Precopulatory mate guarding and mating behaviour in the rotifer *Epiphanes senta* (Monogononta: Rotifera)

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Epiphanes senta is a littoral rotifer species that occurs in temporary waters and displays a mating behaviour which has not, to my knowledge, so far been described for monogonont rotifers. Monogonont rotifers show distinctive periods within their life cycle during which mictic females appear. Mictic females produce haploid eggs that develop into males or into diapausing eggs if fertilized. The females of *E. senta* are mostly stationary on the substrate while males are more active swimmers. If they encounter eggs with female embryos of their own species, they attend them and mate with the hatching female. Experiments showed that males are able to discriminate between male, female and diapausing eggs. They exhibit a strong preference for female eggs that are only a few hours away from hatching compared with eggs in early developmental stages. Further experiments did not show any significant differences in male attendance of mictic and amictic eggs. It is hypothesized that males judge the age of a female egg by sensing a chemical that is produced by the growing embryo and diffuses through the egg shell. The male mating behaviour is similar to precopulatory mate guarding known from arthropods but it lacks the monopolization of the female by the male.

Keywords: mating behaviour; mate choice; cyclic parthenogenesis; mate guarding; rotifer

1. INTRODUCTION

Epiphanes senta is a monogonont rotifer that colonizes littoral habitats, which are often temporary (Klement 1960; Pejler 1995; Schröder 2001). Monogonont rotifers are opportunistic organisms that can rapidly colonize a habitat via parthenogenetic reproduction of amictic females that produce only female offspring. However, sexual reproduction occurs periodically and results in the production of diapausing eggs that are able to survive harsh environmental conditions (Gilbert 1974, 1993; Pourriot & Snell 1983). During sexual periods, mictic females appear whose oocytes undergo meiosis (Gilbert 1983). Haploid males are produced parthenogenetically by unfertilized mictic females. When mictic females are fertilized by males they produce diploid diapausing eggs. As diapausing eggs are the only stage that are capable of surviving adverse environmental conditions such as the terrestrial phases in temporary water bodies, there should be a strong selective pressure for an efficient production of diapausing eggs during the sexual phase of the life cycle. The males in most monogonont rotifer species are dwarf males whose morphology and behaviour are designed to increase their reproductive success (Gilbert & Williamson 1983). They lack a functional intestine and all their energy reserves derive from the maternal yolk provided to the male egg. The reduction of body size and organs allows the mother to produce more male offspring per unit time and enables the male to swim at an increased speed without using more energy resources (Epp & Lewis 1979), thereby increasing contact probability with fertilizable females. When a male encounters a female in brachionids he starts circling around her, keeping in contact with her body sur-

face with his corona. After a number of circulations he copulates with the female. The sperm are transferred into the female body cavity by hypodermic insemination (Gilbert 1963; Snell & Hawkinson 1983). Females are fertilizable during only a short period after hatching from the egg (Buchner *et al.* 1967; Snell & Childress 1987) and likewise male fertility decreases rapidly with age (Snell & Childress 1987). This imposes a strong selective pressure on the male to choose the right mate, especially as the male lifespan is short and their sperm reserves are limited (Snell 1977; Snell & Hoff 1987). Indeed, Gómez & Serra (1996) have shown that male *Brachionus plicatilis* preferably copulate with young females. However, they are not able to discriminate between mictic and amictic females.

The males of *E. senta* display a unique mating behaviour that has not, to my knowledge, been observed in rotifers before and is completely different from the mating behaviour described from other species. Observations in laboratory cultures have shown that they aggregate on subitaneous eggs and wait on the egg until the female hatches. Then they try to copulate with the hatching female. This behaviour resembles the precopulatory mate guarding found in many arthropod species—a male time investment strategy that optimizes male reproductive success (Parker 1974; Wickler & Seibt 1981). Selection of young mictic females is an essential component of male reproductive success in rotifers. Attending the egg of a prospective mate and copulating after it hatches may be an effective strategy to increase the number of a male's successful fertilizations because it would reduce the risk of copulating with infertile older females. The ability to distinguish eggs developing into mictic females from those developing into amictic females would be of further advantage, because attending eggs of amictic females and copulating with them would be a waste of time and sperm. Both time and sperm may be limited resources as male

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lifespan in monogonont rotifers is short compared with females and fertility decreases rapidly with male age.

