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Effects of acanthocephalan parasites on colour and carotenoid quantities of their intermediate host *Gammarus lacustris* in the context of variable lake productivity

by

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Dedication

To my wife Ana and her dog Laica, who courageously protected us from wild 'beasts' in the field and let me name one of the studied lakes after her.

Abstract

I studied the effects of *Polymorphus paradoxus* Connell and Corner and *Polymorphus marilis* Van Cleave on colour and carotenoid quantities of their host amphipod *Gammarus lacustris* G.O. Sars in seven lakes in Alberta, Canada, that differed in productivity. *Polymorphus marilis* induced 'pigmentation dystrophy' and a higher degree of blueness in both amphipod sexes than did *P. paradoxus*. Colour shifted toward lighter, bluer and greener tones, denoting less carotenoids, in amphipods parasitized by *P. marilis*. This is the first study to measure host-colour changes induced by parasites using consistent, repeatable digital analysis. Maximum carotenoid quantities were reached in hypereutrophic lakes where parasite-induced colour changes were also generally minimal. I developed regression models that accurately predict carotenoid quantities in amphipods by their colour, parasite status and water chemistry. This is the first study showing that carotenoid quantities and parasite-induced colour change in crustaceans depend on lake productivity.

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Table of Contents

List of Tables

List of Figures

Chapter 1 - Introduction

1.1 Life cycle of *Polymorphus paradoxus* and *P. marilis*

Acanthocephalans, or thorny-headed worms, are parasites that are trophically transmited from intermediate to final hosts. In Alberta, Canada, the two best-studied species are *Polymorphus paradoxus* Connell and Corner and *P. marilis* Van Cleave (Palaeacanthocephala: Polymorphida: Polymorphidae). These *Polymorphus* species use mammals and birds as final hosts, and the amphipod crustacean *Gammarus lacustris* G.O. Sars as the intermediate host. The appropriate definitive hosts for the two species differ. While *Polymorphus marilis* develops in waterfowl species including the lesser scaup duck, *Aythya affinis* (Eyton) and the redhead *Aythya americana* (Eyton), *P. paradoxus* infects both waterfowl species, and aquatic mammals such as muskrats *Ondatra zibethicus* (Linnaeus) and beavers *Castor canadensis* Kuhl (Denny, 1969).

Adult acanthocephalans absorb nutrients from the final host intestines. A single final host can suffer infestation of hundreds of *Polymorphus* adults producing intense hemorrhage or blockage of gut lumen as acanthocephalans penetrate and attach themselves to the gut walls of the hosts with their spiny proboscis (Bush *et al*, 2001). The life cycles of the two parasite species are similar (Figure 1-1). Fertilized eggs (and the first larval developmental stage or acanthor) are ingested accidentally along with detrital food material by *G. lacustris*. The acanthor's body consists of three syncytial masses, hooks and and body spines, and absence of any organs or nervous system (Albrecht *et al*, 1997). At this stage acanthocephalan larvae are immotile, and absence of nervous system suggests that the acanthor stage is not fully developed. It is believed that it reaches full development when the acanthor is ingested by the intermediate host and becomes motile (Albrecht *et al*, 1997). Newly hatched acanthors penetrate through the gut wall of the crustacean into the haemocoel where they develop into the acanthella, a stage in which the parasite larva develops distinct tissues and organs (Duclos, 2006). The last larval stage found in amphipods and infective to final hosts is called the cystacanth, which is characterized by complete invagination of the fore-body and posterior end into the hind-body (Denny, 1969). *Polymorphus*

paradoxus and *P. marilis* have lemon-like shaped cystacanths. In other species, the infective stage has a 'sausage-like' shape, in which case only the proboscis and neck invaginate. Besides the shape differences between the infective larvae, they seem to differ also in the degree of development of the sexual organs. The elongated type has sexual organs more developed than the lemon-like type (Mehlhorn, 2008). Encystment seems to be an adaptation to avoid mechanical damage when the intermediate host is consumed by the final host (Mehlhorn, 2008). Cystacanths have a proboscis that is similar in size, shape and armature to that of adults (Nickol *et al*, 2002). When an apropriate final hosts ingests a parasitized amphipod, liberated cystacanths develop into adult male and female acanthocephalans. Acanthocephalan adults attach to the small intestine of birds and mammals but their position is not permanent and they can reposition themselves in different regions of the intestines (Bush *et al*, 2001). In the gut lumen females can be subsequently copulated and inseminated by several males. Females release fully embryonated eggs.

1.2 Manipulative parasites – an overview

Bethel and Holmes (1973, 1977) were the first to describe abnormal phototaxis and escaping behaviour in freshwater amphipods harbouring cystacanths of acanthocephalan parasites. Since then, manipulation of hosts by parasites has become a research topic of wide interest for parasitologists and evolutionary biologists in general (Thomas *et al*, 2005). Studies have described phenotypic alterations (Hindsbo, 1972; Oetinger and Nickol, 1981; Bakker *et al*, 1997, Benesh *et al*, 2009), physiological modifications (Bentley and Hurd, 1993; Plaistow *et al*, 2001; Rojas and Ojenda, 2005; Dezfuli *et al*, 2007) and behavioural manipulations (Cezilly *et al*, 2000; Bauer, 2005; Baldauf *et al*, 2007; Perrot-Minnot, 2007) induced by acanthocephalan cystacanths in aquatic crustaceans. These effects include modified colour, weight loss, increased serotonin and dopamine levels, increased glycogen concentration or decreased fatty acids depending on the amphipod sex, increased predation and feeding rates, altered phototaxis, clinging and escaping behaviour and immunodeficiency. Such modifications are often interpreted as being adaptive manipulation by the parasite

in order to increase the likelihood of its transmission to a final host (i.e. Medoc *et al*, 2006; Seppala *et al*, 2008). Thomas *et al* (2005) highlighted the progress made in the research topic of manipulative parasites and underlined some directions for future studies, concluding that parasitic manipulation still remains an exciting and promising research area based on its complexity.

1.3 Colour, a phenotypic trait 'manipulated' by parasites

Colour is one of the phenotypic traits affected by parasitism. Colour modifications in parasitized invertebrate hosts have seldom been studied in detail, and it is not clear how changes are produced, what are their effects on the parasitized host, and whether they truly represent an evolutionary manipulation enhancing trophic transmission of the parasite. Although behavioural modification of parasitized gammarid amphipods has been a hot topic (Thomas *et al*, 2005), colour modifications in these particular parasitized hosts have been reported only by one study, Hindsbo (1972). The author observed that the amphipod *Gammarus lacustris* G.O. Sars parasitized *by Polymorphus minutus* (Goeze) from ponds in Denmark appeared bluer than non-parasitized ones. He also noticed modified phototropism and increased predation by domestic ducklings on the blue amphipods. Other authors have reported different colour modifications in parasitized crustacean hosts. Benesh *et al* (2009) described a darker abdominal colouration in isopods *Asellus aquaticus* (Linnaeus) parasitized by *Acanthocephalus lucii* (Muller) cystacanths. Increased light transmission through the opercula of mature isopods *Asellus intermedius* Forbes infected by *Acanthocephalus jacksoni* Bullock suggested "pigmentation dystrophy" (i.e., general colour loss, as in becoming paler or 'whiter') in parasitized hosts (Oetinger and Nickol, 1982). A similar pigmentation dystrophy induced by the acanthocephalan parasite *Centrorhynchus* sp. was recorded in the terrestrial isopod *Atlantoscia floridana* (Van Name) (Amato *et al*, 2003).

In addition to reporting the colour modifications in parasitized hosts, a few researchers have investigated the role of colour in parasitic cystacanths that accumulate carotenoids. These authors usually address only the influence of colour of the parasite itself, and not also that of the host, in determining its

possible adaptive role of colour in parasite trophic transmission to final host. Despite the increasingly large body of research on manipulative parasites, phenotypic modification, including of colour, is a phenomenon whose role in trophic transmission of parasite larvae to final hosts is still debated. Thus, Bakker *et al* (1997) were able to demonstrate that *Gammarus pulex* (Linnaeus) parasitized by *Pomphorhynchus laevis* (Zoega) were preyed on more by stickleback fish than were non-parasitized amphipods. However, their study had two main design flaws (Cezilly *et al*, 2010). In order to mimic parasitized amphipods, some non-parasitized individuals were painted red on a body side but without considering the match in colour between real parasites and the paint, and second, sticklebacks are not the appropriate final host for *P. laevis* (Hine and Kennedy, 1974). Kaldonski *et al* (2009), experimented with the same parasite-host system but used spectrophotometry to match the paint and the parasite colours as well as an appropriate final host, brown trout. In this case the mimicked colour of the acanthocephalan cystacanths did not have a role in increasing predation of amphipods by trout. To date there is no clear proof that colour alteration associated with infection is directly responsible for increased predation of parasitized crustaceans. There could be other characteristic aspects of the parasitized host, like reduced stamina and physical condition that could actually make the host more vulnerable to predation (Cezilly *et al*, 2010).

Colour-measuring techniques vary considerably, from colour evaluation by eye, to comparison to standards, to computer- or spectrophotometer-aided quantifications. Computer aided image analysis technique seems to be the preferred method in food and agriculture industry (i.e. Brosnan and Sun, 2002; Mendoza *et al*, 2006; Lana *et al*, 2006) where processed food or fresh fruits and vegetables can be quickly scanned and their quality determined based on colour. This offers a quick and reliable method for colour measurement, and when solely a scanner lamp alone is used, it addresses the problem of colour constancy. Increased quality of colour reproduction and easily accessible software packages make computer analysis an attractive colour measurement method. Stevens *et al*

(2007) reviewed and described the appropriate methods and protocols to follow when using a digital camera for studying animal colouration.

1.4 Parasite induced modifications in colour, linked to carotenoid composition

Many studies suggest that carotenoid composition could influence the phenotype of various taxa of animals, including birds, fish and crustaceans. In birds, carotenoids are associated with plumage and skin colouration and the colour of sexually selected traits in males can potentially predict the ability to deal with future parasitic infections (Dawson and Bortolotti, 2006). In house finches *Carpodacus mexicanus* (Statius Muller), females prefer redder males, a secondary sexual character that is strongly related to carotenoid uptake from food (Lozano, 1994). Redder males would seem to be able to forage better and at the same time avoid predators, besides showing up a healthier status or be able to deal better with health issues. Hamilton and Zuk (1982) proposed that male secondary sexual traits indicate heritable variation in parasitic resistance. Zuk *et al* (1990) showed that parasitized and non-parasitized male jungle fowl *Gallus gallus* (Linnaeus) differ in sexual but not non-sexual traits, suggesting that parasitism has a particularly strong effect over male features associated with mating success. Parasitism in birds can induce a trade-off in carotenoids allocation between immunologic functions and degree of ornamentation, e.g. parasitized blackbirds *Turdus merula* Linnaeus show reduced bill colouration (Baeta *et al*, 2008). A similar phenomenon was recorded in male guppies *Poecilia reticulata* Peters. Male guppies have orange spots on their skin, a secondary sexual trait, where they deposit excess absorbed carotenoids with a better efficiency than females (Grether *et al*, 1999; Hudon *et al*, 2003). Infection with the monogean parasite *Gyrodactylus turnbulli* Harris, diminishes the carotenoids in orange spots, and infected male guppies receive less attention from females.

In aquatic crustaceans, carotenoids are involved in exoskeleton colouration in the form of carotenoprotein complexes. This complex molecular association produces colour variations that depend on constituent amino acids and/or carotenoids (Czeczuga and Krywuta, 1981). Several carotenoproteins have

been chemically characterized but probably the crustacyanin group has received the most attention. Three types of crustacyanins have been identified to date and they are described as blue carotenoproteins, invariably having astaxanthin as the prosthetic group. Other crustacean carotenoproteins contain carotenoids like phoenicoxanthin and canthaxanthin, having a grey colour (Czeczuga, 1996), or zeaxanthin and astaxanthin with a yellow colour (Milicua *et al*, 1986).

1.5 Parasite induced modifications in carotenoid composition

Hindsbo (1972) compared colour changes in parasitized and non-parasitized amphipods while drying at room temperature and reported a decrease in total carotenoid quantity in the parasitized blue *G. lacustris*. In contrast, Gaillard *et al* (2004) used the HPLC method for comparing total carotenoid concentration in parasitized and non-parasitized female amphipods, and concluded that *Polymorphus minutus* had no effect on carotenoids of *Gammarus pulex* and *G. roeseli* Gervais.

There clearly is a carotenoid based interaction between pigmented acanthocephalan cystacanths and their crustacean hosts, given that the carotenoids in the cystacanths had to have come originally from the host (see below). However, white acanthocephalan cystacanths of *Centrorhynchus* sp. can induce 'pigmentation dystrophy' in some of their intermediate isopod hosts *Atlantoscia floridana* (van Name) (Amato *et al*, 2003). This particular phenotypic modification has not been yet related to biochemical changes in the host. Also, no research has been done on the parasite-host interactions between the white acanthocephalan cystacanth of *Polymorphus trochus* Van Cleave and its common host *Hyallela azteca* (Podesta and Holmes, 1970). For pigmented acanthocephalan taxa, biochemical analysis of pigment molecules in acanthocephalan cystacanths of *Polymorphus minutus* (Goeze) showed that they accumulate carotenoids in their fat deposits, made mainly of wax esters (Barrett and Butterworth, 1968, a, b). Clearly, as animals cannot synthesise *de novo* carotenoids (Davies, 1991), the source of accumulated carotenoids in the parasite during its larval development must be their hosts. However, cystacanths apparently have the ability to modify stolen carotenoids, as carotenoids reported

from these parasites tend to be different from those of the host. Esterified astaxanthin was the main carotenoid in the acanthocephalan *P. minutus*, a lipid that is not found in the body of gammarid amphipods (Barrett and Butterworth, 1968 a); Gaillard *et al*, 2004). It is reasonable to conclude that the parasite larva is able to modify the astaxanthin absorbed from the host's body. Predominance of esterified astaxanthin could be explained by either a preference of acanthocephalan for this specific carotenoid or the ability to transform other carotenoids toward esterified astaxanthin, a hypothesis that has not been further investigated by Gaillard *et al* (2004).

1.6 Parasite effect on carotenoids, more than a 'pigmentation dystrophy' issue

The immunological function of carotenoids in aquatic crustaceans is not well understood but it seems to be one of their main functions, besides their role in colour determination of the exoskeleton (whose colour pattern presumably acts principally as camouflage). Babin *et al* (2010) showed a positive correlation between immune defence mechanisms and concentration of dietary carotenoids of *Gammarus pulex*, through stimulation of humoral responses (non-specific immune mechanism in invertebrates) and increased resistance to bacterial infections. Immune response in *G. pulex* parasitized by *Pomphorhynchus laevis* is reduced either because of direct manipulations of non-specific cellular or humoral effectors responsible for the immune activity, or depletion of energy resources (Cornet *et al*, 2009). Reduced immune response can increase the chance of the host contracting opportunistic diseases or becoming infected with other acanthocephalan species (Cornet *et al*, 2009; Cornet and Sorci, 2010).

Cornet *et al* (2009) could not establish a correlation between immunodeficiency levels and behavioural modifications in *G. pulex* parasitized by *P. laevis*. However, Maynard *et al* (1996) showed that *P. paradoxus*, but not *P. marilis*, changes the anatomical pattern of neurons in the third thoracic ganglion of the host, increasing the secretion of neuromodulators, like serotonin, which regulates both the immune and the nervous systems (Cornet *et al*, 2009). In another parasite-host system, the acanthocephalan *Profilicollis antarcticus*

Zdzitowiecki alters the body posture and increases the oxygen consumption rates in the crab *Hemigrapsus crenulatus* (H. Milne Edwards) (Rojas and Ojeda, 2005). These parasite-induced modifications are related to an increase of hemolymph dopamine level in parasitized crabs. Given the interactions between immune system and nervous system, it appears that acanthocephalans can induce behavioural changes indirectly in the host, through the immune system (Helluy and Holmes, 2005).

1.7 Environment–carotenoid interaction

The environment experienced by growing plants can affect the concentration of carotenoid in their tissues, e.g., high nitrogen in soil is correlated with high carotenoids (Mozafar, 1993). This environmental effect can move through trophic levels, as increasing the concentration of carotenoids in diet can increase carotenoid concentration in the tissue of animals (Bjerkeng, 2008). Grether *et al* (1999) showed that carotenoid concentrations in guppies *Poecilia reticulata* vary along an environment gradient, where rainforest streams with more light have a higher concentration of algae and therefore more carotenoids concentrated food available for guppies. Also, other studies have shown that abiotic factors like temperature and food contribute to the host-parasite system (Blanford *et al*, 2003; Mitchell *et al*, 2005) in ways that influence infectivity and susceptibility of parasites and hosts. Despite these logical connections, no studies have considered the effect of a gradient of environmental factors including nutrients in the interaction between aquatic crustaceans and their carotenoid-'stealing' acanthocephalan parasites.

1.8 Objectives

The aim of this study is to address the effect of two acanthocephalans, *Polymorphus paradoxus* and *P. marilis*, on colour and carotenoids of their common intermediate host *Gammarus lacustris*, from water bodies that differ in nutrient availability. Phosphorus is typically the limiting nutrient for primary producers in standing freshwater bodies (Smith *et al*. 1999). I test the hypothesis that total phosphorus affects carotenoid availability for amphipods, which in turn influences how the carotenoid-stealing parasites affect their host.

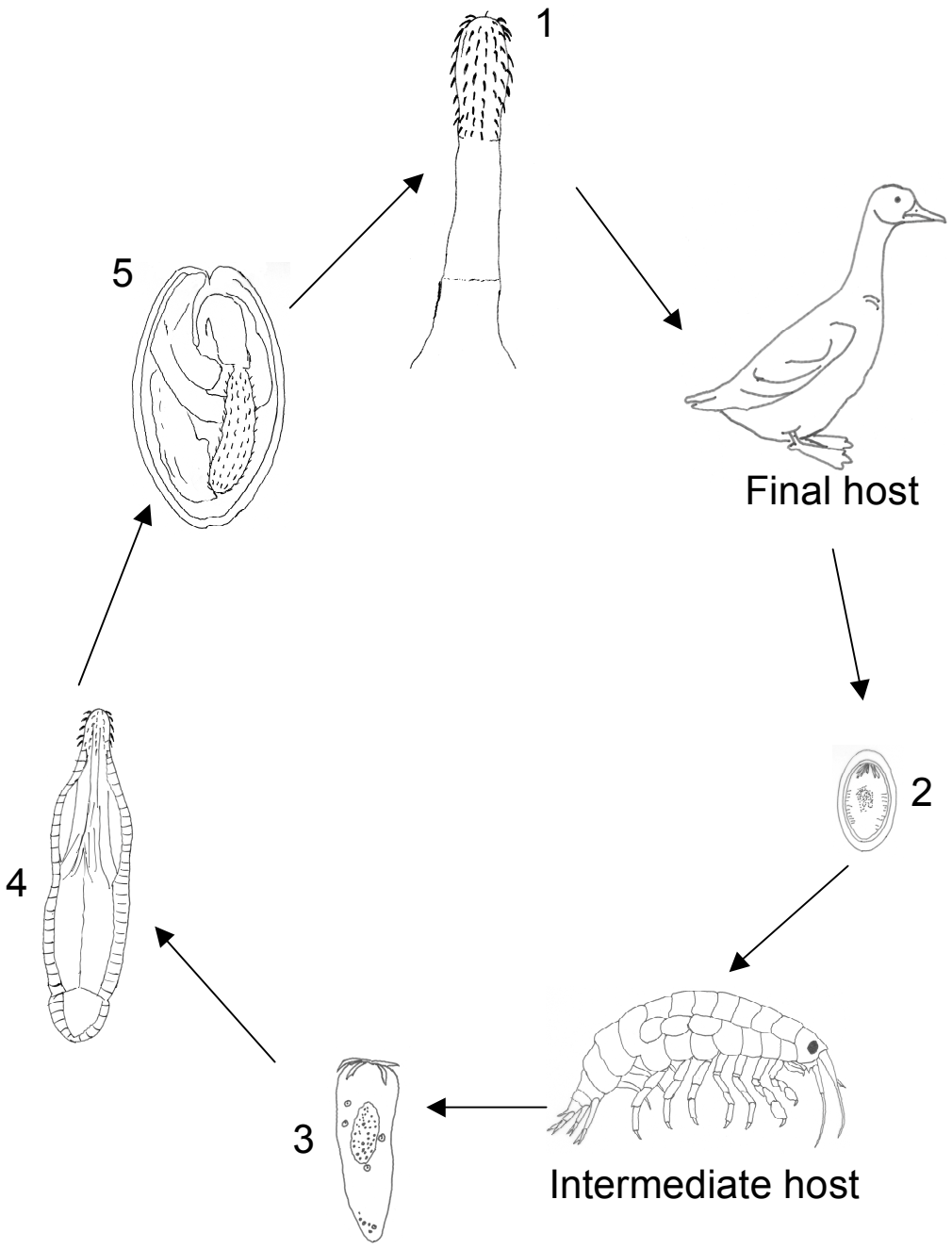

Figure 1-1. Life cycle of a *Polymorphus* species 1= adult; 2= embryonated egg; 3= released acanthor; 4= acanthella; 5= lemon-shaped cystacanth (stages 3, 4 and 5 develop in the intermediate host; see text for details)

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Chapter 2 - Acanthocephalan parasites, water quality and diet affect colour of the amphipod *Gammarus lacustris*

2.1 INTRODUCTION

Colour, an important phenotypic trait

The roles of colour in ecology and natural selection have been studied for more than a century (Bates, 1861; Darwin, 1880). Intraspecific variation in colour has been shown to correlate with survival (Caesar, 2010), reproductive success (Salvador *et al*, 2007), and resistance to pathogens (Joop *et al*, 2006). Such variation may be genetic (Valenzano *et al*, 2009) or it may be induced by diet (Schmalhofer, 2000) or health (Martin and Lopez, 2009). Parasitized hosts often display colour patterns different from non-parasitized individuals (i.e. Ressel and Schall, 1989). Effects of parasites on carotenoid based colours have been of particular interest to biologists, because colour can influence sexual selection in animals (Shykoff and Widmer, 1996) or represent a parasite-induced manipulation that enhances transmission to final hosts (Benesh *et al*, 2009). Such effects have been described both for definitive hosts (those in which the parasite engages in sexual reproduction) and in intermediate hosts. Parasites whose presence has been correlated with changes in host colour include apicomplexans (Ressell and Schall 1989), nematodes (Zuk *et al*, 1990), and acanthocephalans (see below).

Parasitic manipulations in intermediate hosts

Members of the entirely parasitic phylum Acanthocephala (the thorny-headed worms) have complex life cycles using arthropods as their intermediate hosts and relying on predation mechanisms in order to reach their final vertebrate hosts (Bethel and Holmes, 1973). Previous studies have shown that acanthocephalans induce behavioural modification in their intermediate crustacean hosts that make them more prone to predation. Mechanisms used by acanthocephalan parasites include changes in responses to mechanical (Bethel and Holmes, 1973) and light stimuli leading to altered escape mechanisms (Bethel and Holmes, 1973; Bauer *et al*, 2005; Tain *et. al*, 2006; Perrot-Minot, 2007). Acanthocephalan parasites can also induce subtle modifications in host

physiology, such as increasing production of the neurotransmitter serotonin (Maynard, 1996) and decreasing the immune response (Cornet *et al*, 2009). Reduced pairing success in males (Zohar and Holmes, 1997), lower egg count or complete sterility in females, and modifications in feeding behaviour (Hernandez and Sukhdeo, 2008) have also been described as consequences of parasitism.

Bethel and Holmes (1973) studied the interaction between the amphipod *Gammarus lacustris* G.O. Sars and its acanthocephalan parasites *Polymorphus paradoxus* Connell and Corner and *Polymorphus marilis* Van Cleave. They showed that cystacanths of *P. paradoxus* induce modified photic and escape responses in the intermediate host, while *P. marilis* alters the light preferences but not the escape response. The definitive hosts for *P. marilis* are several waterfowl species including the lesser scaup duck, *Aythya affinis* (Eyton) and the redhead *Aythya americana* (Eyton). This acanthocephalan species seems to be a more specialised one than *P. paradoxus*, which infects various waterfowl species, and also mammals like muskrats *Ondatra zibethicus* (Linnaeus) and beavers *Castor canadensis* Kuhl (Denny, 1969).

Although less studied than behavioural changes, colour modifications in aquatic crustacean species induced by acanthocephalan parasites have been previously described in isopods (e.g., *Lirceus lineatus* (Say) infected by *Acanthocephalus dirus* (Van Cleve), Oetinger and Nickol, 1981) and amphipods (e.g., *Gammarus pulex* (Linnaeus) infected by *Pomphorhynchus laevis* (Zoega), Bakker *et. al*, 1997). Acanthocephalan parasites inducing colour modifications in intermediate hosts have orange-red pigments made up of carotenoids (Barrett and Butherworth, 1968). Given that animals are unable to create carotenoids *de novo* (Davies, 1991), they are presumably taken from the tissues of their hosts, with the hosts themselves having obtained their carotenoid precursors from plants or algae in their diets. The acanthocephalan cystacanth is frequently visible as a brightly coloured dot in the body of the intermediate host. Presence of the bright spot is believed to make *Gammarus lacustris* infected by *P. paradoxus* more prone to predation by mallard ducks, muskrats and beavers (Denny, 1969; Holmes and Bethel, 1973) and *G. pulex* infected by *P. laevis* more prone to predation by

stickleback fish *Gasterosteus aculeatus* Linnaeus (Bakker *et al*, 1997). Mimicking the colour of the acanthocephalan parasite on the carapace of uninfected gammarids made them more detectable and preyed on by stickleback fish, a mechanism called "oddity selection" (Bakker *et al*, 1997). In contrast, a more recent study (Kaldosnki *et al*, 2009) showed that the orange acanthocephalan parasites *Pomphorynchus laevis* and *Polymorphus minutus* (Goeze) have no effect of on the susceptibility of *Gammarus pulex* to predation by the brown trout *Salmo trutta* Linnaeus. These studies focused on the orange colour of cystacanths but did not investigate colour modifications of the amphipod host's own body tissues. Only one report (Hindsbo, 1972) described the effect of *Polymorphus minutus* on *Gammarus lacustris*, the parasitized intermediate host being bluer and also more susceptible to predation than the non-parasitized ones. Other acanthocephalan species have different colour effects on their hosts. The cystacanths of *Centrorhynchus* sp. induces "pigmentation dystrophy" in terrestrial isopod *Atlantoscia floridana*, (Van Name), parasitized hosts becoming paler than non-parasitized ones (Amato *et al*, 2003). Similarly, *Oncicola venezuelensis* (Marteau) makes its termite host *Nasutitermes acajutlae* (Holmgren) significantly lighter than non-parasitized termites (Fuller *et al*, 2003). *Acanthocephalus anguillae* (Mueller) makes its isopod host *Asellus aquaticus* (Linnaeus) darker, lighter or does not change the colour at all (Dezfuli *et al*, 1994).

Carotenoids play an important role in colour determination in crustaceans (Zagalski, 1976; Wade *et al*, 2009). Acanthocephalan cystacanths could influence the colour of their host through direct absorption of carotenoids from the host body, or through modifications in physiological processes impeding the host's ability to absorb and/or deposit specific carotenoids in its tissues. A few previous studies on crustacean colour have reported ambient factors to be of high importance in determining the colour of aquatic crustaceans. Juveniles of the shore crab *Carcinus maenas* (Linnaeus) exhibit colour patterns and variations that are similar to the background they live in, possibly with functionality in preventing predation (Todd *et al*, 2006).

Colour measurements in ecological studies

All the aforementioned studies have reported colour differences in the hosts induced by parasites, but the nature of colour differences between parasitized and non-parasitized individuals was quantified using methods of comparison to colour charts or visual cross comparison between parasitized and non-parasitized individuals. Therefore these findings may not be easily replicable among human observers, and were not able to quantify colour differences between parasitized and non-parasitized hosts. However, Benesh *et al* (2009) used digital photography and computer vision to describe changes in colour of the intermediate host *Asellus aquaticus* due to the acanthocephalan parasite *Acanthocephalus lucii* (Muller). They concluded that the change in host colour reached a maximum level after the parasite larvae transformed into the cystacanth stage, and that colour variation was also influenced by the size and sex of the parasite.

Assessing colour is complicated because it depends on many factors associated with the perceived objects. Colour perception by an observer is influenced by other colours from the surrounding environment, or by the capability of the observers themselves in perceiving colours. There are several published methods for quantification of perceived colour. Each has its advantages and drawbacks, and most methods are designed to describe the human visual system or to measure the physical properties of light (i.e. spectrophotometry and colorimetry). The method of visually matching to Munsell colour standards, although widely used by biologists (e.g., Slagsvold and Lifjeld, 1985; Wood and Wood, 1972), it is highly subjective to environmental light characteristics and the observer's visual capabilities (Endler, 1990). Small differences in colour, often not visible to the human eye, can have important roles in signalling individual characteristics in many animals including arthropods (Davis *et al*, 2009), fish (Zion *et al*, 2008) and birds (Bolund *et al,* 2007).

