DISSERTATION

PARASITE-ALTERED BEHAVIORS IN ACANTHOCEPHALAN/AMPHIPOD SYSTEMS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BARBARA J. MAYNARD ENTITLED PARASITE-ALTERED BEHAVIORS IN ACANTHOCEPHALAN/AMPHIPOD SYSTEMS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF SCIENCE.

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ABSTRACT OF DISSERTATION

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Acanthocephalan parasites are known for inducing behavioral alterations in their intermediate hosts. Infection with *Polymorphus paradoxus* causes amphipods, commonly known as scuds, to display altered photic and escape responses. The neurochemical serotonin has been previously implicated in these behavioral responses. This dissertation presents research investigating changes in serotonin correlated with *P. paradoxus* infection.

The pattern of serotonin-like immunoreactivity differed with infection: ventral nerve cords from *P. paradoxus*-infected amphipods contained more visible serotonergic varicosities than did nerve cords from non-infected amphipods. *Polymorphus marilis*, a related parasite which induces only slight changes in photic response and no change in escape behavior, was not associated with any change in serotonergic immunoreactivity. High performance liquid chromatography failed to detect any difference in the amount of serotonin in ventral nerve cords from infected and non-infected amphipods. The absence of a difference, however, could be because the local strain of the parasite does not induce altered behaviors, as evidenced by subsequent behavioral assays.

Another acanthocephalan/amphipod system exists in Italy, where *Pomphorhynchus laevis* infects *Echinogammarus stammeri*. I examined the behavior of infected amphipods in field and laboratory studies. *Pomphorhynchus laevis*-infected amphipods drifted more, were more attracted to light, and were more active than non-infected amphipods. Non-infected amphipods reduced their activity in the presence of fish odors, whereas infected amphipods remained active. *Pomphorhynchus laevis*-

infected amphipods of *E. stammeri* are abundant year round, and would be a suitable system for future investigations of the mechanisms of parasite-altered behavior.

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TABLE OF CONTENTS

Chapter 1

Literature Review

Ants aren't normally aquatic animals. But Maeyama, et al. (1994) found some individuals of a captive *Colobopsis* colony walking into their water dish. If the ants were removed from the water, they would immediately return, floundering in the water until they drowned. These suicidal individuals were all infested with round worms, members of a *Mermis* sp. that develops in ants but must return to water as adults to mate. Several minutes after each ant entered the water dish, one to several worms emerged from the ant's cloaca and entwined itself around other worms at the bottom of the dish.

This anecdote illustrates one of the more bizarre examples of parasites that change their hosts' behavior. Many more such stories are known; this review is restricted to behavioral changes induced in invertebrate hosts. Some altered behaviors are simple, such as reduced activity. Lambert and Farley (1968) found that periwinkles, *Littorina littorea*, infected with the trematode *Cryptocotyle lingua* crawled down a beach much more slowly than did non-infected snails. Williams and Ellis (1975) observed the same reduced movement in this system, and proposed a link between the physiological effects of the parasite and the sluggish behavior. These authors suggested that the migration to lower reaches of the intertidal zone might serve to place the snails into more uniform environmental conditions for breeding. Because the trematode castrates its snail host, infected snails would lack this motivation to migrate.

Another trematode/snail system exhibits a slightly more sophisticated behavioral alteration. Curtis (1987; 1990) found that *Gynaecotyla adunca*-infected snails of *Ilyanassa obsoleta* didn't move less than their non-infected conspecifics. Instead of a change in the amount of movement, these snails showed a change in the direction of

movement. Parasitized snails crawled to the upper shore of the intertidal zone, with this daily migration timed to expose the snails on the shore during the nighttime low tide. In this case, the behavior change could serve to increase the transmission of the trematode to its second intermediate host, beach-hoppers (*Talorchestia longicornis*), which are active on exposed shores at night. The trematode cercariae leave their snail host and infect the beach-hoppers by an unknown method.

Many parasites depend upon predation for transmission: a vertebrate definitive host must eat the infected intermediate host. Correspondingly, many behavioral changes appear to increase rates of predation. For example, Wedekind and Milinski (1996) observed that copepods (*Macrocyclops albidus*) infected with cestodes (*Schistocephalus solidus*) were more active, had lower swimming ability, and were easier to catch. Sticklebacks, the next host of this cestode, preferentially attacked and consumed infected copepods. Poulin, et al. (1992) saw similar behavioral changes in copepods (*Cyclops vernalis*) infected with the cestode *Eubothrium salvelini*. Because the behavioral changes arose approximately coincident with the cestode becoming infective to its next host, the evidence that the behavioral change is a parasite strategy to increase its rates of transmission was strengthened.

Increased activity, even if it results in increased predation by the parasite's next host, is not necessarily a parasite strategy. Jakobsen and Wedekind (1998), working with the *Macrocyclops/Schistocephalus* system, found that the hyperactivity of cestode-infected copepods could be the result of infected copepods trying to meet increased energetic demands. These authors gave copepods a choice between water scented only with sticklebacks and water scented with sticklebacks and copepods. Non-infected copepods chose the latter treatment, presumably to dilute their risk of predation. Infected copepods, on the other hand, avoided the scent of other copepods. The hungrier the infected copepods were (the longer since time of last feeding), the more they avoided their conspecifics. Jakobsen and Wedekind (1998) suggest that the cestodes create

increased energy demands, which the copepods try to meet by avoiding food competition. In this case, one cannot determine whether the lack of predator avoidance originated as a parasite or host strategy. A choice by infected copepods for stickleback-scented over non-scented water would strongly point to parasite control of the behavior; this test was not done.

As the above example illustrates, close examination of an altered behavior in different contexts can alter our interpretation of its adaptive significance. For example, hyperactivity doesn't always represent increased foraging activity. Hechtel, et al. (1993) found that aquatic isopods (*Caecidotea intermedius*) infected with the acanthocephalan *Acanthocephalus dirus* spent more time away from refugia and nearer to predators than did non-infected isopods. On first examination, one might hypothesize that these isopods are in a similar situation as the copepods in the previous example -- taking greater risks to meet the greater energetic demands placed by the parasites. However, because the refugia in this experiment were also food sources (leaf discs), foraging cannot account for the diminished antipredator behavior. In this case, the altered behavior seems more likely to be a parasite strategy, although other host benefits could exist.

The above examples illustrate the subtleties involved in interpreting the fitness consequences of parasite-altered behaviors. Nonetheless, behavioral changes that appear to make a host more vulnerable to predation have commonly been assumed to be parasite adaptations. Poulin (1995) and Moore and Gotelli (1990) cautioned against making this assumption without supporting evidence. Even if the current consequence is increased transmission, they argued the behavior may have originated as a fortuitous result of another aspect of parasite biology. For example, a parasite may have originally invaded the nervous system to avoid the host's immune system. Once there, the parasite could have induced behavioral alterations merely by its presence. More recently, Poulin (1998) proposed two litmus tests to be used in concert to discriminate adaptations from side-effects. First, fitness benefits must be shown. Second, mechanisms must be understood. Specifically, "any specific action of the parasite having no function other than to alter

host behaviour can suggest that the behavioural change is adaptive for the parasite," (Poulin, 1998). He also suggested that evidence of convergent evolution, similar behavioral changes arising in separate host or parasite lineages, supports the hypothesis of an adaptation (Poulin, 1998).

Moore and Gotelli (1996) examined several lineages of cockroaches for evidence of convergent evolution in the behavioral changes induced by the acanthocephalan *Moniliformis moniliformis*. They examined the behavior of seven different species of cockroach, representing four subfamilies. Phylogenetic history did not seem to influence whether or not the parasite altered roach behavior, thus strengthening the evidence that these behavioral changes are adaptive for either host or parasite.

To address the issue of fitness consequences, several authors have tested the hypothesis of increased predation on parasitized animals. Increased predation would presumably enhance parasite fitness by increasing rates of transmission. Moore (1983) found that isopods of *Armadillidium vulgare* infected with the acanthocephalan *Plagiorhynchus cylindraceus* were more frequently in less humid areas, on light colored paper, and in unsheltered areas. Infected females rested less often and moved farther. These differences in microhabitat preference and activity level could make the isopods more obvious to the parasite's definitive host, starlings (*Sturnus vulgaris*). In fact, Moore (1983) found in both laboratory and field studies that starlings preferentially preyed on infected isopods.

In another acanthocephalan example, *Polymorphus paradoxus* infects an amphipod intermediate host *Gammarus lacustris*. Mallard ducks (*Anas platyrhynchos*) serve as the final host after ingesting parasitized amphipods (Petrochenko, 1971). Normally, uninfected amphipods avoid light and scavenge their food from the detritus on the bottom of freshwater ponds; mallards are dabbling ducks that feed primarily at the water surface. Parasitized amphipods, however, are attracted to light, which brings them in close contact with the surface of the water (Bethel and Holmes, 1973; Hindsbo,

1972). In addition, upon being disturbed, these parasitized amphipods skim the water surface or cling to floating objects such as vegetation (Bethel & Holmes, 1973). These behavioral changes could increase the parasite's chances of being transmitted from a bottom-dwelling amphipod to a surface-feeding duck. In fact, mallards preyed preferentially on infected amphipods (Bethel and Holmes, 1977).

The timing of the behavior change relative to the infective life history of the parasite can also help to resolve whether parasites control host behavior. The acanthocephalan enters the amphipod as an egg ingested by the host. Once inside, the parasite migrates through the intestinal wall into the hemocoel and develops into a larval stage known as a cystacanth. Not until the cystacanth develops is the parasite able to infect a mallard duck. Prior to this time, the developing parasite will die if its host is ingested. Thus one would predict that the parasite should attain the cystacanth stage before altering its host's behavior. This is exactly what was observed for *P. paradoxus* (Bethel and Holmes, 1974).

In the above examples, the intermediate hosts exhibited altered behaviors, and were more vulnerable to predation. However, the increased predation was not shown to directly result from the altered behaviors. Other changes could accompany infection that might also influence susceptibility to predation. Bakker, et al. (1997) attempted to distinguish behavioral from color cues in an amphipod/acanthocephalan system much like that described above. A European species of amphipod, *Gammarus pulex*, serves as the intermediate host for the acanthocephalan *Pomphorhynchus laevis*, but in this case, the definitive host is fish, not ducks (Kennedy et al., 1978). Infected amphipods are positively phototactic, spend more time on surface vegetation, and are more likely to be eaten by fish (Kennedy et al., 1978). Bakker, et al. (1997) proposed that while the behavioral alterations seem likely to be responsible for the increased predation, the color of the parasite could also be responsible. *Pomphorhynchus laevis* absorbs carotenoids from its intermediate host. These pigments make the parasite visible

through the host hemocoel as an orange ball. This color spot could provide a visual cue for fish. In order to distinguish behavioral from color differences, Bakker et al. (1997) disguised infected and non-infected amphipods by painting their exoskeletons. In trials designed to test the effect of behavior on predation, both infected and non-infected amphipods were painted with a brown dot over the parasite (or where the parasite would be), so that all amphipod coloration was the same. In trials designed to test the effect of color, the color cues of non-infected and infected amphipods were switched: non-infected amphipods received an orange dot on their exoskeletons, and infected amphipods received a brown dot. Predation trials with sticklebacks showed that both color and infection are responsible for the increased predation on infected amphipods (Bakker et al., 1997).

Demonstrations of increased predation address the issue of fitness consequences of parasite-altered behaviors. According to Poulin (1998), mechanisms underlying these changes must also be elucidated for valid interpretation of their evolutionary significance. In some instances, the mechanism is simple -- the parasite eats or otherwise damages host tissue. Field crickets (*Gryllus integer and G. rubens*) infected by the parasitoid fly *Ormia ochracea* exhibit reduced mating, egg-laying, and fighting behavior (males) (Adamo et al., 1995). These behavioral deficits are not surprising, considering that the fly larvae feed on cricket muscle and fat (Adamo et al., 1995). Poulin (1998) would not classify this behavior change as an adaptation because the mechanism most likely originated for another purpose, to feed the parasite.

Other parasites inflict no gross tissue damage, so more subtle mechanisms must be involved. Haye and Ojeda (1998) measured oxygen consumption and behavior of acanthocephalan- (*Profilicollis antarcticus*)infected estuarine crabs (*Hemigrapsus crenulatus*). Parasitized crabs were more active and spent more time in excited postures; the authors suggest this could increase predation by the parasite's next host, the gull *Larus dominicanus*. The hyperactivity was accompanied by elevated metabolic

rates. The authors report this as "the first experimental demonstration that altered behavior induced by acanthocephalan parasites on their hosts has a physiological basis," (Haye & Ojeda, 1998).

Other parasites may alter host behavior by their physical presence in the hosts' central nervous systems. Amphipods (*Gammarus insensibilis* and *G. aequicauda*) are the second intermediate host for the trematode *Microphallus papillorobustus*. Altered behaviors such as an attraction to light and hyperactive response to disturbance may be caused by metacercariae that lodge themselves in the amphipod's central nervous system: in field trials, 86% of amphipods caught at the water surface had metacercariae in their heads, while only 18% of amphipods caught at the bottom of the water had metacercariae in their heads (Helluy, 1983). One metacercaria was sufficient to elicit the altered behaviors, and behavioral alterations did not occur until the metacercariae were ready to be transmitted to their next host, the gull *Larus cachinnans*. Predation by gulls was greater on infected amphipods (Helluy, 1983).

Perhaps one of the most specific and complex parasite-induced behavioral alterations is also caused by trematode metacercariae that lodge themselves in their host's central nervous system (reviewed by Schmidt and Roberts, 1989). Ants infected with *Dicrocoelium dendriticum* leave their nests and climb blades of grass as the temperature cools in the evening. There the ants remain, latched firmly to the grass with their mandibles, until the air warms again in the morning. This temperature-dependent behavior could increase incidental predation of the ants by grazing sheep, the trematode's next host. After an ant becomes infected with *D. dendriticum* by eating a cercaria-laden slime ball, the metacercariae migrate towards the ant's subesophageal ganglion, near the origin of the mandible nerves. Once one metacercaria reaches this destination, all other metacercariae stop their migration and encyst in the hemocoel.

The evidence that the ant's "brainworm" is responsible for the behavioral alteration is supported by comparison with a closely related system. *Dicrocoelium hospes*

is an African trematode that causes its ant hosts to "assemble in groups at elevated places, preferably on plants, and rest there motionless for a long time," (Romig et al., 1980). These metacercariae also migrate toward the cerebral ganglia of the ant, but encyst in the antennal lobes rather than the subesophageal ganglion. Two metacercariae become encysted, one in each lobe, before the others stop their brain-ward migration. In this system, the mandibles are not involved in the behavior change. Correspondingly, the encysted metacercariae are not located near the origin of the mandible nerves, as *D. dendriticum* are.

