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Genetics, intensity-dependence, and host manipulation in the trematode *Curtuteria australis*: following the strategies of others?

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Manipulation of host phenotype by parasites can require a collective effort from many individuals. The cost of manipulation may only be paid by the individuals actually inducing the manipulation, while its benefits are reaped by all. Here, we determine if there is genetic variation in manipulative effort among different clonal lineages of the trematode *Curtuteria australis*, and whether the decision to manipulate is context-dependent. *C. australis* impairs the burrowing efficiency of its second intermediate host, the cockle *Austrovenus stutchburyi*, by encysting at the tip of the cockle's foot, which facilitates the parasite's trophic transmission to shorebirds. However, manipulative individuals at the tip of the foot are vulnerable to non-host predators (foot-cropping fish); in contrast, those encysted at the base of the foot, although they do not contribute to manipulation, are safe from foot-croppers and can benefit from altered host phenotype. In an experimental study, different clonal lineages showed no significant variation in their tendency to encyst in the tip versus the base of the foot, with only the former contributing to host manipulation. However, the decision to manipulate was intensity-dependent: the greater the number of parasites already committed to manipulation (i.e. already encysted in the foot tip), the more likely newly arriving parasites were to join them. These findings indicate considerable intraspecific variation in the strategies adopted by 'manipulator' parasites, with external influences determining what a parasite actually does.

Cooperation and altruistic behaviour have evolved independently in several taxa (Dugatkin 1997). The main framework for understanding the evolution of cooperation has been provided by the theory of kin selection (Griffin and West 2002, Lehmann and Keller 2006), whereby altruistic individuals can receive fitness benefits indirectly through inclusive fitness (Hamilton 1964). The direct fitness benefit and cost of altruistic acts, as well as the genetic make-up of groups of interacting organisms, determine whether cooperative strategies are advantageous or not.

In parasites, for instance, an individual restricting the rate at which it exploits host resources will achieve lower rates of replication that could be offset if its altruistic moderation allows genetically related individuals to prosper. Indeed, infections by single genetic strains often, though not always, result in lower virulence than infection by multiple strains (Taylor et al. 1998, de Roode et al. 2003, Marzal et al. 2008). Cooperation can be manifested as prudence in the use of host resources, but also as the generation of 'public goods' that can benefit conspecifics (Buckling and Brockhurst 2008). This may apply to the well-known ability of parasites to manipulate host behaviour (Moore 2002, Thomas et al. 2005, Poulin 2007) for two main reasons. Firstly, host manipulation usually incurs some form of cost to the individuals responsible for the manipulation (thereafter referred to as 'manipulators') (Poulin 1994, Poulin et al. 2005). Manipulators can be considered as generating a public good, with the potential for 'cheating' individuals to take advantage of any benefits without paying the cost associated with the manipulation effort. Secondly, hosts are usually infected with multiple individuals, some of which may be kin of the manipulators. Often the magnitude of the behavioural change shown by the host is intensity-dependent, i.e. it is a function of the number of manipulators present and thus involves a form of cooperation (Poulin 1994). Importantly, the altered host behaviour induced by the manipulators benefits both manipulators and cheaters, and both kin and non-kin.

Here, we explore the genetic and extrinsic determinants of manipulative and cheating strategies within a parasite species. *Curtuteria australis* (Echinostomatidae) is a marine trematode with a complex life cycle involving three different hosts (Allison 1979). The whelk *Cominella glandiformis* serves as the first intermediate host; it becomes infected when penetrated by a larva derived from a parasite egg. Within the whelk, the parasite undergoes asexual replication, producing numerous free-swimming infective stages known as cercariae, which are released into the water. An important feature of this asexual multiplication is that the many cercariae produced from the same original larva are all genetically identical, i.e. they are genetic clones. The

cercariae then enter the cockle *Austrovenus stutchburyi* through its inhalant siphon, penetrating the host from within the mantle cavity, and encysting as metacercariae in the cockle's foot muscle. The life cycle is completed when an infected cockle is eaten by pied oystercatchers, *Haematopus ostralegus*. Prevalence of infection in cockles in the Otago area of New Zealand's South Island is virtually 100%, although the number of metacercariae per cockle ranges from just a few to over one thousand. As metacercariae accumulate in the foot tissue, the cockle gradually loses the ability to use its foot to burrow into the sediment, as cockles usually do; the foot becomes mechanically impaired by the sheer number of metacercarial cysts (Thomas and Poulin 1998, Mouritsen 2002). As a result, the cockle is stranded on the sediment surface where it incurs a seven-fold increase in its susceptibility to predation by the definitive hosts of *C. australis* (Thomas and Poulin 1998). The parasites encyst exclusively in the cockle's foot, and not in any of the other host tissues available to them (de Montaudouin et al. 2009). This fact, combined with the increased transmission success that results from altered host burrowing, indicate that this strategy has evolved specifically to manipulate host behaviour.

