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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Egg fibrils and transmission in the acanthocephalan *Acanthocephalus dirus*

Alaina C. Pfenning [1,2] · Timothy C. Sparkes [1]

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Abstract
Acanthocephalans have multi-host life cycles that include arthropods as intermediate hosts and vertebrates as definitive hosts. Eggs are dispersed into the habitat from definitive hosts and in some species eggs possess fibrils, which have been proposed to facilitate transmission to intermediate hosts. We examined the potential role of fibrils in transmission of the acanthocephalan *Acanthocephalus dirus* to its intermediate host *Caecidotea intermedius*, a stream-dwelling isopod. We identify three properties of fibrils that could favor transmission. First, there was a slow rate of fibril release, which was dependent on the actions of stream microorganisms. Second, eggs with fibrils were more likely to adhere to the substrate than those without fibrils. Third, in feeding trials, isopods exposed to eggs with fibrils had a higher infection prevalence than isopods exposed to eggs without fibrils. These properties could favor transmission by increasing the likelihood that eggs sink to the sediment occupied by their target hosts before adhering to items on the substrate (e.g., leaves) and by increasing recruitment after the eggs have been consumed.

Keywords Acanthocephala · *Acanthocephalus dirus* · Egg dispersal and transmission · Host feeding · Recruitment

Introduction

Trophically transmitted parasites infect multiple hosts and as a consequence occupy multiple habitats during a life cycle. Significant variation in dispersal and transmission may be expected and there is evidence that this type of variation occurs in acanthocephalans (Nikishin 2001). We examined the potential role of egg fibrils that occur in several helminths (Munson 1972; Marchand 1984; Taraschewski and Peters 1992; Nikishin 2001) in transmission to intermediate hosts in an aquatic acanthocephalan.

Acanthocephalans are trophically transmitted parasites that are found in diverse habitats where they infect arthropod intermediate hosts and vertebrate definitive hosts. Several studies have shown that the morphology of eggs (shelled acanthors) may be related to both properties of the habitat and host type (West 1964; George and Nadakal 1973; Oetinger and Nickol 1974; Uznanski and Nickol 1976; Marchand 1984; Taraschewski and Peters 1992; Nikishin 2001; Wongkham and Whitfield 2004; Arredondo and de Pertierra 2009). Membranes (envelopes) of eggs vary in acanthocephalan classes (West 1964; Whitfield 1973; Marchand 1984; Taraschewski and Peters 1992; Nikishin 2001). Specifically, the second envelope (E2, Marchand 1984) appears to be highly variable in response to habitat properties and host type. In some aquatic acanthocephalans, eggs possess fibrils (filaments) in the E2 envelope, which are released when the eggs are in the environment (Oetinger and Nickol 1974; Uznanski and Nickol 1976; Nikishin 2001). Fibril release facilitates transmission by attaching to vegetation consumed by intermediate hosts (Uznanski and Nickol 1976; Taraschewski and Peters 1992; Barger and Nickol 1998) and slows the passage of eggs through the intestines of intermediate hosts (Whitfield 1973; Oetinger and Nickol 1974).

Studies have shown that fibrils may function in different ways in different species (Oetinger and Nickol 1974; Uznanski and Nickol 1976; Taraschewski and Peters 1992; Barger and Nickol 1998). For example, acanthors of *Leptorhynchoides thecatus* (Linton, 1891) Kostylew, 1924 infect intermediate

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✉ Alaina C. Pfenning
apfenning@huskers.unl.edu

[1] Department of Biological Sciences, DePaul University, 2325 North Clifton Avenue, Chicago, IL 60614, USA

