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Progenesis and facultative life cycle abbreviation in trematode parasites: Are there more constraints than costs?

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ABSTRACT

Complex life cycles provide advantages to parasites (longer life span, higher fecundity, etc.), but also represent a series of unlikely events for which many adaptations have evolved (asexual multiplication, host finding mechanisms, etc.). Some parasites use a radical strategy where the definitive host is dropped; life cycle abbreviation is most often achieved through progenesis (i.e. early maturation) and reproduction in the second intermediate host. In many progenetic species, both the typical and abbreviated life cycles are maintained. However, conditions that trigger the adoption of one or the other strategy, and the pros and cons of each parasite life history strategy, are often complex and poorly understood. We used experimental infections with the trematode *Coitocaecum parvum* in its fish definitive host to test for potential costs of progenesis in terms of lifespan and fecundity. We show that individuals that adopt progenesis in the intermediate host are still able to establish in the definitive host and achieve higher survival and fecundity than conspecifics adopting the typical three-host life cycle. Our results and that of previous studies show that there seems to be few short-term costs associated with progenesis in *C. parvum*. Potential costs of self-fertilization and inbreeding are often suggested to select for the maintenance of both life-history strategies in species capable of facultative progenesis. We suggest that, at least for our focal species, there are more constraints than costs limiting its adoption. Progenesis and the abbreviated cycle may become the typical life-history strategy while reproduction in the vertebrate definitive host is now a secondary alternative when progenesis is impossible (e.g. limited host resources, etc.). Whether this pattern can be generalized to other progenetic trematodes is unknown and would require further studies.

1. Introduction

Many phylogenetically distant groups of parasites develop through complex life cycles and mature through distinct developmental stages using a succession of different hosts (Choisy et al., 2003; Parker et al., 2003a; Lefebvre and Poulin, 2005a; Poulin, 2007). For example, from simple one-host cycles, parasitic helminths have evolved life history strategies comprising two or three, sometimes more, taxonomically distant host species (Parker et al., 2003a). Complex life cycles offer advantages to helminth parasites. First, large and long-lived vertebrate hosts enable increased life span and survival, greater body size, higher fecundity and/or greater access to sexual partners (Brown et al., 2001; Parker et al., 2003a). Second, intermediate hosts may facilitate transmission to definitive hosts (Choisy et al., 2003). However, complex life cycles constitute a combination of unlikely transmission events. Parasites have thus developed adaptations such as high fecundity, asexual multiplication, host-finding mechanisms in free-living larvae, long-lived dormant stages in intermediate hosts and host manipulation to increase the odds of completing a generation (Combes et al., 1994, 2002; Brown et al., 2001; Thomas et al., 2002; Cribb et al., 2003; Poulin, 2007).

Some species have evolved a more radical strategy in which parasites skip one host from the life cycle (Combes, 2001; Poulin, 2007). Fewer hosts are used, decreasing the number of transmission steps and rendering the life cycle easier to complete (Poulin and Cribb, 2002). Such life cycle abbreviation is particularly common in parasites relying on trophic transmission when infected prey have to be consumed by the definitive host for parasites to mature and reproduce. Numerous trematode parasites species from phylogenetically distant families have independently dropped the definitive host from the ancestral three-host life cycle (Font, 1980; Barger and Esch, 2000; Poulin and Cribb, 2002; Lefebvre and Poulin, 2005a). Parasites produce eggs while still in their second intermediate host via precocious maturity (i.e. proge-

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nesis) and self-fertilization (most trematodes are hermaphroditic; Poulin, 2001). Approximately half of trematode genera in which abbreviated cycles are documented include only species that have permanently dropped the definitive hosts and reproduce exclusively through progenesis. However, many other species, in a variety of trematode genera and families, that are capable of progenesis and life cycle abbreviation retain the ability to reproduce in the ancestral vertebrate host (see review by Poulin and Cribb (2002) for details). The developmental plasticity provided by facultative progenesis could thus serve to increase the probability of producing offspring when transmission rates to the definitive host are low (Wang and Thomas, 2002; Davies and McKerrow, 2003). However, conditions that trigger the adoption of one or the other strategy by parasites, and the pros and cons of each life history strategy, are mostly unknown.

Coitocaecum parvum (Opecoelidae) is probably the most intensively studied progenetic trematode. It is a parasite of freshwater fish but both the classical three-host and an abbreviated two-host life cycle can be observed synchronously in parasite populations (MacFarlane, 1939; Holton, 1984a; Poulin, 2001; Lagrue and Poulin, 2008a). Eggs produced by adults in the fish are released in host faeces and hatch into free-swimming larvae (miracidia). Miracidia penetrate the snail *Potamopyrgus antipodarum* in which they develop into colonies of sporocysts. Sporocysts produce free-living larvae that leave the snail and infect an amphipod crustacean where they encyst as metacercariae in the body cavity. Metacercariae can either await ingestion by a fish or reach maturity through progenesis. Progenetic individuals reproduce by self-fertilization (*C. parvum* is hermaphroditic) and lay eggs within their metacercarial cyst (Holton, 1984b; Poulin, 2001). Eggs are released upon death of the intermediate host and hatch into larvae that are infective to the snail first host, allowing the parasite to reproduce even when transmission to the definitive host is impossible. The maintenance of both strategies and the highly variable proportion of progenetic individuals in *C. parvum* populations suggest that they may have equal fitness payoffs and/or that their adoption is highly influenced by external factors (Poulin and Cribb, 2002; Lagrue and Poulin, 2008a).

