Sexual signaling in the European bitterling: females learn the truth by direct inspection of the resource

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In many taxa females appear to base their mate choice on multiple traits. But the relative importance of different traits in mate choice has rarely been determined. Here we show that females of a freshwater fish, the European bitterling, *Rhodeus sericeus,* base their mate choice on multiple traits that differ in their reliability as indicators of expected reproductive success and are used at different stages of the decision process. The initial decision to inspect a male is based on male behavior and red coloration, whereas the final spawning decision is based on the quality of the live unionid mussel, *Anodonta anatina,* that the male is defending as an oviposition site. Male traits may indicate which males are worth inspecting by reflecting male quality, such as reproductive condition and genetic constitution. Male traits do not, however, reflect mussel quality, as bright males also court females vigorously toward mussels that yield a low probability of survival of the offspring. Females, on the other hand, are choosier than males in their choice of spawning site and seem to gain reliable information about the survival probability of the eggs by inspecting the mussel directly. *Key words:* bitterlings, mate choice, multiple ornaments, reliable signaling, resource quality, *Rhodeus sericeus,* sexual selection. *[Behav Ecol 12:407–411 (2001)]*

Sexual signaling involves a conflict of interest as the signaler benefits from misleading the receiver as to its mate quality whereas the receiver gains by accurately assessing the quality of the signaler (Johnstone, 1997). Different male traits are used as signals in mate attraction, and in some cases there is evidence for the traits reflecting direct or indirect benefits of mating with a male (reviewed by Andersson, 1994; Johnstone, 1995). An increasing number of studies show, however, that females of some species also use the quality of the resource that the male is defending as a cue in mate choice (e.g., Alatalo et al., 1986; Balmford et al., 1992; Carranza, 1995; Goulet, 1998). Generally, it can be expected that the importance of inspecting the resource would increase with the influence of the resource on the number or quality of offspring produced. This is especially likely because male traits may be subjected to some cheating even in a stable system (Johnstone and Grafen, 1993), whereas the quality of the resource may be difficult to disguise.

Females may use multiple signals in mate choice if different signals reflect different properties of male quality (the multiple message hypothesis) or if they provide accurate overall assessment of male quality (the back-up signal hypothesis; Iwasa and Pomiankowski, 1994; Johnstone, 1996), or if the use of multiple signals facilitates detection of the male or takes advantage of arbitrary preferences (Johnstone, 1997). However, few studies have investigated the relative importance of different signals in female mate choice. A complicating factor in studies of multiple signaling is that the level of expression of different traits may be correlated within individuals. The quality of the resource may be correlated with male phenotypic traits if, for example, competition among males determines the quality of the resources that the males can defend. In three-spined sticklebacks, for example, males with concealed

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nests have a higher mating success (Sargent, 1982; Candolin and Voigt, 1998; Guderley and Guevara, 1998; Kraak et al., 1999), but it is not known whether this is due to males that are attractive to females occupying the best nest sites or to females preferring concealed nests.

The European bitterling (*Rhodeus sericeus,* Cyprinidae) is a freshwater fish in which the quality of the resource is of crucial importance for successful reproduction but can easily be manipulated. Males typically defend one or more living freshwater mussels (Unionidae) and attract females to spawn in the mussels. The males develop nuptial coloration consisting of red-colored fins and eyes. The intensity of the red color shows large variation among males, and the color of the fins can change within a few minutes during courtship or when confronted with a competing male (Candolin U, personal observation). Males court females with a distinctive quivering display that draws females toward mussels. Females then inspect mussels by pointing their heads down over the exhalent siphon of the mussel, presumably picking up cues about the state of the mussel. If they accept the mussel, they lay two to four eggs at a time into the gills of a mussel by rapidly inserting a long ovipositor into the exhalent siphon of the mussel (Heschl, 1989; Wiepkema, 1961). Males fertilize the eggs by releasing sperm over the inhalent siphon both before and after female spawning. Females spawn repeatedly, and several females may spawn in one mussel. Eggs hatch in 1–2 days, and the embryos remain inside the mussel for 3–6 weeks, depending on temperature (Reynolds et al., 1997). The survival of the offspring depends on both the species of the mussel and on the density of embryos already present in the mussel, with the mortality rate increasing with the density of embryos (Smith et al., 2000). It is therefore important for the female to be able to determine the quality of the mussel. Females have been found to avoid spawning in species of mussels with a high density-dependent mortality rate and in mussels that already contain large numbers of embryos (Smith et al., 2000). It appears that females are able to determine the quality of the mussel by inspecting the water flowing from the exhalent siphon.

