Strong association between parasitism and phenotypic variation in a supralittoral amphipod

C. Lagrue, K. Heaphy, B. Presswell, R. Poulin*

Department of Zoology, University of Otago, Dunedin 9054, New Zealand

ABSTRACT: Phenotypic variation is common among conspecifics from the same population, but its causes are not always clear. Parasites may often play a role, as their ability to induce phenotypic changes in their hosts is well established, though not widely acknowledged among marine ecologists. Here, we tested for a possible role of parasites as determinants of marked colour polymorphism, as well as variation in several distinct behavioural traits in the supralittoral amphipod *Transorchestia chiliensis* (Talitridae). Our results indicate that the juvenile stages of 2 parasites, the acanthocephalan *Plagiorhynchus allisonae* and a dilepidid cestode, are disproportionately common in certain colour morphs (i.e. green and dark grey in the case of acanthocephalans and green and blue in the case of cestodes) relative to other colour morphs. In addition, amphipods preferring light over dark background substrate were more likely to harbour acanthocephalans, whereas amphipods that either stayed at the surface or did not burrow very deeply harboured more cestodes than those that burrowed deep into the sediment. Our findings also indicate that later developmental stages of *P. allisonae* are associated with more pronounced host phenotypic variation, suggesting that phenotypic changes escalate in parallel with parasite growth within the host. Furthermore, the 2 parasite species tend not to co-occur in the same individual hosts; thus, their distinct phenotypic effects apply to different subsets of the host population. We suggest that the role of parasitism in inducing and maintaining phenotypic variation within populations of marine invertebrates is probably more important than currently recognised.

KEY WORDS: Behavioural changes · Colour polymorphism · Host manipulation · Phenotypic variation · Talitridae · Acanthocephalans · Cestodes · *Transorchestia chiliensis*

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INTRODUCTION

The nature and extent of phenotypic variation among individuals in a population is a key determinant of the strength or outcome of intra- and inter-specific interactions as well as natural selection (Wilson 1998, Bolnick et al. 2011). Phenotypic variation results from genetic, epigenetic and environmental influences, as well as interactions among these factors (Mousseau et al. 2000, Bossdorf et al. 2008). The potential role of parasites as agents capable of inducing phenotypic variability among conspecific individuals is now well established (Poulin & Thomas 1999). In particular, trophically transmitted helminth para-sites, whose juvenile stages in intermediate hosts must be transmitted to a definitive host through predation, are notorious for their ability to manipulate host phenotype, causing changes in a host's behaviour or appearance that enhance its susceptibility to predatory definitive hosts (Lafferty 1999, Moore 2002, Thomas et al. 2005, Poulin 2010). Typically, multiple host traits can be affected in parallel, resulting in a markedly altered host phenotype (Thomas et al. 2010). There are a growing number of studies on this phenomenon; however, the majority are focused on a very small number of model systems involving mainly freshwater or terrestrial organisms (Poulin & Maure 2015). Knowledge of the impact of parasitism

on intraspecific phenotypic variability in marine systems remains limited.

Here, we investigate the link between parasitic infection and phenotypic variation in the supralittoral amphipod *Transorchestia chiliensis* (Milne-Edwards, 1840) (Talitridae). This small amphipod is common along South Pacific shorelines from Chile to New Zealand, where it feeds on rotting algae and organic matter (Catenazzi & Donnelly 2007). Previous studies on *T. chiliensis* have made no mention of colour polymorphism among individuals of the same population (Marsden 1984, Marsden et al. 2003, Catenazzi & Donnelly 2007). However, at our study site in Lower Portobello Bay (Otago Harbour, South Island, New Zealand), we observed pronounced variation in body colouration of *T. chiliensis* individuals (see Fig. 1) on a very small spatial scale, i.e. even among individuals collected under the same rock. Dissection of a small subsample of these *T. chiliensis* revealed relatively common infections by the juvenile stages of 2 parasite species: 1 acanthocephalan and 1 cestode. Identification of these parasites (see 'Results') indicated that their definitive hosts were most certainly birds, probably oystercatchers and/or ducks. Transmission of these parasites from amphipods to birds occurs via predation, therefore any parasite-induced change in colouration or behaviour that increases the conspicuousness or vulnerability of the amphipods could benefit the parasites.

If body colouration in *T. chiliensis* is not genetically determined, there are good reasons to believe that one or both of the above parasites may be responsible for the marked colour polymorphism seen in this amphipod population. Several studies have reported a clear association between presence or absence of acanthocephalans and colour variation in amphipods or isopods among conspecific individuals from the same locality (Hindsbo 1972, Camp & Huizinga 1979, Oetinger & Nickol 1981, Benesh et al. 2008). In addition, acanthocephalans frequently manipulate the behaviour of their arthropod intermediate host in ways that enhance predation risk by the parasite's definitive host (e.g. Camp & Huizinga 1979, Moore 1983, Bakker et al. 1997, Lagrue et al. 2007). In fact, the ability to manipulate host phenotype is believed to be an ancestral trait in the phylum Acanthocephala, as most species investigated have proven capable of altering host behaviour or appearance (Moore 1984, 2002). Cestodes are also capable of modifying the behaviour (Poulin et al. 1992, Robb & Reid 1996, Franz & Kurtz 2002, Sánchez et al. 2007) or colouration (LoBue & Bell 1993, Trabalon et al. 2000, Sánchez et al. 2006) of their intermediate hosts.

The occurrence of these 2 parasites in our study population may therefore account for the large variation in body colouration, a phenomenon not previously reported from other populations in New Zealand or elsewhere.

The aim of this study was to test the hypothesis that phenotypic variation in the amphipod population reflects parasite infections. More specifically, we aimed to (1) identify as precisely as possible the 2 parasite taxa found in the study population, (2) quantify population-wide infection levels for both parasite species, stratified by host sex and size, (3) test for a statistical link between parasite infection and host colour polymorphism, and (4) test for associations between infection level and 3 distinct behavioural phenotypes. Overall, our study provides a general test of whether or not parasitism can account for marked phenotypic variation which has no other plausible external causes.

