



Varying levels of melanotic encapsulation of gordiid hairworm cysts (Nematomorpha) by aquatic insect larvae: seasonal and host effects

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ARTICLE INFO

Keywords:
Host immunity
Host-parasite interactions
Trichoptera
Conoosucidae
Gordiida

ABSTRACT

The defence reactions of insects to parasitic invaders are both varied and complex. Melanisation of pathogens is often an important step in insect immunity and can play a key role in isolating parasites. Within samples collected from a subalpine stream in New Zealand during two consecutive seasons (i.e., winter and spring), we observed and categorised different levels of melanotic encapsulation by aquatic insect larvae to dormant *Gordius* sp. hairworm (Phylum Nematomorpha) cysts, a relatively obscure group of parasites. Some of these insect species act as intermediate transport hosts in the complex life cycle of hairworms. Based on these new observations, we calculated the melanisation response for an abundant species of caddisfly larvae (*Olinga* sp.) using the proportion of non-melanised cysts per individual host. We tested the hypothesis that season and total number of cysts in an infected host impact its melanisation response. Also, we explored the effect of host body size on the total number of cysts it carries. We found that the total number of cysts does not affect the melanisation response of the host. Season did have an impact on the melanisation response in *Olinga* sp., with lower levels observed in the spring. Additionally, larger caddisfly larvae harboured more cysts than smaller ones. Since little is known about the cryptic interactions between hairworms and their intermediate hosts, this new information adds some complexity to this poorly understood group of parasites.

1. Introduction

Parasites with complex life cycles have to overcome multiple challenges during their transmission from one host to the next (Poulin and Lagrue, 2015; Thieltges et al., 2013). In the case of trophic transmission, the capacity of a parasite to survive the internal defence reaction of its intermediate host may increase the odds of a successful transmission. Conversely, the internal defence reaction of intermediate hosts can considerably hinder the life cycle of parasites by effectively blocking their transmission (Buckling and Read, 2001; Fox et al., 2013). In particular, insect hosts have evolved rapid immune responses to parasites capable of breaching their outer defence barriers (e.g., the cuticle and endothelia) (Schmid-Hempel, 2005). Moreover, the innate immune reactions of insects toward parasitic infections are complex and can result in multiple chemical and cellular responses (Gillespie et al., 1997). One of these responses involves melanotic encapsulation or melanisation, a humoral response resulting from the activation of phenoloxidase in reaction to injury (i.e., the proPO system) (Bidla et al., 2005; Brivio et al., 1996; Nakhleh et al., 2017). Here, we explore the melanisation of freshwater macroinvertebrates, specifically insect larvae, in response to natural parasite infections during two consecutive

seasons in New Zealand.

Freshwater hairworms or gordiids (Nematomorpha: Gordiida) are a relatively obscure group of parasites with a complex life cycle that includes five recognised life stages and multiple invertebrate hosts (Hanelt et al., 2005). Adult gordiids, found mainly within scavenger and/or predatory terrestrial insects (e.g., cave wētā, praying mantids, and cockroaches), are recognised as manipulative parasites capable of inducing their definitive host to enter water so they can exit the host, mate, and lay their eggs (Ponton et al., 2011; Thomas et al., 2002). The larvae that hatch from these eggs do not swim efficiently and become part of the benthos, where they are consumed by a myriad of aquatic animals, mostly invertebrates but also including vertebrates (e.g., Torres et al., 2017). With the use of specialised mouthparts, gordiid larvae can move through and encyst indiscriminately within the tissues of these intermediate hosts (Hanelt and Janovy, 2003). Some intermediate hosts, such as aquatic snails or fish, never exit water and act as “dead-end” hosts, which could represent population sinks for gordiids (Hanelt et al., 2001). Aquatic insect larvae (e.g., mayflies, midges, and stoneflies) are presumably the true paratenic hosts responsible for transporting cysts to dry land after they metamorphose, where they are eventually consumed by definitive hosts (Hanelt and Janovy, 2004).

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<https://doi.org/10.1016/j.jip.2019.107258>

Received 28 June 2019; Received in revised form 8 October 2019; Accepted 9 October 2019

Available online 11 October 2019

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Few studies have looked at the cryptic interactions between gordiid larvae/cysts and their intermediate hosts (Hanelt and Janovy, 2003; Poinar and Doelman, 1974). Therefore, relatively little is known on the effects of gordiid infections in aquatic macroinvertebrates.

