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Taken to the limit — Is desiccation stress causing precocious encystment of trematode parasites in snails?

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ABSTRACT

When hosts experience environmental stress, the quantity and quality of resources they provide for parasites may be diminished, and host longevity may be decreased. Under stress, parasites may adopt alternative strategies to avoid fitness reductions. Trematode parasites typically have complex life cycles, involving asexual reproduction in a gastropod first intermediate host. A rare phenomenon, briefly mentioned in the literature, and termed 'precocious encystment' involves the next stage in the parasites' life cycle (metacercarial cyst) forming within the preceding stage (redia), while still inside the snail. In the trematode *Parorchis* sp. NZ using rocky shore snails exposed to long periods outside water, we hypothesised that this might be an adaptive strategy against desiccation, preventing parasite emergence from the snail. To test this, we first investigated the effect of prolonged desiccation on the survival of two species of high intertidal snails. Secondly, we measured the reproductive output (cercarial production) of the parasite under wet and dry conditions. Finally, we quantified the influence of desiccation stress on the occurrence of precocious encystment. Snail mortality was higher under dry conditions, indicating stress, and it was somewhat exacerbated for infected snails. Parasite reproductive output differed between wet and dry conditions, with parasites of snails kept in dry conditions producing more cercariae when placed in water. Little variation was observed in the occurrence of precocious encystment, although some subtle patterns emerged. Given the stresses associated with living in high intertidal environments, we discuss precocious encystment as a possible stress response in this trematode parasite.

1. Introduction

Trematode parasites have maintained close relationships with gastropod hosts for millions of years [1]. This intimate association typically involves the gastropod playing first intermediate host to the trematodes, which reproduce asexually inside the snail tissue. The parasite reproduces by producing free-swimming stages known as cercariae, which are produced by parasite structures called sporocysts or rediae, depending on the trematode species. Once fully developed, cercariae emerge from the snail and must find their next host to continue the parasite's life cycle [2]. Some degree of plasticity has been reported in trematode life cycles in response to varying environmental conditions. For example, some trematodes can skip their definitive host through progenesis when the latter host is rare in the local environment [3,4]. This enables the parasite to bypass a transmission event and complete two life stages within a single host, most likely enhancing transmission or survival under specific conditions. In our study system, we observed another sort of abbreviated life cycle, here termed 'precocious encystment'. This might also be termed 'intraredial encystment'. While encystment within the asexually reproducing stage inside the snail first intermediate host has often been reported for trematodes with sporocyst stages [5], it has rarely been observed in species with rediae. The cercariae, instead of emerging from the snail, remain inside the rediae and form the next life stage, a metacercarial cyst (Fig. 1). The first record of precocious encystment dates back almost a century [6], with a few additional records published in the 1960's [7–11]. However, there exists very little information on the causes or implications of this phenomenon. Precocious encystment in the studies listed above was only observed in trematodes of the superfamily Echinostomatoidea and, thus far, only from those infecting freshwater snails. It has been suggested that the conditions under which precocious encystment might occur include: development under some form of stress, in a heavy infection, or in an unsuitable host [12].

A wide range of trematode species, including echinostomatoids, utilise littorinid snails, or periwinkles, as their first intermediate host [13–16]. These gastropods achieve high abundances in the intertidal environment, and have a global distribution on rocky shores [17]. The intertidal environment exposes its inhabitants to stress from wave action and air exposure, as well as huge variation in salinity and temperature. The majority of studies investigating these factors have focused on conspicuous organisms, such as littorinids. In this article, we delve deeper and investigate the effect of desiccation stress on trematode infections within these snails.

