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Increased haemolymph osmolality suggests a new route for behavioural manipulation of *Talorchestia quoyana* (Amphipoda: Talitridae) by its mermithid parasite

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Summary

1. Hairworms (phylum Nematomorpha) and mermithid nematodes (phylum Nematoda) have independently evolved almost identical life cycles: both parasites cause their terrestrial arthropod hosts to seek water, required for their aquatic free-living adult stage. Hairworms achieve this by altering host neurotransmitters, but the mechanisms used by mermithids remain unknown.

2. The physiological effects of the mermithid nematode *Thaumamermis zealandica* on its host, the supralittoral talitrid amphipod *Talorchestia quoyana*, are investigated. The parasite develops in the haemocoel, and induces the host to burrow more deeply than healthy amphipods and the adult *T. zelandica* emerges from the host into moist sand at these greater depths.

3. Parasitized amphipods had higher haemolymph osmolality than unparasitized amphipods. There was no difference in haemolymph Na^+, K^+ or Mg^{2+} concentrations between parasitized and unparasitized amphipods.

4. Water content did not differ between parasitized and unparasitized amphipods. Lipid reserves were lower in parasitized male amphipods than in unparasitized males; there was no difference among females.

5. Increase of host haemolymph osmolality by *T. zealandica* could induce 'thirst', explaining why parasitized amphipods seek water-saturated sand. This mechanism appears more parsimonious than that used by nematomorphs to achieve the same change in host behaviour.

Key-words: Amphipod, haemolymph, lipid reserves, manipulative parasite, water-seeking

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Introduction

Parasite-induced changes in host behaviour are well documented in many different taxa (see Hurd 1990; Moore & Gotelli 1990; Moore 2002). In many instances, they appear adaptive for the parasite, serving to facilitate its transmission to the next host in its life cycle, or its release in an environment suitable for the next phase of its development. Manipulation is generally thought to act on the nervous system, and to be driven by parasite-produced molecules (neurotransmitters like serotonin, dopamine and octopamine) (Helluy & Holmes 1990; Øverli *et al*. 2001), or through action on opioid systems (Kavaliers & Colwell 1994; Thompson & Kavaliers 1994).

Hairworms (phylum Nematomorpha) cause their terrestrial insect hosts to seek water and jump into it (Thomas *et al*. 2002), allowing the parasite to complete its life cycle as an aquatic free-living adult. Mermithids (phylum Nematoda) have independently evolved an almost identical life cycle, and are also known to induce water-seeking in their terrestrial hosts (Maeyama, Terayama & Matsomoto 1994; Vance 1996). Crickets infected with the nematomorph *Paragordius tricuspidatus* (which enter water and drown, Thomas *et al*. 2002) show increased brain titres of several amino acids, especially taurine, valine and tyrosine, all important neurotransmitters in insects (Thomas *et al*. 2003). Whether similar changes are induced by mermithid nematodes in their hosts is unknown. The manipulation of neurotransmitters is a complex explanation for the alteration of host behaviour – neurotransmitters tend to be relatively large molecules,

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requiring synthesis by the parasite, and must then travel from the parasite in the haemocoel into the arthropod's neural system. Here we investigate a more parsimonious alternative in a nematomorph–crustacean system: that the parasite changes host behaviour by directly altering some parameters of the haemolymph.

The mermithid nematode parasite *Thaumamermis zealandica* grows to large sizes (200 mm or more) in the haemocoel of the supralittoral sandhopper *Talorchestia quoyana* (Amphipoda: Talitridae), which is up to 21 mm long, *c*. 3 mm wide, often completely filling the body cavity before emerging from its host into moist sand for the free-living adult stage of its life cycle (Poinar, Latham & Poulin 2002). Amphipods infected with *T. zealandica* burrow more deeply than uninfected amphipods (into sand layers with greater water content); and burrowing depth is proportional to parasite length (Poulin & Latham 2002). How the nematode induces this is unknown.

