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Operational sex ratio and alternative reproductive behaviours in the European bitterling, *Rhodeus sericeus*

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Abstract We investigated the effects of male population density and male-biased operational sex ratio (OSR) with constant and limited resource density on male mating tactics shown by a freshwater fish, the European bitterling, *Rhodeus sericeus*. This species spawns inside living unionid mussels. Large males defended territories and were aggressive towards conspecifics under equal sex ratios. They also monopolised pair spawnings with females, releasing 98% of all sperm clouds during mating. However, the mating tactic changed at high male density where large males ceased to be territorial and instead competed with groups of smaller males to release sperm when females spawned. Large, medium and small males now obtained 61%, 33%, and 6% of sperm releases respectively, thereby reducing the opportunity for sexual selection by half. Females spawned at equal rates in the two densities of males, despite lower courtship at high density. These results run counter to the usual expectation that an increasingly male-biased OSR should lead to higher variance in male mating success. Instead, the use of alternative reproductive behaviours by males can lead to lower resource competition and mating variance at high male densities.

Keywords Sexual selection · Density dependence · Alternative reproductive behaviour · Cyprinidae

Introduction

Classical sexual selection theory predicts that as the operational sex ratio (OSR: the ratio of sexually active males to fertilisable females) increases, males compete more intensely for females and/or for the resources that females need (Emlen and Oring 1977; reviewed in Kvarnemo and Ahnesjö 1996; Reynolds 1996). Females, in turn, can afford to be more selective of their mates. Variance among males in mating success will usually be higher with more males per female, and the opportunity for sexual selection on males will therefore be greater. This theory explains the evolution of male ornamentation and elaborate courtship displays in many species in which males provide little to the young, and are sexually active for longer time periods than females (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992; Andersson 1994; Kvarnemo and Ahnesjö 1996; Parker and Simmons 1996; Reynolds 1996). Additional factors which skew the OSR towards males have been shown to cause similar increases in sexual selection on males. These include increasing numbers of adult males relative to females (Kvarnemo et al. 1995; Parker and Simmons 1996; Debuse et al. 1999), variation in the distribution of sexually mature adults in space and time (Reynolds et al. 1986; Madsen and Shine 1993; Grant et al. 1995; Otronen 1996), greater female dispersal (Venkataraman 1998) and environmental influences on the potential reproductive rates of the sexes (Kvarnemo 1994, 1996).

It is not always sufficient simply to count the sexually mature members of each sex in the population when predicting the intensity and form of sexual selection (Oliveira et al. 1999). For example, if nest sites are limited and some mature males are prevented from competing for mates, then females may compete more intensely and be less choosy than males (Oliveira et al. 1999; Ahnesjö et al. 2001). Recently Kokko and Monaghan (2001) and Kokko and Johnstone (2002) have introduced a life history perspective on OSR to include the costs of breeding, mate quality variation and parental investment in predicting the direction of sexual selection.

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Sexual selection theory is focused on increased benefits of allocations toward courtship and resource defence as the OSR becomes skewed towards one sex. A central feature of this framework for predicting the intensity of sexual selection is that mates, or resources used to attract mates, must be economically defensible (Emlen and Oring 1977). Theories of resource defence (Brown 1964; Grant 1993) point out that as intrusion pressures on territories increase, the size of the territory that males defend decreases (Myers et al. 1979; Warner and Hoffman 1980; Chapman and Kramer 1996; Grant et al. 2000). Indeed, when taken to extremes, resources become undefensible and males may forego territoriality altogether (Grant et al. 1995) or adopt an alternative reproductive behaviour with direct competition to gain access to females without courtship (Gross 1996; Taborsky 1998). Female choice may therefore become less important than male-male competition.

Since economic defensibility of resources and mates depends on intrusion pressure, it is not immediately obvious how changes in OSR will affect the intensity and form of sexual selection. For example, if five males compete for one breeding site that could attract many females, then resource competition may be intense and the outcome may be high variance among males in mating success. In other words, there will be a positive relationship between OSR and competition for resources critical for breeding, as well as variance among males. On the other hand, if no single male can defend the mating site from the other males, then territoriality may break down and more than one male will be able to mate. This leads to a negative relationship between OSR and resource competition as well as variance among males. Therefore, the direction of correlations between OSR and resource competition and variance in mating success depends on details of the biology of the particular species of interest.