We investigated the relative importance of mussel quality

and male traits as cues in female spawning decisions. Females have a lower potential reproductive rate than males, and they may therefore be choosier than males in their choice of spawning site. Moreover, as the last eggs spawned into a mussel suffer most from density-dependent mortality (Mills and Reynolds, unpublished data), males may have little to lose from attracting females to a mussel that contains a large number of embryos independent of their paternity of the embryos. We determined whether male behavior and coloration honestly indicate the number of embryos present in a mussel. If a male does not adjust his signaling level to the quality of a mussel, a female might benefit from basing her mate choice on direct inspection of the mussel.

METHODS

Mussels, *Anodonta anatina,* were collected from Reach Lode, a slow-flowing canal that feeds into the River Cam in Cambridgeshire, England (see Reynolds and Guillaume, 1998; Reynolds et al., 1997). They were collected in mid-March 1999 before the onset of the spawning season to ensure that they did not contain any bitterling embryos. The mussels were transported to the University of East Anglia and kept in outdoor pools. Bitterlings were collected from the same site as the mussels by electrofishing in mid-April 1999 and kept indoors in large aquaria (120×60 cm, 40 cm high) at the university. No damage to the fish from electrofishing was noticed, and all fish remained healthy when brought to the laboratory. The temperature for the fish was increased gradually from $15°$ to $22°$C, and the light cycle was set to match the natural cycle so that the fish would enter reproductive condition. Bitterling females with developing ovipositors were put into aquaria containing mussels and males in breeding condition to stimulate the females to develop their ovipositors to full size (as long as to the end of the tail). We covered the mussels at night to prevent females from spawning in the mussels if they developed the ovipositors overnight.

To determine whether males adjust their behavior and coloration to mussel quality and to determine the relative importance of male traits and mussel quality to female spawning decisions, males were allowed to attract females to spawn in a high-quality (empty) mussel and a low-quality mussel (containing embryos from 8–10 spawnings by other males and females) in alternating order. We assigned mussels to the low-quality treatment after we had chosen them randomly from pairs of mussels that were size matched for body length (± 2 mm). We then exposed them to spawning by males and females that were not used for the subsequent behavioral test (size of mussels without embryos: mean $\pm$ SD $= 93.2 \pm 8.5$ mm; mussels with embryos: 92.9 ± 9.0, paired t test, $t_{13} = 0.69$, $p = .5$). Mussels sometimes ejected eggs and embryos, and these were replaced through new spawnings. The eggs in the mussels were 1–4 days old when the mussels were given to the males. Each experimental male was given a new pair of mussels so that no mussel was used more than once.

To run the experiment, a randomly selected male in breeding condition (i.e., with nuptial coloration) was put into an aquarium (60×60 cm, 40 cm high) with an undergravel filter system and artificial vegetation and containing either an empty mussel or a mussel that contained embryos. After at least 1 day of acclimatization, a female with a fully developed ovipositor was put into the aquarium, and the behavior of the male and the female and the red coloration of the male were recorded as described below. If a female did not try to spawn within 15 min after she had first inspected a mussel, we stopped recording. In the field, inspections last a much shorter time than 15 min (Reynolds JD, personal observation), and females that did not spawn within this time were assumed not

to want to spawn in the mussel. These females were capable of spawning; they all spawned when they were later used for a different purpose (i.e., filling mussels to be offered to other pairs of fish).

After recording, we measured male red coloration as described below, and the mussel and the female were removed from the aquarium. One to three days later, the male was given the other mussel, and a new female with a fully developed ovipositor was put into the aquarium. We recorded male and female behaviors as described below, and the red color of the male was again determined. The experiment was repeated for 14 males; half of the males were first given an empty mussel followed by a mussel containing embryos, and the other half received mussels in reverse order. This yielded a paired design for males. Different females were used each time.

Behavioral observations

For males, we recorded the time until start of courtship quivering, time until first sperm release (which often occurs before spawning), and number of sperm releases until first spawning attempt by the female. For females, we recorded time until first inspection of the mussel and time until first spawning attempt. Not all female spawning attempts lead to successful spawning. Depending on the opening of the siphon, a female may try several times before she succeeds in getting the ovipositor into the mussel.

Time until inspection of a mussel without embryos was square-root transformed to achieve normality. The same results are found if time until inspection of mussels with embryos are square-root transformed as well.

Measurement of red coloration

We determined the coloration of the male by photographing the male under standardized conditions (Candolin, 1999) immediately after spawning or after 15 min had passed since the female first inspected the mussel in cases where the female did not attempt to spawn. The male was dip-netted and put into a small, water-filled glass box ($6 \times 3 \times 6$ cm) that was placed in fixed position in a large dark-box with a lock that excluded outer light. The left lateral side of the male was photographed with a digital camera (Kodak DC 50) with a lateral flash as the only light source. To make sure that the lighting conditions were constant, three plastic strings of red, blue, and green colors on the front side of the glass box served as color references in the analyses. The photography procedure took only a few seconds, and the male was released back into the aquarium afterward. We measured red coloration with image analyzing software (Sigma Scan Pro, 4.0). Five pixels were selected randomly on the anal fin, and five pixels were selected in the eye, and their average red index, R (Frischknecht, 1993), was calculated for the fin and the eye by dividing the brightness value (0–255) of the red component by the sum of the red, blue, and green component values. We measured the intensity of red coloration instead of the area of red because changes in red coloration occur through changes in the intensity of red (Candolin U, personal observation).

