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Smells like sib spirit: kin recognition in three-spined sticklebacks (*Gasterosteus aculeatus*) is mediated by olfactory cues

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Abstract The ability to recognise kin has been demonstrated in several animal species. However, the mechanisms of kin recognition often remain unknown. The most frequently discussed sensory modalities to recognise kin are visual, olfactory and acoustical cues. Three-spined sticklebacks (*Gasterosteus aculeatus*) are able to differentiate between kin and non-kin when presented visual and olfactory cues combined. To elucidate, which cues they use to recognise kin female sticklebacks were given the choice between two identical computer animations of courting stickleback males. Next to one animation, water conditioned by a brother was added, while near the other, water from an unrelated male was added. In half of the experiments, the brother was familiar while in the other half he was unfamiliar to the female. Both scenarios were carried out with both outbred and inbred fish. The results showed that the females adjusted their choice behaviour according to relatedness. Furthermore, they were able to recognise both familiar as well as unfamiliar brothers. Inbreeding did not affect this ability. Hence, three-spined sticklebacks are able to recognise their relatives using olfactory cues alone. The cognitive mechanisms underlying this ability were independent from familiarity and not impaired by inbreeding.

Keywords Mate choice · Sibling · Olfactory cues · Computer animation · Odour · Inbreeding avoidance · Fish

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Introduction

Kin recognition, i.e. the ability to discriminate between related and unrelated individuals, has been demonstrated for a whole range of taxa, from social microbes (*Dictyostelium purpureum)* (Mehdiabadi et al. 2006) to chimpanzees (*Pan troglodytes)* (Vokey et al. 2004). Given the number of studies demonstrating the existence of kin recognition in various species, it is surprising how little is still known about the cues animals use to make such discriminations (see Halpin 1991 for an early review). In general, olfactory, acoustical or visual cues, alone or in combination, have been shown to be important. For example, juvenile zebrafish (*Danio rerio*) (Mann et al. 2003) as well as bluegills (*Lepomis macrochirus*) (Hain and Neff 2006) used olfactory cues to identify related individuals during shoaling decisions. In mate choice, male mice (*Mus musculus*) significantly preferred the odour of an unrelated female to the odour of a sister (Krackow and Matuschak 1991). Kin recognition using vocal cues was demonstrated in several bird species (see Barry and Goth 2006 for an overview) as well as in lambs (*Ovis aries*), which responded more frequently to the bleats of their siblings than to those of non-kin (Ligout et al. 2004). Chimpanzees used similarities in the faces of related but unfamiliar individuals to recognise mothers and their sons (Parr and de Waal 1999).

In the context of kin recognition the cognitive mechanisms underlying the receiver's response to cues produced by the sender within a communicative interchange often remain unknown (see Mateo 2004 for an overview). When siblings remain together for part of their life, familiarity is a reliable measure of kinship (for a review see Pusey and Wolf 1996; Lieberman et al. 2007). When, on the other hand, individuals do not live in family groups, it becomes important to recognise unfamiliar kin. Holmes and Sherman

(1982) suggested that such recognition might be based on "phenotype matching", in which an individual builds a template based on cues from familiar, related conspecifics, then uses that template to evaluate the relatedness of an unknown conspecific. Such a mechanism has been demonstrated for example in golden hamsters (*Mesocricetus auratus*) (Heth et al. 1998) and an African cichlid (*Pelvicachromis taeniatus*) (Thünken et al. 2007). "Self-referent phenotype matching" (Hauber et al. 2000; Mateo and Johnston 2000) is a special case of phenotype matching (see Hare et al. 2003; Mateo and Johnston 2003 for discussion). In this situation, the individual uses its own cues to build the recognition template. The use of these different mechanisms of kin recognition may be context-dependent (Mateo 2004; Hain and Neff 2006) and may depend upon the genetic make-up of the receiver. So, for example in some situations, inbred animals may lose the ability to recognise kin because of inbreeding degeneration concerning the recognition mechanisms (Frommen et al. 2007c). In other cases, such individuals may show a more pronounced recognition of kin than their outbred counterparts because it may be easier for a genetically homogeneous phenotype to recognise a "like" individual than it is for a heterogeneous phenotype. Furthermore, selection should be stronger on inbred individuals to avoid inbreeding since a further reduction of heterozygosity will produce even stronger inbreeding depression (Mazzi et al. 2004).