In this paper we examine the effects of two male population densities on male resource competition and male mating tactics shown by a freshwater fish, the European bitterling (*Rhodeus sericeus*). Our objectives are to test for changes in male resource competition, male reproductive tactics and the mating success of both sexes to evaluate the opportunity for sexual selection at different male densities with constant and limited resource density. The European bitterling is a good model to study the effects of population density and OSR on male mating behaviour because: (1) the mussel spawning site represents a discrete and easily quantified resource, (2) the female's reproductive condition is readily assessed by the length of her ovipositor, (3) males show no paternal care, and (4) males display the alternative reproductive tactics of territorial defence, sneaking and group spawning.

The European bitterling is a member of the subfamily Acheilognathinae (family Cyprinidae) and has an obligate spawning relationship with living freshwater mussels (family Unionidae). This species co-occurs with four freshwater mussels over much of their European range: *Unio pictorum*, *U. tumidus*, *Anodonta anatina*, and *A.*

cygnea (Reynolds et al. 1997; Smith et al. 2000a; Mills and Reynolds 2002a). During the reproductive season large males defend territories around one or more mussels and attract females with courtship displays (Candolin and Reynolds 2001). Males are highly aggressive towards other males, especially after females spawn (Candolin and Reynolds 2002b). Female bitterling insert their long ovipositors through the exhalant siphon of mussels and lodge the eggs in the gills of the host (Wiepkema 1961). The eggs are fertilised when the male bitterling's sperm is drawn into the mussel with the inhalant respiratory current. The eggs develop within the mussel for a period of 2–4 weeks, until the yolk sac is absorbed whereupon the young bitterling swim out of the exhalant siphon (Reynolds et al. 1997; Reynolds and Guillaume 1998; Aldridge 1999). Bitterling populations have been recorded to vary by over two orders of magnitude among lakes that vary in size by only four-fold in the Czech Republic (Smith et al. 2000a). OSRs vary greatly in the wild, with particularly intense male-biases in early spring when 20 or more males may compete for spawning with a single female (personal observation).

We manipulated the population density of males while maintaining a constant resource density in order to test the theory of OSR by investigating whether male territoriality, courtship and spawning rates increased or decreased with a higher male density. We also compared the relative proportion of sperm releases by males adopting different behaviours at different population densities to compare the opportunity for sexual selection.

Methods

Forty individuals of the mussel species *Unio pictorum* were collected in March 2000 from Reach Lode, a tributary of the River Cam, Cambridgeshire, at the point of confluence with Wicken Lode, N.G.R.: TL 545 696. The mussels were collected before the onset of the bitterling reproductive season, to ensure they did not contain bitterling eggs. The mussels were collected by hand along the silt bottoms near to the banks and transported in aerated containers to outdoor plastic pools at the University of East Anglia. Twenty pools (140×90×30 cm high) covered in a 10 cm layer of washed sand and filled to a depth of 30 cm with de-chlorinated freshwater, were divided in half using netting. A rectangular retainer (80×40×10 cm high) was positioned centrally in each half of the pool to maintain mussels within a fixed area. The external dimensions of the mussels' shells were measured to the nearest millimetre and mussels were weighed to the nearest gram. The mussels were paired according to similar shell dimensions and weight and each pair of mussels was assigned randomly to each pool, one mussel per pool half. The distribution of mussels varies in the wild from 1 to 20 individuals per m² (personal observation). The mussels were fed daily with a live algal suspension derived from an outdoor pool that had been seeded with *Chlorella vulgaris*.

Male and female bitterling were collected from the same site as the mussels by electrofishing. The fish were transported in aerated carriers to outdoor stock pools. The standard body length of the fish was measured to the nearest millimetre and fish were then distributed among the 20 experimental pools. One half of each pool contained a low density treatment and the other half contained a high density treatment. The low density treatment consisted of 3 males: 1 large (51–60 mm), 1 medium (41–45 mm), 1 small (36–40 mm) (mean = 45.5±5.2 mm) and 3 females: 1 large (41–60 mm),

1 medium (31–40 mm), 1 small (20–30 mm) (mean=37.2±7.3 mm). The high density treatment had 18 males: 1 large, 4 medium, 13 small (mean=40.1±1.0 mm) and 3 females: 1 large, 1 medium, 1 small. The fish were fed a mixed diet: live *Daphnia pulex*, *Chaoborus* pupae, *Culex* larvae and pupae, *Chironomid* larvae; frozen *D. pulex*, *Tubifex* sp., and *Artemia salina*. The quantities were controlled for the number of consumers.

