

Chemical communication and mate attraction in echinostomes

Sandrine Trouvé*¹, Christine Coustau

Centre de Biologie et d'Ecologie Tropicale et Méditerranéenne, Laboratoire de Biologie Animale, UMR 5555 du CNRS, Université de Perpignan, Av. de Villeneuve, 66860, Perpignan Cedex, France

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Abstract

Mate attraction is widespread among animals and appears to facilitate mating and to prevent hybridisation between closely related species. In this study we investigated mate preference between two geographical isolates of *Echinostoma caproni* (Trematoda, Platyhelminth) and another species of the genus *Echinostoma* *E. sp.* Because previous experiments showed a partial reproductive isolation between echinostome isolates, we examined the possibility that such isolation resulted from differential mate attraction. We compared intra-isolate, inter-isolate and interspecific pairings using two in vitro experimental designs. In the first experiment we compared mate attraction of two individuals belonging to or not belonging to the same isolate, while in the second experiment we examined mate choice when individuals were in the presence of individuals from both the same isolate and from a different isolate or a different species. Distances between worms were measured over a period of 90 min. Results from both experiments suggested that mate attraction was similar for intra-isolate, inter-isolate or interspecific combinations. This lack of mate preference in vitro would therefore support an alternative hypothesis of a reproductive isolation through sperm selection. © 1999 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

Mate preference is one of the important phenomena influencing patterns of genetic vari-

ation, and thus population genetic structure and speciation processes. Because of the prominent role of mate choice in population biology, numerous studies have attempted to determine how mating preference has evolved. Mating preference may provide both short-term and long-term benefits [1]. On the one hand, short-term benefits can be obtained by choosing a partner with high fecundity, high fertility, parental abilities or resources provided for mating. On the other hand, individuals may improve the quality of their offspring (long-term benefits) by choosing

* Corresponding author. Tel: +33-4-68-66-20-50; fax: +33-4-68-66-22-81

E-mail address: trouve@univ-perp.fr (S. Trouvé).

¹Present address: Institut d'Ecologie, Laboratoire de Zoologie et d'Ecologie Animale, Bâtiment de Biologie, Université de Lausanne, 1015 Lausanne, Switzerland. Phone: +41-21-692-41-63. Fax: +41-21-692-41-05. E-mail address: sandrine.trouve@ie-zea.unil.ch (S. Trouvé)

genetically superior mates (good gene hypothesis) or mates with an optimal degree of relatedness. Indeed, pairing with too closely related individuals might give rise to the expression of deleterious recessive mutations resulting in inbreeding depression [2]. In contrast, crosses between lines, populations or species might unfavourably reduce fitness of offspring because of disruption of co-adapted gene complexes [3].

Many parasitic species show a tendency to pair and cluster with each other, behaviours favoured by inter-individual chemical communications. These communications involve excretory–secretory (ES) products released through several pores (tegument, mouth, gonopore, excretory system [4, 5]). Although experimental evidence is lacking, some sensory papillae present on larval and adult trematodes might be involved in chemoreception. Such chemosensory signals may not only play a role in coordinating matings, reducing the cost of copulation (time and energy spent to find mate), enhancing inter-sex recognition, participating to intraspecific sexual stimulation [6] but may also function for species recognition.

In a previous work, the excretory–secretory (ES) products of a simultaneous hermaphroditic parasite *Echinostoma caproni* (Trematoda, Platyhelminth) were studied using SDS–PAGE analyses. Results showed that, although the polypeptide patterns from three isolates (i.e. coming from different geographic areas) shared most major bands, isolate-specific bands could be observed. This study also pointed out that a different species (*Echinostoma* sp.) presented the most distinctive pattern, as none of the major bands were shared with *E. caproni* isolates [7].

In coinfection of mice with two individuals from the same isolate and one from a second isolate, we found elsewhere that both related individuals shared very few outcrossed offspring with the unrelated one [8]. The assortative mating observed for these echinostome isolates may be an adaptive consequence of a postzygotic isolation. Indeed, fecundity of recombinant hybrids of two parental isolates is significantly lower than that of the mid-parent [9].

