Morphological and behavioural sex reversal in mermithid-infected mayflies

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SUMMARY

This study reports the impact of infection by the mermithid nematode *Gasteromermis* sp. on the secondary sexual morphology and behaviour of its adult mayfly host *Baetis bicaudatus*. By applying the technique of flow cytometry I show that both male and female mayflies are infected by the parasite. The external secondary sexual characters of infected male hosts are feminized resulting in the formation of intersexes and it is possible that some infected males undergo complete sex reversal. The external morphology of infected female hosts is not altered. The behaviour of infected hosts is also affected. Infected mayflies join unparasitized females in upstream dispersal and ovipositing behaviour. I suggest that these alterations are induced by the parasite as an adaptive strategy that ensures: (i) upstream dispersal; (ii) return to water for completion of parasite life cycle; and (iii) maximum use of host nutrients.

1. INTRODUCTION

Parasites and pathogens commonly interfere with the reproductive systems of their invertebrate hosts (Hurd 1993). These disturbances can range from a slight reduction in fecundity (Junnikkala 1985; Watson & Petersen 1993) to a complete destruction of reproductive tissues (castration) and irreversible alterations in external appearance (Baudoin 1975; Amat *et al.* 1991). Host mating (Møller 1993; McLennan & Shires 1995), dispersal (Lundberg & Svensson 1975; Kaya *et al.* 1979) and oviposition behaviour (Undeen & Nolan 1977; Tomalak *et al.* 1990) may also be affected.

Mermithid nematodes are well known for their interference with insect host reproduction (Wülker 1975; Popiel & Hominick 1992). Mermithids are exceptional among parasites in that they commonly result in the formation of intersexes in their hosts (individuals exhibiting features intermediate between the male and female sex) (Wülker 1975). In addition to creating taxonomic difficulties (Rubstov 1958), the presence of intersexes makes interpretations about the parasite: host interaction difficult because uncertainty remains over the genetic sex of infected individuals. Intersex individuals may be masculinized females (Rempel 1940; Soldán & Landa 1981) or feminized males (Wülker 1961; Baudoin 1975; Hubschman & Stack 1992). The sex of mermithid-induced intersexes has been cause for debate for over 90 years (Wheeler 1904).

I report the effects of infection by the mermithid nematode *Gasteromermis* sp. Micoletzky (Nematoda: Mermithidae) on the adult mayfly host *Baetis bicaudatus* Dodds (Ephemeroptera: Baetidae). The study represents the first re-examination of the long-standing debate about the original sex of intersexes using the modern technique of flow cytometry. The genetic analysis is combined with morphological descriptions and behavioural field studies to document the impact of mermithid parasitism on male and female mayfly secondary sexual characters and secondary sexual behaviour.

2. STUDY ANIMALS

Specimens of the winged adult *B. bicaudatus* were collected from a third-order section of the East River, Gunnison County, Colorado using emergence traps (Peckarsky & Cowan 1991). *B. bicaudatus* is commonly infected with *Gasteromermis* in this region with infection rates ranging from 1–70 % (Vance & Peckarsky 1996). *Gasteromermis* mermithids parasitize the early instars of the aquatic *B. bicaudatus* host by penetrating the insect integument. They absorb nutrients from the haemocoel and continue to grow until the host has completed its nymphal development. Once the host has moulted to an adult, the large parasite emerges from and kills the mayfly. The life cycle of the mermithid is then completed in the stream bed (Hominick & Welch 1980; Popiel & Hominick 1992; Vance & Peckarsky 1996). Mayflies are unique among the extant insects in having a sub-adult (subimago) stage after nymphal development that moults to the full adult (imago) in 24–48 h (Edmunds & McCafferty 1988).

Mayflies collected from the East River in emergence traps were used for morphological descriptions of the impact of the parasite on the host, genetic analysis of the sex of unparasitized and parasitized mayflies and behavioural observations of parasitized and unparasitized mayflies under controlled conditions.

