship between M-M_P of the fish and 11KT levels in KL population (KL: $r=0.073$, $n=19$, $P=0.765$) but a significant positive correlation existed between 11KT and M-M_P in the VP population ($r_s=0.584$, $n=21$, $P=0.005$). However, this correlation did not exist among non infected fish ($r_s=0.341$, $n=13$, $P=0.255$) or infected fish when analysed alone ($r_s=-0.191$, $n=8$, $P=0.651$). This suggests that the difference between the infection classes in terms of 11KT and M-M_P created this relationship, rather than a true correlation existing.

2.3.9. Body condition comparisons across both populations

A number of physiological variables were inter-correlated among infected fish from the two populations (Table 2.1.) hence, a principal component analysis was performed to condense this variation into a smaller number of variables. The first principal component (PC1) explained 65% of the total observed variation (Table 2.2.) and was selected as an index of physiological condition. A significant difference was found to exist between PC1 values for infected fish from VP and KL, with VP fish having significantly lower PC1 values ($t_{22}=4.9$, $P<0.001$; Figure 2.10.a). The slopes of the relationship between PC1 and I_P differed significantly between the two populations, with infected fish from VP having a lower PC1 value in relation to I_P across the range of I_P values observed (ANCOVA; $F_{20}=4.9$, $P=0.039$; Figure 2.10.b).

Table 2-1 Correlation matrix showing correlations between indicators of body condition and health status among infected fish grouped together from the two study populations. Variables used were fish mass (M-M_P) and length (L_S), spleen (M_S) and liver (M_L) mass and packed cell volume (PCV). Values in first level are r values, whilst italicised values are P values; significant values are in bold.

	M-M _P	L _S	M _S	M _L *†
L _S	0.965 <i><0.001</i>			
M _S	0.543 <i>0.006</i>	0.514 <i>0.01</i>		
M _L *†	-0.840 <i><0.001</i>	-0.798 <i><0.001</i>	-0.623 <i>0.001</i>	
PCV	0.400 <i>0.053</i>	0.412 <i>0.046</i>	-0.376 <i>0.071</i>	-0.330 <i>0.115</i>

*M_L was reflected before being logarithm transformed so this is why the r values are –ve.

†M_L was approaching normality when transformed; P=0.031

Table 2-2 Results of principal component analyses on various measures of body condition in male sticklebacks infected with *S. solidus*. Variables used were fish mass (M-M_P) and length (L_S), spleen (M_S) and liver (M_L) mass and packed cell volume (PCV). Data from 24 infected males from both populations were used. Loadings, eigenvalues and explained variance are given for the emerging axes.

Variable	PC1	PC2
M-M _P	0.537	0.065
L _S	0.528	0.086
M _S	0.355	-0.626
M _L	-0.513	0.033
PCV	0.207	0.772
Eigenvalue	3.265	1.37
Variance explained	0.653	0.274

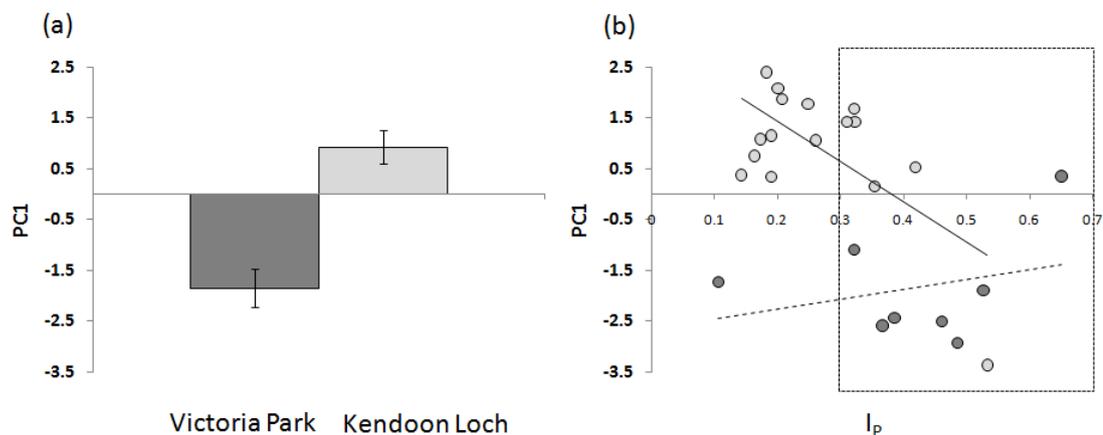


Figure 2.10 Differences in the index of physiological condition in infected sticklebacks across the two study populations, measured using principle component analysis. (a) The mean \pm s.e. PC1 value in fish from Victoria Park (dark grey) and fish from Kendoon Loch (light grey) (b) the relationship between PC1 and parasite index (I_p) in Victoria Park (dark grey) and Kendoon Loch (light grey) fish; the box represents a conservative analysis performed in the discussion.

2.4. Discussion

In this study, male sticklebacks infected with *S. solidus* typically exhibited reduced reproductive behaviour compared with non infected conspecifics; however, the reproductive behaviour and development of infected males differed between host populations. No infected fish from VP built a nest and only the least heavily infected fish in that population exhibited (elementary) courtship behaviour. In contrast, five of 16 infected fish from KL, all with higher I_p (14-32%) than the single courting fish from VP, built nests and exhibited courtship behaviour. VP males, which typically harboured heavy infections, exhibited 11KT titres that never exceeded 6ng.ml^{-1} , compared with non-infected males, which had a mean of 72ng.ml^{-1} . In contrast, the endocrine phenotype of infected males from KL was more variable and closely linked to infection severity. Only the most heavily infected KL males had severely reduced 11KT titres, with some males harbouring smaller infections ($I_p < 19\%$) recording 11KT titres equivalent to non-infected fish.

2.4.1. Are population differences in the condition of infected fish associated with differences in reproductive behaviour?

Population differences in the reproductive behaviour and endocrine status of infected sticklebacks were paralleled by indices of sexual development and health. The intensity of sexual colouration, the relative mass of the kidney and the gonads and the body condition factor were all consistently lower in infected than non infected fish from VP. In contrast, values among infected fish from KL typically spanned the mean values of non infected fish from both populations. Most infected fish from VP had I_s that exceeded the mean values of non-infected fish from both populations. The spleen is an important lymphoid organ in fishes (Kurtz et al., 2007) and its enlargement (splenomegaly) is often associated with immune stimulation (Seppanen et al., 2009).

Hence, population differences in I_S associated with infection may reflect differential responses against infection.

Packed cell volume, the proportion of red blood cells in the blood, is a measure of metabolic condition that indicates the blood's oxygen carrying capacity that is routinely used as an index of fish health (but see Houston, 1997). Typical PCV values for teleost fish are species-specific and range from 23-50% (Moyle and Cech, 2004); non-infected sticklebacks from KL and VP showed PCVs of approximately 40%. PCV was significantly reduced among infected fish from VP with the most heavily infected fish recording PCVs of 7 and 12%. PCV was less severely affected among infected fish from KL, most of which had PCVs $>30\%$. In rainbow trout, *Oncorhynchus mykiss*, which normally have PCVs of 23-33%, fish with PCVs $<22\%$ are classed as anaemic (Gallaughier et al., 1995). Because infected fish are known to have a higher metabolic rate (Arme and Owen, 1967, McPhail and Peacock, 1983, Pennycuik, 1971, Tierney et al., 1996), reduced PCVs are likely to limit metabolic scope in infected sticklebacks. Notably, the five infected fish from KL that exhibited the highest courtship and nesting scores also exhibited the highest PCVs.

Because many of the physiological traits that were quantified co-varied, a multivariate index (PC1) of physiological condition was generated. Infected KL males had significantly higher PC1 scores than those from VP, supporting the hypothesis that KL males apparently experience less severe effects of infection on their physiological condition, possibly permitting their successful reproduction. Infected fish from VP, on the other hand, appeared to be less able to counter the effects of infection.

Two potentially confounding factors limit the ability to draw direct comparisons between infected fish from the two populations. First, during the nesting trials VP fish received a measured ration of 8–10% of their body mass (i.e. 12–38 large *Chironomus*

sp.) daily, whereas fish from KL were fed *ad libitum*; KL fish may therefore have received more food during this period. Both populations, however, were fed *ad libitum* for a month prior to the nesting trials, which should have permitted infected fish to replenish field-depleted energy reserves (Tierney et al., 1996); food was often left uneaten in the nesting tanks of infected fish from both populations, suggesting that even under the lower ration infected fish were receiving sufficient food during nesting trials. Terminal I_H , an indicator of medium-term energetic condition (Chellappa et al., 1995), also did not differ between populations or infection classes. This suggests that both feeding regimes during nesting trials were sufficient to sustain body condition. Furthermore, Candolin & Voigt (2001) documented nesting activity among naturally infected fish when they were fed only five *Chironomus* sp. each day; VP fish were fed substantially more than this. Hence, whilst acknowledging that the population differences in feeding regime were undesirable and have the potential to affect reproductive development and behaviour of infected fish differentially, this is unlikely to be the cause of such changes.

Second, and perhaps more importantly, the distribution of I_p among infected fish differed between populations. Infected fish with $I_p < 30\%$ were frequent among the KL sample, but not among those from VP where only one such fish was tested. This could be important since among parasitized fish from KL, those with smaller I_p typically exhibited the most developed reproductive behaviour and physiology. Hence, the reduced reproductive development and behaviour of infected VP fish might simply reflect a lack of fish with small I_p 's. The slope of the relationship between I_p and the multivariate index of physiological condition (PC1), however, differed statistically between fish from KL and VP, suggesting that differences in the physiology of infected fish from the two sites may not be due solely to population differences in the relative

size of infections. In a conservative analysis, the comparison was limited to infected fish with $I_p > 30\%$ (Figure 2.10.). This also revealed significant differences in the physiology of the most heavily infected fish in the two populations ($t_{12}=-2.9$, $P=0.014$).

If population differences do exist, one possible explanation is that the two populations may differ in their period of evolutionary interaction with the parasite. Both water bodies are man-made; KL was formed after river impoundment in 1935, whereas VP pond was created in the early 1990s. Although the identity of founding populations is unknown, KL is expected to have been colonized from the fish present in the original river. As there are no natural lakes in the region, the VP population is most likely to have been founded from a local stream, where sticklebacks are abundant. Because *S. solidus* is not a characteristic parasite of sticklebacks in flowing waters, the differential effects of infection may result from a longer association time of KL sticklebacks with the parasite. Evidence for such an effect is provided by Kalbe & Kurtz (2006), who showed that sticklebacks from a lake with endemic *Diplostomum pseudospathaceum* infection were better adapted to cope with infections than those from a river, where the parasites do not occur. The reduced I_p of infected fish in the KL population, compared with those from VP, may also support the co-evolutionary time hypothesis. It is also possible, however, that absolute genetic differences between parasite populations, for example, related to parasite serotype, may contribute to the variation in infection phenotypes observed. Alternatively, ecological differences between KL and VP may be important (see chapter 5).

2.4.2. How is the reproductive status of *S. solidus* infected male sticklebacks from the two populations linked to endocrinological phenotype?

Our results show that observed variation in the reproductive potential of *S. solidus* infected male sticklebacks is associated with differences in circulating 11KT. Amongst

all fish, kidney size correlated closely with spiggin and 11KT levels (Borg et al., 1993, Katsiadaki et al., 2002a). We also confirmed previous observations that the level of spiggin in the kidney is closely related to infection level (Rushbrook et al., 2007), being highly divergent between infection classes among VP fish and more variable and dependent on I_p among infected KL males. Both 11KT and spiggin levels correlated closely with nesting and courtship behaviour across all males in the study, but the impact of parasite infection was related to infection severity; whereas heavily infected VP males with significantly reduced 11KT showed no advanced reproductive behaviours (i.e. the phenotype of experimentally castrated fish; Hellqvist et al., 2006), infected KL males with small infections (typically though not exclusively <19%) had 11KT levels similar to non infected conspecifics and built nests and courted females. Thus in our study, whether or not parasitized male sticklebacks engaged in reproductive behavior depended on their level of circulating 11KT. The lack of fish from VP with small infections may explain the uniform effect of *S. solidus* on host reproductive phenotype in that population, though we cannot rule out true population differences occurring as described above. *S. solidus* infections therefore do not appear to be a barrier to male reproduction *per se*, so long as the fish can synthesize sufficient 11KT. Why some infected fish have such low 11KT levels remains unclear. In the case of some non-responsive males from VP, the low observed levels of 11KT may have been due to relatively small testes, but for the rest of the males in both populations there was no strong evidence that testis size plays a role in determining 11KT titres. Hence it seems unlikely that 11KT synthesis was typically limited by testis mass in infected males.

Extreme low 11KT levels could result from an induction of the stress response, leading to the production of cortisol, which potentially interferes with reproduction

(Schreck et al., 2001). Interactions between the immune and endocrine systems are also becoming increasingly recognised (Weyts et al., 1999, Hedger and Meinhardt, 2003), with clear evidence that immune responses can inhibit testosterone production (Lister and Van Der Kraak, 2002, Bornstein et al., 2004, Boonekamp et al., 2008). Lister and Van Der Kraak (2002) demonstrated that murine proinflammatory cytokines (TNF α and IL-1 β) and supernatants from a fish macrophage line were capable of reducing testosterone levels in goldfish (*Carassius auratus*) testis *in vitro*. Invading *S. solidus* trigger an innate immune response, initially involving the production of monocytes at an early stage of infection followed by the respiratory burst reaction later on in infection (Scharsack et al., 2007). In humans, monocytes produce inflammatory cytokines (Neylan et al., 2011); if cytokines are produced by stickleback monocytes during infections then they could potentially have a suppressive effect on 11KT production by hosts. The link between *S. solidus* infection, immunity, stress and sexual development needs further study. Alternatively, it remains a possibility that some unmeasured underlying trait influencing both infection susceptibility and 11KT levels could be responsible for the phenotypic correlations observed. A programme of experimental infection studies is clearly required to tease out the underlying mechanisms here.

2.4.3. Adaptive or non adaptive reproductive disruption by parasites?

Reproductive castration has previously been demonstrated in cyprinid fish infected with *Ligula intestinalis*, a large-bodied pseudophyllidean closely related to *S. solidus* (Arme, 1968, reviewed by Hoole et al., 2010). *Ligula*-infected roach *Rutilus rutilus* have reduced levels of follicle stimulating hormone (FSH β) and luteinizing hormone (LH β) (Carter et al., 2005, Trubiroha et al., 2009), supporting the hypothesis that the parasite interferes with the pituitary-gonadal axis (Arme, 1997). Hecker and Karbe (2005) demonstrated that *Ligula* infected male and female bream (*Abramis brama*) had

significantly reduced circulating levels of 11KT and 17 β estradiol respectively. Their study also investigated testosterone (T) levels in relation to infection status. Although levels of T were significantly reduced in *Ligula*-infected fish, the absolute difference between infection classes – when compared with other sex steroids – was small. T acts as a precursor to 11KT and 17 β estradiol, so high T in combination with low 11KT in infected fish suggests that the parasite interferes with the latter stages of sex steroid synthesis (Hecker and Karbe, 2005, Hecker et al., 2007). Because T increases host appetite, and food conversion efficiency (Woo et al., 1993), such a strategy may be advantageous, as *Ligula* – like *S. solidus* – makes high energetic demands on host fish. The fact that neither I_P nor host condition correlate with reproductive status in infected roach (Trubiroha et al., 2009) also supports the hypothesis that reproduction is not impeded through nutrient theft. A recent study by Trubiroha et al. (2011) showed that housing *Ligula*-infected roach in favourable laboratory conditions attenuated but did not remove the effects of the parasite on the reproductive system, further supporting the view that *Ligula* selectively manipulates host reproductive development by switching it off at an early stage in development.

One prediction of the ‘adaptive manipulation’ hypothesis is that reproductive development should be halted even in hosts harbouring small parasites, since young plerocercoids have most to gain by channelling host energy into parasite growth rather than host reproductive development (Minchella, 1985, Heins et al., 2010a). Conversely, simple ‘nutrient theft’ hypotheses predict that host reproduction should be affected in proportion to worm mass. Are observed patterns of reproductive disruption in *S. solidus* infected sticklebacks consistent with the ‘adaptive manipulation’ hypothesis? Most studies on *S. solidus* female sticklebacks suggest that reproduction is affected proportionally to infection load, i.e. inconsistent with the adaptive manipulation

hypothesis (Heins and Baker, 2003, Schultz et al., 2006, Heins and Baker, 2008, Heins et al., 2010a) (though see Heins and Baker, 2010). The results of our study examining male reproductive development are also inconsistent with the adaptive manipulation hypothesis. Although endocrine phenotypes were substantially altered in the most heavily infected fish, such effects were proportional to I_p and some fish were reproductively active despite harbouring worms of up to 32% of their mass. Furthermore, the relationship between circulating 11KT and kidney spiggin content did not differ between infected and non-infected fish. Spiggin synthesis is expected to be energetically costly (Östlund-Nilsson, 2001) and production of the glue, which is used solely in nest construction, has no obvious benefit to developing parasites. Our data is therefore more consistent with the nutrient theft hypothesis, at least in the population that provided males with a broad range of I_p 's (KL). Although data from VP males appear to be consistent with the 'stepwise' change in reproductive physiology and behaviour expected in selectively manipulated hosts, the lack of fish with small infections in that population prevents statistical comparisons.

2.4.4. Ideas for further work

Experimental infection studies (Barber and Scharsack, 2010) will provide an essential next step in determining population consistency (or otherwise) in the mechanisms underpinning reproductive effects on *S. solidus* on stickleback hosts. Because parasite growth rate in this system is known to be related to host nutrition (Barber, 2005), parental MHC phenotypes (Kurtz et al., 2004), co-evolutionary processes (Kalbe, 2007) and environmental factors such as water temperature (Chapter 5), there is considerable potential for individual and population variation in the effects of infection on host reproduction and population viability. If the effects of *S. solidus* on stickleback reproductive development arise as side effects of a nutritionally demanding infection,

rather than reflecting adaptive parasite manipulation through endocrine disruption, and the degree of disruption depends on parasite size, then future studies should focus on how genetic and environmental factors interact to determine parasite growth rates, reproductive phenotypes of infected fish and subsequent consequences for host population dynamics (Heins et al., 2010b).

3. The effect of *Schistocephalus solidus* infection on the reproductive capacity of wild and laboratory infected sticklebacks



3.1. Introduction

Reduced host reproduction following parasite infection is commonly observed (Hurd, 2001, Minchella, 1985). By diverting host resources away from reproduction, host-derived energy is freed up in a manner that does not reduce host longevity (Hurd, 2001, Lafferty and Kuris, 2009). In the stickleback – *Schistocephalus* system reproduction is an energetically demanding process; males build nests, engage in courtship behaviour and parental care, whilst females produce multiple clutches of energetically expensive eggs throughout the breeding season (Wootton, 1984). Therefore, mechanisms that allow *Schistocephalus* to reduce host reproduction could prove advantageous to the parasite.

Three types of evolutionary explanations are typically used to explain the reduced reproduction that is often associated with parasite infections. Firstly, parasites may be capable of manipulating host resource allocation to prevent reproduction and release energy for parasite growth, or to prolong the survival of their host, thus increasing their transmission potential (Webb and Hurd, 1999, Ebert et al., 2004, Heins et al., 2004). Alternatively reduced reproduction could be a host adaptation if it reduces the effect of the parasite on the host, for example if the host uses the energy available to increase in size and outlive the parasite (i.e. gigantism; Minchella, 1985, Major et al., 1997). Finally, the changes may be non adaptive, and occur as a simple “side effect” of infection (Heins et al., 2010a). Here nutrient theft may be operating, whereby as the parasite grows it requires more nutrients reducing the energy available for reproduction or the immune response that is associated with the parasite infection may reduce the energy available for reproduction (Hurd, 2001).

In chapter 2 the results of studies investigating reproduction in *Schistocephalus* infected wild male sticklebacks were presented. Studies using wild fish are important as

they identify host phenotypes in nature. However, studies using laboratory fish are also extremely useful as they standardise conditions by removing the effects of environmental factors such as food competition and predation risk (Trubiroha et al., 2011). Such studies are possible in the stickleback-*Schistocephalus* system as the life cycle of the parasite can be completed in the laboratory, allowing experimental infections of sticklebacks to be carried out (Smyth, 1946, Barber and Scharsack, 2010). Previous studies have often illustrated differences in results between laboratory and wild studies. Candolin and Voigt (2001) showed that in field studies the proportion of courting males infected with *S. solidus* was significantly lower than those found among shoaling males. Only two infected males courted in the wild, and these were the largest fish with the lowest parasite index, whilst in a laboratory study in which wild fish were brought in to the lab and provided with nesting material, both infected and non infected fish built nests and engaged in courtship behaviour. Another study performed in the *Ligula*-roach (*Rutilus rutilus*) system showed that housing wild *Ligula* infected roach in long term favourable lab conditions did not enable them to reproduce, but did attenuate the parasite's effect (Trubiroha et al., 2011).

Therefore, one of the main aims of the present chapter was to investigate the effect of *S. solidus* on reproduction in experimentally infected male and female sticklebacks. Furthermore, as the effects of *S. solidus* infection on reproduction in wild female sticklebacks had not yet been assessed, another study investigated this. Levels of vitellogenin (VTG) - an egg yolk protein precursor - were assessed in infected laboratory and wild female fish alongside a suite of other reproductive and body condition parameters. Thus four key questions were investigated: 1) Under controlled laboratory experimental infections, how does exposure to *S. solidus* infection interfere with reproduction in male and female sticklebacks 2) How does *S. solidus* infection

interfere with reproduction in wild female sticklebacks 3) How do the results of laboratory and wild studies compare to one another and 4) How do these results support the hypotheses of adaptive and non-adaptive host reproductive disruption by parasites?

3.2. Materials and methods

3.2.1. Wild caught female fish study

Wild fish were collected from Carsington Reservoir (Derbyshire, UK; N53°03'21", W1°37'25") on the 13th April 2010. Suspected infected and non infected female fish (i.e. lacking male sexual ornamentation) were transferred individually to 14L aquaria (36x20x20cm). Each aquarium housed a plastic biofilter and plant and had a gravel and sand substratum. Fish were fed daily, *ad libitum*, to excess with frozen bloodworm (*Chironomus* sp.) larvae and maintained at 16L:8D and 16±1°C. The fish were housed in these laboratory conditions for one month before post mortem analysis. On dissection it was confirmed that 9 *S. solidus* infected and 13 non infected females had been used. During dissections another type of parasite was found alongside *S. solidus*, plerocercoids of *Diphyllbothrium* spp; however these cestodes were extremely small (too small to weigh) and thus were not considered to have important nutritional effects in comparison with *S. solidus*, which were considerably larger and thus considered to be more energetically demanding.

3.2.2. Experimental infection study

Fish supply and husbandry

A total of nine laboratory stickleback broods were produced by IVF (Barber and Arnott, 2000) using wild adults from Carsington Reservoir. They were raised in family groups at 11L:13D and a temperature of 18± 1°C for six months before going through a winter period for three months at 11°C and 8L:16D. Fish were fed *ad libitum* with *Artemia* nauplii and frozen bloodworm (*Chironomus* sp. larvae.). At 9-11 months of age the fish

were exposed to experimental *Schistocephalus solidus* infections before being placed in summer conditions at 16L:8D and 16±1°C.

Experimental infections of copepods and sticklebacks

Single, infective (i.e.>50mg; Tierney and Crompton, 1992); plerocercoids of *S. solidus* were dissected from naturally infected wild sticklebacks (Carsington Reservoir) and cultured singly *in vitro* to produce eggs using a method adapted from Smyth (1946). The plerocercoids were placed in 6.3mm diameter dialysis tubing (Visking, UK), suspended in a glass boiling tube filled with 50ml of horse serum (Sigma UK) and 50ml of RPMI 1640 cell culture medium (Sigma UK). To this mixture, 1ml of penicillin-streptomycin-glutamine solution was added for every 100ml of solution (Thermo Scientific UK). The tubes were placed in a shaking incubator at 40.8°C for 4d. The eggs that were produced by the cestode were then removed and washed with dH₂O. They were stored in a sealed Petri dish, covered in tinfoil and incubated in the dark for 21d. The day before copepods were due to be infected, parasite eggs were removed and exposed to light for 3h. The parasite eggs were then incubated overnight in the dark and exposed to light the next morning to facilitate hatching (Scharsack et al., 2007).

Laboratory reared copepods (*Cyclops strenuus abyssorum*) were exposed to 1-3 hatched coracidia in a drop of water. After visual inspection to identify that the coracidia had been eaten, copepods were kept individually in 5ml of water. At around 14d post exposure (dpe) the copepods were screened to check their infection status and infectivity to sticklebacks, based on the presence of a cercomer (Smyth, 1969). Copepods infected with 1-3 infective proceroids were fed to the fish at 21dpe. The experimental infections took place over three separate days in March 2010.

The fish were starved for 2d prior to experimental infections. Fish were then selected randomly from nine clutches and either sham exposed to a non infected

copepod or exposed to an infected copepod, in small 1.2L aquaria (15.5x9.5x8.5cm). After one hour the aquarium water was inspected visually for the copepod. If the copepod was not located, the water was then sieved through fine mesh (45µm) to determine if the copepod had been eaten. When an infected copepod was recovered, that fish was removed from the study and the copepod was reused with a new fish.

The fish were then placed in “sham exposed” or “exposed” tanks in groups of 10 (on a temperature controlled, filtered re-circulating system) for 2 months. In May, 32 of the exposed fish that appeared to be infected were selected; an attempt was made to try and include equal numbers of suspected male and female fish. Of the sham exposed fish, 24 controls were selected (12 females and 12 males). The fish were transferred into individual plastic aquaria that were split into two sections by a divider with each compartment measuring 17x19x19cm and holding 6L of water. Each tank had a gravel substratum covered with a layer of sand and an airstone. The fish were held individually for a period of 21d before dissection. On dissection it was found that of the 32 exposed fish, 15 were female. Five of these were infected with *S. solidus*, whilst the rest remained non infected (i.e. exposed non infected fish). There were 17 exposed males, of these only three were infected with *S. solidus*.

Nesting Behaviour

All fish were provided with nesting material in the form of two hundred, 70mm black polyester threads and given visual access to gravid females five times per week. Nests were scored every day (ranging from a "0", which represented no nesting behaviour, to a "3", which indicated the completion of a nest; Rushbrook and Barber, 2006). This process was carried out with all fish in case putative females had been sexed incorrectly. The experiment ran for 21d, during which the fish were maintained at

16L:8D and $16\pm 1^\circ\text{C}$ and fed daily to excess, *ad libitum*, with frozen bloodworm (*Chironomus* sp.) larvae.

3.2.3. Post mortem analysis (both studies)

At the end of each study the fish were euthanised with an overdose of Benzocaine (Stock solution: 10g prepared in 1L of 70% EtOH). Blood samples were taken in heparinised microhaematocrit capillary tubes by caudal severance. Samples were centrifuged at 11,800rpm for 5 min (13,000g) to separate the red blood cells and plasma. The packed cell volume (PCV; the proportion of the blood that red blood cells account for) was taken by measuring the length of the red blood cells in the tube (L_R) and the length of the whole blood sample (L_B). The PCV was then calculated using the equation $\text{PCV} = 100(L_R/L_B)$. The plasma samples were then stored in an eppendorf tube for approximately 7 months at -20°C for future vitellogenin (VTG; females) or 11-ketotestosterone (11KT; males) analysis. The fish were then weighed (M) and measured (standard length; L_S). On dissection the mass of the liver (M_L), kidney (M_K ; males only), gonad (M_G), spleen (M_S) and the parasite (M_P) were recorded. The hepatosomatic (I_H), kidney (I_K), gonad (I_G) and spleen (I_S) indices were calculated using the equation $I_X = 100[M_X/(M - M_P)]$, where x is the organ considered. The parasite index (I_P) was calculated using the formula $I_P = M_P/(M - M_P)$, where $(M - M_P)$ is the mass of fish excluding the parasite weight. The body condition factor (K) was calculated using the equation: $K = [(M - M_P)L_S^{-3}] \cdot 10^5$ (Pennycuick, 1971). Digital photographs of the throats of male fish taken under standardized light conditions were analysed using Adobe Photoshop 5.5 and a redness index (I_R) was calculated for each fish (Frischknecht, 1993, Barber et al., 2001).

3.2.4. Vitellogenin (VTG) analysis – ELISA (based on the OECD, 2011 test guidelines)

Vitellogenin levels were quantified using a well-established and validated enzyme linked immunosorbent assay (ELISA). The specificity of the antibodies in the ELISA was tested by I. Katsiadaki (Personal communication; Katsiadaki et al. 2000). Previous tests using 2% BSA as a blocking agent identified that a blocking step was not necessary in the ELISA (Katsiadaki et al., 2002a).

Creating Vtg standards (Day 1) Stickleback VTG standard (in lyophilised form) was dissolved in carbonate buffer and distilled water (50:50 v/v) in a small vial to provide a working standard of 0.5mg/ml (SS). The vial was gently rolled to mix the solution together. Standard 1 (S1; 10µg/ml) was created by adding 100µl of SS to 4.9ml of assay buffer (Washing buffer, 0.1%BSA (w/v), 0.15mM sodium azide) and vortexing.

Standard 2 (S2; 2µg/ml) was produced by adding 1ml of S1 to 4ml of assay buffer.

Adding assay buffer and standards to LPB 96 well plates (Day 1) Plasma samples were thawed and diluted 1:10 with assay buffer; these samples were used in the VTG ELISA. A second dilution was also made at 1:1000 and used in the VTG ELISA in case VTG levels were very high, as the fish were reproductive. 15µl of the samples were added to low protein binding (LPB) (Polypropylene) 96 well microplates. 135µl of assay buffer was then added to all of the wells by reverse pipetting using a 12 channel pipette. The plasma samples underwent a further three serial dilutions on the microplates –giving 1/10, 1/100 and 1/1000 dilutions. 15µl of S1 and S2 were added in duplicate to their allocated wells and mixed with the assay buffer. Serial dilutions of the standards were made as mentioned above.

Incubating samples with 1° antibody (LPB plates) (Day 1) The polyclonal anti-stickleback VTG (raised in rabbit) was diluted with assay buffer to provide a dilution of

1/60,000. 65µl of antisera was then added to all the wells on the 96 well LPB plates. Plates were shaken on a plate shaker for approximately 3 min. Plates were sealed and incubated overnight at 4°C. This allowed for the antibody to bind to any of the VTG in the plasma samples.

Coating high protein binding (HPB) 96 well plates with VTG coating material (Day 1)

The stickleback VTG standard (SS=0.5mg/ml) was diluted with carbonate buffer to provide a dilution of 0.5µg/ml. 100µl of diluted VTG standard was added to each well by reverse pipetting. Plates were sealed stored overnight at 4°C.

Transfer incubations to HPB plates (Day 2) The HPB plates that were coated with VTG material were washed three times with wash buffer (0.1M sodium phosphate (72mM di-basic, 28mM monobasic), 140mM NaCl, 27mM potassium chloride, 0.05% Tween 20 (v/v)) using an automated plate washer; leaving VTG coating the surface of the wells. From the LPB plates 150µl of the sample were transferred to the HPB VTG coated plates. This was done by the use of an 8-channel pipette and starting with the weakest dilution and transferring it to the corresponding wells on the HBP VTG coated plates. This allows the antibody to bind competitively with the sample and the VTG coated wells. Plates were sealed and left to incubate at room temperature for 4-6 hours. If there was a high amount of VTG in the sample, the antibody will have bound more to the sample and less to the VTG coating (or vice versa).

Incubating samples with 2° antibody (Day 2) The HPB plates were washed three times again with wash buffer, this removed the entire bound sample leaving the VTG coated material and any of the 1° antibody that was bound to it. The 2° antibody (alkaline phosphate conjugated anti-rabbit IgG whole molecule; Sigma) was diluted 1/15,000. 150µl were then added to all the wells by reverse pipetting. Plates were sealed and

wrapped in wet paper and incubated overnight at 4°C. This allowed for the 2° antibody to bind to the VTG coating-1° antibody complex.

The addition of a VTG tracer molecule (pNPP Kit) (Day 3) The pNPP kit (Sigma Chemicals, Poole, UK) was removed from freezer, and allowed to warm to room temperature. The HPB plates were washed 3 times with distilled water (NOT wash buffer). A suitable number of pNPP tablets were dissolved in the 0.2M Tris buffer as per manufacturer's instructions. The flask was covered in foil while dissolving as light affects the colour development. 150µl of the tracer molecule, pNPP was added to each well by reverse pipetting. This molecule breaks down and part binds to the antibody complex and the other part turns yellow. Therefore the brighter the sample (a higher optical density) the less VTG there was in the sample as a lot of the antibody was attached to the VTG coating material. The clearer the sample (a lower optical density) the more VTG in the sample, as there is no antibody complex for the tracer to bind to. The colour was allowed to develop until the standards read 2-2.4 optical density (OD). The OD reading was then taken (using an ELISA plate reader) and the concentration of VTG in each sample calculated automatically with the plate reader's software. The VTG standards allow a standard curve to be produced for each plate (See chapter 2 Figure 2.1.). By knowing the concentration that the standards were and measuring the optical density of these, the curve can be used to calculate the concentrations of VTG in the samples. The more accurate reading was used for each sample depending on its position in relation to the standard curve. The most accurate part of the curve is the steepest part. For samples that had low levels of VTG out with the standard curve, they were given the value of the lowest standard – 0.002 – and therefore are on the conservative side. This value was multiplied by the dilution factor (i.e. X10 or X1000) to provide the amount of VTG ug/ml of plasma.

3.2.5. Quantification of kidney spiggin and 11KT levels

Refer to chapter 2 for the spiggin (Section 2.2.4.) and 11KT quantification methods (Section 2.2.5); the spiggin ELISA was carried out by the Cefas collaborator. In this study the nine 11KT standards used ranged from 2-500pg/100µl (different from chapter 2). This meant that a detection threshold of 2ng.ml⁻¹ existed. In our analysis one sample had an 11KT concentration below the detection threshold and was conservatively allocated the concentration of 2ng.ml⁻¹.

3.2.6. Statistical analysis

Proportional data (K, I_R, I_H, I_S, I_G, I_K, I_P) were arcsine square root transformed and tested for normality using the Kolmogorov-Smirnov statistic. Non parametric data was normality transformed using inverse, square root and logarithm transformation or reflected before being logarithm transformed; if normality was still not achieved non parametric statistical tests were used.

Wild caught female fish study

T tests or Mann Whitney U tests investigated the effect of infection status (infected / non infected) on measured variables. A Pearson's or Spearman's correlation was used to identify if correlations existed between variables. Throughout the analysis four samples (two non infected and two infected) were removed from the VTG and PCV analysis due to blood samples being unobtainable.

Experimentally infection study

One way ANOVAs were used to test the effect of exposure status (non exposed / exposed non infected / infected) on reproductive parameters (in males and females) and condition (in females only). A Pearson's or Spearman's correlation test identified correlations between variables and I_P in fish. Regression analysis was used (with larger samples) to test the relationship between reproductive parameters.

In males, differences in the time taken to reach a nest score of 3 between exposed non infected and non exposed fish were tested using a Mann Whitney U test. ANCOVA was used to test the relationship between 11KT and I_R in exposed non infected and non exposed fish. T tests were used to compare body condition parameters in the two groups (exposed non infected / non exposed).

Some fish were excluded from certain parts of the analysis meaning that sample sizes are reduced slightly due to difficulty in getting blood samples and measuring the spleen.

Comparison of lab and wild female studies

T tests or Mann Whitney U tests were used to test the effect of study group (lab or wild) on reproductive and condition parameters. A Pearson's or Spearman's correlation test was then used to identify if correlations existed between parameters.

3.3. Results

3.3.1. Wild caught Carsington Reservoir females

The effect of infection status on sexual development

In comparison to non infected females, *S. solidus* infected females had reduced hepatosomatic indices (I_H ; $t_{20}=5.6$, $P<0.001$; Figure 3.1.a). The I_H has been included here as an index of sexual development rather than condition, as in females this is the site of VTG synthesis before it is transported to the ovaries via the blood (Clemens, 1974, Sumpter and Jobling, 1995). A near to significant negative correlation between I_H and parasite index (I_P) also existed ($r=-0.664$, $n=9$, $P=0.051$; Figure 3.1.a). Infected females had significantly reduced gonad indices (I_G) compared to non infected fish ($t_{20}=6.8$, $P<0.001$; Figure 3.1.b) but there was no correlation between I_G and I_P ($r=0.437$, $n=9$, $P=0.239$). Infected fish had significantly reduced vitellogenin (VTG)

levels ($W_{11,7}=28$, $P<0.001$) and a strong negative correlation existed between VTG levels and I_p ($r=-0.982$, $n=7$, $P<0.001$; Figure 3.1.c).

Amongst all fish (i.e. both infected and non infected) positive correlations existed between VTG levels and both I_H ($r_s=0.725$, $n=18$, $P=0.001$; Figure 3.2.a) and I_G ($r_s=0.666$, $n=18$, $P=0.003$; Figure 3.2.b). However, these correlations were not significant among non infected fish ($n=11$; I_H : $r=0.249$, $P=0.46$; I_G : $r=-0.35$, $P=0.291$), nor did they exist among infected fish alone ($n=7$; I_H : $r=0.635$, $P=0.126$; I_G : $r=0.618$, $P=0.139$), suggesting that the main effect was of being infected or non infected.

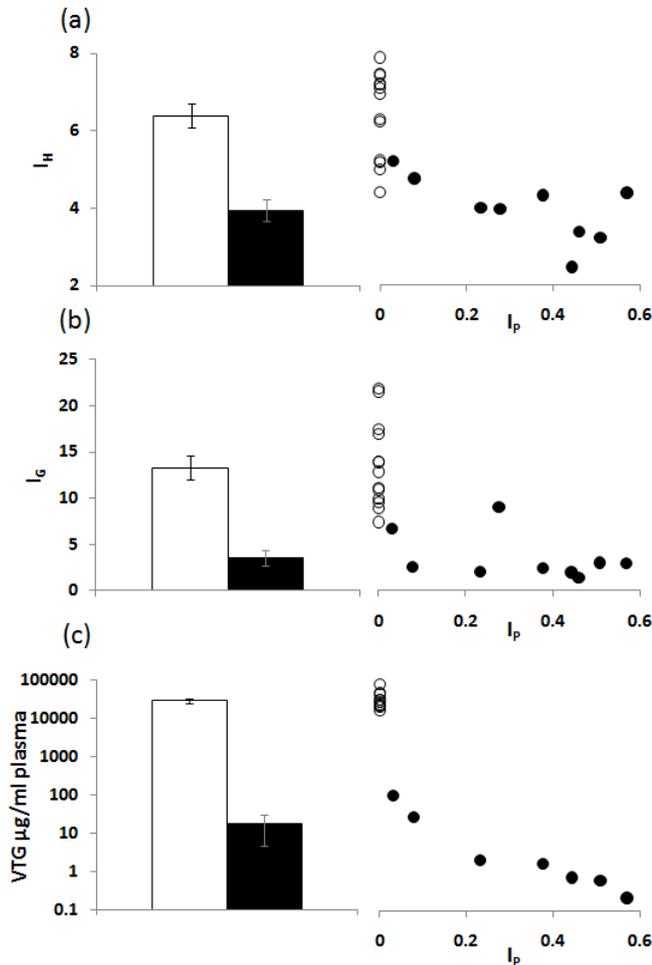


Figure 3.1 The effect of *S. solidus* infection on sexual development in female wild caught sticklebacks from Carsington Reservoir. Histograms show the mean \pm s.e. (a) hepatosomatic index (I_H) (b) gonad index (I_G) and (c) vitellogenin levels (VTG) in non infected (open bars) and infected (filled bars) females. Scatter plots show the relationship between parasite index (I_p) and (a) I_H (b) I_G and (c) VTG levels in non infected (open circles) and infected (filled circles) fish.

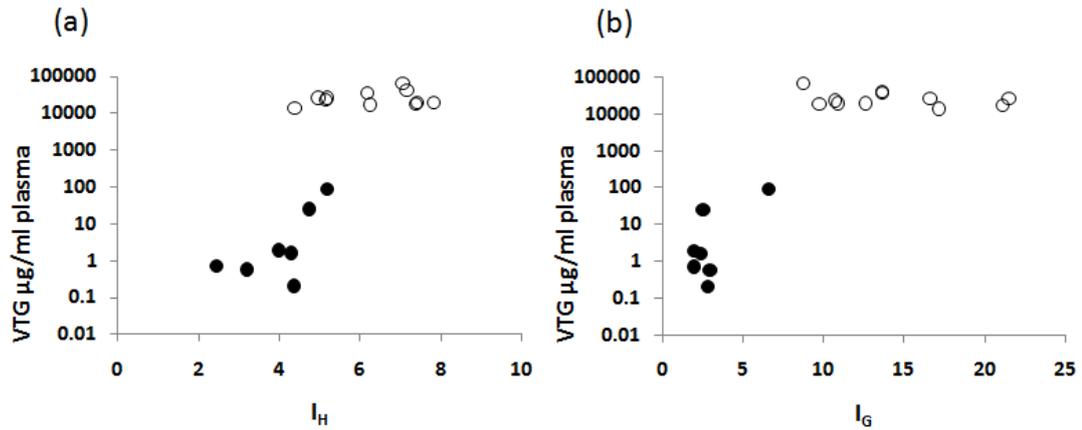


Figure 3.2 Relationships between vitellogenin levels (VTG) and measurements of sexual development in *S. solidus* infected and non infected female wild caught Carsington sticklebacks. The relationship between VTG and (a) hepatosomatic index (I_H) and (b) gonad index (I_G) in non infected (open circles) and infected (filled circles) wild females.