The melanisation of gordiid larvae and/or cysts has been observed in certain species of intermediate insect hosts. Poinar and Doelman (1974) noted that heavy cyst loads can increase host mortality rates in laboratory-induced infections. However, it is unknown how gordiid cysts impact host survivability in nature. Most observations on melanisation of hairworms by intermediate insect hosts were done on gordiid larvae. Early reports found that chironomid *Chironomus* larvae react to the intrusion of gordiid *Chordodes japonensis* larvae by covering them in a special chitinous “cyst wall” (Inoue, 1960). Poinar and Doelman (1974) also noted that late-instar culicid *Culex pipiens* larvae react quickly to penetration of the larvae of the gordiid *Neochordodes occidentalis* by depositing melanin; gordiids encysted normally in early-instar *C. pipiens* larvae. This host instar-dependent reaction was also observed in other dipterans infected with *Paragordius varius* larvae (de Villalobos and Ronderos, 2003; de Villalobos et al., 2006). Lethal haemocyte encapsulation and melanisation was observed in larval *Culex tarsalis* infected with three common gordiid species, although these reactions were independent of host instar (Hanelt and Janovy, 2003). Melanised gordiid cysts were observed in species of immature Trichoptera, Plecoptera, Ephemeroptera, and Megaloptera in New Zealand (Poinar, 1991). It would appear that melanotic encapsulation against gordiids varies greatly between intermediate host species in terms of host development and its lethality toward different gordiid life stages (i.e., larvae and cysts). However, it remains unknown if melanotic encapsulation can prevent excysting of dormant gordiid larvae within their definitive host. Melanisation may be an important factor in the internal defence reaction against cysts and is known to be toxic for other parasites (Volz et al., 2006), but this complex biochemical cascade is only a part of insect immunity toward pathogens (Gillespie et al., 1997; Nakhleh et al., 2017).

Observations of gordiid cyst infections suggest that prevalence levels change according to season and insect taxon. In Japan, differences of infection prevalence were observed in five insect taxa across all seasons (Yamashita et al., 2017). Chironomid larvae collected from a stream in northern Taiwan were found to harbour cysts almost year-round, with a peak of infection prevalence occurring in mid-September (Chiu et al., 2016). In this study, it was noted that some of the cysts were darker in appearance (up to half in one of three identified cyst morphotypes), presumably caused by the melanisation of the cysts by their dipteran hosts. Humoral encapsulation of gordiids by intermediate hosts is known to vary according to host taxon and development stage (see above), but it is not known whether melanisation is affected by seasonal changes. Additionally, seasonality and host taxon may not be the only factors impacting hairworm infection levels and the melanisation in intermediate hosts. Host body size is known to affect parasite prevalence and intensity, considering that a bigger host is more likely to harbour more parasites (Poulin, 2013; Yule and Burns, 2015). Also, parasite load can increase energy costs and weaken the immune system of a host (Hicks et al., 2018; Sheldon and Verhulst, 1996). Therefore, it is possible that host size plays a role in the internal defence reaction against hairworm cysts.

In the current study, we present new observations of melanotic encapsulation in different invertebrate taxa of intermediate hosts infected with *Gordius* sp. hairworms and categorise them according to level of intensity in order to calculate a general host melanisation response. With this new information, we investigated the relationship between the melanisation response, sampling season (i.e., winter and spring), and total cyst load (i.e., the intensity of hairworm infections) for larvae of one insect taxon, *Olinga* sp. caddisfly, that was abundant in both seasonal samples. We hypothesised that a greater parasitic load in a host impacts its capacity to defend itself, resulting in a higher proportion of hairworm cysts that elicit no visible melanisation response.

We also explored the relationship between the size of *Olinga* sp., the total number of hairworm cysts it harbours, and its melanisation response. In this case, we hypothesised that host body size affects both parasitic load and melanisation response and predicted that larger hosts harbour more parasites, and thus are less capable of defending themselves against parasites (i.e., a lower melanisation response). We also present data on seasonal changes in the prevalence of hairworm infections in all sampled aquatic macroinvertebrate host taxa.