[2] Present address: School of Biological Sciences, University of Nebraska-Lincoln, 424 Manter Hall, 1104 T St, Lincoln, NE 68588, USA

hosts that feed in the water column and the eggs enter the habitat with their fibrils released (Uznanski and Nickol 1976). This mechanism appears to favor transmission because it allows the eggs to entangle with suspended vegetation, which is consumed by intermediate hosts (Uznanski and Nickol 1976; Barger and Nickol 1998). In contrast, acanthors of *Acanthocephalus dirus* (Van Cleave, 1931) Van Cleave et Townsend, 1936 infect intermediate hosts that feed on the sediment and the eggs enter the habitat with their fibrils enclosed in the outer membrane (West 1964; Oetinger and Nickol 1974). In this case, the fibrils appear to be released after the membrane has been degraded by microorganisms present in the habitat (Oetinger and Nickol 1974), which may allow the eggs to sink to the sediment before the fibrils are released. Eggs of *Pomphorhynchus bulbocolli* Linkins in Van Cleave, 1919, that lack fibrils, have also been shown to sink to the sediment without entangling in suspended vegetation (Barger and Nickol 1998). In addition, fibrils appear to vary in adherence properties among species (Taraschewski and Peters 1992). Species that disperse in flowing water [*Pomphorhynchus laevis* Zoega in Müller, 1776; *Polymorphus minutus* (Goeze, 1782) Lühe, 1911] appear to have fibrils with more adherent properties than a species that disperses in calm water [*Acanthocephalus anguillae* (Müller, 1780) Lühe, 1911].

The acanthocephalan *A. dirus* infects the stream-dwelling isopod, *Caecidotea intermedius* Forbes, 1876, as its intermediate host and stream fishes as definitive hosts (Camp and Huizinga 1980; Kopp et al. 2011). Infection of juvenile *C. intermedius* occurs when *A. dirus* eggs are consumed along with food (e.g., leaves) located on the stream sediment. The eggs appear to reach the sediment through one of the two routes (Muzzall and Rabalais 1975; Kopp et al. 2011; Wahl and Sparkes 2012). One route, which may also occur in other acanthocephalans [e.g., *P. minutus*; *Echinorhynchus truttae* Schrank, 1788; *Polymorphus marilis* Van Cleave, 1939; *Neorhadinorhynchus nudum* (Harada, 1938), Yamaguti, 1939; *Sclerocollum saudii* Al-Jahdali, 2010] involves the eggs being carried to the sediment in the body of the gravid female (Nicholas and Hynes 1958; Awachie 1966, Denny 1968; Hassanine 2006; Al-Jahdali et al. 2015; respectively). The eggs are then dispersed when the body degrades (Kopp et al. 2011; Wahl and Sparkes 2012). The other route, which appears to be more common in acanthocephalans (Kennedy 2006), involves the release of eggs with the feces of the host. The eggs would then sink through the water column to the sediment.

To examine the role of fibrils in *A. dirus* transmission, we conducted three laboratory-based experiments to quantify the following: (1) The time course of fibril release and the effect of exposure to stream microorganisms on this relationship; (2) Whether the presence of fibrils increased the adherence of eggs to a substrate; and (3) Whether the presence of fibrils increased establishment of *A. dirus* eggs within intermediate hosts.

Materials and methods

For all three experiments, we obtained gravid *A. dirus* females from the intestines of two fish hosts in Buffalo Creek IL, USA (42° 11′ 9″ N, 88° 3′ 27″ W): green sunfish, *Lepomis cyanellus* Rafinesque 1819; creek chub, *Semotilus atromaculatus* (Mitchill, 1818), Bicknell 1886. We then maintained mature *A. dirus* eggs in filtered stream water (8 °C). To remove microorganisms from the stream water, we filtered the water twice, once to remove larger organisms (Whatman Grade 1 filter) and a second filtration to remove any remaining microorganisms (polyethersulfone (PES) filter with a 0.45-μm inclusion; Whatman, Puradisc 25 mm).