The present study addresses the question of whether the behaviour of *E. senta* males is adaptive in the way that it contributes to a higher reproductive success of the males. The behaviour is described in further detail and several experiments are reported that were conducted to test whether males exhibit a preference for different egg types (male and female subitaneous eggs, diapausing eggs) and whether they are able to discriminate between eggs that hatch into mictic females and those that hatch into amictic females. As time seems to be a limiting resource for the male, preferences for different developmental stages of female egg were tested and life table experiments were conducted trying to assess male survivorship and fertility with age.

2. MATERIAL AND METHODS

The clone E1017 of *E. senta* was used for all experiments. This clone originated from a female that hatched from a diapausing egg in a soil sample collected from the floodplains of the Oder River near Schwedt, Germany. Rotifer mass cultures were maintained on *Cryptomonas erosa* var. *reflexa* in ca. 150 ml modified MBL (Marine Biological Laboratory) medium (Stemberger 1981). Male eggs were transferred from the mass cultures to a Petri dish and checked every 2 h for hatching in order to obtain males of a known age. The males used in the experiments were 2–12 h old. Subitaneous eggs and diapausing eggs used in the experiments were also obtained from these mass cultures. All experiments were performed in small Petri dishes (polystyrene; diameter of 35 mm) filled with modified MBL medium.

One experiment was conducted to test whether males are able to discriminate between female and male subitaneous eggs as well as diapausing eggs of their own species. Twenty males were introduced into a Petri dish that contained five diapausing eggs, five male subitaneous eggs, five female subitaneous eggs that had been deposited less than 2 h prior to the experiment and five mature female subitaneous eggs that were close to hatching: these eggs contained embryos that were already fully developed and moving within the eggshell. Every 30 min the number of males that had attached to an egg was recorded as well as the type of eggs they had attached to. The experiment was run in five replicates and lasted for 3 h.

The next experiment tested whether males were able to discriminate between eggs of mictic and amictic females when they attach to an egg. Ten males were transferred into each of four Petri dishes that all contained 20 female subitaneous eggs. These eggs were isolated after they had undergone one to three cleavage divisions and the males were introduced 24 h later. At that time the embryos had already fully developed. The eggs were checked every 30 min and the number of attached males was recorded for each egg individually. The amount of time that each egg was attended was then calculated, assuming that a male that was found attached to an egg at two consecutive checkings was sitting on the egg for 0.5 h. Egg attendance by two males was counted twice, attendance by three males three times and so on. Because some eggs hatched during the observation, which lasted 3 h, the total time of male attendance was corrected for each egg. This was done by dividing the time of attendance through the time until hatching, or if the egg did not hatch, through the total experimental time. Thus the proportional

amount of time that each egg had been attended was calculated. After the experiment, each egg was transferred into a depression of a 96-well tissue culture plate filled with *Cryptomonas* suspension, which was changed every day. The hatching females were raised until they produced offspring so that they could be typed as mictic or amictic.

One experiment was conducted to test whether males would preferably copulate with mictic females and whether males are more likely to copulate with a female that hatched from an egg they had been attending rather than with a female they encountered while swimming around. Two experimental series were set up: in one series two males were put together with a newborn female (less than 30 min old) and observed for 15 min. A small volume of 10 µl of MBL medium was used for these observations to ensure a high probability of several encounters between males and a female during the experimental time period. In the other series two males were allowed to attach to a mature egg. The rotifers were observed until the female hatched and 15 min thereafter. Male copulations with the female were recorded in both experimental series and the females were then raised in a *Cryptomonas* suspension, until they produced offspring and could be typed as mictic or amictic.

Male fertility was determined by placing males of different ages (0–2 h, 24–26 h, 48–50 h and 72–74 h) into 50 µl of a *Cryptomonas* suspension together with a female subitaneous egg containing a fully developed embryo that was about to hatch. Twenty-four hours later the male was removed. The females were then raised until they deposited their first eggs. The relation of fertilized to unfertilized mictic females was taken as a measure of male fertility at each age. Male survivorship was determined by recording the number of surviving males in MBL medium at 15 °C.

The role of egg-shell properties as a signal for the male was assessed by a test of the male response to the egg shell only. Embryos were removed from the egg shell with a glass needle and a micro manipulator. The egg shells of an egg that had been deposited within 4 h prior to the experiment and of a mature egg with a fully developed embryo were transferred to a 10 µl drop of MBL medium. A male was then added to the drop and its behaviour was observed for 10 min. The amount of time the male spent on each egg shell displaying egg recognition behaviour was measured with a stop watch. A series of 21 trials was run in this way. In half of the series the embryo was removed first from the recently deposited egg, in the other half the mature embryo was removed first from its egg shell. This was done to avoid any confounding effects due to the degradation of one egg shell while the other was prepared for the experiment.