We recorded data on reproductive behaviour from 16 May to 30 June 2000. Behavioural observations of event frequency data were made over a 10-min period in each pool with a 5-min delay between pools. The behavioural recording methods were derived from Wiepkema (1961) and frequency estimates were made for large, medium and small males for the following behaviours:

1. Aggression: chasing away males and females (rapid swimming towards another fish which invariably flees), guarding (male patrols an area around a mussel – usually the area of the retainer). The size of the male or female chased away was also noted. We recorded durations for males guarding mussels.
2. Courtship: courtship chasing (male swims rapidly towards a female often followed by quivering and leading), quivering (rapid lateral undulating movement of the body and fins in front of the female), leading (male orientates towards the mussel in front of the female and quivers while swimming towards the mussel).
3. Spawning: mussel inspection (bitterling approach and face the top of the mussel), head down posture (bitterling head is just above the exhalant siphon at about 45° angle or more), sperm release (a white sperm cloud is visible), unsuccessful spawning attempts (female misses the mussel siphons with ovipositor), and successful spawning (ovipositor momentarily rigid in exhalant siphon).

Paternity tests have not been carried out in order to translate ejaculations by different males directly into fertilisation success. Isozyme analyses of a closely related species, the rose bitterling, *Rhodeus ocellatus*, found no significant difference between the fertilisation successes from pre-oviposition ejaculations by small sneaking males and that of territorial males (Kanoh 1996). However, it is risky to generalise this or any other paternity analysis across species and across spawning situations. So while our indices of opportunity for selection are based on sperm releases, we treat conclusions for these results cautiously. We calculated the percentage of sperm releases for each male by dividing the number of sperm releases by an individual by the total number of sperm releases by all males during a spawning. The opportunity for sexual selection was calculated as the variance in the proportion of sperm releases per male (s^2) divided by the square of the mean proportion of sperm releases (\bar{X}^2) (Arnold and Wade 1984; McLain and Boromisa 1987; Jirotkul 1999a).

Results

Female reproductive success

The male population density did not significantly affect the reproductive rate of females, though there were more unsuccessful spawning attempts at high male density (Wilcoxon matched pairs signed ranks test on the mean number of successful spawnings: $T_+ = -0.32$, $n=20$, $P=0.752$; unsuccessful spawning attempts $T_+ = -1.99$, $n=20$, $P=0.047$; Fig. 1). At high male density female spawning behaviour was frequently interfered with by males competing to release sperm over the mussel.

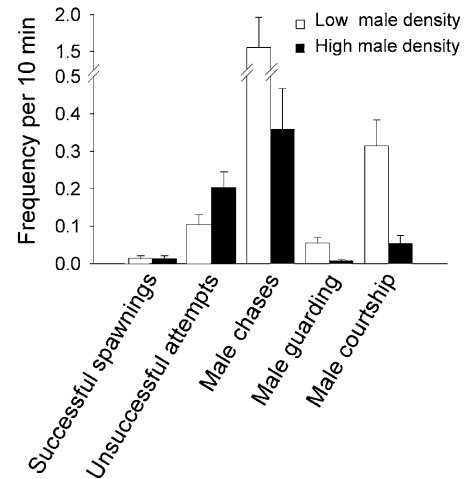


Fig. 1 Mean (+1SE) frequency of successful spawnings and unsuccessful spawning attempts by females, and chases and courtship by the large males in high and low male density. Male guarding refers to the mean (+1SE) duration of time males spent guarding mussels. ($n=20$ replicates). Data are given per 10-min interval

Aggression by males

At low male density all aggressive behaviours (chasing other males and mussel guarding) were performed by the large male, whereas at high density 87% of the chases were performed by large males, and the remaining 13% were performed by small males. In both male population densities, large males performed significantly more chases than the medium- and smaller-sized males than were expected by chance (chi-square test, low density: $\chi^2_{2,18}=509.3$, $P<0.001$; high density: $\chi^2_{2,18}=158.3$, $P<0.001$). Large males chased other males much more often in low male population density than in high male density ($T_+ = -2.85$, $n=20$, $P=0.004$; Fig. 1). The pattern of territorial guarding was similar to that of chasing where large males spent significantly more time guarding in low male population density than in high male density ($T_+ = -3.14$, $n=20$, $P=0.002$; Fig. 1).