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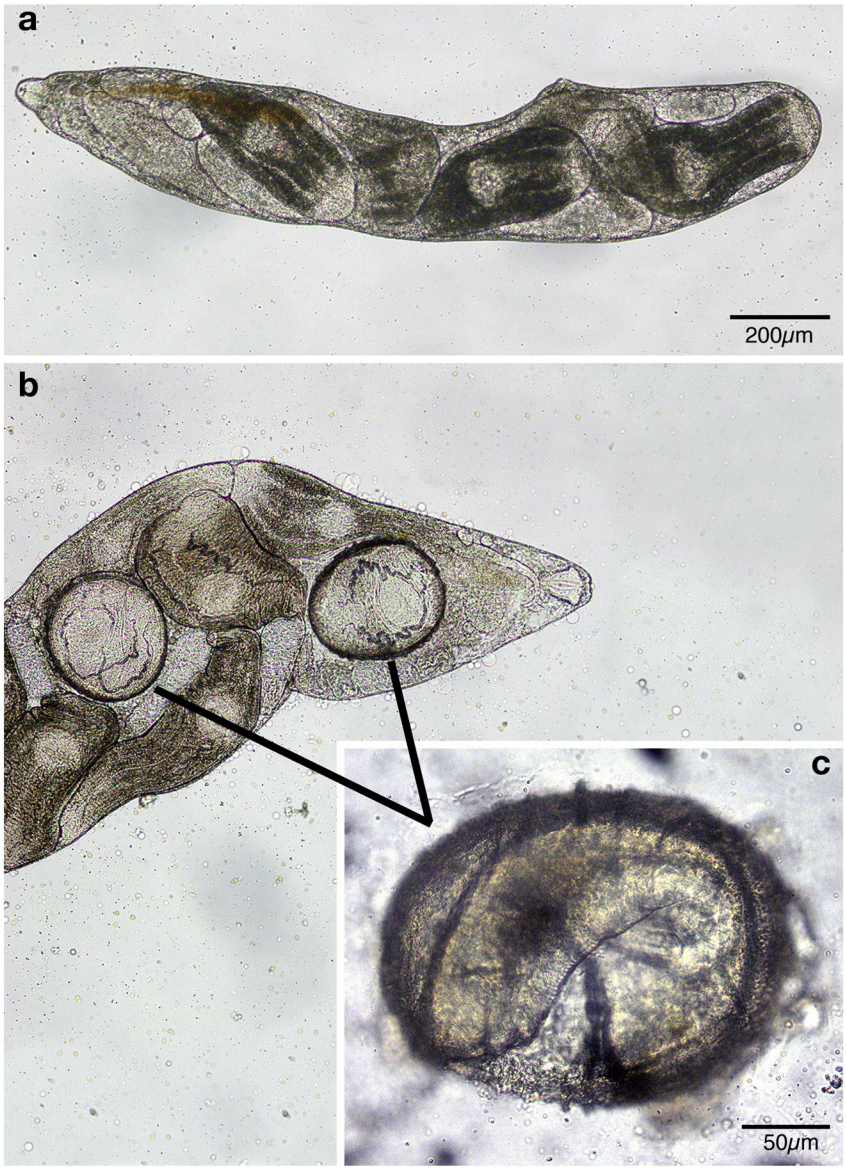

Fig. 1. Precocious or intraredial encystment. a = Normal redia, scale bar 200 μm; b = Redia with precociously encysted cercariae; c = Encysted cercaria; scale bar 50 μm.

Prior research has focused mostly on the combined effects of stress due to parasite infection and other types of environmental stress on the host, in the context of multiple stressors having cumulative effects on hosts (see review [18]). For example, desiccation stress, in combination with trematode infection, results in higher mortality for some gastropods [19]. Fewer studies have focused on the effects of environmental stress on the parasites themselves. For instance, temperatures and salinities outside the normal range negatively affect the survival, development and transmission of trematodes, by reducing cercarial output, encystment or survival of the encysted stage [20–22]. In trematode species using snails that are not permanently submerged, the presence of water triggers cercarial emission [2]. Therefore, although the outcome of environmental stress is often reduced cercarial output, the effect of desiccation is more likely to concentrate cercarial emergence during periods of immersion. This is because trematodes in snails kept in dry conditions are unable to release their cercariae until water is present. While earlier studies have investigated cercarial output as the response of the parasite to varying conditions, it remains unclear how external stresses can induce parasites within hosts to alter their developmental strategies and life cycles, in ways that may enhance their transmission success under difficult conditions [23].

Here, the effect of stress associated with desiccation in the intertidal zone was the main focus. Firstly, the effect of desiccation on host survival was investigated, in combination with parasite infection, and secondly, the effects of desiccation stress experienced by the host on the parasites it harboured were quantified. The high intertidal zone provides an ideal system for studying prolonged desiccation effects on trematode parasites and their hosts, as it is likely a common risk for organisms living there. It was hypothesised that (i) under desiccation stress, infected snails would incur higher mortality rates than uninfected ones; (ii) more cercariae would be released from snails when placed in water if they were previously in dry conditions; and (iii) cercariae would be more likely to encyst within snails. The focal study system consisted of the two species of New Zealand littorinids, the blue-banded *Austrolittorina antipodum* and the brown *Austrolittorina cincta*, which host the same parasite, *Parorchis* sp. NZ, described in [24]. The results show that desiccation stress increased mortality in the littorinid host and affected the development of the parasite's transmission stages.

