Evolutionary Ecology Research, 2016, **17**: 291–300

No evidence of sex reversal by means of experimentally altered sex ratios in threespine stickleback

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ABSTRACT

Background: Among fishes, sex reversal by abiotic or social factors is well documented even in species with genetic sex determination. All species of the family Gasterosteidae studied thus far show genetic sex determination, and natural sex reversal is most likely rare. In threespine stickleback (*Gasterosteus aculeatus* L.), exposure to sex hormones or endocrine disrupters can induce functional intersexuality or even sex reversal.

Hypothesis: In the presence of a shortage of reproductive males (i.e. a female-biased operational sex ratio), female threespine stickleback may become males.

Methods: A classical male-removal experiment, which induces sex reversal in many protogynous fishes. In six large male-removal tanks, each containing a full-sibling group, every time that a reproductive male appeared, it was removed. In the respective yoked-control tank (one control tank for each male-removal tank), a random fish was removed at the same time. The sex of all removed fish was determined by visual inspection of the gonads.

Results: Approximately 16 months after the appearance of the first reproductive male, the total number of fish (removed and remaining fish) in each male-removal and yoked-control tank showed no significant deviation from an even sex ratio (except for a single female-biased control tank). The male-removal and yoked-control tanks did not differ significantly in sex ratio. Threespine stickleback thus failed to show sex reversal under the applied male-removal regime.

Keywords: *Gasterosteus aculeatus*, male removal, sex determination, sex ratio, social cues, threespine stickleback.

INTRODUCTION

Sex determination systems vary widely among animal species, ranging from pure genetic to pure environmental sex determination and mixtures of the two (Devlin and Nagahama, 2002; Stelkens and Wedekind, 2010; Pandian, 2011; Bachtrog *et al.*, 2014; Beukeboom and Perrin, 2014). In fishes, which show the full spectrum of sex determination systems, 98% of species are gonochoristic – that is, they have separate sexes (Pandian, 2011). At least 350 fish species belonging to 34 families are sequential hermaphrodites, which are mostly coral reef fishes (Pandian, 2011). The most

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frequent form of sex reversal is protogyny [first functional female, then functional male (Warner, 1984)], which is mediated by social interactions. There are several hypotheses for the proximate control of protogynous sex change (reviewed in Lutnesky, 1994). The most established classes of hypotheses are the social group composition and the social group density hypotheses (for a complete overview of epigenetic sex differentiation, see Beukeboom and Perrin, 2014). The social group composition hypothesis comprises hypotheses such as the sex-ratio threshold hypothesis [the dominant female changes sex when the sex ratio in the group reaches a threshold (Shapiro and Lubbock, 1980)] and the size-ratio threshold hypothesis [a given female will change sex when the ratio of smaller-to-larger individuals in the group reaches a threshold (Ross *et al.*, 1983)]. The social group density hypothesis includes hypotheses such as the encounter-rate threshold hypothesis [the dominant female changes sex when the threshold level of stimulation from encountering smaller females exceeds the threshold level of inhibition from encountering a larger male (Lutnesky, 1994)].

In sequential hermaphrodites, several cues may trigger sex reversal (Beukeboom and Perrin, 2014). Even in various gonochorists with genetic sex determination, particular cues during sensitive periods in development may promote environmental sex reversal [ESR: reversal of the primary genotypic sex by environmental conditions during ontogeny (Stelkens and Wedekind, 2010)], such as temperature, pH, hypoxia, and population density (reviewed in Devlin and Nagahama, 2002; Godwin *et al.*, 2003; Stelkens and Wedekind, 2010; Beukeboom and Perrin, 2014). Temperature seems to be the most important environmental factor in many fishes (Baroiller and D'Cotta, 2001). For example, in zebrafish (*Danio rerio*), lower temperatures during early life caused a male-biased sex ratio, while at higher temperatures the sex ratio became female biased (Sfakianakis *et al.*, 2012). Hypoxia also caused a male-biased sex ratio in zebrafish by affecting the synthesis of sex hormones and inducing male phenotypic sex in genotypic females (Shang *et al.*, 2006). These environmental effects occur even though wild zebrafish have WZ/ZZ sex determination (Wilson *et al.*, 2014), while sex determination in domesticated strains is a complex genetic trait governed by several loci (Bradley *et al.*, 2011; Liew *et al.*, 2012).

