Behavioral Ecology Vol. 10 No. 5: 493–497

No sibling odor preference in juvenile three-spined sticklebacks

Nicole Steck, Claus Wedekind, and Manfred Milinski
Abteilung Verhaltensökologie, Universität Bern, Wohlenstrasse 50a,
CH-3032 Hinterkappelen, Switzerland

Laboratory-bred juvenile three-spined sticklebacks from 11 sibships did not prefer to shoal with their siblings when they were offered the choice between odor from unfamiliar siblings and non-kin in a fluviarium, although the power for finding a significant preference was very high (0.99). The test fish preferred the side where odor from the heavier shoal was supplied; this shows that they could appreciate odor cues from conspecifics in our apparatus and should have preferred their siblings if such a preference exists. Our results are compatible with theoretical predictions but are at variance with previous findings by other authors. We used independent replicates in a blind protocol with strict randomization of fish and procedures. *Key words: Gasterosteus aculeatus,* kin preference, odor, shoaling behavior, sticklebacks. *[Behav Ecol 10:493–497 (1999)]*

A fish that joins a shoal of conspecifics benefits from antipredator advantages such as dilution and confusion effects (see Pitcher and Parrish, 1993, for a review). There is accumulating evidence that fish prefer to shoal with familiar conspecifics (Brown and Smith, 1994; Griffiths, 1997; Griffiths and Magurran, 1997; Van Havre and FitzGerald, 1988). This behavior can be adaptive; for example, experimentally composed shoals of familiar fathead minnows exhibited better antipredator behavior when subjected to chemical stimuli from pike and a pike model than comparable shoals of unfamiliar fish (Chivers et al., 1995).

Should fish also prefer to shoal with kin? The evidence so far does not support the hypothesis that natural shoals of fish are composed of related individuals (Avise and Shapiro, 1986; Naish et al., 1993; Peuhkuri and Seppä, 1998). Theoretical studies of the effects of genetic relatedness on the predicted size of social groups come to the general conclusion that increasing relatedness will ordinarily decrease, and never increase, equilibrium group size under free entry (Giraldeau and Caraco, 1993; Higashi and Yamamura, 1993; see also Rannala and Brown, 1994). It may actually often be advantageous to group with non-kin (Grafen, 1992). A recent study (Griffiths and Magurran, 1999) with Trinidadian guppies, *Poecilia reticulata,* a species whose reproductive biology favors the association of kin groups, tested experimentally for a potential preference to associate with kin and found that juveniles reared together were able to recognize one another on the basis of either visual or chemical cues but showed no preference for schooling with unfamiliar kin.

Reviewers seem to agree that we have a very poor understanding of the adaptive value of kin discrimination in most species that have been investigated (Barnard, 1990; Blaustein et al., 1991; Grafen, 1990; Waldman et al., 1988). Nevertheless, experimental studies have shown that fish prefer to shoal with their siblings. Sibling discrimination by chemical cues has been demonstrated in several species of salmonids (review in Olsén and Winberg, 1996). Three-spined sticklebacks (*Gasterosteus aculeatus*) prefer unfamiliar siblings and even half-siblings over unfamiliar nonsiblings when they can see and smell both alternatives (FitzGerald and Morisette, 1992; Van Havre and FitzGerald, 1988).

We thus have a discrepancy between expectations and experimental findings. Because in the stickleback studies test fish could not only smell but also see the two shoals, they could have preferred those fish that matched their own size best (i.e., potentially their siblings); sticklebacks have been shown to prefer to shoal with conspecifics of similar size (Keenleyside, 1955; Peuhkuri et al., 1997; Ranta et al., 1992), probably to avoid becoming the odd target (Ohguchi, 1981). Although Van Havre and FitzGerald (1988) do not mention whether the size of test and stimulus fish were matched, FitzGerald and Morisette (1992) state that they matched the size of the test fry and stimulus fry but do not present substantiating data.