The effect of infection status on body condition

Infected females from Carsington Reservoir were in significantly poorer body condition (K) than non infected fish ($t_{20}=5.7$, $P<0.001$; Figure 3.3.a). The mass (M- M_P) of infected females was reduced compared to non infected females ($t_{20}=2.9$, $P=0.009$; Figure 3.3.b) and this difference remained significant when the gonad mass of each fish was removed from their overall mass ($t_{20}=2.3$, $P=0.031$). whilst there was no significant difference in the length (L_S) of infected and non infected females ($t_{20}=1.8$, $P=0.086$; Figure 3.3.c). Infected fish had significantly larger spleen indices (I_S) compared to non infected conspecifics ($t_{20}=-2.1$, $P=0.046$; Figure 3.3.d) whilst the packed cell volume (PCV) was significantly higher in infected fish ($t_{16}=-3.3$, $P=0.005$; Figure 3.3e). No correlations existed between I_P and K, M- M_P , L_S , I_S and PCV (K: $r_s=-0.500$, $n=9$, $P=0.170$; M- M_P : $r=-0.484$, $n=9$, $P=0.186$; L_S : $r=-0.42$, $n=9$, $P=0.26$; I_S : $r=-0.150$, $n=9$, $P=0.7$; PCV: $r=-0.373$, $n=7$, $P=0.41$).

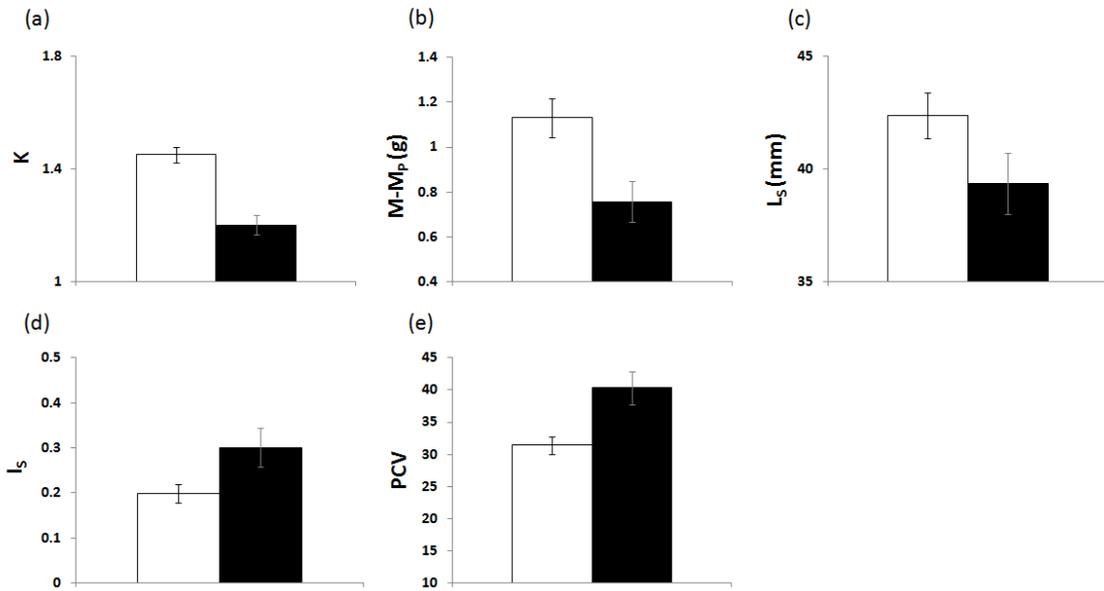


Figure 3.3 Effect of *S. solidus* infection status on indices of body condition in female wild caught Carsington females. The mean \pm s.e. (a) body condition factor (K) (b) fish mass ($M-M_p$) (c) fish length (L_s) (d) spleen index (I_s) and (e) packed cell volume (PCV) in non infected (open bars) and infected (filled bars) wild caught females.

3.3.2. Experimentally infected Carsington Reservoir females

The effect of infection on sexual development

There was a significant effect of exposure status on I_H ($F_{2,24}=7.5$, $P=0.003$; Figure 3.4.a). Infected fish had significantly smaller I_H 's than exposed, non infected ($P=0.003$) and non exposed fish ($P=0.007$). I_G was significantly different across the three exposure classes ($F_{2,24}=15.2$, $P<0.001$; Figure 3.4.b), with infected fish having significantly lower I_G s than exposed non infected ($P<0.001$) and non exposed fish ($P<0.001$). There was a significant effect of exposure status on VTG production ($F_{2,21}=5.8$, $P=0.01$; Figure 3.4.c); variances were not equal ($P=0.041$) therefore a stringent α level of 0.01 was used. Infected fish had significantly reduced VTG levels compared to both exposed non infected ($P=0.048$) and non exposed females ($P=0.008$).

There was no relationship between I_H and VTG among the global sample ($F_{1,22}=1.2$, $r^2=0.053$, $P=0.281$; Figure 3.4.d). There was a significant relationship between I_G and VTG among all fish ($F_{1,22}=5.3$ $r^2=0.194$, $P=0.031$; Figure 3.4.e), but this

relationship did not exist when excluding all infected fish ($F_{1,17}=0.2$, $r^2=0.01$, $P=0.684$).

There were no significant correlations between I_P and I_G , I_H or VTG ($n=5$; I_G : $r=0.167$, $P=0.788$; I_H : $r=0.425$, $P=0.476$; VTG: $r_s=0.447$, $P=0.450$).

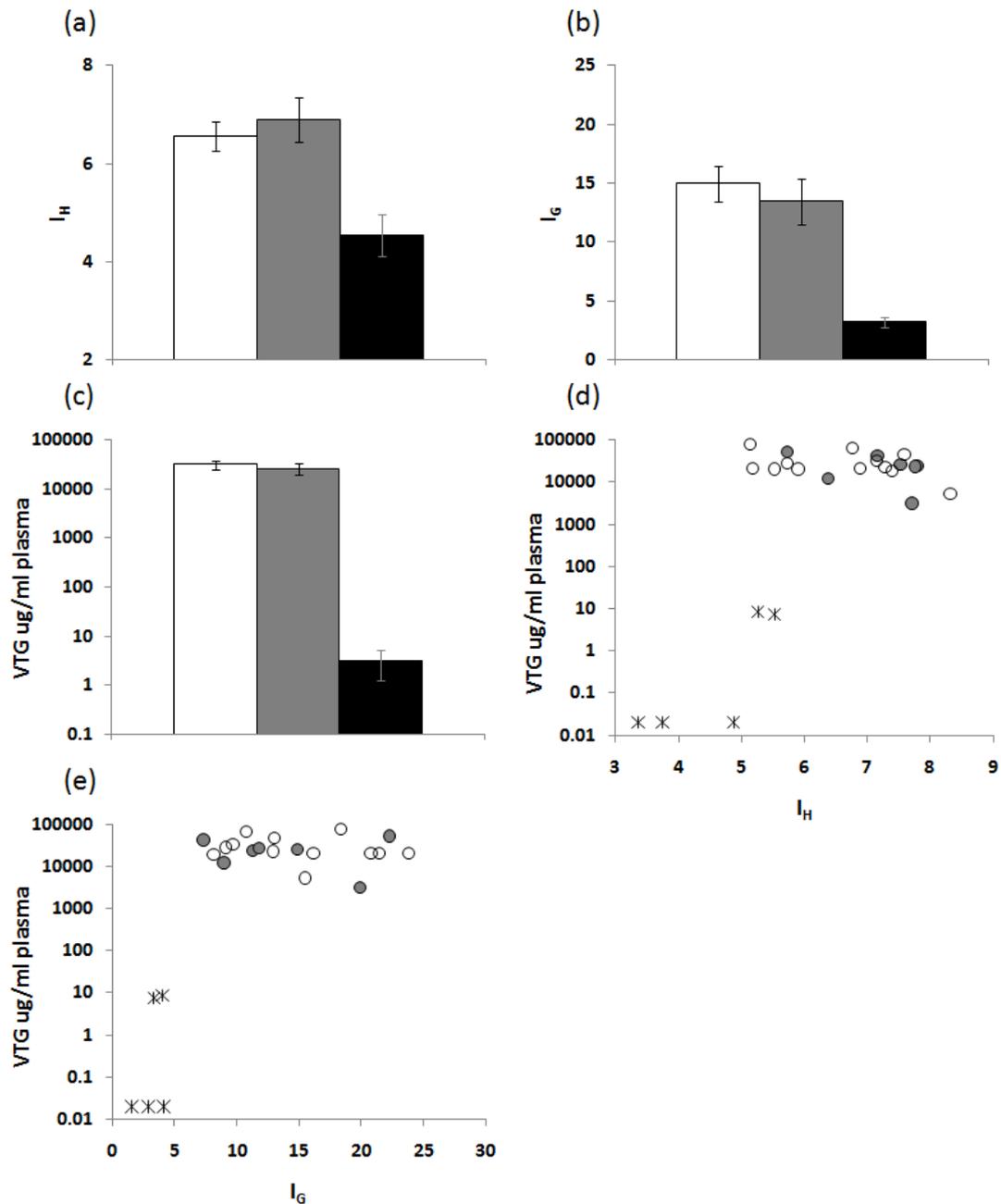


Figure 3.4 The effect of experimental *S. solidus* exposure status on measurements of sexual development in lab raised Caringston females. The mean \pm s.e. (a) hepatosomatic index (I_H) (b) gonad index (I_G) and (c) vitellogenin (VTG) levels in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) lab bred females. The relationship between VTG levels and (d) I_H and (e) I_G in non exposed (open circles), exposed non infected (grey circles) and infected (*) lab bred females.

The effect of infection on body condition

There was a significant effect of exposure status on K ($\chi^2_2=8$, $n=27$, $P=0.018$; Figure 3.5.a). Among fish that had been exposed, infected fish had a lower K than exposed non infected fish ($W_{10,5}=18$, $P=0.005$). There was no significant effect of exposure status on female size (M-M_P: $F_{2,24}=2.5$, $P=0.101$; L_S: $F_{2,24}=2.6$, $P=0.095$; Figure 3.5.b.c).

Exposure status had a significant effect on I_S ($F_{2,19}=19.1$, $P<0.001$; Figure 3.5.d) with infected fish having significantly larger I_S compared to non exposed and exposed non infected fish ($P<0.001$). There was no significant effect of exposure status on PCV ($F_{2,20}=1.9$, $P=0.174$; Figure 3.5.e). There were no correlations between I_P and K, M-M_P, L_S, I_S and PCV ($n=5$; K; $r=0.147$, $P=0.813$; M-M_P; $r=-0.594$, $P=0.29$; L_S; $r=-0.599$, $P=0.286$; I_S; $r=0.261$, $P=0.672$; PCV; $r=-0.721$, $P=0.169$).

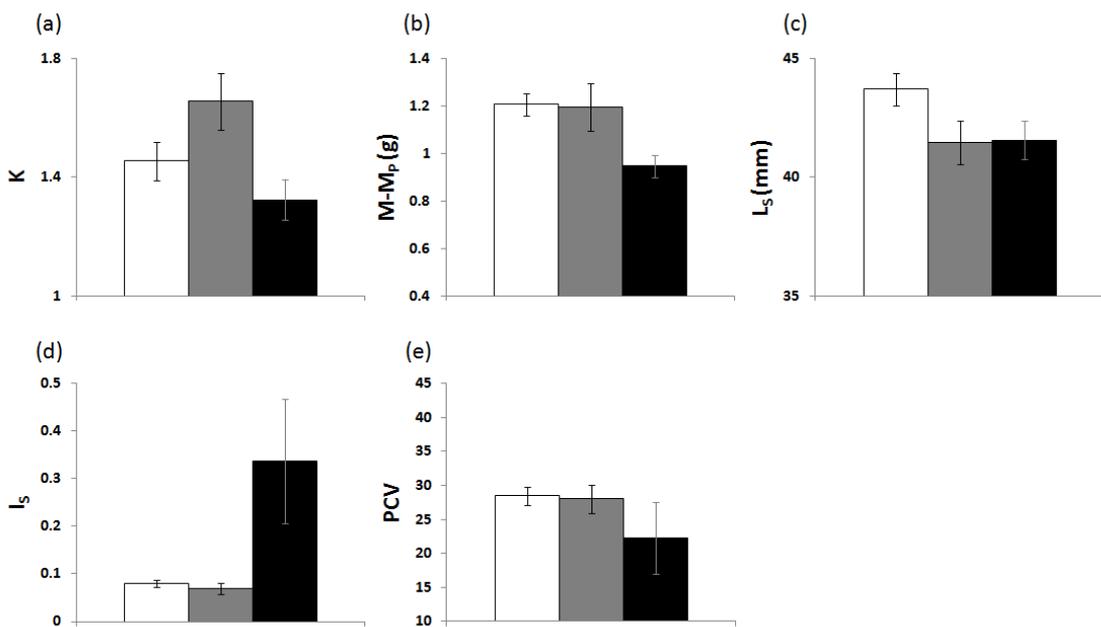


Figure 3.5 The effect of experimental *S. solidus* exposure status on indices of body condition in lab raised Carsington females. The mean \pm s.e. (a) body condition factor (K) (b) fish mass (M-M_P) (c) fish length (L_S) (d) spleen index (I_S) and (e) packed cell volume (PCV) in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) fish.

3.3.3. Comparison of lab bred and wild caught Carsington Reservoir

females

Sexual Development

Combining the female lab and wild fish, a significant positive correlation was found to exist between VTG levels and both I_H ($r_s=0.495$, $n=42$, $P=0.001$; Figure 3.6.a) and I_G ($r_s=0.569$, $n=42$, $P<0.001$; Figure 3.6.b). A significant relationship existed between $M-M_p$ and VTG, with larger females producing higher levels of VTG ($r_s=0.414$, $n=42$, $P=0.006$; Figure 3.6.c.). However this correlation did not occur among non infected fish ($r_s=-0.055$, $n=30$, $P=0.772$) or infected fish alone ($r=-0.153$, $n=12$, $P=0.634$) suggesting the differences were due to small body size and low VTG levels of infected fish rather than fish mass alone.

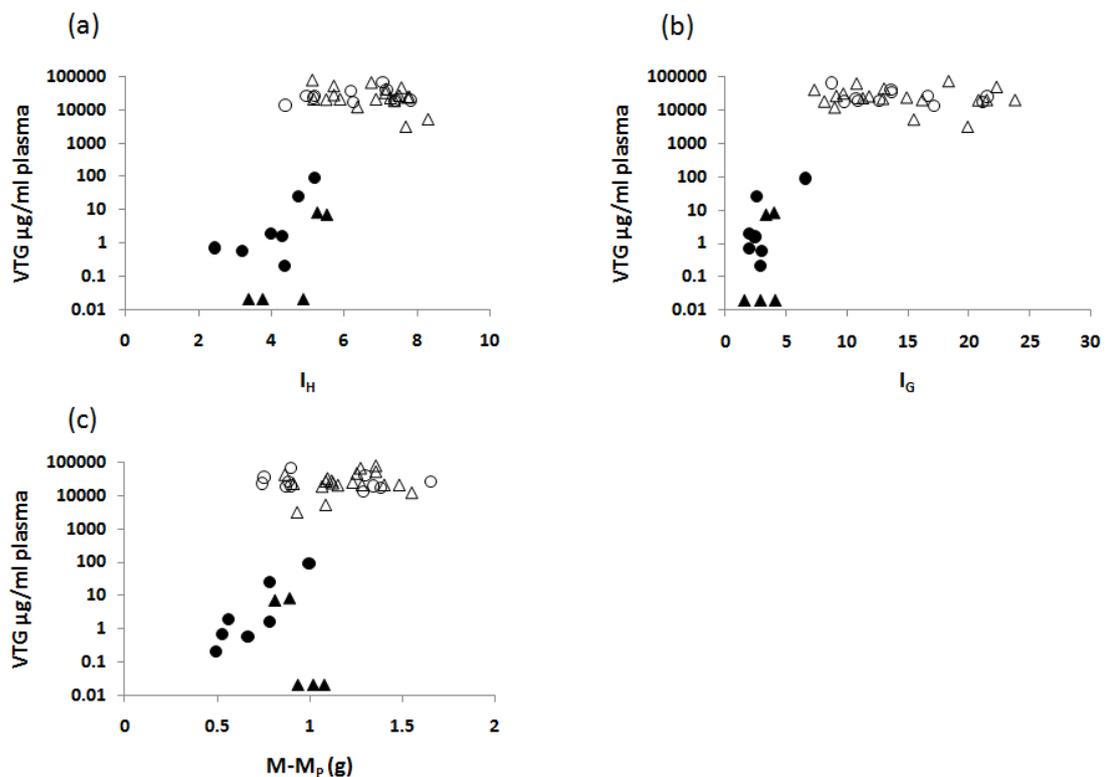


Figure 3.6 Relationships between measurements of sexual development and body condition in *S. solidus* infected and non infected wild and lab female Carsington sticklebacks. The relationship between vitellogenin (VTG) levels and (a) hepatosomatic index (I_H) and (b) gonad index (I_G) and (c) fish mass ($M-M_p$) in wild (circles) and lab (triangles) fish separated by *S. solidus* infection status: non infected (open) and infected (filled).

Relationships between parasite index (I_P) and reproductive parameters and body condition (K)

There was no effect of study type (wild / lab) on I_H , I_G , VTG levels or K in infected fish (I_H ; $t_{12}=-1.2$, $P=0.251$; I_G ; $t_{12}=0.059$, $P=0.954$; VTG; $t_{10}=1.6$, $P=0.149$; K ; $W_{9,5}=58$, $P=0.205$). On combining the infected fish from both studies correlations did not exist between I_P and I_G , VTG levels or K (I_G ; $r=-0.347$, $n=14$, $P=0.224$; VTG; $r=-0.287$, $n=12$, $P=0.366$; K ; $r_s=-0.481$, $n=14$, $P=0.081$; Figure 3.7.b.c.d). A significant negative correlation between I_P and I_H was found to exist ($r=-0.570$, $n=14$, $P=0.033$; Figure 3.7.a). There was no correlation between VTG levels and K of infected fish ($r=-0.158$, $n=12$, $P=0.623$).

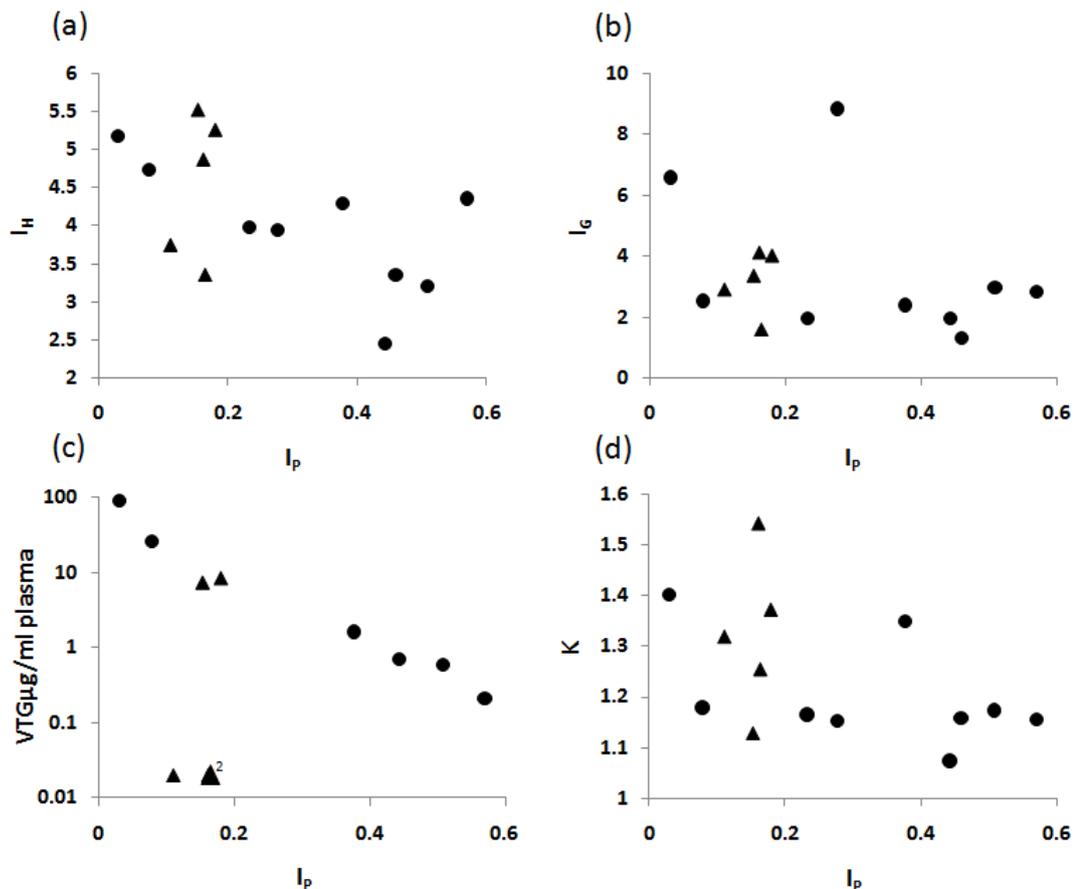


Figure 3.7 Relationships between parasite index and reproductive and body condition parameters in *S. solidus* infected wild and lab female sticklebacks. The relationship between parasite index (I_P) and (a) hepatosomatic index (I_H) (b) gonad index (I_G) (c) vitellogenin (VTG) levels and (d) body condition (K) in wild (filled circles) and lab (filled triangles) female fish.

3.3.4. Experimentally infected Carsington Reservoir males

Sexual development – effect of infection on gonad (I_G), kidney (I_K) and redness (I_R) indices, 11-ketotestosterone (11KT) and spiggin levels

There was no effect of exposure status on I_G ($F_{2,26}=1.4$, $P=0.273$; Figure 3.8.a). Redness index was significantly different across the three groups ($F_{2,26}=6.6$, $P=0.005$; Figure 3.8.b); infected fish had a significantly reduced I_R compared to both exposed non infected ($P=0.004$) and non exposed fish ($P=0.006$). A significant effect of exposure status on 11-ketotestosterone (11KT) levels existed ($F_{2,24}=6.1$, $P=0.007$; Figure 3.8.c); infected individuals had significantly lower 11KT levels compared to exposed non infected ($P=0.047$) and non exposed fish ($P=0.005$). There was a significant effect of exposure status on kidney index (I_K) ($F_{2,26}=7.03$, $P=0.004$; Figure 3.8.d) with infected fish having significantly reduced I_K compared to exposed non infected ($P=0.005$) and non exposed fish ($P=0.003$). There was a significant effect of exposure status on spiggin levels ($F_{2,26}=4.7$, $P=0.018$; variances approaching normality $P=0.046$; Figure 3.8.e). Infected fish had significantly lower spiggin levels compared to exposed non infected ($P=0.045$) and non exposed fish ($P=0.013$). A significant relationship existed amongst all the fish between 11KT and spiggin levels ($F_{1,25}=8.9$, $r^2=0.263$, $P=0.006$; Figure 3.8.f), but did not exist when the three infected males were excluded ($F_{1,22}=2.9$, $r^2=0.115$, $P=0.105$).

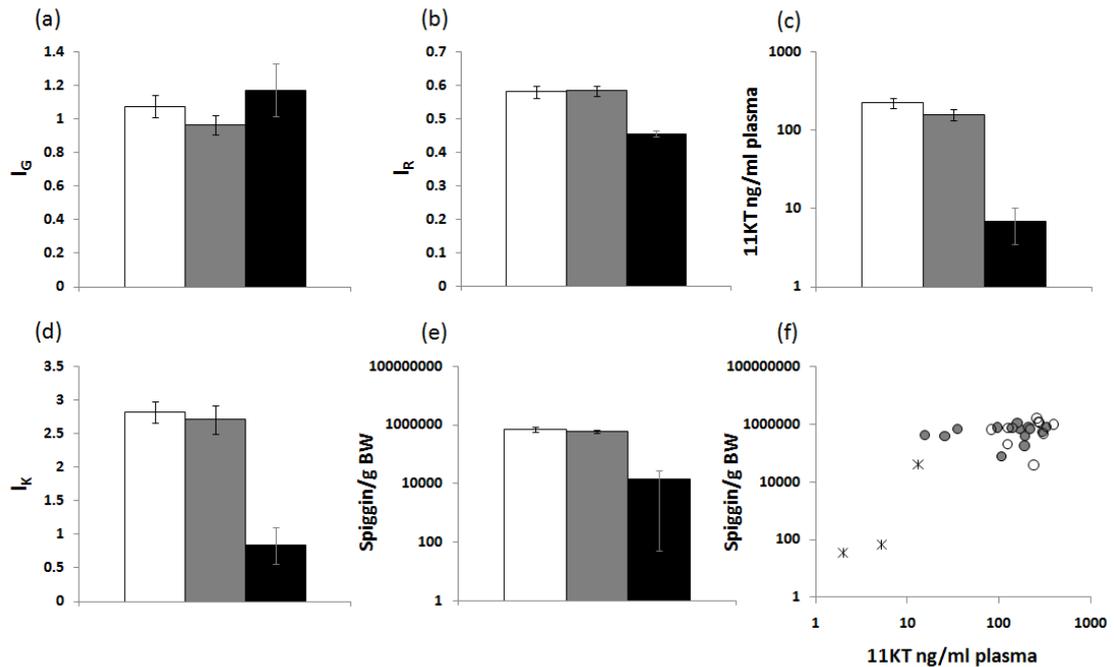


Figure 3.8 The effect of experimental *S. solidus* exposure status on measurements of sexual development in lab raised Carpsington males. The mean \pm s.e. (a) gonad index (I_G) (b) redness index (I_R) (c) 11-ketotestosterone levels (11KT) (d) kidney index (I_K) (e) spiggin levels/g BW in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) male fish and (f) the relationship between 11KT and spiggin levels in non exposed (open circles), exposed non infected (grey circles) and infected (*) fish.

The effect of exposure status on nesting behaviour

Infected males only achieved a maximum nest score of 1, indicating that the fish moved threads from the pile that they were presented with. In contrast, all non exposed and exposed non infected fish built nests, and within the 3 week experimental period had achieved a nest score of 3 indicating that they completed their nest. There was a significant difference in the time taken to obtain a nest score of 3 between exposed non infected and non exposed males ($W_{14,12}=119.5$, $P=0.026$; Figure 3.9.a). Exposed, non infected fish took longer to obtain a nest score 3 compared to non exposed fish. To determine if the fish varied in condition, differences in body condition parameters across the two groups were tested, however there were no significant differences in any of the measured parameters (K ; $t_{24}=1.2$, $P=0.247$; $M-M_P$; $t_{24}=1$, $P=0.304$; L_S ; $t_{24}=0.3$, $P=0.783$; PCV ; $t_{22}=0.7$, $P=0.483$; I_H ; $t_{24}=0.6$, $P=0.527$; I_S $t_{21}=1$, $P=0.334$). The only

other difference found with respect to exposed non infected and non exposed fish was the relationship that I_R had with 11KT. There was a significant difference in the slope of this relationship ($F_{1,20}=8.2$, $P=0.01$; Figure 3.9.b) with non exposed fish having a positive relationship, whilst there was no relationship between 11KT and I_R in exposed non infected fish.

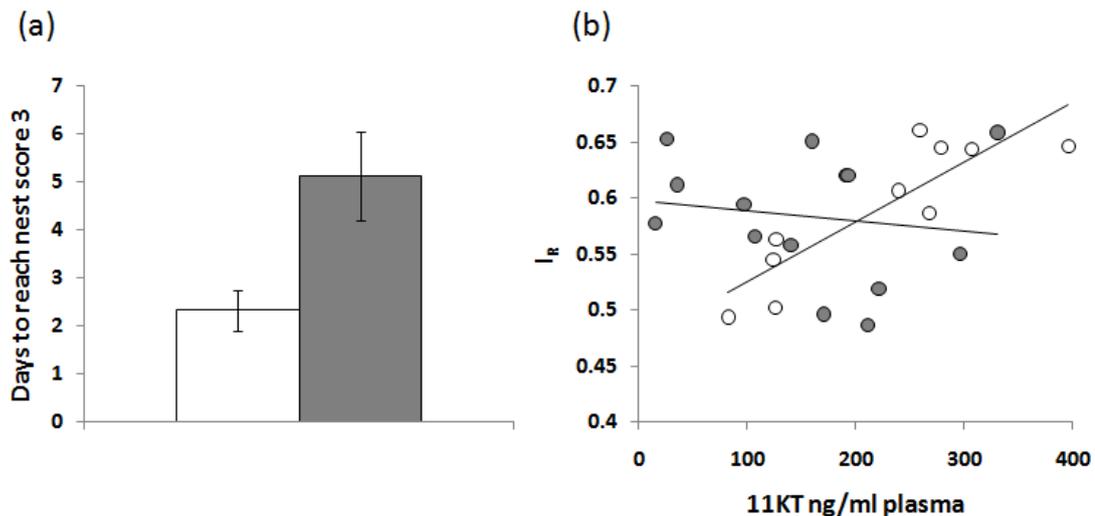


Figure 3.9 The effect of *S. solidus* exposure status on reproductive behaviour and physiology in non infected male lab raised sticklebacks. (a) The mean \pm s.e. days taken to reach a nest score of 3 in non exposed (open bars) and exposed non infected (grey bars) male fish (b) the relationship between 11-ketotestosterone (11KT) and redness index (I_R) in non exposed (open circles) and exposed non infected (grey circles) male fish.

3.4. Discussion

3.4.1. Effects of *S.solidus* infection on the reproductive development of female sticklebacks

The results of both the wild and experimental studies illustrated that *Schistocephalus solidus* infection in female sticklebacks is associated with severely reduced vitellogenin (VTG) synthesis. VTG is an egg yolk protein precursor that is produced in the liver under estrogenic control before being transported to the ovaries where it used to produce egg yolk (Clemens, 1974). *S. solidus* infected females also had reduced liver and gonad sizes and amongst all fish (infected and non infected) a positive relationship existed between VTG levels and both liver and gonad size. Thus both organs play an

important role in reproduction in female sticklebacks, and *S. solidus* interferes with these roles. In the wild study, infected fish had parasite indices (I_P) that ranged from 3% to 57% with total parasite weights ranging from 30-338 mg. Only two infected fish had measurable levels of VTG above $2\mu\text{gml}^{-1}$ plasma; both of these fish had single *S. solidus* infections with I_P s less than 10% and a parasite weight of 30 or 61mg. These fish had VTG levels of 90 and $26\mu\text{gml}^{-1}$ plasma respectively, whereas the mean VTG level of non infected wild fish was $28,281\mu\text{gml}^{-1}$ plasma. Unfortunately there were no wild fish that harboured I_P s that ranged from 10-20%. However, despite obtaining a small number of experimentally infected female fish ($n=5$) these fish all had I_P s that ranged from 11-18% and total parasite weights ranging from 118-167mg. The laboratory fish had been bred and raised under favourable conditions where food competition was relaxed in contrast to wild conditions, making it more likely that these fish would be able to breed. However, none of these fish had VTG levels exceeding $8.5\mu\text{gml}^{-1}$, in comparison with non infected fish which had a mean VTG level of $29,254\mu\text{gml}^{-1}$ plasma. There was no difference in VTG levels between wild caught and experimentally infected females, thus the infected fish from the two studies could be combined to make a comparison across all the recorded I_P s. Amongst all the infected fish there was no correlation between I_P and VTG levels. Thus, among females the parasite appears to prevent VTG production over the whole spectrum of I_P s in both experimentally infected lab and wild fish. It also became clear on comparing laboratory and wild studies that they both show similar results with respect to reproductive physiology.

3.4.2. Effects of *S.solidus* infection on the body condition of female sticklebacks

Infection with *S. solidus* was associated with several changes in body condition, with similar patterns being seen in both the wild and laboratory studies. In the study of wild-caught fish, the non infected fish potentially include both fish that have not been exposed to *S. solidus* infection and those that have been exposed but have not become infected. This needs to be recognised when making comparisons between the lab and field studies. For example, when comparing body condition across the two studies, the wild infected fish are in lower K than non infected fish. Similarly, in the lab studies when the exposed non infected and non exposed fish are combined together, the K of infected fish is significantly lower ($W_{5,22}=33$, $P=0.021$). The wild infected females have a mean K of 1.2, whilst the laboratory infected fish have a slightly higher K of 1.3. In both studies spleen size was increased in infected individuals; spleen enlargement is often associated with an immune response (Seppanen et al., 2009).

In the lab study, infection did not influence the packed cell volume (PCV), whilst in wild fish the PCV was significantly higher in infected fish compared with non infected conspecifics. As discussed in chapter 2, the PCV (or haematocrit) is indicative of the blood's oxygen carrying capacity (Houston, 1997). Here, the results found are in contrast to what was found in Chapter 2. However, a review by Houston (1997) suggests that PCV is not a reliable measure of blood oxygen carrying capacity due to it being influenced by the maturation of red blood cells and sampling stresses. Thus the differences observed may be due to these inconsistencies.

A significant negative relationship existed between VTG and parasite index in the wild study, however this was due to the two fish that produced VTG levels above $2\mu\text{gml}^{-1}$ plasma. When combining the data from both studies there were no correlations

between parasite index and K, I_G and VTG levels. A negative relationship did exist between I_H and parasite index. Thus it seems that parasite infection leads to a reduction in several reproductive parameters regardless of the severity of infection. In contrast the I_H varied with severity of infection, possibly due to its dual role of synthesising VTG and acting as a medium term energy store for fish (Chellappa et al., 1995). In addition, no relationship existed between the condition of the fish and reproduction (VTG) illustrating that body condition is not responsible for the reduced reproduction that is seen.

3.4.3. Evolutionary significance of reduced reproductive capacity of

***S. solidus* infected female sticklebacks**

As described in chapter 2, cyprinid fish often become infected with *Ligula intestinalis*, a close relative of *S. solidus*. This parasite exerts a range of effects on host reproduction (reviewed by Hoole et al., 2010). Several studies have now shown that endocrinological parameters are reduced in the presence of the parasite including reduced gonadotrophin expression (FSH β and LH β), 17 β estradiol (E2) and progesterone levels and aromatase activity (Carter et al., 2005, Hecker and Karbe, 2005, Hecker et al., 2007, Trubiroha et al., 2009, Geraudie et al., 2010). In agreement with the findings of the present study, VTG levels or gene expression have also been shown to be significantly reduced in infected females (Hecker and Karbe, 2005, Geraudie et al., 2010, Trubiroha et al., 2010, Trubiroha et al., 2011). Due to reduced reproduction occurring irrespective of parasite index, it is proposed that *Ligula*, adaptively manipulates host resources to prevent reproduction by disrupting the endocrine system (Carter et al., 2005, Trubiroha et al., 2009, Trubiroha et al., 2010, Trubiroha et al., 2011). However, Geraudie et al. (2010) found that occasionally infected female and male roach did mature, illustrating for the first time that gonad development is possible in the presence of *Ligula*.

In the present study reproduction appears to be reduced irrespective of parasite index, as even where VTG production was above $8.5\mu\text{gml}^{-1}$ the levels that infected fish were producing were significantly lower than that of non infected reproductive females. Thus it seems likely that infected females in Carsington were unable to produce a clutch of eggs. In the experimental infection study, non infected gravid females were recorded at dissection to have gonad indices ranging from 18-24%. Experimentally infected lab fish had I_{GS} that did not exceed 4.1%, whilst wild fish had I_{GS} no greater than 9%. To be able to prevent reproduction early on in infection is a trait associated with parasite manipulation of resource allocation; as small parasites have a lot to gain from preventing reproduction and using the available energy to grow in size (Heins et al., 2010a).

Heins and Baker (2010) illustrated that female nine spined sticklebacks (*Pungitius pungitius*) infected with *Schistocephalus pungitii* were unable to reproduce except when harbouring very small infections; female fish that had I_{Ps} greater than 10% were not able to produce a clutch of eggs (Heins et al., 2004). The authors propose that here, the reduced reproductive capacity is due to adaptive manipulation of the hosts energy reserves. This is in contrast to several other studies carried out on the three spined stickleback and *S. solidus*. Heins et al. (2010a) showed that there was a 59% chance that *S. solidus* infected females harbouring parasite indices greater than 40% could produce a clutch of eggs. They also showed negative relationships between I_p and clutch size, mass and ovum mass. Heins and Baker (2008) illustrated that female sticklebacks infected with *S. solidus* from three different Alaskan lakes were capable of reproduction with between 44-80% of the infected females producing clutches. These females had mean I_{Ps} ranging from 14-24%. Thus studies of Alaskan three spined sticklebacks support the idea that reproductive disruption occurs via nutrient theft and

support the “side effect” hypothesis (Heins and Baker, 2003, Schultz et al., 2006).

Although the side effect hypothesis supports the idea that reproduction can occur in fish with light infections, negative relationships should exist between reproductive parameters and the infection load (I_p); as when parasites increase in size they will require more nutrients, leading to reduced reproduction and condition of the host (Heins et al., 2010a). Bagamian et al (2004) showed in an Alaskan population that a positive relationship existed between body condition and clutch production.

Studies in the UK and British Columbia tend to support the idea that infected females are incapable of reproducing, unless they harbour only very small parasite loads. McPhail and Peacock (1983) found that only 1% of gravid females collected were infected. Similarly, Tierney et al. (1996) found low levels of infected fish reached sexual maturity.

One study illustrated the possibility for host increased investment in gonad growth in response to parasite infection. Experimentally infected female fish were found to have larger gonads than non exposed fish (Barber and Svensson, 2003). One hypothesis for these results is that on becoming infected the fish invest more heavily in reproduction as a counter adaptation to parasite infection before the parasite reduces energy available for reproduction. However, these fish weren't reproductive and if they did become sexually mature the gonads of non infected fish would become enlarged probably removing this difference or reversing it. In the present study, when the laboratory bred fish were dissected the hosts were incapable of producing clutches based on VTG analysis and gonad size. However, in the run up to these dissections the fish were held for 2 months in mixed tanks and then 3 weeks individually, during this time it was not noted if individual fish were becoming gravid. Thus there is the possibility that infected fish had spawned earlier before the parasite effectively

castrated the fish and the reduced I_{GS} seen are post reproductive. However, the fact that fish with I_p s of 3% and 8% in the wild fish were not capable of reproducing makes this seem unlikely. However, future experimental infection studies that track the gravid status of infected individuals are needed to rule out host adaptation as a strategy.

3.4.4. How does *S. solidus* exposure interfere with reproductive parameters in male sticklebacks?

At the end of the experimental infection study it was found that only three males were infected. Therefore this small sample size makes it difficult to make conclusions from the study, however it is still interesting to compare the results to those presented in Chapter 2. The present study, using experimentally infected Carsington males, showed that under favourable lab conditions *Schistocephalus* infections reduced reproductive behaviour and androgen levels. The infected fish did not build a nest over a three week period and they had significantly reduced spiggin levels. These results are thus consistent with those described in Chapter 2, whereby the ability of infected fish to reproduce is dependent on their levels of 11KT (Figure 3.10.).

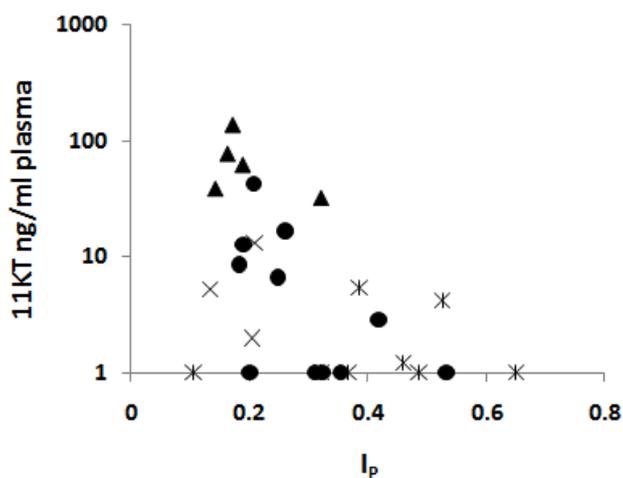


Figure 3.10 The effect of *S. solidus* parasite index on levels of 11-ketotestosterone (11KT) in different populations of male sticklebacks. The relationship between 11KT and parasite index (I_p) in wild fish from Kendoon loch (Filled symbols; triangles: nesters, circles: non nesters) and Victoria Park (*) and laboratory Carsington reservoir (X).

Comparing the nesting behaviour of non exposed and exposed non infected fish revealed an interesting result; exposed non infected fish took significantly longer to nest than non exposed fish. This occurred despite the fact that both classes of fish had similar levels of 11KT and spiggin and were in comparable body condition. Exposure to *Schistocephalus* infective stages is likely to involve the upregulation of the immune response (Scharsack et al., 2007). Thus this would require energy and could lead to energy being diverted away from reproduction and towards the immune response instead. However, it is surprising that no differences occur in any of the other measured parameters. It is also interesting to note that the experimental infections took place approximately two months before the nesting study, so it seems that resisting *S. solidus* infection has long lasting costs. It may be that 11KT and spiggin levels took longer to build up in these fish, but as these measurements were taken at the end of the study when the fish were nesting, such differences would be missed. The evolution of resistance to infection can incur costs (Minchella, 1985). Snail lines (*Biomphalaria glabrata*) that were resistant to *Schistosoma mansoni* produced less offspring than susceptible lines in the absence of parasites, despite having similar fecundity measurements (Webster and Woolhouse, 1999). The reduced fertility may result from the absence of some internal component that is involved in both the development of embryos and parasites i.e. a pleiotropic effect. Something similar could be operating here and other costs of resistance may have been found if males were allowed to breed.

