Sexual behaviour of immature male eastern mosquitofish: a way to measure intensity of intra-sexual selection?

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Immature males of eastern mosquitofish Gambusia holbrooki start to be sexually active well before their copulatory organ (gonopodium) has completely developed and before they become able to transfer sperm. Sexual activity of males, consisting of copulatory attempts tending to bypass female acceptance, is intense (one attempt per minute) and is likely to be energetically very costly. The sexual behaviour of immature males relative to their maturation stage is described and tested against two possible adaptive explanations. Sexual activity was present in males from the beginning of the development of their gonopodium and increased during the following stages of maturation. Two to three weeks before gonopodium development was completed, sexual activity of immatures was as high as that of adults. Adult males showed aggressive behaviour against a male attempting a copulation, irrespective of the maturity of the latter. Since previous studies have shown that the reproductive success in this species is negatively correlated with male size when male–male competition is low (i.e. when the sex ratio is female biased), but decreases with male size when competition is high, the hypothesis was tested that sexual activity of immature males functions as a way to predict their future reproductive success if they mature at a given size. A second hypothesis tested was that precocious sexual experience improves the efficiency of copulatory attempts. Results were more in agreement with the first hypothesis, since size at maturity of males was influenced by the sex ratio experienced during maturation and precocious experience gave very little advantage.

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Key words: Gambusia holbrooki; sexual behaviour; immature males; aggressive behaviour; sex ratio; experience.

INTRODUCTION

An increasing body of evidence indicates that, as assumed by life history theories, individuals generally pay costs associated with reproduction (Clutton-Brock, 1991; Marconato, 1991). Selection should therefore favour strategies that reduce costs to a minimum, and individuals are expected to reproduce only when advantages exceed costs in terms of reduced future fitness. In many bird species, for example, secondary sexual traits develop only during the breeding season (Andersson, 1983), or, as in many fish species, breeding coloration is shown or intensified only at the appearance of females (e.g. Kodric-Brown, 1983; Gronell, 1989).

An obvious corollary to the above statements is that mating behaviour and manifestations connected with reproduction (territoriality, breeding coloration, courtship behaviour, etc.) should not appear until individuals reach functional sexual maturity. The rare exceptions known so far have an adaptive explanation. In several primates, including humans, young infertile individuals

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include sexual acts in their play and in some cases a complete coitus is achieved (Ford & Beach, 1951; Mason, 1965). Among chimpanzee (Pan troglodytes Blumenbach), young with no previous sexual experience are not immediately able to copulate successfully, which suggests a possible role for sexual play. In aquarium studies, immature males of the eastern mosquitofish (Gambusia holbrooki Girard) exhibit precocious copulatory activity, and their rate of copulatory attempts was comparable to that of adults (Bisazza, unpublished data).

Gambusia holbrooki is a small poeciliid fish commonly found in brackish and fresh waters of Italy, where it was introduced from North America at the beginning of this century. Fertilization is internal and the male uses its modified anal fin (gonopodium) to transfer sperm to the female. As in most poeciliids (Constantz, 1975; Farr, 1984), male sexual activity mainly consists in approaching the female from behind, moving the gonopodium forward and trying to introduce forcibly the gonopodial tip into her genital pore (gonopodial thrusting). In this species, females rarely if ever show receptivity. Males make copulatory attempts at a very high rate, but only a small proportion of them is successful (Martin, 1975; Bisazza & Marin, 1995). Male mosquitofish, typical of most poeciliid species, stop growing when they become sexually mature (Schultz, 1961; Borowsky, 1973a; Zulian, 1990) and body size attained at maturation changes in response to the social environment (Borowsky, 1973b; Sohn, 1977).

