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Periwinkles and parasites: the occurrence and phenotypic effects of parasites in *Littorina saxatilis* and *L. arcana* in northeastern England

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Short running head: PERIWINKLES AND PARASITES

ABSTRACT

Littorina saxatilis is a common intertidal gastropod on shores of the North Atlantic, and an important study species for evolutionary investigations. Its congener *L. arcana* is much less widely distributed, but both species are common at Old Peak, Yorkshire, UK. The parasite profiles of *L. saxatilis* and *L. arcana* from this shore were determined histologically, revealing a ciliated protist, *Protophrya ovicola*, an unidentified apicomplexan (present in <1% *L. saxatilis*) and three trematode parasites tentatively assigned to *Renicola* sp., *Microphallus similis* and *M. pygmaeus*. The profile data include prevalence information and associated histology. *Protophrya ovicola* associated predominantly with the wave ecotype of *L. saxatilis* (65%) rather than the crab ecotype (16%). *Microphallus similis* occurred at a higher prevalence in the *L. arcana* population (38%) in comparison with the *L. saxatilis* population (11%). Overall, there appeared to be a lower prevalence of trematodes in the high-shore *L. saxatilis*. By modelling occurrence of individual parasites and shell morphometrics, an assessment of parasite-associated morphological change was conducted. We conclude that parasitism appeared not to cause shell-shape change, but rather that snails of a certain shell shape were more likely to display infection. Records of parasites in *L. saxatilis* and *L. arcana* are briefly reviewed, showing that the diversity of parasites reported here is relatively low.

INTRODUCTION

Marine gastropods are host to numerous parasites and are a common focus for research on host-parasite interactions (Coen & Bishop, 2015). Parasites are crucial factors in ecosystem function: their prevalence and pathogenicity can drastically alter animal population sizes, ecosystem services and the environment (Marcogliese, 2005; Hudson, Dobson & Lafferty, 2006). Besides resulting in pathology and mortality, parasitism may be associated with changes in host behaviour (e.g. Bunke *et al.* 2015) or morphology (e.g. Gorbushin & Levakin 1999). Reports of such manipulations of the host date back to 1931 (reviewed by Thomas, Adamo & Moore, 2005) and since then it has become axiomatic that infected individuals show phenotypic modifications to be construed as benefiting the parasite—often to the detriment of the host.

Littorina saxatilis, and its congener *Littorina arcana*, are common intertidal gastropods on North Atlantic coasts. These species have been the subject of much parasitological study, resulting in considerable knowledge of their trematode parasite diversity (Galaktionov, 2012). The effects of parasitism on the host, in this case, are rarely reported. We are aware of two reports of manipulation of the host's shell shape by trematode parasites in the snail *Littorina saxatilis*. Panova, Sergievsky & Granovitch (1999) considered that infection with trematodes caused an increase in spire height of *L. saxatilis*, although this was apparently not an inevitable consequence. They suggested that infected snails that did not show a change in shape had been recently infected and drew attention to a possibly confounding effect of microhabitat: snails from upper shore levels had higher spires than those lower down the shore. McCarthy, Fitzpatrick & Irwin (2004) considered that infection with *Microphallus piriformes* induced changes in the shape of the shell; again, infected snails had taller spires, presumably to the advantage of the parasite by providing more internal shell space for maturation of the metacercariae.

Microparasites in these hosts have received less attention. *Digyalum oweni*, a gregarine parasite (Apicomplexa) principally infects *L. obtusata*, but is found in all Atlantic *Littorina*

72 species including *L. saxatilis* and *L. arcana* (Dyson, Evennett & Grahame, 1992). Other
73 periwinkle-associated organisms include the ciliated protists, which are often commensal
74 organisms on/in many hosts (Morado & Small, 1994; Sokolova, 1995). In *L. saxatilis*, it is
75 unclear whether the ciliated protist *Protophrya ovicola* is a symbiont, a commensal or a
76 parasite (Sokolova, 1995). Apart from the occasional occurrence of *D. oweni*, neither *L.*
77 *saxatilis* nor *L. arcana* are currently associated with other microparasites, including viruses,
78 bacteria or microsporidians—many of which have been noted in other molluscan species
79 (e.g. Sagristà *et al.*, 1998; Barbosa-Solomieu *et al.*, 2005; Beaz-Hidalgo *et al.*, 2010).

80 Our aims in this study were twofold. First, we used a histological screen to determine
81 parasite profile (i.e. all trematodes and microbes within the host) for *L. saxatilis* and *L.*
82 *arcana* sampled across their full tidal range (the high- and midshore) at Old Peak, Yorkshire,
83 UK. Histology is an established tool for parasite, commensal and symbiont detection and is
84 capable of detecting a large suite of organisms living within host tissues (e.g. Bojko *et al.*,
85 2013). Populations of *L. saxatilis* at Old Peak have been argued to be undergoing
86 ecologically-driven diversification, which may be an early stage of speciation (Butlin *et al.*,
87 2014). Microevolutionary studies at this site have not yet included parasitological
88 investigation, so we sought to address this aspect of the ecology of the divergent ecotypes.
89 In addition we sought comparative data from its sister species *L. arcana* (Reid, 1996).

90 Second, we addressed the question of shape variation in the snails. The divergent
91 ecotypes are referred to here as 'wave' (on the high shore) and 'crab' (on the mid shore),
92 following the usage of Butlin *et al.* (2014), reflecting the likely agents of selection involved in
93 the diversification process. The shapes of the shells are considered to be important in the
94 evolution and adaptation of the ecotypes. Given the reports of shape variation due to
95 trematode infection and the argument that this may be adaptive manipulation by the
96 parasites, there are both methodological and conceptual reasons for investigating the
97 possible effects of parasitic infection on shell shape, and potential confounding effects of
98 ecotypic divergence and parasites.

99

100 ## MATERIAL AND METHODS

101 *Sampling and preliminary processing of material*

102 *Littorina* species ('rough periwinkles') were collected from the intertidal shore of Old Peak at
103 the southern end of Robin Hood's Bay, Yorkshire (BNG NZ984021), in October 2013 (n =
104 236), to assess the parasite profile and collect morphological data. In January of 2015 an
105 opportunistic sample of 14 snails was taken from a single high-shore boulder to be used in
106 the analysis of shape.

107 Animals were held in the laboratory at 5 ˚C for no more than 3 d before diagnosis and
108 processing. First, snails were imaged, after which the shell was broken and the body
109 removed. The presence of external signs of pathology was recorded together with any
110 obvious signs of parasitic infection. The snails were recorded as male, female or immature.
111 Females were identified according to the form of the pallial oviduct as *L. arcana* (with a jelly
112 gland) or *L. saxatilis* (with a brood pouch) (Reid, 1996). With the exception of only one
113 female, the pallial oviduct was sufficiently well formed to reach a diagnosis. Crab and wave
114 ecotypes of *L. saxatilis* were classified according to collection site on the mid- and high-
115 shore, respectively. The total number of females confidently identified was 169, in the three
116 categories: *L. arcana*, and *L. saxatilis* crab or wave ecotype. In order to identify males
117 (indistinguishable anatomically), we then used the measurements for the identified female
118 shells as a training set in a discriminant function cross-validation analysis (DFA). First
119 examining the training set, we calculated a linear discriminant function (Proc DISCRIM; SAS
120 Institute, 1990) allowing classification of the already identified snails on solely morphometric
121 criteria. We found that >70% of the snails assigned *a priori* to the three groups classified
122 back to those groups. Then, using DFA with the more conservative criterion of 80% certainty
123 of classification, we classified unassigned snails (there were 67: males, immature snails and
124 the single parasitized individual not hitherto classified) to one of the three categories. Only

two snails failed to classify on the 80% criterion, these were rejected from further analysis, leaving a total dataset of 248 individuals of both species and both sexes.

Histological detection of parasites

Fixation for histology was in 1-2 ml of Davidson's saltwater fixative (Hopwood, 1996) followed by 70% industrial methylated spirit (IMS) after 24 h. Each specimen underwent wax infiltration using an automated tissue processor (Polaris, Leica Microsystems) and was subsequently embedded in a wax block. A single section (3 µm thickness) was taken through the centre of the animal using a rotary microtome (Thermofisher), to standardise the screening approach. Sections were placed onto glass slides and stained with haematoxylin and alcoholic eosin (H&E) before being mounted. Slides were examined using standard light microscopy (Nikon/Leica Eclipse E800). Images were annotated using LuciaG computing software (Nikon), which provides accurate scale bars and allows the addition of arrows and other annotation.