MATERIALS AND METHODS

Field sampling and laboratory processing

Amphipods were collected by hand at low tide from the gravel and silt around piles of rocks frequented by shorebirds in Lower Portobello Bay, Otago Harbour, South Island, New Zealand (45° 52′ S, 107° 42′ E). There were 3 collections. First, on 25 November 2015, 4 samples were taken by placing a quadrat (25 × 25 cm; 0.0625 m^2 surface area) in a haphazardly chosen location, pushing it into the sediment and exhaustively collecting all amphipods within the quadrat area to a depth of 10 cm. Rocks within the area were lifted to uncover all amphipods. These 4 samples were used to quantify the average density of amphipods at the site, structured by sex and size, to determine population-level measures of infection and to assess the frequency of different colour morphs in the population. Second, a further 4 samples were obtained between 10 and 22 December 2015; these consisted of selectively hand-picking amphipods mostly belonging to rare colour morphs. By combining individuals from this second collection with those obtained from the initial 4 quadrat samples, we had sufficient numbers of individuals of all colours to test for an association between parasitism and body colour. Third, we collected a final set of amphipods between 4 and 29 January 2016, by haphazardly gathering as many individuals as possible from all colour morphs. These were used in the behavioural tests described below.
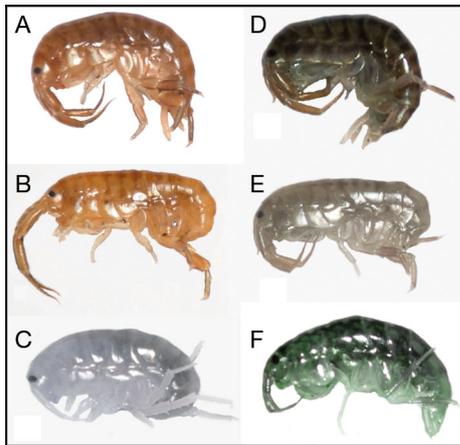
Fig. 1. Colour morphs in the population of the amphipod *Transorchestia chiliensis* from Lower Portobello Bay, Otago Harbour, New Zealand. Shown are individuals of (A) brown, (B) light brown, (C) blue, (D) dark grey, (E) grey and (F) green morphs

Amphipods were returned alive to the laboratory in 2 l plastic containers lined with substrate taken from the collection site; the substrate was kept moist by regular water spraying. In the days following capture (or after the completion of the behavioural tests in the case of the third collection), each individual was measured (height of the 4th coxal plate, to the nearest 0.1 mm; Bollache et al. 2000) and sexed (males have enlarged gnathopods used for mate guarding) under a dissecting microscope. In the case of gravid females, the number of offspring held within the marsupial pouch was counted and recorded. All amphipods were also assigned to 1 of 6 colour categories: grey, dark grey, brown, light brown, blue, and green (Fig. 1), always by the same observer to ensure consistency. Photographs were taken of the first individuals assigned to each category, and used as benchmarks for later individuals to ensure standardised colour matching. Finally, the amphipods were dissected and all cestodes and acanthocephalans found within their body cavity were counted. Acanthocephalans were separated into 3 distinct and successive developmental stages: acanthella, early cystacanth and late cystacanth (Kennedy 2006), and counted separately.

Parasite identification

Acanthocephalan specimens found in *Transorchestia chiliensis* were of variable age, ranging from young acanthellae to mature cystacanths. Represen-

tatives of all stages were manually excysted, with the proboscis everted by pressure in some cases. No proboscis was seen fully everted, but the inverted hooks could easily be seen through the wall of the proboscis. Specimens were cleared and temporarily mounted in beechwood creosote, either stained with acetic iron carmine or unstained, for microscopy and measurements. All recovered cestodes were at the metacestode stage; they were lightly squashed under a coverslip in saline temporary mounts for microscopy and measurements.

Three acanthocephalan specimens (1 acanthella, 1 early cystacanth and 1 late cystacanth) were characterised molecularly. Genomic DNA was extracted from ethanol-fixed isolates in 200 μl of a 5% suspension of Chelex® in deionised water and containing 0.1 mg ml^{-1} proteinase K followed by incubation at 56°C for 5 h, boiling at 90°C for 8 min, and centrifugation at 14 000 × g for 10 min. Partial fragments of the large ribosomal subunit (28S) were amplified (1800 bp; primers U178F: 5′-GCA CCC GCT GAA YTT AAG-3′ and L1642R: 5′-CCA GCG CCA TCC ATT TTC A-3′; Lockyer et al. 2003). Polymerase chain reaction (PCR) amplifications were performed in 25 μl reactions containing 5 μl of extraction supernatant, 1× PCR buffer (16 mM [NH$_4$]2SO$_4$, 67 mM Tris-HCl at pH 8.8), 2 mM MgCl$_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 0.5 mM each primer, and 0.7 units BIOTAQ™ DNA polymerase (Bioline). Thermocycling conditions used for amplification of the 28S region followed Blasco-Costa et al. (2009). PCR amplicons were purified prior to sequencing using exonuclease I and shrimp alkaline phosphatase enzymes (Werle et al. 1994). Amplicons were cycle-sequenced from both strands using PCR primers, employing BigDye® Terminator v.3.1 Ready Reaction Cycle Sequencing Kit, alcohol-precipitated and run on an ABI 3730XL Analyser (Applied Biosystems). Contiguous sequences were assembled and edited using BioEdit v.7 (Hall 1999) and submitted to GenBank (accession number KU922939).

Newly generated 28S rDNA sequences together with published sequences of the acanthocephalan order Polymorphida from GenBank were analysed using ClustalW implemented in MEGA v.6 (Tamura et al. 2013). The extremes of the alignments were trimmed to match the shortest sequence prior to phylogenetic analyses. The final 28S dataset included 18 representative sequences of polymorphid species retrieved from GenBank. Three sequences of species belonging to the order Echinorhynchida were included as outgroups. Phylogenetic analysis was conducted in MEGA v.6 and inferred using the maximum

likelihood method based on the Tamura-Nei model. The analysis involved 891 bp, and only the tree with the highest log likelihood (−8690.43) was retained.

DNA was also extracted from one cestode specimen and sequenced for 28S using the same primers and conditions. This produced a sequence 600 bp long that was used in a BLASTn search (http://blast. ncbi.nlm.nih.gov/) on GenBank.