All experiments were analysed statistically with the JMP statistics package (SAS Institute 2001).

3. RESULTS

(a) *Description of the male egg recognition and mating behaviour*

Males who normally swim along substrate surfaces make contact with female eggs at random. While swimming around, they constantly move their corona from one side to the other, which results in a zigzag movement. The males are more active swimmers than the females, who mostly remain stationary on the substrate and deposit their eggs on the substrate surface. However, there seems to be no directed search of the males for eggs. A male that passes an egg in a distance of a few micrometers will not

Figure 1. Egg recognition behaviour of the *Epiphanes senta* male. See § 3a for further explanations.

make any changes in his swimming direction, but pass the egg without noticing it. If he hits an egg while swimming around, he first displays an avoidance reaction and tries to move around the object (figure 1a), but then he immediately stops, contracts his body and attaches to the substrate (figure 1b). He then starts to spin around in circles and slowly moves his position. Doing this he may finally come back to the egg. When his corona comes into contact with the egg again the male starts to inspect the egg (figure 1c). He moves his corona back and forth on the egg surface. The male finally moves onto the egg, attaches with his foot on the egg surface (figure 1d), and waits for the female to hatch. During this time he repeatedly moves his corona over the egg surface. When the female hatches and the male comes into contact with her, he leaves the egg shell and moves onto the female's body. He explores the female integument with his corona for a few seconds and then copulates with the female. Hypodermic insemination can take place anywhere on the female's body. The copulation lasts between 35 s and 3.5 min, $1.5 \text{ min} \pm 15 \text{ s}$ on average (mean $\pm 1 \text{ s.e.}$, $n = 12$).

(b) Male selectivity in egg attendance

Figure 2 shows that males clearly recognized and selectively attached to female subitaneous eggs, whereas male subitaneous eggs and diapausing eggs were almost completely neglected. After 3 h, differences in the numbers of attached males were highly significant (ANOVA: $F_{3,16} = 351.56$, $p < 0.0001$). Also the males strongly preferred mature female subitaneous eggs that were going to hatch within a few hours to eggs that had just recently been deposited. These were no more attractive than male eggs or diapausing eggs.

However, males were not able to discriminate between subitaneous eggs hatching into mictic females and those hatching into amictic females. Amictic eggs were attended for as long as mictic eggs when males were given a choice of 20 mature subitaneous eggs (figure 3). Differences in

Figure 2. Males attaching to diapausing eggs (open inverted triangles) and to subitaneous eggs with young female embryos (squares), mature female embryos (circles) or male embryos (closed triangles). Symbols represent means $\pm 1 \text{ s.e.}$ of five replicate experiments with 20 males added to a Petri dish containing 20 eggs (five eggs of each type).

Figure 3. The proportional amount of time that eggs developing into amictic and mictic females were attended by males. Bars are means $\pm 1 \text{ s.e.}$

the amount of time that mictic and amictic eggs were attended by males were not statistically significant (Student's t -test; $t_{63} = 0.464$, $p = 0.64$).

(c) Effect of the female reproductive status and egg attendance on mating

Table 1 summarizes the results of observations of males copulating with newborn females. Males that were attached to the egg shell while the female hatched copulated more often with that female than did males who encountered a recently hatched female while swimming around. In a substantial number of cases males copulated with amictic females. The effects of female reproductive status (mictic or amictic) and of the male–female encounter mode (male attached to the egg or encountering the female while swimming) on the initiation of a copulation were examined in a nominal logistic regression model using likelihood ratio tests with female reproductive status and male–female encounter mode as effects (SAS Institute 2001). Female reproductive status had no significant

Table 1. Copulations with hatching mictic and amictic females (a) after males had been attending the egg and (b) with newborn mictic and amictic females that were encountered by males while swimming.

(a) males attached to the egg when the female hatches		
	percentage of observed copulations	n
amictic female	61.5	26
mictic female	63.2	29

(b) males encountered a newborn female while swimming		
	percentage of observed copulations	n
amictic female	20.8	24
mictic female	50.0	6

Figure 4. Survivorship and age-specific fertility of male *Epiphanes senta*. The survivorship (solid line) was recorded for $n = 61$ males. Male fertility (open circles) is expressed as the proportion of mictic newborn females that were fertilized by males of the given age that attended the eggs (age 0–2 h: $n = 22$; age 24–26 h: $n = 15$; age 48–50 h: $n = 11$; age 72–74 h: $n = 17$).

effect ($\chi^2_{1,1} = 0.7045$, $p = 0.40$), but male–female encounter mode was significant ($\chi^2_{1,1} = 7.9224$, $p = 0.005$).