However, this host manipulation comes at a cost. While *C. australis* can encyst anywhere within the cockle's foot, only metacercariae close to the tip of the foot cause impaired burrowing (Mouritsen 2002), though any resulting increase in transmission benefits all metacercariae, regardless of where they are encysted. However, metacercariae at the foot tip are vulnerable to the foot-cropping fish *Notolabrus celidotus*, which is an unsuitable host for the parasite; parasites at the base of the foot are safe from foot-croppers (Mouritsen and Poulin 2003). At one locality, more than 80% of surface-stranded cockles had on average 21% of their foot missing following cropping, with all cropping limited to the foot tip area; the net result was that over 17% of metacercariae in the population end up dying following ingestion by fish (Mouritsen and Poulin 2003). These numbers are similar at the nearby sites within Otago Harbour (South Island, New Zealand) from which cockles and parasites were collected for the present study.

Thus, metacercariae at the base of the foot benefit from greater transmission without incurring the associated risk. These cheaters reap the benefits of manipulation without paying the cost. On the one hand, the population may consist of individuals with genetically determined manipulator or cheater strategies. On the other hand, adoption of either strategy could also be context-dependent (Thomas et al. 2002). Intraspecific variation in manipulative ability among individual parasites has been documented with respect to variables such as age (Franceschi et al. 2008). However, given that the manipulation of cockles by *C. australis* is intensity-dependent, a cercaria entering a cockle that is already heavily parasitised might opt to encyst at the base of the foot and let the pre-existing metacercariae take the risk associated with manipulation. In contrast, in a host harbouring few parasites, a newly-arrived cercaria might be more likely to cooperate and participate in host manipulation. Preliminary data indicate that individuals with the same genotype represent only a small portion of all parasites in the same cockle in nature (Leung unpubl.), therefore ruling out any major role for kin selection. The payoffs of cooperating or cheating for a cercaria may depend solely on the current numbers of manipulators and cheaters in the host.

This study tackles three main questions. Firstly, is there intraspecific variation in encystment site preference between clonal lineages? We determine whether certain clones are genetically predisposed toward either manipulation (encysting in the foot tip) or cheating (encysting at the base of the foot). Secondly, are there differences in infectivity among clones, and how do these relate to encystment site? Because of how water flows inside a cockle, it may be easier to encyst at the foot tip, whereas attempts to encyst elsewhere may lead to cercariae being expelled in outflowing water (Mouritsen 2002); we determine whether cheating clones experience lower infection success. Thirdly, how do pre-existing infections affect encystment site and infection success? We test if the number of metacercariae already committed to host manipulation affects whether newcomers will join in the manipulation effort or cheat.

Material and methods

Screening for infected whelks

Approximately 800 mud whelks, *C. glandiformis*, were collected from Lower Portobello Bay, South Island, New Zealand, in October 2007. The whelks were screened for *C. australis* infection by placing them individually into a clear plastic cylinder (60 mm high $\times$ 40 mm wide) filled with seawater and incubated at 25°C under constant illumination for 18 h to encourage cercarial emergence. Whelks shedding *C. australis* cercariae were transferred to a separate plastic container (300 mm long $\times$ 130 mm wide $\times$ 150 mm high) filled with seawater and approximately 30 mm of fine sand, and aerated with an airstone. Whelks infected with a single clonal lineage of *C. australis* were identified by pooling 25 cercariae, from each snail separately, into a 1.5 ml tube; DNA was then extracted from the cercariae in 500 µl of 5% chelex containing 0.1 mg ml^{-1} proteinase K, incubated at 60°C for 4 h and boiled at 100°C for 8 min. Cercariae DNA samples were genotyped at six microsatellite loci (Cau1, Cau5, Cau11, Cau13, Cau15, Cau19) as described in Leung et al. (2008). The loci were selected on the basis of their level of polymorphism and their statistical power to identify true genetic clones. The probabilities of observing at least as many identical genotypes by chance based on the loci used were estimated using GENCLONE ver. 2.0 (Arnaud-Haond and Belkhir 2007). GENCLONE can take into account any departure from Hardy–Weinberg equilibrium (HWE) when calculating the probability that identical multilocus genotypes were produced via sexual reproduction instead of being true genetic clones. Analysis by GENCLONE indicated that cercariae with identical multilocus genotypes found in the same host can be reliably considered as true genetic clones (all $p_{sex} < 0.0003$).