Previous studies have shown that many factors influence the adoption of progenesis in *C. parvum*. Information about transmission opportunities present in the environment (predator chemicals cues) can be used by parasites to preferentially adopt one or the other strategy (Poulin, 2003). For example, presence (or absence) of fish definitive hosts or non-host predators influence the proportion of *C. parvum* metacercariae adopting progenesis in experimental parasite populations (Lagrue and Poulin, 2007). However, in natural populations, fish abundance, and thus transmission opportunities, did not seem to influence parasite strategy, indicating that other factors may affect the *C. parvum* life-history strategy (Lagrue and Poulin, 2008a). Intra- and interspecific competition in amphipod hosts can also constrain the adoption of progenesis in *C. parvum* metacercariae (Lagrue and Poulin, 2008b). For example, the intensity of intraspecific competition (i.e. number of co-infecting *C. parvum*) limits the ability of metacercariae to adopt progenesis and restricts growth and egg production; within-host resource limitations likely affect the *C. parvum* life strategy (Ruiz-Daniels et al., 2013). Even kin selection and genetic similarities among co-infecting metacercariae may influence their life history strategies; genetically similar individuals are significantly more likely to adopt the same life history strategy when co-infecting the same intermediate host (Lagrue et al., 2009; Joannes et al., 2014). Eventually, *C. parvum* individuals remaining in their intermediate host for a long time often adopt progenesis and the abbreviated cycle, regardless of the presence of fish definitive hosts or other environmental factors (Lagrue and Poulin, 2009a). The parasite's environment thus contains many layers of heterogeneity, each influencing or constraining the *C. parvum* life-history strategy in different ways.

Abbreviated life cycles provide the insurance to produce at least some offspring when transmission to the definitive host is impossible, too low or highly unpredictable. Although, as described above, some constraints on progenesis and the large parasite size required for early maturation may modulate its adoption, it could be argued that most if not all trematodes are faced with low and/or highly variable probabilities of transmission. It is thus surprising that life cycle abbreviation has not actually evolved in more trematode lineages (Poulin, and Cribb, 2002). As for species where progenesis allows facultative life cycle abbreviation, why is life cycle abbreviation not adopted by all individuals that apparently could (Lefebvre and Poulin, 2005b)? Potential costs associated with progenesis and reproduction in intermediate hosts may counterbalance the advantages provided by a shorter, hence easier to complete, life cycle. For example, progenetic trematodes such as *C. parvum* reproduce through self-fertilization, leading to the production of offspring with low genetic heterogeneity and potentially reduced viability (Font, 1980). Self-fertilization can be advantageous by removing the need for a sexual partner, as for trematodes isolated in their larval cyst, but is also a severe case of inbreeding. By increasing homozygosity, self-fertilization can result in inbreeding depression (Charlesworth and Charlesworth, 1987; Trouvé et al., 1996). The maintenance of the typical three-host life cycle could be driven by inbreeding depression and strong selection pressures against self-fertilization (Rauch et al., 2005). However, despite signs of low genetic heterogeneity and heterozygote deficiencies characteristic of self-fertilization (Lagrue et al., 2007, 2009), no short-term deleterious effects of inbreeding have been detected in *C. parvum* (Lagrue and Poulin, 2009b).

Another hypothesis for the maintenance of both life strategies is linked to parasite fecundity. Egg production inside a small, short-lived, invertebrate host is often assumed to be much lower than that achieved inside the vertebrate definitive host. There may thus be a trade-off between the insurance of producing a few offspring in intermediate hosts and the high risk-high reward strategy leading to potentially much higher fecundity in vertebrate hosts. In an article specifically dedicated to trematode life cycle abbreviation, Poulin and Cribb (2002) wrote "progenetic metacercariae of *Coitocaecum parvum* can produce a maximum of ~200 eggs in their amphipod host; the lifetime fecundity of their normal conspecifics inside their fish definitive host is unknown, but is probably much higher". This is a crucial point and remains an outstanding question as the survival, average life span and fecundity of *C. parvum* in fish remain unknown.

Here, we designed a novel experimental approach using controlled infections to assess survival, growth and fecundity (daily and overall) of *C. parvum* metacercariae after transmission to fish definitive hosts. Our main aim was to compare progenetic and non-progenetic (i.e. normal (Poulin and Cribb, 2002)) individual fitness and test whether parasites adopting the typical three-host life cycle achieved higher fecundity in fish than progenetic individuals in intermediate hosts, as suggested by Poulin and Cribb (2002). We subdivided this main goal into three experimentally testable questions. (i) Are progenetic metacercariae still able to establish in fish or is progenesis a clear-cut strategy? In other words, are the progenetic and non-progenetic strategies mutually exclusive? Progenesis may require physiological adaptations rendering *C. parvum* individuals unable to establish or survive in the ancestral definitive hosts, making it a clear-cut strategy. (ii) Does survival in definitive hosts differ between *C. parvum* individuals originating from progenetic or non-progenetic (i.e. non-egg producing) metacercariae? Even if progenetic metacercariae remain capable of infecting fish, they may incur costs in terms of survival, growth or life span. (iii) Do daily and overall fecundity differ

between progenetic and non-progenetic individuals? If survival is similar between *C. parvum* individuals using different strategies, then the answer to that question becomes a key factor in the evolution and maintenance of facultative life cycle abbreviation. Generally, answers to these three questions should thus give a clear picture of the potential costs of progenesis and life cycle abbreviation in *C. parvum*, and shed light on the potential factors driving the evolution of shorter life cycles in parasites.