Behavioural tests

After 1 to 4 d to recover from capture and acclimate to lab conditions (18 to 20°C, 12 h light:12 h dark photoperiod) in groups within 2 l plastic containers lined with moistened substrate and food from the field, all individuals from the third collection were each put through 3 behavioural tests. The order of tests was alternated among amphipods, to avoid any bias due to habituation or other factors related to time in captivity. All tests were carried out under an artificial light (standard fluorescent tube, 2.5 m above the dish) at room temperature (20°C). Between tests, amphipods were individually maintained for 2 to 10 h in labelled plastic containers (4.5 cm diameter, 6 cm high) with a 1.5 cm thick layer of moistened substrate at the bottom.

The first behavioural test allowed the amphipod to choose between light and dark background colourations. The test arena consisted of a plastic doughnut-shaped enclosure (outside and inside diameter: 25 and 5 cm, respectively), open at the top, with 10 cm high sides. The arena was divided into 8 angular sections of equal size (i.e. sections shaped like pieces of pie); the bottom and sides of half of them were covered in black tape, and the other half were left uncovered showing the enclosure's white colour. This produced 4 zones with light and 4 with dark background colouration. The base of the arena was covered with a sprinkling of moist sand from the field to provide a more natural substrate while still allowing the light or dark background colourations to come through clearly. An amphipod was placed on a randomly chosen location within the arena, and after 10 min of ac-climation, the background colouration on which it stood was then recorded 6 times, at 10 min intervals. A sighting on the light background was scored as 0, and one on the dark background was scored as 1; the sum of these scores across all 6 recordings gave an index ranging from 0 (always on the light background) to 6 (always on the dark background).

The second test measured the amphipod's preference for being hidden under cover versus exposed on the surface. The arena consisted of a clear plastic Petri dish (8.5 cm diameter), with half of the lid and sides covered in black tape and the other half uncovered. Here again, the base of the dish was covered with moist sand from the field to provide a more natural substrate. An amphipod was placed in the centre of the dish, and after 10 min of acclimation, whether it was exposed or under cover was recorded 6 times at 10 min intervals. An exposed sighting was scored as 0 and being under cover was scored as 1; the sum of these scores across all 6 recordings gave an index ranging from 0 (always exposed) to 6 (always hidden under cover).

The third behavioural test measured an amphipod's tendency to burrow under the sediment. A plastic tube (1.5 cm diameter, 10 cm high) was filled almost to the top with a 9 cm thick layer of moist sand from the collection site. An amphipod was placed in the centre of the tube, the lid was screwed on, and the amphipod was given 15 min to acclimate. Following this, whether it was at the surface or buried under sediment was recorded 8 times, at 15 min intervals. A surface sighting was scored as 0 and being buried under sediment was scored as 1; the sum of these scores across all 8 recordings gave an index ranging from 0 (always at the surface) to 8 (always buried). The depth at which the amphipod was buried at the end of the experiment was also recorded and scored from 0 (at the surface, not buried) to 4 (buried to the maximum allowed depth). Thin slots on the side of the tube at 1.5 cm intervals allowed rigid plastic sheets to be inserted, which divided the sediment into 5 depth zones from which the amphipod was recovered (amphipods never occurred in the fifth and deepest zone).

Statistical analyses

All analyses were conducted in JMP v.11.0 (SAS Institute). Population trends based on the 4 quadrat samples obtained during the first field collection were tested using either 1-way ANOVAs to compare body sizes among particular groups, or correlation coefficients (Spearman's rho, r_S) testing for a relationship between pairs of variables such as body size, numbers of parasites per individual host, or number of offspring per female.

To test for an association between parasitism and host body colouration, we used a multinomial logistic regression, since our response variable was a nominal variable with 6 categories (grey, dark grey, brown, light brown, blue and green). The predictors

were amphipod body size, sex, number of acantho-cephalans and number of cestodes (both $\log_{10} + 1$ transformed). We focused on possible biological determinants of body colouration, and did not include the sample of origin as a predictor since the analysis combined the first (random quadrat samples) and second (samples targeting mostly rare colour morphs) field collections. We repeated the multinomial logistic regression by replacing the number of acanthocephalans as a predictor, with the separate numbers of acanthellae, early cystacanths and late cystacanths (all log-transformed), and compared the 2 models using their corrected Akaike information criterion (AIC_c) scores.

To test for an association between parasitism and host behaviour, we used generalised linear mixed models (GLMMs) with Poisson error structure separately for each behavioural measure (choice of substrate colour, use of cover, tendency to burrow and burrowing depth). The predictors were amphipod body size, sex (3 categories: males, females with offspring and females without offspring), number of acanthocephalans and number of cestodes (both $\log_{10} + 1$ transformed); colour morph was used as a random factor to account for possible indirect influences of body colouration on behaviour. Again, we repeated the GLMMs by replacing the number of acanthocephalans as a predictor with the separate numbers of acanthellae, early cystacanths and late cystacanths (all log-transformed), and compared the 2 models using their AIC_c scores.

RESULTS

Amphipod population characteristics

The 4 quadrat samples yielded 541 *Transorchestia chiliensis* individuals, for an average density (mean ± SE) of 2164 ± 287 ind. m^{-2}. Mean body size (measured as height of the 4th coxal plate) was 2.12 ± 0.02 mm, though the size frequency distribution showed a hint of bimodality, suggesting either sexual size dimorphism or that there may have been 2 cohorts within our samples (Fig. 2). The female-to-male sex ratio across the 4 samples was 4.96 ± 0.48, although this may be an overestimation since some younger males may have been misclassified as females because they had not yet developed enlarged gnathopods. Males were significantly larger than females (2.44 ± 0.04 vs. 2.05 ± 0.02 mm; ANOVA, $F_{1,539} = 65.676$, p < 0.0001). Among the 447