Arguably the most intriguing behavior-altering mechanims are those in which neuroendocrine manipulation has been implicated. The best understood neuroendocrine tale involves parasitic castration. In a system similar to the sluggish snails described at the beginning of this review, snails (*Lymnaea stagnalis*) infected by the avian schistosome *Trichobilharzia ocellata* do not develop normal reproductive structures (reviewed in Hurd, 1990). By three months post-infection, gonads were only 1% of average control volume; gametogenesis was reduced but not blocked. Supplementation of reproductive hormones such as dorsal body hormone, which directs reproductive development, produced increased fecundity in control snails, but had no effect on infected animals. The findings that gametogenesis was not completely inhibited and that hormone supplementation was ineffective indicated that the normal reproductive hormones must be present in infected snails, but the trematode sporocysts somehow reduce its activity. A novel peptide, schistosomin, is produced by the snail's central nervous system in response to trematode infection, and interferes with gonadotropic hormones at the receptor level. Thus, even though infected snails produce greater amounts of dorsal body hormone, the hormone's actions are diminished and the gonads do not develop. Recently Hoek, et al. (1997) reported that neuropeptide gene expression is altered in the brains of infected snails. We have yet to learn how the parasite might interfere with gene expression.

Less is known about the role of neurohormones in a parasitoid/insect system. Tobacco hornworms (*Manduca sexta*) infected with a parasitoid wasp (*Cotesia congregata*) showed a marked decrease in feeding and voluntary locomotion at the time of parasitoid emergence onto the hornworm's cuticle (Adamo et al., 1997). The hornworms were, however, capable of brief bouts of normal feeding and of normal reflexes when provoked. This lethargy might protect the wasps: non-infected hornworms ate wasp cocoons presented to them or glued onto their backs; infected hornworms did not. Coincident with parasitoid emergence was a significant increase in levels of octopamine in the hornworms' hemolymph (Adamo et al., 1997). However, injection of the octopamine antagonist phentolamine did not restore normal behavior. The correlation of elevated octopamine titers with lethargy is opposite to the typical pattern in insects and to the effects of injecting non-parasitized hornworms with octopamine: octopamine is normally associated with increased locomotion. How octopamine might be related to the altered behaviors is yet to be determined.

Indoleamines have also been implicated in the behavioral changes induced by *P. paradoxus* in its amphipod intermediate host. Helluy and Holmes (1990) noticed that the clinging posture of parasitized amphipods resembled the dominant posture of lobsters, with the abdomen curled and legs flexed. Work in the 1980's had shown that serotonin injections elicit prolonged dominant postures by lobsters and crayfish (Livingstone et al., 1980). Octopamine injections elicit opposite effects, the subordinate posture with abdomen and legs extended. Helluy and Holmes (1990) proposed that these neurochemicals might have similar effects in another crustacean, the amphipods. In fact, injections of serotonin, but not other classical neurotransmitters, induced non-infected amphipods to display a clinging behavior very similar to that exhibited by *P. paradoxus*-infected amphipods (Helluy & Holmes, 1990). Injections of octopamine suppressed the clinging behavior of infected amphipods. From these observations, Helluy and Holmes proposed that "cystacanths of *P. paradoxus* modulate the behavior of gammarids through

the alteration of neural activity in some serotonin-sensitive or serotonergic central pathway, probably one involved in precopulatory clinging in male amphipods," (Helluy & Holmes, 1990, p. 1214). The mate-guarding behavior of male amphipods also resembles the dominant lobster posture, with the abdomen curled and legs flexed. Therefore, Helluy and Holmes (1990) suggested that the most likely pathway for a parasite to induce clinging behavior would be to take advantage of a pre-existing neural pathway.

Helluy and Holmes' (1990) observations demonstrate that serotonin is sufficient to induce the parasitized form of behavior. However, they do not directly test whether serotonin is actually involved in the behavior change by parasitized amphipods. For example, injection of serotonin could, in some spurious way, be only mimicking the effects of the actual mechanism of parasite-induced behavior change, even if that mechanism did not involve serotonin. Thus, a direct observation of serotonin-related change in the nervous systems of parasitized amphipods is lacking.

The first goal of the present research was to more closely investigate the role of serotonin in behavior changes elicited by the parasite in its amphipod host. An increased serotonin hemolymph titer has been shown to be sufficient to induce these behavioral changes. If serotonin is indeed involved, then how is its action affected within the amphipod? Two reasonable hypotheses are that serotonin changes in fine-scale distribution and (or) in its global titer.

A second goal of the present research was to investigate the behavioral changes induced in another acanthocephalan/amphipod system. As described above, the acanthocephalan *Pomphorhynchus laevis* induces altered behavior in its amphipod host *Gammarus pulex*. In Italy, *P. laevis* uses a different amphipod, *Echinogammarus stammeri*, as its intermediate host (Dezfuli et al., 1991). Does this acanthocephalan produce the same behavioral alterations in two species of amphipod? Infected *E. stammeri* are plentiful year-round (pers. obs.), while the other *Gammarus* systems can be difficult or impossible to find. If this parasite induced behavioral changes in *E. stammeri*,

then this system might be well suited for mechanistic studies and could provide for some interesting comparative studies amongst several amphipod/acanthocephalan systems.

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Chapter 2

Gammarus lacustris harboring *Polymorphus paradoxus* show altered patterns of serotonin-like immunoreactivity

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ABSTRACT: The pattern of serotonin-like immunoreactivity in the central nervous system of individuals of the amphipod *Gammarus lacustris* harboring polymorphid cystacanths was compared to the pattern seen in individuals not infected with acanthocephalans. Ventral nerve cords from both parasitized and non-parasitized amphipods showed the same bilateral pair of immunoreactive cell bodies in the third thoracic ganglion. Significant differences were noted in the fine structure of these cell bodies, with nerve cords from *Polymorphus paradoxus*-parasitized amphipods showing a greater number of bright spots, or localized points of storage of serotonin. The results of this study indicate that infection of *G. lacustris* by cystacanths of *P. paradoxus*, but not *P. marilis*, is correlated with changes in the anatomy of the serotonergic neurons of the amphipod's central nervous system.

INTRODUCTION

Studies of host-parasite interactions have revealed numerous instances in which parasites alter the behavior of their hosts, frequently to the apparent benefit of the parasite (see Moore and Gotelli, 1990 for review). In some cases, these altered behaviors are the direct result of gross mechanical damage to host tissue by the parasite (Holmes and Zohar, 1990). In other cases, the mechanism of behavior change involves a subtle manipulation of physiological processes within the host (see Thompson and Kavaliers, 1994 for

review). The neurochemical serotonin has been indirectly implicated in one such parasite-induced behavioral alteration, the amphipod/acanthocephalan interaction between *Gammarus lacustris* and *Polymorphus paradoxus*.

Individuals of the gammarid amphipod *G. lacustris* serve as the intermediate host for *P. paradoxus*, followed by mallard ducks or muskrats as the definitive hosts. In a series of elegant experiments, Bethel and Holmes (1973, 1974, 1977) found that amphipods harboring cystacanths, the infective larval stage of *P. paradoxus*, exhibited altered photic and escape responses. Parasitized amphipods preferred more brightly lit environments, and did not dive to the darker water bottom upon disturbance. Rather, the parasitized individuals reacted to disturbance by skimming the water surface until encountering a floating substrate, to which they clung for some time after the initial stimulus had passed.

Other species of acanthocephalan parasites also alter the behavior of their amphipod hosts. Infection with cystacanths of *P. marilis* causes *G. lacustris* to prefer lit environments over dark areas, but does not alter the escape response (Bethel and Holmes, 1973). The distinct behavioral changes associated with these two species of *Polymorphus* are correlated with the feeding niches occupied by their respective definitive hosts. The positive phototaxis and clinging induced by *P. paradoxus* would seem to enhance the vulnerability of the amphipod host to predation by dabbling mallard ducks. In fact, *P. paradoxus*-parasitized amphipods were more likely to be eaten by mallards and muskrats than were their non-parasitized conspecifics (Bethel and Holmes 1977). The less drastic behavioral alterations induced by *P. marilis* may serve to increase predation by its definitive host, the lesser scaup, a diving duck that feeds in open waters, but this hypothesis remains to be tested (Bethel and Holmes 1977).

The mechanisms underlying these altered behaviors are not immediately obvious. Polymorphid cystacanths exist in the amphipod's hemocoel as free-floating balls, causing no apparent mechanical damage. This suggests that more subtle neurophysiological

mechanisms are involved. Helluy and Holmes (1990) found that injection of serotonin, but not other classical neurotransmitters (dopamine, norepinephrine, and GABA), caused non-parasitized amphipods to exhibit clinging and photic responses (Helluy, 1988) similar to those of individuals parasitized by *P. paradoxus*. In addition, they found that octopamine, which frequently acts in opposition to serotonin, reduced the clinging response in *P. paradoxus*-parasitized amphipods. These results suggest that serotonin-related changes in the nervous system may underly the parasite-induced changes in behavior. However, they do not eliminate the possibility that serotonin injections mimic the effects of the parasite without normally being involved. Furthermore, they do not tell us how serotonin, if in fact necessary for the behavioral change, is altered.

The present study tests the hypothesis that infection with polymorphid cystacanths is correlated with changes in serotonergic neurons of the amphipod central nervous system. Using immunocytochemical techniques, we compared the pattern of serotonin-like immunoreactivity in the ventral nerve cords of six *P. paradoxus*- and six *P. marilis*-parasitized amphipods to those from non-parasitized individuals. Although there were no differences in the location and number of immunoreactive cell bodies, the fine structure of the neurons was dramatically different in those nerve cords from *P. paradoxus*-parasitized amphipods.

METHODS

Individuals of *Gammarus lacustris* were collected from East Delaney (40° 42' N, 106° 18.9' W) and Cowdrey (40° 50' N, 106° 18' W) Lakes, Jackson County, Colorado and from Cooking Lake (53° 25' N, 113° 00' W) and an unnamed pond near Edmonton, Canada. Amphipods were maintained in pond water at 4 - 12° C with natural vegetation until dissection, 1 to 24 days after collection. Only amphipods containing the infective cystacanth stage, as initially assessed by visual inspection and confirmed during the dissection, were used as infected individuals. Only those amphipods found to contain no

visual sign of acanthocephalan infection were used as uninfected individuals. Amphipods containing the earlier, noninfective acanthellae stage were not used in this study.

Cystacanth species were initially identified by visual inspection of size and color, and later confirmed by identification according to the procedure of Denny (1969). Cystacanths were placed in tap water until the proboscis everted, at which point they were transferred to AFA (10% formalin, 70% ethanol, 5% acetic acid, 15% deionized water) to fix overnight. Fixed cystacanths were dehydrated in successive ethanol baths (50%, 75%, and 95%), then cleared in methyl salicylate and mounted in Permount. The number of hooks per row, rows per proboscis, length of longest hook, and thicknesses of body wall layers were all used to determine species.

The immunocytochemistry procedure of Beltz and Kravitz (1983) was adapted for use with small amounts of tissue from freshwater animals. Amphipods were immobilized at -20° C until movement ceased, approximately 20 min, or killed in 50% ethanol. The ventral nerve cord was dissected from the amphipod in cold freshwater crustacean saline (VanHarreveld, 1936) and pinned out in a Sylgard-lined dish. The nerve cords were fixed in 4% paraformaldehyde overnight, then rinsed several times with 0.3% Triton-X in 0.1M phosphate buffer, pH 7.4. After rinsing, the tissue was incubated in a rabbit serotonin antiserum (Incstar; 1:500) overnight. This antiserum was removed and the tissue rinsed several times in 0.3% Triton-X, followed by incubation in the secondary antibody, a goat anti-rabbit serum labeled with either FITC or CY3 (Jackson Laboratories; 1:500), overnight at 4° C. The tissue was then rinsed in 0.1 M phosphate buffer, followed by a rinse in 4 mM sodium carbonate buffer, pH 9.5, and mounted in *n*-propyl gallate. In all cases, ventral nerve cords were paired in dishes for processing, with a nerve cord from an infected and a nerve cord from an uninfected amphipod, both caught at the same time in the same lake, processed and analyzed together.

To control for non-specific staining, two nerve cords were processed as described above, but with the serotonin antiserum omitted. Another pair of nerve cords were

processed with serotonin antiserum that had been incubated with 1.5 mM serotonin at 4° C overnight prior to application to the tissue. In both cases, no staining was seen in the specimens.

Nerve cords were examined and analyzed with the use of a confocal microscope (Molecular Dynamics CLSM MultiProbe 2001) in conjunction with an inverted Nikon microscope equipped for epifluorescence. The 25 mW argon laser used for excitation had primary emission lines of 457, 488 and 514 nm. FITC was visualized with 488 nm excitation and a longpass emission filter at 510 nm. CY3 was visualized with 488 nm excitation and a longpass emission filter at 535 nm. The intensity of the laser was adjusted by use of neutral density filters, to reduce the intensity to 3% or 10% of maximum. In all cases, the same neutral density values were used for each pair (uninfected and infected) of specimens. Thirty to 40 optical sections of 1 μm thickness were taken through each preparation at 20 or 40X (the same number of sections and magnification used for each member of a pair); these sections were then combined into a composite, three-dimensional image using the Volume Workbench Look Through feature of the Molecular Dynamics software, which allows brightness to be independent of depth within the image. These computerized images were used for later quantification of fine structures (see below).

RESULTS AND DISCUSSION

The only serotonin immunoreactive cell bodies seen in the ventral nerve cords were a bilateral pair located laterally in the third thoracic (T3) ganglion. These neurons showed the same anatomical structure in both non-parasitized and parasitized amphipods. Each projected an axon across the commissure to the contralateral hemiganglion, as well as posteriorly along the ipsilateral, medial region of the length of the nerve cord. Each posterior-running axon branched in each ganglion, sending fine processes into the neuropil. An additional, intertwined pair of immunoreactive axons runs the length of both

sides of the nerve cord lateral to the axons of the T3 neurons. The cell bodies of these lateral axons were not located, but probably lie in the cerebral ganglia.

The observation that parasitized and non-parasitized nerve cords show identical serotonin-like immunoreactivity in terms of number and location of somas stained eliminates the possibility that serotonin titers could be increased by a change in the neurotransmitter phenotype of existing nerve cord cells or by causing the birth of new serotonergic neurons in the nerve cord.

Although the general structure of the T3 neurons was the same in parasitized versus non-parasitized amphipods, there was a dramatic increase in the number of bright "spots", presumably varicosities (Lnenicka, et al. 1991), along immunoreactive fibers in nerve cords from *P. paradoxus*-parasitized individuals (Fig. 2.1); nerve cords from *P. marilis*-parasitized individuals did not show this pattern (Figure 2.2). Varicosities, swollen sites of neurotransmitter release along neural processes, are characterized by a "beads-on-a-string" configuration as seen in Figure 2.1. Electron microscopy should be used to confirm that such swellings are indeed release points, but the appearance is characteristic. We quantified the number of these putative varicosities in 12 pairs of nerve cords by having two observers blind to infection state count all of the "spots" contained within three rectangular areas (50 μm X 50 μm) imposed upon computerized images of each preparation. Placement of the rectangles was based on consistent neuroanatomical reference points in each image. The variability inherent in immunocytochemical processes dictated that the absolute score for any given nerve cord was meaningful only in relation to the other nerve cord processed in the same dish. The total number of varicosities scored by each observer for each infected preparation was divided by the total for its uninfected pair. The resulting ratios for each pair of nerve cords, averaged across the two observers, ranged from 1.50 to 7.01 for the six *P. paradoxus*-infected preparations and from 0.40 to 1.14 for the six *P. marilis*-infected preparations. Thus, the mean ratio of *P. paradoxus*-infected to uninfected varicosities was significantly different

from one (Fig. 2.3; Wilcoxon signed-rank test, $T = 0$; $n = 6$; $p \leq 0.05$, 2-tailed). The mean ratio of *P. marilis*-infected to uninfected varicosities was not significantly different from one (Fig. 2.3; Wilcoxon signed-rank test, $T = 4$; $n = 6$; $p > 0.2$, 2-tailed). The average ratios for *P. paradoxus* versus *P. marilis*-infected amphipods were significantly different (Wilcoxon rank sum test, $T = 57$; $n = 6$; $p < 0.05$, 2-tailed).