Colour analysis using computer vision has been used in ecological studies on topics as diverse as determining readiness for breeding in lobsters *Panulirus cygnus* George (Wade *et al*, 2008), sexual dimorphism in red-legged partridge

Alectoris rufa (Linnaeus) (Villafuerte and Negro, 1998) or aposematic colouration in milkweed bugs *Oncopeltus fasciatus* (Dallas) (Davis, 2009 (b)). In plants, examples include identifying intraspecific colour variations and patterns in flowers (Masco *et al*, 2004), leaf necrosis and chlorosis (Wang *et al*, 2008), and the health condition of in-vitro plant cultures (Aynalem *et al*, 2006). Computer vision is also extensively used as a practical and accurate method of establishing the overall quality of agricultural products, given that colour is an indicator of other chemical, physical or sensory qualities of fruits, vegetables and animal products (Mendoza *et al*, 2006; Lana *et al*, 2006). Probably the most important advantages of using enclosed digital-imaging devices such as flatbed scanners in measuring colour include the ability to reproduce colours with high consistency, while holding a constant light intensity and colour temperature over the scanned objects (Villafuerte and Negro, 1998).

Hypotheses

The present study investigates whether the acanthocephalans *Polymorphus paradoxus* and *Polymorphus marilis* affect the colour of their host *Gammarus lacustris* from water bodies in Alberta, Canada. This research was spurred by earlier observations (H. Proctor, pers. obs.) that *G. lacustris* parasitized by acanthocephalans are sometimes, but not invariably, pale blue instead of the normal greenish-brown, and that this difference in colour appears to vary among lakes in Alberta. Therefore I also test for a relationship between the magnitude of the parasite-induced colour change in amphipods and lake water quality, based on total phosphorus concentration (TP). *Gammarus lacustris* are generally considered herbivorous scavengers (Agrawal, 1965) and detritivores (Moore, 1977), but can also act as a predator (Wilhelm and Schindler, 1999). Total phosphorus concentration is a good indicator of lake productivity (Wetzel, 1975; Theis *et al*, 1978). Total phosphorus is also positively correlated with amounts of chlorophyll in Canadian water bodies. Dillon and Rigler (1974) were able to use TP to predict the amount of clorophyll a from lakes with different productivity levels (R^2= 0.95). Assuming that high levels of TP denote an increased availability of food resources, hypereutrophic lakes would give parasitized *G.*

lacustris individuals the possibility to replenish carotenoid pigments lost due to the infection. Concentration of dietary carotenoids can influence the carotenoid composition of animals (Grether *et al*, 1999; Negro *et al*, 2000; Bjerkeng *et al*, 2008). Therefore I hypothesize that parasitized amphipods from eutrophic and hypereutrophic lakes would suffer comparatively less from altered colouration than the parasitized amphipods originating from oligotrophic lakes.

The colour of amphipods could also be directly affected by water chemistry. Although the sclerotized exoskeleton of crustaceans is considered to be impermeable to water due to its biochemical composition, the soft new integument during and immediately after moulting is permeable to water (Morris *et al*, 1987; Rasmussen *et al*, 1996). Thacker *et al* (1993) found that the colour of the crayfish *Orconectes virilis* (Hagen) is affected by water chemistry, as individuals reared on same diet but different water treatments resulted in different colours. Dissolved organic carbon, affects the colour, pH value and penetration of ultraviolet and visible radiation significant for the photosynthetic process (Pace and Cole, 2002). Fulvic and humic acids colour the water and they could also influence the development of colour in *G. lacustris.*

2.2 MATERIALS AND METHODS

Collection of samples

Gammarus lacustris were collected from water depths up to 1.5 m in seven water bodies from Alberta, Canada (Figure 2.1), during April-May 2009, using a 500 μm mesh dip-net. Although some of the water bodies were small, shallow and densely vegetated, and hence are more appropriately termed 'marshes', for convenience I refer to all of them as 'lakes'. Most of the observed *G. lacustris* individuals were swimming in the proximity of, or clinging to, submerged cattail stems (*Typha latifolia,* Linnaeus) in hypereutrophic lakes, or under patches of benthic detritus in oligo- and eutrophic water bodies. Chemical physical and vegetation characteristics of the sampled lakes are summarized in table 2-1. Size of the water bodies was either measured using Google Earth (Google Inc., 2008) or when available, data were collected from the Atlas of Alberta Lakes (Crosby and Prepas, 1990; Bradford *et al*, 1990). Vegetation was

described based on available resources (Crosby and Prepas, 1990; Brdford *et al*, 1990) and/or field observations. Collected amphipods were taken back to the laboratory at the University of Alberta, where they were kept alive in their native water and 11°C and their colour measured within the next 24 hours. No food was provided to them between time of collection and colour measurement. A total of 800 individuals were analysed for colour, 143 from Beyette (75 males and 68 females), 107 from Narrow (52 males, 55 females), 104 from Long (58 males and 47 females), 64 from Forestry 754 (34 males and 30 females), 146 from Kettle (69 males and 70 females), 127 from Range Road 223 (60 males and 64 females) and 118 from Laica (59 males and 59 females).

Acanthocephalan cystacanths were extracted from sampled amphipods following colour measurement. Twenty non-everted cystacanths of *P. marilis* and 39 of *P. paradoxus* were measured in length and the longest diameter. Some of the extracted parasites were set in tap water for 12-24 hours at 6ºC to encourage the cystacanths to evert, immediately after being extracted from the host body. Species were identified based on the number of hooks per row, the size of the largest hook, the length of the proboscis, the with of the proboscis at the widest point, the length of the neck, the width of the neck at the widest and narrowest points and the total length of the body (Denny, 1969). Measurements were made on 30 everted *P. paradoxus* and on seven everted *P. marilis* from Laica, Kettle, Rg. Rd 223 and Beyette lakes.

Water samples of 400 ml were taken, using white plastic Nalgene bottles, from each studied lake mostly in two consecutive years during the spring and fall seasons of 2008-2009. They were analysed for total phosphorus, dissolved organic carbon, turbidity and colour (details below). The lakes were selected in order to span a wide range in trophic status. They included two oligotrophic lakes (Narrow and Forestry 754), two eutrophic lakes (Long and Beyette), and three hypereutrophic lakes (Range Road 223, Kettle and Laic (Table 2-2 and Figure 2-1; see Water chemistry in Results). Classification into these trophic levels was made based on the total phosphorus concentration in the water. Oligotrophic, mesotrophic, eutrophic and hypereutrophic lakes should have concentrations of

lower than 10 μg L^{-1} P, 10 to 20 μg L^{-1} P, more than 20 μg L^{-1} P, and more than 100 μg L^{-1} P respectively (Wetzel, 1975).

Water analysis

All water analyses took place in the Biogeochemical Analytical Laboratory of the Department of Biological Sciences at the University of Alberta. Turbidity was measured using a HACH turbidimeter model 2100N within 24 hours of water sample collection. Colour was measured in a 10 cm cell at an absorbance of 440 nm using a Shimadzu UV2410 spectrometer. DOC concentration was measured using the Shimadzu model TOC-5000A carbon analyser. All inorganic carbon contained in the water samples was removed from the samples by acidifying with 2 M/L HCl and sparging with hydrocarbon-free air before analyzing DOC. Dissolved organic carbon in water samples was converted to carbon dioxide (CO_2) by catalytic combustion at 680 °C. A non-dispersive infrared detector (NDIR) then detected the resulted CO_2. Total phosphorus was measured using a Lachat QuickChem 8500 FIA automated ion analyser. TP (unfiltered) samples were digested with potassium persulfate ($K_2S_2O_8$) using an autoclave, and then water samples were filtered through a 0.45 μm filter. The orthophosphate reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form an antimony-phosphomolybdate complex. This complex is reduced with ascorbic acid to form a blue complex, which absorbs light at 880 nm. The absorbance is proportional to the concentration of orthophosphate in the sample.

Colour measurement – Scanner characterization

I used an HP DeskJet 7400C colour flatbed scanner to scan the amphipods (details below). The scanner was tested for colour-reproduction consistency by scanning the same colour swatch 25 times a day for 5 consecutive days during a week. The RGB average values and standard deviations were analysed. The temperature at the scanner glass surface was measured using a regular alcohol thermometer. Temperature remained constant at 30.8˚C when the lamp had warmed up, after 4-5 scans.

The scanner records colour in RGB values, which form a device-dependant colour space. Therefore, the measured red, green and blue values in this study can be reproduced only with exactly the same colour measuring system. In order to eliminate this issue the colour information collected by the scanner can be transformed into device-independent colour spaces, providing colour information that does not depend on the experimental device setup. In 1931, the International Commission on Illumination (CIE) defined the device independent colour space called CIE L*, a*, b*. CIE L*, a*, b* colour space is constructed on approximately uniform scale, meaning that the distances (differences) observed by human eye between two colours are plotted inside the colour space with similar distances (HunterLab, 2008). The values spanned by the vectors in CIE L*, a*, b* colour space range from 0 (pure black) to 100 (pure white) for luminosity (L*), from -127 (pure green) to +127 (pure red) for the green-red vector (a*), and from -127 (pure blue) to +127 (pure yellow) for the blue-yellow vector (b*).

The relationship between sRGB and CIE L*, a*, b* colour spaces was determined for the HP DeskJet 7400C using Kodak Q60 IT8.7/2 colour targets (December 2009 batch). The 240 colour patches of the target were scanned with all the image enhancement settings off. The square marquee tool was used to select the patches to be measured in Adobe Photoshop CS4 for Mac. The histograms of the R, G and B channels were normally distributed, therefore the average value of each channels was used for further statistics. No clipped pixels were observed in the selected areas. The size of each selection was 130x130 pixels, which covered about 90% of each colour patch. The published XYZ, another device independent colour space developed by CIE, and L*, a*, b* values for the colour target were used as a reference for sRGB to CIE XYZ transformation (Kodak, 2009). Non-linear polynomial regression was chosen over other transformation methods currently used, such as look up tables and neural networks, due to its satisfactory results, moderate time and computational requirements, and its popularity (Zhihong *et al*, 2001). A multivariate non-linear cubic polynomial analysis was used for determining the relationship between each

vector of the CIE XYZ and the sRGB colour spaces (Sokolowsky, 2003). For statistical computation, the polynomial terms were first calculated in an Excel spreadsheet and then the polynomial regression coefficients between sRGB and each vector of the XYZ colour space were calculated using a multivariate linear regression in SPSS v.17 (SPSS Inc., 2008).

Colour measurement – Amphipods

Adult amphipods were divided into male and female based on their size, morphology of their second gnathopods and pairing position in coupled amphipods. No egg-bearing females were included in the colour analysis. Males and females were separately analysed for colour, as it was possible that the effect of parasitism on colour differs between the sexes, and/or that there is a pre-existing colour difference between non-parasitized male and female amphipods.

The surface of each amphipod was gently dabbed with a Kimwipe in order to reduce the effect of water droplets on light reflection and geometry. Then, amphipods were placed directly on the scanner glass, setting up a small spacer to avoid crushing them with the scanner cover. Previous testing showed that three or more scans of the same amphipods changed the colour of the animal due to the high temperature at the scanner glass level, and therefore each amphipod was scanned only once, at 16.7 million colours and 600 dpi. The JPEG pictures were saved on a PC computer transferred and analysed on a Mac Book Pro with Adobe Photoshop CS4 v.11 software for Mac (Adobe Systems Inc., 2008). First, the picture of the amphipod itself was separated from the background using the 'erase' tool. Also antennae, gnathopods, pereopods, pleopods and uropods, and the last segment of the urosome were erased due to high transparency and mobility of these parts in live amphipods, which produced colour artefacts (Figure 2-2a). Eyes were removed for variations in colour between amphipods. R, G and B values were determined by using the 'quick selection' tool on the remaining amphipod image, which in previous tests proved to be more consistent than the 'magic wand' tool in terms of repeatedly selecting the same number of pixels. If the parasite was visible through the carapace (Fig. 2-2b) it was removed from the

picture in order to reduce the effect of the bright orange colour on the overall colour of the amphipod.

Once the scanned image was obtained, amphipods were transferred to small pieces of aluminum foil, and under a dissecting microscope the sex was confirmed before the amphipod was dissected and any acanthocephalan parasites removed. The amphipods and parasites were kept for further processing in a freezer at -20ºC (see Chapter 3).

Effects of diet and water quality on colour of lab-reared amphipods

To test the influence of food and water quality on the colour of amphipods, young non-parasitized amphipods of approximately the same size (~3 mm long) were collected at the beginning of September 2009 from hypereutrophic Kettle Lake. They were reared in the laboratory in sets of 10 individuals in 8 jars of 2 L for 30 days, which was observed to be sufficient for at least two moulting events to take place (lab conditions are described below). This was deemed sufficient time for any effects of diet and/or water quality (absorbed via soft newly moulted cuticle) to influence the colour of the amphipods). Water and organic substrates were collected from Kettle Lake and from oligotrophic Narrow Lake at the same time as the amphipods were collected. Four combinations of water and diet were tested for effects on amphipod colour: oligotrophic lake water + oligotrophic lake substrate, oligotrophic lake water + hypereutrophic lake substrate, hypereutrophic lake water + oligotrophic substrate and hypereutrophic lake water + hypereutrophic substrate. There were two replicates (= two jars of 10 amphipods each) of each of the four treatment combinations. The jars were maintained in an environmental chamber at 11 h light: 13 h dark (appropriate for the season) at a temperature of 11ºC, and the water was lightly aerated. Previous trials of rearing amphipods in smaller (60 mL) jars without aeration resulted in a high mortality of individuals within 2-3 weeks of captivity, whereas there proved to be little mortality in the 2 L aerated jars. Food, in the form of benthic substrate retained on a 45 μm phytoplankton mesh, was given in weekly rations of 5 g/jar, and water and food was changed once a week. Colour of amphipods was recorded as described above both before and

after the experiment; however, because the amphipods were kept in groups, I was unable to record before and after measurements for individuals. The CIE 1931 L*a*b* data were statistically analysed using multivariate GLM SPSS for determining the effect of each of the treatment categories, diet and water quality, separately (see below).

Statistical analysis

Colour differences between parasitized and non-parasitized and male vs. female amphipods were analysed using multivariate GLM (MANOVA) in SPSS for each lake. For testing the colour differences between non-parasitized amphipods, the variable sex, dummied as '0' for males and '1' for females, was set as the explanatory factor for the observed differences in the CIE L*, a*, b* colour vectors between the two sex groups from a lake. For testing the colour differences within amphipods from a sex group, the parasite load dummied as '0', '1' and '2' for non-parasitized amphipods, amphipods parasitized by *P. paradoxus* and those parasitized by *P. marilis* respectively, was set as the explanatory variable for the observed differences between amphipods from one lake. For a proper interpretation of observed differences between two or more colours, Jackson (1958) recommends the use of multivariate tests because the colour vectors making up a tristimulus colour space are not independent. As colour is made up of the three component vectors, individually none of them describes the overall colour of an object (Jackson, 1958). Based on my observations, all CIE L*, a*, b* colour vectors were significantly correlated ($p < 0.01$) with a stronger relationship between vectors a* and b*. Comparison of two or more colours based on individual CIE L*, a*, b* vectors (e.g., with ANOVA) is neither statistically appropriate nor intelligible. The multivariate analyses were done on measured CIE 1931 L*a*b* values at a standard 2º observer (initial colour observations were made on a 2º field of view). After the GLM multivariate test for colour differences between parasitized and non-parasitized hosts, in which both parasite species were combined to make a single explanatory factor, the colour differences produced by each parasite species were analysed separately using a similar GLM analysis but with only one parasite species at a time.

The distance between two colours was estimated using the Euclidian distance between two points in CIE L*a*b* color space, $\Delta E_{ab} = (\Delta L^* + \Delta a^* + \Delta b^*)^{1/2}$ (hereafter called Equation 2-1). A colour difference (ΔE_{ab}) of at least 2 to 3 units is considered to be detectable by human eye, a parameter also called the Just Noticeable Difference (JND) (Sharma and Trussell, 1997). The differences between two colours in each L*, a* and b* vector can be interpreted as follows (HunterLab 2008)

Eq.2-2 $\Delta a^* = a^*sample - a^*standard$ $-\Delta a^*$ = sample is greener than standard
$+\Delta a^*$ = sample is redder than standard

Eq.2-3 $\Delta b^* = b^*sample - b^*standard$ $-\Delta b^*$ = sample is bluer than standard
$+\Delta b^*$ = sample is yellower than standard

Eq.2-4 $\Delta L^* = L^*sample - L^*standard$ $-\Delta L^*$ = sample is darker than standard
$+\Delta L^*$ = sample is lighter than standard

(HunterLab, 2008)

Individual effects of water and diet on the colour of amphipod juveniles reared in laboratory conditions were tested with SPSS GLM analysis. Water (0= oligotrophic, 1= hypereutrophic) and diet (0= oligotrophic, 1= hypereutrophic) quality were set as dummied independent variables while the three colour vectors were the dependent variables. Interpretations of colour differences were made based on the differences in means between groups of amphipods and equations 2-1 to 2-4.

To test the coupled effect of both water and diet on the colour of amphipods, analysis of similarity (ANOSIM) in PATN v3.11 was used (Belbin, 1989), with each of the four treatment combinations treated as an *a priori* group. Samples were ordinated based on Gower metric distances using semistrong hybrid multidimensional scaling (SSH) in two dimensions, with 5000 starts and the default ratio-ordinal cut value of 0.9. The colour values L*, a*, b* were set as intrinsic vectors (i.e., they were used to create the ordination) while water quality and diet were used as extrinsic vectors for the analysis (i.e., they were simply correlated with the ordination). ANOSIM determines whether the samples in each *a priori* group are clustered more tightly with each other than expected by chance.

Principal axis correlation (PCC) in PATN, a type of multiple-linear regression, was used to determine the correlations between the placement of samples in 2D ordination space and the values of colour vectors L*, a*, b*, water quality and diet. The statistical significance of the relationship between abiotic factors and colour vector values was determined using Monte Carlo Attributes in Ordination (MCAO) in PATN over 5000 randomizations. High values of PCC and MCAO for a variable means that the variable is strongly and significantly correlated with the ordination, and hence important in creating it (for intrinsic variables) or in agreement with the ordination (for extrinsic variables). Examination of direction of vectors on the ordination can indicate potential relationships among variables.

2.3 RESULTS

Occurrence of acanthocephalan cystacanths in amphipods varied from lake to lake. The most affected amphipod populations were those from Laica, Kettle and Range Road 223 water bodies. About 25% of the amphipod sampled amphipods from these water bodies were parasitized. On the other hand, the gammarid populations from Forestry 754, Narrow, Long and Beyette water bodies were less affected by parasitism. Less than 1% of the sampled gammarids from Forestry 754 Lake were infected by acanthocephalans and about 2% of amphipods from Narrow Lake.

Amphipods harbouring the younger acanthella developmental stage, although found among some the amphipods initially sampled, were not included in the analysis. Only the cystacanth developmental stage of the acanthocephalans was found in the parasitized amphipods subjected to colour analysis. Parasitism by two or more acanthocephalan cystacanths of the same species occurred occasionally, but separating these into a different category would have resulted in smaller sample sizes per treatment category, therefore it was not accounted for. Also, approximately 10% of the sampled parasitized amphipods harboured both acanthocephalan species. Amphipods harbouring both acanthocephalans were not included in this study. Parasite status, as a statistical explanatory variable included three nominal categories: non-parasitized amphipods, parasitized by *P. paradoxus* and parasitized by *P. marilis*.

Morphometric characteristics of *P. paradoxus* and *P. marilis* cystacanths

Measurements of acanthocephalan cystacanths were made on unfixed specimens. Inverted cystacanths of *P. paradoxus* had a lemon-like shape, with a yellow-orange tint. The average length of *P. paradoxus* cystacanths was 1.12 ± 0.07 mm and the average width at the widest point was 0.73 ± 0.03 mm. The cystacanths of *P. marilis* were also lemon like shaped but smaller and yellower than those of *P. paradoxus*. The average length of *P. marilis* cystacanths was 0.72 ± 0.03 mm and their average widest point measured 0.47 ± 0.03 mm.

Morphological characteristics of the everted cystacanths are summarized in Table 2-2. The differences between the two species were clear in all measured aspects: length and width of proboscis, number of hooks per row, length of the longest hook, overall body length. Overall, non-everted and everted cystacanths of *P. paradoxus* were larger than the cystacanths of *P. marilis* (Figure 2-3).

Ethanol-preserved exemplars of each species are deposited in the Freshwater Invertebrate Collection of the Department of Biological Sciences, University of Alberta.

Water chemistry

Water quality analysis (Table 2-1) showed that the average TP concentration in Kettle (289 μg L^{-1} P), Range Road 223 (271 μg L^{-1} P) and Laica (250 μg L^{-1} P) placed these lakes as hypereutrophic water bodies. Beyette (46 μg L^{-1} P) and Long (31 μg L^{-1} P) are eutrophic lakes, and Narrow (9 μg L^{-1} P) and Forestry 754 (8 μg L^{-1} P) are oligotrophic water bodies. The analysis for dissolved organic carbon showed similar variations, Kettle, Range Road 223 and Laica had the highest average concentrations of DOC, 38 mg L^{-1}, 33 mg L^{-1} and 40 mg L^{-1} respectively. Forestry 754 and Narrow lakes had the lowest amount of dissolved organic carbon, 2 mg L^{-1} and 10 mg L^{-1} respectively. Beyette and Long lakes showed DOC values that are between the values found in hypereutrophic and oligotrophic water bodies, 18 mg L^{-1} and 12 mg L^{-1} respectively. Turbidity and colour analyses followed a similar pattern with the highest values in the hypereutrophic lakes, moderate values in the eutrophic lakes and the lowest

concentrations in the oligotrophic lakes (Table 2-2). Physical and vegetation characteristics are also summarized in the same table.

Scanner characterization

Based on previous studies (Gindi, 2008) a multivariate cubic polynomial with 20 terms was determined to best suit the Kodak Q60 colour target reference data:

$P(R,G,B) = a_0 + a_1B + a_2G + a_3B + a_4RG + a_5GB + a_6RB + a_7R^2 + a_8G^2 + a_9B^2 + a_{10}\,RGB + a_{11}R^3 + a_{12}G^3 + a_{13}B^3 + a_{14}RG^2 + a_{15}R^2G + a_{16}GB^2 + a_{17}G^2B + a_{18}RB^2 + a_{19}R^2B$. This equation produced the lowest differences between measured sRGB values of the colour patches and the CIE L*, a*, b* values referred by Kodak for the Q60 colour target.

SPSS Multiple Linear Regression analysis showed a strong relationship between each of the CIE XYZ colour vectors (an intermediate step in transforming sRGB to CIE L*, a*, b* colour space) and the measured sRGB values ($R^2 = 0.99$). The colour patches I22 and L13 had sRGB values that were identified as outliers and removed from the regression analysis. The coefficients a_0 to a_{19} were used to calculate the predicted L*, a*, b* values. The average difference between these values and the average values for the December 2009 Q60 IT8/7.2 colour target sheet published by Kodak was 1.58 units with a maximum difference of 7.82 units, a minimum of 0.07 units and 89% of the calculated L*, a*, b* values were under a difference of 3 units (Figure 2-4). These results are similar to the findings of other scanner characterizations using the same method, for example Finlayson (1997) found an average ΔE_{ab} of 1.58 units, and Zhihong (2001) found a ΔE_{ab} of 1.74 units with maximum difference of 7.38 units. Therefore, I conclude that the scanner used in my study was adequately calibrated and the regression would properly transform scanner RGB to CIE L*, a*, b* values .

Repeated scans of the same colour swatch 25 times per day for 5 days during a week produced very similar sRGB values for the JPEG pictures, the average red channel was constant at R = 254.00 ± 0.00SD, G = 92.21 ± 0.54SD and B = 204.19 ± 0.40SD. The largest standard deviation was seen in the green

channel, but such a small variability would not affect the overall, interpreted colour of the scanned swatch. It was concluded that the scanner had a good capability of consistently reproducing the colour of objects both within and between days of.

Male vs. female colour differences

The colour differences between male and female amphipods were tested using a multivariate SPSS GLM analysis, with the probability set at $p<0.05$. Males and females belonging to each of the three nominal groups, non-parasitized, parasitized by *P. paradoxus* and parasitized by *P. marilis* were tested separately (Table 2-3).

The multivariate GLM analysis showed that there were strong *a priori* differences in colour between non-parasitized male and female amphipods, with the significance of the GLM test of $P = 0.01$ or less in all lakes (Table 2-5). A test of between-subjects effects showed the influence of the variable 'sex' within each group of amphipods having the same parasite status ($p< 0.05$). These overall colour differences between were influenced by all or just one of the colour vectors L*, a* and b* (Table 2-4). As the statistical results indicated, there was a high variability between lakes in terms of which colour vectors where responsible for driving overall colour differences. In some cases the test of between-subjects-effects (a SPSS ouput test, similar to ANOVA) showed a probability between 0.05 and 0.09 for one of the three colour vectors (i.e. colour vector L* in Beyette, $p= 0.08$, see Table 2-4) in determining the colour differences between sexes within parasite status group. In such cases I considered that these colour vectors could still have an effect on the overall colour differences. The decision was based on a very low probability value of the multivariate GLM test ($p< 0.01$), suggesting that the colour differences may have been driven by more than one of the colour vectors.

The CIE L*, a*, b* values of male amphipods were used as 'standards' for comparison (see Equations 2 to 4 in Methods), therefore the resultant interpretations of colour is relevant for the CIE L*, a*, b* values of female amphipods relative to the colour values of the males. Non-parasitized females

were redder than the non-parasitized males in Long, Narrow, Forestry 754 and Kettle water bodies (Table 2-3). In all other cases the colour difference seems to not be influenced by the colour vector a*. Also, in all cases where vector b* had an influence on the colour differences, females were bluer than males (Beyette, Long, Narrow and Range Road 223). They were darker than males in Beyette and Long lakes and lighter than males in Narrow, Forestry 754 and Laica water bodies, but the lightness vector was not relevant for producing a colour difference between sexes in Kettle and Range Road 223.

The colour differences that were observed between sexes in non-parasitized amphipods tended to disappear when both males and females were parasitized by *P. paradoxus* (Table 2-3). This phenomenon was observed in Beyette, Long and Kettle water bodies. Among the oligotrophic lakes, only Narrow Lake harboured both females and males parasitized by *P. paradoxus*, and in this case females became lighter than males. In two of the hypereutrophic water bodies, Range Road 223 and Laica, parasitized females were bluer than parasitized males. However, the differences in colour vectors L* and a*, induced by *P. paradoxus* in both sexes, varied between these two water bodies (Table 2-3).

A similar phenomenon of lessening of colour differences between the sexes was also produced by *P. marilis*. In three water bodies, Beyette, Long and Range Road 223, where *P. marilis* was found in both male and female amphipods, the colour differences between the two sexes of parasitized hosts were not statistically significant ($p > 0.1$). The exception was found in Kettle Lake where females infected by *P. marilis* were lighter, yellower and most likely redder than males infected by the same parasite ($p = 0.01$) (Table 2-3).

Colour differences between parasitized and non-parasitized amphipods within a sex group

a) Parasite induced changes in amphipod males

Because the sexes differed in colour (see above), examination of parasite effect was tested on each sex separately. A multivariate general linear model in SPSS was used to quantify the colour differences between parasitized and non-

parasitized *G. lacustris* within each sex. Because the two parasite species potentially have different effects on their common host, the measured colour values of parasitized male amphipods, divided into those parasitized by *P. paradoxus* and those parasitized by *P. marilis*, were compared to the colour values of uninfected individuals (Table 2-6). Both species of parasite had an effect on the colour of the male hosts with GLM $p < 0.05$ in most lakes (Table 2-6), except for those from hypereutrophic Laica ($p= 0.23$, $F= 1.50$, $df = 3; 37$).

In order to test the colour differences between amphipods parasitized by the two acanthocephalan species, the CIE L*, a*, b* values of those parasitized by *P. marilis* were compared to those parasitized by *P. paradoxus*. It was found that the two acanthocephalan species induced statistically different colours in male amphipods in four of the studied lakes ($p<0.05$). Only male amphipods infected by *P. paradoxus* and *P. marilis* originating from Laica Lake were not statistically different ($F= 1.49$, $df= 3; 22$, $p= 0.25$) (Table 2-6).