females, 147 (32.9%) carried offspring in their marsupium, with an average of 9.4 ± 0.3 female^{-1}. Excluding those without offspring, there was no correlation between female size and the number of off-
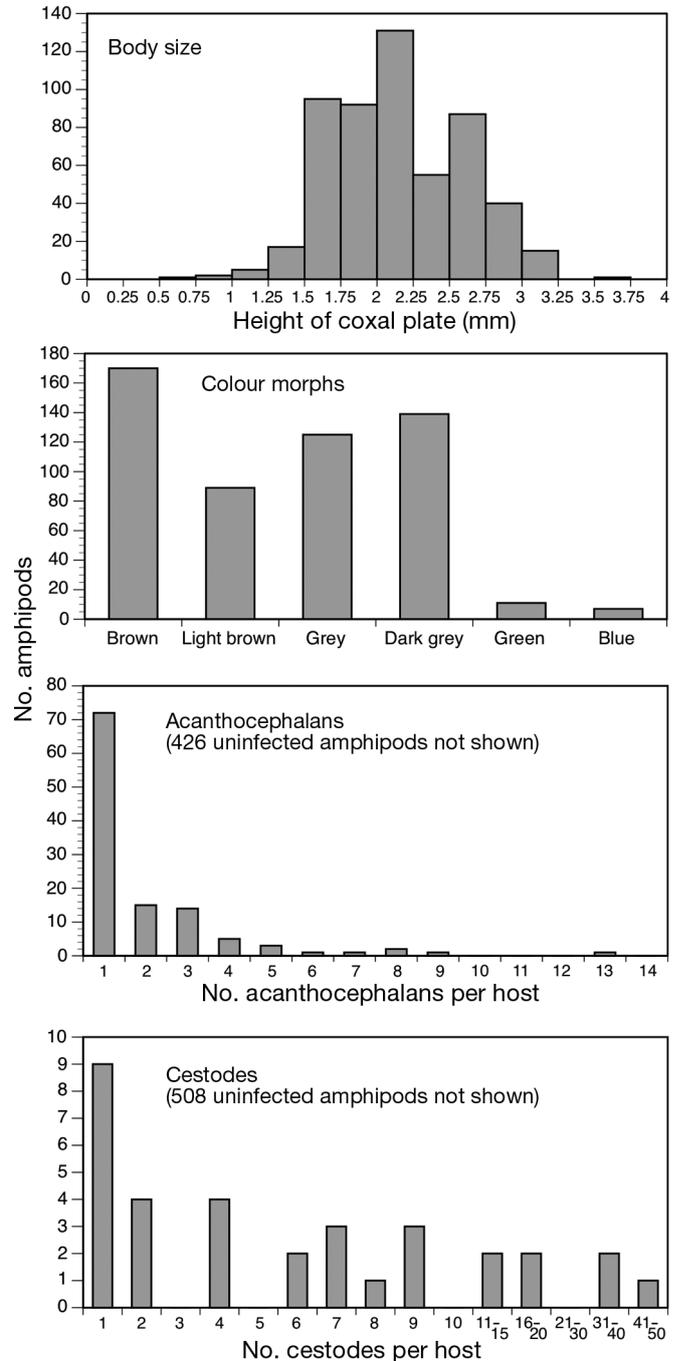

Fig. 2. Frequency distributions of body sizes (measured as height of the 4th coxal plate), colour morphs, numbers of acanthocephalans per host, and numbers of cestodes per host, among 541 individual *Transorchestia chiliensis* amphipods from Lower Portobello Bay, Otago Harbour, New Zealand

spring it carried (Spearman's rho, $r_S = 0.0614$, p = 0.460).

The 6 colour morphs showed unequal frequencies across all 541 amphipods sampled, with blue and green morphs being particularly rare (Fig. 2). Blue individuals ranged from pale to dark, but were lumped into a single category as very few were found in total. Males and females were represented in all colour morphs in roughly similar proportions. There were significant differences in body size among colour morphs ($F_{5,535} = 8.630$, p < 0.0001), though there were only few pairwise differences (Tukey-Kramer HSD post hoc tests) involving either the smallest (light brown) or largest (dark grey) morphs.

Of the 541 amphipods examined, 115 were infected with acanthocephalans, giving a prevalence of 21.3%. The frequency distribution of numbers of acanthocephalans per host was highly skewed, with most individuals harbouring only 1 to 3 parasites (Fig. 2). Of the 230 acanthocephalans recovered, 83 (36.1%) were acanthellae, 103 (44.8%) were early cystacanths, and 44 (19.1%) were late cystacanths. Including only amphipods harbouring at least 1 acanthocephalan, we found generally negative correlations among hosts between numbers of parasites at different developmental stages (acanthellae vs. early cystacanths: $r_S = -0.3072$, p = 0.0008; acanthellae vs. late cystacanths: $r_S = -0.2256$, p = 0.0153; early vs. late cystacanths: $r_S = -0.0399$, p = 0.672). This shows in particular that amphipods harbouring acanthellae are unlikely to also harbour cystacanths, and vice versa. The prevalence of cestodes was much lower, i.e. 6.1% (33 infected amphipods out of 541). Infected individuals often harboured many cestodes; the maximum being 44 cestodes in a single amphipod (Fig. 2). Including only the 135 amphipods harbouring at least 1 parasite of either taxon, there was a clear negative relationship ($r_S = -0.416$, p < 0.0001) between the number of acanthocephalans and the number of cestodes per host: amphipods with acanthocephalans tended not to harbour cestodes, and vice versa (Fig. 3).

Amphipod body size correlated weakly but significantly with both the number of acanthocephalans ($r_S = 0.408$, p < 0.0001) and the number of cestodes per host ($r_S = 0.125$, p = 0.0003), i.e. infected amphipods were generally a little larger than uninfected ones. For the 147 females with at least one offspring in their marsupium pouch, there was no correlation between the number of offspring carried and either the number of acanthocephalans ($r_S = 0.1234$, p = 0.136) or the number of cestodes ($r_S = 0.0864$, p = 0.298) harboured.
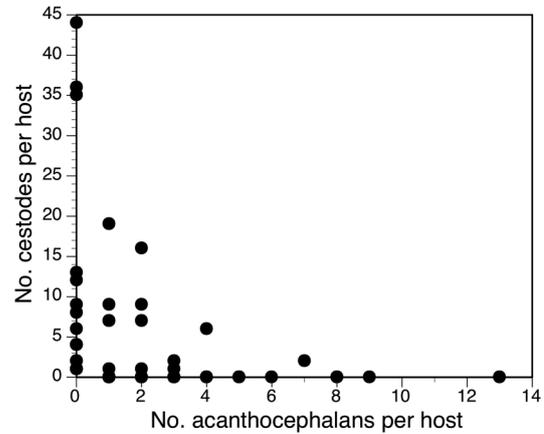

Fig. 3. Number of cestodes per host versus the number of acanthocephalans per host across 135 *Transorchestia chiliensis* amphipods harbouring at least one parasite of either taxa. Note that several points are stacked at coordinates (0,1) and (1,0)