Considering the observation of distinctive ES products, higher intra-isolate cross-fertilisation and hybrid breakdown in these echinostomes, we hypothesise the existence of a mechanism facilitating pairings with partners from the same isolate. In the present work, we examined whether attraction between adult echinostomes was variable and correlated with the degree of genetic relatedness between individuals. This question has been addressed using two in vitro experimental designs. In the first experiment, we compared mate attraction of two individuals belonging either to the same isolate, different isolates or to different species. In the second experiment, we analysed mate choice when individuals were in the presence of individuals from the same isolate and individuals from a different isolate or a different species.

2. Materials and methods

2.1. Organisms

Echinostomes are simultaneous hermaphrodite Trematoda (Platyhelminth), parasitising vertebrate intestines. We considered two geographical isolates of *Echinostoma caproni*; originating, respectively, from Madagascar (Ec) and Egypt (E1). We also used another *Echinostoma* species, from Niger (*E. sp.*: [7]) and previously shown to differ greatly from the two *E. caproni* isolates by nuclear rDNA ITS sequencing [10]. The life cycle of echinostomes includes three successive hosts, and an asexual reproduction occurring in the first intermediate host. These parasites were cycled in our laboratory through *Biomphalaria pfeifferi* snails and mice (Swiss OF1 stock) for *E. caproni*, or *Bulinus globosus* snails and hamsters for *E. sp.*, according to previously described procedures [8].

For each experiment, 30 metacercariae obtained from laboratory infected snails were fed to mice or hamsters. Adult echinostomes were removed from the small intestine of rodents 25 days after infection. Previous observations have shown that multiple fertilisations easily occur during the life of the echinostomes [8]. Adult worms were collected and washed five times in

2 ml Locke's solution before use in the experiments.

2.2. Mate attraction: Experiment 1 (worm-to-worm attraction)

This experiment was designed to determine whether intra-isolate pairing occurs more readily than inter-isolate or interspecific pairing. The experiment was conducted simultaneously for three combinations: 2 Ec, 1 Ec and 1El, or 1 Ec and 1 *E. sp* individuals. To prevent any confounding variable related to the host of origin (similar vs different host) Ec individuals paired in the first combination were recovered from different mice. A total of 64 pairs, representing the three different combinations were tested: 22 Ec–Ec, 21 Ec–El and 21 Ec–*E. sp*. Two worms were placed 20 mm apart from each other in a Petri dish (6 cm diameter), with a 1% nutrient agar substratum and a 10-ml Locke's solution overlay [11]. Dishes were incubated at 38°C under atmospheric conditions. Lighting conditions were standardised to prevent potential left/right bias due to photo-attraction. Distances between worm centres were measured at 5 min intervals over 90 min. Worms were considered paired when they were in contact or within 5 mm of each other and did not show any movement in the opposite direction [12]. No mortality was observed at the end of the experiment.

Since our data did not meet assumptions of parametric analyses, we used non-parametric methods. We tested, with Friedman's method, whether worms of each pair were significantly attracted to each other throughout time. Distances between individuals among combinations were compared using the Kruskal–Wallis test.

For each combination we recorded the number of paired worms at 5 min intervals over 90 min. Therefore, we observed the 22 Ec–Ec combinations 396 times (22×18), the 21 Ec–El combinations 378 times (21×18) and the 21 Ec–*E. sp* combinations 378 times (21×18). The number of paired worms was compared among combinations using a chi-square test.

2.3. Mate choice: Experiment 2 (worm-to-chimney attraction)

Mate choice was examined in a barrier design (Fig. 1) where one mobile worm was in the presence of two sets of worms maintained in perforated chimneys. The tube chimneys were stuck into the 1% nutrient agar substratum and protruded above the Locke's solution overlay, allowing the passage of ES products. The potential mate preferences of Ec worms were analysed using two different combinations. One combination consisted in a single mobile Ec echinostome with chimneys containing Ec and El individuals, respectively. In the other combination the mobile Ec individual was in the presence of Ec and *E. sp* individuals (in chimneys). A total of 62 repetitions, representing both combinations were tested, 31 Ec–Ec–El and 31 Ec–Ec–*E. sp*. In order to optimise diffusion of potential chemical signals, four Ec or El individuals were introduced into the chimneys [13]. Because *E. sp* adults are twice as big as *E. caproni* worms, only two *E. sp* worms were placed in a chimney. As previously, the mobile Ec individuals were recovered from different mice than those placed in chimneys. At the beginning of the experiment,