During histological analysis, infection burden was recorded on an interval scale: 0, no infection; 1, low infection; 2, medium; 3, high. Trematodes were identified at least to genus, using published descriptions (James, 1968, 1969; Granovitch & Johanessen, 2000).

Morphometric data and analysis

Snails were imaged in a standard orientation such that the columella axis was horizontal and the widest dimension across the shell at 90° to this axis was also horizontal. These alignments were achieved by eye, against the reference of a horizontal shelf across the laboratory bench.

Commented [JG1]: FIGURE 1 ABOUT HERE

Images were captured using a JVC Colour Video Camera Head TK-1280E with a Matrox Comet framegrabber card mounted in a PC. Example images are shown in Figure 1, which also illustrates the linear dimensions recorded for a Euclidean Distance Measure approach

151 in shape analysis. We justify the use of this method because we consider that it is closer to
152 growth-model analyses of shell form (e.g. Raup, 1966; Moulton, Goriely & Chirat, 2012) than
153 are geometric morphometric approaches, affording an intuitive grasp of the likely functional
154 significance of changes in turbinate shell shapes. Moreover, other workers reporting on the
155 putative effects of parasitic infection on shell shape have generally used ratios derived from
156 linear distance measures (e.g. McCarthy *et al*., 2004).

157 Data were analysed in the R package v. 3.2.2 (R Core Team, 2013). First the linear
158 dimensions were transformed by expressing each as a ratio of the geometric mean of the
159 dimensions for that shell and then taking the base 10 logarithm of this ratio. This procedure
160 was adopted to minimize the effect of size variation as such; it is the '*DM_LOG*' approach of
161 Jungers, Falsetti & Wall (1995).

162 We used principal component analysis (PCA) to obtain (1) the eigenvectors of the
163 correlation matrix and (2) the scores placing each shell on each of the components. The
164 individual values in the eigenvectors are coefficients, one for each variable. Negative and
165 positive coefficients indicate variables that are inversely related to one another with respect
166 to that principal component (PC). Then, shells may be described as most different on each
167 of the PCs. Thus, the shells illustrated in Figure 1 have extreme negative (Fig. 1A, C) or
168 positive (B, D) scores on the first (and most important) PC in a PCA of all shells together. In
169 fact, if two separate analyses are executed, one on *L. saxatilis* and the other on *L. arcana*,
170 the same shells are identified as extremes in those two analyses as in the single global one.
171 Notwithstanding that shape variation in the two species seems to have much in common, for
172 investigation of possible effects of parasites on shape, we performed separate PCAs for the
173 two species and, usually, for the two ecotypes of *L. saxatilis*. Three-dimensional graphs of
174 the PCA axes were drawn using scatterplot3d (Ligges & Mächler, 2003) within R.

175 In further analyses, the scores for the shells in PCs 1, 2 and 3 were used as response
176 variables in linear mixed-effects models (LMM) implemented in nlme (Pinheiro *et al.* 2013)
177 within R. We used the occurrence of the parasites and shore zone as fixed effects, while

sample site was a random effect. Where a parasite/symbiont was very rare in one or both of the hosts (*Microphallus pygmaeus* was rare in both hosts and *Protophrya ovicola* was rarely detected in *L. arcana*), these were excluded from the analysis.

RESULTS

Parasite profile determined by histology

A ciliated protist (Fig. 2A) and an apicomplexan-like microparasite (Fig. 2B) constituted the microfauna identified from histological analysis. Three trematode macroparasites were found in 32/178 *Littorina saxatilis* (18%) and 25/56 *L. arcana* (45%)

The ciliated protist was identified as *Protophrya ovicola,* based on its location in the host (mostly in the brood pouch) and ciliated morphology. It was found in both snail species, with a greater prevalence in *L. saxatilis* than in *L. arcana* (36% and 4%, respectively; proportion test $\chi2 = 20.49$, $P < 0.0001$). In both host species *P. ovicola* was either found in small numbers on the exterior of the snail or, in female *L. saxatilis*, most commonly within the brood pouch (Fig. 2A). No immune responses such as granuloma formation or melanization reactions were observed in response to *P. ovicola*. The protist was more common in high-shore *L. saxatilis* (proportion test $\chi2 = 43.03$, $P < 0.0001$; Table 1) and female *L. saxatilis* were favoured over males for the population as a whole (2/31 males and 62/136 females; proportion test $\chi2 = 10.37$, $P = 0.0013$).

Apicomplexan-like protists were present in a single *L. saxatilis* from the mid-tidal zone, causing infection in the gut epithelium of the host (Fig. 2B), where infected cells were observed with one to two parasite inclusions (Fig. 2B inset). Parasites in the gut lumen were closely associated with the epithelial cells and not free within the lumen. No host immune response was observed in relation to the presence of this parasite.

Three trematodes were present in each snail species. The first appears to be an encysted metacercariae of *Renicola* sp. based upon the size, development stage and eosinophilic cyst

204 layers containing the trematode (Fig. 2C). The second is a first intermediate trematode
205 infection (type 1) tentatively assigned to *Microphallus similis* based on morphology, host
206 choice and development stage (mother and developing daughter sporocyst; Fig. 2D). The
207 third trematode is a first intermediate infection (type 2) representing a mother sporocyst
208 containing several daughter sporocysts belonging to a species within the *M. pygmaeus*
209 complex, differing in size, shape, presence of tegumental spines and staining from the
210 previously assigned *M. similis*; this is tentatively identified as *M. pygmaeus* (Fig. 2E-G).

211 *Renicola* sp. was present in both *L. saxatilis* (11%) and *L. arcana* (38%) from both tidal
212 zones. It had a higher prevalence in *L. arcana* than *L. saxatilis* (proportion test $\chi^2 = 18.56$, P
213 < 0.0001). The incidence in each host ranged from one to several encysted parasites. This
214 parasite encysted within the digestive gland (Fig. 2C), gut, muscle and epidermal tissues,
215 causing displacement of tissues and organs. A thick eosinophilic layer was commonly
216 observed around the parasite, but it is unclear whether this was produced by the host (an
217 immune response to segregate the parasite from host tissues) or formed by the parasite as a
218 protective layer. Other than the potential immune response to segregate parasites from host
219 tissues, no other immune responses were observed.

220 The mother and daughter sporocysts of *M. similis* (Fig. 2D) were present in both *L.*
221 *saxatilis* (7%) and *L. arcana* (13%) from both tidal zones. Infections caused by this
222 trematode were pathologically consistent in both host species and limited to the digestive
223 gland. The parasite was often present in large numbers contained within a single mother
224 sporocyst, causing the digestive gland to turn completely white or mottled white and brown.
225 The host tissues wwere displaced, but no host immune responses were observed.

226 Four animals were infected with *M. pygmaeus* (Fig. 2E-G) across both host species
227 (Table 1). The parasites were present in the digestive gland and in some cases elicited an
228 inflammatory immune response resulting in the aggregation of haemocytes (Fig. 2E).

229 Our external examination recorded only 21 cases of visible parasitism, 20 of which were
230 confirmed in the histological screen. This screen showed an additional 37 cases of

parasitism that had not been detected initially. In only two instances did we observe a snail lacking sexual organs or gonad: both were *L. saxatilis* crab ecotypes, about 12 mm in columella length (therefore not juvenile), but in only one did we find parasites. This snail was co-infected with both *M. similis* and *Renicola* sp., both with a high burden (score 3).

Shell morphology, zone and parasites

The distribution of shells of *L. arcana* and *L. saxatilis* in PCA ordinations are shown in Figure 3. The first three eigenvalues were >1 and thus eligible for consideration as meaningful (Everitt & Dunn, 2001); we therefore show three-dimensional plots. To assist with visualising these in three dimensions, animations are provided as Supplementary Material animations 3A and 3B. Snails from the mid-tidal zone were larger than those from the upper shore in *L. saxatilis* (Kruskal-Wallis rank sum test (K-W) $\chi^2 = 24.47$, $P < 0.0001$), but not in *L. arcana* (K-W $\chi^2 = 0.16$, $P = 0.69$).