2. Materials and methods

2.1. Collection and maintenance

Large numbers of snails of both *A. antipodum* and *A. cincta* were collected haphazardly from a boulder beach in Lower Portobello Bay, Otago Harbour, South Island, New Zealand (45°520 S, 170°420 E) on 28 October 2012. The snails were transported to the laboratory and allowed to acclimate for 48 h in shallow seawater. They were then kept individually in 12-well tissue culture plates, with 3 ml filtered seawater. To induce cercarial release, they were placed on an orbital shaker, at 80 rpm, for 7 h. The plates were screened using a stereomicroscope to identify snails releasing cercariae of the philophthalmid parasite *Parorchis* sp. NZ [24]. As one-off screenings often miss infected snails, this was repeated three times, with approximately 10 days between successive screenings. Between screenings, the snails were kept in containers with rocks from the collection site and filtered seawater. The experiment was started on 24 November 2012 with infected and uninfected snails divided into separate groups and assigned to plastic containers (17 cm × 12 cm × 7 cm), with either 'dry' or 'wet' conditions. Three replicate containers were used for each snail species and for infected and uninfected individuals, resulting in 24 containers in total, each containing approximately 20 individual snails. Overall, *A. antipodum* individuals used in the experiment had shell lengths ranging from 6.34 to 14.04 mm (mean ± SD: 9.71 ± 1.52 mm), and *A. cincta* individuals had shell lengths ranging from 6.24 to 19.98 mm (14.55 ± 2.62 mm).

Seawater (2 cm deep, adequate for full immersion of littorinid snails) was added to all containers in the 'wet' conditions; the water was changed at weekly intervals, while the snails in 'dry' conditions, without any water, were disturbed at the same time and to a similar extent, to control for disturbance of the 'wet' snails. For both treatments, a slanted ceramic tile was added to each container to provide a more natural grainy substrate for the snails, also allowing them to exit the water at will. The containers, with mesh lids, were arranged in a randomised fashion on a bench top, and maintained at ~17 °C and in a naturally lit area of the laboratory. Snails, totalling 211 individuals of each species, were kept in these conditions for 12 weeks; they were checked every few days and any dead snails were recorded and removed.

2.2. Cercarial release

At 6, 9 and 12 weeks after the start of the experiment, approximately five infected snails per container were assigned again to individual wells in 12-well plates with 3 ml seawater and left overnight. This allowed the snails to acclimatise to the presence of seawater and for cercariae to begin to emerge. In the morning they were put in new 12-well plates and placed on an orbital shaker at 80 rpm to induce cercarial emergence. Every hour for the first 3 h, and later at 5 and 7 h, the plates were screened for free-swimming and/or encysted cercariae, which were counted under a stereomicroscope. The snails were not returned to their container of origin but were dissected for further data acquisition (see below). In total 84 individuals of *A. antipodum* (31 from dry conditions; 53 from wet conditions) and 105 individuals of *A. cincta* (44 from dry conditions; 61 from wet conditions) were measured for cercarial release across the 6, 9 and 12 week periods.

2.3. 'Precocious encystment' within rediae

At each of the 6, 9 and 12 weeks intervals, following the cercarial release observations (details above), snails were dissected and the rediae inside were counted. This was done by first dissecting each snail in a petri dish and removing the upper portion of body tissue, containing the gonad and digestive gland, for closer inspection. The snail tissue was then dissected to separate the parasite tissue. This was compressed gently between two glass plates, with the addition of Neutral Red dye. The rediae were counted, the presence or absence of encysted cercariae within rediae was recorded and, when present, the number of rediae containing cysts was noted. As some snails did not shed cercariae but still contained infections sample sizes were slightly greater here. In total 95 *A. antipodum* individuals were measured (35 from dry conditions; 60 from wet conditions) and 124 *A. cincta* individuals (52 from dry conditions; 72 from wet conditions) across the three measurement time periods.

2.4. Data analysis

All analyses were carried out in R version 3.1.0 [25]. Data were analysed separately for each snail species and for datasets from 6, 9 and 12 week measurements. The variable 'snail length' was centred before inclusion in the models.

Snail mortality data were analysed using binomial bias-reduced generalised linear models. Bias reduction was required due to snail groups which did not experience mortality, and so the same value (alive) was recorded for all snails in those categories. This was done using the function *brglm* from the package *brglm* [26]. Due to the use of bias reduced models, the random effect of tub identity could not be included. The main effects used in the models were treatment, infection status and snail length. The interaction treatment × infection status was also included.

