Multidimensionality and intra-individual variation in host manipulation by an acanthocephalan

D. P. BENESH[1]*, E. T. VALTONEN[1] *and* O. SEPPÄLÄ[2]

[1] *Department of Biological and Environmental Science, POB 35, FI-40014 University of Jyväskylä, Finland*
[2] *EAWAG, Swiss Federal Institute of Aquatic Science and Technology, and ETH-Zürich, Institute of Integrative Biology (IBZ), Überlandstrasse 133, PO Box 611, CH-8600, Dübendorf, Switzerland*

(*Received 27 August 2007; revised 22 October 2007 and 11 January 2008; accepted 11 January 2008; first published online 25 February 2008*)

SUMMARY

Trophically-transmitted parasites frequently alter multiple aspects of their host's phenotype. Correlations between modified characteristics may suggest how different traits are mechanistically related, but these potential relationships remain unexplored. We recorded 5 traits from individual isopods infected with an acanthocephalan (*Acanthocephalus lucii*): hiding, activity, substrate colour preference, body (pereon) coloration, and abdominal (pleon) coloration. Infected isopods hid less and had darker abdominal coloration than uninfected isopods. However, in 3 different experiments measuring hiding behaviour (time-scales of observation: 1 h, 8 h, 8 weeks), these two modified traits were not correlated, suggesting they may arise via independent mechanisms. For the shorter experiments (1 h and 8 h), confidence in this null correlation was undermined by low experimental repeatability, i.e. individuals did not behave similarly in repeated trials of the experiment. However, in the 8-week experiment, hiding behaviour was relatively consistent within individuals, so the null correlation at this scale indicates, less equivocally, that hiding and coloration are unrelated. Furthermore, the difference between the hiding behaviour of infected and uninfected isopods varied over 8 weeks, suggesting that the effect of *A. lucii* infection on host behaviour changes over time. We emphasize the importance of carefully designed protocols for investigating multidimensionality in host manipulation.

Key words: altered host phenotype, plastic/flexible behaviour, repeatability, *Asellus aquaticus*, Acanthocephala, intermediate host, isopod.

INTRODUCTION

Trophically-transmitted parasites often alter their host's phenotype in ways which presumably increase transmission to the next host in the life-cycle (reviewed by Moore, 2002). Theoretical studies have examined parasitic investment into host manipulation, but have not addressed whether or not this investment targets multiple host traits (Poulin, 1994; Brown, 1999). Many, if not most, parasites affect several aspects of their host's phenotype, including behaviour, appearance and physiology (e.g. Hindsbo, 1972; Moore, 1983; Bakker *et al*. 1997), i.e. parasitic manipulation of hosts is multidimensional. Thus, a comprehensive picture of the extent that host phenotype is altered by individual parasites requires the simultaneous quantification of multiple traits (Thomas *et al*. 2005). Moreover, this approach permits potential relationships between traits to be explored, which may be important for understanding the profitability of a given manipulation strategy. Two altered traits may, for example, have the same underlying mechanism, resulting in positive correlations between trait magnitudes and perhaps lower costs of manipulation (Cézilly and Perrot-Minnot, 2005). Alternatively, modifications could arise via independent physiological processes, presumably leading to uncorrelated trait intensities. In this case, parasites may need to devote more energy to alter both traits, depending on the costs associated with the mechanism of each trait modification (Cézilly and Perrot-Minnot, 2005).

To confidently correlate the magnitudes of different modified characteristics, representative trait values should be obtained for individual hosts. Thus, an important prerequisite for studies examining the relationships between manipulated traits is an experimental design with high measurement repeatability (Cézilly and Perrot-Minnot, 2005). When individuals are observed multiple times, a repeatable experimental set-up would be expected to yield data with low within-individual variation relative to that between individuals, i.e. it should give individually representative trait values. Repeatability will naturally be related to the level of intra-individual variation in the measured trait. For example, if an altered trait's magnitude varies stochastically over time, short experiments may only

* Corresponding author: Current address: Department of Evolutionary Ecology, Max-Planck-Institute for Evolutionary Biology, August-Thienemann-Strasse 2, 24306 Plön, Germany. Tel: +49 45227 63258. Fax: +49 45227 63310. E-mail: benesh@mpil-ploen.mpg.de

doi:10.1017/S0031182008004216 Printed in the United Kingdom

capture a portion of an individual's trait variability. Consequently, the recorded trait values from such experiments will deviate randomly from actual individual trait averages. Repeatable experimental set-ups and representative trait measurements are necessary not only to study multidimensionality in host manipulation, but also to investigate the potential sources of between-host variation in altered traits, e.g. varying parasite manipulative ability or differing host resistance to manipulation (Thomas *et al*. 2005).

In this study, we investigated multiple features of the manipulation strategy of an acanthocephalan. *Acanthocephalus lucii* is a common parasite of freshwater fish in Europe, particularly European perch, *Perca fluviatilis*. Adults live in the intestine where they mate and release eggs into the environment with the host faeces. Eggs are ingested by the intermediate hosts, freshwater isopods (*Asellus aquaticus*). The parasite develops in the isopod to the infective cystacanth stage, and the life-cycle is completed when a cystacanth-harbouring isopod is ingested by an appropriate definitive host. Infection with *A. lucii* cystacanths does not affect isopod response to light or a disturbance (Lyndon, 1996). The respiratory opercula of infected isopods become conspicuously darker as the parasite reaches infectivity, and infected isopods are more susceptible to predation by perch (Brattey, 1983). Thus, this may be a suitable system to study multidimensionality in host phenotype alteration because relatively few traits seem affected by the parasite, making the measurement of multiple traits in succession more manageable, yet some aspect of infection renders isopods more susceptible to fish predation.