3. INTERSEX FORMATION IN PARASITIZED INDIVIDUALS

Unparasitized male mayflies possess a pair of gonopods (or 'claspers') on the ninth abdominal tergite typical of the Ephemeroptera (Needham *et al.*

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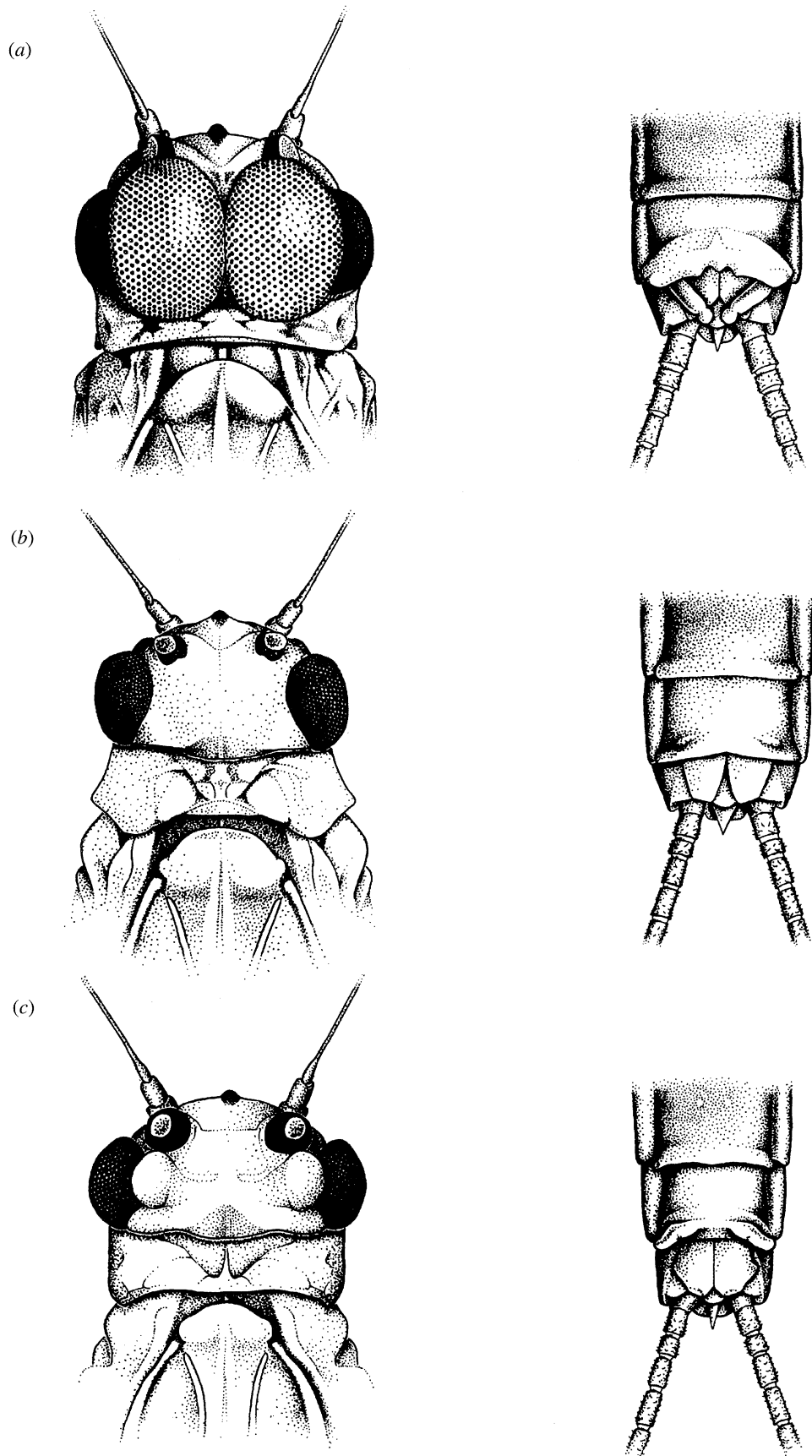*Printed in Great Britain*

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(a)

(b)

(c)

Figure 1. Dorsal view of the head and ventral view of the external genitalia of: (a) unparasitized male; (b) unparasitized female; and (c) parasitized intersex imagoes of *B. bicaudatus*.