(d) **Male lifespan and fertility**

Males have a mean lifespan (i.e. the time that 50% of the males survive) of 74.5 h at 15 °C. The developmental time of a female subitaneous egg is 36–40 h, which is ca. 50% of the mean male lifespan. During most of the lifespan male fertility does not decrease significantly (figure 4). Males of age 0–2 h were able to fertilize 73% of mictic females successfully, which is not significantly different from the fertilization success of 48–50 h and 72–74 h old males (G -test: $\chi^2_{3,61} = 0.399$, $p = 0.94$). Therefore, males that are able to detect female eggs that are hatching soon will spend less time on an egg and in turn have more time to fertilize other females.

(e) **The role of egg-shell properties as signals for males waiting on the egg**

The signal that causes a male to wait on a female subitaneous egg until it hatches are at least in part associated

Figure 5. Male egg recognition behaviour elicited by egg shells of female embryos in early developmental stages (recently deposited eggs; $n = 21$) and in late developmental stages (eggs close to hatching; $n = 21$). Bars represent means ± 1 s.e. The embryos were removed from the egg shells prior to the experiment.

with the properties of the egg shell. This is shown in experiments with egg shells from which the embryo had been removed. They showed that male egg recognition behaviour can be elicited by empty egg shells only. Males exhibited egg recognition behaviour more often and for a longer time when they encountered shells from eggs which were close to hatching. Egg shells from recently deposited eggs only elicited egg recognition behaviour for very short time periods (figure 5). This difference was statistically significant (ANOVA: $F_{1,40} = 8.3591$, $p = 0.006$). Nevertheless males spent much less time waiting on the empty egg shells than they did on mature eggs in the other experiments and in cultures. In only a few cases did males spend almost the whole time of the experimental period on the egg shell. In most cases males repeatedly examined the egg shells of mature eggs for periods of between 20 s and 3 min. By contrast, once a male has come into contact with a mature egg, he usually stays waiting on the egg until the female hatches.

4. DISCUSSION

This study demonstrates that male mating behaviour in rotifers can be more complex than previously known and that there may be considerable variation in behavioural patterns among species. All species studied so far for their mating behaviour only exhibit mating behaviour when they encounter a female (Gilbert 1963; Aloia & Moretti 1973; Snell & Hawkinson 1983; Rico-Martínez & Snell 1995, 1997; Gómez & Serra 1996; Rico-Martínez *et al.* 1996; Velázquez-Rojas *et al.* 2002), none has been described to attend female eggs until the female hatches. The females of most species that have been investigated for their mating behaviour carry the eggs until the offspring hatches, or they are viviparous so that it would be difficult or impossible for the male to attach to a female egg. As in *E. senta*, two of the species studied (*Euchlanis dilatata* and *Trichocerca pusilla*) deposit their eggs on the substratum. However, their mating behaviour is similar to that of the other species studied (Rico-Martínez & Snell 1997) and not comparable to the mating behaviour of *E.*

senta. The egg attendance of the male in *E. senta* may be adaptive in a way that this behaviour increases the fertilization success of the male. Work on *Asplanchna sieboldi* and *B. plicatilis* has shown that mictic females are only fertilizable for a short time during the pre-reproductive period of their life after they have hatched from the egg (Buchner *et al.* 1967; Snell & Childress 1987), and there is some evidence that the same is true for *E. senta* (see Plate 1885, p. 41). Female fertility should therefore be an important criterion for mate choice of males (Searcy 1982). Because a male initiated mating behaviour and copulated significantly less often when he encountered a female that had already hatched, even if she was just a few minutes old, waiting on the egg until the female hatches is an effective mechanism to ensure that the male will mate with a female during her fertilizable period. This behaviour is consistent with the findings of Gómez & Serra (1996) for *B. plicatilis*, whose males also discriminate against older females.