This increase in putative varicosities in the ventral nerve cords of *P. paradoxus*-parasitized amphipods may represent actual increases in the number of serotonergic varicosities, or it may represent an increase in the amount of serotonin stored in each varicosity, rendering those points more easily seen. Regardless of whether serotonin is altered in amount or in number of local storage sites, the difference in putative varicosity number between *P. paradoxus*-parasitized and non-parasitized amphipods provides new evidence that serotonin plays a key role in this altered behavior, and suggests that the parasite is effecting a change in central nervous system function. The absence of this trend in *P. marilis*-parasitized amphipods indicates that distinct strategies, not just degree of effect, may be responsible for the different behavioral changes induced by each of these parasites.

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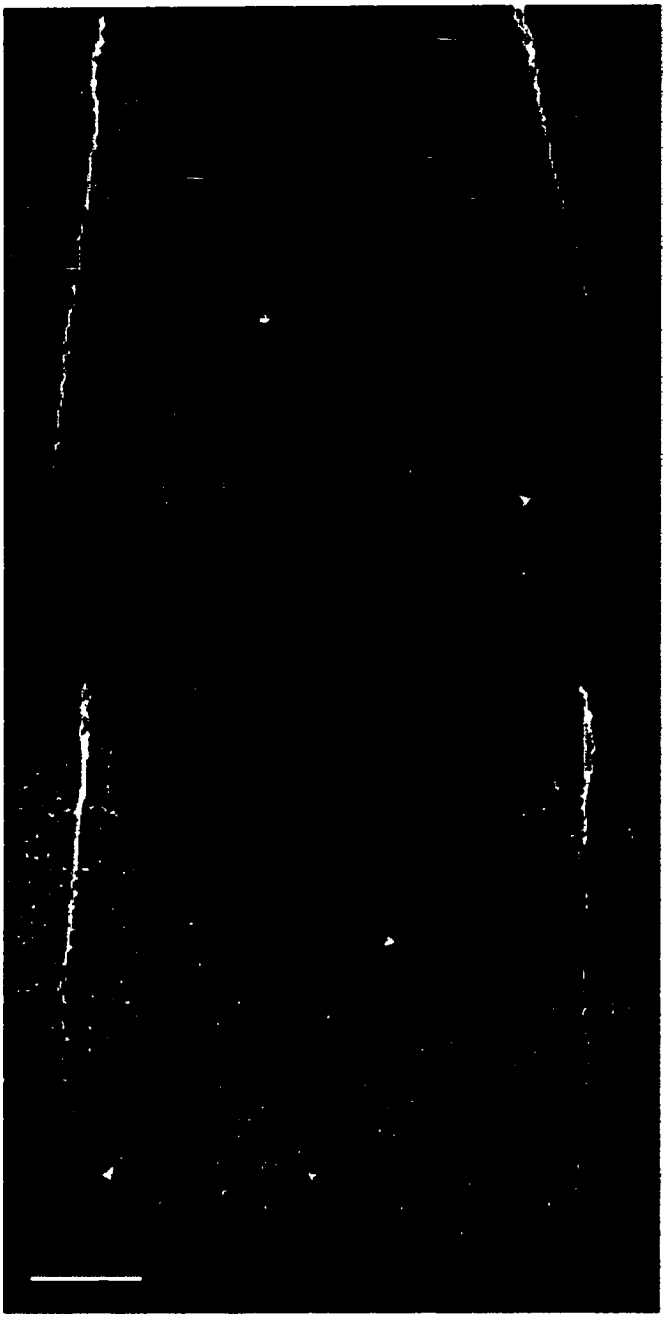

Figure 2.1. Serotonin-like immunoreactivity in the third thoracic ganglia of a non-parasitized (A) and a *P. paradoxus*-parasitized (B) amphipod. These two preparations were processed in the same dish, so differences cannot be attributed to variability in preparation. Arrowheads point to examples of putative varicosities. T3 cell bodies are out of the field of view. The parasitized ganglion shows 1.7 times more visible varicosities than its unparasitized counterpart.

Scale bar is 20 μm.

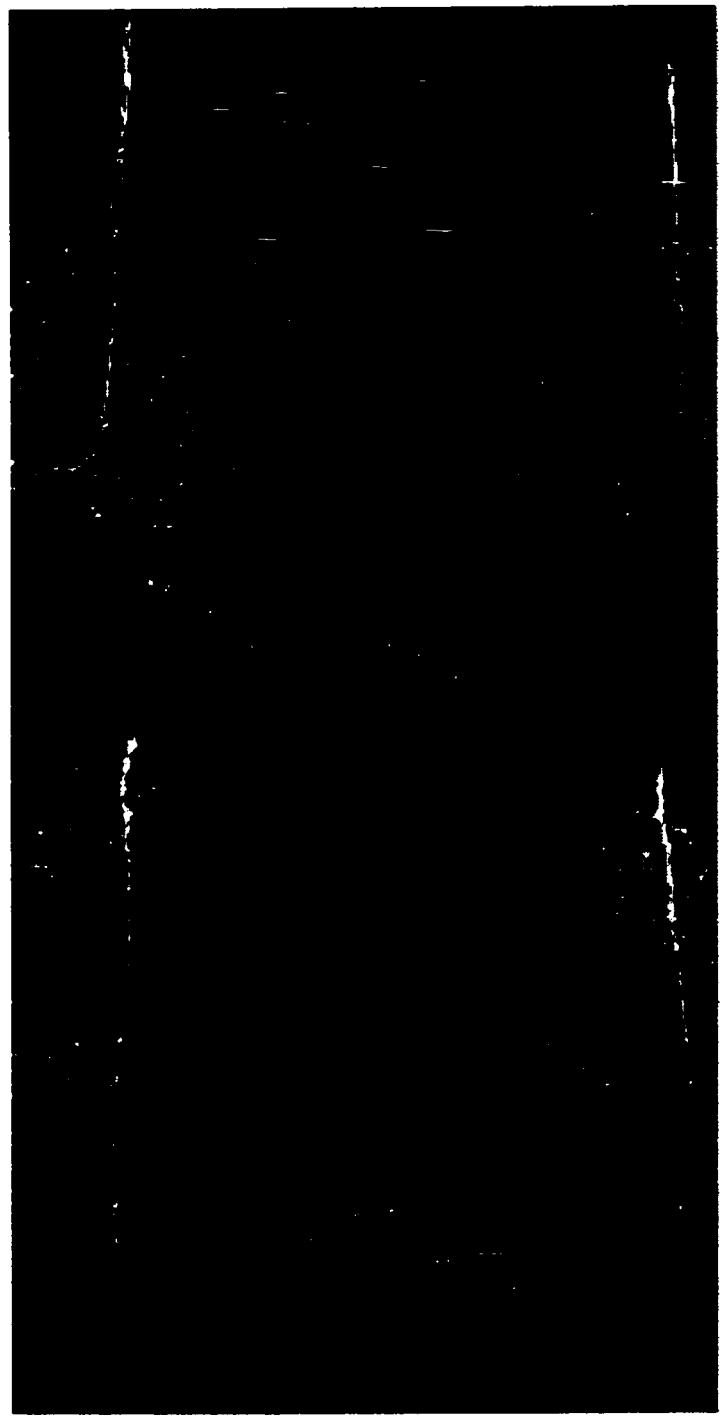

Figure 2.2. Serotonin-like immunoreactivity in the third thoracic ganglia of a non-parasitized (A) and a *P. marilis*-parasitized (B) amphipod. In contrast to the *P. paradoxus*-parasitized preparation, there is no increase in the number of putative varicosities in the infected preparation.

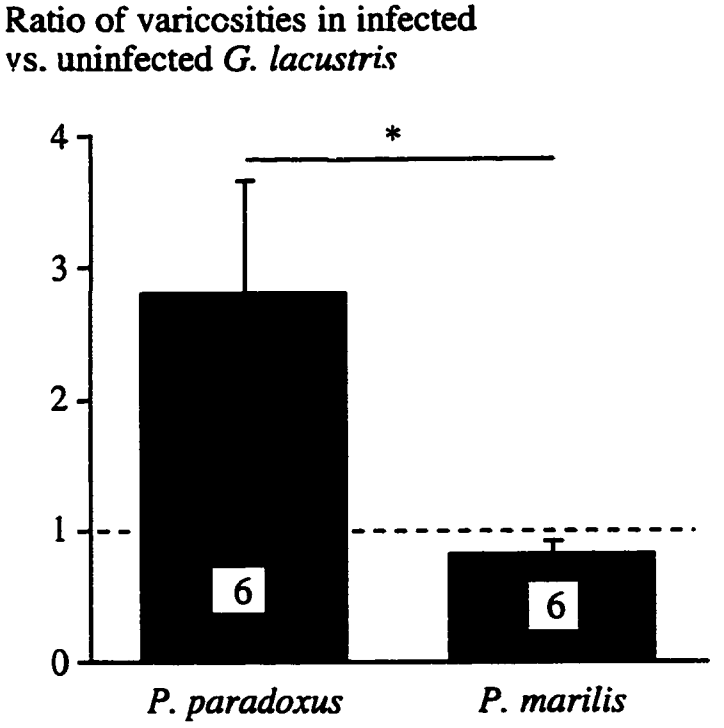

Figure 2.3. *P. paradoxus*-parasitized ganglia showed significantly more varicosities than did their unparasitized counterparts; *P. marilis*-parasitized ganglia did not show this trend. Shown is the mean ratio (number of varicosities) of each parasitized ganglion to its unparasitized counterpart for the two species. The dashed line represents the null hypothesis, a ratio of 1. Sample sizes are shown a the base of bars, error bars represent ± one standard error, and asterisks indicate $p \leq 0.05$.

Chapter 3

Serotonin levels in parasitized and non-parasitized amphipods quantified by HPLC

INTRODUCTION

The acanthocephalan parasite *Polymorphus paradoxus* markedly alters the photic and escape behavior of its amphipod intermediate host *Gammarus lacustris* (Bethel & Holmes, 1973). Parasitized amphipods show an increased preference for more brightly lit environments in comparison with non-infected individuals. In response to disturbance, non-infected amphipods will dive to the bottom of the water column. In contrast, *P. paradoxus*-infected amphipods swim to the top of the water column, where they skim the surface until contacting an object to which they cling for some time after the disturbance has passed.

The neurochemical serotonin has been implicated in these *P. paradoxus*-induced behavioral alterations. Helluy and Holmes (1990) found that injection of serotonin, but not other neurotransmitters, would cause non-parasitized amphipods to exhibit the photic and escape behaviors of parasitized animals. The evidence of the role of serotonin in this host-parasite system was strengthened by Maynard, et al. (1996), who found changes in the pattern of serotonin immunoreactivity in the ventral nerve cords of *P. paradoxus*-infected amphipods. The number and location of serotonergic cell bodies was the same, but nerve cords from infected amphipods had more visible varicosities than did cords from non-infected individuals. These changes were specific to this species of parasite. Nerve cords from amphipods parasitized by *P. marilis*, a species that causes only minor changes in photic behavior and no change in escape behavior, showed no change in the number of visible varicosities.

An increased number of visible varicosities could reflect a change in the number

of varicosities. However, the strength of an immunoreactive response depends upon the quantity of antigen present. Therefore, more varicosities could be made visible if more serotonin is stored in each, either from increased production of serotonin or from redistribution of serotonin from the cell bodies. If more serotonin is produced, then we would expect an increase in the amount of serotonin in the nerve cords of *P. paradoxus*-, but not *P. marilis*-, infected amphipods.

We used high pressure liquid chromatography (HPLC) with electrochemical detection to measure the amount of serotonin in the ventral nerve cords of *P. paradoxus* -infected, *P. marilis*-infected, and non-infected amphipods.

METHODS

Animals

Amphipods were collected from East Delaney Lake (4,506,966N, 377,303E, Zone 13), Cowdrey Lake (4,521,483N, 389,170E, Zone 13), and Lake John (4,515,051N, 375,778E, Zone 13), Jackson County, Colorado. Amphipods were maintained in aerated pond water at 6 - 11° C with natural vegetation for food. Light was provided by a 25 W incandescent bulb on a 12 hour light/ 12 hour dark schedule.

Only amphipods containing the infective cystacanth stage, as initially assessed by visual inspection and confirmed during the dissection, were used as infected individuals. Only those amphipods found to contain no visual sign of parasite infection were used as uninfected individuals. Amphipods containing the earlier, non-infective stages of acanthocephala or any stage of cestode were not used in this study.

Cystacanths removed during dissection of the amphipod were initially identified by visual inspection of size and color, and later confirmed by identification according to the procedure of Denny (1969). Cystacanths were placed in tap water until the proboscis everted, at which point they were transferred to AFA (10% formalin, 70% ethanol, 5% acetic acid, 15% deionized water) to fix overnight. Fixed cystacanths were dehydrated in

successive ethanol baths (50%, 75%, and 95%), then cleared in methyl salicylate and mounted in Permount. The number of hooks per row, rows per proboscis, length of longest hook, and thicknesses of body wall layers were all used to determine species (Denny 1969).

Tissue Preparation

Amphipods were immobilized at -20° C for approximately 20 min. The sex, length (in mm), and coloration of the amphipod were noted before dissection. The ventral nerve cord was removed in cold freshwater crustacean saline (VanHarreveld, 1936) and promptly transferred to 20 µl 0.2 M perchloric acid on ice. Samples were kept on ice during sonication for approximately 20 s, then centrifuged at 8,000 rpm for 5 min in a 4° C chamber. The supernatant was removed for analysis by HPLC.

Chromatography

The mobile phase contained 75 mM sodium phosphate, 10 µM ethylenediaminetetraacetic acid, and 1.4 mM 1-octanesulfonic acid in deionized water. This mixture was adjusted to pH 3.2 with phosphoric acid before adding 10% acetonitrile. The chromatography apparatus consisted of a Bioanalytical Systems (BAS) 481 isocratic chromatograph with a UniJet™ injector, single channel LC-4C amperometric controller, and UniJet™ detector cell. The mobile phase was pumped at a flow rate of 1 ml/min. Samples of approximately 20 µl were injected into a 5 µl loop. Separation was achieved with a 100 x 3.2 mm I.D. analytical column (BAS MF-6213) packed with octadecyl-bonded spherical 3 µm silica particles and protected by a 3.2 x 15 mm guard column (BAS MF-6206). All separations were done at room temperature. Serotonin was detected by electrochemical oxidation using a thin-layer amperometric detector at 675 mV versus a Ag/AgCl reference electrode. Output was recorded on a two-pen strip chart recorder. All chemicals and reagents were HPLC quality.