The relationship between 11KT and redness index (I_R) also differed between non exposed and exposed non infected fish. Non exposed fish had a positive relationship between 11KT and I_R whereas exposed non infected fish had similar I_{RS} , regardless of 11KT levels. Exposed non infected fish may transfer their carotenoids to

produce their nuptial colouration in reproductive conditions regardless of their 11KT levels, to avoid missing out on reproduction if they become exposed to infection again.

3.4.5. Conclusions

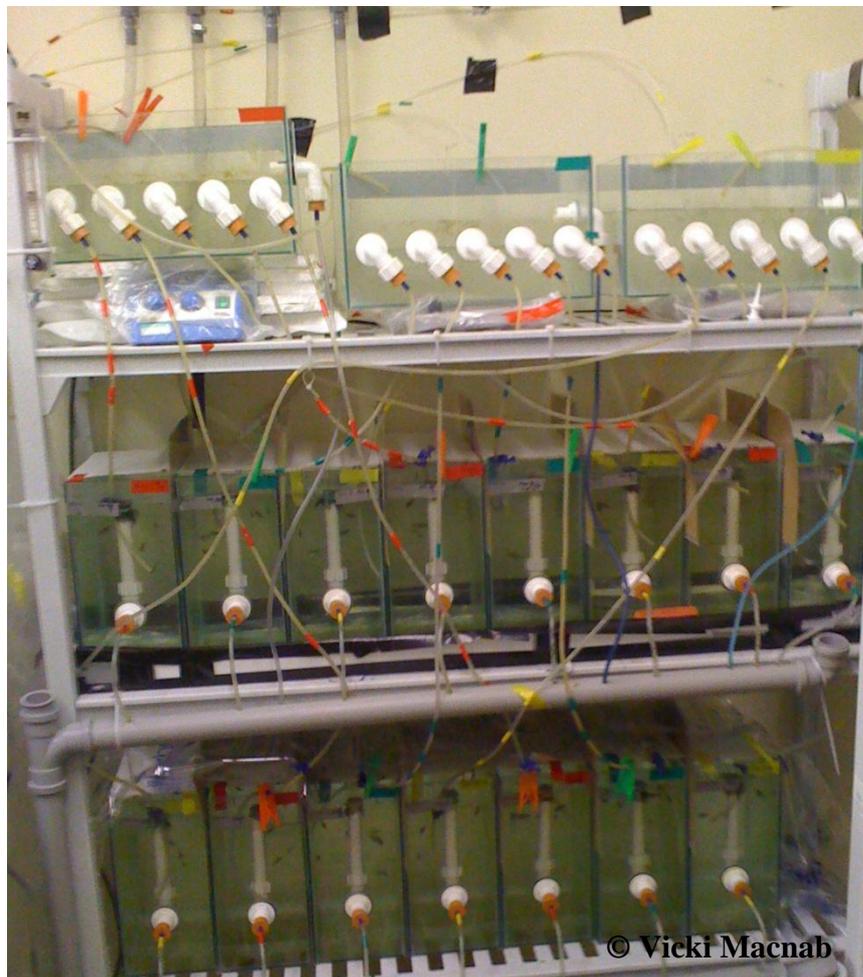
In conclusion, *Schistocephalus* infections led to reduced reproductive capacity in infected females. The wild and laboratory studies showed similar results; both experimentally and naturally infected females from Carsington exhibited reduced reproductive physiology as a result of parasite infection. Therefore being bred and raised under favourable laboratory conditions did not change the outcome of parasite infection in terms of host reproductive fitness. The wild study did use fish that had been in laboratory conditions for a month, however, if anything this should have encouraged the fish to breed, which it didn't.

S. solidus infections irrespective of parasite index resulted in severely reduced VTG levels and gonad and liver masses. In contrast to the results of chapter 2, the results of this chapter are not consistent with the nutrient theft hypothesis, whereby fish with light infections are capable of reproducing and reproductive and condition parameters correlate with the severity of infection (but note the negative correlation between I_H and I_P). These results suggest that reduced female reproduction may arise as an adaptation on either the part of the parasite or the host. Due to wild fish with I_P s less than 10 % being unable to reproduce it seems likely that the parasite is manipulating its host resources preventing reproduction and using the energy made available from this to increase its growth. However, we can also not rule out the possibility that host adaptation is occurring, whereby infected individuals reproduce before parasite infection removes the energy available for reproduction; as we may have missed the point where these individuals were becoming gravid. Future studies are needed to elucidate this. The differences that we see in Chapters 2 and 3 with respect to the effect

that *S. solidus* has on reproduction may occur due to population differences described in Chapter 2, ecological differences in sites or genetic differences in host or parasite population. Also as described in Chapter 2 co-evolutionary processes could be responsible as Carsington Reservoir was formed in 1992, giving these sticklebacks a short association time with *S. solidus* parasites compared to Kendoon Loch where the sticklebacks have had 76 years to become adapted to coping with *S. solidus* infection. Also fish from the Alaskan populations tend to live to 2 or 3 years (Heins and Baker, 2008), whereas our population breeds at one year before dying (personal observations). Thus differences in energy availability in longer lived populations may enable them to reproduce even when harbouring heavy infections.

The experimental infection study yielded few infections that were male sticklebacks, making it difficult to make conclusions regarding this study. However, the results did support those found in Chapter 2 whereby infection reduced 11KT levels resulting in a reduced reproductive capacity. Interestingly exposed non infected fish took longer to obtain a nest score 3 than their non exposed counterparts, illustrating that there may be long-lasting costs of resisting *S. solidus* infection. However, 11KT levels and condition parameters were similar across both groups. Further work could involve investigating energy allocation to the immune response, and then tracking the time it took to nest to see if links exist here. Also by allowing these fish to breed may also yield further costs of resistance, such as reduced attractiveness to females or reduced offspring numbers or parental investment. In addition, tracking 11KT and spiggin levels in the run up to nesting may illustrate that in exposed non infected fish, it took longer for the hormone and nest building glue to build up.

4. The effect of endocrine disrupting chemicals on infection susceptibility and disease progression



© Vicki Macnab

4.1. Introduction

Our reliance on water makes it essential that water is recycled (Sumpter, 2009), and this alongside the fact that water bodies act as sinks for anthropogenic chemicals, increases the number and diversity of stressors that fish and other aquatic organisms are exposed to (Poulin, 1992, Sumpter, 2005). Multiple stressors are therefore a major threat to aquatic animals (Sumpter, 2005). Yet despite this, the effects of stressors are mostly studied in isolation, with test organisms otherwise experiencing ideal conditions; for example, in ecotoxicological studies, organisms are typically exposed to one chemical at a range of concentrations whilst other conditions are kept optimal (Holmstrup et al., 2010). Although such studies detect individual concentration dependent effects, this scenario does not accurately reflect the situation encountered by animals in the wild (Sures, 2008). In particular, such studies cannot tell us about the relative importance of different stressors on animal health and how stressors interact. Stressors can act additively, synergistically or antagonistically (Marcogliese and Pietrock, 2011; reviewed in Chapter 1). Holmstrup et al. (2010) reviewed over 150 studies that investigated the interactive effects of natural stressors and anthropogenic chemicals on test organisms and illustrated synergistic effects occurred in over 50% of the studies.

4.1.1. Endocrine disruption in natural environments

The issue of endocrine disruption in the aquatic environment has become a ‘hot’ topic of research since the late 1990’s and a large body of literature now exists (Colborn et al., 1993, Purdom et al., 1994, Jobling et al., 1998). The feminisation of male fish in UK rivers receiving sewage treatment plants effluent, led to the discovery that estrogenic chemicals were present in the environment and affecting wildlife (Purdom et al., 1994). These male fish were producing significant amounts of the egg yolk protein precursor vitellogenin (VTG). Naturally, VTG only occurs in females, with estrogens

leading to the production of VTG in the liver, which is then transported to the ovaries to be used in the production of egg yolk (Clemens, 1974). Later studies by Desbrow et al. (1998) confirmed the estrogenic activity of the effluent to be due to the natural steroids 17β estradiol (E2) and estrone (E1) and the synthetic estrogen 17α ethynylestradiol (EE2); the active ingredient of the female contraceptive pill. Since then estrogens have been shown to have a wide range of effects on the reproduction and development of wildlife (Jobling et al., 1998).

Lifelong exposure to EE2 at a concentration of 5ngL^{-1} in zebrafish (*Danio rerio*) resulted in reproductive failure due to feminised males not producing sperm and leaving eggs unfertilised (Nash et al., 2004). Interestingly, reproductive behaviour was unaffected in these fish, which meant that feminised males interfered with the spawning attempts of normal males which could ultimately have effects at a population level. The application of similar concentrations of EE2 ($5\text{-}6\text{ngL}^{-1}$) to a lake containing wild populations of fathead minnow, *Pimephales promelas* led to gonadal abnormalities, VTG production in male fish and greatly reduced young-of-the-year numbers which almost caused the extinction of the species after the second season of EE2 application (Kidd et al., 2007). Recent studies have also indicated that estrogens can have a range of effects on the immune system of fish (Filby et al., 2007, Thilagam et al., 2009, Milla et al., 2011). The ability of estrogens to modulate the immune response raises the possibility that they could have important effects when animals encounter parasites.

4.1.2. Parasites and pollution

Parasites are ubiquitous in fish, however ecotoxicological studies using fish as models often don't consider the effects of parasites, and several researchers have criticised these studies as a result (Moller, 1987, Hamilton et al., 1990, Poulin, 1992, Lafferty and Kuris, 1999, Thilakaratne et al., 2007, Marcogliese et al., 2010). This is surprising, as

parasites may modify the biomarker responses that are currently measured in such studies. Marcogliese et al. (2010) illustrated that parasite resistance in yellow perch (*Perca flavescens*) did not differ across sites that varied in contamination levels; however the pathological effects of two parasites (*Diplostomum spp.* and *Apophallus brevis*) was higher at the contaminated sites in terms of biomarkers of oxidative stress (often used as a biomarker of pollution). Due to the universal presence of parasites in wild fish, this study and others illustrate the importance of including parasites in ecotoxicological studies (Thilakaratne et al., 2007, Sures, 2008). It has been proposed that parasites could also be used as biomarkers of pollution; however different parasite groups vary in their response to pollutants (Lafferty, 1997).

Chemical pollutants can also have a variety of effects on parasites. Pollution could modulate the host's immune response and thus render them more susceptible to parasite infections (Khan, 1990, Hoole, 1997, Thilakaratne et al., 2007, Morley et al., 2006). Conversely if any part of a parasites life cycle is adversely affected as a result of chemical exposure then this could reduce parasite success (Poulin, 1992, Lafferty and Kuris, 1999, Sures, 2004). Heavy metal pollution appears to have a consistently negative effect on parasite numbers (Lafferty, 1997). A chemical could also show a range of these effects depending on its concentration. Gheorghiu et al (2005) showed that guppies (*Poecilia reticulata*) exposed to intermediate levels of zinc ($15 - 60\mu\text{gL}^{-1}$) prior to the challenge with the ectoparasite *Gyrodactylus turnbulli* were less likely to develop an infection. They proposed this was due to the presence of mucus on the fish epidermis as a result of zinc exposure which made it difficult for the parasite to attach. Where fish did develop infections their recovery time increased with zinc concentration suggesting that high zinc levels lead to the eventual depletion or acclimation of the mucous response. At the highest concentration ($240\mu\text{gL}^{-1}$), zinc had a toxic effect on

the parasite reducing infection loads. This study also illustrated the synergistic effects of parasites and pollutants; high zinc concentrations did not induce mortality in non infected fish, whilst mortality increased with zinc levels in *Gyrodactylus* infected guppies. Links between parasites and pollutants in the wild have been highlighted; a higher prevalence of the cestode *Ligula intestinalis* was found in bream *Abramis brama*, from the most heavily polluted regions of the River Elbe, Germany (Hecker and Karbe, 2005).

Parasites can also modify the effect that chemicals have on their hosts. Pascoe and Cram (1977) illustrated that cadmium was more toxic to *Schistocephalus* infected sticklebacks than control fish. This was due to parasitized hosts being unable to deal with additional stressors effectively (Sures, 2004). However, some endoparasites, such as acanthocephalans, are capable of accumulating heavy metals (Sures, 2004, Sures, 2007). Sures and Siddall (1999) showed that the acanthocephalan *Pomphorhynchus laevis* accumulates concentrations of lead much higher than those found in the organs of its fish host, chub (*Leuciscus cephalus*). In addition infected fish had lower levels of lead in their intestinal wall than non infected conspecifics, suggesting a possible positive effect of the parasite in heavy metal polluted environments. In addition, the cestode *L. intestinalis* was also capable of accumulating heavy metals at higher levels than those observed in their host and this process appears to be dependent on the age of the parasite (Tenora et al., 2000, Barus et al., 2001, Tenora et al., 2002, reviewed by Hoole et al., 2010).

At a population level, the effect of anthropogenic stress may oppose that of what we see at an individual level. For example stress may increase the susceptibility of individuals to parasites. However at a population level, if this stress leads to a reduction

in host density (through mortality of parasitized individuals) then disease levels should fall (Lafferty and Holt, 2003).

4.1.3. Aims of current study

This chapter describes an experimental study investigating the interaction of two stressors that are known to affect fish in isolation, namely endocrine disrupting chemicals and parasites. Experimental studies examining the combined stress of more than a single factor are scarce, yet are essential in understanding the impact that anthropogenic stressors may have on fish already under disease threat.

This study aimed to assess the effects of a natural parasite and an anthropogenic chemical stressor on a model fish host; the three spined stickleback, *Gasterosteus aculeatus*. The natural steroid 17β estradiol (E2) was chosen as an example of an endocrine disrupting chemical due to the large amount of existing literature and the presence of many chemicals with estrogenic modes of action (Purdom et al., 1994, Jobling et al., 1998, Sumpter, 2005). Estrogens have been shown to affect the reproductive development of wildlife (Jobling et al., 1998) and natural estrogens have been proven to be present in sewage treatment effluent as a result of incomplete degradation at sewage treatment plants (Purdom et al., 1994, Desbrow et al., 1998, Sumpter, 2005). In addition, man made xenoestrogens have also been shown to be present in the environment and these include chemicals used in pesticides, plastifiers and detergents and can enter the aquatic environment via run off, or incomplete degradation at sewage treatment plants (Sumpter, 2005, Combalbert and Hernandez-Raquet, 2010). The cestode *Schistocephalus solidus* was used as a natural stressor due to its abundance in natural stickleback populations and because its life cycle can be manipulated in the lab to allow experimental infections to be performed (Barber and Scharsack, 2010).

Fish were exposed to E2 and parasite infections in a controlled laboratory experiment. Two nominal E2 concentrations were utilised; 10ngL^{-1} represented an environmentally relevant concentration (Williams et al., 2003) and a higher concentration of 100ngL^{-1} was used as the other treatment.

4.2. Materials and Methods

4.2.1. Experimental design

A total of 14 laboratory stickleback broods were produced using IVF techniques (Barber and Arnott, 2000) using wild-caught adult broodstock from Carsington Reservoir (Derbyshire, UK; N53°03'21", W1°37'25"). The fish were reared in family groups and fed *ad libitum* with *Artemia* naupli and kept under a photoperiod of 16L: 8D and a temperature of $18\pm 1^\circ\text{C}$. At 5-6 months of age, fish were randomly selected from all broods and allocated to one of three treatments. Fish were exposed either to a solvent control, 10ngL^{-1} or 100ngL^{-1} of E2. To achieve the E2 concentrations required, a stock solution was produced by dissolving the chemical in ethanol. From this, working solutions of E2 (dissolved in dH_2O) were generated every 6d at $20\mu\text{gL}^{-1}$ and $200\mu\text{gL}^{-1}$. These solutions were transferred to mixing vessels using a peristaltic pump at a rate of $50\mu\text{lmin}^{-1}$. Here, they were diluted with water (1/2000) into the aquaria at a rate of 100mlmin^{-1} , regulated by flow meters, to achieve the target concentrations in the tanks of 10ngL^{-1} and 100ngL^{-1} of E2 respectively. The chemical exposure ran for at least a week prior to fish entering the tanks to allow the target concentrations to stabilise. The fish were continuously exposed to their chemical treatment via a flow-through aquarium system. There were nine replicate aquaria tanks per treatment. On exposure day 18 the fish were subjected to experimental parasite infections with *S. solidus* (Figure 4.1.).

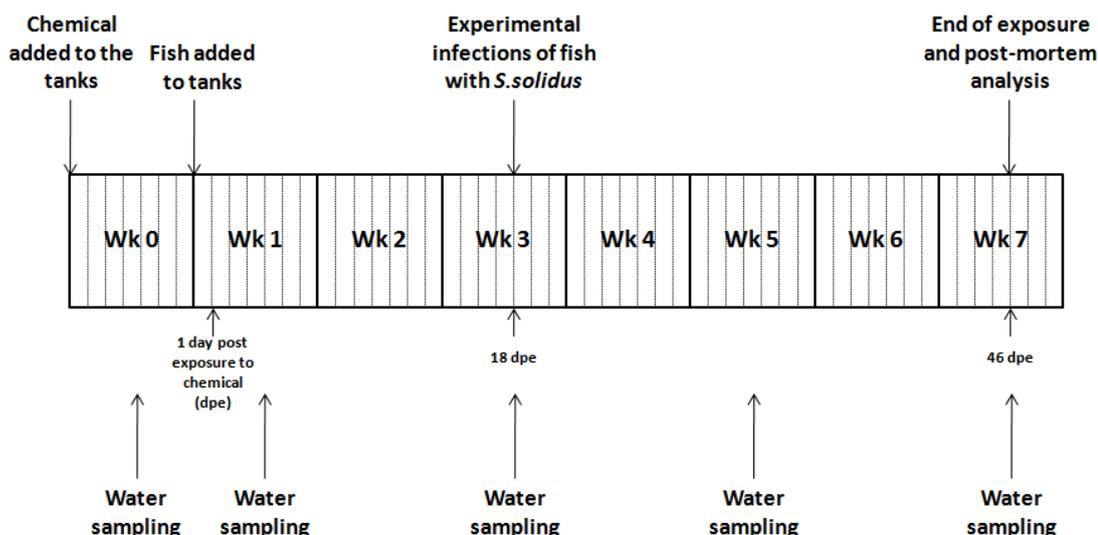


Figure 4.1 The experimental design where sticklebacks were exposed to chemical E2 treatments before undergoing experimental *Schistocephalus solidus* infections.

4.2.2. Experimental infections of copepods and sticklebacks

Single infective plerocercoids of *S. solidus* were dissected from naturally infected wild sticklebacks from Carsington Reservoir and cultured *in vitro* to produce eggs (Smyth, 1946, Wedekind, 1997). Chapter 3, methods section 3.2.2. provides a detailed experimental infection protocol. Briefly, laboratory reared copepods were fed 1 – 2 hatched coracidia. After visual inspection to identify that the coracidia had been eaten, copepods were kept individually and screened 7 days post exposure (dpe) to check infection status and infectivity based on the presence of a cercomer (Smyth, 1969). Copepods infected with a single proceroid were fed to the fish at 17-19dpe.

After experimental infections the fish were placed back in their treatment tanks for 28d and fed daily *ad libitum* to excess with frozen bloodworms (*Chironomus* sp. larvae). Across the three treatments at the end of the study 203 fish remained that had undergone an experimental parasite infection with *S. solidus*; 68 fish in the solvent control group, 66 fish in the 10ngL⁻¹ group and 69 fish in the 100ngL⁻¹ E2 group. Over the entire exposure period (i.e. before and after experimental infections) there were 19 mortalities across the three chemical treatment groups.

4.2.3. Post mortem analysis

At the end of the study fish were euthanised with an overdose of Benzocaine (Stock solution: 10g prepared in 1L of 70% EtOH). Fish were blotted, measured (standard length, L_s , to 0.1mm) and weighed (Mass, M , to 0.001g). A fin clip was taken and placed in 1ml of pure EtOH, for future molecular sex determination. The fish were then frozen at -20°C for approximately 30d at which point the head and tail of each fish was cut, pooled, weighed and stored at -20°C for future vitellogenin (VTG) analysis. The rest of the fish was dissected and the blotted mass of the liver (M_L , to 0.000001g) was measured and where possible the sex of the fish was noted. The infection status of the fish was recorded and the mass of any parasites (M_P , to 0.000001g) was taken. The total mass of the parasite in infected fish allowed for the parasite index to be calculated:

$I_P = 100(M_P/(M-M_P))$. The body condition factor of the fish was calculated from the following equation $K = [(M-M_P)L_S^{-3}] \times 10^5$ (Pennycuick, 1971). Hepatosomatic index (I_H) was calculated using the equation $I_H = 100[M_H(M-M_P)^{-1}]$.

4.2.4. Vitellogenin (VTG) analysis

Sample preparation for VTG analysis The average mass of the head and tail pooled from each individual fish was calculated to be 180mg. To identify the volume required to produce a 2.5X dilution, 180mg was multiplied by 2.5 giving a dilution required of 450 μl . The exact dilution factor of each sample was back calculated by dividing the weight of each head and tail pool by 450 μl . For those samples which fell within the dilution factor acceptable range (1.5- 4X) 450 μl of homogenisation buffer (50mM Tris-HCl pH7.4, 1% Protease inhibitor cocktail (Sigma)) was added to break down the tissue and release the VTG in to the liquid. To those samples that fell out with the prescribed dilution factor range (1.5-4X) the amounts of homogenisation buffer added were adjusted; 600 μl was added to heavier samples and 200 μl was added to the lighter

samples in order not to under or over dilute them. The homogenisation buffer was stored on ice during use. Once the correct amount of buffer was added to each sample, a homogenate was created by the use of disposable pestels. This was immediately centrifuged at 13,500rpm (1600 g at 4°C for approximately 15min. 15µl of supernatant was then added to low protein binding (LPB) (Polypropylene) 96 well microplates; samples were plated in duplicate and were immediately frozen.

VTG ELISA An ELISA was used to quantify VTG levels. See chapter 3 (section 3.2.4) for full description of VTG ELISA technique. Once VTG levels were quantified using the ELISA plate reader this value was multiplied by the dilution factor (how much the sample was originally diluted with the homogenisation buffer) to provide the amount of VTG (µg/ml) in the whole homogenate. This was finally converted into ng and divided by the body weight, to provide the amount of VTG (ng) present per gram of body weight.

4.2.5. Water sampling

Collection and pumping During the exposure, water samples were taken at week 0 (when no fish were present), week 1 and approximately every 2 weeks after this to verify if the target (nominal) concentrations were achieved. For this, at least 250ml of water was taken from each replicate tank. A peristaltic pump was used to pump water samples through C18 solid phase extraction (SPE) cartridges (Sep-pak Plus, Waters Ltd, Watford, UK). Prior to pumping, the SPE cartridges were primed using 5ml of methanol followed by 5ml of distilled water, labelled and attached to the pump tubing. Water was then pumped through the cartridges at a rate of ca. 10ml/min into pre-weighed flasks. When approximately 250ml of water had been pumped, the pump was stopped and the cartridges were stored on ice. The flasks were then weighed with their contents to obtain the exact volume of each pumped sample. The cartridges were then

washed with 5ml of dH₂O, air dried using a syringe, sealed using Parafilm and were then stored at -20°C for future chemical analysis.

Sample preparation for radioimmunoassay (RIA) SPE cartridges were removed from the freezer and allowed to defrost. The Parafilm was removed and they were placed in a vacuum to ensure they had dried out. Using a syringe, 6ml of methanol was pushed through the cartridge into a labelled tube. Additional methanol was added to samples to ensure each sample was 6ml in volume. 2ml of each sample was then placed in a smaller tube, and dried under N₂ steam at 45°C. 2ml of RIA buffer (0.5M phosphate buffer containing 0.2% bovine serum albumen, 0.8% sodium chloride, 0.03% EDTA and 0.01% sodium azide) was added to the dried extracts and vortexed. 100µl of the samples from the low and control treatment was then added to duplicate glass tubes for the assay. Samples from the high treatment were diluted 1:10 before adding 100µl of the dilution to the duplicate tubes.

The radioimmunoassay (RIA) See chapter 2 section 2.2.5. for description of RIA process. The only difference in the procedure is that here the radio-labelled antigen was E2, and antibody for this antigen was selected. Also nine E2 standards of known concentrations were added to the assay (0.5 – 125pg/100µl).

4.2.6. Molecular sex determination

Fin clips were taken from each fish and stored in 1ml of EtOH. They were processed approximately 3 months after collection and were genotyped for their sex using polymerase chain reactions (PCRs) with stickleback specific primers designed for the sex linked marker, isocitrate dehydrogenase (IDH); forward primer :5'GGGACGAGCAAGATTTATTGG 3'; reverse primer 5'TATAGTTAGCCAGGAGATGG 3' (Peichel et al., 2004).

DNA extraction The samples were transferred into 100µl of dH₂O and left for approximately 30 mins; this step washes ethanol from the samples as this can lead to contamination of the PCR product. The fins were then moved to a new Eppendorf tube containing 400µl of DNA extraction buffer (200mM Tris pH 7.5, 25mM EDTA pH 8, 250mM NaCl, 0.5% sodium dodecyl sulphate (SDS)). To this 7µl of 20mg/ml proteinase K was added. The sample was then vortexed for 10-15 s before being placed on a dry heat block at 55°C overnight (approx 16 hours). This step degrades the cell membranes and opens up the cells, allowing the extraction buffer to draw the DNA into the liquid. The following day the samples were then heated gradually to 94°C and left at this temperature for approximately 10minutes, allowing for the denaturation of proteinase K. This is important to prevent any DNA degradation by the enzyme at future steps. Following this the samples were vortexed for 10-15 s and then centrifuged at 13,500 rpm for 2 min (Eppendorf Centrifuge 5415C). Using a pipette, 300µl of the supernatant was added to a new Eppendorf tube and to this 300µl of isopropanol was added. The tubes were then mixed 3-5 times, before being placed at -80°C overnight. This procedure brings the DNA out of the extraction buffer, allowing it to be spun down into a DNA pellet at a later step. On day 3 the samples were taken out of the -80°C freezer and allowed to defrost before they were centrifuged for 10 min. A DNA pellet had now formed at the bottom of the Eppendorf tube and the supernatant was removed by pipetting, leaving only the pellet in the tube. To this 198µl of 70% ethanol was added and the sample was centrifuged for 2 min. The supernatant was removed again from the tube leaving the DNA pellet remaining. This step removes any isopropanol in the sample. The tubes were then left open, covered with micropore tape for over 3 hours. This allows for any alcohol to be evaporated. At this point 100µl of ddH₂O was added to each sample and they were placed at 4°C overnight.

Polymerase chain reaction (PCR) The PCR components were taken out of the freezer and defrosted on ice. Reactions of 10µl were used. A single reaction is made up of 3µl of ddH₂O, 5µl of Sigma Red Taq master mix (dNTPs, magnesium chloride, buffer, and Taq polymerase) and 0.5µl of the forward and reverse primer (Peichel et al., 2004), which were added in this order. A master mix of this was made up for the required number of samples. From this 9µl was added to each PCR tube and to this 1µl of the DNA was added. The samples were briefly spun down before loading them into the PCR machine. The machine was programmed to:

- 1 cycle of: 5 min at 94°C (denaturing of DNA – splits the DNA strands)
- 40 cycles of: 30 s at 95°C (denaturing of DNA – splits the DNA strands)
30 s at 56°C (annealing of the primers)
30 s at 72°C (extension of the new DNA with polymerases)
- 1 cycle of: 10 min at 72°C (Final extension – makes as many new strands of the product as possible)

Agarose gel set up For each gel we ran a 5% agarose gel (high resolution for DNA/RNA fragments 10-500bp; Melford MB1250) and 0.5µl of ethidium bromide was added. Once the gel had set, the samples were removed from the PCR machine and spun down briefly. The gel was placed in the gel tank and covered in 1xTAE. The samples were then loaded into the gel and 2µl of a DNA ladder was added. The gel was run at 60 volts for approximately one hour, before transferring the gel to gel doc to check the image.

Molecular sex bands The samples were genotyped using the marker isocitrate dehydrogenase (IDH). This marker is closely linked to sex with females producing a single band of approximately 300bp, whilst males produce two products of 270bp and 300bp due to a small deletion (Withler et al., 1986, Peichel et al., 2004). Therefore fish

that show two bands on the gel are males, whilst single bands represent females (Figure 4.2.).

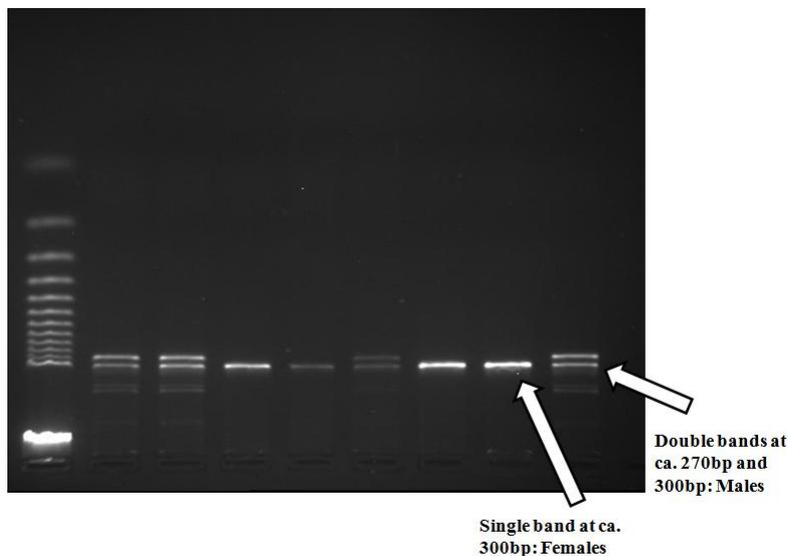


Figure 4.2 Molecular sex determination gel showing female sticklebacks producing a single band of approximately 300bp and males producing two products of 270bp and 300bp.

4.2.7. Statistical analysis

A Chi squared test for independence was used to investigate if E2 treatment or fish sex had an impact on infection susceptibility. Proportional data were (I_P , K , I_H) arcsine square root transformed and normality was tested using the Kolmogorov-Smirnov statistic. Non normal data was log or square root transformed or, if the data was skewed to the right, then it was reflected and then log or square root transformed. For parametric data, two way ANOVAs were used to investigate if E2 treatment, fish sex and infection status had a significant impact on a measured variable ($M-M_P$, L_S , I_P , K , I_H). The VTG data could not be normally transformed so a Kruskal Wallis test was used to assess the effect of E2 treatment, and Mann Whitney U tests were used for post hoc analysis with P values undergoing Bonferonni adjustment ($\alpha= 0.017$). Within an individual E2 treatment VTG could be normality transformed (solvent control group approached normality; $P=0.049$) and therefore the effect of infection status and fish sex on VTG production was tested using two way ANOVAs. Regression analysis showed

that a significant relationship existed between parasite mass (M_P) and fish length (L_S) so a two way ANCOVA was used to investigate if E2 treatment and sex had a significant effect on M_P , taking into account the measured covariate (L_S). The residual values from the relationship between parasite mass and fish length were calculated and are displayed as $rL_S M_P$. ANCOVA analysis was used to identify if E2 treatment had a significant effect on the relationship between I_P and measured variables ($M-M_P$, L_S , K , I_H , VTG). The relationship between the covariate (I_P) and the measured variables among all the infected fish is also presented.

One fish (infected from the high E2 treatment) was removed from the entire analysis (apart from the Chi squared test for E2 infection susceptibility) due to a fish sex ambiguity; it had been identified visually as a female but molecularly as a male. Throughout the liver analysis tests 7 additional fish were removed: 3 non-infected and 4 infected; this was due to the organ being too small to measure in these fish.

4.3. Results

4.3.1. Water analysis

The concentrations of E2 in the tanks were shown to be close to the nominal concentrations. Over the entire exposure the mean concentration for the nominal 10ngL^{-1} E2 was 7.8ngL^{-1} and 100ngL^{-1} E2 was 65.5ngL^{-1} (Figure 4.3.). There were negligible amounts of E2 in the solvent control (0.8ngL^{-1}). This data was not corrected for recovery rates, and therefore the values include any losses during the water sampling procedure.

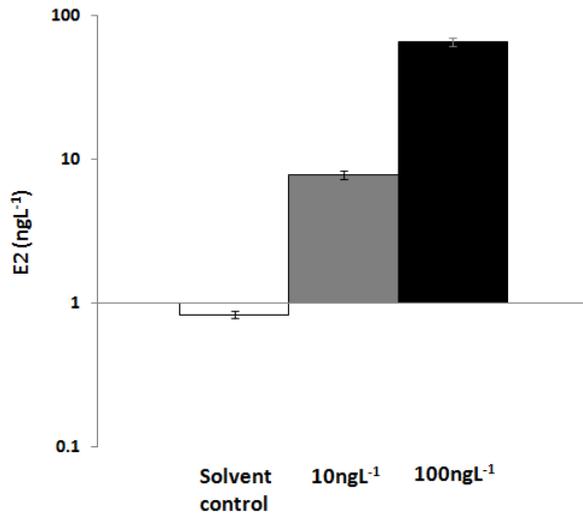


Figure 4.3 The E2 steroid concentrations achieved in tanks across the three treatments over the course of the exposure; bars represent mean \pm s.e.

4.3.2. Effect of E2 treatment on infection susceptibility, condition and vitellogenin production of all fish

Infection Susceptibility

Overall, 93 of the 203 fish that were fed infective proceroids developed plerocercoids (45.8%). There was no effect of E2 treatment on infection susceptibility (Solvent control: 29/68; 10ngL⁻¹: 36/66; 100ngL⁻¹: 28/69; n=203, $\chi^2_{2}=3.1$, P=0.216). There was no effect of sex on infection susceptibility (Females: 48/107; Males: 44/95; n=202, $\chi^2_{1}=0.004$, P=0.947).

Body condition

There was no effect of E2 treatment or infection status on fish mass (excluding parasite mass; M-M_P); but a significant effect of sex on fish size existed; with females being heavier and longer (Figure 4.4.ab.; Table 4.1.). E2 treatment had a near to significant effect on fish length (L_S; Figure 4.4.b; Table 4.1) with fish in the high E2 treatment being longer than those in low E2 treatment (Tukey P=0.097) and the solvent control group (Tukey P=0.059). Amongst all fish, there was no main effect of infection status on condition (K); however there was a significant main effect of sex and E2 treatment

(Figure 4.4.c; Table 4.1.); males had a significantly lower K than females. Fish in the 100ngL⁻¹ E2 group had significantly lower K than those in the solvent control and 10ngL⁻¹ E2 treatment (Tukey post hoc; P<0.001 and P=0.001). There was no significant effect of E2 treatment and infection status on hepatosomatic index (I_H), though a significant effect of sex did exist (Figure 4.4.d; Table 4.1.) with females having higher I_Hs. There were no significant interaction effects between sex, infection status and E2 treatment with respect to the measured variables above.

Vitellogenin (VTG) production in all fish

There was a significant effect of E2 treatment on VTG production among the whole sample ($\chi^2_2=135$, n=202, P<0.001; Figure 4.4.e). The high 100 ngL⁻¹ group was significantly different from the solvent control and low 10ngL⁻¹ group (solvent control: $W_{68,68}=2346$, P<0.001; 10ngL⁻¹E2: $W_{66,68}=2212$, P<0.001; Bonferonni adjusted $\alpha=0.017$). Within the solvent control and individual E2 treatments the effect of infection status on VTG levels was not significant (Table 4.2.). The effect of sex on VTG production was not significant for the solvent control and 100 ngL⁻¹ E2 treatment. There was a significant effect of sex on VTG production in the 10ngL⁻¹ E2 treatment with females producing higher amounts compared to males (Table 4.2.; Figure 4.4.e).

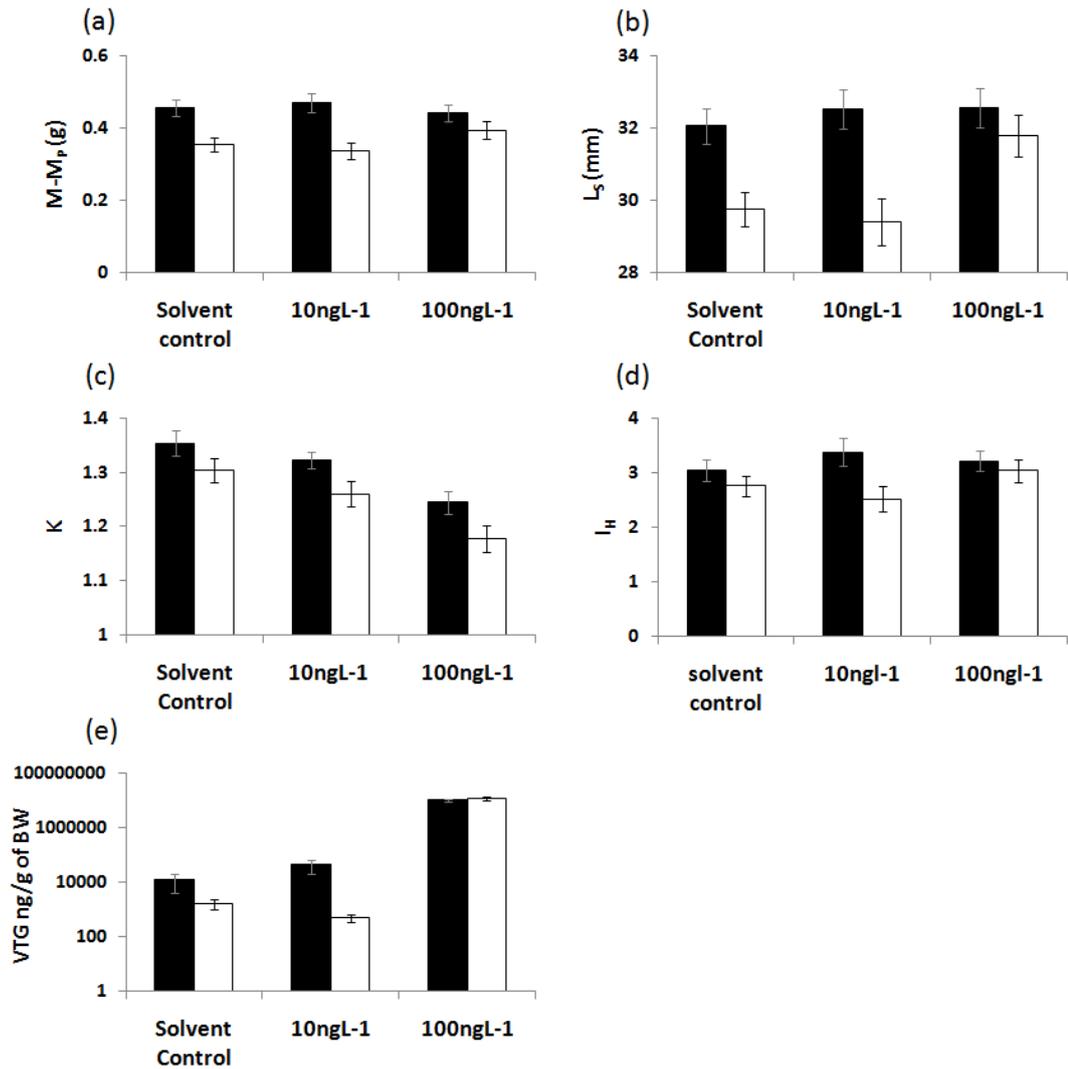


Figure 4.4 The effect of E2 treatment and fish sex on measurements of body condition in sticklebacks exposed to *S. solidus* infection. The mean \pm s.e. (a) mass ($M-M_p$) (b) length (L_s) (c) body condition (K) (d) hepatosomatic index (I_H) and (e) vitellogenin (VTG) levels across the three treatments separated by fish sex (females: filled bars; males: open bars).

Table 4-1 Results of two way ANOVAs performed to investigate the impact of infection status, E2 treatment and fish sex on measurements of body condition; mass (M-M_P), length (L_S), condition (K) and hepatosomatic index (I_H). There were no significant interactions for any of the measured variables. P values that are significant are in bold, whilst italicised values are near to significant.

	M-M _P			L _S			K			I _H		
Source	Infection status	E2 Treatment	Sex	Infection status	E2 Treatment	Sex	Infection status	E2 Treatment	Sex	Infection status	E2 Treatment	Sex
d.f.	1, 190	2, 190	1, 190	1, 190	2, 190	1, 190	1, 190	2, 190	1, 190	1, 183	2, 183	1, 183
F	0.05	0.22	23.8	0.53	2.98	19.64	0.89	14.48	10.75	0.6	0.43	5.06
P	0.825	0.801	<0.001	0.466	<i>0.053</i>	<0.001	0.346	<0.001	0.001	0.44	0.652	0.026

Table 4-2 Results of two way ANOVAs performed to investigate the impact of infection status and fish sex on vitellogenin (VTG) production within a single E2 treatment group. P values that are significant are in bold.