Talitrid amphipods are strong osmoregulators (Morritt 1988; Morritt & Spicer 1998). Na^+ is the most important ion in terms of its contribution to the osmotic pressure of the haemolymph (Moore & Francis 1986; Zerbst-Boroffka *et al*. 2000), and is closely regulated over a wide range of external concentrations, in contrast to haemolymph $[K^+]$, which tends to remain above that of the external media, steadily increasing with increasing external concentrations (Spicer & Taylor 1987; Morritt 1989). In every phylum that has been investigated an increase in the osmolality of body fluids leads to water-seeking behaviour (e.g. Johnson & Thunhorst 1997; Stricker & Sved 2000). We suggest that the observed water-seeking behaviour in *T. quoyana* (Poulin & Latham 2002) could be induced by changes in haemolymph composition, and test the hypothesis that parasitized amphipods have higher haemolymph $[Na^+]$, $[K^+]$ and osmolality than uninfected amphipods. Since an increase in haemolymph osmolality could be a consequence of desiccation, we also compare water content between parasitized and unparasitized amphipods.

Amphipod burrowing activity may also be influenced by haemolymph manipulation via other mechanisms, for example, by mimicking seasonal responses. Mg^{2+} has a narcotizing effect on many marine invertebrates, and there is a general negative relationship between extracellular $[Mg^{2+}]$ and activity in crustaceans (Morritt & Spicer 1993). In talitrid amphipods, Mg^{2+} is maintained at very low levels during the summer months, while in winter, when the amphipods have reduced activity and stay in deep burrows, $[Mg^{2+}]$ is significantly higher (Spicer, Taylor & McMahon 1990). A modification of haemolymph $[Mg^{2+}]$ in the host could therefore result in the observed deeper burrowing in *T. quoyana*, so we compare haemolymph Mg^{2+} between parasitized and unparasitized amphipods.

In addition to providing a vehicle to the adult habitat, *T. quoyana* provides resources for the growth of *T. zealandica.* Energy demands imposed by parasites have been quantified in some host–parasite systems (Munger & Karasov 1989; Kearns, Hurd & Pullin 1994; Sorensen & Minchella 1998; Kristan & Hammond 2000); however, in several systems parasites appear to make no impact on host energy reserves (Amat *et al*. 1991; Franz & Kurtz 2002). In these cases, the energy needed by the parasite for its growth appears instead to be diverted from host reproduction, leaving lipid stores untouched (Franz & Kurtz 2002). *Thaumamermis zealandica* castrates its host, making energy usually devoted to reproduction available, but since it increases in size to 3–5% of the host's body weight at emergence, its demands may be greater than the energy that would otherwise be invested in reproduction. We quantify the energetic cost to *T. quoyana* of parasitism by *T. zealandica* by measuring lipid content of infected and uninfected amphipods.

Overall, we examine potential mechanisms of host manipulation by testing two non-exclusive hypotheses about haemolymph parameters of *Talorchestia quoyana* infected and uninfected with the mermithid nematode *Thaumamermis zealandica*. We hypothesized that (1) parasitized amphipods will have higher haemolymph $[Na^+]$ and $[K^+]$, higher haemolymph osmolality, and/or lower water content than unparasitized amphipods; and (2) parasitized amphipods will have higher haemolymph Mg^{2+} concentrations than their unparasitized counterparts. We also hypothesized that the energetic cost of the mermithid nematode parasite is greater than the cost of reproduction for *T. quoyana*, such that lipid reserves will be lower in parasitized than in unparasitized individuals.

Methods

ION CONTENT AND OSMOLALITY

Amphipods (*Talorchestia quoyana*) were collected from beneath kelp and tidal debris above the high-tide line at Long Beach, north of Dunedin, New Zealand. Prevalence of *Thaumamermis zealandica* at this location has been found to range from 2 to 14% in November 1999 (Poulin & Rate 2001), to over 30% in November 2002 (Poulin & Latham 2002). Owing to a prevalence well below that of previous years, 50 amphipods per trip were collected on several occasions between November 2002 and March 2003. On each occasion, the 50 individuals were taken from at least five different patches of kelp at least 5 m apart, as infection rates vary between patches (Poulin & Rate 2001). Amphipods larger than 10 mm body length (likely to be mature) were handpicked without regard for sex and were transported back to the laboratory and kept in their groups of 50 in covered clear 15 cm × 40 cm plastic containers containing a 10 cm layer of sifted sand which was collected at the same time as the amphipods. The amphipods were acclimated for 4–12 days under a natural photoperiod at 20 °C ± 1, the sand was sprayed with 100 ml water daily, and kelp was provided for food. There was no relationship between acclimation

time and haemolymph parameters (Pearson's r, $P > 0{\cdot}2$ in all cases).