2. Materials and methods

2.1. Animal collection and husbandry

The main definitive host of *C. parvum* is *Gobiomorphus cotidianus* (Lagrue et al., 2011; Cirtwill et al., 2016). Due to its small size, abundance and ease of acclimation in laboratory settings, it is an ideal study model for experimental infections. Fish were captured in Conroys Dam (New Zealand, 45°16′53.7″S 169°19′08.4″E); this site was chosen for the absence of *C. parvum* due to the lack of crustacean intermediate hosts. All fish were captured on the same date (1st of April 2016) using minnow traps. Fish recovered from the traps were sorted by size; only adult fish between 60 and 75 mm (mean ± S.E. = 65.2 ± 0.4 mm) in total body length (n = 130) were brought back to the laboratory. Fish were maintained in two large tanks (100 l) filled with continuously aerated lake water and maintained at room temperature (16 ± 1 °C) under a controlled photoperiod (12:12 h dark:light). Fish were fed commercial pellets every 2 days until required for experimental infections. Pieces of polyvinyl chloride (PVC) plumbing pipes were also provided as refuges. Fish were given 2 weeks to acclimate to laboratory conditions before controlled infections.

Paracalliope fluviatilis, the amphipod intermediate host of *C. parvum*, were collected in Lake Waihola (South Island, New Zealand, 46°01′09.8″S 170°05′49.6″E) using dip nets and brought back to the laboratory. Amphipods were maintained under the same conditions as the fish and provided with macrophytes (*Elodea canadensis*) for food.

2.2. Fish experimental infections

Parasites used for experimental infections of fish were obtained by inspecting amphipods under a microscope and selecting all individuals that showed signs of *C. parvum* infection, i.e. an opaque mass in the body cavity (Lagrue and Poulin, 2007). Because we wanted to measure individual parasites and count the numbers of eggs laid in amphipod hosts before infecting fish, amphipods were dissected to recover *C. parvum* metacercariae. Amphipods were killed in 70% ethanol and rinsed thoroughly in saline solution. This method kills the amphipod but not their parasites (Lagrue and Poulin, 2008b). Each amphipod was then dissected in saline solution to keep parasites alive; only *C. parvum* metacercariae found alone (i.e. single infections) in the amphipod were used for experimental infections to remove any potential effect of intra-host competition on life history strategy, growth and egg production (Lagrue and Poulin, 2007, 2008a). Furthermore, amphipod host size can influence *C. parvum* metacercariae size and fecundity (Lagrue and Poulin, 2008a; Ruiz-Daniels et al., 2013). To limit potential confounding effects, we extracted *C. parvum* metacercariae only from adult male amphipods of similar size (approximately 3 mm in body length).

Metacercariae of *C. parvum* were classified as either non-progenetic (non-egg producing) or progenetic (egg producing) according to the presence of eggs in the metacercarial cyst or still in utero (i.e. visible by transparency within the metacercaria). Each metacercaria was measured to the nearest 0.01 mm (length and width) and eggs counted when present. The body surface of each parasite was determined using the formula for an ellipsoid, $(\pi LW)/4$, where L and W are the length and width of the metacercaria. Surface area was used as a surrogate for body size (Lagrue and Poulin, 2007). Live *C. parvum* were then transferred to a small Petri dish filled with saline solution. Within 15 min of amphipod dissections, a small group of *C. parvum* metacercariae (between six and eight individuals) was used to experimentally infect a single individual fish. Because parasites had been removed from their amphipod hosts, metacercariae were injected (i.e. force fed) into the fish with a small amount (5 μl) of saline solution using a micropipette. The tip of the pipette was gently inserted into the fish mouth, past the throat and down to the stomach before metacercariae were injected into the fish stomach. The method is comparable to reversed stomach flushing and completely harmless to the fish (Lagrue and Bollache, 2006). Each fish was then transferred to a small container (400 ml) filled with lake water and isolated for an hour in the dark to limit stress; during that time, containers were screened every 10 min for any *C. parvum* metacercariae regurgitated by the fish. Within the first 10 min p.i., a small proportion of parasites were expelled before being able to securely attach onto the gastric epithelium of fish. The number of metacercariae lost by each fish was subtracted from the number injected for later survival estimations. Fish were fasted for 4 days and, on day 5, each fish was experimentally infected by either non-progenetic (48 individual fish) or progenetic metacercariae (48); because we could not individually track each parasite, we did not use mixed groups of parasites (i.e. non-progenetic + progenetic). After 1 h, fish were transferred to a larger container (2 l) filled with continuously aerated lake water. Each fish was provided with a PVC tube for cover and fed every 2 days until dissections.

Amphipod dissections in the previous experiment showed 100% success of parasite screening for single infections by progenetic *C. parvum* metacercariae. We thus ran a second experiment to test for the potential effects of our parasite injection protocol (i.e. artificial infection) on *C. parvum* survival, body size and fecundity in fish. A subsample of fish (n = 24) was isolated in 2 l tanks filled with continuously aerated lake water and provided with a PVC tube. Fish were then fasted for 4 days. On day 5, each fish was offered eight live *P. fluviatilis* amphipods infected with a single progenetic *C. parvum* metacercaria. All amphipods were consumed (i.e. natural infection) by the fish within 1 h. Tanks were checked for rejected parasites as described previously but none were found and we assumed that all parasites had been able to establish in the fish. Fish were maintained individually in their container under the same conditions as artificially infected fish.