To explain the behaviour of immature male mosquitofish, two hypotheses were proposed. The first one postulates that, as observed in apes and other vertebrates, experience gained when immature increases the efficiency of later sexual behaviour as adults. The second hypothesis, which will be discussed in more detail later, is derived from the peculiar reproductive biology of this species. The success of mating attempts in G. holbrooki is in fact negatively correlated with the male's size (Bisazza, 1993; Bisazza & Marin, 1995), a relationship observed in other poeciliid species and possibly a general phenomenon in this family (Farr et al., 1986). Conversely, both intrasexual selection and female preference tend to favour the largest in a group of males (Bisazza & Marin, 1991; 1995). Computer simulations indicate that when males are rare and the encounter rate between them is low, males should mature at a small size, but when population density is high and the sex ratio is male biased, the influence of intra- and intersexual selection increases and males should delay maturation to attain a large size (Bisazza & Marin, 1995). It is suggested that by attempting to copulate with females, immature males gain information about their probable reproductive success at their present body size, and accordingly ‘decide’ whether to delay maturity or not.

In the first part of this paper information is given about the ontogeny of male sexual behaviour and the interactions between adults and immature males described in relation to their developmental stage. In the second part, two experiments aimed at testing the two explanations for the presence of intense sexual activity in immature males are presented.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The individuals used for this work were captured during the breeding season in three different bodies of water of Northern Italy. Mostly handnets were used, with a 2·5 mm
mesh that retained specimens of every size. Before starting the experiments, individuals were acclimatized for some days in 130-l aquaria (stock aquaria), maintained at a constant photoperiod (illuminated from 06.00 to 20.00 hours) and temperature (26 ± 1°C). Fish were fed twice a day with commercial fish food (TetraMin). For testing the influence of precocious experience on male sexual behaviour at maturity (Experiment 3), offspring born in captivity to some of the wild females were used. All individuals to be used in the experiments were anaesthetized with MS222, sexed and measured to the nearest 0.5 mm total length (T.L.).

MATURATION STAGES OF MALES

Maturation stage of males was determined by examining the development of the gonopodium under a dissecting microscope. Ten stages were identified (Dulzetto, 1931). Briefly, the development of the gonopodium starts with the elongation of the 3rd, 4th and 5th rays of the anal fin (stage 1 to 4); at stage 5 the length of the gonopodium is similar in length to that of mature males, and rays 3 to 5 begin to develop distally bifurcated articules that, at stages 8 and 9, form a series of hooks. At stage 8 the gonopodium can be turned forward. At stage 9 the gonopodium, previously opaque, becomes transparent. At stage 10 the distal articles of the gonopodium become fused. The maturation of the gonopodium is paralleled by the progressive maturation of the testes, which is completed at stage 9, but males are able to inseminate females only at stage 10, when the gonopodium is completely developed (Dulzetto, 1933).

FREQUENCY OF COPULATORY ATTEMPTS IN IMMATURE MALES

One hundred and forty-six males at different stages of sexual maturation were tested. The day before each test, one male was introduced in one of six 60 × 36 × 36 cm aquaria, each containing two acclimated adult females. Only females that had not delivered young recently were used, because the frequency of attempts by males is significantly higher with post-partum females (Bisazza et al., 1989). Each male was observed for two periods of 10 consecutive minutes, at least 30 min apart, and copulatory attempts were counted. At the end of each test, the males were anaesthetized, measured, and the stage of maturation of the gonopodium was determined.

FREQUENCY OF AGGRESSIVE BEHAVIOUR OF ADULT RESIDENT MALES TOWARD IMMATURE MALES

One hundred and thirty-one males in different stages of sexual maturation were tested. Experimental conditions were similar to those of above, the only difference being that one adult male (resident) was also present in each experimental aquarium. Thus, each aquarium contained three adults (one male and two females). The test male (from stage 1 to 10 of maturation) was introduced 1 day before being tested. The resident male was always larger than the test male. During the two periods of observation recordings were made of the number of attacks directed by the resident male to the test male, the number of attacks from the test male to the resident, and the number of copulatory attempts by the test male. At the end of each test, total length and maturation stage were determined for both males.