The circumstances in which individuals respond differently on the basis of kinship are as diverse as the groups of organisms, which show the ability to recognise kin (Hepper 1991). In general, situations in which kin recognition is of importance can be divided into two groups: (1) social interactions like shoaling behaviour, territoriality and aggression or brood-care, and (2) mating behaviour and the avoidance of inbreeding. Close inbreeding is known to be disadvantageous in many animal taxa (e.g. Crnokrak and Roff 1999; Armbruster and Reed 2005; Kempenaers 2007). Such "inbreeding depression" has been documented frequently (for a summary of the fish database, see Waldman and McKinnon 1993). One way to avoid inbreeding would be to recognise relatives and reject them as mates (e.g. Mateo 2004). Although this mechanism has only been studied in a handful of vertebrate taxa thus far, the results are intriguing (for a review see Pusey and Wolf 1996).

Fish are still underrepresented in cognitive research despite their high potential to answer questions concerning a wide array of cognitive abilities (Bshary et al. 2002). In this study three-spined sticklebacks (*Gasterosteus aculeatus*) were used as study species. They are an excellent system to study the mechanism of kin recognition for several reasons: Their use of both visual (e.g. McLennan and McPhail 1990; Bakker and Milinski 1993; Baube et al. 1995) and olfactory cues (McLennan 2003; Milinski 2006;

Rafferty and Boughman 2006) in mating decisions has been well documented, as has the importance of olfactory cues to social recognition (Ward et al. 2004, 2005) and foraging (Webster et al. 2007). Outbred sticklebacks are able to use the distinction between either familiar or unfamiliar kin and non-kin to make shoaling decisions (Frommen and Bakker 2004; Frommen et al. 2007c). The ability to recognise unfamiliar kin in a shoaling context was lost in inbred fish (Frommen et al. 2007c). Furthermore, outbred as well as inbred females avoided mating with familiar brothers when both visual and olfactory cues were presented, although it is currently unclear whether this result was based on familiarity or on kinship (Frommen and Bakker 2006). Finally, sticklebacks from the population used in this study indeed suffer from inbreeding depression. Inbred fish had reduced fertilisation success, egg survival, and fry survival to adulthood (Frommen et al. 2008). They also displayed a higher rate of morphological asymmetries (Mazzi et al. 2002), which may effect both brood-caring behaviour (Künzler and Bakker 2000) and mate choice (Mazzi et al. 2003).

Given the preceding data, this study aimed to answer the following three questions: (1) do female sticklebacks recognise kin based solely on olfactory cues? (2) do they recognise kin independent from direct familiarity? and (3) if the answer is yes to either one or both of these questions, does inbreeding influence the female's ability to recognise kin? The experiments were conducted using olfactory cues of a familiar/unfamiliar brother and an unfamiliar unrelated male in combination with two identical computer animations as visual stimuli (Künzler and Bakker 1998).

Materials and methods

Experimental subjects

Fish from an anadromous, genetically heterogeneous population (Heckel et al. 2002) were caught during their spring migration in April 2002, 2003 and 2004 on the island of Texel, the Netherlands. Test fish were outbred and one or two generation inbred descendents of these wild-caught ancestors. Outbred fish were the F1 of haphazardly crossed fish. Inbred fish were produced through one or two generations of brother–sister matings. Thus, one generation inbred fish were the F2, and two generation inbred fish the F3, progeny of wild caught fish (see Frommen et al. 2008 for details). Outbred eggs were laid in April and May 2004, inbred eggs in December 2004 and January 2005. Clutches were taken out of the nests directly after fertilisation and divided into two sub-groups that were reared separately, producing familiar and unfamiliar kin for each of the sib group. At two months post hatching, group sizes were reduced to 20 full sibs in each tank (see Frommen et al.