2.3. Fish dissection and data collection

A subsample of fish (n = 10) was dissected immediately after collection to confirm that *C. parvum* was absent from the host population. We found some common species of trematodes infecting the body cavity, gonads, muscles and eyes of these fish (Lagrue and Poulin, 2015). However, no gastrointestinal parasite was found and the population was considered free of trophically transmitted helminths, including *C. parvum*, due to the lack of crustacean intermediate hosts in the system. Of the 120 fish used in the two infection experiments (artificial and natural), 11 fish died before dissection and were discarded from the analyses; eight had been artificially infected with non-progenetic *C. parvum* metacercariae and three fish with progenetic individuals. These numbers are not outside of the normal mortality rates for *G. cotidianus* in captivity (personal observations) and the experimental protocol had no obvious adverse effect on fish.

In both experiments (artificial and natural infections), one-third of the fish were haphazardly selected for dissection after 2 days.

After 7 days, half of the remaining fish were dissected and the remaining fish after 21 days. All fish were killed prior to dissections by severing the spine behind the skull and crushing the brain stem using scissors, following University of Otago (New Zealand) Animal Ethics Committee guidelines (permit N° AEC2/16). Fish were measured (total length in mm), weighed to the nearest 0.01 g and dissected. The gastrointestinal tract, from oesophagus to anus, was removed and placed in saline solution for parasite recovery. The digestive tract was cut open length-wise and examined for parasites.

Coitocaecum parvum individuals recovered were counted and measured to the nearest 0.01 mm (length and width) to estimate survival and growth. Parasite body size (mm^2) was calculated as described previously. As fish were tracked individually from infection to dissection, we were able to estimate accurate parasite survival. Parasites were transferred to 96-well plates and kept individually in saline solution (200 μl) for 24 h at the same temperature (16 ± 1 °C). After 24 h, eggs released by *C. parvum* individuals were counted to estimate parasite daily fecundity. Daily fecundity was then used to approximate total fecundity (i.e. overall egg production over the time period spent in the fish definitive host) as a factor of daily fecundity and time p.i. (i.e. number of days between artificial or natural injection and fish dissection).

Note that none of the non-progenetic metacercariae had matured into egg laying adult *C. parvum* after 2 days in the fish but all of them were producing eggs after 7 days. We thus assumed that egg production started on day 3 and overall egg production in individuals originating from non-egg producing metacercariae was approximated as daily egg production multiplied by the number of days since transmission minus the 2 days required for maturation. This may lead to an overestimation of total fecundity in the case of non-progenetic individuals since the actual start of egg production may have been later than day 3. By estimating the high end of fecundity for non-progenetic individuals, we likely reduced the actual differences between non-progenetic and progenetic individuals. This approximation is thus conservative and so are our interpretations of the results.

2.4. Statistical analyses

Fish size and weight did not vary among groups of fish used in artificial or natural infections, *C. parvum* life history strategy (non-progenetic or progenetic metacercariae) or time of dissection (two, seven or 21 days; ANOVAs, all $P > 0.05$). At day 0 (i.e. time of fish infection), *C. parvum* metacercariae recovered from amphipods were used to infect fish that were subsequently dissected after two, seven or 21 days (i.e. time of dissection), generating three haphazard groups of fish. To ensure that parasite body size at the time of infection did not influence results at the time of dissection, we tested for potential differences in parasite size among the different fish groups. At day 0, non-progenetic *C. parvum* size was similar among groups of fish (ANOVA, $F_{2,242} = 0.986$, $P = 0.375$). Similarly, at day 0, progenetic individuals showed no difference in body size or egg production among fish groups (ANOVA, $F_{2,284} = 0.34$ and 2.534, $P = 0.712$ and 0.081, body size and egg production, respectively). Fish size and weight, and parasite size and fecundity, at the time of fish infection (day 0) were thus unlikely to have influenced our results and were not included as factors in the following analyses.

Our analyses had two main goals. The first was to compare parasite survival, growth and fecundity between progenetic *C. parvum* metacercariae from artificially and naturally infected fish. Results were used as an indication of potential confounding effects of artificial infections and whether the method may have influenced the results of the second goal, comparing survival, growth and fecundity between adult parasites originating from progenetic and non-progenetic *C. parvum* metacercariae. All analyses were performed using STATISTICA Software 6.0 (StatSoft Inc., France).

First, potential effects of the *C. parvum* life history strategy in amphipod hosts (non-progenetic or progenetic), infection mode (artificial or natural) and time spent in the fish (i.e. dissection time; two, seven or 21 days) on parasite survival were tested using Fisher's exact tests; proportions of surviving parasites recovered from fish were compared in a pair-wise manner among relevant groups. Second, effects of infection mode (artificial or natural), *C. parvum* life history strategy (non-progenetic or progenetic), and time spent in the fish (two, seven or 21 days) on *C. parvum* body size (dependent variable) were tested using a General Linear Model (GLM). Third, effects of infection mode, *C. parvum* life history strategy and time spent in fish on *C. parvum* daily fecundity (dependent variable) were also tested using a GLM. Finally, potential differences in overall fecundity (estimated total egg production for adults in fish and actual egg counts for metacercariae in amphipods) between progenetic metacercariae in amphipod and adult *C. parvum* in fish were tested using a GLM. Similarly to metacercariae in amphipods where egg production is highly related to the size of the parasite (Lagrue and Poulin, 2007, 2009a), both daily fecundity and overall egg production in fish definitive hosts increased significantly with parasite size ($r = 0.639$ and 0.387, respectively, $n = 129$, both $P < 0.0001$). Parasite size was thus included as a continuous predictor (i.e. covariate) in the GLMs examining egg production. The number of eggs was log-transformed before analyses to normalize the data. In all GLMs, least significant difference post-hoc tests (i.e. Fisher's LSD) were used when appropriate.

