Inbreeding Affects Female Preference for Symmetry in Computer-Animated Sticklebacks

Dominique Mazzi,[1,4] Reto Künzler,[1] Carlo R. Largiadèr,[2] and Theo C. M. Bakker[3]

Fluctuating asymmetries are small random deviations from perfect symmetry in bilateral traits caused by the inability of individuals to cope with stress during development. The degree of asymmetry of secondary sexual characters is supposed to convey information about a male's phenotypic and/or genetic quality, and females are thus expected to use bilateral symmetry as a cue in mate choice. We offered female three-spined sticklebacks (*Gasterosteus aculeatus* L.) that had been inbred for one generation and outbred control females the choice between computer-animated male models differing exclusively in the symmetry of their pelvic spines. Inbred females exhibited a significantly stronger preference for the symmetric model than outbred females, suggesting that females of relatively poor quality are more prepared to pay the costs of choosiness and obtain higher marginal benefits from their discrimination than females of better quality.

KEY WORDS: Computer animation; fluctuating asymmetry; *Gasterosteus aculeatus;* inbreeding; sexual selection; three-spined sticklebacks.

INTRODUCTION

Fluctuating asymmetries (FA) are subtle random deviations from perfect symmetry in bilateral traits caused by the inability of individuals to buffer their development against disruptive genetic and/or environmental perturbations (Ludwig, 1932; Palmer and Strobeck, 1986; Van Valen, 1962). Because the development of a trait on both sides of the body is the product of the same genome, deviations from perfect symmetry represent an epigenetic measure of developmental homeostasis, reflecting the ability of individuals to withstand accidents occurring during development (Waddington, 1942). The often reported negative association between

FA and various fitness parameters (reviewed in Møller, 1997; but see also Clarke, 1998; Møller, 1999) has led to the hypothesis that FA is used in signaling contexts as a surrogate measure of individual quality (Møller, 1990). Secondary sexual characters are particularly sensitive to the effects of stress (Møller and Pomiankowski, 1993; Watson and Thornhill, 1994)—supposedly as a consequence of a recent history of persistent directional selection and condition-dependent expression (Møller and Pomiankowski, 1993)—and their FA therefore is especially suited as a predictor of individual performance. If the degree of asymmetry of secondary sexual characters conveys information about the overall quality of potential mates (Møller, 1990), females are expected to use bilateral symmetry as a criterion in their mate choice decisions, and females mating with symmetric partners may benefit in terms of direct and/or indirect advantages. Recently, a number of observational studies have advocated a role for the symmetry of ornaments in sexual selection (Møller and Thornhill, 1998). However, in order to infer that females actually rely on symmetry *per se* for assessment of male quality, rather than merely on correlates

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[1] Division of Behavioural Ecology, Institute of Zoology, University of Bern, CH-3032 Hinterkappelen, Switzerland.

[2] Division of Population Genetics, Institute of Zoology, University of Bern, CH-3012 Bern, Switzerland.

[3] Institute for Evolution and Ecology, University of Bonn, D-53121 Bonn, Germany.

[4] To whom correspondence should be addressed at Department of Biological and Environmental Science, University of Jyväskylä, P. O. Box 35, FIN-40014 Jyväskylä, Finland. Tel: +358 14 2604239. Fax: +358 14 2602321. e-mail: domazzi@cc.jyu.fi

thereof, experimental manipulation of trait asymmetry independently of other confounding variables is essential. Practical difficulties have often precluded a conclusive interpretation of manipulative studies, for the manipulation either resulted in differences far larger than those naturally occurring or simultaneously affected other potential aspects of quality (e.g., Bennett *et al.,* 1996; Møller, 1992; Swaddle and Cuthill, 1994). We circumvented the pitfalls of an experimental approach to the issue of female preference for symmetry by using computer animations, an innovative noninteractive test paradigm that allowed us a rigorously standardized and repeatable presentation of a pair of male stimuli that were identical in all respects but the symmetry of their pelvic spines.

Pelvic spines are one of several bilateral structures (e.g., pectoral fins, armor plates) whose symmetry is expected to be favored by natural and/or sexual selection in sticklebacks. Male three-spined sticklebacks, *Gasterosteus aculeatus,* often erect their pelvic spines during courtship (Symons, 1965), and the characteristic zig-zag dance performed by courting males may act as an amplifier (Hasson, 1989; Hasson, 1990) giving females the opportunity to assess simultaneously both sides of a bilateral trait.