Our specific goals were to (1) document isopod traits altered by *A. lucii*, (2) evaluate intra-individual variation in a manipulated trait (hiding behaviour) and its apparent dependence on the experimental design used to measure it and (3) assess whether any altered traits may be related.

MATERIALS AND METHODS

Animal collection and maintenance

All experimental isopods were collected in September and October, 2005 from Lake Jyväsjärvi, Central Finland (62°14′N 25°44′E). Isopods infected with *A. lucii* cystacanths were initially identified by their darkened respiratory opercula (Brattey, 1983). Thus, all the infected isopods used in the experiments presumably carried parasites capable of infecting fish, because the alteration of opercular coloration is associated with parasites reaching the infective cystacanth stage (Brattey, 1983). The morphology of cystacanths dissected from isopods was consistent with previous descriptions of larval *A. lucii* (Andryuk, 1979). In the lab, animals were maintained at approximately 16–18 °C under constant illumination. Because we used naturally infected isopods, infection was not a randomly assigned treatment. Thus, there may be pre-existing differences between uninfected and infected isopods, and we acknowledge the possibility that such differences might impact the measured phenotypic traits. Natural infections, however, are advantageous because the observed isopod phenotypes are probably similar to those encountered by definitive host fish predators in the field. Experimental isopod infections could circumvent the mentioned problem, but higher-than-natural *A. lucii* intensities are often produced in these experiments (Brattey, 1986; Hasu *et al*. 2007; Benesh and Valtonen, 2007).

Experiment 1 – traits altered by infection and their inter-relationships

We recorded 5 traits from each individual isopod [hiding behaviour, activity, substrate colour preference, body (pereon) coloration and abdominal (pleon) coloration], and checked whether they differed between infected and uninfected isopods. Hiding behaviour was assessed by placing individual isopods into a Petri dish (8·5 cm diameter) with 100 ml of water. In the centre of the dish an unconditioned, and therefore unpalatable, piece of birch leaf (*Betula pendula*; approximately 7 cm^2) acted as shelter. Leaves generally need to be 'conditioned' in lake water for a few weeks to allow microbial colonization before being palatable for isopods (Graca *et al*. 1993). Every 3 min for 1 h, isopods were recorded as being under the leaf or exposed and visible from above. Refuge use was summarized as the proportion of time an isopod spent exposed. Isopod activity was evaluated by placing an individual into a Petri dish and counting the number of times it crossed a centre line in 5 min. For the substrate colour preference experiment, Petri dishes were divided in half; one side had a white substrate and the other a black substrate. Substrates were created using coloured paper placed under the Petri dish. Each background colour extended 2–3 cm beyond the edge of the Petri dish to reduce the possibility that isopod behaviour was affected by substrates visible outside the Petri dish. Over the course of 1 h, isopod substrate choice was recorded every 3 min. The proportion of time spent on the white background was calculated for each individual isopod. Acclimation times for the behavioural trials were 1 min for the activity assay and 5 min for the hiding and substrate choice observations. All behavioural observations were made directly, i.e. not via camera recordings. Although care was taken to avoid any change in light conditions and any disturbance to the water during observation, movements of the observer could have affected isopod behaviour. Infected and uninfected isopods

were observed in an identical fashion, although, any disturbances presumably influenced both groups to a similar degree.

For all isopods, we assessed hiding behaviour first, then activity and finally substrate choice. Two traits were never recorded from individual isopods in the same day. The interval between observations was kept as short as possible, usually 1 day, but ranged up to 4 days because of the time required to collect, handle, and observe all the isopods. During this interim, isopods were maintained individually in plastic containers ($10 \times 15 \times 5$ cm) with 400 ml of water and fed a diet of conditioned leaves, primarily of alder (*Alnus glutinosa*). For each of the 3 behavioural traits, we observed a subsample of infected and uninfected isopods ($n = 9$–27) a second time, 1–3 days after the original observation, to evaluate experimental repeatability and determine whether the relatively short 1-h observation period was sufficient to characterize an individual's behaviour. Repeatability for each trait was assessed with intra-class (unordered) correlations (ICC). ICCs are used to test the agreement of multiple quantitative measurements; high levels of correlation are indicative of high measurement repeatability (Müller and Büttner, 1994). It should be noted that identical values for multiple measurements are not necessary for high repeatability. For instance, the trait average across individuals could change between the repeated measurements, yet variation within individuals could remain low relative to that between individuals, e.g. individuals with high trait values in the first trial also have high values in the second trial, albeit at a different magnitude. ICCs were performed separately for infected and uninfected isopods for each trait. At the end of the behavioural experiments, isopods were frozen at $-20\,^{\circ}$C. Freezing has been used as a preservation method in previous studies examining isopod pigmentation (Hargeby *et al.* 2004).