2007b for details). The holding tanks measured $50 \times 30 \times 30$ cm and were separated from each other by grey opaque partitions. Water in the tanks was cleaned and aerated through an internal filter, and a third of the water volume was replenished with tap water once a week. Fish were kept in an air-conditioned room under standardised winter light-regime (day length 8L:16D, temperature $17 \pm 1°C$). For simulating the start of the breeding season the light regime was changed to summer-conditions (16L:8D, $17 \pm 1°C$) 4 weeks before the experiments started (Borg et al. 2004). During the experiments fish were fed daily on frozen *Chironomus* spp. larvae.

Computer animation

Stickleback males show multiple traits that make them attractive to females (Künzler and Bakker 2001; Candolin 2003), such as blue eyes and red throat colouration, body symmetry, body size and courtship behaviour. It is thus difficult to test the influence of a single trait on female's mating preferences. The use of computer animations in mate choice helps to eliminate this problem (Künzler and Bakker 1998; Baldauf et al. 2008), because the researcher can focus on a single trait while keeping all other factors constant. In sticklebacks, computer animations are well-established and worked in diverse studies (e.g. McKinnon and McPhail 1996; Bakker et al. 1999; Künzler and Bakker 2001; Mazzi et al. 2003, 2004; Zbinden et al. 2003, 2004). The computer animation used in the present study was a modified version of the one described in detail by Künzler and Bakker (1998). It consisted of a reoccurring 133 s sequences of a pale-coloured, courting stickleback male that was zig-zagging towards the test female and fanning at his nest (Fig. 1a, b). After each sequence, the computer-animated male left the arena for 9 s and then reappeared.
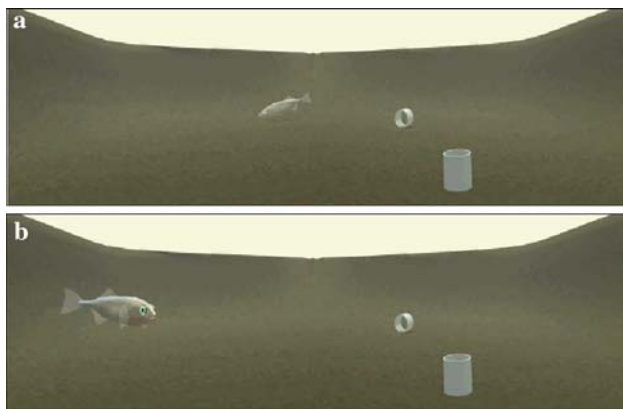

Fig. 1 Screenshots of the computer animation (see also Künzler and Bakker 1998). The computer-animated male had a *pale red*-coloured throat and slightly *blue* eyes. The landscape itself was *brownish*. **a** The fanning male near the nest and **b** the male in front of the test female after zig-zagging towards her

Experimental design

Experiments were conducted between September and November 2006. Mate choice preferences were tested in a glass tank measuring $45 \times 40 \times 30$ cm. The tank was filled with one-day-old tap water and lit by a 36 W fluorescent lamp placed 91 cm above the bottom of the tank. Interactions with the environment were prevented by a black curtain tightened around the tank and by placing grey plastic plates on all sides. Two small windows (7.5×30 cm) on opposite sides allowed the test female to see the two computer animations. These were shown using two identical monitors (Sony, Trinitron, Multiscan 200Ps, $1,024 \times 768$ Pixel, 85 Hz) (Baldauf et al. 2008) that were placed at a distance of 2.5 cm from each side (Fig. 2).

In the experiment, a receptive female was given the choice between two identical computer-animated males. Water conditioned by a courting brother was added on one side, water conditioned by an unrelated, courting male was added on the other. The conditioned water was released 3 mm under the water surface via a peristaltic pump (Ismatec, MS-CA4/640) with a flow rate of 4 ml/min. This amount of odour-conditioned water was sufficient to cause a reaction of the female in preliminary experiments (Mehlis 2007). The side at which the odour of the brother was added alternated between the trials. In half of the experiments the brother was familiar ($N = 19$) while in the other half he was unfamiliar to the test female ($N = 19$). Both scenarios were performed with outbred ($N = 22$) and inbred fish ($N = 16$). Thus, the total sample size was 38.