Parasite identification

Acanthocephalan cystacanths found in *T. chiliensis* are almost certainly assignable to *Plagiorhynchus* (*Plagiorhynchus*) *allisonae* Smales, 2002. Adults of this species have previously been found in the South Island pied oystercatcher *Haematopus finschi* Martens and the variable oystercatcher *H. unicolor* Forster in New Zealand (Smales 2002). Except for overall size (trunk length and width), which is a function of maturity (i.e. cystacanths are smaller than adults), all morphometrics were identical (Table 1). In addition, our specimens agreed with the original description of the aspinous trunk, cylindrical pro-

Table 1. Comparative morphometrics of adult *Plagiorhynchus allisonae* (data from Smales 2002) and a single female cystacanth from the amphipod *Transorchestia chiliensis*. Only female morphometrics are shown

| | *P. allisonae* | |
	adults Smales (2002)	cystacanth This study
Proboscis hook no. rows	18–23	18–22
Proboscis no. hooks row^{-1}	14–20	ca. 16
Largest hook length (μm)	49–50	47–50
Smallest hook length (μm)	30–33	30–35
Trunk length (mm)	3.23–9.5	2.5
Trunk width (mm)	1.2–2.9	0.72
Proboscis length (mm)	0.97–1.19	1.05
Proboscis width (mm)	0.20–0.30	0.20
Proboscis receptacle length (mm)	0.94–1.11	0.95
Proboscis receptacle width (mm)	0.24–0.34	0.29

boscis, size, shape and distribution of the hooks, and presence of spiral muscle fibres in the proboscis receptacle. The 28S sequences were identical among the 3 specimens analysed, confirming that they were developmental stages of the same species. Although no sequences of *P. allisonae* were available for direct comparison, in a preliminary phylogenetic tree of relationships based on 28S sequences (Fig. 4), the specimens were closely related to *Plagiorhynchus cylindraceus*. However, uncorrected p-distances between the *Transorchestia* acanthocephalan and all other branches were high (between 0.32 and 0.38), suggesting that the relationship with *P. cylindraceus* is not very close. Sequencing of further genes and (most importantly) more species of *Plagiorhynchus* will be needed to elucidate these relationships in more detail.

The larval (metacestode) stage of the cestode found in *T. chiliensis* was of the cysticercoid type (Chervy 2002), with an oval outer capsule (300 × 260 μm) and cercomer. The scolex bore suckers, which were ca. 100 × 80 μm, and there were 20 rostellar hooks in 2 rows that were only slightly offset from each other, the hooks being similar in size and shape and 28 to 30 μm long. The hook size and shape did not correspond to any of the cestodes that have been reported thus far from New Zealand birds (McKenna 2010). A BLASTn search using the partial 28S sequence obtained confirmed the cestode to be a species of the family Dilepididae. A lack of comparable data on GenBank precluded any closer identification than family level.

Parasitism and colour polymorphism

Combining the 4 quadrat samples from the first field collection with the 4 targeted samples from the second collection resulted in 824 amphipods for analysis of the determinants of body colour, although numbers belonging to rare colour morphs were still
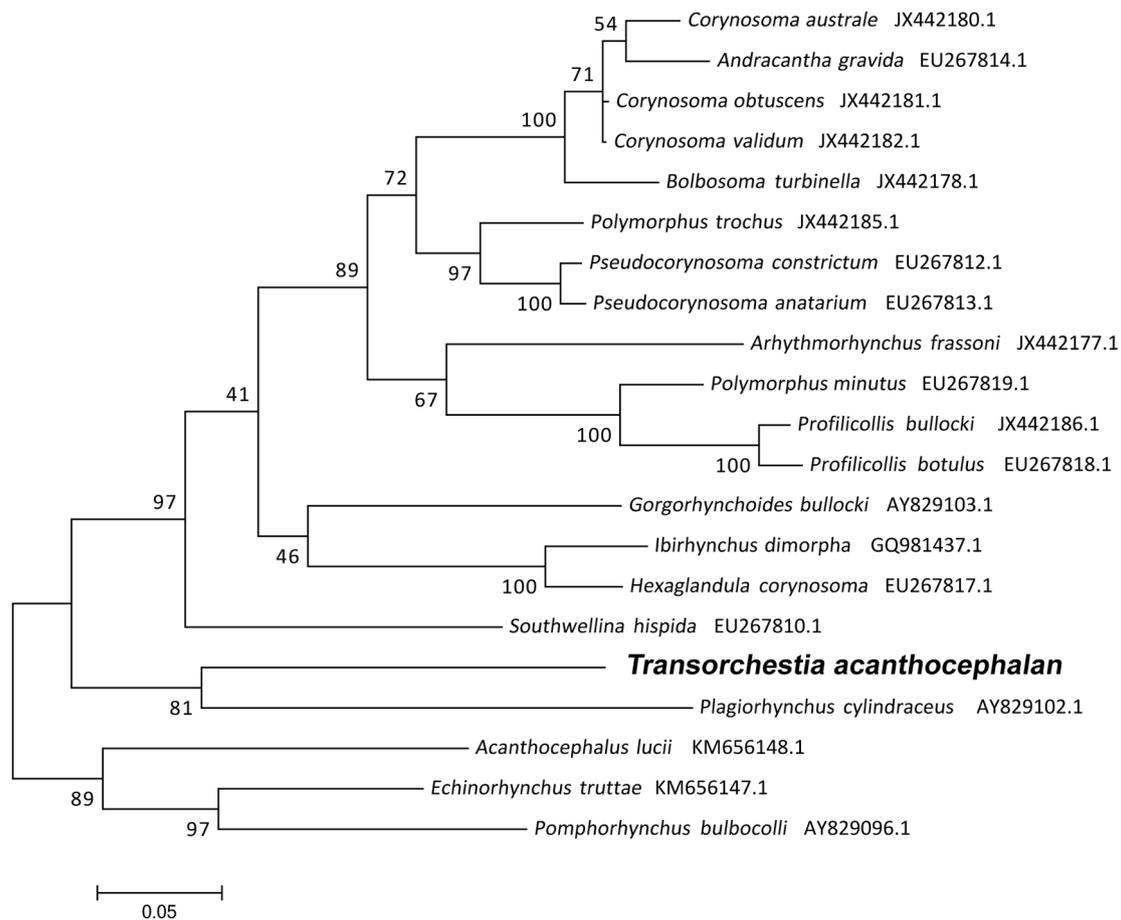