EFFECT OF PRECOCIOUS SEXUAL EXPERIENCE ON THE EFFICIENCY OF MALE COPULATORY BEHAVIOUR

Thirty males were used. Fifteen of them were grown from birth to sexual maturity in 130-l aquaria containing adult females (experienced males). The other 15 males were grown in similar aquaria, but adult females were not present (naïve males). Upon reaching sexual maturity, each male was tested in aquaria (70 × 70 × 36 cm) containing four adult females. Observations started 1 min after the male was introduced, and continued for 30 min. Since the success of the copulatory attempts depends strongly on the ratio of male to female length (Bisazza & Marin, 1991), the four females in the experimental aquaria were similar in size, and the male to female length ratio was kept as
constant as possible. Copulatory attempts and successful gonopodial thrusts (observed contact between gonopodium and female genital pore) were recorded for each male of the two groups. As a measure of the efficiency of the copulation behaviour of a male, the ratio between successful gonopodial thrusts and total number of copulatory attempts was calculated.

EFFECT OF THE SEX-RATIO EXPERIENCED BY IMMATURE MALES ON THEIR SIZE AT MATURITY

The effect of the sex ratio on male size at maturation was tested using 36 immature males (stage 1–2), 72 adult males and 90 adult females. The experimental aquaria were 70 × 70 × 36 cm in dimension. The natural habitat of G. holbrooki is characterized by the presence of dense vegetation. Aquaria were therefore subdivided into six sections, using vertical partitions, in order to simulate the complexity of a natural situation. Partitions allowed fish to move freely from one compartment to any other. Food was provided twice a day ad libitum. One immature male (stage 1 or 2) and two adult males, whose size exceeded by 4 ± 0·5 and 7 ± 0·5 mm that of the immature male, were introduced to each aquarium. To simulate two situations with different sex ratios, one adult female was introduced in some of the aquaria (adult male: female ratio = 2 : 1, 19 replicates), and four adult females in the rest of the aquaria (sex ratio 1 : 2, 17 replicates). Total length of males was measured before the beginning of the experiment by using a microscope with a micrometric ocular. Specimens were observed under the microscope in a Petri dish filled with water, thus avoiding anaesthetizing males before the experiment. 15 days after the immature male matured, it was removed from the aquarium, its total length was measured and its maturation stage assessed as previously described.

The aquaria used for these experiments did not allow the continuous observation of the males' behaviour. To measure the frequency of aggressive and sexual interactions between immature males and adult males, and between immature males and females, similar aquaria were used in the middle of a dimly-lit room. Twenty-eight replicates were run (14 with one female and 14 with four females), using immature males at stages 5 and 6 of gonopodium maturation. For each replicate, immatures were kept under observation for 30 min a day (three periods of 10 min, 10 to 15 min apart from each other), for three consecutive days. Attacks by adult males towards immature, attacks by the immature towards the adults, and gonopodial thrusts attempted by the immature were scored, and the fraction of time that the immature spent in sectors of the aquarium where one or two adult males were also present were measured.

STATISTICAL ANALYSIS

All variables were normally distributed and parametric tests were used. When variance was not homogeneous between groups, data were logarithmically transformed. Proportional data were arcsin-root transformed. Statistics were computed with the SPSS/PC statistical package.

RESULTS

FREQUENCY OF COPULATORY ATTEMPTS IN IMMATURE MALES (Table I)

The size of males was significantly different between groups (ANOVA, F = 17·7; d.f. = 9, 145; P < 0·001), and increased with maturation stage of the gonopodium (trend analysis, F = 146·2; d.f. = 1, 145; P < 0·001). However, differences between groups were not significant among males in stages 6 to 10 (a posteriori analysis, Duncan procedure, P < 0·05), and body size differences did not affect the results of the experiment [ANOVA, stage (factor): F = 17·3; d.f. = 9, 145; P < 0·001; total length (covariate): F = 1·35; d.f. = 1, 145; P = 0·24].
The frequency of copulatory attempts differed significantly between stages (ANOVA, $F=34.3$; d.f. = 9, 145; $P < 0.001$), and increased with the progress of maturation from stage 1 to stage 6 (trend analysis, $F=275.5$; d.f. = 1, 145; $P < 0.001$). After stage 6, differences between groups were not significant (a posteriori analysis, Duncan procedure, $P < 0.05$).