To estimate the time course of fibril release and to determine whether the presence of stream microorganisms affected the rate of fibril release, we obtained mature eggs from gravid females ($n = 34$). In each case, 40 μl of egg solution was added to 960 μl of stream water that was either filtered (i.e., without microorganisms) or unfiltered (i.e., with microorganisms). The solution was mixed, using a micropipetter, then pipetted onto separate counting cell slides (50 × 20 mm, Sedgewick-Rafter Counting Cell slide, Structure Probe, Inc., West Chester, PA) and incubated (8 °C) to mimic natural conditions. We examined each slide daily for 7 days and recorded the presence/absence of fibrils in the first 10 eggs observed using a compound microscope at × 40 (Nikon Eclipse E400, Nikon, Tokyo, Japan). To minimize any systematic biases, we chose a different starting location and therefore subsample of eggs on each day. We then examined the effect of stream microorganisms on the timing of fibril release using two measures. First, we compared the number of females that had at least one egg with fibrils released using a McNemar's test (Samuels and Witmer 2003). Second, we compared the proportion of eggs with fibrils released using a paired *t* test (Systat 13). In both cases, the analysis was paired by maternal group because eggs from the same females were divided between the two treatments ($n = 34$).

To determine whether fibrils increased the ability of eggs to adhere to environmental substrates, we quantified adherence rates across eggs with and without fibrils released. We collected eggs from 13 *A. dirus* females, prepared egg solutions, pipetted (10 μl) onto a counting slide with stream water, and incubated at 8 °C (as outlined above). Each day, we randomly selected a sample of eggs (10–20), recorded the presence/absence of fibrils, and determined whether they were adhered to the cover slide by slowly moving the coverslide 1 cm forward and backward. If the egg did not move with the solution, it was marked as adhered. We calculated the percentage of eggs that were adhered in each treatment. To determine differences in adherence between eggs with and without fibrils, we used a Wilcoxon test for paired, nonparametric data (Conover 1980). Analysis was paired by maternal group because eggs from the same female were compared between groups ($n = 13$). A nonparametric test was used because the data did not meet the assumptions for parametric analysis.

To examine whether the presence of *A. dirus* fibrils increased the likelihood of establishment in intermediate hosts, we exposed uninfected juvenile *C. intermedius* to eggs with and without fibrils in an infection assay. We collected 12 uninfected juveniles from each of 30 maternal lines (to control for any potential maternal effects) and maintained them under standard conditions for 2 months (leaves, microbially conditioned in filtered stream water (Whatman Grade 1 filter), and aerated stream water). Two days prior to the infection assay, we removed the leaves to increase the likelihood of feeding during the trials. We then placed equal-sized leaf disks (6-mm diam.) in each container with an individual isopod and either *A. dirus* eggs with fibrils (held in stream water for a 4-day pre-trial) or *A. dirus* eggs without fibrils (held in filtered water for a 4-day pre-trial). Individual isopods were exposed to either a high (50 μl) or low (20 μl) dose of *A. dirus* eggs, for 2, 3, or 4 days. After the allotted days of feeding, we transferred juveniles to new individual containers with fresh stream water and microbially conditioned leaves, and monitored the containers daily for 2 weeks. After this time, isopods were preserved (70% ethanol), measured body length (mm), and the number of *A. dirus* present recorded. To estimate the number of eggs dispensed in each trial, we estimated egg density for each solution by counting the number of eggs present in two additional samples per maternal group (20 μl, 50 μl). To ensure that infections were due to experimental exposure to eggs, we dissected one additional juvenile per maternal group that had not been exposed to eggs. For the analyses, we calculated infection prevalence (the percentage of infected isopods per maternal group) and infection intensity (median number of *A. dirus* per infected isopod). All values were calculated at the maternal group level to account for the varying exposure conditions (egg density and feeding time) and to maintain independence among data points used in the analysis. For prevalence and intensity of infection, analysis was paired by isopod maternal group because juvenile isopods from the same female isopod were assigned to each treatment ($n = 30$). Wilcoxon tests were used to assess differences in prevalence and intensity between treatments (Conover 1980) because the data did not meet the assumptions for parametric analysis.