Fig. 4. Maximum likelihood tree showing the phylogenetic relationship between the acanthocephalan found in the amphipod *Transorchestia chiliensis* (in **bold**), and other acanthocephalan species (GenBank accession number follows species name), based on 28S rDNA sequences. Bootstrap support values (% from 1000 iterations) shown for each node. Scale bar: number of substitutions site^{-1}

Table 2. Multinomial logistic regressions testing potential predictors of body colouration in the amphipods, showing the likelihood ratio tests for each predictor, the corrected Akaike Information Criterion (AIC_c) score for each model and the difference (ΔAIC_c) from the best model

Model Predictor	df	χ^2	p-value	AIC_c	ΔAIC_c
Best					
Body size	5	79.253	<0.0001	2492.31	0
Sex	5	60.707	<0.0001		
No. acanthellae	5	9.157	0.1030		
No. early cystacanths	5	16.956	0.0046		
No. late cystacanths	5	12.829	0.0250		
No. cestodes	5	26.667	<0.0001		
Other					
Body size	5	77.428	<0.0001	2496.39	4.08
Sex	5	60.227	<0.0001		
No. acanthocephalans	5	14.132	0.0148		
No. cestodes	5	27.057	<0.0001		

included 50 grey, 64 dark grey, 57 brown, 23 light brown, 40 blue and 20 green individuals. Of the 196 acanthocephalans recovered from these amphipod hosts, all were at the early or late cystacanth stage; no acanthellae were found in these individuals. There was no correlation between any pair of behavioural traits across those amphipods (choice of substrate colour vs. use of cover: $r_S = 0.0241$, $p = 0.702$; choice of substrate colour vs. tendency to burrow: $r_S = -0.0874$, $p = 0.165$; use of cover vs. tendency to burrow: $r_S = 0.0378$, $p = 0.549$). However, tendency to burrow and burrowing depth at the end of the test were strongly correlated ($r_S = 0.6914$, $p < 0.0001$), i.e. amphipods that were frequently observed below the surface also tended to burrow deeper. Because these 2 traits were not independent and similar results were obtained for both, hereafter we only report analyses involving burrowing depth.
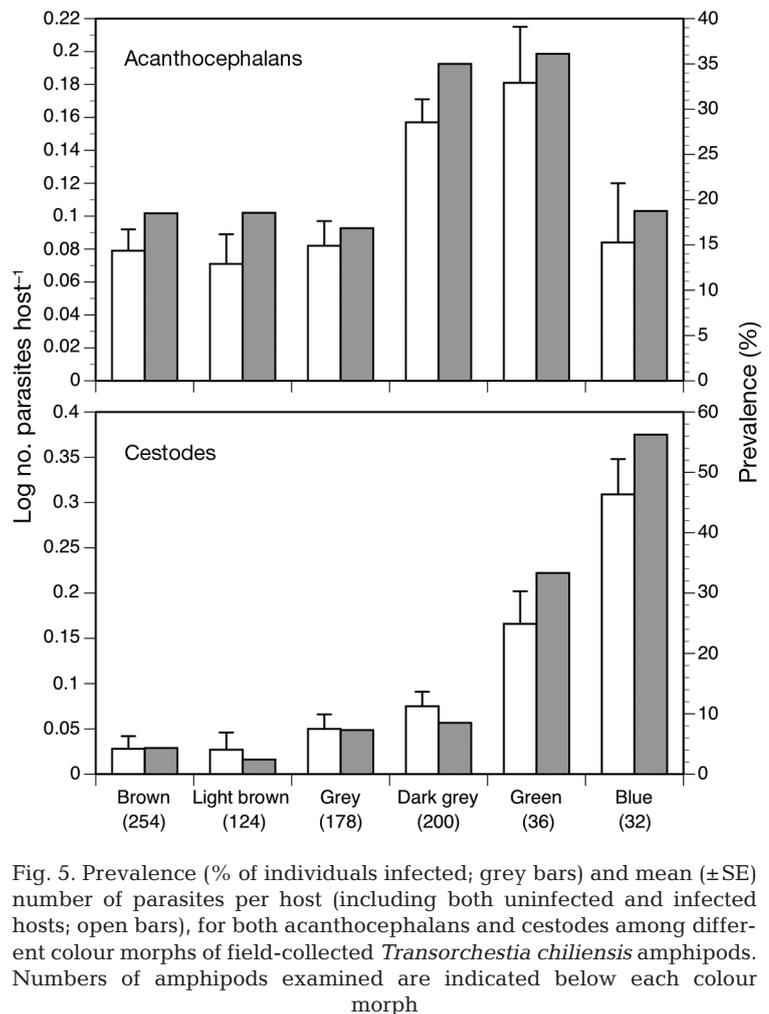
modest (blue: n = 32; green: n = 36). Our analysis revealed effects of both amphipod body size and sex on body colouration (Table 2). Larger individuals were more likely to exhibit a darker shade of grey or brown, or to be blue or green. Male amphipods tended to be more common among the brown, light brown and green colour groups. More interestingly, independent of host size and sex, there were significant associations between infection by both parasite taxa and body colouration of amphipods (Table 2). Acanthocephalan infections were more frequent in dark grey and green individuals than in other colour morphs, whereas cestode infections peaked in green and blue individuals (Fig. 5). Also, the best-fitting model (i.e. lowest AIC_c) was the one in which acanthocephalan developmental stages were considered separately (Table 2). Therefore, although acanthocephalan infections in general are linked with host colouration, this is mostly due to infections by cystacanths than by the earlier acanthella stage.