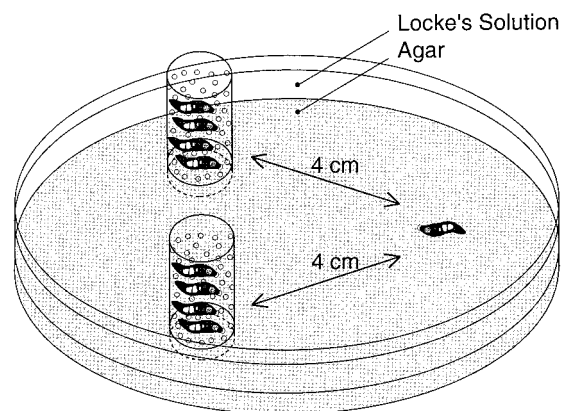


Fig. 1. Petri dish chamber used in the barrier design of experiment 2. This design consists of two perforated chimneys prepared with sections of eppendorf (1.5 ml) of 10 mm high and punctured with common pins. The chimneys were placed 40 mm apart from each other in petri dishes (6 cm diameter), stuck into the 1% nutrient agar substratum and protruded above the Locke's solution overlay.

the mobile *Ec* adult was placed at 40 mm from each chimney (Fig. 1). Dishes were incubated at 38°C under atmospheric conditions. Distances between the mobile worms and each chimney were measured at 7-min intervals over 90 min. Worms were considered 'paired' with a chimney when they were in contact with or within 5 mm of it and did not show any movement in the opposite direction [12]. No mortality was observed at the end of the experiment.

Since our data were not normally distributed, the distance between the mobile worm and the two chimneys was compared at each time period using a sign test. Then, within each combination, an *Ec*'s preference score was calculated as the number of times the *Ec* individual was observed near the native chimney (*Ec*) minus the number of times it was observed near the foreign chimney (either *El* or *E. sp*). Scores could range from -13 to +13 (the number of observations was 13). A diagnosis of choice for each combination was made if the average score was significantly different from zero using two-tailed *t*-tests [14]; these data were normally distributed.

3. Results

3.1. Mate attraction: Experiment 1 (worm-to-worm attraction)

Each combination (*Ec*-*Ec*, *Ec*-*El* or *Ec*-*E. sp*) resulted in a significant attraction between worms (Friedman's method: $P < 0.001$ for each combination). At the beginning of the observation period adult echinostomes were at 20 mm apart from each other and at the end the distance between them averaged only 9 mm (Fig. 2). The first worm contact or non-contact pairing was observed 20, 20 and 10 min after the beginning of the experiment for *Ec*-*Ec*, *Ec*-*El* and *Ec*-*E. sp*, respectively. At each time, 10 to 45% of the worms were paired for any combination considered.

At each time period there was no significant difference among the three combinations in the distance separating individuals (Kruskal-Wallis test: $P > 0.05$ for each comparison). Throughout

time, movements of the echinostomes were similar for the combinations considered (intra-isolate, inter-isolate or interspecific), indicating that the individuals of a pair, originating from the same area or not, are equally attracted to each other.

The number of pairings observed were 85/396 for the *Ec*-*Ec* combination, 83/378 for the *Ec*-*El* one and 108/378 for the *Ec*-*E. sp* one. Striking differences arose in the number of pairings when *Ec*-*E. sp* was compared to *Ec*-*Ec* and to *Ec*-*El* (chi-square: $P < 0.05$ for both comparisons): the partners *Ec*-*E. sp* were observed more often in contact with each other. In contrast no significant difference appeared between the pairing behaviour of the *Ec*-*Ec* and *Ec*-*El* pairs (chi-square: $P = 0.71$).

3.2. Mate choice: Experiment 2 (worm-to-chimney attraction)

For the *Ec*-*Ec*-*El* combination, the first 'pairing' of the *Ec* worm to the *Ec* chimney was observed within 14 min following worm exposure and the first 'pairing' to the *El* chimney was

