Effects of host nutrition on the Host-parasite interactions of *Schistocephalus solidus* infections in sticklebacks

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Abstract

Dietary factors – including the quantity or quality of the food ingested – potentially impact the outcome of host-parasite interactions, through a variety of mechanisms. In this project, host nutritional content is manipulated qualitative and quantitatively, and the influence on host and parasites are examined. The consequences of either quantitative changes in food intake or qualitative changes in diet (i.e. type of food) may potentially benefit the host fish or parasites. The first part of the thesis results (Chapters 3 and 4) documents experiments conducted to evaluate the effect of food type, dietary protein content and ration on host-parasite interactions, focusing on the health, growth and development of fish, and the growth rate of *Schistocephalus solidus* plerocercoids in experimentally infected three-spined sticklebacks *Gasterosteus aculeatus.* The results of these studies indicate that the level of dietary protein had a significant effect on the performance of both infected and non-infected fish in the study and suggest that dietary protein plays an important role in determining the emergent phenotypes of infected fish. The level of host alimentation (i.e. ration) also played an important role in determining fish health indices and suggests that the availability of food might have a significant effect on the performance of both infected and non-infected fish in parasitized populations. The second part of this thesis (Chapter 5) therefore investigated the effect of infection on the preferences of sticklebacks for a certain type of diet; (*Artemia*, bloodworm and Artificial diet). The results of this showed that *S. solidus* infection reduces the proportion of time spent in the *Artemia* zone. In addition, behavioural studies showed that sticklebacks tend to prefer bloodworm as their first choice of food. The third part of this thesis (Chapter 6) therefore investigates the effect of a dietary supplement – carotenoids on host-parasite interactions in sticklebacks. Carotenoids appeared to influence three-spined sticklebacks' investment in splenosomatic index and haematocrit. Female reproductive investment was also influenced by these dietary supplements. *S. solidus*-infected sticklebacks showed a significant increase in their splenosomatic index. This research has demonstrated that the influence of factors such as variation in nutritional composition, level of feeding and additive nutrients in diet have a significant effect on the performance of both infected and non-infected fish in the study.

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Chapter 1 Introduction

1.1 Introduction

With the population of the world increasing dramatically, scarcity of resources means that many people suffer from malnutrition. One of the most fundamental and substantial sources of protein for humans is fish, which according to the United Nations' Food and Agriculture Organization (FAO) (1997), comprises 15.6% of animal protein consumed by humans (Tidwell and Allan, 2001). However, the field of fish production and aquaculture has faced many challenges in recent years, most notably in relation to resistance to issues surrounding diseases and combating parasites, bacteria and viruses, which represent the foremost threats to aquatic plants and animals (Johnson and Paull, 2011). Moreover, global warming, and other environmental issues including chemical pollution have also adversely affected fish production and welfare (Utne et al., 2017).

Fish are exposed to numerous parasites that are present in the aquatic environment, which can cause enormous harm (Begon et al., 1990) and incur a wide range of problems that impact on fish health, growth and reproduction and affect nutritional quality, market value and productivity in aquaculture, including weakness and performance, and also the reduction of nutritional quality and reproductive ability (Scholz, 1999).The strategies for controlling parasitic infections in managed fish populations are based largely on knowledge about how fish interact with their parasites. One of these strategies is to use drug treatments that can be administered through the intake of antiparasitic compounds in the diet, which has been shown to be effective, for example, against the monogenean *Heterobothrium okamotoi* (Hirazawa et al., 2000). An alternative approach is to use pesticides; however, many chemicals have a damaging effect on the environment. Furthermore, using an excessive amount of pesticides can have a toxic effect on fish and causes of oxidative stress and damaged DNA in zebrafish *(Danio rerio)* (Ge et al., 2015). Formalin is a pesticide used against parasites that causes a steep decline in the percentage of dissolved oxygen in water (Klinger and Floyd, 2009). Moreover, control of parasitism using chemotherapeutic treatments is under threat due to the emergence of pathogen resistance. This has stimulated research into alternative control strategies, and

consequently some specialists have developed a number of methods to overcome this problem, one of which is to implement sustainable control strategies by manipulating nutrition. Both macronutrients and micronutrients influence rapid development and growth, yet allow a heathy fish stock to be maintained (Craig et al., 2002). There is abundant evidence that good nutrition supports the immune system of the fish, protecting it from the risks associated with infection. The powerful role played by effective nutrition in promoting health among fish has been thoroughly documented in systematic reviews, scholarly studies, and in the literature (Blazer, 1992, Waagbø, 1994) Nutrients are potentially assigned to different physiological processes, for example to support the immune system. It is a widely held view that some researchers have found that host nutrition has a significant effect on the pathological status as a result of parasitism (Hunter, 1953). An improvement in host resistance against parasites was obtained when adequate feed is provided (Gibson, 1963).

1.1.1 Nutrition and the host-parasite interaction

Parasites are key constituents of all aquatic ecosystems (Scholz, 1999), but can have a detrimental effect on the fitness of the host by assimilating the latter's nutrients(Arme and Owen, 1967). In addition, parasites usually reduce food intake and lower the efficiency of energy utilization by the host (Thompson et al., 2005b). Previous studies have showed that parasites may have significant effects on reproduction (Bagamian et al., 2004), growth (Barber et al., 2000), mortality (Lester and Adams, 1974), behaviour (Barber, 2013), susceptibility to predation (Brassard et al., 1982), and swimming performance (Coleman, 1993), amongst others.

Parasite infection can have serious physical or economic impact on aquaculture and nature, and so it is important to develop strategies for enhancing responses by using knowledge of nutritional effects on the interaction between fish and parasites. It is postulated that the nutritional status of the host is likely to interfere with the pathogenesis of infection of host. This is investigated by manipulating host diets to study the effect of nutritional factors on the interaction between fish and their parasites.

1.2 Nutritional effects on susceptibility to acquiring infections

Many factors increase the susceptibility to infection. Climate change can increase pathogen development (Harvell et al., 2002), as can seasonal variation, the occurrence of infection is higher in the rainy season whilst there is a lower prevalence during the dry season (González-Tokman et al., 2011) depending on the genetic background (Karvonen et al., 2016).

The nutritional status of the host can be considered as one of the principal factors influencing resistance to infection (Athanasiadou et al., 2008). Host can suffer from malnutrition and, consequently, become immunocompromised, which, can increase their susceptibility to infection. Parasitic infection is also considered to be reliant on the quality of the host's feeding patterns, where energy intake provides resources for the immune response. Susceptibility to parasitic infection could affect by nutritive dietary provision, which can be countered through the use of macronutrients and micronutrients, as these known to affect the immune response (Calder and Kew, 2002). Indeed in studies of the immune health of Pacific Salmon (*Oncorhynchus tshawytscha*), for example, an insufficient quantity and quality of food was determined as the cause of their weakened resistance to disease. (Alcorn et al., 2003)**.** Poor host nutrition might also make the effect of parasitic infection more severe, in terms of host survival and parasite proliferation, and result in a decrease in the resistance of hosts against pathogens. Indeed, parasites often have an impact on their hosts, and these effects can result in a reduction in nutrient absorption in the intestine (Holmes, 1993), and increased utilisation of energy reserves of the host (Schultz et al., 2006), reduced food intake (Cunningham et al., 1994) and reduced deposition of fat and protein (Coop et al., 1982).

Previous studies have reported that certain constituents of food can influence non-specific immune functions in fish. For instance, carotenoids are natural pigments that fish cannot synthesize themselves, and which can be an important factor in immune activation (Jeney et al., 1997, Kolluru et al., 2006), with high levels of carotenoids being able to influence resistance to bacterial and fungal diseases in salmonids (Czeczuga, 1979). The nutritional status of the host is composed of both qualitative and quantitative aspects of the diet, with greater

levels of feeding being found to influence the growth of cestodes; an observation made with fish fed a 16 % body weight bw.d^{-1} food ration in comparison to fish fed an 8 % body weight ration (Simmonds, 2015).

1.2.1 Nutrition and parasite encounter (Figure 1a)

Parasites are a highly diverse group of organisms that can have significant effects on the biology of individual hosts, populations and ecosystems (Hurd, 2001, Hudson et al., 2006). It has been established that certain factors might increase the potential rate of encounters between hosts and their parasites, thus increasing the probability of encounters.

The host's characteristics influence parasite infection patterns, such as whether the fish are genetically modified (Xiong et al., 2017) . Meanwhile, the outcome of infections depend on a range of other factors including nutrition and climate change. The likelihood of encounters is increased by greater intake of food; more specifically, hosts may encounter stages of higher degree of infection of parasites acquired through food when individuals of larger size assimilate greater amounts of food (Des Clers, 1991).

Most commonly, some parasites are acquired through the diet; exposure is increased by greater food intake. According to Johnson et al. (2007) trematode (*Ribeiroia ondatrae*) cercariae were released faster from snails owing to eutrophication. Proliferation of infected snails was maximised by high parasite load and levels of nutrients in water; each snail released a higher proportion of cercariae, increasing the occurrence of infected amphibian definitive hosts.

One contributing factor is that snail hosts have access to a greater abundance of algae food, which has a high nutrient load, thus leading to a proliferation of snail intermediate hosts.

Food intake with a high nutritional value tends to convey greater advantage to the parasite, as compared to the host. This has become evident in various ecosystems, whereby the immediate host is more likely to be exposed to infection, according to Bruno et al. (2003), the severity of coral diseases can be significantly increased by moderate increases in the concentration of nutrients. In a less obvious way, the types of parasites that a host population are exposed to

are determined by the types of prey that are available. Three-spined sticklebacks provide an example. They have an abundant and varied diet, which is primarily made up of insect nymphs, zooplankton, benthic crustaceans such as, *Daphnia, Asellus aquaticus* and Cyclops, Chironomid larvae and *Tubifex* (Hynes, 1950, Wootton, 1984). Consequently, three-spined sticklebacks are vulnerable to diverse parasites present in aquatic ecosystems (Barber, 2007a)

Other factors increase the chance of hosts encountering parasites in the environment. In particular, evidence in the literature seems to indicate that outbreaks of disease, and/or the severity of diseases, may be caused by nutrient loading in amphibians (Johnson et al., 2007) and fish (Lafferty, 1997). The adverse impact of global climate change may raise in the form of different taxa becoming more prone to infection (Harvell et al., 1999), partially due to the increased temperature associated with climate change (Harvell et al., 2002).

For example, the increased prevalence of metacercariae in the Sutchi catfish (*Pangasianodon hypophthalmus*) was highest in the rainy season and lowest in the dry season (Thuy et al., 2010). In the study of the effect of ration and temperature, Allen and Wootton (1982) found that sticklebacks show an increased growth rate with increased temperature as a result of the range expansion of hosts to diets more susceptible to infection.

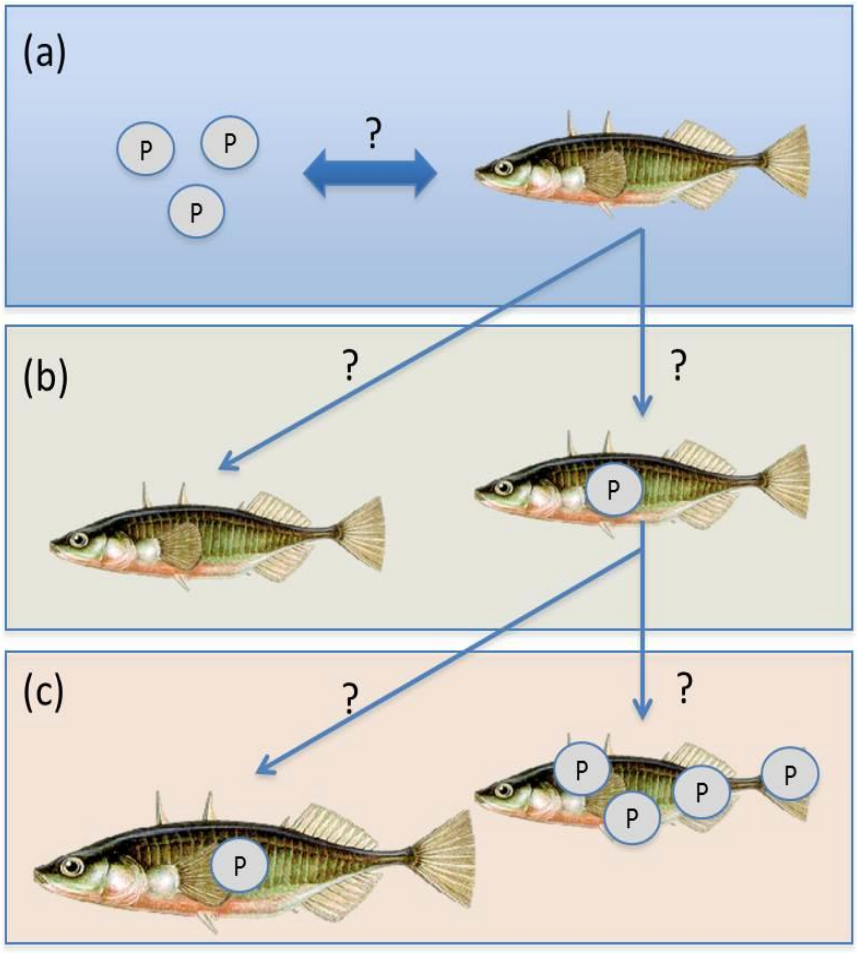


Figure 1.1 Schematic diagram showing how host nutrition might affect the interactions of fish with parasites

(a) Host nutrition might influence the rate at which hosts encounter parasites. For example, fish with a higher rate of food intake (larger individuals, fish modified genetically for fast growth or fish with higher BMR) might encounter food-borne infections at a higher rate. (b) Host nutrition might affect the likelihood of a fish becoming infected following encounter, for example by influencing the fish's immune response. (c) Host nutrition might affect the infection phenotype, for example by influencing rate, at which parasites grow or reproduce, by influencing the ability of the fish to grow or develop following infection.

1.2.2 Nutrition and host resistance (figure 1b)

Resistance can be defined as the capacity of a host to restrict increases in the weight and fertility of parasites, or to restrict the persistence of a parasite population (Coop and Holmes, 1996). Fish diseases and infections also appeared when fish had insufficient sources of nutrition to maintain health and prevent stress (Lall, 2000). The quality or quantity of food is, therefore, also important to disease resistance, and it therefore seems likely that a host will be susceptible to infection when food is inadequate in terms of quantity and/or quality. (Coop and

Kyriazakis, 1999, Zhou et al., 2015). In studies by Holmes (1993), (Coop and Holmes, 1996), the study found that during infection exposure, the host's capacity to develop immunity was influenced by the levels of dietary protein provided; the experimental evidence for which is consistent with the position of the framework. Feeding Coho salmon (*Oncorhynchus kisutch*) a corn gluten diet caused these fish to experience difference bacterial kidney disease, leading to a difference in mortality compared to fish fed cottonseed meal (Wedemeyer and Ross, 1973).

As well as providing the fish with the essential nutrition for growth, commercial or natural diets confer health benefits, protecting the fish against parasitism (Wang et al., 2014). Supplementation with micronutrients is considered essential in providing nutrients that affect host susceptibility (Ingale et al., 2010). A number of recent researches have shown that intake of vitamins and minerals can have an impact on disease resistance and immunity in fishes , as reviewed by Oliva-Teles (2012) and (Trichet, 2010) A vitamin C rich diet decreases the risk of infection in fish caused by multiple bacteria and viruses.(Waagbø, 1994). Fish's resistance against pathogens is also influenced by additive nutrients (Trichet, 2010). In salmonids, resistance to bacterial and fungal diseases can be promoted by high levels of carotenoids (Czeczuga, 1979). Fish that receive sufficient nutrition can be healthier and have the ability to resist parasite infestation. The influence of nutrition on immunity regarding these processes must be analysed to gain an understanding of the interactions between diet and resistivity to infectious disease (Lall, 2000).

1.2.3 Nutritional effects on immune performance

The function of the immune system is to protect the body from pathogens and toxic substances that attack it and cause diseases and health problems. The strength of the immune system is determined by its ability to resist inflammation and resistance against bacteria, viruses or parasites. Gaining insight into the processes by which nutrition has an impact on the immune system is crucial because it can illuminate appropriate ways in which to examine the subtle relationships between diet and resistivity to infectious disease (Lall, 2000).

As is the case with other animals, including humans, fish immune systems can be classified into specific (or acquired) immunity and nonspecific (or innate)

immunity, for the most part, fish are more dependent on acquired immunity comparedwith mammals (Lall, 2000).

For example, several studies have demonstrated that food intake in fish has a direct impact on the development of both the nonspecific (Fletcher, 1986, Blazer, 1991, Waagbø, 1994) and specific immunity (Landolt, 1989, Waagbø, 1994).

Many studies have also investigated the functional role of macronutrients and micronutrients in the resistance of fish to pathogen infection. These studies have examined the role of dietary factors – including amino acids, fatty acids, minerals and vitamins – that affect immunity. Both macronutrients and micronutrients can influence immunity; where there is a lack of macronutrients and micronutrients in the diet, animals show an increased susceptibility to infection (Calder and Kew, 2002).Amino acids (both essential and nonessential) are of dietary benefit to fish, despite the fact that fish do not have a true protein requirement (Wilson, 2002) . Amino acids are one of the key factors for maintaining immunity (Trichet, 2010). Protein sources used in aquafeeds can differ markedly. Animal sources (fishmeal) are a major ingredient in many feeds (Rumsey, 1993), though vegetable protein can also be used. According to (Sitjà-Bobadilla et al., 2005) alterations in the innate immunity of sea bream (ranging between 50-100% fishmeal) sometimes can occur when fish meal is substituted with graded levels of plant protein mixtures. Evidence also suggests that arginine, along with nine other amino acids, is essential for fish (Buentello et al., 2007) identified the impacts of heightened dietary arginine on the haematology and immune function of the immature channel catfish *Ictalurus punctatus*. It was noted that dietary levels of arginine exceeding 4% corresponded to statistically significant increases in haemoglobin, haematocrit, and circulating erythrocytes.

Fatty acids may also be important for immunity. For example, one study reported that a diet rich in unsaturated fatty acids improved the performance of the immune system, development and resistance to parasites and other pathogens in juvenile large yellow croaker (Zuo et al., 2012).

1.2.3.1 Effects of food ration (quantity of food) on resistance

Food ration has a major effect fish weight (Brett, 1979) and also aihas a direct impact on the development of innate immunity (Fletcher, 1986, Blazer, 1991, Waagbø, 1994) and adaptive immunity (Landolt, 1989, Waagbø, 1994).

A high level of index of health, as opposed to low levels of satiation, are displayed in Chinook salmon (*Oncorhynchus tshawytscha*) being fed to high levels of satiation (Alcorn et al., 2003).

After examining the impact that food intake has on the development of gastrointestinal parasitism in a population of growing lambs, the volume of food voluntary consumed by lambs was noted as decreasing when they were infected. In view of this, it can be concluded that the immune responses can be associated with the lambs consuming more energy when compared to lambs with a smaller diet (Valderrábano et al., 2002).Similarly, Blunt Snout Bream (*Megalobrama amblycephala*) showed that more frequent feeding was associated with a higher disease immunity, higher growth, and greater chance of survival (Li et al., 2014).One notable study examined the impact that more frequent feeding had on macrophage functions in tilapia (*Oreochromis niloticus L.*). The outcomes indicated that the groups which engaged in feeding at the rate of eight times per day could control bacterial infections in a more efficient way than their counterparts, which fed only two times per day (Garcia and Villarroel, 2009)

1.2.3.2 Effect of nutritional composition on resistance (i.e., quality of food)

Adequate nutrition is essential to maintain health as well as to reduce disease susceptibility and pathological changes. A study by Blazer and Wolke (1984) This study reported that less effective immune responses were associated with those fish fed with a commercial diet, especially when comparatively examined against control diet fish. However, it should be noted that no deaths occurred as a result of infection over the course of the experiment.In some studies, it has been found to some extent that the use of high levels of nutrition in the form of protein can have a beneficial effect on hosts. In a study exploring acquired resistance to *Trichostrongylus colubriformis* infection and the associated immune responses

lambs . Kambara et al. (1993) fed one group of lambs a low-protein diet, whilst another group was fed a high protein diet.

The animals on the high protein diet developed better resistance. a study by Dobson and Bawden (2009) showed that the influence of protein intake on the immunity of sheep to *Oesophagostomum columbianum* adult worms was detrimental in sheep fed on low protein diets in comparison to those fed on high protein diets.

The relationship between dietary carbohydrates, intestinal bacterial flora and fishes' lymphoid tissue emphasises their significance in relation to immune responses. Pathogenic bacteria can become trapped by insoluble dietary fibres which blocks their entry into the gut mucosa (Trichet, 2010).

Fatty acids have considerable potential to affect immune cells through a variety of mechanisms. Firstly, through production of energy; and secondly, via the influence on immune receptor activity, which may affect the probability of gene expression among cells (Calder, 2007).

The influence of the protein component and of the sources of energy in the diet of a host play a fundamental and substantial part in the acquisition of immunity in sheep (Brown et al., 1991, Kambara et al., 1993, Coop et al., 1995). Feeding Coho salmon (Oncorhynchus kisutch) a corn gluten diet caused these fish to experience difference bacterial kidney disease, leading to a difference in mortality compared to fish fed cottonseed meal (Wedemeyer and Ross, 1973).

1.3 Nutritional effects on infection phenotypes (Figure 1c)

Responsive and adaptive measures to parasitism, as well as the potential to attain immunity thereof, vary dramatically between species (Gray, 1991). In part, this is highly likely to stem from genetic differences (Coltman et al., 1999). However, nutritional status has also been suggested to play a role in determining parasite growth (Bedhomme et al., 2004).

Parasites can have a depressive effect on the growth of hosts (Barber et al., 2008, Kuris et al., 2008, Schultz et al., 2006). In some cases, parasitised fish consumed a larger dietary volume than non-parasitized fish (Tierney, 1994). Extensive parasitic growth and reproduction with minimal harm to the host is

possible if the host has adequate nutrition, enabling it to supply rich resources to parasites (Simmonds, 2015). It has been known that the growth of parasites is achieved by deriving energy directly from host. However, as reported by Barber (2005), fish with the quickest growth had the largest parasite mass, implying that the host is a direct source of energy for parasite development.

Nutrition causes many changes in the phenotypes of the parasite. Improved nutrition, particularly protein, can ameliorate many phenotypes expressed as a result of infection. The major effect of nutritional supplements may be a loss of the ability for larvae to grow (Balic et al., 2000). In studies that experimentally infected channel catfish with the pathogenic bacterium, *Edwardsiella tarda*, Durve and Lovell (1982)) noted there was a correlation between the dietary levels of ascorbic acid levels and mortality.

1.3.1 Effects of host nutrition on host performance

For adequate growth and resistance to pathogens, an adequate supply and balance of nutrients are required for proper efficiency of the host defences (Trichet, 2010). When nutrition is inadequate, this can cause deterioration in health and affect growth, susceptibility to infection and disease and other behavioural and health conditions (Oliva-Teles, 2012).

The overall goal of nutritional support to hosts is to maintain or improve their nutritional status and thereby improve body condition. An additional goal is to reduce disease or improve functional capacity against parasites (Chandra, 1993).The high cost of protein in the diet of carnivorous fish is the source of their rapid growth rates (McGoogan and Gatlin, 1999), while lipids are an important component in feed due to host need access to sources of energy (Lee et al., 2002, De Silva et al., 2001) Lipids are responsible for generating much of the energy potential in a number of fish, particularly in carnivorous fish (Tocher, 2003). Fatty acids are the preferred source of metabolic energy for reproductive development (Henderson et al., 1984). It was also found that the number of mature eggs increased with increasing dietary protein levels in the diet (Al Hafedh et al., 1999).

1.3.2 Effects of host nutrition on parasite performance

Parasites are found in all living creatures (Windsor, 1998). Parasitism can impair growth in fish, and affect the immune system and physical condition of the host (Pascual et al., 2004, Wang et al., 2014, Guagliardo et al., 2009). In this context, host nutrition is considered to be a vital factor in the interaction between host and parasite; several studies have shown that an improved nutrition status of the host can reduce mortality associated with infection (Walkey and Meakins, 1970, Pascoe and Mattey, 1977, Durve and Lovell, 1982)

It has been well established that the nutritional status of the host can influence the growth and development of parasites and thus, in general, the quantity and quality of diet may be of significant influence on effective infection by a parasite. For example, there have been experiments with specific carbohydrates which may influence the growth of the cestode *Hymenolepis diminuta*. The results of these studies have shown that with increased glucose intake, the weight of worms was greater than those fed on other carbohydrates (Dunkley and Mettrick, 1969).

The diet was has also been shown to have a profound influence on the establishment and survival rate of parasitic nematodes (Chandler, 1953, Geiman, 1958). Improving nutritional value to allow for the synthesis of essential proteins, hosts are able to expel worms more easily (Steel and Symons, 1982). In addition, food availability has been associated with worm egg counts in faecal matter in a study by (Valderrábano et al., 2002), which also showed that a restricted diet produced higher mean faecal egg counts than those fed at a high plane of nutrition.

Some studies have suggested that the use of protein is a more important factor in influencing parasitism than any other dietary element (Coop and Kyriazakis, 1999, Kyriazakis and Houdijk, 2006).

Recently investigators have examined the effects of protein in parasite establishment in Finn Dorset lambs, where dietary protein may have influenced parasite establishment. The lambs which were fed a diet low in protein had a higher faecal egg output four weeks after infection and more severe clinical signs than infected lambs of the same breed on a high protein diet (Abbott et al., 1985).

protein supplements had a significant impact on decreasing the concentrations of eggs in faeces (Van Houtert et al., 1995, Datta et al., 1998). According to the above logic, amino acids such as methionine have a powerful impact on protecting hosts against parasites (Coop et al., 1997).

1.4 Effect of additive nutrients on host-parasite interactions in fish

Current evidence indicates that one of the key limitations to aquaculture production is the phenomenon of disease outbreak. It affects the way in which the industry develops economically, thereby bringing about a variety of negative impacts (Yunxia, 2001). Additive nutrients such as carotenoids (Amar et al., 2001, Pike et al., 2007b) and ascorbic acid (vitamin C) (Dabrowski, 1990) are considered an optimal feed by which to improve growth performance, feed efficiency, and disease resistance in aquaculture (Yin et al., 2006, Franks et al., 1990, Shakya and Labh, 2014).

The effects of dietary supplements may be important in regulating the host-parasite interactions in fish. Wang et al. (2014) demonstrated that a group of fish fed chromium polynicotinate (Cr-Nic) in their diet improved their growth performance and were protected from *C. irritans.*

An inadequate supply of vitamin E to channel catfish *Ictalurus punctatus* leads to reduced immunological activity and in turn to non-specific immunological outcome such as a lack of response to infection (Wise et al., 1993). One set of research investigated the correlation between the non-specific immune reaction of gilthead seabream (*Sparus aurata* L.) and vitamin C, revealing that the higher the levels of vitamin C, the stronger the non-specific immune response became and, additionally, the greater the resistance to several bacterial and viral pathogens (Ortuno et al., 1999).

(Durve and Lovell, 1982) indicated that an increase in the dose of ascorbic acid reduces fingerling channel catfish (*lctnlurus punctata*) mortality as a result of infection with the pathogenic bacterium *Edwardsiellcr tarda.* In addition, the immune response arising from the consumption of supplements with soybean isoflavones improved and increased the growth of juvenile Golden Pompano*, Trachinotus ovatus* (Zhou et al., 2015, Waagbø, 1994).

As mentioned above, previous studies on fish supplementation has focussed on the effects of added nutrients on the interaction between fish and their parasites. Fish, as with other animals, cannot synthesis carotenoids, which are widely used as pigment as females prefer carotenoid coloured males as mates. (Bourne et al., 2003); carotenoid supplements in the diet of fish have also been shown to improve immune response (Kolluru et al., 2006).

1.5 Nutritional composition of natural and artificial commercial diets

1.5.1 Nutritional composition of natural diet

As diet is the subject of autonomous monitoring and intake-balancing, when in the wild, fish are able to respond to, and regulate from nutritive deficits through natural behaviours. A plentiful supply of food is particularly important for rearing larval fish (Abi-Ayad and Kestemont, 1994). Aquaculture depends upon diverse live foods for protein, including blood worms (*Chirinomidae*), shrimp and *Tubifex* worms (Chong et al., 2002) A wide range of information about the diet of three-spined sticklebacks is detailed below

1.5.1.1 Nutritional content of *Artemia*

Artemia is a common and widespread species worldwide (Lavens and Sorgeloos, 1996). Live *Artemia*, as a dietary component, have many vitamin and mineral over a synthetic diet, such as having balanced nutritional characteristics and being distributed throughout the water column (Cahu and Infante, 2001). Indeed, *Artemia* have become the most important food source in aquaculture (Helland et al., 2000). There are arguments in favour of using *Artemia*; firstly, it is available throughout the year and secondly, it is a suitable food source of high nutritionally value for fish (Léger et al., 1986).The dry mass of *Artemia* constitutes 31% protein in enriched *Artemia* nauplii, of which an estimated 14% comprises free amino acids (FAA) (Helland et al., 2003). However, the digestion rates of *Artemia* among fish is low because it has about 50-80% chitin in its dorsal exoskeleton (Shiau and Yu, 1999)

1.5.1.2 Nutritional content of *Daphnia*

Daphnia are planktonic freshwater crustaceans belong to family Cladocera, and a widespread distribution throughout freshwater (Steiner, 2002). It is known that Daphnia are closely associated with primary producers and fish (Reichwaldt and Abrusán, 2007).

The high levels of phosphorus, which is limited in the aquatic ecosystem (Schindler, 1977), can accumulate in cladocerans, particularly Daphnids (Hessen, 1990). *Daphnia* is also considered to be a key source of protein for fish, being comprised of 30.8 - 60% protein (Bogatova et al., 1971).

1.5.1.3 Nutritional content of Bloodworms (*Chironomus spp.* larvae)

Bloodworms (*Chironomus spp.* larvae) are a major food source for fish and other vertebrates or invertebrates (Lee et al., 2006b) *Chironomidae* larvae live in aquatic ecosystems and many species of animal depend on them as their principal form of dietary intake (Sharifian Fard et al., 2014). Bloodworm (*Chironomus)* larvae contain high quantities of lipids and vitamins (McLarney et al., 1974) The protein content is 26.06%, while the proportion of dietary lipids varies (0.33 g×100g-1). Meanwhile, the percentage of energy content and carbohydrate content found in Blood worms were 23.80 kcal×100 g^{-1} and 0.97%, respectively (Chittapun et al., 2013).

1.5.2 Nutritional composition of artificial commercial diets

In fish production and aquaculture, nutrition is the most critical factor in achieving economic production, and accordingly the development of an understanding of nutrition has received considerable research attention. Previous investigations have demonstrated that optimising the composition of an artificial diet in terms of fish protein levels (Sitjà-Bobadilla et al., 2005, Rodríguez-González et al., 2006, Thompson et al., 2005a), protein source (Kissil et al., 2000, Sitjà-Bobadilla et al., 2005), lipids (John et al., 2002), carbohydrate (Dong et al., 2018, Borba et al., 2006), and minerals and vitamins (Ortuno et al., 1999, Ortuño et al., 2001). Many studies have been designed, and indeed replicated other experiments, finding that acceptable or optimal levels of achievement have been determined.

1.5.2.1 Fishmeal

The substantial protein intake of high value carnivorous fish is often best provided (in supplementary terms) via the use of feed (fishmeal) consisting of various high protein marine forage species.(Tacon and Metian, 2008).fishmeal is commonly used as a sources certain amino acids ; specifically lysine, methionine and cysteine, (Ariyawansa, 2000). Fishmeal is a major ingredient in fish aquafeeds due to its palatability and protein quality (Lovell, 1988). Fishmeal is very rich in vitamins B12, riboflavin, choline and niacin; in addition, it has high amounts of minerals such as calcium, copper, and iron as well as a low fibre content (Ariyawansa, 2000), and is used on a large scale for aquaculture as well as the farming of animal livestock (Tacon and Metian, 2008); Fishmeal is rich in natural protein (including both acids and curd proteins), often containing 60-70% protein content. (Perez-Velazquez et al., 2018), in 2010 , the utilization of fishmeal in aquaculture feed accounted for approximately 56% (Olsen and Hasan, 2012).

1.5.2.2 Casein

Casein is emerging as a high-quality protein source, containing significant amounts of essential amino acids, which are required as part of a well-balanced diet. Thus, according to El-Sayed (1989) it is replacing fish meal as a potential alternative nutritional source for fish. Rawling et al. (2014) concurred that it is a suitable substitute to use, again on the basis of its high-protein content. Notwithstanding its overall favourable nutritional composition, El-Sayed (1989) identified casein's low arginine content as one of its sole deficits. Similarly, Shin-ichi Teshima and Kanazawa (1978), have recognised its important dietary contribution in terms of protein intake, so as to aid growth and satisfy the appetite, as well as to achieve high feed efficiency.

1.5.2.3 Cod liver oil

The weight of a number of species of fish is affected with intake of dietary lipids (Velasco-Santamaría and Corredor-Santamaría, 2011).In dietary terms, fish require the provision of fatty , The major source of energy in the diet fish is fish oil . Cod liver oil , extracted from the livers of Atlantic cod- (Bayraktar and Bayır, 2012) The fatty acid n-3 (found in cod liver oil) is essential for the health of fish

(Sargent et al., 1997) ; in general fish need some fatty acid especially n-3 fatty acids (Velasco-Santamaría and Corredor-Santamaría, 2011).

1.5.2.4 Sucrose

The evidence indicates that dietary carbohydrates are important in feeding fish for immune responses. and play critical roles in immunological function in interactions with the gastrointestinal bacteria flora and the gut-associated lymphoid tissue (Trichet, 2010). Fish differ in their ability to use carbohydrates (Wilson, 1994). With some restricted their ability to digest and metabolise carbohydrates (Zhou et al., 2013). Carnivorous fish do not utilize carbohydrates as their main conventional energy sources, but gain valuable energy from protein (Oliva-Teles, 2012).

1.6 The three-spined stickleback (*Gasterosteus aculeatus)*

1.6.1 General biology

The following section provides a brief overview of the life cycle of the three-spined stickleback. The three-spined stickleback *Gasterosteus aculeatus* is a small teleost fish belonging to the Family Gasterosteidae (Class: Actinopterygii, Super-Order: Teleostei, Order: Mesichthyes) (Heuts, 1947) which contains five genera (Wootton, 1976). It is a small fish (length between 3 cm and 8 cm (Falter, 1987) and has the special characteristic that it can be identified by their three sharp spines on the back and in front of the dorsal fin (Wootton, 1976).

The three-spined stickleback is an excellent model species through which to study questions about parasitic ecology. There are several reasons for this; firstly, they have a wide global distribution in diverse types of aquatic ecosystems, including marine, brackish and even fresh water, however, typically it is limited to marine coastal waters (Wootton, 1976, Kennedy, 1974, Barber, 2013). Secondly, it has become a species of choice in experimental biology laboratories due to its high suitability for laboratory studies. Third, sticklebacks are easy to breed in lab environments (Barber and Arnott, 2000, Barber, 2013). For these reasons, Numerous studies have used the three-spined sticklebacks as the model species to explore behaviour, comparative immunology, developmental and comparative

biology (Barber, 2013), and, based on research on genetics and evolution, has emerged to become a supermodel organism (Foster and Baker, 2004) and has become an important model organism in parasitology (Barber, 2013).

1.6.2 Feeding of Three-spined sticklebacks

Three-spined sticklebacks are carnivorous feeders that can eat a wide variety of foods (Wootton, 1976) although the relatively small size of this animal limit its choice of prey. An assessment of the diet and feeding patterns of fish can provide useful insights. This process generally comprises undertaking an analysis of fish stomach contents as an indicator of food availability, as well as monitoring habitual feeding behaviour (Andrian, 1996).

The dietary choice of sticklebacks is influenced by the way, in which, they select their prey. This is determined by a. In this regard, Wootton (1984) identifies features such as size, type of movement and perceived colour variations as being strongly correlated with the capacity to locate their prey.

The diet of three-spined sticklebacks has been the focus of many studies.

(Wootton, 1976, Allen and Wootton, 1984) number of factors including the prey's visual appearance in terms of colouration, texture, size and shape, as well as its movement capability (Ibrahim and Huntingford, 1989), and this constitutes a wide variety of zooplankton, especially copepods larvae and the pupae of chironomids (Hynes, 1950). Juveniles at one week post-fertilization begin to feed (Artemia, nauplii), whilst adults can eat a variety of foods including live feed crustaceans such as Cyclops and *Daphnia* sp., *chironomid* larvae, tubifex, Asellus and Duphniu.(Wootton, 1984), and even other stickleback eggs (Allen and Wootton, 1984).

Eating habits changes according to season in three-spined stickleback (Allen and Wootton, 1984). Sticklebacks have the capacity to feed on surface-floating food, such as commercial dried fish or on food bottom habitant such as tubificid and enchytraeid. (Wootton, 1976).

1.6.3 The stickleback *S. solidus* host-parasite model

1.6.4 Life cycle and general biology

Several species of freshwater fish have been recorded as harbouring *S. solidus* plerocercoids, although there are suggestions that the plercerocoids that infect three-spined sticklebacks are of a different species to those that infect other fish, such as nine-spined sticklebacks (Bråten, 1966, Nishimura et al., 2011). The life cycle of *Schistocephalus solidus* involves transmission through three separate hosts (Barber and Scharsack, 2010) (Figure 1.3).The first intermediate hosts are cyclopoid copepods, the second are three-spined sticklebacks (Bråten, 1966), and the definitive hosts – which are used to host development to the sexually mature adult worms – are typically piscivorous birds, where approximately 40 species of aquatic birds can be used as definitive hosts (Heins et al., 2002, Nishimura et al., 2011). Being hermaphrodites, individual adult *Schistocephalus solidus* worms have both male and female sex organs (Lüscher and Wedekind, 2002). The process of infection begins when sticklebacks ingest copepods, which are infected with procercoids in their haemocoel; *S. solidus* then changes morphology, achieving the procercoid form. The gut wall of the fish is penetrated by the procercoids, which then pass into the body cavity of the fish; *S. solidus* sheds its cercomer, and 60-80 proglottids (Smyth, 1946) are released, and a very distended body is sometimes visible as a result (Arme and Owen, 1967). The plerocercoid grows to a large size relative to their hosts, sometimes even exceeding the mass of the host itself (Hopkins and Smyth, 1951, Cunningham et al., 1994, Barber et al., 2008); when the plerocercoid reaches 50 mg in size, it is capable of infecting its definitive host. (Tierney and Crompton, 1992).

Following ingestion of the second intermediate host by the definitive host, the plerocercoids develop into sexually mature adults in the alimentary canal of more than forty types of fish-eating birds (piscivorous birds) including pelicans, ravens and herons. The eggs of the parasite are then transferred into bodies of water by the bird's faeces(Smyth, 1994). The hatching of these eggs is temperature-dependent, but usually occurs following a developmental period of eight days, releasing free-swimming larvae (coracidia), (Smyth, 1994).

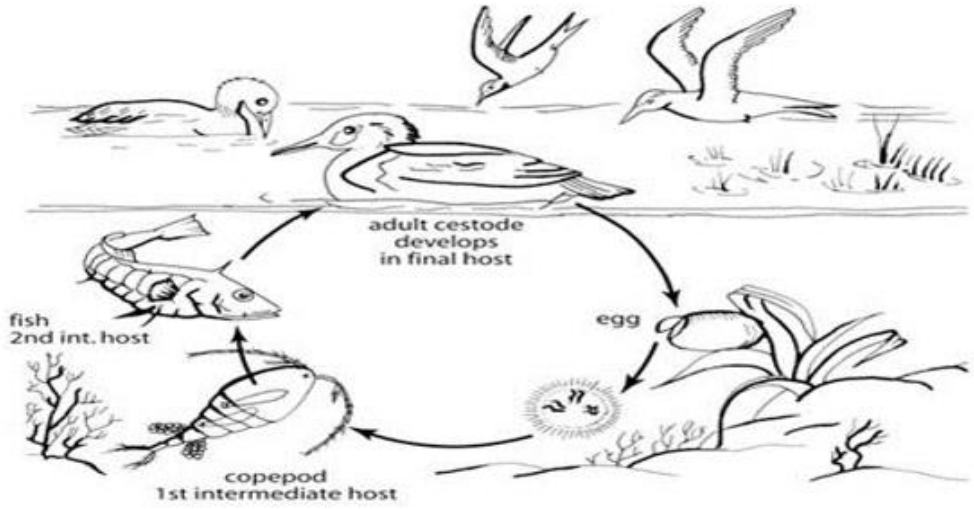


Figure 1.2 The life cycle of *Schistocephalus solidus*

1.6.5 Effects of infections on the biology of host

1.6.5.1 Effects of infection on host growth and energetics

The damaging effect of pathogenic infection upon fish fitness has been observed in multiple species (Schultz et al., 2006, Barber and Arnott, 2000, Markle et al., 2014, Seppälä et al., 2008). It is widely accepted that there are generally negative effects due to the interactions between host and parasite. Negative effects on host fitness have been demonstrated where infection is present, and usually involves such issues as reduced growth and an impact on energy (Barber et al., 2008, Kuris et al., 2008, Schultz et al., 2006), which is essentially due to parasites fulfilling their energetic requirements by utilising host-derived energy sources.

As previously mentioned, natural growth rates can be reduced by infections (Tierney et al., 1996, Goater et al., 2013, Yin et al., 2014). The mechanism by which parasites can impair growth have been studied extensively, and can be generalized as being the result of the drain in energy available to the host (Tierney et al., 1996), though, Consistent with the literature, that the infection affects the growth , inability to forage for food resources in a more competitive

manner (Barber, 2007b). Plerocercoids of the *cestode Schistocephalus* solidus are known to have a deleterious effect on the growth of their second intermediate host, the three-spined stickleback, *Gasterosteus aculeatus* (Tierney et al., 1996, Rushbrook et al., 2007).

It has also been suggested that the infection affects the energy of fish. Parasite infection produces a significant increase in the drain on available energy, in which immunological responses to the parasitic infection play a major part (Demas et al., 1997).

In some cases, an increase in host respiration can occur as a result of infection (Meakins and Walkey, 1975). Parasitic infection can result in a significant increase in the energy required for respiration (Lettini and Sukhdeo, 2010). The occurrence of *Schistocephalus solidus* in three-spined sticklebacks has been associated with increased respiration rates.

1.6.5.2 The effect of infection on reproductive development

Parasites often impair the reproduction of their hosts. Fundamental consequences that have been observed when parasitic organisms infect the germinal tissue are the impairment and inhibition of mature and normal gonads, where the propagation of the parasitic organism within the oocyte results in their collapse (Gbankoto, 2001).

There is increasing evidence that a large number of parasites can reduce the reproductive capacity of fish, such as Roach (*Rutilus rutilus*) and Gudgeon (*Gobio gobio*) (Arme, 1968, Bean and Winfield, 1989). Parasites probably cause significant damage to sexual maturation in some species of sticklebacks(McPhail and Peacock, 1983). In some cases, body condition, being an important element of the physiological state, affects reproductive success (Bagamian et al., 2004).

The reproductive capability of three-spined sticklebacks is directly suppressed by *Schistocephalus solidus* parasite infections.(Arme, 1968, Macnab et al., 2009) . This reduction results from infection, which is evident from a variety of cases; one example being that the presence of the plerocercoid is likely to reduce vitellogenin (VTG) synthesis in females (Macnab, 2011).

The process of infection by *S. solidus* has a major effect on the condition, reproductive development and energy consumption of host sticklebacks (Barber et al., 2008). It seems that three-spined sticklebacks, when infected, suffer effects such as weight loss and reduced body condition, particularly with respect to their liver mass, and also show decreased production of erythrocytes (Arme and Owen, 1967). *S. solidus* impairs the stickleback's reproduction, and few infected sticklebacks reach a normal level of sexual maturity (Tierney et al., 1996). Stages of oocyte development are sometimes late, and in extreme cases of infection, spawning does not occur(Arme and Owen, 1967). Early death may occur in severe cases in infected fish (Threlfall, 1968).