Pooled cercariae DNA samples that did not possess more than two alleles at any locus were then selected for further genotyping at the level of individual cercariae, to confirm that the whelks from which they originated were indeed infected with a single clonal lineage. This was done to

protect against missing rare genotypes that may have amplified poorly and the presence of multiple homozygous genotypes that could have been misidentified as single-clone heterozygotes. Cercariae shed from infected whelks were collected and placed individually into 1.5 ml tubes for DNA extraction. The DNA extraction procedure was identical to that of pooled cercariae except that individual cercariae were each in 200 µl of 5% chelex. A total of 24 cercariae were genotyped from each whelk, consisting of two separate batches of 12 cercariae shed from the whelks 4 weeks apart, to ensure that multi-clone infections would be identified even if there are any temporal differences in the shedding patterns among clones.

Ten whelks identified as having a single clonal infection of *C. australis* were marked with a number written on their shell with a permanent marker and kept in a container identical to the one previously described for holding *C. australis*-infected whelks. The container was held in a temperature-controlled room at 24°C with a 12/12 day/night period to encourage cercarial production. The whelks were fed with cockle flesh ad libitum every four days.

Cockle experimental infections

Approximately 150 cockles, *A. stutchburyi*, were collected from a sand flat at Otakou, Otago Harbour, South Island, in October 2007. The infection intensity of *C. australis* is known to be relatively low at that site (Mouritsen 2002; Poulin unpubl.), which is only about 1 km from where the whelks were collected. Prior to experimental infection, the cockles were held in plastic containers (300 mm long × 130 mm wide × 150 mm high) filled with seawater and approximately 60 mm of fine sand, and aerated with an airstone.

To obtain cercariae, the 10 whelks were induced to shed in the manner described above, and the cercariae were collected at least 3 h after emerging from the whelk. This period between initial cercarial emergence and collection was necessary due to the behavioural pattern of the *C. australis* cercariae. Within their first few hours, these cercariae display energetic swimming, positive phototatic and negative geotactic behaviour that make them difficult to collect, possibly corresponding with a 'dispersal phase' in the natural habitat. After the first few hours, the cercariae begin displaying positive geotactic behaviour that, in their natural environment, would probably bring them closer to the open siphons of their second intermediate host (Leung unpubl.). At this point the cercariae were collected with a 200 µl pipette and transferred into a 60 mm petri dish filled with 5 ml of artificial seawater.

For labelling individual cercariae and tracking their encystment site within the cockle, we used fluorescent dyes, i.e. fatty acid analog probes BODIPY FL C_{12} (green dye) and BODIPY 558/568 C_{12} (red dye). Following purchase of the dyes, 10 mM stocks and 100 µM working stocks of both dyes were created by dissolving the dyes in DMSO. The BODIPY dyes have been found to have no effect on cercarial survival or infectivity (Keeney et al. 2008). The cercariae were transferred with a 200 µl pipette into a 1.5 ml tube, filled with 1 ml of artificial seawater with 200 nM concentration of the dye. The tube with cercariae was then incubated in the dark at 25°C for 60 min. The cercariae were removed from the tube with a pipette, and rinsed to decrease dye carryover by sequentially transferring them into three wells in a 96-well plate, each well containing 75 µl of artificial seawater. The cercariae were then used for experimental infection of their assigned cockles.

For experimental infection, approximately five cockles were assigned to each single *C. australis* clone (the number varied between 4–8 depending on the availability of cercariae), with each cockle initially receiving 30 cercariae and then another 30 cercariae two weeks later, for a total of 60 cercariae per cockle, all 60 being clones. Each cockle was exposed to these two batches of cercariae to allow for any temporal variation in infectivity and encystment pattern within each clone. For infection, cockles were placed individually in plastic cylinders (60 mm high × 40 mm wide) and completely covered with fine sand so that only the cockle's siphons protruded above the substrate. The container was then filled with 15 mm of seawater. The cockles were given at least 60 mins to acclimatise to the container and start filtering the water layer, before a single-clone batch of 30 dye-treated cercariae was added to the water with a 200 µl pipette.