Mice are known to discriminate between conspecifics through the products encoded by the highly polymorphic loci of the major histocompatibility complex (MHC) (e.g., Yamazaki et al., 1983; review in Penn and Potts, 1999). In addition to being part of the vertebrate immune system, the MHC affects an individual's odor profile (Singer et al., 1997; review in Penn and Potts, 1998). The MHC of three-spined sticklebacks has been analyzed (Sato et al., 1998). If sticklebacks discriminate their siblings by odor cues, a correlation of this preference with shared MHC alleles could be addressed in a future study, the present study being a prerequisite.

The aim of the present study was to extend the previous work of shoaling preferences for siblings versus non-kin in sticklebacks by using only olfactory cues in a blind procedure with strict randomization of fish and procedures using fish from a European population. We used juvenile fish to exclude mate choice, which might be correlated with the MHC. Furthermore, a potential antipredator advantage of shoaling with siblings was tested by using olfactory cues of pike (*Esox lucius*).

METHODS

Test and stimulus fish

The test fish were bred in the laboratory from 11 female and 11 male sticklebacks (*Gasterosteus aculeatus*) that had been caught in Bielefeld, Germany. One to two hours after each clutch of eggs had been spawned and fertilized, it was removed from the male's nest and split into halves. We transferred each half to a small tank in which the sticklebacks

Address correspondence to M. Milinski. E-mail: manfred.milinski@esh.unibe.ch.

Received 4 September 1998; revised 12 November 1998; accepted 31 January 1999.

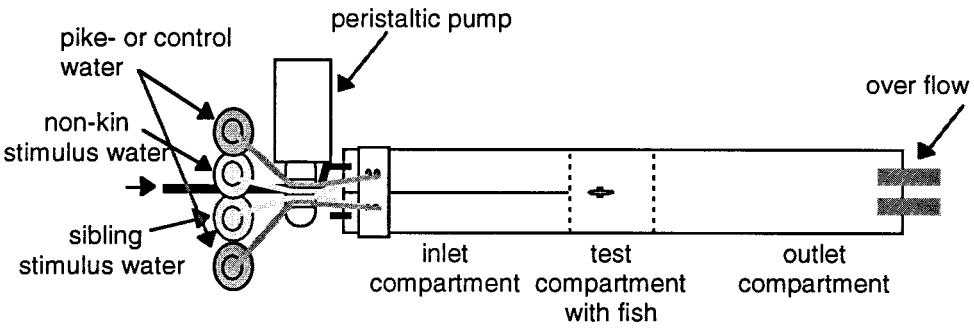

Figure 1
Experimental setup from above. See text for details.

hatched after 10–12 days. Thus there were 2 separate subgroups of each of the 11 sibling groups. After hatching each subgroup was placed in a tank (40×20 cm, 23 cm water level, constant supply of spring water, temperature 13°C, 16 h illumination by a fluorescent 30-W tube). To avoid environmental variation between the two tanks of a sibling group being smaller than among all tanks, the two tanks of each sibling group were placed neither in the same row nor in the same or in a neighbor column of the shelf in the culture room. The fish were fed live *Artemia* and *Daphnia* and frozen *Bosmia* and *Artemia*.

We tested the fish between 61 and 91 days of age (standard length between 1 and 2 cm); the youngest sibship was 20 days younger than the oldest. One subgroup (''stimulus fish'') of each sibship was used for producing stimulus water, the other for providing the ''test fish'' (decided by drawing lots for each sibgroup). We reduced each subgroup to 10 fish by a chance procedure. Because the fish of different sibships differed in size (standard length of stimulus fish were determined before that of test fish after the experiment), the shoals of different sibships also differed in total weight. During the experimental period the fish were kept in tanks (2 l, aerating stone) without constant supply of water. The position of the tanks on the shelf was randomized. The fish were fed daily with as many frozen *Daphnia* and *Artemia* as they could consume within 10 min. Both in the morning and at noon 0.5 l of water was removed from each stimulus-fish tank and kept in glass bottles for 10–135 min until the experiment. In the evening all tanks were filled up with spring water and the glass bottles were cleaned with hydrochloric acid.