1.6.5.3 Effects on host behaviour

Many parasites affect their hosts in ways that change their behaviour (Barber, 2013, Giles, 1983, Seppälä et al., 2008). Atypical behaviours are often observed as a result of parasitism. Studies have shown that parasite infections can lead to changes in the behaviour of host fish. Parasites can modify their host's behaviour in ways that enhance their likelihood of infecting the final host (Øverli et al., 2001b). The intermediate host becomes more susceptible to predators because of the modulation of their behaviour by parasites (Lafferty and Morris, 1996). As a result of their increased need for energy (Godin and Sproul, 1988), the parasitzed fish's behaviour changes as result of an imbalance in their endocrine systems (Øverli et al., 2001b). Parasitized fish consume more oxygen than uninfected fish, thus infected fish tend to swim nearer the surface of the water (Lester, 1971). One of the effects of a Schistocephalus infection in sticklebacks, is the host fish adopts a greater swimming height to spend more time at the surface. (Meakins and Walkey, 1975).

Three-spined sticklebacks infected with cestoda Schistocephalus solidus exhibit changes in behaviour(Giles, 1983, Barber and Huntingford, 1995). This is largely due to the parasites in the host (Ackman and Gjelstad, 1975), which affect the relationship between prey and predators, with the fish ordinarily having a camouflage colour that makes detection difficult (Bone et al., 1995). Morphological characterization reveals the presence of described changes such

as black or white spots(Milinski, 1985) and abdominal distension(Barber, 1997) . These changes make the fish visible to predators.

1.7 Aims and objective of the thesis

The biological functioning of a host, including its growth and reproductive ability, is typically compromised by parasites, which divest the host of at least some of its nutrition. The host's ability to withstand the pressures imposed by parasitic infections is potentially influenced in part by its nutrition. Adequate nutrition to support the development and functioning of the immune system has a considerable impact on the host body's interactions with parasites. Diet potentially influences a number of mechanisms influencing the outcome of interactions with parasites, including resistance and resilience.

The overall aim of the research presented in this thesis was to investigate the effect of a range of host nutritional factors on the interactions between hosts and parasites using the three-spined stickleback - *Schistocephalus solidus* system as an experimentally amenable model.

Changes in husbandry practices or in availability of prey can lead to changes in food types and availability. It is necessary to comprehend the manner in which the biology of interplay between host and parasite can be affected by such changes.

The first objective was to examine the effects of host nutrition on interactions between three-spined sticklebacks and the parasite *Schistocephalus solidus*. In Chapter 3, fish captured from the wild, either naturally infected or non-infected with *S. solidus*, were fed a diet of either *Artemia* sp., *Daphnia* or bloodworms (*Chironomus* sp. larvae). The aim of this study was to determine whether the level of feeding on a particular food type (i.e. the quantity of food ingested) and the subsequent body condition of the host influence the susceptibility to acquiring parasites following exposure to infective stages?

The second objective was established to study how host diet affected the growth and development of *Schistocephalus* infections in sticklebacks. In these experiments, I studied the influence of nutritional quality and quantity on host growth, health status, immune status and sexual development, not only in fish

but in the parasite thought study the effect of diet on parasite growth and fecundity. In the first experiment, the effects of dietary protein content on the growth, development and health indicators of three-spined sticklebacks that had been bred and reared under standardised, controlled conditions in the laboratory were studied, and experimental parasite challenges were used to study the effects of host diet on the growth of *Schistocephalus solidus* plerocercoids in experimentally infected fish. In the second experiment, I examined the effect of host food intake on the interaction between fish and parasite. Fish were fed one

of two standard artificial diets with high levels of satiation (giving %bw.d-1) and medium levels of satiation (giving %bw.d-1), respectively. The effects on host and parasite growth were studied, in terms of condition and development. In addition, plerocercoids were cultured to the adult phase for a period of 12 weeks to test the hypothesis that host diet may have implications for parasite fitness (chapter 4).

Additionally, the research reported in chapter 5 aimed to investigate the effects of host ration on the growth of a common and ecologically important fish parasite; plerocercoids of the diphyllobothriidean cestode Schistocephalus solidus. Presumably, the infection could affect the preference or selectiveness of fish for various diets. Therefore, the research question set out in chapter 5 aims to establish whether being infected by parasites altered the host fish's dietary preferences in terms of food quality. They select, as well as whether these changes benefit hosts, parasites or neither.

A final objective, addressed in Chapter 6, was to study the effect of additive nutrients on host parasite interaction. The use of additive nutrients was increased in response to improving the quality of nutrients by adding a supplementary diet during aquaculture production. Carotenoid pigments have attracted great interest as pigments for attracting mates and conferring certain immunological benefits, to determine whether the carotenoid supplemented could affect susceptibility to infection and parasite growth.

Chapter 2 Materials and methods

2.1 Sources of experimental fish

To explore how the fish-parasite interaction was affected by host nutrition, the present study employed the species three-spined stickleback *Gasterosteus aculeatus* as a model (Barber and Svensson, 2003, Arnott et al., 2000)

2.1.1 Wild fish

Three-spined sticklebacks were caught using hand nets and minnow traps from the River Soar at Abbey Park, Leicester (52°38'43.6"N 1°08'01.6"W),. In early December 2014. The sticklebacks were then transferred to the aquarium facility of the Department of Neuroscience, Psychology and Behaviour at the University of Leicester. The experiment, which will be discussed in chapter 3, was carried out using these fish.

2.1.2 Lab breeding using *in vitro* fertilization techniques

In nature, three-spined sticklebacks breed under a wide variety of conditions of aquatic environments (Östlund-Nilsson, 2006) , and the reproductive cycle of the three-spined stickleback is seasonal (Wootton, 1976), beginning in late spring to early summer (Hellqvist et al., 2006). When the breeding season starts, in the middle of April, sexually mature males and females are selected according to certain features; the male exhibits secondary sexual characteristics such as nuptial colouration, which comprises of a red throat, and the develops a bright blue colour, whilst the patterns on the females' bodies are more dark (Wootton, 1976). Barber and Arnott (2000) give the characters by which adult males can be identified by the red nuptial coloration around their throats.

Most of the experiments in this project used fish that were produced using published *in vitro* fertilisation techniques (Barber and Arnott, 2000). Parental stock were collected as juveniles from the Clatworthy Reservoir population (N 51°07'29, W 3°36'92), and reared in the lab until they were sexually mature. They were then used to produce families via standard *in vitro* fertilisation techniques (Barber and Arnott, 2000) during the 2015 breeding season (May to August) (Östlund-Nilsson, 2007)

Gravid females that exhibited lateral swelling of the abdominal area caused by egg production were selected for reproduction. Sexually mature males were then euthanized by immersion in an overdose of benzocaine solution (benzocaine ethyl-aminobenzoate, stock solution: $10g.L^{-1}$). Each male was placed in a dissecting dish, and immediately the skin was cut from the anal fin using scissors and an incision made horizontally toward the head; a second incision was then made very carefully from the body wall to the dorsal spines along the dorsal side of the fish no higher than the lateral line. The muscles were carefully exposed, and the paired testes gently were removed, by macerating forceps. After extraction of the sperms from each male, they were placed in a watch glass with normal ice and macerated with a little distilled water. Clutches of eggs were then carefully squeezed from each female into the watch glass containing the macerated testes, making sure that they were loose inside the watch glass, thus allowing the sperm to combine with the eggs in the watch glass.

Then, the potentially fertilised eggs were checked under the microscope to ensure that fertilisation had occurred by observing segmentation which should have taken place approximately 2 hours after fertilisation.

The fertilised eggs were transferred to a small plastic aquarium of (1 litre volume) and aerated using an air stone. Methylene Blue stock solution (2ml, concentration 1 mg/ml) was added to reduce the risk of fungal contamination. Eggs where checked daily to make sure the air was being properly suppled and hatching was monitored; eggs normally hatched 8-10 days after fertilisation*,* the temperature was monitored between 18^{o} -19^{o} C. Three-quarters of the water was changed on day 6, then on day 7 and each day subsequently until the fry hatched, when 50% of the water was changed daily. Newly-hatched fry were fed *ad libitum* with a succession of Liquifry No 1™ (Interpet, UK), using one drop for the first week, and fed with *Artemia* sp. nauplii when one day old and, after a week, were placed on two-day-old frozen or live *Artemia* nauplii, after which the fish were transferred to a rearing tank (holding tank) for five months. In the meantime, the fish were fed bloodworm *ad libtum* twice a day.

2.2 Experimental infections of copepods and sticklebacks

2.2.1 In *vitro* culture of *Schistocephalus solidus* plerocercoids

In this study*, Schistocephalus solidus* plerocercoids were recovered from the body cavity of infected three-spined sticklebacks that had been sampled from the River Soar at Abbey Park, Leicester 52°38'43.6"N 1°08'01.6"W). The fish were killed by immersion in Benzocaine solution (10g Benzocaine dissolved in 70% industrial methylated spirit), rinsed and botted dry, and a ventral incision opened the abdominal cavity. Worms were recovered using sterilised forceps and cultured using standard *in vitro* techniques (adapted from the original technique described by Smyth, 1954). Single plerocercoids were placed in semi-permeable dialysis tubing) that mimics the conditions inside the gut of a piscivorous endotherm, and incubated individually in boiling tubes containing highly buffered media comprising a 50:50 mixture of horse serum and cell culture medium, with additional antibiotics including to reduce contamination (Barber and Scharsack, 2010). The culture tubes were sealed using screw-top lids, and left in a lateral shaking incubator for 7 days at a temperature setting of 40˚C (which is typical for a bird) (Tierney and Crompton, 1992) . After 7 days, the tubes were checked to make sure that the worms had produced eggs, after which the eggs were isolated using sterile procedures.

Preferably, eggs were collected before the adult worms had died. Before the eggs were collected, all glassware was cleaned, and then the tapeworm along with the content of the culture membrane was transferred to a Petri dish. The remnants of the worm were removed and the metabolic products that had accumulated in the vicinity of the eggs were removed by successively rinsing the egg suspension in sterile dH_20. After being washed several times, the eggs were transferred in water to sterile Petri dishes which were sealed and covered in aluminium foil, before being incubated in the dark at 20^oC for 21 days, This protocol was used in the experiment in chapter 3.

In some cases, different pairs of worm from different infected fish were used in vitro cultures of *S. solidus* plerocercoids, each pair were placed in a single tube

as can be seen in figure 2.1. Then, the same protocol which was used with the single worm was applied to the pairs.

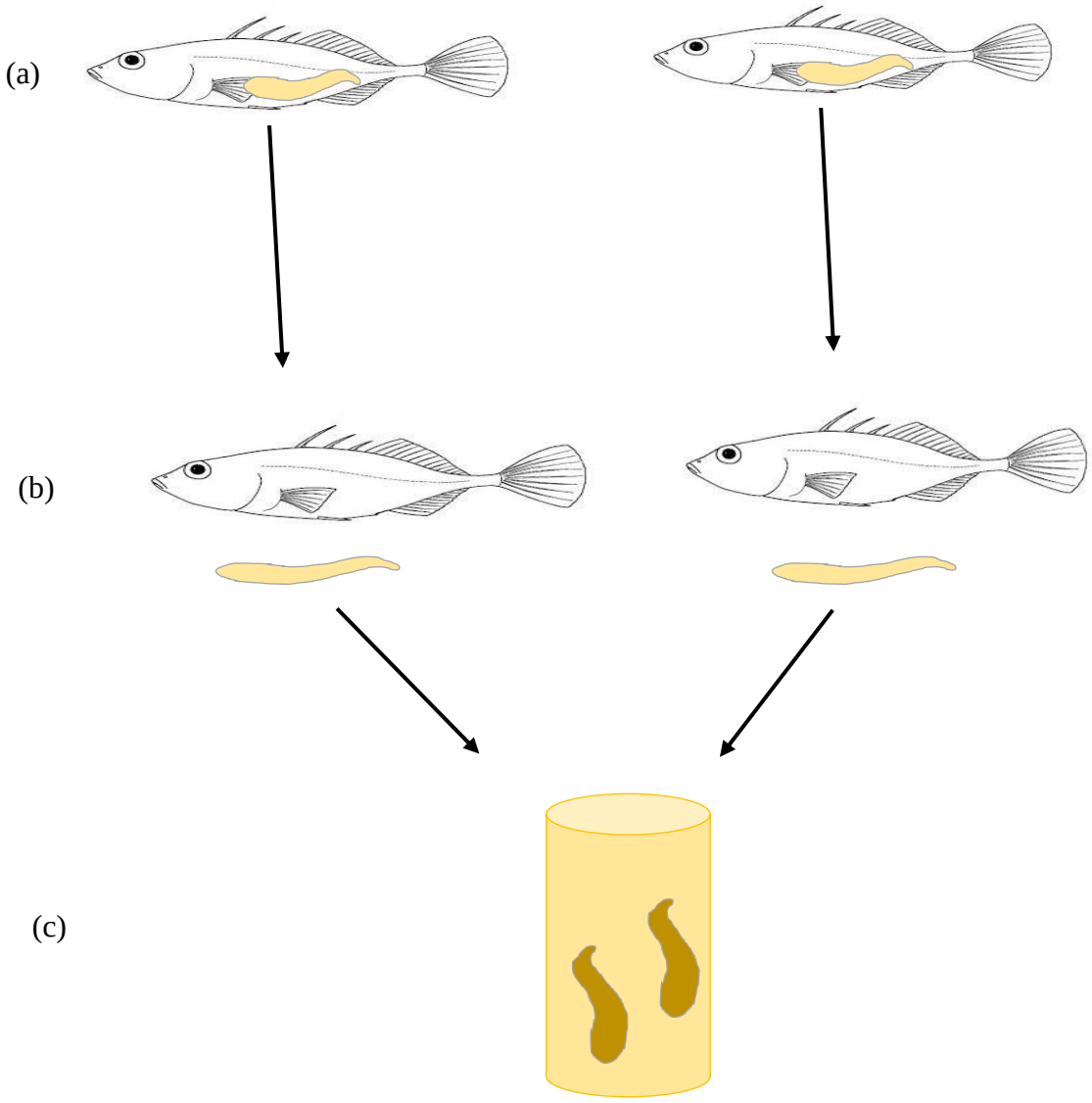


Figure 2.1 Culture pairs of *Schistocephalus solidus* plerocercoids in vitro in the laboratory.

(a) three-spined sticklebacks infected with s. solidus (b) s. solidus plerocercoids recovered from the body cavities of infected fish (c) culture vessels containing pairs of plerocercoids

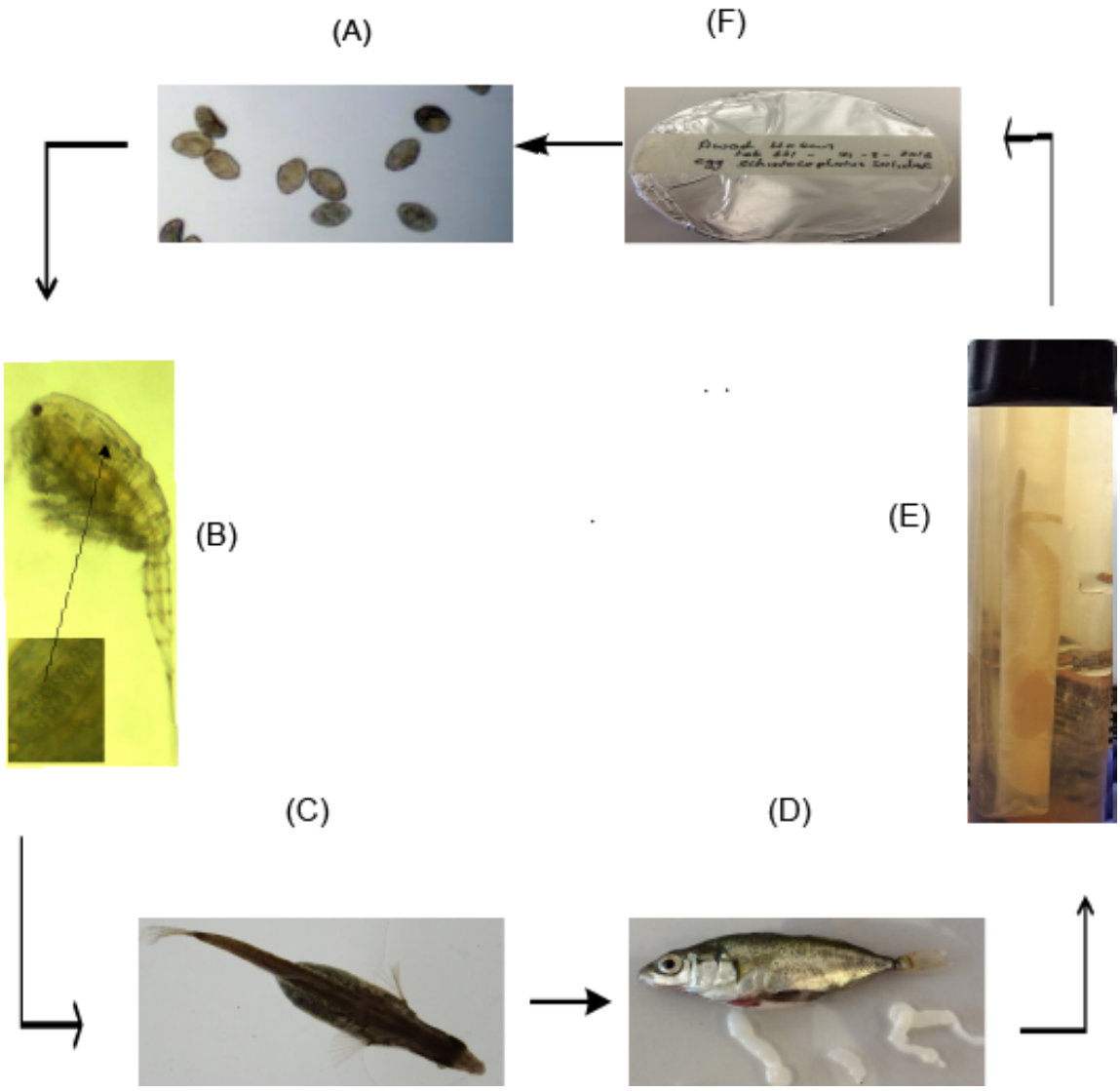


Figure 2.2 The life cycle of *Schistocephalus solidus* in vitro laboratory.

(A) parasite eggs hatched in water after exposed to light and releasing coracidia (B) Copepod (*Cyclops strenuus abyssorum)* experimentally infected with *Schistocephalus solidus* procercoids (arrow inset) after ingesting coracidia (C) Three spined sticklebacks infected become infected after ingesting infected copepod , the plerocercoid grows to a large size (D) *Schistocephalus solidus* plerocercoid recovered from infected sticklebacks (E) the pairs plerocercoids worm combined in culture tube (F) petri dish in incubated in the dark 20^{o}C. Parasite eggs photo by (Simmonds, 2015)

2.3 Counting eggs

In this project, we tested whether there was an effect of diet on egg output in order to estimate parasite fecundity.

Schistocephalus solidus plerocercoids were recovered from sticklebacks that had been sampled from infected fish in our research study. Plerocercoids were cultured using *in vitro* techniques, previously described in section 2.2.1 (Barber and Svensson, 2003) .The eggs of the dialysis membrane were transferred to a petri dish in order to make a visual check of whether there was any waste. The remnants of adult parasite collection were removed, after being washed several times with sterile distilled water, and were transferred in this water into sterile petri dishes 9 cm in diameter. The suspension was centrifuged in 10 ml tubes at 1,500 rpm for 4 min. The contents of the tubes were reduced to 2 ml and the eggs were resuspended by vigorous pipette action (Dörücü et al., 2007), For each parasite, the total egg output was estimated by undertaking separate haemocytometer counts of the eggs in 10 1.8 µ1 samples drawn from the resuspended egg solution, each counted under x100 magnification. Ten values were taken from the egg counts for each parasite, giving a total egg output. The highest and lowest egg counts from each worm were excluded in order to calculate the total egg output of each individual parasite from the mean count of the eight samples remained. For statistical analysis, a Log10 transformation was utilised to normalise the plerocercoid mass and egg output, thereby facilitating parametric examination.

2.4 Experimental infection of copepods

A laboratory population of cyclopoid copepods (*Cyclops strenuus abyssorum*: initially purchased from Sciento, UK*)* was maintained throughout the study. The copepods were sorted once in two weeks, and divided into three sizes of nauplii, copepodites and adult copepods by different sized sieves (45, 150 and 250 µm). In the lab, all sizes were then placed in flasks and fed once a week with 100-200 ml of alfalfa infusion (*Medicago sativa*)

The Alfalfa infusion was prepared by adding 1 g of alfalfa powder (Naturally Green, reading UK) to 400 ml of distilled water in a flask; then, the solution was put on a hotplate for approximately 5 minutes until it reached boiling point. The solution (flask) was left to boil on a hotplate for approximately 5 minutes. The solution was filtered into a 400 ml bottle and 0.2 Na2HPO4 was added. The solution was autoclaved, after becoming cold, then 20 ml of yeast /glucose

solution was added and 50 ml of stock protozoa food *colpidium striatum*. The solution was left at room temperature for 24 h then stored at 4^{o} until it was used for feeding .One week prior to planned experimental infection, copepodites were separated from the laboratory culture and placed into a 200 ml flask, where they were starved for one week before exposure to free-swimming coracidia. Parasite eggs were then removed from the incubator one day before experimental infection and exposed to natural sunlight to stimulate hatching for 24 hours. The presence of free-swimming coracidia was verified under a binocular microscope (Scharsack et al., 2007) and, after these were fed on by copepodites, copepodites from a lab population of copepods were exposed to batch of newly-hatched coracidia, copepods were left for three weeks and fed a few drops of alfalfa infusion once a week. Copepods were then screened for infection using a compound microscope (x100) 21 days following initial exposure to *S. solidus* coracidia, after being anaesthetised by using carbonated water. After this visual inspection they were checked to identify whether procercoid is infective when the cercomer was observed.

Lab-bred three-spined sticklebacks (see above) were placed in a small tank (1 litre) which filled of water to 2 cm depth and each fish was deprived of food for 24 hours. After that, the fish were exposed individually to infective stages of *S. solidus* by allowing them to eat a single infected copepod. Sham infected controls were fed a non-infected copepod. Following parasitic exposure, experimental fish were transferred to the aquaria in which they were held for the duration of the study.

Figure 2.3 Copepod (Cyclops strenuus abyssorum) experimentally infected with Schistocephalus solidus procercoids.

2.5 Experimental diets and feeding regimes

In this project, two different types of diet were used: a natural diet, and an artificial diet.

2.5.1 Natural diet

Three commercially available and pre-frozen food types were used to feed the fish in this project: adult water fleas *Daphnia* sp., adult brine shrimps *Artemia* sp., and bloodworms, which are larvae of *Chironomus* sp. Midges. All frozen foods were purchased from commercial suppliers, and manufactured by 3F Fish Food (The Netherlands, www.frozenfishfood.nl). Frozen food was thawed in warm water and then blotted dry, this food was used in experiment 1 in chapter 3 the fish were fed bloodworm *ad libtum* twice a day in a husbandry routine daily. Table

1 shows the basic nutritional composition of each frozen food, as supplied by the manufacturer.

Table 2.1 Basic nutritional composition of the three different types of commercially available frozen food used in the experiments.

Constituent	Artemia sp.	Chironomus sp.	Daphnia sp.
Crude Protein	5 %	5%	2.4%
Crude fat	1 %	1%	0.7%
Crude fibre	0.9%	0.9%	0.3%
Ash	0.8%	0.8%	0.7%
Moisture	92.2%	92.0%	96.3%

2.5.2 Artificial diet

Different artificial diets were formulated containing low, medium and high protein. Fishmeal (Haith's Baits, UK) and casein (Bulk Powders, UK) were used as the source of dietary protein, cod liver oil (seven seas , UK) was used as the main lipid source, and sucrose was used as the carbohydrate. Gelatin, vitamins and minerals In addition, a red food colouring dye was added to increase the visibility and attractiveness of food.

The experimental diets were prepared by thoroughly mixing the dry ingredients (fishmeal, casein, cod liver oil, sucrose, vitamins and mineral mix). The feed was then mixed with water to achieve the correct consistency to allow it to be extruded using a syringe. Once mixed, the diet was extruded using a syringe with a 1.2 mm aperture and chopped to accommodate the size of the mouth of the three-spined sticklebacks roughly 2 mm. It was further dried in an incubator at 37℃ for one hour and stored at 4℃ until further use. The fish were fed daily with a measured ration (wet mass) of fish body weight for a week's feeding period, taking into account that the ration would change with changes in body weight.

2.6 Experimental tanks

2.6.1 Holding tanks

Prior to the start of the experiment, fish were housed in glass aquaria, where each aquarium (24cm x 29cm x 40 cm) was a tank containing a plastic plant and supplied with compressed air via an air stone. Water temperature was maintained at 14°C throughout the rearing period and a photoperiod of 12L:12D was implemented. Fish were fed *ad libitum* with bloodworm until the experiment commenced. Each tank contains 40 fish, and all fish were monitored to check their health twice a day.

2.7 Autopsy and terminal data collection

At the end of each experimental study, all fish were weighed, measured and dissected under a binocular microscope to confirm their sex and infection status, and to permit analysis of the various body condition indices. Individual fish were first blotted dry and measured (standard length, *SL*, to 0.1 mm) weighed on an analytical balance (*M*, to 0.001 g), before being deeply anesthetized by immersion in an overdose of Benzocaine solution (benzocaine ethyl-aminobenzoate, stock solution: $10g.L^{-1}$). Under deep anaesthesia, the caudal peduncle was then severed using a sharp scalpel, and whole blood was collected from the caudal vein into heparinised capillary tubes. The end of the tube was then sealed, and the blood sample was centrifuged for 60 s (12,000 rpm) at room temperature in order to calculate the haematocrit value. Each fish was then dissected aseptically. 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 (each to 0·0001 g) using an analytical balance. The carcass was wrapped in a sterile piece of aluminium foil and frozen at -20^{o}C. The liver was saved in RNA later™.

Any *S. solidus* plerocercoids recovered from the body cavities of infected sticklebacks were removed with fine forceps and placed in small sterile pouches made of aluminium foil. They were blotted with filter paper and then transferred to a Petri dish and immediately weighed individually. This process was undertaken using a stereomicroscope with cool, fibre-optic illumination to prevent heating the tissues.

2.8 Measurement of growth, condition, immune activation and reproductive development in fish

2.8.1 Growth of fish

2.8.1.1 Absolute growth of fish

During the experimental trials, individual lengths and weights were taken for all fish after the acclimation period, and weight and length were measured at 14-day intervals. The ability to accurately determine the growth of the fish is an important tool in fish and fisheries biology. The calculation for absolute growth in mass is shown in Equation 1 below:

fish growth (in g) = final fish mass (in g) – initial mass (in g) **(Equation 1)**

2.8.1.2 Specific Growth Rate (SGR)

The specific growth rate (SGR) of each individual fish between week 0 and 8 was calculated as shown by the equation below (Russell et al., 1996, Barber and Svensson, 2003).

SGR (% body mass gain per day) = (ln M_f – ln M_i x100) / t **(Equation 2)**

Where:

ln M_f = the natural logarithm of final mass of the fish

ln M_i = the natural logarithm of initial mass of the fish

t = time interval (in days) between ln M_i and ln M_f

2.8.2 Indices of fish condition

2.8.2.1 Fish mass

The end of experiment, the total mass of each individual fish was recorded, then the fish mass was measured as shown by the equation below

M_f = total fish mass – parasite mass.

2.8.2.2 Body Condition Factor (BCF)

The body condition factor describes the relationship between body mass and length of the fish (Pennycuick, 1971), and has been used to determine the -term energetic status of the fish (Chellappa et al., 1995). In this experiment, fish length and mass was recorded every two weeks, and used to calculate the body condition factor. At the end of the experiment, body condition factor was calculated according to the following equation:

$$K = [M \text{ (in g)} / L^3 \text{ (in mm)}] \times 100{,}000 \quad \textbf{(Equation 3)}$$

Where M is the fish mass (in g) and L is fish length (in mm)

2.8.2.3 Hepatosomatic index (HSI)

The Hepatosomatic Index (HSI) is defined as the mass of the liver as a percentage of total body mass, and is used to give an indication of the of the condition and the energetic status of the fish (Chellappa et al., 1995). At the end of each experimental study, the HSI was calculated, according to the following equation.

$$HSI = M_L / M_f * 100 \quad \textbf{(Equation 4)}$$

Where M_L and M_f represent liver mass and fish mass (grams), respectively.

2.8.2.4 Haematocrit

Haematocrit is defined as the percentage (%) of red blood cells by volume in the blood, and defines the blood oxygen carrying capacity.

Under deep anaesthesia, the caudal peduncle was severed using a sharp scalpel, and the whole blood was collected from the caudal vein into heparinised capillary tubes. After sealing the end of the tube, the blood sample was centrifuged for 60s (12,000 rpm) at room temperature in order to calculate the haematocrit value.

2.8.3 Assessing immune activation in sticklebacks

2.8.3.1 Splenosomatic Index (SSI)

In order to estimate the level of immune activation, the splenosomatic index (SSI) was calculated. The spleen is an organ involved in immune defence (phagocytosis) and in blood cell synthesis (erythropoiesis). The SSI is calculated as the mass of the spleen expressed as a percentage of total fish body mass, and gives an indication of the overall immune system health of the fish (Ruane et al., 2000, Hadidi et al., 2008, Henrich et al., 2014). Enlargement of spleen reflection is investment in immune function (John, 1994)

The splenosomatic index was calculated according to the following equation:

$$SSI\ (\%) = M_s / M_f * 100 \qquad \textbf{(Equation 5)}$$

Where M_s and Mf represent spleen weight and fish mass (grams), respectively.

2.8.4 Measurement of sexual development of male and female fish

2.8.4.1 Males: Kidney somatic index (KSI)

KSI is often used as a measure of sexual maturation in males. In male sticklebacks, it is often used as a measure of sexual maturation in male

The kidney was weighed to calculate the kidney-somatic index, as follows:

$$KSI\ (\%) = [M_k / M_f] \times 100 \qquad \textbf{(Equation 6)}$$

2.8.4.2 Females: Gonadosomatic index (GSI)

Gonads were weighed to calculate Gonadosomatic index, as follows. The Gonadosomatic index (GSI) is often used as a measure of sexual maturation in female Paired gonads were weighed to calculate the Gonadosomatic index, as follows:

$$GSI\ (\%) = [M_g / M_f] \times 100 \qquad \textbf{(Equation 7)}$$

2.9. Calculating parasite growth during the studies

2.9.1 Total parasite mass (*M*p)

The mass of each *S. solidus* plerocercoid recovered from experimentally infected fish were recorded at the termination of each study. Any plerocercoids recovered were blotted dry and weighed individually on an analytical balance (to 0.001 g). The total mass of plerocercoids recovered from each fish was calculated (*M*p).

2.9.2 Parasite index (*I*p)

Fish mass was used to calculate the parasite index (*I*p) as the total parasite mass divided by the fish mass and multiplied by 100

$$I\text{p}\ (\%) = [M\text{p} / M_f] \times 100 \quad \textbf{(Equation 8)}$$

Chapter 3 Impact of food quality type on fish health and parasite growth

Abstract

Diseases caused by parasites, bacteria and viruses represent a major threat to fish, both in natural and managed (aquaculture) populations, and the importance of diseases is likely to be exacerbated in the face of rapid global environmental change. Alterations in husbandry practices or in availability of prey can lead to modifications in food types and availability. It is necessary to comprehend that the manner in which the biological interplay between host and parasite can be affected by such changes represents an important challenge. In the current study, I examine the effects of host nutrition on interactions between three-spined sticklebacks and the parasite *Schistocephalus solidus*. Here I present the results of a diet manipulation study in which we fed infected and non-infected sticklebacks on a diet of either *Artemia* spp., *Daphnia* or bloodworms (*Chironomus* larvae) at a rate 10% fish body mass per day for 8 weeks under controlled lab conditions. I report the consequences of diet for the growth rate, energetic condition (body condition, hepatosomatic index) and health status (splenosomatic index, haematocrit) and sexual development (ornamentation, kidney somatic index, gonadosomatic index). Our results demonstrate that diet type has consequences for the growth, energetic condition (body condition factor, hepatosomatic index) and health status (haematocrit) of sticklebacks kept under laboratory conditions, our results support the hypothesis that dietary factors may play an important role in determining emergent infection phenotypes in fish.

3.1 Introduction

3.1.1 Impact of parasite infections on fish in natural and managed populations

Fish can be affected by various types of infection, including protozoans, arthropods, and helminths. Such pathogens and parasites can profoundly affect the biology of fish in the wild and in aquaculture, and the successful establishment of a parasite within a host can significantly influence the host's energy levels, growth rate and sexual development (Schultz et al., 2006) and the physiology of locomotory performance (Holmes et al., 1990). Additionally, parasites can indirectly influence host feeding through their effects on behaviour. For example, in species of dace such as *Leuciscus leuciscus*, metacercariae of the trematode *Diplostomum spathaceum in* the eye impair vision and influence foraging behaviour by reducing the ability to capture prey (Crowden and Broom, 1980). Infection in sheepshead minnows, *Cyprinodon variegatus, by* the trematode *Ascocotyle pachycystis* causes a reduction in swimming performance because parasites reduce the energy resources available to host growth. Fish belonging to the family Salmonidae are plagued by external parasites, known as salmon lice *(Lepeophtheirus salmonis*). As a result of the proliferation of such parasites, sea trout (*Salmo trutta*) suffer a decrease in individual body size when they travel to freshwater environments to spawn, affecting commercial fisheries in the most impacted areas (Arechavala-Lopez et al., 2015).

3.1.2 Nutritional factors influencing susceptibility to infection in fish

The main limitation to aquaculture production is the phenomenon of disease outbreak, which affects the way that the industry develops economically, thereby bringing about a whole variety of negative impacts (Thompson et al., 1996, Yunxia, 2001).

The immune response is considered one of the most important factors in determining the ability of a fish to resist infection by bacteria, viruses or parasites (Trichet, 2010). The ability of host organisms to effectively damage existent parasitism, or to effect immunity thereof, has been associated, in both innate and adaptive immunity, with the host's nutritive intake. (Li and Gatlin, 2006, Li et al.,

2004, Calder, 2007). For example, the growth of Hymenolepis diminuta in the intestine could be affected by the availability some of dietary elements such as glucose (Dunkley and Mettrick, 1969) in some cases, while infection may enhance the growth of a parasite. There is extensive evidence that good nutrition supports parasitic growth such as in cestode (*Ligula intestinalis*) infections of roach (*Rutilus rutilus*) (Loot et al., 2002), evidence for which has recently, been corroborated by (Simmonds, 2015). The availability of food plays a critical role in the growth of Schistocephalus solidus.

Increased immune response due to high levels of vitamin C in gilthead sea bream (*Sparus aurata)* led (Ortuno et al., 1999) and A high vitamin C intake could function as an effective supplement to the dietary elements known to facilitate growth , haematology (Ibrahem et al., 2010), innate immunity and disease resistance in Nile tilapia (*Oreochromis niloticus*). In grass carp ,Negative outcomes (including disease,early morbidity and depressed growth) were observed as being causally related to a deficiency in vitamin E. (Pan et al., 2017). The same study also found that vitamin E deficiency decreased lysozyme, as well as the weights of kidneys and spleens of grass carp *Ctenopharyngodon idella*.

According to Sitjà-Bobadilla et al. (2005) grading fishmeal with plant proteins has been observed to effect between 50-100% augmentation of the immunity of sea bream to parasitism. The evidence reported by the researchers indicated that phagocytes' oxidative burst was improved with a 75% dietary substitution of plant protein instead of fishmeal.

In comparison to most other animals, fish lack a genuine protein requirement, but they do require effective balancing with respect to essential (i.e., indispensable) and non-essential (i.e., dispensable) amino acids. (Wilson, 2002) As previously noted, nutrition is a key determinant in the nature of host-parasite interaction, not least because immunity and nutrition are intimately connected. The consequence of this is that host resistance can improve, often accompanied by an improvement in the degree to which they are resilient. The effect of nutritional factors on immune responses showed that innate immune of blunt snout bream, *Megalobrama amblycephala*, infected with *Aeromonas hydrophila* showed a greater levels of leukocyte and increasing feeding frequency (Li et al., 2014).

The dietary nutrition of three-spined sticklebacks has been investigated in many studies (Allen and Wootton, 1984, Maitland, 1965). Three-spined sticklebacks have a varied diet(Barber, 2013). Patterns of food consumption by three-spined stickleback changes seasonally (Allen and Wootton, 1984). Sticklebacks have the capacity to feed on surface-floating food, such as commercial dried fish or on food bottom habitant such as tubificid and enchytraeid (Wootton, 1976) more details in chapter one section 1.6.2

3.1.3 Aims

As is evident from above, the nutritional type and availability of food play critical roles in the health and behaviour patterns of fish, and an understanding of how this can impact the biology of host-parasite interactions represents an important challenge. In this chapter, the effect of host nutrition on interaction between three-spined sticklebacks and the parasite *Schistocephalus solidus* is investigated. The results demonstrate the effects of diet manipulation in which infected and non-infected sticklebacks are fed on a diet of either *Artemia* spp., *Daphnia* or bloodworms (chironomus sp. larvae) at a rate of 10% fish body mass per day for 8 weeks under controlled lab conditions, which was hypothesised to potentially impact upon physiology or infection.

3.2 Methods

3.2.1 Fish collection and husbandry

The experimental fish were offspring of wild, naturally-spawning parents. Juvenile, young-of-the-year (0+) sticklebacks were collected using hand nets and minnow traps from the River Soar at Abbey Park, Leicester (52°38'43.6"N 1°08'01.6"W)in early December 2014. These fish were transferred to the aquarium facility of the Department of Neuroscience, Psychology and Behaviour at the University of Leicester. The fish were inspected for characteristic signs of *S. solidus* infection (Barber, 1997) and their fins inspected for any visible ectoparasites.

The investigation was carried out in an experimental aquarium system consisting of 66 individual plastic aquaria (each 14× 25×13 cm) placed within a filtered

recirculating system, with water depth of 10 cm. Each aquarium was supplied with compressed air which was delivered through an air stone and a plastic plant for shelter. Water temperature was maintained at 14°C and controlled thermostatically, with a photoperiod of 12 hours of light: 12 hours of darkness (12L: 12D) implemented. The fish were distributed among the aquaria with one fish being placed in each aquarium.

3.2.2 Experimental diets and feeding regime

Three commercially available and pre-frozen food types were used to feed the fish in this experiment: adult *Daphnia* sp. water fleas, adult *Artemia* sp. brine shrimps, and bloodworms (larvae of *Chironomus* sp. Bloodworms) (3F, The Netherlands, www.frozenfishfood.nl). The fish were fed a measured ration of 10% (wet mass) of fish body weight per day, for an eight-week feeding period. Table 3.1 shows the basic nutritional composition of each food type, as supplied by the manufacturer. Frozen food was defrosted in water then blotted dry and weighed on an analytical balance 24-well plastic microtiter plate using lab tweezers. Absolute ration was adjusted at two weeks to take account of changes in body mass as the fish grew.

3.2.3 Experimental procedure

At the start of the experiment and at 14d intervals thereafter (i.e. at weeks 0, 4 and 8), individual fish were photographed in dorsal profile above a light box to maximize contrast. Basic measurements of standard length (*SL*, from the snout to the end of the fleshy caudal peduncle, measured to 0.1mm) and wet mass (*M*, to 0.001g) were also recorded on each occasion.

Table 3.1 Basic nutritional composition of the three different food types used in Experiment 1 Data provided by the food manufacturer

Constituents	*Artemia* sp.	*Chironomus* sp.	*Daphnia* sp.
Crude Protein	5 %	5%	2.4%
Crude fat	1 %	1%	0.7%
Crude fibre	0.9%	0.9%	0.3%
Ash	0.8%	0.8%	0.7%
Moisture	92.2%	92.0%	96.3%

3.2.4. Monitoring of water quality

Maintaining acceptable water condition is paramount to the success of long-term experiments, the water supply was from the same source, with line water quality monitoring (filtration recirculating system); each aquarium had one fish and the temperature was controlled.

3.2.5 Autopsy

At the end of the experiment, after 8 weeks, all fish were dissected under a binocular microscope, to confirm their sex and infection status, and to permit analysis of the various body condition indices. Each fish was anesthetized by immersion in an overdose of benzocaine solution (benzocaine ethyl-aminobenzoate, stock solution: $10g.L^{-1}$). Individual fish were blotted dry, weighed on an analytical balance (terminal mass, M_{56}, to 0.001g), and measured (terminal SL_{56}, to 0.1mm). Absolute growth rate of fish was calculation as fish growth (in g) = final mass (in g) - initial mass (in g). Spleen mass (*MS*) was also recorded (to 0.001g) and the spleen somatic index (SSI=*MS*/*Mf* *100) was calculated. Haematocrit is defined as the percentage volume (%) of red blood cells in the blood. Liver mass (*ML*) was recorded (to 0.001g) and the hepatosomatic index (HSI=*ML*/*Mf* *100). Kidney mass = (MK-M_F) *100. Under deep anaesthesia, the

caudal peduncle was severed using a sharp scalpel, and whole blood was collected from the caudal vein into a heparinised capillary tube. The end of the tube was sealed and the blood sample was centrifuged for 60s (12,000 rpm) at room temperature in order to calculate the haematocrit value. Each fish was then dissected aseptically. 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 (each to 0·0001g) using an analytical balance. The carcass was wrapped in a sterile piece of aluminium foil and frozen at -20C. The liver was fixed in RNAlater™.

Any *S. solidus* plerocercoids recovered from the body cavity of infected sticklebacks were removed with fine forceps and placed in small sterile pouches made from aluminium foil. They were blotted with filter paper and then transferred to a Petri dish and immediately weighed individually. This process was undertaken using a stereomicroscope with cool, fibre-optic illumination to prevent heating the tissues. The total mass of plerocercoids (M_p, to 0.001g) was determined, allowing the parasite index (I_p) to be calculated as (M_p / M_{56}) * 100)

3.2.6 Statistical analysis

All data were tested for normality and homogeneity of variance and subjected to transformation (Johnson transformation) if necessary prior to using parametric statistical analysis. General Linear Models (GLMs) were then used to test effects of food type and infection status and the interaction between food type and infection status on output variables.

One -way ANOVA was run to examine the effects of food type on fish condition and health status, Post hoc pairwise Tukey tests were then used to identify significant differences between treatments. For all boxplots in this chapter , the dark line represent the median, the box shows the Q1-Q3 interquartile range (IQR), and the Whisker plots represent the greatest value and lowest value , while ● represents an outlying observation , All statistical analyses were carried out using Minitab (version 17).