Results

The percentages of female *A. dirus* that had at least one egg with fibrils released in stream water and filtered water are shown in Fig. 1a. Exposure to stream microorganism affected fibril release (McNemar's test: $\chi^2_1 = 12.0$, $p < 0.001$). Of the 34 female *A. dirus* examined, 74% (25/34) had at least one egg with fibrils released during the experiment. In stream water, this number increased steadily from 15% (day 1) to 71% (day 7). In filtered water, the number also increased from 6% (day 1) to 35% (day 7). For the 25 females that had eggs with fibrils released in at least one of the two groups (filtered, unfiltered water), there was also a difference in the number of eggs with fibrils released (paired t test: $t_{24} = 9.2$, $p < 0.001$, Fig. 1b). Eggs exposed to stream water were more likely to release fibrils than eggs exposed to filtered water (day 7, stream water = 60%, filtered water = 6%).

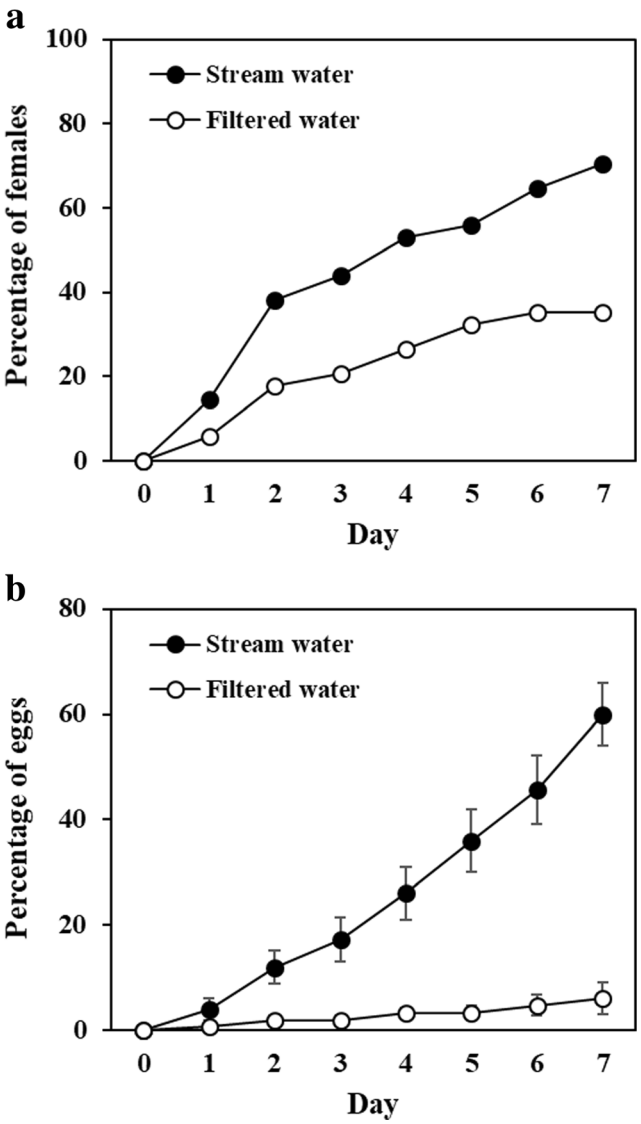

Fig. 1 The timeline of fibril release for *A. dirus* eggs in stream water and in filtered water. **a** The percentage of females with at least one egg with its fibrils released per day ($n = 34$). **b** The percentage of eggs with fibrils released per day (mean ± standard error, $n = 25$)

The adherence properties of eggs that differed in fibril presence were examined for eggs from 13 different maternal groups (Fig. 2). Eggs from two maternal groups did not release fibrils during the trials and were excluded from the analysis. For the remaining 11 maternal groups, a total of 1030 eggs were assessed for adherence (fibrils, $n = 334$; no fibrils, $n = 696$). Percent adherence values per maternal group differed between eggs with fibrils and those without fibrils (Wilcoxon test, $T = 2.9$, $p < 0.05$, $n = 11$). Overall, eggs with fibrils adhered to the substrate in 94% of cases (per maternal