Commented [JG2]: FIGURE 3 ABOUT HERE, TABLE 2 ABOUT HERE

In both hosts, PC1 most contrasts lip length and aperture width—lip length may be large and aperture width relatively small, or the reverse—and thus separates the distinctive crab and wave ecotypes of *L. saxatilis* (Fig. 1A and B, respectively), for example. We have not included the relevant eigenvectors here, but the shape extremes of both species are clear in Figure 1. There is a marked difference in how these shapes are distributed on the shore in the two species. For *L. saxatilis* most upper shore snails conform to the shape typical of the wave ecotype (Fig. 1B), whereas most mid-shore snails are the shape of the crab ecotype (Fig. 1A), as reflected in the separation along PC1 according to tidal level (Fig. 3B). In contrast, the shape extremes of *L. arcana* (Fig. 1C, D) are not segregated by tidal level (Fig. 3A).

The scores for the shells on the PCs were then treated as shape variables and used as response variables in a LMM approach. This analysis showed that for *L. arcana*, tidal zone has no significant effect with any of PCs 1, 2 or 3; the only significant effect is with *M. similis*

and PC2 (Table 2). For both ecotypes of *L. saxatilis* considered together, the importance of zone as a factor was overwhelming on PC1 (P = 0.0000; confirming inspection of Fig. 3B); the only significant effect was for *Renicola* sp. (P = 0.0053; Table 2).

This makes the exploration of possible shape differences with or without parasites different for the two host species. It is reasonable to proceed by treating *L. arcana* as a whole (as in Table 2), but for *L. saxatilis* the very strong influence of zone on shell shape (reflected in the recognition of two ecotypes) justifies treating the wave and crab ecotypes separately. For the wave ecotype only PC3 was associated with any significant effect and that was with *P. ovicola* (Table 3). For the crab ecotype, again there was only one PC associated with any significant effect, in this case PC1 with *Renicola* sp. and *M. similis* (Table 4). Notably the sign of the coefficients is opposite for these two trematode species.

In trying to understand why there might be links between shell shape and parasites, we turn to consideration of the shell variables as picked out by the PCA—those variables at or near the negative and positive extremes of the eigenvectors. There are three that are of interest: the eigenvector for PC2 for *L. arcana*, that for PC3 for *L. saxatilis* wave ecotype, and that for PC1 for *L. saxatilis* crab ecotype. These eigenvectors are shown in Table 5. For intuitive ease and comparison with other studies, the shell variables may now be expressed as ratios one of the other, when this demonstrates an attribute of shell shape. We also use the standardized size of a variable—its value as a ratio to the geometric mean of all the other linear variables—when a simple shape ratio such as 'tallness' is not intuitively useful.

As shown, in *L. arcana*, the only relationship found in modelling parasite presence and shape PCs was for PC2: there is an effect when infected with *M. similis* (Table 2). The most contrasted shell variables here (Table 5) are lip length and whorl width 2. Noting that aperture width has a coefficient almost as large as that for lip length, we use the ratio of whorl width 2 to aperture width as an expression of the relative spire height (or tallness) of the shell. Snails infected with *M. similis* have a greater relative spire height (Fig. 4A; K-W χ^2

283 = 11.925, *P* = 0.0006). Example shells from the extreme ends of PC2 are shown in
284 Supplementary Material Figure S1.

285 Turning to *L. saxatilis*, we consider the ecotypes separately. In the wave ecotype, *P.*
286 *ovicola* is significantly associated with PC3 (Table 3); this PC contrasts whorl width 0 with
287 aperture width (Table 5). Here there are two ratios that are significant, namely for whorl
288 widths 0 and 1, where the standardized size of these variables is larger for snails with *P.*
289 *ovicola* (K-W χ^2 = 7.282, *P* = 0.007, for whorl width 0; χ^2 = 4.27, *P* = 0.039 for whorl width 1).
290 Table 5 shows that the variables whorl width 0 and aperture width form the extremes of
291 eigenvector three for *L. saxatilis*; this would lead to the expectation that their ratio might be
292 different depending on the presence of *P. ovicola*, since it is associated with PC3. This
293 expectation is not met: the ratio for whorl width 0 to aperture width is numerically greater in
294 snails with the ciliates, but this difference is not significant (K-W χ^2= 1.419, *P* = 0.234).
295 Example shells from the extreme ends of PC3 are shown in Supplementary Material Figure
296 S2. It is intriguing to note that PC3 here is substantially the same as the PC3 identified by
297 Walker & Grahame (2011) as being associated with a change in relative brood size (in that
298 study, a proxy for reproductive effort). The correlation between the two eigenvectors from the
299 independent datasets of that study and our own is 0.782 (*P* = 0.038).

300 Finally, for the *L. saxatilis* crab ecotype, PC1 is associated with infection with both
301 *Renicola* sp. and *M. similis* (Table 4); Table 5 shows that the most contrasted variables on
302 this PC are whorl width 2 and aperture width (in this respect resembling PC2 for *L. arcana*).
303 Using the same index of relative spire height (the ratio of whorl width 2 to aperture width) we
304 find that again the snails infected with *M. similis* are relatively higher-spired (Fig. 4B; K-W χ^2
305 = 3.849, *P* = 0.0498), while those infected with *Renicola* sp. are relatively lower-spired (Fig.
306 4C; K-W χ^2 = 10.239, *P* = 0.0014). We note that the effect for *M. similis* is only just significant
307 (at $P \leq 0.05$), but is in the same direction as for *L. arcana* with *M. similis* (Fig. 4A). Example
308 shells are shown in Supplementary Material Figure S3.

Commented [JB3]: Table 5 here

We do not have quantitative data for the numbers of parasites in the host snails, but we did score the apparent level of infection in categories 0 to 3 (see Methods, above). Including these scores in the LMMs shows that in no case does the level of infection make a significant contribution to the model, using the criterion of $P \leq 0.05$. Only in the case of *L. arcana* infected with *M. similis* was there a result close to significance ($P = 0.075$). However, of the 17 cases of infection of this snail with this trematode, 14 were scored as 3 (the heaviest level); thus 'level' is not very different from 'present' in this instance, and the additional information makes no meaningful contribution. We conclude that if there is any possible effect of degree of infection, our data do not show it.

In expressing the relationships between parasitic infection and shell shape as simple ratios, we gain in comparability with other studies that have used such ratios, but lose in the simplification of a multivariate system to single axes. Therefore, we show figures expressing the relationships of the presence of parasites to shape as three-dimensional ordinations from the PCAs (Fig. 5). Animations are provided as Supplementary Material.

For *L. arcana* and *M. similis*, the relationship of infection to PC2 is apparent in the ordination (Fig. 5A; Animation 5A). The figure also suggests that we might expect a relationship with PC1, but this was not supported by modelling. The figure shows an interesting feature, namely that while the most extreme shapes on PC1 are of infected snails, there are uninfected ones near them, and that infected snails are scattered among uninfected ones in the shape space defined by the ordination. Co-infections numbered only three, all with *Renicola* sp. and one also with *M. pygmaeus* (this was not included in the modelling due to low numbers observed; see Methods). *Renicola* sp. makes no contribution to shape as revealed by modelling the PCs, and including coinfection as a term in the modelling showed this to make no contribution either. Thus, we conclude that infection by other parasites does not account for the presence in the plot of snails uninfected with *M. similis* among those so infected.

For the crab ecotype of *L. saxatilis*, the ordination (Fig. 5B; Animation 5B) again shows uninfected snails sharing shape space with those infected by either *Renicola* sp. or *M. similis*, while the latter two categories occupy largely different portions of the shape space (this reflects the different sign of the coefficients in the LMM for PC1; Table 4). We note that when shape is expressed by the scores of shells on PC1 the model gives a very significant coefficient with *M. similis* (P = 0.0018, Table 4), whereas the simpler index of relative spire height is not significantly different between the infected and uninfected snails (P = 0.085, see above).

These results show that while there is an association between shape and parasites in a number of instances, it is also true that there is shape variation that is not accounted for by parasitic infection. There are hosts that have 'infected shapes' but are not infected and *vice versa* (Fig. 5).