Threespine stickleback (*Gasterosteus aculeatus* L.) have an XY sex-determination system with the sex chromosome being linkage group 19 (Peichel *et al.*, 2004; Ross and Peichel, 2008; Urton *et al.*, 2011). Sex reversal is most likely rare in *G. aculeatus* but there are several reports of intersex in the wild (Craig-Bennett, 1931; Borg and van den Hurk, 1983; Gercken and Holmer, 2002; Katsiadaki, 2005). The exposure of *G. aculeatus* to sex hormones or endocrine disrupters may change phenotypic sex, with effects ranging from feminization and masculinization, as measured by biochemical markers such as vitellogenin and spiggin (Katsiadaki *et al.*, 2002; Hahlbeck *et al.*, 2004b; Andersson *et al.*, 2007; reviewed in Katsiadaki *et al.*, 2007), to functional intersex or total sex reversal (Hahlbeck *et al.*, 2004a; Bernhardt *et al.*, 2006).

If it is possible for a sex change to occur through extraneous chemical treatment in stickleback, then maybe there is the potential for adaptive plasticity in gonad development as a result of extreme changes in the social situation due to a shift in the operational sex ratio. I aimed to experimentally test whether *G. aculeatus* is capable of exhibiting sex reversal under manipulation of the operational sex ratio. This goal was inspired by anecdotal observations of P. Sevenster and E. Feuth-de Bruijn (personal communication) in the laboratory in Leiden, where the present study was performed. During a selection experiment, Sevenster and 't Hart (1974) sequentially collected a conspicuously disproportionate number of males in some large holding tanks with full-siblings. As reproductive males had been successively removed for breeding purposes in these tanks, this would have resulted in female-biased operational sex ratios. The impression that these tanks continued to produce

reproductive males until almost no fish were left suggested that sex reversal had occurred (P. Sevenster and E. Feuth-de Bruijn, personal communication). The approach that I followed here was removal of the dominant male, which in many protogynous fish triggers the largest female in a polygynous group to change sex (reviewed in Godwin, 2010). If *G. aculeatus* is capable of sex reversal from female to male under male shortage, then after a period of removal of reproductive males one would expect a male-biased sex ratio for the sample of fish (including removed ones) in the male-removal tanks, compared with the yoked-control tanks where randomly chosen individuals were removed. This is the first time that such an experiment has been performed in stickleback.

MATERIALS AND METHODS

Study population

Threespine stickleback from a Dutch anadromous population were caught during the 1985 spring migration in Den Helder (52°57′N, 4°46′E) and transported to the laboratory in Leiden, where they were bred randomly in the first two weeks of July the same year. Fertilizations of paired experimental and control groups (see below) were at most 6 days (median 1 day, range 0–6 days) apart. Eggs were removed from the father's nest about one hour after fertilization and hatched artificially (for details, see Bakker, 1986). Crosses using 12 different mothers and seven different fathers were made. The half-sib families were randomly distributed across the experiment.

Rearing conditions

The F_1 fish were raised in full-sibling groups in small tanks (length × width × height: $34 \times 17 \times 20$ cm) under simulated summer conditions (light/dark cycle of 16/8 hours, 18–20°C) and fed various food items *ad libitum* (live *Tubifex* worms, *Artemia*, *Chironomus* larvae, and defrosted *Artemia* and *Mysis*) twice a day. Groups were visually isolated from each other. Group size was haphazardly and gently (by not catching individual fish) reduced to roughly 40 juveniles (median 39.5, range 31–55) about 2 months after fertilization and groups transferred to larger tanks (length × width × height: $60 \times 35 \times 40$ cm), in which they stayed until dissection. Each tank was illuminated by a 100-W bulb, fixed about 20 cm above the water surface. Fish were kept under simulated summer conditions (see above), and fed the same food items as mentioned above. Tanks were opaque on three sides, had a sand bottom, and were rather densely planted with long-leafed plants (*Vallisneria*, *Allisma*, etc.) and tufts of green, filamentous algae. Tanks were equipped with internal filter aeration. One-third of the water in the tanks was replenished regularly, but as few times as possible in order not to remove chemical signals that may facilitate sex reversal (e.g. Cole and Shapiro, 1995). Water changes were performed for the first time about 4 months after transfer, and about once per month thereafter. Excess plants were removed such that all fish could be observed but enough cover was still available to them.