	Solvent control			10ng L ⁻¹ E2			100ng L ⁻¹ E2		
Source	Infection status	Sex	Interaction	Infection status	Sex	Interaction	Infection status	Sex	Interaction
d.f.	1, 64	1, 64	1, 64	1, 62	1, 62	1, 62	1, 64	1, 64	1, 64
F	2.47	0.69	0.13	0.04	7.34	1.85	0.15	0.269	0.007
P	0.121	0.409	0.717	0.835	0.009	0.179	0.7	0.605	0.935

4.3.3. Effect of E2 treatment on parasite size and relationships between parasite index and body condition measurements

The effect of E2 treatment and sex on parasite index (I_P) and parasite mass (M_P)

Among infected fish, there was a near to significant effect of E2 treatment on I_P with fish in the 100ngL⁻¹ having higher I_P s (Figure 4.5.a; Table 4.3.). A significant effect of sex existed with male fish having higher I_P s (Figure 4.5.a; Table 4.3.). Regression analysis showed that there was a significant relationship between L_S and M_P ($F_{1,90}=8.3$, $r^2=0.084$, $P=0.005$, Figure 4.5.b). The relationship between fish mass and M_P was also considered ($F_{1,90}=5.9$, $r^2=0.062$, $P=0.017$); however L_S explained more of the variation so this was used for further analysis. A two way ANCOVA with L_S as a covariate, illustrated that there was a significant interaction between fish sex and E2 treatment on M_P (Figure 4.5.c; Table 4.3.); all fish in the solvent control and 10ngL⁻¹ E2 treatment had similar M_P s for a given L_S across the sexes, whereas males in the 100ngL⁻¹ E2 treatment had a significantly higher M_P for a given fish L_S .

Table 4-3 Result of two way ANOVA (I_P) and ANCOVA (M_P) performed to investigate the impact of E2 treatment and fish sex on *S. solidus* parasite load; parasite index (I_P) and parasite mass (M_P). P values that are significant are in bold, whilst italicised values are near to significant.

Source	I_P			M_P		
	E2 Treatment	Sex	Interaction	E2 Treatment	Sex	Interaction
d.f.	2, 86	1, 86	2, 86	2, 85	1, 85	2, 85
F	3.06	9.38	1.59	2.54	3.31	4.08
P	0.052	0.003	0.21	0.085	0.072	0.02

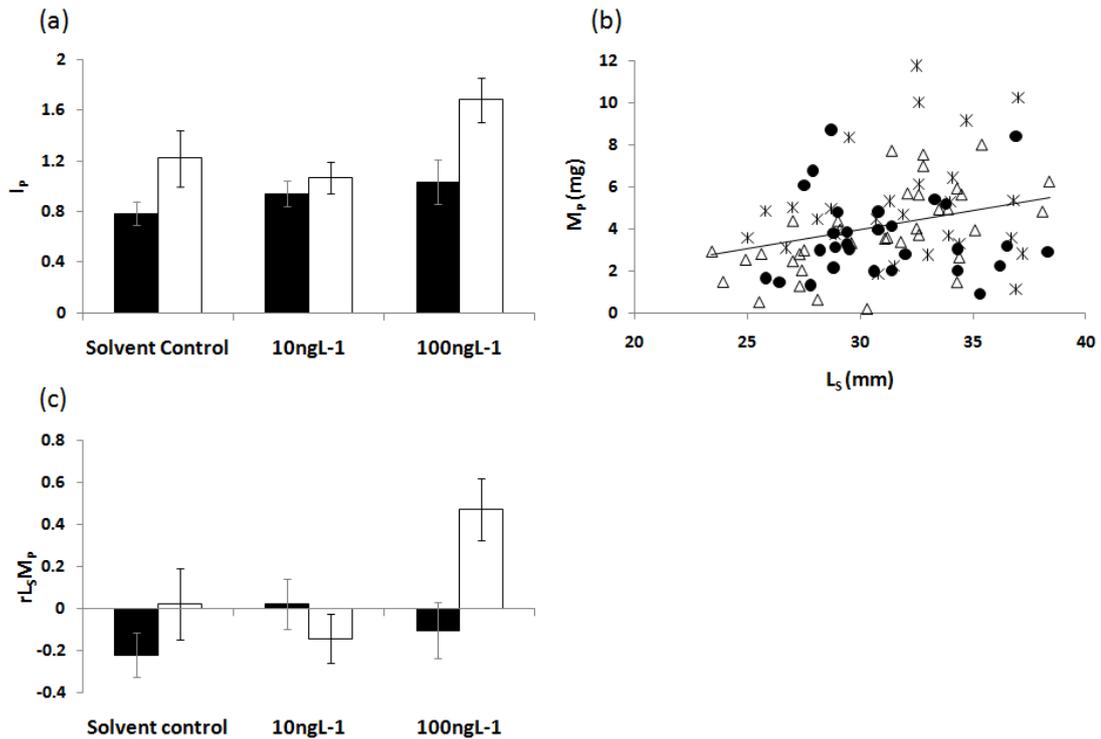


Figure 4.5 The effect of E2 treatment and fish sex on *S. solidus* parasite load (a) the mean \pm s.e. parasite index (I_P) across the three treatment groups (females filled bars, males open bars) (b) the relationship between parasite mass (M_P) and fish length (L_S) in the solvent control (filled circles), 10ngL^{-1} E2 (open triangles) and 100ngL^{-1} (*) treatment group (c) the residuals from the relationship between M_P and L_S ($r_{L_S}M_P$) across the three treatments (females filled bars, males open bars).

Relationships between fish weight ($M-M_P$), length (L_S) and condition indices (K , I_H) with parasite index (I_P)

There was no effect of E2 treatment on the relationships between I_P and size or I_H ($M-M_P$: slope $F_{2,86}=0.8$, $P=0.441$, elevation $F_{2,88}=0.8$, $P=0.412$; L_S : slope $F_{2,86}=1.2$, $P=0.304$, elevation $F_{2,88}=2.6$, $P=0.077$; I_H : slope $F_{2,82}=1.3$, $P=0.271$, elevation $F_{2,84}=1.1$, $P=0.337$; Figure 4.6.a.b.d). Treatment had a significant effect on the relationship between I_P and K with fish from the high E2 treatment being in lower K for a given I_P (slope $F_{2,86}=0.5$, $P=0.617$, elevation $F_{2,88}=4.2$, $P=0.018$; Figure 4.6.c). A significant relationship was found to exist between I_P and $M-M_P$ ($F_{1,88}=17$, $P<0.001$), L_S ($F_{1,88}=14$, $P<0.001$) and K ($F_{1,88}=6.7$, $P=0.011$), but did not exist with I_H ($F_{1,84}=3.3$, $P=0.073$) (Figure 4.6).

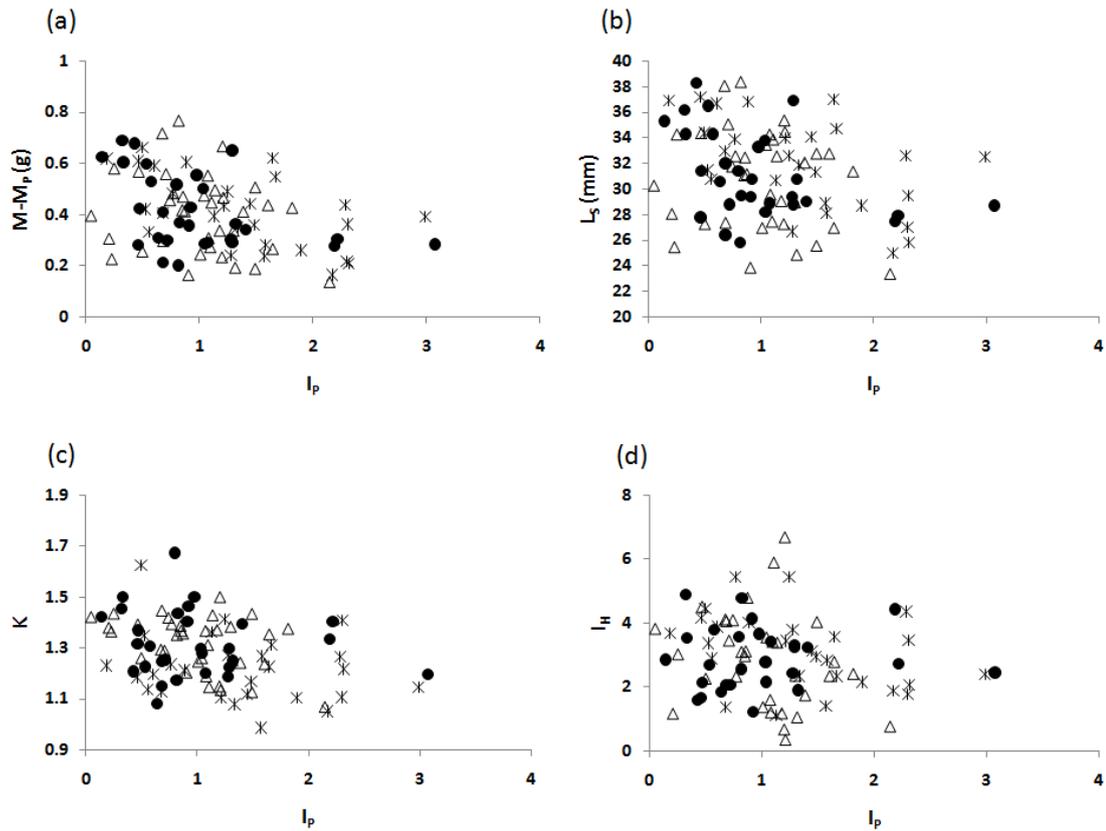


Figure 4.6 Relationships between parasite index and measurements of body condition in experimentally *S. solidus* infected sticklebacks from different E2 treatments. The relationship between parasite index (I_p) and (a) fish mass ($M - M_p$) (b) fish length (L_s) (c) body condition (K) and (d) hepatosomatic index (I_H) in fish from the solvent control (filled circles), 10 ng L^{-1} (open triangles) and 100 ng L^{-1} (*). treatment.

Relationship between vitellogenin production (VTG) and parasite index (I_p)

As the VTG data could not be normalised due to fish in the high E2 treatment having much higher VTG levels compared to the other treatments, ANCOVA analysis using the three treatments could not be performed, but it is clear that the high E2 treatment differed from the other groups. ANCOVA analysis was performed for the low E2 treatment and solvent control group showing that treatment group did not influence the relationship between I_p and VTG levels (slope $F_{1,61}=0.227$, $P=0.635$, elevation $F_{1,62}=0.355$, $P=0.553$; Figure 4.7.a). The relationship between the covariate (I_p) and VTG was not significant ($F_{1,62}=0.3$, $P=0.577$; Figure 4.7.a). A significant positive

relationship existed between I_p and VTG production in the 100ngL^{-1} treatment ($F_{1,25}=4.8$, $r^2=0.162$, $P=0.038$; Figure 4.7.b).

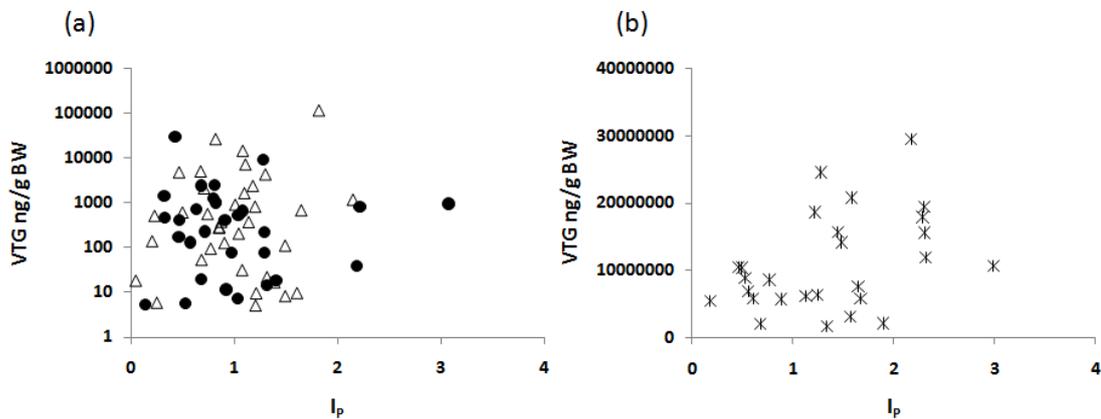


Figure 4.7 Relationships between parasite load and vitellogenin (VTG) levels in experimentally *S. solidus* infected sticklebacks from the different E2 treatments. The relationship between VTG levels and parasite index (I_p) in (a) the solvent control (filled circles) and 10ngL^{-1} E2 (open triangles) treatment and (b) the 100ngL^{-1} E2 (*) treatment.

4.4. Discussion

4.4.1. The impact of E2 treatment on parasite growth, condition and physiological measurements

These results indicate that E2 exposure modifies parasite growth rate and host body condition in a dose and sex dependent manner. Males were in poorer condition compared to females and fish from the high E2 treatment were in the lowest condition. Parasite growth was also highest in males from the high E2 treatment. In the solvent control and low E2 treatment, male fish were significantly smaller than females, but this difference disappeared in the high E2 treatment group, possibly due to the feminising effects of the high E2 treatment.

E2 exposure did not affect hepatosomatic index amongst all fish, but fish sex did have a significant effect. This appears to be due to females in the low E2 group possessing larger livers than males, which is likely to result from increased VTG production in the livers of some females but not males in this group (Clemens, 1974). VTG production was upregulated in the high E2 group, but was not affected by fish sex,

with both males and females exhibiting similar levels. The reduced condition of fish in the high E2 group could result from this increased VTG production as this process will take energy away from somatic growth and development in a similar way that reproduction reduces energy availability for other functions (Tierney et al., 1996, Bagamian et al., 2004). As male physiology has not evolved to deal with high levels of VTG they may suffer disproportionately as a result (Zaroogian et al., 2001).

Negative relationships existed between parasite index and the mass, length and condition of host fish. Fish with higher parasite indices exhibited reduced size and condition, most likely due to the increased nutrient demands of larger parasites. However, it is interesting to note that the plerocercoids recovered were relatively small in comparison to the size they can achieve in their host. The observed plerocercoid weights and indices were <12mg and <3.5% respectively. This parasite becomes infective to its definitive host at 50mg in size (Tierney and Crompton, 1992) and parasite indices of up to 50% have been regularly reported (Arme and Owen, 1967, Smyth, 1994). The study lasted only four weeks after parasite infection, so the parasites were in the early stages of growth and development. It is perhaps surprising that even at this early stage a relationship between condition and parasite index is detectable. It seems likely that this effect would have been enhanced if the study had lasted longer and parasites were allowed to increase in size. Similarly, the lack of a detectable relationship between the hepatosomatic and parasite index may have changed if this study had continued for longer (Arme and Owen, 1967).

4.4.2. Possible mechanisms behind increased parasite size at high E2 levels in males

There are a number of non-mutually exclusive mechanisms that could be responsible for generating our main result – that increased parasite growth occurs in males under the high E2 regime – these will be discussed in the following paragraphs (Figure 4.8).

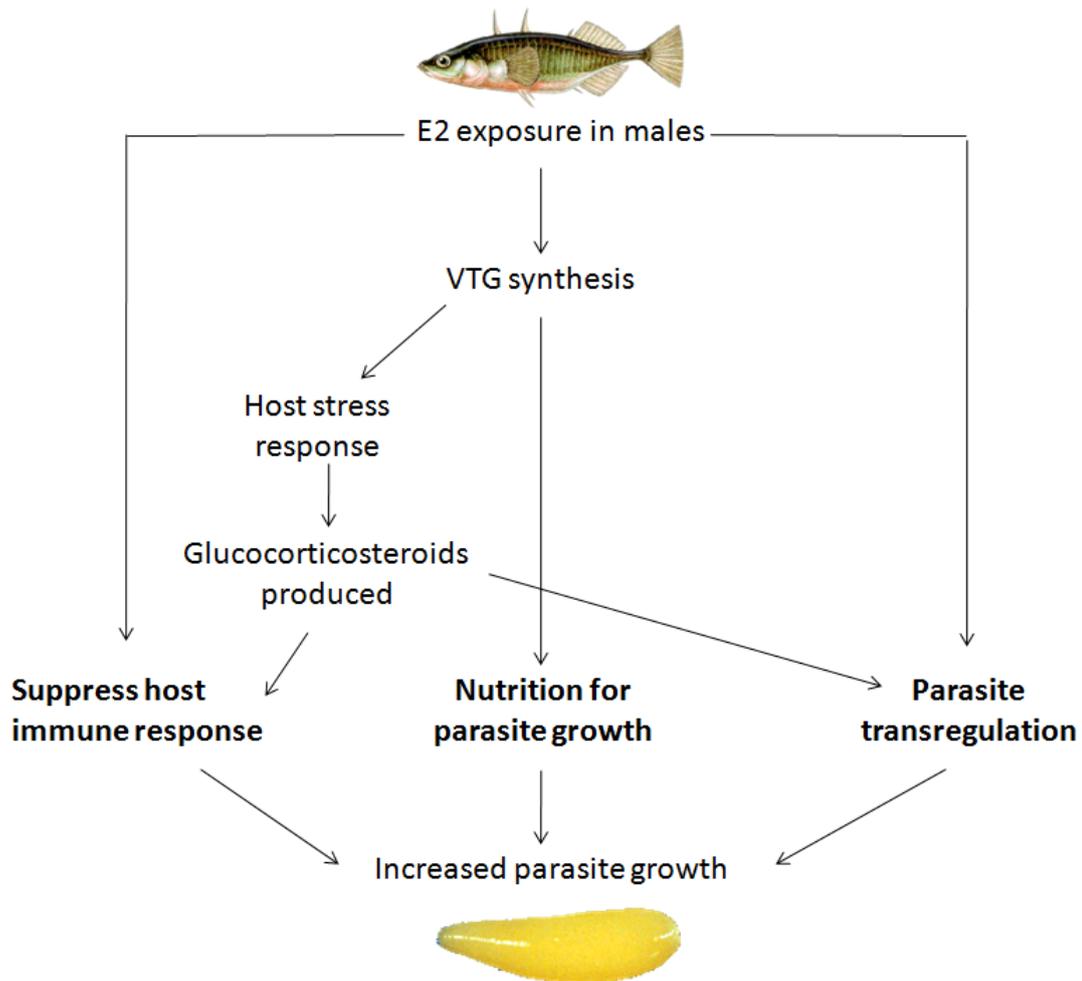


Figure 4.8 The potential mechanisms involved in increased parasite growth in male fish in the high E2 group.

Immunosuppressive effects of estrogens

Exposure to high levels of E2 may interfere with the immune response of the fish. It is now well recognised that bidirectional interactions occur between the endocrine and immune systems; the existence of hormone receptors on immune cells re-emphasises

this (Weyts et al., 1999, Ahmed, 2000, Thilagam et al., 2009). Early studies indicated that E2 had an immunosuppressive effect. Goldfish (*Carassius auratus*) fitted with implants that produced physiologically relevant levels of E2 in serum exhibited significantly reduced lymphocyte proliferation and suffered from higher parasitemia and mortality when they were challenged with the hemoflagellate *Trypanosoma danilew* (Wang and Belosevic, 1994). More recent studies have indicated that estrogens have many different effects on fish immune systems (Milla et al., 2011), including reductions in circulating lymphocytes and an increase in granulocytes (Filby et al., 2007), enhanced respiratory burst activity (Thilagam et al., 2009), the suppression of immune genes (Williams et al., 2007) and a reduction in phagocytic activity (Watanuki et al., 2002). However results are often not consistent between species and between estrogens (Thilagam et al., 2009, Milla et al., 2011).

The discovery that estrogens can modulate the immune response has led to concern regarding environmental chemicals that are putative EDCs. In one study, four commonly used herbicides, recognised as possible EDCs (Atrazine, Simazine, Diuron, Isoproturon; ASDI) were mixed together and administered to goldfish at environmentally relevant concentrations which showed an increase in non specific immunity, whilst the specific immune response was reduced (Fatima et al., 2007). When fish treated with the ASDI mixture for 12 weeks were subjected to an additional stressor, the pathogen *Aeromonas hydrophila*, lysozyme activity decreased in response to higher pathogen doses in contrast to control fish whose lysozyme activity increased.

Fish in the high E2 group therefore may be undergoing modulation of their immune response. Immune responses were not measured in this study; however if immunomodulation by estrogens was solely responsible for increased parasite growth, females in the high E2 treatment should also exhibit higher parasite indices but this was

not the case. It is possible that high levels of E2 are not as immunosuppressive to females, which have evolved to withstand high levels of circulating estrogens in the blood during vitellogenesis.

It is possible that sex differences in immunity are operating here, with males and females differing in their immune responses. The prevalence of parasitic infections is typically higher in males compared to females, in both humans and animals (Folstad and Karter, 1992, Klein, 2004). This appears to be due to females having stronger immune responses than males (Ahmed, 2000), and the immunosuppressive effects of testosterone (Folstad and Karter, 1992, Zuk and McKean, 1996, Klein, 2004). Another line of thought is based upon Bateman's principle, whereby males gain fitness by increasing their number of mating events, and females increase their fitness via longevity (Rolff, 2002). It is therefore proposed that females should invest more in immunity than males (Rolff, 2002). However, if differences in parasite load were associated solely with sex differences in immunity, males in all E2 and control groups would be expected to harbour larger parasites. Although there was a significant effect of sex on parasite index, when the analysis was performed with parasite mass these groups didn't appear to have significantly larger parasites.

Vitellogenin (VTG) production in males

Pathological effects of VTG production: Males do possess the gene for VTG synthesis, but in general won't produce measurable amounts as this is an estrogen dependent protein that is utilised by females to generate egg yolk (Folmar et al., 2001). Estrogens lead to the production of VTG in the liver and this is transported via the blood to the ovaries where it will enter the developing oocyte and is stored for subsequent use by the developing embryo (Clemens, 1974, Purdom et al., 1994). Thus male fish have not

evolved to utilise VTG and have no capacity to store the protein (Zaroogian et al., 2001).

Studies have illustrated that VTG synthesis in males can lead to a build up of VTG material in the kidney and liver (Folmar et al., 2001, Zaroogian et al., 2001, Hahlbeck et al., 2004) and this can lead to adverse health effects (Zaroogian et al., 2001, Thorpe et al., 2007). In the present study kidney and liver pathology were not studied and the concentrations used in the above studies were a lot higher than the present study, therefore it is unlikely that these changes would have been seen to this extent here. However the studies do indicate that in males, increased VTG synthesis has negative health effects. Folmar et al. (2001) stated that lower estrogenic concentrations could potentially lead to liver and kidney function being impaired leading to a reduction in the ability to resist disease. Interestingly, VTG has recently been shown to have an immune function in teleosts; with the protein being capable of recognition of microbes and initiating phagocytosis (Li et al., 2008). However, in the present study increased VTG production did not lead to a decrease in disease progression rates.

VTG production as a stressor: Stress is defined as a condition in which the equilibrium of homeostasis is disrupted in a living organism, due to internal or external factors known as stressors (Wendelaar Bonga, 1997). Fish react to a stressor with a suite of behavioural and physiological responses known as the stress response. A stress response results in the release of catecholamines (CAs) and glucocorticosteroids (GS; cortisol is the major corticosteroid in fish), both of which cause a range of physiological effects. They are involved in the increased uptake of oxygen across the gills, and changes in the allocation of energy (gluconeogenesis) away from activities such as growth and reproduction; instead the energy is used to restore homeostasis via actions

on processes such as respiration and tissue repair (Wendelaar Bonga, 1997, Weyts et al., 1999). Exposure to EE2 resulted in an increase in cortisol and cortisone in the gonads of roach (*Rutilus rutilus*) and the authors proposed this may cause some of the gonad histopathology observed during estrogen exposure (i.e. feminisation) (Flores-Valverde et al., 2010). Perch (*Perca flavescens*) and pike (*Esox lucius*) sampled from sites contaminated with polycyclic aromatic hydrocarbons, polychlorinated biphenyls and mercury were unable to elevate their cortisol levels in response to the stress of capture (Hontela et al., 1992). In addition, their pituitary corticotropes that release corticotrophin and lead to cortisol production in the interrenal glands seemed to be atrophied. Conversely, fish from reference sites showed elevated cortisol levels in the range expected for acute stressors (>100ng/ml). The inability of fish to raise their cortisol levels in response to chronic pollutant stress may be due to the exhaustion of the stress response probably as a result to prolonged hyperactivity.

In the present study cortisol levels were not tracked. However, under normal conditions males would not experience exposure to these high E2 levels and its associated VTG synthesis can lead to pathological effects (Zarogian et al., 2001). Therefore it could be proposed that these conditions are disturbing homeostasis and inducing a stress response in males in the high E2 treatment. If this were the case the suppressive action of glucocorticosteroids on the immune response could be causing the increased parasite load that we see (Maule et al., 1989, Wendelaar Bonga, 1997, Weyts et al., 1999).

Increased nutrient availability for parasites: VTG is a protein that could essentially be used by the parasite for growth. As mentioned, male fish do not normally produce VTG and thus lack an organ for the storage and utilisation of it in contrast to females which

incorporate VTG into their ovaries (Zaroogian et al., 2001). Therefore in male fish taken from the high E2 group there may be high levels of VTG available to the growing parasite compared to females. Male fish could also benefit by reducing VTG levels and one way would be by actively transferring the protein to the parasite. Therefore both the fish and parasite may benefit from the transport of VTG to the parasite at an early stage. However, in this study infected fish did not have reduced VTG levels compared to non infected conspecifics. Yet there is evidence to suggest that VTG is involved in parasite growth, as fish with the highest VTG levels had bigger parasite indices. It may be that these fish were in poorer condition enabling the parasite to grow faster, however no relationship between body condition and VTG levels was detected in infected fish. Conversely, if the study had lasted longer larger parasites may have resulted in reduced VTG levels in infected fish. As described in the introduction some acanthocephalans can accumulate heavy metals, reducing levels in their host (Sures, 2004, Sures, 2007). It is possible that a similar transport mechanism exists in the stickleback-*Schistocephalus* system whereby VTG is accumulated by the cestode.

Cestodes do not possess a digestive tract and thus nutrients must pass across their external body surface before they can be utilised (Pappas and Read, 1975). Molecules can be transported into cestodes by diffusion, facilitated diffusion and active transport (Charles and Orr, 1968, Pappas and Read, 1975). Pinocytosis was also considered to be used by *S. solidus* to internalise macromolecules (Hopkins et al., 1978, Threadgold and Hopkins, 1981) however this was later disputed by Conradt and Peters (1989). Polzer and Conradt (1994) illustrated aminopeptidase activity in the surface tegument of plerocercoids of *S. solidus*. They suggest that the parasite utilises this to degrade proteins as they are not capable of ingesting such macromolecules. Nutrition in cestodes is mainly through the absorption of monosaccharides and amino acids (Pappas

and Read, 1975, Polzer and Conradt, 1994), thus proteins like VTG could be broken down into amino acids before being transported into the cestode for utilisation. *S. solidus* inhabits the body cavity of the stickleback, and what still remains unclear is the exact mechanism by which they obtain their nutrients. Their position alongside internal organs like the liver and gut may facilitate their access to host nutrients or nutrients present in the perivisceral fluid of the fish may be used. In addition the parasite could tap into the blood circulatory or excretory system to obtain their nutrients. The answers to these questions remain unclear, however the fact that the parasite can reach such a large size in its fish host illustrates that they are highly adapted to obtaining nutrients. Thus it seems possible that the parasite can utilise available VTG during its growth, and with a more readily obtainable supply of VTG in males compared to females; this could explain the increased parasite size that we see in male fish from the high E2 treatment.

Parasite Transregulation

Another possible mechanism that could lead to increased parasite growth is that the parasite could directly use the host's hormonal environment to increase its own growth and reproductive rates in a process called transregulation (Escobedo et al., 2005). The effects of host sex steroids on parasite growth are often thought to be indirect, via the regulation of the immune response (Escobedo et al., 2004). However host-derived sex steroids have also been shown to have direct effects on parasites *in vitro*. E2 promoted the proliferation of *Taenia crassiceps* cysticerci *in vitro* and it was shown that isoforms of the classic estrogen receptor (ER- α and ER- β) were expressed by the parasite (Escobedo et al., 2004). Maunder et al (2007b) illustrated that sticklebacks exposed to E2 had high bioaccumulation levels of the steroid; however it was similar across the sexes. If male fish somehow had increased estrogen availability in the high E2

treatment then there is a possibility that this hormonal environment could have a direct effect on parasite growth. The direct effects of adrenal hormones on parasites have also been documented. *In vitro* culture with cortisol increased the growth of *Entamoeba histolytica* trophozoites (Carrero et al., 2006), whilst a different study showed that cortisol reduced the viability of cercariae, schistosomula, adult worms and egg laying of *Schistosoma mansoni* (Morales-Montor et al., 2001). If cortisol levels were elevated in males during the high E2 treatment, then there is a possibility that the parasite could be utilising this in some way to increase its growth; *in vitro* studies with the parasite are required to elucidate this.

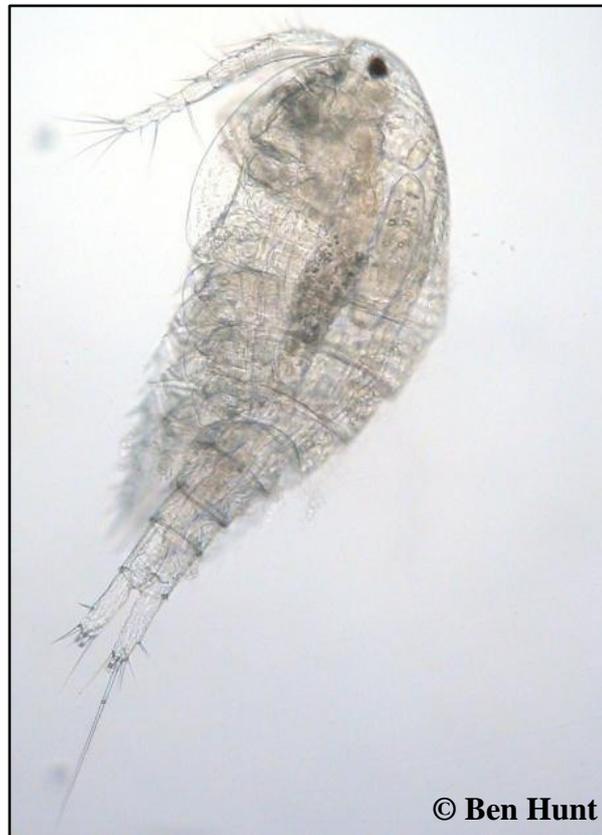
4.4.3. Conclusions and future studies

Treatment with environmentally relevant concentrations of E2 (10ngL⁻¹) for six weeks did not affect health aspects of the fish significantly, while exposure to higher concentrations (100ngL⁻¹) had a significant impact on condition, VTG production and parasite load. Male fish in the high E2 treatment developed significantly larger parasites for their size compared to female fish in this group and all other treatments. Although it is unlikely that fish would experience such high E2 concentrations in the wild (although concentrations higher than the low E2 concentration have been recorded in the wild; Duong et al., 2010, Yang et al., 2011) these results indicate some of the potential effects that estrogens can have on host-parasite interactions in general and raises questions on its mode of action. Furthermore, since fish are almost never exposed to a single environmental chemical but a mixture of tens or even hundreds of them simultaneously (Sumpter, 2009), it is possible that environmentally relevant lower concentration chemical mixture scenarios may exert similar effects. In addition, it would be interesting to investigate intermediate concentrations to identify the point at which estrogens begin to affect the condition of the fish and their defence against parasites. It

will also be important to perform studies that expose fish to E2 from hatching through to parasite infection, since long term exposure to environmentally relevant concentrations of E2 may produce similar effects. The most likely mechanism for increased parasite growth in males at high E2 levels seems to be that parasites are able to use VTG as a nutrient for growth. Future studies investigating VTG levels in parasites from E2 treated fish are needed to confirm this mechanism. Another strong possibility is that the pathological effects of high E2 / VTG levels in males that have not evolved to handle them causes a stress response that adversely affects immunity. However other mechanisms that cannot be ruled out include the chemicals having a direct toxic effect on immune parameters and parasites directly using the host's hormonal environment to increase in size.

Importantly this study indicates that E2 exposure had sex-dependent health implications. If wild fish were experiencing estrogenic conditions that led to increased parasite loads in males, this could have serious implications at a population level. This is especially true if the parasites reduce the reproductive capability of male fish (Chapter 2 and 3), as alongside EDCs this may render fish unable to reproduce. Future studies are required to elucidate the mechanisms behind this and investigate the effects of different concentrations / chemicals to reveal the true risk that EDCs pose to disease resistance in the wild.

5. The effect of temperature on disease progression in the stickleback-*Schistocephalus* system



5.1. Introduction

A host and its parasites co-exist over evolutionary time scales, leading to adaptations evolving in hosts to remove or avoid parasites, and counter adaptations in parasites to facilitate their survival in the host (Poulin, 1995, Lafferty, 1999, Mostoway and Engelstadter, 2011). Therefore the outcome of a host-parasite interaction will depend on the battle between resistance and virulence (Lafferty, 1999, Mostoway and Engelstadter, 2011). However, it is also becoming clear that environmental conditions have important effects on the outcome of a host-parasite interaction (Thomas and Blanford, 2003).

Climate change and the temperature increases associated with it have considerable implications for host-parasite interactions (Harvell et al., 2002). This is particularly true for ectothermic hosts, as their body temperature matches their external environment, which in turn denotes the conditions under which a parasite must survive and develop under (Thomas and Blanford, 2003, Deutsch et al., 2008, Ward et al., 2010). The ability for ectotherms to perform a particular function, such as reproduction or growth, over a range of different temperatures is shown by a thermal performance curve, with minimum, maximum and optimum temperatures for any given physiological process (See Introduction section 1.2.3.1.) (Deutsch et al., 2008). This curve also exists for parasites, and if the parasite and host curves overlap, changes in temperature will affect both in the same direction. However, these curves may show some degree of separation meaning that altered temperatures may have different impacts on the success of hosts and their parasites (Thomas and Blanford, 2003). For example, locusts (*Schistocerca gregaria*) maintain their body temperature at 38-40°C, whilst their natural fungal pathogen *Metarhizium anisopliae* var. *acridum* grows optimally at 27-30°C (Elliot et al., 2002).

In the wild it is difficult to disentangle the direct effects of elevated temperatures on host-parasite interactions since many other stressors exist alongside temperature, and controlled experimental studies remain the best way of investigating such questions. The present study uses this approach in the stickleback-*Schistocephalus* host-parasite system. The cestode *Schistocephalus solidus* has a three host life cycle, being transmitted trophically from a cyclopoid copepod to a three spined stickleback (*Gasterosteus aculeatus*) and finally reproducing in its definitive host, usually a piscivorous bird (Smyth, 1946). Whilst in its stickleback host, plerocercoids can grow to large sizes and have considerable impacts on the fitness of the host (Arme and Owen, 1967, Milinski, 1985, Tierney et al., 1996, Rushbrook et al., 2007, Heins and Baker, 2008). Studies have already shown that sticklebacks, one of the UKs hardiest fish species, are adversely affected by temperature with a rise of 4°C reducing the population biomass of sticklebacks by 60% in experimental mesocosms (Moran et al., 2010). Therefore it seems likely that temperature will influence host-parasite interactions in this species.

The size of *S. solidus* plerocercoids dictates parasite fitness with plerocercoids consistently becoming infective to their avian definitive host on reaching 50mg in size (Tierney et al., 1996). Furthermore the reproductive potential of the adult worm is also governed by parasite size, with larger plerocercoids producing more eggs as adults (Dörücü et al., 2007). Because plerocercoid mass correlates negatively with host fitness and positively with parasite fitness, the growth rate attained by *S. solidus* plerocercoids indicates the outcome of conflict between the host and its parasite.

In this study, we test the hypothesis that altered temperatures affect the outcome of this conflict, with consequences for parasite growth that potentially impact the rate of parasite life cycle completion. To test the effect of temperature, lab-bred sticklebacks

that had been experimentally exposed to *Schistocephalus* infected copepods or sham exposed to non infected copepods by feeding, were housed at either 15°C or 20°C over an 8-week post-exposure period. Temperature regimes were selected in line with predictions of a rise in temperature in temperate regions of 3-5°C over the next 100 years (IPCC, 2007). Three key questions were addressed: (1) how do elevated temperatures affect the growth of parasites and hosts in the stickleback-*Schistocephalus* system; (2) what are the likely mechanisms underpinning temperature effects on patterns of parasite and host growth; (3) what are the implications of temperature-associated differences in parasite growth for reproductive output of parasites.

5.2. Materials and methods

5.2.1. Experimental infections

Single infective *S. solidus* plerocercoids (i.e. > 50mg; Tierney and Crompton, 1992) were dissected from naturally infected wild caught sticklebacks from Carsington Reservoir (Derbyshire, UK; N53°03'21", W1°37'25") and cultured *in vitro* to produce eggs (Smyth, 1946, Wedekind, 1997). Eggs were then collected, washed and incubated in the dark for three weeks. Laboratory reared copepods (*Cyclops strenuus abyssorum*) were exposed to a single hatched coracidium. After visual inspection to identify that the coracidium had been eaten, copepods were kept individually in 5ml of water and screened 7 days post exposure (dpe) to check infection status and infectivity based on the presence of a cercomer (Smyth, 1969). Infective copepods were exposed to the fish from 15-17 dpe (See chapter 3 section 3.2.2 for detailed experimental infection protocol).

Laboratory bred sticklebacks were generated from crosses of wild caught adults from Carsington reservoir using IVF techniques (Barber and Arnott, 2000), and reared in four family groups under a day length period of 16L:8D and a temperature of

18±1°C. At 6 months of age (0.235g ± 0.05 SD), fish were randomly sham exposed to a non infected copepod or exposed to an infected copepod in a small 1.2L aquarium (15.5x9.5x8.5cm).

5.2.2. Temperature treatments

Large glass aquaria (60x40x30cm) were used as water baths, each housing up to six smaller plastic tanks (17x19x19cm; 6L) containing individual, randomly placed exposed or sham-exposed sticklebacks. There were five replicate water baths for each temperature treatment. A re-circulating, temperature-regulated water supply was used in conjunction with thermostatically controlled aquarium heaters to maintain a mean (±sd) water temperature at 20.0 ± 0.47°C in the baths housing the high-temperature ('20°C') treatment fish and 14.8 ± 0.22°C in those housing low-temperature ('15°C') treatment fish. Water in the small plastic tanks housing fish was continually filtered and aerated via airlift sponge biofilters and temperature in each water bath was recorded daily.

5.2.3. Experimental design

Fourteen exposed and 12 sham-exposed fish were held under the 20°C treatment, and 13 exposed and 12 sham-exposed fish were held under the 15°C treatment, for 8 weeks. During this time, fish were fed *ad libitum* to excess daily, with frozen bloodworms (*Chironomus* sp. larvae) under a photoperiod of 11L: 13D. Plerocercoid growth causes an increase in the dorsal profile area of infected fish (Barber, 1997) that can be used to track *in vivo* parasite growth (Barber and Svensson, 2003). Fish were measured, weighed and photographed in dorsal profile for subsequent quantification of parasite-induced swelling at weeks 0, 3, 6 and 8 (see below). Three fish (2 exposed, 20°C; 1 sham-exposed, 20°C) died before completion of the study and are not included in the analysis.

5.2.4. Data collection

At the end of the study, fish were killed using a lethal dose of Benzocaine anaesthetic (Stock solution: 10g prepared in 1L of 70% EtOH) before being blotted, measured (standard length, L_S , to 0.1mm) and weighed (wet mass, M , to 0.001g). Fish were frozen individually in zip-lock bags at -20°C for 2d, thawed and dissected to allow the blotted mass of the liver (M_L) and spleen (M_S) to be measured (to 0.0001g) and the sex and infection status of the fish to be recorded. Any plerocercoids recovered were also weighed (M_P , to 0.0001g). Parasite index (I_P), which describes parasite mass as a proportion of infected host mass, was calculated as $I_P = 100(M_P/M - M_P)$. Fish body condition factor (K) was calculated as $K = [(M - M_P)L_S^{-3}] \times 10^5$ (Pennycuik, 1971) and hepatosomatic (I_H) and splenosomatic (I_S) indices were calculated using the equation $I_x = 100[M_x(M - M_P)^{-1}]$, where x refers to the organ under consideration.

Dorsal profile photographs allowed the extent of the parasite induced swelling to be quantified. *ImageTool 3.0* (available at <http://ddsdx.uthscsa.edu/dig>) was used to quantify the dorsal profile area (A) of each fish at each sampling point (Barber, 1997, Barber and Svensson, 2003). Residual values from the significant relationship between square root transformed A ($A^{1/2}$) and standard length were then calculated to produce an index of swelling ($rA^{1/2}$).

The specific growth rate (G) of each individual fish between week 0 and 8 was calculated as $G = 100(\ln(M - M_P) - \ln(M_0))/t$, where M_0 is the mass at the start of the experiment and $(M - M_P)$ is the parasite-corrected mass of the fish at the end of week 8 and t represents the number of days between these measurements (Barber and Svensson, 2003). As expected, M_0 and G correlated significantly and negatively ($G = -2.861.M_0 + 2.077$, $r^2 = 0.25$, $P < 0.001$), so analysis is carried out on residual values (rG).

Plerocercoid mass and adult fecundity (i.e. egg output, F) of *S. solidus* in culture are related by the equation $\log_{10}(F)=7.33 + 2.11*\log_{10}(M_P)$ (Dörücü et al., 2007). We used this equation to estimate the number of eggs that would have been produced by adult worms developing from all plerocercoids grown in sticklebacks experiencing the divergent thermal regimes in this study.

5.2.5. Statistical analysis

Proportional data (I_P , K , I_H , I_S) were arcsine square root transformed and tested for normality using the Kolmogorov-Smirnov statistic. A mixed between-within subjects ANOVA was carried out to investigate if there was an effect of exposure status (non exposed / exposed non infected / exposed infected), temperature treatment (15°C / 20°C) or week number (weeks 0, 3, 6 and 8) on parasite-induced swelling (i.e. $rA^{1/2}$). Differences in I_P and absolute plerocercoid mass recovered from hosts reared under the high and low treatments, and the estimated egg output of adult parasites developing from these plerocercoids, were tested using parametric t-tests. The effects of temperature treatment (15°C / 20°C) and exposure status (non exposed / exposed non-infected / infected), and their interactions, on fish growth and indices of condition were tested using 2-way ANOVA. All statistical analyses were carried out using SPSS (version 18).