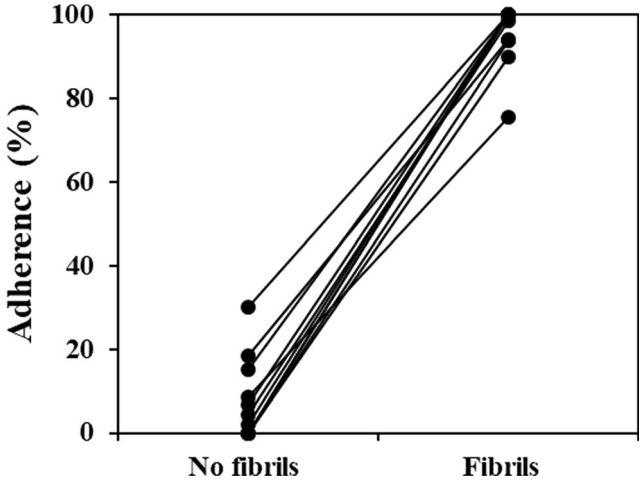

Fig. 2 Adherence properties of egg fibrils in *A. dirus*. Each line represents eggs from the same maternal group ($n = 11$)

group median = 100%, range = 75–100%) and eggs without fibrils adhered to the substrate in 8% of cases (per maternal group median = 4%, range = 0–30%).

Juvenile isopods were exposed to eggs on leaves to determine the effect of fibril presence on establishment success. Estimates of the number of eggs dispensed in each trial revealed that approximately 20 eggs were dispensed per microliter of egg solution (SE = 2.7, $n = 30$), which would result in approximately 400 eggs being dispensed in the 20 μl treatment and approximately 1000 eggs in the 50 μl treatment. The juvenile isopods that were raised in the laboratory but not exposed to eggs were not infected by *A. dirus* parasites indicating that infection occurred only if the isopods were exposed to eggs experimentally. There was no difference in *C. intermedius* body size between the two water treatment groups (paired t test, $t_{29} = 0.2$, $p > 0.5$).

Of the 360 individuals of *C. intermedius* used, 313 (87%) survived for the duration of the experiment. In cases where an individual isopod died, the paired sibling that represented the same treatment combination from the other water treatment group was removed from further analysis. This resulted in the inclusion of data for 276 isopods (77%), which included all 30 of the maternal groups originally exposed to parasite eggs. Members of only one of the 30 isopod maternal groups exposed to *A. dirus* eggs evaded infection in all treatment groups. A summary of prevalence and intensity values for specimens of *A. dirus* is shown in Table 1. At the maternal group level, median prevalence was 67% for stream water (range = 0–100%) and 50% for filtered water (range = 0–83%). Prevalence was greater in the stream water group than the filtered water group (Wilcoxon test, $T = 2.6$, $p < 0.01$, $n = 30$). At the maternal group level, median intensity of infection was 3.0 for stream water (range = 1–11) and 2.7 for filtered water (range = 1–8). There was no difference in infection intensity between the groups (Wilcoxon test, $T = 1.7$, $p > 0.05$, $n = 25$).