**FREQUENCY OF AGGRESSIVE BEHAVIOUR OF ADULT MALES TOWARDS IMMATURE MALES (Table II)**

The total length of immature males increased with gonopodium maturation from stage 1 to 6, but thereafter did not change significantly (ANOVA, $F=20.8$;
d.f. = 9, 130; $P < 0.001$, a posteriori analysis, Duncan procedure, $P < 0.05$). No significant difference in length between resident males was found (ANOVA, $F = 1.4$, d.f. = 9, 130; $P = 0.19$).

Adult males attacked immatures at all stages of maturation of the gonopodium. Variance between groups for attacks of adult males towards immatures was not homogenous and so a logarithmic transformation was used. Aggressive behaviour of adult males towards immatures increased with the maturation of gonopodium (ANOVA, $F = 12.4$; d.f. = 9, 130; $P < 0.001$; trend analysis, $F = 105.6$; d.f. = 1, 130; $P < 0.001$). After stage 7, the number of attacks by adults towards immatures did not change significantly (a posteriori Duncan procedure, $P < 0.05$). Attack frequency by immatures towards adults was always very low ($0.2 \pm 0.58$ min$^{-1}$) and did not differ significantly between groups (ANOVA, $F = 0.80$; d.f. = 9, 130; N.S.).

The number of copulatory attempts performed by the immature males was negatively correlated with the number of attacks received from the adult male ($r = -0.36$, $n = 131$, $P < 0.001$). In order to compare males at different stages of maturation, the number of attempts was standardized as the ratio between the number of attempts by each immature male at a given stage of maturation, and the average number of attempts of immature males at the same stage. Similar results were obtained when the absolute number of attempts by males at similar stages of maturation were considered (stages 7 to 10, $r = -0.49$, $n = 50$, $P < 0.001$).

**EFFECT OF PRECOCIOUS SEXUAL EXPERIENCE ON THE EFFICIENCY OF MALE COPULATORY BEHAVIOUR (Table III)**

Experienced and naïve males did not differ in total length ($t = 0.75$, $n = 30$ N.S.; Student’s $t$-test). Naïve males made more copulatory attempts than experienced males ($t = 5.46$, $n = 30$, $P < 0.001$), and more attempts were successful ($t = 3.14$, $n = 30$, $P = 0.003$). None the less, copulation efficiency (ratio between successful attempts and total attempts) did not differ significantly between the two groups ($t = 0.54$, $n = 30$, N.S.). If the observation period is divided into three parts of 10 min each (Fig. 1), in the first 10 min of observation experienced males did better than naïve males ($t = 2.31$, $n = 30$, $P = 0.028$), whereas this difference was absent in the subsequent two periods ($t = 0.73$, $n = 30$, N.S., and $t = 0.48$, $n = 30$, N.S., respectively).

**Table III. Effect of precocious sexual experience on the efficiency of copulatory attempts of adult males (mean number ± s.d.)**

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Ratio between male T.L. and females T.L. (mm)</th>
<th>Copulatory attempts</th>
<th>Successful copulatory attempts</th>
<th>Frequency of successful copulatory attempts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve males</td>
<td>15</td>
<td>0.62 ± 0.039</td>
<td>136.46 ± 26.53</td>
<td>7.26 ± 3.71</td>
<td>5.49 ± 2.8</td>
</tr>
<tr>
<td>Experienced males</td>
<td>15</td>
<td>0.63 ± 0.040</td>
<td>75.73 ± 33.95</td>
<td>4.06 ± 1.33</td>
<td>6.01 ± 2.5</td>
</tr>
</tbody>
</table>
EFFECT OF THE SEX RATIO EXPERIENCED BY IMMATURE MALES ON THEIR SIZE AT MATURITY (Tables IV and V)