Preparation of pike water

A pike (*Esox lucius,* about 20 cm standard length) was fed daily with three freshly killed sticklebacks (about 3 cm standard length) in its home tank during 5 days. Thereafter it was placed in a smaller tank (22 l) for 52 h without being fed. The water from this tank was filled into plastic bags (Tangan, Migros) for preparing ice cubes and frozen at a temperature of −20°C (Gelowitz et al., 1993). Tap water (without any water treatment chemicals) used as ''control water'' was frozen in the same way. On the evening before an experiment a person (not the experimenter) removed ice cubes of both ''pike water'' and ''control water'' from the bags, filled them into marked Pet bottles, each type into a separate one, and sealed the bottles with Parafilme. The experimenter did not know the code. On the next day the water, at room temperature (15°C), was poured into 0.5-l glass bottles.

Apparatus

In the experiment a test fish maintained its position in a current to which stimulus water was continuously added (i.e., water from siblings on one side and water from non-kin on the other side). The fluviarium (100 cm×10.4 cm, water level 8 cm; see Figure 1) was similar to that used in experiments on kin recognition in salmonids (Brown et al., 1993; Höglund, 1961). It was divided into an inlet, a test, and an outlet compartment by nets (1 mm mesh). A pump (380 l/h) produced a constant current (1 cm/s). The inlet compartment (40 cm) was divided laterally into halves by a gray PVC partition. A peristaltic precision pump (Reglo Analog MS-4/8) supplied stimulus water (7 ml/min) from a sibling group and a non-kin group through silicon tubes to the halves of the inlet compartment (Figure 1). Additionally, either pike or control water was supplied at the same rate through two other channels of the pump. So a test fish in the test compartment (15×10.4 cm) had always the choice between a sibling and a non-kin side either with pike water added to both sides or control water. A test with colored water showed that the two types of water hardly mixed in the test compartment. The fluviarium was illuminated from above by a fluorescent tube of 30 W and visually isolated by black cloth from all sides. A video camera was suspended above the test compartment.

Procedure

On each of 10 consecutive days we tested 1 fish of each of the 11 sibling groups, the sequence being varied between days. The stimulus water from each shoal was used both as sibling water and as non-kin water on each day. A block randomization design guaranteed that the stimulus water from each shoal was used equally often as sibling with pike, sibling with control, non-kin with pike, and non-kin with control water. Each of the 10 test fish of 1 sibling group was tested with another non-kin group, so that each possible combination of sibling group and non-kin group occured once.

Each test fish was gently caught with a glass pipe (see Milinski and Bakker, 1992) and placed in the current of the test compartment. After 1 min the supply of both sibling and non-kin water and of either pike or control water started. After 3 min the supply was stopped for 1 min, and the sides of sibling and non-kin water were reversed. This was repeated until each type of stimulus water had been supplied twice on each side. Thereafter the standard length of each test fish was measured. The weight of the test fish had not been measured directly, so we estimated it from measuring 20 other sticklebacks with a similar size distribution from breeds of the same population. From the relation between standard length and log weight (Pearson's $r = .67$, $p = .0015$, $N = 20$), an exponent of 1.4 was determined to transform length (millimeters) to weight (milligrams).

The video tapes were encoded so that they could be analyzed blindly with respect to origin of test fish, side of sibling water, and mode of predator stimulus (pike or control). On the video screen the test compartment was subdivided into equal quarters (7.5×5.2 cm). We measured the time the test fish (snout) spent in each quarter from the record.