348

349 # DISCUSSION

350

351 *Parasite profiling of Littorina saxatilis and L. arcana*

352 The histological screen revealed five associated organisms present in both *L. saxatilis* and *L.*
353 *arcana*: three trematode parasites, one ciliated protist (*Protophrya ovicola*) and one
354 unidentified apicomplexan. This is a comparatively low diversity of parasites in molluscan
355 hosts. The Mollusca in general are known to host a variety of parasites(McClymont *et al.*,
356 2005; Carnegie & Engelsma, 2014) and histology is capable of detecting a wide suite of
357 parasites, commensals and symbionts (e.g. Bojko *et al.*, 2013). The surprisingly low
358 incidence of parasites observed in this study could perhaps indicate that the *L. saxatilis* and
359 *L. arcana* populations at Old Peak are relatively healthy and free from many of the parasites
360 that have been identified in previous studies of *Littorina* species (Table 6).

Commented [DR4]: Table 6 here

361 The parasites detected here are predominantly trematodes, tentatively identified as an
362 encysted *Renicola* sp. metacercaria, a first intermediate sporocyst (type 1) of *Microphallus*
363 *similis* and a first intermediate sporocyst (type 2) of *M. pygmaeus*. More certain identification
364 would require molecular taxonomy (Galaktionov *et al.*, 2012). Here, we opted for a
365 histological approach with the aim of observing many parasite groups at low cost rather than
366 apply primers for specific parasite groups in a PCR diagnostics approach.

367 *Protophrya ovicola* was found externally, but also and most frequently in the brood pouch
368 of *L. saxatilis*. Sokolova (1995) found no evidence that it was associated with incidence of
369 embryo abnormality and in this study we observed no immunological response by the host to
370 its presence. The presence of *P. ovicola* may be beneficial for the snail by providing a
371 service, such as removing fungi and bacteria from the developing young, while itself
372 benefiting from the protected environment within the brood pouch in the harsh high-tidal
373 zone.

374 The detection of a rare apicomplexan infecting the gut epithelium of a single *L. saxatilis*
375 hints at some microparasite diversity. This gregarine apicomplexan shows some pathological
376 and morphological similarities (based on histology) to *Digyalum oweni* discovered in the gut
377 of *L. obtusata* (Koura, 1982; Koura *et al.* 1990; Dyson *et al.* 1992). Without genetic evidence
378 for the originally described species, or for that found here, or sufficient material fixed for
379 electron microscopy, no taxonomic conclusion is possible. A PCR or metagenomic screen
380 for gregarines may help to reveal gregarine diversity in these hosts in the future.

381 The parasite profiles of the two *Littorina* species differ from each other. Although they
382 were collected alongside each other, each appears to have a different susceptibility to
383 infection. For example, 11% of *L. saxatilis* were infected by *Renicola* sp., whereas the figure
384 for *L. arcana* was 36%. This difference could be related to relative genetic, and resultant
385 phenotypic, resistance of the two hosts to this trematode. Alternatively, there could be a
386 difference in their niches, whereby the niche of *L. arcana* could promote increased infection
387 with *Renicola* sp.

388 In addition, differences can be noted between the parasite profiles of snails from high-
389 and mid-tidal zones. Wave-ecotype *L. saxatilis* on the high shore had a high prevalence of *P.*
390 *ovicola* within their brood pouch (65%), while this was lower in crab-ecotype *L. saxatilis* on
391 the mid shore (16%). This organism only occurs externally on *L. arcana* (since it lacks a
392 brood pouch), with a prevalence of only 4% in both tidal zones. Other than its high
393 prevalence of *P. ovicola*, the wave-ecotype *L. saxatilis* had a lower prevalence of both
394 *Renicola* sp. and *M. similis*, relative to the crab ecotype (Table 1). Although this is not
395 statistically significant, it parallels the data of Granovitch & Johannesson (2000) from the
396 Swedish coast. The difference in parasite profile between the wave and crab ecotypes of *L.*
397 *saxatilis* suggests that high-shore wave ecotype could be 'escaping' its parasites to some
398 extent by moving away from the more frequently inundated mid-tidal zone, while in the
399 process it may have acquired a likely symbiont, *P. ovicola*. This suggestion requires further
400 research, but we speculate that disease will likely have been a factor in past and current

401 littorinid evolution. Speciation in the two littorinid hosts is a well-studied topic that currently
402 does not take parasitism into account (Galindo & Grahame, 2014).

403

404 *Parasites and shell morphology*

405 The distribution of shell shapes across the intertidal habitat were as expected for *L. saxatilis*
406 at Old Peak, much investigated since Hull *et al.* (1996) first reported evidence of a partial
407 reproductive barrier within this *L. saxatilis* population, separating it into high-shore H morphs
408 (equivalent to 'wave ecotype' as used here, following the usage of Butlin *et al*., 2014) and
409 mid-shore M morphs (i.e. 'crab ecotype') (see Galindo & Grahame, 2014, for review). Our
410 focus in this work was on the possible effects of parasite infection on shape. *Microphallus*
411 *pygmaeus* was very rare in our samples, precluding investigation of its possible effects on
412 shell shape. Both *Renicola* sp. and *M. similis* were more common, and there are three
413 instances where there might be considered a *prima facie* case for an effect of a parasitic
414 trematode on the shell shape of its host. In addition, our data show an apparent relationship
415 between occurrence of the ciliate *P. ovicola* and shell shape.

416 For the crab ecotype of *L. saxatilis* infected with either *M. similis* or *Renicola* sp., there
417 was an effect on PC1 (Table 4). This component reflects the relative height of the spire of
418 the shell (and conversely, its overall roundness or globosity); *L. saxatilis* infected with *M.*
419 *similis* tended to be higher-spired (Fig. 4B) and those infected with *Renicola* sp. lower-spired
420 (Fig. 4C). This is reminiscent of the findings of Panova *et al*. (1999) and McCarthy *et al.*
421 (2004), where *L. saxatilis* infected with trematodes were usually a different shape from
422 uninfected individuals. But, there is a difference; those authors found infected snails to be
423 consistently higher-spired, whereas we found infected snails to be either higher-spired or
424 lower-spired, depending on the species of parasite.

425 McCarthy *et al*. (2004) studied the trematode *M. piriformes*, which, as they noted, has an
426 abbreviated life cycle, alternating between snail and bird hosts. These authors suggested

427 that this might put an additional demand on the snail host to accommodate an enhanced
428 volume of parasite tissue in the absence of a free-swimming cercarial stage. Thus, they
429 favoured an adaptationist explanation in this case—the parasite was manipulating host
430 shape to its advantage.

431 In our study, those *L. saxatilis* with trematode infections are not a unique shape, or even
432 always an extreme one, but rather there are many individuals with no infection that share the
433 shape characteristics of the infected ones. The data suggest that the explanation in this
434 instance is that certain snail phenotypes are more likely to become infected than others. This
435 was an idea considered (but rejected) by McCarthy *et al.* (2004). Interestingly, Panova *et al*.
436 (1999) did suggest a microhabitat contribution for the shape changes they reported. At Old
437 Peak there is a simple possibility: *L. saxatilis* on the sides and upper parts of intertidal
438 boulders are slightly, but significantly, rounder than those on the cobbles and bedrock
439 around the boulders (B. Fairclough, K. Fisher & J. Grahame, unpublished). It is plausible that
440 this microhabitat difference may make the snails more or less susceptible to trematode
441 infection by particular parasites, though why this might be so remains unknown.

442 For *L. arcana* infected with *M. similis,* there is also evidence of an association of shell
443 shape and infection, and again the infected snails are broadly mixed in shape space with
444 uninfected ones (Fig. 5A). *Littorina arcana* apparently resembles the crab ecotype of *L.*
445 *saxatilis* in showing a higher-spired shell when *M. similis* is present (with the caveat that the
446 simple index of spire height is not significantly greater in *L. saxatilis*, so this interpretation
447 rests on the similarities revealed by the LMM analyses). But for *L. arcana* we have no
448 information on microhabitat distribution, and so do not know whether the speculation above
449 for *L. saxatilis* might apply.

450 Finally, returning to *P. ovicola*, which shows a shape association on PC3 in the wave
451 ecotype of *L. saxatilis*. This axis of shape variation is much the same PC identified by Walker
452 & Grahame (2011) as associated with their estimate of reproductive effort. It is intriguing that

this association should emerge for *P. ovicola*, which largely inhabits the brood pouch of the snails.