The test started by placing a receptive female in the test tank for an acclimatisation period of 30 min, during which an empty landscape was shown on the monitors. After 30 min the two animations were started and the odour was added simultaneously. Females' movements were recorded using a webcam (Creative, Creative Webcam Live!) that was attached 89 cm above the bottom of the tank and connected to a laptop behind the curtain. After the experiment, the test female was allowed to spawn with an unrelated male. One female was excluded from analysis because she failed to spawn within 24 h after the trial. This criterion ensured that the 38 females used in the analysis were ready to spawn (Bakker et al. 1999; Mazzi et al. 2003). After each experiment the test tank was emptied, the whole set-up was cleaned using a 3% solution of hydrogen peroxide, rinsed with clear water and the test tank was refilled with one-day-old tap water. This ensured that odours from previous tests would not remain to contaminate the new test water (McLennan 2004).

Producing the olfactory stimuli

Males that showed breeding colouration in the holding tanks were isolated in separate tanks ($40.5 \times 20.5 \times 25$ cm).
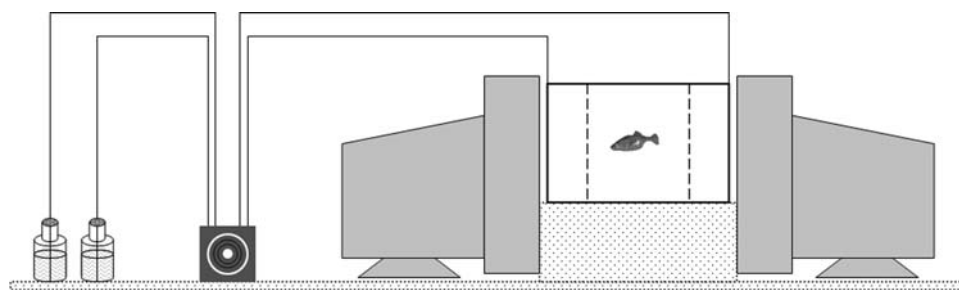
Fig. 2 Experimental set-up. The test female was allowed to choose between two identical computer animations. In front of the computer-animated males water conditioned with the odour of a brother or an unrelated male was steadily added using a peristaltic pump. Choice zones of 10 cm in front of the monitors used in the analysis are indicated. For a detailed description see "Materials and methods"

Each tank was equipped with an airstone, 2 g of dark-green wool for nest-building (Schachenmayr nomotta, polyacryl, colour-no. 0072) cut into pieces of 30 ± 10 mm (Schütz 1980), and a petri-dish (Ø 9 cm) filled with washed sand. Before the experiment started the whole set-up was washed with a 3% solution of hydrogen peroxide, rinsed with tap water, filled with 15 l of one-day-old tap water and covered from above with transparent plastic sheets so that the tanks could not be contaminated by air-borne odours (McLennan 2004). The tanks were isolated from each other with grey, opaque plastic partitions to avoid visual contact between the males.

All males built their nest within 6 days after introduction. Water from tanks in which the nest had been finished for at least 8 days was used in the experiments. Two size-matched males, a brother of the test female and an unrelated male were selected. Both were stimulated for 15 min with an unrelated, receptive female enclosed in a clear plastic box ($10 \times 7 \times 17$ cm) placed outside in front of the stimulus tank (Frommen and Bakker 2006). This was done to increase the production of odours used in courtship. After stimulation 200 ml of water was drawn from 1 cm above the nest using a tube and filled in a bottle. The tube and the bottles had previously been cleaned with a solution of 3% hydrogen peroxide and rinsed with clear water. The resulting two bottles were connected to the peristaltic pump and the trial started immediately. Standard length (SL) and body mass (M) of the males were measured and their condition factor (CF = 100 M/SL^3) (Bolger and Connolly 1989) was calculated. Brothers and non-brothers did not differ significantly in SL, M and CF (paired t tests, $N = 38$, all t between -0.559 and -0.112, all $p \geq 0.579$).

Analysis

Recordings were analysed for 30 min after the animations began. On the monitor, choice zones were marked 10 cm in front of each stimulus monitor (Fig. 2). The measurement of female choice started as soon as the head of the test fish had entered both choice zones. If the test female did not

enter both choice zones within 10 min, the experiment was discarded (Frommen and Bakker 2006) ($N = 6$). Three females spontaneously spawned during the experiment and were discarded. Stimulus males of all discarded experiments were used again in a later trial, but females were excluded from further testing.