Frozen isopods were thawed and individually photographed with a Nikon Coolpix 4500 digital camera (scene mode: close up, focal length: 96 mm, aperture: F3.5, shutter speed: 1/30, sensitivity: ISO100, image size: 1600×1200 pixels, image quality: fine, focus mode: auto). The camera was attached to an Olympus SZX9 dissecting microscope (Olympus Europa, Hamburg, Germany) with an $M28 \times 0.75$ digital coupler (Thales Optem Inc., Fairport, NY, USA). Light for the photographs came from a fluorescent lamp situated 12 cm above the microscope stage (light intensity on the stage was 6000 lx). Photographs were analysed using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA). All pictures were converted to greyscale for analysis. Reflectance measurements were taken from the dorsal side of isopods (Fig. 1), because isopods are probably observed by predators in this orientation. For instance, Hargeby *et al.* (2004, 2005) have found that dorsal isopod pigmentation matches substrate conditions in natural populations, suggesting selection for isopod crypsis, most likely to avoid predators. In each photograph, reflectance was measured from 4 circular areas along the isopod: the dorso-lateral portion of the first, fourth, and seventh segment (all part of the pereon) as well as along the dorso-lateral side of the abdomen (i.e. the pleon) (Fig. 1). The size of the analysed circle was adjusted according to isopod size so that it filled nearly the entire anterior-posterior length of the segment but did not overlap the intestine (i.e. 20 pixel diameter for isopods less than 6·5 mm long, 40 pixels for isopods 6·5 to 7·5 mm, and 60 pixels for isopods greater than 7·5 mm). The scale of reflectance in the software ranged between 0 (black, 100% saturation) and 255 (white, 100% reflectance). Histograms of reflectance of individual pixels within the selected areas resembled a normal distribution, so we took the mean value of reflectance from each area as a measure of isopod coloration. Reflectance values for the first, fourth and seventh segments were averaged to give a mean value for pereon pigmentation, whereas abdominal reflectance was treated separately. The ranges of reflectance values were 55·1 to 126·2 for the pereon and 43·8 to 117·3 for the abdomen. We photographed several isopods twice (uninfected: $n = 8$ and infected: $n = 11$) to evaluate the repeatability of the method. After being photographed, all isopods were sexed, measured to the nearest 0·5 mm and dissected to determine whether they were infected.

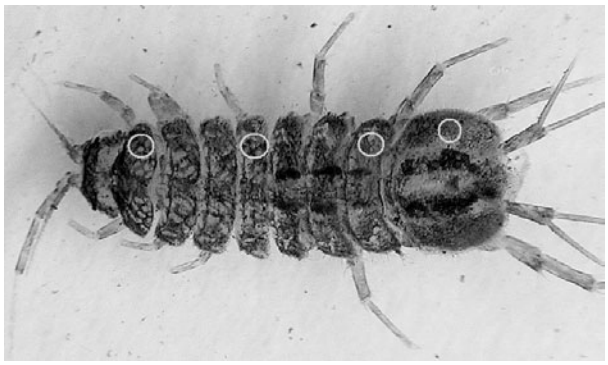

Fig. 1. Analysis of isopod photographs. For individual isopods, reflectance was measured from 4 places (indicated with circles): the lateral portion of the first, fourth, and seventh segment (all part of the pereon) as well as along the lateral side of the abdomen (i.e. the pleon). The values for the first, fourth and seventh segment were averaged to give a mean value for body (pereon) coloration.

Generalized linear models (GLZ) were used to assess which of the 5 traits differed between infected ($n = 62$, mean parasite intensity = 1·08, mean length = 6·48 mm) and uninfected ($n = 90$, mean length = 6·31 mm) isopods. In GLZs, the error structure of the data can be explicitly defined, and a link

function is used to relate the expected values of the response variable to the predictor variables (Wilson and Grenfell, 1997). This approach is especially useful for evaluating proportion and count data, because they often deviate from normality. Models were defined with the GENLIN function in SPSS 15.0 (SPSS Inc., Chicago, Illinois). Infection and isopod sex were included as predictors. Isopod hiding and substrate choice were modelled using binomial errors and a logit link function, whereas activity was evaluated using Poisson errors and a log link function. For isopod body and abdominal coloration, the error structure was considered normally distributed, and an identity (untransformed) link function was used. Using other probability distributions and link functions for the analyses did not affect our conclusions, i.e. the statistical significance of model terms was rather insensitive to model specifications. Spearman correlations were performed between the traits that differed between infected and uninfected isopods to evaluate whether they may be related. A few infected isopods harboured multiple cystacanths ($n=3$), and, if parasites cooperate to manipulate host behaviour (e.g. Poulin *et al*. 2003), these individuals could be a source of bias. Exclusion of these individuals from the data, however, had no effect on the results, so the multiply-infected isopods were also included in the analyses.

Longer observation periods and the relationship between altered traits

In 2 additional experiments, the traits altered by infection in Exp. 1 (hiding behaviour and abdominal colouration; see Results section) were recorded from individual isopods, but longer lengths of observation were used to measure isopod hiding behaviour. This was done because hiding was not measured with high repeatability in the 1-h experiment (see Results section). Our aim was to assess whether longer periods of observation result in more repeatable measurements of behaviour, permitting more reliable correlations between traits altered by infection to be conducted.

Experiment 2 – hiding behaviour measured on an intermediate time-scale (h)

Infected ($n=48$, mean intensity$=1{\cdot}13$) and uninfected ($n=42$) isopods were placed individually in plastic containers ($10\times15\times5$ cm) containing 400 ml of lake water and an unconditioned alder leaf (approximately 14 cm^2) for shelter. They were given 1 h to acclimate. After the acclimation period, isopods were recorded as being exposed (visible from above) or hidden (under the leaves) every 10 min for 8 h. At the end of the first trial of the experiment, unconditioned leaves were removed from the containers and replaced with palatable, conditioned leaves. Isopods were allowed to feed and recover for 2 days before the experiment was repeated. One hour before the second trial of the experiment, conditioned leaves were replaced with unconditioned ones and the water in the containers was changed to remove isopod faeces. This was done in order to have similar conditions for both trials of the experiment. After the second trial, isopods were frozen before being photographed at a later date, in a manner identical to that described for the animals from Exp. 1. In both trials, the 8-h observation period was between 16.00 and 24.00.

Experimental repeatability was assessed using ICC and generalized estimating equations (GEE). GEEs are extensions of generalized linear models that permit the incorporation of repeated-measures (Liang and Zeger, 1986). The GEE tested whether the proportion of time isopods spent exposed changed between the two recording times. The model was defined as having a binomial error structure and a logit link function, and was implemented with the GENLIN function in SPSS. Infection was used as a fixed factor in the model and Bonferroni-adjusted *post hoc* tests were used to compare infected and uninfected isopod behaviour in each trial. The relationship between an individual's abdominal coloration and hiding behaviour, averaged over both trials, was assessed with Spearman correlations. Inclusion or exclusion of isopods harbouring more than 1 cystacanth ($n=5$) in the analyses had no effect on the results, so data from all infected isopods were utilized.