Courtship and mating success

The large male courted females far more often at low than at high male density ($T_+ = -3.23$, $n=20$, $P=0.001$; Fig. 1). Spawning behaviours by females in the low male population density were positively related to the frequency of courtship by large males ($r_s=0.83$, $F=71.45$, $P<0.001$; Fig. 2a). However, there was a trade-off for large males between courtship and guarding mussels from other males ($r_s=0.82$, $F=17.74$, $P=0.014$; Fig. 2b). At high density there was no significant correlation between the frequency of courtship and spawning behaviours ($r_s=0.01$, $F=0.19$, $P=0.67$). At high density guarding was too infrequent for analyses of relationships with courtship (Fig. 1).

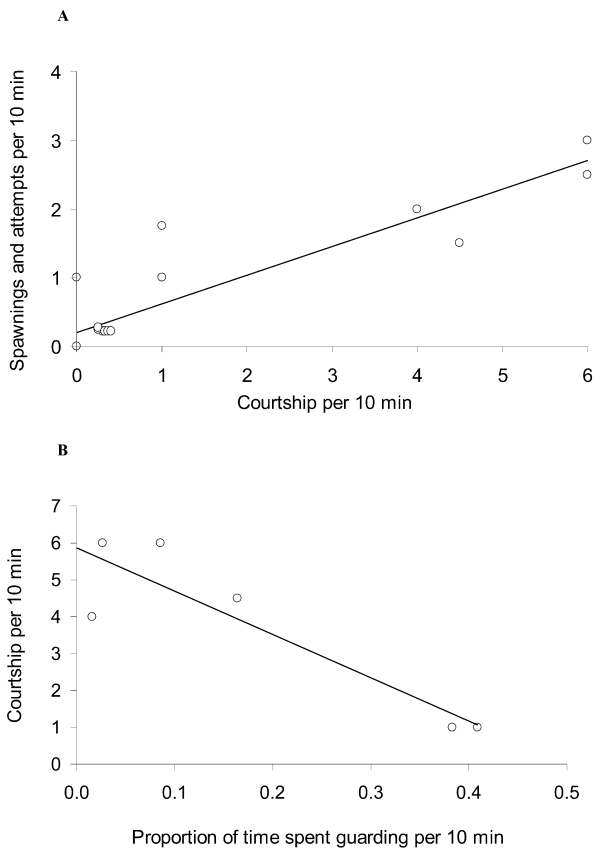


Fig. 2 For the low male density treatment, relationship between **a** the frequency of courtship by the large male bitterling and the frequency of successful spawnings and unsuccessful spawning attempts by the courted female ($Y=0.42X+0.197$, $n=17$) and **b** the proportion of time spent guarding mussels by the large male bitterling and his frequency of courtship ($Y=-11.73X+5.87$, $n=6$)

Despite the presence of six times as many males in the high density treatment, the mean number of sperm releases during a spawning event by all males in each replicate was not significantly higher than in the low density treatment (low male density: 8.23 ± 2.6 ; high male density: 13.91 ± 4.1 ; t -test for equal variances: $t_{16} = -1.08$, $P=0.29$; Fig. 3a). The sample size was 16 because no spawning occurred in four pools. The large male monopolised sperm releases at low male density (Friedman's test $\chi^2_{2,5} = 12.8$, $P=0.002$; Fig. 3a). However, at high male density, medium- and small-sized males joined in releasing sperm with the large male, so that most of the males present were competing to release sperm at the same time over the mussel. Although larger males pushed smaller ones away from the exhalant siphon, there was no significant difference in the mean number of sperm releases by males during each spawning event at high male density (Friedman's test $\chi^2_{2,7} = 0.333$, $P=0.846$; Fig. 3a).