Because previous studies only used computer animations for short time spans (between 2 and 5 min) (e.g. McKinnon and McPhail 1996; Bakker et al. 1999; Künzler and Bakker 2001; Mazzi et al. 2003, 2004; Zbinden et al. 2003, 2004), we investigated the effects of a prolonged exposure to the images and scent by subdividing the analysis in six blocks lasting 5 min. The observer was naïve with respect to the side where the odour of the test fish's relative was added.

Parametric statistics were used as data did not significantly deviate from normal distributions according to Kolmogorov–Smirnov tests with Lilliefors correction. For analysis, linear mixed effect models were conducted using the "lme" function in the "nlme" library of the R 2.4.1 statistical package. The relative time the test female spent on each side of the test tank during the experiment was used as dependent variable. Fixed factors were kinship (brother or non-brother), familiarity (familiar or unfamiliar), and breeding regime (inbred or outbred). Furthermore, we included tank side (right or left) to reduce the impact of any side effect. Interactions between kinship × breeding regime × familiarity, kinship × familiarity and kinship × breeding regime were included. Non-significant factors and interactions after Bonferroni correction were removed from the analysis (Engqvist 2005). Tests of significance were based on likelihood-ratio tests ("LRT") that follow a χ^2-distribution, hence, degrees of freedom always differed by one. The test probabilities are two-tailed throughout. Twelve families provided the test fish twice, once in the familiar and once in the unfamiliar treatment. Eight families provided two unrelated males, one male in each treatment. All stimulus males and test females were used only once to avoid pseudoreplication. Consequently, the influence of the families on the test fish's behaviour was tested prior to the analysis. Neither the related nor the unrelated family had a

significant influence on the test female's choice (LRT, all $\chi^2 < 3.729$, all $P > 0.05$). Family was, thus, excluded from further analysis.

Results

Analysing the first 5 min block of the trial indicated that females spent significantly more time near the odour of the unfamiliar, non-kin male than near their brother (Table 1, Fig. 3). This preference switched gradually over the next 25 min, such that females spent significantly more time near the odour of their brother in the sixth 5 min block (Table 1, Fig. 3). These results remained significant after

Bonferroni correction with a Bonferroni adjusted α-level of 0.008. Only the tank side additionally influenced female preferences. Neither familiarity (kinship × familiarity) nor inbreeding (kinship × breeding regime) had a significant effect (Table 1). The time females spent in both choice zones combined did not differ significantly between the consecutive 5 min blocks (ANOVA, $F_{5,222} = 0.324$, $P = 0.898$).

As female preferences switched during the experiment, analysing the total 30 min revealed no significant preference for a certain male (Table 1). Females spent $50.35 \pm 3.59\%$ (mean ± SE) in the choice zone where the odour of an unrelated male was added and $49.65 \pm 3.59\%$ in the choice zone where the odour of a related male was

Table 1 Results of the analysis of female preferences for male odours during the consecutive 5 min blocks and the total 30 min

Factors	Time blocks													
	First 5 min		Second 5 min		Third 5 min		Fourth 5 min		Fifth 5 min		Sixth 5 min		30 min	
	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P	χ^2	P
Kinship × breeding regime × familiarity	3.166	0.075	1.250	0.264	0.109	0.741	2.056	0.152	2.344	0.126	0.295	0.587	0.348	0.555
Kinship × breeding regime	0.277	0.599	0.321	0.571	0.809	0.369	0.888	0.346	0.216	0.642	<0.001	0.990	0.020	0.886
Kinship × familiarity	0.160	0.689	0.170	0.680	4.038	0.045	1.043	0.307	0.222	0.638	3.078	0.079	1.709	0.191
Kinship	8.237	**0.004**	0.273	0.602	0.006	0.939	0.535	0.464	3.492	0.062	7.179	**0.007**	0.133	0.716
Tank side	14.408	**<0.001**	13.745	**<0.001**	3.606	0.058	9.653	**0.002**	2.410	0.121	8.442	**0.004**	14.312	**<0.001**