Experiment 3 – hiding behaviour measured on a long time-scale (weeks)

Infected ($n=43$, mean intensity$=1{\cdot}55$) and uninfected ($n=42$) isopods were placed individually in plastic containers ($10\times15\times5$ cm) with 400 ml of lake water. Isopods were continuously provided conditioned alder leaves, which acted as both shelter and an *ad libitum* food supply. For 8 weeks, individuals were recorded twice a day, once in the morning and once in the afternoon, as being exposed or hidden. At the end of the experiment, isopods were frozen before being photographed as described previously. After photographs were taken, isopods were dissected and infection status was noted.

A GEE was used to determine whether the behaviour of infected and uninfected isopods varied between weeks. The proportion of time isopods spent exposed was modelled using binomial errors and a logit link function. Infection was used as a fixed factor in the model and Bonferroni-adjusted *post hoc* tests were used to compare infected and uninfected isopod behaviour for each week. ICCs between weeks were calculated for infected and uninfected

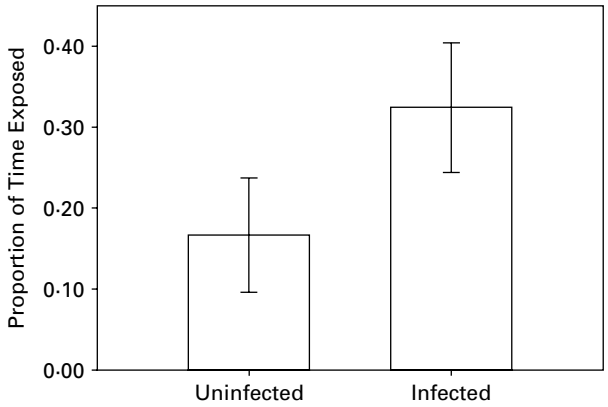

Fig. 2. Average proportion of time uninfected ($n=90$) and infected ($n=62$) isopods spent exposed, not under a leaf shelter, during 1 h of observation. The experiment was conducted in Petri dishes. Bars represent ± 2 standard errors.

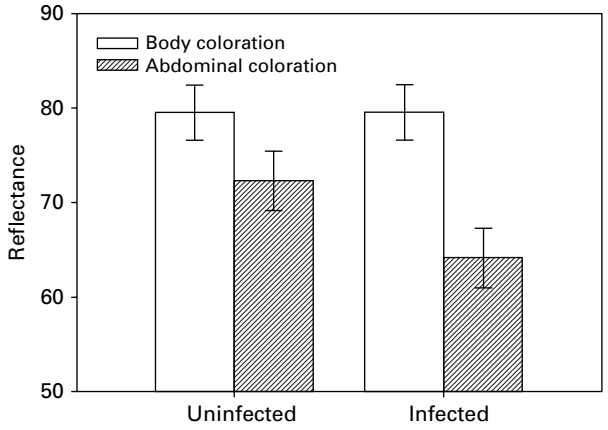

Fig. 3. Average body (pereon) and abdominal (pleon) coloration of uninfected ($n=90$) and infected ($n=62$) isopods. Coloration was measured by taking the mean value of pixel reflectance in photographs of individual isopods. Reflectance values for the first, fourth, and seventh segment were averaged to give a single measure for body coloration. Coloration is lighter at higher values on the scale. Bars represent ± 2 standard errors.

isopods. Two Spearman correlations were used to assess the relationship between hiding behaviour and abdominal coloration. In the first, the average amount of time an individual spent exposed over the entire 8 weeks was used, while in the second only the proportion of time an individual was exposed during the last week of observation was used. Data from the eighth week of observation reflects individual behaviour shortly before abdominal coloration was measured. Inclusion or exclusion of isopods harbouring multiple cystacanths ($n=17$) had no effect on the results, so data from all infected isopods were utilized in the analyses.

All analyses were conducted using SPSS 15.0 (SPSS Inc., Chicago, Illinois) statistical software.

Table 1. Experimental repeatability of the 5 traits recorded from uninfected and infected isopods in Exp. 1

(Intra-class correlation coefficients (ICC) indicate the extent that individual isopods behaved similarly during repeated observations. *F*-tests assess whether ICCs are significantly greater than 0, i.e. whether the association between repeated measurements was greater than expected by chance.)

	n	ICC	*F*	D.F.	*P*
Uninfected					
Hiding	9	0·41	2·39	8, 9	0·108
Activity	15	−0·21	0·66	14, 15	0·779
Substrate choice	15	0·12	1·27	14, 15	0·323
Body coloration	8	0·99	343·65	7, 8	<0·001
Abdominal coloration	8	0·99	219·47	7, 8	<0·001
Infected					
Hiding	27	0·02	1·04	26, 27	0·460
Activity	19	0·70	5·58	18, 19	<0·001
Substrate choice	19	0·01	1·21	18, 19	0·343
Body coloration	11	0·98	95·95	10, 11	<0·001
Abdominal coloration	11	0·99	236·15	10, 11	<0·001

RESULTS

Experiment 1 – traits altered by infection and their inter-relationships

Infected isopods spent less time hiding than uninfected isopods (GLZ, Wald $\chi^2_1=7{\cdot}17$, $P=0{\cdot}007$; Fig. 2), and they had darker abdominal coloration (GLZ, Wald $\chi^2_1=13{\cdot}60$, $P<0{\cdot}001$; Fig. 3). Activity, substrate choice and body pigmentation of infected and uninfected isopods did not differ (GLZs, all Wald $\chi^2_1<3{\cdot}17$, $P>0{\cdot}075$). There were no differences between male and female isopods for any of the 5 traits (GLZs, all Wald $\chi^2_1<1{\cdot}15$, $P>0{\cdot}283$), nor were there any significant interactions between isopod sex and infection (GLZs, all Wald $\chi^2_1<2{\cdot}59$, $P>0{\cdot}107$). Experimental repeatability was low for the 3 behavioural traits, and only the activity of infected isopods seemed to be measured in a repeatable manner (Table 1). The photographic method for quantifying isopod coloration, though, was highly repeatable (Table 1). Hiding and abdominal coloration, the two traits differing between infected and uninfected isopods, were not correlated (Table 2).