Individual amphipods were selected at random and placed under Cargille's Immersion Oil (Type A) (Cargille Laboratories, Cedar Grove, NJ, USA) to prevent evaporation during haemolymph collection. The fourth peraeopod (walking leg) was removed and the exuding haemolymph was drawn into a capillary tube (1 mm inner diameter), sealed with immersion oil and stored at –80 °C until further use. The amphipod was then decapitated, measured (anterior end of cephalon to anterior tip of the telson), sexed and dissected. If *T. zealandicus* were present they were extracted from the amphipod, counted, straightened without stretching and measured to the nearest mm. Since few parasitized amphipods were found among the samples collected for this study, haemolymph was analysed from randomly selected unparasitized amphipods from the same collection. Two external symbionts were observed on the amphipods – a rhabditid nematode and a mite – but these are not thought to be parasitic (see Poulin & Rate 2001). A total of 237 amphipods were dissected to provide the 9 infected individuals for haemolymph studies.

Haemolymph osmolality (unparasitized: $n = 5$ females, 2 males, parasitized: $n = 7$ females, 2 males) was measured by optical measurement of the melting point on a Nanolitre Osmometer (Otago Osmometers, Dunedin, New Zealand). Thawed haemolymph samples and oil were transferred into a haematocrit capillary by syringe and centrifuged for 3 min to separate the haemolymph from the surrounding oil. Triplicates of each sample were transferred in a micropipette in Cargille's Immersion Oil (Type A) to wells filled with Cargille's Immersion Oil (Type B) on the stage of the Nanolitre Osmometer. Calibration was checked regularly using distilled, deionized water and standards of known osmolality (Wescor, Logan, UT). Samples were snap-frozen, and the melting point of the haemolymph determined from the temperature at which the last ice crystal melted, and the mean of the triplicate samples converted to osmolality (mOsmoles) by the formula mOsm = (MP/ –1·86) × 1000, as the osmolal melting point depression is 1·86 °C. Osm^{-1} (Zachariassen 1985).

From the remaining haemolymph, 1 µl was pipetted into 1·999 ml 0·2% nitric acid solution to fix heavy ions, then the concentrations of Na^+, K^+ and Mg^{2+} in the haemolymph were determined using a Perkin Elmer-Sciex Elan-6100 DRC Plus ICP-Mass Spectrometer (Perkin Elmer, Boston, MA, USA). Triplicates were run for each sample, and the mean of these triplicates was used for further analysis. Sample sizes, unparasitized, $n = 5$ females, 2 males; parasitized, $n = 7$ females, 2 males).

Only individuals for which both osmolality and ion concentrations were measured were used in analyses. Haemolymph osmolality and ion concentrations were compared between infected and uninfected amphipods and between collection dates, using analysis of variance (ANOVA), and male and female values were pooled (haemolymph parameters were not significantly correlated to body size of the amphipods). All size measurements (amphipod length and parasite length) were $\log_{10}$-transformed so that they met the assumptions of normality. Total osmolality and concentrations of Na^+, K^+ and Mg^{2+} in the host haemolymph were related to total parasite length for the infected individuals only, and Pearson's correlation coefficients used assess the significance of the relationships.

WATER AND LIPID CONTENT

Amphipods were collected in a single, large sample of several hundred individuals, on 10 October 2003, and housed in the laboratory for 1 week as previously described, without kelp to ensure that the gut was empty. Individual amphipods were then chosen at random from the container without regard to sex, and each amphipod was decapitated and opened over a Petri dish to determine whether it housed a parasite, ensuring that all haemolymph remained in the Petri dish. Any parasites present were removed, counted, straightened without stretching, and measured to the nearest mm. All parasitized amphipods were used, until a total of 56 parasitized amphipods was reached, and of the unparasitized amphipods approximately equal numbers of males and females were kept to a total of 52; the rest were discarded.