Immature males that grew in aquaria with a low sex ratio increased on average their body size at maturation by about 35% of their original size. Males that grew in aquaria with a high sex ratio increased their size by about 50%; the
difference between the groups was highly significant ($t=2.84$, $n=35$, $P<0.01$, Table IV). In 13 out of 19 cases, males grown in aquaria with a high sex ratio matured at a size which was greater than that of both adult males present in the same aquarium. Only six out of 17 immatures in the aquaria with a low sex ratio matured at a size larger than that of the largest adult male. The difference was only marginally significant ($\chi^2=2.74$, $P<0.10$).

Aggressive interactions between immature (stage 5 and 6) and adult males did not differ in aquaria with low and high sex ratios, in terms of attacks received ($t=0.4$, $n=30$, N.S.), attacks performed ($t=0.51$, $n=30$, N.S.), and total number of attacks ($t=0.13$, $n=30$, N.S., Table V). In addition, the time spent by immatures in sectors of the aquaria where at least one adult male was present did not differ in the two conditions ($t=1.49$, $n=30$, N.S.). However, immatures in aquaria with a low sex ratio made a higher number of copulatory attempts than those in aquaria where the sex ratio was high ($t=2.75$, $n=30$, $P=0.01$).

**DISCUSSION**

The results demonstrate that a male’s sexual behaviour (copulatory attempts) starts well before it is able to fertilize females. On average, gonopodium maturation from stage 1 to 10 takes about 20 to 30 days in the laboratory (Zulian, 1990). Even during very early stages of maturation some sexual activity was observed, and the frequency of attempts increased during the first five stages. From stage 6, immatures were approximately as active as adults. The dynamics of copulatory attempts were roughly constant during the entire maturation period, the main difference being that the forward rotation of the gonopodium occurred only after the 8th stage. As sexual activity of immature males increased, they received an increasing number of attacks by adult males. From stage 6 of gonopodial maturation onward, the frequency of attacks by adults did not change significantly. It therefore seems that the frequency of attacks by adult males depended on the sexual activity of the immatures and not on gonopodial maturation stage, and that adult males do not discriminate between immature males that are incapable of fertilizing the females and other adult males. They apparently treat all males which are attempting to copulate with females as possible competitors.

It is difficult to find an explanation for the high rate of copulatory attempts in immature males. In theory, this behaviour could be non-adaptive, a consequence, perhaps, of increasing levels of blood androgen hormones. Precocious copulatory activity can be induced by administration of testosterone in fish (Liley & Seghers, 1975) and other vertebrates (Stone, 1940; Andrew, 1975). Adult sexual behaviour can be elicited also in young of several birds and mammals, but in most cases it appears to be a reflex response to peculiar circumstances, and is observed very infrequently under natural conditions (Andrew, 1966). A non-adaptive explanation is difficult to test, but in this case it seems at least unlikely. During the breeding season adult male *G. holbrooki* make on average one copulatory attempt per min, throughout the whole day (Martin, 1975; Bisazza & Marin, 1991). It seems likely that such an intense activity is energetically very expensive. Moreover, males engaging in sexual activities are more visible, pay less attention to approaching predators (Endler,
1982), and probably suffer high mortality. Indirect evidence for these costs has been found by Krumholz (1963) in populations of Gambusia manni (Hubbs). He found that populations living in habitats with a high density of predators had a sex ratio biased towards females, and a low mean size of adult males, compared to those observed in populations living in habitats with low predator density.