The total numbers of sperm releases in each size category were divided by the number of males in that size category to show sperm release per individual male (Fig. 3b). This yielded a pattern that was similar to that

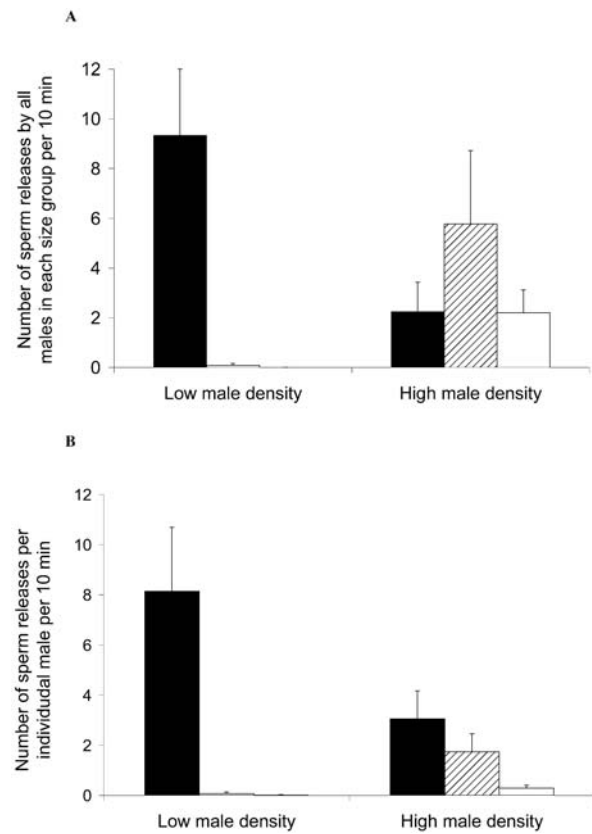


Fig. 3 Mean (± 1 SE) number of sperm releases by **a** all males in each size group and **b** per individual male in each size group; low male density: 1 large, 1 medium and 1 small male; high male density: 1 large, 4 medium and 13 small males. $n=7$ and 9 for low and high density respectively. ■ Large male, ▨ medium-sized male, □ small male

discussed above. At low male population density the proportion of sperm releases by the large male was 98%, which was significantly greater than that by smaller males (Friedman's test $\chi^2_{2,5} = 12.78$, $P=0.002$; Fig. 3b). This proportion was significantly reduced to 61% at high male density (t -test for unequal variances: $t_{12} = 5.32$, $P=0.001$; Fig. 3b). However, unlike the pattern for absolute numbers of sperm releases, large males still released a significantly higher proportion of sperm clouds per individual than both medium-sized and small males ($F_{2,18} = 20.8$, $P < 0.001$, one-way ANOVA; Tukey HSD, large and medium males: $P < 0.05$; large and small males: $P < 0.001$). At high male density medium-sized males also obtained a significantly higher proportion of sperm releases than small males (Tukey HSD, $P=0.014$). At high density medium-sized males released a significantly higher proportion of sperm clouds than at low density (low male density: $0.6\% \pm 0.6$; high male density: $33\% \pm 7.5$, $t_{12} = 4.34$, $P=0.005$) and small males showed a similar pattern (low male density: $1\% \pm 1.0$; high male density: $6\% \pm 1.9$, $t_{12} = 2.32$, $P=0.045$).

The decreased variance among males in proportions of sperm releases at high male density resulted in a

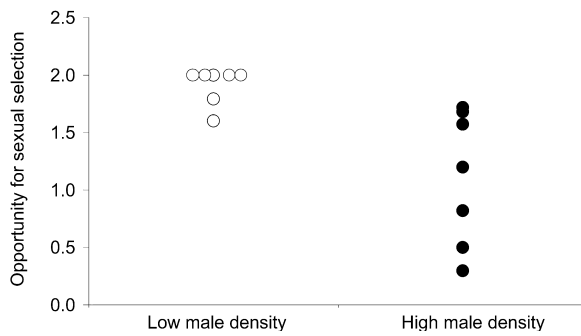


Fig. 4 Opportunity for sexual selection (variance in sperm releases per male divided by the square of the mean number of sperm releases) in relation to male population density

significantly lower opportunity for sexual selection [low density: $1.91 (\pm 0.06)$, high density: $1.11 (\pm 0.22)$; t -test for unequal variances: $t_{12}=3.51$, $P=0.01$, Fig. 4].