Interpretation of colour change was based on the GLM multivariate analysis (Table 2-6), and results of the test of between-subjects effects indicated which of the colour vectors produced the colour difference observed in the multivariate analysis (Table 2-7). *Gammarus lacustris* males infected by *P. marilis* became greener (less red) than non-parasitized males. This effect was constant in all cases where colour vector a* had a role in determining the colour differences (Long, Range Road 223 and Laica). A more common effect of this parasite was to turn its host blue. With the exception of Laica Lake, in all other cases where the parasite was found, parasitized amphipods became bluer than non-parasitized ones. And finally, the luminosity vector was changed to lighter in four out of six lakes where *P. marilis* was found (Beyette, Long, Forestry and Laica). In amphipods infected by *P. marilis* originating from Range Road 223 and Kettle water bodies, the luminosity vector played no role in determining the colour differences. Based on the differences DL* and Db* it seemed that the colour vectors L* and b* had the most important role in determining the colour differences between parasitized and non-parasitized male amphipods. Overall, when there was a colour change, amphipods parasitized by *P. marilis* became

lighter, greener and bluer (less yellow) than the non-parasitized ones (Table 2-8). The pigmentation dystrophy produced by *P. marilis* in amphipods from oligotrophic and eutrophic water bodies, Forestry 754 (ΔE_{ab}= 11.43), Long (ΔE_{ab}= 13.76) and Beyette (ΔE_{ab}= 8.99), was more accentuated than that in amphipods from hypereutrophic water bodies, Range Road 223 (ΔE_{ab}= 8.42) and Laica (ΔE_{ab}= 2.29) (Table 2-8). Considering that the 'Just Noticeable Difference' is somewhere between 2 and 3 units (Sharma and Trusell, 1997), the colour change produced by *P. marilis* in infected *G. lacustris* would be clearly visible to humans in all cases.

The luminosity vector does not seem to be influenced by *P. paradoxus* (Table 2-8). Parasitized male amphipods changed luminosity (turned darker in this case) only in Long Lake (Table 2-7). This species of parasite may change the colour direction but appears not to induce "pigmentation dystrophy" in *G. lacustris*. For those cases in which colour vector b* was significant (all lakes except Kettle and Laica), it was shifted toward a bluer value. The colour vector a* was not significant for Beyette, Laica or Narrow. When significant, parasitized amphipods had either a redder value than non-parasitized amphipods, in Kettle and Range Road 223 water bodies or a greener one in Long Lake.

Considering the just noticeable difference parameter, the degree of colour change produced by *P. paradoxus* in infected *G. lacustris* ranges from no change (Laica), to changes that, although statistically significant, would not be visible to the human eye (Kettle and Narrow), to changes that are above 2 units and hence should be visible to the human eye (Long Lake, Range Road 223, Beyette) (Table 2-8).

b) Parasite induced changes in amphipod females

Colour differences induced by *P. marilis* in female *G. lacustris* had a similar trend to that observed in *P. marilis*-infected males. Parasitized females usually turned greener, bluer, and, most commonly, lighter when compared to uninfected females (Table 2-9). In Kettle Lake the colour vector b* had no role in determining the colour differences, but parasitized females from Beyette, Long and Range Road 223 turned blue. The colour vector a* had no role in determining

the colour differences in Range Road 223 and Kettle water bodies. *P. marilis* was not found in samples from Narrow, Forestry 754 and Laica water bodies. This species seems to induce about the same degree of difference in colour of females from hypereutrophic and eutrophic lakes, although the smallest difference is seen in hypereutrophic Kettle Lake (ΔE_{ab}= 1.07), and the largest colour difference is observed in eutrophic Long Lake (ΔE_{ab}= 14.06). Applying the same rule of 'Just Noticeable Difference', the colour differences produced by *P. marilis* in female amphipods from Kettle Lake (ΔE_{ab}= 1.07) would not be visible to human eye, but would be highly visible in the other three lakes in which it was found (Table 2-9).

The effect of *P. paradoxus* on amphipod females is similar to the effect produced by *P. marilis*. Females parasitized by *P. paradoxus* became lighter than non-parasitized ones, with the exception of those from Laica Lake where parasitized females turned darker (Table 2-9). *Polymorphus paradoxus* had a different effect on the luminosity vector in females than males. While in *paradoxus*-infected male *G. lacustris* luminosity generally didn't change, or amphipods turned darker in one instance (in Long Lake), females generally turned lighter, excepting in Laica Lake. A change in colour vector b* is observed only in Long and Laica lakes, where its value was shifted towards blue. The colour vector a* is affected by *P. paradoxus* only in Long Lake, where parasitized female amphipods turned greener than non-parasitized females. Also, *P. paradoxus* seems not to produce a change in colour in all parasitized females (Table 2-10). There was no difference in colour between parasitized and non-parasitized females in Narrow Lake (p=0.87, F=0.24, df=3; 51) or Kettle Lake (p=1.00, F <0.01, df=3; 50). Based on the JVD parameter the colour change produced by *P. paradoxus* in female amphipods ranges from no effect in Kettle Lake to a visible colour change to the human eye in the Beyette (ΔE_{ab}= 2.80), Range Road 223 (ΔE_{ab}= 3.04), Laica (ΔE_{ab}= 2.91) and Long (ΔE_{ab}= 4.75) (Table 2-8). Just like in male gammarids, the effect of *P. marilis* on the colour of the female hosts was stronger than the effect produced by *P. paradoxus*.

The relationship between water quality parameters TP and DOC and the colour of *G. lacustris*

The degree of colour change in parasitized males and females was plotted against two of the water quality parameters, total phosphorus (Figures 2-5 and 2-6) and dissolved organic carbon (Figures 2-7 and 2-8). Colour and turbidity, two other water quality parameters that were measured followed a similar pattern to TP and DOC. They all describe the trophic status of a water body and are strongly correlated, therefore plotting the colour differences against two of water quality parameters would be enough to depict an image of the relationship between colour and lake productivity. The intensity of the colour change produced by *Polymorphus paradoxus* on both sexes of *G. lacustris* seemed insensitive to variations in total phosphorus (Figure 2-5) and dissolved organic carbon concentration (Figure 2-7) in the studied lakes. Although the trend seemed negative, the correlation between parasite-induced colour changes and TP or DOC concentrations was not significant (Table 2-11). However, the highest difference in colour was observed in the eutrophic Long Lake, and the smallest was observed in the hypereutrophic Kettle Lake. This parasite had no effect on the colour of females originating from Kettle and Narrow lakes or on males originating from Laica Lake (Figure 2-5 and 2-7).

The colour change produced by *Polymorphus marilis* seemed greater in amphipods from oligotrophic and eutrophic lakes than in those originating from hypereutrophic lakes, showing a negative relationship with TP (Figure 2-6) and DOC (Figure 2-8). This phenomenon is observed in both male and female *G. lacustris*, but a strong significant correlation was detected only between parasite-induced colour changes in males and TP or DOC (Table 2-11). TP and DOC concentrations were highly correlated with the degrees of colour differences between *P. marilis* parasitized and non-parasitized male amphipods (Figure 2-6 and 2-8). An increase in TP or DOC decreased the amount of colour difference induced by *P. marilis* in male amphipods. At the same time, parasite-induced colour changes in female amphipods seemed to not be correlated with concentrations of TP or DOC. The maximum parasite-induced colour difference

was found in eutrophic Long Lake (ΔE_{ab}=13.77 in males and ΔE_{ab}=14.06 in females), followed by oligotrophic Forestry 754 (ΔE_{ab}=11.43 in males) and the smallest colour difference is observed in hypereutrophic Kettle Lake (ΔE_{ab}=1.95 in males, ΔE_{ab}=1.07 in females). This pattern was not consistent, however, as the colour difference between non-parasitized and *P. marilis* parasitized female amphipods from hypereutrophic Range Road 223 Lake is higher than the colour difference produced by the same parasite in Beyette, a eutrophic lake.

Colour change produced by diet and water quality parameters in non-parasitized *G. lacustris* juveniles, a laboratory experiment

Amphipods were initially scanned, before entering the treatment period, and the CIE L*, a*, b* values were statistically analysed using SPSS GLM multivariate analysis. They were randomly divided into four groups. The multivariate analysis of colour differences between the four groups of amphipods showed that there was no difference between them before experiment began (F=1.07, df =9; 180, P> 0.1) (Table 2-12, Figure 2-9a). After a month under treatment conditions (water and diet from the oligotrohic lake, water and diet from the hypereutrophic lake and the two water and diet treatments resulted as combinations between the two lakes) the amphipods were scanned again and the resulting colours were statistically analysed.

The possibility of individual jars influencing the colour of amphipods, which would reduce the replication of treatments, was considered and tested using PATN v.3.11. If the treatments had a stronger influence on colour than did the jars, the amphipods from the two replicates (= jars) per treatment should cluster tightly with each other in multivariate space. All amphipods from a single jar were treated as a single *a priori* group. The Analysis of Similarity (ANOSIM) indicated that amphipods from two jars receiving the same treatment were not statistically different (Table 2-13) therefore the container had no significant influence on the colour of amphipods. Because amphipods were kept in groups, individuals could not be tracked for before and after colour measurements. Instead, treatment effects on final realized colour were compared between two amphipod test groups at a time. Colour interpretation is provided for amphipods

reared in conditions of water and/or food from Kettle Lake, relative to amphipods reared on conditions of water and/or diet from Narrow Lake.

In order to test the effect of diet on colour, amphipods under the same water treatment but different diets were compared using a multivariate GLM model in SPSS. This revealed that diet type affects colour, both when amphipods were held in water from Kettle Lake ($F=45.56$, $df=3, 31$, $P< 0.01$) and from Narrow Lake ($F= 4.69$, $df=3.31$, $P<0.01$). Intensity and direction of observed colour changes were determined through equations 2 to 4 applied to the average colour values of amphipods from the two food treatments (Table 2-15). The test of between-subjects effects indicated which of the colour vectors are responsible for the observed colour differences (Table 2-16).

When two groups of amphipods were maintained in the same water conditions from the hypereutrophic Kettle Lake, but one was fed substratum from the same hypereutrophic lake and the other substratum from the oligotrophic Narrow Lake, the former became darker, greener and bluer than the latter (Table 2-17). The average colour distance between the two diet groups was $\Delta Eab= 11.35$ units. Two other groups of amphipods were maintained in water from oligotrophic Narrow Lake, but one group was fed substratum from the hypereutrophic Kettle Lake and the second one received substratum from the oligotrophic Narrow Lake. In this case the amphipods on a 'hypereutrophic' diet became darker than amphipods fed with an 'oligotrophic' diet (Table 2-17). At the same time colour vectors a* and b* had no role in determining the colour differences between the two groups of amphipods. The average colour distance in amphipods fed on the two different diets and maintained in water from Narrow Lake was 1.85 units.

Although, the same two diet treatments were applied in both of these treatment combinations, the average colour distances between the two groups of amphipod was different. The only manipulated factor that could cause this difference was the water chemistry. The maximum colour distance between the two diet groups was observed when amphipods were placed in water from Kettle Lake (11.35 units), a colour difference that would be clearly visible to human eye. The colour distance between the two diet groups was only 1.85 units when

amphipods received water from the oligotrophic Narrow Lake. It is unlikely that this colour distance would be perceivable by human eye.

The colour differences between the groups of amphipods fed a hypereutrophic diet but reared in different water treatments were statistically significant ($F= 17.23$, $df = 3, 32$, $P< 0.01$) (Table 2-14). Amphipods fed an oligotrophic diet were also significantly affected by water treatment ($F=4.24$, $df = 3, 30$, $P= 0.01$) (Table 2-14). Amphipods on a hypereutrophic diet and kept in hypereutrophic water became darker and greener than the amphipods on the same diet but reared in water from the oligotrophic Narrow Lake. The average colour difference between these two groups was $\Delta E_{ab}= 6.29$ (Table 2-14), a difference clearly perceivable by human eye. Those amphipods fed an oligotrophic diet and kept in hypereutrophic water became redder and yellower than those reared in water from Narrow Lake. The average difference between the colour of the two groups of amphipods was $\Delta E_{ab}= 5.04$, a difference that is also perceivable by human eye.

All the above data showed that there was an interaction between water and diet affecting the colour in *G. lacustris* reared under laboratory conditions (Table 2-9 b). The distinctiveness of the four treatment groups was tested using ANOSIM in PATN. It was found that two groups of amphipods were not statistically different ($p=0.22$, Table 2-20). These amphipods were maintained in water from the oligotrophic Narrow Lake but one fed on substrate from the hypereutrophic Kettle Lake and the other on substrate from the oligotrophic Narrow Lake. All other pair-wise comparisons were statistically different. An ordination was used in order to express the colour differences of the amphipods in 2-D space, and to plot on vectors of L*, a*, b* to visualize their covariance and direction relative to the four treatments. All three colour vectors had a strong influence on the ordination (PCC correlation coefficients: $L^*= 0.80$, $a^*=0.89$, $b^*= 0.78$ and MCAO probability of correlation coefficients were all smaller than 0.001), which is not surprising given that these three variables alone were entirely responsible for creating the ordination. The group centroids for amphipods treated with water and diet from Kettle and those treated with water from Kettle and diet

from Narrow were at opposite loci on the colour space vectors (Figures 2-10a and 2-10b). Vectors for L*, a*, b* increased towards the amphipods reared in hypereutrophic water with oligotrophic food, indicating that they are lighter, redder and yellower than the rest of the 3 groups of tested amphipods. On the other hand the amphipods fed on substrate and swimming in water from Kettle Lake are in the direction of decreasing vector values, meaning that they are darker, greener and bluer than the rest of the groups.

To test whether these lab results were corroborated by field data, previously collected colour data for non-parasitized adult amphipods sampled from Kettle and Narrow lakes, were compared for differences in colour using a multivariate GLM analysis. CIE L*, a*, b* values were the dependent factors and the overall lake trophic quality was the independent nominal factor. The two sexes were separately analysed. The differences in colour between wild amphipods from the two lakes were statistically significant, both in males ($F= 69.38$, $df= 3; 60$, $p< 0.01$, Power 1.00) and in females ($F= 47.43$, $df= 3; 61$, $p< 0.01$, Power= 1.00). The test of between-subjects effects showed which of the colour vectors had a role in determining the colour differences detected by the multivariate test (Table 17). Males from Kettle Lake were greener and bluer than those from Narrow Lake (Table 19). Vector L* had no power in determining the colour differences between the two groups. In females, all three colour vectors had a role in determining the colour differences between the two groups, with females from hypereutrophic Kettle Lake being darker, greener and bluer than those from Narrow Lake. Thus, field data support the lab observations of colour differences in the two extreme combinations of water quality and food source.

2.4 DISCUSSION

Although there are several published studies on the colour effect of acanthocephalans on invertebrate hosts (Hindsbo, 1972; Oetinger and Nickol, 1981; Bakker *et al*, 1997), this is the first to focus on quantifying colour differences in amphipod hosts. I observed clear effects of parasitism by *Polymorphus* sp. on colour of *Gammarus lacustris*, although the strength of the effect varied within host sex, parasite species, and water body of origin. Colour

formation in juvenile amphipods was dependent on both water quality and supplied diet. Using a flatbed scanner provided a standardized method for comparing individuals originating from several lakes. The parameters of the scanner remained constant along the experiment, which allowed for detection of small, sometimes not noticeable by human eye, differences among and between groups. A drawback of the scanner was that repeated scannings of the same individual would likely cause damage due to heat on the platen, and perhaps also from the intense light.

Colour differences between non-parasitized *G. lacustris* males and females

The sex of the amphipod had a strong effect on colour, in both parasitized and non-parasitized individuals. Generally non-parasitized females were redder and bluer than non-parasitized males. However, the luminosity vector shifted between lower or higher value in females than in males in the various lakes included in the study. This made female amphipods either darker or lighter than males. Assuming that colour intensity is given by the concentration of carotenoids in the exoskeleton and/or body (see Chapter 3), this suggests that the total carotenoid content in females could be higher than in male amphipods from the two eutrophic lakes Beyette and Long, and lower in the oligotrophic Forestry 754 and Narrow and the hypereutrophic Laica water body. Females could have an overall higher concentration of carotenoids, as astaxanthin and esterified astaxanthin have been found as the main carotenoids involved in the lipovitellin-carotenoid complex occurring in the yolk (Mantiri *et al*, 1996; Berticat *et al*, 2000).

Colour differences between parasitized and non-parasitized amphipods

The measured higher luminosity values (pigmentation dystrophy) in female amphipods support the observation that parasitized females are unable to produce eggs due to carotenoid depletion (Dezfuli and Giari, 1999). Accepting a 'Just Noticeable Difference' between 2 and 3 units, *P. marilis* induced colour changes that were strongly visible to humans in *G. lacustris* originating from Beyette, Long, Forestry and Range Road 233 water bodies. At the same time the colour differences induced by the same parasite would be hardly visible to human eye in amphipods from the hypereutrophic lakes, Laica and Kettle. *Polymorphus*

paradoxus produced a clearly visible difference in colour only in the eutrophic Long Lake. In all other lakes, visible colour differences induced by this parasite species in parasitized amphipods are either debatable or lacking completely (i.e. amphipods from Narrow and Kettle lakes). This supports the original impression (H. Proctor, pers. obs.) that acanthocephalan-infected amphipods were different in colour only in some water bodies; however, it provides the added knowledge that this is not just related to the water body, but also to the species of parasite. It would be interesting to determine if the colour differences detectable to humans were also detectable by the waterfowl and rodent final hosts of these parasites

Polymorphus paradoxus had an effect on colour that was dependent on the sex of the host. In parasitized males it did not induce pigmentation dystrophy (generally luminosity vector didn't change), while parasitized females had higher luminosity values suggesting a pigmentation distrophy. *Polymorphus marilis* had the same effect on both male and female *G. lacustris*. It turned its hosts, greener and bluer with a high degree of pigmentation dystrophy. *Polymorphus marilis* induced colour differences between parasitized and non-parasitized amphipods that were more intense than the colour differences induced by *P. paradoxus* in both sexes and in all lakes. Therefore *P. marilis* must have more efficient mechanisms of influencing the colour in amphipods most likely through removing carotenoids and/or impeding the hosts from depositing them (see Chapter 3).

Parasitized amphipods tend to become bluer as a result of infection by both parasite species. Even though there is not a consistent increase of blueness difference between *P. marilis* parasitized and non-parasitized amphipods in-between oligotrophic, eutrophic and hypereutrophic lakes, the blueness change in colour is higher in oligotrophic + eutrophic lakes compared to the hypereutrophic ones. The luminosity values in amphipods parasitized by *P. marilis* from oligotrophic and eutrophic are generally higher than those in parasitized amphipods from hypereutrophic lakes. Higher luminosity coupled with more intense blue values measured in parasitized amphipods from oligo- and eutrophic lakes is consistent with my observations of more commonly found blue

parasitized amphipods in this type of lakes. This colour change made the bright red-orange acanthocephalan more contrastive with the colour of the host.

Effect of water and diet quality on the colour of wild and laboratory-reared amphipods

I showed in the lab study that both water chemistry and diet (Figure 2- 10 a and b) affected amphipod colour, but the exact mechanism remains unknown. Dissolved pigmented molecules may interact with the soft surface of the carapace right after moulting influencing the colour of amphipods. Dissolved organic carbon affects the colour and transparency of lake water through the effect of humic and fulvic acids (Pace and Cole, 2002). DOC influences the amount of radiation transmited through the water column. High concentrations of DOC from hypereutrophic lakes screen against most of the UV transmited radiations. Although the effect of radiation on colour formation of amphipods remains unknown, more intense UV radiations induce darker pigmentation in daphnids (Luecke and O'Brien, 1983). The influence of diet quality could be explained by the amount of carotenoids available in the substrates provided, which can influence the overall colour of amphipods. Higher concentration in dietary carotenoids can increase carotenoid concentration in animals (Grether *et al*, 1999; Bjerkeng, 2008) and influence their colour (Negro *et al*, 2000).

This study showed that both TP and DOC influenced all three CIE L*, a*, b* colour vectors. The influence of water quality over the colour of amphipods is ambiguous, as the two water treatments in the lab produced contradictory results. Based on the PATN analysis amphipods reared on different diets but same water from oligotrophic Narrow Lake were not different (Table 2-19). At the same time, the same two diets but water from hypereutrophic Kettle Lake induced different colour in amphipods. Similar observations were made by Wade *et al* (2008) when they tried to replicate the change of colour in *Panulirus cygnus* wild caught red individuals to the white stage under laboratory conditions.

Concluding remarks

The acanthocephalan-associated behavioural modifications (clinging behaviour or altered escaping response) were not observed in any of the

oligotrophic and eutrophic lakes from the present study but they were very strong in many amphipods from all three hypereutrophic lakes (pers. obs.). Similarly to my observations, Bethel and Holmes (1973) also described modified clinging and escaping behaviour in parasitized amphipods originating from two hypereutrophic lakes from Alberta, Hastings and Cooking lakes. This may be due to differences in fish presence in these water bodies. There may be selection on the acanthocephalans to not alter host behaviour in habitats where a predator other than the correct final host is likely to consume the intermediate hosts. Hypereutrophic lakes are less likely to harbour fish due to winterkill (winter anoxia) (Danylchuk and Tonn, 2003).

High variations in observed colour differences, modified behaviour and contested hypotheses regarding the purpose of carotenoids in cystacanths (i.e. Duclos, 2006; Kaldonski *et al*, 2009) suggest that acanthocephalans affect their hosts differently in various populations. Acanthocephalan parasites have a high mobility between lakes, by means of waterfowl movement. Although studied parasites should have similar genotypes, for the reason previously mentioned, the colour differences induced by *P. paradoxus* in amphipods from Long and Narrow Lake, geographically two very close lakes, are indeed different. Wolinska and King (2009) recognised the heterogeneity of environmental factors and their influence on the host-parasite interactions.

Given that this study considered several populations of amphipods and their parasites in environments with different trophic parameters, and showed that the parasite-host interactions are not the same between different water bodies, future studies should consider and incorporate environmental factors when investigating these interactions.

Table 2-1. Chemical, physical and biological characteristics of the seven sampled lakes from Alberta, Canada

Lake	TP µg/L	DOC mg/L	Colour mg/L Pt-Co	Turbidity NTUs	Area km^2	Max depth m	Dominant shore vegetation	Dominant aquatic macrophytes
Forestry 754	8.4	1.70	6.20	0.83	0.04	6	*Picea* sp.	*Water moss*
Narrow	9.3	17.2	10.90	0.47	1.14	38	*Populus* sp.	*Chara* sp. *Utricularia* sp. *Potamogeton* spp.
Long	31.0	11.6	16.88	1.83	1.62	28	*Picea* sp. *Populus* sp.	*Myriophyllum* sp. *Chara* sp. *Ceratophyllum* sp.
Beyette	45.7	17.7	13.12	1.27	0.40	4	*Picea* sp. *Populus* sp.	*Drepanocladus* sp. *Myrriophyllum* sp.
Laica	248.5	39.6	94.53	6.45	0.15	4	*Typha* sp. *Populus* sp.	*Typha* sp. *Nuphar* sp. *Myriophyllum* sp. *Typha* sp.
Range Rd 223	271.0	32.8	84.92	2.38	0.05	3	*Typha* sp. *Populus* sp.	*Typha* sp. *Myriophyllum* sp
Kettle	289.4	38.0	77.13	0.17	0.13	10	*Typha* sp.	*Typha* sp. *Myriophyllum* sp.

Table 2-2 Measurements of *Polymorphus paradoxus* and *P. marilis* everted cystacanths. The specimens were collected from Laica, Kettle, Range Road 223 and Beyette lakes, Alberta

ID	Proboscis length /width (µm)	Neck length/ wide base/ width at proboscis (µm)	Body length (mm)	# of hooks/row	Longest hook (µm)
P. paradoxus	0.56/0.3	0.71/0.3/0.22	3.13	9 (alternate 8)	70
P. marilis	0.38/0.17	0.33/0.18/0.13	2.00	7	55

Table 2-3. Colour differences between female and male *G. lacustris* when both are parasitized by *P. paradoxus* or *P. marilis*, or non-parasitized in seven lakes from Alberta. The colour differences ΔL*, Δa*, Δb* and ΔEab are calculated using equation 1-4 (Introduction) on the averages displayed by SPSS GLM descriptive statistics, using the colour of males as the standard colour

Site	CIE Lab 1931	No parasite		*P. paradoxus*		*P. marilis*	
		Female vs. Male	Colour	Female vs. Male	Colour	Female vs. Male	Colour
Beyette	ΔL*	-1.63	Darker	-	No difference	-	No difference
	Δa*	-0.58	-	-		-	
	Δb*	-5.25	Bluer	-		-	
	ΔE_{ab}	5.53					
Long	ΔL*	-1.85	Darker	-	No difference	-	No difference
	Δa*	1.19	Redder	-		-	
	Δb*	-2.14	Bluer	-		-	
	ΔE_{ab}	3.07					
Narrow	ΔL*	5.61	Lighter	5.65	Lighter	-	No samples
	Δa*	0.60	Redder	0.48	-	-	
	Δb*	-2.59	Bluer	-0.64	-	-	
	ΔE_{ab}	6.21		5.71			
Forestry 754	ΔL*	4.42	Lighter	-	No samples	-	No samples
	Δa*	1.12	Redder	-		-	
	Δb*	-0.75	-	-		-	
	ΔE_{ab}	4.62					
Kettle	ΔL*	0.07	-	-	No difference	1.62	Lighter
	Δa*	0.82	Redder	-		0.65	Redder
	Δb*	0.19	-	-		1.77	Yellower
	ΔE_{ab}	0.84				2.49	
Rg. Rd. 223	ΔL*	0.30	-	2.47	Lighter	-	No difference
	Δa*	0.00	-	-1.37	Greener	-	
	Δb*	-5.28	Bluer	-2.39	Bluer	-	
	ΔE_{ab}	5.29		3.70			
Laica	ΔL*	2.71	Lighter	-2.13	Darker	-	No samples
	Δa*	0.32	-	-0.58	-	-	
	Δb*	-0.72	-	-3.61	Bluer	-	
	ΔE_{ab}	2.82		4.23			

Table 2-4. Test of between-subjects effects (similar to ANOVA) showing the effect of variable 'sex' on the CIE L*, a*, b* vectors within a group, non-parasitized *G. lacustris*, parasitized by *P. paradoxus* and parasitized by *P. marilis*. $\alpha < 0.05$. * indicates a significant difference

Site	CIE 1931	df; df error	F	Sig.	Power	df; df error	F	Sig.	Power	df; df error	F	Sig.	Power
		No parasite				Parasitzed by *P. paradoxus*				Parasitized by *P. marilis*			
Beyette	L*	1; 71	3.25	0.08	0.43	1; 60	2.33	0.13	0.32	1; 6	2.05	0.20	0.23
	a*	1; 71	1.26	0.27	0.20	1; 60	2.50	0.12	0.34	1; 6	2.22	0.19	0.24
	b*	1; 71	31.33	0.00*	1.00	1; 60	3.79	0.06	0.48	1; 6	1.92	0.22	0.22
Long	L*	1; 50	3.66	0.06	0.47	1; 30	4.33	0.05	0.52	1; 19	2.04	0.17	0.27
	a*	1; 50	4.65	0.04*	0.56	1; 30	3.12	0.09	0.40	1; 19	0.32	0.58	0.08
	b*	1; 50	4.19	0.05*	0.52	1; 30	0.85	0.36	0.14	1; 19	0.03	0.87	0.05
Narrow	L*	1; 65	81.55	0.00*	1.00	1; 38	36.81	0.00*	1.00				
	a*	1; 65	5.89	0.02*	0.67	1; 38	0.56	0.46	0.11	No samples			
	b*	1; 65	17.81	0.00*	0.99	1; 38	0.28	0.60	0.08				
Forestry 754	L*	1; 58	25.78	0.00*	1.00								
	a*	1; 58	6.89	0.01*	0.73	No samples				No samples			
	b*	1; 58	0.92	0.34	0.16								
Kettle	L*	1; 60	0.03	0.85	0.05	1; 45	0.09	0.77	0.11	1; 25	13.01	0.00*	1.00
	a*	1; 60	11.44	0.00*	0.91	1; 45	0.29	0.59	0.14	1; 25	3.42	0.08	0.40
	b*	1; 60	0.16	0.69	0.07	1; 45	0.34	0.56	0.16	1; 25	5.36	0.03*	0.67
Rg. Rd. 223	L*	1; 60	0.20	0.66	0.07	1; 52	4.17	0.05	0.52	1; 6	3.54	0.11	0.35
	a*	1; 60	0.00	1.00	0.05	1; 52	6.78	0.01*	0.72	1; 6	0.02	0.89	0.05
	b*	1; 60	46.49	0.00*	1.00	1; 52	3.68	0.06	0.47	1; 6	0.85	0.39	0.12
Laica	L*	1; 64	13.66	0.00*	0.95	1; 32	3.90	0.06	0.48				
	a*	1; 64	0.94	0.33	0.16	1; 32	0.79	0.38	0.14	No samples			
	b*	1; 64	0.61	0.44	0.12	1; 32	7.38	0.01*	0.75				

Table 2-5. CIE L*a*b* average values ± SD for scanned pictures of *G. lacustris* females and males from seven lakes from Alberta based on SPSS GLM output. $\alpha < 0.05$