Male-removal experiment

There were six experimental and six yoked-control tanks; each yoked-control tank was situated next to its experimental tank. In the experimental tanks, the appearance of

reproductive males (based on the extensive development of nuptial coloration, sometimes supported by observations of aggressive displacement of other fish as a sign of territory establishment) was checked almost daily. Each time a reproductive male appeared in the experimental tanks, it was removed before nest building. The first reproductive male appeared on 9 November 1985, 124 days after fertilization. Fish were removed using a glass pipe (Fig. 1), which allows the selective and gentle catching of one particular fish with minimal disturbance to the caught individual and the other fish in the tank. This was common practice in the laboratory in Leiden but is hardly ever mentioned in publications (but see Jenni *et al.*, 1969; Jenni, 1972). In the respective yoked-control tank, a randomly chosen fish was removed from the tank on the same day. All fish that were removed from the tanks were quickly killed by decapitation. Sexes of all removed fish in both treatments were checked by macroscopic inspection of the gonads. Regular observations (about once a week) of reproductively active fish (females and males) were made in the control tanks and of ripe females in the male-removal tanks. The first ripe female appeared on 5 November 1985, 111 days after fertilization. Ripe females were marked by cutting the tip of their right pelvic spine, whereas males (in control tanks only) were marked by cutting the tip of the second dorsal spine. In the control tanks, nests were removed once a week. All remaining fish (145 fish in total) were dissected on 30 March 1987 (about 20 months after fertilization and 18 months after group size reduction).

Statistical analyses

Analyses were performed using the R v.3.1.3 statistical software package (R Development Core Team, 2014). In view of the relatively small sample size (six male-removal and six yoked-control tanks), non-parametric statistics were applied. Comparisons between treatment groups were done with paired-sample Wilcoxon signed-rank tests. Differences in sex ratio (proportion of males: the number of males divided by the total number of individuals) between separate pairs of tanks (male-removal and yoked-control tanks) were tested with Fisher's exact tests. Deviations of sex ratio from parity were tested with either a one-sample Wilcoxon signed-rank test (treatments) or binomial tests (single groups). *P*-values cited are two-tailed throughout. The level of significance was set at 0.05.

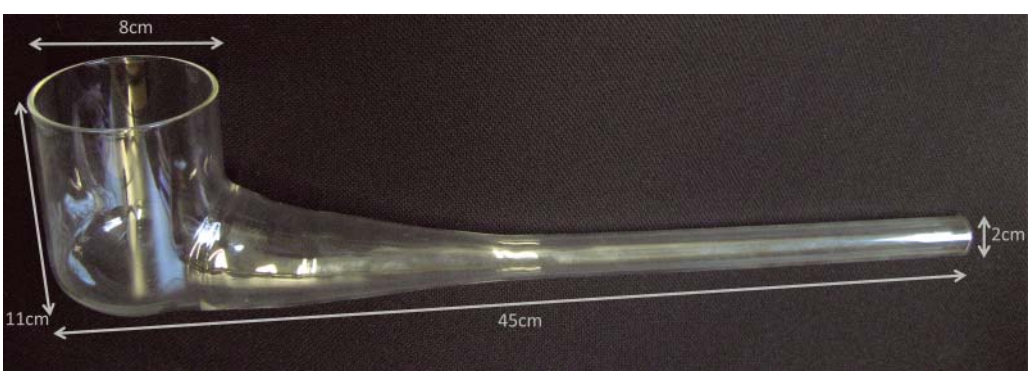

Fig. 1. Glass pipe [called a 'glass catching funnel' by Jenni (Jenni *et al.*, 1969; Jenni, 1972); usually called *vangklok* in Dutch and *Fangglocke* or *Fischfangglocke* in German) used to selectively catch fish with minimal disturbance. The thickness of the glass is 2 mm.

RESULTS

Initial group sizes were not significantly different between the male-removal treatment and the yoked-control treatment [median (quartiles): male-removal: 40 (38, 42); yoked-control: 37.5 (32, 43); Wilcoxon signed-rank test, $V = 5.5$, $N = 6$, $P = 1.0$]. A similar percentage of fish died in both treatments during the ~18 month period between group size reduction and the termination of the experiment [median (quartiles): male-removal: 13.4% (7.9, 19.5); yoked-control: 14.4% (8.8, 24.4); Wilcoxon signed-rank test, $V = 11$, $N = 6$, $P = 1.0$]. A few fish (16 fish, 3.4%) that died during the experiments could not be sexed as they were detected too late and had not been marked; this involved half of the tanks [median (quartiles): dead unsexed fish in two male-removal and four yoked-control tanks: 2 (2, 3)].