Table 1. *The morphological appearance of the external secondary sexual characters of unparasitized and parasitized* B. bicaudatus *imagoes*

(No external abnormalities were found in unparasitized male and female mayflies. Parasitized mayflies looked either 'female' or showed an array of intersex states in the appearance of host eyes and genitalia.)

	unparasitized imagoes	parasitized imagoes
male eyes & male genitalia	518	0
female eyes & female genitalia	490	82
female eyes & intersex genitalia	0	6
male eyes & intersex genitalia	0	6
intersex eyes & female genitalia	0	18
intersex eyes & male genitalia	0	5
intersex eyes & intersex genitalia	0	9
total	1008	126

1935; figure 1). Claspers are absent in the female (see figure 1). Males can also be readily distinguished from females by the presence of large turbinate eyes (Needham *et al.* 1935; figure 1). A total of 1134 imago individuals were preserved in 70% alcohol and dissected under a dissecting microscope. The state of external morphology, internal reproductive tissues and presence of nematodes were recorded for all mayflies to document the extent of abnormalities in parasitized individuals.

Of the 1134 total, 1008 were unparasitized and 126 parasitized (each by a single mermithid nematode). All unparasitized females were gravid ($n = 490$), and all unparasitized males had visible testes ($n = 518$). In addition no external morphological abnormalities were observed in unparasitized individuals.

All parasitized individuals ($n = 126$) contained neither visible eggs, ovaries nor testes. In addition the external morphology of the parasitized mayflies was often altered. Of the 126, 82 (or 65%) possessed eyes and genitalia indistinguishable from those of unparasitized females. No parasitized individuals showed complete male sexual characters. The remaining 44 parasitized individuals showed an array of intersex morphologies (see table 1). External genitalia of intersexes (figure 1) were smaller and had less complex claspers than the unparasitized males. Intersex eyes (figure 1) were considerably reduced in size compared with the male state (one half to one eighth of normal size), sometimes with altered colour (white instead of red in 14 cases).

4. SEX OF PARASITIZED INDIVIDUALS

Flow cytometry can be used to determine the genetic sex of animals based on differences in the genome size between the two sexes (Tiersch *et al.* 1989; Marescalchi *et al.* 1990). In this study this technique was used to measure the DNA content of 10 000 individual nuclei from each individual mayfly (unparasitized male, unparasitized female and parasitized individuals of unknown sex). The average amount of DNA per
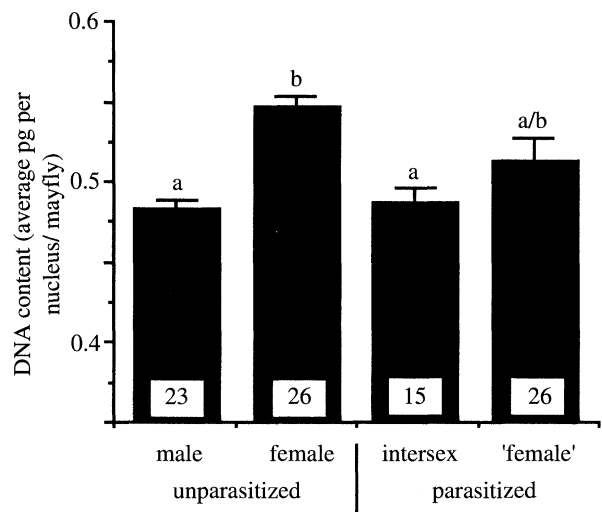
Figure 2. DNA content (average pg per nucleus/ mayfly) for unparasitized male, unparasitized female, parasitized intersex and parasitized 'female' imagoes of *B. bicaudatus*. Data are shown as mean ± 1 s.e. with sample sizes at the base of each column. Statistical differences (ANOVA, $p < 0.05$) are indicated by differing letters.

nucleus for each mayfly was then given by the cytometer. To correct for variation that occurs between samples, the standard practice of adding an internal standard (sample of known DNA content) to every sample was followed so that accurate comparisons could be done between samples (see Ormerod 1994 for more details of flow cytometry techniques). I then compared the genome size of male and female unparasitized adult *B. bicaudatus* with those of parasitized individuals to determine the genetic sex of the infected individuals.

Mayflies were collected as described above and shipped live to Ithaca, New York where they were placed in an ultralow (-80 °C) freezer. One mayfly at a time was removed, and the state of its external morphology, internal reproductive tissues and presence of nematodes were recorded. Any parasites were removed and the mayfly was transferred to a 4 °C solution containing an internal genome-size standard of 10 μm of a 1% PBS suspension of chicken red blood cells (obtained from Cornell University Poultry & Avian Sciences Research Program). To this a 0.5 ml solution containing 25 μg propidium iodide, 0.1% sodium citrate, 25 μl RNAase in buffer (1 mg ml^{-1}), and 0.1% Triton X-100 was added and the mayfly was minced with a scalpel. After filtration through a 41 μm nylon mesh samples were run on an Epics Profile flow cytometer (Coulter Cytometry, Hialeah, Florida) with a 15 mW argon ion laser to produce a 457, 488 and 514 nm wavelength excitation beam. Fluorescence was measured through a 457–514 nm blocking filter as well as 550 and 590 nm long pass filters. Fluorescence values were converted to picograms by multiplying by 2.5 picograms, the chicken red blood cell genome size (Tiersch *et al.* 1989).