Site	CIE Lab 1931	No parasite		*P. paradoxus*		*P. marilis*	
		Male	Female	Male	Female	Male	Female
Beyette	L*	41.95 ± 3.72	40.32 ± 4.02	41.36 ± 4.54	43.05 ± 4.02	48.74 ± 3.5	44.93 ± 4.02
	a*	-1.06 ± 2.57	-1.64 ± 1.73	-1.02 ± 2.01	-1.85 ± 2.09	-2.27 ± 2.6	-4.25 ± 0.8
	b*	19.63 ± 4.22	14.38 ± 3.79	17.51 ± 5.15	14.92 ± 5.23	13.87 ± 4.98	9.55 ± 3.77
	Sig.	<0.01		0.08		0.33	
	F; df	26.42; 3, 69		2.55; 3, 58		1.54; 3, 4	
Long	L*	39.58 ± 2.33	37.73 ± 4.55	37.97 ± 2.50	40.43 ± 4.18	43.57 ± 2.15	45.79 ± 4.24
	a*	-2.85 ± 2.11	-1.66 ± 1.74	-4.27 ± 1.53	-3.3 ± 1.53	-4.91 ± 0.84	-4.69 ± 0.91
	b*	18.08 ± 4.29	15.94 ± 2.78	11.1 ± 4.22	12.4 ± 3.43	5.07 ± 3.80	4.83 ± 2.82
	Sig.	<0.01		0.13		0.60	
	F; df	11.61; 3, 48		2.08; 3, 38		0.64; 3, 17	
Narrow	L*	38.84 ± 2.53	44.45 ± 2.55	38.22 ± 1.53	43.87 ± 3.79	-	-
	a*	-3.25 ± 1.16	-2.65 ± 0.85	-3.11 ± 2.18	-2.63 ± 1.87	-	-
	b*	15.76 ± 2.95	13.17 ± 1.98	14.06 ± 3.97	13.42 ± 3.63	-	-
	Sig.	<0.01		<0.01			
	F; df	75.34; 3, 63		12.91; 3, 63			
Forestry 754	L*	35.72 ± 2.75	40.14 ± 3.90	-	-	-	-
	a*	-1.96 ± 1.97	-0.84 ± 1.26	-	-	-	-
	b*	16.09 ± 3.84	15.34 ± 1.91	-	-	-	-
	Sig.	<0.01					
	F; df	14.19; 3, 56					
Kettle	L*	39.70 ± 1.70	39.77 ± 1.49	39.65 ± 1.87	39.81 ± 1.93	39.05 ± 1.06	40.67 ± 1.20
	a*	-5.82 ± 0.91	-5.00 ± 1.00	-5.16 ± 0.97	-5.00 ± 1.05	-5.75 ± 0.82	-5.10 ± 0.95
	b*	8.66 ± 1.76	8.85 ± 1.93	9.20 ± 2.06	8.85 ± 1.05	6.82 ± 2.06	8.59 ± 1.87
	Sig.	0.01		0.66		0.01	
	F; df	4.5; 3, 58		0.54; 3, 45		4.78; 3, 23	
Rg Rd. 223	L*	37.07 ± 2.85	37.37 ± 2.37	37.88 ± 5.32	40.35 ± 3.54	39.51 ± 4.18	45.36 ± 0.17
	a*	-4.21 ± 1.99	-4.21 ± 1.84	-2.97 ± 2.29	-4.34 ± 1.57	-6.44 ± 2.08	-6.66 ± 0.1
	b*	20.44 ± 3.35	15.16 ± 2.73	18.10 ± 4.65	15.71 ± 4.50	12.70 ± 5.89	8.51 ± 3.49
	Sig.	<0.01		<0.01		0.44	
	F; df	48.88; 3, 58		5.05; 3, 50		1.12; 3, 4	
Laica	L*	39.92 ± 3.57	42.63 ± 2.24	42.50 ± 3.58	40.37 ± 2.34	-	-
	a*	-3.09 ± 1.12	-2.77 ± 1.55	-2.87 ± 1.85	-3.45 ± 1.55	-	-
	b*	14.48 ± 3.74	13.76 ± 3.67	15.67 ± 4.44	12.06 ± 2.89	-	-
	Sig.	<0.01		<0.01		-	
	F; df	6.99; 3, 62		4.63; 3, 30		-	

Table 2-6. Average CIE L*a*b* results for scanned pictures of parasitized and non-parasitized *G. lacustris* males in seven lakes from Alberta. p value, F test value and degrees of freedom are based on the General Linear Model (α <0.05) output in SPSS. P0= non-parasitized *G. lacustris*, P1= *G. lacustris* parasitized by *P. paradoxus* and P2= *G. lacustris* parasitized by *P. marilis*

Site	CIE L*a*b*	*P. paradoxus*	Non-parasitized	*P. marilis*	GLM P1 vs.P2
		GLM P0 vs. P1		GLM P0 vs. P2	
Beyette	L*	41.36 ± 4.54	41.95 ± 3.71	48.74 ± 3.50	
	a*	-1.02 ± 2.01	-1.06 ± 2.57	-2.22 ± 2.60	
	b*	17.51 ± 5.15	19.63 ± 4.22	13.87 ± 4.98	
		p<0.01, F= 4.57, df=3; 67		p<0.01, F=10.23, df=3;36	p=0.02, F=3.75, df=3;35
Narrow	L*	38.22 ± 1.53	38.84 ± 2.53	-	
	a*	-3.11 ± 2.18	-3.25 ± 1.16	-	
	b*	14.06 ± 3.97	15.76 ± 2.95	-	
		p=0.03, F=3.11, df=3; 48		-	-
Long	L*	37.97 ± 2.50	39.58 ± 2.33	43.57 ± 2.15	
	a*	-4.27 ± 1.53	-2.85 ± 2.11	-4.91 ± 0.84	
	b*	11.10 ± 4.22	18.08 ± 4.29	5.07 ± 3.80	p<0.01,
		p<0.01, F=12.98, df=3; 45		p<0.01, F=57.75, df=3; 35	F=20.65, df=3; 24
Forestry 754	L*	-	35.72 ± 2.75	40.75 ± 2.89	
	a*	-	-1.96 ± 1.97	-3.27 ± 2.25	
	b*	-	16.09 ± 3.84	5.92 ± 7.68	
		-		p<0.01, F= 17.29, df=3; 30	-
Rg Rd 223	L*	37.88 ± 5.32	37.07 ± 2.85	39.51 ± 4.18	
	a*	-2.97 ± 2.29	-4.21 ± 1.99	-6.44 ± 2.08	
	b*	18.10 ± 4.65	20.44 ± 3.35	12.70 ±5.89	p=0.01,
		p<0.01, F=12.19, df=3; 50		p<0.01, F=11.59, df=3; 32	F=4.28, df=3; 26
Kettle	L*	39.66 ± 1.84	39.70 ± 1.70	39.05 ± 1.06	
	a*	-5.15 ± 0.95	-5.82 ± 0.91	-5.75 ± 0.82	
	b*	9.22 ± 2.02	8.66 ± 1.76	6.82 ± 2.06	
		p=0.02, F=3.36, df=3; 53		p=0.03, F=3.34, df=3; 38	p=0.02, F=3.64, df=3; 33
Laica	L*	42.50 ± 3.58	39.92 ± 3.57	41.53 ± 1.89	
	a*	-2.87 ± 1.85	-3.09 ± 1.12	-3.66 ± 0.93	
	b*	15.67 ± 4.44	14.48 ± 3.74	12.95 ± 2.14	p=0.25,
		p=0.23, F=1.50, df=3; 37		p=0.01, F=4.11, df=3; 47	F=1.49, df=3; 22

Table 2-7. Test of between-subjects effects (ANOVA) showing the effect of the two species of parasites, *P. paradoxus* and *P. marilis* on the CIE L*, a*, b* colour vectors of parasitized amphipods when compared to non-parasitized ones within a sex group; $\alpha < 0.05$. * indicates a significant difference

Site	Parasite	Colour vector	df; df Error	F	Sig	Power	df; df Error	F	Sig.	Power
			Male *G. lacustris*				Female *G. lacustris*			
Beyette	*P. paradoxus*	L	1; 69	0.36	0.55	0.09	1; 62	7.23	0.01*	0.75
		a	1; 69	0.01	0.94	0.05	1; 62	0.20	0.65	0.07
		b	1; 69	3.58	0.06	0.46	1; 62	0.24	0.63	0.08
	P. marilis	L	1; 38	12.15	0.00*	0.92	1; 39	4.76	0.04*	0.57
		a	1; 38	0.73	0.40	0.13	1; 39	8.69	0.01*	0.82
		b	1; 38	6.49	0.01*	0.70	1; 39	5.86	0.02*	0.66
Narrow	*P. paradoxus*	L	1; 50	0.93	0.34	0.16	1; 53	0.45	0.51	0.10
		a	1; 50	0.09	0.77	0.06	1; 53	0.00	0.96	0.05
		b	1; 50	3.07	0.09	0.40	1; 53	0.11	0.74	0.06
Long	*P. paradoxus*	L	1; 47	5.20	0.03*	0.61	1; 33	3.05	0.09	0.40
		a	1; 47	6.47	0.01*	0.70	1; 33	7.86	0.01*	0.78
		b	1; 47	31.26	0.00*	1.00	1; 33	11.16	0.00*	0.90
	P. marilis	L	1; 37	21.03	0.00*	0.99	1; 32	25.47	0.00*	1.00
		a	1; 37	8.09	0.01*	0.79	1; 32	31.25	0.00*	1.00
		b	1; 37	66.86	0.00*	1.00	1; 32	123.16	0.00*	1.00
Forestry 754	*P. marilis*	L	1; 32	11.69	0.00*	0.91				
		a	1; 32	1.54	0.22	0.23	No samples			
		b	1; 32	19.33	0.00*	0.99				
Rg. Rd. 223	*P. paradoxus*	L	1; 52	.51	0.48	0.11	1; 60	15.42	0.00*	0.97
		a	1; 52	4.52	0.04*	0.55	1; 60	0.09	0.76	0.06
		b	1; 52	4.58	0.04*	0.56	1; 60	0.34	0.56	0.09
	P. marilis	L	1; 34	3.12	0.09	0.40	1; 32	22.20	0.00*	1.00
		a	1; 34	6.26	0.02*	0.68	1; 32	3.47	0.07	0.44
		b	1; 34	20.38	0.00*	0.99	1; 32	10.91	0.00*	0.89
Kettle	*P. paradoxus*	L	1; 56	0.11	0.74	0.06	1; 52	0.01	0.93	0.05
		a	1; 56	8.62	0.00*	0.82	1; 52	0.00	0.99	0.05
		b	1; 56	2.24	0.14	0.31	1; 52	0.00	1.00	0.05
	P. marilis	L	1; 40	1.39	0.25	0.21	1; 43	6.88	0.01*	0.73
		a	1; 40	0.05	0.83	0.06	1; 43	0.22	0.65	0.07
		b	1; 40	8.15	0.01*	0.80	1; 43	0.08	0.78	0.06
Laica	*P. paradoxus*	L	1; 39	3.35	0.07	0.43	1; 57	14.23	0.00*	0.96
		a	1; 39	0.20	0.66	0.07	1; 57	2.83	0.10	0.38
		b	1; 39	0.61	0.44	0.12	1; 57	3.80	0.06	0.48
	P. marilis	L	1; 49	3.14	0.08	0.41				
		a	1; 49	3.37	0.07	0.44	No samples			
		b	1; 49	2.53	0.12	0.34				

Table 2-8. Colour differences in *G. lacustris* males infected by *P. paradoxus* and *P. marilis* cystacanths when compared to non-parasitized males. ΔL*, Δa*, Δb* and ΔEab are calculated based on the L*, a* and b* averages displayed by the SPSS GLM analysis descriptive statistics for each group of amphipods, non-parasitized, *P. paradoxus* and *P. marilis* parasitized, and using the equations 1-4 (Introduction). P0= no. of non-parasitized *G. lacustris*, P1= no. of *G. lacustris* parasitized by *P. paradoxus* and P2= no. of *G. lacustris* parasitized by *P. marilis*

Site	Colour differences	*P.pardoxus* vs. non-parasitized		*P.marilis* vs. non-parasitized	
Beyette	ΔL*	-0.59	-	6.79	Lighter
P0=36	Δa*	0.04	-	-1.21	-
P1=35	Δb*	-2.12	Bluer	-5.76	Bluer
P2=4	ΔE_{ab}	2.20		8.99	-
Narrow	ΔL*	-0.62	-	-	No samples
P0=33	Δa*	0.14	-	-	
P1=19	Δb*	-1.70	Bluer	-	
P2=0	ΔE_{ab}	1.81		-	
Long	ΔL*	-1.61	Darker	3.99	Lighter
P0=31	Δa*	-1.42	Greener	-2.06	Greener
P1=20	Δb*	-6.98	Bluer	-13.01	Bluer
P2=8	ΔE_{ab}	7.31		13.76	
Forestry 754	ΔL*	-	No samples	5.03	Lighter
P0=30	Δa*	-		-1.32	-
P1=0	Δb*	-		-10.17	Bluer
P2=4	ΔE	-		11.43	
Rg. Rd. 223	ΔL*	0.81	-	2.44	-
P0=30	Δa*	1.24	Redder	-2.23	Greener
P1=24	Δb*	-2.34	Bluer	-7.74	Bluer
P2=6	ΔE_{ab}	2.77		8.42	
Kettle	ΔL*	-0.05	-	-0.65	-
P0=40	Δa*	0.66	Redder	0.07	-
P1=28	Δb*	0.54	-	-1.84	Bluer
P2=11	ΔE_{ab}	0.87		1.95	
Laica	ΔL	-	No difference	1.61	Lighter
P0=33	Δa	-		-0.57	Greener
P1=8	Δb	-		-1.53	-
P2=18	ΔE_{ab}	-		2.29	

Table 2-9. Colour differences in *G. lacustris* females infected by *P. paradoxus* and *P. marilis* cystacanths when compared to non-parasitized females. ΔL*, Δa*, Δb* and ΔEab are calculated based on the L*, a* and b* averages displayed by the SPSS GLM analysis descriptive statistics for each group of amphipods, non-parasitized, *P. paradoxus* and *P. marilis* parasitized, and using the equations 1-4. P0=no. of non-parasitized *G. lacustris*, P1= no. of *G. lacustris* parasitized by *P. paradoxus* and P2= no. of *G. lacustris* parasitized by *P. marilis*

Site	Colour differences	*P.pardoxus* vs. non-parasitized		*P.marilis* vs. non-parasitized	
Beyette	ΔL	2.73	Lighter	4.61	Lighter
P0=37	Δa	-0.21	-	-2.61	Greener
P1=27	Δb	0.55	-	-4.83	Bluer
P2=4	ΔE_{ab}	2.80		7.17	
Long	ΔL	2.70	Lighter	8.06	Lighter
P0=22	Δa	-1.64	Greener	-3.03	Greener
P1=13	Δb	-3.54	Bluer	-11.12	Bluer
P2=12	ΔE_{ab}	4.75		14.06	
Narrow	ΔL	-		-	
P0=34	Δa	-	No	-	No
P1=21	Δb	-	difference	-	samples
P2=0	ΔE_{ab}	-		-	
Forestry 754	ΔL	-		-	
P0=30	Δa	-		-	No
P1=0	Δb	-	No samples	-	samples
P2=0	ΔE_{ab}	-			
Rg. Rd. 223	ΔL	2.99	Lighter	8.00	Lighter
P0=32	Δa	-0.13	-	-2.45	-
P1=30	Δb	0.55	-	-6.65	Bluer
P2=2	ΔE_{ab}	3.04		10.68	
Kettle	ΔL	-		1.01	Lighter
P0=31	Δa	-	No	-0.14	-
P1=23	Δb	-	difference	-0.31	-
P2=16	ΔE_{ab}	-		1.07	
Laica	ΔL	-2.26	Darker	-	
P0=33	Δa	-0.68	-	-	No
P1=26	Δb	-1.71	Bluer	-	samples
P2=0	ΔE_{ab}	2.91			

Table 2-10. Average CIE L*a*b* ± SD for scanned pictures of parasitized and non-parasitized *G. lacustris* females in six lakes from Alberta. Significance p value, F test value and degrees of freedom are based on the General Linear Model output in SPSS (α <0.05). P0= non-parasitized *G. lacustris*, P1= *G. lacustris* parasitized by *P. paradoxus* and P2= *G. lacustris* parasitized by *P. marilis*; '-'no data available

Lake site	L*a*b*	Colour space average values ± SD			
		P. paradoxus GLM P0 vs. P1	Non-parasitized	*P. marilis* GLM P0 vs. P2	GLM P1 vs.P2
Beyette	L*	43.05 ± 4.02	40.32 ± 4.02	44.93 ± 4.02	
	a*	-1.85 ± 2.09	-1.64 ± 1.73	-4.25 ± 0.80	p=0.07,
	b*	14.92 ± 5.23	14.38 ± 3.79	9.55 ±3.77	F=2.62,
		p=0.01, F=3.98, df=3; 60		p=0.01, F=7.18, df=3; 37	df=3; 27
Narrow	L*	43.87 ± 3.79	44.45 ± 2.55	-	
	a*	-2.63 ± 1.87	-2.65 ± 0.85	-	
	b*	13.42 ± 3.63	13.17 ± 1.98	-	
		p=0.87, F=0.24, df=3; 51		-	-
Long	L*	40.43 ± 4.18	37.73 ± 4.55	45.79 ± 4.24	
	a*	-3.30 ± 1.53	-1.66 ± 1.74	-4.69 ± 0.91	
	b*	12.40 ± 3.43	15.94 ± 2.78	4.83 ± 2.82	p<0.01,
		p<0.01, F=4.76, df=3; 31		p<0.01, F=57.38, df=3; 30	F=11.47, df=3; 21
Rg Rd 223	L*	40.35 ± 3.54	37.37 ± 2.37	45.36 ± 0.17	
	a*	-4.34 ± 1.57	-4.21 ± 1.84	-6.66 ± 0.10	
	b*	15.71 ± 4.50	15.16 ± 2.73	8.51 ± 3.49	p=0.03,
		p<0.01, F=7.35, df=3; 58		p<0.01, F=21.57, df=3, 30	F=3.64, df=3; 28
Kettle	L*	39.81 ± 1.93	39.77 ± 1.49	40.78 ± 1.12	
	a*	-5.00 ± 1.05	-5.00 ± 1.00	-5.14 ± 0.95	
	b*	8.85 ± 2.10	8.85 ± 1.93	8.54 ± 1.92	p=0.22,
		p=1.0, F<0.01, df=3; 50		p=0.02, F=2.66, df=3; 43	F=1.56, df=3; 35
Laica	L*	40.37 ± 2.34	42.63 ± 2.24	-	
	a*	-3.45 ± 1.55	-2.77 ± 1.55	-	
	b*	12.06 ± 2.89	13.76 ± 3.65	-	
		p<0.01, F=5.16, df=3; 55		-	-

Table 2-11. Correlation between amounts of colour differences induced by *P. paradoxus* and *P. marilis* in amphipods and total phosphorus (TP) and dissolved organic carbon (DOC) from the sampled lakes

Parasite status			Correlation	
			TP	DOC
Male amphipods	*P. paradoxus*	Pearson's r	-0.42	-0.51
		p-value	0.48	0.38
		n	5	5
	P. marilis	Pearson's r	-0.89	-0.91
		p-value	0.02	0.01
		n	6	6
Female amphipods	*P. paradoxus*	Pearson's r	-0.54	-0.66
		p-value	0.46	0.34
		n	4	4
	P. marilis	Pearson's r	-0.55	-0.70
		p-value	0.45	0.30
		n	4	4

Table 2-12. Average ± SD values of CIE L*, a*, b* 1931 colour space of images of non-parasitized juvenile *G. lacustris* from Kettle Lake before entering the laboratory experiment. Values are obtained from the descriptive statistic of GLM in SPSS. $\alpha < 0.05$

CIE 1931	Water Kettle + Diet Narrow	Water Kettle + Diet Kettle	Water Narrow + Diet Kettle	Water Narrow + Diet Narrow	Multivariate GLM	
L*	44.40 ± 2.59	43.9 ± 3.28	44.29 ± 2.27	44.73 ± 2.24	df; df error	9; 180
a*	-0.59 ± 1.54	-0.23 ± 1.66	-1.47 ± 1.56	-0.81 ± 1.94	F	1. 07
b*	22.37 ± 2.96	21.56 ± 2.50	20.42 ± 3.29	21.73 ± 4.80	Sig.	> 0.1

Table 2-13. PATN ANOSIM p-values for the colour differences in amphipods from 8 jars receiving 2 water treatments (WK and WN) and 2 food treatments (DK and DN). p-values for the pair-wise test between jars receiving the same water and food treatments are highlighted in grey. The cut-off for significance is 0.05 (SSH, MDS, Gower-Metric, Stress = 0.084 in 2 dimensions. WN,DN – water and diet from Narrow Lake; WK,DK – water and diet from Kettle Lake; WN,DK and WK, DN – combinations of water and diet from Narrow and Kettle lakes

	Jar 1 WK,DN	Jar 2 WK,DN	Jar 3 WK,DK	Jar 4 WK,DK	Jar 5 WN,DK	Jar 6 WN,DK	Jar 7 WN,DN
Jar 2 WK,DN	0.22						
Jar 3 WK,DK	0.00	0.00					
Jar 4 WK,DK	0.00	0.00	0.98				
Jar 5 WN,DK	0.10	0.01	0.00	0.03			
Jar 6 WN,DK	0.01	0.00	0.00	0.00	0.28		
Jar 7 WN,DN	0.59	0.01	0.00	0.00	0.37	0.08	
Jar 8 WN,DN	0.07	0.00	0.00	0.00	0.34	0.44	0.33

Table 2-14. Average CIE L*, a*, b* values for amphipods under treatments of water (WN) and diet (DN) from Narrow Lake and water (WK) and diet (DK) from Kettle Lake. GLM output for testing the effect of different diets under similar water conditions and of the effect of different water under similar diet treatment is presented. $\alpha < 0.05$

Diet /	DK			DN			GLM
Water	L*	a*	b*	L*	a*	b*	DK vs. DN
WK	43.14 ± 3.27	-3.23 ±1.14	15.00 ±2.22	52.88 ± 3.60	0.52 ± 1.68	19.47 ± 3.91	F= 45.56 df= 3; 31 p< 0.01 Power= 1.0
WN	49.18 ± 2.69	-1.98 ±1.27	16.22 ± 2.22	50.73 ± 2.82	-1.45 ± 1.55	15.36 ± 3.04	F= 4.69 df= 3; 31 p< 0.01 Power= 0.9
	GLM WK vs. WN F= 17.23; df= 3; 32 p < 0.01; Power= 1.00			GLM WK vs. WN F= 4.24; df= 3; 30 p= 0.01;Power= 0.81			

Table 2-15. The colour differences produced by treatments of diet (D) and water (W) from the oligotrophic Narrow Lake (DN and WN) and the hypereutrophic Kettle Lake (DK and WK) on the colour of *G. lacustris* juveniles. Differences in colour vectors and distance between two colours were calculated based on equation 1-4 (Introduction) and on the descriptive statistic output of GLM analysis in SPSS at $\alpha < 0.05$ (Table 12). $X^{standard}$ - the standard colour for comparison purposes

Treatment	DK			$DN^{standard}$			Colour differences - Diet treatment			
	L*	a*	b*	L*	a*	b*	ΔL*	Δa*	Δb*	ΔEab
WK	43.14	-3.23	15.00	52.88	0.52	19.47	-9.74	-3.75	-4.47	11.35
$WN^{standard}$	49.18	-1.98	16.22	50.73	-1.45	15.36	-1.55	-0.53	0.86	1.85
Colour differences	ΔL*	Δa*	Δb*	ΔL*	Δa*	Δb*				
- water treatment	-6.04	-1.25	-1.22	2.15	1.97	4.11				
ΔEab		6.29			5.04					

Table 2-16. Test of between-subjects effects (ANOVA) showing the effect of the four treatments on the CIE L*, a*, b* colour vectors of juvenile amphipods in laboratory conditions. Each test, diet from Kettle Lake (DK) vs. diet from Narrow Lake (DN) and water from Kettle Lake (WK) and water from Narrow Lake (WN) was run under different water conditions (WK or WN) and diet conditions (DK and DN) respectively. $\alpha < 0.05$. * significant difference

GLM/ Colour vectors		df; df error	F	Sig.	Power	GLM/ Colour vectors		df; df error	F	Sig.	Power
Treatment			DK vs. DN			Treatment			WK vs WN		
WK	L*	1; 33	70.53	<0.01*	1.00	DK	L*	1; 34	37.69	<0.01*	1.00
	a*	1; 33	59.82	<0.01*	1.00		a*	1; 34	9.52	<0.01*	0.85
	b*	1; 33	17.55	<0.01*	0.98		b*	1; 34	2.69	0.11	0.36
WN	L*	1; 33	2.83	0.10	0.37	DN	L*	1; 32	3.78	0.06	0.47
	a*	1; 33	1.25	0.27	0.19		a*	1; 32	12.54	<0.01*	0.93
	b*	1; 33	.90	0.35	0.15		b*	1; 32	11.69	<0.01*	0.91

Table 2-17. Interpretation of colour change in amphipods subjected to diet and water treatments, based on the averages displayed by the multivariate GLM output (Table 12) and the test of between-subjects effects of the same multivariate test (Table 14). The described colour changes are seen in amphipods reared on conditions of water (WK) and diet (DK) from Kettle Lake Water relative to amphipods reared on conditions of water (WN) and diet (DN) from Narrow Lake (standard). $X^{standard}$ - the standard colour for comparison purposes

Treatment/Colour vectors/ Constant	DK vs. $DN^{standard}$			Treatment/Colour vectors/ Constant	WK vs. $WN^{standard}$		
	ΔL*	Δa*	Δb*		ΔL*	Δa*	Δb*
WK	Darker	Greener	Bluer	DK	Darker	Greener	-
WN	Darker	-	-	DN	-	Redder	Yellower

Table 2-18. GLM SPSS analyses of colour differences between adult non-parasitized amphipods originating from Narrow and Kettle water bodies, and between juvenile amphipods exposed to treatments of water and diet from Narrow (WN,FN) and water and diet from Kettle Lake (WK,DK). $\alpha < 0.05$

Statistic test	Colour vectors	df; df error	F	Sig.	df; df error	F	Sig.	df; df error	F	Sig.
		WN,DN vs. WK,DK juvenile amphipods			Narrow vs. Kettle male amphipods			Narrow vs. Kettle female amphipods		
GLM		3; 31	37.48	< 0.01	3; 60	69.38	< 0.01	3; 61	47.43	< 0.01
ANOVA	L*	1; 33	53.71	< 0.01	1; 62	2.50	0.12	1; 63	79.32	< 0.01
	a*	1; 33	15.05	< 0.01	1; 62	96.66	< 0.01	1; 63	105.50	< 0.01
	b*	1; 33	0.16	0.69	1; 62	134.20	< 0.01	1; 63	79.31	< 0.01

Table 2-19. Colour differences between wild caught adult amphipods from Narrow and Kettle lakes and those between the juveniles reared under laboratory conditions of water and diet from Narrow Lake (WN,DN) and water and diet from Kettle Lake (WK,DK). Values are based on the GLM output at a $p < 0.05$ (Table 16) and intensity and direction of colour change was based on equation 1-4 and the test between-subjects effects of the GLM analysis (Table 16). $X^{standard}$ - the standard colour for comparison purposes

	Treatment	Males			Females		
		L*	a*	b*	L*	a*	b*
In the wild	$Narrow^{standard}$	38.84	-3.25	15.76	44.45	-2.65	13.17
	Kettle	39.70	-5.82	8.66	39.77	-5.00	8.85
	Colour differences	0.86	-2.57	-7.09	-4.67	-2.35	-4.32
		-	Greener	Bluer	Darker	Greener	Bluer
	Juvenile amphipods						
In the lab	$WNDN^{standard}$	50.73	-1.45	15.36			
	WKDK	43.14	-3.23	15			
	Colour differences	-7.59	-1.78	-.36			
		Darker	Greener	Bluer			

Table 2-20. PATN ANOSIM p-values for the colour differences in amphipods from 8 jars receiving 2 water treatments (WK and WN) and 2 diet treatments (DK and DN). The cut-off for significance is 0.05 (SSH, MDS, Gower-Metric, Stress = 0.096 in 2 dimensions

Treatment	WK,DN	WK,DK	WN,DK
WK,DK	<0.001		
WN,DK	<0.001	<0.001	
WN,DN	<0.001	<0.001	0.21