The container holding the cockle and the cercariae was kept in the dark for 24 h to minimise the influence of light on the fluorescence of the dye. The small volume of water and the long exposure period ensured that the cercariae had ample opportunity to infect the cockle. After the 24 h exposure period, the cockles were removed from their containers and each given a unique number on the shell with a permanent marker denoting which *C. australis* clone they received. The cockles were then haphazardly assigned to a holding container. These were opaque plastic containers (240 mm long × 190 mm wide × 120 mm high) filled with seawater and 20 mm of fine sand for substrate and aerated with an airstone. Up to 10 cockles were kept in each container.

Two weeks after being exposed to the initial 30 red dye-treated cercariae, the cockles were exposed to a further 30 freshly shed cercariae from the same clone, as above, though this time the cercariae were dyed green. Two weeks after exposure to the green-dyed cercariae, cockles were dissected to determine the infection success and site selection of the parasites. The shell length of each cockle was measured to the nearest 0.1 mm with digital callipers before they were opened. The foot was removed by cutting along the narrow bridge between the gonad and foot basis, and placed between two glass plates that were pressed firmly together to gently flatten the foot. All encysted metacercariae were visible through the transparent foot tissue. The foot was then cut into three sections (Fig. 1); the sectioning of the foot was performed in a standard way, by cutting at 45° from the bottom margin of the foot, once starting from the distal edge, and once from one-third of the way toward the proximal end. The tip area matched roughly the part of the foot protruding from the shell when a cockle attempts to burrow, i.e. the part most likely to be cropped by fish, whereas the base area is never exposed to cropping (Fig. 1). While flattened between the glass plates, each section of the foot was examined with a fluorescence stereomicroscope equipped with a digital camera system and GFP2 and DiI

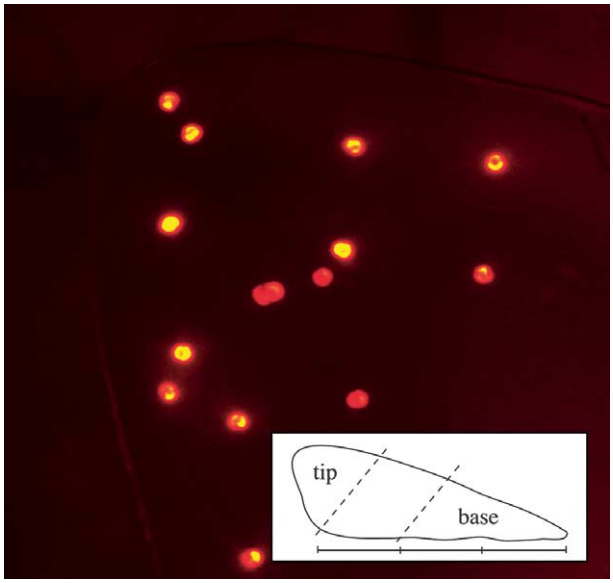

Figure 1. Experimentally dyed metacercariae in the foot tip of a cockle seen under a fluorescence stereomicroscope. Insert: a diagram of the cockle's foot showing how the foot muscle was cut (broken lines) into three sections.

fluorescent filter sets suitable for BODIPY FL C_{12} and BODIPY 558/568 C_{12}, respectively (Fig. 1). All metacercariae (red, green, or non-dyed previously encysted metacercariae) from each section were counted.

A separate experiment was performed to verify that the dyes have no effect on choice of encystment sites by cercariae. Cercariae were obtained simultaneously from 14 whelks, and were well-mixed in a petri dish to obtain a genetically homogeneous mixture. A third of these were then dyed red (as above), a third were dyed green, and the rest were treated as the previous two groups but not exposed to any dye. Each of eight cockles was then exposed to 20 cercariae from each of the three groups (total 60 cercariae per cockle). The cockles were dissected after 24 h (when non-dyed cercariae could be distinguished from pre-existing infections because the former were not yet fully encysted), and the distribution of red, green and non-dyed parasites between the tip and base of the foot was determined.

Statistical analyses

Comparisons of frequencies of metacercariae encysted in different parts of the foot, replicated among cockles or among dye treatments, were performed using replicated tests of goodness-of-fit with the G_H statistic for homogeneity (Sokal and Rohlf 1995, pp. 715–722). All other analyses were parametric tests conducted with JMP ver. 7.0; the only variable that did not conform to the assumptions of normality, i.e. the number of prior metacercariae per cockle (either the total number or those in the foot tip only), was log-transformed. In our main analyses, where we tested for differences among clones, we adopted analysis of covariance (ANCOVA) using clonal identity as the main effect, and shell length and prior infections as covariates. This was done to distinguish between the effects of clonal identity and prior infections while controlling for the potentially confounding effect of host size.