Each amphipod was weighed (± 0·01 mg) to determine fresh mass (FM), and dried over silica gel at 60 °C for 3 days before being reweighed to determine dry mass (DM), and lipids dissolved in three changes of chloroform prior to being dried and weighed to determine lipid-free mass (LFM). Water content was the difference between DM and FM, and lipid content the difference between LFM and DM. Length and mass of unparasitized amphipods are significantly correlated ($r = 0{\cdot}937$, $P < 0{\cdot}000\,01$, $n = 30$), so we used mass in place of length as a measure of body size.

Water and lipid contents (in g) of amphipods were compared between sexes and between parasitized and unparasitized individuals using a two-way analysis of covariance, with fresh mass as the covariate on Statistica 6·1 (Statsoft Inc., Tulsa, OK). To examine the relationship between lipid and water content and total parasite length, lipid and water content were regressed against dry mass of the amphipod, and the correlation between the residuals of these regressions and $\log_{10}$-transformed total parasite length was computed.

Results

OSMOLALITY AND ION CONTENT

Haemolymph osmolality was higher in amphipods parasitized by *Thaumamermis zealandica* than in unparasitized amphipods ($F_{1,14} = 2{\cdot}83$, $P < 0{\cdot}0001$, Table 1). Body length did not differ between unparasitized and parasitized amphipods ($F_{1,14} = 2{\cdot}83$, $P = 0{\cdot}115$). Among all amphipods, there was no relationship between osmolality and amphipod body length ($r = -0{\cdot}248$,

Table 1. Haemolymph osmolality, ion concentrations, water and lipid content of the amphipod *Talorchestia quoyana* as a function of parasitism by *Thaumamermis zealandica.* Mean ± standard error (*n*) is shown. An asterisk indicates a significant difference between values for parasitized and unparasitized amphipods (see text). Different superscript letters within water or lipid content indicate values that are significantly different (Tukey's *post-hoc* test, $P < 0{\cdot}0002$, see text). Although water and lipid content are expressed as mass-specific units, all analyses were conducted using ANCOVA with fresh mass (water) or dry mass (lipid) as covariates

	Unparasitized	Parasitized
Osmolality (mOsm)	757 ± 6 (7)	848 ± 14 (9)*
Na^+ mmol l^{-1}	347 ± 29 (7)	374 ± 23 (9)
K^+ mmol l^{-1}	10·4 ± 0·5 (7)	13·1 ± 1·1 (9)
Mg^{2+} mmol l^{-1}	5·3 ± 0·6 (7)	6·1 ± 0·5 (9)
Water content (g g^{-1} fresh mass)		
Male	0·737 ± 0·007 (30)[a]	0·739 ± 0·007 (15)[a]
Female	0·699 ± 0·008 (23)[b]	0·717 ± 0·005 (41)[b]
Lipid content (g g^{-1} dry mass)		
Male	0·209 ± 0·014 (30)[a]	0·133 ± 0·013 (15)[b]
Female	0·161 ± 0·013 (23)[b]	0·147 ± 0·010 (41)[b]

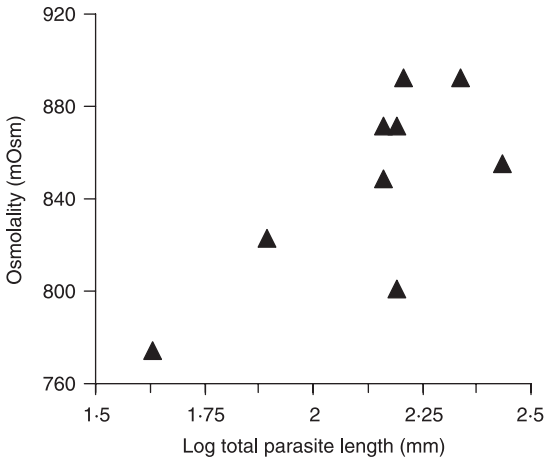

Fig. 1. Positive relationship between haemolymph osmolality of *Talorchestia quoyana* and total length of *Thaumamermis zealandica* parasites harboured. See text for statistics.