3. Results

3.1. Parasite survival

Survival of progenetic *C. parvum* in fish definitive hosts did not differ between infection modes (artificial vervus natural) at any time of dissection (two, seven or 21 days; Fisher's exact tests, $\chi^2 = 0.03$, 0.40 and 0.03, all $P > 0.05$, respectively). Overall, survival of progenetic *C. parvum* in fish was relatively low (Fig. 1). Survival after 1 week remained very similar to post-transmission survival (i.e. after 2 days; Fisher's exact tests, $\chi^2 = 0.01$ and 0.13, $P = 0.95$

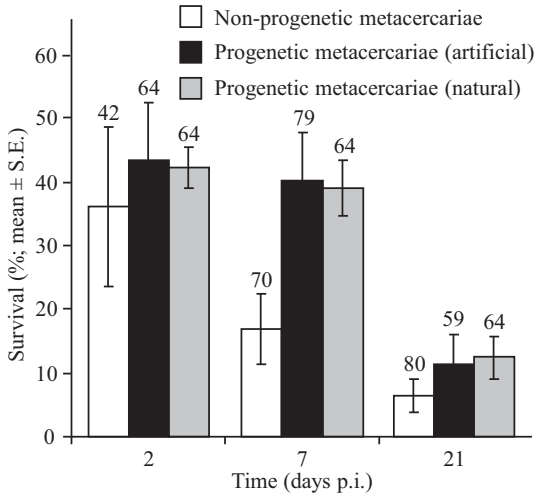

Fig. 1. Proportion (%, mean ± S.E.) of surviving *Coitocaecum parvum* individuals within each life history strategy – infection mode (artificial or natural) after two, seven and 21 days in fish definitive hosts (days p.i.). Numbers above bars are sample sizes (i.e. total number of metacercariae per category at the time of fish infection, day zero).

and 0.72, artificial and natural infections, respectively; Fig. 1). Parasites surviving the transition from intermediate host prey to predator and successfully establishing in the fish experienced very low mortality during the first week. However, survival clearly decreased after that time and the proportion *C. parvum* individuals still alive after 3 weeks in fish was significantly lower (Fisher's exact tests, $\chi^2 = 14.88$ and 11.8, $P = 0.0001$ and 0.0006, artificial and natural infections, respectively; Fig. 1).

The survival pattern of non-progenetic (i.e. non-egg producing) metacercariae in fish was slightly different from that of progenetic individuals. Post-transmission survival (i.e. after 2 days) of non-progenetic *C. parvum* was similar to that of progenetic individuals (Fisher's exact test, $\chi^2 = 0.33$, $P = 0.56$; Fig. 1). However, their survival decreased significantly in the first week in fish (between two and 7 days; Fisher's exact test, $\chi^2 = 7.19$, $P = 0.0073$) but not between seven and 21 days (Fisher's exact test, $\chi^2 = 2.51$, $P = 0.12$; Fig. 1), which contrasts with survival patterns observed in progenetic individuals. As a result, after 7 days, survival of non-progenetic individuals was significantly lower than that of their progenetic conspecifics (Fisher's exact test, $\chi^2 = 14.21$, $P = 0.0002$). However, this difference disappeared after 21 days (Fisher's exact test, $\chi^2 = 1.38$, $P = 0.24$; Fig. 1), by which time survival percentage was low for all groups.

3.2. Parasite body size

Both parasite strategy and time p.i. had significant effects on parasite size (GLM, $F_{1,650} = 58.1$ and $F_{2,650} = 13.1$, respectively, both $P < 0.0001$). There was also a significant interaction between the two factors (GLM, $F_{5,650} = 10.8$, $P < 0.0001$). There was no difference in body size between progenetic *C. parvum* individuals in artificially or naturally infected fish at any time p.i. (Fisher's LSD, degrees of freedom (df) = 650, $0.276 < P < 0.919$; Fig. 2A), further demonstrating that artificial infections did not influence data recorded here. Generally, the size of progenetic individuals did not increase over time in fish (Fig. 2A); there was no difference in body size among progenetic *C. parvum* from the different fish groups at any time p.i. (all possible pair-wise comparisons; Fisher's LSD, df = 650, $0.311 < P < 0.802$). Contrastingly, non-progenetic individuals significantly increased in size over time (Fig. 2A). Although there was no measurable growth in body size between the time of infection (day 0) and 2 days p.i. (Fisher's LSD, df = 650, $P = 0.832$), non-progenetic individuals were significantly larger after 7 days (compared with day 2; Fisher's LSD, df = 650, $P = 0.0007$). Body size increased further between day 7 and day 21 (Fisher's LSD, df = 650, $P = 0.0006$; Fig. 2A). Non-egg producing individuals were thus significantly smaller than their progenetic conspecific at the time of infection (day 0) and after 2 days in fish (Fisher's LSD, df = 650, all $P < 0.0001$; Fig. 2A). Although they had increased in size by day 7, non-progenetic *C. parvum* individuals were still smaller than progenetic parasites after 1 week in fish (Fisher's LSD, df = 650, both $P = 0.002$, artificial and natural infections, respectively; Fig. 2A). However, after 21 days, adult *C. parvum* from non-progenetic metacercariae had reached a body size similar to that of their progenetic conspecifics and no difference in size could be detected (Fisher's LSD, df = 650, $P = 0.383$ and 0.679; Fig. 2A).