Parasitism and amphipod behaviour

The third field collection resulted in 254 amphipods for behavioural tests; these

Fig. 5. Prevalence (% of individuals infected; grey bars) and mean (±SE) number of parasites per host (including both uninfected and infected hosts; open bars), for both acanthocephalans and cestodes among different colour morphs of field-collected *Transorchestia chiliensis* amphipods. Numbers of amphipods examined are indicated below each colour morph

Table 3. Generalised linear mixed models testing potential predictors of amphipod behaviour, showing the fixed effect tests for each predictor (significant predictors in **bold**), the corrected Akaike information criterion (AIC$_c$) score for each model, and the difference (ΔAIC$_c$) from the best model. The percentage of variance not explained by the fixed effects that was accounted for by the random factor 'colour morph' did not exceed 3 % in any model

Model Predictor	df	F-ratio	p-value	AIC$_c$	ΔAIC$_c$
Choice of colour background					
Best					
Body size	1	0.423	0.5160	834.21	0
Sex	2	0.199	0.8199		
No. early cystacanths	1	0.003	0.9540		
No. late cystacanths	**1**	**16.170**	**<0.0001**		
No. cestodes	1	0.837	0.3613		
Other					
Body size	1	0.736	0.3917	843.56	9.35
Sex	2	0.187	0.8298		
No. acanthocephalans	**1**	**6.417**	**0.0119**		
No. cestodes	1	0.8916	0.3460		
Use of cover					
Best					
Body size	1	1.467	0.2270	963.40	0
Sex	2	0.860	0.4253		
No. early cystacanths	1	0.001	0.9984		
No. late cystacanths	1	0.736	0.3918		
No. cestodes	1	0.084	0.7720		
Other					
Body size	1	1.498	0.2222	963.56	0.16
Sex	2	0.961	0.3851		
No. acanthocephalans	1	0.195	0.6591		
No. cestodes	1	0.099	0.7537		
Burrowing depth					
Best					
Body size	**1**	**7.231**	**0.0077**	689.55	0
Sex	2	0.224	0.7995		
No. acanthocephalans	1	2.124	0.1463		
No. cestodes	**1**	**3.911**	**0.0491**		
Other					
Body size	**1**	**7.656**	**0.0061**	689.83	0.28
Sex	2	0.247	0.7814		
No. early cystacanths	1	3.711	0.0552		
No. late cystacanths	1	0.306	0.5809		
No. cestodes	**1**	**4.072**	**0.0447**		

Fig. 6. Mean (±SE) number of acanthocephalans per host (including both uninfected and infected hosts), for all acanthocephalans as well as for early and late cystacanths separately among *Transorchestia chiliensis* amphipods showing different preferences for background substrate colouration. Low scores (0–1) indicate a preference for light backgrounds; high scores (5–6), a preference for dark backgrounds. Numbers of amphipods are indicated below each group

Our analyses indicated that whether the amphipods were males, females with offspring or females without offspring had no influence on their choice of background substrate colour, use of cover or burrowing depth (Table 3). Body size had an effect on burrowing depth, with smaller individuals tending to burrow deeper into the sediment (Table 3). There were also associations between parasite infection and amphipod behaviour, independent of host size and sex (Table 3). Amphipods choosing the light over the dark background were more likely to harbour
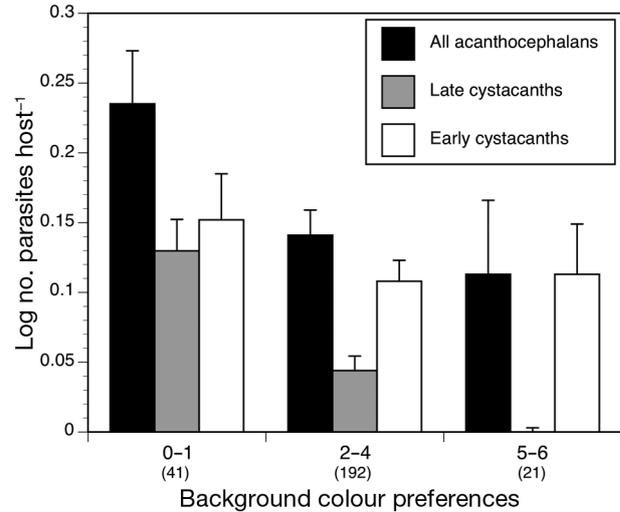
acanthocephalan infections (Fig. 6). The best-fitting model (i.e. with the lowest AIC$_c$) was the one in which acanthocephalan developmental stages were considered separately (Table 3), and therefore the association between background colour choice and infection is mainly due to infections by late, well-developed cystacanths rather than by earlier, less-developed cystacanths (Fig. 6). Also, cestode infections were more common in amphipods that either stayed at the surface or did not burrow very deeply, rather than in those that burrowed deep into the sediment (Fig. 7), although this was not a statistically strong effect (Table 3).

DISCUSSION

Phenotypic variation among individuals in a population can affect the strength or direction of intra- and interspecific interactions in natural communities (Bolnick et al. 2011). Although parasites have long been known to generate phenotypic variation among conspecific hosts (Poulin & Thomas 1999), their importance is still not widely recognised. In this study, we showed that 2 different species of parasites have distinct and independent associations with body colouration and behavioural traits in the supra-littoral amphipod *Transorchestia chiliensis*. There
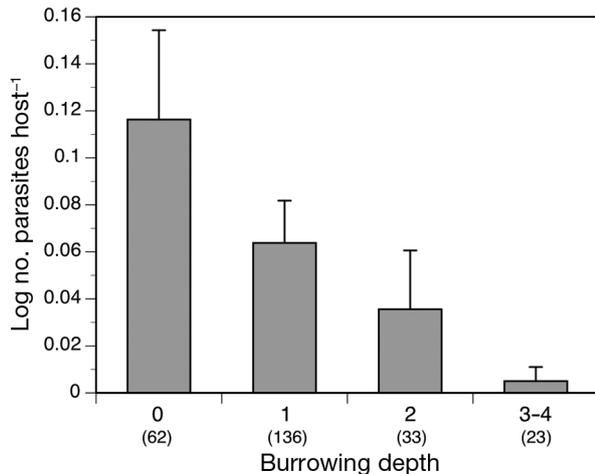
Fig. 7. Mean (±SE) number of cestodes per host (including both uninfected and infected hosts) among *Transorchestia chiliensis* amphipods burrowing to different depths under the substrate. A score of 0 indicates no burrowing (i.e. staying at the surface); higher values indicate increasingly deep burrowing. Numbers of amphipods are indicated below each group

are also good reasons to suspect that the parasites actually induce these phenotypes. These findings have implications not only for the ecology of the amphipod (i.e. its use of the substrate and small-scale distribution among microhabitats), but also for parasite transmission, as the phenotypic characteristics of infected amphipods may increase their probability of predation by the parasites' definitive hosts.