Table 1 Summary of prevalence and intensity of infection of *A. dirus* for each treatment group

Egg density	Days	n	Prevalence (%)		Median intensity (range)	
			Filtered	Stream	Filtered	Stream
400	2	26	27	42	2 (1–6)	2 (1–6)
(20 μl)	3	25	52	56	3 (1–12)	4 (1–16)
	4	21	42	52	4 (1–12)	2 (1–12)
1000	2	25	52	64	2 (1–2)	3 (1–8)
(50 μl)	3	18	50	50	2 (1–5)	2 (1–15)
	4	23	48	61	2 (1–7)	4 (1–18)

Egg density refers to the average number of eggs dispensed into the feeding arena when supplied with either 20 μl (400 eggs) or 50 μl (1000 eggs) of egg solution. Days refer to the number of days *C. intermedius* juveniles were exposed to *A. dirus* eggs. n indicates the number of maternal groups (of the original 30) included in the analysis

Discussion

Eggs of *A. dirus* have to locate to the stream sediment to contact their hosts. These eggs can either sink through the water column or be carried to the sediment by gravid females (Oetinger and Nickol 1974; Muzzall and Rabalais 1975; Kopp et al. 2011; Wahl and Sparkes 2012). For the eggs that sink through the water column, a delay in fibril release may be beneficial because it provides time for the eggs to reach the sediment before the fibrils are released. The results showed that fibril release required several days (3–6) and that the rate of release was dependent on the actions of stream microorganisms (e.g., bacteria, fungi, protists). Sinking (settling) rates are not known for eggs of *A. dirus* but rates of 0.2–0.5 m per hour have been recorded for other helminth eggs (Sengupta et al. 2011). Based on these results, it would appear that eggs of *A. dirus* should have ample time to sink to the sediment. The results also showed that fibril release increased the adherence properties of the eggs. Collectively, these findings indicate that eggs of *A. dirus* would likely sink to the sediment before the fibrils are released and by doing so avoid entanglement in suspended vegetation. After reaching the sediment, the outer membrane would naturally degrade and release the fibrils, which could adhere to items on the sediment, e.g., leaves, periphyton. This adherence is expected to increase encounter rates with target hosts by allowing the eggs to maintain a position in the microhabitat occupied by hosts and by attaching to food items consumed by hosts.

Oetinger and Nickol (1974) proposed that egg fibrils in *A. dirus* may increase establishment success by slowing the passage of eggs through the intestines (see also Whitfield 1973). We tested this hypothesis by exposing isopods to eggs that had been held in either stream water (increased fibrils) or filtered water (decreased fibrils) prior to trial. We assumed that both types of eggs would remain in place since they were laid on the surface of flat leaf disks, which were undisturbed during the trials. The

results obtained provided some support for the hypothesis in that prevalence was increased in the isopods exposed to eggs from stream water. However, we cannot exclude the possibility that the increased number of eggs with fibrils allowed more of the eggs to remain on the leaves in the stream water group. Despite this limitation, isopods exposed to higher numbers of eggs with fibrils had a higher prevalence of infection, providing support for the hypothesis that fibrils increase establishment success. Uznanski and Nickol (1976) showed that both prevalence and intensity were increased in amphipods when they were exposed to eggs of *L. thecatus* that were given the opportunity to entangle in vegetation. We did not examine this hypothesis directly (i.e., eggs with fibrils were not provided with the opportunity to sink and attach to leaves) but we would predict a similar outcome in *A. dirus*.

Several authors have proposed that the variation in egg morphology in acanthocephalans may be explained by factors such as properties of the habitat and the feeding biology of hosts (e.g., Marchand 1984; Taraschewski and Peters 1992; Nikishin 2001). The results presented are consistent with the interpretation that both of these factors may have played a role in the evolution of fibrils in eggs of *A. dirus,* which may be the case in *L. thecatus* (Uznanski and Nickol 1976; Barger and Nickol 1998). Similar types of relationships may also explain some of the variation in egg morphology identified in other acanthocephalans. For example, eggs of *Pallisentis nagpurensis* (Bhalerao, 1931) Baylis, 1931 and *Pallisentis rexus* Wongkham and Whitfield, 1999 expand and become buoyant in water, which may increase encounter rates with their copepod hosts (George and Nadakal 1973; Wongkham and Whitfield 2004; respectively).

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Compliance with ethical standards

The study was performed in compliance with national laws and regulations.

Conflict of interest The authors declare that they have no conflict of interest.

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