RESULTS

Males did not change their behavior or coloration depending on the presence of embryos in a mussel (Table 1). However, male behavior and coloration did influence the time until a female first inspected a mussel: the time decreased the sooner the male had deposited sperm into a mussel and the more

Table 1

Male behaviors and coloration (mean ± SE) when courting a female to a mussel without embryos and to a mussel with embryos

Variable	Without embryos	With embryos	t_{13}	p
Time until quivers (s)	20.2 ± 4.6	20.6 ± 4.9	0.06	.96
Time until sperm release (s)	51.2 ± 13.1	55.4 ± 14.7	0.21	.84
No. of sperm releases/min	1.12 ± 0.13	1.09 ± 0.15	0.19	.85
Fin red color	0.398 ± 0.016	0.397 ± 0.015	0.08	.94
Eye red color	0.543 ± 0.024	0.547 ± 0.019	0.14	.89

Paired t tests were used for analyses.

colorful he was (Table 2). Note that red coloration of the fin showed only a tendency to influence female behavior when the mussel was empty. Females never inspected a mussel or showed any interest in a male before the male had started to quiver and release sperm.

Male traits had no significant effect on the probability that a female would attempt to spawn (Table 2). For mussels without embryos, logistic regression of probability of spawning against male traits could not be carried out because all but one female spawned. There was also no significant relationship between male traits and the time between inspection and first spawning attempt, though the number of replicates was smaller for this analysis (Table 2). Instead, the probability of a spawning attempt was determined by the presence of embryos in the mussel: females avoided spawning in mussels containing embryos (Figure 1a; $\chi^2 = 4.76$, df = 1, $p = .029$), and when they did spawn, females spawned sooner in mussels that lacked embryos than in ones containing embryos (Figure 1b; t test for unequal variances, $t_{9,2} = 4.41$, $p = .002$). In three cases, eggs were ejected by the mussel after spawning. Ejected eggs were always the newly deposited ones, and it was always from mussels that already contained embryos.

DISCUSSION

Bitterling females used multiple signals in their spawning decision, with different signals being used at different stages of the process. Male behavior and coloration influenced the initial decision to inspect a mussel, but mussel quality determined whether the female tried to spawn in the mussel. The relationship between male traits and female inspection behavior was most likely due to females responding to male traits and not to males responding to differences in female willingness to spawn, as females did not inspect mussels before being courted by the male.

Females may have used male traits as cues in the initial decision of whether to inspect a mussel because the traits may indicate male quality. Mate choice most likely carries costs in terms of time and energy expenditure and perhaps increased predation risk (Jennions and Petrie, 1997; Reynolds and Gross, 1990), and a female may therefore only approach a male and inspect his mussel if this is likely to result in successful reproduction with a high-quality male. Male traits may here signal direct benefits to the female, such as that the male is in breeding condition and able to fertilize the embryos, or

Table 2

Multiple linear and logistic regression models of the dependence of female spawning decisions on male traits