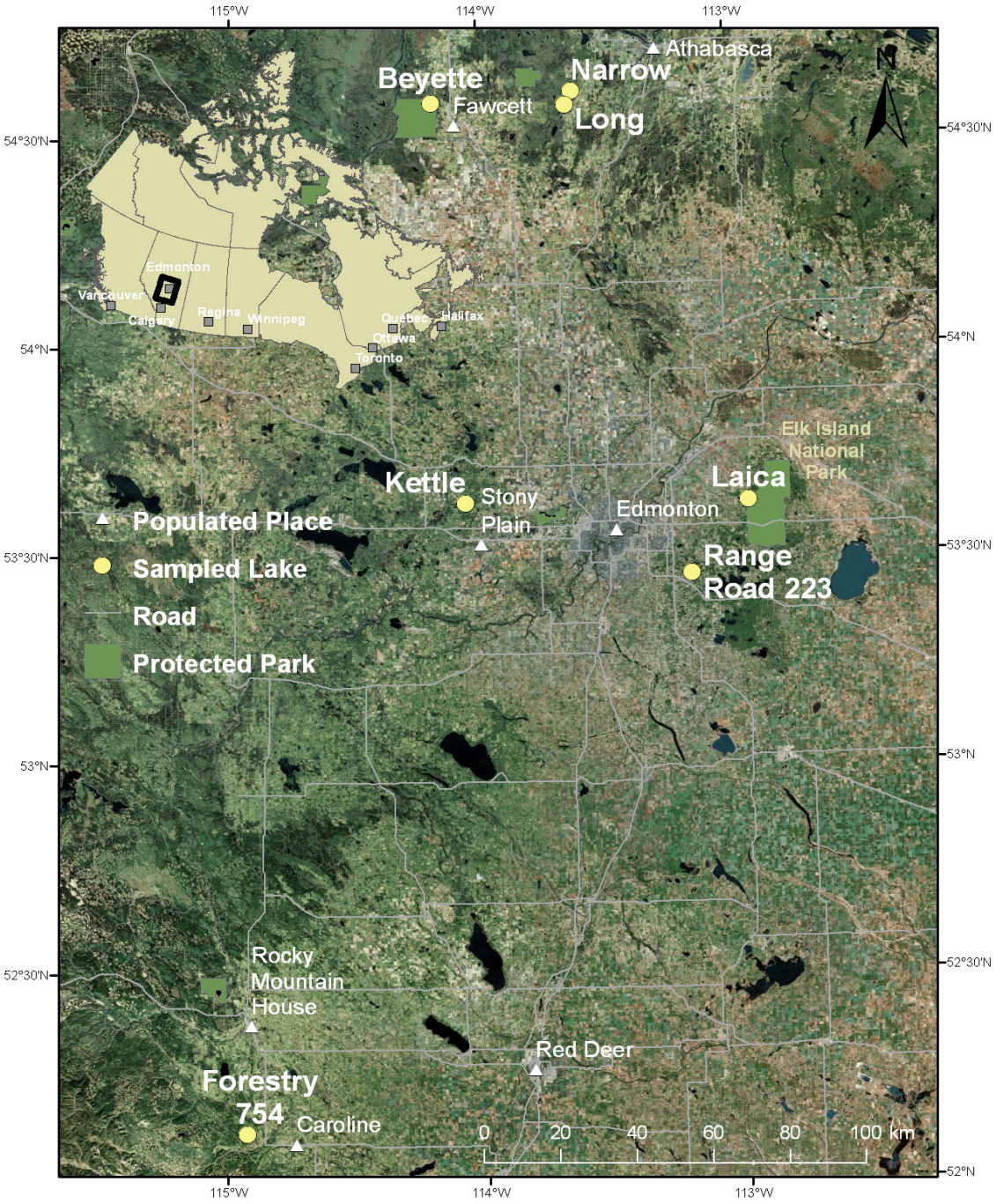
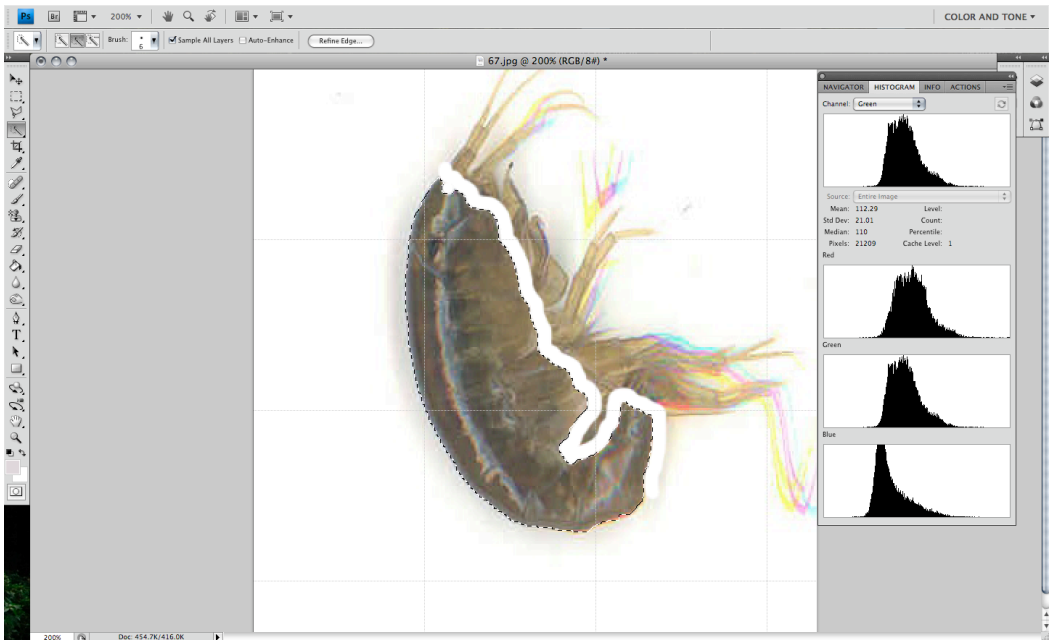
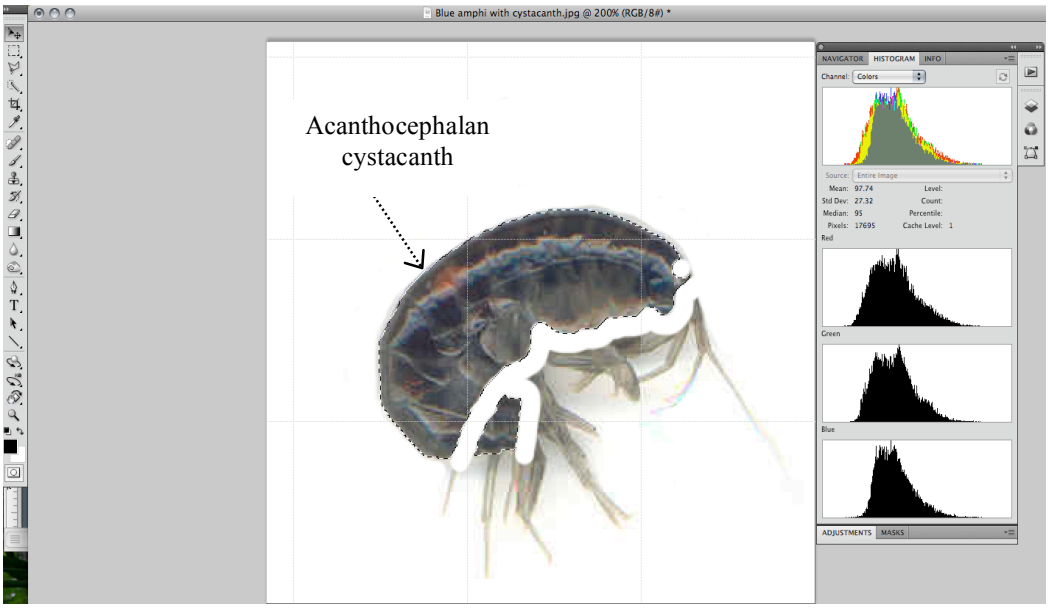


Figure 2-1. GIS Map of the sampled lakes in Alberta, Canada. Beyette (114°10'50"W; 54°35'16"N), Long (113°38'32"W; 54°34'54"N), Narrow (113°36'51"W; 54°36'55"N), Kettle (114°3'36"W; 53°37'43"N), Laica (112°56'37"W; 53°37'43"N), Range Road 223 (113°10'18"W; 53°27'16"N) and Forestry 754 (114°55'32"W; 52°7'10"N)



2a).



2b).

Figure 2-2. Colour measurement of amphipods. Figure 2a. A non-parasitized amphipod. Figure 2b. Blue amphipod with a visible acanthocephalan cystacanth

Figure 2-3. Scanning electron microscope image of *P. paradoxus* (top) and *P. marilis* (bottom). The scale is 100 µm

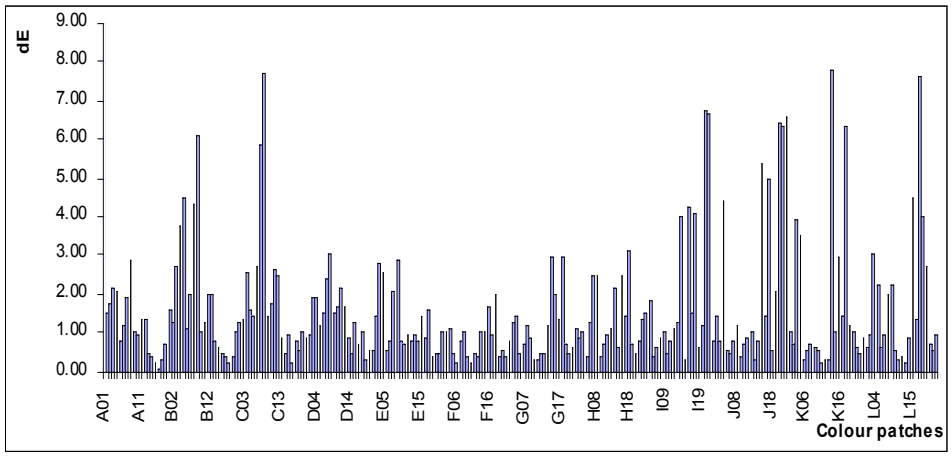


Figure 2-4 ΔE_{ab} values between calculated and measured L*a*b* values of 238 colour patches from Kodak Q60 IT8/7.2 colour target

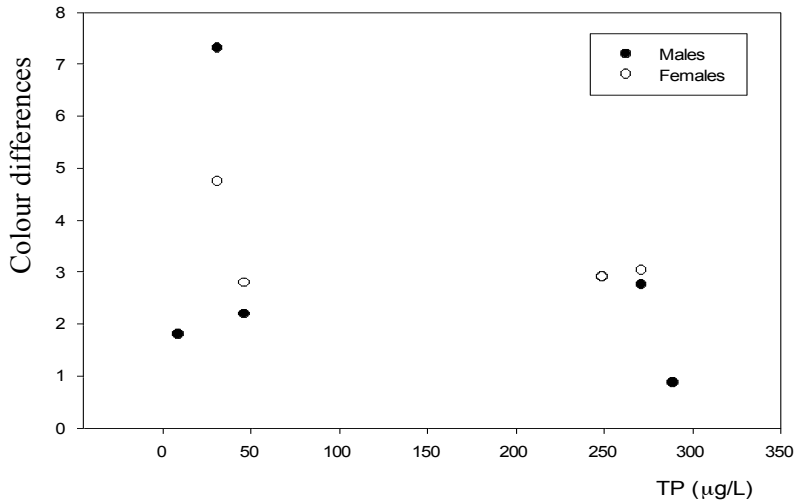


Figure 2-5. Colour differences (ΔE_{ab}) induced by *P. paradoxus* in *G. lacustris* males and females plotted against total phosphorus concentrations. No parasitized male amphipods were found in Forestry 754. There was no statistical difference between parasitized and non-parasitized male amphipods in Laica, nor in female amphipods within Narrow and Kettle water bodies

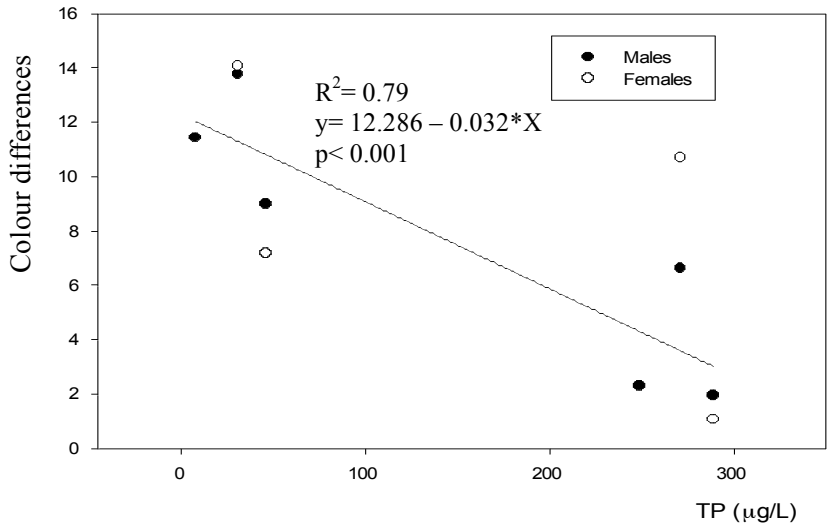


Figure 2-6. Colour differences (ΔE_{ab}) induced by *P. marilis* in *G. lacustris* males and females plotted against total phosphorus concentrations. No parasitized male amphipods were found in Narrow Lake. No parasitized females were found in Forestry 754, Narrow and Laica water bodies. Regression line between parasite-induced colour differences in males and total phosphorus concentrations

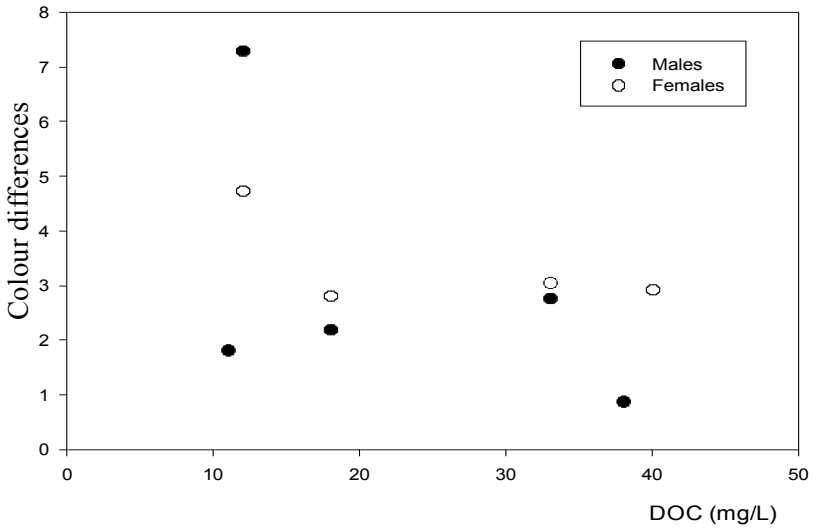


Figure 2-7. Colour differences (ΔE_{ab}) induced by *P. paradoxus* in *G. lacustris* males and females plotted against dissolved organic carbon concentrations. No parasitized male and female amphipods were found in Forestry 754. No statistical difference was observed between parasitized and non-parasitized male amphipods in Laica, or between female amphipods within Narrow and Kettle water bodies

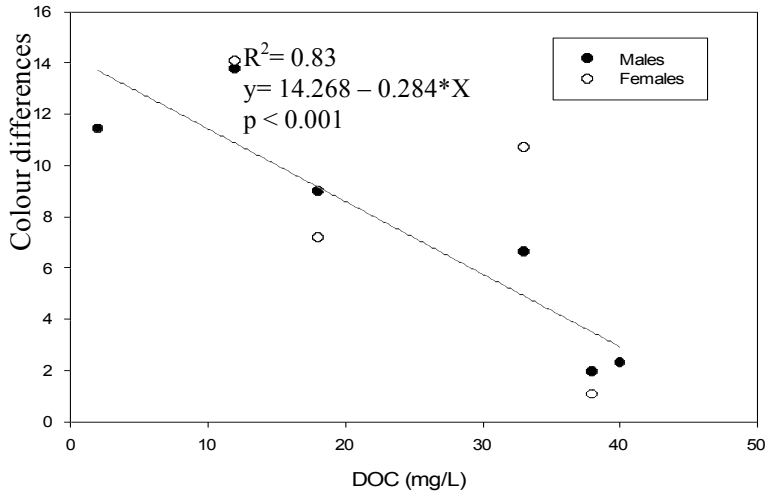


Figure 2-8 Colour differences (ΔE_{ab}) induced by *P. marilis* in *G. lacustris* males and females plotted against dissolved organic carbon concentrations. No parasitized males and females were found in Narrow Lake. No parasitized females were found in Forestry 754, Narrow and Laica water bodies. Regression line between parasite-induced colour differences males and total phosphorus concentrations

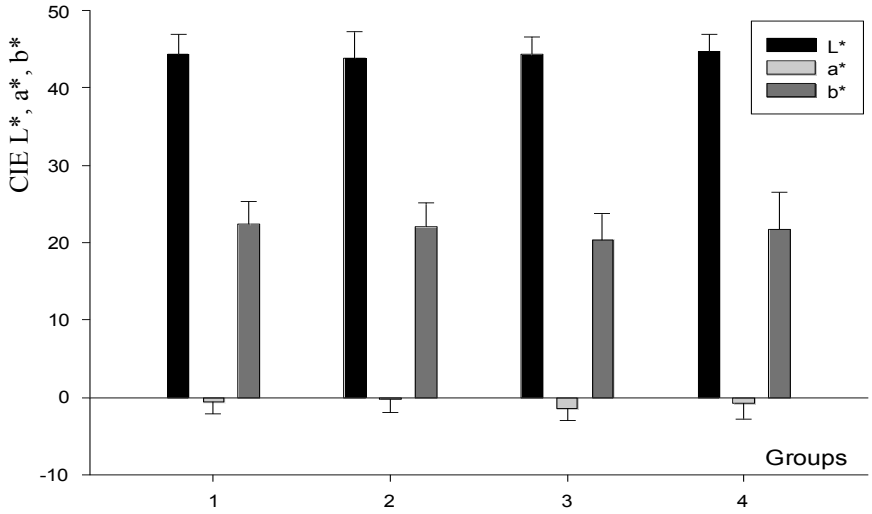


Figure 2-9a. Average CIE L*, a*, b* ± SD for the four groups of *G. lacustris* juveniles collected from Kettle Lake prior to water and diet treatments. Average and standard deviations were obtained using SPSS GLM ($p<0.05$). There is no significant difference between groups ($p> 0.05$)

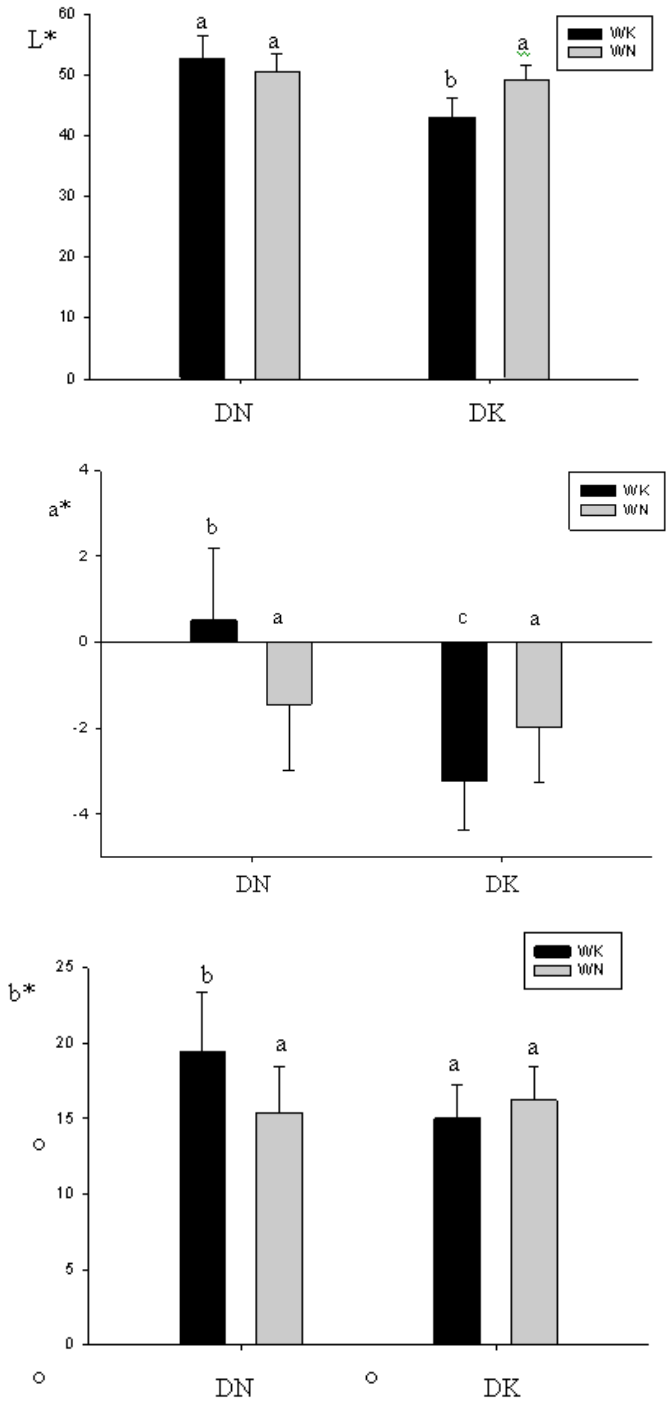
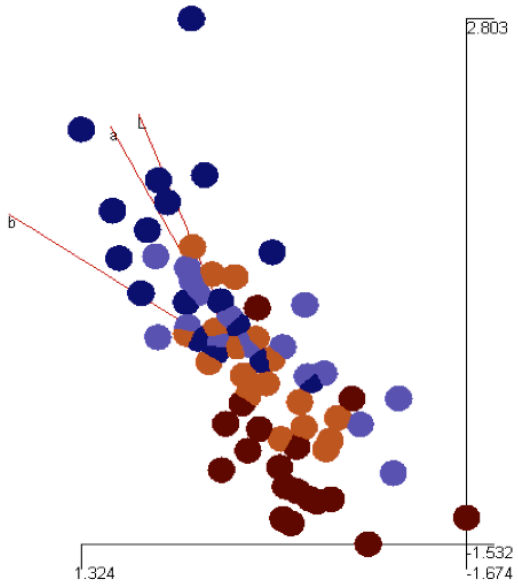
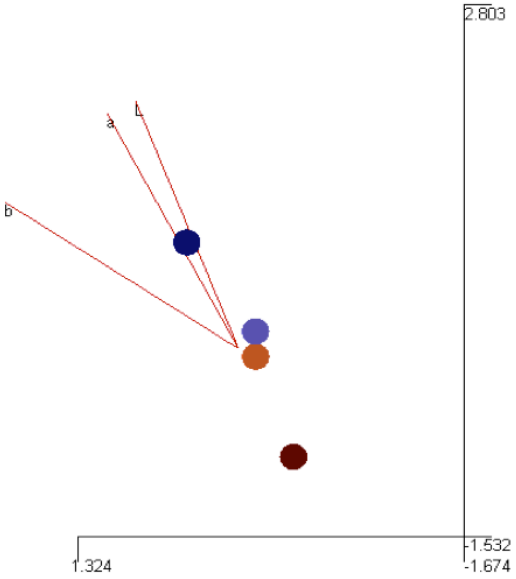


Figure 2-9b. Average CIE L*, a*, b* ± SD for the four groups of *G. lacustris* juveniles subjected fo a mont to treatments of water and diet from the oligotrophic Narrow Lake (WN and DN) and the hypereutrophic Kettle Lake (WK and DK). Colour vectors that are significantly different between the four groups ($p < 0.05$) are marked by distinct letters



10a).



10b).

Fig. 2-10. PATN Ordination showing the relationship between the colour of *G. lacustris* juveniles reared in two water and two diet treatments. Water and diet from Kettle Lake = dark brown, water and food from Narrow Lake = light blue, water from Narrow and diet from Kettle Lake = orange and water from Kettle and diet from Narrow = dark blue (SSH, Gower-Metric, Beta= -0.1, Flexible UPGMA, Stress= 0.096 in 2 dimensions). Figure 2-10b showing the row-group centroids only

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Chapter 3 - Effects of acanthocephalan parasites on carotenoid composition and body weight of *Gammarus lacustris* and a proposed carotenoid-colour relationship

3.1 INTRODUCTION

Roles of carotenoids

Carotenoids are coloured lipophilic biomolecules synthesized exclusively by plants, algae, fungi and bacteria. They are categorized as carotenes if their molecules are made up exclusively of carbon and hydrogen atoms, or as xanthophylls if they have one or more oxygen functional groups attached to the carbon-hydrogen chain (Bhosale and Bernstein, 2007). About 750 carotenoids have been identified up to 2004, with approximately 20 more being reported each year (Maoka, 2009). Many functions have been attributed to carotenoids and their cleavage products (apocarotenoids). In plants, carotenoids and apocarotenoids play roles as diverse as precursors of hormones, pigments involved in photosynthesis, and chemical defence compounds of the roots against fungi (Giuliano *et al*, 2003). In animals, they seem to have antioxidant (Perez-Rodriguez, 2009), immunological (Babin *et al*, 2010) or pigmentary roles (see bellow). Environmental abiotic factors can influence the allocation of carotenoids in animals, affecting their apparent roles. In zebra finches *Taeniopygia guttata* (Vieillot) carotenoids are a limiting resource under cold exposure, absorbed carotenoids being diverted toward self-maintenance functions like the antioxidant barriers and immunity, at the expense of ornamental coloured bill (Eraud *et al*, 2007). Microorganisms living in low temperature conditions have adapted bio-physiological mechanisms to improve their cold-tolerance. Antarctic bacteria change their carotenoid composition during cold periods, increasing the synthesis of polar carotenoids and decreasing the synthesis of non-polar ones in order to stabilize the membrane (Margesin *et al*, 2007). Carotenoids have also UV photoprotectant functions in arctic zooplankton species, which are heaviliy pigmented in order to offset the high amount of radiation penetrating water bodies with low dissolved carbon concentrations from this region (Raution *et al*, 2009).

Carotenoids in amphipods and acanthocephalans: effects of diet and parasitism

Amphipod crustaceans, just like other animals, rely on dietary sources for acquiring carotenoids (Davies, 1991). After being absorbed from the diet, carotenoids are either bound to protein complexes that circulate with the hemolymph through the hemocel, or are deposited in the exoskeleton after having been modified in the epidermis (Duclos, 2006). Once bounded to proteins, the natural orange colour of carotenoids is hidden, and carotenoproteins are described as grey (Czezuga, 1996), yellow (Milicua *et al*, 1986), green, blue or purple (Zagalsky, 1976).

Several studies have described the carotenoids of various crustacean species including *Gammarus* spp. (Amphipoda: Gammaridae). Czeczuga (1980) found three carotenes and fifteen xanthophylls in *Gammarus lacustris* G.O. Sars, and demonstrated seasonal variation in the amount of carotenoids. A more recent study (Gaillard *et al*, 2004) investigated the effect of the parasitic acanthocephalan *Polymorphus minutus* (Goeze) on the carotenoids in females of its common amphipod hosts *Gammarus pulex* (Linnaeus) and *G. roeseli* (Gervais). Gaillard *et al* (2004) found seven main carotenoids in each amphipod species. They found the most abundant carotenoids in *G. pulex* to be lutein and astaxanthin, confirming results of previous investigations (Barrett and Butterworth, 1968). The scant research on the carotenoids of gammarid amphipods depicts a wide range of qualitative and quantitative differences. Gaillard *et al* (2004) found that total carotenoid quantity was 20 to 100 times larger in *G. pulex* and *G. roeseli* than Czeczuga (1980) reported from *G. lacustris*. Differences in environmental sources of carotenoids may explain some of this variation. Laboratory experiments have shown that carotenoids from diet influence the amount of carotenoids acquired by aquatic crustaceans (Gilchrist and Green, 1959). In addition to diet, the importance of abiotic factors in determining carotenoid concentration in aquatic crustaceans should be considered, especially given the large differences among published results regarding the carotenoid composition of closely related species. Total phosphorus is a good

estimator of lake productivity (Wetzel, 1975) and a good predictor for total chlorophyll 'a' within lakes (Dillon and Rigler, 1974) and therefore it could be correlated with the amount of available dietary carotenoids for wild amphipod populations.

Polymorphus paradoxus Cornell and Corner and *P. marilis* Van Cleave use amphipods such as *G. lacustris* as intermediate hosts, with their final hosts being waterfowl or muskrats (Denny, 1969). They are usually located in a central position in the dorsal region of the thorax of their amphipod host, although cystacanths are also occasionally found in odd places like the first segment of the pereon or the first segment of one of the pereopods (pers. obs). Odd locations of cystacanths, including gills, antennae and legs, have been previously reported in *Gammarus pseudolimnaeus* Bousfield, experimentally infected with *Pomphorhynchus bulbocolli* Linkins. Parasites settling in any of these low-haemocyte microhabitats may have increased chances of surviving host immune responses (Taraschewski, 2000). An acellular envelope, functioning as a barrier against a host's immune reaction, surrounds the cystacanth (Dezfuli and Giari, 1999), preventing it from becoming quickly melanized.

Often cystacanths can be observed through the host exoskeleton as an orange spot situated in the hemocel. This intense orange colour denotes the fact that their carotenoids are no longer bound to proteins but are rather found in a free or esterified state, unlike those found in the host haemolymph or exoskeleton. The functions of carotenoids in larval acanthocephalans are debated. Two common hypotheses attribute to carotenoids either a sunscreen role or a visual mean of attracting the predators of amphipods that act as final hosts for the acanthocephalans (Bakker *et al* 1997). Both hypotheses have been called into question by subsequent studies (Duclos, 2006; Kaldonski *et al*, 2009). Lack of carotenoids in the diet of host amphipod *Hyalella azteca* Saussure causes delays in development of the acanthocephalan *Corynosoma constrictum* Van Cleave and potentially reduces the infectivity success of cystacanths in final hosts (Duclos, 2006). Because acanthocephalan larvae take carotenoids from the host haemocel, they must have mechanism for removing the protein portion from the

carotenoprotein complexes found in the host body. However, little is known about the metabolism of acanthocephalan cystacanths. Adult acanthocephalans, living in the gut lumen of vertebrate animals, anaerobically metabolize specific amino acids and carbohydrates that can easily pass through their integument (Duclos, 2006). However, these two developmental stages of the parasite live in completely different environments, therefore the substances they feed on and their metabolism are likely to be considerably different. Acanthocephalan larvae are in close contact with circulating haemolymph, and it has been suggested they have a protein-based aerobic metabolism with enzymes capable of hydrolyzing proteins from the host's body (Duclos, 2006). The parasite seems to absorb a protein that fixes astaxanthin in the host's exoskeleton and thus, free astaxanthin is accumulated as a metabolic by-product (Duclos, 2006). Gaillard *et al* (2004) and Barrett and Butterworth (1968) suggested that acanthocephalan larvae target certain carotenoids, mainly astaxanthin, as they found only different esters of astaxanthin in *P. minutus* cystacanths.