Data for cercarial release (numbers of cercariae released per snail during the full period on the orbital shaker) were overdispersed, as confirmed by the observation of the residual deviance being much larger than the residual degrees of freedom. Zero inflation was not observed, however, and the data were log-transformed. Analysis of these data was carried out using negative binomial generalised linear mixed models. The random effect of tub identity was included in the model to control for snails coming from three different containers per treatment, and the main effects were treatment, snail length and the interaction of both. The analysis was done using the function *glmmadmb* in the R package *glmmADMB* [27].

The proportion of infections which contained cysts (i.e., proportions of infected snails in which encysted cercariae were found in at least some rediae) were analysed using binomial generalised linear mixed models using the function *glmer* in the R package *lme4* [28]. The random effect of tub identity was included, and the main effects were: treatment, snail length.

3. Results

3.1. Snail mortality

Both snail species suffered greater mortality when exposed to prolonged dry conditions (Tables S1 and S2 [Supplementary material]; Fig. 2). This difference between treatments was only significant for *A. antipodum* after 12 weeks of desiccation stress ($z = -3.02$), although a similar trend was observed for *A. cincta* whereby mortality increased with time spent under stress by desiccation (Tables S1 and S2; Fig. 2). The effect of infection status did not significantly affect the mortality of snails (Table S1 and S2), although infected snails tended to succumb more, especially in dry conditions (Fig. 2). Due to small sample sizes, as outlined in the Methods Section 2, the model results are less clear than the trends apparent in Fig. 2.

3.2. Cercarial release and encystment

After exposure to dry conditions, more cercariae were released from snails when these were returned to water compared to snails kept in wet conditions throughout the 12 weeks (Tables S3 and S4; Fig. 3). Cercarial output from snails in wet conditions was much less variable than that from snails kept in dry conditions. However, this was less apparent for *A. cincta* (Fig. 3). For both species, the effect of treatment was

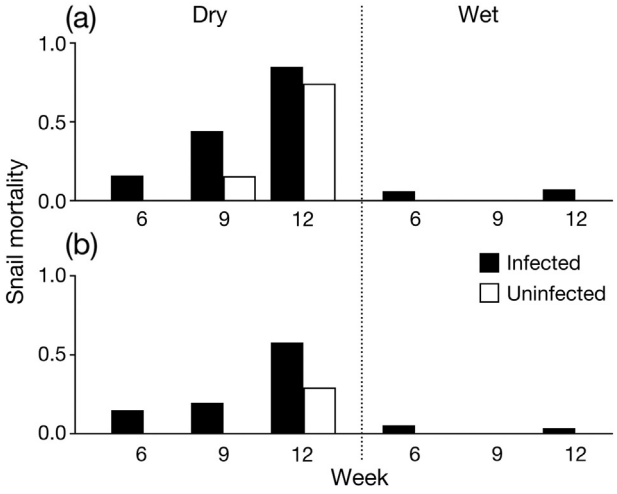

Fig. 2. Snail mortality, expressed as a proportion of the initial number, for infected [black] and uninfected [white] a) *Austrolittorina antipodum* [total N = 211] and b) *A. cincta* [total N = 211], in dry conditions and wet conditions at 6, 9 and 12 weeks.

significant for all weeks (z = −2.31 to −5.83; Tables S3 and S4), except after 12 weeks for *A. cincta* when no significant difference was observed (z = 0.13; Table S4).

3.3. Precocious encystment in rediae

The occurrence of precocious encystment in trematode infections was not found to vary with desiccation, i.e. it did not differ between wet and dry treatments (Tables S5 and S6; Fig. 4). Snail length was found to be a significant predictor of precocious encystment in *A. cincta*