5.3. Results

5.3.1. Effect of temperature on parasite establishment, growth and estimated adult fecundity

Temperature treatment had no effect on the establishment of parasites in exposed fish; infections developed in ~50% of exposed fish held under both temperature treatments (20°C: 7/12, 15°C: 6/13). No sham-exposed fish developed infections.

There was a significant interaction effect between week number, exposure group and treatment on the dorsal profile area ($rA^{1/2}$; $F_{6,80}=2.28$, $P=0.044$; Figure 5.1.a). Therefore the effect that week number had on $rA^{1/2}$ depended on the exposure and treatment group. By week 8 infected fish had dramatically larger $rA^{1/2}$ than non exposed and exposed non infected fish under the 20°C treatment and compared to infected fish held under the 15 °C treatment (Figure 5.1.a). On dissection, infected 20°C fish were found to harbour significantly larger plerocercoids than infected 15°C fish ($t_{11}=-6.13$, $P<0.001$; Figure 5.1.b) and had a correspondingly higher I_p ($t_{11}=-7.8$, $P<0.001$; Figure 5.1.c).

Using the published relationship linking plerocercoid mass to adult fecundity (Dörücü et al., 2007), the mean egg output of adult worms developing from plerocercoids after 8 weeks growth in hosts kept at 15°C was estimated to be $12,253 \pm 3719$, compared with $199,574 \pm 42,832$ from those developing from those growing in hosts at 20°C ($t_{11}=-4.35$, $P=0.005$).

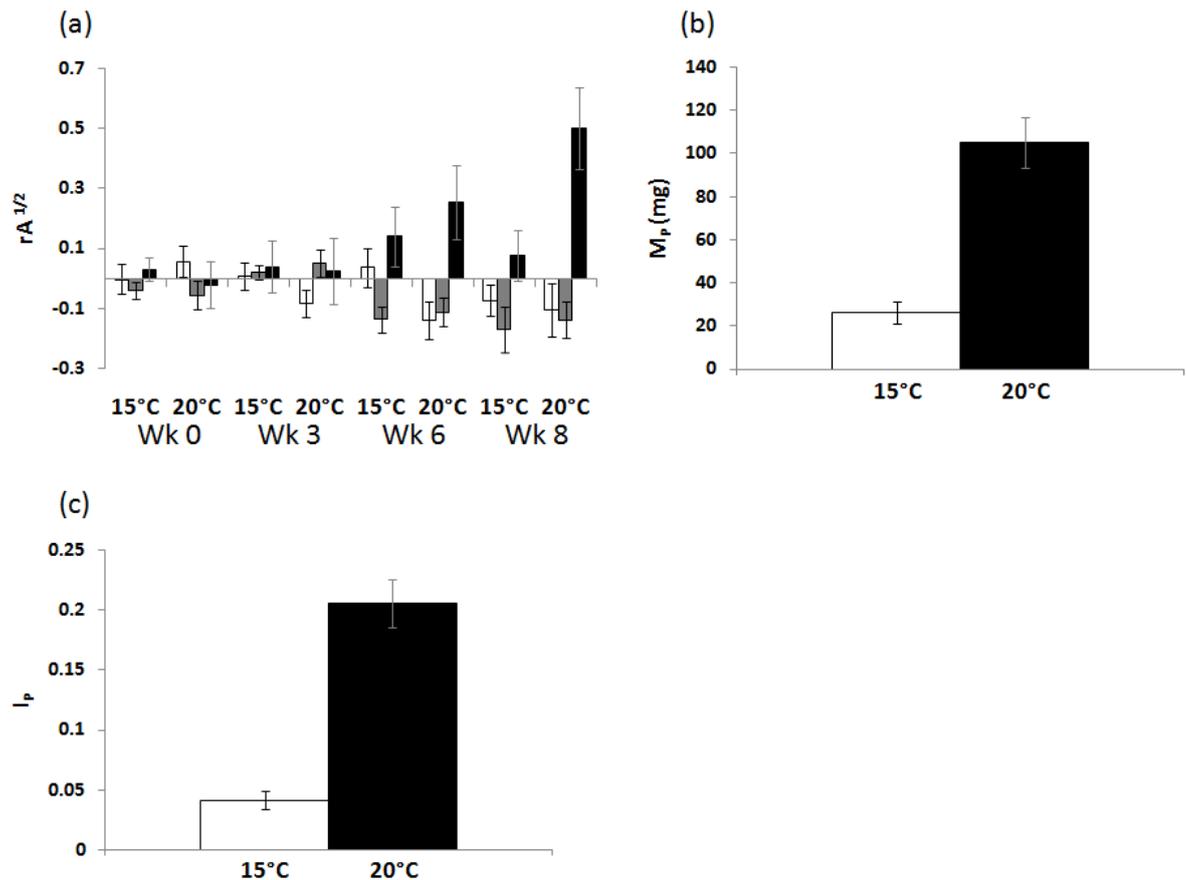


Figure 5.1 The effect of thermal regime on disease progression in sticklebacks. The mean \pm s.e. (a) dorsal profile area ($rA_{1/2}$) in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) fish at weeks 0, 3, 6 and 8 at 15°C and 20°C (b) parasite mass (M_p) and (c) parasite index of plerocercoids taken from fish held at 15°C and 20°C.

5.3.2. Effect of temperature on fish growth and condition

There was a significant main effect of rearing temperature on fish growth (calculated as rG), with 20°C fish growing more slowly than those held at 15°C (Table 5.1; Figure 5.2.a). There was no significant main effect of infection status or interaction effect. As a previous study by Guderley and Leroy (2001) showed that family (i.e. full sibling fish) influenced growth under different temperature regimes, the effect of family and temperature treatment on growth was tested (exposure status could not be included, as each group did not incorporate all of the families). There was a significant main effect of temperature treatment and family, but no interaction effect existed (Table 5.1; Figure 5.2.b). Both results remained significant when Bonferonni adjusted ($\alpha=0.025$).

Table 5-1 Results of two way ANOVAs investigating the impact of temperature treatment (15oC / 20oC) and exposure status (non exposed / exposed non infected / infected) on growth and the effect of family and temperature treatment on growth (rG). Significant P values are in bold.

	rG			rG		
Source	Temp treatment	Infection status	Interaction	Temp treatment	Family	Interaction
d.f.	1,42	2,42	2,42	1, 40	3, 40	3, 40
F	11.0	1.2	0.3	17.4	7.9	1.4
P	0.002	0.318	0.719	<0.001	<0.001	0.253

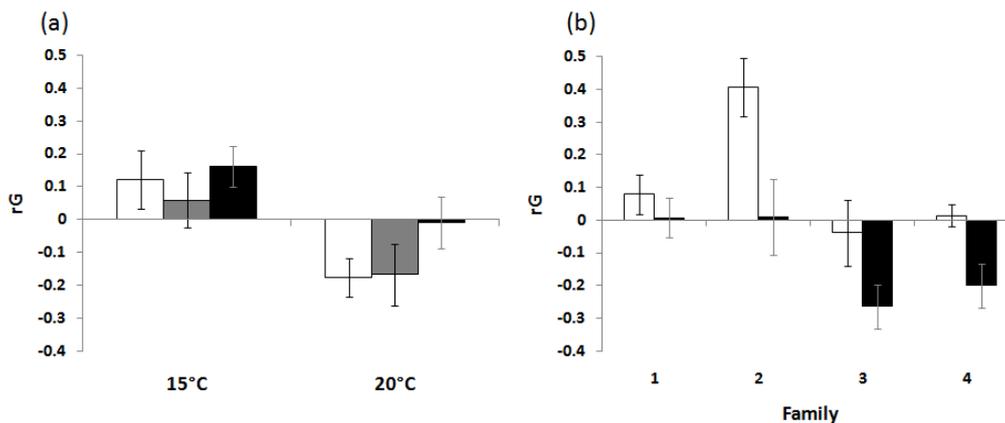


Figure 5.2 The effect of temperature regime on growth in sticklebacks. (a) The effect of exposure status and rearing temperature on growth (rG) in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) fish taken from the 15°C and 20°C treatment; bars are means \pm s.e. (b) The effect of family on growth in sticklebacks held at 15°C (open bars) and 20°C (filled bars); bars are means \pm s.e.

There was a non-significant trend ($P < 0.055$) for parasite exposed fish, and those kept under 20°C, to exhibit reduced I_H , and for infected fish to have a higher K than parasite exposed non infected fish (Table 5.2.; Figure 5.3.a.b). There were no significant main effects of rearing temperature or infection status on I_S of the fish (Table 5.2.; Figure 5.3.c). Furthermore the interaction effect between treatment and infection status was not statistically significant with respect to host I_H , I_S or K (Table 5.2.).

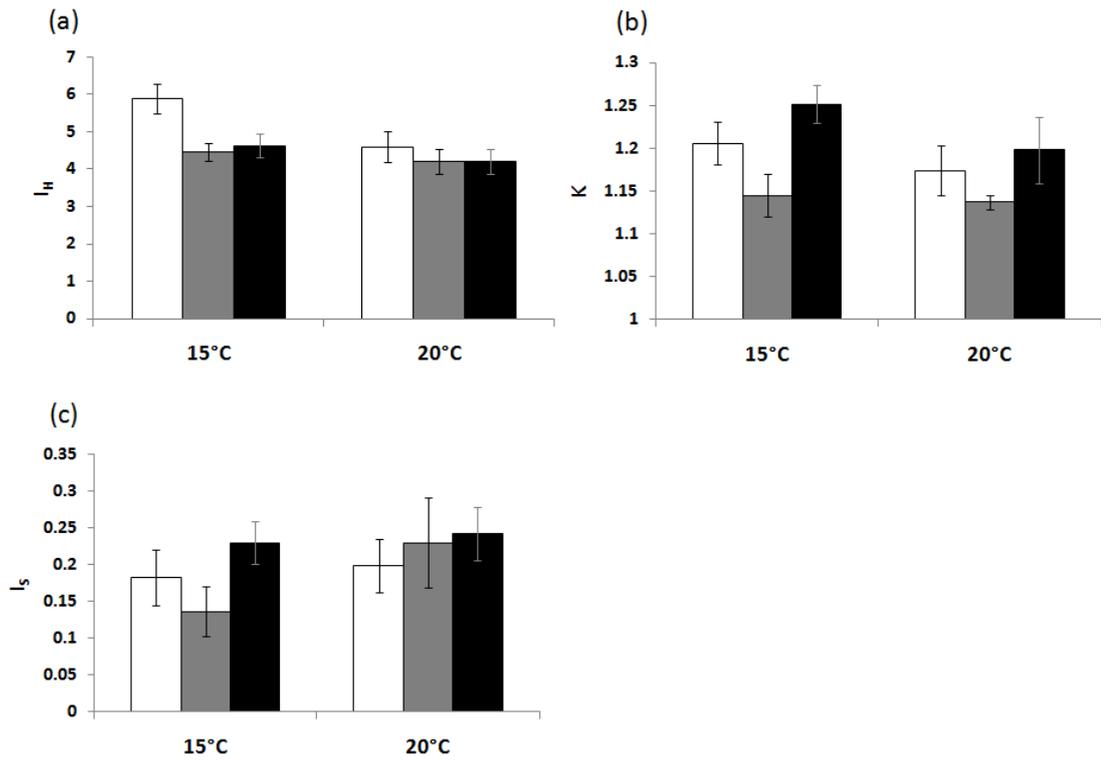


Figure 5.3 The effect of exposure status and rearing temperature on indices of stickleback condition. Mean \pm s.e. (a) hepatosomatic index (I_H); (b) condition (K) and (c) spleen index (I_S) in non exposed (open bars), exposed non infected (grey bars) and infected (black bars) fish taken from the 15°C and 20°C treatment.

Table 5-2 Results of two way ANOVAs investigating the impact of temperature treatment (15oC / 20oC) and exposure status (non exposed / exposed non infected / infected) on indices of condition; hepatosomatic index (I_H), condition (K) and splenosomatic index (I_S). Significant P values are in bold, whilst italicised values are near to significant.

	I_H			K			I_S		
Source	Temp treatment	Infection status	Interaction	Temp treatment	Infection status	Interaction	Temp treatment	Infection status	Interaction
d.f.	1, 42	2, 42	2, 42	1, 42	2, 42	2, 42	1, 40	2, 40	2, 40
F	4.0	3.2	1.0	1.6	3.2	0.3	1.6	1.4	0.6
P	<i>0.054</i>	<i>0.053</i>	0.379	0.216	<i>0.053</i>	0.778	0.207	0.269	0.542

5.4. Discussion

Temperature regime had a dramatic effect on the growth of *S. solidus* plerocercoids, which in turn will affect the rate of completion of the parasite life cycle. All parasites taken from fish held at the high temperature regime had reached infectivity after 8 weeks, whereas none taken from the fish held at 15°C had done so. Critically, the greater size that plerocercoids reached at 20°C predicts that these parasites would have a higher reproductive potential at any given age; at least an order-of-magnitude increase in the output of infective stages of *S. solidus* into aquatic environments is predicted (Dörücü et al., 2007). In addition, temperature had an important effect on host growth, with both control and exposed fish growing significantly slower at higher temperatures. The potential mechanisms underlying this, and the ecological significance, will now be discussed.

5.4.1. What underlying mechanisms might generate increased parasite growth at higher temperatures?

A number of possible mechanisms may be responsible for increased parasite growth at elevated temperatures. These mechanisms, which are not mutually exclusive, include the acquisition of nutrients by growing parasites, effects of immune and stress responses or differences in thermal performance of hosts and their parasites (Figure 5.4.).

Acquisition of nutrients by growing parasites

First, the change in thermal regimes may have influenced the food intake or conversion efficiency of host sticklebacks (Guderley and Leroy, 2001), leading to temperature-associated differences in the availability of nutrients to growing parasites. In this study, there was an effect of temperature on the growth rate of the fish with those in the 15°C

treatment growing faster than those held at elevated temperatures. One explanation for these findings is that at the higher temperature, energy was directed towards maintaining the high metabolic rates that are associated with increased temperature, rather than sustaining growth. This is in contrast to a study by Allen and Wootton (1982) who showed that growth rate of sticklebacks increased with temperature, whilst Guderley and Leroy et al. (2001) showed that four out of six stickleback families studied grew better and had higher food conversion efficiencies at 8°C compared to those at 23°C. This suggests at low temperatures more energy was put into growth compared to at 23°C where more energy would be spent maintaining the high metabolic rate. In the present study, all of the families were found to grow better at cooler temperatures. In addition, analysis of the hepatosomatic index (I_H), a measure of medium-term energy reserves in fish (Chellappa et al., 1995), also supports this; with fish in the 20°C treatment having lower I_H s (near to significant) suggesting that these fish had less energy available to support growth. In the 15°C treatment, non exposed fish had the highest I_H , whilst exposed individuals had comparably lower I_H 's. This could be due to the nutritional requirements of the parasite and immune activation in exposed fish. In the 20°C treatment the unexposed fish had similar I_H 's to the exposed fish, suggesting that elevated temperatures do reduce short term energy reserves in unexposed fish; probably due to the increased metabolic rate at higher temperatures. It is unclear why the exposed fish don't have a reduced I_H at 20°C compared to their counterparts held at 15°C. It may be that exposed fish sit at a baseline level with regard to I_H , and that this cannot be reduced any further. Alternatively, increased feeding may be governed by an increased metabolic rate (Guderley and Leroy, 2001) and by the parasite / immune system activation, keeping the I_H at a similar level to those in the low treatment. In the present study, fish were fed *ad libitum*, allowing those at higher

temperatures to increase their food intake to meet their metabolic demands. Therefore the high parasite growth observed under this treatment may have been reduced under a more competitive feeding regime or under food restriction. In addition, the I_H of exposed fish may have been reduced compared to those held at 15°C if they had been fed a restricted diet. Differences in the body condition of exposed non infected and infected fish were almost significant. Interestingly, the exposed non infected fish were in lower condition compared to infected fish, suggesting that resisting invading parasites has effects on the long term nutritional status of the fish. This may be due to the energetic requirements of producing an immune response that is effective at eliminating the parasite. It is now well established that costs are associated with resisting parasite infection (Minchella, 1985, Webster and Woolhouse, 1999).

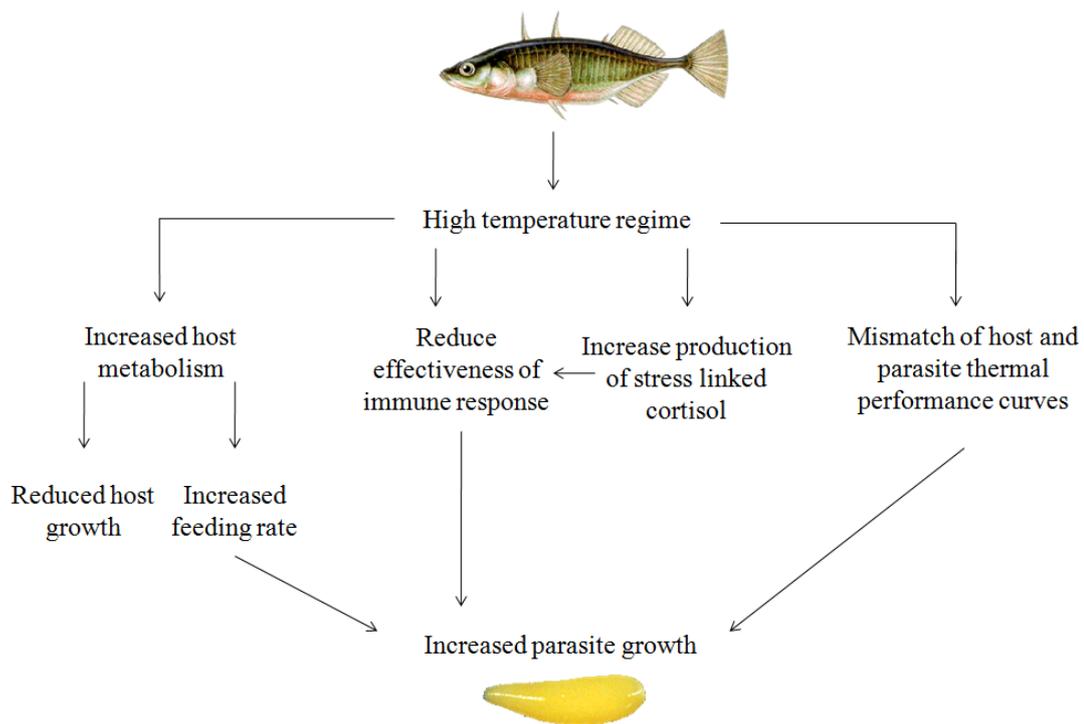


Figure 5.4 The potential mechanisms behind increased parasite growth at elevated temperatures.

The immune response

Altered thermal regimes could impact the efficacy of the fish immune response, altering the host’s capacity to resist infection or regulate the growth of plerocercoids.

Suboptimal temperatures could affect the immune response directly, for example by altering the efficacy of the innate immune response (Kollner and Kotterba, 2002, Nikoskelainen et al., 2004, Chettri et al., 2010). Rainbow trout (*Oncorhynchus mykiss*) held at temperatures greater than 15°C following exposure to the *Aeromonas salmonicida* antigen had increased leukocyte activation compared to those held at lower temperatures (<12°C) (Kollner and Kotterba, 2002). Sticklebacks show an innate immune response towards invading *S. solidus* parasites, mediated largely through monocyte upregulation (Scharsack et al., 2007) which, in other fish species, is more active at higher temperatures (Kollner and Kotterba, 2002). However, we found no effect of rearing temperature on the susceptibility of sticklebacks to infection, nor on splenosomatic index, a commonly used (though relatively crude) indicator of immune activity (Press and Evensen, 1999, Seppanen et al., 2009).

The stress response

If a fish is exposed rapidly to a temperature to which it is not acclimatised, it may induce a stress response. During a stress response, fish produce cortisol which is thought to have an immunosuppressive effect (Esteban et al., 2004, Chettri et al., 2010). Esteban et al. (2004) illustrated that the respiratory burst reaction was suppressed when exposed to high cortisol levels. Cold treated tilapia (*Oreochromis aureus*) had higher cortisol levels than control fish acclimatised to 25°C and phagocytic activity and immunoglobulin M (IgM) levels were reduced (Chen et al., 2002). In this study, 20°C was not an optimal temperature for the stickleback (shown by reduced growth at this temperature) and therefore may have been stressful. Cortisol levels were unfortunately not quantified; however if a stress response had occurred as a result of the high temperature treatment, high cortisol levels could have suppressed the immune response

early on in the course of infection allowing the parasite to proliferate. This is the most important time for parasite elimination, as later on in the infection course the parasite is too big to remove (Scharsack et al., 2007). However, it is important to note that fish were acclimatised to $18\pm 1^{\circ}\text{C}$ prior to the experiment, so on entering the low and high temperatures on the day of parasite exposure both treatments may have been considered stressful.

Differences in thermal performance curves of host and parasite

A fourth possibility is that parasites and their hosts might have different thermal performance curves (Deutsch et al., 2008), and the observed divergent effects of the two thermal regimes may stem from a mismatch between these (Blanford et al., 2003).

Although parasites might be expected to have evolved identical thermal performance curves to their hosts, the ability of parasites that infect both endo- and ectotherms to match host thermal performance may be constrained by the costs of plasticity.

Therefore *Schistocephalus* may be predisposed to exploiting higher temperatures, which are more closely matched to those experienced in the definitive host. Sinha and Hopkins (1967) showed that plerocercoids cultured *in vitro* had maximal growth rates between $23\text{-}27^{\circ}\text{C}$ and between the temperatures of $4\text{-}23^{\circ}\text{C}$ an increase in temperature was accompanied by increased parasite growth. The authors proposed that whilst in the stickleback the parasite enzyme system has an optimal temperature of 23°C that facilitated growth and on movement to an avian host a second enzyme system operating best at approximately 40°C led to maturation. However, it seems likely that *S. solidus* originally evolved as a parasite of either copepods or sticklebacks and that the bird was incorporated at a later stage as the definitive host to increase transmission over a much wider geographic range (Poulin, 2007). Therefore it seems likely that increased parasite

growth at high temperatures results from an across the board metabolic response rather than being pre adapted to an endotherm.

5.4.2. Ecological consequences

It is becoming increasingly recognised that parasites are important components of food webs, accounting for biomasses comparable to top predators (Lafferty et al., 2006, Lafferty, 2008). They can strengthen the links between predators and their prey as they often use manipulation to increase their transmission to their next host (Lafferty et al., 2006). Lafferty and Morris (1996) showed that a small behavioural change by larval trematodes in host killifish *Fundulus parvipinnis* resulted in over a 30 fold increase in predation susceptibility. The results of the present study illustrate that warmer temperatures substantially reduce the time it takes for plerocercoids to attain a size that is infective to avian hosts. In the stickleback-*Schistocephalus* system when plerocercoids reach infectivity a number of behavioural changes in host fish occur including reduced antipredator behaviour (Milinski, 1985, Giles, 1983, Tierney et al., 1993, Barber et al., 2004). These behavioural changes have been proposed to increase predation rates of *Schistocephalus* infected sticklebacks; however, it is unclear if these changes increase transmission to the avian definitive host (Moore, 2002). Regardless, parasitized fish inhabiting warm waters will display these behavioural changes earlier in infection, with the potential to increase transmission rates of the parasite where avian piscivores are major predators of sticklebacks. Therefore a rise in temperature in this system has important implications for host behaviour and predator-prey relationships. Plerocercoid mass also dictates egg output of adult worms, so with larger parasites in warmer environments there is the potential for an increase in the release of parasite eggs into the environment. In turn this could lead to an increase in the prevalence of *Schistocephalus* infections in the first intermediate host, cyclopoid copepods. Therefore

these results predict a dramatic increase in the frequency of successful life cycle completions by the parasite in thermally enhanced aquatic ecosystems.

However, further work is required to determine the effect of increased temperature on other life cycle components including survival and infectivity of the free living coracidium stage. Because the worms were not cultured in the present study, the effect of temperature experienced during plerocercoid growth on the reproductive potential of adult worms also requires clarification. Furthermore the effect of temperature on the parasite proceroid stage and its interaction with its copepod host needs clarified. In addition, the effects observed are unlikely to occur independently of food intake, and a study is required where experimentally infected fish are held under high and low temperatures and are given a fixed maintenance feeding regime i.e. they are not fed *ad libitum*.

5.4.3. Conclusions

In summary, elevated temperatures dramatically increased the growth rate of *S. solidus* plerocercoids in their stickleback intermediate host. This reduced the time taken for the parasites to achieve an infective size, and the reduced antipredatory behaviour associated with reaching this stage would be expected to occur earlier. In addition, the larger size of parasites from higher temperature regimes means that for any given age they potentially have a higher reproductive output compared to those taken from fish in cooler environments (as long as high temperatures don't have a negative effect on parasite reproduction). Overall, the results predict that the frequency of *S. solidus* parasites completing their life cycle will increase. Parasites are important components of ecosystems, with representatives infecting most animal groups (Studer et al., 2010). Given their ubiquitous presence, it is important to understand the potential for climate change to influence the dynamics of parasite infection. These results indicate the

important effects of environments on hosts and their parasites and illustrate how increased parasite growth at elevated temperatures could have potential knock on effects on other components of the parasite life cycle, ultimately increasing the success of the parasite.

As well as findings that show increased parasite growth at high temperatures it will be important to elucidate the mechanisms behinds such changes, as they might illustrate general trends that we will see with climate change, i.e. if the immune system of ectotherms may directly be affected by temperature change or if parasites that place severe energetic demands on their host may benefit from high temperatures due to an increased feeding response as a result of high metabolic rates. In addition, many studies focus on the impact of temperature change on animals that host parasites without considering parasites, but the host's response to temperature may change when it is infected with a parasite.

6. The effect of *Schistocephalus solidus* infection on host thermal preferences in sticklebacks



© Vicki Macnab

6.1. Introduction

Hosts and their parasites participate in arms races over evolutionary time scales, whereby both evolve adaptations to shift the balance of the host-parasite interaction in their favour (Poulin, 1995, Lafferty, 1999). For example, host immune systems target novel parasites, whilst parasites continuously respond by evolving surface antigens to evade such responses (Poulin, 1995). Parasite infections are often associated with a behavioural change in their hosts. Such changes could arise as evolutionary neutral side effects of infection, or they may be adaptive to either the host or the parasite (Robb and Reid, 1996). An adaptation is a feature of an organism that has evolved, or is evolving, because it provides some fitness advantage (Poulin, 1995). Adaptations in hosts and their parasites would evolve if they conferred some fitness advantage, for example in hosts if the resulting behaviour reduced the success of the parasite (Hart, 1994, Moore, 2002) or in parasites if the host's behavioural change increased their transmission to a successive host (Poulin, 1994, Lafferty, 1999, Barber et al., 2000). Parasite behavioural manipulation could even begin as a side effect of infection but over time could proceed to a manipulation technique utilised by the parasite (Poulin, 1994).

Poulin (1995) set out four different criteria to determine if behavioural changes are adaptive (on the part of the parasite or host). The changes should: (1) be complex (2) show a "purposiveness of design" (3) have evolved independently in different lines (4) have a fitness advantage for either the host or the parasite. Some of these suggestions can be difficult to test, but the guidelines provide universal rules that can be followed when investigating postulated adaptations. Researchers normally believe adaptive behavioural manipulation by the parasite is occurring when the parasite uses energy to produce the changes, thus it is costly to the parasite (Maure, 2011), or if the change produces a highly specific fitness advantage for the parasite (Thomas et al.,

2005, Campbell et al., 2010). Whilst adaptations by the hosts should occur when parasites have a significant impact on the host health and such changes should minimise the negative effects of the parasite, if not remove them (Hart, 1994, Moore, 2002).

6.1.1. Host adaptive behavioural change

An interesting example of a behavioural change related to parasite infection that is likely to be host mediated is that of the self medication of chimpanzees using plants (Huffman, 1997). Sick chimpanzees have been observed consuming pith (a bitter substance found in plants for nutrient transport and storage) from *Veronia* sp of plant whilst healthy conspecifics nearby don't show this behaviour (Huffman and Seifu, 1989, Huffman, 1997). Subsequent studies have linked this behaviour with recovery from nematode infection (Huffman et al., 1993, Huffman, 1997). The swallowing of whole rough leaves covered in hairs offers little nutrition to chimpanzees, and it has been proposed to aid the removal of nodular worms (*Oesophagostomum stephanostomum*) with a significant relationship existing between the presence of leaves in the dung and the removal of adult *O. stephanostomum* worms (Huffman et al., 1996, Huffman and Caton, 2001). Huffman et al (1996) proposes that the physical structure of the leaves aids in the removal of the worms via their attachment to the hair like structures or folded within the leaves.

6.1.2. Parasite adaptive behavioural change

Parasites can change the behaviour of their host through direct or indirect mechanisms. The parasite could produce secretions that directly interfere with the nervous system or muscle of the host and create changes in behaviour (Thompson and Kavaliers, 1994, Thomas et al., 2005). However, it can often be difficult to determine whether secretions are produced by parasites or hosts, as a consequence of complex immune reactions (Thomas et al., 2005). Indirect effects can occur when the parasite exerts its effects on

tissues not associated with the nervous system. For example parasites can cause a range of effects on the reproductive development, metabolism and immune parameters of the host, and in turn these changes could lead to alterations in behaviour (Barber et al., 2000, Thomas et al., 2005). Both types can still be manipulative on the part of the parasite. Manipulation will have costs associated with it; for example parasitoid wasps (*Dinocampus coccinellae*) of spotted lady beetles (*Coleomegilla maculate*) on emergence change the behaviour of their host causing them to act as “body guards” to their cocoons. However, there is a negative correlation between the length of time that the lady beetle acts as a guard (= its survival in most cases) and the parasitoid’s potential fecundity (Maure, 2011).

The well studied host-parasite system of rats (*Rattus norvegicus*) and their protozoan parasite *Toxoplasma gondii* is ideal for testing behavioural changes associated with parasite infection in experimental setups. Berdoy et al. (2000) showed that rats infected with *Toxoplasma gondii* showed reduced antipredatory behaviour towards cats, the definitive host, in an experimental “open field” type study. The infected rats did not avoid cat odours, and in fact showed a preference for them compared to non infected controls, which avoided them. In addition, the study illustrated that the odour preferences of infected and non infected rats did not differ when exposed to scents from themselves or rabbits and the authors propose that this shows the change in odour preference with respect to cats was not due to disorders with the olfactory system. Webster et al. (1994) showed that *T. gondii* infected rats were less neophobic than their non infected counterparts. Infection status did not affect body condition of the rats, suggesting that behavioural changes are not pathological side effects of infection. Berdoy et al. (1995) showed that the parasite had no significant impact on other energetically expensive aspects of host behaviour. Infection did not

have an effect on the social status, mating success or testosterone levels of rats whilst infected rats did show higher levels of exploratory behaviour that is suggested to cause increased transmission of the parasite to the next host (Berdoy et al., 1995). The authors conclude that *T. gondii* has specific effects on host behaviour, again supporting the behavioural manipulation by parasites hypothesis. Changes in the predation rates of infected individuals by cats has yet to be proven due to practical and ethical problems; however a previous study has shown that *T. gondii* infected rats are more likely to be caught in traps compared to non infected individuals (Webster et al., 1994). Humans can also become infected with *T. gondii* if they consume the parasites oocytes, although this prevents the completion of the parasite life cycle (Berdoy et al., 2000). Behavioural changes have been found in infected humans with personality changes (Flegr and Hrdy, 1994), longer reaction times (Havlicek et al., 2001) and a higher risk of being involved in a traffic accident being associated with infection. Therefore there appears to be strong evidence supporting the idea that *T. gondii* is capable of behavioural manipulation in mammal hosts in general (Berdoy et al., 2000).

6.1.3. Altered host temperature preferences as adaptations to parasite infection

Altered thermal preferences of host organisms could reflect either host or parasite adaptations to infection. Much research has focused on the use of temperature by the host to limit parasite success and in fewer cases for the parasite to enhance their success (Fialho and Schall, 1995, Campbell et al., 2010). Behavioural fever or chills, whereby animals can raise or reduce their body temperature by residing at such temperatures, could be advantageous to hosts if they reduce parasite success, for example by improving the efficiency of the host's immune response to the parasite or reducing parasite development (Poulin, 1995, Moore, 2002). Müller and Schmid-Hempel (1993)

illustrated that bumblebees (*Bombus terrestris*) infected with parasitoids (conopid larvae) did not return to their nest during the night; instead they stayed outside where they would experience cooler temperatures compared to the nest temperature (~ 30°C). They showed that cooler temperatures (19°C) increased the life span of infected hosts and reduced the development of the parasitoid and that parasitized bumblebees spent significantly more time in the cooler temperatures when given a choice. Thus by choosing cooler temperatures infected bumblebees can reduce the development of the parasitoid.

One example where a parasite obtains an advantage from altered host thermal preferences is shown in sandflies (*Lutzomyia vexator*) which are vectors of the malaria parasite *Plasmodium mexicanum* that infect vertebrate lizard hosts, *Sceloporus occidentalis*. Fialho and Schall (1995) demonstrated that sandflies that had fed on non infected lizards selected a temperature 1.6°C higher than that of unfed flies probably to help digestion. Sandflies that had fed on infected *Plasmodium mexicanum* lizards selected temperatures 3.6°C higher than unfed flies. Higher temperatures were associated with reduced developmental times of the parasite, meaning that it was ready for transmission to its vertebrate lizard host earlier.

Apart from the study described above there are few examples where behavioural modifications of thermal preferences are adaptive to parasites. Chapter 5 illustrated fitness advantages for *Schistocephalus solidus* that are associated with high temperatures. The parasite grew faster at higher temperatures, reducing the time at which it took to become infective to its definitive host. In this parasite, larger sizes are associated with higher egg output (Dörücü et al., 2007) therefore if parasites are capable of growing bigger at warmer temperatures their reproductive rate may be correspondingly higher. Thus on becoming infected with *S. solidus* there is potential for

the parasite to manipulate the thermal preferences of the stickleback causing it to select higher temperatures providing them with these fitness advantages. Alternatively, fitness advantages exist for host sticklebacks that select cooler temperatures, as these were associated with slower parasite growth and higher host growth. Therefore the aim of the present study was to investigate if parasite infection altered host temperature preferences in the stickleback-*Schistocephalus* system, and if it does, to determine the likely adaptive function of such changes.

6.2. Materials and methods

6.2.1. Fish supply and husbandry (wild caught fish)

Forty-six three spined sticklebacks (*Gasterosteus aculeatus*) caught from a naturally infected population in the River Welland, Leicestershire UK (N52°28'36" W0°55'23") in November 2010 were held in 72L stock aquaria. The fish were held at 13°C ± 1°C for 5 days, before being moved to 15°C ± 1°C for another 5 days. Finally 2 weeks prior to behavioural trials, fish were kept under a photoperiod of 16L: 8D and an average temperature of 17.8°C (range 16.9 – 18.7°C). Each tank was fitted with a power filter, gravel substratum and plastic plants for shelter and fish were fed *ad libitum* to excess with frozen bloodworm (*Chironomus* sp. larvae) daily. Food was withheld for 1d prior to testing, to prevent alimention effects on thermal preferences (Ward et al., 2010).

6.2.2. Fish supply and husbandry (experimentally infected fish)

We also tested the thermal preferences of 14 experimentally infected and 20 sham exposed lab-bred sticklebacks. Fish were bred in the lab from wild-caught parents from Carsington Reservoir (Derbyshire, UK; N53°03'21", W1°37'25") and reared in 6 family groups at at 14L: 10D and 16°C ± 1°C. At four months of age fish were either exposed to infective *S. solidus* stages (copepods infected with 1-4 procercooids; two sets of infected copepods from two sets of selfed Carsington worms) or sham-exposed to a non infected

copepod (See section 3.2.2. for detailed experimental infection protocol). The fish were then housed in a tank that had been divided into two compartments each measuring 17 x19x19cm and holding 6L of water, at 16L:8D and kept at a mean temperature of 18°C (range: 16.7-18.9°C). Each compartment had a plastic shelter, air stone and gravel substratum and the fish were fed *ad libitum* to excess with frozen bloodworm (*Chironomus* sp. larvae) daily. Behavioural trials occurred on weeks 0, 1, 2, 6 and 7 with food being withheld for 1d prior to testing, again to prevent alimentionation effects on thermal preferences (Ward et al., 2010).

6.2.3. Screening thermal preferences

The thermal preference apparatus comprised three interconnected flow-through aquaria; the cool and warm aquaria were supplied with a thermostatically controlled water supply to offer discrete temperatures (mean [range]: ‘cool’ = 15.2°C [13.8-16.5°C]; ‘intermediate’ = 17.9°C [16.0-19.6°C]; ‘warm’ = 20.9°C [19.5-23.5°C]; Figure 6.1.). Six replicate thermal preference systems were constructed, with alternate orientation of warm and cool ends to mediate any directional preferences of test fish. Three sets of temperature measurements were taken in each of the tanks at the start and end of the trial. Fish could pass between the tanks via 25 mm diameter portals. Test fish were transferred individually to the ‘intermediate’ temperature tank and guided gently around the apparatus using a hand net (moving at $<1\text{cm}\cdot\text{sec}^{-1}$), to ensure that all available temperatures were experienced, before returning to the intermediate tank. After a 300s settling period the subsequent movements of the fish around the thermal choice tank were recorded remotely by a webcam, programmed using software (*Active WebCam* v8.6, PY Software, Ontario, Canada) to record a still image every 10s over a 3h period.

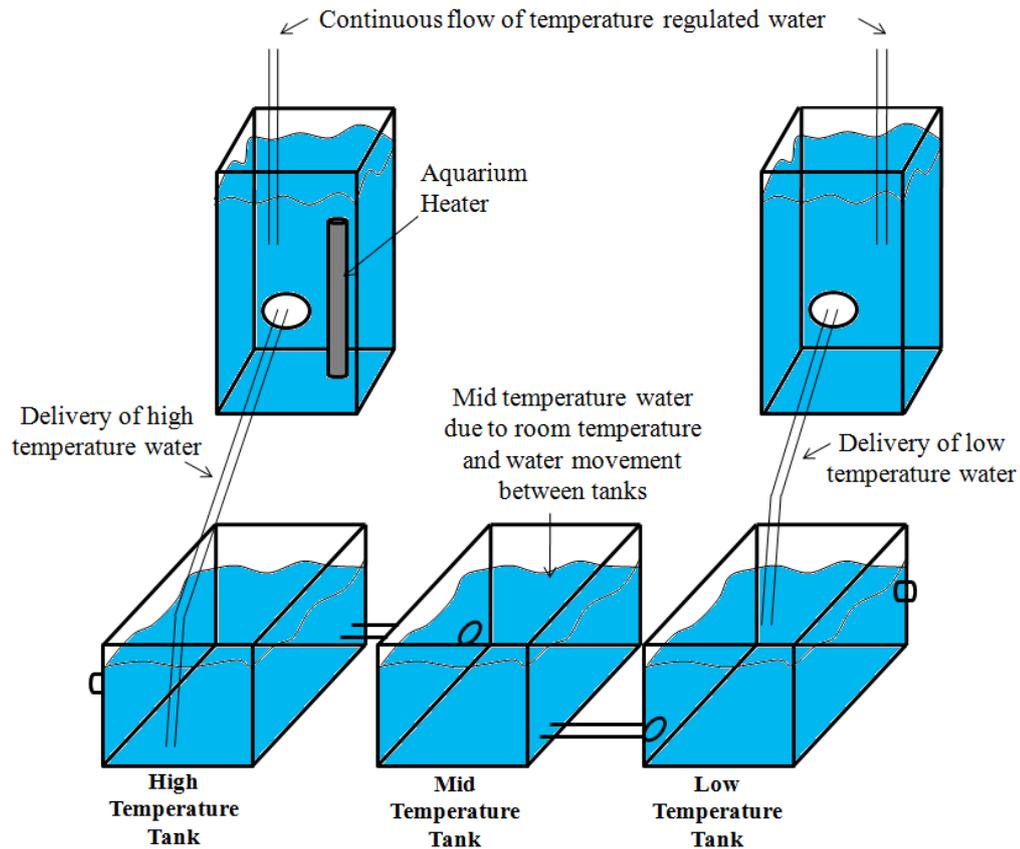


Figure 6.1 The experimental set up used to test thermal preferences of sticklebacks.

6.2.4. Post mortem analysis

At the end of the studies (following the behavioural trial for wild fish and after the behavioural screen at Week 7 for lab fish), fish were exposed to a lethal Benzocaine overdose (Stock solution: 10g prepared in 1L of 70% EtOH). Individual fish were blotted, measured (standard length, L_S , to 0.1 mm) and weighed (wet mass, M , to 0.001 g). The total mass of plerocercoids recovered from infected fish was quantified (M_P , to 0.0001g) permitting the calculation of parasite index: $I_P = 100[M_P (M - M_P)^{-1}]$.

6.2.5. Behavioural trial analysis

For each trial, the proportion of observations each fish spent in the ‘cool’, ‘intermediate’ and ‘warm’ aquaria was multiplied by the mean temperature recorded in each tank before and after the trial. The sum of these values (\sum_{deg}) was then calculated to provide a value reflecting the overall thermal preferences of the fish during the trial.

6.2.6. Statistics and data analysis

Trials using wild caught fish

Proportional data (I_p) was arcsine square root transformed and normality was tested using the Kolmogorov-Smirnov statistic. Non normal data was either inverse or logarithm transformed for parametric testing.