Discussion

The results of this study confirm that the OSR cannot be used on its own to predict how resource competition translates into opportunity for sexual selection in European bitterling. This is because at high male density when competition for limited resources was intense, male territoriality and pair spawning broke down in the face of high male intrusion pressure. Rates of courtship, chasing away males and time spent guarding the mussel by the large male decreased when the male population density was high relative to resource density, leading to group spawning and a lower opportunity for selection. Therefore, although high densities of males relative to females may be expected to increase resource competition and the opportunity for sexual selection, the loss of control of spawning sites by dominant males coincided with males adopting alternative reproductive behaviours, reduced courtship and more opportunities for smaller males to compete.

These results can be readily accommodated by classical mating system theory, which emphasises the concept of economic defendability of resources (Emlen and Oring 1977; Reynolds 1996). When resources cease to become economically defendable, territorial males become unable to capitalise on the environmental potential for polygamy. In the case of European bitterling, high-quality spawning sites (mussels) are in short supply at the peak of the spawning season. Females prefer certain species over others (e.g. *Unio pictorum* over *Anodonta cygnea*), and these preferences match differences among species in survival of bitterling embryos (Mills and Reynolds 2002a). Females also prefer empty mussels over ones that contain bitterling embryos (Smith et al. 2000a; Candolin and Reynolds 2001), again reflecting better survival of embryos (Smith et al. 2000b). Therefore, males compete intensely for mussels, and our experiments using biased ratios with large numbers of males per

mussel reflect the situation in the wild when many males compete for a single spawning with a loss of territorial control and sneaking by 20 or more males.

We found that males faced a trade-off between territorial guarding and courtship behaviour at low density, corroborating the results of a previous study of this species (Candolin and Reynolds 2002b) as well as a study of rose bitterling by Kanoh (2000) and a study of Japanese medaka (*Oryzias latipes*) by Grant et al. (2000). This study also supports results for other fish species, with more courtship at low density than at high density (Warner and Hoffman 1980; Jirotkul 1999b; Kanoh 2000) and at low competitor-to-resource ratio (Grant et al. 2000).

As territoriality broke down at high male density with unaltered resource density, sperm competition became more important, with a decrease in the proportion of sperm releases by the large male from 98% to 61%. A previous study has shown that dominant males increase their sperm releases when in the presence of one competitor, but reduce them with more competitors (Candolin and Reynolds 2002a), as predicted by game theory models (Parker and Simmons 1996). The main beneficiaries of this change in sperm competition were medium-sized males, which increased their per individual proportions of sperm releases from 0.6% to 33%, whereas small males only increased from 1% to 6%. If these changes in sperm releases were correlated with changes in paternity, these would generate increased variance in mating success at high male density, as suggested by theoretical studies by O'Donald (1978) and empirical studies by French and Cade (1989), Cade and Cade (1992) and Jirotkul (1999b).

Although female bitterling did not change their rate of successful spawnings with male density, there were density-dependent changes in their responsiveness to courtship. At low male density, spawning frequency increased with increasing courtship by territorial males, yet at high population density there was no correlation between courtship and spawning. Female bitterling have previously been shown to be attracted to males that court at high frequency (Candolin and Reynolds 2001), but courtship is only an initial cue, with mussel characteristics becoming important for the final decision (Candolin and Reynolds 2001; Mills and Reynolds 2002b). When courtship cues were unavailable at high density, females may have been able to use sperm competition through multiple matings as a method of post-copulatory choice (e.g. Evans and Magurran 2000). Thus, while sexual selection theory predicts that females may become more selective when more males are available (e.g. Balshine-Earn 1996), the reduction in courtship may have hampered pre-spawning choice.

Our study demonstrates that the OSR cannot always predict the intensity of various forms of sexual selection when males adopt alternative mating tactics. In the case of bitterling this is because at high male density there is a break-down in territorial defence and courtship by dominant males. This loss of control entails a change in

the form of sexual competition from resource competition to sperm competition, with an apparent decrease in variance among males in mating success through increased numbers of sperm releases by smaller males. Both forms of competition may result in sexual selection but not necessarily for the same traits.

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