3.3 Results

3.3.1 Survival in the experiment

Fish were checked each day and mortalities recorded. Table 3.3. shows the survival rate of fish fed on the different experimental diets. Nine of the 66 fish died before the end of the 56-day study, = (86.4 %)

Table 3.3. Survival rate of fish fed on different diets in Experiment 1

Type of diet	Stocked fish	Dead fish	Survival rate*
Bloodworm	22	3	86.36%
Artemia	22	0	100%
Daphnia	22	6	72.72%

* Survival rate = (stocked fish – dead fish) / stocked fish) * 100

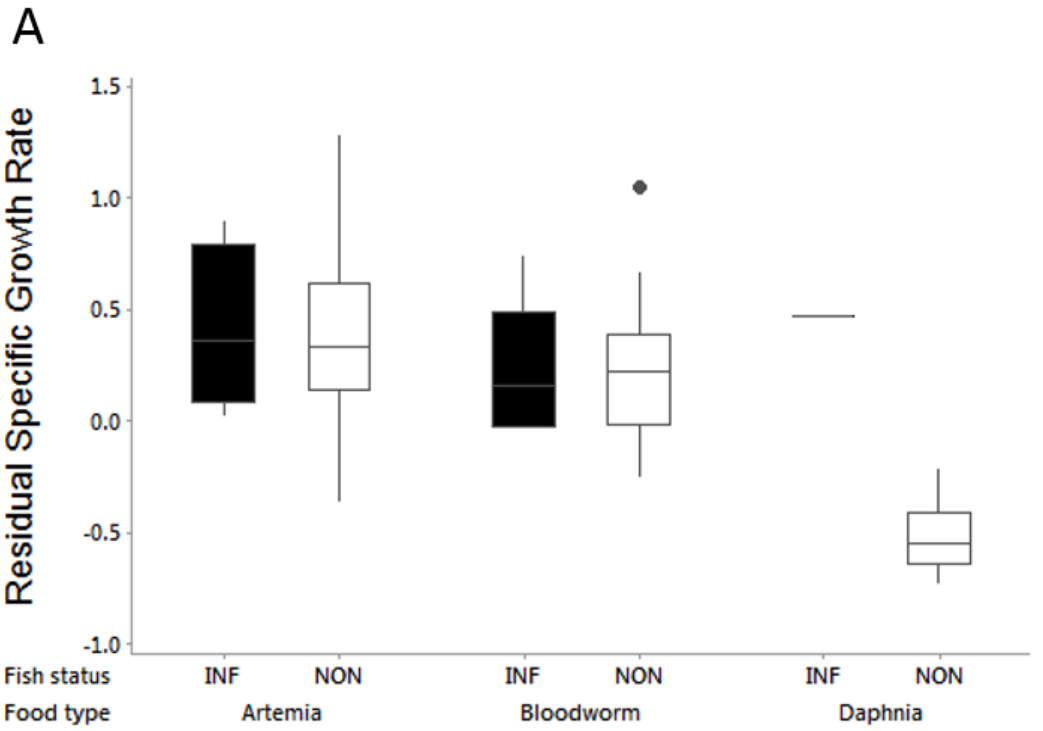
3.3.2 Effects of infection status on fish growth and health'

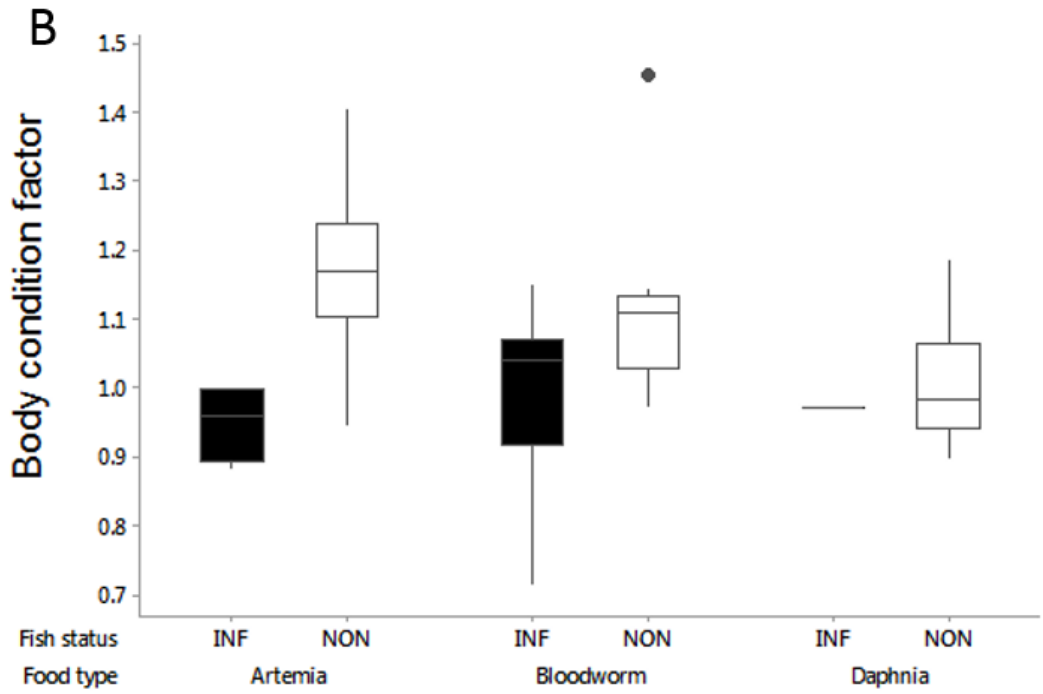
At the termination of the study, 2-way ANOVA tested the effects of food type and infection status on Residual specific growth rate. Further statistical tests revealed that r-SGR values were affected by food type. ($F_{2,50}$ = 28.57, P < 0.001) Additionally,there was no significant effect of infection status ($F_{2,50}$ = 2.40, P = 0.128) and there was no interaction between food type and infection status on growth ($F_{2,50}$ = 0.91, P = 0.409).

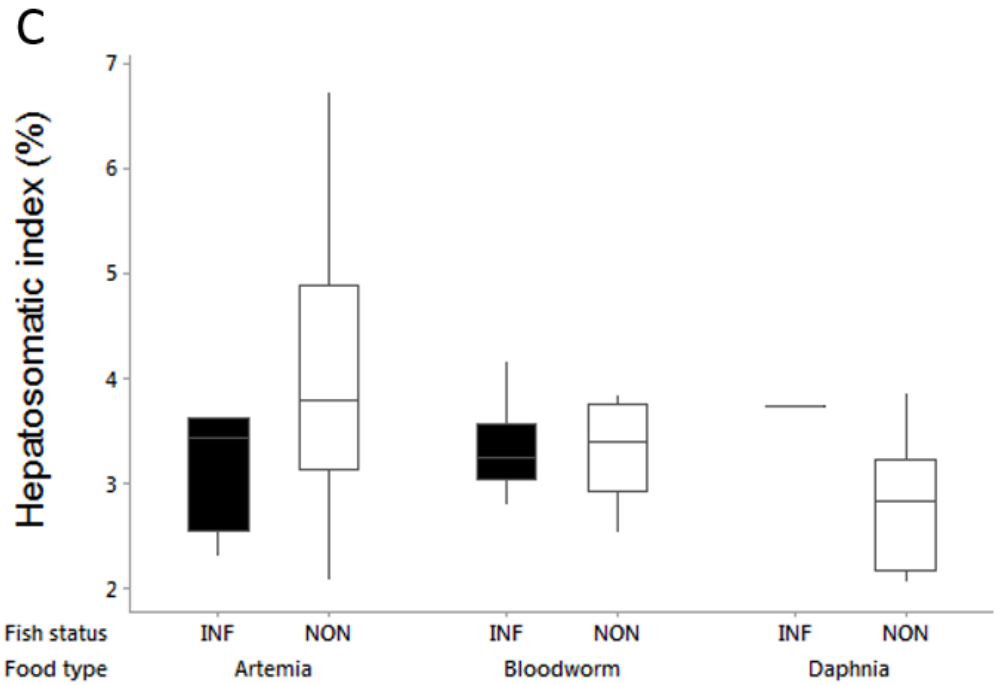
The result from study the effect of food type and infection status on host body condition factor were measured. The result showed the body condition factor was not affected by food type ($F_{2,55}$ = 0.51, P = 0.605), but was significantly affected by infection status ($F_{2,55}$ = 6.33, P = 0.015), with non-infected fish having higher body condition values compared with the infected group. There was no significant interaction between infection status and food type on body condition ($F_{2,55.}$ = 1.25, P = 0.296). In contrast, hepatosomatic index values were not affected by food type or by infection status, and there was no statistically significant interaction

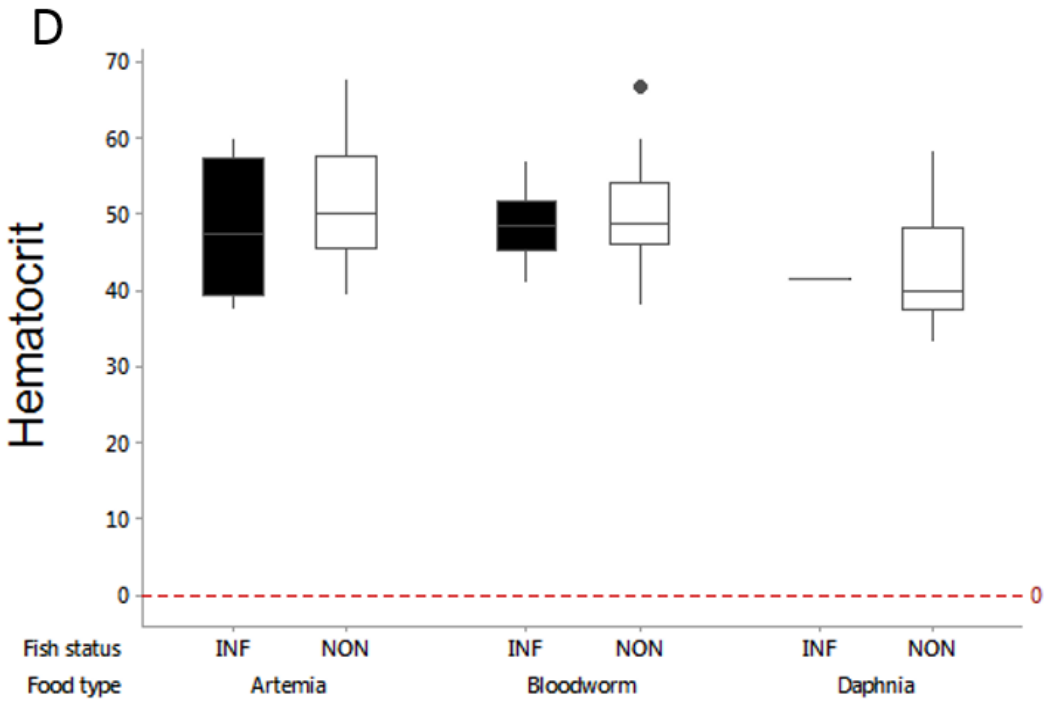
between these factors (food type: $F_{2,55} = 0.32$, $P = 0.729$; infection status : $F_{2,55} = 0.42$, $P = 0.628$; interaction: $F_{2,55} = 2.17$, $P = 0.125$).

The effect of food type and infection status on splenosomatic index and haematocrit was also investigated. Splenosomatic index was significantly affected by food type ($F_{1,52} = 3.40$, $P = 0.019$), but not infection status ($F_{1,52} = 0.88$, $P = 0.352$). However, there was a significant interaction between food type and infection status ($F_{1,52} = 3.63$, $P = 0.034$). Viewing the data suggests that this was because infection was associated with elevated splenosomatic index only in some diet treatments (notably among fish fed *Artemia*). There was no effect of food treatment or infection status on haematocrit, and there was no significant interaction between these factors (ANOVA: ration: $F_{1,52} = 1.17$, $P = 0.193$; infection: $F_{1,52} = 0.31$, $P = 0.577$; interaction: $F_{1,52} = 0.05$, $P = 0.950$).


A
Residual Specific Growth Rate
1.5
1.0
0.5
0.0
-0.5
-1.0
Fish status
INF
NON
INF
NON
INF
NON
Food type
Artemia
Bloodworm
Daphnia


B
Body condition factor
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
Fish status
INF
NON
INF
NON
INF
NON
Food type
Artemia
Bloodworm
Daphnia


C
Hepatosomatic index (%)
7
6
5
4
3
2
Fish status
INF
NON
INF
NON
INF
NON
Food type
Artemia
Bloodworm
Daphnia


D
Hematocrit
70
60
50
40
30
20
10
0
0
Fish status
INF
NON
INF
NON
INF
NON
Food type
Artemia
Bloodworm
Daphnia

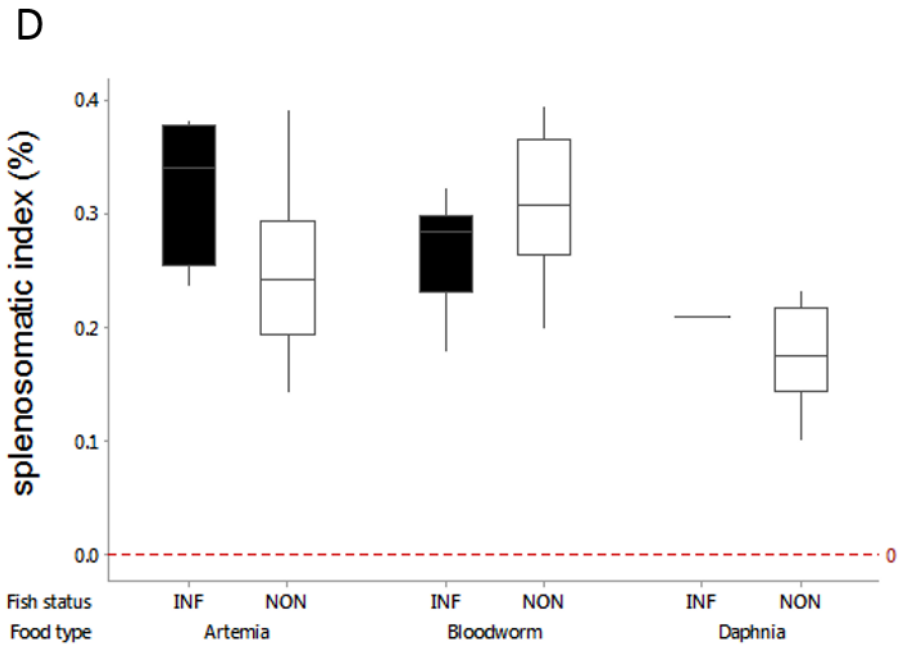


Figure 3.1 Boxplot showing the effect of food type and infection status on indices of three-spined sticklebacks condition (a) residual specific growth rate (b) body condition factor (C) Hepatosomatic index (HSI) and (d) Haematocrit.(e)splenosomatic index

3.3.3 Measurements of growth rate

3.3.3.1 Absolute growth of fish

These results suggest that an increase in mass is observed for fish fed on the *Artemia* and bloodworm diet in the study, however, fish fed the *Daphnia* diet did not show such growth, as shown in figure 3.1. Further statistical analysis revealed growth data show that food source had a significant effect on the growth in mass attained by the fish in the study (1-way ANOVA: $F_{2,45}$=33. 94, P<0.001). The highest increases in absolute mass were seen in fish groups fed the *Artemia* and bloodworm diets, where the lowest values were in fish groups fed *Daphnia* and there were differences among them (Figure 3.2).

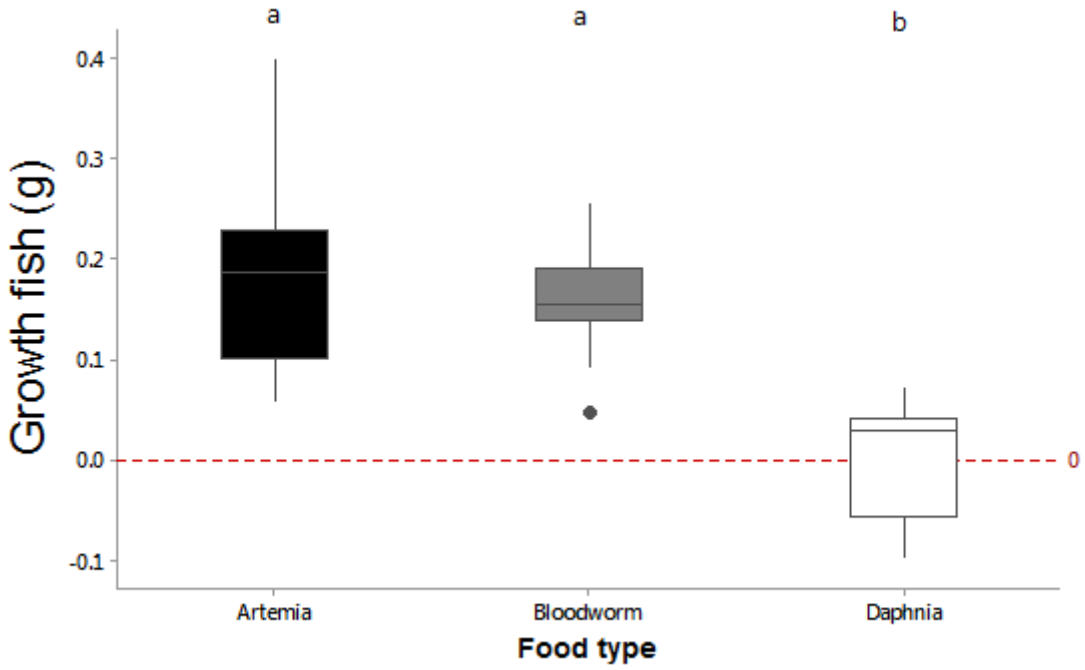


Figure 3.2 The effect of food type (*Artemia*, bloodworm and *Daphnia*) on absolute growth of stickleback fish in the experiment.

3.3.3.2 Residual Specific Growth Rate (rSGR)

The specific growth rate (SGR) of each fish individual fish over the 56 days of the experiment was calculated. As expected, SGR was found to correlate closely with initial body size, with small fish showing higher SGR values than larger individuals (Figure 3.3) do. Residual values (rSGR) from the relationship between initial standard length were calculated, to correct SGR values for initial body size. A 1-way ANOVA was run to examine the effects of food type on rSGR. The results showed that rSGR values were strongly affected by food type ($F_{2,53}$=30.71, *P*<0.001), and revealed that rSGR was significantly higher among *Artemia*- and bloodworm-fed fish than in *Daphnia*-fed fish.

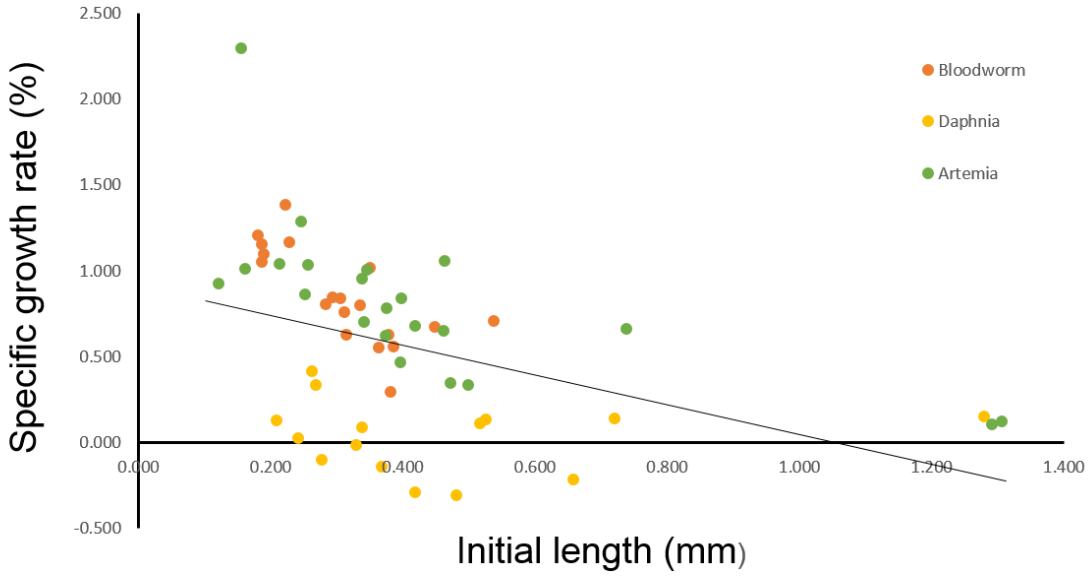


Figure 3.3 Relationship between specific growth rate and initial stander length in three-spined stickleback fed three different of diet (*Artemia*, bloodworm and *Daphnia*)

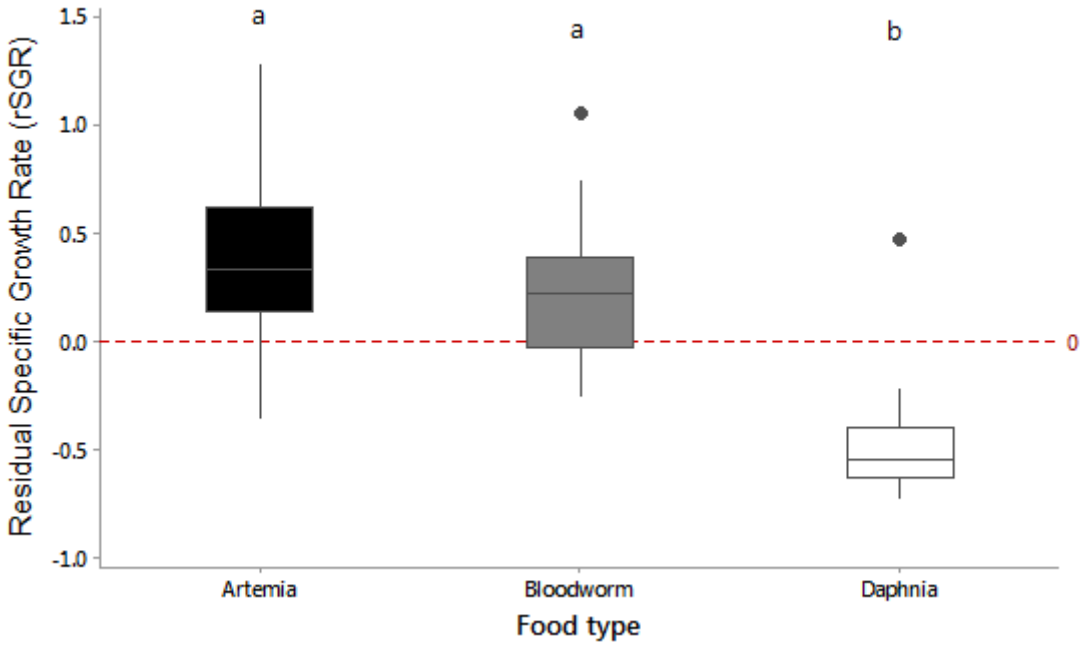


Figure 3.4 The effect of different food types (*Artemia*, bloodworm and *Daphnia*) on residual Specific Growth Rate (rSGR) of sticklebacks.

3.3.4 Indices of fish condition

3.3.4.1 Body Condition Factor (BCF)

At the end of the experiment. Body condition factor (BCF) was significantly affected by the food type fed to fish in the study, a one-way ANOVA testing the main effect of food type (F_2, 53=3.91, *P*=0.026; Figure 3.5). The highest BCF values were recorded among fish fed *Artemia*. There were significant differences between *Artemia* and *Daphnia*. Compared to fish fed bloodworm and *Daphnia,* there was no significant difference between fish fed bloodworm and *Daphnia;* neither was there a significant difference between *Artemia* and *Daphnia;*

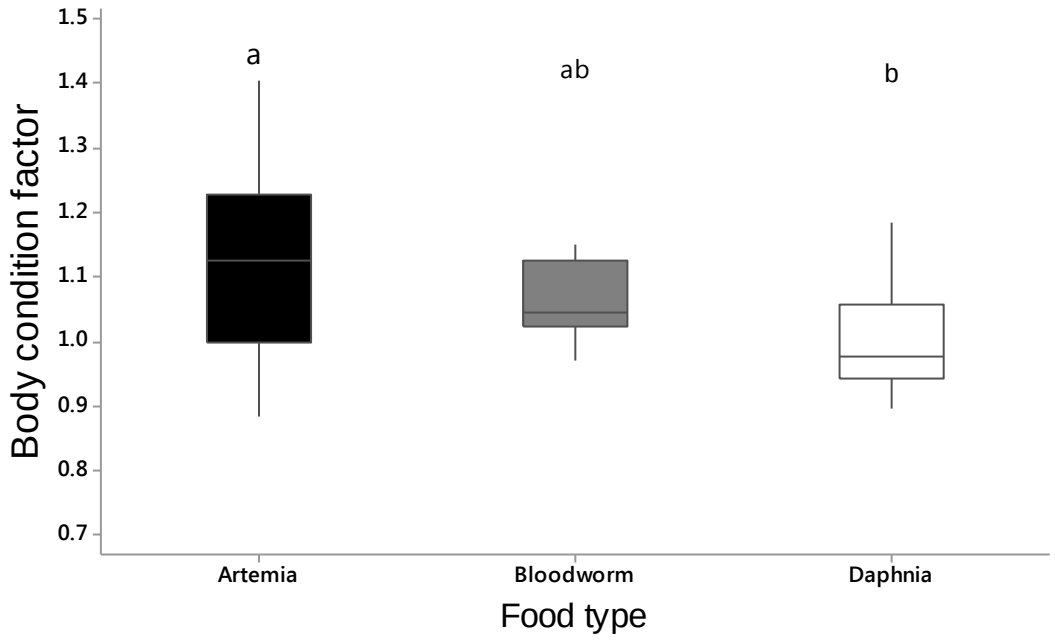


Figure 3.5 The effect of food type on body condition factor of three-spined sticklebacks fed *Artemia,* bloodworm and Daphnia in the experiment.

3.3.4.2 Hepatosomatic index (HSI)

The HSI was measured at the end of the experiment. Statistical analysis showed that differences within each diet throughout the study and between diets throughout the study were significant. Fish fed *Artemia* and bloodworm exhibited the greatest HSI values compared to the fish fed *Daphnia*. There was a significant

difference between *Artemia* and *Daphnia.* Furthermore, additional statistical tests revealed that there were significant differences between fish fed bloodworm and fish fed Daphnia (F2,53=6.93, P=0.002 Figure 3.6).

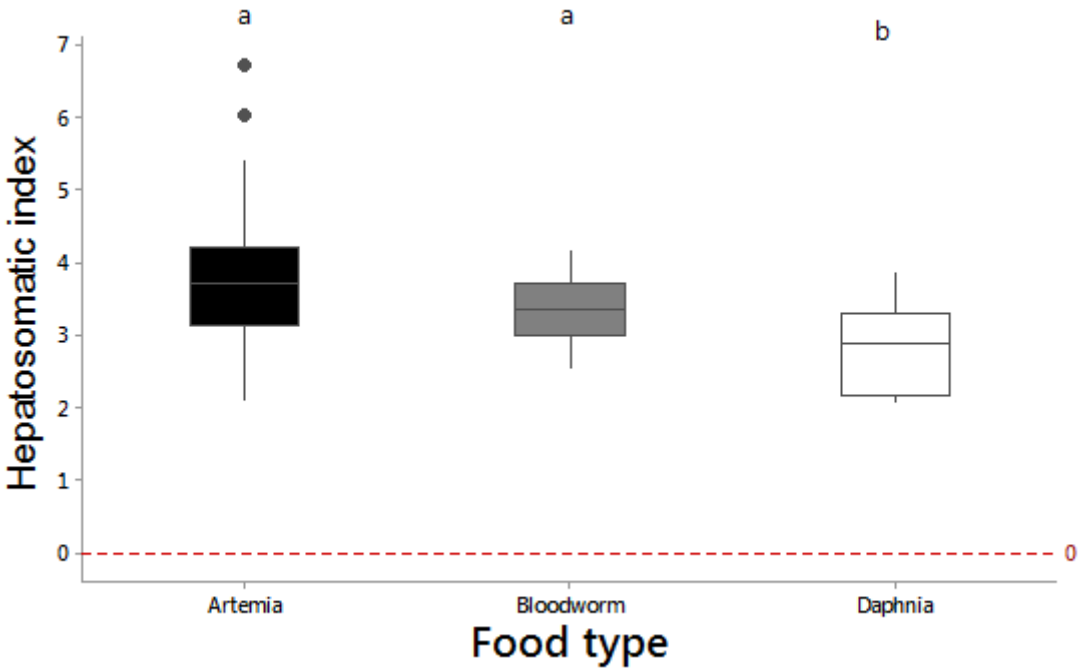


Figure 3.6 The effect of food type on Hepatosomatic index (HSI), i.e. the mass of the liver in relation to body mass, in sticklebacks which were fed different type of diet (*Artemia,* bloodworm and *Daphnia*).

3.3.5.2 Haematocrit

It can be seen in the Figure 3.6. Showed that the differences in Haematocrit were significant for fish fed on the different diets over the course of the study (1-way ANOVA: $F_{2,50} = 6.25$, $P < 0.004$). Haematocrit appeared to be highest when fish were fed *Artemia* and bloodworm compared to those fed *Daphnia*. Further statistical tests revealed that there were no significant differences between fish fed bloodworm and those fed *Artemia*

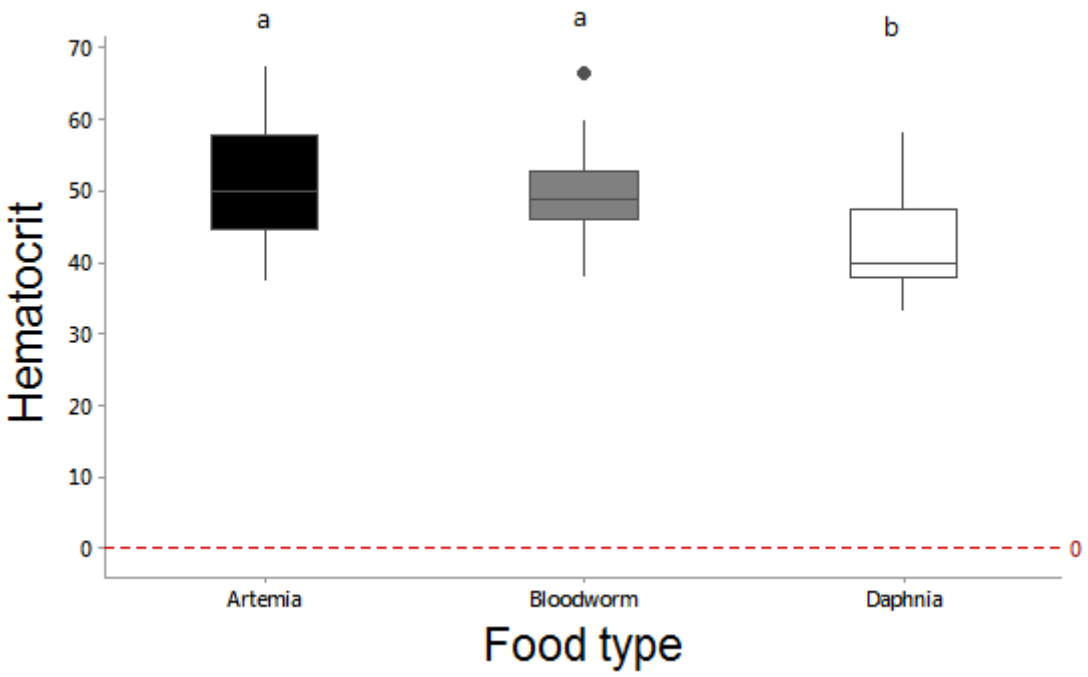


Figure 3.7 The effect food type on Haematocrit (% red blood cells) in sticklebacks fed different type of diet (*Artemia*, bloodworm and *Daphnia*).

3.3.5 Effect of food type on indicators of immune function

3.3.5.1 Splenosomatic Index (SSI)

The results showed that there were no differences among treatments in fish fed bloodworm and Artemia and that these two groups achieved a maximum value SSI. On the other hand, there were significant differences between fish fed *Daphnia* and the fish fed each of the other two diets: *Artemia* and bloodworm.1-way ANOVA ($F_{2,50} = 15.17$, $P < 0.001$ Figure 3.8.).

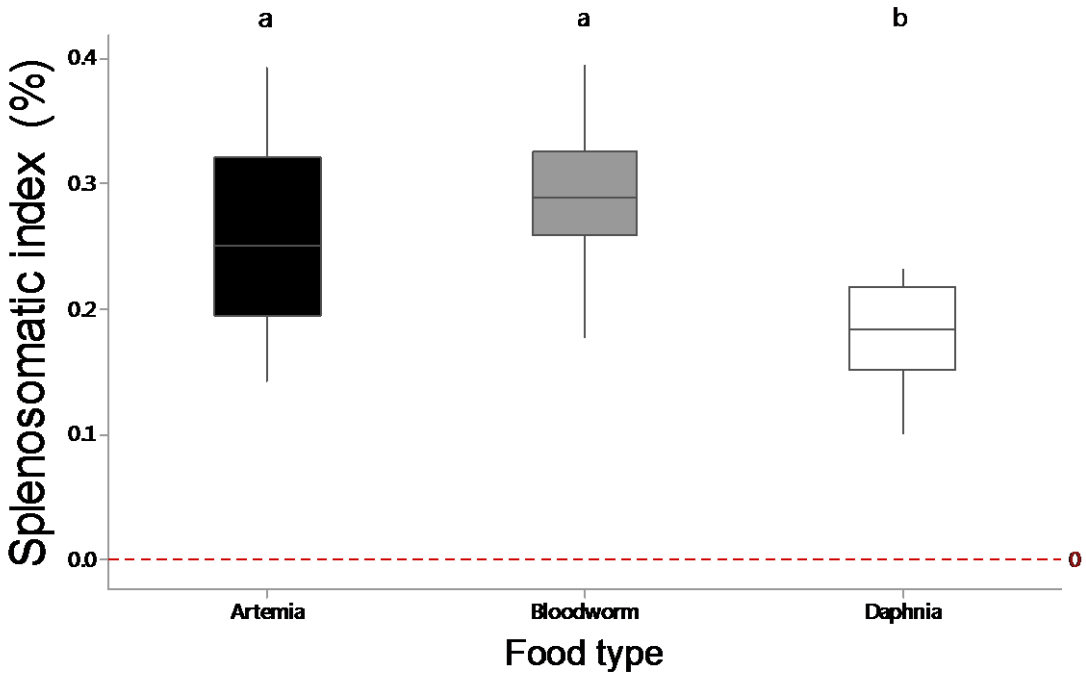


Figure 3.8 The effect food types (*Artemia,* bloodworm and *Daphnia*) on the splenosomatic index (SSI) of sticklebacks in the experiment

3.3.6. Effect of prey type on reproductive development of male and female fish

3.3.6.1 Males: Kidney somatic index (KSI)

A 1-way ANOVA was used to test the effect of food type *(Artemia*, bloodworm and *Daphnia*) on male sexual development. The kidney somatic Index (KSI) did not differ between fish fed the different diet treatments ($F_{2, 24} = 0.98$, $P = 0.389$; Figure 3.9.).

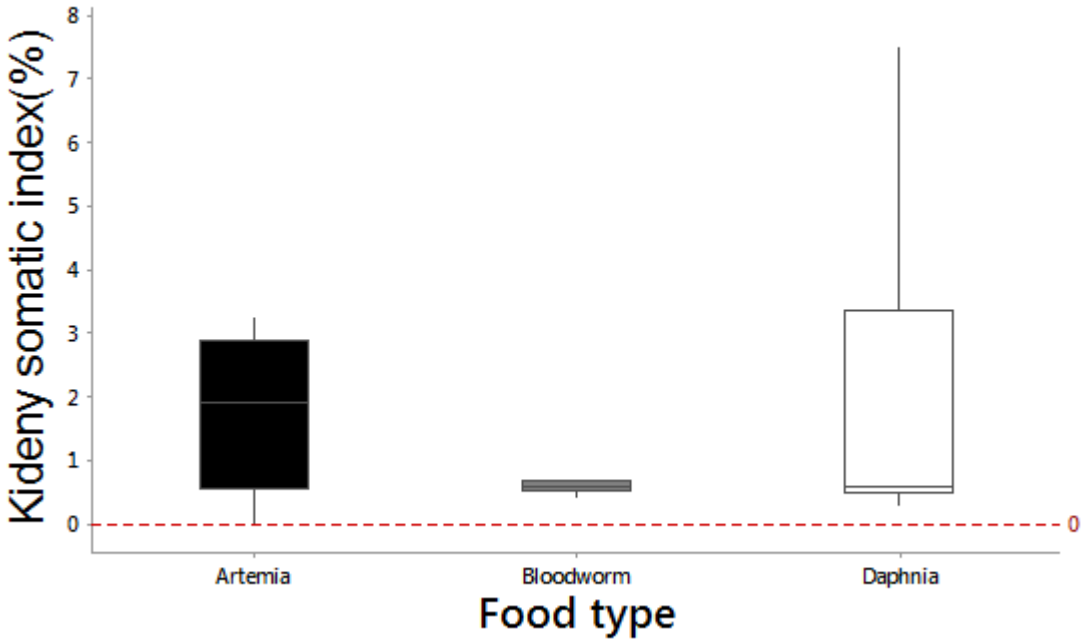


Figure 3.9 The effect of different type of food (*Artemia,* bloodworm and *Daphnia*) on measurement of sexual development (measured as kidney somatic index) in male sticklebacks in the experiment.

3.3.6.2 Females: Gonadosomatic index (GSI)

A 1-way ANOVA was used to test the effect of food type *(Artemia*, bloodworm and *Daphnia*) on female sexual development. The results showed that there were significant differences between fish fed bloodworm and those fed *Daphnia*. On the other hand, the Gonadosomatic Index (GSI) did not differ between fish fed bloodworm and *Artemia* or between fish fed *Daphnia* and *Artemia* ($F_{2,27} = 3.87$, $P = 0.033$; Figure 3.10.).

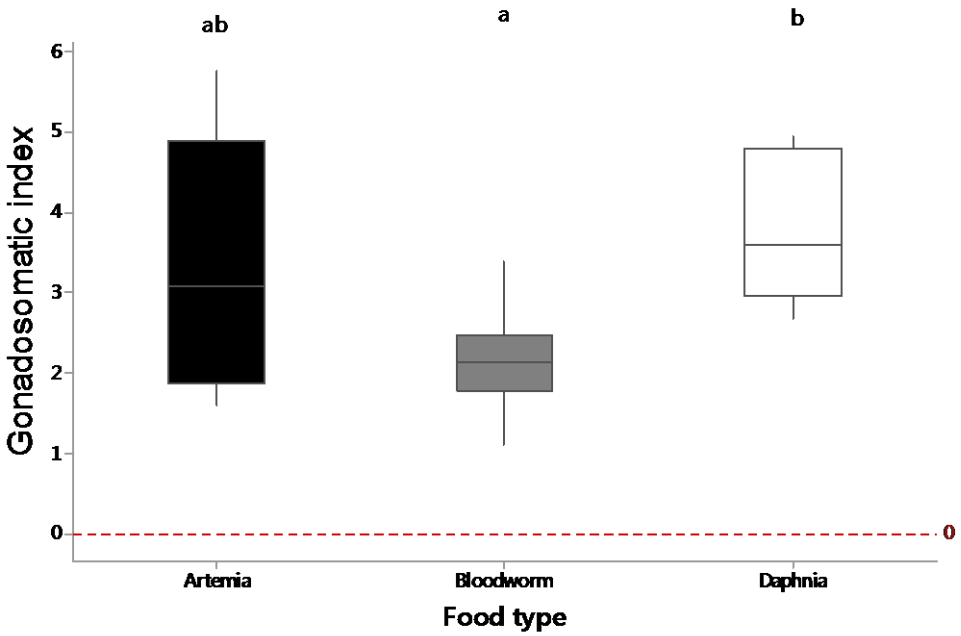


Figure 3.10 The effect of the three different types of food (*Artemia*, bloodworm and *Daphnia*) on reproductive development (measured as gonadosomatic index, GSI) of female sticklebacks in the experiment.

3.4 Discussion

3.4.1 Influence of nutrition on growth

In this study, controlled rations of three different natural diets – *Artemia*, bloodworms (*Chironomus* spp. larva) and *Daphnia* were fed to wild-caught three-spined sticklebacks, and parameters that indicate the growth, health and immune status of these fish were measured. The results showed that, overall, the sticklebacks fed with *Artemia* and bloodworms showed significantly high growth and had health indices than those fed with *Daphnia.* Therefore, the results suggest that the type of food can potentially affect growth in fish.

The findings show that the growth and specific growth rate among fish fed bloodworm and *Artemia* were significantly higher than fish fed with *Daphnia*. This indicates that *Daphnia* are not an optimal foodstuff for three-spined sticklebacks. The reduced weight gain associated with this diet was probably due to the low percent of protein in *Daphnia* compared with other diets (see Table 3.1). On the other hand, Bloodworms often form a major component of the diet of three-spined sticklebacks (Allen and Wootton, 1984), though bloodworms provide a major source of amino acids, are readily digestible (Armitage, 1995), are an

appropriately sized prey for these fish (Watz and Piccolo, 2011), and are of a size that is suitable to fish with smaller-sized mouths such as sticklebacks, and which prefer bloodworm due to their ability to discriminate colour (Popham, 1966).

The *Artemia* diet had similar effects on growth as bloodworm. This is perhaps not surprising ,research undertaken by Stejskal et al. (2018) has shown that *Artemia* sp. comprises an easy-to-digest, high-protein food suitable for fish larvae . *Artemia* contain high levels of fatty acids which are essential for the survival and growth of fish (Sargent et al., 1999, Hamre et al., 2002). Consistent with this. Onura et al. (2018) found that the final weight and specific growth rate of African catfish, *Clarias gariepinus*, was highest when fed a diet of *Artemia* nauplii combined with dry feed. Indeed, Garcia et al. (2008); reported that a diet consisting solely of enriched *Artemia* helped fish to grow properly and survive.

(Przybyla et al., 2014) found that the composition of food can a have considerable impact on the growth of fish.

According to the basic nutritional composition of the three different food types used in this experiment (3.1), the moisture content in Daphnia is higher than other dietary source.

Even though several researches conducted on species of marine fish have projected better growth, the results of this study, in the specified growth range, do not support the theory that a better growth can be achieved with more moisturized feed (Chatzifotis et al., 2005).

3.4.2 Influence of nutrition on health status

The body condition factor (BCF) and hepatosomatic index are important parameters in terms of their ability to provide indirect indications as to the energy and nutritional status in fish (Chellappa et al., 1995). These are fast and simple assessments that can signal the fish's physiological condition (Cui and Wootton, 1988). The liver is a useful organ to detect responses to nutritional and physiological status in fish (Storch and Juario, 1983), In this study, as illustrated in figure 3.6., the hepatosomatic index of fish increased over the course of the study. Interestingly, the body condition factor and hepatosomatic index were higher in fish fed with *Artemia* than those fed with *Daphnia* and bloodworm were.

This suggests that the *Artemia* diet provided a greater amount of energy than bloodworm and *Daphnia*; in addition, the palatability and digestibility of *Artemia* was high (Stejskal et al., 2018).

Both *Artemia* and bloodworm are rich in protein content, which is important for building liver mass (Chellappa et al., 1995). It is not clear why feeding with *Daphnia* did not increase the hepatosomatic index and body condition factor to similar levels as were obtained when feeding with *Artemia*, though this has also been reported to be the case for other species of fish. For example, a study of Atlantic halibut larvae which were fed either *Artemia* or zooplankton showed that good health and high survival rates were associated with provision of *Artemia* macronutrients (Hamre et al., 2002). Furthermore, Fontagne et al. (1998) found that feeding common carp (Cyprinus carpio) *Artemia* resulted in increased larger liver size.

On the other hand, the protein content of the diet might not affect the hepatosomatic index of fish. It has been reported that hepatosomatic index was higher in fish fed with a low protein diet than with a high protein diet (Webster et al., 1995). Also, (Jiang et al., 2016) showed that the hepatosomatic index of fish decreased as dietary protein levels increased.

These results suggested that three-spined sticklebacks could use *Artemia* and bloodworm to increase growth compared to *Daphnia.* In this study, the group fish fed *Artemia* and bloodworm showed the highest levels of growth. There are some components that were not measured in this study, but which could admittedly affect their growth, such as vitamins, minerals and fatty acids. Similar to all other vertebrates, fish are reliant on a number of amino acids to survive. To be specific, they need three long-chain polyunsaturated fatty acids (PUFAs); docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid are also vital to aid growth, progression and are required in the reproductive process (Sargent et al., 1999). Enhanced *Artemia* have been shown to contain significantly lower levels of n-3 HUFA and DHA, which might be a contributory factor to prompting progressive issues in the development of a number of species, including Atlantic halibut (Hamre et al., 2002) One possible explanation is that food palatability and digestibility can also influence food efficiency. The evidence in our study supports

previous results by Stejskal et al. (2018) *Artemia* to be highly digestible .Another possible explanation of increase growth might be that level of protein of diet affects growth. Proteins are the most important compounds in the diet in terms of encouraging rapid growth rates (McGoogan and Gatlin, 1999). The level of protein that can be obtained from *Daphnia* is 3%, but is much higher in *Artemia* and bloodworm (5%). The level of protein can have an impact on growth (see Table 3.1 - basic nutritional composition).

The Haematocrit index (relating to red blood cells) can indicate the physiological status (Kalbe and Kurtz, 2006). Haematological parameters is important indices for evaluating fish health (Schütt et al., 1997). In addition, the Splenosomatic Index (SSI) is a good indicator of the immunity status of the fish; an immune response is generally associated with an increased spleen size (Seppanen et al., 2009) Large spleens indicate a heightened activation or investment in the immune response (Ruane et al., 2000, Hadidi et al., 2008, Henrich et al., 2014)

In this study, food type influenced both SSI and haematocrit values, with spleen mass increasing in size with the *Artemia* and bloodworm diets. Immune responses in fish can be modulated by nutrients including antioxidant vitamins, carbohydrates, carotenoids, minerals, lipids and fish oil substitutes, proteins and their constituent amino acids and nucleotides (Trichet, 2010) The enhancement in immunity depends on the quality of the diet. Review studies have found that proteins and lipids can result in immune enhancement (Trichet, 2010). In this investigation, the SSI as an immunity parameter was found to be increased in fish fed *Artemia* and bloodworm, which were rich in protein compared to *Daphnia* (see table 3.1). It is possible that inadequate amounts of protein or amino acids may have a deleterious effect on fish health overall, including a reduction in the efficacy of the immune response (Li et al., 2007); thus, protein deficiencies may result in depressed immune function. This current study, in agreement with research by Pascual et al. (2004), found that the lower protein diet could be correlated with a negative impact on immune performance.