Results

Fifty-three cockles were each individually exposed to single clone infections of *C. australis* cercariae; only four of these cockles did not become infected with new metacercariae. The mean shell length $\pm$ SE of cockles used was 30.5 ± 0.4 mm. The total number of metacercariae already encysted in the foot of cockles prior to the experiment ranged from 2 to 210 per cockle. One-way ANOVAs found no significant difference in shell length ($F_{9,43} = 1.146$, $p = 0.3531$) or prior infections ($F_{9,43} = 0.646$, $p = 0.7512$) between the groups of cockles assigned to the 10 different clones. There was a correlation between the number of prior infections and cockle shell length (Pearson product–moment correlation: $n = 53$, $r = 0.618$, $p < 0.0001$), i.e. larger cockles tend to harbour more metacercariae, a pattern commonly seen in field-collected samples (Poulin et al. 2000, Leung and Poulin 2007a).

In the experiment testing for any effect of the dyes on choice of encystment sites by cercariae, we recovered 83% of the red, 69% of the green, and 86% of the non-dyed metacercariae in the foot tip of the eight cockles. Among these three groups, the relative numbers of metacercariae in the tip and at the base were homogeneous (replicated test of goodness-of-fit, $G_H = 4.31$, $DF = 2$, $p > 0.10$). In other words, the distribution of metacercariae between the tip and the base of the foot did not differ significantly among red, green and non-dyed individuals.

In the main experiment, a total of 702 dyed cercariae acquired experimentally were recovered from the 53 cockles (range 44 to 130 per clone, though numbers of cockles per clone vary). Infection success (mean percentage of successful cercariae $\pm$ SE) of the first wave of 30 cercariae was $20.3 \pm 2.5\%$, while that of the second wave was $23.9 \pm 2.9\%$, and this difference was not statistically significant (paired t-test: $t = 0.899$, $DF = 52$, $p = 0.373$). Among those parasites that were successful at infecting cockles, the proportion of cercariae encysting in the foot tip did not differ between the first and second waves (mean $\pm$ SE, using only the 37 cockles in which cercariae from both waves were recovered: first wave, $58.3 \pm 4.6\%$, second wave, $55.6 \pm 3.9\%$; paired t-test, $t = 0.180$, $DF = 36$, $p = 0.858$). Therefore, since there were no differences in site selection between cercariae from the two waves of infection, data for the first and second infection exposures are pooled hereafter.

To investigate potential clonal differences in infection success, we used an ANCOVA using clonal identity as the main effect and the proportion of cercariae established as the dependent variable, with shell length and total prior infections as covariates. The analysis found that the 10 clones did not differ in infection success ($F_{9,41} = 1.349$, $p = 0.243$), and that cockle size did not affect infection success ($F_{1,41} = 2.127$, $p = 0.152$). However, the number of pre-existing metacercariae in a cockle was found to have a marginally significant positive effect on the establishment success of new metacercariae ($F_{1,41} = 4.090$, $p = 0.0497$).

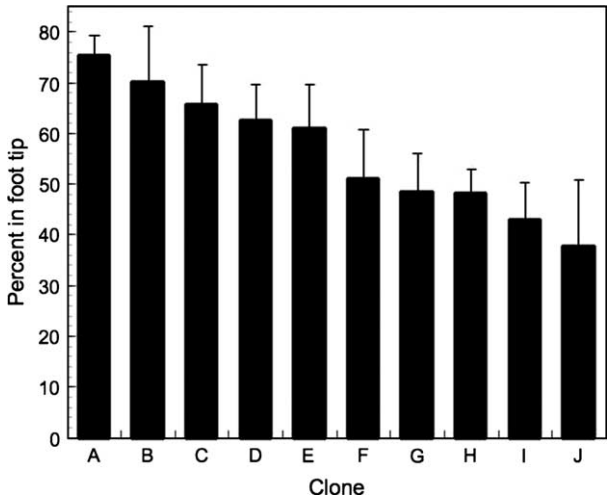

Figure 2. Mean (±SE) percentage of experimentally-acquired cercariae of *Curtuteria australis* that encysted in the foot tip of cockles, for each of 10 different clones. Mean values are computed across all cockles (4 to 8) used for each clone.