Acanthocephalans had previously been reported, but not identified, from *T. chiliensis* at our study site (Koehler & Poulin 2010). Here, we not only identified the acanthocephalan as *Plagiorhynchus allisonae*, previously known solely from its adult form occurring in oystercatchers *Haematopus* spp. (Smales 2002), but we have also completed its life cycle by confirming *T. chiliensis* as the intermediate host. The cestode could only be identified to family level. To our knowledge, there has been only one report (from Hawaii; Alicata 1936), of a juvenile cestode in a talitrid amphipod. In New Zealand, dilepidid cestodes have been reported from several shorebirds; for instance, *Anomotaenia ciliata* in the mallard duck *Anas platyrhynchos*, the paradise duck *Tadorna variegata*, and the grey duck *Anas superciliosa* (McKenna 2010). Both the acanthocephalan and cestode are probably specific to *T. chiliensis* because neither has been observed in an earlier comprehensive survey of all other supralittoral and intertidal crustaceans sympatric with *T. chiliensis* at our field site (Koehler & Poulin 2010).

The negative correlations observed between infections by different acanthocephalan developmental stages and between infections by acanthocephalans and cestodes suggest that amphipods acquire parasites mainly by a single exposure to occasionally large numbers of infective stages. Repeated exposure to one or a few infective stages each time would lead to frequent co-occurrence of different developmental stages in the same host, or of different parasite taxa, and to a frequency distribution of cestode numbers among hosts more skewed toward low values. In contrast, the segregation of acanthocephalan developmental stages and of parasite species among hosts observed here probably reflects a patchy distribution of infective stages among the bird droppings on which the amphipods feed, leading to infrequent but potentially severe infection. Acanthocephalan infections also seem to occur predominantly early in the austral summer, as acanthellae (the earliest developmental stage following infection) were only found in the November and December samples and not in January.

Colour polymorphism has been observed within populations of small crustaceans such as amphipods, isopods and ostracods. It is believed to arise from multiple mechanisms, including genetic determinism, different stages in the moult cycle or environmental influences, and it impacts several life functions ranging from mate choice to microhabitat selection (e.g. Heath 1979, Merilaita & Jormalainen 1997, Hull & Rollinson 2000, Devin et al. 2004). In the *T. chiliensis* population studied here, the significant association between parasitism and body colouration suggests that infection by acanthocephalans or cestodes may generate colour variation among conspecifics. However, only experimental infections could establish a causal role for parasitism. Indeed, it is conceivable that colour polymorphism arises for other reasons, and that different colour morphs are then exposed to different risks of infection through differences in behaviour. For instance, genetically determined colour morphs might have different vulnerability or resistance to infection, or experience different levels of parasite-induced mortality, leading to patterns such as the ones we observed. However, there are good reasons to suppose that parasites induce colour variation. Firstly, both acanthocephalans (Hindsbo 1972, Camp & Huizinga 1979, Oetinger & Nickol 1981, Benesh et al. 2008) and cestodes (Trabalon et al. 2000, Sánchez et al. 2006) have previously been linked to alteration in the colouration of arthropod hosts, a phenomenon attributed to the ability of many parasites to manipulate host phe-

notype to enhance their transmission success (Moore 2002, Poulin 2010). Secondly, in the case of acanthocephalans, the link between infection and host colour is significant for the cystacanth stage but not for the earlier acanthella stage, consistent with the host phenotype changing over time after infection in parallel with parasite development (see Bethel & Holmes 1974, Oetinger & Nickol 1982, Dianne et al. 2010, Franceschi et al. 2010). This may occur via the parasites interfering with pigment deposition in the host cuticle after a moult (Oetinger & Nickol 1982). The host's bluish haemocyanin may thus become more visible from the outside, resulting in a body colouration closer to the grey-blue-green range than to brownish hues. The outcome may well be a mismatch between the host's body colouration and that of the natural substrate, which could lead to increased risk of capture by visual predators (Merilaita et al. 2001) and thus perhaps enhanced transmission of the parasites to avian definitive hosts. Our analysis also indicates that each parasite species has independent effects on host colouration, suggesting that they could have additive or synergistic effects (see Poulin et al. 2003) that would boost their transmission success if they share the same definitive hosts.

Parasite manipulation of intermediate hosts often occurs along multiple phenotypic axes, causing a suite of host traits to be jointly altered by infection (Thomas et al. 2010). Manipulation of host behaviour appears to be an ancestral ability shared by extant acanthocephalans (Moore 1984). Multiple amphipod species are known to be behaviourally modified by acanthocephalans (e.g. Bethel & Holmes 1973, Maynard et al. 1998, Bauer et al. 2000, see Moore 2002 for review), and the only other member of the acanthocephalan genus *Plagiorhynchus* previously studied in this context has become a textbook example of a behavioural manipulator (Moore 1983). Cestodes are also capable of manipulating the behaviour of their arthropod intermediate hosts (Poulin et al. 1992, Robb & Reid 1996, Franz & Kurtz 2002, Sánchez et al. 2007). Therefore, it is perhaps not surprising that in addition to host colouration, both parasites studied here were also associated with specific behavioural phenotypes in the host. Amphipods preferring the light over the dark background were more likely to harbour acanthocephalan infections, whereas amphipods that either stayed at the surface or did not burrow very deeply were more likely to harbour cestode infections than those that burrowed deep into the sediment. The latter effect may be partially linked with amphipod body size, since body size is associated with the number of cestodes per host

and also affects burrowing depth. Nevertheless, in combination with parasite-induced changes in body colouration, it is plausible that these behavioural differences could enhance parasite transmission to bird definitive hosts, and may represent instances of multi-trait phenotypic manipulation by trophically transmitted parasites (Lafferty 1999, Thomas et al. 2005, 2010, Poulin 2010).

In conclusion, we uncovered marked phenotypic variation within a population of the amphipod *T. chiliensis*, which involved multiple independent traits and was linked with infection by the juvenile stages of 2 trophically transmitted parasites. These findings are consistent with the widespread ability of parasites to adaptively manipulate host phenotype to improve their transmission (Lafferty 1999, Thomas et al. 2005, Poulin 2010), although experimental infections would be required to confirm this. Regardless of whether host phenotype is modified by parasites or infection is a consequence of host phenotype, parasites were non-randomly distributed among individual hosts with different body colouration, substrate colour preferences and burrowing tendencies. This has implications for the transmission dynamics of the 2 parasite species, but possibly also for the host population through altered mate choice patterns or increased mortality of certain phenotypic variants. The sort of phenotypic structuring we observed in *T. chiliensis* is likely common among marine invertebrates, and the role of parasites and diseases in generating this variance requires greater investigation and wider recognition.

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