$P = 0{\cdot}354$, $n = 16$). Haemolymph osmolality increased as total parasite length increased (Fig. 1) ($r = 0{\cdot}731$, $P = 0{\cdot}0025$, $n = 9$).

Among all amphipods, haemolymph [K^+] was significantly and positively correlated with haemolymph osmolality ($r = 0{\cdot}796$, $P = 0{\cdot}0001$, $n = 16$). In contrast, haemolymph [Mg^{2+}] and [Na^+] were not significantly correlated with haemolymph osmolality (Mg^{2+}, $r = 0{\cdot}362$, $P = 0{\cdot}169$, $n = 16$; Na^+, $r = 0{\cdot}482$, $P = 0{\cdot}059$, $n = 16$), although the latter was very close to significance, given the small sample size. There was no correlation between haemolymph cations and amphipod body length (Na^+, $r = 0{\cdot}433$, $P = 0{\cdot}094$, $n = 16$; K^+: $r = -0{\cdot}053$, $P = 0{\cdot}845$, $n = 16$; Mg^{2+}, $r = 0{\cdot}219$, $P = 0{\cdot}415$, $n = 16$). Mean haemolymph cation concentrations did not differ between infected and uninfected amphipods (Na^+, $F_{1,12} = 1{\cdot}19$, $P = 0{\cdot}296$; K^+, $F_{1,12} = 3{\cdot}28$, $P = 0{\cdot}095$; Mg^{2+}, $F_{1,12} = 1{\cdot}117$, $P = 0{\cdot}311$, Table 1). Sampling occasion also had no significant effect on haemolymph cation concentrations (Na^+, $F_{1,12} = 0{\cdot}020$, $P = 0{\cdot}890$; K^+, $F_{1,12} = 0{\cdot}338$, $P = 0{\cdot}572$; Mg^{2+}, $F_{1,12} = 0{\cdot}053$, $P = 0{\cdot}822$).