T tests were used to identify if differences existed between infected and non infected fish in their \sum_{deg} and one sample T tests identified if their \sum_{deg} differed from their acclimatisation temperature of 17.8°C. The parasite becomes infective to its definitive avian host at 50mg in size (Tierney and Crompton, 1992) and host behavioural changes have been reported to occur on becoming infective (e.g. Tierney et al., 1993). Therefore to test the hypothesis that worm infectivity influenced host thermal preferences, the behaviour of fish that harboured infective (>50mg) and non infective (<50mg) worms was considered separately. A two way ANOVA was used to test the effect of infection class and sex on \sum_{deg} and one sample T tests were used to identify if the mean \sum_{deg} of each infection group differed significantly from the acclimatisation temperature. An ANCOVA was used to determine if the relationship between the mass of the heaviest parasite and host \sum_{deg} differed between fish harbouring infective or non infective worms. Regression analysis was used to identify if relationships existed between \sum_{deg} and total parasite weight or I_p , and \sum_{deg} and the mass of the heaviest parasite in fish harbouring infective or non infective worms. All statistical analyses were carried out using SPSS (version 18).

Trials using laboratory bred fish

Proportional data (I_p) was arcsine square root transformed and normality was tested using the Kolmogorov-Smirnov statistic. All of the data was normal and did not need transformed for parametric testing.

A mixed between-within subjects ANOVA was carried out to investigate if there was an effect of exposure status (between subjects; non exposed / exposed non infected/ exposed infected) or week number (within subjects) on \sum_{deg} (note week 1 data could not be normally transformed). A one way ANOVA was used to investigate the effect of exposure status on \sum_{deg} . One sample t tests were used for different exposure groups to identify if \sum_{deg} differed from their acclimatisation temperature (18°C).

6.3. Results

6.3.1. Trials using wild caught fish

Temperature preference in non infected and infected fish

After the 300s settling period, fish in each group did exhibit exploratory behaviour moving from the middle tank to at least one of the different temperature tanks, if not both (Figure 6.2.).

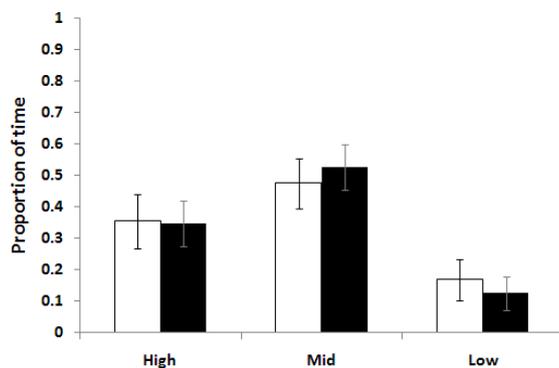


Figure 6.2 The proportion of time that *S. solidus* infected and non infected wild fish spent in the three thermal regimes. Bars represent the mean \pm s.e. in non infected (open bars) and infected (filled bars) fish in the three thermal zones.

There was no significant difference in \sum_{deg} between infected and non-infected wild-caught fish ($t_{44}=-0.213$, $P=0.832$; Figure 6.3.); however, whereas the mean \sum_{deg} of non-infected fish indicated their thermal preferences were matched to the temperature experienced during acclimatisation ($t_{19}=1.4$, $P=0.168$), infected fish preferred a warmer temperature ($t_{25}=2.5$, $P=0.021$; Figure 6.3.).

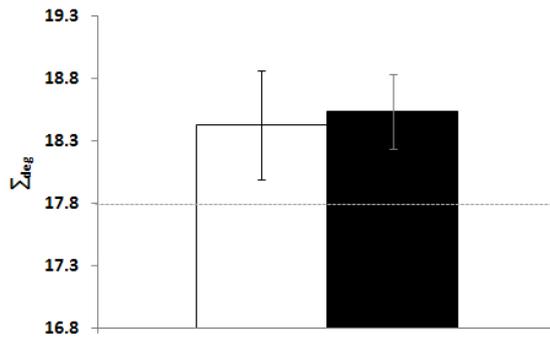


Figure 6.3 The effect of *S. solidus* infection status on wild stickleback thermal preferences. The mean \pm s.e. thermal preference (Σ_{deg}) of non-infected (open bars) and infected (filled bars) fish; dotted line represents acclimatisation temperature (17.8°C).

Temperature preferences of fish harbouring infective or non infective worms

On splitting the fish into three infection classes: non infected fish and infected fish whose heaviest plerocercoid was non infective (<50mg) or infective (>50mg), there was no significant interaction or main effect of infection class or sex on Σ_{deg} (Table 6.1.; Figure 6.4.a). The preferences of fish with non-infective plerocercoids did not differ significantly from their acclimatisation temperature ($t_{19}=1.1$, $P=0.271$), whereas those harbouring infective plerocercoids showed a significantly higher mean Σ_{deg} ($t_5=4.7$, $P=0.005$; Figure 6.4.a).

Table 6-1 Result of two way ANOVA performed to investigate the impact of fish sex and infection class on thermal preferences (Σ_{deg}) of sticklebacks.

Source	Σ_{deg}		
	Sex	Infection status	Interaction
d.f.	1, 40	2, 40	2, 40
F	0.2	1.6	0.04
P	0.660	0.208	0.957

Among all parasitized fish there was no overall relationship between Σ_{deg} and total parasite weight ($F_{1,24}= 0.671$, $r^2=0.027$, $P=0.42$) or I_p ($F_{1,24}= 0.287$ $r^2=0.012$, $P=0.597$); however, the relationship between Σ_{deg} and the mass of the heaviest parasite differed between fish with infective and non-infective plerocercoids (Slope: $F_{1,22}=0.9$, $P=0.352$; Elevation: $F_{1,23} = 14.1$, $P=0.001$; Figure 6.4.b). A significant negative

relationship existed between the mass of the heaviest parasite and \sum_{deg} in fish with non-infective parasites ($F_{1,18}= 5.794$, $r^2= 0.244$, $P=0.027$), whereas among fish with infective plerocercoids there was no relationship between parasite weight and \sum_{deg} ($F_{1,4}= 0.303$, $r^2=0.07$, $P=0.611$).

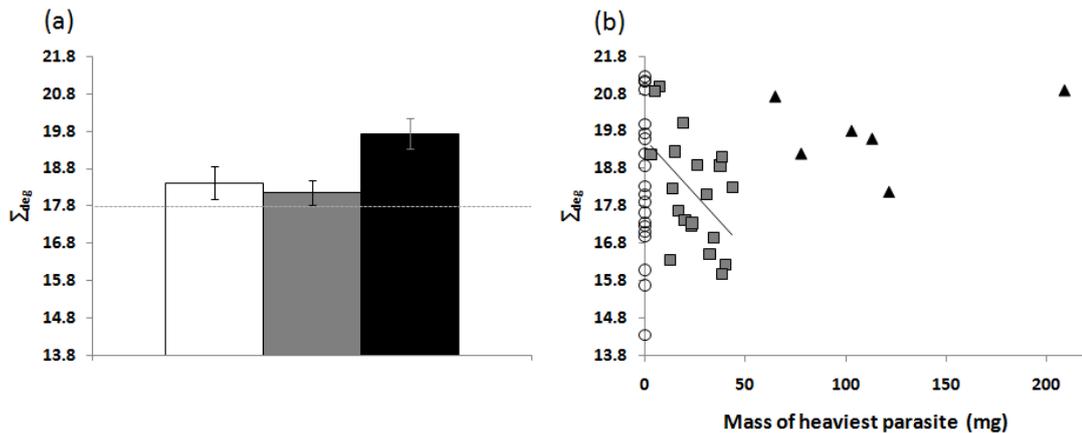


Figure 6.4 The thermal preferences of wild caught sticklebacks that differ in their *S. solidus* infection status. (a) The mean (\pm s.e.) thermal preference (\sum_{deg}) in non infected (open bars) fish and fish harbouring non infective (grey bars) and infective (black bars) parasites and (b) the relationship between the mass of the heaviest parasite and thermal preference (\sum_{deg}) in non infected (open circles) fish and fish harbouring non infective (grey squares) and infective (black triangles) parasites.

Thermal preferences and host weight ($M-M_P$)

Among infected fish the relationship between thermal preference and $M-M_P$ was shown to be significant, with larger bodied infected fish showing a preference for higher temperatures ($F_{1,24}=5.8$, $r^2=0.194$, $P=0.024$), whereas in control non infected fish no relationship existed ($F_{1,18}=0.3$, $r^2=0.019$, $P=0.563$).

6.3.2. Trials using laboratory fish

Temperature preference and exposure status

Fish in each exposure group across all the weeks did exhibit exploratory behaviour moving from the middle tank to at least one of the different temperature tanks, if not both during the trial period (Figure 6.5.).

There was no main effect of week number ($F_{4,25}=1.03$, $P=0.413$) or exposure group ($F_{2,28}=0.61$, $P=0.553$) or interaction effect ($F_{8,50}=0.45$, $P=0.882$) of the two

variables on \sum_{deg} (Figure 6.6.). This analysis was carried out again without the week 0 data due to some missing values, but there were still no significant effects.

There was no significant difference in \sum_{deg} on termination of the experiment at week 7 across the three exposure classes ($F_{2,31}=0.6$, $P=0.573$; Figure 6.7.). The temperature preferences of the three exposure classes at week 7 did not differ from their acclimatisation temperature of 18°C (non exposed $t_{19}=-1$, $P=0.32$; exposed non infected $t_8=0.4$, $P=0.682$; infected $t_4=-0.6$, $P=0.555$; Figure 6.7.).

Figure 6.8.a shows the data separated into the three infection classes (controls, heaviest parasite < 50mg and > 50mg); statistical testing could not be used to compare this data due to small sample sizes in the infected fish. Figure 6.8.b shows the relationship between \sum_{deg} at week 7 and the mass of the heaviest parasite; statistical analysis could not be performed again due to small sample sizes.

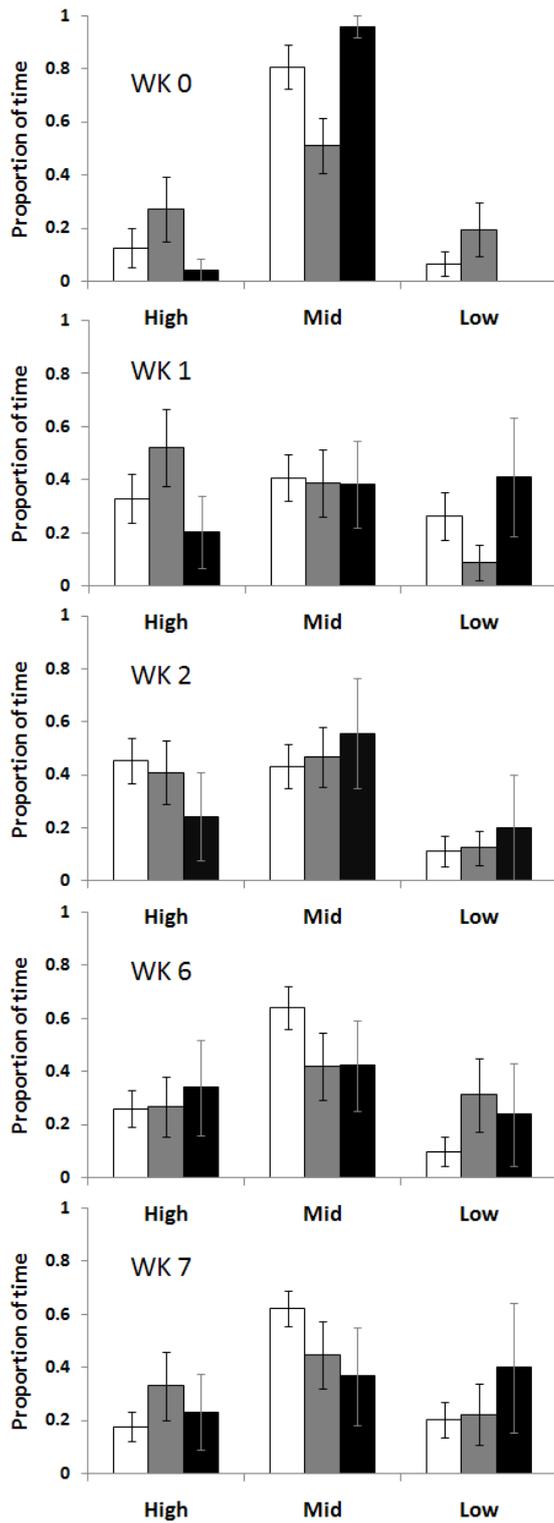


Figure 6.5 The proportion of time that *S. solidus* exposed and non exposed lab fish spent in the three thermal regimes over the 5 tested time periods. The mean \pm s.e. proportion of time spent in the different temperature regimes by the three exposure classes: non exposed (open bars), exposed non infected (grey bars) and exposed infected (black bars).

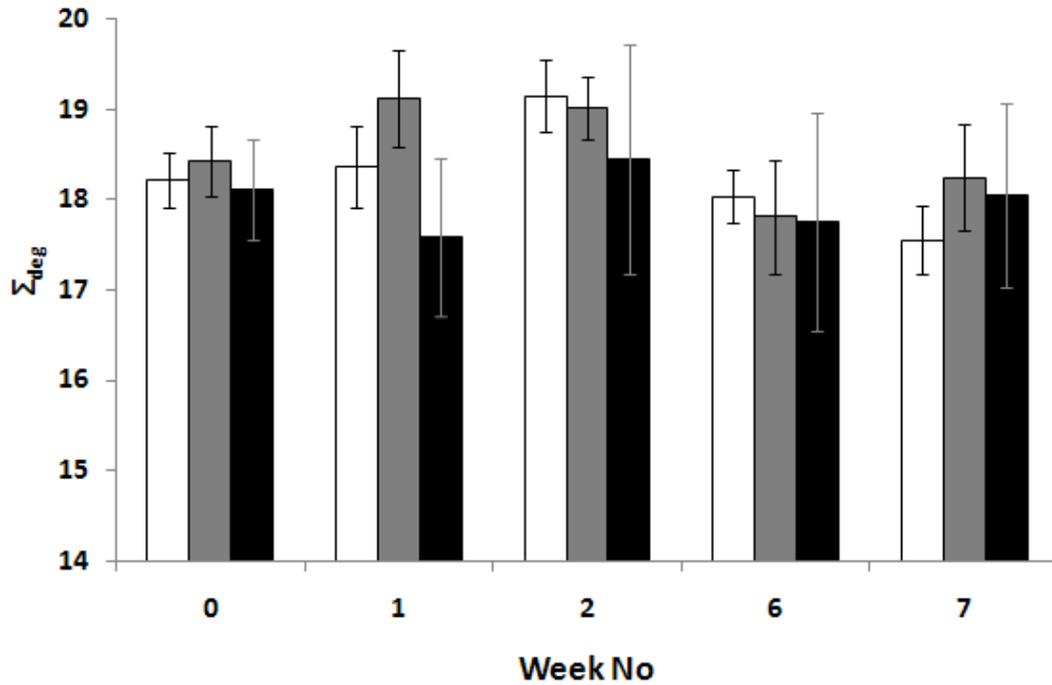


Figure 6.6 The effect of experimental *S. solidus* exposure status on thermal preferences of lab sticklebacks over the 5 tested time points. The mean \pm s.e. thermal preference (Σ_{deg}) at each week across the three exposure classes: non exposed (open bars), exposed non infected (grey bars) and exposed infected (black bars).

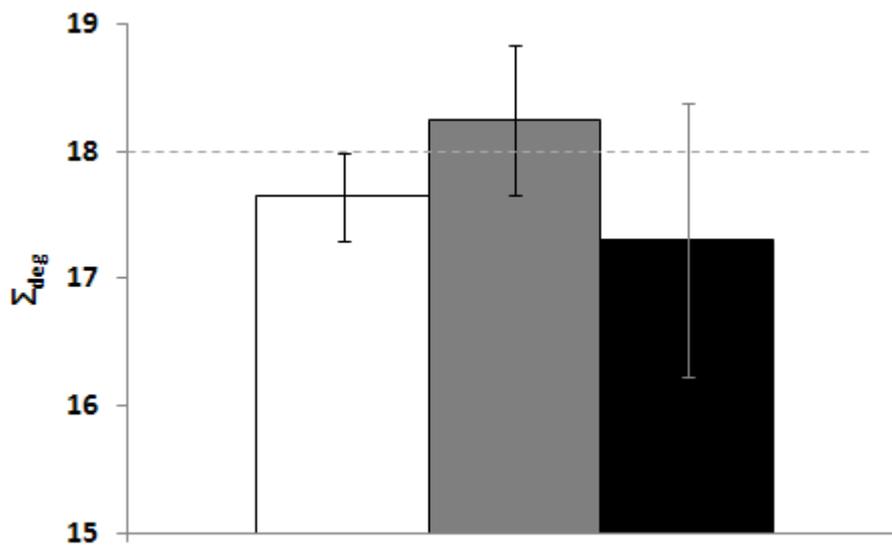


Figure 6.7 The effect of experimental *S. solidus* exposure status on thermal preferences of sticklebacks at the final tested time point (Week 7). The mean \pm s.e. thermal preference (Σ_{deg}) of non exposed (open bars), exposed non infected (grey bars) and exposed infected (black bars) fish. Dotted line indicates acclimatisation temperature.

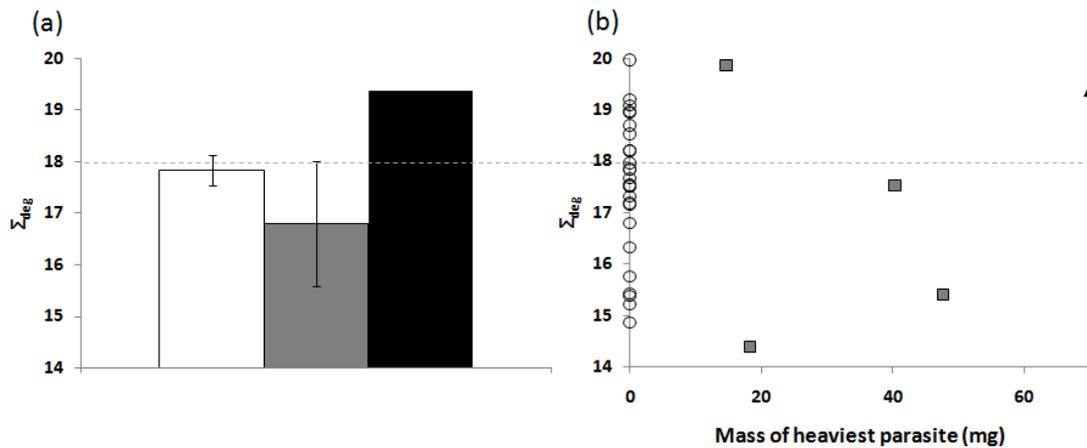


Figure 6.8 The thermal preferences of laboratory bred three spined sticklebacks that differ in their *S. solidus* infection status. (a) The mean (\pm s.e.) thermal preference (Σ_{deg}) in non infected fish (non exposed and exposed non infected; open bars) and fish harbouring non infective (grey bars) or infective (black bars) parasites; (b) the relationship between the mass of the heaviest parasite and thermal preference (Σ_{deg}) in non infected fish (non exposed and exposed non infected; open circles) and fish harbouring non infective (grey squares) or infective (black triangles) parasites. Dotted line indicates acclimatisation temperature.

6.3.3. Comparisons between wild and lab trials: thermal preferences

The thermal preference data from trials using the wild caught naturally infected and laboratory bred experimentally infected fish have been combined in figure 6.9. to illustrate that the results from the lab fish studies are consistent with those from the field caught fish studies, but no attempt is made to compare the datasets statistically.

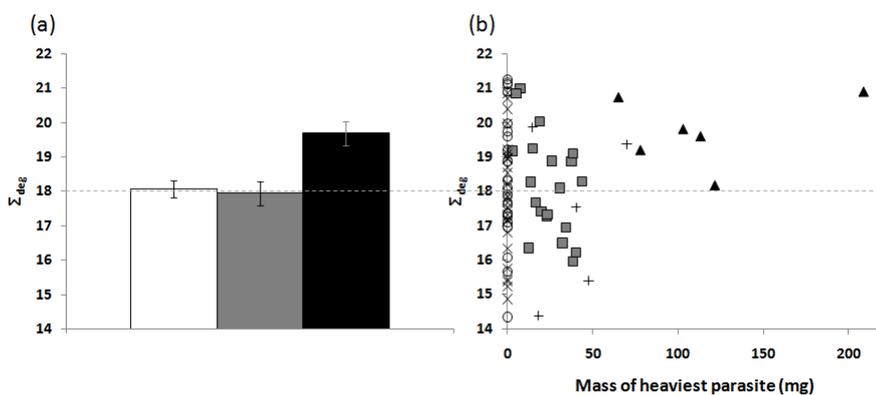


Figure 6.9 The thermal preferences of wild caught and laboratory bred three spined sticklebacks that differ in their *S. solidus* infection status. (a) The mean (\pm s.e.) thermal preference (Σ_{deg}) of non infected (open bars) fish and fish harbouring non infective (grey bars) and infective (black bars) parasites and (b) the relationship between thermal preference (Σ_{deg}) and the host's heaviest parasite in the wild caught fish trials (non infected fish (open circles) and fish harbouring non infective parasites (grey squares), or infective parasites (black triangles)) and lab bred fish trials (non exposed and exposed non infected fish (X) and infected fish (+)). Dotted line indicates acclimatisation temperature.

The data from individual laboratory infected fish is shown in Figure 6.10. with figures a - e representing each individual fish. The first column of plots shows the relationship between \sum_{deg} and disease progression in each fish, as their thermal preferences were tested at weeks 0, 1, 2, 6 and 7 following parasite exposure. The mass of their heaviest parasite (HP) on dissection at week 7 is shown to illustrate that the thermal preferences over the course of the 7 weeks was not directly comparable between the fish as they all harboured parasites that were at different stages of growth. The second column of plots in Figure 6.10. show the data obtained from the behavioural trials using wild caught fish, illustrating the negative relationship between \sum_{deg} and the mass of the heaviest parasite in fish harbouring non infective plerocercoids and the preferences for higher temperatures in fish harbouring infective plerocercoids. The \sum_{deg} of the laboratory infected fish at week 7 is plotted on this graph to show where they fall in relation to the wild fish. For each individual fish the \sum_{deg} over the 7 week period shown in the first graph can be related to where they fall on the second graph. For example, Figure 6.10.a shows a fish harbouring a non infective plerocercoid (14.7mg) and a positive relationship is shown between \sum_{deg} and disease progression. This relationship is consistent with the second graph, as this parasite was very small and falls within those fish that all prefer warmer temperatures in the second graph. Figure 6.10.b shows that from week 6 to 7 the thermal preferences of a fish harbouring an intermediate sized plerocercoid (18.3mg) go from a high temperature to a low temperature, similar to the negative relationship seen in non infective plerocercoids with \sum_{deg} . Figure 6.10.c and d illustrate fish that have plerocercoids very close to infectivity, and show that the fish have a preference for warmer temperatures at weeks 1 and 2 and for cooler temperatures at week 6 and 7, again consistent with what is observed in the second graph. Finally Figure 6.10.e shows the only parasite that had

reached infectivity, and illustrates that the preference for warmer waters existed early on in infection at week 1, before dropping at week 2 to cooler waters and by weeks 6 and 7 high temperatures are preferred, again consistent with the relationships that are seen in the second figure.

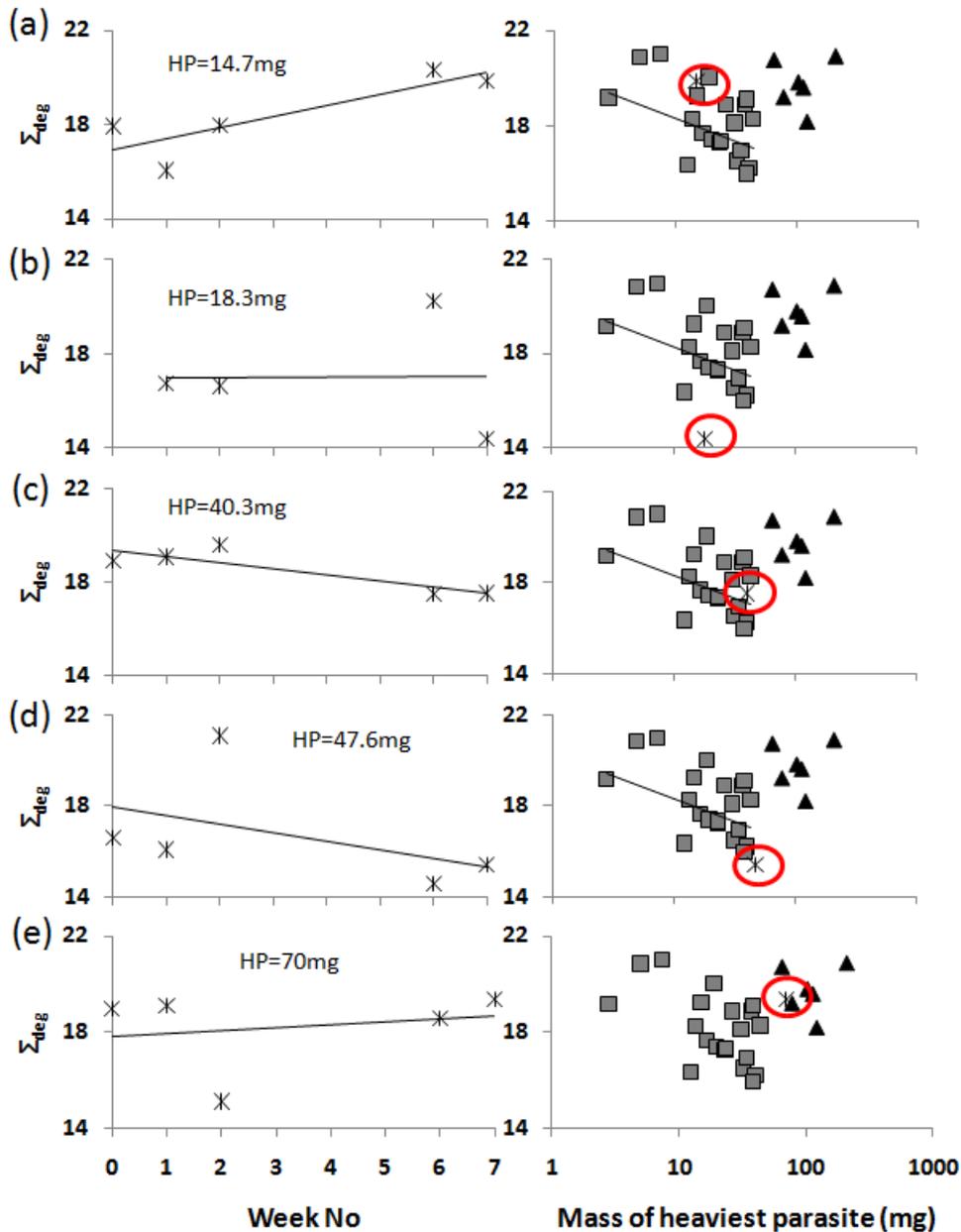


Figure 6.10 The relationship between thermal preferences (Σ_{deg}) and disease progression in laboratory infected fish. (a) – (e) shows data from an individual infected fish. Column 1 illustrates the relationship between Σ_{deg} and disease progression (week no); the mass of the heaviest parasite on dissection at week 7 is displayed (HP). Column 2 shows the thermal preferences of wild caught fish in relation to their heaviest parasite in fish harbouring non infective (grey squares) and infective (black triangles) parasites. The red circle illustrates where the lab fish (*) lie on the graph in comparison.

6.4. Discussion

6.4.1. Behavioural change: side effects or adaptations of host / parasite

Behavioural changes associated with parasite infection can result from non adaptive pathological side effects of infection or adaptations by the host or parasite to improve their fitness (Robb and Reid, 1996) and as described in the introduction Poulin (1995) set out four criteria that can be used to determine if behavioural changes are adaptive. The changes should: (1) be complex (2) show a “purposiveness of design” (3) have evolved independently in different lines (4) have a fitness advantage for either the host or the parasite.

Chapter 5 illustrated that in the stickleback-*Schistocephalus* system, high temperatures increased parasite growth allowing them to reach infectivity to their definitive host quicker. This larger size associated with higher temperatures could potentially increase the reproductive potential of the parasite in their definitive host providing a fitness advantages for the parasite. Whilst, cooler temperatures were associated with reduced parasite growth and increased host growth. Therefore this thermal mismatch between host and parasite performance raises the intriguing possibility that hosts or parasites could use temperature as a “weapon” against the other. The mismatch of thermal performance curves of hosts and their pathogens has previously been discussed in Chapter 5 with the example of locusts *Schistocerca gregaria* preferring warmer temperatures compared to their fungal pathogen, *Metarhizium anisopliae*. Elliot et al. (2002) provided a classic example of how hosts can use temperature against their pathogen. Locusts infected with the fungal pathogen that were allowed to produce a behavioural fever (i.e. access a high temperature area) were able to reproduce whereas locusts in two other groups that were kept at suboptimal

temperatures (could not access high temperature areas) and low temperatures (not able to thermoregulate or produce fever) died before sexual maturation.

In the case of the stickleback-*Schistocephalus* system if the host was capable of using temperature to limit the effects of the parasite, we would predict that they should select cooler water that reduces parasite growth. However, our results do not provide strong evidence that sticklebacks use temperature regimes to limit *S. solidus* growth. The effect of infection status on temperature preference was complex. Initial comparisons directly between the thermal preferences of infected and non infected fish indicated that they did not differ. However, differences were found to exist when the groups were compared to their acclimatisation temperature; with infected fish preferring warmer temperatures, whilst non infected fish had preferences that matched what they were acclimatised to. *Schistocephalus* becomes infective to its avian definitive host at around 50mg in size (Tierney and Crompton, 1992) and fish harbouring infective (>50mg) worms showed a preference for higher temperatures compared to those with non infective worms (<50mg) that had preferences that matched the acclimatisation temperature. A negative relationship was identified between temperature preference and the mass of the heaviest parasite among hosts with non infective worms. Fish with very small parasites were found occupying warmer temperatures, whilst as parasites approached 50mg in size, their hosts had a tendency to select cooler temperatures. A switch seemed to occur at around 50mg in size when fish harbouring infective plerocercoids showed a uniform preference for warmer temperatures, regardless of parasite weight.

A small sample size of infected fish in the laboratory study made it difficult to determine if the laboratory fish showed similar behaviour as the wild fish. However, their thermal preferences fell within the expected ranges of those shown for wild fish

harbouring non infective and infective parasites (Figure 6.9.). Figure 6.10. also illustrated that the thermal preferences over the course of infection could not be directly compared across the 5 infected individuals, as the parasites were all are different stages of growth. Differences in parasite growth rate would occur due to differences in host size and genetics, parasite sibships (copepods were infected with eggs taken from two worms) and the number of worms that hosts were infected with. Nevertheless, this figure illustrated that the thermal preferences for a given fish over the course of infection showed similarities to the relationships that were found in the wild fish behavioural trials. This study needs to be repeated with more fish to generate a higher sample size of infected fish. Also fish would be exposed to singly infected copepods from one batch of selfed parasite eggs; to allow direct comparison of the time points and thermal preferences across the groups.

6.4.2. Potential mechanisms underlying thermal preferences in hosts

harbouring non infective parasites (<50mg)

The initial movement of fish into warmer waters after infection could be explained as either a parasite adaptation to maximise growth, or a host adaptation to maximise the efficacy of immune responses. From the parasite's perspective, rapid growth during the early stages of infection could reduce the likelihood of reaching infectivity.

Hammerschmidt and Kurtz (2005a) showed that *S. solidus* sibships that had a higher infection success rate in their fish host were smaller in size compared to sibships with lower infection rates. Thus it seems likely that slower growth during the early stages of infection to enhance host survival is a trait that has been selected for in the parasite.

Thus it seems unlikely that the selection of high temperatures early during infection would be adaptive on the part of the parasite. From the host's perspective, during the early stages of infection there will be strong selective pressure to produce an efficient

immune response as there is evidence to suggest that *S. solidus* establishment can only be prevented at this time (Scharsack et al., 2007). Scharsack et al. (2007) have described the immune response that occurs following experimental *S. solidus* infection. Early on in infection, monocyte production was upregulated in a cyclical manner and the fish may choose warmer temperatures if this enhances immune activity as it does in other fish species (Kollner and Kotterba, 2002) (Figure 6.11).

The observation that fish with larger, yet still non infective, parasites showed similar thermal preferences to their acclimatisation temperature may be due to the waning of the immune response. Scharsack et al (2007) illustrated that innate immunity remained low following monocyte upregulation till the parasite reached 50mg in size. Whilst the trend that we see for fish with parasites approaching 50mg in size to prefer cooler temperatures may result from the reduced energy requirements associated with colder water (Figure 6.11). Increasing preferences for cooler temperatures could therefore represent either a host or a parasite adaption (or both) to limit the energetic demands of the parasite, which would benefit the survival prospects of both host and parasite. On the other hand, the variation in temperature preferences of fish harbouring non infective worms, may not have anything to do with infection and simply represent the variation that is seen in control fish with respect to temperature preferences. However, the fact that a relationship between thermal preferences and the mass of the heaviest parasite exists makes this seem unlikely.

Host weight was shown to have a significant positive relationship on temperature preferences in parasitized fish, with larger fish preferring warmer temperatures. However, as this relationship was not shown to exist in non infected wild fish, the relationship we see in infected fish is likely to be correlated with parasite size.

Bigger fish have larger worms and thus are more likely to be infective and prefer warmer temperatures.

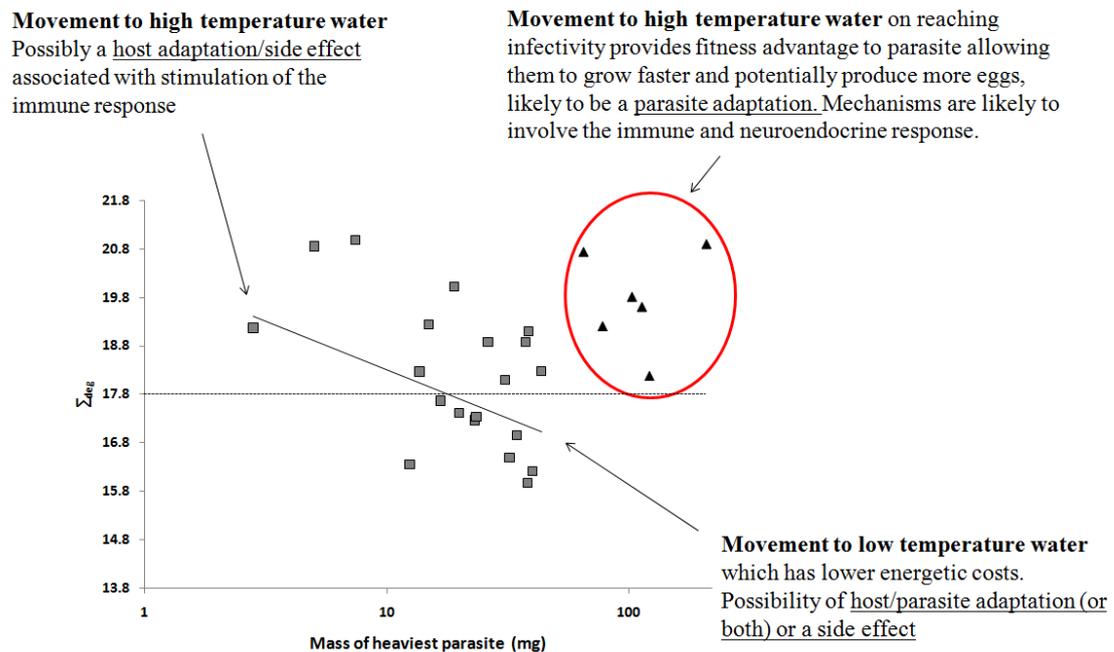


Figure 6.11 The potential mechanisms associated with temperature preferences at different parasite sizes in wild caught fish. The dotted line represents acclimatisation temperature (17.8°C).

6.4.3. Potential mechanisms for thermal preferences in hosts harbouring infective parasites (>50mg)

A switch in temperature preference seems to occur at 50mg in size, the point at which the parasite becomes infective to its definitive host (Tierney and Crompton, 1992) (Figure 6.11). There does not seem to be any fitness advantage for hosts associated with such a move; high temperatures cause increased metabolism and thus would increase the energetic demands of hosts already suffering from energy depletion due to the parasite (Arme and Owen, 1967, Lester, 1971). In addition, parasitized hosts often suffer from reduced survival at high temperatures (Moore, 2002). Conversely, the change offers a clear fitness advantage to the parasite and a “purposiveness of design” (Poulin, 1995). Up until the point of infectivity is reached, the parasite would benefit by reducing its impact on host survival, to permit its progression to infectivity. However,

on reaching 50mg in size such constraints on host exploitation would be removed. This could enable the parasite to cause the host to select warmer temperatures (surface and shallow water), where they should grow faster and be more likely to encounter susceptible predators. In singly infected fish, plerocercoid size dictates adult egg output in its definitive host with larger adult worms producing higher numbers of eggs (Dörücü et al., 2007). Thus parasites that are capable of manipulating their host to seek out warmer temperatures could benefit from increased growth and egg output in their definitive host.

The potential use of the immune response to change thermal preferences

To cause such changes the parasite may use the host's immune response. Scharsack et al (2007) showed that when the parasite reached around 50mg in size there was an upregulation of the host respiratory burst reaction. At this point the cestode cannot be removed and such a response is likely to have a harmful effect on the host. The authors also state that evoking such a response could also harm the parasite; thus it potentially has costs associated with it for the parasites. The parasite could cause the host to produce this immune response to encourage the fish to seek out high temperatures. The respiratory burst reaction has been shown to be more active at high temperatures in some species of fish (Nikoskelainen et al., 2004). Therefore by unveiling themselves to the host's immune response, the fish would select higher temperatures where the response is more active. Hammerschmidt and Kurtz (2005b) illustrated that *S.solidus* changed its surface carbohydrate composition when it moved to the stickleback host, possibly allowing it to evade detection by the immune system (Scharsack et al., 2007). If such features exist, it seems reasonable to presume that adaptations are present to allow them to unveil themselves to host immunity (Barber and Scharsack, 2010). The fish would not be successful in changing the outcome of infection at this point, but the

parasite would benefit from the high temperatures if it allowed them to increase in size rapidly. This could lead to increased egg output if the parasite was transmitted to its definitive host, a trait that would be selected for. The stickleback host may not have evolved a counter adaptation to this if these infected fish do not breed, as they will represent an evolutionary dead end. However, some populations look like they could be capable of breeding (see Chapter 2) and thus in such populations there may be the potential for hosts to evolve counter adaptations. Further work is needed to clarify if this is the mechanism used, and if so to determine if population differences occur.

The potential for reduced antipredator behaviour to be responsible for changes in thermal preferences

Several behavioural changes in *S.solidus* infected hosts have been investigated. Milinski (1985) illustrated that heavily *S.solidus* infected sticklebacks did not show antipredator behaviour in the presence of a predatory fish (cichlid; *Tilapia mariae*). They fed at a similar rate to that when there was no predator present and foraged close to the predator. Whilst non infected and *Glugea anomala* infected fish showed antipredator behaviour feeding less and further away from the predator. Tierney et al. (1993) established that the reduced antipredator behaviour associated with *S.solidus* infection occurred only when parasites had reached infectivity. Infected fish have also frequently been found inhabiting surface or shallow waters (Arme and Owen, 1967, Lester, 1971, Moore, 2002). Increased oxygen demands have been proposed to be involved in this; however Smith and Kramer (1987) and Arme and Owen (1967) suggest a possible involvement of temperature as surface shallow waters where infected fish have been observed are warmer in temperature. These changes have all been proposed to increase predation rates of parasitized hosts; what remains unclear is whether they increase transmission to the avian definitive host and whether the behavioural alterations are

manipulated by the parasite. Here, the behavioural changes found in hosts harbouring infective parasites may be acting alongside parasite temperature preferences; as reduced antipredator behaviour results in fish swimming in shallow and surface waters that are warmer in temperature. Such an effect could mean that the temperature choice could be occurring as a side effect. The mechanism responsible for the reduced antipredator behaviour is unclear. Øverli et al. (2001) showed that brain monoaminergic activity was changed in infected fish and the authors propose that this could be due to a chronic stress response or linked to the immune response. The authors also cannot rule out the idea the parasite is actively manipulating these changes to alter host behaviour.

Scharsack et al. (2007) proposed that the upregulation of the respiratory burst reaction at 50mg in size could cause the neuroendocrine responses shown by Øverli et al. (2001) and this in turn could lead to the reduced antipredator behaviour that we see in infected hosts. Conversely, the neuroendocrine response could lead to the upregulated immune response (Barber and Scharsack, 2010). Therefore it seems likely the immune and neuroendocrine responses are linked and both involved in host behavioural changes (Barber and Scharsack, 2010). Adamo (2002) describes the potential for parasites to manipulate the host's immune system to release large amounts of neurotransmitters which in turn can change host behaviour. It remains unclear whether manipulation has evolved to cause reduced antipredator behaviour or change thermal preferences of the host or both.

Finally, as the definitive hosts of the parasite are endothermic typically birds, they will experience high temperatures (40°C) within this host. It may be that high temperatures chosen on becoming infective may allow the parasite to undergo physiological changes that allow it to rapidly develop into an egg producing adult following transmission.