2. Material and methods

2.1. Sampling natural hairworm infections in intermediate hosts

Samples were collected on 18 June 2018 (winter) and 2 October 2018 (spring) from Grasmere Stream near Cass Field Station (43°02'7" S, 171°45'29" E), in the Canterbury region of New Zealand. In a 40-meter section of the stream, fine mesh dip nets were lightly dragged across the aquatic vegetation to collect macroinvertebrates; bottom fauna was collected downstream in the nets by kicking upstream rocks. These samples were kept alive in small containers of aerated river water until counted in the laboratory, where they were identified to genus or species level. Afterwards, each macroinvertebrate was flattened between a microscope slide and cover glass for examination under the microscope at 100x magnification, in order to count hairworm cysts and/or larvae. For aquatic snails, tissues were separated from the shell using fine tweezers before flattening. Caddisfly larvae were also removed from their protective cases. In order to maintain osmotic pressure around cysts, a drop of Ringer's solution was added to the slide preparation (Barbosa et al., 2015). Hairworm larvae were identified to genus level, based on their folding pattern and morphological characteristics (Szymygiel et al., 2014).

2.2. Categorising melanotic encapsulation

Hairworm cysts were visually categorised into three levels of melanisation using the colouration caused by humoral encapsulation as an indicator of intensity (Fig. 1A). A cyst without colouration nor the presence of haemocytes surrounding it was recorded as non-melanised; dormant hairworm larvae were clearly visible through the cyst wall (Fig. 1B). Cysts with melanin deposits forming a characteristic “barrel-shaped” pattern were considered partially melanised; colour varied from light to dark amber and larvae were still visible through the cyst wall (Fig. 1A and C). The third group showed no discernable melanisation pattern and cysts were completely covered in melanin deposits, resulting in an opaque amber colour. These cysts were categorised as fully melanised and larvae were difficult to observe unless a very bright light was used (Fig. 1D).

2.3. Melanisation response in caddisfly larvae

To explore the relationship between the melanisation response in an intermediate host, the sampling season (i.e., winter and spring), and the total cyst load (i.e., number of hairworm cysts per infected individual), we selected larval *Olinga* sp. (Trichoptera: Conoesucidae) as a representative group for two reasons: (1) this species was the most abundant of all insect taxa sampled on both dates; (2) they had variable levels of melanisation corresponding to all three categories described above. The melanisation response was calculated as the proportion of non-melanised cysts per total number of cysts in each infected caddisfly. Therefore, the proportion of non-melanised cysts is inversely proportional to the melanisation response of the host. Because caddisfly larvae are soft-bodied and it is difficult to measure their body length accurately, the protective cases of *Olinga* sp. sampled in June were measured to the nearest 0.5 mm as a proxy for host body size.