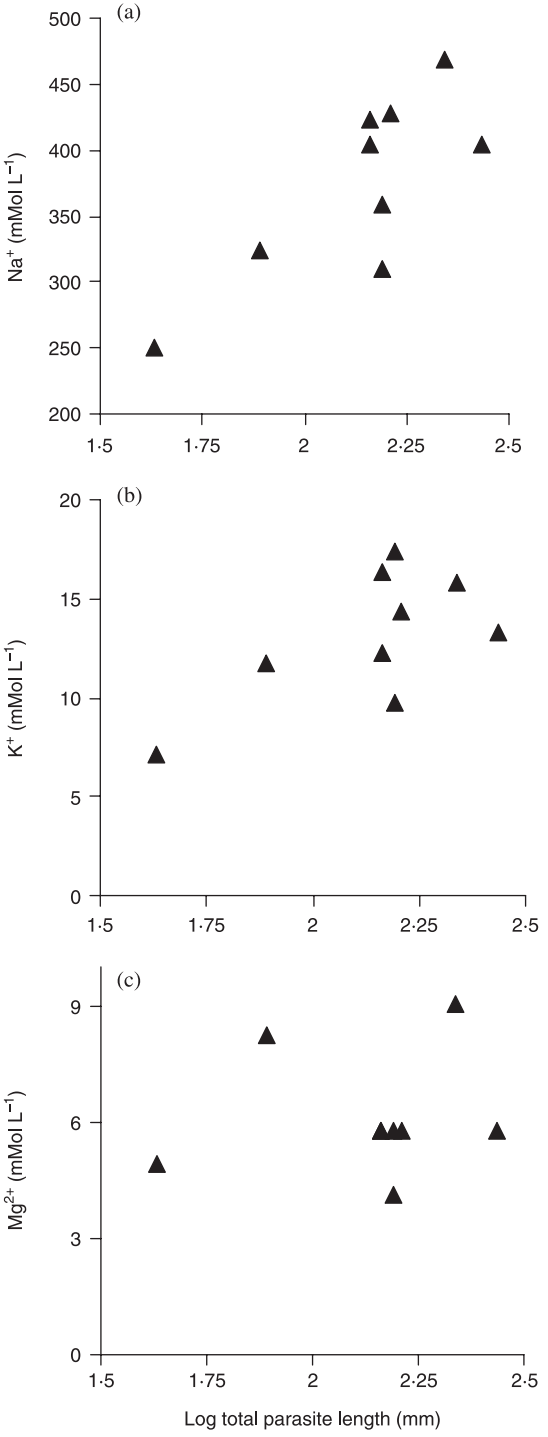

Fig. 2. Relationship between haemolymph concentrations of (a) Na^+, (b) K^+ and (c) Mg^{2+} ions in the haemolymph of *Talorchestia quoyana* parasitized by *Thaumamermis zealandica* and total length of *Thaumamermis zealandica* parasites harboured. See text for statistics.

Haemolymph [Na^+] was significantly positively correlated with parasite length ($r = 0{\cdot}791$, $P = 0{\cdot}011$ $n = 9$; Fig. 2a). K^+ concentrations also showed a trend

towards a positive relationship with total parasite length, although this case the relationship fell just short of significance ($r = 0{\cdot}655$, $P = 0{\cdot}055$, $n = 9$; Fig. 2b). Mg^{2+} concentrations did not show any relationship with total parasite length ($r = 0{\cdot}135$, $P = 0{\cdot}73$, $n = 9$, Fig. 2c).

WATER AND LIPID CONTENT

There was no difference in fresh mass between parasitized and unparasitized amphipods ($t_{(108)} = 1{\cdot}07$, $P = 0{\cdot}286$). The majority (44 out of 56) of infected amphipods contained only one mermithid, with a few amphipods containing either two or three worms. There was no correlation between amphipod fresh mass and total parasite length ($r = 0{\cdot}045$, $P = 0{\cdot}744$, $n = 56$). Neither lipid content nor water content residuals were significantly correlated with total parasite length (water content: $r = 0{\cdot}161$, $P = 0{\cdot}236$, $n = 56$; lipid content: $r = -0{\cdot}185$, $P = 0{\cdot}172$, $n = 56$).

Water content differed between the sexes ($F_{1,104} = 5{\cdot}60$, $P = 0{\cdot}020$, Table 1), with males having a higher water content than females. This difference, although consistent, is small, on average equating to approximately 4% more water in males than in females. Water content did not differ significantly between infected and uninfected amphipods ($F_{1,104} = 0{\cdot}111$, $P = 0{\cdot}740$, Table 1).

Lipid content of amphipods was significantly positively related to the size of the amphipods ($F_{1,104} = 121{\cdot}7$, $P < 0{\cdot}0001$). There was a significant interaction between sex and infection status ($F_{1,104} = 6{\cdot}06$, $P = 0{\cdot}015$), such that uninfected males had significantly higher lipid contents than uninfected females and parasitized amphipods of either sex ($P < 0{\cdot}001$, Tukey's HSD, Table 1).

Discussion

Water-seeking behaviour by arthropods infected with haemocoel-dwelling worms is a striking example of behavioural manipulation of a host by its parasite. Although neurotransmitter titres have been shown to change in crickets infected with nematomorph parasites (Thomas *et al*. 2003), we chose to address a more conservative and parsimonious hypothesis, namely that water-seeking behaviour is a known consequence of 'thirst' (mediated by hyperosmolality or decreased water content), and that since the parasite resides in the haemocoel, it is located in a position ideal to manipulate haemolymph composition.

We found that amphipods infected with *T. zealandica* had increased haemolymph osmolality, but that water content does not differ with infection status. Haemolymph $[Na^+]$, $[K^+]$ and $[Mg^{2+}]$ did not differ between infected and uninfected individuals, the latter suggesting that the behavioural change is not a consequence of manipulation of the effects of Mg^{2+} on activity and burrowing depth (Spicer *et al*. 1990, Morritt & Spicer 1993).