Both observational and experimental evidence indicate that pelvic spine symmetry is subject to sexual selection by female choice in sticklebacks. First, in the field, spine symmetry is associated with reproductive success measured in terms of eggs present in the nest of caring males, and males with longer spines exhibit lower degrees of FA (T. C. M. Bakker, S. B. M. Kraak, and D. Mazzi, unpublished data), a common pattern for secondary sexual characters (Manning and Hartley, 1991; Møller, 1990; but see also Hunt and Simmons, 1997, 1998). Moreover, in an earlier study (Mazzi *et al.,* 2003), we independently and simultaneously manipulated the length and the relative symmetry of the pelvic spines of computer-animated male models. We created four virtual male variants differing in either the length, the relative symmetry, or both the length and the relative symmetry of their spines (whereby realistic values of both variables were used) and tested females for their preference in the six possible pairwise combinations of male models. In all comparisons involving a difference in relative spine symmetry, females spent more time in close association with the symmetric of the two models, regardless of differences in spine length. However, differences only reached statistical significance at the $\alpha = 0.05$ level when both models had short spines, that is, when a model with short symmetric spines was opposed to a model

with short asymmetric spines. The differences were only marginally significant ($0.05 < p < 0.08$) for the other three combinations where relative spine symmetry distinguished the models, that is, a model with long symmetric spines *versus* a model with long asymmetric spines, a model with long symmetric spines *versus* a model with short asymmetric spines, and a model with short symmetric spines *versus* a model with long asymmetric spines. Female choice was random when only spine length was available as a discriminatory criterion, that is, a model with long symmetric spines *versus* a model with short symmetric spines, and a model with long asymmetric spines *versus* a model with short asymmetric spines.

In this study, we tested females that had been inbred for one generation and control outbred females for their preference for symmetric male spines. Inbreeding (i.e., matings between close kin) reduces fitness in most species of plants and animals, a phenomenon known as inbreeding depression (Charlesworth and Charlesworth, 1987; Darwin, 1876; Wright, 1921). Inbreeding increases the proportion of polymorphic loci at which an individual is homozygous (Wright, 1921) and leads to the unmasking of recessive deleterious alleles (East and Jones, 1919). Both heterozygote advantage and the fixation of harmful mutations will reduce fitness, though their relative contribution to the decline in vigor observed in the offspring of consanguineous is still a matter of controversy (Charlesworth and Charlesworth, 1999). Here, inbred and outbred females are assumed to differ in quality as a result of the difference in the magnitude of the genetic stress imposed on them during development. We offered the two sets of females a choice between two computer-animated courting males differing exclusively in the symmetry of their pelvic spines in order to assess whether the quality of the choosing females influences the costs and/or benefits of mate selectivity and thus the strength of the preference for an *a priori* high-quality partner.

MATERIALS AND METHODS

The females were second-generation (F_2) laboratory-reared descendants of fish from a large and genetically heterogeneous anadromous population collected during the 1998 spring migration on the island of Texel (The Netherlands). The parental wild fish were transported to the facilities of the University of Bern in Hinterkappelen (Switzerland), where they were housed individually in 10 L plastic aquaria ($33 \times 18 \times 19$ cm, water level 15.5 cm) with a 3-cm-deep gravel layer. Continuous flow from a well kept the water temperature in the tanks at

16°C to 19°C. Males were additionally supplied with filamentous algae as nesting material. Neighboring aquaria were separated by gray opaque partitions to prevent visual interactions. The tanks were illuminated by 36-W fluorescent tubes mounted ca. 15 cm above the water surface timed to turn on at 0700 and off at 2300 hours.

F_1 sibships were obtained by crossing sexually mature wild fish at random. Egg clutches were removed from the male's nest 1 hour after fertilization and placed in aerated plastic beakers with water from a well at 17°C refreshed twice a day. Following hatching, in spring 1998, each full-sib group was evenly distributed between two 10 L aquaria held under summer conditions (16L:8D, water from a well at 16°C to 19°C) and spatially interspersed in random order. Group densities were regularly reduced and equalized by indiscriminately removing some of the fish with a small net in order to standardize the effects of social interactions during rearing. Fry were fed for the first 3 days on infusoria cultures, then switched to a diet of live *Artemia* nauplii, and later of frozen *Artemia* and chironomid larvae. Fish were fed *ad libitum* twice daily. From December 1998 to March 1999, conditions were switched to winter (water temperature 3°C to 6°C, 8:16 hours light:dark regime, food in excess once every 2 days). In spring 1999, a random sample of 6 fish (three per rearing tank) for each of 20 F_1 sibships were individually isolated as described for the parental generation. Unrelated F_1 fish were paired at random to produce full-sib clutches of outbred offspring, whereas inbred lines resulted from full-sib matings. All fish contributed offspring to one sibship only. F_2 fish were raised following the standardized procedure outlined above. In December 1999, 10 fish per tank (i.e., 20 per sibship) were transferred to winter conditions (described above).