Male removal did not change significantly the adult sex ratio in full-sibling groups [median proportion of males (quartiles) in male-removal: 0.493 (0.345, 0.512) vs. yoked-control: 0.516 (0.441, 0.621); Wilcoxon signed-rank test, $V = 13$, $N = 6$, $P = 0.69$] (Fig. 2). The paired tanks had similar sex ratios (Fisher's exact test, all $P > 0.065$) (Fig. 2). Sex ratio did not deviate significantly from parity in either the male-removal or yoked-control treatment (one-sample Wilcoxon signed-rank test, $V = 4$, $N = 6$, $P = 0.42$, and $V = 12$, $N = 6$, $P = 0.84$, respectively), or in the single tanks (binomial test, all $P \geq 0.108$), except for one female-biased yoked-control group (binomial test, $P = 0.003$) (Fig. 2).

At the end of the experiment, all six male-removal tanks only contained females (median 14, range 6–15), while in five of six yoked-control tanks both sexes (median remaining fish 13, range 3–18) were still present. The proportion of remaining fish that were males at the end of the experiments tended to differ between the male-removal and yoked-control treatments [median proportion males (quartiles): male-removal 0 (0, 0); yoked-control: 0.320 (0.250, 0.400); Wilcoxon signed-rank test, $V = 15$, $N = 6$, $P = 0.060$]. The sex ratio of the remaining fish was significantly female biased in both the male-removal and yoked-control treatments (one-sample Wilcoxon signed-rank test, $V = 0$, $N = 6$, $P = 0.020$, and $V = 0$, $N = 6$, $P = 0.031$, respectively).

DISCUSSION

Although the experiment was run about 16 months after the appearance of the first reproductively active male, removing sequentially appearing reproductive males did not trigger sex change in females, as the total sex ratio (of removed and remaining fish) in male-removal tanks was similar to that in yoked-control tanks. The stickleback population used is likely a yearly population, in which fish reproduce during one season (van Mullem and van der Vlugt, 1964; Mehlis and Bakker, 2013), so the experiment was run beyond the length of the natural breeding season. It is thus very unlikely that we stopped the experiment too soon to identify sex reversal, especially when one takes into account that adult hermaphroditic fishes are able to reverse functional sex in only 7–25 days (Pandian, 2011).

Sex ratio in the male-removal experiment did not point to sex change in threespine stickleback. As fish were sexed by gonad inspection, it cannot be excluded that we failed to recognize cases of sex transition. In hermaphrodites, signs of sex transition can only be identified in very advanced stages with macroscopic methods, but early signs of sex change can be detected microscopically by histology of gonad tissue (Pandian, 2011; Klibansky and Scharf, 2015). In the present study, only macroscopic phenotypic sexing was performed. Assessing genotypic sex may have revealed cases of sex change, but molecular sex tests were not to be

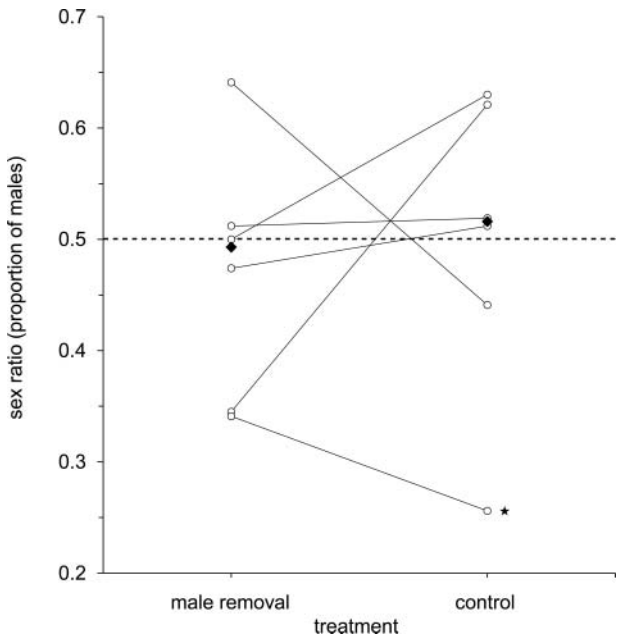

Fig. 2. Total sex ratio (proportion of males) – i.e. the sex ratio of removed and remaining fish – in six male-removal tanks and corresponding yoked-control tanks. Tanks contained initially about 40 full siblings and were tracked and manipulated for about 18 months. Lines connect the paired male-removal and yoked-control tanks. Solid squares indicate median sex ratio of male-removal and yoked-control treatments. The broken line indicates an even sex ratio. Sex ratio differences between male-removal and yoked-control tanks, as well as the deviation of the sex ratio of single tanks from parity, were all non-significant, except for a female-biased sex ratio in one yoked-control tank (indicated by an asterisk).