In accordance with the present results, research has indicated that fish fed protein enhanced the immunological activity, (Sitjà-Bobadilla et al., 2005). The quality of

Artemia as form of sustenance and, indeed, as a staple of aquaculture is attributed to the amount of polyunsaturated fat it contains (Millamena et al., 1988).

3.4. 4 The effect of food type of the host in the interaction between fish and parasite

The parasitic plerocercoid relies on the nutrients from the host fish's diet to grow; as a result, the host lacks the resources required for growth and development to become sexually mature. (Meakins and Walkey, 1975, Barber et al., 2008)

In addition, the present results showed that the food type and infection status have an effect on BCF. This could be the result of increased *S*. *solidus* plerocercoid size. A probable consequence of their growth in the body cavity is that the stomach's capacity to expand is reduced, physically limiting the amount that the host can eat at any given time (Cunningham et al., 1994).

On the other hand, there were no differences in spleen somatic index between infected and non-infected fish. Unexpectedly, the results of this study contrast with the assumption that infection would nominally lead to the stimulation of an immune reaction (John, 1994). One possible reason for there being no significant difference between infected and non-infected fish could be that the fish used in this experiment were caught from the wild, and so could have already been infected by another parasite; for example, infection by the eye fluke *Diplostomum spathaceum,* bacterial infection, or viral infection.

The findings presented here demonstrate the importance of the level of protein and level of lipids in increased growth, immune index and health status. This study has been conducted within numerous limitations that can be further explored to arrive at more comprehensive results. One major limitation was that the fish studied were from the wild, and either infected or non-infected fish, therefore, the size of the parasites was not known at the start of the study. There were differences in the number of infected and non-infected fish, and overall these limitations were necessary in order to develop my methodology. For an appropriate experimental study, it would be useful to use lab-bred fish and so that exposure can be investigated in controlled laboratory conditions. A second

limitation is the nutritional composition of the three different food types used. Futures studies might aim to control the protein content.

Chapter 4 How does host diet impact parasite development and host health in Schistocephalus infected sticklebacks?

Abstract

Dietary factors – including the quantity or quality of the food ingested – have considerable potential to influence the outcome of host-parasite interactions, through a range of mechanisms. For example, increased nutrition may lead to improved resistance to parasite infections if it improves host immune responses; however, it could also increase the supply of nutrients available to parasites, improving their growth and development. The aims of these experiments were to investigate the effects of the protein content of the host diet on the growth of *Schistocephalus solidus* plerocercoids in experimentally infected three-spined sticklebacks *Gasterosteus aculeatus*. Additionally, since it has been suggested that the quality and quantity of diet might improve host immune responses, the experiment aimed to study whether an increase in food intake could improve resistance to parasite infections. On the other hand, increased food intake by the host could make nutrients more available to parasites, benefiting parasite growth and development. Understanding the influence of host food intake on host-parasite interactions is important in the context of environmental changes that may impact food availability.

The aim of this study was to investigate the effect of dietary protein content and host ration on growth of a common and ecologically important fish parasite, plerocercoids of the diphyllobothriidean cestode *Schistocephalus solidus*. Lab-bred three-spined sticklebacks *Gasterosteus aculeatus* were either exposed to infective stages of *S. solidus* by feeding them copepods containing infective parasites, or were sham-exposed. Fish were dissected and important indices for fish health and infection status were quantified. The results indicate that the level of dietary protein had a significant effect on the performance of both infected and non-infected fish in the study, and suggest that the level of dietary protein plays an important role in determining the emergent phenotypes of infected fish. On the other hand, the results indicate that host ration played an important role in determining the value of the indices in the study, and suggest that the availability of food might have a significant effect on the performance of both infected and non-infected fish in parasitized populations. The change in the size of parasite over the experimental feeding was unaffected by host ration.

4.1 Introduction

Nutritional factors can play an important role in determining the outcome of a range of different types of parasitic infections. Dietary factors have the ability to effectively resist gastrointestinal nematodes, which attests to the importance of diet in terms anti-parasitism outcomes (Athanasiadou et al., 2008).

Dietary components play an important physiological role in determining the health and disease status of fish, with type of nutrition having a certain role in resistance to pathogens. The ability of fish to resist invasion by viral, bacterial, parasitic and fungal pathogens is influenced by macro- as well as micro-nutrients (Trichet, 2010). Both macronutrients and micronutrients can influence immunity; when they are lacking in the diet , animals can have increased susceptibility to infections (Calder and Kew, 2002). Hence, food quality can have a direct impact on the nutritional and health status of hosts, which is associated with a high quality diet, especially the level of protein, upon which the fish are reliant to combat the effect of parasites (Abbott et al., 1988, Brown et al., 1991). In the same context, the quantity and ration size of food can influence fish growth, and feeding efficiency.

Nutrition leads to affect the host status, by influencing rate of host growth, reproduction and development (Wootton, 1994). Thus, animal growth may be stunted and health risks are increased under inadequate nutrition, leading to reductions in both behavioural and health condition.

Increasing host ration might increase the capacity of parasitised fish to fight infection (Aeschlimann et al., 2000). Diet was demonstrated have profound influences on the establishment and survival rate of parasitic nematodes (Chandler, 1953, Geiman, 1958). It therefore seems likely that hosts will be susceptible to infection when food is inadequate in terms of quantity or quality; in general, the animal invest diets provide nourishment to sustain the growth and development of fish and that may occur as a consequence of parasite infection.

Parasites typically deprive the host of nutrition and therefore infections can have a significant impact on various biological functions, including growth and reproductive development. For example, the reproductive capacity of female

Pacific hake *Merluccius productus* is adversely affected by myxosporean parasites of the genus *Kudoa* that feed directly on the host's gonads (Adlerstein and Dorn, 1998). Additionally, parasites can exert influences on host feeding indirectly, through their effects on behaviour. For example, in the case of species of dace *Leuciscus leuciscus* infected by metacercariae of the trematode *Diplostomum spathaceum*, parasitism leads to diminished capability of food competition through impaired vision (Crowden and Broom, 1980). As (Coleman, 1993) notes, in sheepshead minnows *Cyprinodon variegatus*, encysted *Ascocotyle pachycystis* metacercariae in the bulbus arteriosus of the heart impair host motility , and hence thus affects the potential ability to capture prey . The development of the host also suffers as a result of parasitism, because parasites exploit the host's energy resources.Since parasites also deplete the host's energy resources, the host's development is disadvantaged as a consequence of parasitism.

The overall goal of providing new strategies support to hosts through dietary manipulation is to maintain or improve nutritional status, body condition and other aspects of host health. In the particular case of fish, the diets which they are fed must be tailored to their nutritional needs to ensure that they can withstand the effects of stressors and pathological agents and prevent and health developmental problems (Oliva-Teles, 2012).

Prevention of deficiencies and maintenance of high performance and health are dependent on a suitable nutritional status. Thus, poor nutritional intake can reduce developmental growth rates in animals and also increase the likelihood of health-related threats occurring. In addition, it can contribute to changes in behaviour and vital health functioning (Oliva-Teles, 2012). In the particular case of fish, the diets which they are fed must be tailored to their nutritional needs to ensure that they can withstand the effects of stressors and pathological agents and prevent and health developmental problems (Trichet, 2010). What is more, studies have shown that the pathological effects of parasitism can depend on the host's nutritional status (Hunter, 1953).

The innate immune system describes a series of key defensive mechanisms to protect the fish against pathogens (Trichet, 2010) and can be influenced by

dietary factors (Jeney et al., 1997). One study investigated the correlation between the non-specific immune reaction of gilthead seabream (*Sparus aurata*) and vitamin C, revealing that the higher the levels of vitamin C, the greater the non-specific immune response (Ortuno et al., 1999). Furthermore, in a study reported that high intake with soybean isoflavones can increase plasma lysosomes (Zhou et al., 2015). Wang et al. (2014) reported that the development of juvenile large yellow croaker (*Larmichthys crocea*), and possibly their resistance to *Cryptocaryon irritans* infections, was increased when their diet was supplemented with chromium polynicotinate (Cr-Nic). Diet can also mediate the negative impacts of infections on host behaviour. For example, the monogenean parasite *Gyrodactylus turnbulli* were found to adversely affect the fecundity and reproduction rate of guppies *(Poecilia reticulata),* changing their mating behaviour so that males with poor nutritional status were less capable of pursuing females than males with good nutritional status (Kolluru et al., 2006).

4.2 Experiment 1: the effect of dietary protein on host and plerocercoid growth in *Schistocephalus solidus* infected sticklebacks

4.2.1 Aims

The aim of the first study was to investigate the effects of dietary protein content on the growth, development and health indicators of three-spined sticklebacks, and to use experimental parasite challenges to study the effects of host diet on the growth of *Schistocephalus solidus* plerocercoids in experimentally infected fish. The quality of diets was manipulated by the level of protein (low and high) fed to lab-bred three-spined stickleback exposed to infective stages of *Schistocephalus solidus*. The following questions were addressed.

1- How does dietary protein affect growth of infected and non-infected fish?

2- What is the effect of dietary protein level on a range of health indicators (immune status, energetic status) and reproductive development of infected and non-infected fish?

3- What is the effect of host dietary protein on the growth and development of *S. solidus* plerocercoids?

4.2.2 Methods

4.2.2.1 Sources of experimental fish

Sexually mature male and female three-spined sticklebacks collected from Clatworthy Reservoir were used as parental stock to produce families via standard *in vitro* fertilisation techniques, previously described in Chapter 2 (Barber and Arnott, 2000) during the 2015 breeding season (May to August). Newly hatched fry were fed *ad libitum* with a succession of Liquifry No 1™ (Interpet, UK) for one week, then newly-hatched *Artemia* sp. nauplii and frozen bloodworm (*Chironomus* sp. larvae.) for five months.

4.2.2.2 Experimental infection of copepods and sticklebacks

Schistocephalus solidus plerocercoids were recovered from the body cavities of infected three-spined sticklebacks sampled from the River Soar at Abbey Park, Leicester 52°38'43.6"N 1°08'01.6"W). Worms were cultured using standard *in vitro* techniques as described in Chapter 2 (adapted from the original technique described by Smyth, 1954). A lab population of cyclopoid copepods (*Cyclops strenuus abyssorum)* were exposed to newly-hatched coracidia, and after 21 days idividuals were screened for infection status using a compound microscope under 100x magnification. Lab-bred three-spined sticklebacks (see above) were exposed individually to infective stages of *S. solidus* by being fed a single copepod containing an infective procercoids, or were sham exposed by feeding a non-infected copepod. Experimental fish were then transferred to the aquarium (25cm x 15cm x 30 cm) in which they were held for the duration of the study (see 4.2.2.4 below).

4.2.2.3 Diet Formulation

Two different artificial diets were formulated, containing low and high protein level. The composition of the basal diet is given in Table 4.1. Fishmeal and casein were used as the source of dietary protein, cod liver oil was used as the main lipid source, and sucrose was used as carbohydrate. All diets were supplemented with a vitamin mix and a mineral mix.

Table 4.1. Composition of the experimental diets. Table showing the two types of diet formulation details used in experiments. (i) High protein and (ii) low protein, with the seven ingredients with proportion which was used .

Ingredient g/100g	High protein	Low protein
Fishmeal	33.28	5.9
Casein	46.72	4.1
Sucrose	10	80
Cod liver oil	5	5
Gelatine	2	2
Vitamin mix	1.5	1.5
Mineral mix	1.5	1.5

Table 4.2 Proximate composition of experimental diets

Items	High protein	Low protein
Crude protein	60.06	17.78
Crude lipid	5.41	4.37
Crude ash	10.57	4.45
Dry matter	92.46	94.26

The experimental diets were prepared by thoroughly mixing the dry ingredients (fishmeal, casein, cod liver oil, vitamins and mineral mix). The feed was then mixed with water to achieve the correct consistency to allow it to be extruded through a syringe. Once mixed, the diet was extruded using a 1.2 mm diameter syringe and chopped into segments of about 2 mm to accommodate the size of the three-spined sticklebacks' month. It was then dried in the incubator at 37℃ for one hour and stored at 4℃ until further use. The fish were fed daily with a measured ration of 10% of fish body weight per day for a 16 week feeding period. Absolute ration was re-calculated using updated body mass data every 14 days.

4.2.2.4 Fish husbandry

A total of 96 lab bred sticklebacks were used in the study; 32 were assigned to each dietary treatment (low protein, high protein or bloodworms), and within each dietary treatment 16 fish were parasite-exposed and 16 were sham-exposed. Following transfer to the feeding aquaria, fish were acclimated to laboratory conditions and the experimental diets for 14 days before being exposed to the parasite. The experiment was organized such that each of the three dietary treatments was fed to replicate aquarium populations. Each aquarium (25cm x 15cm x 30 cm) contained 8 fish, which had either been exposed to the parasite (2 aquaria per diet treatment) or sham exposed (2 aquaria per diet treatment), giving a total of 12 experimental aquaria. These aquaria were held on a filtered, aerated recirculating aquarium system. Each aquarium contained a plastic plant for shelter. Water temperature was controlled at 10°C and a photoperiod of 14L: 10D was maintained for 16 weeks until the end of the experiment.

The fish were held under the experimental diets for 112 days, with absolute rations being recalculated every 14 days. The procedure for recalculating ration was as follows. All fish in the group of eight were weighed individually and then the sum of all individual weights was calculated. 10 % of the sum of the weight of fish in each aquarium was calculated, which gives the value of the amount of diet to be used to feed all fish in that group.

4.2.2.5 Data collection

At the end of the trial, all experimental fish were photographed in dorsal profile (Barber, 1997) , weighed (wet mass, M_{112} to 0.001g) and measured (standard length; L_S, to 0.1mm).the fish mass than was calculated as (M_f = total fish mass – parasite mass). The Specific Growth Rate (SGR) between the start and the end of the study was calculated as =((Ln(M_0)-Ln(M_{112}))/112)*100 , where M_0 is the wet mass of fish at the start of the study and M_{112} is the wet mass of the fish at the end of the 112 day study (excluding parasite mass) . Fish were then sacrificed by exposure to an overdose of Benzocaine anaesthetic, and blood samples were taken post-mortem by caudal severance, to measured haematocrit. The fish were then dissected to record spleen mass (M_S), liver mass (M_L), kidney mass (M_K, males only), gonad mass (M_G, females only), to allow a range of health and body condition indices to be calculated (see Chapter 2). Any *Schistocephalus solidus* plerocercoids recovered from infected fish were blotted dry and weighed to calculated parasite index and total parasite mass. Schistocephalus plerocercoids were then cultured to the adult stage using in vitro techniques adapted from (adapted from the original technique described by (Smyth, 1954)(see chapter 2 for details).

4.2.2.6 Statistical analysis

All statistical analysis were undertaken in Minitab 17 statistical software. Two-way ANOVA tests were used, to test the effects of diet treatment and infection status, and any interaction, on measured response variables. Non-normal data were transformed using appropriate transformation (e.g. Johnson) to normalise and to stabilise variance. Non-continuous data were analysed using suitable non-parametric statistics. Kruskal-Wallis tests were used to assess the fish SGR, and fish mass. For all boxplots in this chapter, the dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR), and the Whisker plots represent the greatest value and lowest value, while ● represents an outlying observation.

4.2.3 Results

4.2.3.1 Specific growth rate (SGR)

There was a significant effect of diet treatment on the specific growth rate (SGR) of fish, which was higher in fish fed the high protein and bloodworm diets, and lower in fish fed the low protein diet (ANOVA: $F_{2,51}$=37.45 P < 0.001; Figure 4.1.). There was no effect of infection status on SGR (ANOVA: $F_{1,51}$= 0.05 P = 0.825; Figure 4.1.) , but a marginally significant interaction existed between diet treatment and infection status (ANOVA: $F_{2,51}$= 3.29 P = 0.045; Figure 4.1.).

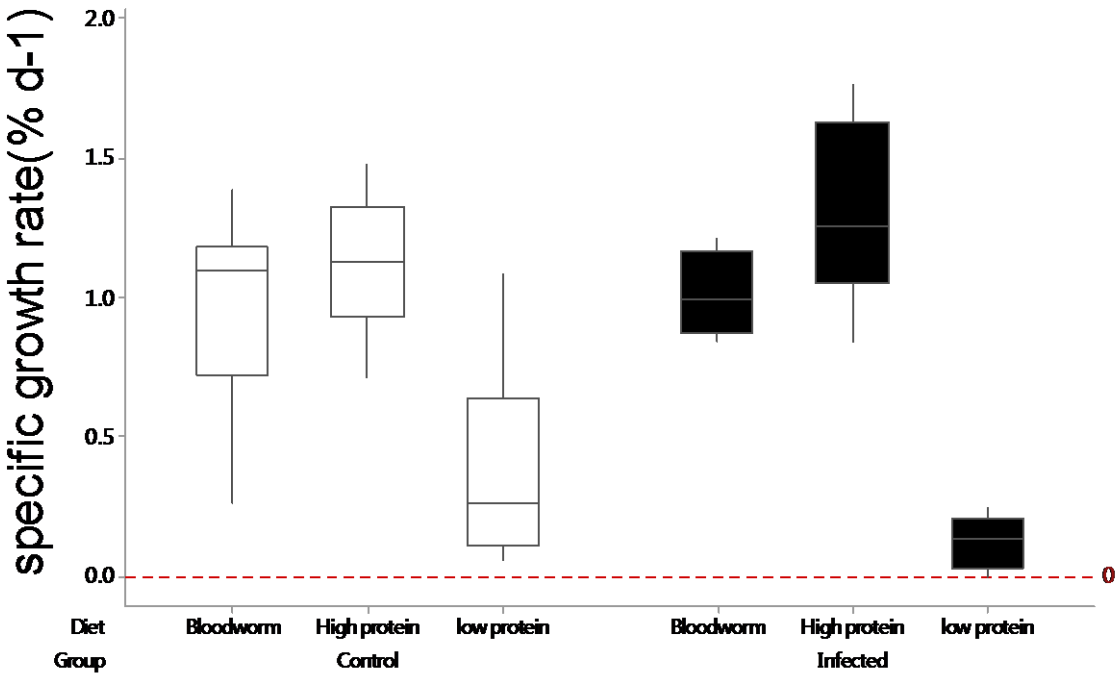


Figure 4.1 Specific growth rates of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.2 Fish mass

Diet treatment had a significant effect on fish mass (Kruskal-Wallis test, H^2 34.24 ,d.f. =2 , P < 0.001; Figure 4.2.) with the mass of fish fed, the high protein and

bloodworm diets exceeding that of fish fed the low protein diet. In addition, there was no significant effect of infection status (Kruskal-Wallis test, H^2 0.21, d.f. =1, *P* = 0.644)**.**

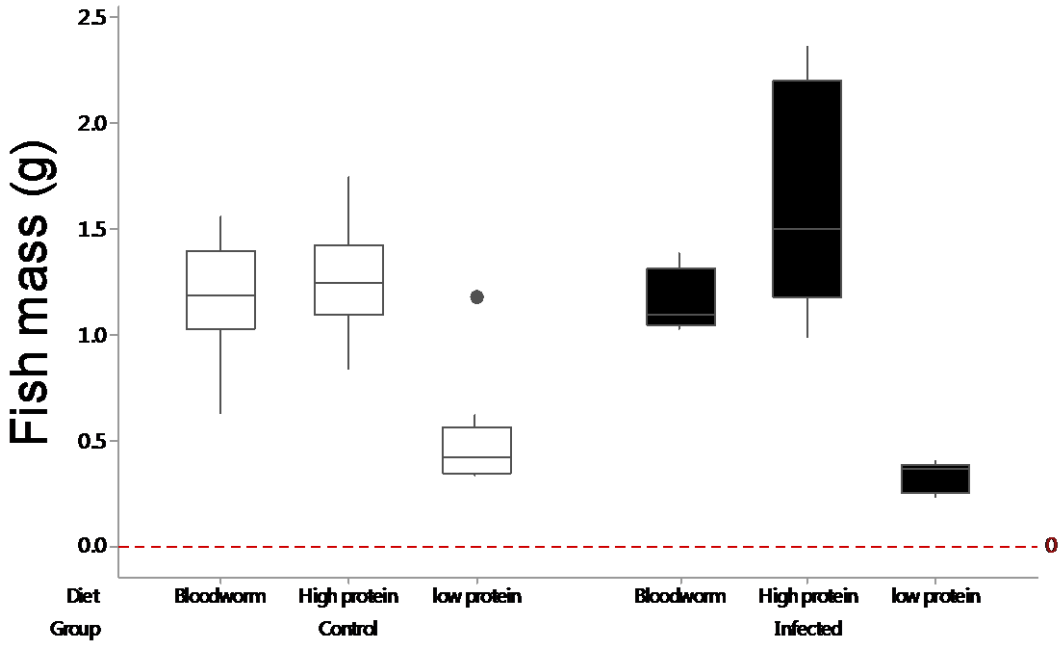


Figure 4.2 Fish mass of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.3 Body Condition Factor (BCF)

At the end of the 16 weeks of study, there was a highly significant effect of diet treatment on body condition factor (ANOVA: $F_{2,51}$ = 17.41 *P* <0.001; Figure 4.3). with fish fed the high protein diet exhibiting the highest BCF and fish fed low protein the lowest values .There was a significant effect of infection on BCF, with infected fish having lower BCF values under all diet treatments (ANOVA: $F_{1,51}$ = 9.50 *P* =0.003; Figure 4.3), and there was no significant interaction between diet treatment and infection (ANOVA: $F_{2,51}$ = 0.15 *P* = 0.861; Figure 4.3).

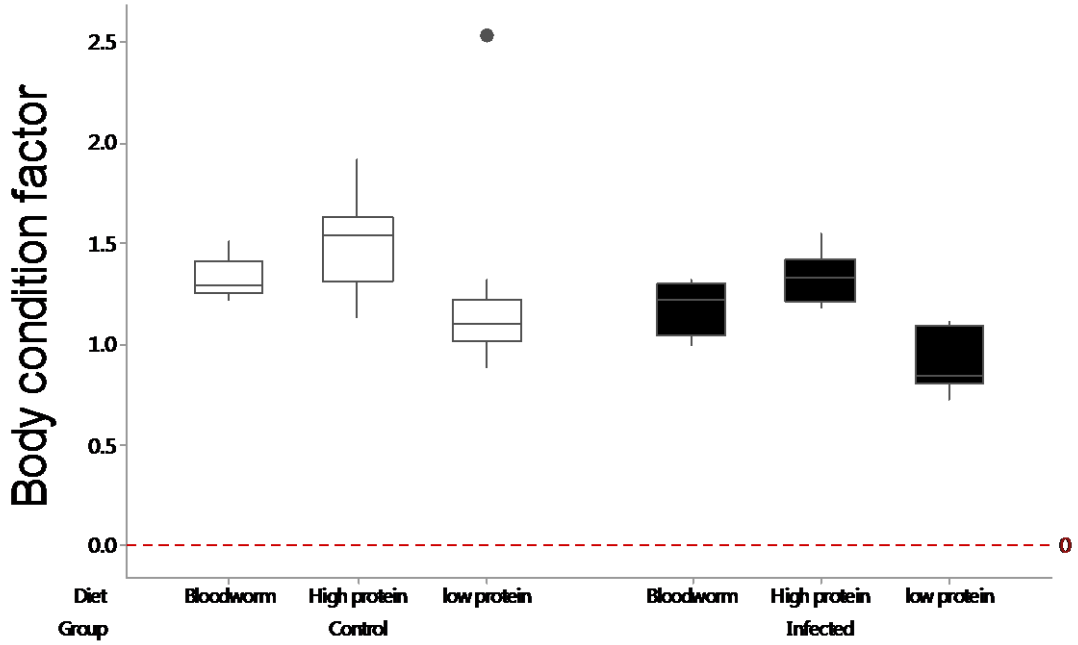


Figure 4.3 Body condition factor of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.4 Hepatosomatic index (HSI)

Diet had a significant effect on HSI (ANOVA: $F_{2,51} = 9.50$ $P< 0.001$; Figure 4.4), with the highest HSI values being recorded among fish fed the bloodworm diet, compared to fish fed high protein and low protein. There was no effect of infection group on HSI, (ANOVA: $F_{1,56} = 0.51$ $P =0.468$; Figure 4.4) and no significant interaction between diet and infection group (ANOVA: $F_{2,51}= 1.37$ $P =0.263$; Figure 4.4)

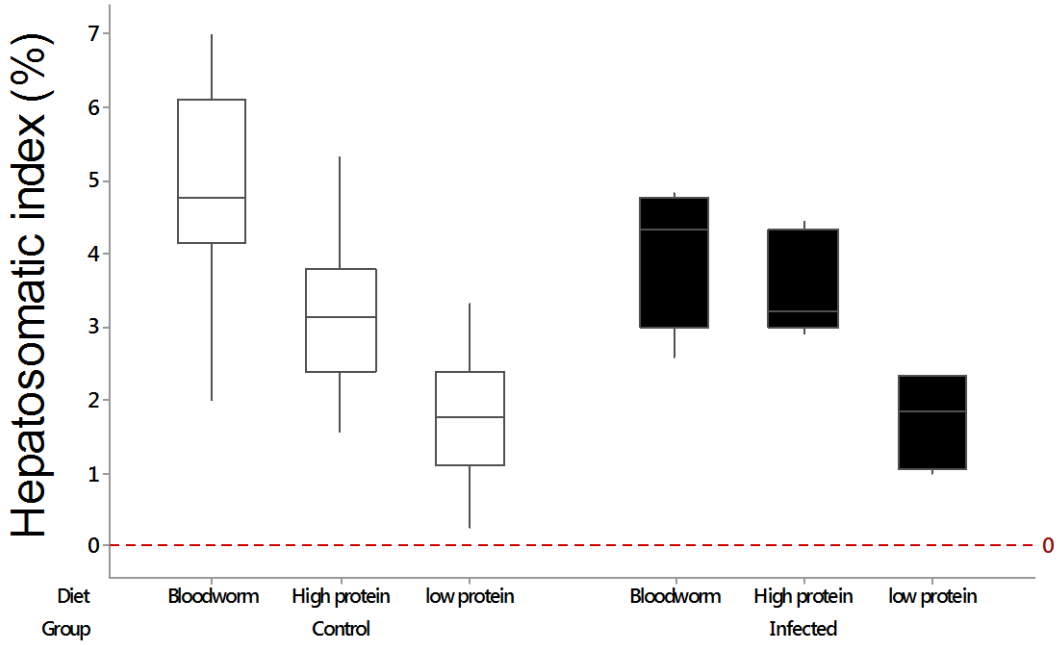


Figure 4.4 Hepatosomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.5 Splenosomatic Index (SSI)

Result from this study have shown that diet treatment had a significant effect on spleen mass (ANOVA: $F_{2,51} = 18.46$ $p < 0.001$; Figure 4.5), with fish fed the low protein diet having the highest SSI values. Infection group also had a significant effect on spleen mass, with the largest spleen mass being recorded among infected fish (ANOVA: $F_{1, 51} = 67.67$ p =<0.001; Figure 4.5) and there was no interaction between food type and infection status (ANOVA: $F_{1,51} = 0.46$ p =0.636.; figure 6. The maximum SSI values were observed among experimentally infected fish fed the low protein diet.

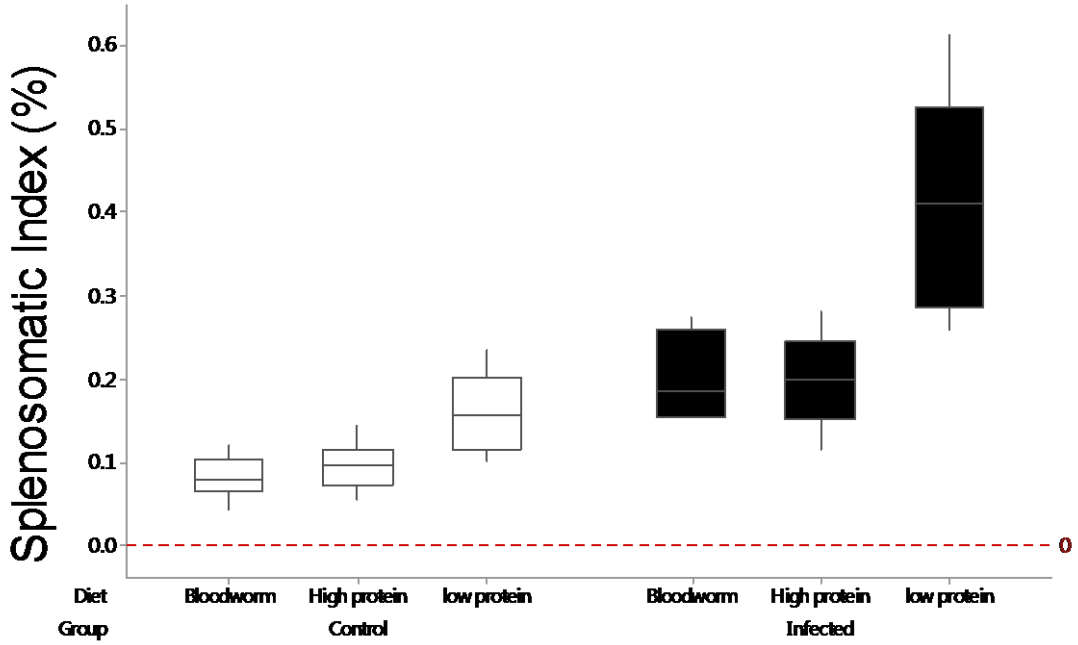


Figure 4.5 Splenosomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level) in protein content).

4.2.3.6 Haematocrit

Haematocrit was significantly affected by diet treatment (ANOVA: $F_{2,50}$= 3.97 P < 0.001; Figure 4.6), being highest among fish fed the bloodworm and high protein diets and lowest among low-protein diet fish. Haematocrit was not influenced by infection status (ANOVA: $F_{1,50}$ = 24.59 P = 0.052; Figure 4.6) there was no interaction between diet and infection group (ANOVA: $F_{1,50}$ = 2.30 P = 0.111; Figure 4.6).

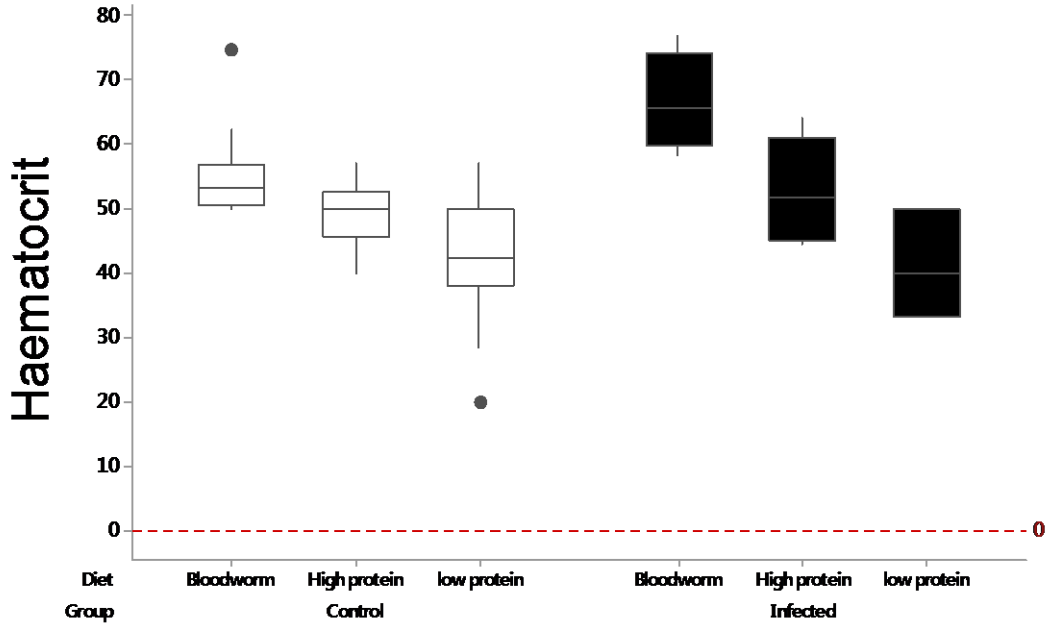


Figure 4.6 Haematocrie of control (sham-infected) and xperimentally *Schistocephalus solidus*-infected three spinedsticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.7 Males: Kidney somatic index (KSI)

The KSI of male fish was affected significantly by diet (ANOVA: $F_{2,23} = 10.23$, $P = 0.001$; Figure 4.7), with males fed the low protein diet exhibiting the lowest KSI values. It can be seen in figure that the value of KSI is higher than in fish fed high protein than fish fed low protein and bloodworm diet, even among infected fish.

There was a significant effect of infection group, with infected fish having the lowest KSI values (ANOVA: $F_{2, 23}=11.38$ $P =0.003$.; figure 4.7) there was no significant interaction between the factors (ANOVA: $F_{1,23} = 1.49$ $P =0.249$.; figure 4.7.

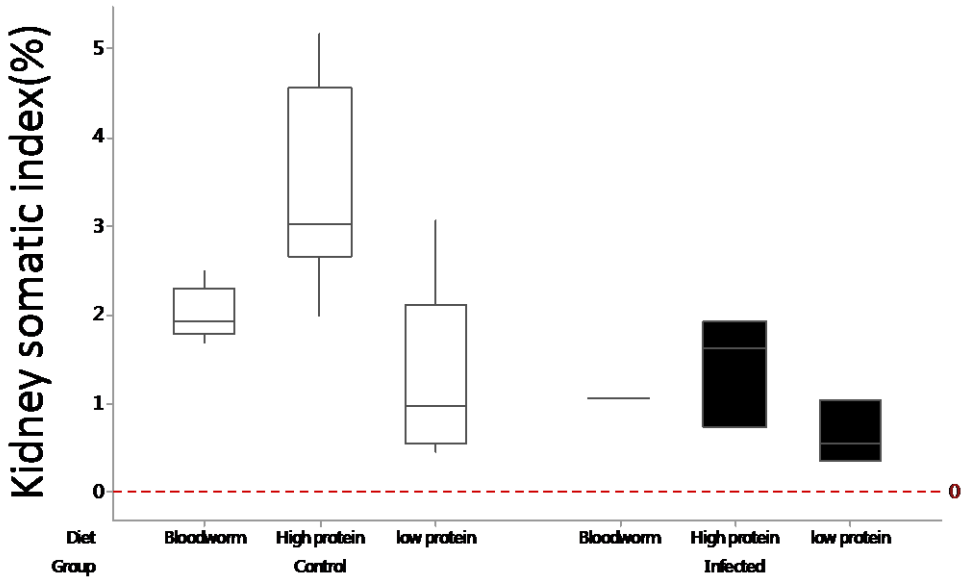


Figure 4.7 Kidney somaticindex of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.8 Females: Gonad somatic index (GSI)

There was a significant effect of diet treatment on the GSI of female fish in the study (ANOVA: $F_{2,25} = 12.59$ $P < 0.001$; Figure 4.8), with fish fed the bloodworm diet developing the highest GSI values and those fed the low-protein diet developing the smallest gonads. There was also a significant effect of infection group on gonad development (ANOVA: $F_{1,25} = 13.20$ $p = 0.001$; Figure 4.8), with infected fish developing small gonads. There was no significant interaction between the factors (ANOVA: $F_{1,25} = 0.44$ $P = 0.649$; Figure 4.8).

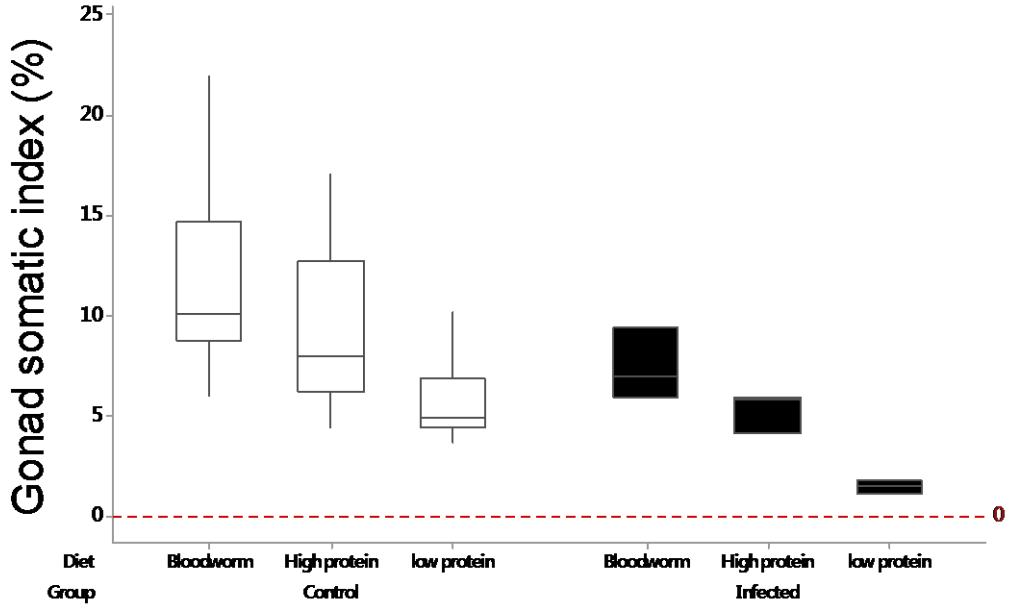


Figure 4.8 Gonad somatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.9 Total parasite mass

The mass of the plerocercoid recovered from experimentally infected fish at the termination of the study was strongly affected by the host diet treatment, with the heaviest parasite mass observed among fish fed the high protein and bloodworm diets, and the lowest values among fish fed the low protein diet (ANOVA: $F_{2, 13} = 19.57$, $P<0.001$). Post-hoc pairwise Tukey tests revealed that total parasite mass did not differ significantly between infected fish fed the high protein and bloodworm diets, but was significantly lower in fish fed the low protein diet ($p<0.05$).

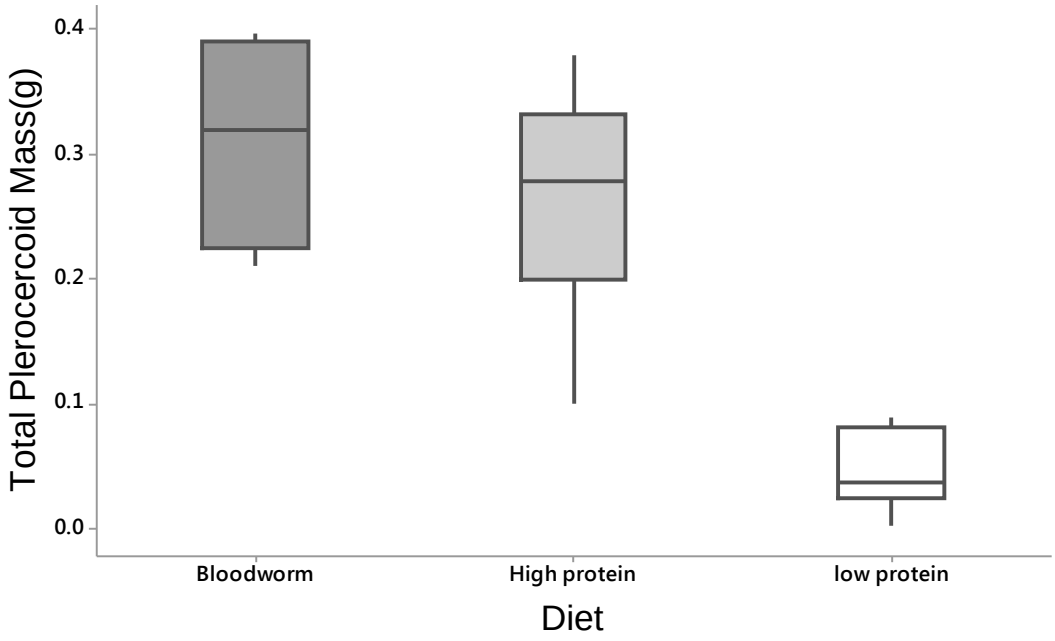


Figure 4.9 Total Plerocercoid mass of experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.3.10 Parasite index (I_p)

There was a significant effect of diet treatment on the I_p values of infected fish (1-way ANOVA: $F_{2,13} = 4.06$, $P = 0.043$). Post-hoc Tukey tests revealed no difference in the I_p values of bloodworm and high-protein diet host fish, but those fed on low protein diet had lower I_p values than those fed on the high-protein diet.

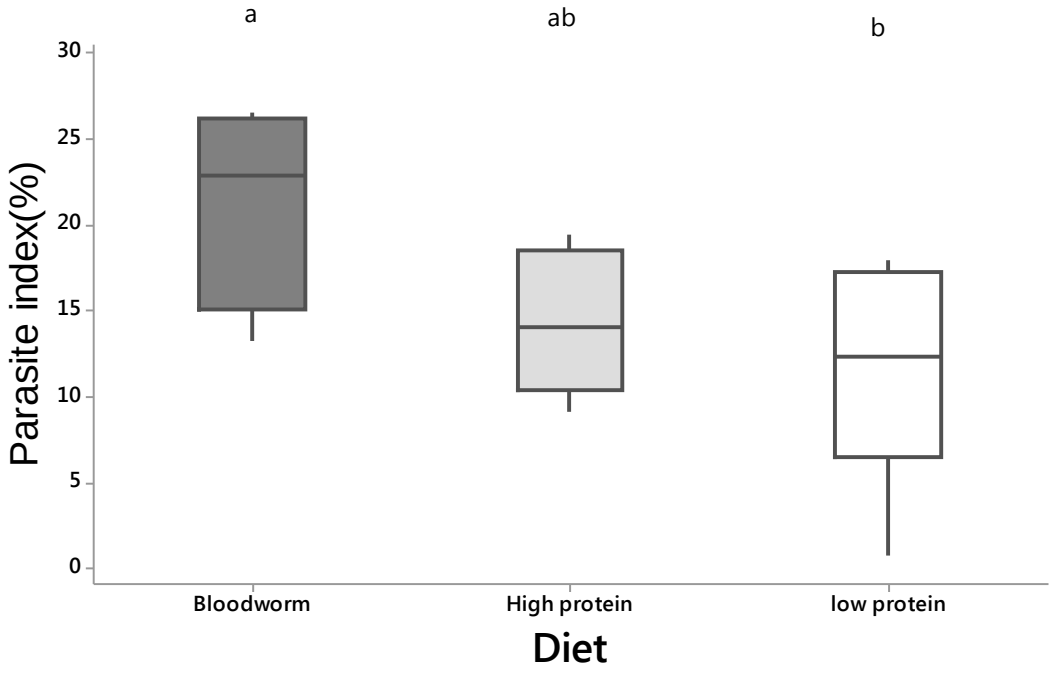


Figure 4.10 parasite index of experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed three type of diet (bloodworms, or an artificial diet that was either high or low in protein level).

4.2.4 Discussion

4.2.4.1 Influence of dietary protein on health Host growth

The results showed that the type of food had a significant effect on all aspects of host growth, sexual development and health status that were measured. Both the bloodworm diet and high protein diet were generated the highest fish growth, with a low-protein diet fish growing significantly more slowly.

The present study shows that the dietary protein level markedly affects the specific growth rate; the increase in growth fish fed with high level of protein obtained in the present study was consistent with studies in which examine the impact of various levels of protein(Webster et al., 1995, Abdel-Tawwab et al., 2010).

Some factors including temperature, salinity, dietary composition and the properties of protein have a role in determining protein needs between species

(NRC, 1983) As cited in Webster et al. (1995), dietary composition plays the major role in increasing growth in sunshine bass (*Morone chrysops X M. saxatilis*) (Webster et al., 1995). Casein and fishmeal were used as the sources of protein in this experiment.