Standard serotonin solutions (20 pg/µl in mobile phase) were run through the HPLC prior to running samples each day. Serotonin peaks were recognized by their time

of elution. Sample peak heights were compared to standard peak heights to determine the picograms per sample.

Serotonin Manipulations

As a measure of chromatography efficacy, serotonin-depleted and serotonin-enhanced nerve cords were examined. The serotonin-specific toxin 5,7-dihydroxytraptamine (5,7-DHT) was injected into amphipods to deplete serotonin. This toxin takes a couple of days to take effect (Glanzman and Krasne, 1986), so amphipods were dissected 1 hour, 1, 2, or 3 days after injection to follow the progression of the toxin. The metabolic precursor to serotonin, 5-hydroxytryptophan (5-HTP) was injected to enhance serotonin levels. Dissections were performed within 30 min of 5-HTP injections.

Each amphipod was injected with 2 µl of solution. Control animals for the 5-HTP experiment received freshwater crustacean saline (VanHarreveld, 1936); treatment animals received 5-HTP (13mM) in saline. 5,7-DHT was mixed up in ascorbic acid to prevent oxidation. Control amphipods received 5.7 mM ascorbic acid in dionized water; treatment animals received 90 mM 5,7-DHT in the ascorbic acid solution.

Injections were performed in a wax-lined, saline-filled tray under a dissecting microscope. The amphipod was held still under fiberglass window screen and a 10 µl Hamilton syringe with 30 gauge needle was inserted laterally between overlapping exoskeletal plates. Care was taken to keep the needle parallel to the sides of the exoskeleton so as not to puncture any internal organs. After injection, the needle was held inside the amphipod for 10 sec to allow the solution to flow through the hemocoel before withdrawing the needle. The syringe was thoroughly flushed with deionized water after each injection.

Amphipods in the 5,7-DHT experiments were individually marked with colored Liquid Paper applied to the exoskeleton and held in aerated 11° C pond water until the time of dissection.

Statistical Analysis

Multiple regression was used to assess differences in the amount of serotonin in the three treatment groups (*P. paradoxus*--infected, *P. marilis*-infected, and not infected). Length and sex were also included in the model to account for any differences due to amphipod size or sex. Plots of studentized residuals were used to check that the assumptions of regression held. A probability less than 0.05 was required to declare statistical significance in all analyses.

Mann-Whitney U tests were used to compare serotonin levels in 5,7-DHT and 5-HTP versus control nerve cords. Values for two and three days post-5,7-DHT injection were combined.

RESULTS

Infection with *P. paradoxus* or *P. marilis* cystacanths did not detectably affect serotonin levels in amphipod ventral nerve cords (Fig. 3.1; multiple regression, $F = 1.2$, d.f. = 4, 43, $p = 0.32$). Neither sex ($\beta = 7.1$, d.f. = 1, , $p = 0.43$) nor length ($\beta = 1.3$, d.f. = 1, , $p = 0.54$) of the amphipod was a significant predictor of serotonin in the ventral nerve cord.

5,7-DHT and 5-HTP detectably decreased and increased serotonin levels, respectively (Fig. 3.2 and 3.3). Two to three days after injection, 5,7-DHT-treated amphipods had significantly less serotonin in their nerve cords than did control animals ($U = 63$, $n = 8, 8$, $p < 0.05$). 5HTP-injected amphipods had significantly more serotonin in their nerve cords than did saline-injected amphipods ($U = 16$, $n = 4, 4$, $p < 0.05$).

DISCUSSION

In the present study, amphipods infected with acanthocephalan parasites did not show increased levels of serotonin in their ventral nerve cords. Tests with 5-HTP and 5,7-DHT injections indicated that our chromatography was sufficiently sensitive to detect the differences induced by these chemical manipulations. Thus, if the acanthocephalans do

induce any changes in nerve cord serotonin levels, they must be less than the differences induced by the precursor and by the toxin.

Regardless of the lack of a detectable change in serotonin levels, I cannot draw any conclusions about the role of serotonin in *Polymorphus*-induced behavioral changes from these results. Concurrent with the HPLC studies, I conducted behavioral trials with parasitized and non-parasitized amphipods, looking for the behavioral differences originally reported by Bethel and Holmes (1973). I failed to find any acanthocephalan-induced behavioral differences in amphipods collected in Colorado (Fig. 3.4). I do not believe that this failure is due to fault in the behavioral studies because I saw the obvious behavioral differences in *P. paradoxus*-infected amphipods collected from the original study sites in Canada. The clinging response of these animals could not be easily overlooked.

Several possible explanations could account for the lack of parasite-altered behaviors in Colorado amphipods. The most likely seems to me that while the parasites found here are morphologically similar to *P. paradoxus*, they are a different strain from those found in Canada. *Polymorphus paradoxus* uses both muskrats or other mammals and mallard ducks as final hosts. Holmes (pers. comm.) suggested that this parasite species might have diverged into two reproductively isolated strains: those that infect mammals and those that infect birds. Those that infect mammals might not have the same selective pressures to induce behavioral changes in their hosts because muskrats are likely to forage in muddy pond bottoms and find amphipods in their normal state.

The possibility also exists that nutritional differences exist between Canadian and Colorado amphipods. Assuming the behavioral changes seen in Canadian amphipods are actively induced by the parasite, it could use compounds extracted from the diet for physiological manipulation of the host. If the amphipods collected locally did not have access to the same foods, then any such compounds would be unavailable to parasites.

Genetic comparisons of parasites collected in Canada and Colorado could help to

understand the differences in their behavioral effects. Unfortunately, several years have passed since anyone has found acanthocephalan-infected amphipods in the Canadian lakes where Bethel, Holmes, and Helluy originally worked.

Despite the lack of parasite-induced behavior changes in local *P. paradoxus/G. lacustris* populations, the pattern of serotonin-like immunoreactivity was altered in both Canadian- and locally-collected animals (Chapter 2). The increased number of visible varicosities together with no detectable change in serotonin levels in ventral nerve cords points toward a redistribution of serotonin, out of the cell bodies into varicosities. The significance and mechanism of this redistribution are yet to be understood.

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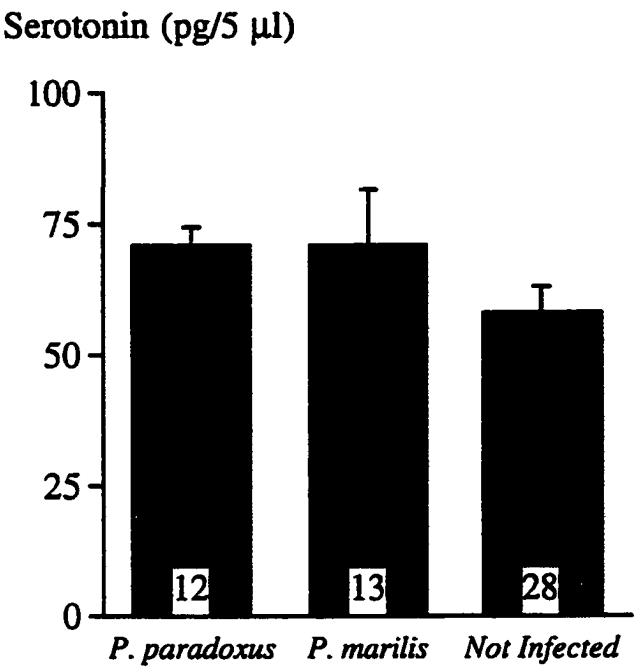

Figure 3.1. The amount of serotonin in the ventral nerve cord did not differ between *P. paradoxus*-infected, *P. marilis*-infected, and non-infected amphipods collected in Colorado. Sample sizes are shown a the base of bars; error bars represent ± one standard error.

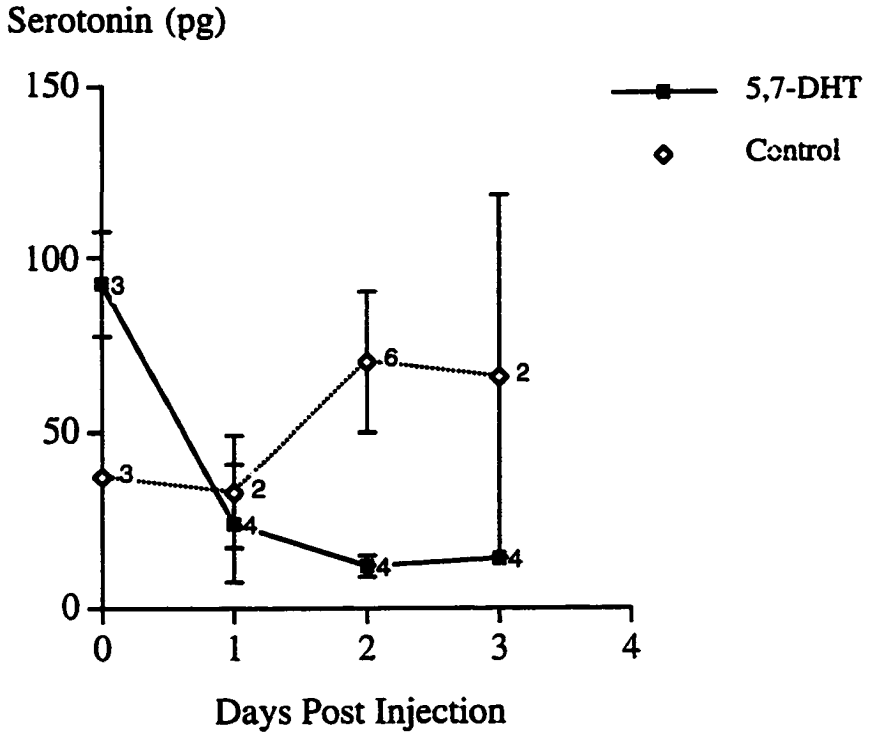

Figure 3.2. 5,7-DHT caused detectable depletion of serotonin in ventral nerve cords two days after injection. Serotonin and 5,7-DHT co-eluted; the measurements on Day 0 include both substances. Sample sizes are shown beside symbols; error bars represent ± one standard error.

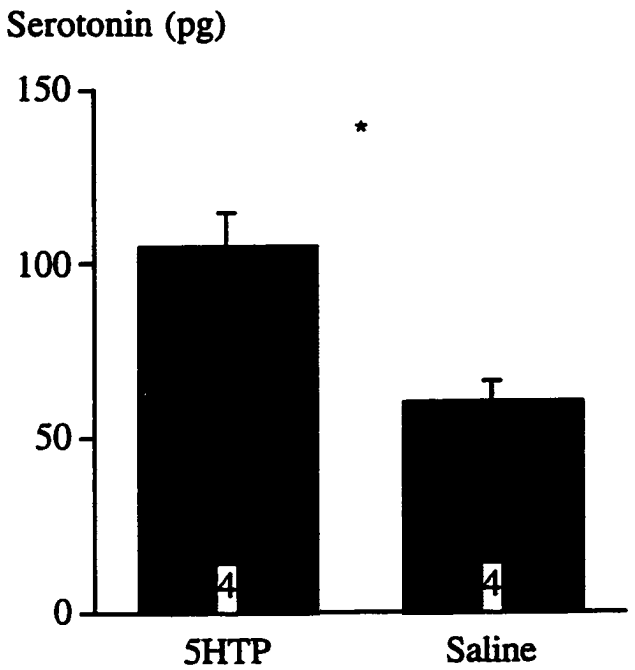

Figure 3.3. 5-HTP injections increased serotonin concentrations in ventral nerve cords. Sample sizes are shown at the base of bars, error bars represent ± one standard error, and the asterisk indicates $p \leq 0.05$.

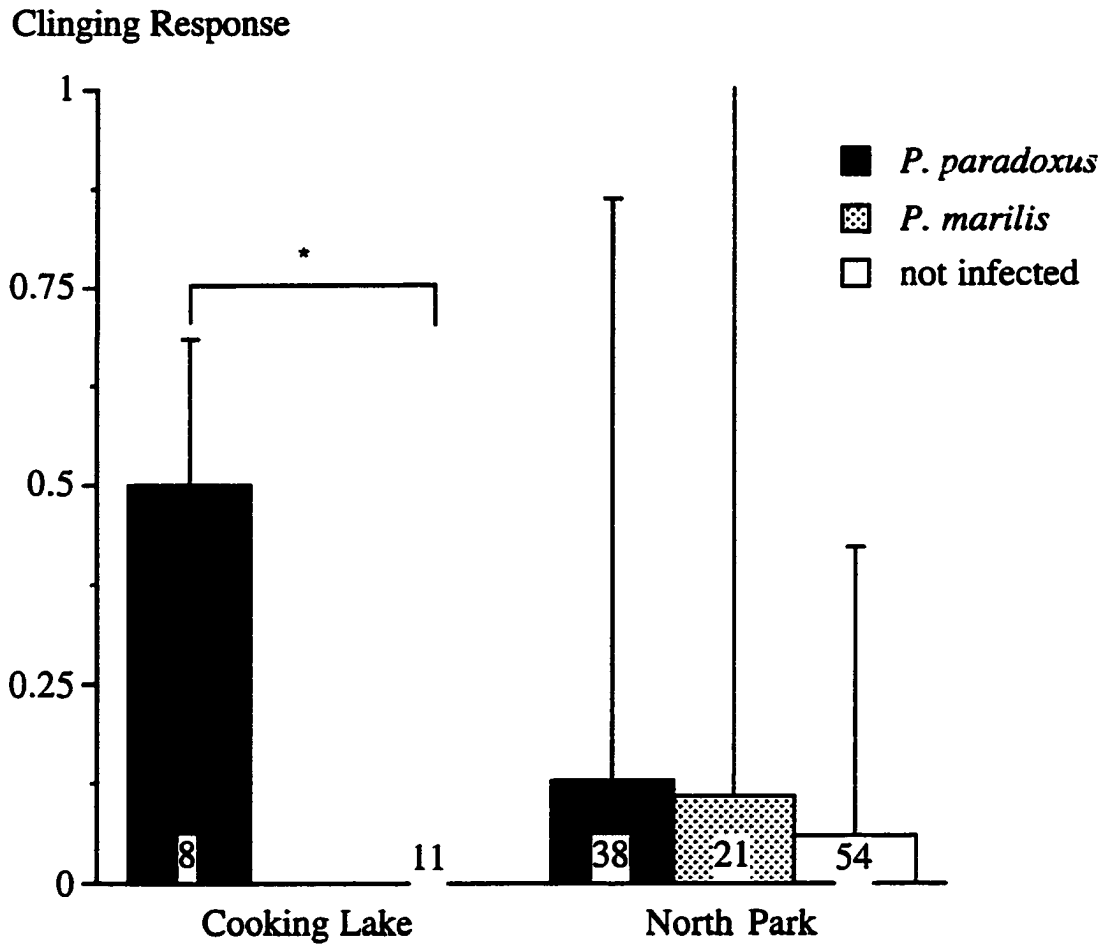

Figure 3.4. *Polymorphus paradoxus*-infected amphipods collected from North Park, Colorado, did not show the same clinging behavior as did *P. paradoxus*-infected amphipods collected from Cooking Lake, Edmonton. The asterisk indicates a significant difference between the clinging response of *P. paradoxus*-infected and non-infected Canadian amphipods (Wilcoson rank sum, $P < 0.05$). Numbers in the bars indicate sample sizes; error bars respresent one standard error.