Concluding remarks

A wide variety of changes have been associated with parasitic infections in molluscan hosts. Reviewing these, Cézilly *et al.* (2013) sounded "a friendly note of caution" about "the endless formulation of *ad hoc* adaptive scenarios for which, most often, no critical test is available."

In the present study, it appears that some parasites do prefer particular areas, and a particular host, in the intertidal zone at Old Peak. The relatively lower infection rate by trematodes in wave-ecotype *L. saxatilis* may indicate they have partly 'escaped' the risk of infection by occupying the high shore, acquiring a likely symbiont (*P. ovicola*) in the process. We note that these host-parasite interactions may be influencing the evolutionary divergence, and even speciation, of the ecotypes of *L. saxatilis*. We have provided evidence of association of shell shape and parasitic infection in *L. saxatilis* and (less certainly) in *L. arcana*. There is, however, no characteristic shape attributable to parasitism by *Renicola* sp. or *M. similis*: rather, it seems that the parasites simply occur in a relatively restricted range of shapes (phenotypes) and we suggest that this could be linked to the likelihood of becoming infected. Based on these results, we recommend that future studies of the ecotypes of *L. saxatilis*, a model microevolutionary system, should include host-parasite effects and consider that hosts may present behaviours or phenotypes that make them more susceptible to parasitism. These attributes may not be caused by the presence of the parasite, but rather are part of the biology of the host. In other words, the parasites and symbionts investigated here follow host shape, they do not cause it.

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622

623 Figure captions

624 **Figure 1.** Specimens of *Littorina saxatilis* and *L. arcana* identified by PCA as extreme on the
625 first PC (see Results). **A, B**, *L. saxatilis*; **C, D**, *L. arcana*. Abbreviations: al, aperture length;
626 aw, aperture width; cl, columella length; ll, lip length; ww0, whorl width 0;, ww1, whorl width
627 1; ww2, whorl width 2. Scale bar = 5 mm.

628

629 **Figure 2.** Parasites from *Littorina* hosts at Old Peak, UK. **A**. Ciliated protist, *Protophrya*
630 *ovicola* (white arrow) within brood pouch of *L. saxatilis*. White stars identify host embryos.
631 Inset shows a single ciliate with highlighted cilia (white triangle) and nucleus (black triangle).
632 **B.** Apicomplexan parasites in gut of *L. saxatilis* (white arrows). Black arrow identifies host
633 nucleus; white star identifies smooth muscle of host. Inset image two parasites in detail
634 (white triangles). **C.** Metacercaria of trematode, *Renicola* sp., encysted within digestive gland
635 of *L. saxatilis*. External pearling is beginning to form around parasite (white triangle). A
636 pharynx or external sucker is present in section (white arrow). **D.** *Microphallus similis*
637 daughter sporocysts in section (one indicated with white arrow), infecting *L. saxatilis*. **E.**
638 Daughter sporocysts of *Microphallus pygmaeus* (white arrow) within digestive gland of *L.*
639 *saxatilis*. H identifies host inflammatory response to parasites. **F, G.** Spines of *M. pygmaeus*
640 have a hooked structure (white arrow in **F**) and cover entire body of the trematode (white
641 arrow in **G**).

642

643 **Figure 3.** Shells of *Littorina arcana* and *L. saxatilis* in PC ordinations. **A.** *L. arcana* from
644 upper shore (solid triangles) and mid shore (open triangles). **B.** *L saxatilis* of wave ecotype
645 (solid triangles) and crab ecotype (open triangles).

646 **Figure 4.** A. Relative spire height of *Littorina arcana* depending on *Microphallus similis*
647 infection status. B. Relative spire height of *Littorina saxatilis* crab ecotype infected or not with

Microphallus similis. C. Relative spire height of *Littorina saxatilis* crab ecotype infected or not with *Renicola* sp. In the box and whisker plots, the rectangle represents the upper and lower quartiles of the data; the horizontal line is drawn at the median. The whiskers extend across the third and fourth quartiles of the data, with extreme outliers (where these occur) shown as open circles.

Figure 5. Ordinations of host shells with or without parasites. **A**. *Littorina arcana* with and without *Microphallus similis*. **B.** *L. saxatilis* crab ecotype with and without *M. similis* or *Renicola* sp. Symbols: open diamond, no infection; solid square, *M. similis* infection; solid triangle, *Renicola* sp. infection; star, coinfection with both parasites.

Tables:

Table 1. Parasite prevalence in *Littorina* host populations according to habitat (zone).

	Renicola sp.	*Microphallus similis*	*Microphallus pygmaeus*	Apicomplexa	*Protophrya ovicola*
L. saxatilis wave ecotype (high shore)	4/72	4/72	1/72	0/72	47/72
L. saxatilis crab ecotype (mid shore)	16/106	8/106	1/106	1/106	17/106
Totals	20/178	12/178	2/178	1/178	64/178
L. arcana high shore	7/28	3/28	0/28	0/28	1/28
L. arcana mid shore	14/28	4/28	2/28	0/28	1/28
Totals	21/56	7/56	2/56	0/56	2/56

664

665 **Table 2.** Results of linear mixed modelling for *Littorina arcana* and *L. saxatilis* (crab and
666 wave ecotypes together). Coefficients considered significant are highlighted in bold type.
667 See text for further explanation.

668 **A.** *L. arcana*, principal component 2.

669 Fixed effects: PC2 score ~ zone + *Microphallus similis* + *Renicola* sp.

	Value	Std error	DF	*t*	*P*
Intercept	-0.2288	0.2990	54	-0.7652066	0.4475
zone	0.2760	0.3227	8	0.8552	0.4174
M. similis	0.7795	0.3481	54	2.2390	**0.0293**
Renicola sp.	-0.4771	0.3119	54	-1.5297	0.1319

670

671 **B.** *L. saxatilis*, both ecotypes together, principal component 1.

672 Fixed effects: PC1 score ~ zone + *M. similis* + *Renicola* sp. + *Protophrya ovicola*

	Value	Std error	DF	*t*	*P*
Intercept	1.5076	0.2180	161	6.9157	**0.0000**
zone	-2.6808	0.2161	161	-12.4079	**0.0000**
M. similis	-0.1948	0.2449	161	-0.7955	0.4275
Renicola sp.	0.5911	0.2092	161	2.8248	**0.0053**
P. ovicola	-0.0821	0.1550	161	-0.5296	0.5971

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677 **Table 3.** Results of linear mixed modelling for *Littoprina saxatilis* wave ecotype. Coefficients
678 considered significant are highlighted in bold type.

679 Fixed effects: PC3 ~ *Renicola* sp. + *Microphallus similis* + *Protophrya ovicola*

	Value	Std error	DF	*t*	*P*
Intercept	0.5212	0.2467	61	2.1128	**0.0387**
Renicola sp.	0.0809	0.5237	61	0.1545	0.8777
M. similis	-0.3497	0.5565	61	-0.6282	0.5322
P. ovicola	-0.7613	0.2736	61	-2.7821	**0.0072**

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683 **Table 4.** Results of linear mixed modelling for *Littorina saxatilis* crab ecotype. Coefficients
684 considered significant are highlighted in bold type.

685

686 Fixed effects: PC1 ~ *Renicola* sp. *+ Microphallus similis + Protophrya. ovicola*

	Value	Std error	DF	*t*	*P*
Intercept	-0.1884	0.2414	94	-0.7805	0.4370
Renicola sp.	--0.9982	0.37915	94	-2.6374	**0.0098**
M. similis	1.4941	0.4733	94	3.1573	**0.0021**
P. ovicola	0.6469	0.3575	94	1.8093	0.0736

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690 **Table 5.** Eigenvectors and their variances of the principal components; the eigenvectors
691 shown are those that relate to components demonstrated as having a relationship with one
692 or other of the parasites, and are ranked in order of the magnitudes of the coefficients in the
693 vectors. For abbreviations of shell dimensions (variables) see Fig. 1.
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L. arcana		*L. saxatilis* wave ecotype		*L. saxatilis* crab ecotype	
variable	PC2, 1.390	variable	PC3, 1.056	variable	PC1, 1.500
ll	-0.281	ww0	-0.822	aw	-0.553
aw	-0.279	ww1	-0.297	al	-0.547
al	-0.215	ll	-0.022	ww0	-0.003
ww0	-0.052	cl	0.077	ww1	0.041
ww1	0.291	ww2	0.078	cl	0.28
cl	0.405	al	0.193	ll	0.237
ww2	0.738	aw	0.431	ww2	0.509

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700 **Table 6.** Review of the parasites currently associated with *L. saxatilis* and *L. arcana*.