The result of this study emphasise that not only higher protein intake improves the fish's growth, but also bloodworm diet had shown similar effect. These results endorse the opinion that feeding fish a diet that is rich in amino acids, such as fishmeal and casein, is important.(El-Sayed, 1989, Buentello et al., 2007) The constituent amino acids, digestibility and palatability all contribute to protein quality (Webster et al., 1995).

On the other hand, fish fed low level of protein showed low growth due the insufficient amount of protein in the diet with high contents of carbohydrate. In fact, as three-spined stickleback are carnivorous fish (Wootton, 1976), which lack some important enzymes involved in carbohydrates metabolism (Krogdahl et al., 2010). However, amylase activity was reported in carnivores but with low levels of enzymatic activity in comparing with omnivores (Hidalgo et al., 1999).

Regarding infection status, the results showed no significant effect on the growth of fish due to the presence of infection; there was no effect of infection status on specific growth rate. However, there was a difference between protein levels in the growth of *Schistocephalus solidus*, in which fish fed higher protein diets had large parasites compared with fish fed with low protein diets. Similar trends were observed for fish fed bloodworm. These results suggest that feeding high levels of protein resulted in increased parasite growth.

There are many studies that have investigated the negative impact of infection in fish (Barber and Arnott, 2000, Schultz et al., 2006, Seppälä et al., 2008, Giles, 1983). One anticipated finding was that infections will reduce natural growth rates (Goater et al., 2013, Yin et al., 2014). The nutrients from the food ingested by the host fish fuels the growth of parasitic plerocercoids, depriving the host of the essential resources it needs itself to grow (Barber et al., 2008). However, the results of this research indicated that the infected sticklebacks were able to withstand the pathophysiological consequences of infection with *Schistocephalus*

solidus thus the diet was sufficient to supply both hosts and parasites with sufficient energy to allow growth.

4.2.4.2 The effect of dietary protein on health of the host in *Schistocephalus solidus* infected sticklebacks

In this study, the indices of the host body's condition were addressed such as hepatosomatic index (HSI), body condition factor, and fish fitness. HSI is given as an indicator to determine the nutritional condition of fish (Cui and Wootton, 1988) and the liver is used to give an indication of medium energetic status of fish (Chellappa et al., 1995).

Fish under the low protein-feeding regime had significantly reduced HSI values, thus the liver mass was reduced by decreased levels of protein, likely because fish obtained energ y for their growth from the digestion of dietary protein and stored this (as glycogen) in the liver. These results indicated that high protein level was sufficiently to reserve energy in liver (Chellappa et al., 1995) this result stands in contrast with previous studies (Abdel-Tawwab et al., 2010, Gallagher, 1999, Webster et al., 1995) which found that the hepatosomatic index was higher for fish fed diet low protein than for fish fed diet high protein. Also, (Jiang et al., 2016) showed that the hepatosomatic index of fish decreased as dietary protein levels increased, diets containing high carbohydrate levels could be caused increasing liver glycogen than those fish fed low dietary protein(Ali and Jauncey, 2005).

There was no difference in the liver size between infected and non-infected fish, suggesting that the infection by plerocercoid *S. solidus* did not influence HSI. Although parasites can reduce the energy of host (Barber et al., 2008), the indication is that sticklebacks allocated the same proportion of resources to their energy reserves, regardless of parasitic infection status. This result conflicts with findings of Tierney et al. (1996) which showed that parasite exhibited curtailment of energy in three-spine stickleback.

One possibility is that the fish invest more protein in storing energy in their liver to overcome the effects of S. solidus infection, whereas in previous study, the fish invest the protein energy more into somatic growth than liver growth

The second index for examining energy status is the body condition factor, which describes the relationship between body mass and length of fish. This parameter is used to determine the long-term energetic status of the fish (Chellappa et al., 1995). The results of this study showed that there was a significant difference in Comparison of the body condition factor of control fish and infected fish shows that infected fish with S. solidus have lower BCF values than the non-infected fish, suggesting that the energetic status of infected fish is detrimentally affected.(Tierney et al., 1996, Bagamian et al., 2004). Moreover, the body condition factor was high in fish fed high levels of protein compared with fish fed low levels of protein. The low protein diet may negatively influence the mass and length of fish and result in poor body condition, as fish fed a low protein diet appeared to obtain insufficient energy from the food. These results are in agreement with the study of Yang et al. (2002) found that body condition factor was significantly higher in fish fed a high protein diet.

Haematological parameters have been recognised as valuable tools for the monitoring of fish health (Schütt et al., 1997) Haematocrit (packed cell volume) is a health indictor that indicates the red blood cell content of the blood. In the current study, low-protein diets generated low haematocrit levels in both infected and non-infected sticklebacks. Although there was reduction in the haematocrit levels in fish fed low protein, this was within reasonable limits. The normal packed cell volume measurements for teleost fish vary from 23-50% and are specific to the species (Moyle and Cech, 2004). Statistically, there was no significant difference in haematocrit levels between infected and non-infected fish. However, these results are in contrast to studies of three-spined sticklebacks (Arme and Owen, 1967) that showed the decline of packed cell volume in infected fish.

Regarding to Influence of nutrition on immune response splenosomatic index was measured to estimate the immune response in threes spined stickleback. The enlargement of spleen (splenomegaly) is the result of the infection of three-spined sticklebacks; this is generally an indication of immune response. (Seppanen et al., 2009) Sticklebacks infected with *S. solidus* show a marked increase in spleen size. It is a possible reflection of investment in immune function (John, 1994).

In this experiment, the size of the spleen was higher in infected fish under all diet treatments, in common with the results reported in previous studies (Macnab et al., 2009) Furthermore, the spleen size was also affected by diet, with fish fed the low protein diet having the largest spleens. Taking into consideration evidence found so far risks associated with poor nutrition are augmented which can lead to variations in behaviour and health In fact, infection and diet effects appeared to be additive, with the largest spleens being observed amongst experimentally infected fish fed low protein diets. In nature, infected fish typically are poor competitors and so are likely to have low protein diets, and thus, may be significantly affected

This result is consistent with studies carried out in other hosts, in sheep fed high-protein and low protein diets, where hyperplasia was detected in the macrophage-lymphocyte series of cells, which were reduced, especially in hosts fed with low-protein diets (Dobson and Bawden, 1974).

The greater enlargement of spleen size of fish fed low protein compared with fish fed high protein diets and bloodworm was not related to the difference in the establishment of plercercoids. In our study, parasite mass in host fish fed bloodworm and high-protein diet was higher than that for fish fed on a low protein diet, indicating the fish under lower protein conditions have increased spleen size. This led to the suggestion that fish are exposed to risk as well when fed insufficient diets such as those low in protein. Moreover, the depression of the splenosomatic index response in fish fed bloodworm and high protein diets were not important factors contributing to the lowered resistance of these animals to infection by *S. solidus.* According to our results, fish mass fed high protein and bloodworm diets gained mass and health status at a significantly higher rate than fish fed low protein diets (parasite mass was excluded from the total fish mass).

4.2.4.3 The effect of dietary protein in sexual development of the host in *Schistocephalus solidus* infected sticklebacks

The results of experimental studies illustrated that *Schistocephalus solidus* infection in female sticklebacks is associated with a reduction in gonad size: the highest values in these parameters were observed in the control group as compared with infected fish. As expected, these results are in agreement with

many studies in the literature (Heins and Baker, 2008, Schultz et al., 2006, Tierney et al., 1996) as a result of parasitic, the absolute amount of energy allocated to reproduction could be affected by infections.

All parasites recovered from fish held under high protein and bloodworm dietary regimes had an infective stage at the end of experiment of more than 50 mg. At this size, plerocercoids become consistently infective to definitive hosts (Tierney and Crompton, 1992) the stickleback-*Schistocephalus* system, the ovary constitutes a significant proportion of the entire body weight of the female during the oocyte maturation period . The ovaries will constitute approximately 2% of the entire body weight by early autumn (Wootton, 1976) . The results of the current experiment illustrate that gonad values observed more than two percent above the normal weight in their natural environment even among the fish fed low protein.

In this experiment, an elevated kidney somatic index was observed in non-infected fish , while kidney mass was lower in the group exposed to infection, as might be expected , This is in consistent with a recent study, which indicated that infected males showed a significant decrease in kidney somatic index compared to non-infected fish(Macnab et al., 2009). This observation may support the hypothesis that the impact of infection have influenced in reproductive development as result of energetic drain of growth of parasite.

Furthermore, the effect of diet on kidney somatic index, with fish fed high protein and bloodworm more likely to develop kidney mass than fish fed low protein, these results suggest that lower level of protein do delayed sexual maturation and development in male.

4.2.4.4 The effect of dietary protein in parasite growth

Dietary protein had a dramatic effect on the growth of *S. solidus* plerocercoids, under the dietary protein regime, parasites grew in proportion to the fish host. This phenomenon may arise so that high protein diets appear to provide sufficient nutrients to allow higher growth. Meanwhile, the proportion of parasite mass was higher, but the infection did not affect the indices of health in fish fed high protein

and bloodworm diets, or adverse consequences such as less weight and deterioration of immunity represented in spleen somatic index. The parasitic plerocercoid develops on the basis of the nutrients derived from the food uptake of the host fish, and therefore the latter loses the key resources required for growth and becoming sexually mature (Barber et al., 2008).

4.3 Experiment 2: How does host ration affect plerocercoid growth in three spined stickleback infected with *Schistocephalus solidus*

4.3.1 Aims

The aim of this study was to investigate the effects of host ration (i.e. the amount of food available) on the growth of *Schistocephalus solidus* plerocercoids in experimentally infected three-spined sticklebacks *Gasterosteus aculeatus*. Lab-bred sticklebacks were either exposed to infective stages of *S. solidus*, by feeding them copepods containing infective parasites, or were sham-exposed, by feeding a non-infected copepod. Experimental fish were subsequently fed either 6% or 3% body mass per day, respectively for a period of 12 weeks. At the termination of the study, fish were dissected and infection status and plerocercoid mass was quantified, along with a range of indices of fish growth, energetic status and health. The worms were cultured using techniques adapted from (Smyth, 1954)

4.3.2 Methods

4.3.2.1 Experimental fish

Eight families were generated during Spring 2015, using in standard *in vitro* fertilisation techniques (Barber and Arnott, 2000), using eight male and eight female sexually mature adult three-spined sticklebacks from laboratory stocks (Clatworthy Reservoir provenance) as parents. Newly-hatched fry were maintained in groups in 1 L tanks and fed *ad libitum* with a succession of Liquifry No 1™ (Interpet, UK) and live *Artemia* sp. nauplii, before being transferred to 30 L holding aquaria (40cm x 25cm x 30cm) held on a filtered, temperature-controlled recirculating water system. Fish were fed once daily *ad libitum* on frozen bloodworms, and at five months of age fish were transferred to the

aquarium (25cm x 15cm x 30 cm) in which they were held for the duration of the study

4.3.2.2 Experimental infection of copepods and fish *in vitro* culture of *S. solidus* plerocercoids

Multiply infected three-spined sticklebacks were selected from the stock infected fish from Clatworthy Reservoir, which were infected by *Schistocephalus solidus* plerocercoids. *Schistocephalus.s* were recovered from body cavity and cultured *in vitro* by using standard techniques (adapted from the original technique described by Smyth, 1954). Previously described in Chapter 4 section 4.2.2.2. (Barber and Svensson, 2003) Three-spined sticklebacks selected for the study were starved the day before exposure to promote feeding and exposed to infective stages of *S. solidus* by being fed a single infected copepod, presented in a 1-L plastic aquarium. Sixty sticklebacks were fed a copepod containing an infective *S. solidus* procercoids, and sixty sticklebacks were sham-exposed, being subjected to the same treatment but not fed an infected copepod. Exposure to parasite infective stages was carried out under a Home Office licence (Project licence: 70/8184, Personal Licence: I29280977).

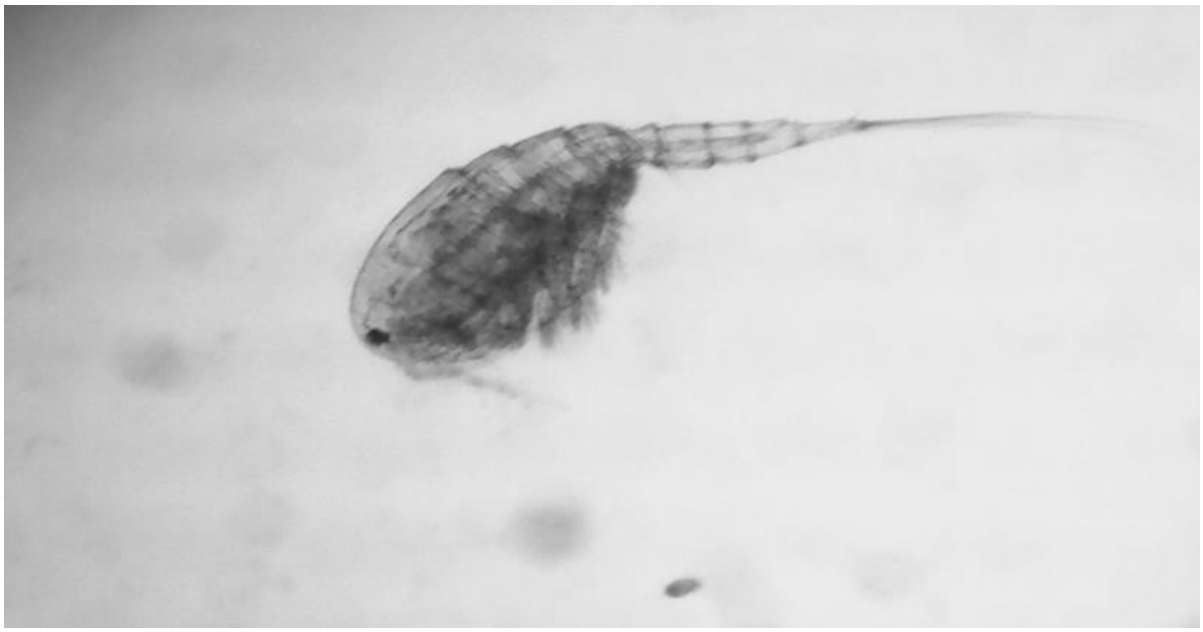


Figure 4.11 Copepod (*Cyclops strenuus abyssorum*) experimentally infected with *Schistocephalus solidus* procercoid.

4.3.2.3 Fish husbandry

After experimental exposure or sham exposure, the 120 fish were transferred to the feeding aquaria, which were held within a filtered recirculating aquarium system. Four replicate tanks were used for each diet treatment. Each aquarium (25cm x 15cm x 30 cm) contained a plastic plant and was stocked with 15 fish. Sixty fish (30 parasite-exposed and 30 sham-exposed) were assigned to each dietary treatment. Fish were acclimated to conditions in the aquarium facility for 1 week prior to the start of food ration manipulation. Fish were randomly assigned to either to medium ratio (3% body weight per day (bw.d-1) food ration and the other experiment at high ratio (6% bw.d-1 food ration). Water temperature was maintained at 14°C and controlled thermostatically, with a photoperiod of 12 hours of light: 12 hours of darkness (12L: 12D). During the trials, the weight, length and a digital dorsal profile photograph was taken of each fish at 14d intervals.

4.3.2.4 Diet Formulation

Experimental diets were formulated, containing high protein. The composition of the basal diet is given in Table 4.2. Fishmeal and casein were used as the source of dietary protein, cod liver oil was used as the main lipid source, and sucrose was used as carbohydrate. In addition, an essential vitamin mix, along with minerals and gelatin as a gelling agent.

Table 4.3 Formulation of the experimental diets, Table showing the diet formulation details used in experiments. With seven ingredients with proportion which was used.

Ingredient	% mass
Fishmeal	57.15
Casein	22.8
Sucrose	10
Cod liver oil	5
Gelatine	2
Vitamin mix	1.5
Mineral mix	1.5

The experimental diets were prepared by thoroughly mixing the dry ingredients (fishmeal, casein, cod liver oil, vitamins and mineral mix). See Chapter 4, methods section 4.2.2.3.)

The fish were fed daily with a measured ration of either 3% or 6% of fish body weight per day for a 12 week feeding period. Absolute ration was adjusted to take changes in body mass into account as the fish grew at two weeks

4.3.3.5 Determined the host ratio

The host ratio was estimated to determine the high and medium ratio; Lab-bred three-spined sticklebacks were used in this experiment, as derived from the Clatworthy population. Fish were selected from the stock tanks and to avoid any body size effect, and were of the same family generation, weight and length, when they had reached a mean length and weight of 41. 5 mm and 0.382 g, respectively. Twelve fish were placed individually in small 1 litre tanks (17 x 11 x 8 cm, length × width × height) and each fish was deprived of food for 24 h to increase appetite and reduce food residue in the gut (standardization of hunger). Fish were fed different of diets (3%, 4%, 5%, 6% and 7% wet mass) levels relative to fish body weight. The feeding period was continued for 10 minutes, with this protocol repeated a large number of times to confirm the optimal ration; after

these repeated experiments, 6% was chosen as the high ratio and 3% as the medium ratio.

4.3.2.6 Post mortem analysis

At the end of the trial, after 12 weeks, fish were sacrificed by immersion in an overdose of Benzocaine solution (benzocaine ethyl-amino benzoate, stock solution: 10g.L-1). Individual fish were blotted dry, and measured (standard length, SL, to 0.1mm) and weighed (mass, M, to 0.001 g). The fish mass than was calculated as (Mf = total fish mass – parasite mass). The Specific Growth Rate (SGR) between the start and the end of the study was calculated as $=((\text{Ln}(M_0)-\text{Ln}(M_{84}))/84)*100$, where M_0 is the wet mass of fish at the start of the study and M84 is the wet mass of the fish at the end of the 84 day study (excluding parasite mass). Under terminal anaesthesia, the caudal peduncle was severed using a sharp scalpel, and whole blood was collected from the caudal vein into heparinised capillary tubes. The end of the tube was sealed and the blood sample centrifuged for 60s (12,000 rpm) at room temperature to calculate the haematocrit value. 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 0·0001g). The carcass was then wrapped in a sterile piece of aluminium foil and frozen at -20C. The liver was saved in RNA later™. The body condition factor (K) was calculated with the equation: $K = (\text{fish mass [in g]} / \text{standard length [in cm]}^3) \times 100000$.

4.3.2.7 Parasite culture

Any *S. solidus* plerocercoids recovered from the body cavity of infected sticklebacks were removed with fine forceps and placed in small sterile pouches made of aluminium foil. They were, blotted with filter paper and then transferred to a Petri dish and immediately weighed (M_p). The parasite index (I_p) was calculated using formula $I_p = M_p / (M-M_p)$, where $(M-M_p)$ is the mass of fish excluding the parasite weight . Plerocercoids were cultured using *in vitro* techniques, previously described in Chapter 4 section 4.2.2.2. (Barber and Svensson, 2003).

4.3.2.8 Statistical analysis

All data Growth fish, body condition, spleen mass, fish mass factor were transformed by (Johnson transformation) then tested for normality and homogeneity of variance prior to using parametric statistical analysis. General Linear Models (GLMs) were then used to test effects of host ration and infection status and the interaction between host ration and infection status on output variables. Analysis of Covariance (ANCOVA) were used to examine the effect of host ration on the relationship between plerocercoid mass and egg output of adult parasites developing from these plerocercoids. One way ANOVA was used to test to compare the mass of plerocercoids, and the parasite index, of fish held under the 3% and 6%, For all boxplots in this chapter, the dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR), and the Whisker plots represent the greatest value and lowest value, while ● represents an outlying observation. All statistical analyses were carried out using Minitab (version 17).

4.3.3 Results

4.3.3.1 General observations

At the end of the experiment, the survival of three-spined sticklebacks in all the trials exceeded 85%. Over the 12 week period of the study, 13 fish died: 2 sham-exposed and 3 parasite-exposed fish from fish fed 3% treatment; 3 sham-exposed and 5 parasite-exposed fish from fish fed 6 % treatment, with 107 fish completing the study. Of the 60 sticklebacks that were exposed to infective stages of *S. solidus* by ingestion of a single infected copepod, 48 harboured plerocercoids at the end of the study. All fish surviving to 84d increased in mass and length, demonstrating that even the 3% ration treatment was sufficient to sustain fish growth and development.

4.3.3.2 Measurements of fish growth

4.3.3.2.1 Specific growth rate (SGR)

Following the calculation of SGR for each fish in the study. SGR in all treatment groups was positive, indicating all infected fish grew over the course of the study.

Infected fish achieved significantly SGR higher than non-infected fish (ANOVA: $F_{1,93} = 7.73$ $p = 0.007$; Figure 4.12), There was consistent effect of host ration on fish SGR, fish held under the 6% ration exhibited higher SGR than conspecifics fed the 3% ration, **(**ANOVA: $F_{1,93} = 4.32$, $P = 0.040$; Figure 4.12). There was no a significant interaction between food intake and infection group on SGR (ANOVA: $F_{1,93} = 2.69$, $P = 0.104$; Figure 4.12)

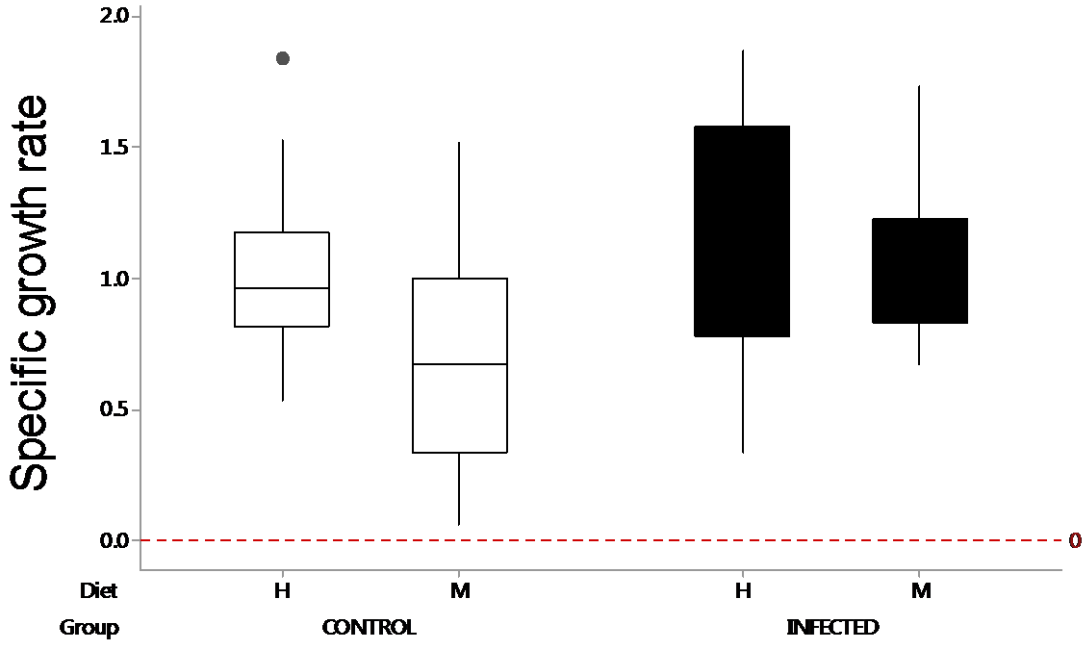


Figure 4.12 Boxplots showing Specific growth rate (SGR) achieved over the 98d for sham-exposed and experimentally-infected three-spined sticklebacks fed either 3% body weight per day or 6% body weight per day ration.

4.3.3.3 Indices of fish health and condition

4.3.3.3.1 Fish mass

Ration had only a marginally non-significant effects on fish mass (ANOVA: $F_{1,93} = 3.73$, $P = 0.054$; Figure 4.14). The results obtained from these experiments further showed that there were on effect of ration between treatments. However, there was a significant effect of infection status (ANOVA: $F_{1,93} = 7.14$, $P = 0.009$; Figure 4.14), with infected fish weighing more than non-infected at the end of the study. There was no interaction between ration and infection status on fish mass (ANOVA: $F_{1,93} = 3.09$, $P = 0.082$; Figure 4.13).

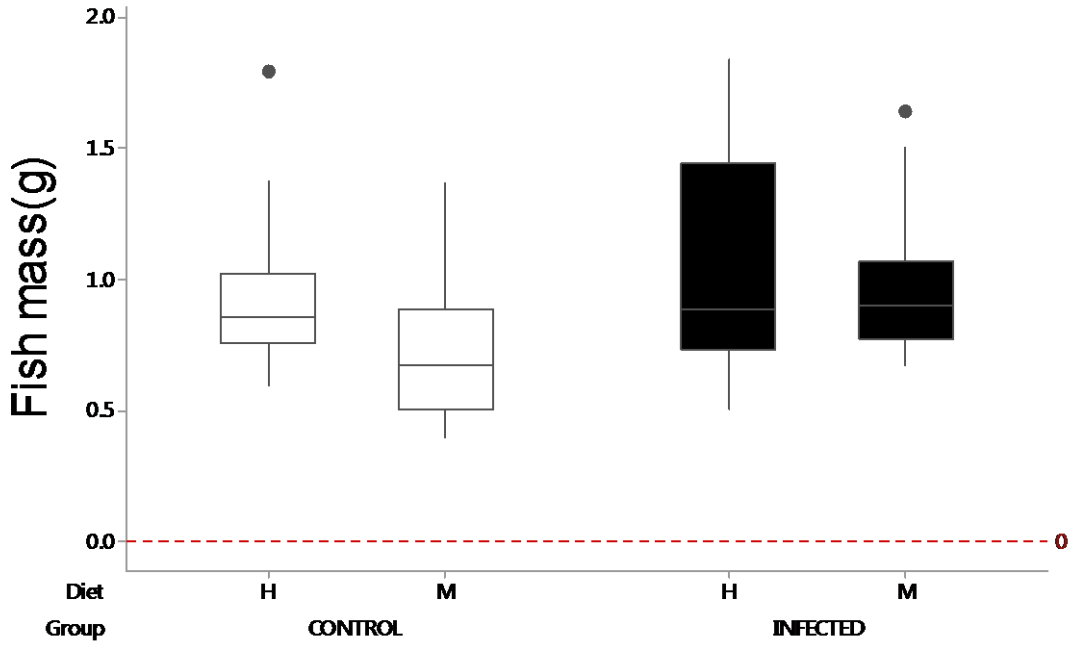


Figure 4.13 Fish mass of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day

4.3.3.3.2 Body condition factor (BCF)

Ration had no significant effect on fish body condition factor (ANOVA: $F_{1,93}$ = 0.14, P = 0.709; Figure 4.15). However, infection status had a significant effect on body condition factor, with infected fish having lower BCF values than non-infected fish (ANOVA: $F_{1,93}$ = 9.57, P = 0.003; Figure 4.15). There was no significant interaction between diet and infection status on BCF (ANOVA : $F_{1,93}$ = 1.22, P=0.272; Figure 4.14)

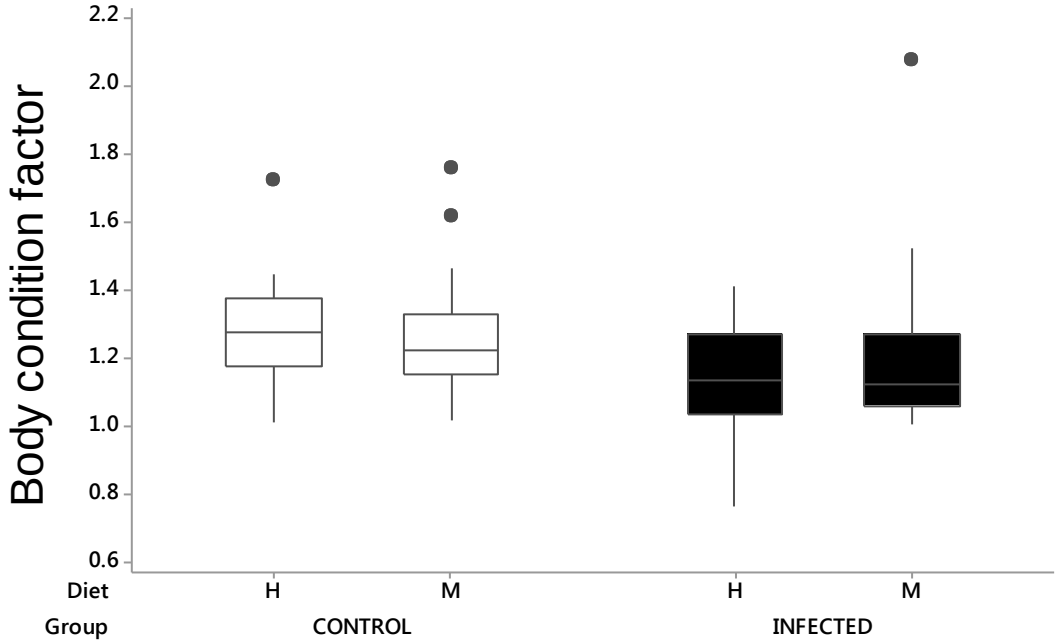


Figure 4.14 Body condition factor of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day

4.3.3.3.3 Hepatosomatic index (HSI)

There was no significant effect of host ration treatment on HSI (ANOVA: $F_{1,93}$ = 0.25, P = 0.617; Figure 4.16). Infection status had a significant effect on HSI (ANOVA: $F_{1,93}$ = 14.64 P < 0.001; Figure 4.16), with infected fish having smaller liver mass than non-infected fish. There was no significant interaction between ration and infection status (ANOVA: $F_{1,93}$ = 0.21, P = 0.651; Figure 4.15).

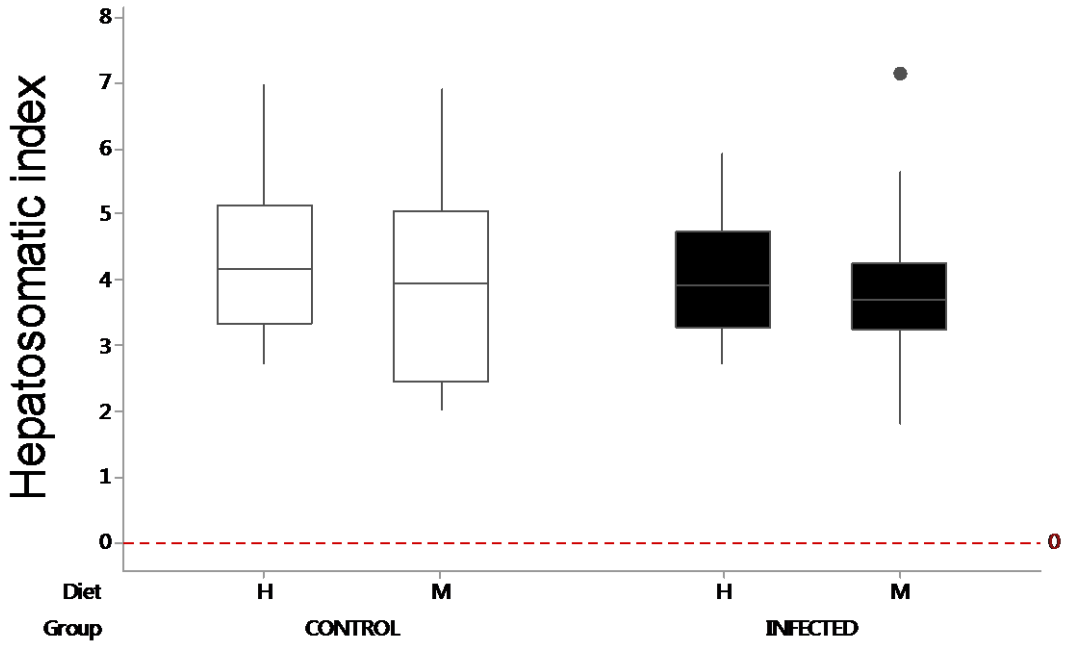


Figure 4.15 Hepatosomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.3.3.4 Haematocrit

Haematocrit was not affected by infected status (ANOVA: $F_{1,91} = 0.00$, P = 0.953; Figure 4.16), but the haematocrit values were strongly affected by ration (ANOVA: $F_{1,91} = 19.76$ P < 0.001 ;Figure 4.16), with fish held under the 3% ration having relatively higher haematocrit values than those held under the 6% ration. There was no significant interaction between ration and infection status (ANOVA: $F_{1,91} = 2.60$, P = 0.110; Figure 4.16).

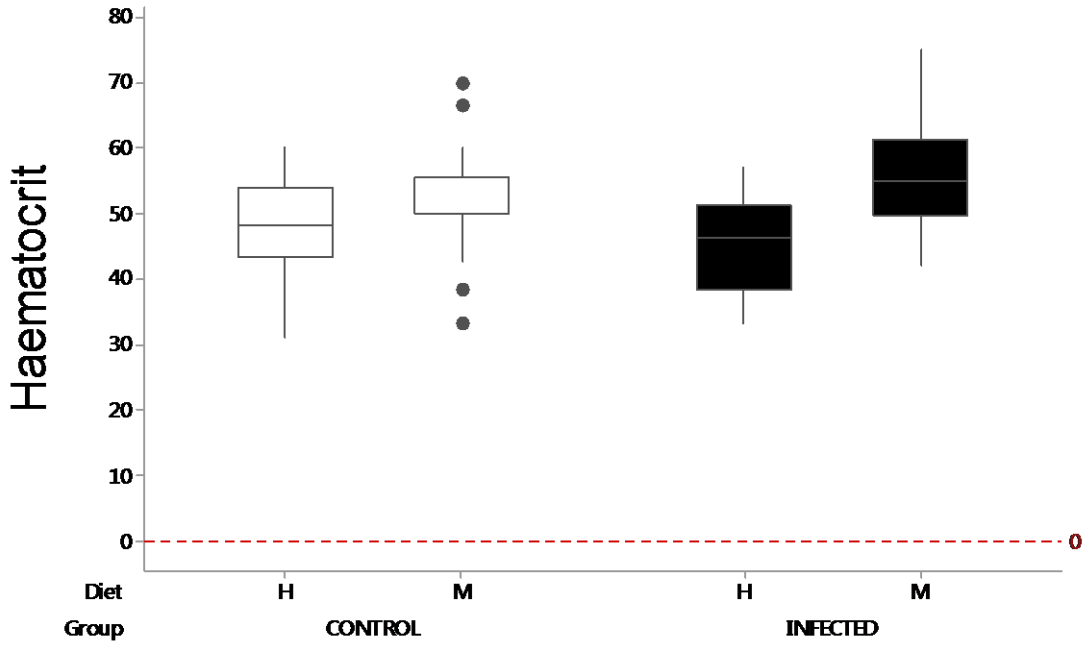


Figure 4.16 Haematocrit of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.3.3.5 Splenosomatic Index (SSI)

Infection status had a significant effect on SSI (ANOVA: $F_{1,93} = 58.18$, $P < 0.001$; Figure 4.17), with infected fish having larger SSI values than sham-exposed fish. Ration also had a significant effect on spleen mass, with the largest SSI values being recorded among fish fed the 3% ration (ANOVA: $F_{1,93} = 8.44$, $P < 0.001$; Figure 4.17).There was no significant interaction between ration and infection status (ANOVA: $F_{1,93} = 0.01$, $P = 0.927$; Figure 4.17).

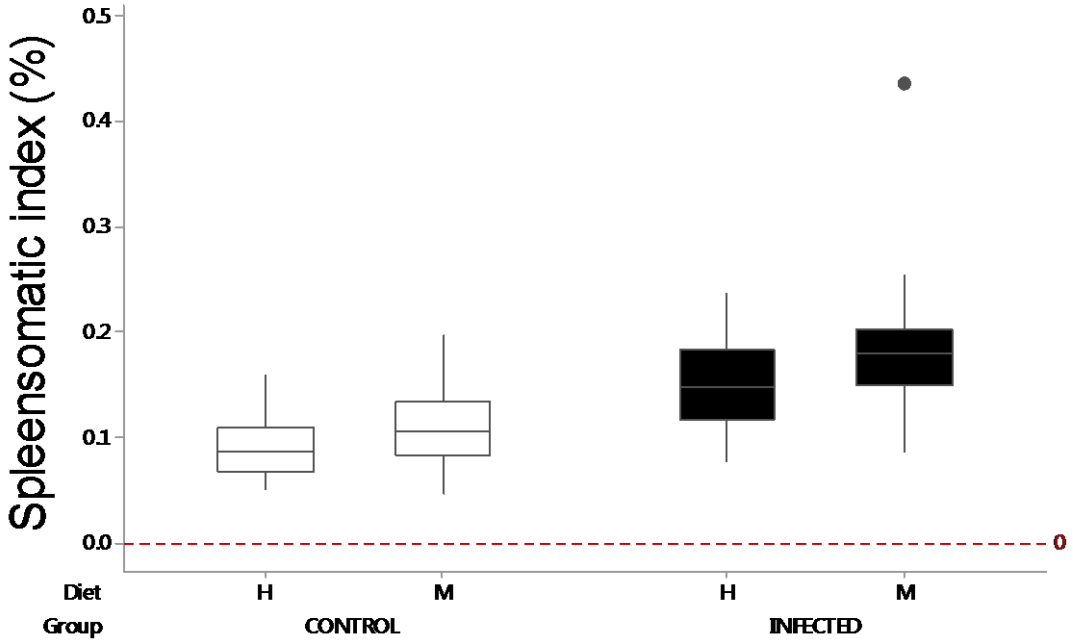


Figure 4.17 Spleensomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.3.4 Effect of diet and infection on host reproductive development

4.3.3.4.1 Females

There was a highly significant effect of infection status on gonad size in females (ANOVA: $F_{1,43}$ = 12.68, P = 0.001), with infected females having smaller gonad mass than non-infected females. There was also a marginally significant effect of ration on the gonad mass of females (ANOVA: $F_{1,43}$ = 4.36, P = 0.043), with those fed the higher ration developing smaller gonads. However, there was also a marginally significant infection status x diet interaction (ANOVA: $F_{1,43}$= 4.37, P = 0.042), which meant that the ration effect being seen exclusively among sham-exposed females; infected females developed small gonads irrespective of ration (Figure 4.18).

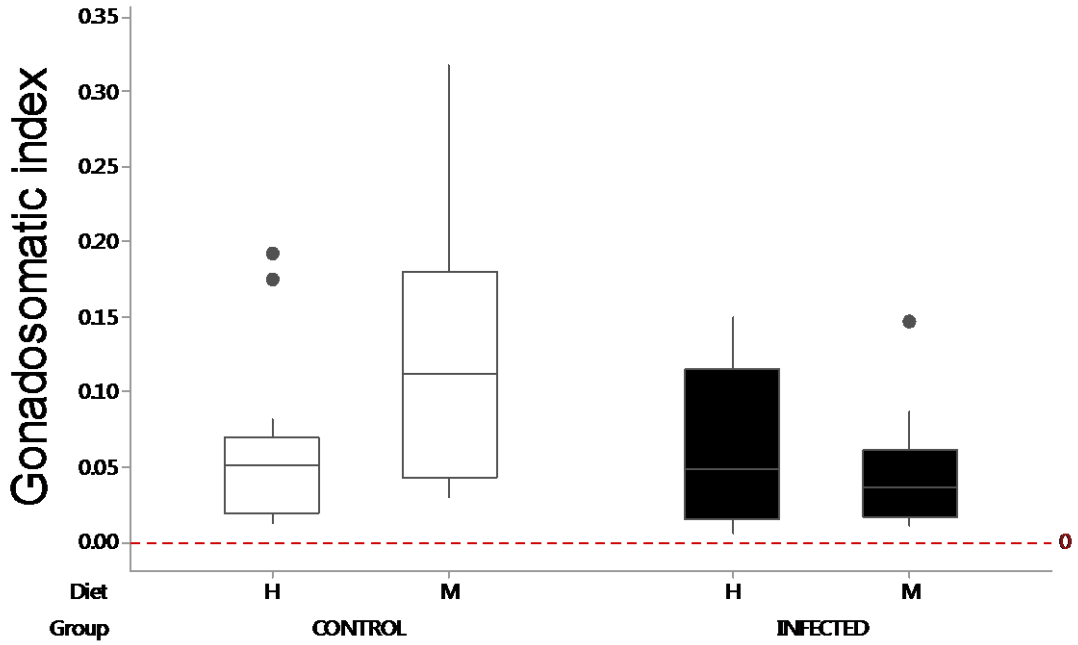


Figure 4.18 Gonadosomssatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.3.4.2 Males

The Kidney somatic index (KSI) of male fish was not affected consistently by ration (ANOVA: $F_{1, 41} = 0.16$ $P = 0.691$), or by infection status (ANOVA: $F_{1,41} = 1.07$ $P = 0.307$). However, there was a significant interaction between diet and infection status (ANOVA: $F_{1,41} = 6.32$ $p = 0.016$).

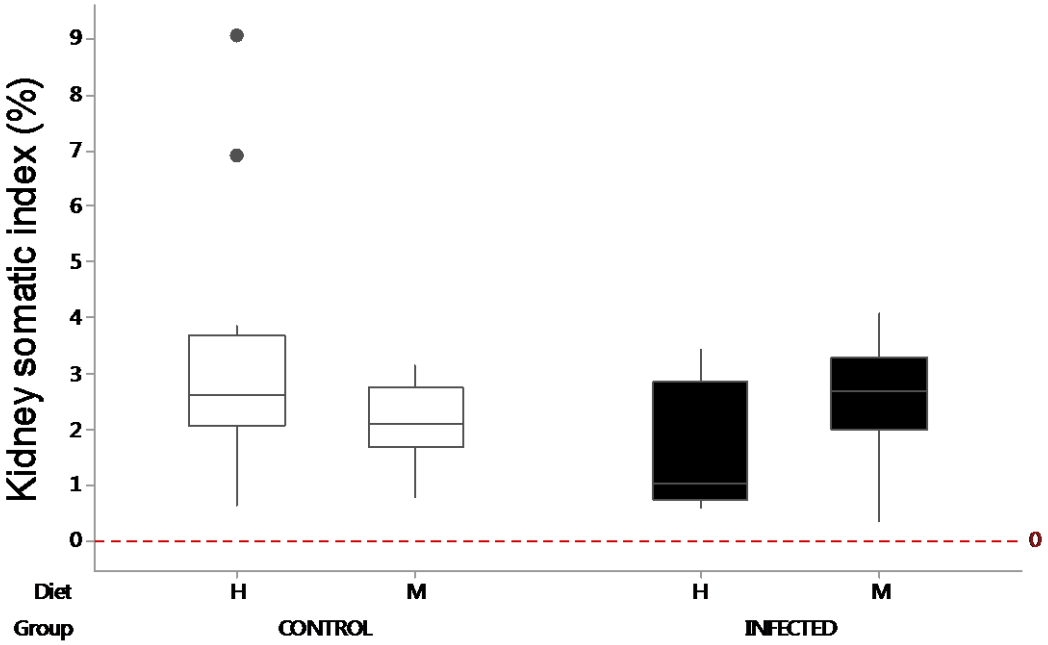


Figure 4.19 Kidney somatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.3.5 Effects of host diet on parasite fitness

4.3.3.5.1 Total parasite mass

The mass of plerocercoids removed from fish after dissection was calculated at the end of experiment. The mean total parasite mass among infected fish in the study was 0.1520g.

Total parasite mass did not differ between fish that were maintained under the high ration and the medium ration (1-way ANOVA: $F_{1,41} = 0.43$, $P = 0.514$).

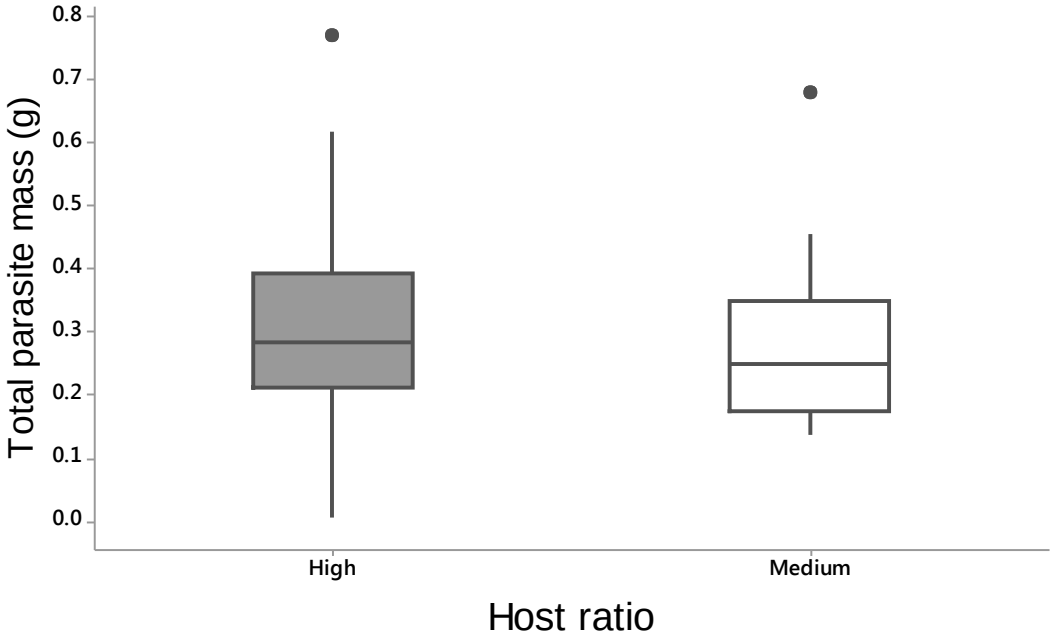


Figure 4.20 Bar graph showing the total mass of *Schistocephalus solidus* plerocercoids recovered from Clatworthy Reservoir three-spined sticklebacks at 84 d.