Chapter 4

Parasite-altered behavior in a crustacean intermediate host: field and laboratory studies

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ABSTRACT

The effects of the acanthocephalan parasite *Pomphorhynchus laevis* on the behavior of its crustacean intermediate host, the amphipod *Echinogammarus stammeri*, were studied. A drift study revealed that infected amphipods were disproportionately represented in drift samples taken throughout a 24-hour period; infection with more than one parasite enhanced this effect. Infection also interacted with the daily timing of drift, with parasitized amphipods beginning to drift earlier in the evening. Two distinct behaviors quantified in laboratory settings may play a role in this increased drifting behavior: parasitized amphipods showed (1.) an increased preference for an illuminated environment and (2.) increased activity in comparison to non-parasitized conspecifics. These results are consistent with previous studies on the effects of *P. laevis* on another amphipod host, *Gammarus pulex*, and provide new data on the activity level of *P. laevis*-infected amphipods.

INTRODUCTION

The transformations of host behavior brought on by certain parasites range from the bizarre, e.g., terrestrial insects that cast themselves into rivers in response to mermithid infections (Maeyama et al., 1994), to the behaviorally simple, e.g., decreases in locomotor activity by gastropods infected with trematodes (Lambert and Farley, 1968)

and the list of examples has rapidly grown in the last few decades. Our understanding of these phenomena has not kept pace with their discovery, however, and several authors (Hurd, 1990; Moore & Gotelli, 1990; Poulin, 1995) have called for an increased focus on the adaptive significance and the physiological mechanisms that underlie these intriguing behavioral changes.

One approach toward testing hypotheses of adaptation or mechanism is a phylogenetic comparative analysis (e.g. Moore and Gotelli, 1996). Such comparative approaches realize their full potential with a full complement of behavioral data on related species of hosts and parasites. One system well-suited for studies on the mechanisms and the evolution of parasite-induced alterations in host behavior involves the acanthocephalan parasites that infect freshwater amphipods. This group of parasites includes the genera *Polymorphus*, *Pomphorhynchus*, and *Corynosoma*, each of which contains species which induce altered responses to light in their amphipod hosts (Bethel & Holmes, 1973; Kennedy et al., 1978). The extent of this altered photic response differs between parasite species, as do other behavioral changes associated with infection. As part of a continuing study on the mechanisms underlying parasite-induced alterations in behavior (Maynard et al., 1996), we investigated the behavioral changes induced by the acanthocephalan *Pomphorhynchus laevis* (Echinorhynchida) in an intermediate host not previously studied, *Echinogammarus stammeri* (Amphipoda).

Pomphorhynchus laevis is an intestinal parasite of stream-dwelling fish, especially chubs (*Leuciscus cephalus*), barbels (*Barbus barbus*), and rainbow trout (formerly *Salmo gairdneri*, new designation *Oncorhychuss mykiss*; Kennedy et al. 1978). These fish acquire the infection by ingesting the parasite's intermediate host, freshwater amphipods, in which *P. laevis* undergoes its larval development. In England and parts of continental Europe, *Gammarus pulex* serves as the amphipod intermediate host, and the effect of *P. laevis* on its behavior has been studied (Kennedy et al., 1978; McCahon et al., 1991). Individuals of *G. pulex* infected with larval *P. laevis* spend more time in brighter

water, in open water, and on surface vegetation (Kennedy et al., 1978) and are disproportionately represented in the drift (McCahon et al., 1991). These changes in behavior would seem to make the amphipods more vulnerable to predation by fish, and, indeed, under laboratory conditions, infected *Gammarus pulex* were more susceptible to predation by dace and grayling (Kennedy et al., 1978) and by sticklebacks (Bakker et al., 1997) than were non-infected individuals. So dramatic and consistent are the changes in behavior and susceptibility to predation that this system has been recommended for use in teaching laboratories (Brown and Thompson, 1986).

Recently, *P. laevis* has been reported in the River Brenta of northern Italy (Dezfuli, Zanini et al., 1991), but here the intermediate host is *Echinogammarus stammeri*, a much smaller species of amphipod. The presence of *P. laevis* in two different intermediate host species raises the question of whether the parasite induces the same behavioral changes in both species, or whether the changes are specific to *G. pulex*. We tested the hypotheses that *P. laevis*-infected individuals of *E. stammeri* would show (1.) increased drifting behavior and (2.) an increased attraction to light in comparison to non-infected individuals. During the course of testing these hypotheses, casual observations indicated that infected amphipods were hyperactive. The effect of *P. laevis* on amphipod locomotor activity has not been studied, but other acanthocephalans are known to increase the activity level of their intermediate hosts (e.g. Moore, 1983a; 1983b). We, therefore, tested the hypothesis that parasitized individuals were more active than non-infected individuals.

METHODS

Study site

All amphipods were collected from a 30 m stretch of the Brenta, a clear-water river draining alpine and subalpine catchments, near the town of Grantorto in northern Italy. This section of the River Brenta supported a diverse assemblage of macroinvertebrates, but *Echinogammarus stammeri* was the predominant taxa in both drift and benthic

samples (T. Wellnitz, pers. obs.). The three experiments were conducted in February and March of 1996 using amphipods with natural infections.

Drift study

The study was performed in the River Brenta over a 24 hr period on 29 Feb and 1 Mar 1996. The Brenta is 62 m wide at the study site, with substrata comprised mainly of cobbles, gravel, and interstitial sand. Current velocity, measured 29 Feb and 1 Mar 1996, ranged from 0.3 - 0.5 m/sec at the water surface.

To quantify the number of drifting amphipods, six drift nets (200 µm mesh) having 33 x 15 cm openings were placed along a transect extending from 7 to 13 m from shore and perpendicular to the flow. Water depth along the transect ranged from 23 to 37 cm. Drift samples were collected for 15 min every 4 hr from 1800 to 1000 and every 2 hr from 1000 to 1800. Nets were suspended above the water on steel reinforcing bars between samples and lowered to the river bottom for the duration of each sampling period. Following each 15 min sample, amphipods were removed from the nets and preserved in 6% formalin for later examination.

To assess the infection prevalence in the population, 12 bottom samples were collected with a box sampler (Merritt and Cummins, 1996) 10 m downstream of the nets between 1400 and 1600 on 29 Feb 1996. The box sampler sampled a 20 x 20 cm area to a depth of 5 cm; amphipods and other macroinvertebrates were collected by scraping the bottom and over-turning cobbles by hand and allowing current to sweep animals into a 250 µm net attached to the downstream side of the box sampler. All macroinvertebrates collected were preserved in 6% formalin for later examination.

Amphipod infection in drift and bottom samples was assessed by inspection of individuals under a dissection microscope. Amphipods were broken apart or cleared in lactic acid (Dezfuli, Zanini et al., 1991), and the number, species, and developmental stage (acanthellae versus cystacanths) of all parasites was recorded. Because amphipods of less than 6 mm in length were not typically infected (B. Maynard, pers. obs.) and were

less likely to be caught in the drift (4% of individuals caught in the drift versus 10% of individuals caught in the bottom), all individuals of this size were discarded to avoid having their numbers bias the sample.

To determine whether or not parasitized amphipods were disproportionately represented in the drift, a Mann-Whitney *U* test was used to compare the proportion of non-parasitized amphipods caught in each of the six drift nets (summed over the nine sampling times) to the proportion of non-parasitized amphipods in the overall population (as represented by the 12 bottom samples). A Mann-Whitney *U* test was also used to determine whether the presence of more than one cystacanth had a greater effect on drift behavior than did one cystacanth alone. An analysis-of-variance was used to determine whether or not infection state (parasitized versus not) interacted with time of day. The response variable (number of parasitized or non-parasitized amphipods caught in each net at each sampling time) was transformed using the square-root + 0.5 transformation (Sokal and Rohlf, 1981) in order to meet the assumptions of homogeneity of variances and normal distribution; net number (1 - 6) was included as a random factor (Sokal and Rohlf, 1981) to preclude any difference between nets from affecting the analysis.

Light/dark choice experiment

Two aquaria (34.5 x 17.5 x 21.0 cm, l x w x h) were filled with well water (11° C) to a depth of 9.5 cm. Each tank was divided horizontally into an illuminated and a darkened half, with black plastic covering the sides, top, and bottom of the darkened half. A black plastic lip extending down from the top of the tanks to the water surface further divided the two halves. A 60 W tungsten lamp centrally placed above the two aquaria illuminated them. Light measurements taken at noon on 8 Feb 1996 with a hand-held Graseby Optronics Optical Power Meter 371 showed a mean (± SE) value of 48 ± 12 μW for the illuminated halves of the aquaria and 0.62 ± 0.24 μW for the darkened halves.

Amphipods were collected from the River Brenta on 7 Feb 1996 and maintained overnight in well water (11° C) under the natural photoperiod. Daytime water

temperatures of the River Brenta ranged from 8 to 10.6° C on 29 Feb and 1 Mar 96. Seven trials were run between 1000 and 1900 on 7 and 8 Feb 1996. For each trial, eight amphipods were placed gently into the center of each tank, parasitized individuals in one tank and non-parasitized in the other. Thus, 112 amphipods were tested over the course of the experiment. Assignment of treatment group (parasitized vs. not) to aquaria alternated between trials. Infection was determined by visual inspection under a dissection microscope: parasitized amphipods included those with one or more cystacanths and no visible parasites of another species, non-parasitized amphipods were those with no visible parasites of any species or developmental stage. The amphipods were given 5 min to acclimate to the tanks before observations began. Every 10 sec for 10 min, the number of amphipods in the illuminated half of each tank was recorded. A 10 min break ensued, followed by another 10 min of observations on the same individuals.

For each of the seven trials, the mean number of amphipods in the illuminated portion of each aquarium was calculated over the entire 20 min of observations. A Wilcoxon signed-rank test was used to compare the means for parasitized versus non-parasitized groups. To determine whether either group (parasitized and not) showed a preference for lighter or darker halves of the tank, a Wilcoxon signed-rank test was used to compare the group median against random.

Activity assay

Amphipods were collected from the River Brenta on 1 Mar 1996 and maintained under a 12 hr light/dark cycle in an aquarium containing aerated, 15° C stream water until the time of the experiment. Food was provided in the form of leaf litter and algae collected from a local stream.

Twenty-four hr before observations, amphipods were placed into drinking cups (one amphipod per cup) containing 200 ml stream water. A black line had been drawn down the middle of the bottom of each cup. For each of two trials (17 and 19 Mar 1996), two observers each recorded the activity of a separate set of ten amphipods, five

parasitized and five not. Infection was determined as in the light/dark study, and was confirmed after the study by dissecting the amphipods. In every case, the determination of infection made on the live amphipods was found to be correct. Each amphipod was observed for three bouts of 2 min, during which the observers counted the number of times an amphipod crossed the line on the bottom of the cup. Every animal was observed for one, 2-min bout before the next bout of observations began. Thus, a total of 40 amphipods were observed for 6 min each. The total number of line crosses in 6 min of observation was tallied for each amphipod, and the mean number of crossings for parasitized versus non-parasitized animals compared using a Mann-Whitney *U* test.

RESULTS

Drift study

Two species of acanthocephalan parasites, *P. laevis* and *Acanthocephala clavula*, use *E. stammeri* as their intermediate host in the River Brenta (Dezfuli, Rossetti et al., 1991; Dezfuli et al., 1994). The number of amphipods found with *A. clavula* was too low (13 of 2,316 amphipods, 5 co-existing with *P. laevis*) to reliably assess its effects on host behavior, so all amphipods containing this species were removed from this data set. *Pomphorhynchus laevis* was the only other parasite found in the amphipods. Likewise, the number of amphipods found with acanthellae was too low (87 of 2,316 amphipods, 44 co-existing with cystacanths of *P. laevis*) to assess their effects, so all amphipods with acanthellae and no cystacanths were disregarded.

The mean number of amphipods caught in the drift at each sampling time is shown in Figure 4.1. Regardless of infection, many more amphipods were caught in the 1800 samples than at any other time of day. There was a significant interaction between infection state (parasitized versus not) and sampling time (ANOVA, $F_{\text{time * infection state}} = 5.17$, d.f. = 7, 35, $P = 0.0004$, 2-tailed), indicating that infection affected the time of day that amphipods drifted. Figure 4.1 shows that the evening peak in drift activity

began earlier for parasitized amphipods.

Summing across sampling times, infected amphipods were disproportionately represented in the drift (Fig. 4.2). Not only were infected amphipods more likely to be caught in the drift, but also the presence of more than one cystacanth increased this effect (Fig. 4.2; Mann-Whitney U; $U = 62$; n = 6 drift samples, 12 bottom samples; $P < 0.02$, 2-tailed).

Light/dark choice experiment

Parasitized amphipods were in the illuminated half of the aquarium significantly more than in the dark (Wilcoxon signed-rank test; $T = 0$, n = 7, $P = 0.02$, 2-tailed), and significantly more than non-parasitized amphipods were (Fig. 4.3). Non-parasitized amphipods showed no preference for lighter versus darker halves of the aquarium (Wilcoxon signed-rank test; $T = 13$, n = 7, $P > 0.5$, 2-tailed). Neither treatment group showed a different response in the first versus second 10 min of observation (Wilcoxon signed-rank test; parasitized: $T = 7$, n = 7, $P = 0.24$, 2-tailed; non-parasitized: $T = 3$, n = 7, $P = 0.12$, 2-tailed), indicating that the difference between groups did not represent a difference in short-term response to the disturbance of being moved to the new tank.

Activity assay

Parasitized amphipods were significantly more active than were non-parasitized amphipods, as indicated by the mean number of times they crossed the mark in 6 min of observations (Fig. 4.4). One confounding factor was noticed on the first trial of observations. On this day, each amphipod was housed in a paper cup. Some amphipods, upon encountering the seam of the paper cylinder, would stop swimming and cling to this seam. On the second day of observations, plastic cups with no seams were used to eliminate this problem. Since this behavior would tend to decrease the observed difference, statistics presented here include the data from those eight parasitized and one non-parasitized amphipods that exhibited this clinging behavior at some time during the observations.

DISCUSSION

Amphipods parasitized by *Pomphorhynchus laevis* showed several differences in behavior, as compared to non-parasitized amphipods. It should be noted that all experiments used amphipods with natural infections, and we therefore cannot rule out the possibility that these behaviors lead to increased likelihood of infection, rather than the converse. Only observations with experimentally infected amphipods could rigorously resolve this issue; however, given the nature of the behavioral changes (increased drift, increased activity, and positive response to light) and the means by which amphipods acquire the infection (ingesting a parasite larva, most likely from the stream bottom), this alternative hypothesis seems unlikely.