3.3. Parasite fecundity

Parasite daily fecundity (i.e. number of eggs produced in 24 h) in fish was significantly affected by *C. parvum* strategy in amphipods (GLM, $F_{2,119} = 132.9$, $P < 0.0001$) and time spent in fish (GLM, $F_{2,119} = 110.3$, $P < 0.0001$). There was also a significant interaction between the two factors (GLM, $F_{4,119} = 63.97$, $P < 0.0001$). Daily fecundity was virtually identical between progenetic metacercariae in artificial and natural infections (days 2, 7 and 21; Fisher's LSD, df = 119, all $P > 0.05$). Daily fecundity of progenetic individuals also remained stable over time (mean ± S.E. = 5.48 ± 0.24, 6.13 ± 0.31 and 6.5 ± 0.51 at days 2, 7 and 21, respectively; Fisher's LSD, df = 119, all $P > 0.05$). Contrastingly, daily fecundity increased significantly over time in non-progenetic *C. parvum* (mean ± S.E. = 0 ± 0, 3 ± 0.33 and 6.6 ± 0.25 at days 2, 7 and 21, respectively; Fisher's LSD, df = 119, all $P < 0.0001$). Daily fecundity of non-progenetic individuals was significantly lower than that of progenetic *C. parvum* after two and 7 days in fish (Fisher's LSD, df = 119, all $P < 0.0001$). After 21 days, however, daily fecundity of non-progenetic individuals was similar to that of progenetic *C. parvum* (Fisher's LSD, df = 119, both $P > 0.05$, artificial and natural infections).

As a result, estimated total fecundity, as a factor of time and daily fecundity, increased over time in all parasite groups (Fig. 2B). Total fecundity was similar in progenetic individuals from artificially and naturally infected fish (Fig. 2B). However, although the daily fecundity of non-progenetic individuals was comparable to that of progenetic individuals, they generally had slightly lower total fecundity estimates than their progenetic conspecifics (Fig. 2B). Finally, total fecundity of adult *C. parvum* in definitive hosts was significantly lower than that of progenetic metacercariae in intermediate hosts, at least for individuals that survived less

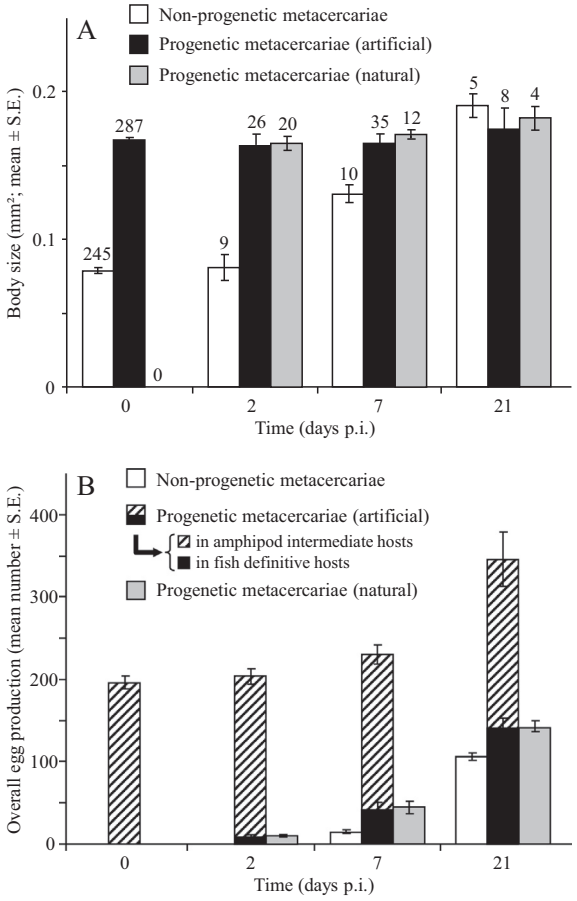

Fig. 2. Effects of *Coitocaecum parvum* life history strategy – infection mode (artificial or natural) and time spent in fish definitive hosts (zero, two, seven or 21 days p.i.) on (A) parasite body size (mm^2, mean ± S.E.) and (B) overall fecundity (estimated total number of eggs produced) in fish definitive hosts (plain bars). Note that B also shows the number of eggs produced in amphipod intermediate hosts, before transmission to fish definitive hosts, by injected progenetic metacercariae (dashed bars) for comparison purposes. Numbers above bars are sample sizes (number of individual parasites per category at the time of fish dissection); samples sizes are the same for A and B.

than 21 days in fish (i.e. *C. parvum* individuals recovered after 2 and 7 days; GLM, $F_{6,408} = 72.81$, $P < 0.0001$; Fig. 2B). This pattern held true for both progenetic and non-progenetic metacercariae (Fisher's LSD, df = 408, all $P < 0.0001$; Fig. 2B). After 21 days in the fish, there was no difference in total fecundity between *C. parvum* individuals in definitive hosts and progenetic metacercariae in intermediate hosts (Fisher's LSD, df = 408, all $P > 0.05$, artificial and natural infections; Fig. 2B).