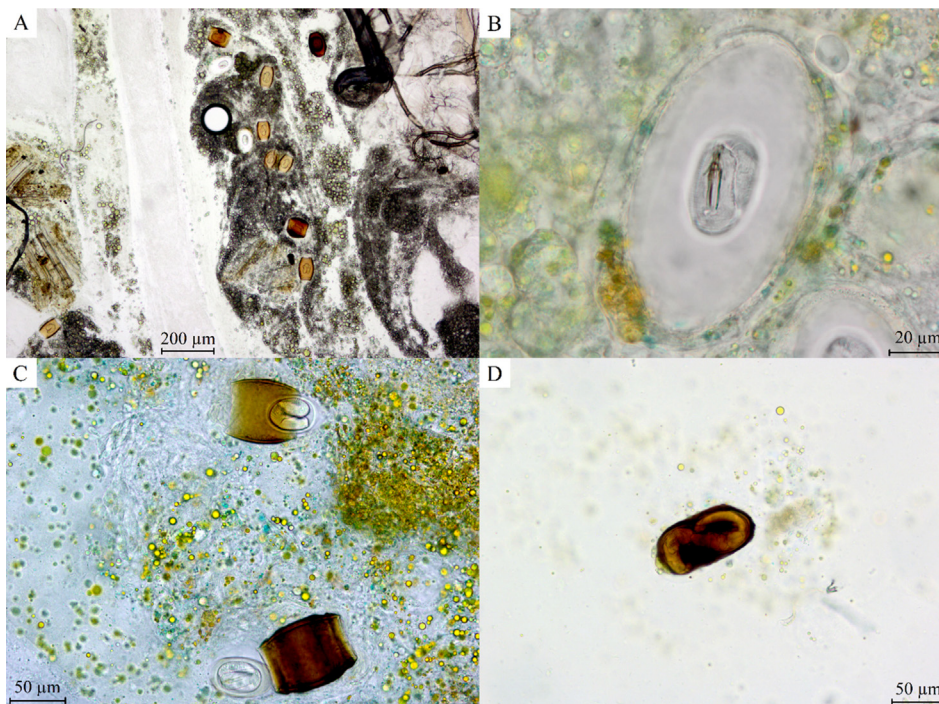


Fig. 1. *Gordius* sp. hairworm (Nematomorpha) cysts found in larvae of *Olinga* sp. sampled from Grasmere Stream, Cass Field Station in Canterbury, New Zealand on 18 June 2018. Host tissues were flattened and pictures taken with a C-Mount microscope camera. (A) Group of cysts with different levels of melanotic encapsulation located around the digestive tract (situated left of group) at 40× magnification. (B) Non-melanised cyst, denoted by a clear cyst wall around a dormant folded hairworm larva (centered) at 400x magnification. (C) Two partially melanised cysts, with the non-melanised portion separating from the melanised portion (top cyst) and completely separated (bottom cyst) at 100× magnification. (D) Fully melanised cyst with an unfolded larva apparently trapped within (centered) at 100x magnification.

2.4. Statistical analyses

All statistical analyses were performed using R version 3.6.0 (R Core Team, 2019). To test the hypothesis that melanisation response varies according to season and total cyst load in *Olinga* sp., we used a beta regression model with the *betareg* package (Cribari-Neto and Zeileis, 2010). This model was selected because melanisation response is a proportion and can only assume values between 0 and 1 inclusively (Ferrari and Cribari-Neto, 2004). Values assuming exactly 0 and 1 were transformed according to Smithson and Verkuilen (2006). Fixed effects were season (winter or spring) and total cyst load (number of cysts per infected caddisfly). Another beta regression was performed to evaluate the effect of host body size and total cyst load on the melanisation response, for *Olinga* sp. collected in June (the only month for which body size data were available). For both analyses, total cyst load was log-transformed. Model selection was done with the AICc and residuals were checked with the “standardised weighted residual 2” for beta regressions suggested by Espinheira et al. (2008). Also, we used a generalised linear model with a gamma distribution (*lme4* package, Bates et al. 2015) to test the hypothesis that host body size positively affects total cyst load. This model was selected because only individuals harbouring cysts were considered, therefore zero counts were excluded from the dataset. No grouping factors were included in the models.

3. Results

3.1. Distribution of hairworm cysts in aquatic macroinvertebrates

The results presented in this section are summarised in Table 1. A total of 307 aquatic macroinvertebrates were examined for hairworm cysts in June (six taxa); 683 were processed in October (15 taxa). Individuals of only five taxa contained either melanised cysts or larvae. Interestingly, some of the encysted larvae were observed either adjacent to or breaking free from the partially melanised part of their cyst (Fig. 1C). However, they remained inactive inside visibly smaller cysts with thinner walls. No haemocyte activity was observed around cysts from all three levels of melanisation. Based on the larval folding pattern, all hairworm cysts were identified to the genus *Gordius*.

The caddisfly *Olinga* sp. was most abundant, representing 31.5% of

both samples. They also harboured 87.6% of the 1466 hairworm cysts counted in the laboratory. In this caddisfly species, cysts were usually located in the haemocoel around the gastrointestinal tract (Fig. 1A).

3.2. Melanisation response in caddisfly larvae

The effects of season and total cyst load on melanisation response in *Olinga* sp. were tested and the beta regression model with the lowest AICc included both explanatory variables. For this model, the proportion of non-melanised cysts nearly doubled in October (Fig. 2A), meaning caddisfly larvae had a significantly lower melanisation response in the spring. The total cyst load did not have any overall effect on the melanisation response (Fig. 2B). In testing the effects of host body size and total cyst load on melanisation response with the June dataset, none of the models were more parsimonious than the null model according to their AICc, meaning that both variables had no significant effect.

3.3. Host body size and total cyst load

For *Olinga* sp. collected in June, case length was measured as a proxy for body size and was tested as an effect on total cyst load. As seen in Fig. 3, host body size had a positive effect on total cyst load (effect size = -0.014 ; SE = 0.003), with larger caddisfly larvae harbouring more cysts on average than smaller ones.