In June 2000, 1 female from each of 19 outbred and 19 inbred F_2 sibships was randomly selected for the preference tests. Females were individually marked by clipping of the dorsal spines and housed in groups of three or four in 20-L plastic aquaria (water temperature 14 ± 1°C, 16:8 hours light:dark regime). They were fed to satiation with frozen chironomid larvae or frozen *Artemia* three times a day to promote egg maturation.

Ripe females, as assessed by the distension of the abdomen and the opening of the cloaca, were selected daily for the preference tests. They were hand-netted and gently released in a 1-L container ($12 \times 7 \times 17$ cm, water level 8.6 cm, corresponding to the height of the test movies displayed on the computer screen) made of clear polyacryl plastic with all the inner walls painted dull gray but for a spare front window (9.5×8 cm). Females were transferred to the test room (room temperature 18 ± 1°C), where they were allowed 30 minutes of acclimatization in a pretest compartment, without computer display, but with the same lighting conditions as in the test chamber. The females were transferred from the holding room to the (warmer) test room in a container with water from their holding tanks in order to avoid abrupt temperature changes. Previous studies using the same test paradigm suggest that the acclimatization time allowed here is enough to evoke sound behavioral decisions (e.g., Künzler and Bakker, 2001; Mazzi *et al.,* 2003). Illumination was provided by a fluorescent tube (18 W/33) mounted 35 cm over the female container and dimmed with sheets of white paper. After acclimatization, the test tank with the female was put in front of the computer screen displaying a still empty scene of a marbled brown mud layer on a green-yellowish background, mimicking the natural breeding ground. The empty scene was shown for 5 minutes to familiarize the female with the test situation. The female was then shown a still image featuring the two males in the back of the scene, at their nest positions. As the males were oriented sideways relative to the choosing female, with their spines retracted, the female was unable to make out any difference between the stimulus males at this time. A gray opaque PVC partition between the middle of the female container and the computer display prevented the female from seeing the left half of the scene when staying in the right half of the container and *vice versa.* Trials were viewed remotely via a video camera mounted above the test container connected to a monitor outside the test chamber. On the monitor, pieces of tape divided the top-view of the female container in two (right and left) thin front sections measuring 4.2×0.7 cm each, and a neutral back section. Movie playback began after the female had spent at least 2 seconds in each front section and had regained the neutral section. All females entered the three sections within 2 minutes of first males' presentation.

Females were given a choice between two identical copies (mirror-images produced by horizontally flipping the original) of a courting stickleback male model differing exclusively in the symmetry of their pelvic spines. The construction of the 3D model and its animation are extensively detailed in Künzler & Bakker (1998). Briefly, a representative male three-spined stickleback was poisoned, fixated, and cast with epoxy resin. The casting was cut into thin slices, scanned and digitized. Vectorized outlines were imported in a 3D program and lined up to the body's length axis.

A colored texture was wrapped around the body outline. Spines and eyes were constructed and attached to the body at adequate positions, along with the scanned fins. The model's motion pattern was based on the path of a courting real male video-recorded from above. A standard courtship sequence, including a sequence featuring fanning bouts at the nest, was produced, and a test movie consisting of variable repeatments of the different parts of the standard sequence (e.g., zig-zagging toward the front of the scene, swimming back to the nest, fanning) was composed.

For this study, the original model with symmetric spines of average length described in Künzler and Bakker (1998) was opposed to a model whose left spine had been elongated by 5%, the right spine shortened by 5%, relative to the original length. The spines are sketched in Figure 1. Relative asymmetry values of 10% as obtained by such scaling fall within the range of natural variation observed in this population. Asymmetries of 10% of the size of a trait are large though, and reflect the extreme values in a sample of wild males, with 2 out of 45 measured specimen exhibiting as large or larger asymmetries in spine length (see Mazzi *et al.,* 2002 for details on the natural variation in asymmetry). The spines of the models used in this study are of intermediate length when compared to the values in Mazzi *et al.* (2003), whereas the relative asymmetry values are identical. Furthermore, the original model had its pelvic spines animated so that it raised and retracted them continuously while courting. The dorsal spines moved synchronously. The models had their spines raised at each turning point between zigs and zags, that is, whenever facing the choosing female. Males regularly erect their spines at the angular turns of their courtship path, as inferred from video recordings of live courting males. Full details of the test procedure are given in Künzler and Bakker (1998).