4. Discussion

Our main goal was to test whether individual parasites adopting the ancestral three-host life cycle and reproducing in the vertebrate definitive host attain higher lifetime fecundity than their progenetic conspecifics reproducing in intermediate hosts, as it is often suggested (Poulin and Cribb, 2002). In particular, we tested whether progenetic and non-progenetic strategies are mutually exclusive; are progenetic individuals still able to survive and establish in the definitive host? Results show that both non-progenetic and progenetic metacercariae are able to establish in fish. Early maturation and reproduction in the intermediate host did not prevent infection of, or reproduction in, the definitive host. This may seem logical a posteriori but there is evidence that minor morphological or physiological changes could be necessary to achieve progenesis and early reproduction in intermediate hosts (Grabda-Kazubska and Bayssade-Dufour, 1999; Poulin and Cribb, 2002). Such changes may in turn prevent successful infection of the definitive host. However, no such evidence was found in our study. Furthermore, survival of progenetic individuals after transmission to definitive hosts was significantly higher than that of non-progenetic *C. parvum*. Noticeably, mortality of non-progenetic metacercariae in fish was high in the first week after infection. It is thus possible that some non-progenetic individuals had not developed enough to survive in the definitive host, and were thus not infective to fish. Consumption of the intermediate host, even by the appropriate host, before the parasite is capable of establishing in the definitive host, results in the parasite's death (Chubb et al., 2010, Médoc and Beisel, 2011). Overall, not only are progenetic and non-progenetic strategies not mutually exclusive, but progenetic individuals also had a higher survival in definitive hosts than their non-progenetic conspecifics.

It is often assumed that vertebrate definitive hosts are advantageous to parasites by providing more energy and space than smaller intermediate hosts, thus allowing parasites to reach larger adult sizes (Parker et al., 2003a). Although non-progenetic *C. parvum* did increase in body size in fish, they did not achieve larger adult size than their progenetic conspecifics. In addition, progenetic metacercariae did not grow further once established in the fish definitive hosts, indicating that the higher resources available in fish definitive hosts do not influence *C. parvum* maximum body size (Lefebvre and Poulin, 2005b). Long-lived vertebrate definitive hosts may also provide parasites with the opportunity of an extended life span compared with short-lived intermediate hosts (Parker et al., 2003a). Here we found that not only was survival generally low p.i. (~40% after 2 days) but also the *C. parvum* lifespan in definitive hosts was relatively short, with only ~10% of parasites still alive after 21 days in fish (Fig. 1). A direct consequence of the short *C. parvum* life span in fish is that the parasite lifetime fecundity may not be higher in definitive hosts, another classical argument for the maintenance of vertebrate hosts in parasite complex life cycles (Parker et al., 2003a). Although we did not test the maximum life span of *C. parvum* in fish and thus we cannot estimate the maximum fecundity of the parasite in its definitive host, only 10% of individuals were still alive after 21 days. This means that our estimation of lifetime fecundity for *C. parvum* in fish is valid or overestimated for 90% of individuals successfully transmitted to the definitive host. Our results show that *C. parvum* progenetic metacercariae actually produced more eggs (mean number ± S.E. = 196 ± 8) in amphipod intermediate hosts than most individuals in fish definitive hosts (Fig. 2B).

The time spent by progenetic *C. parvum* in amphipods to reach the fecundity observed here is unknown. However, previous studies showed that the total fecundity of progenetic metacercariae doubled every 2 weeks once egg production had started. Total fecundity reached around 20 eggs after 5 weeks in the amphipod, 40 after 7 weeks and 80 after 9 weeks (Lagrue and Poulin, 2007, 2009a). By extrapolation, progenetic metacercariae would have to spend approximately 12 weeks in their intermediate host to attain the fecundity shown by our present results. It required 21 days in fish, a time at which only ~10% of parasites still survived, for progenetic and non-progenetic individuals (mean number ± S.E. = 136.5 ± 6.6 and 107.4 ± 3.4, respectively) to reach overall fecundities in fish approaching that of progenetic metacercariae in their intermediate hosts. Even though the daily fecundity of progenetic metacercariae in amphipods is likely lower than that of adult individuals in fish, the overall fecundity of progenetic individuals in intermediate hosts is at least equivalent to that of adult parasites in definitive hosts if transmission to the fish fails and at best double if transmission occurs (Fig. 2B). In that case, progenetic metacercariae actually "hit the ground running" and just continue producing eggs in fish definitive hosts while their non-egg producing conspecifics need several days to achieve the size and degree of maturation required to start producing eggs. Generally, in *C. parvum*, non-progenetic and progenetic metacercariae have similar probabilities of transmission to the definitive host and progenetic individuals actually have higher survival and fecundity than non-progenetic ones. As a result, progenesis achieved in the second intermediate host should always be advantageous, with very few apparent costs (Balboa et al., 2001).

So why are all *C. parvum* metacercariae not adopting progenesis early in their intermediate host if it does not hinder their ability to establish in the definitive host while providing a reproductive insurance against failed transmission? Previous studies and our data show that there are few apparent short-term costs to progenesis. Remaining questions about progenesis and life cycle abbreviations in *C. parvum* revolve around the long-term costs of inbreeding depression; potential fitness costs may still arise after multiple generations of progenesis and self-fertilization (Lagrue et al., 2009). The answer to that question partly depends on the rate of outcrossing in fish. To date there is no evidence showing that *C. parvum* individuals either favour outcrossing (i.e. cross-fertilization between sexual partners), carry on self-fertilizing in the definitive host, or use both strategies and in what proportions. The maintenance of vertebrate definitive hosts in parasite complex life cycles relies heavily on the hypothesis that genetic mixing through outcrossing is selectively advantageous (Wedekind et al., 1998; Christen et al., 2002; Rauch et al., 2005). Although *C. parvum* mating pairs have been anecdotally observed in fish before, they are uncommon, even in hosts with high parasite loads where access to mating partners is unlimited (Lefebvre and Poulin, 2005b; personal observations). It is thus likely that *C. parvum* keep producing a large proportion of their eggs through self-fertilization, even in definitive hosts. However, the proportion of eggs produced through outcrossing compared with self-fertilization remains unknown. We have thus far failed to extract enough DNA from *C. parvum* eggs for such a comparison. However, low genetic heterogeneity and strong heterozygote deficiencies in *C. parvum* indicate that self-fertilization is a major method of reproduction for this species (Lagrue et al., 2007, 2009). Overall, it seems like there are no short-term costs and potentially few long-term negative consequences to progenesis in *C. parvum*.