4.3.3.5.2 Parasite Index

The results for the mean mass of plerocercoid extracted from infected fish which survived to 84 d under the medium and high ration diets showed that there was no significant effect of ration treatment on the parasite index of infected fish which under the medium and high ration conditions (1-way ANOVA: $F_{1,41} = 0.01$, $P = 0.916$).

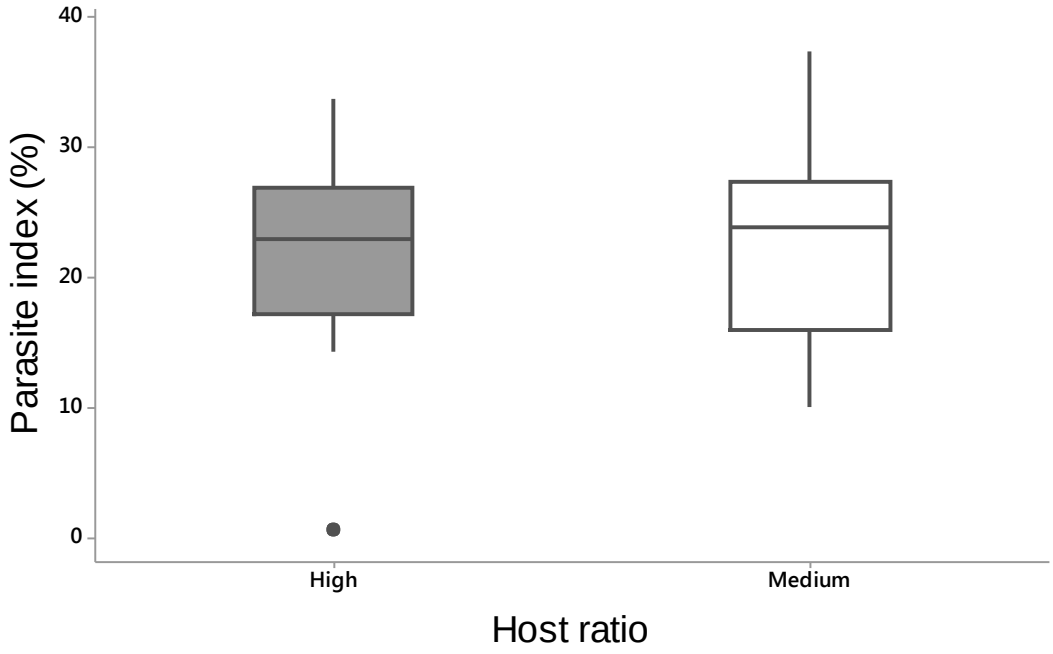


Figure 4. 21 Bar graph showing the parasite index (Ip) that established in three-spined sticklebacks fed the medium and high treatment.

4.3.3.5.3 Parasite fecundity

In total, plerocercoids from 23 fish fed the 3% ration, and 20 from fish fed the 6% ration, were transferred to culture tubes and generated adults that were then quantified for egg output. Host ration was found to have a significant effect on adult egg output, with adults emanating from plerocercoids recovered from infected fish fed the 6% ration producing more eggs across the range of parasite sizes examined than those recovered from fish fed the 3% ration (ANCOVA, with plerocercoid mass as covariate; slope: $F_{1,42} = 0.010$ $P = 0.749$; elevation: $F_{1,42} = 7.13$, P 0.011).

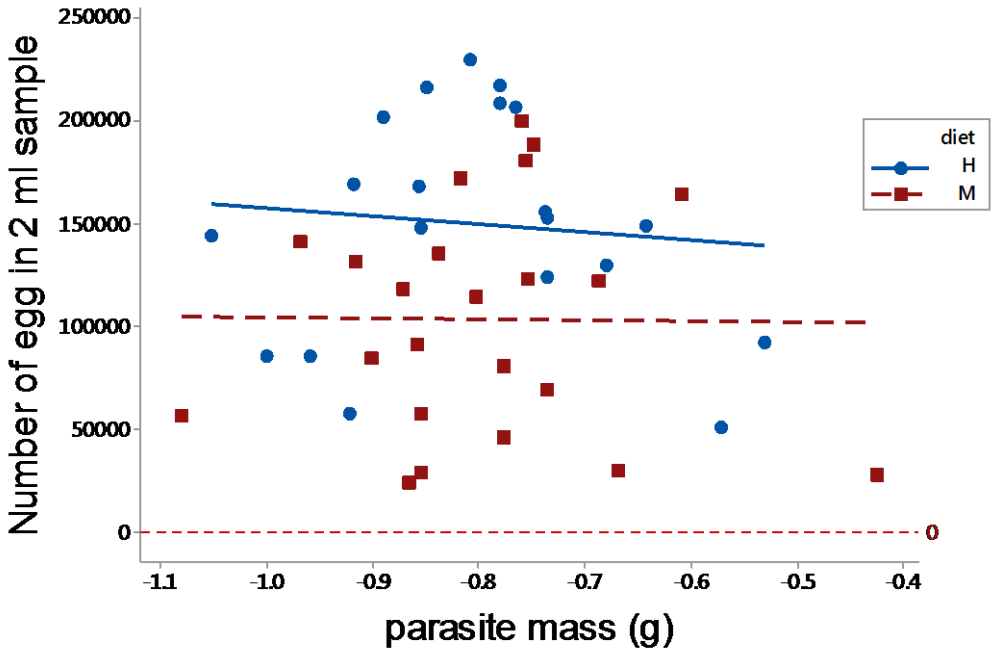


Figure 4. 22 Egg output from of experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either 3% or 6% body weight per day.

4.3.4 Discussion

4.3.4.1 The effect of host ration on host growth

The aim of this study was to assess the effect of host ration on the growth and development of three-spined sticklebacks, which were either experimentally-infected, or not, with *Schistocephalus solidus* plerocercoids. A secondary aim was to examine the impact of host ration on parasite growth and fitness. The results also indicate that the level of host alimentation can play an important role in determining the effects of *Schistocephalus solidus* infection on hosts. In the present study, infected fish exhibited elevated specific growth rates compared to non-infected fish, even when the parasite mass had been removed.

Importantly, it was also found that the fish held under medium ration achieved significantly higher spenosomatic index than their conspecifics fed the higher ratio. Despite the fact that host ration did not impact the size of plerceoirds that developed, the number of eggs of *S. solidus* released by adult worms developing

from plerocercoids recovered from fish fed on the 6% ration was higher than those fed at 3%, suggesting an additional impact of host nutrition on parasite fitness.

Specific growth rate is an important parameter related to the size of a fish and is indicated by percentage increase in a body dimension (e.g. mass, or length) per unit time (Hopkins, 1992, Björnsson and Steinarsson, 2002). In this research, the host ration was found to have an effect on the specific growth rate of the fish, with those in the high ration treatment growing faster than those in the medium ration treatment. Fish held under a high ration reached three times their initial weight, and the differences within the group were marginal, which suggests that the fish fed both high and medium rations were given sufficient energy to allow growth, and that both were able to sustain the high growth rates that give the fish the ability to maintain their reproductive potential. This finding is consistent with that of (Zeng et al., 2012), who found that the specific growth rate of small cyprinids *Tanichthys albonubes* generally increases significantly with ration.

Additionally, the infected fish achieved a significantly higher SGR than non-infected fish, which was probably because the parasites in question transitioning from the procercoid to the large plerocercoid worm in the body cavity of the fish (Barber et al., 2008);

Hence, it is probable that the susceptibility of fish towards *S. solidus* is not affected by the parasitic infection. These results indicate that increased food ration results in enhanced parasite growth, which is consistent with the findings of (Barber and Svensson, 2003) and (Barber, 2005). There are other reasons that the nutrition consumed by infected fish was greater than non-infected fish; first, to sustain metabolism, and second due to stress (Barber et al., 2008, Walkey and Meakins, 1970). The explanation given for this result was the fact that infected fish showed a greater increase in weight than non-infected fish.

4.3.4.2 The effect of ration on host health indices

Food intake and infection status were found to have implications for spleen somatic index, with infected fish fed and these fed the medium ration having the largest spleen, In teleosts, the spleen is a lymphoid organ that is associated with immunocompetence (Zapata et al., 2006, Press and Evensen, 1999). The spleen is subject to enlargement, which is referred to as splenomegaly, which is generally an indication of immune stimulation (Seppanen et al., 2009). In this study, the spleen was increased in size with *S. solidus* infection. These results are in agreement with those obtained by Kalbe and Kurtz (2006) which are associated with immune activation, where this strong evidence revealed the activity of the spleen after being challenged by a parasite.

However, this is inconsistent with the findings reported in (Valderrábano et al., 2002) who found that lambs (*Ovis aries*) with increased feeding rates displayed improved immunocompetence compared to those undergoing dietary restrictions. A recent study by Simmonds (2015) found that the spleen mass of three spined sticklebacks was same under both higher and lower rations feeding regimes. Sitjà-Bobadilla et al. (2003) examined the influence of feeding regime on the sea bream's *(Sparus aurata)* immune system and on parasitic infection, finding that reduced feeding regimes had significantly lower mortality, lower prevalence of infection and no adverse association with immunity.

In our study, fish held on a medium ration showed a larger spleen size than those on the high ration. It could be suggested from the current study that the optimum feeding ration for a immunostimulation of the spleen was below the high ratio ,previous studies found that fish try to adapt their digestion to obtain nutrients more effectively at levels below high satiation (Eroldoğan et al., 2004, Kim et al., 2007).

The other index used to investigate the effect of diet on host health status was the volume of red blood cells in the blood (packed cell volume), also known as haematocrit, which acts as an indicator of the oxygen-carrying capacity of the blood. Thus, it can serve as a basis for fish health evaluation (Houston, 1997).

In the present study, the results showed no apparent association between infection status and haematocrit value between treatments, packed cell volume did not differ between infected and non-infected fish, which results suggests that the host ration was sufficient to allow maturation of red blood cells in both infected and non-infected fish, and hence infection did not influence packed cell volume.

This is in contrast to the results presented in part 1 of this chapter, where high protein diets had an effect on haematocrit. Therefore, haematocrit is more likely to be correlated with other factors, including qualitative properties of diets while not with quantitative dietary properties.

This contrasts with previous findings showing that *S. solidus* infection resulted in a significant reduction in packed cell volume of the host in three-spined sticklebacks (Arme and Owen, 1967). Similarly, sticklebacks infected with the eye fluke *Diplostomum pseudospathaceum* have also been reported to show an increase in packed cell volume (Kalbe and Kurtz, 2006). By contrast, the results of a recent experimental study in three-spined sticklebacks showed that packed cell volume (PCV) was significantly higher in *S. solidus* infected fish than non-infected fish (Macnab, 2011).

The range of packed cell volume was above the ideal range of value for teleost fish from 23-50% (Moyle and Cech, 2004) With respect to ration, the highest packed cell volume haematocrit was found under the medium ration, suggesting that fish were able to adapt their health to the medium ration to a greater extent than on the high ration and assuming that higher haematocrit values signal higher levels of oxygen carrying capacity and hence are associated positively with fish health. This may be because for some teleosts and other fish, efficiently adapt their digestion and nutrient processing at restricted feeding rate (Van Ham et al., 2003). The present study indicated that the optimum feeding levels in three-spined sticklebacks for when inquiring into health was the medium ratio.

Body condition factor is used to estimate energy content per unit body weight (Chellappa et al., 1995) , in our results showed that body condition factor was similar in fish fed the high and medium rations. However, body condition factor decreased in infected fish in comparison with non-infected conspecifics. Since host-derived nutrition serves as the basis for survival and the propagation of

parasitic organisms, parasite infection is typically associated with host growth rate in an inversely proportional manner (Goater et al., 2013). In the present study, reduction in BCF in infected fish was because of parasite-driven energetic drain, so that infected fish generally have greater metabolic demands (Meakins and Walkey, 1975) . Our results are consistent with those of (Grimnes and Jakobsen, 1996), who found that non-infected Atlantic salmon (*Salmo salar*) were larger and had higher body condition factors than infected fish by salmon louse, Lepeophtheirus salmonis. Moreover, significantly lower body condition factors are often associated with *S. solidus* infections in natural populations of sticklebacks (Bagamian et al., 2004, Tierney et al., 1996). However, Simmonds (2015) showed that body condition factor did not differ between infected and non-infected fish fed a natural diet (Bloodworm) of 8% or 16% of body mass per day, which is likely to be sufficient to sustain BCF, whereas, in our study, fish were fed 3% on the medium ration treatment of the artificial diet. This might be because our study used a different type of food (the artificial diet), as well as the fish being from a different population to those of the Clatworthy population.

The hepatosomatic index serves to indicate the organism's metabolic condition and a predictor of energy reserves , Liver mass can be used to measure the short-term energy reserve (Wootton, 1977, Chellappa et al., 1995). In this study, an elevated HSI was observed in the non-infected (control group), while HSI was lower in the groups infected fish. As might be expected, similar findings were demonstrated by (Tierney, 1994) in wild fish, and by (Ritter et al., 2017) in lab offspring sticklebacks, who demonstrated that reduced liver size was a result of infection that could affect nutritional status. Our study also supports these results as non-parasitized fish had a higher hepatosomatic index than parasitized fish (Barber, 2005)

With respect to ration, fish fed medium and high ration diets did not differ significantly in terms of their HSI values, suggesting that both ration levels were sufficient to supply both groups with energy. This is inconsistent with the previous findings of Zeng et al. (2012), who found that the dry liver masses of female Cyprinid Minnow *Tanichthys . albonube* at first sexual maturity all significantly increased with increased feeding levels .

4.3.4.3 The effect of host ration on host sexual development

It is known that parasite infections are often associated with a lower rate of host reproduction across many taxa(Hurd, 2001). Infection with *S. solidus* was associated with changes in gonad mass development: our results show that *Schistocephalus solidus* infection observed in females resulted in a reduction in gonad mass when compared to non-infected fish. Previous studies have revealed that several parasitic infections can affect host reproduction. For example, (Carter et al., 2005) noted that *Ligula intestinalis* caused gonadotrophin disorders, and gonads remained small and blocked at the primary oocyte stage in female roach. Host nutritional and energy resources were significantly impaired in *S. solidus* sticklebacks in a natural population (Heins and Baker, 2008, Tierney, 1994) The parasitic plerocercoid uses the nutrients derived from the food intake of the host fish to grow. Consequently, the host is left without key nutrients that it itself requires for development and sexual maturation (Meakins and Walkey, 1975, Barber et al., 2008).

These results are also supported by other findings, with one study demonstrating that females with parasitic infections could be associated with a reduced probability of carrying fully-matured gametes . Furthermore, these females displayed comparatively reduced ovarian masses in relation to their uninfected counterparts, and were also found to have reduced somatic energy stores (Schultz et al., 2006).

In terms of the effect of diet on sexual development, one unanticipated finding was that female fish fed the medium ration developed significantly larger gonads than fish fed the high ration. One possible explanation for this is that the three-spined stickleback has the capacity to assimilate enough energy from food for gonad growth when supplied with a medium ration ,and fish fed a high ration put more energy into growth and delay reproductive development, However, in other species, it is important to recognise that other studies have suggested that female *Gambusia affinis* subjected to high, medium, and low rations produced a gonad-somatic index (GSI) time sexual maturity and first-time spawning (Zhu et al., 2015).

The results presented in this study clearly indicate that nutritional status and infection did not appear to have any effect on kidney somatic index, as both infected and non-infected fish developed relatively large kidneys. Infection with the plerocercoid *S. solidus* can influence sexual development in males (Macnab et al., 2009). Not only is the development of sexual organs inhibited, but most infected fish are also incapable of reproduction-related behaviour like nest construction, territory protection, mate selection, and spawning (Arme and Owen, 1967, Tierney et al., 1996)

In respect to parasite-derived nutrients required by the host for growth and survival (Hurd, 2001), despite the effect of infection in the reduction of host reproduction, our results did not indicate any differences between infected and non-infected fish in terms of kidney somatic index. These results suggest that both ratios provided sufficient food to act as a complete source of energy for both hosts and parasites. Another possible explanation is that the study period of this experiment was during acceptable circumstances and environmental conditions, such as with regard to the quality of water and availability of food, For instance, conditions of eutrophication triggered by humans increase the likelihood of low-quality and *S. solidus*-infected male sticklebacks engaging in nest construction, territory protection, and selection as mates by females. (Candolin et al., 2007, Heuschele and Candolin, 2010)

The level of nutrition did not show any significant effect on parasite mass and parasite index; suggesting that both feeding rations during the experiment were sufficient to sustain parasite growth.

However, worms recovered from infected fish fed the high ration were found to have produced more eggs than those on the medium ration. These results showed parasites from fish under the high ration can engage in energetically costly egg output, These results are in contrast with previous studies (Valderrábano et al., 2002) in which no significant differences were found in egg output amongst those recovered from infected fish fed the lower and higher rations were observed.

4.5 Conclusion and future work

In conclusion, increasing the level of protein in the diet elevated all aspect of host growth, sexual development and health status in fish. In addition, the mass of the plerocercoids recovered from experimentally-infected fish was affected by the protein content of the host diet, with the heaviest parasite mass observed amongst fish fed the high protein diets. However, the parasite mass and parasite index were not affected by ration. Future studies should focus on the effect of the combination of ration and level of protein

It is important to recognise that these results indicate that the high protein and bloodworm diets provide adequate nutrition for hosts and their parasites. These results indicate that further research is crucial to determine the appropriate nutritional level of protein diets and the consequences for the health of fish in long-term feeding studies.

Chapter 5 Does parasite infection affect the prey preferences of three-spined sticklebacks?

5.1 Introduction

The term ‘parasitism’ refers to a non-reciprocal symbiotic relationship between two different species in which one species (the host) is taken advantage of by another (the parasite). Parasites constitute a group of multifarious organisms that have the potential to infect and considerably affect the biological aspects of individuals, populations, and even the ecosystem (Hurd, 2001, Hudson et al., 2006). Parasites typically have a negative impact on aspects of host biology, including behaviour (Maitland, 1994), growth (Karvonen and Seppälä, 2008, Brinker and Hamers, 2007), reproduction (Sitjà-Bobadilla, 2009, Hurd, 2001) and ultimately resulting in a reduction in survival (Lafferty and Kuris, 2009).

Since host-derived nutrition serves as the basis for the survival and propagation of parasitic organisms, consensus has been established around the point that parasite infection is associated with normal host growth rate in an inversely proportional way(Goater et al., 2013) and therefore the successful establishment of a parasite within a host influences on the host’s energy levels, growth rate, and sexual maturity (Schultz et al., 2006) and so have considerable possibility to affect aspects of diet and foraging behaviour.

5.1.1 Impacts of parasite infections on quantitative aspects of host food intake

Infected individuals may need more energy because they need to support a large, growing parasite or parasite population, which makes energetic demands on them, a host may be fed an increased food intake leading to an increase in immune response. Following infection, it might be expected that patterns of food consumption will alter. Changes in feeding behaviour may therefore be caused by infection (González-Tokman et al., 2011) As researchers such as Huffman (2003) have shown, in order to achieve nutrient balance, consumption may be increased by parasitism, Exposure to parasites may also alter host energetics. Moret and Schmid-Hempel (2000) explained that immune responses require greater energy expenditure, therefore expansion of food consumption following infection may be a potential offsetting shift. Empirical research suggests that the amount of time guppies (*Poecilia reticulata*) spend foraging may also be

determined by infection with the monogenean parasite Gyrodactylus turnbulli.Those males with greater food intake spent less time foraging than males with less food intake, while uninfected males engaged in less foraging than infected males (Kolluru et al., 2009)

5.1.2 Impacts of parasite infections on qualitative aspects of host food intake

Dietary qualitative factors potentially have a considerable impact on the outcome of host-parasite interactions, through a variety of mechanisms including changes in the total quantity of food ingested. Parasite infections might also affect the types of foods ingested by their hosts. For example, in an unusual feeding behaviour, to avoid parasitic intestinal infections, wild chimpanzees and baboons have been observed to consume plant species with both antimalarial and antileishmanial properties (Krief et al., 2006) or to select for other aspects of food. For example, there is some evidence that primates ingest plants with spiny leaves to help physically remove parasites.

As observed by Shurkin (2014) anti-parasitic behaviours are common to a number of species, the majority of which (i.e. elephants, sparrows,), when affected thereby, ingest compounds to prevent or counteract infection. Such behaviours can be identified clearly, as the animals in question will stop to consume when they recovery.

The change might be driven by the parasite rather than the host, if the parasite can change the diet of the host to increase the benefit received by the parasite from the ingested nutrients. For example, parasites might change host behaviour to stop hosts from ingesting foods with anti-parasitic properties, or they might increase the host's preference for foods that contain nutrients that benefit parasite growth.

Furthermore, there is research evidence that animals may display an innate need to balance and/or obtain nutrients that counteract pathogenic infection which results in high selectivity in their choice of diet (Adamo et al., 2010, Povey et al., 2009) Thus, the greater need for energy may result in increased foraging as part of the infected individuals' behaviour (Baldauf et al., 2007) Given that immune

system capacity is strengthened through protein-rich foodstuffs, such items may be sourced by infected individuals, thus resulting in consumption changes and nutritional alterations due to infection. A study by (Lee et al., 2006a) showed that infected caterpillars chose diets that were significantly more protein-biased.

5.1.3 Energetics and host food intake in the stickleback-Schistocephalus host-parasite system

Barber (2013)identified the three-spined stickleback as an attractive model, being well suited to the diversity of parasitological modelling. Thus, as demonstrated in the works of Barber and Nettleship (2010) and Östlund-Nilsson (2006), they often function as a model for investigation in biological research and parasitology. Plerocercoids of the species *Schistocephalus solidus* (Cestoda: Pseudophyllidea) are frequent parasites of the three-spined stickleback (Wootton, 1976, Barber, 2007a). The three-spined stickleback serves as a second host for *Schistocephalus solidus* (Cestoda: Diphyllobothriidea); a parasite with natural presence in aquatic environments (Kennedy, 1974, Wootton, 1976, Wootton, 1984, Barber, 2007b) Occupying three host organisms (cyclopoid copepods, three-spined sticklebacks, and piscivorous birds) throughout their life cycle, *Schistocephalus solidus* reaches sexual maturity in its definitive hosts (birds), as confirmed by Bråten (1966) and Barber and Scharsack (2010).

Infection by *S.Solidus* can impact host growth, behaviour and reproductive development (Macnab et al., 2009). It is generally expected that infections will diminish natural growth rates; however, (Barber, 2005) indicated that fast growing fish tend to host fast-developing parasites. This suggests that increased food intake benefits parasites as well as fish. therefore assume that infection should increase the rate of food intake, but also note that the parasite constrains stomach size and limits the amount of food that can be eaten in one meal (Wright et al., 2006). It is apparent that different behaviour is displayed by sticklebacks as part of their changed foraging conduct when infected with Schistocephalus (Milinski, 1984, Ranta, 1995).

As mentioned in the introductory chapter, recent studies have shown that three-spined sticklebacks are mainly carnivorous fish and eat a wide range of small

animals including crustaceans, the larvae of *chironomids*, and fish eggs; they even consume other stickleback eggs (Wootton, 1976).

5.1.4 Aims and objectives of the study

This chapter describes an experimental study investigating the effect of qualitative differences in prey choice associated with *S. solidus* infection on food preference type. Presumably, the infection could affect the dietary preferences or selectiveness of fish; therefore, the study aims to understand whether this parasite infection changes the dietary preferences of host fish in terms of the quality of food they select. This study was the first step towards understanding whether preferences for particular types of food are altered in parasitised sticklebacksThe main objective of this study was to evaluate the effect of infection by plerocercoids of the parasite *Schistocephalus solidus* on the prey choices of host three-spined sticklebacks. This was investigated by undertaking replicated experimental host diet preference tests, carried out before and after exposure (or sham exposure) to infective S. solidus parasites. By comparing the changes in food preferences among experimentally infected and control (sham exposed) fish, the experiment tested the hypothesis that experimental infection with *S. solidus* was associated with concomitant changes in host prey preferences, which might indicate either host- or parasite-induced behavioural change.

5.2 Methods

5.2.1 Fish supply and husbandry

Three-spined sticklebacks were bred from the Clatworthy population, in the laboratory, during May-June 2016. As outlined in chapter 2, a total of 40 juvenile three–spined sticklebacks from five different families were bred using standard IVF techniques (Barber and Arnott, 2000).Fry were fed *ad libitum* with a succession of Liquifry No 1™ (Interpet, UK) for the first 5-7 days, After an initial three-week period of family rearing and feeding on *Artemia* nauplii 2 days old, these fish were transferred to the aquarium facility into three different 55L aquaria, where fish were allowed to grow and fed bloodworms (*Chironomus* sp. larvae) ad libitum on a daily basis. To avoid any body size effect, when they had reached a mean length and weight of 42.5 mm and 0.653 g, respectively, only

adult males and females with intermediate size ranges were selected, then fish transferred to a rearing tank, where they were maintained in two groups; those fish that were to be exposed to the parasite and control (sham exposed) fish.

5.2.2 Holding aquarium fish

Experimental fish were housed individually in one of ten separate compartments (10 x 16 x 9 cm) within four glass aquaria (32 x 16 x 25 cm). Each compartment contained a plastic plant and was supplied with compressed air via an air stone. Water temperature was maintained at 14°C throughout the study and a photoperiod of 12L: 12D was implemented. Fish were acclimatized to the experimental conditions for two weeks, during which were fed daily on a mixture on frozen *Artemia*, frozen bloodworms and artificial diet, to satiation; the same food options were also used in the prey preference experiment. The fish therefore had experience with the experimental prey choice options prior to the experimental tests, to acclimates the fish to tank conditions and food.

Figure 5.1. Aquarium setup for holding experimental fish in the diet preference study.

5.2.3 Tank preference trials

The experimental diet preference trials were undertaken in small aquaria, measuring 20 × 35 × 20 cm (length × width × height), which were filled to a depth of 30 cm with water and divided into two equal compartments (a resting

compartment and a test compartment measuring 20 × 17.5 × 20 cm (length × width × height) which divided by plastic divider. To minimize disturbance, the external sides of the aquaria were concealed obscured with laminated brown card. Each aquarium contained compressed air delivered through an air stone, a plastic plant for shelter and gravel. Three watch glasses were placed in the aquaria on one side (the test compartment) filled with experimental diet. Fish were placed individually in the test aquaria.

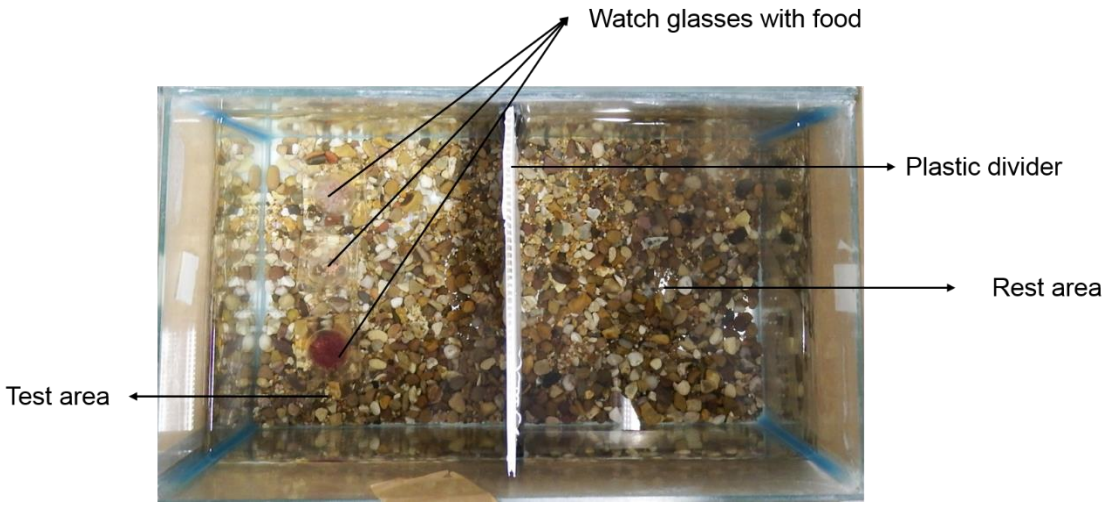


Figure 5.2 Experimental test tank (Rest area, Test area with three glasses dishes with experimental food and the white plastic divider in the middle)

Three types of food were offered to the sticklebacks in each diet preference test. Artificial diet and two commercially available pre-frozen food types; adult *Artemia* sp. brine shrimps, and bloodworms (larval chironomus sp.) (3F Fish Food, the Netherlands, www.frozenfishfood). In addition, they provide a wide range of different physical stimuli (such as colour, movement, shape, and size).

Each test fish was deprived of food for 24 h, to increase appetite and reduce food residue in the gut (standardization of hunger). Immediately prior to the preferences trials, individual fish were moved from their holding tank to the test tank where they left to acclimatise for 60 minutes. Following the settling period prior to the experiment, in watch glasses which were introduced into the experimental tanks via pipette. Each food type covered a similar within each watch glass, to avoid food partially or complete food depletion, and fish were fed

ad libitum. After 10 min of acclimation, the divider was removed giving the fish access to the feeding compartment. A megapixel USB webcam was fixed above the tanks and connected to a computer, enabling the movements and behaviour of the fish to be recorded without disturbing the fish. The observations of each fish were continued for 10 minutes. Individual prey items were clearly visible on the screen; all experimental trials were video-taped, and each trial replicated three times per fish, with the experiment being conducted for 40 fish in total.

5.2.6 Video analysis

Behaviour preferences were assessed in two rounds: the first round of trials were undertaken before the exposure (pre-exposure) in week 0, and the second round of trials were undertaken after exposure (post-exposure), at the end of experiment in week 12. Moreover, the diet preferences of each fish was recorded during each round on three separate occasions, with the physical position of the food type being switched each time.

During each diet preference trial, the following variables were recorded: the total number of seconds spent by the fish in the rest area; the proportion of time spent in each food type 'zone'; the food type towards which the fish first moved at the start of the trial; the total number of prey items taken by the fish; the proportion of bites directed to each food type; the food type towards which the first bite was directed.

5.2.7 Experimental parasite infection procedure

The *S. solidus* plerocercoids used to generate infective stages in the experiment originated from three-spined sticklebacks from the Clatworthy population, which had been experimentally infected in the laboratory from the Clatworthy population. These worms were cultured in pairs *in vitro* to produce eggs (Smyth, 1954); eggs were then incubated in the dark at 21°C for 3 weeks. Hatching was induced by exposure to light (Scharsack et al., 2007). Laboratory-reared copepods were fed 1-2 hatched coracidia. See more detail in chapter 2 section 2.4.

On the day of parasite exposure / sham exposure, individual sticklebacks were placed in separate, 1-L aquaria, half filled with water, for 24h procercoids each

fish was exposed to infected copepod. Thirty fish were fed copepods containing infective parasites, and 10 were sham exposed (i.e. fed non-infected copepods, but were otherwise treated identically). Exposures to parasite infective stages were carried out under UK Home Office licence (Project licence: 70/8148, Personal Licence: I29280977).

5.2.8 Termination and dissection

At the end of the study, following the completion of the second (post exposure) round of preferences tests, individual fish were euthanized by exposure to a lethal overdose of benzocaine anaesthetic and dissected to obtain physiological data, including standard length (to 0.1 mm), total mass, spleen, kidney, gonad and liver mass (all to 0.0001 g) to calculate indices. The total mass of plerocercoids recovered from infected fish was also quantified (MP, to 0.001 g), as described previously (Section 2.7.). On dissection, it was found that, of the 30 exposed fish, 10 had become infected with *S. solidus*, whilst the rest remained non-infected (i.e., there were 20 parasite-exposed yet non-infected fish).

5.2.9 Data analysis

Paired t-tests were used to test the differences between the proportion of time spent in zones diet in pre-exposure at the beginning and the post-exposure at the end of experiment, as well to test the proportion of bites consumed by fish in both rounds of diet preference testing.

Wilcoxon signed rank tests were used when the data did not meet the assumption of normality. A non-parametric Kruskal-Wallis ANOVA was used to examine the preferences of food choices between (control, exposed non-infected, and infected fish) at the end of experiment. Chi-squared tests were used to compare the identity of the first food types consumed.

5.3 Results

5.3.1 Outcome of experimental parasite challenges.

Among the 10 fish that had developed infections, of the 30 exposed fish (33.33 %) the mean total mass of *S. solidus* plerocercoids present in infected fish was 0.045 mg.

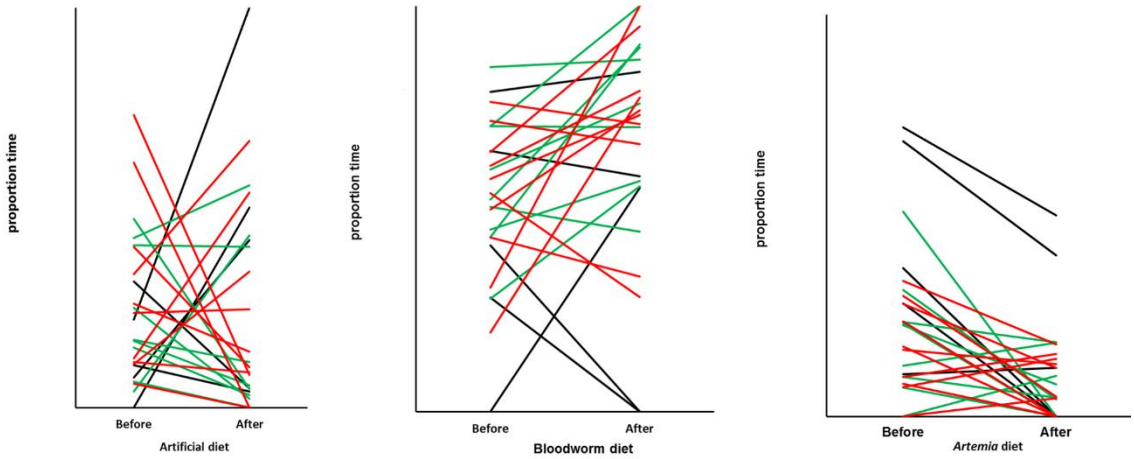
5.3.2 Proportion of time spent in zone diet

The mean proportion of time spent by sham-exposed control, exposed non-infected and experimentally infected in the test compartment (diet area) pre-exposure and post-exposure are presented in Figures 5.3 and 5.4.

Among infected fish, there were no significant differences in the proportion of time spent in the area containing the artificial diet between pre-exposure (week 0) and post-exposure (week 12) (paired t-test, n=10, $P = 0.513$). The same pattern was observed in the area containing bloodworm, with no significant difference in time spent in this area between pre-exposure and post-exposure (paired t-test, n=10, $P=0.109$). Finally, the proportion of time spent by infected fish in the *Artemia* zone was significantly different between week 0 and week 12 (Wilcoxon signed ranks test, $P = 0.017$), with fish spending more time in the Artemia zone during pre-exposure at the end of the experiment (Figure 5.4A).

For parasite-exposed, but non-infected fish, the proportion time spent in the artificial diet zone was significantly different between pre-exposure and post-exposure sessions (paired t-test, n=9 p=0.004). Specifically, there was an decrease in the proportion of time spent in this area post-exposure. In contrast, pre-exposure fish spent less time in the Bloodworm area than they did during post-exposure trials (paired t-test,n=9, $p = 0.017$). No significant statistical difference was detected between the proportion of time spent in the Artemia zone between pre-exposure and post-exposure trials (paired t-test,n=9, $p = 0.092$) (Figure 5.4B).

The sham-exposed control fish spent a similar amount of time in the artificial diet zone and bloodworm zone between the pre-exposure and post-exposure phases of the experiment (artificial paired t-test n=5, P = 0.245), (bloodworm paired t-test n=5, P = 0.864 . However, there were significant differences in the time spent in



the Artemia zone, with fish spending more time in the zone during pre-exposure than during post-exposure (paired t-test,n=5 p = 0.025).

Figure 5.3 The proportion of time spent by fish in the three feeding zones in the experimental diet preference tests. Individual data are shown, with lines connecting data points for the same fish, pre- and post exposure to *S. solidus* (or sham-exposure). Black lines: sham-exposed control fish; Red lines: experimentally-infected fish; Green lines: parasite exposed, but non-infected fish.

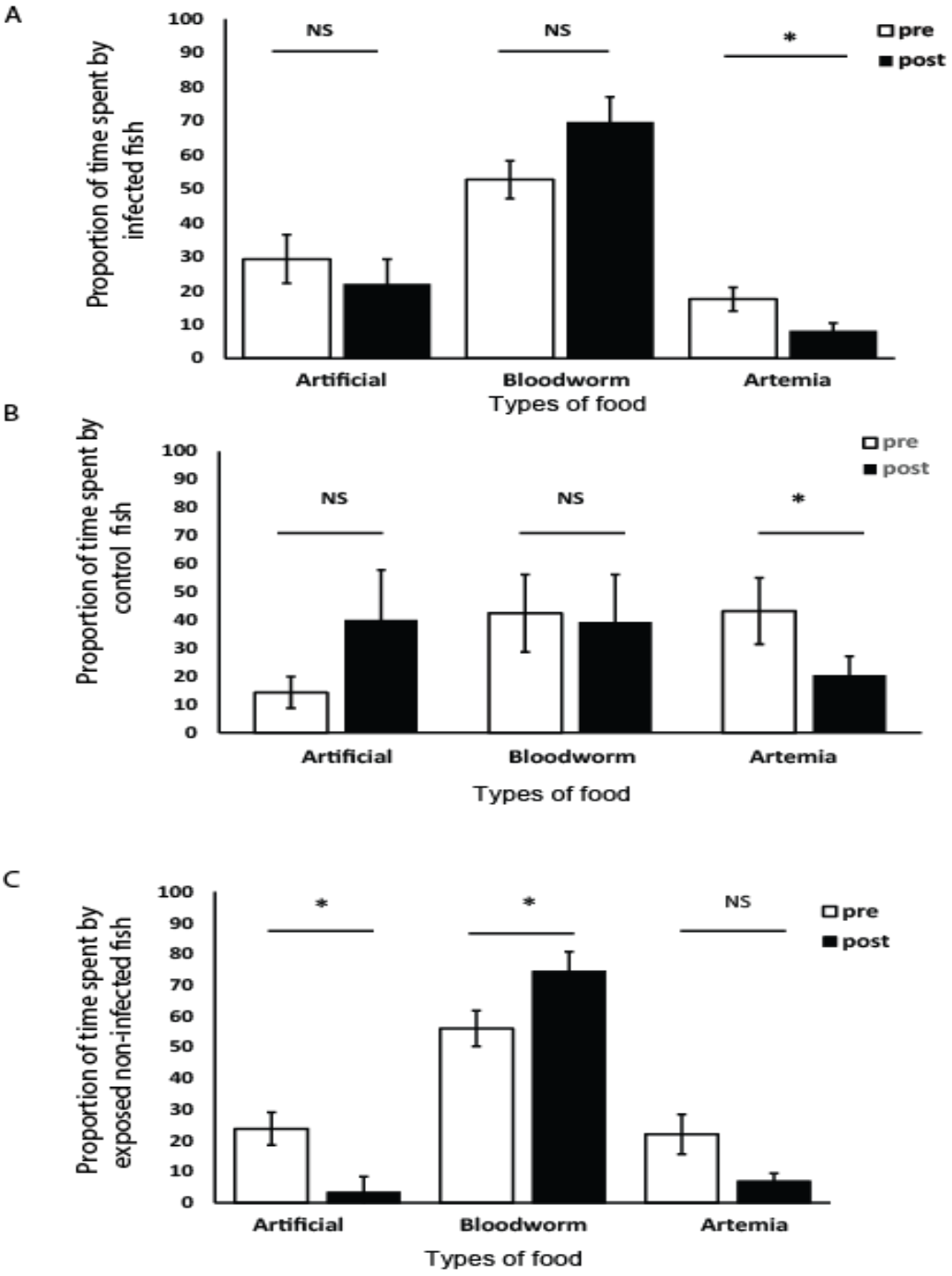


Figure 5.4 The proportion of time spent by a) experimentally parasitised, b) sham exposed, control and c) parasite-exposed but non-infected fish in the various feeding zones during the experimental diet preferences trials. Open bars show pre-exposure (or sham-exposure) values, filled bars show post-exposure (or sham exposure) values. * indicates $P<0.05$.

5.3.3 Prey ingestion number

The mean number of prey ingested by the experimental fish across the three replicate trails undertaken pre- and post exposure / sham exposure are presented in Figures 5.5 and 5.6.

For experimentally infected fish, there was no significant difference in the number bites of artificial diet (paired t-test ,n=10, P = 0.391), bloodworm (Wilcoxon signed ranks test, p=0.919) or *Artemia* (Wilcoxon signed ranks test, p=0.107) that was ingested during pre-exposure and post-exposure trials, suggesting that diet preferences were unchanged.

Parasite-exposed but non-infected fish showed no change in the number of artificial diet ingested during pre-exposure and post-exposure trials (Wilcoxon Rank p = 1.000), but there were significant differences in terms of the preferences of the proportion of *Artemia* and bloodworm taken by pre-exposed and post-exposed fish. The proportion of bloodworm consumed decreased significantly in post exposure trails (paired t-test n=9, p = 0.00). A similar pattern of selectivity was in the proportion of *Artemia* bites eaten in post-exposure trials (Wilcoxon Rank p=0.042). The proportion number of food items eaten pre-exposure higher than those the proportion consumed in post exposure at the end of experiment.

Among the sham-exposed control fish, there were no significant differences in the proportion of prey type consumed of bloodworm, artificial diet and *Artemia* between the first and second trails artificia (paired t-test, *p*=1000), bloodworm (paired t-test,*n=5, p* = 0.347) and *Artemia* exposure (Wilcoxon Rank *p*=0.106)

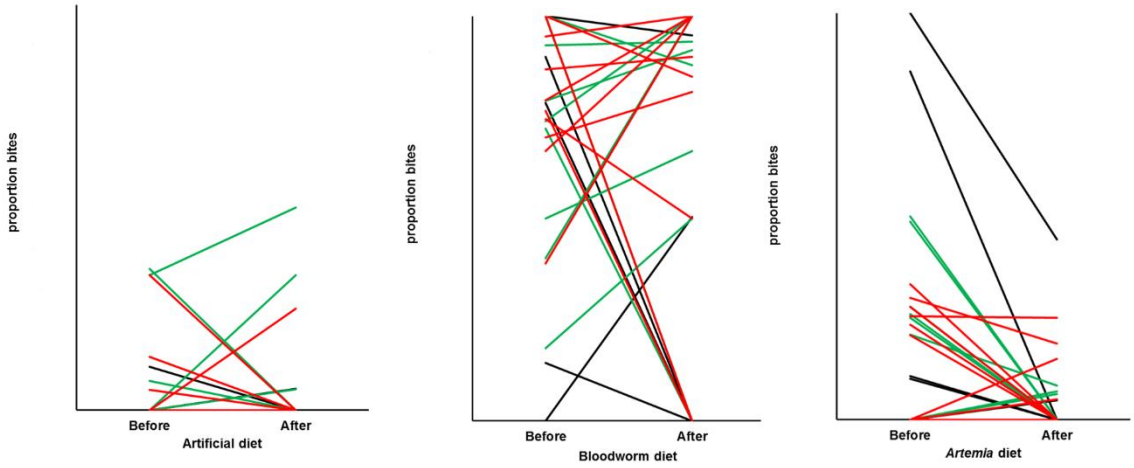


Figure 5.5 The proportion of bites taken by fish in the three feeding zones in the experimental diet preference tests. Individual data are shown, with lines connecting data points for the same fish, pre- and post exposure to *S. solidus* (or sham-exposure). Black lines: sham-exposed control fish; Red lines: experimentally-infected fish; Green lines: parasite exposed, but non-infected fish.