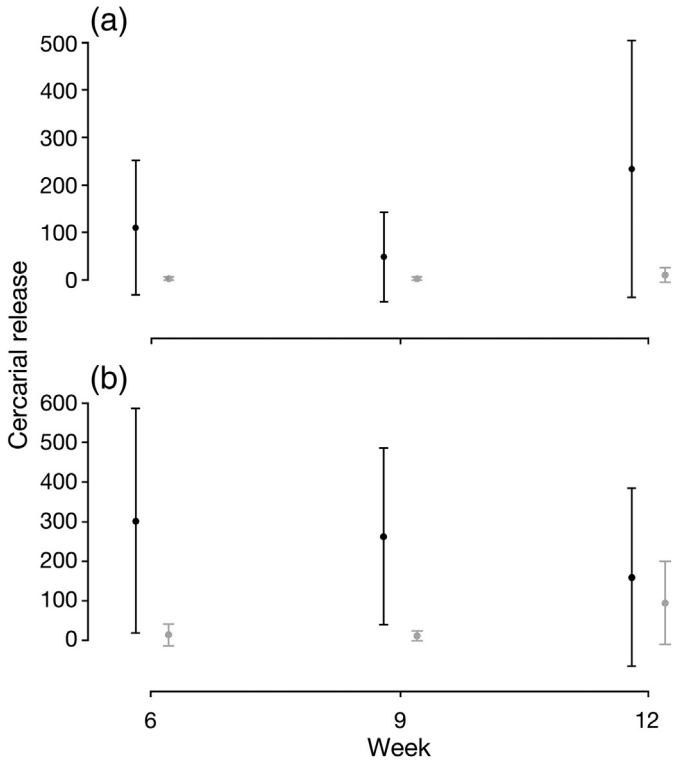

Fig. 3. Mean cercarial release, i.e. mean number of cercariae emerged per snail during the full period on the orbital shaker, for a) *Austrolittorina antipodum* [total N = 84] and b) *A. cincta* [total N = 105], in both wet and dry conditions at 6, 9 and 12 weeks. Grey circles and error bars correspond to wet conditions, and black circles and error bars, to dry conditions. Error bars represent standard error (SE).

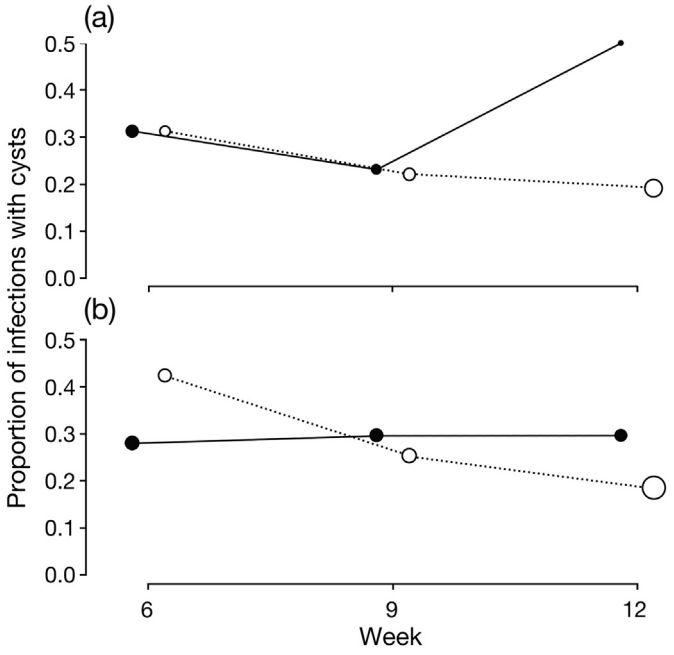

Fig. 4. The proportion of infections with rediae containing cysts, for a) *Austrolittorina antipodum* [total N = 95] and b) *A. cincta* [total N = 124], in both wet and dry conditions at 6, 9 and 12 weeks. Open circles correspond to wet conditions and solid circles to dry conditions. Circle size is proportional to sample size, which ranges from 6 to 33 snails.

after six weeks (z = 2.692; Table S6) but this effect was not found after nine and 12 weeks. In general, in wet conditions the occurrence of cysts in rediae within both snail species decreased over the 12 weeks (Fig. 4), while in dry conditions, although non-significant, more infections contained cysts at 12 weeks for *A. antipodum* (Fig. 4a). The occurrence of cysts in infections of *A. cincta* remained relatively unchanged over the 12 weeks, whether in wet or dry conditions (Fig. 4b).

4. Discussion

Due to large fluctuations in the tidal cycle, organisms in the high intertidal zone may be out of the water for substantial periods of time (e.g. months) [29,30]. Several species of littorinid, including the two New Zealand species studied here, inhabit the extreme high shoreline and, hence, are more likely to experience stress due to desiccation than species inhabiting the lower levels of the shore.