Among the possible adaptive explanations is the ‘training’ hypothesis. Copulatory experience during the stages preceding maturation may enhance the copulatory efficiency of males. As far as we know, in fish there are no examples of experience enhancing performance. However, it is known that young three-spined sticklebacks (Gasterosteus aculeatus L.) fed in the laboratory with live prey are more efficient foragers when they become adults than individuals fed with frozen food (Ibrahim & Huntingford, 1992). Our results do not seem to support an analogous explanation for copulatory activity. In fact, mosquitofish males with precocious experience were more efficient in their copulatory attempts than males grown in aquaria where females were not present, but only in the first 10 min after meeting a female. Later on, naïve males were as efficient as experienced males. It seems therefore unlikely that immature males are sexually so active for weeks in order to get trained, if after a few minutes of copulatory attempts naïve males become capable of normal sexual behaviour.

The other possible explanation tested is that immature males, through an intense sexual activity, gain information about the probable reproductive success they would attain by maturing at their actual size. As in most poeciliid species, male mosquitofish stop growing or grow little after reaching sexual maturity, but considerable variation in this trait is generally found within populations. Previous studies indicate that in G. holbrooki the success of copulatory attempts is inversely related to male size when competition is absent (Bisazza, 1993; Bisazza & Marin, 1995). On the contrary, when sex ratio is biased towards males, larger males monopolize females and keep subordinate males far from females (Bisazza & Marin, 1991). The reproductive success of males therefore depends critically on the size at which they mature in relation to the social environment: at low population densities and when the sex ratio is biased towards females, males are expected to mature at a small size, and vice versa, as shown by computer simulations and field study (Bisazza & Marin, 1995; Zulian et al., 1995). In natural populations, the proportion of males and population density usually increase from the beginning of the breeding season onwards. None the less, there are frequent large variations both between and within populations in different years (Zulian, 1990). A simple rule of thumb, e.g. mature small at the beginning of the breeding season and at a larger size later on, would not be very efficient.

Immature males that are able to test their potential reproductive success and consequently ‘decide’ whether to mature or to keep growing, would therefore be favoured. The present results seem to support this hypothesis: the size attained at maturity by males is influenced by the frequency of sexual interactions with females. Social control of body size at maturity is a well known phenomenon in poeciliids. It has been shown that the frequency of aggressive interactions with adult males influences the size of sexual maturation of males in other poeciliid species (Borowsky, 1973b; Sohn, 1977; Farr, 1980), as well as in G. holbrooki (Zulian et al., 1993). In the present experiments, the frequency of aggression by
adult males did not change significantly between treatments (with one or four adult females). Nonetheless, the body size attained at maturation by males was negatively correlated with the frequency of copulatory attempts, that, in turn, depended on the sex ratio in the aquarium where they grew. These results therefore suggest that social control mechanisms, at least in G. holbrooki, may be more complicated, and perhaps more precise, than previously thought. In fact, it could be argued that, for a maturing male, aggressive behaviour by adult males may not be a precise measure of his possible reproductive success. During the breeding season, adult males spend most of their time around females, attempting to copulate or trying to keep other males away from them (McPeek, 1992; Bisazza & Marin, 1995). For this reason, the best way for an immature male to test his potential reproductive success is by trying to copulate with females, and by gaining information about copulations attempted, frequency of successful copulations and of attacks received. Preliminary studies (Bisazza, unpublished data) suggest that immature males also show sexual behaviour in other poeciliids. In three species, Belonesox belizanus (Kner), Girardinus falcatus (Eigenmann) and Phalloceros caudimaculatus (Hensel), sexual behaviour was observed at an early stage of gonopodium development. Conversely, in Poecilia reticulata (Peters) and in Heterandria formosa (Agassiz) males started to be sexually active from 5 to 10 days after the gonopodium was completely developed. Histological examination of the gonads showed that mature sperms were present in P. reticulata and in H. formosa, but not in G. falcatus and P. caudimaculatus. Interestingly, body size is the major factor determining the outcome of male contests in G. holbrooki and in the other four species that we found to have precocious sexual behaviour (Bisazza & Marin, 1995), but not in the two species lacking it (Kodric-Brown, 1983; Zannini, 1994).

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References