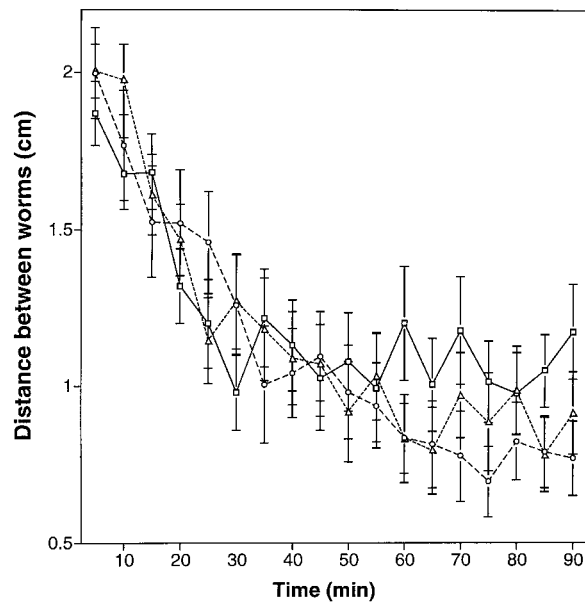


Fig. 2. Distances between worm pairs (mean \pm 1 S.E.). Squares: pair *Ec*-*Ec*, triangles: pair *Ec*-*El* and circles: pair *Ec*-*Echinostoma sp.*

observed within 21 min. For the Ec–Ec–*E. sp* combination, the first Ec worm ‘paired’ to the Ec chimney 42 min after the beginning of the experiment and to the *E. sp* chimney after 35 min.

For the Ec–Ec–El combination, 0–6.5% of Ec worms were ‘paired’ to the Ec chimney and 0 to 9.5% were ‘paired’ to the El chimney at each time period. For the Ec–Ec–*E. sp* combination, 0 to 12.9% of Ec worms were ‘paired’ to the Ec or the *E. sp* chimney at each time period.

At each time period, the distances between the free-worm and one or other chimney were compared (Fig. 3). The difference was not significant for either the combination Ec–Ec–El or for the Ec–Ec–*E. sp* one (sign test: $P > 0.05$ for each comparison).

If we consider the number of times the free-worm was observed in contact, or within 5 mm, of one or other chimney, no significant difference appears. In both combinations, the Ec worm spent approximately the same time next to one

or other chimney (for both combinations: t -test: $P > 0.05$).

4. Discussion

In laboratory conditions, adult digeneans are usually found paired or clustered in the definitive host and are rarely isolated [4]. Adult *E. caproni* were shown to be localised exclusively in a restricted portion of the mouse ileum, either paired or clustered [8]. To eliminate definitive host-mediated factors potentially interfering with worm attraction and migration, in the present study we examined pairing in vitro, on agar plates. Echinostome in vitro pairing was very similar to what we already observed in mice [8]: adult echinostomes are significantly attracted to each other and they tend to pair. This suggests that this pairing behaviour can occur in the absence of intestinal factors.

Results from the first experiment showed that, in the absence of possible choice of a partner, interspecific and inter-isolate attractions were as frequent as intra-isolate attractions. In all cases, attraction was clearly a function of time, with most pairings occurring within 35 min. These data are consistent with a previous report on the absence of habitat segregation within the host intestine for two different isolates of *E. caproni* (Ec and El), meaning that these parasites do not repel each other [8]. This tendency to cluster, between adult echinostomes may represent a beneficial behavioural adaptation leading to nutritive, social or developmental advantages [4]. However, the pairs were not formed randomly, as Ec–*E. sp* pairs were observed more often than Ec–Ec or Ec–El pairs. It is possible that the larger size of *E. sp* individuals induce an intrinsic rate of movement greater in this species. However, this higher velocity would imply more rapid pairing for this couple, which is not the case (Kruskal–Wallis test: $P > 0.05$ for each comparison). In addition, the larger size of *E. sp* probably results in a higher production of excretory–secretory (ES) products. Although ES polypeptides secreted by *E. sp* are known to be different from those of *E. caproni* species [7], the