In this study, desiccation was found to be stressful for both snail species, as evidenced by the higher mortality rate recorded for snails kept in dry conditions, in comparison to those housed in wet conditions. Snails kept in dry conditions were also found to release much greater numbers of cercariae, at each measured time point, than those in wet conditions. This is to be expected because snails in wet conditions are likely to be continuously releasing cercariae. The stress due to desiccation did not result in higher rates of within-snail cyst formation by the parasite, as we found little difference in the frequency of cyst formation inside trematode rediae between snails from dry or wet conditions. However, we did observe a trend for increased cyst formation under dry conditions after twelve weeks, while the prevalence of cyst formation in snails in wet conditions appeared to subtly decrease over the twelve week period. The difference in mortality between the two snail species, as well as the difference between them in the extent to which precocious parasite encystment increased over time, may be related to their natural distribution on rocky shores. The snails are thought to have slightly different distributions on the shore, with *A. antipodum* favouring the upper levels of the high intertidal zone compared to *A. cincta* [31]. This could perhaps explain differences in the occurrence of precocious encystment between the two species, with the two *Austrolittorina*

having different tolerances to desiccation. Furthermore, the parasites could behave differently in the two snail species. For example, varying degrees of pathogenicity of the same parasite species in different snail host species have been reported [32], suggesting that the same parasite could also follow different developmental routes in different snail hosts.

Periods of prolonged desiccation may have selected for an adaptive and facultative abbreviation of the life cycle by the parasite when it is without access to water for cercarial release. This strategy could allow eventual transmission to a bird final host via direct predation on snails, without the need for emergence of cercariae under difficult conditions. Many littorinid species, including those from the high intertidal zone, have been well-studied for their trematode parasites but encystment in rediae has never been observed (K. Galaktionov, pers. comm.). Although precocious encystment was first reported in the literature almost one hundred years ago [6], no studies have thus far attempted to elucidate the reasons for this behaviour in the parasite.

The trematode investigated in this study, commonly found infecting New Zealand high shore littorinids, is a member of the family Philophthalmidae which belongs to the Superfamily Echinostomatoidea. It is interesting to note that, following extensive observations of another trematode (Notocotylidae sp. 1 NZ), also infecting New Zealand littorinids and using redial stages, no cysts were ever recorded inside the rediae of that other trematode (K O'Dwyer, pers. Obs.). This report of cercariae encysting within rediae by the parasite *Parorchis* sp. NZ is in agreement with the few previous reports found, which only recorded this phenomenon from trematodes in the Echinostomatoidea [7–11]. However, all earlier records were from freshwater snail hosts, and to the authors' knowledge, this is the first report of precocious encystment from an echinostomatoid trematode infecting a marine snail.

As mentioned earlier, it has been suggested that precocious encystment is observed when the infection appears somehow compromised or in poor health [12]. No significant effect of desiccation stress on the occurrence of precocious encystment was found in this study. Throughout our observations, we recorded precocious encystment in rediae from both seemingly 'healthy' and 'not so healthy' infections, based on the numbers, size and appearance of rediae, although this was not directly measured. It is also worth noting that snails in both wet and dry conditions equally experienced starvation in this experiment, however snail mortality was greater, albeit non-significantly so, under dry than wet conditions, indicating higher stress experienced by these snails. The slight decrease in the frequency of precocious encystment in snails kept in wet conditions, along with a subtle increase under dry conditions, suggests that there exists a natural baseline level of precocious encystment in infections of this parasite, which may then respond to some degree to external conditions.

The context-dependent nature of host-parasite interactions has been highlighted in many recent studies [33–35]. It may be that the slight but noticeable increase in precocious encystment of parasites in snails from dry conditions is indeed a response to the absence of water. This could enable parasites of snails that remain out of water for extended periods of time, to remain viable for longer. Furthermore, the local abundance of predators or definitive hosts in the environment, which may directly prey on the snail hosts of trematodes, might affect the level of precocious encystment in the population. The diversity of trematode developmental strategies has long been assumed to be due to changes in transmission opportunities [36,37]. This is clearly supported by the existence of progenesis or other types of life cycle truncation in multiple trematode species from 20 different families, and the fact that the adoption of these alternative life cycle routes is often cued by external factors [38]. In the present study, the reasons behind the occurrence of intra-redial cysts, whether adaptive or not, remain unclear as yet, but certainly deserve further study. An interesting avenue for future research would be to use comparisons among geographically distinct populations to assess the influence of the local intensity of direct predation on snails by definitive hosts on the occurrence of within-snail precocious encystment.

5. Conclusions

Precocious encystment has been described here in *Parorchis* sp. NZ and illustrations are provided as a reference for future studies. The effect of prolonged desiccation on the occurrence of this phenomenon was investigated. Although prolonged desiccation was found to be stressful for the snail hosts, no strong relationship was found between prolonged desiccation stress and precocious encystment in this trematode-snail system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2015.09.001.

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