A total of 23 unparasitized male, 26 unparasitized female, 15 parasitized intersex and 26 parasitized 'female' mayflies were analysed in this way.

The genome size (DNA content) of unparasitized male and female mayfly nuclei were distinct from each other (figure 2). Mean DNA content (pg/mayfly nucleus) for unparasitized males = 0.483, s.e. = 0.006, $n = 23$; for unparasitized females, mean = 0.547, s.e. = 0.006, $n = 26$. Parasitized individuals that showed intersex morphology were statistically indistinguishable from unparasitized male mayflies (figure 2) (mean = 0.487, s.e. = 0.01, $n = 15$). Parasitized individuals that showed 'female' morphology were statistically indistinguishable from either unparasitized male or female mayflies (figure 2) (mean = 0.513, s.e. = 0.01, $n = 26$). An ANOVA on the four groups showed a significant differences in sex (DNA content (pg) per mayfly nucleus) between groups, $F_{3,89} = 13.32$, $p < 0.01$. Contrast among groups, unparasitized male versus unparasitized female Scheffe F-test = 11.11, $p < 0.05$; unparasitized male versus parasitized intersex Scheffe F-test = 0.05, $p > 0.05$; unparasitized male versus parasitized 'female' Scheffe F-test = 2.53, $p > 0.05$; unparasitized female versus parasitized intersex Scheffe F-test = 7.4, $p < 0.05$; unparasitized female versus parasitized 'female' Scheffe F-test = 2.23, $p > 0.05$. This suggests that parasitized intersexes are genetically male, and that the group of parasitized individuals with 'female' phenotypes contains both genetically male and genetically female individuals.

5. SEXUAL BEHAVIOUR OF PARASITIZED INDIVIDUALS

The behaviour of unparasitized mayflies differs according to sex. Males form swarms near the river ranging from 20 to several hundred individuals and do not return to the water after they have emerged (Allan & Flecker 1989; Harker 1992; Savolainen *et al.* 1993). Mating occurs within or near to the swarm after which the females return to the river and oviposit on the submerged side of protruding rocks (Harker 1992). Before ovipositing the females of many species of mayfly (including *B. bicaudatus*) show a bias towards upstream flight which is not seen in males (Madsen *et al.* 1977; Flecker & Allan 1988). Upstream movement by females is believed to be an important mechanism by which the recolonization of upstream reaches and headwaters of rivers is achieved (Müller 1982; Hershey *et al.* 1993).

Field samples of male swarms were collected using sweep nets when swarms were formed in the early morning near the East River. Flight movements of *B. bicaudatus* were monitored by placing sticky traps across the river 2 m above the water surface. Sticky traps were made of clear acetate sheets (28 cm × 22 cm) sprayed on each side with Tanglefoot® and attached to a rope at their top and base. Ropes crossed the river in two places 100 m apart. Each crossing had eight sticky traps. Mayflies were removed from the traps daily and I noted whether the insect had flown into the traps from an upstream or downstream direction. Individual rocks in the East River were rolled over in the early morning (and later replaced) to expose females in the process of ovipositing. The state of external morphology, internal reproductive tissues and presence of

Table 2. *Behaviour of unparasitized and parasitized* B. bicaudatus *imagoes*

(Parasitized individuals (both genetically male and female) adopt female dispersal and ovipositing behaviours.)

	unparasitized		parasitized		total
	male	female	'female'	intersex	
swarming	418	0	0	0	418
downstream flight	26	29	2	2	59
upstream flight	22	69	11	6	108
ovipositing behaviour	0	43	5	3	51

nematodes were recorded for all mayflies from the above collections.

Of the 418 swarming adults, all were unparasitized males with fully developed eyes, claspers and testes (table 2). Sticky traps suggested that unparasitized females and also parasitized mayflies showed a bias for upstream flight (table 2). This upstream movement was not apparent in unparasitized males (table 2). Chi-squared of numbers flying upstream versus downstream according to male, female or parasitized = 11.3, 2 d.f., $p < 0.05$.