Taxa	Species	Host	Relationship	Reference
Trematoda	*Microphallus piriformes*	*L. saxatilis, L. arcana*	Parasite	Galaktionov, 1980, 1983, 2012; Granovitch *et al.*, 2000; Granovitch & Johannesson, 2000
	Microphalllus similis	*L. saxatilis, L. arcana*	Parasite	Jägerskiold, 1900; Granovitch et al. 2000; Granovitch and Johannesson, 2000; Galaktionov *et al.* 2012
	Microphalllus triangulatus	*L. saxatilis, L. arcana*	Parasite	Galaktionov, 1984, 2012; Granovitch et al. 2000
	Cercaria littorinae saxatilis	*L. saxatilis*	Parasite	Sannia and James, 1977; Granovitch and Johannesson, 2000
	Microphallus pygmaeus	*L. saxatilis*	Parasite	Levinsen, 1881; Granovitch et al. 2000; Granovitch and Johannesson, 2000; Galaktionov *et al.* 2012
	Microphallus pseudopygmaeus	*L. saxatilis, L. arcana*	Parasite	Granovitch et al. 2000; Galaktionov, 2009, 2012
	Paramonostomum chabaudi (=*Cercaria lebouri*)	*L. saxatilis*	Parasite	Stunkard, 1932; Granovitch and Johannesson, 2000
	Parapronocephalum symmetricum	*L. saxatilis*	Parasite	Belopolskaia, 1952
	Cercaria emasculans	*L. saxatilis*	Parasite	Pelseneer, 1906
	Cercaria brevicauda	*L. saxatilis*	Parasite	Pelseneer, 1906
	Cercaria roscovita	*L. saxatilis*	Parasite	Stunkard, 1932
	Cercaria quadriramis	*L. saxatilis*	Parasite of male	Chubrik, 1966
	Parvatrema homoeotecnum	*L. saxatilis*	Parasite of female	James, 1964
	Podocotyle atomon	*L. saxatilis*	Parasite	Rudolphi, 1802; Granovitch et al. 2000; Granovitch and Johannesson, 2000
	Himasthla elongata	*L. saxatilis*	Parasite	Ishkulov, 2000; Granovitch et al. 2000; Granovitch and Johannesson, 2000
	Renicola roscovita	*L. saxatilis*	Parasite	Stunkard, 1932; Granovitch et al. 2000; Granovitch and Johannesson, 2000
	Cryptocotyle lingua	*L. saxatilis*	Parasite	Creplín, 1825; Granovitch et al. 2000; Granovitch and Johannesson, 2000
	Notocotylus sp.	*L. saxatilis*	Parasite	Granovitch et al. 2000
Ciliophora	*Protophrya ovicola*	*L. saxatilis*	Commensal/Symbiont	Sokolova, 1995
Apicomplexa	*Digyalum oweni*	*L. saxatilis, L. arcana*	Parasite	Dyson *et al.* 1992

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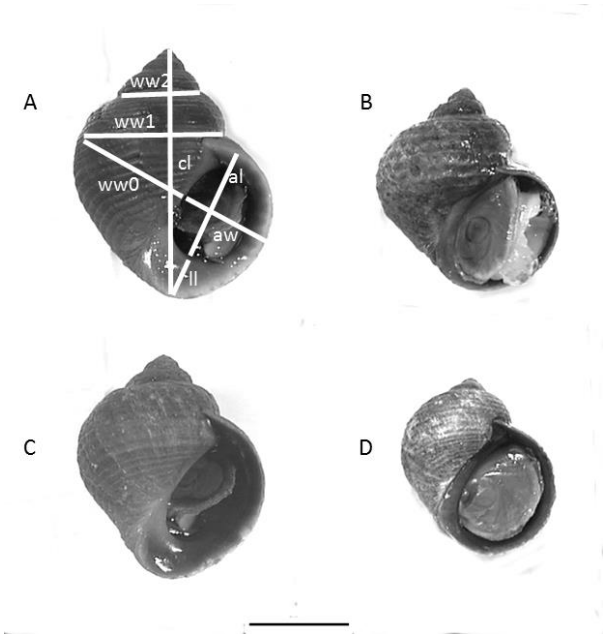

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705 Figure 1

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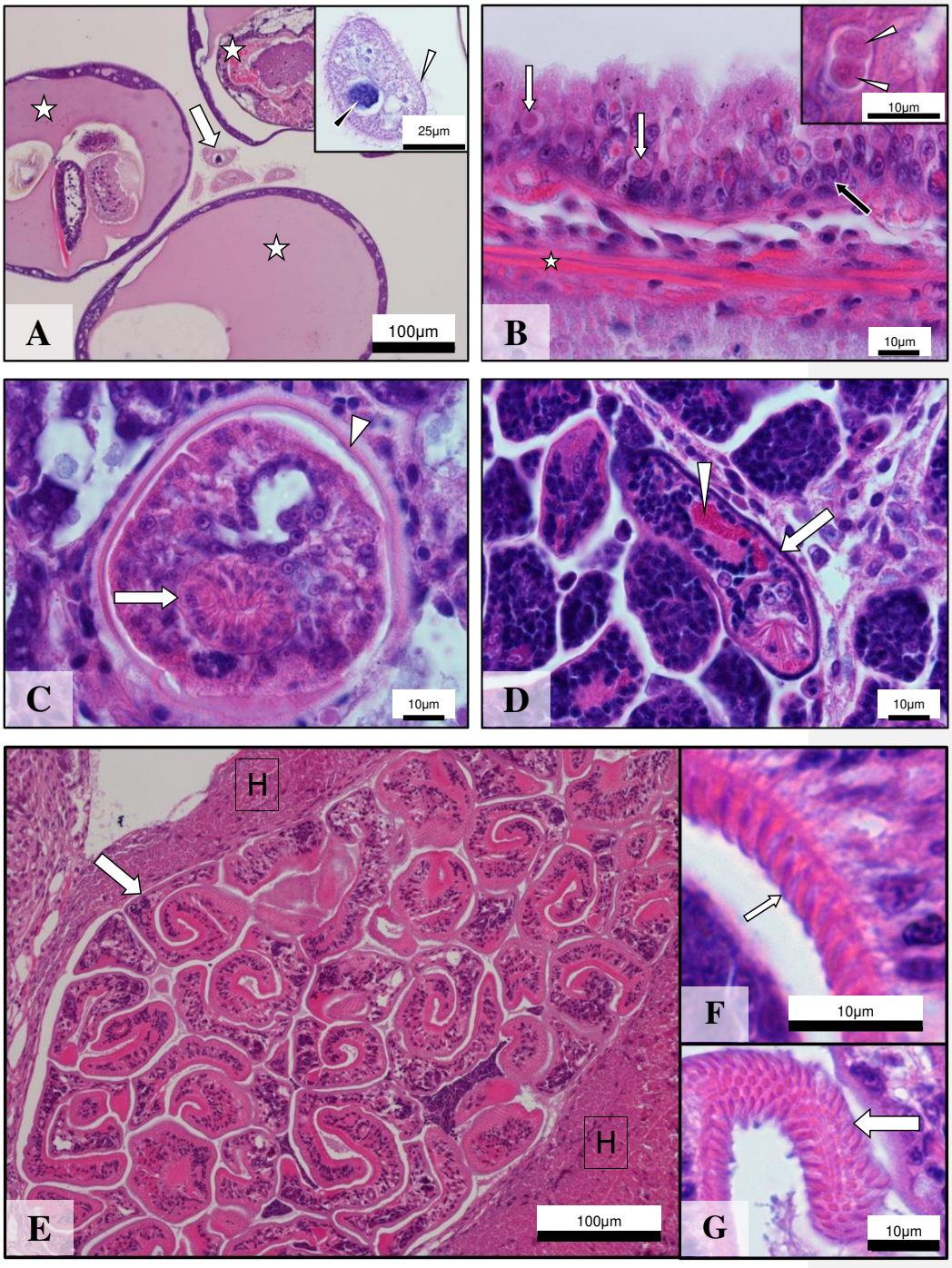