The absence of apparent short-term costs to progenesis in *C. parvum* indicates that the strategy should be advantageous under all environmental conditions. While the proportion of progenetic individuals can be high, there is still a large portion of *C. parvum* metacercariae in natural populations that seems to arrest their development and not produce eggs in intermediate hosts (Lefebvre and Poulin, 2005b; Lagrue and Poulin, 2008a). Since there is also no heritability of the strategy (Lagrue and Poulin, 2009b), other factors must influence the *C. parvum* life-history strategy. The most parsimonious explanation is that non-progenetic metacercariae in intermediate hosts are young individuals that will eventually grow and mature into progenetic individuals. In fact, consistent with the developmental time hypothesis, a large majority of *C. parvum* metacercariae remaining in amphipod hosts for a relatively long time eventually adopted progenesis (Lagrue and Poulin, 2009a). However, some individuals never did, hinting at potential constraints to the adoption of progenesis preventing some individuals from growing larger, maturing and producing eggs in intermediate hosts. Because progenetic *C. parvum* are significantly larger than non-egg producing individuals, the acquisition of resources from the intermediate host (energy and space) necessary for accelerated growth, larger body size and egg production may not be possible in all *C. parvum* individuals (Lefebvre and Poulin, 2005b). Within-host limitation on resource availability seems to be the main constraint in the adoption of progenesis in *C. parvum*. Amphipod size, and thus resources, strongly influences the ability of *C. parvum* metacercariae to adopt progenesis but also individual parasite size and fecundity (Ruiz-Daniels et al., 2013). Some hosts may not provide the resources necessary for *C. parvum* metacercariae to adopt progenesis. Within-host competition among co-infecting parasites may further reduce the limited space and energy available to each individual parasite, and influence their development and life-history strategy (Brown et al., 2003; Parker et al., 2003b; Fredensborg and Poulin, 2005). Co-infecting *C. parvum* metacercariae compete for the same limited resources and the number of co-occurring conspecifics could influence the size and fecundity of each individual (Lefebvre and Poulin, 2005a,b; Jäger and Schjørring, 2006; Michaud et al., 2006). Thus, intense competition among parasites may prevent some or all co-infecting *C. parvum* metacercariae from achieving progenesis (Poulin and Lefebvre, 2006). Both intra- and interspecific competition influence the ability of *C. parvum* to achieve progenesis in amphipod intermediate hosts (Lagrue and Poulin, 2008b).

In conclusion, it seems that there are few, if any, short-term costs to progenesis and life-cycle abbreviation in *C. parvum*. The maintenance of both life-history strategies in parasite populations seems largely due to constraints on the adoption of the abbreviated life cycle and the high-energy requirement of progenesis. While facultative progenesis is often regarded as a reproductive insurance against failed transmission to the definitive host, in some cases it may actually be the opposite. The ancestral three-host life cycle might only be maintained as a secondary way of producing offspring in situations where adopting progenesis is impossible. It may also allow an extended life span and fecundity if the intermediate host within which progenetic individuals have already produced eggs is consumed by the definitive host. Progenesis may be becoming the primary life-history strategy in *C. parvum* and the ancestral three-host life cycle a reproductive insurance or bonus, not the other way around. Our study is a snapshot taken at one point in the evolutionary time scale of *C. parvum* and the species may be evolving towards obligatory progenesis similar to other trematode species (Poulin and Cribb, 2002). Alternatively, long-term costs of self-fertilization and inbreeding depression may maintain the ancestral three-host life cycle in parasite populations.

While our findings are only valid for *C. parvum*, a similar balance between costs, or absence thereof, constraints and advantages may modulate the evolution of abbreviated life cycles and progenesis in other trematode species. For example, the two trematodes *Stegodexamene anguillae* and *Telogaster Opisthorchis* use *G. cotidianus* as intermediate hosts; short and long finned eels (*Anguilla dieffenbachi* and *Anguilla australis*) are the definitive hosts (MacFarlane, 1945, 1951; Holton, 1984a). In these species, the adoption of progenesis is influenced by several factors such as the encystment site in the intermediate host, water temperature, and fish size and body condition (Herrmann and Poulin, 2011a,b, 2012a). In contrast to *C. parvum*, predator cues from the environment did not influence the *S. anguillae* life-history strategy, hinting at the existence of both universal and species-specific factors influencing life cycle abbreviation in parasites with facultative progenesis (Herrmann and Poulin, 2012b). However, the actual balance between costs, constraints and advantages, and their effects on the adoption of progenesis and maintenance of definitive hosts in trematode species with facultative progenesis, are not fully understood. It is only through thorough studies of specific cases that general trends on the selective forces shaping a major step in the evolution of parasite life cycles will be elucidated.

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