The effects of *Polymorphus minutus* on the carotenoids of its common hosts, *Gammarus pulex* and *G. roeseli*, have been tested on female gammarids by Gaillard *et al* (2004). They suggested that the parasite had no effect on qualitative and quantitative aspects of carotenoid composition in the two host species. The small number of individuals used in their statistical analysis, 4 parasitized and 5 non-parasitized *G. pulex* and 7 parasitized and 7 non-parasitized *G. roeseli*, as well as choosing only females as their study subjects might have affected their conclusions. Considering they collected female amphipods during the spring season, a seasonal increase in total carotenoids in females as a result of vitellogenesis might have influenced their results. Carotenoids like astaxanthin and esterified astaxanthin are known to have an important role in crustacean vitellogenesis (Mantiry *et al*, 1996).

Acanthocephalans affect the weight of their intermediate amphipod hosts

Gammarus spp. are typically classified as herbivorous scavengers (Agrawal, 1965) and detritivores (Moore, 1977) but also as predators on diaptomid copepods and daphniids, being able to modify the zooplankton community structure in

small fishless alpine lakes of the Canadian Rockies (Wilhelm and Schindler, 1999). The same authors found a positive relationship between total phosphorus concentration in water and *G. lacustris* density in the small lakes within their study site, possibly because the larger numbers of amphipods released more dietary phosphate concentration sequestrated by its prey, *Daphnia middendorffiana* Fischer.

Acanthocephalan parasites can affect the physiological demands of their amphipod hosts by increasing overall host activity levels (Dezfuli *et al*, 2003), inducing positive phototaxis (Tain *et al*, 2006) and by increasing movements in response to disturbances (Holmes and Zohar, 1990). Bentley and Hund (1993) found increased levels of haemocyanin in amphipods parasitized by *Pomphorhynchus laevis* (Zoega), possibly as a response to the increased oxygen-carrying capacity needed for satisfying the higher energetic demands of parasitized hosts. These effects of parasitism on amphipods suggest that parasitized hosts would require more food to sustain their elevated metabolism; however, results of published research are not entirely supportive of this prediction. In a laboratory study, Fielding *et al* (2003) found that although parasitized amphipods on a diet of frozen chironomids lost weight over time, feeding rates in non-parasitized *G. pulex* and those parasitized by *Echinorhynchus truttae* Schrank did not differ. In the same experiment no weight loss was detected in parasitized amphipods fed on leaves. But, the conclusions of another study regarding the same host-parasite system suggested that parasitism may increase the feeding rates of the hosts. *Gammarus pulex* in Northern Ireland infected by *E. truttae* consumed significantly more prey, *Asellus aquaticus* (Linnaeus), than did non-parasitized amphipods (Dick *et al*, 2010). The same study reported an increase in body size of parasitized compared to non-parasitized individuals. However, Zohar and Holmes (1997), reported significantly smaller *G. lacustris* males parasitized by *Polymoprhus paradoxus* and *P. marilis* than non-parasitized males from Cooking Lake, a hypereutrophic lake from Alberta (Bradford, 1990).

The effect of *Pomphorhynchus laevis* on the lipids of its intermediate host *G. pulex* depends on sex and reproductive state of the amphipod (Plaistow *et al*,

2001). Infected gravid female amphipods had lower lipid content than males but no difference was observed between males and non-gravid females infected by this acanthocephalan. This could be explained by some capability of *P. laevis* to manipulate the large lipid quantities and therefore lipid-dissolved carotenoids stored by gravid female amphipods prior to laying eggs (Plaistow *et al*, 2001).

The strength of sexual selection differs between gammarid sexes, with significant pressure on the size of male amphipods, which have to hold on to and carry females during the pre-copulatory phase, whereas body size doesn't have an effect on mating success of females (Ward, 1988). Thus, male amphipods would have an advantage in increasing their body size, by investing digested food in body growth. There is no accumulation of lipids in male amphipods but glycogen is likely the source of energy used when guarding females (Plaisto *et al*, 2001). The different reproductive strategies of male and female amphipods could render the effect of parasitism on weight a sex-based phenomenon.

Relationship between colour, carotenoids and abiotic factors

As shown in the previous chapter, the acanthocephalans *Polymorphus paradoxus* and *P. marilis* modify the pigmentation of *Gammarus lacustris* in several water bodies from Alberta. These parasite-induced colour modifications vary among populations and differ between the sexes of *G. lacustris*, and I demonstrated that abiotic factors, like total phosphorus (TP) and dissolved organic carbon (DOC) may also play a role in defining the direction and intensity of the colour change. Colour manipulation in gammarid hosts by the two acanthocephalan species could be related to modifications in carotenoid quantities. Colour of the crustacean exoskeleton is mostly due to its carotenoprotein complexes. Different carotenoprotein complexes produce colour variation among individuals (Cheesman *et al*, 1967). A well-described carotenoprotein that produces a blue colour in crustaceans is crustacyanin. Czeczuga and Kriwuta (1981) reported the presence of g- and b-crustacyanin having astaxanthin as the prosthetic group in *G. lacustris*. Colour modifications in parasitized *G. lacustris* have been previously described as an increase in blueness associated with a decrease in carotenoids (Hindsbo, 1972). These parasite-induced

colour variations could be a result of removal of carotenoids or proteins from carotenoprotein complexes in the amphipod exoskeleton. As a result, the remaining blue crustacyanin shows up as a relatively more prevalent carotenoprotein. Removal of carotenoprotein complexes from exoskeleton coupled with an elevated concentration of circulating haemocyanin could also produce the observed increase in blueness in parasitized amphipods. The lightness colour vector described in the previous chapter (parasitized amphipods tended to be lighter) could be strictly related to total concentration of carotenoids, rather than to variation in a specific carotenoid. A high total carotenoid concentration most likely results in a low lightness value, i.e., dark amphipods. No previous study has tried relating total or individual carotenoids to colour vector values in crustaceans, and thus the relationship between these factors is in need of investigation. However, several studies have determined the relationships between tristimulus colour vectors (e.g. CIE L*, a*, b*) and carotenoids in various food products (Ahmed *et al*, 2002; Melendez-Martinez *et al*, 2003). These studies demonstrated that colour values could predict carotenoid quantities with accuracy higher than 90%.

Hypotheses

In this chapter I test the hypothesis that the amount of carotenoids in body tissues of the amphipod *G. lacustris* can be predicted by the total phosphorus (TP) concentration within the water body in which amphipods developed. A higher TP value denotes a water body with increased availability of food sources for amphipods and therefore the animals can easily acquire and store larger amounts of carotenoids. Amphipods found in hypereutrophic water bodies should have a higher amount of total carotenoids when compared to amphipods from oligo- and eutrophic water bodies.

I also propose that parasitism by *P. paradoxus* and *P. marilis* affect the carotenoids of their common host *G. lacustris* in a quantitative way. Parasitized male hosts should have less total carotenoids than the non-parasitized ones. Based on previous findings related to carotenoid composition of acanthocephalan parasites, astaxanthin might be the carotenoid with a higher quantitative drop in

parasitized amphipods, but other component carotenoids of the host may also be affected. Due to possible seasonal variability in carotenoid composition of females, only male amphipods will be considered for the carotenoid analysis.

Parasitism should also influence the weight of amphipods and I propose that parasitized male amphipods should wieght less than non-parasitized ones.

I also test the relationship between total carotenoids and colour in the context of parasitism by the two acanthocephalan species and the different trophic status of sampled lakes. I propose that lower CIE L* values (darker), higher a* (redder) and higher b* (yellower) predict increasing amount of total carotenoids in amphipods.

3.2 MATERIALS AND METHODS

Biological material: amphipods, exoskeletons, acanthocephalans

Gammarus lacustris were collected from a water depth of up to 1.5 m in seven water bodies from Alberta, Canada during April-May 2009, using a 500 μm mesh dip net. Water analysis was carried out in the Biogeochemical Analytical Laboratory of the Biological Sciences Department, University of Alberta, following the procedures described in previous chapter. The lakes were selected to span a wide range of trophic status. They included two oligotrophic lakes, Narrow and Forestry 754, two eutrophic lakes, Long and Beyette, and three hypereutrophic lakes, Range Road 223, Kettle and Laica (Table 3-1 and Figure 2-1). Classification into these trophic levels was made based on the total phosphorus concentration in the water. Oligotrophic, mesotrophic, eutrophic and hypereutrophic lakes have concentrations of lower than 10 μg L^{-1} P, 10 to 20 μg L^{-1} P, more than 20 μg L^{-1} P, and more than 100 μg L^{-1} P, respectively (Wetzel, 1975).

A total of 125 male amphipods was analysed for carotenoids (Table 3-1), 19 from Beyette (10 non-parasitized and 9 parasitized), 19 from Narrow (9 non-parasitized and 10 parasitized), 20 from Long (10 non-parasitized and 10 parasitized), 9 from Forestry 754 (5 non-parasitized and 4 parasitized), 20 from Kettle (11 non-parasitized and 9 parasitized), 19 from Range Road 223 (9 non-parasitized and 10 parasitized) and 18 from Laica (9 non-parasitized and 9

parasitized). Each amphipod had their surface blatted prior to dissection. Dissections were carried out on a flat piece of aluminium foil in order to retain body fluids for subsequent weighing. Acanthocephalans were removed from the host body and both parasite and host were analysed separately for carotenoids. At the same time the gut was emptied by gently squeezing out their contents. This method provided an easy and accurate way of making sure that non-assimilated dietery items found in amphipod guts did not affect the carotenoid analysis. After dissection, amphipods were frozen at -20° C, to prevent carotenoid degradation during the short time (up to 2 months) (Su *et al*, 2002) prior to high-pressure liquid chromatography (HPLC) analysis. Seventyfive cystacanths of *P. paradoxus* sampled from the Range Road 223 Lake, were extracted from hosts, washed with distilled water to remove any traces of host haemolymph, and kept frozen at -20° C until they were analysed by HPLC. The acanthocephalans *P. paradoxus* and *P. marilis* were identified based on the same method used in the previous chapter, following the diagnoses provided by Denny (1969).

Moulted exoskeletons were collected from amphipods originating from Kettle water body that were reared in laboratory conditions for a month with food and water from their native lake. After any individual moulted, the exoskeleton was collected, washed in distilled water and kept frozen at -20°C before being analysed for carotenoids. Twenty moulted exoskeletons of non-parasitized *G. lacustris* were processed similar to the whole bodies of *G. lacustris*, and all were analysed in a single RP-HPLC sample for carotenoids qualitative composition only.

Individual amphipods and the parasites wrapped in aluminium foil were placed on a Labeonco Freezone 12 stoppering tray dryer and freeze-dried for 24 hours at vacuum and tray temperatures of 0° C and -47° C, respectively. Vacuum pressure was maintained at $67*10^{-3}$ mBar. In order to determine the dry weight of the HPLC-processed biomass, the weights of dried amphipods, parasites wrapped in the aluminium foil, and the foil alone after the carotenoid extraction process took place were measured to the nearest 0.1 mg using a Mettler AT261 balance.

The difference between the two weights represented the weight of the biological sample that was analysed for carotenoids.

Carotenoids extraction

All samples were extracted and HPLC analyzed during the first two months after being collected from the studied water bodies. Specimens were randomly selected for each HPLC run in order to avoid systematic error due to fluctuations in the measuring environment. Each freeze-dried amphipod was ground using a ceramic mortar and pestle together with a solution of HPLC grade acetone-methanol 3:1. The aluminium foil pieces stained with body fluids were also flushed with the same solvent solution to remove carotenoid traces. The resulting solution was transferred to glass vials and left for 24 hours in the dark at room temperature. Breaking down the tissues is a method that greatly improves the extraction efficiency of carotenoids from biological samples by facilitating the contact between the biological sample and extraction solution (Rodriguez-Amaya 2001; Rodriguez-Amaya and Kimura, 2004). The extraction solution with carotenoids and ground tissue was filtered through Millipore Millex sterile syringe filter units with a Fluropore hydrophobic PTFE membrane with a pore size of 0.22 mm, for filtration of non-aqueous solutions. The filtered samples were dried out under nitrogen gas and carotenoids were reconstituted in 500 or 1000 mL of injection solution (70% acetone: 25% ion-pairing reagent: 5% methanol) before injection into the HPLC column (Vinebrooke & Leavitt,1999). The ion-pairing reagent consisted of 0.75 g tetrabutylammonium acetate and 8 g ammonium acetate in 100 mL deionised water.

Reversed phase high-pressure liquid chromatography (RP-HPLC) analysis

Pigment concentrations were quantified using a standard reverse phase high-pressure liquid chromatographic technique (Vinebrooke & Leavitt, 1999). Chromatographic analysis was performed on a Hewlett-Packard (Hewlett-Packard Canada Ltd., Mississauga, Ont.) 1100 Series HPLC system equipped with a 100 x 4.6 mm Agilent Eclipse Plus C18 reversed-phase column, with a 3.5 mm particle size. For each analyzed sample, a total of 100 mL of the reconstituted pigments were run through the HPLC system. Pigments were detected using an inline HP

Series 1100 diode array detector (435-nm detection wavelength) and a fluorescence detector (435-nm excitation wavelength, 667-nm detection wavelength). Analytical separation involved constant delivery (1.0 $mL \cdot min^{-1}$) of a mobile phase A (10% IPR in methanol) for 1.5 min, a linear succession to 100% solution B (27% acetone in methanol) over 7 min, and isocratic hold for 12.5 min. The column was re-equilibrated by continued isocratic delivery for 3 min, a linear return to 100% solution A over 3 min, and isocratic delivery for a final 4 min. HP ChemStation for LC v.10.02 (Agilents Technologies, Palo Alto, CA, USA) software plotted the chromatograms at 435 nm, and the carotenoid peaks were identified by the elution order, retention times and absorption properties (l_{max} values) with pure standards.

PeakFit chromatogram analysis

Because a slight overlap and tailing of some of the detected carotenoids made their identification difficult, chromatograms were exported as .csv files into Peak Fit v.4.12 for Windows (Systat Software Inc., 2003) for additional analysis. Each peak was reanalysed and deconvoluted using the minimal residual method and the Exponential Modified Gauss function. The EMG model is a widely used model capable of modelling tailing and overlapping in carotenoids (Jeansonne and Foley, 1991). Other methods used for deconvoluting overlapped chromatographic peaks (i.e. geometrical methods and perpendicular drop at the valley, triangular correction and tangent skimming of the overlapped tailing peaks) have also been widely used, but in contrast to EMG based equations, these methods can produce errors that depends considerably on the asymmetry and the difference of peak heights of the overlapped chromatographic peaks (Pap and Papai, 2001).

Quantification of pigments

Pigment concentrations were quantified via separate calibration curves for astaxanthin, lutein, zeaxanthin, echinenone and an electronic spectral library constructed using standards purchased from DHI Water and Environment (Agern Alle 5, DK-2970 Hoersholm, Denmark). Although I was unable to definitively identify two of the detected carotenoids, the calibration curves for diadinoxanthin and violaxanthin were used for quantifying their corresponding quantities due to

spectral similarities between the two unknown carotenoids and these standards. Geometric or positional isomers of the same carotenoids were added to the total quantity of a single carotenoid. Area under each carotenoid peak was read on the fitted PeakFit chromatograms. As there is a numerical difference in measured area under the peak between ChemStation and PeakFit, chromatograms of pure astaxanthin and echinenone at various concentrations were also imported into PeakFit to determine a conversion factor. The conversion factor, 63.6734 mAU, was determined by dividing the area under the peak of the same standard measured in ChemStation by the area of the same peak measured in PeakFit. Each area under the peak measured in PeakFit was then multiplied by this conversion factor, to adjust for numerical differences between the two chromatogram-generating programs. All quantities were expressed as μg carotenoids/ g dry mass of animal tissue.

Colour measurements

CIE L*, a*, b* values for all collected male amphipods were measured according to the procedure described in previous chapter.

Statistical analysis

The differences in carotenoid concentrations between parasitized and non-parasitized amphipods within each lake were statistically analysed using multivariate general linear models (GLM) in SPSS v17 (SPSS Inc, 2008). A multivariate analysis was necessary, as multiple dependent variables were compared between three independent groups of male amphipods: non-parasitized, parasitized by *P. paradoxus*, and parasitized by *P. marilis*. The analysis showed the overall differences between the groups for all carotenoids as well as differences in each identified carotenoid. A similar analysis was used for testing the effects on carotenoids of parasitism and water quality expressed as units of total phosphorus (TP). An analysis of variance (ANOVA) in SPSS was also used for comparing the total amount of carotenoids between the three groups within each lake.

The differences in weight and total carotenoids between all sampled parasitized and non-parasitized amphipods, accounting for lake differences in TP,

were tested using a 'mixed effects restricted maximum likelihood' analysis in Stata v.11 (StataCorp, 2009). Parasite status of amphipods (the three groups described above) and total phosphorus (TP) were set as explanatory factors for the weight differences between parasitized and non-parasitized amphipods. Water body was set as the random factor. A mixed-effects analysis considers also the possible effect of sampling site on the overall differences between parasitized and non-parasitized amphipods, with random slope and intercept for each lake. Thus the results will describe trends in weight differences, which are a combination between the fixed effects (parasitism and TP) and the variation of that trend, or random effects, for each lake (Hamilton, 2009).

Mixed effects restricted maximum likelihood analysis was also used to test the differences in total and individual carotenoids among the three groups of male amphipods. Sampling site was set as the random effect, and parasitism and TP were set as the explanatory factors for the differences between parasitized and non-parasitized amphipods.

The relationship between carotenoids and amphipod colour was determined using both multivariate linear regressions, for exploratory purposes only, and partial least square regression (PLS) in SPSS. Partial least square regressions is a predictive technique, an alternative to structural equation modelling (SEM). Compared to other regression analyses, PLS is not restricted by common assumptions like sample size restrictions, missing data, or multicollinearity (Garson, 2009). Total carotenoid quantities were set as the dependant variable while CIE colour vectors L*, a* and b* as independent factors along with TP, DOC and parasite status. Thus total carotenoid quantities could be predicted based on known colour and abiotic factor values. The relationship was interpreted based on the PLS parameter output, and efficiency of the developed models was tested using one way bivariate correlations between predicted and measured carotenoid quantities.

3.3 RESULTS

Carotenoids from parasitized and non-parasitized *G. lacustris*

Grinding amphipod tissues did not produce complete extraction of pigments, a result that was consistent across all samples. The remaining pigments were extractable in double distilled water without further mechanical processing, resulting in a pale pink solution. Carotenoids are lipids and in their pure form they do not dissolve in water, but once they are bound to proteins to form carotenoproteins, their chemical and physical properties change, and they become water-soluble. Therefore it seems that carotenoids from some carotenoprotein complexes are not completely extractable in pure methanol and/or acetone. Czeczuga and Krywuta (1981) identified carotenoproteins in *G. lacustris* from the crustacyanin groups b- and g containing astaxanthin. Using acetone is a common way of liberating carotenoids from their binding to protein complexes, and I experimented with several methanol-acetone ratios for the best carotenoid extraction results, but none of them were able to extract the pigments completely. Unsaturated carotenoids are prone to isomerization and oxidation, with the degree of oxidative degradation depending on the amount of available oxygen (Sajilata *et al*, 2008). The sensitivity to photo-oxidation varies among carotenoids, lutein being a more labile one (Rodriguez-Amaya, 2001). In order to minimize the exposure time of the extraction solution to environmental factors, usage of water as a solvent was avoided, hence freeze-drying the samples. In contrast to extraction from host amphipods, extraction of carotenoids from acanthocephalans appeared to be complete.

All detected carotenoids had a retention time between 6 and 14 minutes, with similar chromatographic peaks for both parasitized and non-parasitized amphipods (Figure 3-1). Six main carotenoids were identified in *G. lacustris*. By comparing the retention times and the spectral signatures with the standards recorded in the electronic library, I was able to precisely identify four out of the six carotenoids as astaxanthin, lutein, zeaxanthin, and echinenone. Two of the carotenoid pigments remained unidentified: “peak 2” eluting at 7.2 min with a shoulder at 424 nm and two peaks at 446 nm and 476 nm, and “peak 5” eluting at

10.8 min with a shoulder at 418 nm and two peaks at 440 nm and 466 nm. A carotenoid with a similar spectrum to "peak 2" was previously found in *G. pulex* and *G. roeseli* females by Gaillard *et al* (2004), who suggested that this carotenoid could be β,β-carotene 3,4,3'–triol with a ionization base peak at m/z 582. The first peak, running at 6.8 minutes, had a spectrum identical to astaxanthin, with a single wide peak with an absorption maximum at 478 nm. The third peak, eluting at 8.3 minutes, was identified as zeaxanthin, with a shoulder at 428 nm and two peaks at 450 nm and 478 nm. This carotenoid eluted before lutein, the fourth peak, in our samples. Lutein, eluted at 9.2 minutes with a shoulder at 422 nm and two peaks at 446 nm and 474 nm. Although the elution order of the two carotenoids is not backed up by their chemical properties (lutein normally elutes before zeaxanthin in a reversed-phase HPLC system), there is enough evidence to conclude that the first out of these two peaks is correct. The ChemStation carotenoid library matched zeaxanthin spectra from biological samples and the spectrum library with a score above 990 out of 1000, indicating an identical object. The matching score coupled with a similar elution time of zeaxanthin when compared to standards led to the conclusion that this carotenoid is indeed zeaxanthin. The sixth peak, eluting at 13.8 minutes, had a broad peak, with absorption maximum at 464 nm, similar in spectrum and elution time to that of the echinenone standard.

Another carotenoid found in traces and in only some of the male amphipods was b-carotene. Its occurrence was not related to parasitism or water body trophic status. It eluted at 14.7 minutes with a shoulder at 428 nm and two peaks at 450 nm and 474 nm.

The total amount of carotenoids in non-parasitized *G. lacustris* from the seven studied lakes varied greatly. The smallest quantity of carotenoids/amphipod was found in those from Long Lake, a slightly eutrophic lake, and the maximum in Laica Lake, a hypereutrophic water body (Table 3-1). Within each lake the amount of each identified carotenoid varied in non-parasitized amphipods. The most abundant carotenoid was lutein, accounting for 28% up to 37% of the total carotenoids (Table 3-2). The exception was found in Laica, where zeaxanthin

accounted for an average 55% of the total carotenoids in non-parasitized amphipods. Astaxanthin, reported as one of the most abundant carotenoid in crustaceans (Czeczuga, 1980; Gaillard *et al*, 2004) came as the second most abundant carotenoid, following lutein, in all oligo- and eutrophic lakes. However, Barrett and Butterworth (1968) also reported lutein as the main carotenoid in *Gammarus pulex*. The average percentage of this carotenoid out of the total amount of carotenoids varied between 17% and 23% in the aforementioned water bodies. Different results were observed in the hypereutrophic lakes. In Kettle and Range Road 223 water bodies, zeaxanthin was the second most abundant carotenoid after lutein, counting for 28% to 32% of the detected carotenoid quantities. In Laica water body, where zeaxanthin was the most abundant carotenoid at 55%, lutein followed with an estimated 20% out of the total carotenoid quantities. In these hypereutrophic lakes astaxanthin was represented by only 8% to 12% of the total carotenoid amount.

Carotenoid quantity and composition in parasitized amphipods varied between lakes similarly to the quantities observed in non-parasitized amphipods. The maximum amount of carotenoids in parasitized amphipods was observed in hypereutrophic lakes, notably in individuals parasitized by *P. paradoxus* in Laica Lake (Table 3-1). The minimum amount of carotenoids in amphipods parasitized by this parasite species was observed in Long Lake. The same trend was also observed in amphipods parasitized by *P. marilis* (Table 3-1), with a maximum carotenoid quantity in Laica Lake and a minimum in Long Lake.

In parasitized individuals, lutein remained the dominant carotenoid in oligo- and eutrophic lakes, accounting for 30% to 33% in amphipods parasitized by *P. marilis* and 29% to 34% in amphipods parasitized by *P. paradoxus* (Table 3-2). Astaxanthin was the second most abundant carotenoid in parasitized amphipods from oligo- and eutrophic lakes, accounting for 22% to 25% in amphipods parasitized by *P. marilis* and 17% to 35% in amphipods parasitized by *P. paradoxus* (Table 3-2). In amphipods from the hypereutrophic Kettle and Laica, zeaxanthin was the most abundant carotenoid in those parasitized by *P. paradoxus* with 41% and 57% respectively, and in amphipods parasitized by *P.*

marilis with 37% and 58%. In hypereutrophic Range Road 223, lutein (36%) and zeaxanthin (25%) were the main carotenoids in amphipods parasitized by *P. paradoxus*. In amphipods parasitized by *P. marilis*, zeaxanthin and lutein accounted for about 30% each.

Carotenoids in the acanthocephalan *P. paradoxus* and in moulted exoskeletons of *G. lacustris*

Only the cystacanths of *P. paradoxus* were tested for carotenoids. A minimum quantity of *P. marilis* cystacanths that could produce HPLC chromatograms with little or no noise was not achievable, due to their relatively low frequency in sampled amphipod populations. RP-HPLC detected 6 carotenoids in *P. paradoxus* (Figure 3-1B and Table 3-8). Free astaxanthin was detected in trace quantities only. The most abundant carotenoids found in *P. paradoxus* were lutein and zeaxanthin. In contrast to the carotenoid composition in *G. lacustris*, *P. paradoxus* had a substantial quantity of esterified astaxanthin. Other detected carotenoids were echinenone and the same unidentified carotenoid eluting just after astaxanthin that was found in the amphipod hosts ("peak 2"). A total carotenoid quantity of 88.56 mg/ g dry weight was found in the lumped sample of 75 cystacanths of *P. paradoxus*, but because a single dried parasite weighs on average about only 0.11 mg the amount of carotenoids per parasite was minute, an average of 9.74 ng of carotenoids per dried cystacanth.

Moulted exoskeletons of non-parasitized amphipods were analysed for qualitative purposes only. The HPLC histogram of detected carotenoids (Figure 3-1C.) looked very similar to that of whole bodies of *G. lacustris*. Six main carotenoids were found in moults: astaxanthin, unidentified "peak 2", zeaxanthin, lutein, unidentified "peak 5", c and echinenone. No esterified astaxanthin was found in moulted exoskeletons.

Differences in carotenoid quantities between parasitized and non-parasitized amphipods within a lake

Individual multivariate GLM analyses were conducted on amphipods for each sampled lake (n = 7 analyses). The multivariate analysis considered all carotenoid quantities in non-parasitized amphipods, amphipods parasitized by *P.*

marilis and amphipods parasitized by *P. paradoxus* as dependent variables, and parasitic status as an independent nominal variable. The results of Wilk's Lambda test were considered as more than two groups were compared. The results varied between lakes with a statistically significant difference among the categories of amphipods in overall quantities of carotenoids found in Long Lake ($F= 6.46$, $df= 12; 24$, $p< 0.01$), and Kettle Lake ($F= 2.69$, $df= 12; 24$, $p= 0.02$) (Table 3-1). No statistical differences in overall carotenoid quantities were found in the remaining five lakes included in the study (see GLM and ANOVA statistical results, Table 3-1).

The differences between individual carotenoids induced by the two acanthocephalan species were determined based on subsequent multivariate GLM analyses considering only one of the parasite species at a time, and on the test between-subjects effects following each GLM analysis. In hypereutrophic water bodies Kettle, Range Road 223 and Laica, parasitism affected the constituent carotenoids of amphipods in different ways. In Kettle Lake, *P. paradoxus* induced a significant increase of zeaxanthin while *P. marilis* had no effect on any of the constituent carotenoids (Table 3-1). In Range Road 223, *P. paradoxus* had no effect on parasitized amphipods, while *P. marilis* significantly lowered the amount of four of the host's carotenoids (Table 3-1). In Laica, none of the parasitic species had an effect on the host's carotenoids (Table 3-1). In eutrophic Beyette Lake, *P. paradoxus* affected the quantities of astaxanthin, lutein and unidentified carotenoid from "peak 5" of the chromatograms. In this lake, the amount of lutein slightly increased with parasitism while the carotenoid from peak 5 slightly decreased. Only one *G. lacustris* infected by *P. marilis* from this lake was analysed, therefore it was not included in the GLM analysis. In Narrow Lake, amphipods parasitized by *P. paradoxus* had a significantly lower amount of lutein. No other carotenoid was affected by parasitism in this lake, and no amphipods parasitized by *P. marilis* were tested (Table 3-1). Amphipods parasitized by *P. paradoxus* from eutrophic Long Lake had lower amounts of three carotenoids: unidentified "peak 2", lutein, and unidentified "peak 5". In the oligotrophic water body Forestry 754 an overall difference in carotenoids between

non-parasitized amphipods and those parasitized by *P. marilis* was not detected (GLM p> 0.05). However each carotenoid was individually affected, excepting for peak2 (Table 3-1).