Infection with *Pomphorhynchus laevis* was correlated with several aspects of the behavior of *Echinogammarus stammeri*. Amphipods with one cystacanth drifted significantly more than did non-parasitized individuals; amphipods of *E. stammeri* with two or more cystacanths showed an even greater propensity to drift. Infected amphipods also showed an altered diel pattern of drift. McCahon et al. (1991) found that amphipods of *Gammarus pulex* carrying one cystacanth of *P. laevis* showed increased drifting behavior, but did not statistically address the questions of whether multiple infections had an increased effect on drift, or whether infection altered the diel pattern of drift.

What is different about the behavior of infected *E. stammeri* or *G. pulex* that makes them more likely to be caught in the drift? Drifting could be the result of many different kinds of behavior. For instance, an amphipod weakened by infection could be less able to maintain its position in or amongst cobbles and be swept away by normal river currents. The results of our laboratory studies, whereas not refuting that interpretation, suggest two other aspects of amphipod behavior that may account for the change in drift behavior.

McCahon et al. (1991) suggested that the increased drift of *P. laevis*-infected *G.*

pulex could be due to a parasite-induced preference for brighter water. Evidence reported here that parasitized *E. stammeri* spent more time in illuminated waters than did non-parasitized individuals suggests that parasite-induced positive phototaxis could also play a role in the drift of infected *E. stammeri*. Whereas this test was conducted in aquaria with still water and a horizontal light/dark choice, a similar response in the river could result in the amphipods swimming toward surface (brighter) waters, and thus being carried by the current.

The results in our light/dark assay could be indicative of responses to factors other than light. By covering one half of the aquaria with black plastic, we not only darkened this half, but also altered the background or visual stimuli in the two halves. However, a similar difference would exist in a river, between the dark environment in the stream bottom and the brighter environment in the open water.

An altered photic response by infected amphipods may contribute to their disproportionate representation in the drift during the day, but does not explain the persistence of this trend during night samples seen in this study and by McCahon et al. (1991). A second behavioral change that could influence drift is the increased activity level of infected *E. stammeri*. Hiroki (1980) found a positive correlation between the diel patterns of drift and activity level for two species of amphipod (*Anisogammarus annandalei* and *A. jesoensis*) and concluded that drifting is a direct result of activity for those species. Amphipods that spend more time swimming might find themselves suspended in the water column and thus subject to current more often than less active individuals. Our observations on activity level were all conducted during the evening. Whether or not the activity level of infected or uninfected *E. stammeri* fluctuates throughout the day and whether or not *P. laevis* changes the activity level of *G. pulex* are not known.

The increase in host activity raises the question of where the host obtains the energy required for the increase. Poulton and Thompson (1987) reported that female *G.*

pulex infected with *P. laevis* produced fewer eggs than did non-infected females. One of us (B. Dezfuli, pers. obs.) has found the same trend with female *E. stammeri*. The possibility exists that energy not used for reproduction is available for the increased activity.

The altered response to light and anecdotal observations from the activity assay suggest similarities of the *P. laevis*/amphipod interaction to that of another acanthocephalan cystacanth, *Polymorphus paradoxus* (Polymorphida), with its amphipod intermediate host. *Polymorphus paradoxus* is a North American parasite that uses *Gammarus lacustris* as its intermediate host and mallard ducks and beavers as definitive hosts (Bethel & Holmes, 1977). Individuals of *G. lacustris* infected with *P. paradoxus* show an abnormal preference for brighter waters and cling to floating material in response to disturbance. In the first day of our activity assay, we noticed that eight of the ten infected *E. stammeri* were clinging to the seams of the paper cups, the only substratum available to them. Only one non-parasitized amphipod displayed this behavior. Whereas this observation is anecdotal, the similarities in photic and clinging responses raise the possibility that two different acanthocephalan parasites may employ the same mechanism(s) to alter the behaviors of their amphipod intermediate hosts. Some evidence suggests that the neurochemical serotonin may be important for the *P. paradoxus*-associated behavioral changes (Helluy & Holmes, 1990; Maynard et al., 1996). The questions of what role serotonin plays in the *P. paradoxus*/ *G. lacustris* system, and whether it is involved in the *P. laevis*/ *E. stammeri* system remain to be answered.

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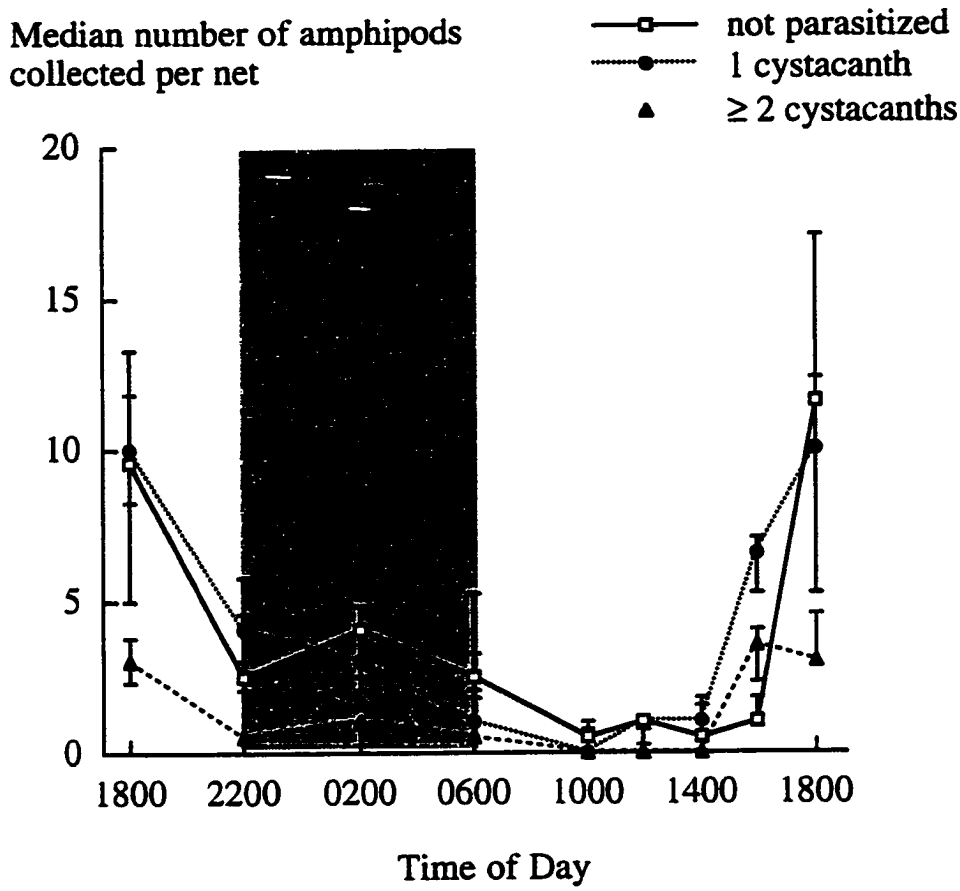

Figure 4.1. The number of amphipods collected in drift nets showed a diel pattern. Regardless of infection, the greatest number of amphipods was caught in the evening. Each point represents the median number of amphipods with 0, 1, or ≥ 2 cystacanths caught in the six drift nets at the denoted sampling time. Error bars represent the interquartile range; shading indicates samples taken during the night.

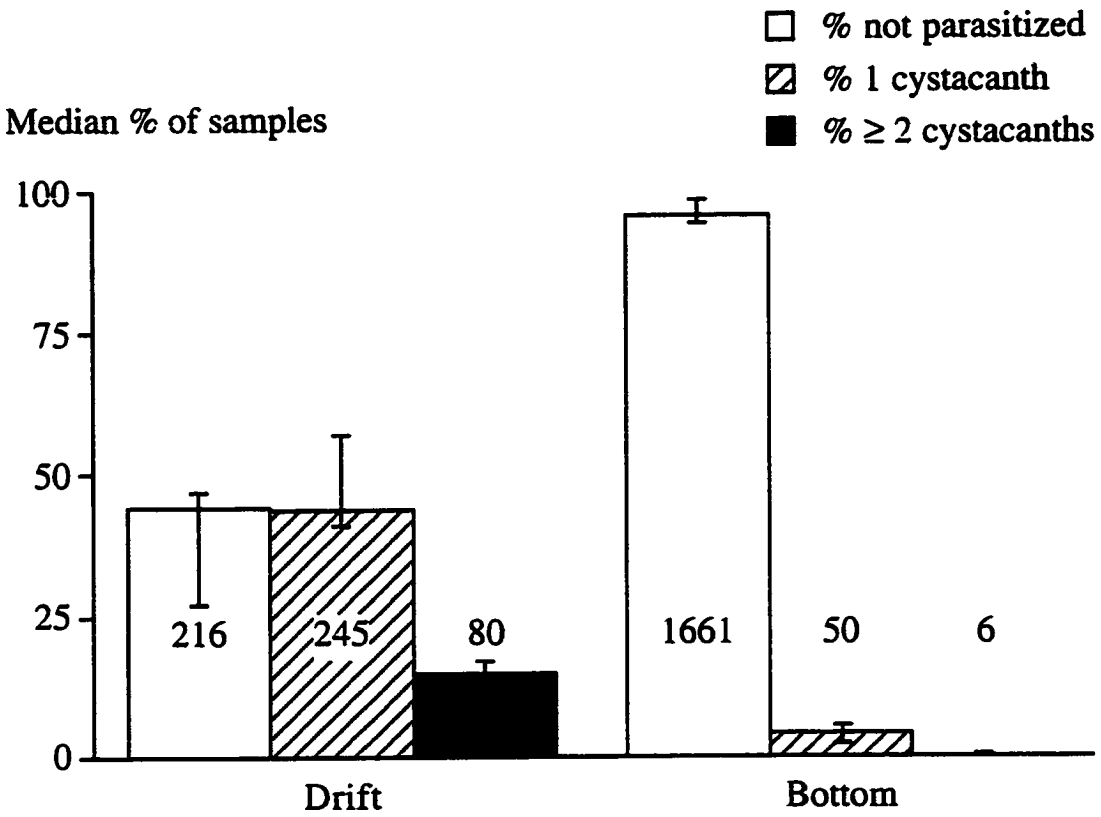

Figure 4.2. Infected amphipods were disproportionately represented in the drift (Mann-Whitney U; $U = 72$; n = 6 drift samples, 12 bottom samples; $P < 0.001$, 2-tailed). The bars representing drift samples depict the median percentage of amphipods with 0, 1, or ≥ 2 cystacanths caught in the six drift nets, each net summed over the nine sampling times; bars representing bottom samples depict the median percentage of amphipods with 0, 1, or ≥ 2 cystacanths caught in the 12 bottom samples. Numbers within or above the bars represent the total number of amphipods caught in either the six drift nets or the 12 bottom samples with 0, 1, or ≥ 2 cystacanths. Error bars represent the interquartile range.

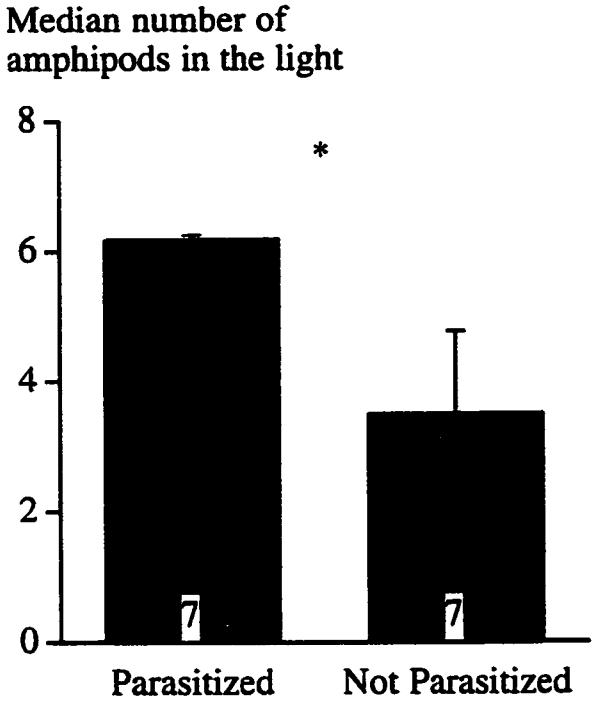

Figure 4.3. In light/dark choice experiments, parasitized amphipods showed a preference for light relative to uninfected amphipods. Bars represent the median number of eight amphipods that were in the light half of a divided aquarium over 20 min of observation (Wilcoxon Signed Rank test, $T = 0$, n = 7 trials, $P =$ 0.02, 2-tailed). Numbers in the bars represent the number of trials; error bars represent the interquartile range.

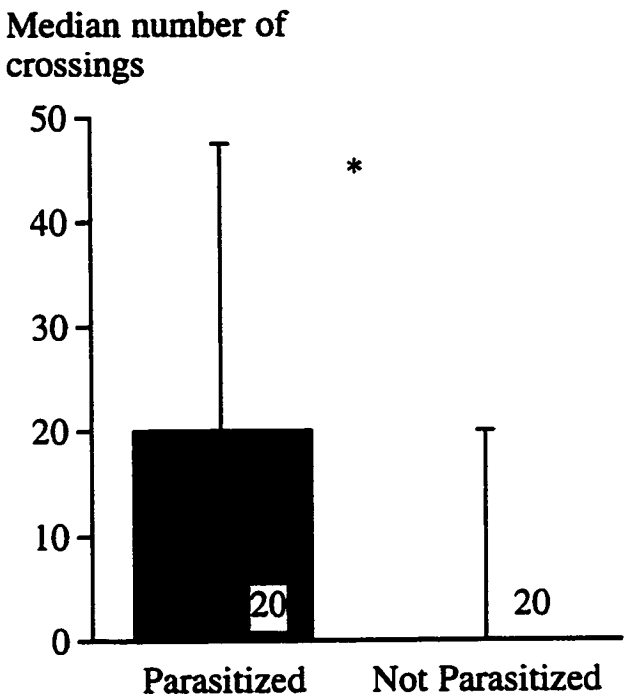

Figure 4.4. Parasitized amphipods were much more active than were non-parasitized amphipods, as assessed by the median number of times they crossed a line drawn across the bottom of their holding cups (Mann-Whitney U, U= 277, n = 20, $P < 0.05$, 2-tailed). Numbers in or above the bars represent the number of amphipods observed; error bars represent the interquartile range.

Chapter 5

Activity levels and predator detection by amphipods infected with an acanthocephalan parasite

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ABSTRACT

The acanthocephalan parasite *Pomphorhynchus laevis* uses freshwater amphipods as its intermediate host. In order to complete the life cycle, the infected amphipod must be consumed by a fish, where the acanthocephalan will mature and reproduce. Parasite transmission, and therefore fitness, could be enhanced if infective amphipods fail to detect or avoid predatory fish. We compared the activity levels of infected and non-infected amphipods in both the presence and absence of fish (*Leuciscus cephalus*) odors. Throughout the experiment, infected amphipods were more active than were non-infected individuals. The non-infected amphipods reduced their activity after the addition of fish odors, but the infected amphipods failed to show a significant decrease. The failure of infected amphipods to reduce activity levels in the presence of fish odor may reflect a parasite strategy to increase its chances of transmission by making its amphipod host more vulnerable to predation by fish.