4. Discussion

In this study, we report new observations on varying melanisation levels of hairworm cysts in aquatic insect larvae. An interesting finding is the characteristic pattern formed by melanin deposits on the surface of cysts, which was observed in three taxa from two distinct insect orders, Trichoptera and Ephemeroptera. Partially melanised cysts all had the same “barrel-shaped” pattern, which could be due to surface conformation. Because photographs of hairworm cysts are usually two-dimensional (caused by the flattening of host tissues for microscope slide preparation), they do not inform us on their three-dimensional shape. Ultrastructural photographs of hairworm cysts could provide information about their natural shape, which may help explain why

Table 1

Prevalence and intensity (number of cysts per infected host ± SE) of *Gordius* sp. hairworm (Nematomorpha) cyst infections in aquatic macroinvertebrates captured by dip net from Grasmere Stream, Cass Field Station in Canterbury, New Zealand on 18 June 2018 and 2 October 2018.

Class	Order ¹	Family	Genus or species	June 2018			October 2018			
				Sample size (n)	Infected (prevalence)	Number of cysts per infected individual ± SE	Sample size (n)	Infected (prevalence)	Number of cysts per infected individual ± SE	
Insecta	Trichoptera	Conoesucidae	<i>Olinga</i> sp.*	177	96 (54.2%)	12.0 ± 0.9	135	19 (14.1%)	6.8 ± 1.8	
			<i>Pycnocentria</i> sp.*	0	–	–	38	16 (42.1%)	7.6 ± 1.6	
			<i>Pycnocentroides</i> sp.	0	–	–	1	1 (100%)	2.0 ± 0.0	
		Hydrobiosidae	<i>Hydrobiosis</i> sp.	0	–	–	6	0 (0%)	–	
	<i>Orthopsyche</i> sp.		0	–	–	7	0 (0%)	–		
	Ephemeroptera	Coloburiscidae	<i>Coloburiscus</i> sp.**	13	0 (0%)	–	116	11 (9.5%)	1.7 ± 0.5	
			<i>Deleatidium</i> sp.*	0	–	–	49	10 (20.4%)	2.2 ± 0.7	
		Nesameletidae	<i>Nesameletus</i> sp.	0	–	–	1	0 (0%)	–	
	Plecoptera	Gripopterygidae	<i>Zelandoperla</i> sp.	7	2 (28.6%)	1.5 ± 0.7	1	0 (0%)	–	
	Diptera	Chironomidae	NA	0	–	–	10	0 (0%)	–	
	Coleoptera	Elmidae	<i>Hydora</i> sp.	0	–	–	9	0 (0%)	–	
	Megaloptera	Corydalidae	<i>Archichauliodes diversus</i> **	2	0 (0%)	–	25	4 (16.0%)	2.8 ± 1.0	
	Gastropoda	Hygrophila	Physidae	<i>Physella acuta</i>	76	1 (1.3%)	2.0 ± 0.0	90	1 (1.1%)	1.0 ± 0.0
		Littorinimorpha	Tateidae	<i>Potamopyrgus antipodarum</i>	32	0 (0%)	–	189	0 (0%)	–
Clitellata	Haplotaxida	Lumbricidae	<i>Eiseniella</i> sp.	0	–	–	6	1 (16.7%)	1.0 ± 0.0	

* Taxa with melanised and non-melanised cysts.

** Taxa with melanised larvae.

¹ Clades were used for both gastropod species, due to paraphyly in traditional orders.

melanin appears to form initially around the midsection. Another interesting observation is the apparent interaction between the melanin deposits and the outer cyst wall. Partially melanised cysts flattened under the cover glass were sometimes separated from the non-melanised portion of the cyst. This may be an artifact from the slide preparation because hairworm cysts could have separated from the partial melanin layer due to the physical pressure used to flatten host tissues, which may have forced the inner layers of the cyst wall to separate from the outer layers. This would support previous observations that the hairworm cyst wall is a multilayered structure (Poinar, 2010; Poinar and Doelman, 1974).

However, the separation of partially melanised cysts raises questions about the lethality of melanisation on encysted hairworms.