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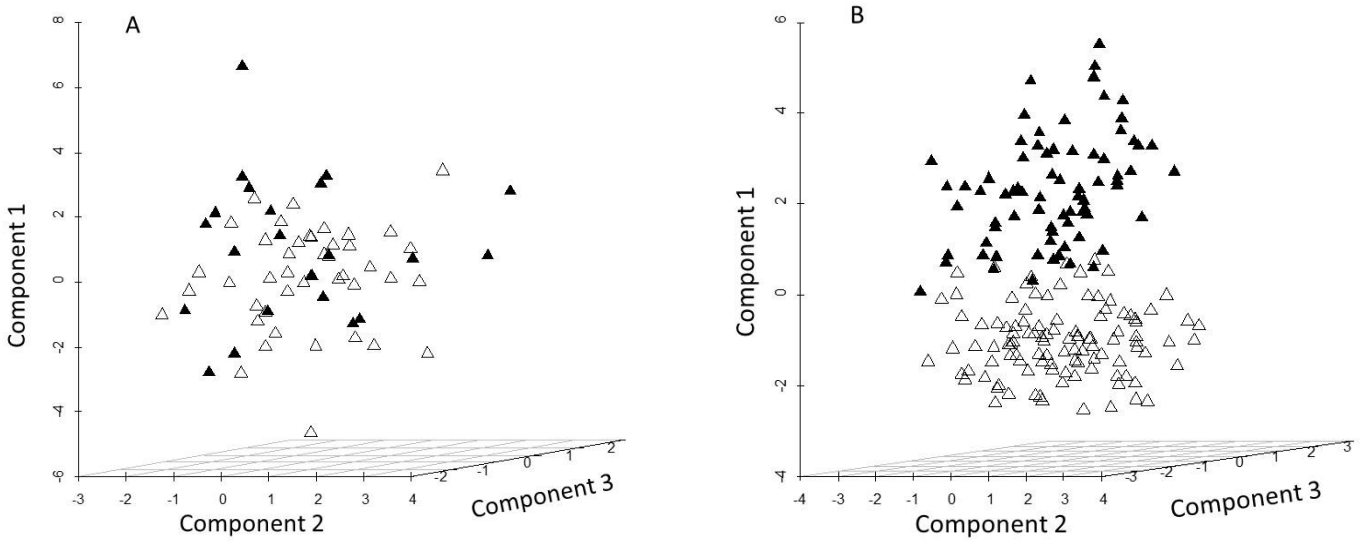
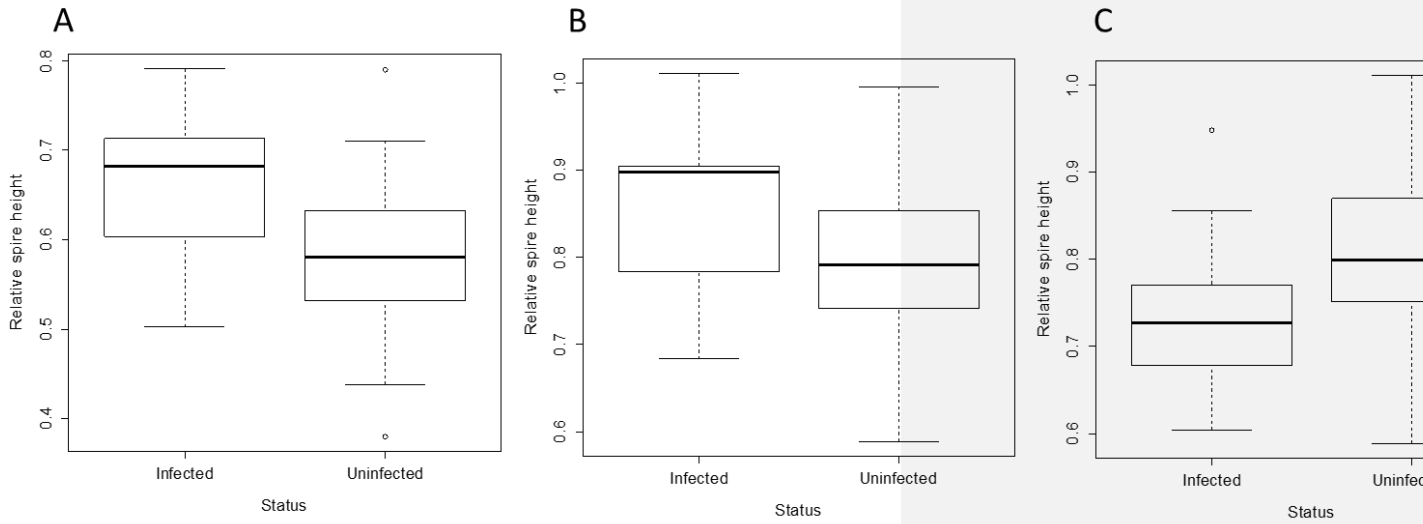

A
Component 1
Component 2
Component 3
B
Component 1
Component 2
Component 3

Figure 3



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728 Figure 4.

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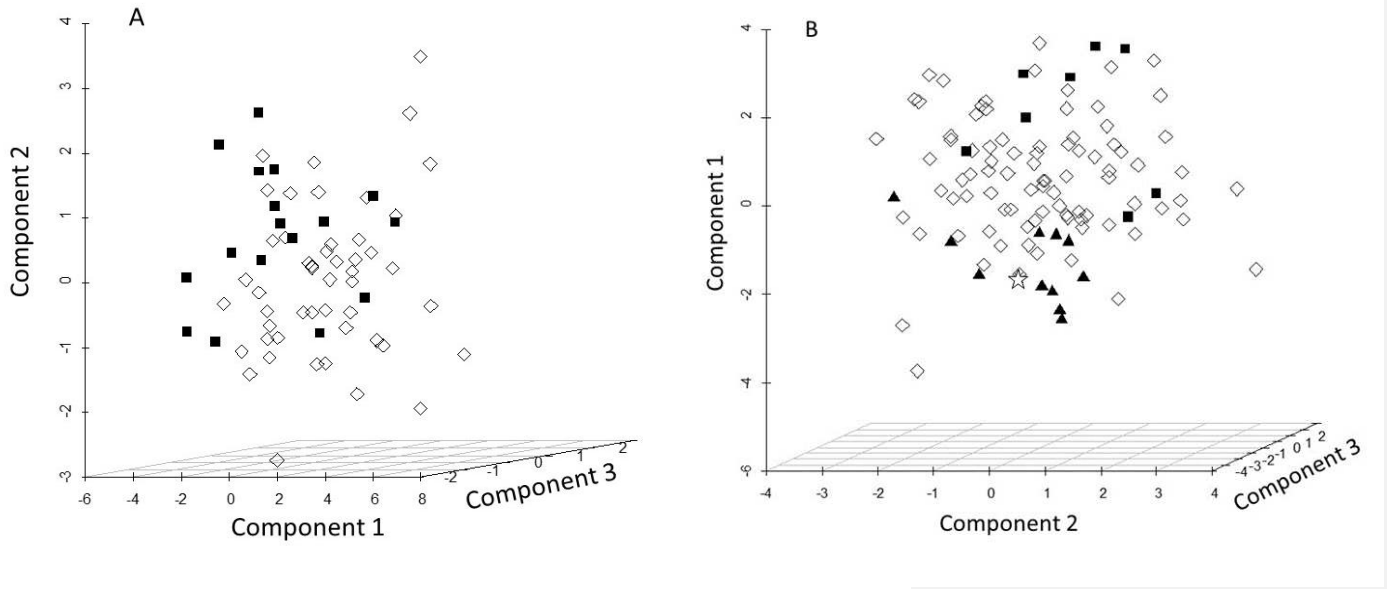
A
Component 2
Component 1
Component 3
B
Component 1
Component 2
Component 3

Figure 5

748 Supplementary information: Figures 1-3

Fig. S1. Specimens of *L. arcana* ordered on PC2, indicated as being associated with infection by the trematode *Microphallus similis*. Snails which are relatively tall (**B, D**) are more likely to be infected, though only **D** here is. **D** is the largest specimen shown, but there are other specimens of the same size which lack the trematode. Further there is no correlation between the geometric mean size of the shells and PC2 ($r = 0.127$, $P = 0.308$). We illustrate four specimens to show that allometric growth cannot itself be responsible for the transition between the squat, globose shape (**A, C**) to the more high-spired shape (**B, D**). The arrow indicates the 'direction' of the principal component, and indicates the scale of 5 mm.