Experiment 2 – hiding behaviour on an intermediate time-scale

Overall, infected isopods hid less than uninfected isopods (GEE, Wald $\chi^2_1=12{\cdot}92$, $P<0{\cdot}001$). This was primarily the case during the second 8-h trial; *post hoc* tests indicated that there was a significant difference between infected and uninfected isopods in the second trial, but not the first (Fig. 4).

Table 2. Spearman correlations (r_s) between the 2 traits (hiding behaviour and abdominal coloration) found to differ between infected and uninfected isopods

(In separate experiments, isopod hiding behaviour was recorded for 1 h, 8 h, or over 8 weeks. For the isopods observed for several weeks, 1 correlation was performed using hiding behaviour averaged over the entire 8-week observation period and 1 correlation was conducted in which only behaviour from the last week of observation was considered.)

	n	r_s	*P*
Uninfected			
1 h	90	−0·06	0·54
8 h*	42	0·12	0·46
8 weeks	42	−0·19	0·22
8th week	42	−0·03	0·86
Infected			
1 h	62	−0·19	0·14
8 h*	48	−0·01	0·94
8 weeks	43	−0·10	0·54
8th week	43	0·03	0·84

* The time isopods spent exposed in two 8-h trials was averaged and used as a measure of hiding behaviour in the correlation.

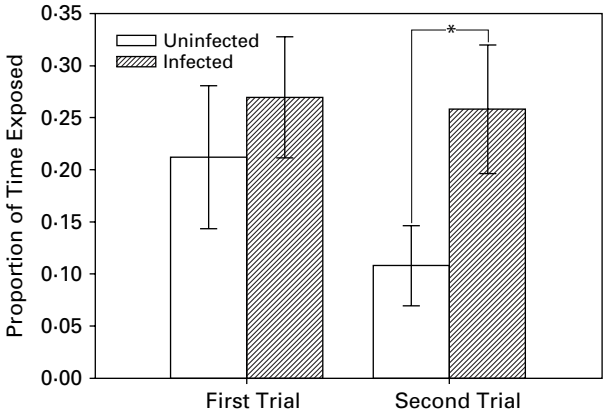

Fig. 4. Average proportion of time uninfected ($n=42$) and infected ($n=48$) isopods spent exposed, not under a leaf shelter, during 8 h of observation. The experiment was conducted twice with 2 days between the trials. Bonferroni-adjusted *post hoc* tests indicated a significant difference, noted by a *, between infected and uninfected isopods during the second trial. Bars represent ±2 standard errors.

In general, isopods spent more time hiding during the second trial (GEE, Wald $\chi^2_1=6{\cdot}49$, $P=0{\cdot}011$; Fig. 4). This result can be largely attributed to increased time spent hiding by uninfected isopods (GEE, interaction between infection and trial, Wald $\chi^2_1=4{\cdot}80$, $P=0{\cdot}028$; Fig. 4). The repeatability of isopod hiding behaviour between the two trials was low (Table 3). As in experiment 1, hiding behaviour and abdominal coloration were not correlated for either infected or uninfected isopods (Table 2).

Table 3. Experimental repeatability of isopod hiding behaviour on 2 time-scales

(Intra-class correlation coefficients (ICC) indicate the extent that individual isopods behaved similarly during repeated observations. *F*-tests assess whether ICCs are significantly greater than 0, i.e. whether isopod behaviour was repeatable on the observed time-scale. In the first case, isopods were observed for 8 h twice and repeatability was calculated from these two trials. In the second case, repeatability statistics were calculated using 8 weekly observations of isopod behaviour. All pairwise combinations of weeks were considered simultaneously in the analysis, i.e. behaviour was not just compared between consecutive weeks. For instance, refuge use in the first week was compared with that in the second, third, fourth weeks, etc.)

	n	ICC	*F*	D.F.	*P*
Uninfected					
8 h	42	0·06	1·13	41, 42	0·343
8 weeks	43	0·13	2·21	42, 301	<0·001
Infected					
8 h	48	0·07	1·14	47, 48	0·323
8 weeks	42	0·24	3·59	41, 294	<0·001

Experiment 3 – hiding behaviour on a long time-scale

On the scale of weeks, isopod hiding behaviour varied over time (GEE, Wald $\chi^2_7=171{\cdot}63$, $P<0{\cdot}001$) and, generally, the time isopods spent exposed increased throughout the experiment (Fig. 5). The interaction between time and infection was significant (GEE, Wald $\chi^2_7=41{\cdot}48$, $P<0{\cdot}001$), indicating that the temporal pattern of behaviour differed between infected and uninfected isopods. During the first 6 weeks of observation, there was not a significant difference between the behaviour of infected and uninfected isopods (Fig. 5). From the fourth week until the end of the experiment, refuge use by uninfected isopods remained at a relatively constant, average level. The time spent exposed by infected isopods, however, continued to increase throughout the experiment, so that by the final weeks of observation infected isopods spent significantly more time exposed than uninfected isopods (Fig. 5). When all 8 weeks of observation were considered jointly, measurements of isopod hiding behaviour were somewhat repeatable (Table 3). Neither an individual's average hiding behaviour over all 8 weeks of observation nor the proportion of time it spent exposed in the final week was correlated with abdominal colouration (Table 2).