Reports of immunity to hairworms in intermediate insect hosts are scarce and focus mainly on the melanisation of larval hairworms (de Villalobos et al., 2006; Poinar et al., 1969; Poinar, 2010). One report by Inoue (1962) found that melanised hairworm cysts in chironomid larvae fed to definitive mantid hosts resulted in a lower abundance of juvenile hairworms than that obtained when using non-melanised cysts, suggesting that melanisation hinders the transmission of hairworms. But it is unknown whether these cysts were fully or partially melanised. Poinar (1991) also reported melanised hairworm cysts in several insect orders in New Zealand, but no distinction was made regarding the level of melanisation. Therefore, it remains unknown whether a hairworm larva can excyst from a partially melanised cyst once inside its definitive host. Feeding partially melanised cysts to definitive hosts could

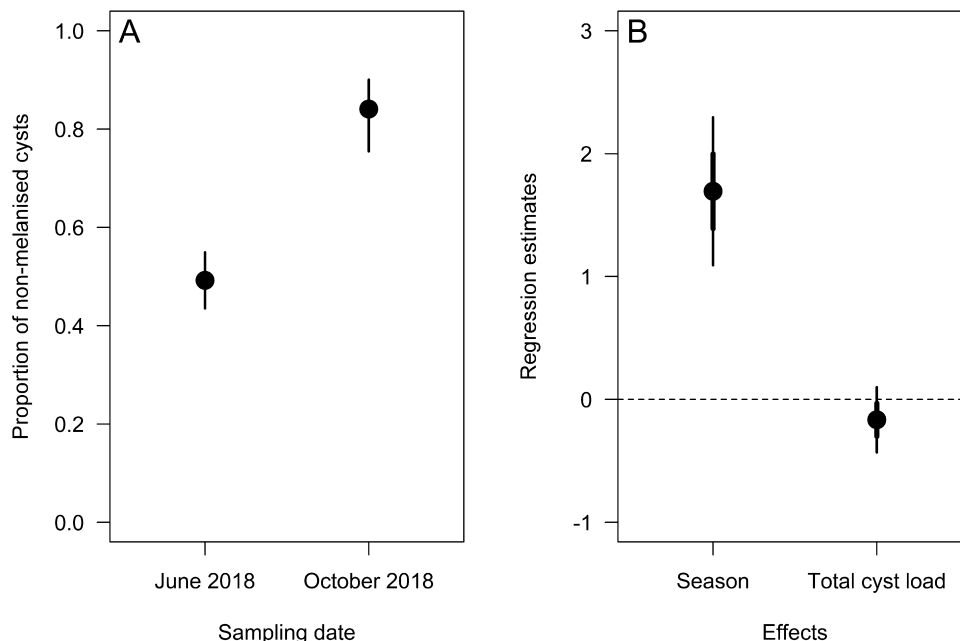


Fig. 2. (A) Estimated proportions (± 95% confidence intervals) of non-melanised *Gordius* sp. hairworm (Nematomorpha) cysts in *Olinga* sp. caddisfly larvae between sampling dates based on beta regression modelling. Samples were collected on 18 June 2018 and 2 October 2018. (B) Beta regression estimates for the fixed effects in the selected model with 95% error bars. A regression estimate different from 0 (including error bars) indicates a significant effect.