Unparasitized gravid females were most commonly found ovipositing on protruding rocks (table 2). No males were found in these samples. In addition to females, however, parasitized mayflies that were female in appearance and parasitized mayflies that were intersexes were also found 'ovipositing'. Thus it appears that genetically female and genetically male mayflies that are parasitized by *Gasteromermis* undertake female flight patterns and adopt mock ovipositing behaviours.

Laboratory studies confirmed many of these patterns. Mayflies collected in emergence traps (described above) were kept in 18 cm × 28 cm enclosed plastic cages containing gravel, river water (changed daily) and a protruding stone. Parasitized individuals (16 that looked 'female', and nine that were intersexes) became very agitated shortly after moulting to the imago. Within 3–6 h all parasitized mayflies had crawled into the water down the side of the rock. The mermithid could then clearly be seen escaping through a puncture wound in the mayfly's abdomen. Once free of the host the mermithid would burrow into the gravel at the bottom of the chamber. The mayfly was killed by the emergence of the nematode. Unparasitized male ($n = 20$) and unparasitized female mayflies ($n = 20$) used as controls did not exhibit any of these behaviours and lived for 3 d as adults. These observations suggest that the 'ovipositing' behaviour of parasitized mayflies (both genetic males and females) results in the return of the mermithid to water.

6. DISCUSSION

Infections by mermithid nematodes have been reported to produce a confusing array of alterations in their insect hosts (Titmus & Badcock 1981; Wülker 1985; Hubschman & Stack 1992). Some of this

confusion stems from the uncertainty over the genetic sex of the host since genetic analyses of the sex of parasitized individuals have rarely been done (Wülker 1961). The only previous report of mermithid-induced alterations in adult mayflies hypothesizes that inter-sexes are masculinized females (Soldán & Landa 1981). In other host taxa infected with mermithid nematodes, studies have been interpreted to both support this hypothesis (Rempel 1940), and also to suggest that infected individuals are instead feminized males (Hubschman & Stack 1992). This study is the first to apply modern genetic techniques to this long-standing debate. Results from my analysis support the suggestion that intersexes are feminized males. Further, the demonstration in this study that parasitized individuals that appear 'female' cannot be genetically distinguished as being either male or female suggests that there may be both male and females in this group. If *Gasteromermis* is able to cause complete sex reversal in some genetic males as is suggested by this result, it should prompt further investigations into other parasite systems where morphology alone as been used to determine the sex of infected individuals. That para-sites are able to cause complete sex reversal has rarely been reported (Rasmussen 1959; Orr 1966) and may be a result of this confusion rather than a true indicator of the frequency of sex reversal.

The alterations observed in this study are not apparent in earlier stages of infection (Vance & Peckarsky 1996). This observation combined with the large sample size examined in this present study leads me to suggest that feminization of infected male *B. bicaudatus* hosts is a parasite-mediated alteration. I further hypothesize that the alterations increase the fitness of the parasite in three ways. First, the adult flight activity of aquatic hosts is believed to be an important source of upstream dispersal for mermithid parasites (Colbo & Porter 1980; Molloy 1981). The normal behaviour of male mayflies does not result in any net upstream movement. In contrast, the flight direction of female mayflies is strongly directional. Female flight, therefore, would provide the mermithid with considerable upstream dispersal. Second, mermi-thids need to return to water to complete their lifecycle. As males swarm over land, the mermithids would not be returned to the river. The normal behaviour of uninfected males would result in the death of all parasites. Female behaviour, in contrast, ensures the parasite's return to water as the female undergoes ovipositing behaviour. Third, normal male swarming behaviour requires considerably more energy from fat reserves than the dispersal and ovipositing behaviour of females (Sartori *et al.* 1992). *Gasteromermis* may therefore benefit from female mating behaviour as this is a more conservative use of host nutrients.

It appears that the mermithid *Gasteromermis* obtains considerable fitness benefits from the secondary sexual behaviours of female *B. bicaudatus* hosts. The secondary sexual behaviours of uninfected male hosts, in contrast, would result in the death of the parasite. This study suggests that the benefits the parasite derives from female behaviours are secured in infected male hosts by parasite-mediated feminization of males.

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