The models were compared with likelihood-ratio tests (LRT) that follow a χ^2-distribution. Hence, degrees of freedom always differed by one. After Bonferroni correction with an adjusted α-level of 0.008, only the factors "kinship" and "tank side" remained significant: Kinship significantly influenced female choice during the first and last 5 min block. While test females preferred the unrelated male during the first 5 min, they preferred the side of their brother at the end of the experiment. Significant results are printed in *bold*
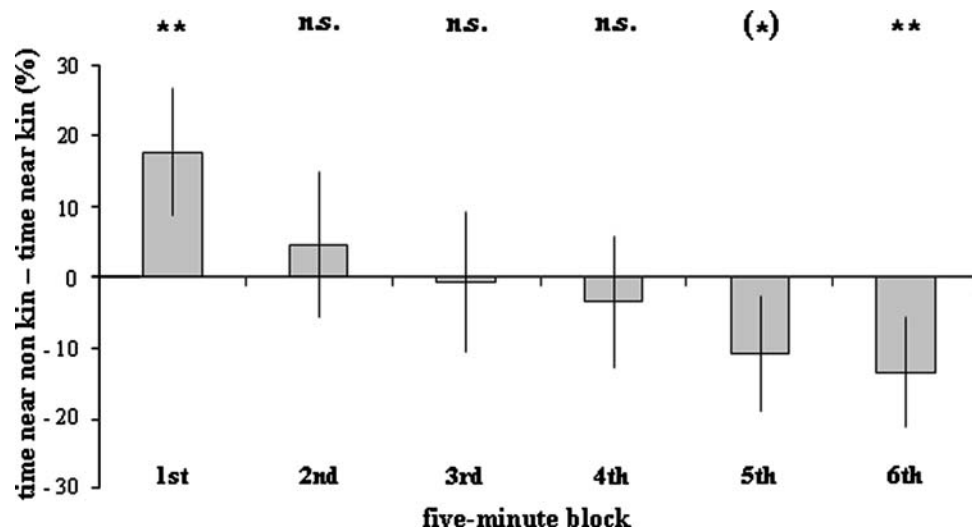

Fig. 3 Preferences of the females during the respective 5 min blocks, calculated as relative time spent near non-kin minus relative time spent near kin. Plotted are mean differences and standard errors. *Positive values* indicate a preference for the unrelated male. Females spent significantly more time near the odour of the unfamiliar non-kin male than near her brother in the first 5 min. This preference switched during the following 5 min blocks, so that females spent significantly more time near the odour of their brother in the sixth 5 min block.** $P < 0.01$, (*) $P < 0.1$, n.s. $P > 0.1$

added. Again, familiarity and inbreeding did not significantly influence female preferences (Table 1). However, females significantly preferred the right side of the test tank (Table 1).

Discussion

Inbreeding depression has been shown to reduce individual fitness in many animal species (Crnokrak and Roff 1999). One way to avoid inbreeding is to recognise kin using information from a variety of cues, then to avoid breeding with them (e.g. Pusey and Wolf 1996). Previous research in three-spined sticklebacks demonstrated that both inbred and outbred females prefer unfamiliar, unrelated males over familiar brothers when visual and olfactory cues were provided simultaneously (Frommen and Bakker 2006). However, the cognitive basis of this ability remained unknown. The results of the present study showed that females are capable of recognising both familiar and unfamiliar brothers based on olfactory cues alone. Time spent near a male is generally accepted as a good indicator of mating preference in this species (McLennan and McPhail 1990; Milinski et al. 2005). Given that the female spent a significant proportion of her time near the non-kin male, it appears that, as expected, she is using olfactory cues to avoid mating with her brother. Females only showed this preference for the non-kin male, however, in the first 5 min of the 30 min trial. As time progressed they moved from no preference to a significant preference for the scent of brother in minutes 25–30. This time effect can be interpreted in two different ways. First, a female may lose interest in the preferred computer-animated male because courtship did not proceed to completion, and so may have moved to the second, less preferred male even though it smelled like her brother. Thus far, stickleback experiments using computer animations have not lasted longer than 5 min (McKinnon and McPhail 1996; Bakker et al. 1999; Künzler and Bakker 2001; Mazzi et al. 2003, 2004; Zbinden et al. 2003, 2004), so that this possibility cannot be excluded. If true, one would expect that with test durations longer than 30 min, females would either reverse their preference again or simply show no preference at all. Second, females may have habituated to courtship, lost interest in spawning and switched to shoaling behaviour. Male odour accumulated on each side of the female's tank over the course of the experiment, which may eventually have suggested the presence of a fish shoal to her. As sticklebacks prefer to shoal with kin outside a reproductive context (Frommen and Bakker 2004) shoaling may also explain female preferences during the last period of the experiment (but see Steck et al. 1999). However, as a previous study has shown that outbred fish preferred to shoal with either familiar or unfamiliar kin, but inbred fish