developed for another 15 years (Griffiths *et al.*, 2000; Peichel *et al.*, 2004). One case of a mismatch between phenotypic sex at different stages of the experiment was detected. In a control tank, an individual that was clearly a male by gonad inspection at the end of the experiment had been marked by a shortened right pelvic spine, which was used for marking females that became ripe during the experiment. So this may be a case of sex reversal from female to male, but occurred in a control tank in which no sex reversal was expected. It is, however, more likely that the first sexing was done erroneously. The assessment of females on the basis of appearance alone is not free from error, even to the experienced eye. So maybe the sex on the first occasion was misjudged. It cannot also be excluded that among the well over 200 markings that were applied, one fish was marked incorrectly.

At the end of the experiment, no males were left in the male-removal tanks, so male removal had been successful. Most control tanks still contained both sexes at the end but the sex ratio was female biased in that about twice as many females as males were left at the end. Although fish were randomly removed from the control tanks, apparently more males had been removed probably because the reproductively active ones were more conspicuous and active, and reproductive females were harder to catch.

In almost all tanks, the overall sex ratio (removed and remaining fish) did not deviate significantly from parity in accordance with the expectation of sex ratio theory for sexually

reproducing diploid species (Fisher, 1930). One out of 12 tanks showed a significantly female-biased sex ratio, which was unexpected. In zebrafish, for example, sex ratios varied greatly and consistently across families (Liew *et al.*, 2012). Such variation is unknown for sticklebacks but there is a lack of studies on family sex ratios. An equal sex ratio was assumed when I planned the present experiment, which is supported by personal observations and by the few sexing studies that exist using laboratory-bred juveniles (Albert *et al.*, 2008; Ramler *et al.*, 2014; T.C.M. Bakker *et al.*, unpublished data). Because of small sample sizes, the power of the present experiment was too low to detect moderate deviations from an equal sex ratio or moderate differences in sex ratio between treatments. In field samples of sticklebacks, equal sex ratios have often been observed, such as in young-of-the-year in a Scottish pond (Arnold *et al.*, 2003) and in samples from diverse stickleback populations (e.g. Dauod *et al.*, 1985; Crivelli and Britton, 1987; Niksirat *et al.*, 2010; but see Hagen and Gilbertson, 1973). During the breeding season, one may find female-biased adult sex ratios. However, this is probably due to decreased ability to trap males (e.g. Arnold *et al.*, 2003; Blais *et al.*, 2004; Alexandre and Almeida, 2009; Niksirat *et al.*, 2010), or a shift in the operational sex ratio during the breeding season towards females (e.g. Kynard, 1978; Mori, 1993; but see Whoriskey *et al.*, 1986; Tinghitella *et al.*, 2013).

In summary, with the male-removal scenario used here, no sex reversal could be induced in threespine stickleback. As phenotypic sex is sensitive to environmental sex hormones in stickleback (Katsiadaki *et al.*, 2007), a test in which phenotypic sex is tracked from all genotypic male or all genotypic female groups that have been reared together would be a more rigorous test. Molecular sex tests (Griffiths *et al.*, 2000; Peichel *et al.*, 2004) on DNA from spine clips, fin clips or body mucus swabs (Le Vin *et al.*, 2011) would make such an approach feasible.

ACKNOWLEDGEMENTS

I am very grateful to Enja Feuth-de Bruijn for assistance in performing the experiments, to the late Piet(er) Sevenster for suggesting the study and discussing the set-up, to Claus Wedekind for discussion and stimulating publication, to Marion Mehlis, Katie Peichel, and two anonymous referees for helpful comments on the manuscript, to Mike Bell for organizing the 8th International Stickleback Conference on Behavior and Evolution, and to Susan Foster, Iain Barber, and Felicity Jones for inviting me to give the Pieter Sevenster address at the stickleback conference, which included the sex reversal study. The experiments complied with the laws of the country in which they were performed.

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