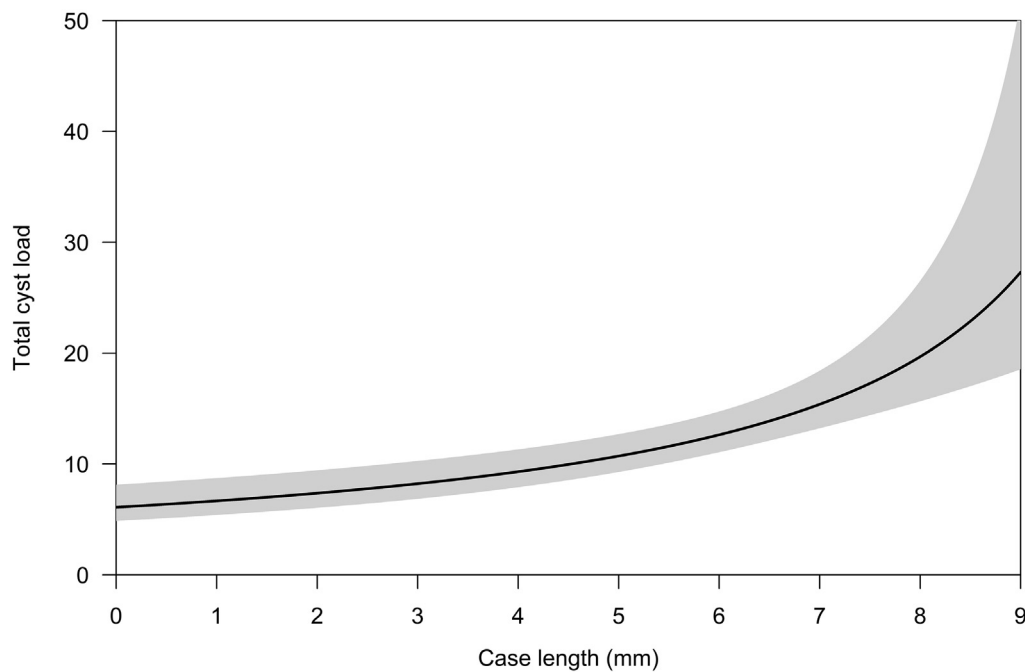


Fig. 3. Predicted effect of case length (proxy for host body size) of caddisfly larvae *Olinga* sp. on total *Gordius* sp. hairworm (Nematomorpha) cyst load. The grey area represents the 95% confidence band of the fitted gamma regression model.

help determine the impact of intermediate host immunity on the life cycle of hairworms.

It is evident from the two seasonal samples that the diversity and abundance of macroinvertebrates in Grasmere stream increased in October. This is likely caused by an increase in general activity due to warmer springtime temperatures. However, certain groups were completely absent from the June collection, which could be partly explained by minor differences in the specific areas sampled from the stream section on the two collection dates. The groups harbouring the highest number of cysts per infected individual (Trichoptera and Ephemeroptera) are part of the benthos macrofauna and thus are most likely to consume hairworm larvae and act as true paratenic intermediate hosts (Hanelt and Janovy, 2003). Interestingly, the mega-lopteran *Archichauliodes diversus* also harboured cysts, but this species is predatory and probably ingested them by consuming primary paratenic hosts (Poinar, 1991). In October, the prevalence was higher in likely paratenic host taxa, except for *Olinga* sp. and *Zelandoperla* sp., which may be due to a “dilution effect” caused by an increase in host density and diversity in the spring, meaning more potential hosts consuming hairworm larvae between June and October (Keesing et al., 2006). The prevalence was lower in *Olinga* sp. collected in October, which could indicate that interspecific competition increased and hairworm larvae were less available for consumption by immature caddisflies between collection dates.

As the prevalence and intensity of hairworm cysts decreased in *Olinga* sp. between seasons, so did the melanisation response. Temporal differences could play a role in the proportion of non-melanised cysts found in immature caddisflies. It was reported that species of immature *Olinga* can be found all year with several generations overlapping in the same stream (Burrell and Ledger, 2003; Cowley, 1978). Therefore, if new generations of *Olinga* sp. appeared before or after the June sampling date, larvae could have consumed hairworm larvae much closer to the October sampling date, thus impacting the proportion of non-melanised cysts. These findings indicate that the temporal and generational dynamics between intermediate hosts and hairworm cysts require further investigation. Regular sampling throughout an extended period could help explain the complex interactions between aquatic macroinvertebrates and the hairworm cysts they harbour.

We also investigated the effect of host body size on total cyst load. The cases of *Olinga* sp. sampled in June were measured as a proxy for body size and the results support our hypothesis that a larger host harbours more cysts on average than a smaller one. As a host feeds and grows, it is likely to have consumed more hairworm larvae and accumulated more cysts. Of course, as these results pertain to only one host species, they do not inform us about the effect of body size in other paratenic host taxa, but it is reasonable to speculate that it plays a role regardless of host taxon. In fact, the positive relation between host size and parasite intensity has been reported in a varied number of host-parasite systems (Poulin, 2000; Yule and Burns, 2015).

5. Conclusions

The new observations presented here on the different levels of hairworm cyst melanisation in intermediate hosts adds a new layer of complexity to the dynamics between hairworms and their paratenic hosts. We showed that melanotic encapsulation varies and it is possible to visually categorise different levels in order to calculate a melanisation response that is inversely proportional to the relative quantity of non-melanised cysts in a host. This melanisation response varied significantly between winter and spring for one species of caddisfly, which may be attributed to some temporal and generational dynamics of the host between both sampling dates. The total cyst load did not have a significant impact on the melanisation response for this species. However, this cyst load was affected by host body size; larger caddisfly larvae harboured more cysts on average than smaller ones. These new results add to our limited knowledge of the cryptic interactions between hairworms and their intermediate hosts and suggest further study to tackle some mysteries of this relatively understudied group of parasites.

Acknowledgements

The authors thank Eddy Dowle and Alan Eriksson for help in field sampling and Antoine Filion for help with statistical analyses in R. This work was supported by a University of Otago Doctoral Scholarship to JFD and a University of Otago Summer Research Scholarship to XC.

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