DISCUSSION

Like many, if not most, trophically-transmitted parasites (see Moore, 2002) the alteration of host phenotype associated with *A. lucii* infection is multidimensional. Of 5 examined host traits, 2 were

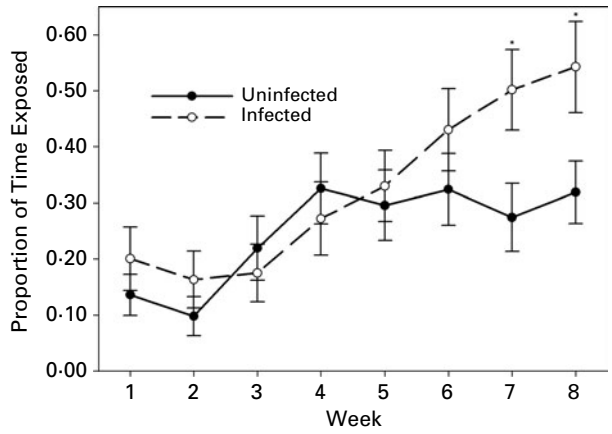

Fig. 5. Average proportion of time spent exposed by uninfected ($n=43$) and infected ($n=42$) isopods over 8 weeks of observation. Isopod hiding behaviour (exposed or hidden) was recorded twice a day and then averaged for each week. Significant differences (Bonferroni-adjusted *post hoc* tests) between infected and uninfected isopods are indicated by a *. Bars represent ± 2 standard errors.

affected by infection, abdominal coloration and hiding behaviour. It is unlikely that these modifications increase isopod fitness. *Acanthocephalus lucii* castrates female hosts (Brattey, 1983) and probably impairs male reproductive behaviour, e.g. male isopods infected with the congener *A. dirus* do not readily engage in pre-copula (Sparkes *et al.* 2006). Thus, in an evolutionary sense the host has died and more or less become an expression of the parasite's phenotype (Kuris, 1997). Moreover, the altered traits likely predispose infected isopods to predation (Brattey, 1983), so they probably reflect adaptive host manipulation by *A. lucii*.

Some *Acanthocephalus* species affect the pigmentation of their intermediate host's whole body, either increasing it (Lyndon, 1996) or decreasing it (Oetinger and Nickol, 1981). There is often variation within species, however, in the frequency that host appearance is altered, e.g. *A. dirus* (Seidenberg, 1973; Amin *et al.* 1980; Oetinger and Nickol, 1981). Infection with *A. lucii* results in darker host respiratory opercula (Brattey, 1983) and abdominal coloration, but host body pigmentation seems unaffected. Infected isopods, thus, tend to have less consistent coloration than uninfected isopods. Unlike other *Acanthocephalus* species (Muzzall and Rabalais, 1975; Camp and Huizinga, 1979; Hetchtel *et al.* 1993; Lyndon, 1996), *A. lucii* was not known to alter the behaviour of its intermediate host. Although additional work is clearly necessary to establish the full scope of variation in host manipulation strategies among *Acanthocephalus* species, a phylogenetic approach may be helpful in explaining which, if any, altered host traits are conserved among species and which may be part of species-specific transmission strategies (Lyndon, 1996).

Cézilly and Perrot-Minnot (2005) suggested that the different host traits manipulated by parasites may be related in various ways, e.g. through a shared mechanism, or a trade-off, or not at all. Here, the two traits differing between infected and uninfected isopods appear unrelated. Regardless of the experimental set-up, the time an individual spent hiding did not have any apparent relationship with its abdominal coloration. This suggests that these traits may be modified via independent mechanisms which are unconstrained by potential trade-offs, e.g. through distinct physiological pathways (Tain *et al.* 2006). Moreover, if these traits are mechanistically and genetically unlinked, they could have been favoured by selection via independent, positive effects on parasite transmission (Bakker *et al.* 1997), even though each alteration may entail distinct energetic costs. For the 1- and 8-h experiments, however, confidence in this null relationship is undermined by the low repeatability with which hiding behaviour was recorded. Individual isopods did not behave similarly in repeated trials of the experiments, so the observed behaviours may or may not be representative of individual average trait values. On the scale of weeks, though, the proportion of time spent exposed each week was relatively consistent within individuals. This is more evident when consecutive weeks are compared rather than considering all 8 weeks jointly. The average of the 7 ICCs between consecutive weeks was 0·55 (range 0·38–0·7) for infected isopods and 0·36 (range 0·06–0·67) for uninfected isopods. This suggests that individually representative measurements of hiding behaviour were more likely obtained from this experiment than the shorter experiments. Therefore, the absence of a correlation between hiding behaviour and abdominal coloration in the long-term experiment suggests, less equivocally, that these traits are unrelated.

It should be noted that slight methodological differences between experiments may have impacted the recorded behaviours, possibly confounding between-experiment comparisons. For example, in the 8-week experiment, leaves did not only serve as shelter for isopods, but also as food. Thus, this experiment may have recorded, to some degree, isopod foraging behaviour rather than refuge use. However, isopods could feed on leaves from either above or below, so their recorded position is likely more indicative of their hiding behaviour than their foraging behaviour.