| Source	Without embryos		With embryos	
	t	p	t	p
Time until inspection (linear regression)				
Time until male quivers (s)	−0.46	.657	−1.85	.102
Time until sperm release (s)	2.69	.027	12.03	<.001
No of sperm releases/min	−0.93	.378	1.59	.151
Fin red color	−2.20	.059	−4.04	.004
Eye red color	1.07	.317	0.81	.442
Whole model	$F_{5,8} = 8.34$, $r^2 = .84$, $p = .005$		$F_{5,8} = 52.38$, $r^2 = .97$, $p < .001$	
Probability of spawning attempt (logistic regression)				
Time until male quivers (s)			0.03	.978
Time until sperm release (s)			−0.62	.537
No of sperm releases/min			0.32	.745
Fin red color			0.64	.525
Eye red color			0.09	.928
Whole model			$\chi^2 = 4.56$, df = 5, $p = .47$	
Time from inspection to spawning attempt (linear regression)				
Time until male quivers (s)	−1.44	.193	2.97	.097
Time until sperm release (s)	−0.20	.848	−2.09	.172
No of sperm releases/min	1.01	.344	−1.39	.300
Fin red color	−1.72	.129	1.50	.272
Eye red color	−1.56	.129	−1.53	.265
Whole model	$F_{5,7} = 1.51$, $r^2 = .52$, $p = .30$		$F_{5,2} = 2.93$, $r^2 = .88$, $p = .27$	
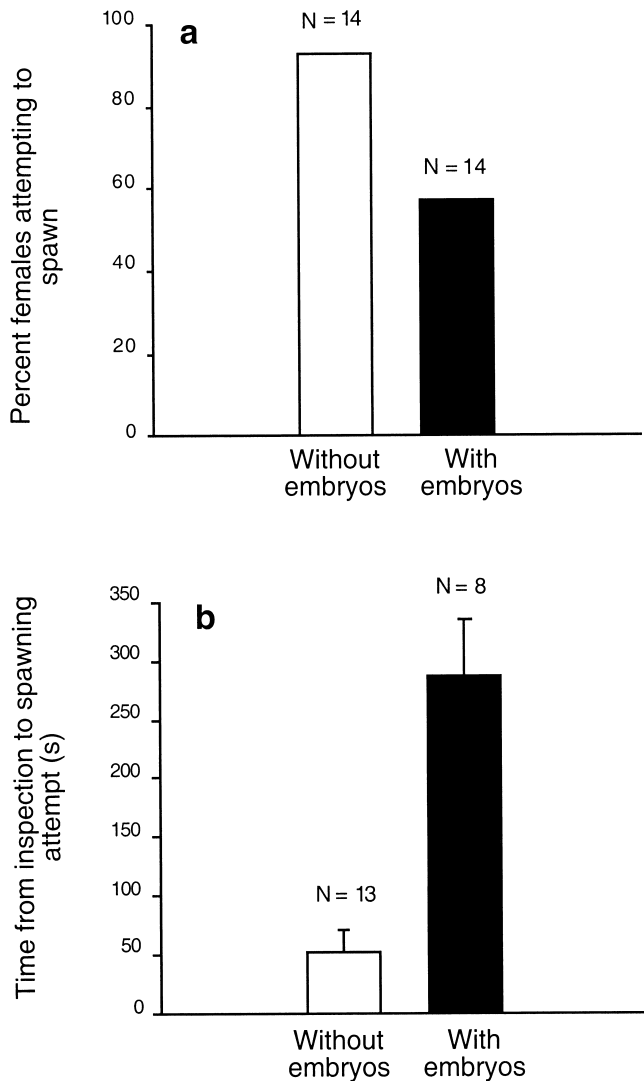
Figure 1
(a) Proportion of females that attempted to spawn in a mussel within 15 min of having inspected the mussel and (b) mean time (+ SE) elapsed between first inspection of a mussel and first spawning attempt, when the mussel did not contain embryos and when it did.

the traits could reflect additional genetic benefits to the young. In particular, the red carotenoid-based coloration of the fins may reflect the nutritional condition and quality of the male, as has been suggested for other species with carotenoid-derived coloration (Hill and Montgomerie, 1994; Kodric-Brown, 1989; Milinski and Bakker, 1990).

Male traits may, however, not reflect mussel quality. When males were given mussels of different quality, they did not adjust their courtship behavior or red coloration to the quality of the mussels. A female may therefore need to inspect the mussel directly to determine the quality of the mussel and the survival probability of the eggs. Males are probably less choosy then females in their choice of spawning site because of their higher potential reproductive rate, and they may continue to attract females to a mussel as long as the marginal benefits of attracting females to the mussels are higher than the marginal benefits of searching for other empty mussels. This requires that some females spawn in low-quality mussels, which might be the case if females differ in their choosiness due to differences in the costs of searching for better mussels.

In the field, male–male competition might increase the correspondence between male traits and mussel quality if males compete for the best mussels and the expression of the traits matches male dominance and fighting ability. In several species male traits have been found to be used both as cues in mate choice and as status or threat signals in male-male competition (Berglund et al., 1996). It is possible that the traits of the bitterling male also play this dual role in sexual selection and that a male with a large trait expression usually defends the best mussels. However, the correlation between mussel quality and male quality may decrease as the defended mussels become filled with embryos. At the peak of the spawning season, all mussels of the species used here (*A. anatina*) greater than approximately 75 mm in length contain bitterling embryos (Reynolds and Guillaume, 1998). Three other species of mussels are available, two of which are used regularly (*Unio pictorum* and *U. tumidus*), and one of which is avoided (*Anodonta cygnea*; Reynolds et al., 1997; Smith et al., 2000). The costs to the male of searching for high-quality mussels with a low number of embryos may therefore become high as the best mussels become filled. A possibility remains that males change their traits with time when they are aware of the number of females that have spawned in the mussel or when the number of females that reject the mussel increases.

In conclusion, this study shows that bitterling females use multiple cues in mate choice, with different cues being used at different stages of the decision process. The cues vary in their value as indicators of the mortality risk of the eggs, and the final spawning decision is based on the cue that most reliably reflects the survival probability of the eggs.

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