6.4.4. Ecological consequences of parasite altered host thermal preferences

The finding that heavily infected sticklebacks exhibit behavioural changes that lead them to seek out warmer waters suggests positive feedback mechanisms between parasite size and host thermal preferences. If parasites grow faster at high temperatures and hosts are consistently seeking out higher temperatures this could lead to dramatic increases in parasite size and reduce the time taken to complete the parasite life cycle. A recent study by Bates et al. (2011) illustrated that snails (*Zeacumantus subcarinatus*) parasitized by the trematode *Maritrema novaezealandensis* become more heat tolerant on infection and seek out warmer temperatures in a thermal gradient. The development of this parasite and its infectivity increases at higher temperatures (up to 25°C; Studer et al., 2010) suggesting that the parasite is adaptively manipulating its hosts thermal preferences (Bates et al., 2011). Therefore, this suggests that this phenomenon may be widespread among parasites.

In general, research in to manipulative parasites is important as they are potentially regulators of ecosystem function (Lefevre et al., 2009). They can have important effects on food webs, as manipulation often results in increased predation. There is also the potential for parasites to influence intra and inter specific competition. For example, behavioural changes associated with *Schistocephalus* infections have the potential to increase predation rates by piscivorous fish; this introduces an easier source of prey for these fish. This would also lead to a reduction in the population of sticklebacks, which would have important implications for competitors that live in sympatry (Lefevre et al., 2009).

6.4.5. Ideas for further work

Further work is required to elucidate the mechanisms responsible for the observed changes in thermal preferences and to determine whether such changes more likely

reflect side effects of infection or adaptations of the host or the parasite. Tracking the thermal preferences of larger numbers of experimentally infected fish will help identify if the negative trend between parasite size and temperature choice occurs over the course of an infection in individual fish, and investigate if the switch does occur at 50mg in size. Increasing the sample sizes of fish harbouring infective parasites would strengthen the idea that thermal preferences are altered on becoming infective. Furthermore, future studies investigating the mechanisms that are causing these effects would include tracking the immune response alongside the temperature preferences of fish. Also the possibility that infective parasites prefer warmer temperatures to allow them to reproduce quicker or better in the definitive host could be tested by taking worms of similar sizes from low and high temperature acclimatised hosts to identify if there are differences in reproductive output. Finally fish that were experimentally infected could be set up in one of three conditions, either they could be kept under constant high or low temperatures or they could be allowed to choose their temperatures; this would determine if hosts that choose their temperature can limit their parasites growth.

Ligula intestinalis is closely related to *Schistocephalus* and a study by Van Dobben (1952) showed that *Ligula* infected roach made up 7% of fishermen's catches, whereas 15-30% of roach taken by cormorants were infected with *Ligula* (a definitive host); suggesting increased predation of parasitised roach. It would be interesting to investigate if temperature has a similar effect on thermal preferences and parasite growth in this species. This would help identify if this adaptation has evolved in different lines. If it did it would strengthen the case for behavioural manipulation by the parasite.

6.4.6. Conclusions

This study has illustrated a complex effect of *S. solidus* infection on the thermal preferences of host sticklebacks. Overall, fish harbouring non infective parasites show a similar temperature preference to the one they were acclimatised to. However, a significant negative relationship between parasite mass and temperature choice among fish harbouring non infective parasites, suggests that certain temperatures are preferred over the course of infection; whether these changes are adaptive or side effects of infection remains unclear. Fish harbouring parasites infective to their definitive host showed a consistent and strong preference for warmer temperatures. Such a behavioural change around the point of parasite infectivity shows a fitness advantage and “purposiveness of design” that Poulin (1995) states should occur if behavioural changes are true parasite adaptations.

Our results are important as they illustrate the potential for adaptations to develop whereby parasites or hosts can use their environmental conditions to increase their success. These results suggest that if environments get warmer, parasite development and transmission may be affected in at least two ways. Firstly, by enhanced growth as a result of an overall temperature increase (Chapter 5) and secondly by increasing the likelihood that infected hosts will seek out warmer microhabitats, thus increasing parasite growth again. The results seem to indicate that *Schistocephalus* is capable of manipulating its host’s behaviour an idea that highlights the importance of parasites in the evolution of host behaviour.

7. General Discussion



7.1. Summary of main findings

Parasite infections have the potential to interfere with host reproduction and biology through a number of diverse mechanisms. However, as well as dealing with natural stressors like parasites, hosts now face the added challenge of coping with a range of anthropogenic stressors in their environment. This is especially true of animals inhabiting aquatic habitats (Sumpter, 2005). Understanding how these anthropogenic factors effect host-parasite interactions are important in determining how man made global change will affect wildlife and disease characteristics. In this thesis the stickleback-*Schistocephalus* model host-parasite system was used to investigate a range of related questions, firstly identifying the effects of parasite infection on host reproduction and secondly investigating how anthropogenic stress influences the host-parasite interaction. In this general discussion the key results will be summarised and emerging research questions will be highlighted.

7.1.1. Variation in the effects of infection on host reproduction in sticklebacks

The effects of parasite infection on reproduction in the stickleback-*Schistocephalus* system were investigated in Chapters 2 and 3 and the physiological basis behind the reduced reproduction was determined. In males, parasite infection reduced the host's ability to produce 11-ketotestosterone, which resulted in reduced reproductive behaviour and sexual development. In females, vitellogenin (VTG) production was reduced as a result of parasite infection, which prevented egg maturation. This could be potentially due to reduced production of estrogenic chemicals that induce VTG, however this was not investigated. The main outcome from this research appears to be the variability in the effects that *Schistocephalus* infection has on reproduction. In Chapter 2, reduced reproduction in males from Kendoon Loch appears to result as a

side effect of infection, probably as a result of nutrient theft. Whilst in the Victoria Park population the evidence suggests that there is potential for adaptive manipulation by the parasite, however the data is inconclusive and thus further studies are needed to determine the strategy in this population. Finally, the effects of infection on female fish from the Carsington population in wild and laboratory investigations support the hypothesis that the reduced reproduction may be adaptive either on the part of the host or the parasite. It is important to understand the reasons behind population differences in reduced host reproduction in infected individuals, as it will help determine factors that influence parasite virulence. Once the mechanisms that determine parasite virulence in a population have been identified, the effects that anthropogenic stressors will have on parasite virulence can be elucidated

There is the potential that different strategies of parasite exploitation have evolved in different host / parasite populations. However, there are a number of further factors that need to be investigated. Cross infection studies are required to investigate how infection outcomes are influenced by the combination of host and parasite genotypes in the different populations ($G_H \times G_P$ interaction). For example the parasite strain infecting the Carsington population may be more pathogenic than those in the Kendoon Loch population, and therefore when fish from Kendoon are infected with it, both populations would have similar host reproductive phenotypes. Conversely, the particular host strain in Kendoon Loch may have been better adapted to deal with parasite infection, and thus when infected with parasites from a different population there will still be individuals capable of reproducing. It is also important to note that when comparing Carsington population with Kendoon Loch, we are really comparing females with males (as there was a very small sample size of males in the Carsington study), thus it may be that the sexes are affected differently by infection.

However, the idea that the parasite or host is locally adapted to the other party is dependent on what constitutes a *S. solidus* population. The definitive host of *S. solidus* is usually a bird, making it likely that the parasites are constantly transported to new populations which could prevent the establishment of genetic barriers and local adaptation. Hoole et al. (2010) state a similar argument in the case of the *Ligula intestinalis* parasite, where avian definitive hosts are also present. It has been illustrated in the case of *S. solidus* that significant gene flow does seem to occur between geographically distinct populations such as Oregon and Alaska, supporting the idea that local adaptation is unlikely (Nishimura et al., 2011). Whereas differentiation does seem to occur between *S. solidus* parasites at a global scale, with differences observed between parasites from Alaska and Wales (Nishimura et al., 2011).

Alternatively, the population differences observed may be due to the co-evolutionary time hypothesis. All the water bodies that were sampled are man-made. Kendoon Loch was formed after river impoundment in 1935; likewise Carsington Reservoir was formed in 1992 by this process, whilst the Victoria Park pond was created in the early 1990s. Although the founding populations for Kendoon Loch and Carsington Reservoir are not known, they are likely to have been colonized from fish present in the original river. The Victoria Park population is also likely to be founded from a local river, as there are no natural lakes in this region. *S. solidus* is not a characteristic parasite of sticklebacks in flowing water, therefore these founding dates should represent the association time between the host and its parasite. Therefore, in the populations where adaptive manipulation by the parasite is suspected, the sticklebacks have had a shorter association time with the parasite, compared to the Kendoon Loch population. Therefore the differential effects of infection in populations may be related to this. This idea is backed up by a study by Kalbe and Kurtz (2006) which showed that

sticklebacks from a lake with endemic *Diplostomum pseudospathaceum* infection were better adapted to cope with infection and had a higher immunocompetence than sticklebacks from a river where the parasite does not occur.

7.1.2. Environmental effects on host – parasite interactions

The effects of environmental conditions on the host-parasite interaction are likely to be important. For example, certain conditions may influence genotypes differently leading to genotype x environment (G x E) interactions.

7.1.2.1. *The effects of EDCs on host – parasite interactions*

Chapter 4 showed that chemicals with estrogenic modes of action have the potential to increase disease progression, and that this may occur in a sex dependent manner. Future studies over longer time periods (8 weeks post parasite exposure) are now needed, as these will probably show that bigger differences exist between the sexes under estrogen exposure. Furthermore, studies that use different estrogens are required, to determine if synthetic estrogens and xenoestrogens have a similar effect. Depending on the mechanism behind this effect there is potential for androgenic chemicals to have a similar effect, but on females. For example, if parasites in male fish are actively using VTG as a nutrient for growth there is the potential that parasites can use other hormone dependent proteins for growth. The exposure of female sticklebacks to androgenic chemicals results in the production of the glycoprotein nest building glue, spiggin (Katsiadaki et al., 2002a). Here there is potential for parasites in infected females from androgen polluted environments to use spiggin as a nutrient for growth. Similarly, if the results observed are due to immunosuppression (or cortisol induced immunosuppression) it seems plausible that similar mechanisms may exist in females dealing with levels of androgens that they would normally not experience. Further work is needed to elucidate the underlying mechanisms of increased parasite growth in males

exposed to estrogens. In addition, experiments with androgenic chemicals will determine if similar effects are observed in exposed females.

How these stressors influence host reproduction and parasite reproduction will ultimately determine their effect on parasite virulence. Therefore, further studies investigating the effect of EDCs on the parasite life cycle will identify how parasite reproduction will be influenced. It seems likely that the increased parasite growth in males associated with estrogen exposure could lead to a scenario where host reproduction is halted in infected individuals, preventing hosts from coevolving with parasites since in order to evolve a counter adaptation to reduce virulence offspring need to be produced, which would not occur in castrated populations.

7.1.2.2. *The effects of temperature on host – parasite interactions*

Chapter 5 showed that elevated temperatures increased disease progression in sticklebacks infected with *Schistocephalus*. As mentioned above, the effect that temperature has on host reproduction and parasite reproduction will determine its effect on parasite virulence.

Temperature could lead to the differences seen in terms of fecundity reduction in infected fish from the various populations. For example, the Kendoon Loch population is from a higher latitude, and is therefore likely to experience cooler environmental temperatures. Chapter 5 illustrated that parasites grew slower at cooler temperatures; therefore in supporting this growth a slower supply of nutrients will be required than those from warmer temperatures. This may result in fish from Kendoon Loch experiencing conditions that facilitate parasite growth without requiring for them to reduce the reproductive output of its host until the parasite is large in size. If for example, the Carsington population was experiencing warmer temperatures their parasites would grow faster and would require a faster supply of nutrients which could

be obtained by the fecundity reduction of hosts. Again, laboratory studies could help identify the effects of environment. If offspring are raised in a common garden environment the effects of the environment would be removed. If differences still occurred when cross infection studies were performed, this could illustrate the evolutionary basis of the differences, for example, hosts could differ in their adaptation to their environment.

Studies that track the temperature in natural environments and relate these to the reproductive ability of infected populations will be extremely useful in identifying if temperature could create population differences in fecundity reduction in infected fish. Further studies are required to identify the mechanism causing increased parasite growth at high temperatures. In addition, the effects of temperature on aspects of the entire life cycle will help to determine how parasite reproduction is influenced and help identify how parasite virulence will be affected from an evolutionary perspective.

The potential for anthropogenic stress to interfere with host behaviour is demonstrated in Chapter 6. The hypothesis that hosts and / or parasites can use their environmental conditions to their own advantage relies on the idea that one party gains a selective advantage over the other in terms of the conditions experienced. In Chapter 5, elevated temperatures increased parasite growth and Chapter 6 illustrated the potential for infective parasites to manipulate its host, by altering host thermal preferences. This provides a feedback mechanism, whereby increased temperatures increase parasite growth and fish harbouring infective parasites seek out the warmest temperature leading to parasites growing at their highest possible rate and potentially exacerbating the effects of temperature rises on parasite growth. Future studies are required to elucidate if true parasite manipulation of host behaviour is occurring. In addition, the mechanisms underlying the changes need to be determined i.e. is it

immune or neuroendocrine based (or both). In addition, it will be interesting to study the behaviour of different populations. As fish in warmer environments are likely to experience faster growing worms there should be high selective pressure on the host to evolve a way to limit parasite growth. Therefore hosts from warmer environments may have evolved a mechanism to try and limit parasite growth this may make them more likely to seek out cooler temperatures given the choice. Or they may have developed stronger immune responses that limit parasite growth. However, although selective pressure should exist for hosts to reduce parasite growth in warm environments, counter adaptations may not exist if these fish are unable to reproduce. Conversely, fish in cooler environments are more likely to experience slower growing worms and thus there would be less selective pressure on them to limit parasite growth, and their immune responses may not be as developed as fish from warmer environments. In addition, if the thermal preference for warmer waters in fish harbouring infective parasites is immune based, population differences in immune responses may lead to differences in behavioural responses to temperature.

Therefore our results suggest that if environments get warmer, parasite development and transmission may be affected in at least two ways. Firstly, by enhanced growth as a result of an overall temperature increase (Chapter 5) and secondly by increasing the likelihood that infected hosts will seek out warmer micro habitats, thus increasing parasite growth again (Chapter 6).

7.1.2.3. *Ideas for further work*

This thesis illustrates the potential for two individual anthropogenic stressors to have substantial effects on disease progression. It is now becoming clear that organisms face multiple anthropogenic stressors in their environment, and the combined effects of these stressors could have a significant effect on disease in parasitized hosts. This model

system therefore appears ideal for studying the combined effects of temperature and endocrine disrupting chemicals on disease progression. Recent research has highlighted the potential for increased temperatures and precipitation, two outcomes of climate change, to influence the effect of pollutants by changing their distribution and usage (Bailey, 2003, Noyes et al., 2009). Studies have also illustrated that temperature has the potential to increase the VTG response of estrogen exposed fish (Ishibashi et al., 2001, Brian et al., 2008). This alongside the results from this thesis suggests that there is real potential for EDCs and temperature to interact in a synergistic or additive manner increasing parasite growth rates and future studies should focus on this. Also the effects of combined anthropogenic stressors on the entire parasite life cycle should be investigated to identify if parasite transmission will be altered and how this will influence the parasite life cycle.

7.2. Main conclusions

The major result of this thesis is that anthropogenic stressors affect disease progression in the stickleback-*Schistocephalus* system. This is likely to have a knock on effect on host reproduction, reducing the reproductive potential of infected populations regardless of whether adaptive or non adaptive mechanisms exist, as large worms are always associated with reduced reproduction. The increased growth of parasites as a result of anthropogenic stress also suggests that parasite reproduction will be increased, potentially leading to the evolution of higher virulence in the host – parasite interaction.

8. References

- ADAMO, S. A. (2002) Modulating the modulators: Parasites, neuromodulators and host behavioral change. *Brain Behavior and Evolution*, 60, 370-377.
- AHMED, S. R. (2000) The immune system as a potential target for environmental estrogens (endocrine disruptors): a new emerging field. *Toxicology*, 150, 191-206.
- ALLEN, J. R. M. & WOOTTON, R. J. (1982) The effect of ration and temperature on the growth of the 3-spined stickleback, *Gasterosteus-aculeatus* L. *Journal of Fish Biology*, 20, 409-422.
- ALLEN, Y. T., KATSIADAKI, I., POTTINGER, T. G., JOLLY, C., MATTHIESSEN, P., MAYER, I., SMITH, A., SCOTT, A. P., ECCLES, P., SANDERS, M. B., PULMAN, K. G. T. & FEIST, S. (2008) Intercalibration exercise using a stickleback endocrine disrupter screening assay. *Environmental Toxicology and Chemistry*, 27, 404-412.
- ANDERSON, R. M. & MAY, R. M. (1982) Coevolution of hosts and parasites. *Parasitology*, 85, 411-426.
- ARME, C. (1968) Effects of the plerocercoid larva of a pseudophyllidean cestode, *Ligula intestinalis*, on the pituitary gland and gonads of its host. *Biol.Bull.*, 134, 15-25.
- ARME, C. (1997) *Ligula intestinalis*: Interactions with the pituitary-gonadal axis of its fish host. *Journal of Helminthology*, 71, 83-84.
- ARME, C. & OWEN, R. W. (1967) Infections of the three spined stickleback, *Gasterosteus aculeatus* L., with the plerocercoid larvae of *Schistocephalus solidus* (Muller, 1776), with special reference to pathological effects. *Parasitology*, 57, 301-314.
- BAGAMIAN, K. H., HEINS, D. C. & BAKER, J. A. (2004) Body condition and reproductive capacity of three-spined stickleback infected with the cestode *Schistocephalus solidus*. *Journal of Fish Biology*, 64, 1568-1576.
- BAILEY, S. W. (2003) Climate change and decreasing herbicide persistence. *Pest Management Science*, 60, 158-162.
- BARBER, I. (1997) A non-invasive morphometric technique for estimating cestode plerocercoid burden in small freshwater fish. *Journal of Fish Biology*, 51, 654-658.
- BARBER, I. (2005) Parasites grow larger in faster growing fish hosts. *International Journal for Parasitology*, 35, 137-143.
- BARBER, I. (2007) Host-Parasite Interactions of the Three-Spined Stickleback. IN ÖSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. (Eds.) *Biology of the Three-Spined Stickleback*. London, CRC Press.
- BARBER, I. & ARNOTT, S. A. (2000) Split-clutch IVF: A technique to examine indirect fitness consequences of mate preferences in sticklebacks. *Behaviour*, 137, 1129-1140.
- BARBER, I., ARNOTT, S. A., BRAITHWAITE, V. A., ANDREW, J. & HUNTINGFORD, F. A. (2001) Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasitic infections. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 268, 71-76.
- BARBER, I., HOARE, D. & KRAUSE, J. (2000) Effects of parasites on fish behaviour: a review and evolutionary perspective. *Reviews in Fish Biology and Fisheries*, 10, 131-165.

- BARBER, I. & NETTLESHIP, S. (2010) From 'trash fish' to supermodel: the rise and rise of the three-spined stickleback in evolution and ecology. *Biologist*, 57, 15-21.
- BARBER, I. & SCHARSACK, J. P. (2010) The three-spined stickleback-*Schistocephalus solidus* system: an experimental model for investigating host-parasite interactions in fish. *Parasitology*, 137, 411-424.
- BARBER, I. & SVENSSON, P. A. (2003) Effects of experimental *Schistocephalus solidus* infections on growth, morphology and sexual development of female three-spined sticklebacks, *Gasterosteus aculeatus*. *Parasitology*, 126, 359-367.
- BARBER, I., WALKER, P. & SVENSSON, P. A. (2004) Behavioural responses to simulated avian predation in female three spined sticklebacks: The effect of experimental *Schistocephalus solidus* infections. *Behaviour*, 141, 1425-1440.
- BARUS, V., TENORA, F., KRACMAR, S. & PROKES, M. (2001) Accumulation of heavy metals in the *Ligula intestinalis* plerocercoids (Pseudophyllidea) of different age. *Helminthologia*, 38, 29-33.
- BATES, A. E., LEITERER, F., WIEDEBACK, M. L. & POULIN, R. (2011) Parasitized snails take the heat: a case of host manipulation? *Oecologia*, Published online ahead of press.
- BELL, A. M. (2001) Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*. *Animal Behaviour*, 62, 775-780.
- BELL, A. M. (2004) An endocrine disrupter increases growth and risky behavior in threespined stickleback (*Gasterosteus aculeatus*). *Hormones and Behavior*, 45, 108-114.
- BELL, M. A. & FOSTER, S. A. (1994) *The Evolutionary Biology of the Threespine Stickleback*, Oxford, Oxford University Press.
- BERDOY, M., WEBSTER, J. P. & MACDONALD, D. W. (1995) Parasite-altered behaviour: is the effect of *Toxoplasma gondii* on *Rattus norvegicus* specific. *Parasitology*, 111, 403-409.
- BERDOY, M., WEBSTER, J. P. & MACDONALD, D. W. (2000) Fatal attraction in rats infected with *Toxoplasma gondii*. *Proceedings of the Royal Society B-Biological Sciences*, 267, 1591-1594.
- BERNHARDT, R. R., VON HIPPEL, F. A. & CRESKO, W. A. (2006) Perchlorate induces hermaphroditism in threespine sticklebacks. *Environmental Toxicology and Chemistry*, 25, 2087-2096.
- BIRON, D. G., PONTON, F., JOLY, C., MENIGOZ, A., HANELT, B. & THOMAS, F. (2005) Water-seeking behavior in insects harboring hairworms: should the host collaborate? *Behavioral Ecology*, 16, 656-660.
- BLANFORD, S., THOMAS, M. B., PUGH, C. & PELL, J. K. (2003) Temperature checks the Red Queen? Resistance and virulence in a fluctuating environment. *Ecology Letters*, 6, 2-5.
- BOONEKAMP, J. J., ROS, A. H. F. & VERHULST, S. (2008) Immune activation suppresses plasma testosterone level: a meta-analysis. *Biology Letters*, 4, 741-744.
- BORG, B. (1987) Stimulation of reproductive behaviour by aromatizable and non-aromatizable androgens in the male three-spined stickleback, *Gasterosteus aculeatus* L. *Proceedings of V. Congr. Europ. Ichthyol., Stockholm*, 269-271.
- BORG, B. (1994) Androgens in teleost fishes. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*, 109, 219-245.

- BORG, B. (2007) Reproductive Physiology of Sticklebacks. IN ÖSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. (Eds.) *Biology of the Three-Spined Stickleback*. London, CRC Press.
- BORG, B., ANTONOPOULOU, E., ANDERSSON, E., CARLBERG, T. & MAYER, I. (1993) Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male 3-spined sticklebacks, *Gasterosteus aculeatus*. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 71, 2327-2329.
- BORNSTEIN, S. R., RUTKOWSKI, H. & VREZAS, I. (2004) Cytokines and steroidogenesis. *Molecular and Cellular Endocrinology*, 215, 135-141.
- BOXALL, A. B. A., SINCLAIR, C. J., FENNER, K., KOLPIN, D. & MAUD, S. J. (2004) When synthetic chemicals degrade in the environment. *Environmental Science & Technology*, 38, 368A-375A.
- BRIAN, J. V., AUGLEY, J. J. & BRAITHWAITE, V. A. (2006) Endocrine disrupting effects on the nesting behaviour of male three-spined stickleback *Gasterosteus aculeatus* L. *Journal of Fish Biology*, 68, 1883-1890.
- BRIAN, J. V., HARRIS, C. A., RUNNALLS, T. J., FANTINATI, A., POJANA, G., MARCOMINI, A., BOOY, P., LAMOREE, M., KORTENKAMP, A. & SUMPTER, J. P. (2008) Evidence of temperature-dependent effects on the estrogenic response of fish: implications with regard to climate change. *Science of the Total Environment*, 397, 72-81.
- BRIAN, J. V., HARRIS, C. A., SCHOLZE, M., BACKHAUS, T., BOOY, P., LAMOREE, M., POJANA, G., JONKERS, N., RUNNALLS, T., BONFA, A., MARCOMINI, A. & SUMPTER, J. P. (2005) Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. *Environmental Health Perspectives*, 113, 721-728.
- CALOSI, P., BILTON, D. T. & SPICER, J. I. (2008) Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biology Letters*, 4, 99-102.
- CAMPBELL, J., KESSLER, B., MAYACK, C. & NAUG, D. (2010) Behavioural fever in infected honeybees: parasitic manipulation or coincidental benefit? *Parasitology*, 137, 1487-1491.
- CANDOLIN, U. & VOIGT, H. R. (2001) No effect of a parasite on reproduction in stickleback males: a laboratory artefact? *Parasitology*, 122, 457-464.
- CARRERO, J. C., CERVANTES, C., MORENO-MENDOZA, N., SAAVEDRA, E., MORALES-MONTOR, J. & LACLETTE, J. P. (2006) Dehydroepiandrosterone decreases while cortisol increases in vitro growth and viability of *Entamoeba histolytica*. *Microbes and Infection*, 8, 323-331.
- CARTER, V., PIERCE, R., DUFOUR, S., ARME, C. & HOOLE, D. (2005) The tapeworm *Ligula intestinalis* (Cestoda : Pseudophyllidea) inhibits LH expression and puberty in its teleost host, *Rutilus rutilus*. *Reproduction*, 130, 939-945.
- CHARLES, G. H. & ORR, T. S. (1968) Comparative fine structure of outer tegument of *Ligula intestinalis* and *Schistocephalus solidus*. *Exp Parasitol*, 22, 137-49.
- CHELLAPPA, S., HUNTINGFORD, F. A., STRANG, R. H. C. & THOMSON, R. Y. (1995) Condition factor and hepatosomatic index as estimates of energy status in male 3-spined stickleback. *Journal of Fish Biology*, 47, 775-787.
- CHEN, W. H., SUN, L. T., TSAI, C. L., SONG, Y. L. & CHANG, C. F. (2002) Cold-stress induced the modulation of catecholamines, cortisol, immunoglobulin M, and leukocyte phagocytosis in tilapia. *General and Comparative Endocrinology*, 126, 90-100.

- CHETTRI, J. K., HOLTEN-ANDERSEN, L. & BUCHMANN, K. (2010) Factors influencing in vitro respiratory burst assays with head kidney leucocytes from rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases*, 33, 593-602.
- CHRISTE, P., OPPLIGER, A. & RICHNER, H. (1994) Ectoparasite affects choice and use of roost sites in the great tit, *Parus major* *Animal Behaviour*, 47, 895-898.
- CHRISTENSEN, M. R., GRAHAM, M. D., VINEBROOKE, R. D., FINDLAY, D. L., PATERSON, M. J. & TURNER, M. A. (2006) Multiple anthropogenic stressors cause ecological surprises in boreal lakes. *Global Change Biology*, 12, 2316-2322.
- CLARK, L. & MASON, J. R. (1985) Use of nest material as insecticidal and anti-pathogenic agents by the European Starling. *Oecologia*, 67, 169-176.
- CLAYTON, D. H. & MOORE, J. (1997) Introduction. IN CLAYTON, D. H. & MOORE, J. (Eds.) *Host - parasite evolution*. Oxford, Oxford University Press.
- CLEMENS, M. J. (1974) The regulation of egg yolk protein synthesis by steroid hormones. *Prog Biophys Mol Biol*, 28, 69-108.
- COLBORN, T., SAAL, F. S. V. & SOTO, A. M. (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*, 101, 378-384.
- COMBALBERT, S. & HERNANDEZ-RAQUET, G. (2010) Occurrence, fate, and biodegradation of estrogens in sewage and manure. *Applied Microbiology and Biotechnology*, 86, 1671-1692.
- CONRADT, U. & PETERS, W. (1989) Investigations on the occurrence of pinocytosis in the tegument of *Schistocephalus solidus*. *Parasitology Research*, 75, 630-635.
- COORS, A. & DE MEESTER, L. (2008) Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. *Journal of Applied Ecology*, 45, 1820-1828.
- DESBROW, C., ROUTLEDGE, E. J., BRIGHTY, G. C., SUMPTER, J. P. & WALDOCK, M. (1998) Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. *Environmental Science & Technology*, 32, 1549-1558.
- DEUTSCH, C. A., TEWKSBURY, J. J., HUEY, R. B., SHELDON, K. S., GHALAMBOR, C. K., HAAK, D. C. & MARTIN, P. R. (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 6668-6672.
- DÖRÜCÜ, M., WILSON, D. & BARBER, I. (2007) Differences in adult egg output of *Schistocephalus solidus* from singly- and multiply-infected sticklebacks. *Journal of Parasitology*, 93, 1521-1523.
- DUONG, C. N., RA, J. S., CHO, J., KIM, S. D., CHOI, H. K., PARK, J. H., KIM, K. W. & INAM, E. (2010) Estrogenic chemicals and estrogenicity in river waters of South Korea and seven Asian countries. *Chemosphere*, 78, 286-293.
- EBERT, D., CARIUS, H. J., LITTLE, T. & DECAESTECKER, E. (2004) The evolution of virulence when parasites cause host castration and gigantism. *American Naturalist*, 164, S19-S32.
- EBERT, D. & HERRE, E. A. (1996) The evolution of parasitic diseases. *Parasitology Today*, 12, 96-101.
- EIZAGUIRRE, C., LENZ, T. L., SOMMERFELD, R. D., HARROD, C., KALBE, M. & MILINSKI, M. (2011) Parasite diversity, patterns of MHC II variation and olfactory based mate choice in diverging three-spined stickleback ecotypes. *Evolutionary Ecology*, 25, 605-622.

- ELLIOT, S. L., BLANFORD, S. & THOMAS, M. B. (2002) Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 1599-1607.
- ESCOBEDO, G., LARRALDE, C., CHAVARRIA, A., CERBON, M. A. & MORALES-MONTOR, J. (2004) Molecular mechanisms involved in the differential effects of sex steroids on the reproduction and infectivity of *Taenia crassiceps*. *Journal of Parasitology*, 90, 1235-1244.
- ESCOBEDO, G., ROBERTS, C. W., CARRERO, J. C. & MORALES-MONTOR, J. (2005) Parasite regulation by host hormones: an old mechanism of host exploitation? *Trends in Parasitology*, 21, 588-593.
- ESTEBAN, M. A., RODRIGUEZ, A., AYALA, A. G. & MESEGUER, J. (2004) Effects of high doses of cortisol on innate cellular immune response of seabream (*Sparus aurata* L.). *General and Comparative Endocrinology*, 137, 89-98.
- EUSTACHE, F., MONDON, F., CANIVENC-LAVIER, M. C., LESAFFRE, C., FULLA, Y., BERGES, R., CRAVEDI, J. P., VAIMAN, D. & AUGER, J. (2009) Chronic Dietary Exposure to a Low-Dose Mixture of Genistein and Vinclozolin Modifies the Reproductive Axis, Testis Transcriptome, and Fertility. *Environmental Health Perspectives*, 117, 1272-1279.
- FATIMA, M., MANDIKI, S. N. M., DOUXFILS, J., SILVESTRE, F., COPPE, P. & KESTEMONT, P. (2007) Combined effects of herbicides on biomarkers reflecting immune-endocrine interactions in goldfish immune and antioxidant effects. *Aquatic Toxicology*, 81, 159-167.
- FERRARI, J., MULLER, C. B., KRAAIJEVELD, A. R. & GODFRAY, H. C. J. (2001) Clonal variation and covariation in aphid resistance to parasitoids and a pathogen. *Evolution*, 55, 1805-1814.
- FIALHO, R. F. & SCHALL, J. J. (1995) Thermal ecology of a malarial parasite and its insect vector: consequences for the parasites transmission success. *Journal of Animal Ecology*, 64, 553-562.
- FILBY, A. L., NEUPARTH, T., THORPE, K. L., OWEN, R., GALLOWAY, T. S. & TYLER, C. R. (2007) Health impacts of estrogens in the environment, considering complex mixture effects. *Environmental Health Perspectives*, 115, 1704-1710.
- FLEGR, J. & HRDY, I. (1994) Influence of chronic toxoplasmosis on some human personality factors. *Folia Parasitologica*, 41, 122-126.
- FLORES-VALVERDE, A. M., HORWOOD, J. & HILL, E. M. (2010) Disruption of the Steroid Metabolome in Fish Caused by Exposure to the Environmental Estrogen 17 alpha-Ethinylestradiol. *Environmental Science & Technology*, 44, 3552-3558.
- FOLMAR, L. C., GARDNER, G. R., SCHREIBMAN, M. P., MAGLIULO-CEPRIANO, L., MILLS, L. J., ZAROOGIAN, G., GUTJAHR-GOBELL, R., HAEBLER, R., HOROWITZ, D. B. & DENSLOW, N. D. (2001) Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquatic Toxicology*, 51, 431-441.
- FOLSTAD, I. & KARTER, A. J. (1992) Parasites, bright males, and the immunocompetence handicap. *American Naturalist*, 139, 603-622.
- FRISCHKNECHT, M. (1993) The breeding coloration of male 3 spined sticklebacks (*Gasterosteus aculeatus*) as and inidicator of energy investment in vigor. *Evolutionary Ecology*, 7, 439-450.

- GALLAUGHER, P., THORARENSEN, H. & FARRELL, A. P. (1995) Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respiration Physiology*, 102, 279-292.
- GERAUDIE, P., BOULANGE-LECOMTE, C., GERBRON, M., HINFRAY, N., BRION, F. & MINIER, C. (2010) Endocrine effects of the tapeworm *Ligula intestinalis* in its teleost host, the roach (*Rutilus rutilus*). *Parasitology*, 137, 697-704.
- GHEORGHIU, C., MARCOGLIESE, D.J. AND SCOTT, M. (2005) Concentration-dependent effects of waterborne zinc on population dynamics of *Gyrodactylus turnbulli* (Monogenea) on isolated guppies (*Poecilia reticulata*). *Parasitology* 132, 225-232.
- GILES, N. (1983) Behavioral effects of the parasite *Schistocephalus solidus* (Cestoda) on an intermediate host, the 3-spined stickleback, *Gasterosteus aculeatus* L. *Animal Behaviour*, 31, 1192-1194.
- GREYTAK, S. R., TARRANT, A. M., NACCI, D., HAHN, M. E. & CALLARD, G. V. (2010) Estrogen responses in killifish (*Fundulus heteroclitus*) from polluted and unpolluted environments are site- and gene-specific. *Aquatic Toxicology*, 99, 291-299.
- GRIFFITHS, R., ORR, K. J., ADAM, A. & BARBER, I. (2000) DNA sex identification in the three-spined stickleback. *Journal of Fish Biology*, 57, 1331-1334.
- GUDERLEY, H. & LEROY, P. H. (2001) Family origin and the response of threespine stickleback, *Gasterosteus aculeatus*, to thermal acclimation. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 171, 91-101.
- GUDERLEY, H. E. (1994) Physiological ecology and evolution of the threespine stickleback. IN BELL, A. M. & FOSTER, S. A. (Eds.) *The Evolutionary Biology of the Threespine Stickleback*. Oxford, Oxford University Press.
- GUILLETTE, L. J., CRAIN, D. A., GUNDERSON, M. P., KOOLS, S. A. E., MILNES, M. R., ORLANDO, E. F., ROONEY, A. A. & WOODWARD, A. R. (2000) Alligators and endocrine disrupting contaminants: A current perspective. *American Zoologist*, 40, 438-452.
- GUILLETTE, L. J. & GUNDERSON, M. P. (2001) Alterations in development of reproductive and endocrine systems of wildlife populations exposed to endocrine-disrupting contaminants. *Reproduction*, 122, 857-864.
- HAHLBECK, E., KATSIADAKI, I., MAYER, I., ADOLFSSON-ERICI, M., JAMES, J. & BENGTSSON, B. E. (2004) The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption - II - kidney hypertrophy, vitellogenin and spiggin induction. *Aquatic Toxicology*, 70, 311-326.
- HAMILTON, W. D., AXELROD, R. & TANESE, R. (1990) Sexual reproduction as an adaptation to resist parasites (a review). *Proceedings of the National Academy of Sciences of the United States of America*, 87, 3566-3573.
- HAMILTON, W. D. & ZUK, M. (1982) Heritable true fitness and bright birds - a role for parasites. *Science*, 218, 384-387.
- HAMMERSCHMIDT, K. & KURTZ, J. (2005a) Evolutionary implications of the adaptation to different immune systems in a parasite with a complex life cycle. *Proceedings of the Royal Society B-Biological Sciences*, 272, 2511-2518.
- HAMMERSCHMIDT, K. & KURTZ, J. (2005b) Surface carbohydrate composition of a tapeworm in its consecutive intermediate hosts: Individual variation and fitness consequences. *International Journal for Parasitology*, 35, 1499-1507.

- HAMMERSCHMIDT, K. & KURTZ, J. (2007) *Schistocephalus solidus*: Establishment of tapeworms in sticklebacks - fast food or fast lane? *Experimental Parasitology*, 116, 142-149.
- HART, B. L. (1994) Behavioural defense against parasites: interaction with parasite invasiveness. *Parasitology*, 109, S139-S151.
- HART, B. L. (1997) Behavioural defence. IN CLAYTON, D. H. & MOORE, J. (Eds.) *Host-parasite evolution*. Oxford, Oxford University Press.
- HARVELL, C. D., MITCHELL, C. E., WARD, J. R., ALTIZER, S., DOBSON, A. P., OSTFELD, R. S. & SAMUEL, M. D. (2002) Ecology - Climate warming and disease risks for terrestrial and marine biota. *Science*, 296, 2158-2162.
- HAVLICEK, J., GASOVA, Z., SMITH, A. P., ZVARA, K. & FLEGR, J. (2001) Decrease of psychomotor performance in subjects with latent 'asymptomatic' toxoplasmosis. *Parasitology*, 122, 515-520.
- HECKER, M. & KARBE, L. (2005) Parasitism in fish - an endocrine modulator of ecological relevance? *Aquatic Toxicology*, 72, 195-207.
- HECKER, M., SANDERSON, J. T. & KARBE, L. (2007) Suppression of aromatase activity in populations of bream (*Abramis brama*) from the river Elbe, Germany. *Chemosphere*, 66, 542-552.
- HEDGER, M. P. & MEINHARDT, A. (2003) Cytokines and the immune-testicular axis. *Journal of Reproductive Immunology*, 58, 1-26.
- HEINS, D. C. & BAKER, J. A. (2003) Reduction of egg size in natural populations of threespine stickleback infected with a cestode macroparasite. *Journal of Parasitology*, 89, 1-6.
- HEINS, D. C. & BAKER, J. A. (2008) The stickleback-*Schistocephalus* host-parasite system as a model for understanding the effect of a macroparasite on host reproduction. *5th International Conference on Stickleback Behavior and Evolution*, 625-645.
- HEINS, D. C. & BAKER, J. A. (2010) Castration of Female Ninespine Stickleback by the Pseudophyllidean Cestode *Schistocephalus pungitii*: Evolutionary Significance and Underlying Mechanism. *Journal of Parasitology*, 96, 206-208.
- HEINS, D. C., BAKER, J. A., TOUPS, M. A. & BIRDEN, E. L. (2010a) Evolutionary significance of fecundity reduction in threespine stickleback infected by the diphyllbothriidean cestode *Schistocephalus solidus*. *Biological Journal of the Linnean Society*, 100, 835-846.
- HEINS, D. C., BIRDEN, E. L. & BAKER, J. A. (2010b) Host mortality and variability in epizootics of *Schistocephalus solidus* infecting the threespine stickleback, *Gasterosteus aculeatus*. *Parasitology*, 137, 1681-1686.
- HEINS, D. C., ULINSKI, B., JOHNSON, J. & BAKER, J. A. (2004) Effect of the cestode macroparasite *Schistocephalus pungitii* on the reproductive success of ninespine stickleback, *Pungitius pungitius*. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 82, 1731-1737.
- HELLQVIST, A., SCHMITZ, M., MAYER, I. & BORG, B. (2006) Seasonal changes in expression of LH-beta and FSH-beta in male and female three-spined stickleback, *Gasterosteus aculeatus*. *General and Comparative Endocrinology*, 145, 263-269.
- HOLMSTRUP, M., BINDESBOL, A. M., OOSTINGH, G. J., DUSCHL, A., SCHEIL, V., KOHLER, H. R., LOUREIRO, S., SOARES, A., FERREIRA, A. L. G., KIENLE, C., GERHARDT, A., LASKOWSKI, R., KRAMARZ, P. E., BAYLEY, M., SVENDSEN, C. & SPURGEON, D. J. (2010) Interactions

- between effects of environmental chemicals and natural stressors: A review. *Science of the Total Environment*, 408, 3746-3762.
- HONTELA, A., RASMUSSEN, J. B., AUDET, C. & CHEVALIER, G. (1992) Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs and mercury. *Archives of Environmental Contamination and Toxicology*, 22, 278-283.
- HOOLE, D. (1997) The effects of pollutants on the immune response of fish: Implications for helminth parasites. *Parassitologia (Rome)*, 39, 219-225.
- HOOLE, D., CARTER, V. & DUFOUR, S. (2010) *Ligula intestinalis* (Cestoda: Pseudophyllidea): an ideal fish-metazoan parasite model? *Parasitology*, 137, 425-438.
- HOPKINS, C. A., LAW, L. M. & THREADGOLD, L. T. (1978) *Schistocephalus solidus* - Pinocytosis by plerocercoid tegument. *Experimental Parasitology*, 44, 161-172.
- HOUSTON, A. H. (1997) Review: Are the classical hematological variables acceptable indicators of fish health? *Transactions of the American Fisheries Society*, 126, 879-894.
- HOWELL, W. M., BLACK, D. A. & BORTONE, S. A. (1980) Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: Evidence for environmentally-induced masculinisation. *Copeia*, 676-681.
- HUEY, R. B. & STEVENSON, R. D. (1979) Integrating thermal physiology and ecology of ectotherms - discussion of approaches. *American Zoologist*, 19, 357-366.
- HUFFMAN, M. A. (1997) Current evidence for self-medication in primates: A multidisciplinary perspective. *Yearbook of Physical Anthropology, Vol 40 - 1997*, 40, 171-200.
- HUFFMAN, M. A. (2010) Self-Medication: Passive Prevention and Active Treatment. IN M.D., B. & J., M. (Eds.) *Encyclopedia of Animal Behavior*. Oxford, Academic Press.
- HUFFMAN, M. A. & CATON, J. M. (2001) Self-induced increase of gut motility and the control of parasitic infections in wild chimpanzees. *International Journal of Primatology*, 22, 329-346.
- HUFFMAN, M. A., GOTOH, S., IZUTSU, D., KOSHIMIZU, K. & KALUNDE, M. S. (1993) Further observations of the use of the medicinal plant, *Veronia amygdalina* (Del), by a wild chimpanzee, its possible effect on parasite load, and its phytochemistry. *African Study Monographs*, 14, 227-240.
- HUFFMAN, M. A., PAGE, J. E., SUKHDEO, M. V. K., GOTOH, S., KALUNDE, M. S., CHANDRASIRI, T. & TOWERS, G. H. N. (1996) Leaf-swallowing by chimpanzees: A behavioral adaptation for the control of strongyle nematode infections. *International Journal of Primatology*, 17, 475-503.
- HUFFMAN, M. A. & SEIFU, M. (1989) Observations on the illness and consumption of a possibly medicinal plant *Veronia amygdalina* (DEL.), by a Wild Chimpanzee in the Mahale Mountains National Park, Tanzania. *Primates*, 30, 51-63.
- HURD, H. (2001) Host fecundity reduction: a strategy for damage limitation? *Trends in Parasitology*, 17, 363-368.
- IPCC (2001) Climate Change 2001: impacts, adaptation and vulnerability. Contribution of Working Group II to the Third Assessment Report. IN MCCARTHY, J. J.,

- CANZIANI, O. F., LEARY, N. A., DOKKEN, D. J. & WHITE, K. S. (Eds.). Cambridge, Cambridge University Press.
- IPCC (2007) Summary for policymakers. IN SOLOMON, S. Q. D., MANNING, M., CHEN, Z., MANNING, M., AVERYT, K.B., TIGNOR, M. & MILLER, H.L. (Ed.) *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge and New York, Cambridge University Press.
- ISHIBASHI, H., TACHIBANA, K., TSUCHIMOTO, M., SOYANO, K., ISHIBASHI, Y., NAGAE, M., KOHRA, S., TAKAO, Y., TOMINAGA, N. & ARIZONO, K. (2001) *In vivo* testing system for determining the estrogenic activity of endocrine-disrupting chemicals (EDCs) in goldfish (*Carassius auratus*). *Journal of Health Science*, 47, 213-218.
- JAKOBSSON, S., BORG, B., HAUX, C. & HYLLNER, S. J. (1999) An 11-ketotestosterone induced kidney-secreted protein: the nest building glue from male three-spined stickleback, *Gasterosteus aculeatus*. *Fish Physiology and Biochemistry*, 20, 79-85.
- JANEWAY, C. A., TRAVERS, P., WALPORT, M., SHLOMCHIK, M.J. 2005 (2005) *Immunobiology: the immune system in health and disease*. , New York, Garland Science Publishing.
- JOBLING, S., NOLAN, M., TYLER, C. R., BRIGHTY, G. & SUMPTER, J. P. (1998) Widespread sexual disruption in wild fish. *Environmental Science & Technology*, 32, 2498-2506.
- KALBE, M., JAKOBSEN, P.J., SCHARSACK, J.P., (2007) Sympatric and allopatric combinations of sticklebacks and the tapeworm *Schistocephalus solidus* - mutual adaptations of hosts and parasites. *Parassitologia*, 49, 318.
- KALBE, M. & KURTZ, J. (2006) Local differences in immunocompetence reflect resistance of sticklebacks against the eye fluke *Diplostomum pseudospathaceum*. *Parasitology*, 132, 105-116.
- KATSIADAKI, I. (2007) The Use of the Stickleback as a Sentinel and Model Species in Ecotoxicology. IN ÖSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. (Eds.) *Biology of the Three-Spined Stickleback*. London, CRC Press.
- KATSIADAKI, I., MORRIS, S., SQUIRES, C., HURST, M. R., JAMES, J. D. & SCOTT, A. P. (2006) Use of the three-spined stickleback (*Gasterosteus aculeatus*) as a sensitive *in vivo* test for detection of environmental antiandrogens. *Environmental Health Perspectives*, 114, 115-121.
- KATSIADAKI, I., SANDERS, M., SEBIRE, M., NAGAE, M., SOYANO, K. & SCOTT, A. P. (2007) Three-Spined Stickleback: an Emerging Model in Environmental Endocrine Disruption. *Environmental Sciences*, 14, 1-16.
- KATSIADAKI, I., SCOTT, A. P., HURST, M. R., MATTHIESSEN, P. & MAYER, I. (2002a) Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicology and Chemistry*, 21, 1946-1954.
- KATSIADAKI, I., SCOTT, A. P. & MAYER, I. (2002b) The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental Research*, 54, 725-728.