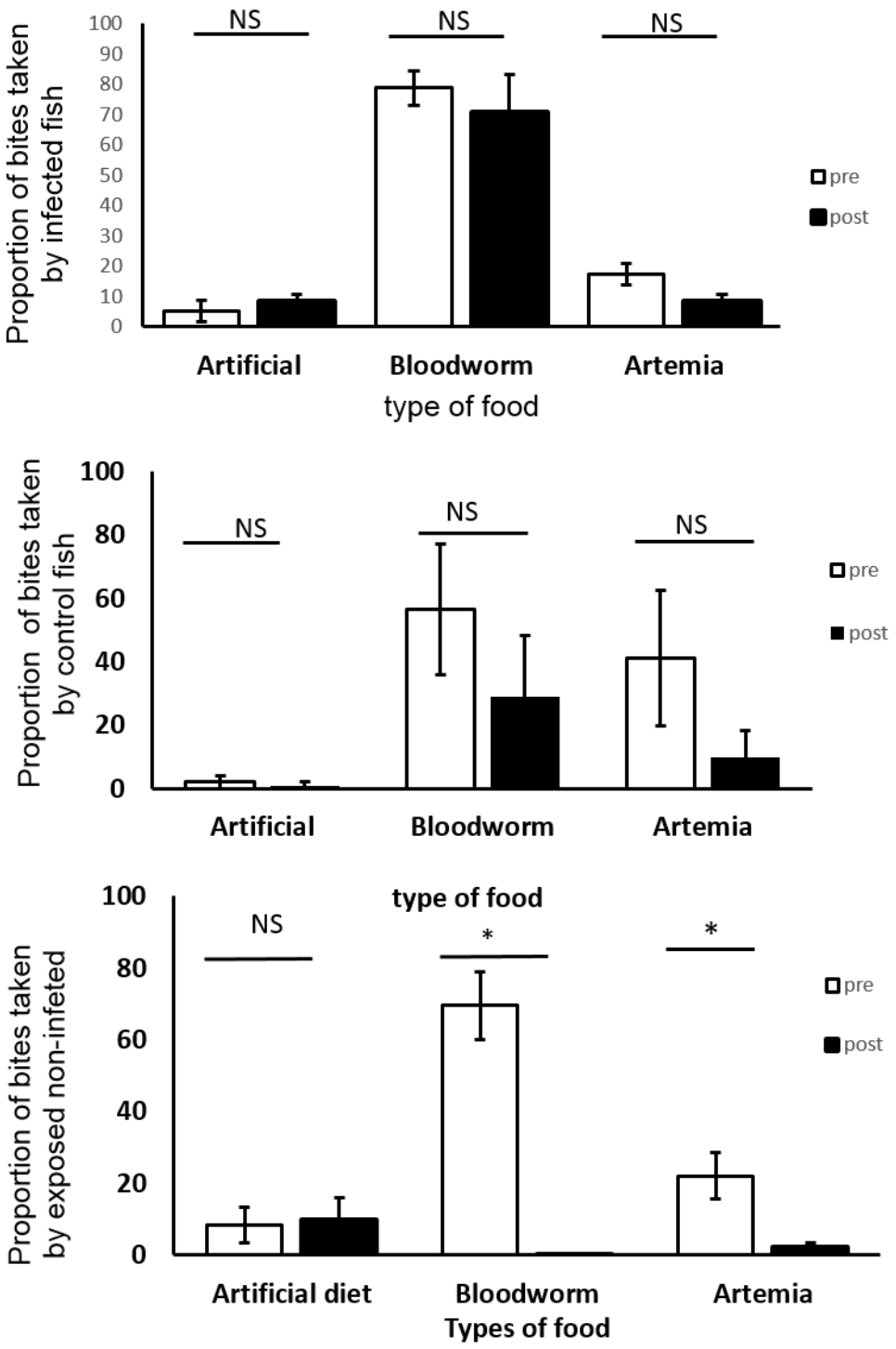


Figure 5.6 The proportion proportion of bites eaten by, a) experimentally parasitised, b) sham exposed, control and c) parasite-exposed but non-infected fish in the various feeding zones during the experimental diet preferences trials. Open bars show pre-exposure (or sham-exposure) values, filled bars show post-exposure (or sham exposure) values. * indicates $P<0.05$.

5.3.4 The first food three-spined sticklebacks prefer in pre-exposure and post-exposure

At the beginning of each trial the first food consumed by fish were recorded in the first trial and second trial. Chi-squared tests were used to compare the first food consumed, the result showed that the bloodworm were the first food stickleback prefer to consume ($P < 0.001$) in both the first and second trial ($P < 0.001$).

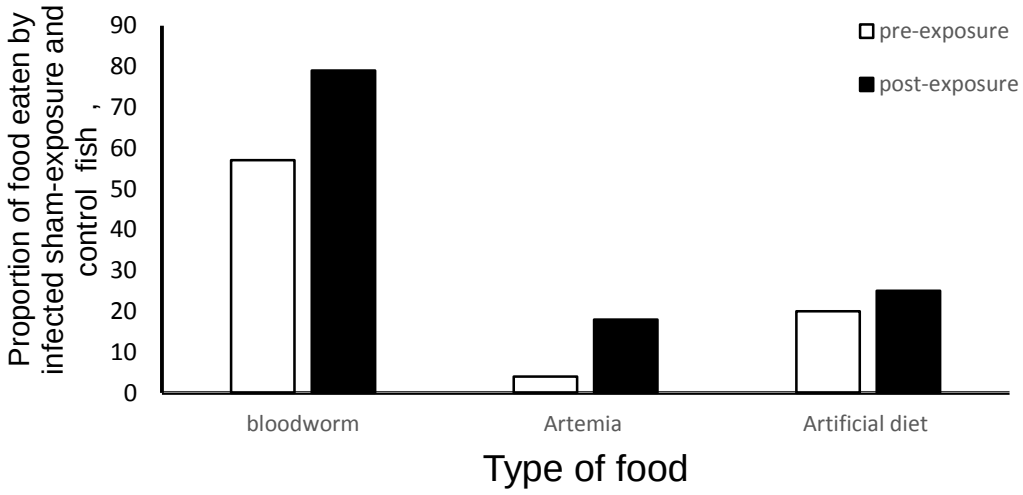


Figure 5.7 The first prefer food by three-spined stickleback at pre-exposure (open bar) and post-exposure (filled bar)

5.4 Discussion

The type of nutrient used in the test of dietary preference of three-spined sticklebacks used in this study consisted of aquatic crustaceans (*Artemia*), the larvae of *chironomids* (bloodworms) and an artificial diet (protein content: 50%). Bloodworms were selected because they have previously been used in preference tests on sticklebacks (Barber and Huntingford, 1995) Also, they were used because they are routinely fed to fish in aquaria, and are natural components of stickleback diets, and were found to facilitate fish growth and health in other experiments as part of this project.

Meanwhile, *Artemia* were chosen because they have previously been used in feeding the fry of three-spined sticklebacks (Hahlbeck et al., 2004) they represent an alternative prey source replicating other natural components of the fish's diet. The artificial diet was used in this experiment, as previous observations with other fish species showed that this diet has high protein contents to provide a high-protein option for the fish (Povey et al., 2009) Work undertaken in this project (chapter 4) has demonstrated that the artificial diet had a positive effect on all aspects of host growth, sexual development and health status that were measured.

Previous studies have shown that infection can impact host behaviour (Maitland, 1994) as well as other physiological characteristics (Karvonen and Seppälä, 2008, Sitjà-Bobadilla, 2009, Lafferty and Kuris, 2009), and infection have been shown to influence the preference of hosts in terms of their choice of food in a way that enhance the amount of protein to benefit the functioning of their immune systems (Povey et al., 2009). Hence, any changes in diet selection could be host- or parasite induced, and could have positive or negative implications for hosts or parasites.

Studies of the preference of S. *solidus*-infected sticklebacks shows that experimentally-induced parasite infections only affect the proportion of time spent in the *Artemia* zone, with infected fish spending less time here post exposure than pre-exposure. However, control fish were also showed same results.

Despite the result that the three-spined stickleback is infected, this study does not bring forth any evident effect of prey choices on infected fish. Nevertheless, the preferences towards *Artemia* decline throughout the study.

The theory about modification of prey or food choices that has been explained in extant literature as a by-product of parasitic infection can be better argued from the point of view of changes in the behaviour patterns of the host, as well as their physiology and immunity and both qualitative and quantitative aspects of the diet

In their work, Kolluru et al. (2009) posited that foraging times in the case of guppies (*Poecilia reticulata*) could be affected as a result of infection by the monogean parasite *Gyrodactylus turnbulli*.

Shurkin (2014) proposes that in species, other than the fish, the members consume mixtures to either stop or neutralize the infection. Such feeding habits are easy to spot as the animals stop eating in this way during their recovery period

In contrast with our study, in the work of Povey et al. (2009), reported that host susceptibility to infection is associated with levels of protein in their diet. Generally, the results do not support our prediction that infection by *Schistocephalus solidus* affects the preference of fish to the bloodworm and artificial diet.

Regarding to the intake of artificial diet, there were no differences either the proportion number of bite or the proportion of time spent in artificial diet between pre exposure and post-exposure, this results suggest that parasite infection did not affect the preference of artificial diet. it may be the case that infected fish, being poor preferences toward artificial diet , Investigation of the behavioural impact of infection revealed that infection led to more time being spent on foraging (Giles, 1987).

The findings of this research also demonstrate that the preferences towards *Artemia* decline with time through the study, the reason behind this observation is not obvious. a possible explanation for this might be that *Artemia* is genus of Crustacea (Triantaphyllidis et al., 1998, Nunes et al., 2006). Therefore, the exoskeleton of this species is approximately 50-80% chitin (Shiau and Yu, 1999) The main structure of the exoskeleton is chitin (Talens-Perales et al., 2017) The carnivorous fish lack the carbohydrate-degrading enzymes such as amylase and glucanase, therefore, they probably consume few amount of chitin (German et al., 2009)

As well as. The effect of infection on preference, diet for Bloodworm there were no difference in the proportion of bite and the proportion of spent time in Bloodworm diet. It suggest that hypothesis predicts that parasitize fish should increase metabolic demands. The reason for that there no different between pre-exposure and post-exposure. In pre-exposure stage, fish were small size and in

post exposure the fish were infected , other studies indicated that the parasitic infection can reduce the stomach capacity (Tierney, 1994) Moreover, unparasitized fish record a higher swimming speed than their parasitized counterparts(Lester, 1971, Arme and Owen, 1967).

It's a reasonable postulation that infection did not affect the dietary preferences, though the situation in the wild might differ, where there are many different prey choices available as in this experiment there are only three dietary options (Wootton, 1976, Allen and Wootton, 1984).

In this study, tests of prey preference were carried out on individual fish in isolation. This contrasts with the behaviour of three-spined sticklebacks in the wild which live in large shoals (Poulin and FitzGerald, 1989**)**. Compared to individual fish, shoaling fish were observed to be quicker in detecting limited sources of food. (Pitcher and Parrish, 1993) Therefore, the reduced foraging I observed is likely to be due to keeping fish in isolation during experiments.
One additional justification for the same can be given by the fact that fish have flexible eating habits that are subject to food availability and they turn to consuming food that is available in abundance in the environment around them (Azevedo, 1972).

Regardless of infection status, in study, the first bite consumed were recorded. In the first recorded and the second recorded, thus, three-spined sticklebacks showed a tendency to select bloodworm as their preferred diet. The present investigation was designed to discover the food preferences of three-spined sticklebacks in relation to prey profitability. Experiments have shown that there are clear differences in feeding behaviour between the three types of diet (*Artemia*, Bloodworm and artificial diet) with an overall preference for Bloodworm.

The higher selection of Bloodworm may be due to the fact that three-spined sticklebacks prefer prey whose colour is discernible (Popham, 1966); the colour of Bloodworm is red, as a result of the red pigment of Blood haemoglobin (Armitage et al., 2012). In support of the present study, similar results have been reported for Thinlip mullet (*Liza ramada*) larvae fed a diet with different coloured food. The best performance and survival were achieved in fish fed on dark-coloured diets (red, dark blue and dark brown) (El-Sayed and El-Ghobashy,

2011). However, the artificial diet offered to three-spined sticklebacks was of the same colour as bloodworm, as it was coloured using commercial food colourants. Therefore, this suggests that three-spined sticklebacks may not be visual feeders and the food colour was not a factor in food preference. Nevertheless, the influence of the colour of diet in preference of fish might be controversial, Jegede and Olusola (2010) demonstrated that tilapia zillii fed feeds with different colours showed better growth and feed efficiency with yellow and light-green food than those fed on dark-coloured diets (Jegede and Olusola, 2010). Conversely, Nile tilapia larvae, for example, are visual feeders that favour food that is dark in colour, although their fingerlings will consume food of any colour (El-Sayed et al., 2013). In a previous study by (Johannesen et al., 2012), three-spined sticklebacks use their sense of smell to find food, especially when visibility is poor as in turbid water. Conversely, in the indigenous habitat, augmented algal turbidity results in a higher dependence on olfactory signals in the mating process in contrast with clear waters (Heuschele et al., 2009). In addition, other non-visual characteristics such as size, form, and palatability are considered to be some of the important factors when foraging for food (Villamizar et al., 2009, Gibson, 1980, Wootton, 1984). This is supported by earlier studies; for example, (Gibson, 1980) found that the foraging behaviour of three-spined sticklebacks showed a preferred related to size, where the fish select larger, rather than smaller, prey.

Multiple investigations concur with the contention and designated that supplementary physiological procedures, excluding the vision-feeding relationship, assume a greater role in encouraging the behaviour of juvenile and adult developed fish.

(Kallayil et al., 2003) It was discovered that even in non-sensory surroundings, foraging behaviour of cod could be artificially stimulated by utilizing bait odour.

However, in this experiment, sticklebacks were selecting prey according to physical appearance, over the study period this experiment was conducted in acceptable circumstances and to aquarium maintenance, water was changed regularly. In our results, the three-spined sticklebacks showed a tendency to select Bloodworm. Our results suggest that three-spined stickleback relied more on olfactory cues than visual in clear water, although this may not always

compensate for the reduction in visual cue availability caused by turbidity. Our results suggest that sticklebacks, use their olfactory sense to indicate their prey location, and their eyes for discovering sites.

As the water was stable in this specific experiment, visual perception was used effectively to detect prey. Prevalent signs from the olfactory senses signal to the fish that their prey is in the direct surroundings. A possible explanation for this is that the Bloodworm have a strong odour that attract the host.

5.5 Conclusions and future studies

The empirical study carried out yielded the finding that the eating habits of three-spined stickleback, infected with *Schistocephalus solidus,* are not affected by an infected host. The observed reduction in the proportion of time spent on the *Artemia* diet by infected and non-infected fish might therefore more likely be due to Artemia not being a common diet for sticklebacks. Nevertheless, further studies of the relevant factors are required to determine the reason for such variations in published results, focus on the period of experiment maximum six week.

Chapter 6 Effect of additive nutrients on Host-Parasite interaction in fish

Abstract

Food additives are natural extracts that are mixed with food to perform specific functions. Even the aquaculture sector has made use of several such food additives in the form of supplements that increase immunity and also result in more health benefits for the host in some of the cases.

In the field of agriculture, additive nutrients make crops resistant to diseases, augment growth, and function as immunostimulants. Some of the best potential options for augmenting skin colour, sexual signalling, as well as attainment and maintenance of immunity, are lutein and astaxanthin.

It was found that 4 weeks of dietary supplementation showed an effect of the feeding period on the infection response of three spined sticklebacks. The infection was determined after a period of feeding. In the first round, fish were raised on an optimal feeding level of 200 mg (containing lutein and astaxanthin)/kg, or a control non-supplemented diet carotenoid until 28 days pre-exposure. After that, the fish were exposed to the parasite infective stage of *Schistocephalus solidus.* In the second round of feeding, all the fish were then fed bloodworm *ad libitum* every day for six weeks. At 14d intervals the individual fish were weighed, their lengths measured, and they were individually photographed.

Feeding history influences the immune response of infected fish. Fish fed the carotenoid supplement had higher SSI values than those fed the control diet, and infected fish had higher SSI values than non-infected fish. However, the dietary carotenoid supplements did not affect growth fish and specific growth rate; there was no apparent effect of carotenoid on health condition or parasite growth.

6.1 Introduction

Food additives are ingredients that are added to foods to carry out particular functions, and can be extracted from natural sources. Additive nutrients play an important role in aquaculture in that they can enhance growth, improve disease resistance and can act as immunostimulants (Yin et al., 2006, Shakya and Labh, 2014). Many such additives have been used as food supplements in aquaculture to enhance immunity, and the utilization of these supplements has in some cases been shown to confer health benefits to the host.

Using vitamin in feeding fish, such as vitamin E, as fish food additives. Lin and Shiau (2005) found that vitamin E enhances the immune response. It should be noted that there are some vitamins that fish cannot synthesize, such as vitamin C (Zhou et al., 2012), which plays an important role in growth and immune response (Gabaudan et al., 2000, Ai et al., 2004, Zhou et al., 2012) .

Carotenoids are natural pigments that vertebrates, including fish, cannot synthesize themselves (Amar et al., 2001, Pike et al., 2007a), and therefore must be acquired entirely through diet (Goodwin, 1980). Accordingly, there is evidence that supplementary carotenoids can enhance the quality of the diet, with positive consequences for growth rate, reproductive development, and immune response. For example, Meilisza et al. (2017) found significant effects due to carotenoids with regards to the growth and survival rates of Lake Kurumoi rainbowfish, *Melanotaenia parva.* Carotenoid supplementation might also be able to enhance immunity.

Carotenoids are beneficial not only for their physiological functions but also as a vitamin A source.(Lorenz and Cysewski, 2000). Vitamin A plays a role in the function of normal growth, (Halver, 2002) Dietary carotenoids have also been shown to enhance immunological function (Kolluru et al., 2006) and provide protection against parasitic infection across a wide range of taxa (Olson and Owens, 1998).

Phagocytosis improved, alongside other aspects of fishes' humoral immune systems and cell-based defences, which are crucially affected by astaxanthin and

other carotenoids. Combatting of infections, larval development and endurance are all facilitated by such factors (Amar et al., 2004, Yanar et al., 2007).

A carotenoid supplemented diet could not only regulate the immunity of the common carp (*Cyprinus carpio*) but also make it less susceptible to *Aeromonas hydrophila.* (Anbazahan et al., 2014). (Amar et al., 2004) showed that an improvement of the non-specific immune system in rainbow trout (*Oncorhynchus mykiss Walbaum*) was associated with the dietary intake of carotenoids. Furthermore, experimental studies have investigated the possible enhancement in immunity and promote resistance to infection diseases as associated with carotenoids in the diet in a number of species of fish. In an experimental study investigating the effects of carotenoids and food availability in guppies (*Poecilia reticulata*), parasite (*Gyrodactylus turnbulli*) loads were found to be lowest in males raised on a diet containing medium carotenoid concentration, whereas the low - and high-carotenoid diet groups were very small (Kolluru et al., 2006). However, Czeczuga (1979) have shown that resistance to bacterial and fungal diseases could be correlated with high levels of carotenoids in salmonids. According to Anbazahan et al. (2014) giving common carp (*Cyprinus carpio*) carotenoid dietary supplements protects the fish against *Aeromonas hydrophila,* and potentially enhances the carp's immune defences

As well as acting as immunostimulants and other roles in the promotion of growth and reproduction, carotenoids are also pigments and typically the compounds responsible for yellow, orange and red sexual ornamentation in the animal kingdom (Olson and Owens, 1998) (Kolluru et al., 2006). The capacity to develop sexual ornamentation therefore depends on adequate level of carotenoids in the diet. Carotenoid-based ornamentation is often used in inter-sexual displays; typically, though not exclusively, by males. There some studies showed that female preference for mating with males with high levels of carotenoid was observed in several species (Olson and Owens, 1998, Kodric-Brown, 1989).Female fish (Poecilia parae) often prefer more brightly coloured males, especially where those with colours are carotenoid-dependent (Bourne et al., 2003).

Females sticklebacks typically prefer males with bright nuptial colouration, which may indicate their condition (Milinski and Bakker, 1990), and the former may be able to avoid becoming infected themselves (Houde and Torio, 1992).

As well as playing a direct role in choice of mate, carotenoid-based ornamentation may also provide an honest signal of a male's capacity to produce offspring that resist parasitic infection. The effects can be significant; for example, Eurasian perch *Perca fluviatilis* have demonstrated greater resistance to being infected with parasites (Eckmann et al., 2017).

This study focussed on carotenoids as one of the additive nutrients that potentially enhance immunity in order to determine whether the carotenoid supplemented could evaluate the effect of carotenoid supplements in the diet on the immune response and disease resistance of sticklebacks. In this study, three-spined sticklebacks were given a diet supplemented with immunoenhancing carotenoid (Lutein and Astaxantnin) (Kolluru et al., 2006, Jagruthi et al., 2014, Bédécarrats and Leeson, 2006, Lorenz and Cysewski, 2000) where preliminary studies with these two supplements produced high immune response. Three-spined sticklebacks were fed either a carotenoid-supplemented diet (containing lutein and astaxanthin) or a control, non-supplemented diet for four weeks around the time of being exposed to infective stages of *S. solidus.*

6.2 Aims

The aim of the study was to test the hypotheses that (a) dietary supplementation with carotenoids affects the probability that three-spined stickleback become infected following exposure to the parasite, and that (b) dietary carotenoid supplementation affects the infection phenotype that emerges in infected fish (c) that carotenoids may enhanced immune response such as splenosomatic index.

6.3 Methods

6.3.1 Fish supply and husbandry

In total, 10 families were represented in breeding season 10 male and 10 female, sexually mature adult three-spined sticklebacks were selected from Clatworthy Reservoir background lab stocks and used as parents in standard in *vitro*

fertilisation techniques (Barber and Arnott, 2000) to produce fry during Spring 2016. Newly-hatched fry were maintained in groups in 1 L tanks and fed *ad libitum* with a succession of Liquifry No 1™ (Interpet, UK) and *Artemia* sp. nauplii, before being transferred to 30 L holding aquaria (40x25x30cm) held on a filtered, temperature controlled recirculating water system. Fish were fed once daily *ad libitum* on frozen bloodworms, and at five months of age.

At six months of age, 88 juvenile sticklebacks were selected from the lab-bred Clatworthy Reservoir stock (see Chapter 2 and 2.1.2 above). Fish were transferred from the holding tank to the experimental tank two weeks prior to the experiment to allow them to acclimatise to the new environment.

A commercially available, pre-frozen food type was used to feed fish prior to the experimental study *Chironomus* sp. larvae (bloodworms: 3F Fish food, Netherlands (www.frozenfishfood.nl)).

6.3.2 Experimental tanks

At the beginning of the experiment (day 0), all fish were weighed (blotted wet mass, to 0.001g) and measured (standard length, to 0.01mm) and each was photographed individually before being replaced into the same tanks. The mean standard length of the fish on day 0 was 36. mm and the mean mass was 0.64g. Fish were then housed individually in 2 L plastic aquaria (L x W x H: 14×13×20 cm; water depth: 12 cm); each aquarium was divided into two equal compartments and a plastic plant was provided for shelter. Each tank contained aerated recirculated freshwater, the water temperature was controlled at 14°C and a photoperiod of 14L: 10D was implemented.

6.3.3 Experimental design

The whole experiment comprised two rounds of supplementary feeding and one round of parasite exposure. During the first round of feeding, a total of 88 sticklebacks were used, with 44 being assigned to each dietary treatment group. One group was fed with a carotenoid-supplemented diet and the other group was fed with a non-supplemented control diet.

Details of both diets are provided in Table 6.1. All fish were fed daily, *ad libitum* on their experimental diet for four weeks. After the 4-week feeding period, each fish was either exposed to an infective *Schistocephalus solidus* parasite by feeding an experimentally infected copepod (N=28), or was sham-exposed by feeding a non-infected copepod (N=16). The fish were then weighed, length measured, and individually photographed.

Following parasite exposure / sham exposure, all fish were subsequently fed Bloodworms (*Chironomus* spp. larvae) *ad libitum* every day, for six weeks, giving plerocercoids time to develop in sticklebacks that had become infected.

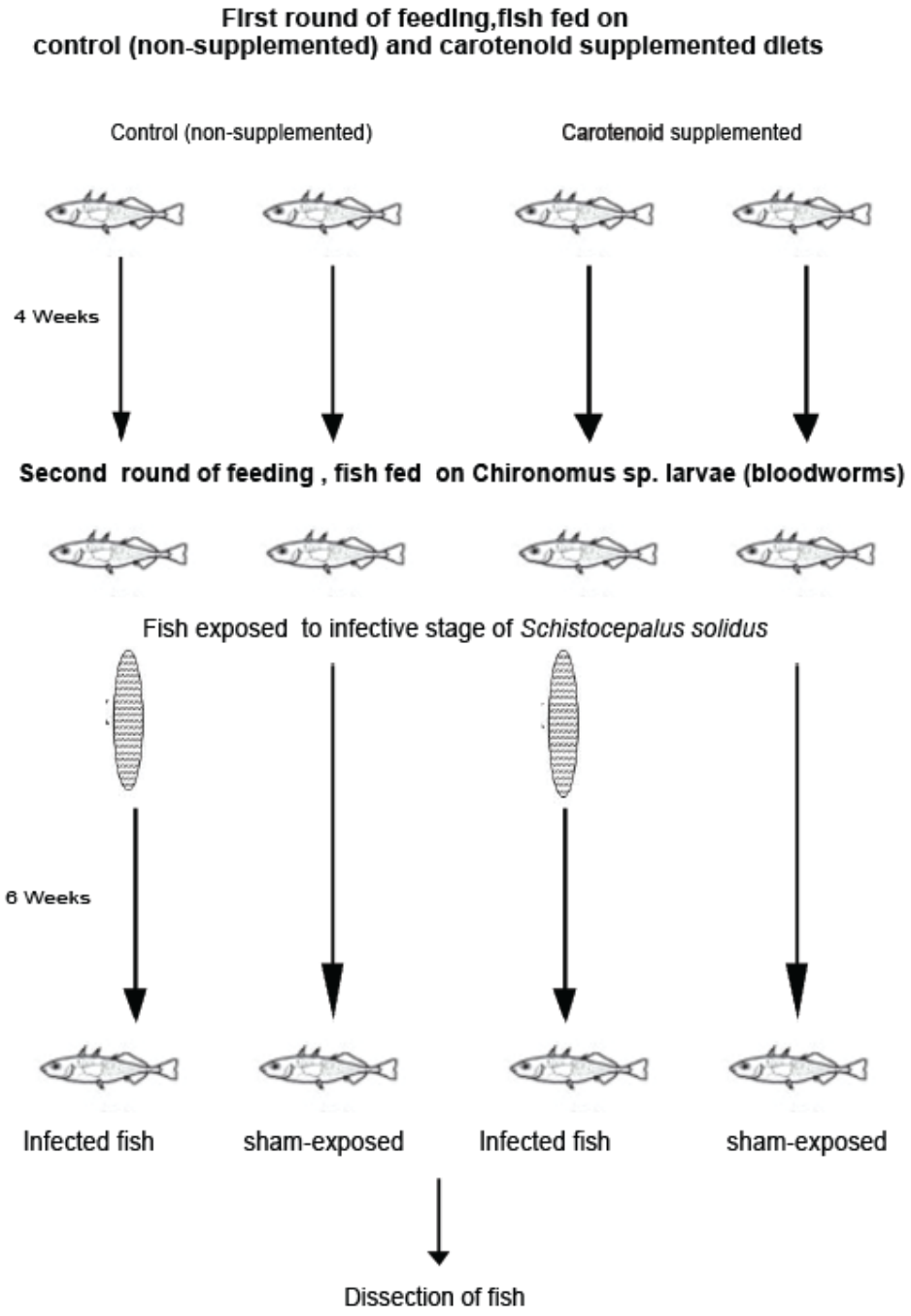


Figure 6.1 Graphical representation of the experimental design.

The whole experiment comprised 2 round of feeding and I round of infection first 4 weeks fish fed diet either control non-supplemented or carotenoid supplemented than fish were exposed to infective stage of *Schistocephalus solidus.* After that second of feeding, fish were then fed Bloodworms (*Chironomus* spp. larvae)

6.3.4 Experimental diets

Two experimental diets were formulated, with individual fish either being fed the control (non-supplemented) diet or a diet supplemented with carotenoids. Both diets comprised approximately of 60 % crude protein, with casein and fish meal as protein sources, and 10% lipid, with cod liver oil as the source of lipid and sucrose as the main source of carbohydrate. Astaxanthin and lutein were used as supplementary carotenoids, added to the supplemented diet at a concentration of 200 μg g^{-1}.(SIGMA-UK).

Table 6.1 Formulation of the experimental diets, Table showing the diet formulation details used in experiments. With seven ingredients with proportion which was used.

Ingredient g/100g	Control	Carotenoid supplemented (%)
Fishmeal	57.2	57.2
Casein	22.8	22.8
Sucrose	10	10
Cod liver oil	5	5
Gelatine	2	2
Vitamin mix	1.5	1.5
Mineral mix	1.5	1.5

6.3.5 Experimental parasite exposure

Schistocephalus solidus plerocercoids were obtained by dissecting experimentally-infected sticklebacks from the Clatworthy lab population. Plerocercoids were cultured *in vitro,* in pairs, to produce eggs (Smyth, 1954) see chapter 2 Egg clutches were removed and incubated in a Petri dish in the dark at 21°C for 3 weeks, after which eggs were exposed to sunlight to induce hatching (Scharsack et al., 2007). Coracidia were then transferred to a flask containing lab-cultured copepods (*Cyclops strenuus abyssorum*) and copepods were screened after 21 days to confirm their infection status and ensure that procercoids they harboured were infective (i.e., they possessed a cercomer;

(Smyth, 1969)Three-spined sticklebacks were then exposed to the infective parasites by ingestion of an infected copepod possessing a cercomer (N = 56).

6.3.6 Fish dissection and physiological data collection

At the end of the trial, all fish were weighed, measured and individually photographed , before being euthanized using a UK Home Office Schedule I method (immersion in an overdose of Benzocaine solution followed by destruction of the brain) and dissected under a binocular microscope. Specific growth rate was calculated as =((Ln(M_0)-Ln(M_{70}))/70)*100 , where M_0 is the wet mass of fish at the start of the study and M_{70} is the wet mass of the fish at the end of the 70 day study (excluding parasite mass) the fish mass than was calculated as (M_f = total fish mass – parasite mass). Physiological data, in cluding total mass, spleen, kidney, gonad and liver mass (each weighed to 0.0001 g), were used to calculate hepatosomatic index (HSI), splenosomatic index (SSI), gonadosomatic index (GSI), and kidney somatic index, (KSI). Blood samples were taken from each fish by severing the caudal peduncle and collecting the whole blood in a heparinised microcapillary tube. The blood sample was centrifuged for 60s (12.000) at room temperature to calculate the haematocrit (percentage by volume of red blood cells in the whole blood).

Any *S. solidus* plerocercoids recovered from infected fish were also weighed (to 0.0001g) and this was used to calculate the parasite index, which descirbes the proportion of fish mass contributed by the parasite. These methods are described in detail in Chapter 2, section 2.9.2

6.3.7 Statistical Analysis

Chi-squared tests were used to compare the prevalence of parasite infection between groups of fish fed the different experimental diets. A general linear model was used to investigate if there was any effect of parasite infection or carotenoid supplementation on the specific growth rate, body condition factor, health status, immune statuss or reproductive development of sticklebacks in the experiment. Among infected fish, the effect of diet treatment on parasite mass and parasite index were tested using one-way ANOVA. For all boxplots in this chapter, the dark line represents the median, the box shows the Q1-Q3

interquartile range (IQR), and the Whisker plots represent the greatest value and lowest value, while ● represents an outlying observation.

6.4 Results

In feeding trials, survival across all feeding groups ranged from 94% in fish fed the control diet to 89% in fish fed with a diet supplemented with carotenoids.

All fish increased in length and mass during the experiment. Eleven of the 88 (12.5%) fish died before the end of the study for unknown reason. These fish were distributed throughout experimental groups (two from the sham-exposed, control diet (ShC-) group; one from the parasite-exposed, control diet group (ExC-); three from the sham-exposed, carotenoid-supplemented (ShC+) group; five from the parasite-exposed (ExC+), carotenoid-supplemented group. Data for these fish were omitted from statistical analysis. Final sample sizes were therefore ShC+ = 13, ExC+: 21, ShC-: 14, ExC-: 25.

6.4.1 Effect of carotenoid supplementation on *S. solidus* infection rates

Overall 56 of the 88 fish exposed to infective stages of *S. solidus* developed plerocercoid infections. The probability of a plerocercoid establishing in a fish host was not significantly associated by dietary supplementation, with infections developing in 25 of the 28 (89.2%) parasite-exposed fish that had been fed the control (non-supplemented) diet, and in 21 of the 28 (75%) of exposed fish fed the carotenoid-supplemented diet (x^2=0.028, p=0.867).

6.4.1 Specific growth rate (SGR)

There was no significant effect of carotenoid supplementation on the SGR of host sticklebacks ($F_{1, 69}$ = 0.00, P = 0.996). There was no effect of *S. solidus* infection status on stickleback SGR ($F_{1, 69}$ = 0.01, P = 0.911), there was no significant interaction between the two factors ($F_{1,69}$ = 0.40, P = 0.527; Figure 6.2.)

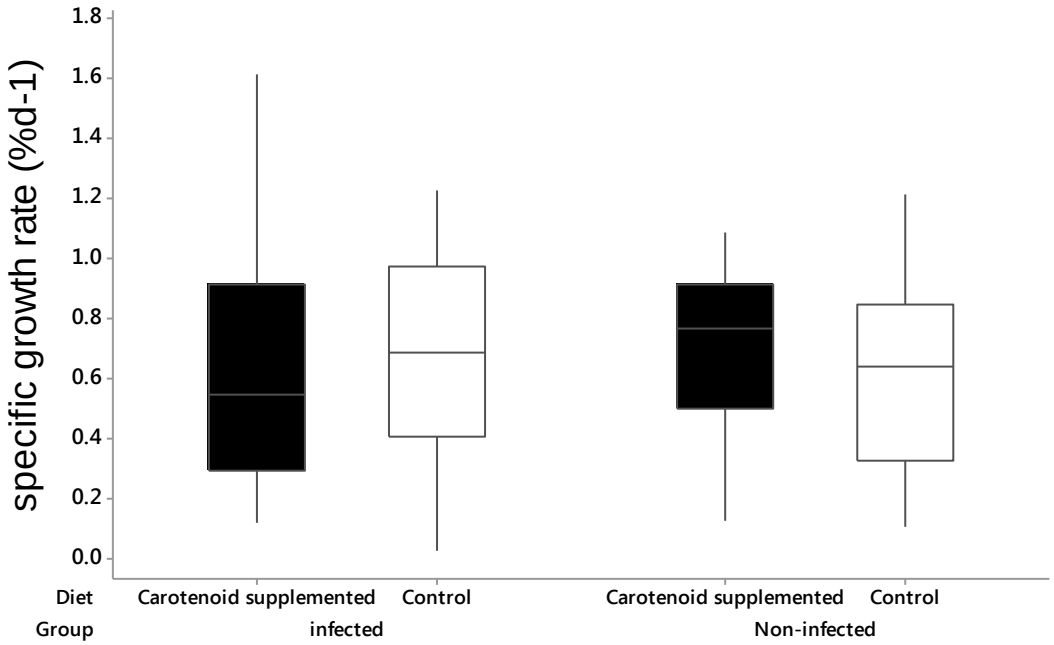


Figure 6.2 Specific growth rate of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented

6.4.2 Fish condition and health status and immunity

6.4.2.1 Fish mass

The statistical analysis of data pertaining to terminal fish mass (excluding parasite for infected fish) showed that there was no significant effect of diet type or infection status, and no interaction between the factors (ANOVA: diet: $F_{1, 69} = 0.29$ $P = 0.592$; infection: $F_{1, 69} = 0.09$ $P = 0.768$; interaction: $F_{1, 69} = 0.94$ $P = 0.334$; see Figure 6.3).

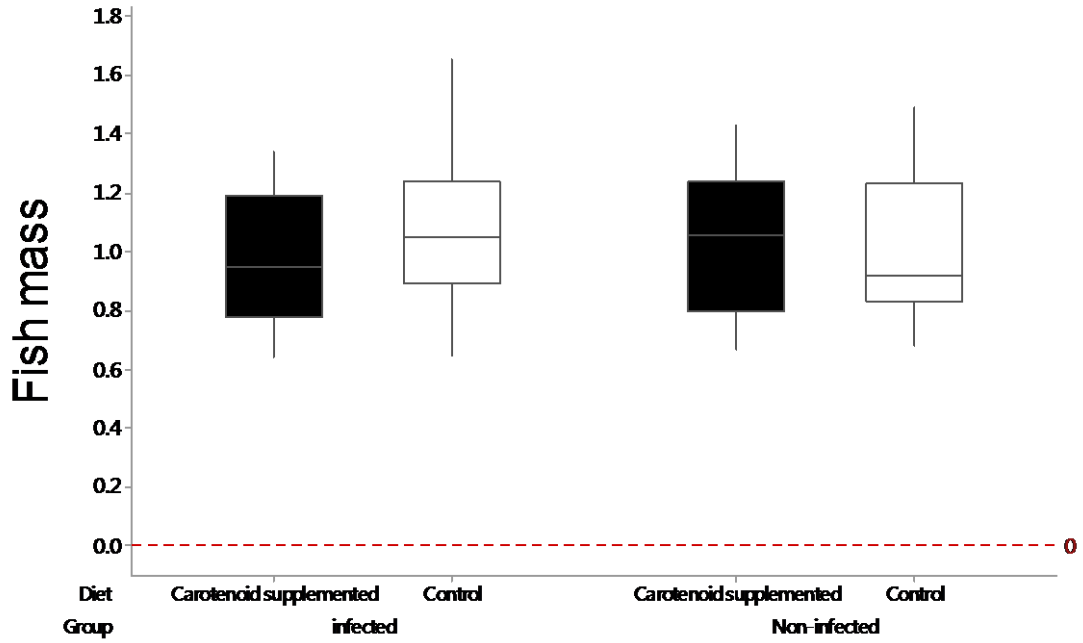


Figure 6.3 Fish mass of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented

6.4.2.2 Body condition factor (BCF)

The result of the general linear model comparing body condition factor among all treatments indicated no significant effect of carotenoid supplementation on K ($F_{1, 69}$ = 0.30, P = 0.584) and no effect of infection status ($F_{1, 69}$ = 0.54, P = 0.467). There was no significant interaction effect between the two factors ($F_{1,69}$= 1.32, P = 0.255; Figure 6.4).

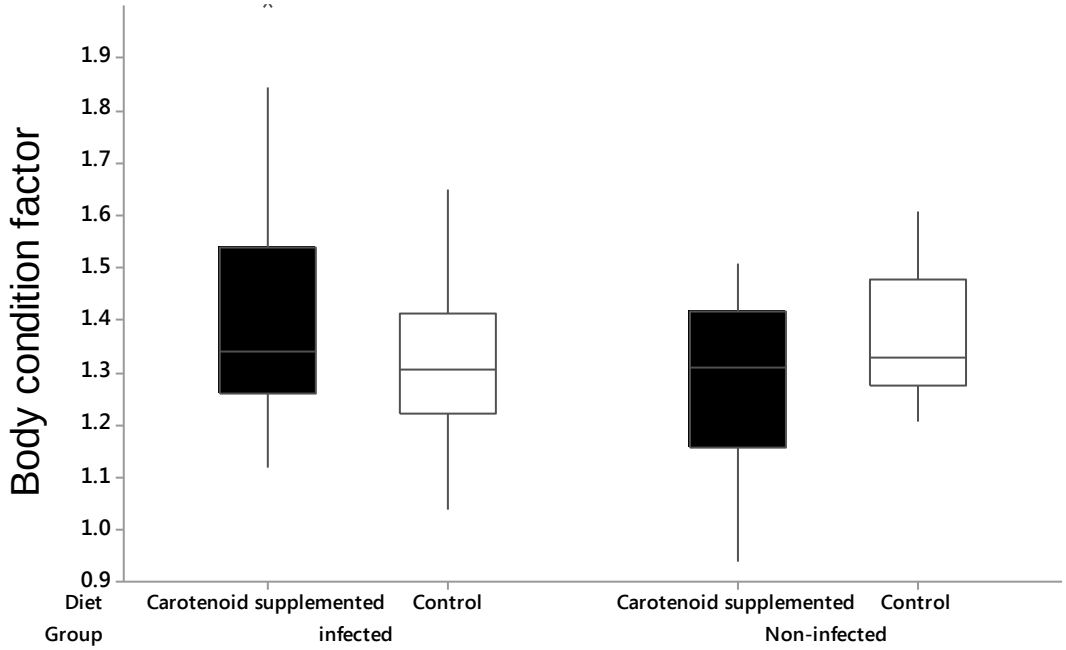


Figure 6.4 Body condition factor of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented.

6.4.2.3 Hepatosomatic index (HSI)

At the termination of the study, there was a no significant effect of carotenoid supplementation ($F_{1,69}$ = 0.14, P = 0.709) and only a marginally non-significant effect of infection on HSI ($F_{1,69}$ = 3.28, P = 0.075). There was no significant interaction between infection status and diet ($F_{1,69}$ = 3.10, P = 0.083; Figure 6.5).

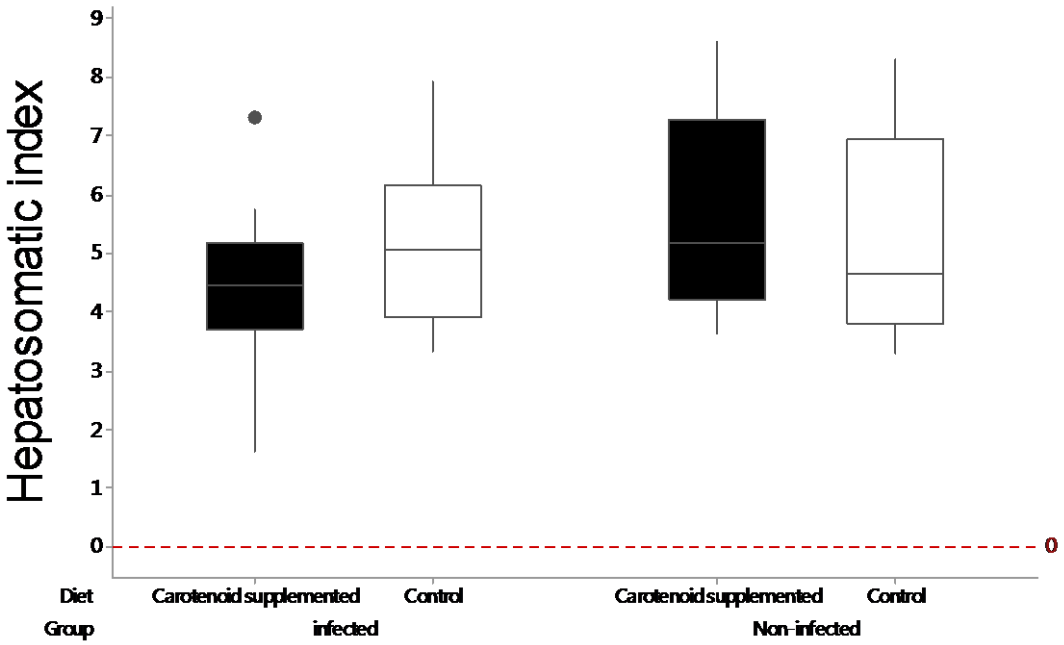


Figure 6.5 Hepatosomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented

6.4.2.4 Splenosomatic index (SSI)

In this study, both carotenoid supplementation ($F_{1, 69} = 11.20$, $P = 0.001$) and *S. solidus* infection status ($F_{1, 69} = 45.60$, $P < 0.001$) significantly affected SSI (Figure 6.7). Fish fed the carotenoid supplement had higher SSI values than those fed the control diet, and infected fish also had higher SSI values than non-infected fish. The effects appeared to be additive, with no statistical interaction between diet and infection status on SSI ($F_{1, 69} = 11.20$, $P = 0.255$).