only responded to familiar kin (Frommen et al. 2007c), one might have expected differences in the preferences of inbred and outbred fish during the last 5 min block, which was not the case. Regardless of the explanation for the temporal pattern of response, the results show clearly that females are able to recognise kin using olfactory cues alone.

Females were able to recognise their brothers irrespective whether they were familiar or not. Using this experimental design, there is no way to determine whether they recognise their unfamiliar siblings on the basis of earlier experiences with kin (phenotype matching) or some kind of self-reference (Mateo 2004). Stickleback fry stay in the nest for the first days of their life where they live in close association with other full-sibs and half-sibs (Wootton 1976). During this phase, they have the opportunity to become familiar with the olfactory cues of their kin, which may be used later in life as a template to recognise relatives. Frommen et al. (2007a) showed that early learning plays a major role in stickleback's kin recognition in shoaling decisions, so phenotype matching might be a plausible mechanism to explain the findings of the recent study. On the other hand, female sticklebacks use self-reference to optimise their MHC allele number during mate choice (Aeschlimann et al. 2003), making self-referent phenotype matching a possible alternative to phenotype matching in this system. A female stickleback chooses a mate whose MHC complex coupled with her own produces an intermediate distribution of alleles in the offspring (Reusch et al. 2001; Aeschlimann et al. 2003; Milinski 2006). Strongly heterogeneous females may thus not avoid, and perhaps even prefer, inbreeding in order to limit the MHC variability of their young. Given this, we would have predicted that there should be differences in the mate choice behaviour of inbred and outbred fish used in this study, which was not the case (see also Frommen and Bakker 2006). Cues other than MHC complement may have affected female choice in this experiment. For example, previous studies have demonstrated that shoaling decisions are influenced by habitat- or diet-based odour cues (Ward et al. 2004, 2005). This explanation is untenable in our study because all fish in the experiments were maintained under the same food and habitat conditions. It is also possible that the olfactory cue itself may transmit information based on differences in the individual's bacterial flora (Brown 1995), differences that may be smaller in related individuals. The composition of an individual's bacterial flora is, however, not constant through time, making bacterial-derived cues a highly unpredictable measure of relatedness (Thom and Hurst 2004). Overall, then, it seems unlikely that MHC complement, habitat, diet or bacterial influences on the olfactory cue affected the results of this study, leaving the composition of the olfactory cue open to further investigation.

Inbreeding effects on mate choice have been investigated in only a few studies thus far (Kempenaers 2007), with ambiguous results. Mazzi et al. (2004), for example, showed that inbred female sticklebacks were choosier during mate choice than outbred ones. In contrast, Frommen and Bakker (2006) found that both inbred and outbred females equally preferred unfamiliar, unrelated males over familiar brothers. Similar results were found in the present study, too. However, Frommen et al. (2007c) showed in a recent study that inbred and outbred sticklebacks differed in their preferences for unfamiliar kin in a shoaling context. This may imply that sticklebacks use different recognition mechanisms during shoaling decisions and mate choice, for example phenotype matching and self-reference, respectively. While the former was affected by only one generation of inbreeding the latter was not.

In summary, this study showed that sticklebacks use olfactory cues to recognise kin. This ability was independent from familiarity and not affected by inbreeding. These results deepen the knowledge of the cognitive mechanisms of kin recognition in fish.

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