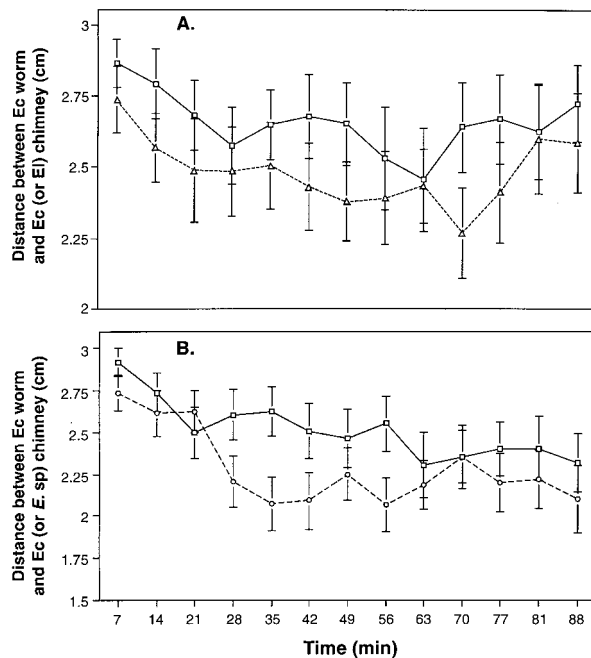


Fig. 3. (A) Distances (cm) between Ec worm (mean \pm 1 S.E.) and Ec (squares) or El (triangles) chimney. (B) Distances (cm) between Ec worm (mean \pm 1 S.E.) and Ec (squares) or *Echinostoma sp* (circles) chimney.

attracting molecules may be other than polypeptidic and may be functionally close for these two species. In order to test for this apparent inter-specific preference we carried out the second experiment.

In the choice design, *Ec* flukes showed no significant preference for either intra-isolate, inter-isolate or inter-specific partners (in chimneys). Indeed, no difference in attraction as well as no difference in the number of pairings was observed. This result differs from another observation where echinostomes have shown consistent patterns of choice behaviour. In that case intra-specific attractions of either *Echinostoma trivolvis* or *E. caproni* were significantly greater than inter-specific attractions [11].

Other studies on mate choice in helminths brought mixed evidence, for both the occurrence, or absence of choice. When associated with *Zygodotyle lunata*, in vitro intraspecific attraction of *E. trivolvis* is greater than interspecific attraction. In contrast, *Z. lunata* does not exhibit a greater intraspecific than interspecific attraction [15]. Similarly, when associated with *Haematoloechus medioplexus*, *E. trivolvis* presents the same intraspecific than interspecific attraction, while *H. medioplexus* presents a lower intraspecific than interspecific attraction [16]. There is no evidence that schistosomes discriminate between native and foreign partners since *Schistosoma mansoni*, *Schistosoma japonicum* and *Schistosoma haematobium* showed equivalent intraspecific and interspecific attraction [17]. In the absence of choice of mate, *S. mansoni* and *S. intercalatum* do not exercise any preference and heterologous pairings occur in vivo. However, when choice is possible, matings occur preferentially between conspecifics [18–20]. Among nematodes, although *Nippostrongylus brasiliensis* worms pair infrequently and transiently in vitro to *Strongyloides ratti* (parthenogenetic female), they pair as readily with *Heligmosomoides polygyrus* and *Trichinella spiralis* as they do with conspecific partners [21].

Our results indicate a lack of attraction preference in *E. caproni*. However, a previous genetic study showed that when mice were infected with two individuals from the same isolate and one

from another, intra-isolate cross-fertilisation was higher than the inter-isolate one [8]. Although these results appear contradictory, they can be explained by three alternative hypotheses. First, the preferential intra-isolate cross-fertilisation could be interpreted as postzygotic incompatibility between the two isolates. However a previous work with no mate choice [22] compared the inter-isolate and intra-isolate outcrossing rate and did not show a significant difference. This means that there is no postzygotic incompatibility. Second, adult echinostomes can be attracted to each other in a non-specific way as was observed here, but they may avoid crossing with individuals from a different isolate or species. Nollen's [23] observations are consistent with this hypothesis. Although *E. caproni* and *Echinostoma paraensei* partially share the same habitat in mice intestine, there is no cross-insemination between these two species [23]. However, in the case of co-infection with two *Ec* and one *El*, although *Ec* individuals produce most of their offspring via intra-isolate outcrossing, a small proportion of their offspring are produced via inter-isolate outcrossing [8]. In concert with the results of the present study, this suggests that attraction, pairing and sperm transfer take place. Thus the third hypothesis is that a post-copulatory prezygotic isolation mechanism occurs. This process, called sperm selection, can be regarded as inter-male competition, in which ejaculates from different males compete for access to a female's eggs [24], or as a real female choice [25, 26] involving the behaviour of females, blockage of sperm movement [27, 28], sperm digestion [29] or sperm precedence [30, 31]. Finally, sperm selection can also result from sperm-transport difficulties or divergence in gamete recognition systems. These qualitative differences vary from one individual to another and from one population to another [32]. Although sperm competition has been largely investigated during the last decade over a wide range of taxa, to our knowledge no study has been conducted to date on platyhelminth parasites. Such studies would be of great importance in providing conclusive evidence for postcopulatory and prezygotic isolation in such organisms.

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