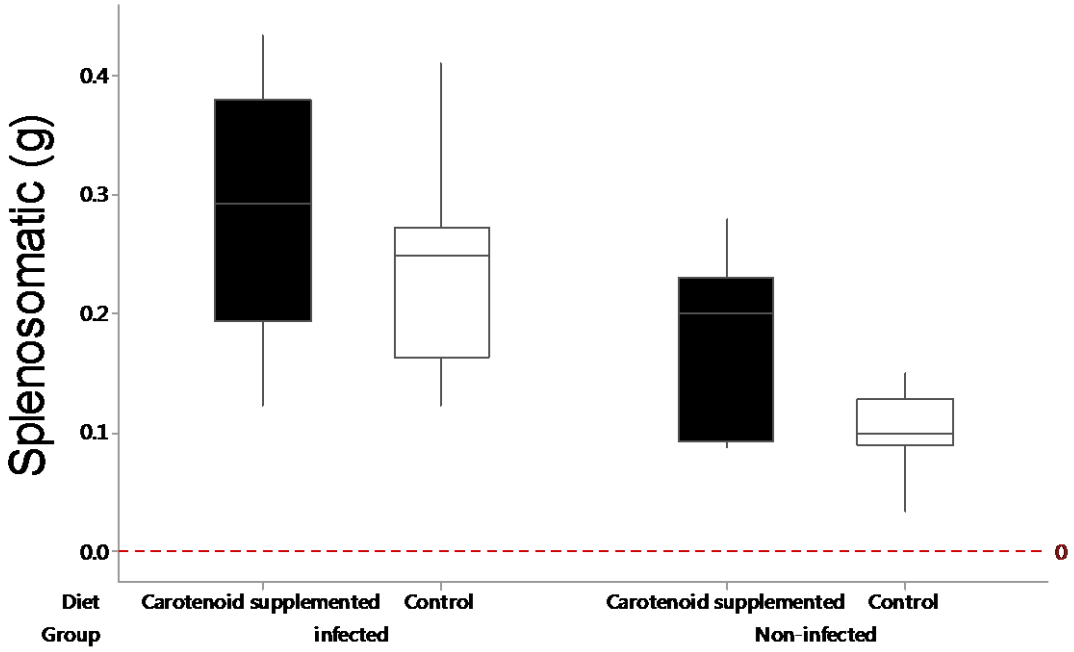


Figure 6.6 Splenosomatic of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control , non-supplemented or Carotenoid supplemented

6.4.2.5 Haematocrit

The results indicated that haematocrit was not influenced by carotenoid supplementation ($F_{1, 65}$ = 0.87, P = 0.354), but there was a significant effect of infection status ($F_{1, 65}$ = 9.52, P = 0.003), with infected fish showing higher haematocrit values than non-infected fish. There was no statistical interaction between the factors ($F_{1, 65}$ = 0.40, P = 0.532; Figure 6.7).

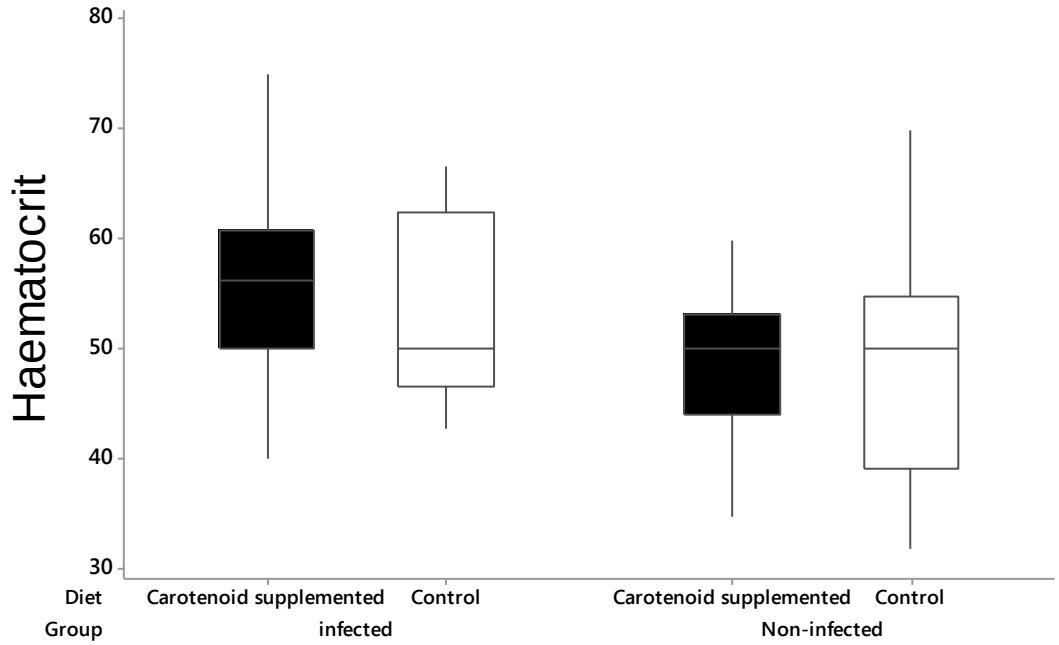


Figure 6.7 Haematocrit of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented

6.4.3 Effect of carotenoid supplementation and infection status on reproductive development of female and male fish

6.4.3.1 Females: Gonadosomatic index (GSI)

There was a significant effect of infection status on gonadosomatic index of female fish ($F_{1,29} = 6.17$ $P = 0.019$), with non-infected females having larger GSI values than infected fish. However, carotenoid supplementation was found to have no apparent effect on GSI ($F_{1,29} = 0.03$, $P = 0.854$). Furthermore, there was no statistically significant interaction between treatment and infection status ($F_{1, 29} = 1.52$, $P = 0.228$; Figure 6.8).

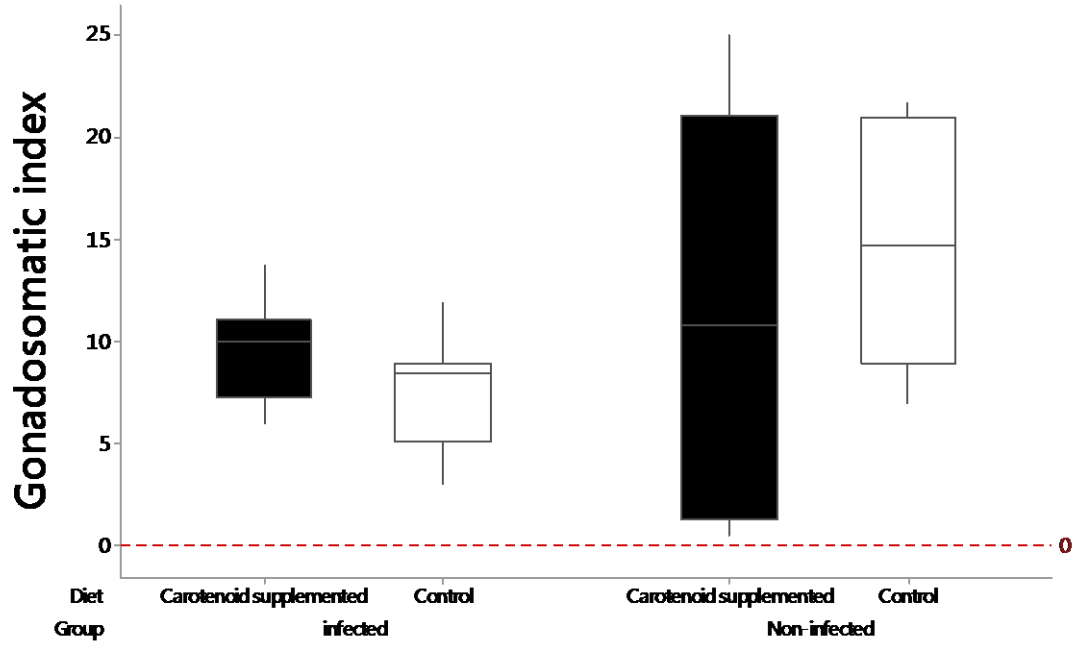


Figure 6.8 Gonadosomatic index of control (sham-infected) and experimentally *Schistocephalus solidus*-infected three spined sticklebacks in the study fed either Control, non-supplemented or Carotenoid supplemented

6.4.3.2 Males: Kidney somatic index (KSI)

The results showed that carotenoid supplementation had no significant effect on the KSI of male fish ($F_{1,36}$ = 0.83, P = 0.367), nor was there any effect of infection status ($F_{1,36}$ = 0.44, P = 0.509). There was no interaction between diet and infection status ($F_{1,36}$ = 1.94, P = 0.172; Figure 8).

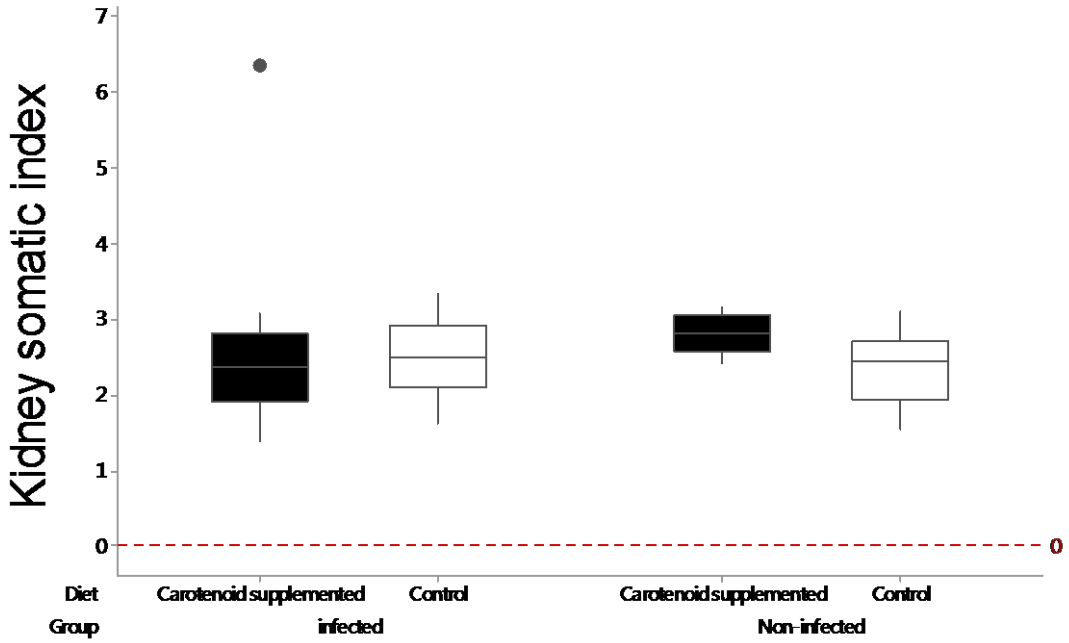
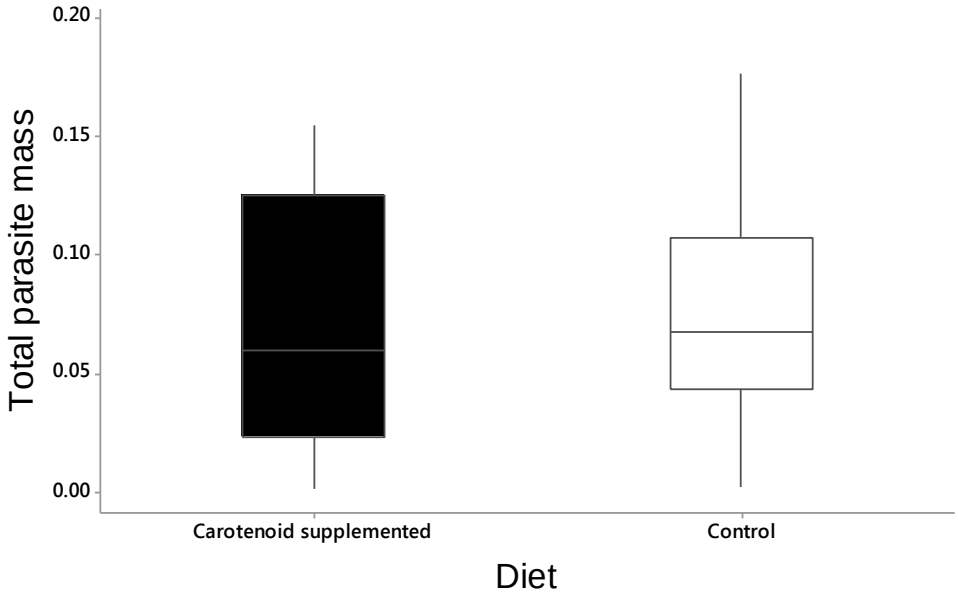


Figure 6.9. The effect of diet (dietary carotenoid and control) and exposure status on kidney somatic index (KSI) on Clatworthy Reservoir three-spined sticklebacks

6.4.4 Effects of host carotenoid supplementation on parasite growth

The mean mass of plerocercoids recovered from the infected fish in the study was 0.0724 mg, which exceeds the mass typically assumed to be associated with infectivity 50mg, (Tierney and Crompton, 1992). The mass of plerocercoids recovered from infected fish did not differ between infected fish maintained under the carotenoid-supplemented diet and under the control, non-supplmneted diet treatments (ANOVA: $F_{1,44}$ = 0.00, P = 0.948, Figure 6.10a). When the plerocercoid mass was expressed as parasite index, reflecting the proportional contribution to the mass of the infected fish, there was no significant difference found between the two dietary treatments, carotenoid supplemented and control ($F_{1,44}$ = 0.30, P = 0.584; Figure 6.10b).

(a)



(b)

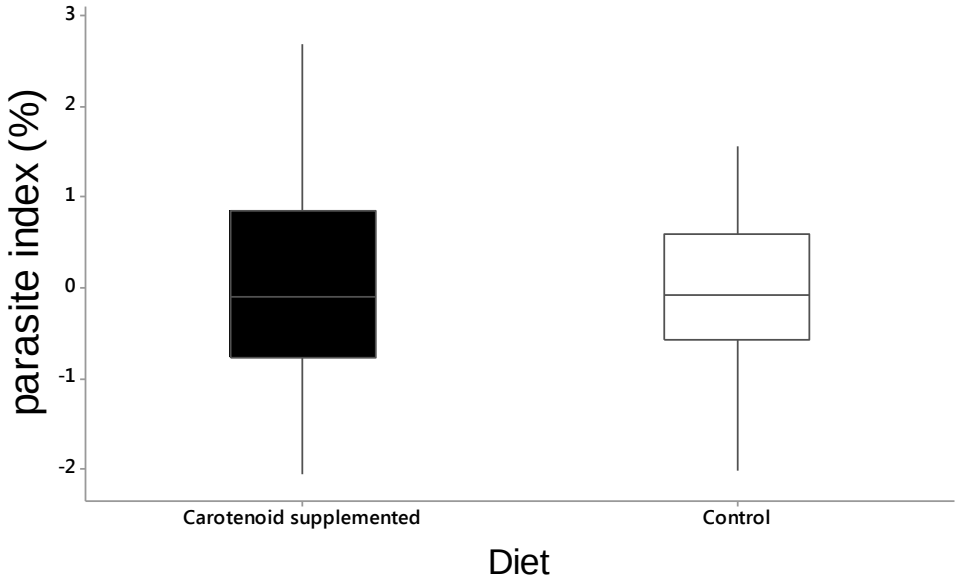


Figure 6.10. The size of *Schistocephalus solidus* plerocercoids recovered from infected three spined sticklebacks A; Total parasite mass giving absolute parasite size. B; Parasite index (*Ip*), showing parasite mass relative to host mass in the study fed diet carotenoid supplemented (filled bar) and control non supplemented (open bar).

6.5 Discussion

6.5.1 Effect of carotenoid supplementation on fish growth

Regarding the specific growth rate of sticklebacks in the study, the results suggest that supplementary addition of carotenoids to the artificial diet had no significant effects on specific growth rate. These results are consistent with the studies of Amar et al. (2004) and Baker et al. (2002), which showed that the astaxanthin diet did not affect growth. It could be possible from these results that three-spined sticklebacks limited access to dietary carotenoid can impose restrictions in specific growth rate. In agreement with this finding, specific growth rates were also found not to be affected by dietary carotenoid supplementation in rainbow trout (*Oncorhynchus mykiss Walbaum*) (Amar et al., 2004) or Atlantic salmon (*Salmo salar*) (Bell et al., 2000). Similarly, (Yi et al., 2014) found there were no differences in either absolute growth and specific growth rates among large yellow croaker (*Larimichthys croceus*) between control fish and those fed a carotenoid supplement. However, several studies have reported that carotenoids can influence fish growth. For example, Segner et al. (1989) found that carotenoid supplements had a positive effect on growth, whilst in shrimp, *Litopenaeus vannamei* dietary carotenoids have also been reported to enhance growth (Niu et al., 2009). The reasons for these discrepancies between studies are not clear. It could be that the type of carotenoid, or the species of fish involved, may be important, with fish growth being influenced in some combinations of carotenoid and species, but not in others.

On the other hand, the lack of effect observed in the current study might simply be related to the length or timing of the rearing period selected. In the present study, the period of supplementary carotenoid feeding was shorter than that typically used when compared to those of previous studies. For example, in a study of rainbow trout (*Oncorhynchus mykiss*), fish were fed carotenoid supplements for nine weeks (Amar et al., 2004). Studies on Australian snapper (*Pagrus auratus*) (Doolan et al., 2008), red devil, *Cichlasoma citrinellum* (Pan and Chien, 2009), farmed red porgy (*Pagrus pagrus*) (Chatzifotis et al., 2011) also

showed similar results to those reported here , although the feeding period in previous studies was more than 4 weeks.

Another possible explanation is that different carotenoids may have different effects on growth, with Korean rose bitterling (*Rhodeus uyekii*), fish fed on astaxanthin-supplemented diets showed a higher growth rate than those fed β-carotene or lutein and the control (Kim et al., 1999)

Several reports have shown that parasites can have a negative impact on host growth, in fish, in a wide range of animals, ectoparasite and endoparasite have considerable impacts on host by decreased growth rate. (Goater et al., 2013, Karvonen and Seppälä, 2008, Brinker and Hamers, 2007). in case the high parasite burden , the infection of *Diplostomum spathaceum*, can lead to negative effects in in white fish , which contributes to weight loss (Karvonen and Seppälä, 2008).

According to the results, it seems that a correlation exists between infection and fish mass. It is known that *S. solidus* infections impose energetic costs on their hosts and therefore can impact host growth, with infected fish often exhibiting reduced growth as a consequence (Barber et al., 2008) . It was therefore surprising, in the current study, that no differences in growth were found between experimentally infected and non-infected fish.

It seems reasonable to assume that sticklebacks infected with *S. solidus* can elevate regulation of feeding behaviour in fish in different ways. There are likely causes for these results; the non-effect on growth serve as an attempt to boost the species existence by bettering the threat of malnutrition, thus. the diet was sufficient for both fish and parasite and enabled the host to outlive infection (Ballabeni, 1994). Another possible explanation is that the lack of competition meant that the fish were able to support parasite growth without impacting their own growth rates.

This finding is contrary to a previous one, where enhanced growth was identified in different animals infected with trematodes (Probst and Kube, 1999, Miller and Poulin, 2001). The findings of the current study support previous research (Øverli et al., 2001a), which found that fish mass (excluding parasite mass) of infected and non-infected were not different statistically.

6.5.2 Influence of carotenoid supplementation and *S. solidus* infection on indices of health in sticklebacks

Regarding the various health indices of sticklebacks examined in the current study, the results demonstrated that the diet used in this experiment during the pre-exposure period (control and carotenoid supplemented artificial diets) and the diet provided during the post-exposure period (bloodworms) were sufficient to support growth and health among both infected and non-infected fish.

In this study, neither carotenoid supplementation nor infection by *S. solidus* had an effect on liver mass. In previous studies, *S. solidus* infections were found to be associated with a reduction in liver size (Tierney et al., 1996), although a reason for this may be that the study was carried out with wild-caught fish, which have to compete for access to food, and so this is the most likely reason for the more significant effect.

The fact that liver mass was not reduced even in infected fish, may indicate that it is more likely that the feeding regime in the experiment was sufficient to sustain parasite growth, host growth and energy storage in infected fish. (Segner et al., 1989).

The spleens of infected fish were enlarged compared to those of non-infected fish. Evidence from other studies indicates that spleen size among infected sticklebacks increase in relation to the mass of infecting plerocercoids (Arnott et al., 2000, Kalbe and Kurtz, 2006). The spleen is a lymphoid organ, and its enlargement (splenomegaly) typically indicates greater levels of activation associated with immune stimulation (Seppänen et al., 2009) and potentially reflects the immunocompetence status of fish (Zapata et al., 2006, Seppanen et al., 2009). The enhanced spleen sizes shown by infected sticklebacks in the present study most likely reflects the increased activation of immune responses as a result of the experimental parasite challenge (Arnott et al., 2000). Thus, supplemented carotenoids increased spleen size which might indicate that they promote immune response, similar to results also found in Kolluru et al. (2006) , (Blount et al., 2003) ,(McGraw and Ardia, 2003) and (Grether et al., 2004)

In addition, the data presented here showed higher spleen mass in fish fed supplemented carotenoid compared with fish control diet. Carotenoid is an essential supplement for physiological functions such as encourage the growth, pigment source and enhancement of the resistance of diseases in fish (Amar et al., 2001, Christiansen et al., 1995b, Pike et al., 2007a).

As well as having physiological functions, carotenoids are a valuable source of vitamin A. (Lorenz and Cysewski, 2000). Some types of carotenoids, including xanthophylls, astaxanthin, canthaxanthin, and isozeaxanthin, act as precursors for the synthesis of vitamin A in fish and birds (Olson, 1983). The immune response may be affected physiological functions by the use of carotenoid precursors of vitamin A as diet supplements. Processes such as normal development, (Halver, 2002). Some studies have reported evidence for the immunostimulant effects of carotenoids (Christiansen et al., 1995a, Bédécarrats and Leeson, 2006, Saino et al., 1999). The findings of the current study support the previous research by (Tachibana et al., 1997), who found that the supplementation of carotenoid caused increase in spleen mass.

In the present study, *S. solidus* infection caused an effect on haematocrit, the data presented here show that infection caused a significant increase in haematocrit, with infected fish showing higher haematocrit than uninfected fish. These results showed the improvement of fish health which increased their ability to fight off infections through increasing the haematocrit. However, research by (Řehulka, 2000) was conducted to examine the impact of carotenoids, results indicated that no significant total red blood cell count variations occurred in shrimp assigned to the treatment group.

On the other hand, carotenoid supplementation had no an effect on haematocrit the data presented here show that no difference between control and carotenoid supplemented , this finding was also reported by Eeva et al. (2008) who showed that carotenoid supplementation in common carp, *Cyprinus carpio*, did not affect haematocrit levels, while the white blood cell count was higher compared with those fed the control diet in in common carp as well (Sowmya and Sachindra, 2015). A possible explanation for these results might be that the lutein and astaxanthin used in this study had no effect on haematocrit compared with other

type of carotenoid. Our results do not agree with previous observations by (Yonar, 2012), who found that haematocrit increased in group offered carotenoids compared to a control group. Similarly, in a study of the hematological parameters of hybrid catfish (*Clarias macrocephalus* × *C.gariepinus*), the haematocrit was lower for non- carotenoid supplemented compared to fish fed carotenoid supplemented (Chow et al., 2016)

There are a number of ways that parasites affect their hosts. The fish's reproductive health is often adversely influenced (Hurd, 2001). The results of the present experiment demonstrate that *S. solidus* infection can lead to a reduction in the size of the gonads of females. In general the infection of S. solidus is often associated with reduced sexual development of female stickleback hosts (Hurd, 2001, Sitjà-Bobadilla, 2009). This may be because to the continuous energy drain effect created by the parasite, resulting in the diseased stickleback being unable to ensure that its ovaries proceed through the pre-spawning stage. (Arme and Owen, 1967).

In this study, the lowest gonad somatic index values were associated with S. solidus infections these results are in accordance with the previous findings of (Heins, 2012, Arme and Owen, 1967). Plerocercoids grow using host-derived nutrients (Barber et al., 2008), and therefore Infectious pathogens can decrease overall energy levels, which can, in turn, adversely impact upon an organism's growth, sexual development and capacity to store energy. This can ultimately lead to alterations in egg production patterns, characterised by diminished or total depletion of supply (Hurd, 2001) related to host nutritional status.

The results obtained revealed that, compared to non-infected fish, infected fish displayed a smaller gonad size, probably owing to the higher parasite mass and the fact that parasites of larger size have greater nutrient requirements.

There was no significant effect of carotenoid supplementation on the gonadosomatic index of sticklebacks. It is possible that in the current study, the duration of feeding carotenoid was not sufficient to enhanced development of gonads.

In males, there was no significant effect of either *S. solidus* infection or carotenoid supplementation on kidney somatic index, which is used as an indicator of male

sexual development (Borg et al., 1993) On the other hand, males display the effect of carotenoid as sexual nuptial colouration on throat, the current study did not evaluate the level of carotenoid deposits on the skin.

6.5.3 Parasite growth and host carotenoid supplementation

In this investigation, the results showed that pre-feeding with dietary carotenoid supplements had no significant effect on the subsequent growth of parasites within infected stickleback hosts. Thus, there was no difference in the size attained by plerocercoids recovered from infected fish that had been fed the control diet or the carotenoid-supplemented diet pre-exposure. In this experiment, sticklebacks were fed carotenoid supplements (lutein and astaxanthin) for four weeks prior to parasite exposure. This result suggested that the pre-exposure carotenoid supplemented had no significant impact on subsequent parasite growth. All plerocercoids recovered from experimentally infected fish exceeded 50 mg, the mass considered infective to definitive hosts (Tierney and Crompton, 1992).

This finding is inconsistent with that of Baeta et al. (2008) European blackbirds (Turdus merula) were fed a diet that for four weeks was supplemented with carotenoid. As a consequence, the bird's immune response was effective in reducing the rate of replication of the parasite, Isospora. . Another possible explanation for these findings is that at the amount of carotenoid in the diet used in this study, which was 200 µg g /100g, was sufficiently high level for three spined sticklebacks according to Pike et al. (2007a) but did not affect parasite mass. Despite the large size of parasites in this study, they did not affect the specific growth rate, or health status of fish

6.5.4. Conclusions and future studies

This study that no effect of carotenoid supplemented diet (containing lutein and astaxanthin) on susceptibility to S. solidus infections and in fish growth. However, the results show that carotenoid supplemented diets fed for four weeks post-parasite exposure / sham exposure, and experimental *S. solidus* infections, both generated higher splenosomatic index values in sticklebacks. It would be useful to know what the mechanistic link to be between carotenoid supplementation and

fish growth. because carotenoids boost immune protection and therefore protect the fish from expending energy fighting infections Further investigations on the carotenoid supplemented diet in three-spined sticklebacks the effect of carotenoid supplemented for a longer time and the determine whether infection *S. solidus* affects the supplement of carotenoid-associated sexual ornamentation in male sticklebacks infected with *Schistocephalus solidus*.

Chapter 7 General discussion

7.1 Overall aims of the research and the approach taken

The management of disease, including viral, bacterial and parasitic infections, has become an issue of increasing concern in fish farming and aquaculture-related industries (Johnson and Paull, 2011). A group of organisms displaying great diversity, parasites can extensively impact hosts, populations and ecosystems from a biological perspective (Lemly and Esch, 1984, Hurd, 2001, Hudson et al., 2006). The term 'parasitism' refers to a non-reciprocal symbiotic relationship between two different species in which one species (i.e., the host) is taken advantage of by another (i.e., the parasite). Parasites typically deprive the host of nutrition and therefore infections can have a significant impact on various biological functions, including growth and reproductive development.

Numerous scientific studies have explored various methodologies to combat resistant aquatic health issues, with the management and relevant adjustment of diet posed as amongst the most effective ways to combat disease and parasitic infestation. Through a series of experimental investigations using the three-spined stickleback-*Schistocephalus solidus* experimental host-parasite model, the effect of host nutrition on the interaction between fish and their parasites was studied.

In this thesis, the three-spined stickleback is an attractive model for investigating a wide range of questions in the biological sciences, to investigate the effect of host nutrition interaction between fish and parasite

Firstly, identifying the impact of food quality and quantity on fish health and parasite growth was investigated in chapters 3 and 4.

Furthermore, they determine the effect which infection has on dietary choices and whether it has any effect on food choices of the host fish, with regards to quality. They also determine whether these changes are a benefit for parasite, host, or neither. Finally, in respect to the effect of additive nutrients on interactions between fish and parasites, I will summarise the effect of host nutrition as an important factor to host-parasite interactions, the main results will be summarised, and future research will be suggested.

7.2 Summary of main findings of each study

The main aim of the research was to understand how aspects of host nutrition influenced parasitism in fish, including the impact on indicators of host condition and health, the ability of host fish to withstand parasite infections, and the impact on emergent host-parasite interactions, including host phenotypes and patterns of parasite growth and fitness. One of the most successful and sustainable control approaches thus far is through the management of adequate nutrition, which can substantially strengthen the ability of fish to protect themselves against disease. Poor diet is a major source of immunodeficiency, which leaves fish vulnerable to infections. Moreover, factors that contribute to the stress of fish populations, such as a limited or poor quality food supply, are regularly attributed to the outbreak of disease. Diet is, therefore, one aspect of fish life that may be managed and manipulated in aquaculture operations in order to sustain the good health of cultivated fish (Lall, 2000). the physical properties of diet, associated with nutritional content, can increase the risk of infection occurring in fish (Lall, 2000).

7.2.1 Effects of food type on fish health and host-parasite interactions

The food generally can have important biotic factors for interactions between fish and parasites. The aim of this portion of the research is to study which type of food affects the interaction between the hosts and parasites, with regards to growth, development, and health of the fish and also the rate at which *Schistocephalus solidus* plerocercoids grow by using common natural diets of three sticklebacks.

The results showed that the type of food can have an impact on fish growth, and that the effect of a diet where fish were fed *Artemia* and bloodworm was that of increased growth in fish and the specific growth rate of fish. Improved health indices were observed in fish held under the *Artemia* and bloodworm diet treatments, compared to the *Daphnia*-fed fish. This could potentially be due to the high digestibility and palatability (Stejskal et al., 2018). An addition the levels of protein and lipids contained in these diets could play an important role in increased growth, and have been recognised as a major factor in increased growth. In the studies of (Armitage, 1995, Stejskal et al., 2018), composition of diet was found to be an important factor in determining fish growth ((Webster et

al., 1995, Abdel-Tawwab et al., 2010). With the moisture content of food being a factor which could lead to a decrease in fish growth (3.1).

In addition, clearly the results of this study indicate that the type of food can affect disease progression in body condition factor only, but other parameters are not affected by infection. Generally, many studies have investigated the negative impact on various biological functions (Giles, 1983, Barber and Arnott, 2000, Schultz et al., 2006, Seppälä et al., 2008) . In our study, infection was found to have no significant effect on fish condition. This was an expected result, and might possibly be due to the fish being caught from the wild and therefeore these results suggest that there are other factors that might influence the infection status of the fish, which may be affected by other parasites, viruses or bactaria.

there is evidence from previous studies that decreased immune response can be associated with inadequate of protein and amino acids (Li et al., 2007). There is abundant evidence that Natural or commercial diet increased growth and development of fish,protecting it from the risks associated with infection . In addition, the studies presented in chapter 3 provide evidence that parasitic infection is also considered to be reliant on the quality of the host's feeding patterns, In studies of the immune health of Pacific Salmon, for example, an insufficient quantity and quality of food was determined as the cause of their weakened resistance to disease (Alcorn et al., 2003).

7.2.3 Impact of parasite infections on the dietary preferences of sticklebacks

The effect of parasite infection is one of affecting the preferences of three-spined sticklebacks' prey types, as investigated in chapter 5,

Fish harboring infective parasites show reduced antipredator behaviour that could increase predation susceptibility and may potentially arise from adaptive parasite manipulation,

Where previous studies have shown that parasites may have significant effects on many of the physiological characteristics, showing altered behaviour that could ultimately lead to fish mortality (Bagamian et al., 2004, Barber et al., 2000, Lester and Adams, 1974).

In this chapter, I focused in the effect of parasitic infection in terms of preference for certain types of food, where parasitic infection reduces preference for the *Artemia* diet which resulted in a reduced proportion of time spent in the *Artemia* zone and non-infected fish also showed a decreased preference for *Artemia*

However, infection did not affect the food preference in terms of artificial diet or Bloodworm. In nature, a wider range of food types would be available and so there may be variations in the diet seen under more natural foraging situations

Another possible explanation is that a limited range of food types were presented in this study, and prior familiarisation with some of the foods (bloodworms) could have played an important role.

In addition, the first prey item chosen by the fish was bloodworm. Moreover, as the explanation for the preference of many fish species for particular types of prey. The visual characteristics of prey may stimulate sticklebacks to consume (movement, colour, size, odour) a particular prey type, though the colour of the food could contribute considerably to the feeding preferences of sticklebacks (Popham, 1966) . In the present study, the artificial diet offered to three-spined sticklebacks had the same colour as their preferred prey.

In a previous study by (Johannesen et al., 2012) three-spined sticklebacks use their sense of smell to find food, especially when visibility is poor as in turbid water. Other factors could stimulate their preference in diet, for instance palatability (Villamizar et al., 2009, Gibson, 1980, Wootton, 1984).

As an almost logical outcome from this research, there appears to be a preference amongst sticklebacks to rely on visual and olfactory senses due to their eyes being highly developed (Beukema, 1968). This is not the only factor, however, as there are clearly other factors that contribute to dietary preference. The most crucial factor probably relates to features of the prey that might be expected to be important in prey selection.

The shape of the prey in the artificial diet offered to fish was the same as that of Bloodworm; the diet was extruded using a syringe with a 1.2 mm orifice and chopped to accommodate the size of the mouth of the three-spined sticklebacks (chapter 2).

7.2.4 Effects of carotenoid supplementation on fish health and host-parasite interactions

In considering the potential impact of carotenoid supplements, the effect of carotenoid properties is determined to be pivotal in increasing the nutritional value of fish food in particular, and animal feed in general (Jeney et al., 1997, Spolaore et al., 2006, Kolluru et al., 2006, Meilisza et al., 2017). Dietary carotenoids of various types have been used as dietary supplements (containing lutein and Astaxanthin). Carotenoids, as precursors of vitamin A (Olson and Owens, 1998), can have an impact on immunity in fish (Halver, 2002, Trichet, 2010, Oliva-Teles, 2012).

In this current study, the utilisation of additive nutrient such as carotenoids, represent source to enhance host health and physiology in fish.

It appears that fish fed supplementary carotenoids showed higher splenosomatic indexes as one of indictor immunity in fish (Seppanen et al., 2009) which could be an indication that carotenoid may stimulate immune responses that are associated with enlargement of the spleen , in agreement with studies Consistent with other research, Tachibana et al. (1997) reported that feeding parrot fish larvae with rotifers enriched with b-carotene led to greater numbers of spleen lymphocytes.

In addition, infected fish had higher splenosomatic indexes than non-infected fish, which could be an indication that fish health may influence immunological processes. Previous studies have shown that size is larger in infected fish than non-infected fish (Kalbe and Kurtz, 2006), carotenoid supplementation have potential to affect the immunity response (Kolluru et al., 2006),

Moreover, haematocrit was used as an index of physiological status (Kalbe and Kurtz, 2006), and in this investigation the observed significant increase in haematocrit in infected fish compared to non-infected fish reflects a strong physiological status in fish. These results suggest that carotenoid can influence the increasing of red blood cell levels as a response against infection in sticklebacks. Our results are consistent with those of a recent study (Jagruthi et al., 2014)

Despite these obvious beneficial effects towards the immune status of three-spined sticklebacks. Even though there was no effect on growth and fish condition, which did not vary significantly between the infected and control sticklebacks, the present results indicate that at 200 mg/ kg, carotenoids do not significantly affect in their ability to growth. This result was in agreement with those in previous studies. (Yi et al., 2014, Baker et al., 2002)This may be a result of the small quantities of carotenoid used in this experiment. Similar results were also reported by (Amar et al., 2001) because, The significant contributions of some micronutrients, such as carotenoids, are not as obvious as size development. A possible explanation for this could be that carotenoid were presented to fish for 4 weeks, these results are in agreement with previous work (Baeta et al., 2008)

One possible explanation here is that fish invested the supplemented carotenoid toward affecting flesh coloration rather than investing in growth (Baker et al., 2002), although colour deposition in skin was not measured in this experiment

7.3 Synthesis and main conclusions

The major results of this thesis are that host-parasite interactions are influenced by host nutrition. In summary, the current study indicates that food restriction, either in terms of quantity or quality has physiological implications that may impact the functioning of immune systems. Under certain conditions of feeding fish may have impact on growth of parasite

The present study suggests that there is the potential for using bloodworm and *Artemia* as part of the sticklebacks' rations as a natural diet that could prevent the build-up of plerocercoids, whilst at the same time leading to fish growth with good conformation. The increased immune response as a result of carotenoid supplements also suggests that sticklebacks showed increased immunity characteristics such as an increased splenosomatic index and haematocrit.

7.2.2 Effects of host dietary ration and protein content on fish health and host-parasite interactions

The experiments undertaken in Chapter 4 were designed to investigate the effect of (a) the level of dietary protein contained in artificial food and (b) host ration (i.e.

quantitative differences in the amount of food ingested), on the health of sticklebacks and their host-parasite interactions.

Recently, it has been reported that protein could play an important role in the functional induction of the immune response in fish (Trichet, 2010, Pascual et al., 2004) The present study discovered that the spleen-somatic index depended significantly on the level of protein. It seems that the energy possessed by the fish was sufficient to stimulate the spleen to enlargement in size.

In terms of the effect of dietary protein in infection , the results showed that the effect of *Schistocephalus solidus* on spleen enlargement which were more severe in the infected fish which received lower amounts of protein (Dobson and Bawden, 1974). Infected fish fed high protein diets and bloodworm had significantly higher condition factors. It can be assumed that the fish had sufficient usable energy to both support the parasite and let it grow.

Increased levels of protein were found to increase *S. solidus* plerocercoid size in experimentally infected sticklebacks.

This points to the possibility that the fish in this study had the capability to procure enough nutrition to sustain both themselves and the parasites they were harboured. One of the most probable explanations could be the fact that there was no shortage of food, contrary to the natural situation.

One limitation of this study may be that the low protein levels fed to both the control fish and those infected with *S. solidus*, challenged their overall nutritional status resulting in the fish and parasites' slower rate of growth, thereby imparting a potentially detrimental effect on the research outcomes.

On the other hand, increases in health, immune indices and growth were more pronounced, among fish fed high protein and bloodworm diets. Likewise, the bloodworm diet used in Chapter 3 showed a positive effect on growth fish and Some research has indicated that fish on high protein level have higher the Immunological indicators of proten (Sitjà-Bobadilla et al., 2005).

This is also in accordance with earlier observations identified in Chapter 3, which showed the health implications associated with improving the health and welfare of fish based on nutritional value

Nevertheless, infection did not affect the health indices of fish fed the high protein diet or the bloodworm diet, or result in adverse consequence such as reduced weight and decrease of splenonsomatic index. Obviously, a balance should be struck between the beneficial aspects of high quality feed and its expense. The exact mechanism connecting host protein with parasitic growth is not yet known and should be additionally researched.

As expected, the results of studies described in chapter 4 showed that low protein limited growth in fish and parasites, however, increases in the spleen somatic index, as an indicator of immune activation, were observed in these groups. This result suggested that increased spleen size of fish that are kept on low protein diets could be caused by inadequate levels of protein. Another intervening factor could be the effect on health condition; carbohydrate diets are known to affect growth in carnivorous fish (German et al., 2009).

Food ration may also have important implications for both parasite and host. There were no significant effects of ration treatment on the parasite index of infected fish (chapter 4, experiment 2)

Parasitic growth and reproduction rates are subject to the availability of food for the host. Immune strength and responses rely upon energy from the food ingested by the host and hence it can be said that vulnerability to infections depends directly on host nutrition .Fish could choose to increase or decrease their food intake after they become infected , Frost et al. (2008) posits that in the case of *Daphnia* magna, infected by Pasterium ramosa, supplying the host with food options rich in phosphorous reduced the spread of infection , also it might be the case that infection limits the fish's ability to compete for food , previous research noted that the fastest swimmer in a group of sticklebacks feeding on cladocerans accessed more food than the other individuals (Milinski, 1982).

Infection may also reduce swimming performance, Infection by trematode *Ascocotyle pachycystis* encysts obstructed the flow of blood in sheepshead

minnows (*Cyprinodon variegates*) thus, the fish were unable to swim normally (Coleman, 1993).

In fact, the highest recorded of Specific growth rate and fish mass were in infected fish. This was an unexpected result, because the parasite impaired growth (Karvonen and Seppälä, 2008, Brinker and Hamers, 2007), This finding may be indicative of health status. The evidence presented here suggests that rather than reducing the growth of infected fish, it increased it, because the infected fish expend less energy in locomotion (maybe they swam less) (Coleman, 1993) Another explanation, however, is that the growth parasite from procercoid stage to plerocercoid stage did not affect the fish growth and fish mass

which is in disagreement with previous studies (Grimnes and Jakobsen, 1996) who determined that uninfected fish were greater in condition factors than those who had been infected, due to *S. solidus* infected fish are known to have a higher metabolic demands than non-infected fish (Arme and Owen, 1967) Although , in this study, there were no significant differences in parasite mass between treatments.

The evidence presented here suggests that host ration can influence the Splenosomatic Index in fish. Interestingly, the host medium ration food has more impact on the Splenosomatic Index, while the results showed that this appears to affect physiological status as higher haematocrit levels were found under the medium ration, suggesting that fish were able to adapt their health status to the medium ration more than the high ration.

For some teleost and other fish, digestion is optimised for the purpose of facilitating nutrient extraction in an efficient way at reduced feeding rates

(Van Ham et al., 2003, Zoccarato et al., 1994). As the study presented in this thesis in chapter 4 the immune parameters, (splenosomatic index) finding that reduced feeding regimes had no adverse association with immunity.

7.4 Suggestions for future work

Overall, the results presented in this thesis illustrate how the improvement of host nutrition might contribute to the improvement in fish response to infection by parasites.

I have shown a medium ratio rather than high ratio is the most important factor for three-spined stickleback in acquiring an optimal immune response and physiological status. The mechanism of this could be due to fish being able to adapt their health condition with the medium ration to a greater extent than the high ration.

The main factor influencing both host sensitivity to infectious diseases and parasite growth and development is the body size of the host. (Poulin, 2011)

As a consequence of their under-developed immune response, the mortality rate for juvenile fish is higher than that for adults.(Sol et al., 2003).Future studies should focus on how the age of the host and host ratio factors interact to determine overall immune response.

The results indicate that high protein diets and bloodworm provide adequate nutrition for both host and parasite. These results indicate that further research is crucial to the determination of the appropriate nutritional level of dietary protein and its consequences for the health of fish in long-term feeding studies.

The effects of additive nutrients on host parasite interactions in fish was demonstrated in chapter 6 by using two types of carotenoid, lutein and astaxanthin, for four weeks prior to exposure to infection. In view of this, future research should aim to investigate the effects of carotenoid supplementation for longer periods. In fact, carotenoids are known to enhance the immune response and are largely responsible for producing the bright colour in the body which motivates attraction by females (Olson and Owens, 1998, Kolluru et al., 2006, Amar et al., 2004). Further studies are required to investigate whether carotenoids could play role in dietary preference of fish..

In this research, I have not focused in any way on the effects of carotenoid and parasite infection in the nuptial colouration.

elaborate carotenoid-based sexual adornments are created by the males of numerous fish and bird species (Olson and Owens, 1998).

The result of an empirical infection study indicated that the kidney somatic index was not affected by carotenoid supplements. By contrast, GSI was significantly affected by carotenoid supplements to diet. However, this study did not measure

the quantity of carotenoid accumulated in the skin. Further studies are required to investigate the ability of carotenoids to enhance the immune system with ornamental carotenoid-based coloration.

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