INTRODUCTION

Behavioral avoidance of predators is an important survival strategy for many animals. For animals infected with parasites, however, the behavioral phenotype of the host animal may be the result of two competing genotypes: that of the host, which benefits from

avoiding predators in order to survive and reproduce; and that of the parasite, which benefits from being eaten by a predator, its next host, where it will mature and reproduce (Moore & Gotelli, 1990). There are numerous examples of parasites that change their host's behavior in ways consistent with increased predation (Bakker et al., 1997; Bethel & Holmes, 1973; Lafferty and Morris, 1996; Moore, 1983b).

One common response to infection is hyperactivity (Poulin et al., 1992; Urdal et al., 1995; Wedekind & Milinski, 1996). Over-active individuals are likely to be more visible and exposed to predators, so this could be a strategy on the part of the parasite to increase its transmission. On the other hand, increased activity could also reflect the host's attempts to meet the greater nutritional demands imposed by infection (Jakobsen & Wedekind, 1998). One potential clue to determining whether an altered behavior is a parasite or a host strategy is whether the behavior persists in the presence of predators. If the intermediate host diminishes its activity in the presence of predators, then this would challenge the idea that the hyperactivity was a parasite strategy, since the altered behavior isn't performed when it would benefit the parasite most. However, if the host remains hyperactive in the presence of a predator, then this would suggest that the hyperactivity is either the result of behavioral manipulation by the parasite, or a host strategy in which the benefits of hyperactivity outweigh the risks of predation. Increased hyperactivity in the presence of a predator would suggest even more strongly that the behavior change is a parasite, rather than a host, strategy.

This study examines the behavior of amphipods infected with an acanthocephalan parasite in the absence and presence of chemical cues from fish, a major predator of amphipods and definitive host of the parasite. Freshwater amphipods have been shown to decrease their activity in the presence of fish (Andersson et al., 1986; Wooster, 1998), and chemical cues are sufficient to invoke reduced activity levels in lab and field studies (Holomuzki and Hoyle, 1990; Williams and Moore, 1985). We asked whether parasitic infection influences this predator avoidance behavior.

In the River Brenta of northern Italy, the helminth *Pomphorhynchus laevis* uses the amphipod *Echinogammarus stammeri* as its intermediate host (Dezfuli et al., 1991a), where the worm undergoes its larval development. Once the worm has reached an encysted stage known as a cystacanth, it is then infective to its next host, which includes fish of several species (Hine and Kennedy, 1974). Once a fish eats an infected amphipod, the cystacanth is released into the intestine, where it attaches, matures, and reproduces. The mature parasite's fertilized eggs eventually pass out of the digestive tract to be eaten by amphipods, thus beginning the life cycle anew.

Previous studies have shown that *P. laevis* induces behavioral alterations in both *E. stammeri* (Maynard et al., in press) and in *Gammarus pulex*, an amphipod host in other parts of Europe (Kennedy et al., 1978; McCahon et al., 1991). In both host species, parasitized amphipods display increased drift behavior, altered response to light, and increased activity levels. The drift studies were conducted in rivers with fish, indicating that increased drifting persists in the presence of predators, but predator presence was not measured or controlled (Maynard et al., in press; McCahon et al., 1991). The present study was conducted in artificial stream channels where exposure to chemical cues from fish could be manipulated experimentally and the behavior of individual amphipods compared before and after such exposure.

This study was designed to assess whether infected *E. stammeri* are more active than non-infected amphipods, whether non-infected *E. stammeri* decrease their activity level in response to chemical stimuli from predatory fish, and whether *E. stammeri* infected with *P. laevis* respond in a similar manner.

METHODS

Study Organisms

Amphipods were collected from the River Brenta on 8 March 97, and transported in aerated stream water to Duebendorf, Switzerland, where they were maintained in

artificial stream channels (see below) and fed algae and aquatic vegetation collected locally.

Chubs (*Leuciscus cephalus*) were collected from the River Toess, Switzerland, on 13 March 97, by electroshocking. Throughout the experiment the fish were held in 22 l tanks of aerated, non-chlorinated tap water and fed stream invertebrates (primarily mayflies) collected locally. Stream vegetation and terra cotta pot shards provided refugia. Fresh water continuously flowed through tanks at a rate of approximately 2 l/min. Seven chubs of 15 - 20 cm length were held in a 22 l holding tank until the beginning of the fish treatment. At that time, four chubs were moved to the 22 l exposure tank, and maintained as before. All fish were released at the end of the experiment.

Artificial stream design and operation

The channel design has been described previously (Wellnitz and Ward, 1998). Sixteen artificial streams were constructed of Plexiglas™. Each circular channel was 17 cm in diameter, 8 cm deep, and had a 4 x 9 cm (diameter x height) standpipe mounted at the center (Fig. 5.1). Water entered the streams through submerged nozzles that were angled sideways to provide current, and exited through drainage holes cut into the standpipes. The drainage holes maintained a water depth of 5.5 cm and were covered with screen collars (1 mm mesh) to prevent amphipods from being washed out of the channels. Water exchange rates in channels were 11.2 ± 0.44 ml/s (mean $\pm$ SE; $n = 16$).

Non-chlorinated tap water was fed to the channels through a PVC pipe fitted with spigots along its length. Tygon™ tubing connected individual spigots to the 16 channels; current velocity within each stream channel was controlled by adjusting the spigots. Channel current was kept at approximately 1.3 ± 0.1 cm/s (mean $\pm$ SE; $n = 16$), a speed which allowed the amphipods to swim and drift normally. Approximately one-fifth of the bottom of each channel was covered with a single layer of gravel (8 - 16 mm diameter particles) to provide refugia for amphipods.

Water from the fish exposure tank was siphoned into each channel throughout the

experiment. The rate of flow from the tank to each channel was 2.8 ± 0.1 ml/s (mean ± SE).

The experiment was conducted in a greenhouse illuminated by natural lighting. Water temperature, measured with a calibrated thermister, showed diurnal fluctations between 8.5 and 10.5° C. Light levels, measured with a Licor™ spherical quantum PAR sensor, oscillated between 1000 μmol/m^2/s during the day and 0.001 μmol/m^2/s at night.

Experimental Procedure

Three amphipods were placed into each of 16 channels on 21 March 97 (day 0). All three amphipods in each channel were either infected with one cystacanth or not infected, as determined by observing the parasites through the amphipods' exoskeletons (previous work has found this technique to be reliable and accurate, Maynard, pers. obs.). Field and preliminary observations in the channels indicated that amphipods are inactive during the majority of daylight hours, with activity increasing in the evening. Light was too dim for observations after 2200, therefore observations were conducted from 1900 - 2130. Observations were conducted each evening on days 1 through 4, and again on days 6 through 8, allowing the amphipods a full day to acclimatize to the channels before the beginning of the experiment. During each half hour from 1900 - 2130, each channel was observed for 1 min, yielding 6 min of observations per channel per evening. During each observation, the number of up- and downstream movements by any amphipod in each channel was counted. Once each morning between 0900 and 0940, all channels were scanned to see how many amphipods were active in each channel during the daylight. Throughout the experiment, amphipods in the channels were fed a clump of green algae in the mornings, which was removed in the afternoon.

On day 5 at 1835, four chubs were introduced into the fish exposure tank, thus beginning the fish treatment for each channel. This tank contained stream vegetation, stream invertebrates, and terra cotta pot shards throughout the experiment; thus, the only change in the exposure tank at the beginning of the fish treatment was the addition of the

fish.

Analysis

The total number of laps in each channel was summed over observation times and days for each fish treatment. Upstream and downstream laps were summed separately. The effects of infection and fish stimuli on the number of up- or downstream laps made were assessed with a two factor, repeated measures analysis of variance, using the MANCOVA module of Statistica™. Tukey's Honest Significant Difference (HSD) was used for multiple comparisons within treatments. Lap sums were square-root transformed in order to meet the assumption of homogeneity of variance, as determined by the Cochran C test. For all statistical tests, a probability of less than 0.05 was required to find a significant difference.

To examine more closely the effect of fish odor on amphipod movement, we computed each channel's mean daily number of up- or downstream laps prior to fish treatment. For each day after fish treatment, a paired t-test was used to compare the number of laps on that day to the mean daily number of laps prior to fish treatment. These paired t-tests were calculated separately for channels containing parasitized and non-parasitized amphipods and for up- and downstream laps. Also, to compare parasitized and non-parasitized amphipods, the total number of up- or downstream laps on each day for each channel was standardized by dividing by that channel's mean daily number of up- or downstream laps prior to fish treatment. Each day's standardized lap scores for channels with parasitized versus non-parasitized amphipods were compared to each other using a two-sample t-test.

Morning activity levels of infected and non-infected amphipods were compared using the Mann-Whitney U test. This non-parametric test was used because the data were not normally distributed.

RESULTS

Amphipod mortality for both infected and non-infected amphipods was very low. Over the course of the experiment with 48 amphipods, one non-infected and two infected amphipods died, one each on the fifth, seventh, and eighth days of observations. To account for the losses, the number of laps made in any channel in which an amphipod had died was multiplied by 3/2. One precopula pair formed in each of five channels containing non-infected amphipods. Any lap made by a precopula pair was counted as two laps, one for each of the two in the pair.

Throughout the study, parasitized amphipods seemed to be hyperactive. Infected amphipods made more downstream laps than did non-infected amphipods (Fig. 5.2; MANOVA, df = 1, 14; F = 11.5, p = 0.004); this pattern held both before (Fig. 5.2; Tukey HSD, p = 0.002) and after (p = 0.004) the addition of chemical stimuli. During the daytime observations, the parasitized amphipods were more active than non-infected amphipods both before (Fig. 5.3; Mann-Whitney U, T = 13.2, n = 8, 8, $p < 0.05$) and after (Fig. 5.3; Mann-Whitney U, T = 0.67, n = 8, 8, $p < 0.05$) the addition of fish stimuli.

The addition of fish chemicals also had significant effects on activity. Taken together, the two groups of amphipods made fewer downstream laps after the fish tank water was added to the channels (Fig. 5.2; MANOVA, df = 1, 14; F = 11.8, p = 0.004). Considered separately, neither parasitized (Fig. 5.2; Tukey HSD; p = 0.08) nor non-parasitized (Fig. 5.2; Tukey HSD; p = 0.16) amphipods made significantly fewer downstream laps after the addition of fish odors. There was no significant interaction between fish treatment and infection (Fig. 5.2; MANOVA, df = 1, 14; F = 0.08, p = 0.8). However, looking at responses over days shows a different pattern.

The greatest change in response to fish odors was downstream laps by non-parasitized amphipods one (Fig. 5.4; paired t-test, n = 8, T = -5.22, p = .0013) and two days (paired t-test, n = 8, T = -4.0, p = 0.0053) after addition of fish odors. Non-parasitized amphipods returned to their pre-fish levels of downstream activity on day 8.

The parasitized amphipods did not show a significant drop in the number of

downstream laps on any day after the addition of fish odors. The between groups difference in standardized scores for parasitized versus non-parasitized amphipods was significant only on day 6 (two-sample t-test, n = 8, 8, T = 2.76, p = 0.017). The addition of fish odors did not significantly affect the number of non-parasitized (Fig. 5.3; Wilcoxon signed rank, T = 8, n = 8, p > 0.05) or parasitized (T = 15, n = 8, p > 0.05) amphipods active during daytime observations.

The overall number of upstream laps was not different for infected versus non-infected amphipods (Fig. 5.5; MANOVA, df = 1, 14; F = 1.0, p = 0.33), nor did the two groups change their upstream activity in the presence of fish stimuli (Fig. 5.5; MANOVA, df = 1, 14; F = 1.1, p = 0.32). Although the number of upstream laps by parasitized amphipods seemed to go up relative to non-parasitized amphipods after addition of fish odors (Fig. 5.5), there was no significant interaction between fish treatment and infection (MANOVA, df = 1, 14; F = 0.50, p = 0.49). Looking at responses over days, only the difference between infected and non-infected amphipods on day 7 was significant (Fig. 5.6; two-sample t-test, n = 8, 8, T = 2.35, p = 0.034). The parasitized amphipods made more laps on day 7 than they did on average before the addition of fish stimuli, but this difference was not significant.

DISCUSSION

Infected amphipods were more active than uninfected individuals, regardless of whether or not fish odors were present. These findings are consistent with a previous drift study in the River Brenta: infected amphipods were much more likely to be caught in drift nets throughout the day than were non-infected individuals (Maynard et al., In press). This hyperactivity, present during the day as well as during the more active evening hours, could make the infected individuals more susceptible to predation by fish.

Non-infected amphipods decreased their activity after the addition of fish odors, as previously reported (Holomuzki & Hoyle, 1990; Williams & Moore, 1985). Infected

amphipods, however, did not. Whether the amphipods were unable to detect the fish odors, or whether they detected them but didn't respond accordingly, remains to be determined. Regardless, the persistence of hyperactivity by infected amphipods in the presence of fish odors leaves open the possibility that this behavior is a parasite strategy.

It could be argued that the decreased downstream activity by non-infected amphipods in the presence of fish stimuli was due to an order effect, rather than to the fish treatment. Because we had no effective means to wash the fish stimuli from the Plexiglas™ channels, the fish treatment was applied to all channels only once, after the behavior in the absence of fish stimuli had been observed. Thus, a progressive decrease in activity levels might be due to a progressive decrease in activity while in the experimental set-up. However, several species of freshwater amphipods are known to decrease their activity in response to chemical cues from fish (Holomuzki & Hoyle, 1990; Williams & Moore, 1985). In addition, the non-parasitized amphipods showed a sharp decrease in downstream activity on the observation day immediately following the addition of fish odors, followed by a return to pre-fish levels after three days of exposure, a pattern we would not expect if the decreased activity was due to extended time in the channels. Finally, amphipods either maintained or increased their number of upstream laps after the addition of fish odor. Since swimming upstream would presumably be more energetically expensive than downstream, we would not expect this pattern if the amphipods were stressed from being in the channels.

Parasitized and non-parasitized amphipods showed different up- versus downstream responses. In flow-through systems, amphipods have been shown to detect downstream fish odors and drift accordingly (Dahl et al., 1998). However, with our circular channels, it is difficult to determine whether the cues would have been interpreted as coming from up- or downstream, or both, which makes it difficult to interpret the disparity in up- versus downstream responses by the two groups of amphipods.

The hyperactivity of parasitized amphipods in all experimental conditions could reflect a host strategy, for example to find more or more varied food, or it could be a parasite strategy to increase the likelihood of transmission. A significant change in this activity in the presence of fish odors could have given us a strong indication as to whether it is a parasite or a host strategy. An increase would have indicated that hyperactivity is a parasite manipulation of host behavior, since it is difficult to imagine how becoming even more active while predators are present would benefit the amphipod. A decrease would have provided strong support for the idea that hyperactivity is a host strategy, because the behavior change would have failed to persist when it would benefit the parasite most. The absence of any such significant response to fish odors makes it difficult to label the hyperactivity exhibited by infected amphipods as a host or parasite strategy.