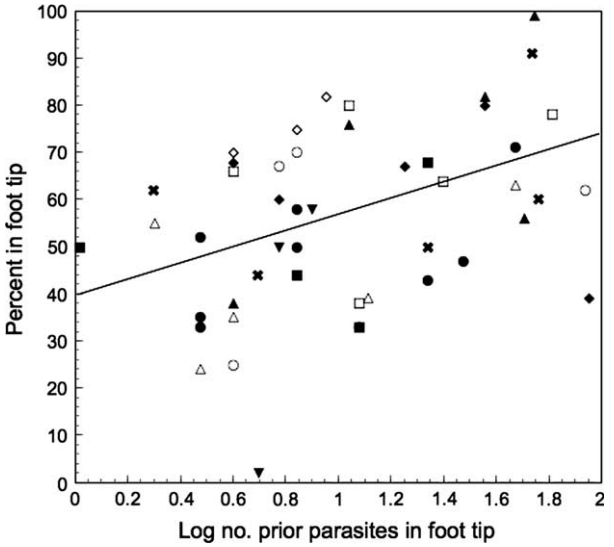

Figure 3. Percentage of experimentally-acquired cercariae of *Curtuteria australis* that encysted in the foot tip of cockles as a function of the number of metacercariae already present at the foot tip, across 49 cockles. Cockles infected by each of the 10 parasite clones are indicated by different symbols. The line is that given by a linear regression ($r^2 = 0.157$).

Among successfully established cercariae acquired during the experiment by the 49 cockles that did pick up new parasites, an overall mean (±SE) of 55.9±2.8% became established in the tip of the foot, whereas 23.2±2.5% settled at the base of the foot. The proportion of cercariae settling at the foot tip showed a two-fold variation among the 10 different clones, ranging from 38 to 76% (Fig. 2); this variation was not quite significant, however.

The number of pre-existing metacercariae in the foot tip ranged from 1 to 90 per cockle, with a geometric mean (±SE) of 10.8±1.2. On average, there were about two times more pre-existing metacercariae in the foot tip than at the base, and the numbers in both parts of the foot are positively correlated with each other across all cockles ($n = 53$, $r = 0.823$, $p = 0.0001$). Cockle shell length did not correlate with the proportion of pre-existing metacercariae that were encysted in the foot tip ($n = 53$, $r = -0.141$, $p = 0.312$). Importantly, among the 21 cockles harbouring at least 30 pre-existing metacercariae, the relative numbers of metacercariae in the tip and at the base were homogeneous (replicated test of goodness-of-fit, $G_H = 22.12$, $DF = 20$, $p > 0.10$). In other words, the distribution of metacercariae between the tip and the base of the foot showed no significant inconsistency among cockles; the cockles used were therefore comparable with respect to their susceptibility to infections in different parts of the foot.

These prior infections can nevertheless affect site selection by incoming cercariae. To control for this, we performed an ANCOVA using clonal identity as the main effect, the percentage of experimentally-acquired metacercariae in the foot tip as the dependent variable, and three covariates: the number of prior infections in the foot tip, the number of prior infections at the base of the foot, and cockle shell length (two-way interactions were non-significant and are not reported here). This analysis found no significant difference among the clones in terms of their preference for encysting at the foot tip ($F_{9,36} = 1.923$, $p = 0.0798$). Cockle shell length ($F_{1,36} = 0.324$, $p = 0.573$) and the number of prior infections at the base of the foot ($F_{1,36} = 1.667$, $p = 0.205$) also did not covary with the percentage of experimentally-acquired metacercariae ending up in the foot tip. However, the number of metacercariae already encysted at the tip was found to have a positive effect on the proportion of newly arriving cercariae opting to encyst at the tip of the foot ($F_{1,36} = 5.344$, $p = 0.0266$) (Fig. 3). A similar ANCOVA testing for factors influencing the percentage of cercariae opting to encyst at the base of the foot, showed that neither clonal identity ($F_{9,36} = 0.795$, $p = 0.623$), prior infections in the base of the foot (range, 0 to 83 per cockle; $F_{1,36} = 0.045$, $p = 0.833$), cockle shell length ($F_{1,36} = 0.032$, $p = 0.859$), nor the number of prior infections in the foot tip ($F_{1,36} = 0.736$, $p = 0.397$) affected the proportion of new metacercariae encysting in that 'safe' part of the foot; all two-way interactions were non-significant.