Differences in total amount of carotenoids between non-parasitized amphipods, amphipods parasitized by *P. paradoxus*, and those parasitized by *P. marilis*, were tested using ANOVA. A statistically significant difference in total carotenoids was detected only in eutrophic Long Lake and oligotrophic Forestry 754. No amphipods parasitized by *P. marilis* were analysed for Beyette and Narrow lakes. A post-hoc Tukey analysis, set at an experiment-wise error rate of 0.05, indicated that the statistical differences in total carotenoids were driven by the differences between non-parasitized amphipods and amphipods parasitized by *P. marilis*.

Effects of water quality on differences in carotenoid quantities between parasitized and non-parasitized amphipods

Mixed effects restricted maximum likelihood analyses in Stata v.11 were used for testing the effect of water quality expressed as mg of total phosphorus (TP) per litre, and parasitic status of amphipods on the carotenoid composition of all sampled amphipods. Because the measured water quality parameters (total phosphorus, dissolved organic carbon, turbidity) are highly correlated, resulting in a collinearity issue for regressions, only TP was considered for this analysis. As a limiting factor, phosphorus cycle controls the rates of biological productivity in many lakes. Declining concentrations of phosphorus in productive lakes results in rapid decrease in their productive capacity (Wetzel, 1975); therefore, it is reasonable to use TP alone to reflect lake productivity and availability of dietary carotenoids. Besides testing the effect of the abiotic variable on host's carotenoids, this analysis also increased the sample size for testing the effect of the two parasite species on host carotenoids.

Average TP and the three options for parasite status of amphipods were set as independent variables, while the sampling site was set as the random effect, representing the effect of lake on carotenoid composition of amphipods.

The resulting mixed-effects model implied seven different intercepts. The estimated standard deviation (111.05 mg/ g dry mass) for the random effect of lake was about three standard errors (36.48 mg/ g dry mass) from zero, indicating that there is significant sampling site to sampling site variation in slope coefficients. Results showed that *P. marilis* decreased the total amount of carotenoids in parasitized amphipods on average by 54.13 ± 27.07 (SE) mg/ g dry mass when compared to non-parasitized amphipods ($p < 0.05$). In contrast, *P. paradoxus* did not influence the total amount of carotenoids in parasitized amphipods ($p = 0.45$) (Table 3-3). Total phosphorus had a strong effect on determining the differences in total carotenoids between parasitized and non-parasitized amphipods ($p < 0.01$) (Tables 3-3 and 3-4). The likelihood-ratio test indicated that the mixed effects model offered significant improvements over a linear regression model with fixed effect only ($p < 0.01$).

The estimated average total amount of carotenoids in amphipods, from a theoretical sampling site with an average TP value (Table 3-3a), showed that there were no differences between the average carotenoid quantities in non-parasitized amphipods and those parasitized by *P. paradoxus*, as the 95% confidence intervals overlap completely. On the other hand, the 95% confidence intervals of the average total amount of carotenoids in non-parasitized amphipods and those parasitized by *P. marilis* do not overlap completely indicating a likely difference in carotenoids between them.

Separated mixed- effects REML analysis for each carotenoid, with parasitism status and TP as explanatory variables, and sampling site as random factor, showed that *P. marilis* affected the quantities of each carotenoid but zeaxanthin and "peak 5". It induced a decrease in astaxanthin, 'peak2", and echinenone and at the same time an increase in lutein. Total phosphorus had a strong effect on the observed differences in zeaxanthin, lutein, peak5 and echinenone between parasitized and non-parasitized amphipods (Table 3-4).

The relationship between TP concentration and total carotenoids in parasitized and non-parasitized male amphipods is illustrated in Figure 3-2. Both parasitized and non-parasitized amphipods from oligotrophic and eutrophic water

bodies had significantly lower total amount of carotenoid than amphipods from similar parasite-status groups from the three hypereutrophic water bodies. A linear increase of total carotenoid quantity with TP was not detected, but rather an abrupt increase of carotenoids in amphipods when comparing those from hypereutrophic water bodies Kettle, Range Road 223 and Laica to those from oligo- and eutrophic lakes. Excluding the effect of parasites, a correlation between carotenoid quantities in non-parasitized males and water quality parameters TP and DOC, showed a strong relationship between the carotenoids and the abiotic factors (Table 3-5).

Effect of parasitism on the body weight of amphipods from all lakes

Differences in weight between non-parasitized and parasitized amphipods were tested among individuals within a single lake, and in a separate analysis, among lakes. Preliminary testing of parasite effect on the weight of amphipods within a single lake showed no differences between the weight changes induced by the two parasite species in amphipods. Therefore, in the case of analysis for a single lake, parasite species were pooled in order to increase the sample size of parasitized amphipods. However, parasite species was accounted for in the weight analysis of amphipods from all lakes combined.

Parasitism by a *Polymorphus* sp. had a strong effect on male amphipod weight in most of the sampled sites (Table 3-6). In all these cases, parasitized amphipods were lighter than non-parasitized amphipods by an average of 2.9 (1.3 – 4.4) mg. The weight of amphipods varied considerably among lakes, with the heaviest non-parasitized amphipods being found in eutrophic lakes Beyette and Long where parasitism also induced the largest average loss in weight, 4.36 and 3.20 mg respectively. Parasitism had no significant effect on the weight of amphipods from Narrow ($t= 0.33$, $df= 17$, $p> 0.05$) and Forestry 754 ($t= 1.56$, $df= 7$, $p> 0.05$) (Table 3-6).

Weight analysis of amphipods from all sampled sites was done using a mixed effects restricted maximum likelihood analysis. Amphipod status and TP were the explanatory factors while sampling sites were set as random effect. Both parasite species have a strong effect on the weight of their host at $P < 0.01$ for

both *P. paradoxus* and *P. marilis* (Table 3-7) inducing about the same weight loss of 2.5 mg dry weight on average (2.5 mg). When TP concentration was set at a theoretical average concentration the 95% confidence intervals of the estimated marginal weight means of parasitized amphipods overlaped (Table 3-7a). Therefore, no difference existed between the average weights of amphipods parasitized by the two acanthocephalan species. Total phosphorus had no influence on the weight differences between parasitized and non-parasitized amphipods from all sampled water bodies ($p > 0.05$). But, at the same time, the sampling site had a strong effect on observed weight differences between parasitized and non-parasitized amphipods. The random effect had an estimated standard deviation (2.66 mg) that was more than three standard errors (0.88 mg) from zero, and its value is substantial in the metric of the dependent variable, mg dry weight. The model estimated 7 different intercepts, one for each sampling site, and based on the statistics of the random effect, these intercepts are different. The likelihood-ratio test confirmed that this random-intercept model offered significant improvements over a linear regression model with fixed effects only ($p < 0.01$).

Carotenoid – colour relationship

None of the 125 measured male amphipods were identified as outliers, based on the size of their residual values being smaller than 3 standard deviations. Four PLS analyses were made in order to determine the regression models between carotenoids, colour, parasitism and the abiotic factors TP and DOC. All models considered 5 latent factors (the default number), which are based on crossproduct relations between independent and dependent variables (Garson, 2009). More than five latent factors did not improve the proportions of variance explained by the models. Total phosphorus (TP) and dissolved organic carbon (DOC) were set as ordinal variables, therefore the largest TP and DOC categories (in Kettle Lake, TP= 289 mg/L and DOC= 40mg/L) became reference categories. Also parasite status in amphipods was set as an ordinal variable, dummied '0' for non-parasitized amphipods, '1' for amphipods parasitized by *P. paradoxus* and '2' for those parasitized by *P. marilis.* Amphipods parasitized by *P. marilis*

became the reference category for the other two groups, as a result of default settings of the analysis. Therefore, the relationship between carotenoids and colour was interpreted considering the significance of increasing TP and DOC values and the differences between the first two groups of amphipods ('0' and '1') and the last one ('2'). CIE L*, a*, b* colour space has three colour vectors: L* the luminosity axis varies between 0 (black) and 100 (white), a* the green-red axis and varies between -127 (green) to +127 (red) and finally the colour vector b* describes the blue-yellow axis and varies between -127 (blue) to +127 (yellow).

The first model (Model I) included all explanatory factors and carotenoid quantities of all sampled amphipods. In this case, the proportion of variance explained by latent factors was R^2= 0.84 (adj. R^2= 0.83). The values of TP and DOC regression parameters indicated how much carotenoids are loss or gained at that specific concentration. Total carotenoid quantities are not decreasing constantly with lower TP values. The relationship between total carotenoids and DOC seemed to better reflect the expected decrease in total carotenoids associated with lower lake productivity. Exceptions were found at TP= 250 mg/L (Laica water body) and DOC= 38 (Kettle water body). The analysis considered the highest TP and DOC values as reference for the rest of the values. It was expected that carotenoid quantities would decrease as TP and DOC decrease too. It can be noticed that even though the previously mentioned two values are smaller than the reference TP and DOC concentrations, the amount of carotenoids increased in amphipods from these two lakes (Table 3-9). Non-parasitized amphipods ('0') and those parasitized by *P. paradoxus* ('1') had more carotenoids than amphipods parasitized by *P. marilis*, although the parameters suggested that amphipods parasitized by *P. paradoxus* accumulate more carotenoids than non-parasitized ones (non-parasitized amphipods = 20.31 vs. amphipods parasitized by *P. paradoxus*= 21.94) (Table 3-9). The parameter L* had a negative sign, meaning that at higher L* values (lighter coloured amphipods) total amount of carotenoids decreased. Colour vectors a* and b* were positively correlated with carotenoids, i.e. when total amount of carotenoids increases, vectors a* and b* increase too, and amphipods become redder and yellower (Table 3-9).

Three other PLS models were used for testing the relationship between carotenoids, colour, and abiotic factors within each parasite-status group of amphipods. The proportions of variance explained by latent factors in non-parasitized amphipods (Model II), those parasitized by *P. paradoxus* (Model III) and those parasitized by *P. marilis* (Model IV) were R^2= 0.82 (adj. R^2= 0.80), R^2= 0.91 (adj. R^2= 0.89) and R^2= 0.97 (adj. R^2= 0.96), respectively. TP and DOC parameters are generally negative, meaning that lower concentrations of TP and DOC were correlated with a decrease in the amount of total carotenoids in parasitized and non-parasitized male amphipods. The same exceptions found in Model I, were also present in the last three models. TP and DOC values for Kettle and Laica water bodies had positive signs meaning that although their parameter values were lower than the references, total amount of carotenoids increased.

In all three models the luminosity colour vector was negatively correlated with total carotenoids, indicating that generally total amount of carotenoids decreased when L* values increased, or in other words amphipods became darker at higher carotenoid concentration. However, in Model IV (amphipods parasitized by *P. marilis* only), the value of L* was very close to zero (L*= -0.02), therefore I concluded that there was little difference between luminosity values among this group of amphipods no matter what carotenoid quantities amphipods were able to reach. Moreover, the regression parameters for a* and b* in Model IV were smaller than in the other models, overall indicating less colour changes among this group of amphipods between different concentrations of carotenoids when compared to the other models. Amphipods from Model II, III and IV all became redder (a*= 17.58, a*= 21.82 and a*= 3.27 respectively) when carotenoid concentrations increased (Table 3-9). Amphipods also became yellower when total carotenoids increased with the exception of those parasitized by *P. paradoxus*, which became bluer (b*= 0.54, b*= 0.27 and b*= -0.87 in model II, III, and IV respectively (Table 3-9).

For testing the constructed models, measured and predicted carotenoid amounts and colour values were compared by means of Pearson Correlations (one-tailed). All correlations were statistically significant, with P-values for

Pearson Correlation coefficients smaller than 0.001 for all variables in each model (Table 3-10). Total carotenoid amounts were best predicted by Model IV, while the full Model I is the poorest predictor. However, the correlation coefficients for measured and predicted carotenoids are very strong, with values above 0.90 in all models (Table 3-10).

In contrast to total carotenoids, strong correlations or regression coefficients could not be established between any of the constituent carotenoids and colour vectors.

3.4 DISCUSSION

Short review of results in the context of stated hypothesis

As expected, TP was strongly correlated with total carotenoid content of *G. lacustris*. The mixed-effects REML analysis on total carotenoids showed that TP appears to affect the difference in carotenoids between parasitized and non-parasitized amphipods ($p < 0.01$). At the same time a steep increase of total carotenoids in amphipods collected from all hypereutrophic lakes, when compared to oligo- and eutrophic water bodies can be attributed to the total phosphorus of those water bodies. Total phosphorus regulates the biological productivity in lakes, and based on the statistical results, I can suggest that amphipods originating from hypereutrophic lakes have more available dietary carotenoids that may allow for higher accumulation of carotenoids.

The hypothesized negative effect of parasitism on the host's carotenoid content has been only partially supported. The two parasite species had different effects on their hosts. *Polymorphus paradoxus* did not alter total carotenoid quantities in its host ($p > 0.1$), but *P. marilis* reduced total carotenoids ($p < 0.01$). Parasitism did not completely removed any of the carotenoids from the host body, nor induce synthesis of new ones, but quantities of each constituent carotenoid were differently affected by parasitism. A strong relationship could be established between carotenoids, colour, parasite load of amphipods and abiotic factors TP and DOC. Unlike the effects on carotenoids, both parasite species decrease the weight of amphipods ($p < 0.01$) to a similar degree, but their effects on weight are

different between lakes ($p < 0.01$) and not related to total phosphorus concentration ($p > 0.05$).

Detected carotenoids in amphipods, exoskeleton and acanthocephalan cystacanths

The method used for carotenoid extraction did not yield a complete separation of carotenoids from *G. lacustris* tissues but similar extraction efficiency was achieved in all samples. The incomplete extraction results were not related to the size of tissue particles obtained through grinding, but rather to the chemical property of carotenoproteins that had not completely dissolved in the acetone-methanol solution. Further mechanical tissue fragmentation, although not HPLC analysed and statistically tested, did not seem to produce better results (pers. obs). Rather, micro particles would just float in the extraction solution and therefore having no chromatographic significance. At the same time, their complete extraction into pure water without any further mechanical actions suggests that a certain carotenoid could not be liberated from the carotenoprotein complex using only acetone, a method regularly used by Czeczuga (i.e. see Czeczuga *et al*, 2005). If crustacyanin, a carotenoprotein found in crustacean carapace, was the only carotenoprotein incompletely extracted, then the levels of astaxanthin in the amphipods I examined will have been slightly underestimated. Crustacyanin has been studied extensively and invariably contains one molecule of astaxanthin as the prosthetic group (Zagalski, 1976).

The carotenoids reported by Czeczuga (1980) and Gaillard *et al* (2004) in *G. lacustris* from Poland and in *G. pulex* and *G. roeseli* from France are similar to our findings excepting for echinenone, which was not found in either of the two previously mentioned studies. Similarly to the results reported by Gaillard *et al* (2004), canthaxanthin was not detected in my samples. In spite of this, the most likely chemical pathway of astaxanthin synthesis in *G. lacustris* from Alberta would be from β-carotene through echinenone-canthaxanthin to astaxanthin. This biosynthesis pathway was suggested by Czeczuga (1980), along with an alternative way of astaxanthin synthesis through a transformation of β-carotene and/or lutein through α-doradexanthin and β-doradexanthin intermediate

carotenoids. Traces of β-carotene were found in some amphipods, indicating a rapid metabolization of this carotenoid by amphipods (Gaillard *et al*, 2004), and also confirming the metabolic pathway for astaxanthin starting from β-carotene. Moreover, this metabolic pathway was also suggested by the positive relationship between echinenone and astaxanthin quantities in *G. lacustris*.

Lutein and astaxanthin were the main carotenoids identified in both parasitized and non-parasitized *G. lacustris* from oligo- and eutrophic lakes and zeaxanthin and lutein in amphipods from hypereutrophic water bodies, results supported by Barrett and Butterworth (1968). These differences in carotenoids could be induced by different availability of dietary carotenoids in the sampled lakes and/or by different carotenoid accumulation strategies. Astaxanthin seemed to oscillate between 40 and 60 μg/ g dry weight in all lakes. An increase in lake productivity did not induce a relatively similar accumulation of astaxanthin. A laboratory study on the copepod *Acartia bifilosa* (Giesbrecht) indicated that high feeding rates and eutrophication of water bodies may decrease the amount of accumulated astaxanthin (Holeton *et al*, 2009). However, accumulation of lutein and zeaxanthin didn't seem to be restricted in amphipods, their concentrations increasing along with an increase in lake productivity. Two of the detected carotenoids could not be identified based only on RP-HPLC chromatograms.

A similar carotenoid composition was found in moulted exoskeletons of non-parasitized male *G. lacustris*. The moults presented a pale yellow orange colour, immediately after shedding, suggesting that most if not all present carotenoids were no longer bound to proteins. In contrast to those from full-body preparations, carotenoids from amphipod moults were rapidly and completely extractable in the HPLC grade methanol-acetone extraction solution.

Cystacanths of *P. paradoxus* had similar qualitative and relative quantitative carotenoid composition to the host amphipod. The carotenoids of acanthocephalans included lutein, zeaxanthin, esterified astaxanthin, echinenone, an unidentified carotenoid from the second peak of HPLC chromatograms and traces of free astaxanthin. Most of these carotenoids were found in the host, with the exception of esterified astaxanthin. These findings differ from previous

reports of carotenoids in *Polymorphus* spp.; esterified astaxanthin was the only carotenoid in *P. minutus* cystacanths (Gaillard *et al*, 2004; Barrett and Butterworth, 1968). However, Duclos (2006) also reported a broader carotenoid composition of *Corynosoma constrictum* cystacanths, namely astaxanthin, canthaxanthin, lutein and β-carotene. Because esterified astaxanthin was not detected in hosts, esterification must occur in acanthocephalan larvae, probably during the process of lipid storage. Barrett and Butterworth (1968) found that cystacanths of *P. minutus* store large quantities of wax esters, up to 90% of total body lipids. In light of previous findings, detected esterified astaxanthin in *P. paradoxus* might not reflect the actual quantities. HPLC is not the most appropriate method for measuring esterified astaxanthin (Gaillard *et al*, 2004, Duclos, 2006), and therefore the detected quantities of esterified astaxanthin are unreliable. Astaxanthin can be separated from its bonds to fatty acids through a process called saponification, and thus the amount of total free astaxanthin in parasites would increase. The carotenoid composition of the whole amphipod body, the exoskeleton, and the parasite, indicates that there is a direct relationship between the three. There is no evidence to conclude that *P. paradoxus* actively selects only a certain carotenoid, like astaxanthin, as it was suggested in other parasite-host systems (Gaillard *et al*, 2004; Barrett and Butterworth, 1968).

Parasitism effect on carotenoid quantities of *G. lacustris*

On average, amphipods loss 54.13 μg carotenoids/ g dry weight or about 0.61 μg carotenoids/individual due to parasitism by *P. marilis*. On average *P. paradoxus* contained 9.74 x 10^{-3} μg of carotenoids per dry cystacanth. As *P. marilis* is smaller than *P. paradoxus*, one might expect it to contain an even smaller quantity of carotenoids. Therefore, only 1.06% of carotenoid loss by an individual host can be explained by parasites directly 'stealing' them. Thus it seems logical that carotenoid loss in the host is not actually caused solely by direct 'theft' but rather through other mechanisms that may involve impaired absorption and/or deposition of carotenoids, increased activity and decreased feeding rates. Because only *P. marilis* induced a statistically significant loss of carotenoids in parasitized hosts, it must have mechanisms that strongly impede its

hosts from accumulating and/or depositing carotenoids in their tissues. The figures previously mentioned should be regarded with caution, and be used only as an approximation of an existing phenomenon due to the study limitations. These include the limitations of RP-HPLC method in estimating the quantity of esterified carotenoids in parasites, the variability in statistic data (ie. wide variances about means), and by the assumption that *P. marilis* has a similar carotenoid composition to that of *P. paradoxus*.

Effect of abiotic TP on total carotenoids in *G. lacustris*

There is strong evidence that TP, which characterizes lake productivity , total amount of chlorophyll a and indirectly the amount of dietary carotenoids, affects the total carotenoids in *G. lacustris* and also influences the differences in carotenoid composition between parasitized and non-parasitized amphipods. This is likely related to the amount and type of food available to amphipods in the wild. The sharp increase in zeaxanthin and echinenone quantities in amphipods from hypereutrophic lakes can be traced back to the specific carotenoid composition of cyanobacteria, which in addition to cryptoxanthin, canthaxanthin, and β-carotene, are the main carotenoids of this group (Squier *et al*, 2004). Lutein, zeaxanthin, antheraxanthin and β-carotene are the main carotenoids of Chlorophyta (Squier *et al*, 2004). Total carotenoid quantities in non-parasitized male amphipods from hypereutrophic lakes were almost double that observed in oligotrophic lakes. However, the trend was not linear, as total carotenoids in amphipods from eutrophic lakes Beyette and Long were less than the total carotenoids in oligotrophic lakes. This would suggest that amphipod populations have adapted locally and may employ different feeding strategies depending on availability of nutrients.

Parasitism effect on weight of *G. lacustris* males

Both acanthocephalan species affect the weight of their host in a similar manner. A greater weight loss was recorded in both of the sampled eutrophic lakes, where uninfected amphipods were heavier than amphipods from all other sampled lakes. Moreover, acanthocephalans did not affect the weight of their hosts from either of the oligotrophic lakes. The metabolic demands of the

cystacanths cannot explain the measured weight differences of about 2.4 mg of dry weight in parasitized hosts. Acanthocephalans in the cystacanth stage have already formed their tissues and organs and their nutrient demands, most likely carotenoprotein complexes, have passed the maximum peak found in an acanthellae stage when larvae undergo major changes (Duclos, 2006). This could be explained through a couple of hypotheses: cystacanths still take nutrients from their hosts, or the host is not able to recover from the weight loss suffered during the acanthellae stage of the parasite development. Acanthocephalans are known for changing the behaviour in their amphipod host, making them swim more in open water compared to non-parasitized amphipods, and inducing modified clinging and geotactic behaviour (Bethel and Holmes, 1974; Bauer *et al*, 2005), modifications that suggest an overall increased energetic demand. An increased metabolism in parasitized amphipods could divert glycogen and other nutrients from being converted to body mass, thus impairing the host's growth. On an observational level, it was noted that parasitized amphipods from the oligotrophic water bodies Forestry 754 and Narrow did not present a modified clinging or escaping behaviour, while strong clinging and escaping modified behaviour was observed in parasitized amphipods from eutrophic and hypereutrophic lakes. This could explain the parasite induced weight differences in amphipods from eutrophic and hypereutrophic water bodies, and lack of weight differences in the two oligotrophic water bodies.

Relationship between carotenoids and colour, parasitism and abiotic factors

Colour is a good indicator of the total amount of carotenoids found in amphipods. Abiotic factors and parasite load can influence both colour and carotenoid quantities and therefore they were included in the regression models. Darker, redder and yellower amphipods have higher carotenoid levels. Less complex models (within a group of amphipods with similar parasite load) predicted total carotenoid quantities with higher accuracy (Pearson's r varied between 0.94 and 0.99 in Model II, III and IV) than the more complex Model I, which included parasitism as well (Pearson's r = 0.91). Astaxanthin (Latscha, 1989; Wade *et al*, 2008; Tume *et al*, 2009) or esterified astaxanthin (Wade *et al*,

2005) have been previously nominated as the main carotenoid responsible for colour determination of crustaceans. This seems reasonable considering that crustacyanin is one major pigmentary carotenoproteins in crustaceans and it invariably contains astaxanthin as the prosthetic group. However, colour was only approximated (measured by eye) in previous studies describing the relationship between colour and carotenoids in crustaceans. These colour differences were related to observed differences in carotenoids (i.e. Wade *et al*, 2005). Inability to detect a relationship between colour and any quantitative variations in individual carotenoids could be due to the colour measurement technique that I used. Colour measurements in amphipods were made using a scanner, therefore the overall colour is an expression of both reflected and transmitted light. Thus, the measured colour values in amphipods depict not only the colour of the exoskeleton but also the colour of internal tissues.

Future research

Further investigations of parasite effects on *G. lacustris* should include a quantification of carotenoids in parasitized and non-parasitized female amphipods. This would allow a clear comparison of infected male and female amphipods, and detect potentially sex based parasite induced differences in carotenoid composition of *G. lacutris*. Environmental factors such as phosphorus, affects the host-parasite interactions both in terms of carotenoids concentration and the weight differences, but its effect didn't seem to be linear. Physiological adaptations of local populations to local environment (e.g., to consistent low availability of nutrients) may have played a role in creating the noise, but it is still not clear if the host reacts differently in different environments, if the parasite can sense the external environment and react accordingly, or if there is a more complex interaction between host, parasite and environment. Also, the absence or presence of final hosts, waterfowl and muskrats in this case, or their density at studied sites, might influence the extent of parasitic manipulations observed in amphipods. A study that would consider the interactions between populations of parasites, intermediate and final host could clarify the aforementioned issue. Two of the lakes that I sampled, Long and Narrow, are about 2 km apart, a

geographical distance that should not impede the same waterfowl from visiting both lakes and carrying parasites from one place to the other. Nevertheless, the measured effects of *P. paradoxus* on the carotenoid composition and weight of *G. lacustris* males from the two lakes were different. This suggests that virulence and host reaction were influenced by abiotic factors, but other factors beside total phosphorus and food availability could be also involved. Influence of abiotic factors, such as food and temperature, over the virulence of some parasites and infectivity susceptibility of hosts has been also recognized by Cornet *et al* (2009) in their experimental study on the association between *G. pulex* and *P. laevis*. Blanford *et al* (2003) went further and demonstrated how environmental factors like temperature can influence the expression of genotypic variation in susceptibility in the pea aphid *Acyrthosiphon pisum* (Harris) and virulence in its fungal pathogen *Erynia neoaphidis* (Remaudiere and Henebert). Proteomics or metabolomic studies could provide further information on the host genome activity during infections with acanthocephalan parasites from different locations, elucidating some of the differences observed between individuals or populations. Including such variables may help improve the predictive power of the carotenoid-colour regression models and the measured carotenoid quantities.

Temporal variation within a lake affecting its productivity may also influence the amount of carotenoids deposited by amphipods and have an impact on the overall parasite-host interactions. A laboratory study investigating host manipulation (carotenoids, weight, behavioural modifications) and interactions with gradients of environmental factors would be necessarily to further elucidate the effects of water and food quality on the host-parasite relationship.

Concluding remarks

To date, no study has considered the importance of environmental factors on acanthocephalan-gammarid relationships even though expression of certain host and parasite traits is dependent on the environment (Vale *et al*, 2008). Parasitic manipulation of amphipod hosts includes several mechanisms: modified colour, carotenoids, weight, and behaviour. These changes in hosts can be investigated separately. However, drawing conclusions on increased predation

rates by the final host based on single mechanism analyses is short sighted. It is not clear if changes associated with parasitism by acanthocephalans are indeed adaptations for increasing predation of the intermediate host, or if they are merely secondary effects of parasitism. For example the increased foraging in parasitized host could be caused by an increased energetic demand and not by a neurological manipulation by the parasite (Poulin 1995).

There were variations among parasitized amphipods detected at an individual scale, even between amphipods collected from the same water body and parasitized by the same species of acanthocephalan. The results suggested that parasitism does not induce similar effects in all individuals, and abiotic factors can explain most of the inter-population variations. These variations are disguised by population statistics through averaging of the samples, but at an individual level these existing variations show that the partners involved in this antagonistic relationship can have different genotype expressions, experienced different environment or a combination of the two (Thomas *et al*, 2005).