Predation-risky behaviors that may be host strategies are not unknown. Jakobsen and Wedekind (1998) found that copepods infected with a cestode were less risk sensitive than were non-infected conspecifics. Given a choice between odors of only predators (i.e. no competition for food but predation risk) and odors of both predators and copepods (i.e. lower predation risk due to risk dilution, but more competition for food), infected copepods avoided their conspecifics. While Jakobsen and Wedekind (1998) don't dismiss the possibility of manipulation by the parasite, they consider it more likely that this is a host strategy to decrease competition for food that inadvertantly increases the parasite's chances for transmission.

Regardless of whether or not the increased activity of infected amphipods is a result of active manipulation by the parasites, it most likely results in increased parasite fitness through increased transmission.

ACKNOWLEDGEMENTS

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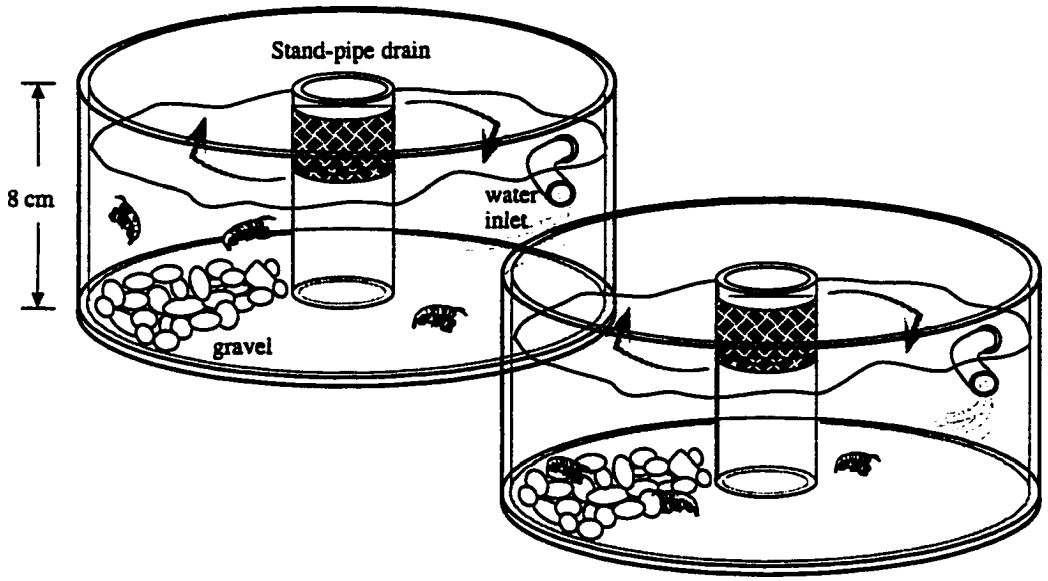
Figure 5.1. Artificial stream channels. Freshwater came in through the water inlet and drained through the mesh standpipe collar; water from the fish exposure tank entered through a separate tube (not shown).



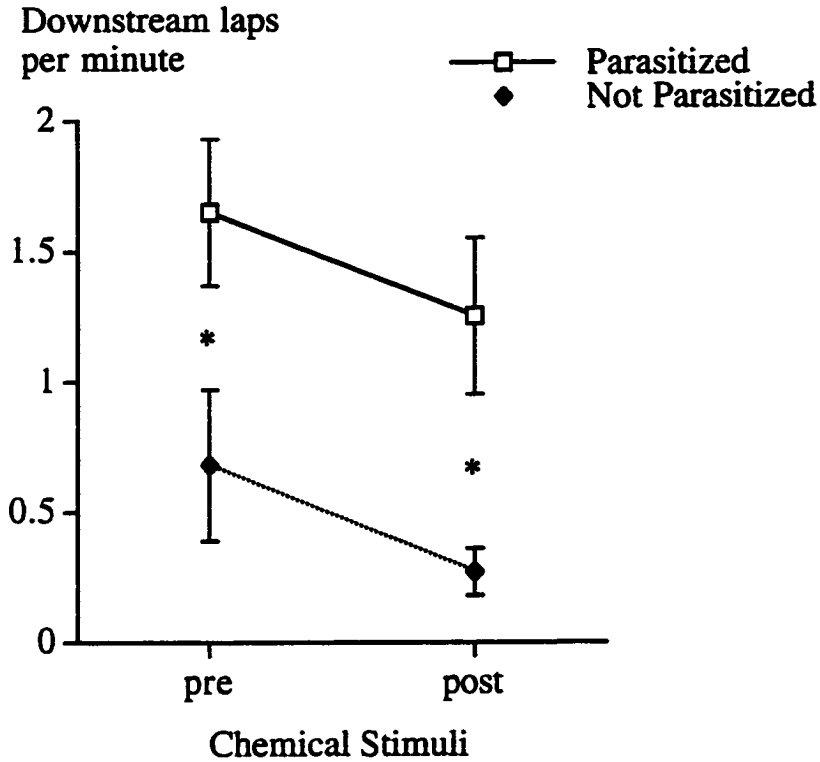


Figure 5.2. The mean number of downstream laps per minute of observation in the absence (pre) or presence (post) of fish chemical stimuli. Error bars in this and subsequent figures represent ± one standard error. Asterisks indicate significant differences ($p < 0.05$) between parasitized and non-parasitized amphipods.

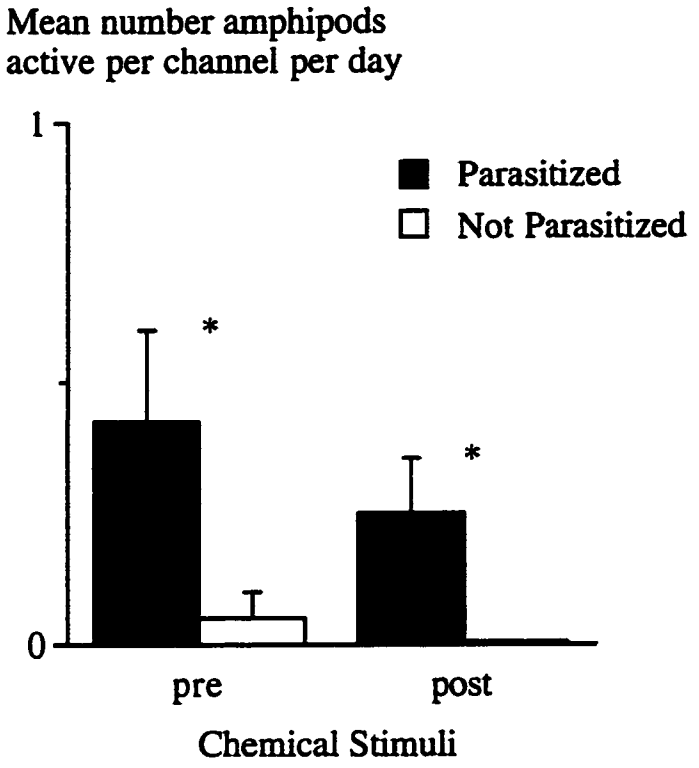


Figure 5.3. The mean number of amphipods active per channel per day during morning observations in the absence (pre) or presence (post) of fish chemical stimuli. Asterisks indicate significant differences ($p < 0.05$) between parasitized and non-parasitized amphipods.

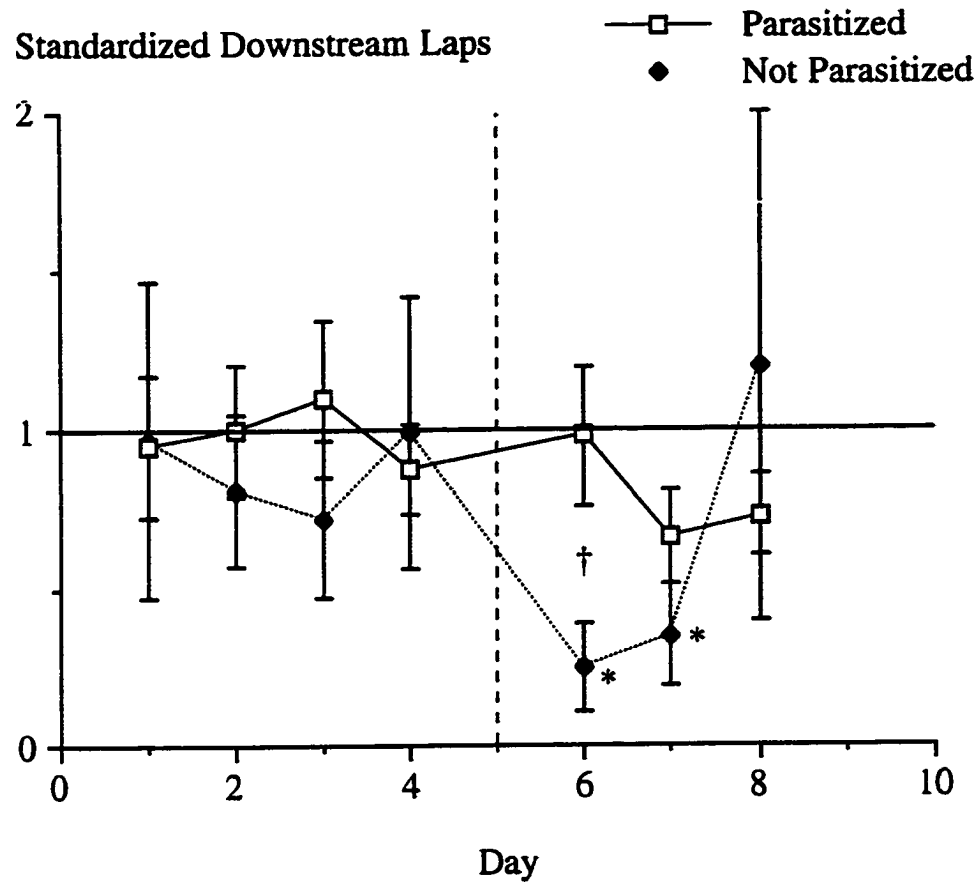


Figure 5.4. Downstream laps, standardized by dividing by each channel's mean pre-fish odor score (see text). Error bars represent ± one standard error. The cross between bars indicates a significant difference ($p < 0.05$) between the parasitized and non-parasitized amphipods; asterisks beside bars indicate means significantly different from one.

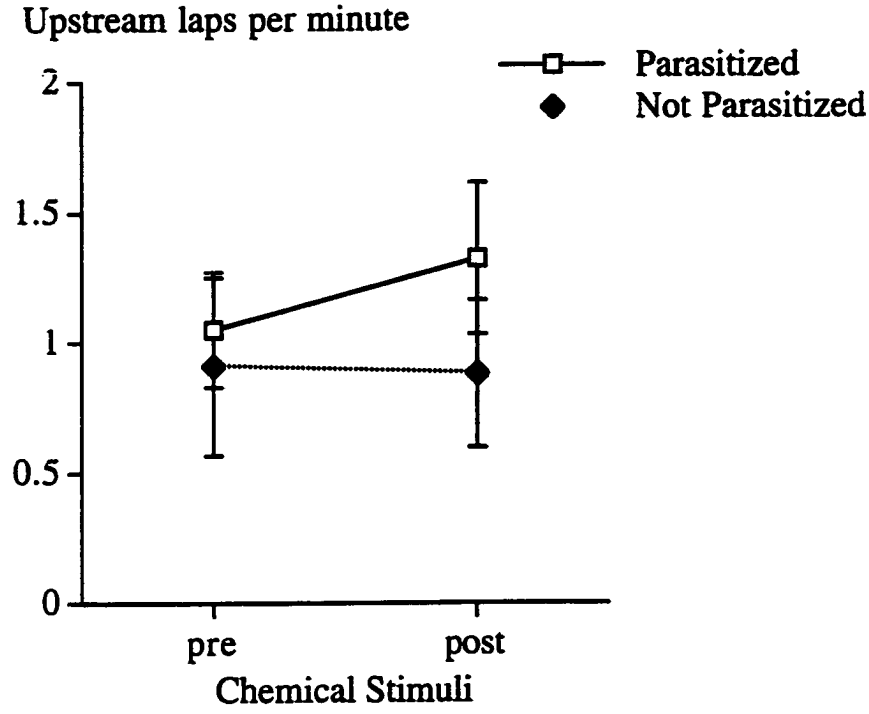


Figure 5.5. The mean number of upstream laps per minute of observation in the absence (pre) or presence (post) of fish chemical stimuli.

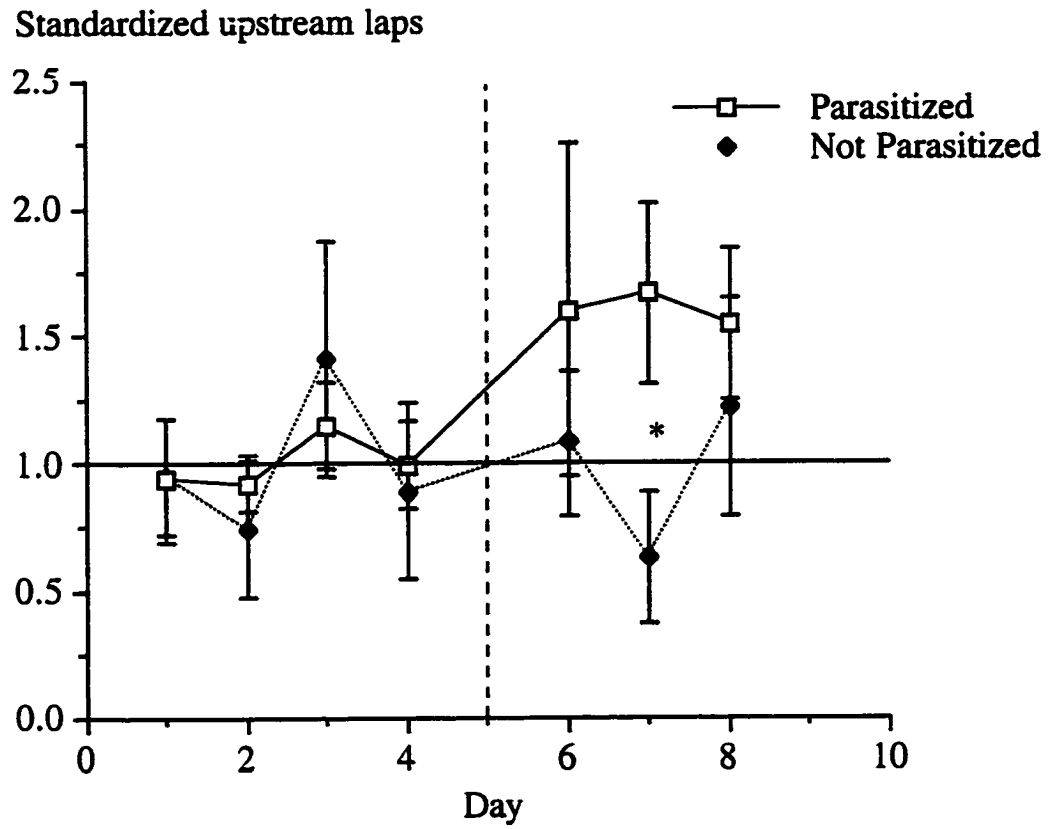


Figure 5.6. Upstream laps, standardized as described in the text. The asterisk between bars indicates a significant difference ($p < 0.05$) between the parasitized and non-parasitized amphipods.