The ecological relevance of the intra-individual variation in hiding behaviour is not known, but phenotypic flexibility can be favourable if the environment changes rapidly and unpredictably (Piersma and Drent, 2003). Thus, considerable within-individual variability in hiding behaviour might be expected if isopod and/or parasite condition fluctuates. For instance, parasite investment in

or host resistance to manipulation may vary intermittently (Thomas *et al*. 2005). A consequence of this seemingly intrinsic behavioural variability is that short-term experiments are likely insufficient to characterize average individual behaviour. When a trait is highly variable, short observations likely only capture a portion of each individual's trait variability, a portion which may not reflect an individual's longer-term trait average. Multiple observations on individuals are thus less likely to be similar and the obtained data will be characterized by high within-individual variation relative to that between individuals, i.e. repeatability will be negligible. Even on the scale of weeks, intra-individual variation in hiding behaviour may have contributed to random inaccuracies in trait measurement. Thus, even with a moderately repeatable experimental design, there is likely noise in the data which could make a weak relationship between hiding behaviour and abdominal coloration difficult to detect. Without accurate trait measurements, repeatability estimates cannot be interpreted in terms of the sources of phenotypic variation, e.g. as a means to estimate trait heritability (Dohm, 2002).

The measurements of isopod coloration were highly repeatable, suggesting that the acquired values are representative for individual isopods. Unlike the behavioural traits, though, the repeated measurements of coloration were taken almost simultaneously, so the temporal constancy of individual isopod coloration could not be evaluated. If measurements were taken days or weeks apart and isopod coloration varies considerably over time, then within-individual variation in coloration may have been higher and repeatability estimates might have thus been lower. The coloration of individual isopods, though, does not seem to change much on short time-scales (e.g. 2 weeks; Hargeby *et al*. 2004), but growth and development on a longer time-scale may lead to changes in coloration (Hargeby *et al*. 2005). In any case, the repeatability of this method provides an opportunity to explore the sources of between-host variation in a manipulated trait.

Unlike the apparently stochastic fluctuations observed on a shorter time-scale, intra-individual changes in hiding behaviour seemed directional on the scale of weeks. The proportion of time infected and uninfected isopods were exposed tended to increase over 8 weeks. This trend, though, was not identical in both groups. As a consequence, the difference between infected and uninfected isopods varied over time, peaking at the end of the experiment. Isopods were maintained at constant light and temperature, so this variability was presumably not caused by changing environmental factors. Acclimation to laboratory conditions, though, could account for some of the behavioural changes over time. The behaviour of uninfected isopods, for instance, became more consistent around week 4, possibly reflecting adjustment to lab conditions. Infected isopod behaviour, however, did not plateau; the time they were exposed continually increased. Thus, lab acclimation is unlikely to explain all of the temporal variation in hiding behaviour.

Alternatively, the level of parasite-induced host alteration may change over time. Whether the temporal changes in infected isopod behaviour are beneficial for *A. lucii* is unclear, but an increasing probability of host mortality over time might promote intensified manipulation. The temporal behavioural changes were apparently not a consequence of parasite ontogeny; at the onset of the experiment parasites were presumably infective cystacanths. Though plastic parasite strategies have been discussed (Thomas *et al*. 2002), they have received relatively little empirical attention (e.g. Davies and McKerrow, 2003; Poulin, 2003; Vizoso and Ebert, 2005; Medoc *et al*. 2006; Lagrue and Poulin, 2007). Because most experiments examining altered traits are conducted on short time-scales, typically less than a day, plasticity in host manipulation could be commonly overlooked. Flexible manipulation strategies may be favoured, however, by the changes in host condition and behaviour associated with long-term developmental processes, e.g. growth, maturation and/or senescence.

Our study suggests that intra-individual variation, over various time-scales, may characterize some manipulated traits. Consequently, measured levels of host alteration may depend on when and how long individuals are observed. Though this temporal variation within individuals may be ecologically significant, it represents a challenge to designing experiments capable of accurately quantifying the phenotype of individual hosts. Such experiments are necessary, however, to investigate possible relationships between manipulated traits, as well as the causes and consequences of between-individual variation in these traits. As suggested by Thomas *et al*. (2005), elucidation of underlying mechanisms may permit more direct measurements of trait manipulation, and thus be a promising way to circumvent these problems.

D.P.B. was supported by the Biological Interactions Graduate School at the University of Turku, and O.S. received support from the Ella and Georg Ehrnrooth Foundation. We thank Jukka Jokela and two anonymous referees for helpful comments on an earlier draft of this manuscript.

REFERENCES

Andryuk, L. V. (1979). Developmental cycle of the thorny-headed worm, *Acanthocephalus lucii* (Echinorhynchidae). *Parazitologiia* **13**, 530–539 (in Russian).

Amin, O. M., Burns, L. A. and Redlin, M. J. (1980). The ecology of *Acanthocephalus parksidei* (Acanthocephala: Echinorhynchidae) in its isopod

intermediate host. *Proceedings of the Helminthological Society of Washington* **47**, 37–46.

Bakker, T. C. M., Mazzi, D. and Zala, S. (1997). Parasite-induced changes in behavior and color make *Gammarus pulex* more prone to fish predation. *Ecology* **78**, 1098–1104.

Benesh, D. P. and Valtonen, E. T. (2007). Effects of *Acanthocephalus lucii* (Acanthocephala) on intermediate host survival and growth: implications for exploitation strategies. *Journal of Parasitology* **93**, 735–741.

Brattey, J. (1983). The effects of larval *Acanthocephalus lucii* on the pigmentation, reproduction and susceptibility to predation of the isopod *Asellus aquaticus*. *Journal of Parasitology* **69**, 1172–1173.

Brattey, J. (1986). Life history and population biology of larval *Acanthocephalus lucii* (Acanthocephala: Echinorhynchidae) in the isopod *Asellus aquaticus*. *Journal of Parasitology* **72**, 633–645.

Brown, S. P. (1999). Cooperation and conflict in host-manipulating parasites. *Proceedings of the Royal Society of London, B* **266**, 1899–1904.

Camp, J. W. and Huizinga, H. W. (1979). Altered color, behavior, and predation susceptibility of the isopod *Asellus intermedius* infected with *Acanthocephalus dirus*. *Journal of Parasitology* **65**, 667–669.