Finally, we looked for correlations among clones between infection success and the proportion of metacercariae that encysted either at the foot tip or at the base; for these, we used clonal mean values computed across all cockles that received particular clones. We found no relationship across the 10 clones between infection success and the proportion of new metacercariae that encysted in either the tip (Pearson product-moment correlation: $r = -0.218$, $p = 0.546$) or the base ($r = -0.126$, $p = 0.729$) of the cockle's foot.

Discussion

Based on where they encyst in their cockle host, individual *Curtuteria australis* can either cooperate with other manipulators and contribute to enhanced transmission though

incurring a significant risk of death from ingestion by fish, or cheat by opting for a safer location where they nevertheless reap the benefits of manipulation at no cost. Our findings point toward weak genetic and strong environmental influences acting on the parasite's transmission ecology and choice of strategy.

We found no evidence of genetically-based differences in infection success among clones, although such differences have been reported for other trematode species (Seppälä et al. 2007). There was also no relationship among the 10 clones between infection success and the tendency to encyst either near the tip or the base of the cockle's foot, i.e. no tradeoff between infection success and the tendency to either manipulate the host or cheat. However, in contrast to what would be expected from density-dependent competitive exclusion, cockles with higher numbers of pre-existing metacercariae were slightly more susceptible to further infections. Since pre-existing metacercariae did not impede newly arrived cercariae from settling in the cockle's foot, we can assume that, at least at the infection levels seen in this experiment, there was no saturation of the foot tissue with metacercariae. Indeed, wild cockles are known to accumulate several hundred to more than a thousand metacercariae in their foot (Poulin et al. 2000, Leung and Poulin 2007a). Uninfected cockles of a size suitable for infection cannot be obtained from the wild or from commercial providers, and thus cockles harbouring prior infections had to be used in this experiment. However, given the low levels of prior infections in those cockles, this did not prevent further experimental infections and allowed to evaluate the influence of pre-existing parasites on incoming ones.

Among the 10 clones used in this study, the proportion of individual parasites becoming manipulators (i.e. encysting in the foot tip) ranged from 38 to 76%. Inter-clone variation was not statistically significant, however; thus, we found no evidence of differences in the genetic tendencies of *C. australis* individuals to either opt for manipulation or cheating, and none of the clones investigated here could be classified either as a specialist manipulator or an absolute cheater. Given that the difference among the clones in terms of their preference for encysting at the foot tip was not far from statistically significant ($p = 0.0798$), including more clones in the analyses may have revealed the expected genetic variation.

More intriguing was the finding that, contrary to expectation, the presence of numerous metacercariae at the tip of the foot did not result in a greater proportion of newly arriving cercariae opting for the less risky option of encysting at the base of the foot, where they would be safe from fish croppers. We expected that cercariae entering a cockle already heavily parasitised might opt to encyst at the base of the foot and let the pre-existing metacercariae incur the risk associated with manipulation. If observed, this pattern could also have been explained by space saturation in the foot tip, had numbers of metacercariae been sufficiently high. Instead, in our experiment the presence of manipulators appeared to encourage new arrivals to also become manipulators. Thus the likelihood of a *C. australis* cercaria becoming a manipulator may be to some extent an intensity-dependent response: the more metacercariae are already committed to manipulation, the more likely new arrivals will join in. A proximate cue is necessary for newly-arrived cercariae to know how many pre-existing parasites are already encysted in a particular part of the foot; this cue may well be the relative abundance of scars or pits left by previous parasites on the surface of the foot, detectable by a cercaria as it crawls over the foot surface prior to penetrating it.

The effectiveness of the manipulation effort is related to the number of metacercariae encysted at the foot tip: burrowing becomes impossible only when infection intensity reaches a certain threshold (Thomas and Poulin 1998, Mouritsen 2002). For cockles of the size used in our experiment, approximately 100–150 metacercariae in the foot tip would be necessary for impaired burrowing. Thus, in this context, regardless of the risk of fish cropping, the closer the number of manipulators is to this threshold, the more beneficial it may be for new cercariae to join in and cooperate with earlier arrivals to quickly achieve impaired burrowing of the host. If, on the other hand, the cockle is only lightly infected, the best option may be to encyst at the base of the foot, and wait for the slow accumulation of enough metacercariae at the foot tip to benefit from increased vulnerability to bird predation. In cockles harbouring very few (25 or less) metacercariae in the tip of the foot, there are nevertheless about 40% of newly arriving parasites opting to encyst there; thus, the accumulation of metacercariae in the foot tip will proceed even in a previously uninfected cockle, but the accumulation rate will increase disproportionately as the intensity of infection increases.