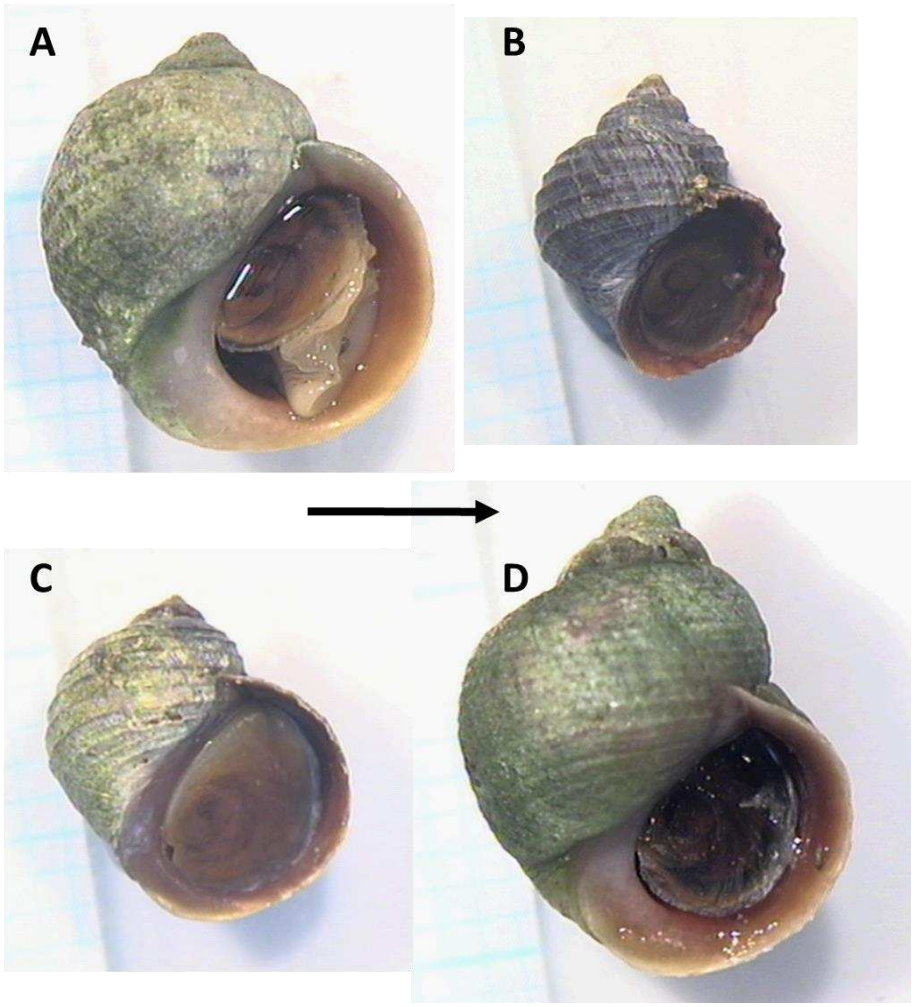

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Fig. S2. Specimens of *L. saxatilis* wave ecotype, ordered on PC3, indicated as being associated with presence of the ciliated protist *Protophrya ovicola*. **A.** Snails with a relatively large whorl width 0 (see Fig. 1 in the printed paper) are more likely to harbour the protist, as this specimen did, compared with those with a smaller ww0 (**B**), which did not. Relative ww0 size is estimated from the ratio of that dimension to the geometric mean size of all seven linear dimensions. For these specimens, that ratio is **A**: 1.241; **B**: 1.078. The arrow indicates the 'direction' of the principal component, and indicates the scale of 5 mm.

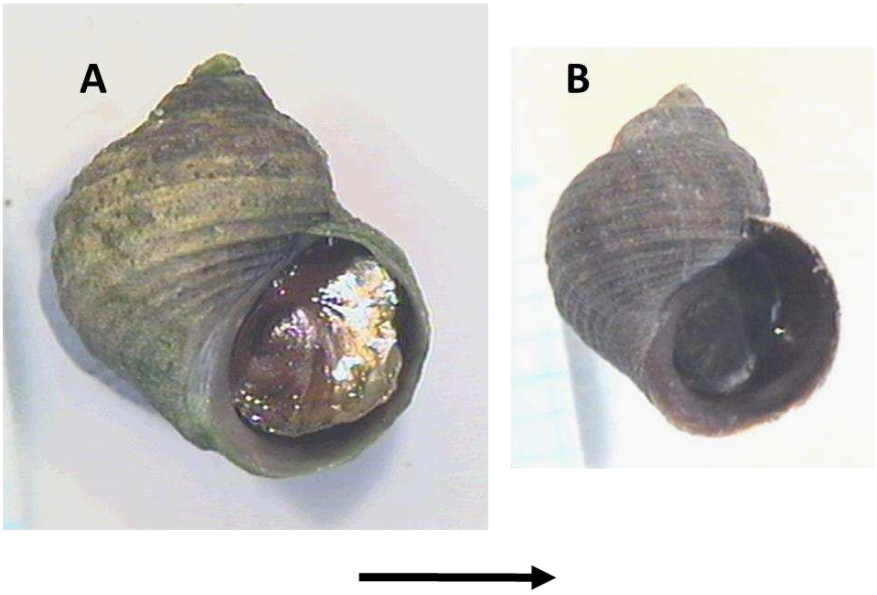

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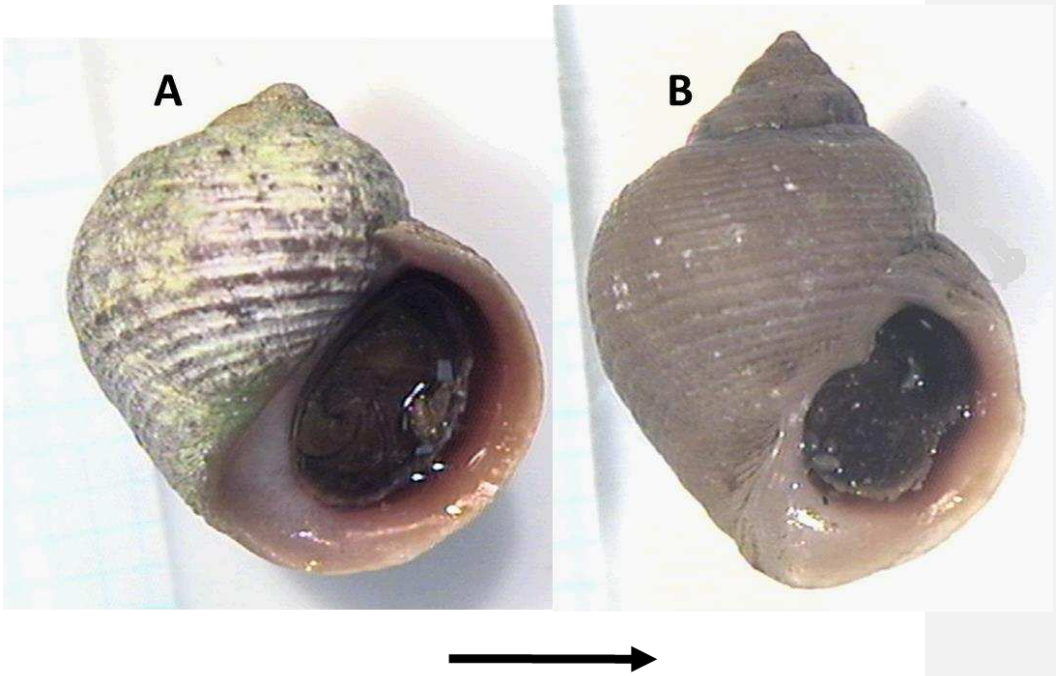
Fig. S3. Specimens of *L. saxatilis* crab ecotype, ordered on PC1, indicated as being associated with infection by the trematodes *Microphallus similis* or *Renicola* sp. **A.** Relatively squat, low-spired shell, more likely to be infected by *Renicola* sp. (though this specimen had no infection). **B.** Relatively high-spired, more likely to be infected by *M. similis* (as this specimen was). The arrow indicates the 'direction' of the principal component, and indicates the scale of 5 mm.



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