- KATSIADAKI, I., SCOTT, A.P & MATTHIESSEN, P., 2000 The use of the three-spined stickleback as a potential biomarker for androgenic xenobiotics. IN NORBERG, B., KJESBU, O.S., TARANGER, G.L., ANDERSSON, E., STEFANSSON, S.O. (Eds.) *The Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish*, Bergen, Norway, pp: 359-361.
- KELCE, W. R. & WILSON, E. M. (1997) Environmental antiandrogens: Developmental effects, molecular mechanisms, and clinical implications. *Journal of Molecular Medicine-Jmm*, 75, 198-207.
- KHAN, R. A. (1990) Parasitism in marine fish after chronic exposure to petroleum-hydrocarbons in the laboratory and to the Exxon Valdez oil-spill. *Bulletin of Environmental Contamination and Toxicology*, 44, 759-763.
- KIDD, K. A., BLANCHFIELD, P. J., MILLS, K. H., PALACE, V. P., EVANS, R. E., LAZORCHAK, J. M. & FLICK, R. W. (2007) Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 8897-8901.
- KIESECKER, J. M. (2002) Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proceedings of the National Academy of Sciences of the United States of America*, 99, 9900-9904.
- KINGSLEY, D. M. (2003) Sequencing the genome of threespine sticklebacks (*Gasterosteus aculeatus*). *National Human Genome Research Institute White Paper*.
- KLEIN, S. L. (2004) Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunology*, 26, 247-264.
- KOLLNER, B. & KOTTERBA, G. (2002) Temperature dependent activation of leucocyte populations of rainbow trout, *Oncorhynchus mykiss*, after intraperitoneal immunisation with *Aeromonas salmonicida*. *Fish & Shellfish Immunology*, 12, 35-48.
- KURTZ, J., KALBE, M., AESCHLIMANN, P. B., HABERLI, M. A., WEGNER, K. M., REUSCH, T. B. H. & MILINSKI, M. (2004) Major histocompatibility complex diversity influences parasite resistance and innate immunity in sticklebacks. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 271, 197-204.
- KURTZ, J., KALBE, M., LANGEFORS, S., MAYER, I., MILINSKI, M. & HASSELQUIST, D. (2007) An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. *American Naturalist*, 170, 509-519.
- LAFFERTY, K. D. (1997) Environmental parasitology: What can parasites tell us about human impacts on the environment? *Parasitology Today*, 13, 251-255.
- LAFFERTY, K. D. (1999) The evolution of trophic transmission. *Parasitology Today*, 15, 111-115.
- LAFFERTY, K. D. (2008) Ecosystem consequences of fish parasites. *Journal of Fish Biology*, 73, 2083-2093.
- LAFFERTY, K. D., DOBSON, A. P. & KURIS, A. M. (2006) Parasites dominate food web links. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 11211-11216.
- LAFFERTY, K. D. & HOLT, R. D. (2003) How should environmental stress affect the population dynamics of disease? *Ecology Letters*, 6, 654-664.

- LAFFERTY, K. D. & KURIS, A. M. (1999) How environmental stress affects the impacts of parasites. *Limnology and Oceanography*, 44, 925-931.
- LAFFERTY, K. D. & KURIS, A. M. (2009) Parasitic castration: the evolution and ecology of body snatchers. *Trends in Parasitology*, 25, 564-572.
- LAFFERTY, K. D. & MORRIS, A. K. (1996) Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. *Ecology*, 77, 1390-1397.
- LAZZARO, B. P. & LITTLE, T. J. (2009) Immunity in a variable world. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 364, 15-26.
- LEFEVRE, T., LEBARBENCHON, C., GAUTHIER-CLERC, M., MISSE, D., POULIN, R. & THOMAS, F. (2009) The ecological significance of manipulative parasites. *Trends in Ecology & Evolution*, 24, 41-48.
- LESTER, R. J. G. (1971) Influence of *Schistocephalus plerocercoids* on respiration of *Gasterosteus* and a possible resulting effect on behaviour of fish. *Canadian Journal of Zoology*, 49, 361-&.
- LI, Z. J., ZHANG, S. C. & LIU, Q. H. (2008) Vitellogenin Functions as a Multivalent Pattern Recognition Receptor with an Opsonic Activity. *Plos One*, 3.
- LISTER, A. & VAN DER KRAAK, G. (2002) Modulation of goldfish testicular testosterone production in vitro by tumor necrosis factor alpha, interleukin-1 beta, and macrophage conditioned media. *Journal of Experimental Zoology*, 292, 477-486.
- LITTLE, T. J., PERUTZ, M., PALMER, M., CROSSAN, C. & BRAITHWAITE, V. A. (2008) Male three-spined sticklebacks *Gasterosteus aculeatus* make antibiotic nests: a novel form of parental protection? *Journal of Fish Biology*, 73, 2380-2389.
- MAHANEY, W. C., HANCOCK, R. G. V., AUFREITER, S. & HUFFMAN, M. A. (1996) Geochemistry and clay mineralogy of termite mound soil and the role of geophagy in chimpanzees of the Mahale Mountains, Tanzania. *Primates*, 37, 121-134.
- MAJOR, M., WEBB, T. J. & HURD, H. (1997) Haemolymph from female beetles infected with *Hymenolepis diminuta* metacystodes retards the development of ovarian follicles in recipient *Tenebrio molitor* (Coleoptera). *Parasitology*, 114, 175-179.
- MARCOGLIESE, D. J., DAUTREMEPUITS, C., GENDRON, A. D. & FOURNIER, M. (2010) Interactions between parasites and pollutants in yellow perch (*Perca flavescens*) in the St. Lawrence River, Canada: implications for resistance and tolerance to parasites. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 88, 247-258.
- MARCOGLIESE, D. J. & PIETROCK, M. (2011) Combined effects of parasites and contaminants on animal health: parasites do matter. *Trends in Parasitology*, 27, 123-130.
- MATTHIESSEN, P., ALLEN, Y., BAMBER, S., CRAFT, J., HURST, M., HUTCHINSON, T., FEIST, S., KATSIADAKI, I., KIRBY, M., ROBINSON, C., SCOTT, S., THAIN, J. & THOMAS, K. (2002) The impact of oestrogenic and androgenic contamination on marine organisms in the United Kingdom - summary of the EDMAR programme. *Marine Environmental Research*, 54, 645-649.
- MATTHIESSEN, P. & JOHNSON, I. (2007) Implications of research on endocrine disruption for the environmental risk assessment, regulation and monitoring of chemicals in the European Union. *Environmental Pollution*, 146, 9-18.

- MAULE, A. G., TRIPP, R. A., KAATTARI, S. L. & SCHRECK, C. B. (1989) Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus-tshawytscha*). *Journal of Endocrinology*, 120, 135-142.
- MAUNDER, R. J., MATTHIESSEN, P., SUMPTER, J. P. & POTTINGER, T. G. (2007a) Impaired reproduction in three-spined sticklebacks exposed to ethinyl estradiol as juveniles. *Biology of Reproduction*, 77, 999-1006.
- MAUNDER, R. J., MATTHIESSEN, P., SUMPTER, J. P. & POTTINGER, T. G. (2007b) Rapid bioconcentration of steroids in the plasma of three-spined stickleback *Gasterosteus aculeatus* exposed to waterborne testosterone and 17 beta-oestradiol. *Journal of Fish Biology*, 70, 678-690.
- MAURE, F., BRODEUR, J., PONLET, N., DOYON, J., FIRLEJ, A., ELGUERO, E., THOMAS, F. (2011) The cost of a bodyguard. *Biology Letters*, Published online ahead of press.
- MAYER, I., BORG, B. & PALL, M. (2004) Hormonal control of male reproductive behaviour in fishes: A stickleback perspective. *Behaviour*, 141, 1499-1510.
- MAYER, I. & PÁLL, M. (2007) Hormonal Control of Reproductive Behaviour in the Stickleback. IN ÖSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. (Eds.) *Biology of the Three-Spined Stickleback*. London, CRC Press.
- MCMICHAEL, A. J., CAMPBELL-LENDRUM, D. H., CORVALÁN, C. F., EBI, K. L., GITHEKO, A. K., SCHERAGA, J. D. & WOODWARD, A. (2003) Climate Change and Human Health. Risks and Responses. Geneva, World Health Organization.
- MCMICHAEL, A. J., WOODRUFF, R. E. & HALE, S. (2006) Climate change and human health: present and future risks. *Lancet*, 368, 842-842.
- MCPHAIL, J. D. & PEACOCK, S. D. (1983) Some effects of the cestode (*Schistocephalus-solidus*) on reproduction in the threespine stickleback (*Gasterosteus aculeatus*), evolutionary aspects of a host-parasite interaction. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 61, 901-908.
- MILINSKI, M. (1985) Risk of predation of parasitized sticklebacks (*Gasterosteus aculeatus* L.) under competition for food *Behaviour*, 93, 203-215.
- MILINSKI, M. (2006) Fitness consequences of selfing and outcrossing in the cestode *Schistocephalus solidus*. *Integrative and Comparative Biology*, 46, 373-380.
- MILINSKI, M. & BAKKER, T. C. M. (1990) Female sticklebacks use male coloration in mate choice and hence avoid parasitised males. *Nature*, 344, 300-333.
- MILLA, S., DEPIEREUX, S. & KESTEMONT, P. (2011) The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. *Ecotoxicology*, 20, 305-319.
- MINCHELLA, D. J. (1985) Host life history variation in response to parasitism. *Parasitology*, 90, 205-216.
- MOLLER, H. (1987) Pollution and parasitism in the aquatic environment. *International Journal for Parasitology*, 17, 353-361.
- MOORE, J. (2002) *Parasites and the Behavior of Animals*, USA, Oxford University Press.
- MOORING, M. S. & HART, B. L. (1995) Costs of allogrooming in impala: distraction from vigilance. *Animal Behaviour*, 49, 1414-1416.
- MORALES-MONTOR, J., MOHAMED, F., GHALEB, A. M., BAIG, S., HALLAL-CALLEROS, C. & DAMIAN, R. T. (2001) In vitro effects of hypothalamic-pituitary-adrenal axis (HPA) hormones on *Schistosoma mansoni*. *Journal of Parasitology*, 87, 1132-1139.

- MORAN, R., HARVEY, I., MOSS, B., FEUCHTMAYR, H., HATTON, K., HEYES, T. & ATKINSON, D. (2010) Influence of simulated climate change and eutrophication on three-spined stickleback populations: a large scale mesocosm experiment. *Freshwater Biology*, 55, 315-325.
- MORET, Y. & SCHMID-HEMPEL, P. (2000) Survival for immunity: The price of immune system activation for bumblebee workers. *Science*, 290, 1166-1168.
- MORLEY, N. J., LEWIS, J. W. & HOOLE, D. (2006) Pollutant-induced effects on immunological and physiological interactions in aquatic host-trematode systems: implications for parasite transmission. *Journal of Helminthology*, 80, 137-149.
- MORRAN, L. T., SCHMIDT, O. G., GELARDEN, I. A., PARRISH, R. C. & LIVELY, C. M. (2011) Running with the Red Queen: Host-Parasite Coevolution Selects for Biparental Sex. *Science*, 333, 216-218.
- MOSTOWY, R. & ENGELSTADTER, J. (2011) The impact of environmental change on host-parasite coevolutionary dynamics. *Proceedings of the Royal Society B-Biological Sciences*, 278, 2283-2292.
- MOYLE, P. B. & CECH, J. J. J. (2004) *Fishes: an introduction to ichthyology*, New Jersey, Prentice-Hall, Inc.
- MÜLLER, C. B. & SCHMID-HEMPEL, P. (1993) Exploitation of cold temperature as defense against parasitoids in bumblebees. *Nature*, 363, 65-67.
- NASH, J. P., KIME, D. E., VAN DER VEN, L. T. M., WESTER, P. W., BRION, F., MAACK, G., STAHLSCHMIDT-ALLNER, P. & TYLER, C. R. (2004) Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives*, 112, 1725-1733.
- NEYLAN, T. C., SUN, B., REMPEL, H., ROSS, J., LENOCI, M., O'DONOVAN, A. & PULLIAM, L. (2011) Suppressed monocyte gene expression profile in men versus women with PTSD. *Brain Behavior and Immunity*, 25, 524-531.
- NIKOSKELAINEN, S., BYLUND, G. & LILIUS, E. M. (2004) Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. *Developmental and Comparative Immunology*, 28, 581-592.
- NISHIMURA, N., HEINS, D. C., ANDERSEN, R. O., BARBER, I. & CRESKO, W. A. (2011) Distinct Lineages of *Schistocephalus* Parasites in Threespine and Ninespine Stickleback Hosts Revealed by DNA Sequence Analysis. *Plos One*, 6.
- NOYES, P. D., MCELWEE, M. K., MILLER, H. D., CLARK, B. W., VAN TIEM, L. A., WALCOTT, K. C., ERWIN, K. N. & LEVIN, E. D. (2009) The toxicology of climate change: Environmental contaminants in a warming world. *Environment International*, 35, 971-986.
- OAKS, J. L., GILBERT, M., VIRANI, M. Z., WATSON, R. T., METEYER, C. U., RIDEOUT, B. A., SHIVAPRASAD, H. L., AHMED, S., CHAUDHRY, M. J. I., ARSHAD, M., MAHMOOD, S., ALI, A. & KHAN, A. A. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, 427, 630-633.
- OECD (2011) Fish Sexual Development Test (FSDT), Test guideline no TG234, Guidelines for testing chemicals. *OECD, Paris*.
- OLLEVIER, F. & COVENS, M. (1983) Vitellogenins in *Gasterosteus aculeatus*. *Annales de la Societe Royale Zoologique de Belgique*, 113, 327-334.

- ÖSTLUND-NILSSON, S. (2001) Fifteen-spined stickleback (*Spinachia spinachia*) females prefer males with more secretional threads in their nests: an honest-condition display by males. *Behavioral Ecology and Sociobiology*, 50, 263-269.
- ÖSTLUND-NILSSON, S. (2007) Reproductive Behaviour in the Three-Spined Stickleback. IN ÖSTLUND-NILSSON, S., MAYER, I. & HUNTINGFORD, F. A. (Eds.) *Biology of the Three-Spined Stickleback*. London, CRC Press.
- ØVERLI, Ø., PALL, M., BORG, B., JOBLING, M. & WINBERG, S. (2001) Effects of *Schistocephalus solidus* infection on brain monoaminergic activity in female three-spined sticklebacks *Gasterosteus aculeatus*. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 268, 1411-1415.
- PÁLL, M. K., MAYER, I. & BORG, B. (2002a) Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus* I. - Changes in 11-ketotestosterone levels during the nesting cycle. *Hormones and Behavior*, 41, 377-383.
- PÁLL, M. K., MAYER, I. & BORG, B. (2002b) Androgen and Behavior in the male three-spined stickleback, *Gasterosteus aculeatus* II. Castration and 11-ketoandrostenedione effects on courtship and parental care during the nesting cycle. *Hormones and Behavior*, 42, 337-344.
- PAPPAS, P. W. & READ, C. P. (1975) Membrane-transport in helminth parasites - review. *Experimental Parasitology*, 37, 469-530.
- PARKS, L. G., LAMBRIGHT, C. S., ORLANDO, E. F., GUILLETTE, L. J., ANKLEY, G. T. & GRAY, L. E. (2001) Masculinization of female mosquitofish in kraft mill effluent-contaminated Fenholloway River water is associated with androgen receptor agonist activity. *Toxicological Sciences*, 62, 257-267.
- PASCOE, D. & CRAM, P. (1977) Effect of parasitism on toxicity of cadmium to 3-spined stickleback, *Gasterosteus-aculeatus* L. *Journal of Fish Biology*, 10, 467-472.
- PATERSON, S., WILSON, K. & PEMBERTON, J. M. (1998) Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries* L.). *Proceedings of the National Academy of Sciences of the United States of America*, 95, 3714-3719.
- PATZ, J. A., CAMPBELL-LENDRUM, D., HOLLOWAY, T. & FOLEY, J. A. (2005) Impact of regional climate change on human health. *Nature*, 438, 310-317.
- PEICHEL, C. L., ROSS, J. A., MATSON, C. K., DICKSON, M., GRIMWOOD, J., SCHMUTZ, J., MYERS, R. M., MORI, S., SCHLUTER, D. & KINGSLEY, D. M. (2004) The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome. *Current Biology*, 14, 1416-1424.
- PENNYCUICK, L. (1971) Quantitative effects of three species of parasites on a population of three-spined sticklebacks, *Gasterosteus aculeatus*. *Journal of Zoology*, 165, 143-162.
- PERSCHBACHER, P. W. (2005) Temperature effects on acute copper toxicity to juvenile channel catfish *Ictalurus punctatus*. *Aquaculture*, 243, 225-228.
- POLZER, M. & CONRADT, U. (1994) Identification and partial characterization of the proteases from different developmental stages of *Schistocephalus solidus* (Cestoda, Pseudophyllidae). *International Journal for Parasitology*, 24, 967-973.
- POULIN, R. (1992) Toxic pollution and parasitism in fresh-water fish. *Parasitology Today*, 8, 58-61.

- POULIN, R. (1994) The evolution of parasite manipulation of host behaviour: a theoretical analysis. *Parasitology*, 109, S109-S118.
- POULIN, R. (1995) "Adaptive" changes in the behaviour of parasitized animals: A critical review. *International Journal for Parasitology*, 25, 1371-1383.
- POULIN, R. (2007) *Evolutionary Ecology of Parasites*, Oxford, Princeton University Press.
- POULIN, R. & FITZGERALD, G. J. (1989) Shoaling as an anti-ectoparasite mechanism in juvenile sticklebacks (*Gasterosteus* spp.). *Behavioral Ecology and Sociobiology*, 24, 251-255.
- POUNDS, J. A., BUSTAMANTE, M. R., COLOMA, L. A., CONSUEGRA, J. A., FOGDEN, M. P. L., FOSTER, P. N., LA MARCA, E., MASTERS, K. L., MERINO-VITERI, A., PUSCHENDORF, R., RON, S. R., SANCHEZ-AZOFEIFA, G. A., STILL, C. J. & YOUNG, B. E. (2006) Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439, 161-167.
- PRESS, C. M. & EVENSEN, O. (1999) The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology*, 9, 309-318.
- PURDOM, C. E., HARDIMAN, P. A., BYE, V. J., ENO, N. C., TYLER, C. R. & SUMPTER, J. P. (1994) Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology*, 8, 275-285.
- PURSE, B. V., MELLOR, P. S., ROGERS, D. J., SAMUEL, A. R., MERTENS, P. P. C. & BAYLIS, M. (2005) Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology*, 3, 171-181.
- REUSCH, T. B. H., HABERLI, M. A., AESCHLIMANN, P. B. & MILINSKI, M. (2001) Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. *Nature*, 414, 300-302.
- RIJKERS, G. T., FREDERIXWOLTERS, E. M. H. & VANMUISWINKEL, W. B. (1980) The immune-system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology*, 41, 91-97.
- ROBB, T. & REID, M. L. (1996) Parasite-induced changes in the behaviour of cestode-infected beetles: Adaptation or simple pathology? *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 74, 1268-1274.
- ROLFF, J. (2002) Bateman's principle and immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 867-872.
- ROS, A. F. H., BECKER, K., CANARIO, A. V. M. & OLIVEIRA, R. F. (2004) Androgen levels and energy metabolism in *Oreochromis mossambicus*. *Journal of Fish Biology*, 65, 895-905.
- ROUTLEDGE, E. J., SHEAHAN, D., DESBROW, C., BRIGHTY, G. C., WALDOCK, M. & SUMPTER, J. P. (1998) Identification of estrogenic chemicals in STW effluent. 2. *In vivo* responses in trout and roach. *Environmental Science & Technology*, 32, 1559-1565.
- RUNDLE, H. D., NAGEL, L., BOUGHMAN, J. W. & SCHLUTER, D. (2000) Natural selection and parallel speciation in sympatric sticklebacks. *Science*, 287, 306-308.
- RUSHBROOK, B. J. & BARBER, I. (2006) Nesting, courtship and kidney hypertrophy in *Schistocephalus*-infected male three-spined stickleback from an upland lake. *Journal of Fish Biology*, 69, 870-882.

- RUSHBROOK, B. J., KATSIADAKI, I. & BARBER, I. (2007) Spiggin levels are reduced in male sticklebacks infected with *Schistocephalus solidus*. *Journal of Fish Biology*, 71, 298-303.
- SCHARSACK, J., KALBE, M., DERNER, R., KURTZ, J. & MILINSKI, M. (2004) Modulation of granulocyte responses in three-spined sticklebacks *Gasterosteus aculeatus* infected with the tapeworm *Schistocephalus solidus*. *Diseases of Aquatic Organisms*, 59, 141-150.
- SCHARSACK, J., KOCH, K. & HAMMERSCHMIDT, K. (2007) Who is in control of the stickleback immune system: interactions between *Schistocephalus solidus* and its specific vertebrate host. *Proceedings of the Royal Society B-Biological Sciences*, 274, 3151-3158.
- SCHMID-HEMPEL, P. (2003) Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 357-366.
- SCHRECK, C. B., CONTRERAS-SANCHEZ, W. & FITZPATRICK, M. S. (2001) Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture*, 197, 3-24.
- SCHULTZ, E. T., TOPPER, M. & HEINS, D. C. (2006) Decreased reproductive investment of female threespine stickleback *Gasterosteus aculeatus* infected with the cestode *Schistocephalus solidus*: parasite adaptation, host adaptation, or side effect? *Oikos*, 114, 303-310.
- SCOTT, A. P., BYE, V. J., BAYNES, S. M. & SPRINGATE, J. R. C. (1980) Seasonal variations in plasma concentrations of 11 Ketotestosterone and testosterone in male rainbow trout, *Salmo gairdnerii* Richardson. *Journal of Fish Biology*, 17, 495-505.
- SEBIRE, M., ALLEN, Y., BERSUDER, P. & KATSIADAKI, I. (2008) The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquatic Toxicology*, 90, 37-47.
- SEBIRE, M., SCOTT, A. P., TYLER, C. R., CRESSWELL, J., HODGSON, D. J., MORRIS, S., SANDERS, M. B., STEBBING, P. D. & KATSIADAKI, I. (2009) The organophosphorous pesticide, fenitrothion, acts as an anti-androgen and alters reproductive behavior of the male three-spined stickleback, *Gasterosteus aculeatus*. *Ecotoxicology*, 18, 122-133.
- SEKI, M., FUJISHIMA, S., NOZAKA, T., MAEDA, M. & KOBAYASHI, K. (2006) Comparison of response to 17 beta-estradiol and 17 beta-trenbolone among three small fish species. *Environmental Toxicology and Chemistry*, 25, 2742-2752.
- SEPPANEN, E., KUUKKA, H., VOUTILAINEN, A., HUUSKONEN, H. & PEUHKURI, N. (2009) Metabolic depression and spleen and liver enlargement in juvenile Arctic charr *Salvelinus alpinus* exposed to chronic parasite infection. *Journal of Fish Biology*, 74, 553-561.
- SINHA, D. P. & HOPKINS, C. A. (1967) Studies on *Schistocephalus solidus*. 4. The effect of temperature on growth and maturation in vitro. *Parasitology*, 57, 555-66.
- SITJA-BOBADILLA, A. (2009) Can Myxosporean parasites compromise fish and amphibian reproduction? *Proceedings of the Royal Society B-Biological Sciences*, 276, 2861-2870.
- SKAKKEBAEK, N. E. (2004) Testicular dysgenesis syndrome: new epidemiological evidence. *International Journal of Andrology*, 27, 189-191.

- SMITH, C. & WOOTTON, R. J. (1999) Parental energy expenditure of the male three-spined stickleback. *Journal of Fish Biology*, 54, 1132-1136.
- SMITH, R. S. & KRAMER, D. L. (1987) Effects of a cestode (*Schistocephalus* sp.) on the response of ninespine sticklebacks (*Pungitius pungitius*) to aquatic hypoxia. *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 65, 1862-1865.
- SMYTH, J. D. (1946) Studies on tapeworm physiology. I. The cultivation of *Schistocephalus solidus* in vitro. *J. Exp. Biol.*, 23, 47-70.
- SMYTH, J. D. (1954) Studies on tapeworm physiology. VII. Fertilization of *Schistocephalus solidus* in vitro. *Experimental Parasitology*, 3, 64-7.
- SMYTH, J. D. (1969) *The physiology of cestodes*, Edinburgh, UK, Oliver & Boyd.
- SMYTH, J. D. (1994) *Introduction to Animal Parasitology*, London, Cambridge University Press.
- SOTO, A. M. & SONNENSCHNEIN, C. (2010) Environmental causes of cancer: endocrine disruptors as carcinogens. *Nature Reviews Endocrinology*, 6, 364-371.
- STUDER, A., THIELTGES, D. W. & POULIN, R. (2010) Parasites and global warming: net effects of temperature on an intertidal host-parasite system. *Marine Ecology-Progress Series*, 415, 11-22.
- SUMPTER, J. P. (2005) Endocrine disruptors in the aquatic environment: An overview. *Acta Hydrochimica Et Hydrobiologica*, 33, 9-16.
- SUMPTER, J. P. (2009) Protecting aquatic organisms from chemicals: the harsh realities. *Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences*, 367, 3877-3894.
- SUMPTER, J. P. & JOBLING, S. (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*, 103, 173-178.
- SURES, B. (2004) Environmental parasitology: relevancy of parasites in monitoring environmental pollution. *Trends in Parasitology*, 20, 170-177.
- SURES, B. (2007) Host-parasite interactions from an ecotoxicological perspective. *Parassitologia*, 49, 173-6.
- SURES, B. (2008) Host-parasite interactions in polluted environments. *Journal of Fish Biology*, 73, 2133-2142.
- SURES, B. & SIDDALL, R. (1999) *Pomphorhynchus laevis*: The intestinal acanthocephalan as a lead sink for its fish host, chub (*Leuciscus cephalus*). *Experimental Parasitology*, 93, 66-72.
- TENORA, F., BARUS, V., KRACMAR, S. & DVORACEK, J. (2000) Concentrations of some heavy metals in *Ligula intestinalis* plerocercoids (Cestoda) and *Philometra ovata* (Nematoda) compared to some their hosts (Osteichthyes). *Helminthologia*, 37, 15-18.
- TENORA, F., BARUS, V. & PROKES, M. (2002) Next remarks to the knowledge of heavy metal concentrations in gravid tapeworm species parasitizing aquatic birds. *Helminthologia*, 39, 143-148.
- THILAGAM, H., GOPALAKRISHNAN, S., BO, J. & WANG, K. J. (2009) Effect of 17-beta-estradiol on the immunocompetence of Japanese sea bass (*Lateolabrax japonicus*). *Environmental Toxicology and Chemistry*, 28, 1722-1731.
- THILAKARATNE, I., MCLAUGHLIN, J. D. & MARCOGLIESE, D. J. (2007) Effects of pollution and parasites on biomarkers of fish health in spottail shiners *Notropis hudsonius* (Clinton). *Journal of Fish Biology*, 71, 519-538.
- THOMAS, F., ADAMO, S. & MOORE, J. (2005) Parasitic manipulation: where are we and where should we go? *Behavioural Processes*, 68, 185-199.

- THOMAS, F., SCHMIDT-RHAESA, A., MARTIN, G., MANU, C., DURAND, P. & RENAUD, F. (2002a) Do hairworms (Nematomorpha) manipulate the water seeking behaviour of their terrestrial hosts? *Journal of Evolutionary Biology*, 15, 356-361.
- THOMAS, K. V., HURST, M. R., MATTHIESSEN, P., MCHUGH, M., SMITH, A. & WALDOCK, M. J. (2002b) An assessment of *in vitro* androgenic activity and the identification of environmental androgens in United Kingdom estuaries. *Environmental Toxicology and Chemistry*, 21, 1456-1461.
- THOMAS, M. B. & BLANFORD, S. (2003) Thermal biology in insect-parasite interactions. *Trends in Ecology & Evolution*, 18, 344-350.
- THOMPSON, S. N. & KAVALIERS, M. (1994) Physiological bases for parasite-induced alterations of host behaviour. *Parasitology*, 109, S119-S138.
- THORPE, K. L., BENSTEAD, R., HUTCHINSON, T. H. & TYLER, C. R. (2007) Associations between altered vitellogenin concentrations and adverse health effects in fathead minnow (*Pimephales promelas*). *Aquatic Toxicology*, 85, 176-183.
- THREADGOLD, L. T. & HOPKINS, C. A. (1981) *Schistocephalus solidus* and *Ligula intestinalis* - Pinocytosis by the tegument. *Experimental Parasitology*, 51, 444-456.
- TIERNEY, J. F. & CROMPTON, D. W. T. (1992) Infectivity of plerocercoids of *Schistocephalus solidus* (Cestoda, Ligulidae) and fecundity of the adults in an experimental definitive host, *Gallus gallus*. *Journal of Parasitology*, 78, 1049-1054.
- TIERNEY, J. F., HUNTINGFORD, F. A. & CROMPTON, D. W. T. (1993) The relationship between infectivity of *Schistocephalus solidus* (Cestoda) and antipredator behaviour of its intermediate host, the 3-spined stickleback, *Gasterosteus aculeatus*. *Animal Behaviour*, 46, 603-605.
- TIERNEY, J. F., HUNTINGFORD, F. A. & CROMPTON, D. W. T. (1996) Body condition and reproductive status in sticklebacks exposed to a single wave of *Schistocephalus solidus* infection. *Journal of Fish Biology*, 49, 483-493.
- TOPPARI, J., LARSEN, J. C., CHRISTIANSEN, P., GIWERCMAN, A., GRANDJEAN, P., GUILLETTE, L. J., JEGOU, B., JENSEN, T. K., JOUANNET, P., KEIDING, N., LEFFERS, H., MCLACHLAN, J. A., MEYER, O., MULLER, J., RAJPERTDEMEYTS, E., SCHEIKE, T., SHARPE, R., SUMPTER, J. & SKAKKEBAEK, N. E. (1996) Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*, 104, 741-803.
- TRUBIROHA, A., KROUPOVA, H., FRANK, S. N., SURES, B. & KLOAS, W. (2011) Inhibition of gametogenesis by the cestode *Ligula intestinalis* in roach (*Rutilus rutilus*) is attenuated under laboratory conditions. *Parasitology*, 138, 648-659.
- TRUBIROHA, A., KROUPOVA, H., WUERTZ, S., FRANK, S. N., SURES, B. & KLOAS, W. (2010) Naturally-induced endocrine disruption by the parasite *Ligula intestinalis* (Cestoda) in roach (*Rutilus rutilus*). *General and Comparative Endocrinology*, 166, 234-240.
- TRUBIROHA, A., WUERTZ, S., FRANK, S. N., SURES, B. & KLOAS, W. (2009) Expression of gonadotropin subunits in roach (*Rutilus rutilus*, Cyprinidae) infected with plerocercoids of the tapeworm *Ligula intestinalis* (Cestoda). *International Journal for Parasitology*, 39, 1465-1473.

- VAN DOBBEN, W. H. (1952) The food of the cormorant in the Netherlands. *Ardea*, 40, 1-63.
- VAN IERSEL, J. J. A. (1953) An analysis of the parental behaviour of the male three-spined stickleback (*Gasterosteus aculeatus* L.). *Behaviour, Supplement III*.
- WANG, R. & BELOSEVIC, M. (1994) Estradiol increases susceptibility of goldfish to *Trypanosoma-danilewskyi*. *Developmental and Comparative Immunology*, 18, 377-387.
- WARD, A. J. W., HENSOR, E. M. A., WEBSTER, M. M. & HART, P. J. B. (2010) Behavioural thermoregulation in two freshwater fish species. *Journal of Fish Biology*, 76, 2287-2298.
- WATANUKI, H., YAMAGUCHI, T. & SAKAI, M. (2002) Suppression in function of phagocytic cells in common carp *Cyprinus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, 132, 407-413.
- WEBB, T. J. & HURD, H. (1999) Direct manipulation of insect reproduction by agents of parasite origin. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 266, 1537-1541.
- WEBSTER, J. P., BRUNTON, C. F. A. & MACDONALD, D. W. (1994) Effect of *Toxoplasma gondii* upon neophobic behaviour in wild brown rats, *Rattus norvegicus*. *Parasitology*, 109, 37-43.
- WEBSTER, J. P. & WOOLHOUSE, M. E. J. (1999) Cost of resistance: relationship between reduced fertility and increased resistance in a snail-schistosome host-parasite system. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 266, 391-396.
- WEDEKIND, C. (1997) The infectivity, growth, and virulence of the cestode *Schistocephalus solidus* in its first intermediate host, the copepod *Macrocyclus albidus*. *Parasitology*, 115, 317-324.
- WEDEKIND, C., MEYER, P., FRISCHKNECHT, M., NIGGLI, U. A. & PFANDER, H. (1998) Different carotenoids and potential information content of red coloration of male three-spined stickleback. *Journal of Chemical Ecology*, 24, 787-801.
- WEDEKIND, C. & MILINSKI, M. (1996) Do three-spined sticklebacks avoid consuming copepods, the first intermediate host of *Schistocephalus solidus*? An experimental analysis of behavioural resistance. *Parasitology*, 112, 371-383.
- WEGNER, K. M., KALBE, M., KURTZ, J., REUSCH, T. B. H. & MILINSKI, M. (2003) Parasite selection for immunogenetic optimality. *Science*, 301, 1343-1343.
- WENDELAAR BONGA, S. E. (1997) The stress response in fish. *Physiological Reviews*, 77, 591-625.
- WEYTS, F. A. A., COHEN, N., FLIK, G. & VERBURG-VAN KEMENADE, B. M. L. (1999) Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish & Shellfish Immunology*, 9, 1-20.
- WIBE, A. E., ROSENQVIST, G. & JENSSEN, B. M. (2002) Disruption of male reproductive behavior in threespine stickleback *Gasterosteus aculeatus* exposed to 17 beta-estradiol. *Environmental Research*, 90, 136-141.
- WILLIAMS, R. J., JOHNSON, A. C., SMITH, J. J. L. & KANDA, R. (2003) Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environmental Science & Technology*, 37, 1744-1750.

- WILLIAMS, T. D., DIAB, A. M., GEORGE, S. G., SABINE, V. & CHIPMAN, J. K. (2007) Gene expression responses of European flounder (*Platichthys flesus*) to 17-beta estradiol. *Toxicology Letters*, 168, 236-248.
- WILSON, V. S., LAMBRIGHT, C., OSTBY, J. & GRAY, L. E. (2002) *In vitro* and *in vivo* effects of 17 beta-trenbolone: A feedlot effluent contaminant. *Toxicological Sciences*, 70, 202-211.
- WITHLER, R. E., MCPHAIL, J. D. & DEVLIN, R. H. (1986) Electrophoretic polymorphism and sexual dimorphism in the freshwater and anadromous threespine sticklebacks (*Gasterosteus aculeatus*) of the Little Cambell River, British Columbia. *Biochemical Genetics*, 24, 701-713.
- WOO, N. Y. S., CHUNG, A. S. B. & NG, T. B. (1993) Influence of oral-administration of estradiol-17 β and testosterone on growth, digestion, food conversion and metabolism in the underyearling red sea bream, *Chrysophrys major*. *Fish Physiology and Biochemistry*, 10, 377-387.
- WOODWARD, G., PERKINS, D. M. & BROWN, L. E. (2010) Climate change and freshwater ecosystems: impacts across multiple levels of organization. *Philosophical Transactions of the Royal Society B-Biological Sciences*, 365, 2093-2106.
- WOOTTON, R. J. (1976) *The Biology of the Sticklebacks*, London, Academic Press.
- WOOTTON, R. J. (1984) *A Functional Biology of Sticklebacks*, Berkeley, University of California Press.
- YANG, M., WANG, K. P., SHEN, Y. & WU, M. H. (2011) Evaluation of Estrogenic Activity in Surface Water and Municipal Wastewater in Shanghai, China. *Bulletin of Environmental Contamination and Toxicology*, 87, 215-219.
- ZAHAVI, A. (1975) Mate selection - selection for a handicap. *Journal of Theoretical Biology*, 53, 205-214.
- ZAROOGIAN, G., GARDNER, G., HOROWITZ, D. B., GUTJAHR-GOBELL, R., HAEBLER, R. & MILLS, L. (2001) Effect of 17 beta-estradiol, o,p'-DDT, octylphenol and p,p'-DDE on gonadal development and liver and kidney pathology in juvenile male summer flounder (*Paralichthys dentatus*). *Aquatic Toxicology*, 54, 101-112.
- ZUK, M. & MCKEAN, K. A. (1996) Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology*, 26, 1009-1023.