One aspect of our study at first appears paradoxical: assuming that parasites follow the strategy already adopted by others, cercariae in the second experimental infection wave should have been more likely to encyst in the foot tip than those in the first wave, since the greater numbers of metacercariae in the foot tip should have reinforced the preference for that location. However, one has to consider the absolute numbers involved. Of the 30 cercariae per cockle used in the first infection wave, only 20% on average successfully infected the host, with 58% of those encysting in the foot tip; this means that, on average, the first wave of infection only added 3–4 metacercariae to the foot tip of each cockle, a number insufficient to alter the encystment decisions of individuals in the second wave.

There are at least four alternative explanations for the positive effect of pre-existing metacercariae on the location of further infections, three involving simple proximate mechanisms and the fourth involving an adaptive scenario. First, as cockles grow larger and become more heavily infected, they may, for whatever reasons, accumulate a disproportionate number of new infections at the tip of the foot. Our analyses, however, show that cockle size does not correlate with the proportion of pre-existing metacercariae that occur in the foot tip. Second, there could exist a polymorphism of susceptibility in the cockle population, with infection in the tip of the foot being easy in some hosts but much more difficult in others. However, the relative distribution of pre-existing metacercariae was homogeneous among cockles in our study, suggesting that there are no differences in susceptibility to infection in

particular tissues among hosts. Third, a greater number of *C. australis* metacercariae at the foot tip could somehow weaken or scar the epidermis, therefore facilitating the penetration of subsequent metacercariae in that section of the foot. Fourth, if trematode cercariae infect bivalves via passive processes (de Montaudouin et al. 1998), it may be that newly-arriving parasites do not actively opt to join pre-existing parasites at the foot tip, but instead the pre-existing parasites may hijack the new arrivals and increase their likelihood of encysting in the foot tip by excessive scarring of the foot epidermis. This alternative hypothesis has an adaptive basis, as it would speed up the accumulation of parasites at the foot tip such that the threshold number for effective manipulation is reached sooner. However, assuming that the tegument structure of the foot is uniform along its length, if pre-existing infections predisposed a part of the foot to further infection, as postulated in both the third and fourth explanations above, it should also apply to the base of the foot, but it did not. The earlier adaptive scenario we proposed thus appears reasonable.

Whereas many cases of density-dependent cooperative behaviour are based upon aggregations composed entirely of kin (Saul-Gershenz and Millar 2006), the apparent cooperation exhibited by *C. australis* in this study is more likely to rely on other mechanisms. Like other trematode systems in which large numbers of metacercariae accumulate in second intermediate hosts, the metacercariae that infect cockles are accumulated over a relatively long period of time (Leung and Poulin 2007b) from a number of different first intermediate hosts (Rauch et al. 2005, Keeney et al. 2007, Leung et al. 2009). Therefore, the population of conspecifics found within a cockle is likely to consist mostly of different genotypes. Our experimental design may have generated slightly greater numbers of clones per cockle than those seen in nature; however, given that these parasites have evolved in situations where other parasites sharing their host are mainly non-clones, and in the absence of known kin recognition mechanisms, it is likely that kin selection has not played a significant role in this system. Thus, in *C. australis*, apparent cooperation for a common goal arises without close genetic relatedness. This is true because the direct benefits of cooperation for an individual increase as the number of cooperative parasites (manipulators) increases. The payoff (transmission) can only be realised if the number of participants involved reaches a critical mass.

Our findings not only provide clear evidence that external influences, and not genetic effects, determine whether a particular parasite will opt to manipulate its host or not, but they also cast new light on the phenomenon of host manipulation. The results of this study demonstrate that not all individuals of what are often referred to as 'manipulator' species are necessarily manipulators of host behaviour. As in other situations, there can be a range of life history strategies that differ between individuals. A parasite population may contain certain individuals adopting a 'hitch-hiker' (Thomas et al. 1998) strategy that exploits the investments of true manipulators: these alternative strategies are not restricted to different species. Our manipulator-cheater system may also have dynamics like those of producer–scrounger models (Vickery et al. 1991) used to predict when and how many foragers should opt to exploit the food discoveries of others rather than searching for food themselves.

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