Male positions were alternated between tests. Females were coded with respect to family group, so that the investigator (D. M.) was unaware of the females' genetics at the time choice tests were run. To ensure sexual receptivity, only females that spawned with a live male within 24 hours of the test were considered for analyses. All females were used only once. The choice trials (of a duration of 2 minutes 25 seconds) were recorded from above, and the recordings analyzed *a posteriori* to avoid observer bias. Preference for symmetry was evaluated as the proportion of time spent oriented toward the symmetric model

A condition factor was calculated as $100 \times$ mass (g)/standard length $(cm)^{2.84}$, whereby the exponent in the formula is the slope of the regression of $\log_{10}$(mass) on $\log_{10}$(length) (Bolger and Connolly, 1989). The measure of condition was uncounfounded by egg mass, as females were weighed immediately after having spawned. Six females (five inbred and one outbred) that ate part or all of the spawned egg were omitted from all analyses involving condition.

The recorded variables were checked for departures from normality and homoscedasticity by using the Shapiro–Wilk *W* test (Shapiro and Wilk, 1965) and Bartlett's test (Bartlett and Kendall, 1946), respectively. As the assumptions of parametric statistics were satisfied, we performed one-sample *t* tests on the preference data for the two separate sets of females to test for the deviation from the null expectation of no preference. The effect of the females' genetic background was quantified by one-way analysis of variance (ANOVA) on the recorded preference indices. Significance levels were Bonferroni-adjusted to account for multiple related tests. Given *p* values are two-tailed throughout. Power analyses were done following Cohen (1988). All statistical analyses were conducted using the JMP IN, v. 4.0.3 (SAS Institute, 2000) package.
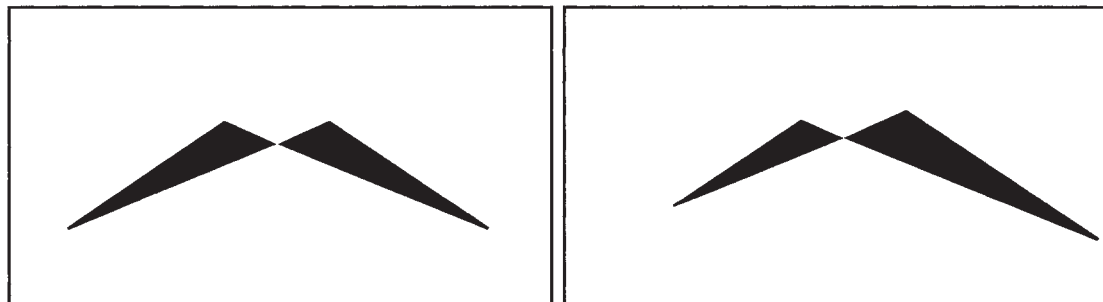

Fig. 1. Schematic representation of the pelvic spines attached to the virtual model of a courting three-spined stickleback male used in the female choice trials.

RESULTS

Inbred females spent a significantly larger proportion of time in close association with the symmetric of the two models than did outbred females (mean proportions of time $\pm$ SD, inbred: 0.60 ± 0.17, outbred: 0.42 ± 0.19; one-way ANOVA, $F_{1,36} = 9.97$, $p = 0.003$; Fig. 2). Inbred females significantly preferred the symmetric male model to the asymmetric one (one-sample t test, $t = 2.66$, df $= 18$, $p = 0.016$), whereas the response of outbred females did not significantly deviate from the null expectation of no preference ($t = -1.87$, df $= 18$, $p = 0.08$). Adjusting the experimentwise significance level to correct for related tests does not alter the above conclusions, either for the effect of the females' genetic constitution on the strength of their preference or for the preferences of the two separate sets of females.