However, attending the egg consumes time. Males of *E. senta*, as males of most other monogonont rotifer species, do not feed and have a lifetime expectancy significantly shorter than female conspecifics. The mean male lifespan is only 17% of the mean female lifespan (T. Schröder, unpublished data). This is even less than in other species where male lifespan can be up to 50% of the female lifespan (Snell 1977; Snell & Childress 1987; Velázquez-Rojas *et al.* 2002). With such a short lifetime expectancy the male ability to discriminate between eggs that have just recently been deposited and eggs that are going to hatch within a few hours should be highly adaptive. Without this ability a male might only mate with two to three females during his life assuming the worst case that he always attends an egg that has just been deposited, and given the fact that the development time of a female subitaneous egg is *ca.* 50% of the mean male lifespan. Therefore the ability to choose eggs that are going to hatch soon, in combination with a long fertile period of the male's lifespan, should considerably increase a male's mating opportunities and hence his reproductive success. Compared with males of other species the fertile period of *E. senta* males in relation to their lifespan is quite long, which may also be a reason that the egg attendance behaviour has not been found in other species so far. This comparison has to be made with caution though because data for other species are very rare. Also, the data derived from experiments at different temperatures, and for comparison it must be assumed that the proportionate relationship between fertile period and lifespan remains the same with changing temperatures. Male fertility in *E. senta* did not change significantly within 72 h, which is *ca.* 71% of the maximum lifespan. In *B. plicatilis* 50% of the males are no longer fertile after 26% of the male lifespan (Snell & Childress 1987); and Gilbert (1963) observed for *Brachionus calyciflorus* that males often would not even initiate mating behaviour after an age of 8 h at 25 °C, which is *ca.* 10–15% of the male lifespan at this temperature.

Nothing is known about the mechanism that enables a male to judge the age of a female egg. The fact that males also display an egg recognition behaviour when they encounter the empty egg shell of a mature egg rules out the possibility that they sense the movements of the mature embryo through the egg shell. It demonstrates that

E. senta males are able to sense chemicals on the surface of the egg, allowing them to distinguish male, female and diapausing eggs.

However, the discrimination between mictic and amictic females should also be important for optimizing the reproductive success of the male. This has been pointed out before by Gómez & Serra (1996). Nevertheless, they found that males of *B. plicatilis*, while slightly but significantly more often initiating mating behaviour with mictic females, still copulate with a considerable number of amictic females, as do males of *E. senta*. This appears to be a widespread phenomenon in monogonont rotifers. Males of species of the genus *Asplanchna* copulate with mictic as well as with amictic females (Whitney 1913; Aloia & Moretti 1973) and so do males of *B. calyciflorus* and *Brachionus angularis* (Gilbert 1963) and males of *Rhinoglena frontalis* (T. Schröder, unpublished data). The fact that males cannot distinguish between amictic and mictic eggs is puzzling because there would seem to be a large selective advantage of a discrimination, because rotifer males are often limited in their fertile time period or in their number of sperm, or both (Snell & Childress 1987). This means that wasting time or sperm is critical to male reproductive success and hence to male fitness. In *E. senta* sperm number is less likely to affect fitness than in other species. A male's testis contains as many as 200 spermatozoa (Whitney 1917; T. Schröder, unpublished data), which is much more than found in other species (Ruttner-Kolisko 1983; Snell & Childress 1987).

Gómez & Serra (1996) modelled the costs of both unsuccessful matings with old unfertilizable mictic females and with amictic females. They come to the conclusion that the age of the female is probably much more critical than her reproductive state. But in their model they assumed that the amount of time that a male spends in copulation is negligible, compared with the length of his fertile period. This may be true for *B. plicatilis* and other species where the mating process with a single female lasts for only seconds to minutes (Snell & Hoff 1987). This assumption is certainly not valid for *E. senta*. Although the threshold age of a female subitaneous egg for a male to choose to attend that egg was not exactly quantified in this study, males were frequently observed to spend 1–2 h waiting on the egg until the female hatches.

The egg attendance behaviour in *E. senta* resembles precopulatory mate guarding behaviour known in crustaceans and some spiders (Fahey & Elgar 1997; Jormalainen 1998), in contrast to postcopulatory mate guarding, which is often found in insects (Alcock 1994). Male precopulatory mate guarding is superior to a strategy of purely searching for a mate, when the male would encounter less than one receptive female if he spent the time searching instead of guarding (Parker 1974; Wickler & Seibt 1981). Both egg attendance in *E. senta* and arthropod precopulatory mate guarding behaviour may have evolved because females are only fertilizable during short periods of their lifetime. The lower female density and the shorter female fertile periods are, the higher is the expected benefit of mate guarding. However, the major difference of egg attendance behaviour in *Epiphanes* is that there seems to be no monopolization of the female by the male, which usually is part of the precopulatory mate guarding strategy in arthropods (Parker 1974; Wickler & Seibt 1981). Males

of *E. senta* do not display any kind of aggressive behaviour against conspecifics. In dense cultures up to eight males can often be observed to attend one female egg together without interfering with each other. When the female hatches several males may copulate with the female. This still leaves the possibility for sperm competition about which nothing is known in rotifers.

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