Table 3-1. Average carotenoid quantities (μg/ g dry weight) ± SD in *G. lacustris* collected from seven water bodies from Alberta. P0, P1 and P2 designate non- parasitized gammarids (sample= n_0), parasitized by *P. paradoxus* (sample size= n_1) and parasitized by *P. marilis* (sample size= n_2) respectively. α= 0.05. * denotes a significant difference

Carotenoid	Parasite	Beyette	Long	Narrow	Forestry 754	Kettle	Rg. Rd 223	Laica
Astaxanthin	P0	50.74±21.37	36.88±8.65	52.65±13.73	55.64±12.27	61.02±14.81	48.75±14.44	47.59±9.95
	P1	48.36±18.07*	36.10±11.56	52.33±15.42	-	49.05±18.75	43.74±20.29	43.86±2.4
	P2	-	25.56±7.41*	-	27.54±4.58*	52.36±7.76	29.58±7.46	46.6±14.56
Peak2	P0	42.85±27.86	20.75±8.74	47.76±15.93	43.67±9.88	36.63±22.39	66.61±23.06	54.63±15.13
	P1	35.71±17.63	11.65±4.60*	42.17±16.99	-	48.49±40.25	49.41±24.87	52.35±3.99
	P2	-	4.31±1.69*	-	23.84±12.64	20.59±10.48	14.78±4.01*	55.45±16.86
Zeaxanthin	P0	35.75±15.13	35.06±7.32	46.52±10.37	61±17.72	155.88±37.34	98.87±27.37	321.86±43.42
	P1	37.82±17.34	34.19±15.20	40.92±14.86	-	216.86±53.52*	77.54±43.54	386.86±100.75
	P2	-	13.81±4.38*	-	16.96±5.43*	168.57±45.26	41.24±11.13*	369.48±55.58
Lutein	P0	60.63±26.98	63.31±10.87	87.48±13.8	116.63±30.86	175.2±48.9	107.11±32.17	117.96±29.04
	P1	63.63±34.74*	47.71±12.45*	72.01±12.36*	-	179.64±28.61	111.31±85.81	154.52±6.08
	P2	-	23.26±6.83*	-	40.14±21.64*	161.57±5.84	39.65±0.05*	129.72±24.88
Peak5	P0	7.58±4.71	6.28±1.59	9.56±3.78	11.29±4.44	19.59±5.59	12.06±3.68	11.09±5.04
	P1	7.43±3.09*	4.26±1.24*	8.24±3.91	-	24.05±9.92	9.86±6.91	9.23±3.17
	P2	-	1.80±0.52*	-	2.16±0.4*	24.44±8.85	3.04±0.28*	9.41±3.28
Echinenone	P0	19.85±8.64	11.69±2.95	19.39±5.57	23.4±7.69	31.03±10.81	19.22±9.52	24.63±5.94
	P1	20.95±10.35	9.36±7.41	15.02±5.13	-	34.51±3.36	15.79±9.32	24.7±1.52
	P2	-	3.71±1.21*	-	6.85±4.69*	21.14±6.99	6.1±0.12	25.5±10.27
GLM F		0.4	6.46	1.028	8.27	2.693	1.713	0.56
GLM df		6; 12	12; 24	6; 12	6; 2	12; 24	12; 22	12; 20
GLM Sign		0.67	<0.01*	0.45	0.11	0.02*	0.13	0.85
n_o		10	10	9	5	11	9	9
n_1		9	6	10	0	6	8	3
n_2		0	4	0	4	3	2	6
Total	P0	215.51±92.79	173.97±25.6	263.35±46.47	311.62±62.37	479.34±111.63	352.63±104.07	577.76±54.79
	P1	210.61±89.8	143.27±39.1	230.69±41.89	-	517.95±93.44	307.66±182.99	671.53±102.56
	P2	-	72.45±18.85*	-	117.49±45.69*	448.66±36.44	134.39±22.39	636.15±70.66
ANOVA F		0.01	17.14	2.60	26.86	0.68	1.94	2.64
ANOVA df		1; 18	2; 17	1; 17	1; 7	2; 17	2; 16	2; 15
ANOVASign		0.91	<0.01*	0.13	<0.01*	0.20	0.18	0.10

Table 3-2. Percentage carotenoid content in *G. lacustris* from seven lakes in Alberta. P0, P1 and P2 identify the parasitized status of amphipods, P0 = non-parasitized, P1= parasitized by *P. paradoxus* and P2= parasitized by *P. marilis*. '- 'no available data

Carotenoid	Parasitic status	Beyette	Long	Narrow	Forestry 754	Kettle	Rg. Rd 223	Laica
Astaxanthin	P0	23.54	21.20	19.99	17.86	12.73	13.82	8.24
	P1	22.96	25.20	22.68	-	9.47	14.22	6.53
	P2	17.61	35.28	-	23.44	11.67	22.01	7.33
Peak2	P0	19.88	11.93	18.14	14.01	7.64	18.89	9.46
	P1	16.96	8.13	18.28	-	9.36	16.06	7.80
	P2	25.65	5.95	-	21.46	4.59	11.00	8.72
Zeaxanthin	P0	16.59	20.15	17.66	19.58	32.52	28.04	55.71
	P1	17.96	23.86	17.74	-	41.87	25.20	57.61
	P2	13.82	19.06	-	14.44	37.57	30.69	58.08
Lutein	P0	28.13	36.39	33.22	37.43	36.55	30.37	20.42
	P1	30.21	33.30	31.22	-	34.68	36.18	23.01
	P2	29.23	32.10	-	34.16	36.01	29.50	20.39
Peak5	P0	3.52	3.61	3.63	3.62	4.09	3.42	1.92
	P1	3.53	2.97	3.57	-	4.64	3.20	1.37
	P2	4.95	2.48		1.84	5.45	2.26	1.48
Echinenone	P0	9.21	6.72	7.36	7.51	6.47	5.45	4.26
	P1	9.95	6.53	6.51	-	6.66	5.13	3.68
	P2	9.97	5.12	-	5.83	4.71	4.54	4.01

Table 3-3a. Mixed-effects restricted maximum likelihood analysis results of total carotenoids differences between non-parasitized *G. lacustris* and parasitized by *P. paradoxus* and by *P. marilis*, with TP as an independent variable and sampling site as the random factor. Number of observations= 125, groups= 7, observations per group= minimum 9, maximum 20, Wald Chi^2= 12.72, Log restricted-likelihood p< 0.01. Random-effects parameters: estimate= 111.05, SE= 36.49

Parasite status of amphipods	Coeficients±SE µg/ g dry mass)	z	P>|z|	95% confidence intervals	
P. paradoxus	-13.93±18.27	-0.76	>0.1	-49.75	21.89
P. marilis	-54.13±27.07	-2.00	<0.05	-107.18	-1.08
TP	1.04±0.35	2.99	<0.01	0.36	1.73

Table 3-3b. Predicted marginal means (μg/ g dry mass) ± SE for total carotenoids in non-parasitized and parasitized amphipods set at an average hypothetical TP concentration, based on the analysis from table 3

Parasite status of amphipods	Margin ± SE . mg/ g dry mass)	z	P>|z|	95% confidence intervals	
Non-parasitized	342.56±43.72	7.84	<0.001	256.88	428.25
P. paradoxus	328.63±44.38	7.4	<0.001	241.65	415.62
P. marilis	288.44±48.73	5.92	<0.001	192.94	383.94

Table 3-4. Mixed effects restricted maximum likelihood showing the effect of parasites *P. paradoxus* and *P. marilis* and environmental factor total phosphorus (TP) on individual carotenoids (±SE) detected in *G. lacustris*. All presented coefficients are differences in carotenoids composition of parasitized amphipods from non-parasitized amphipods expressed in μg carotenoid/g dry weight of crustacean tissue. * -denotes significant difference

Parasite status of amphipods	Astaxanthin	Peak2	Zeaxanthin	Lutein	Peak5	Echinenone
P. paradoxus	-5.16±2.83	-5.52±4.14	7.92±7.08	-6.63±7.04	-1.14±0.92	-2.74±1.49
P. marilis	-9.20±4.17*	-13.32±6.13*	-1.26±10.50	-23.86±10.40*	-2.30±1.36	-6.45±2.20*
P. paradoxus	0.07	>0.1	>0.1	>0.1	>0.1	0.07
P. marilis	0.03*	0.03*	>0.1	0.02*	0.09	<0.01*
TP	>0.1	>0.1	0.03*	<0.01*	0.01*	0.02*

Table 3-5. Bivarite correlation between total quantities of carotenoids in non-parasitized male amphipods and water quality parameters TP and DOC

		TP	DOC
Carotenoids	Pearson correlation	.723**	.741**
	Sig. (2 tailed)	<0.001	<0.001
	N	63	63
TP	Pearson correlation		.931**
	Sig. (2 tailed)		<0.001
	N		63

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3-6. Average weight of freeze-dried non-parasitized and parasitized male amphipods ± SD. The parasitic species was not considered for this analysis. A t-test for independent samples was used for each lake, with a significance cut-off value of 0.05. n_0 and n_x = sample size of non-parasitized and parasitized *G. lacustris* respectively; *- denotes significant difference

Sampling site	Weight ± SD (mg)				Mean difference	t- test	df	Sig.
	Non-parasitized	n_0	Parasitized	n_x				
Forestry	14.02±1.87	5	11.08±3.71	4	-2.95	1.56	7	>0.05
Narrow	10.62±2.6	9	10.31±1.44	10	-0.31	0.33	17	>0.05
Long	16.38±1.36	10	13.19±2.85	10	-3.20	3.19	18	<0.01*
Beyette	18.93±2.99	10	14.57±3.08	9	-4.36	3.21	18	<0.01*
Rg. Rd 223	15.7±2.75	9	12.63±1.37	10	-3.07	3.13	17	<0.01*
Kettle	10.18±1.08	11	8.89±0.93	9	-1.29	2.84	18	0.01*
Laica	11.78±2.69	9	9.08±1.26	9	-2.70	2.73	18	0.02*

Table 3-7a. Mixed-effects restricted maximum likelihood analysis results of mass differences (mg) between non-parasitized *G. lacustris* and parasitized by *P. paradoxus* and by *P. marilis*, with TP as an independent variable and sampling site as the random factor. Number of observation= 125, groups= 7, observations per group= minimum 9, maximum 20, Wald Chi^2= 39.49, Log restricted-likelihood p< 0.01. Random-effects parameters: estimated SD= 2.66, SE= 0.88

Parasite status of amphipods	Coeficients±SE (mg)	z	P>|z|	95% confidence intervals	
P. paradoxus	-2.59±0.46	-5.59	<0.01	-3.51	-1.68
P. marilis	-2.39±0.61	-3.49	<0.01	-3.59	-1.20
TP	-0.01±0.01	-0.97	>0.05	-0.02	-0.08

Table 3-7b. Predicted marginal means ± SE for weight (mg) in non-parasitized and parasitized amphipods set at an average hypothetical TP concentration, based on the analysis from table 3-7

Parasite status of amphipods	Margin ± SE	z	P>|z|	95% confidence intervals	
Non-parasitized	13.87±1.05	13.21	<0.01	11.81	15.93
P. paradoxus	11.27±1.08	10.47	<0.01	9.16	13.38
P. marilis	11.47±1.14	10.08	<0.01	9.24	13.71

Table 3-8. Carotenoid quantities (μg/ g dry weight) detected in acanthocephalan *P. paradoxus* from Range Road 223 water body, Alberta

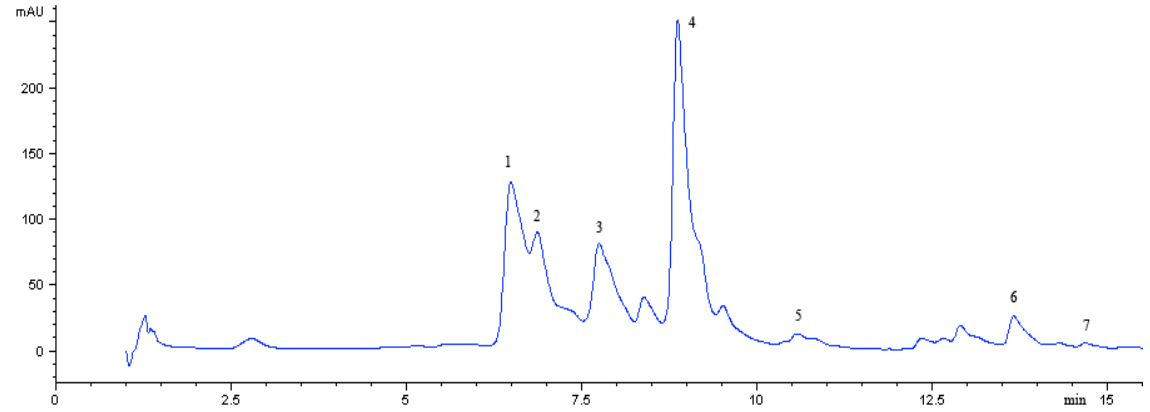
Free astaxanthin	Peak2	Zeaxanthin	Lutein	Esterified astaxanthin	Echinenone
traces	7.88	24.66	33.54	13.80	8.68

Table 3-9. PLS regression parameters estimates, or regression coefficients, for prediction of carotenoid quantities in amphipods collected from 7 lakes from Alberta. TP and DOC were set as ordinal variables based on their concentrations in μg/L. TP and DOC last categories (TP= 289 in Kettle Lake and DOC= 40 in Laica Lake) became reference for the rest of TP and DOC values therefore they are not included among the presented parameters. Parasite status was set as an ordinal variable, dummied 0 for non-parasitized amphipods, 1 for amphipods parasitized by *P. paradoxus* and 2 for those parasitized by *P. marilis.* Parasite status 2 became reference for the rest categories and it is not included among the parameters. Model I includes all 125 sampled amphipods, TP and DOC values, parasite status of amphipods and the CIE L*, a*, b* colour vectors. Model II, III and IV exclude the parasite status. '–' no available data

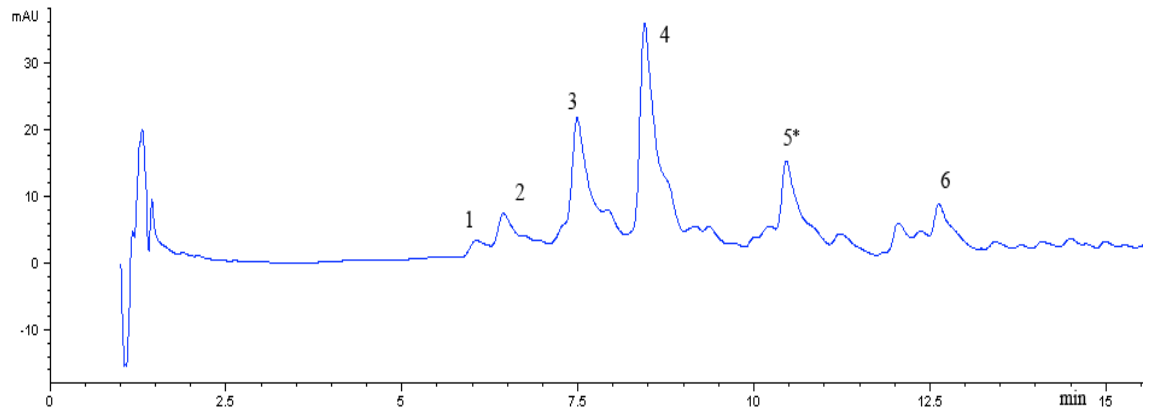
Sampling site	Independent variables	Dependent variable: carotenoid quantities			
		Model I	Model II	Model III	Model IV
	(Constant)	834.79	695.48	1212.70	396.40
Forestry 754	TP= 8	-120.12	-71.89	-	-137.22
Narrow	TP= 9	-65.71	-43.57	-77.56	-
Long	TP= 31	-112.72	-112.70	-118.77	-154.28
Beyette	TP= 46	-97.18	-113.96	-70.94	-80.11
Laica	TP= 250	236.69	183.49	318.95	246.93
Rg. Rd. 223	TP= 271	-45.50	-20.12	-35.28	-116.19
Forestry 754	DOC= 2	-120.12	-71.89	-	-137.22
Narrow/Beyette	DOC= 12	-112.72	-112.70	-118.77	-154.28
Long	DOC= 18	-100.30	-97.58	-105.01	-80.11
Rg. Rd. 223	DOC= 33	-45.50	-20.12	-35.28	-116.19
Kettle	DOC= 38	159.19	144.69	202.88	70.96
	Parasite= 0	20.31	-	-	-
	Parasite=1	21.94	-	-	-
	L*	-11.33	-6.54	-18.95	-0.02
	a*	14.34	17.58	21.82	3.27
	b*	2.16	0.54	-0.87	0.27

Table 3-10. Pearson correlation and significance values between measured and SPSS PLS predicted carotenoid and colour values. Model I includes all 125 measured amphipods from all sampled lakes with carotenoids as dependent variable and CIE L, a*, b*, parasite status, TP and DOC as independent factors. Model II, III and IV test the relationship between carotenoids, colour, TP and DOC within one of the parasite status amphipod groups: non-parasitized amphipods (II), parasitized by *P. paradoxus* (III) and parasitized by *P. marilis* (IV)

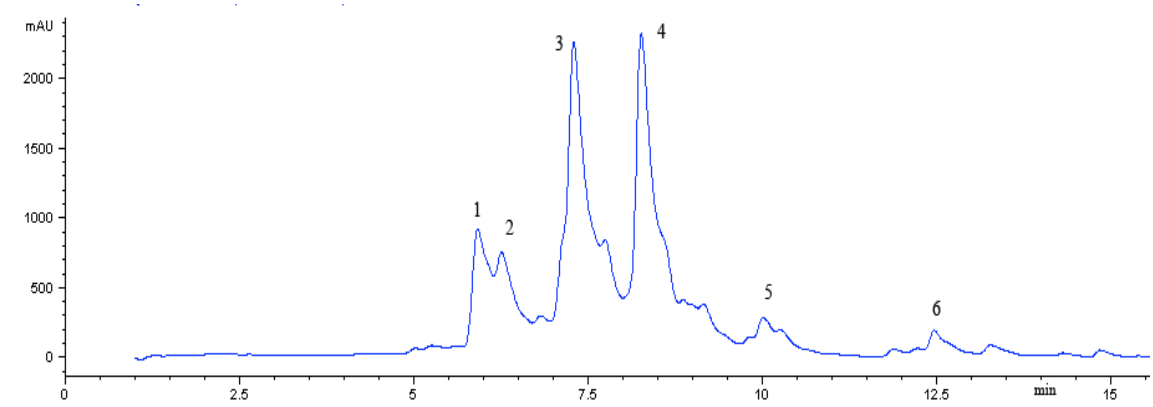
PLS model	Carotenoids	L*	a*	b*
I	0.91 <0.001	0.71 <0.001	0.81 <0.001	0.68 <0.001
II	0.94 <0.001	0.94 <0.001	0.94 <0.001	0.90 <0.001
III	0.95 <0.001	0.98 <0.001	0.88 <0.001	0.92 <0.001
IV	0.99 <0.001	0.97 <0.001	0.92 <0.001	0.84 <0.001



A.



B.



C.

Figure 3-1. HPLC chromatograms of detected carotenoids in a non-parasitized *G. lacustris* (A), *P. paradoxus* cystacanths (B) and moults of non-parasitized *G. lacustris* (C). 1= free astaxanthin, 2= unknown carotenoid, 3= zeaxanthin, 4= lutein, 5= unknown peak, 5*= esterified astaxanthin, 6= echinenone, 7= β-carotene

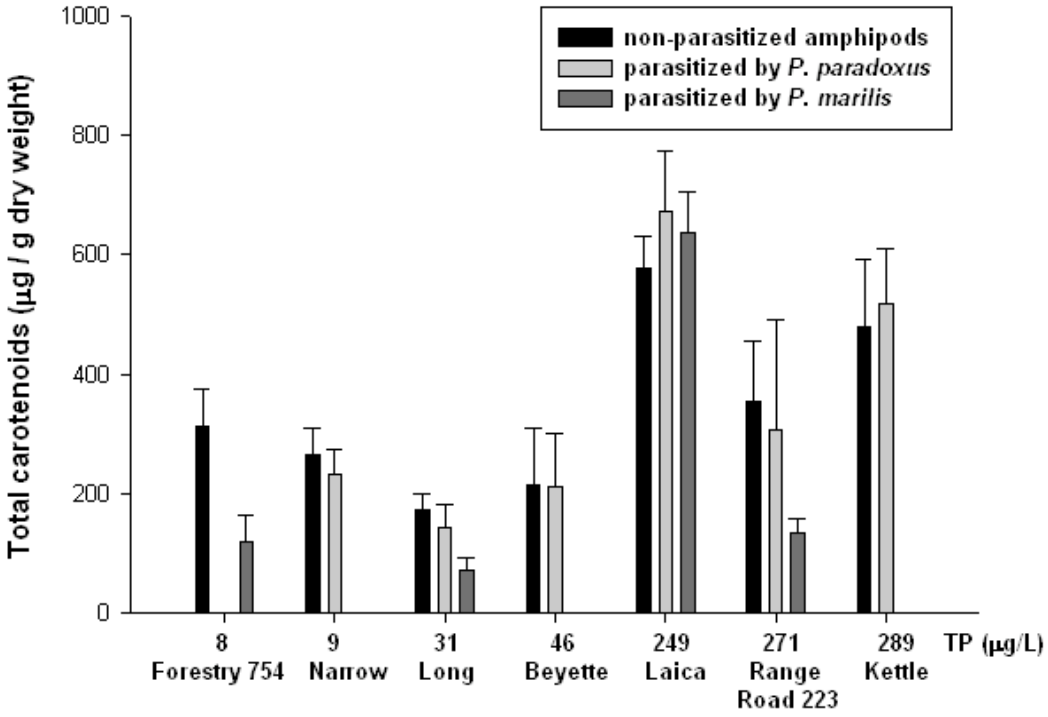


Figure 3-2. Total carotenoid composition of non-parasitized *G. lacustris*, parasitized by *P. paradoxus* and by *P. marilis* from seven lakes in Alberta, Canada, plotted against increasing total phosphous (TP) concentrations

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Chapter 4 - General Discussion

Life cycles of *Polymorphus* species involve a step of trophic transmission from an intermediate invertebrate host to a definitive vertebrate one (Denny, 1969). These parasites can influence many aspects of their intermediate host behaviour, phenotype or physiology. Still, to date it remains unclear to what extent these modifications in intermediate hosts can benefit the parasite, considering that manipulated hosts are not necessarily specific prey targets for the appropriate final hosts (Seppala *et al*, 2008; Cezillly *et al*, 2010).

My research tested the colour and carotenoid modifications induced by two species of acanthocephalans in amphipods from lakes with different productivity. Parasites are influenced by the internal environment of the host (Wolinska and King, 2009) and moreover, abiotic factors may affect expression of traits in both parasite and host (Vale *et al*, 2008). Although environmental factors have been shown to induce variations in resistance and susceptibility to infections (Blanford *et al*, 2003; Wolinska and King, 2009), no previous studies considered the influence of abiotic factors on the interactions between *Polymorphus* species and their gammarid intermediate hosts.

4.1 Colour, carotenoids and influence of acanthocephalans on male and female hosts

I found that colour formation in non-parasitized *G. lacustris* is influenced by both water quality and diet. Besides external environmental factors, there was also a sex based colour determination in amphipods. Non-parasitized male and female amphipods had different colours, and although the differences were not consistent among the studied lakes, females generally were redder and/or bluer than males, and their colour luminosity (CIE L*) vector shifted between lower or higher values than in males. Darker, redder and yellower male amphipods indicated an increase in total carotenoids content along a gradient of TP and DOC concentrations in the studied lakes. These relationships between colour and carotenoids can be extrapolated to colour differences between male and female amphipods. Thus, darker and/or redder females may have more carotenoids than males, while lighter and/or bluer females may have less carotenoids than males.

These differences between sexes were attenuated in amphipods parasitized by either acanthocephalan species, suggesting that parasitism influenced one sex more or differently than the other. All amphipods became bluer as a result of infection by either of the acanthocephalan species; however, the intensity of this change was greater in those hosts infected by *P. marilis*. Also, *P marilis* induced 'pigmentation dystrophy' (weakening of colour intensity) in its hosts, a phenomenon that was not observed in amphipods infected by *P. paradoxus*.

Parasite-induced colour differences in amphipods were reflected in total carotenoid differences between non-parasitized amphipods and those parasitized by the two acanthocephalan species. Although *Polymorphus paradoxus* takes its carotenoids from the body of the host both have a similar carotenoid composition (carotenoids in *P. marilis* were not analyzed) it did not affect the overall carotenoid quantities in hosts. This can potentially explain the reason why no 'pigmentation dystrophy' was observed in amphipod males parasitized by this acanthocephalan species. On the other hand, *P. marilis* had a significant and substantial decreasing influence on total carotenoid quantities in male amphipods and seemed sufficient to explain the overall 'pigmentation dystrophy' observed in male amphipods parasitized by *P. marilis*. Furthermore, the 'pigmentation dystrophy' induced in female amphipods by both acanthocephalan species suggested that they lose carotenoids as a result of infection. Although *P. marilis* is smaller than *P. paradoxus* induced pigmentation dystrophy and reduction in overall carotenoid quantities in male hosts were more accentuated than the effects produced by *P. paradoxus.* These findings suggested that *P. marilis* employs more efficient manipulative mechanisms than *P. paradoxus* does. This needs to be further explored, including measurement of carotenoid composition and content in *P. marilis*.

4.2 Effect of diet and water chemistry on parasite-induced changes in colour, carotenoid quantities and weight

Water chemistry and diet were two other measured factors that influenced the carotenoids and colour of amphipods and the host-parasite colour interaction. Generally, the greatest differences in overall colour (ΔE_{ab}) were observed when

comparing parasite-induced colour differences in amphipods originating from oligotrophic or eutrophic lakes to those in amphipods from hypereutrophic water bodies. However, because the colour difference parameter ΔE_{ab} has no directionality, the differences in luminosity and blue-yellow colour axis provide a better description of the degree of visible blueness induced in parasitized amphipods. As hypothesised, parasitized amphipods became bluer than non-parasitized amphipods to a greater extent in lakes with lower productivity. At a parasite species level, *P. marilis* influenced colour in amphipods according to a pattern that seemed closer to a negative relationship with increasing concentrations of TP and DOC, than did *P. paradoxus*. My observations of parasite-induced colour differences in amphipods are similar to those made by Hindsbo (1972), who noticed an increase blue tint in parasitized *G. lacustris*. However, I was able to measure these differences in a manner independent of human observation, thus eliminating the subjectivity of estimating colour purely by eye.

Beside their effect on the intensity of parasite-induced colour changes in *G. lacustris*, abiotic factors also influenced the quantities of carotenoids accumulated by amphipods. Although, astaxanthin and the 'peak 2' carotenoid levels didn't seem to vary noticeably between lakes, all other main four carotenoids identified in male amphipods are influences by the concentration of TP in lakes (Table 3-4). Amphipods from hypereutrophic lakes accumulate a larger quantity of carotenoids than amphipods from other lakes, the increase being driven mainly by lutein and zeaxanthin (Table 3-1 and Figure 3-2). Their proportions reached a combined maximum of 80% in amphipods parasitized by *P. paradoxus* from hypereutrophic Laica water body. However, dominant carotenoids depended also on the lake productivity. Lutein and astaxanthin were the dominant carotenoids in amphipods from oligo- and eutrophic lakes, results confirmed by previous findings (Barrett and Butterworth, 1968).

The influence of TP on the carotenoid quantities of *G. lacustris* can be associated with an influence of diet on the carotenoid quantities of *G. lacustris*. TP reflects the productivity level of a lake, thus hypereutrophic lakes likely have

more available dietary carotenoids for amphipods. Total carotenoid quantities detected in male *G. lacustris* spanned on a wide range from 173.97 ± 25.6 mg/ g dry weight in Long Lake to 577.76 ± 54.79 μg/ g dry weight in Laica Lake, mostly due to variations in available dietary carotenoids between lakes. These results were very close to the approximated total carotenoid quantities reported by Gaillard *et al* (2004) of 676 μg / g dry weight in *G. pulex* from the Ouche River (France), and exceeded those reported by Czeczuga (1980) for *G. lacustris* from Narrew River (Poland), approximately 70 μg / g dry weight at the maximum annual peak.

Although colour and carotenoids were influenced differently by each of the two parasites, they did not induce different degrees of weight loss in parasitized males. However, the parasite-induced effects on amphipod weight were different between lakes. Weight of amphipods from oligotrophic lakes appeared not to be influenced by parasitism but in all other lakes parasitized amphipods lost an average 2.4 mg of their dry body mass. However, Zohar and Holmes (1997) reported a different effect of the two parasite species on the size of *G. lacustris* from Cooking Lake. *Polymorphus paradoxus* reduced the length of *G. lacustris* while *P. marilis* had no effect on the length of parasitized amphipods.

4.3 Concluding remarks

Demonstrating that abiotic factors can influence various parasite-induced phenotypic modifications in *G. lacustris* cleared up some aspects of variability in published data. Besides affecting available resources for hosts and probably indirectly for parasites, environmental factors can influence the genotype expression (Blanford *et al*, 2003) through the so-called genotype-by-environments interactions (Wolinska and King, 2009). These complex interactions may explain some of the non-linear relationship between environmental factors and parasite-host relationship.

There is a clear correlation between water chemistry parameters, with increasing TP being paralleled by increasing DOC, turbidity and colour. All these factors describe the general trophic state of a lake (Wetzel, 1975) and thus the measured influences of one of the factors on amphipods and their relationship

with the two parasite species really reflects the general environmental conditions in which both host and parasite live.

Parasite-host relationships between *G. lacustris* and its parasites *P. paradoxus* and *P. marilis* proved to be complex indeed, and future studies should consider both the internal (i.e. sex of the host) and external environmental factors (i.e. water quality, diet, temperature, etc.) in describing these relationships. Testing the interactions between parasites, intermediate and final hosts, and environment in lakes with extreme levels of productivity could elucidate the observed differences in parasite-induced behavioural manipulations in *G. lacustris* and other acanthocephalan hosts. The adaptive value of colour for the trophic transmission of acanthocephalans could be elucidated by comparing the predation rate on amphipods parasitized by the same acanthocephalan species but with different colour modifications. Furthermore the environmental conditions would need to alternate between those that would allow and disallow a relevant final host to see the differences in amphipod colours.

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