Inbred and outbred females did not significantly differ in their physical condition ($F_{1,30} = 0.20$, $p > 0.6$) nor in the weight of the eggs they spawned ($F_{1,30} = 0.02$, $p > 0.8$). Female responsiveness as measured by the proportion of time spent oriented toward the male models did not significantly vary depending on the females' genetic make-up ($F_{1,36} = 0.71$, $p > 0.4$). We did not detect any significant relationship between the preference index and body condition, egg
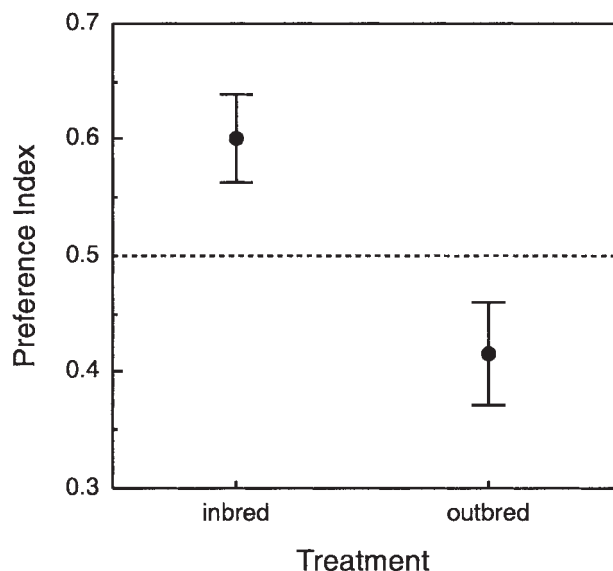

Fig. 2. Mean ($\pm$SE) preference for pelvic spine symmetry for inbred ($n = 19$) and outbred ($n = 19$) three-spined stickleback females given a choice between a computer-animated model of a courting male with symmetric spines and a model with asymmetric spines. The dotted line corresponds to the null expectation of no preference.

load mass, or responsiveness, either within inbred or within outbred females (all $p > 0.1$).

DISCUSSION

Moderate inbreeding—as imposed by one generation of full-sib matings only—had a significant effect on the preference for symmetry of female three-spined sticklebacks. Given the choice between two courting virtual male models differing exclusively in the symmetry of their pelvic spines, inbred females exhibited a significantly stronger preference for the symmetric model than did outbred females. Whereas inbred females significantly favored the symmetric model over the asymmetric one, outbred control females did not significantly discriminate between the two stimuli.

Condition-dependence of male sexual ornaments is well-established and has been invoked as an explanation for the unexpectedly high genetic variance of such traits, in spite of constant directional selection imposed by female choice, and of their close relation to fitness (Pomiankowski and Møller, 1995; Rowe and Houle, 1996). Given a cost to evaluating male quality, aspects of quality can also account for variation in female mating preferences (Bakker, 1999; Bakker *et al.,* 1999). With increasing costs of mate sampling and choosing, females should become less discriminatory (Milinski and Bakker, 1992; Real, 1990). Costs associated with mate sampling are thus a potential constraint on optimal mate choice (Gibson and Bachman, 1992). If only high-quality females can bear the costs of choosiness, positive assortative mating with respect to quality is predicted (Møller, 1994). Conversely, if the payoffs to be derived from selective mate choice differ depending on female quality, females of relatively poorer quality are expected to be more motivated to compensate for their own lacks, and display a stronger preference for high-quality mates (e.g., McLennan and Shires, 1995).

We found no significant difference in the condition of inbred and outbred females, as measured by our condition index. The lack of a difference may likely stem from our feeding regime. The females were kept in small groups and fed several times a day in order to ensure a constant supply of ripe females ready for testing. Leftovers were always present at the bottom of the tanks (personal observation), suggesting that the food was available in excess and thus there was no potential for competition. The finding that five (out of 19) inbred females ate part or all of the eggs they spawned whereas only one (out of 19) outbred female did is

nonetheless curious, as an increased tendency to consume eggs would have more far-reaching fitness consequences than most mating decisions. However, the sample sizes involved do not allow conclusive evidence on the matter, which thus does not deserve more that anecdotic character at the present time.

The symmetry of morphological bilateral traits has been attributed a function as a honest indicator of quality in sexual selection, providing females with accurate information about a male's underlying genetic and/or phenotypic qualities (Møller, 1990). Inbred females should have a keen interest in improving the quality of their offspring and should therefore be particularly responsive to cues of good genes and/or valuable resources.