Cézilly, F. and Perrot-Minnot, M.-J. (2005). Studying adaptive changes in the behaviour of infected hosts: a long and winding road. *Behavioural Processes* **68**, 223–228.

Davies, S. J. and McKerrow, J. H. (2003). Developmental plasticity in schistosomes and other helminths. *International Journal for Parasitology* **33**, 1277–1284.

Dohm, M. R. (2002). Repeatability estimates do not always set an upper limit to heritability. *Functional Ecology* **16**, 273–280.

Graca, M. A. S., Maltby, L. and Calow, P. (1993). Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus* I: feeding strategies. *Oecologia* **96**, 139–144.

Hargeby, A., Johansson, J. and Ahnesjö, J. (2004). Habitat-specific pigmentation in a freshwater isopod: adaptive evolution over a small spatiotemporal scale. *Evolution* **58**, 81–94.

Hargeby, A., Stoltz, J. and Johansson, J. (2005). Locally differentiated cryptic pigmentation in the freshwater isopod *Asellus aquaticus*. *Journal of Evolutionary Biology* **18**, 713–721.

Hasu, T., Holmes, J. C. and Valtonen, E. T. (2007). Isopod size and *Acanthocephalus lucii* infection. *Journal of Parasitology* **93**, 450–457.

Hetchtel, L. J., Johnson, C. L. and Juliano, S. A. (1993). Modification of antipredator behavior of *Caecidotea intermedius* by its parasite *Acanthocephalus dirus*. *Ecology* **74**, 710–713.

Hindsbo, O. (1972). Effects of *Polymorphus* (Acanthocephala) on colour and behaviour of *Gammarus lacustris*. *Nature, London* **238**, 333.

Kuris, A. (1997). Host behavior modification: an evolutionary perspective. In *Parasites and Pathogens, Effects on Host Hormones and Behavior* (ed. Beckage, N. E.), pp. 231–245. Chapman and Hall, New York.

Lagrue, C. and Poulin, R. (2007). Life cycle abbreviation in the trematode *Coitocaecum parvum*: can parasites adjust to variable conditions? *Journal of Evolutionary Biology* **20**, 1189–1195.

Liang, K.-Y. and Zeger, S. L. (1986). Longitudinal data analysis using generalized linear models. *Biometrika* **73**, 13–22.

Lyndon, A. R. (1996). The role of acanthocephalan parasites in the predation of freshwater isopods by fish. In *Aquatic Predators and their Prey* (ed. Greenstreet, S. P. R. and Tasker, M. L.), pp. 26–32. Blackwell Scientific, Oxford.

Medoc, V., Bollache, L. and Beisel, J.-N. (2006). Host manipulation of a freshwater crustacean (*Gammarus roeseli*) by an acanthocephalan parasite (*Polymorphus minutus*) in a biological invasion context. *International Journal for Parasitology* **36**, 1351–1358.

Moore, J. (1983). Responses of an avian predator and its isopod prey to an acanthocephalan parasite. *Ecology* **64**, 1000–1015.

Moore, J. (2002). *Parasites and the Behavior of Animals*. Oxford University Press, New York.

Müller, R. and Büttner, P. (1994). A critical discussion of intraclass correlation coefficients. *Statistics in Medicine* **13**, 2465–2476.

Muzzall, P. M. and Rabalais, F. C. (1975). Studies on *Acanthocephalus jacksoni* Bullock, 1962 (Acanthocephala: Echinorhynchidae). III. The altered behavior of *Lirceus lineatus* (Say) infected with cystacanths of *Acanthocephalus jacksoni*. *Proceedings of the Helminthological Society of Washington* **42**, 116–118.

Oetinger, D. F. and Nickol, B. B. (1981). Effects of acanthocephalans on pigmentation of freshwater isopods. *Journal of Parasitology* **67**, 672–684.

Piersma, T. and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology and Evolution* **18**, 228–233.

Poulin, R. (1994). The evolution of parasite manipulation of host behaviour: a theoretical analysis. *Parasitology* **109**, S109–S118.

Poulin, R. (2003). Information about transmission opportunities triggers a life-history switch in a parasite. *Evolution* **57**, 2899–2903.

Poulin, R., Nichol, K. and Latham, A. D. M. (2003). Host sharing and host manipulation by larval helminthes in shore crabs: cooperation or conflict? *International Journal for Parasitology* **33**, 425–433.

Seidenberg, A. J. (1973). Ecology of the acanthocephalan, *Acanthocephalus dirus* (Van Cleave, 1931) in its intermediate host, *Asellus intermedius* Forbes (Crustacea: Isopoda). *Journal of Parasitology* **59**, 957–962.

Sparkes, T. C., Weil, K. A., Renwick, D. T. and Talkington, J. A. (2006). Development-related effects of an acanthocephalan parasite on pairing success of its intermediate host. *Animal Behaviour* **71**, 439–448.

Tain, L., Perrot-Minnot, M.-J. and Cézilly, F. (2006). Altered host behaviour and brain serotonergic activity caused by acanthocephalans: evidence for specificity. *Proceedings of the Royal Society London, B* **273**, 3039–3045.

Thomas, F., Adamo, S. and Moore, J. (2005). Parasitic manipulation: where are we and where should we go? *Behavioural Processes* **68**, 185–199.

Thomas, F., Brown, S. P., Sukhdeo, M. and Renaud, F. (2002). Understanding parasite strategies: a state-dependent approach? *Trends in Parasitology* **18**, 387–390.

Vizoso, D. B. and Ebert, D. (2005). Phenotypic plasticity of host-parasite interactions in response to the route of infection. *Journal of Evolutionary Biology* **18**, 911–921.

Wilson, K. and Grenfell, B. T. (1997). Generalized linear modelling for parasitologists. *Parasitology Today* **13**, 33–38.