Haemolymph osmolality and cation concentrations for both infected and uninfected amphipods fall within the range reported for other supralittoral amphipods (Morritt 1988). The observed increase in haemolymph osmolality in parasitized amphipods is consistent with the hypothesis that the observed change in amphipod burrowing behaviour when parasitized by the mermithid is caused by water-seeking due to 'thirst'. However, the mechanisms for this remain unclear. Haemolymph $[K^+]$ was positively correlated with osmolality (although it did not differ significantly between the infection groups). Although there is evidence of active outward transport of sodium and potassium ions across the outer hypodermal membrane of nematodes (DeMello & Tercafs 1966; Pax *et al*. 1995; Thompson & Geary 2002), parasites cannot manufacture ions *de novo* for the purpose of excreting them into the host; such excretion would likely be quickly counteracted by the efficient osmoregulatory system of the host, resulting in only minor perturbations (Morritt 1989), and the level of difference in $[K^+]$ is insufficient to explain the changed osmolality. It is more likely that the parasite is manipulating the host's haemolymph osmolality by means of excretion of a less strongly regulated solute, or (less parsimoniously) by acting upon the control of osmoregulation itself.

Haemolymph osmolality and $[Na^+]$ in *T. quoyana* increased with the length (and therefore age) of their *T. zealandica* parasites, and a similar (non-significant) trend was shown for $[K^+]$ ($n = 9$). A relationship between haemolymph osmolality or cation concentrations and parasite size (proportional to age) is consistent with our hypothesis if the behavioural change in the amphipods was dependent on a threshold haemolymph osmolality being crossed, and the change in haemolymph osmolality is dependent on the age of the parasite. Mermithid nematodes rarely grow after emergence, and fecundity in all nematodes is positively correlated with body size (Poinar 2001), so it is likely that the behavioural manipulation will be strongly coincident with maturity of the parasite.

This is the first report of lipid contents of a supralittoral amphipod. Mean lipid contents fall either within or just below the range of those found for aquatic species (Lehtonen 1995; Cavaletto *et al*. 1996; Nalepa *et al*. 2000). Mean lipid content of parasitized males was significantly lower than in unparasitized males (Table 1), even in winter, when lipid levels in unparasitized individuals were at their minimum (Williams 2004). By contrast, lipid content of females was lower than that of unparasitized males, and did not change significantly with parasite infection. Lipid content was expected to be lower in parasitized than in unparasitized amphipods, as the parasite increases in size by up to three orders of magnitude while in the host, and so must impose large energy demands. However, infected female amphipods make no investment in egg

production, and their lipid content does not differ from uninfected females. It would appear therefore that the cost of the parasite is equivalent to the female's (curtailed) investment in reproduction, resulting in no net change in lipid content. By contrast, in male amphipods, whose investment in reproduction lies in guarding females before mating can take place (Craig 1973; Morton & Miller 1973), energy stores are clearly related to fitness. In this case, where males have a low material (but high behavioural) investment in reproduction, the parasite makes a significant impact on lipid reserves.

In summary, it appears that the parasitic mermithid *Thaumamermis zealandica* increases haemolymph osmolality in *Talorchestia quoyana*; this may induce a need for water in the host that would explain their observed water-seeking behaviour. For a parasite to change some parameters of the liquid in which it is bathing would require fewer unexplained steps, and is therefore favoured by parsimonious reasoning over an explanation which required the parasite to synthesize molecules that affect a distant organ with which it has no physical connection; however, the means by which the parasite alters haemolymph osmolality remain unexplained, and the answer may not be any more parsimonious than the neurotransmitter hypothesis. All of the investigations of the mechanisms of host behaviour change have been correlative. Although technically difficult, an experimental approach – including experimental infection of otherwise healthy amphipods and manipulation of the haemolymph of unparasitized amphipods – would markedly strengthen the level of inference in these studies but awaits a sufficiently tractable model system.

Nematomorph parasites show a high degree of convergence with mermithid nematodes, in terms of both their life cycle and the behavioural changes they cause in their host (Thomas *et al*. 2002). This convergence offers an excellent opportunity to explore the evolution of the mechanisms of host manipulation by parasites. Thomas *et al*. (2003) have shown that nematomorphs induce changes in their host's neurotransmitter titres, and here we demonstrate a manipulation of haemolymph parameters. An important next step is to extend both investigations to allow matched comparison of mechanism and behaviour in the two systems to allow comparison within an evolutionary context.

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