A number of studies have reported a negative association between FA and heterozygosity (Mitton, 1993; but see Vøllestad *et al.,* 1998). In three-spined sticklebacks, one generation of inbreeding has been reported to lead to elevated levels of FA in pectoral fins, albeit not in pelvic spines (Mazzi *et al.,* 2002). However, we cannot rule out that the symmetry of the pelvic spines is immune to such genetic stress, nor that females use the asymmetry of one given trait to assess the asymmetry of correlated traits. Heterozygosity is generally beneficial to individuals (Avise, 1994), and females would gain by choosing a mate so that genes in their offspring will be heterozygous at some or many loci (Brown, 1997). Charlesworth's (1988) model of the evolutionary dynamics of female mate choice with respect to the male's genotype at a single diallelic locus revealed that the fitness of progeny of heterozygous males exceeds that of the population as a whole, thus favoring a preference for mating with heterozygotes (at least under weak natural selection). Heterozygous parents produce higher proportions of heterozygous progeny than do homozygous parents at most allelic frequencies (Mitton *et al.,* 1993). Females paying attention to a hypothetical phenotypic indicator of heterozygosity would thus produce genetically diverse offspring that are better competitors by virtue of their heterozygosity (Brown, 1997), and the benefits derived from improved selectivity ought to be higher for inbred than for outbred females.

An analogous argument can be made when nongenetic benefits are considered. In sticklebacks, a male's parental ability crucially affects reproductive success, as the male alone cares for the offspring for about 2 weeks by oxygenating the eggs and defending them against predation (Wootton, 1976). Parental effort is energetically costly and may reduce the chances of surviving to attempt further nesting cycles (Smith and Wootton, 1999). Parental care also exposes males and their nests to heavy inter- and intraspecific predation (Moodie, 1972). By relying on bilateral symmetry as an indicator of quality in their choice of mates, females may select better fathers, and again relatively higher direct benefits may accrue on choosy inbred females if, for instance, their eggs require superior parental care.

The lack of preference for the *a priori* high-quality male of outbred females is puzzling, given that we have already documented a preference for symmetry in an analogous choice experiment (Mazzi *et al.,* 2003), and begs the question of why do not all females prefer the better male to some extent. However, in the aforementioned study, 30 females were tested for each combination of spine length and spine symmetry. With a sample of only 19 females, constrained by the number of families available for the tests, the chance of detecting a preference of the magnitude found earlier is only about 50%. Additionally, the prolonged holding under winter conditions, combined with the artificiality of the test situation (see Kodric-Brown and Nicoletto, 1997, for a comparison of female responsiveness in various test paradigms), may have led to a generalized attenuation of the expressed preference.

The adequacy of video-playback techniques in behavioral research has recently been challenged (Oliveira *et al.,* 2000 and references therein; see also Künzler and Bakker, 2001). The major concerns thereby raised relate to the potential mismatch between the animals' perception of the stimuli displayed on a screen designed for the human eye and the natural counterparts those stimuli attempt to mimic. In this respect, sticklebacks are a suitable study species, as the relevant components of their visual system closely resemble those of humans. The refresh rate (the number of times a display's image is "repainted" in a second) of the computer monitor used in our set-up (120 full frames per second) exceeds the critical flicker-fusion frequency (CFF: the threshold frequency of a flickering light stimulus at which the light appears constantly on, i.e., no flicker is discernible) for both humans and most fish (D'Eath 1998; Fleishman and Endler, 2000). Furthermore, the computer-generated presentation rate (30 frames/s) is above the threshold for motion perception for fish (Fleishman and Endler, 2000; Oliveira *et al.,* 2000), thus ensuring that our test females perceive a flicker-free, continuous, and smooth motion. As for color perception, sticklebacks have three cone classes whose sensitivity maxima closely match those of humans (Lythgoe, 1979; C. L. Baube, personal communication and cited in Rowland *et al.,* 1995). We are not aware of any indications of UV or IR vision in sticklebacks.

In summary, we report an enhanced preference for an *a priori* high-quality, symmetric male in inbred females compared to outbred females, suggesting that inbred females are prepared to pay more careful attention to small length differences between paired structures when assessing the quality of potential mates. Stronger selection pressure for such discriminatory ability on inbred females compared to outbred ones is consistent with the hypothesis that relatively poor-quality females derive higher marginal benefits from choosing high-quality partners. Furthermore, we promote the use of video-playback techniques in behavioral research and suggest that computer animations may be well-suited not only to detect absolute female mating preferences, but also to investigate variation in female mating preferences (see also Bakker *et al.,* 1999), a phenomenon that may crucially concur to the maintenance of substantial additive genetic variance in sexual traits.

ACKNOWLEDGMENTS

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Edited by Lee Ehrman