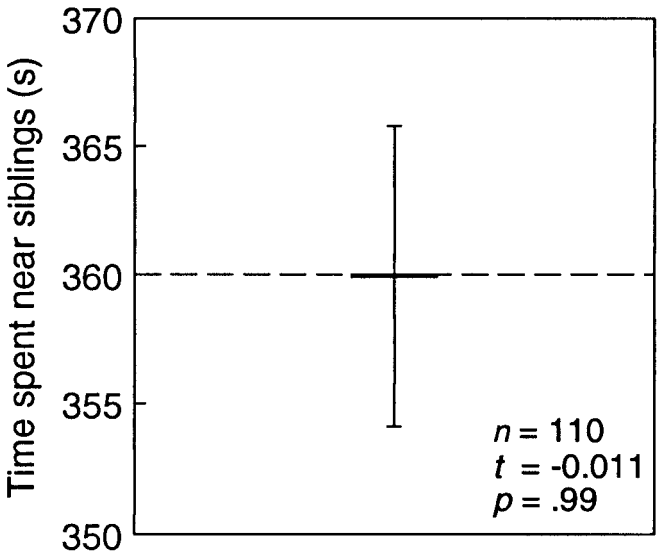

Figure 2
Deviation from the null expectation (no preference at dashed line) of the time spent near siblings (all test fish, mean ±1 SE).

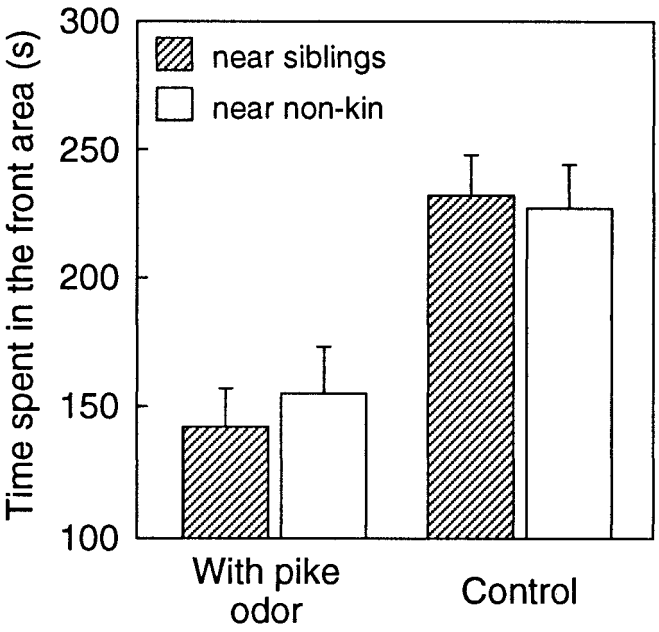

Figure 3
The time spent in the front area near siblings (hatched bars) and near non-kin (open bars) when either the pike water or control water was added (means and 1 SE). Two-way ANOVA with repeated measurements: effect of sibling odor: $F = 0.29$, df = 1, $p = .59$; effect of pike odor: $F = 13.89$, df = 1, $p = .0003$; effect of sib group (not shown in the graph): $F = 1.81$, df = 10, $p = .07$; no interaction significant.

We used SYSTAT for Macintosh (Systat, 1992) for statistical analysis. Power analyses were done following Cohen (1988). All p values are two tailed. Bonferroni corrections were applied when data sets were subdivided.

RESULTS

No preference for siblings

Overall, the sticklebacks did not spend significantly more time on the sibling side than on the non-kin side (Figure 2). Because both Van Havre and FitzGerald (1988) and FitzGerald and Morrissette (1992) found a preference for full sibs in three-spined sticklebacks under various conditions, it is possible to estimate the effect size, d, following Cohen (1988) by an ANOVA performed over all their results. The violation of the assumption of equal variance may have only a slight influence on the p value (Cohen, 1988) and therefore as well on the estimation of the effect size. For a power analysis we use only our data from experiments with control water because the other studies did not test for predator effects. The power of a t test with our data [with a sample size of 55, a critical two-tailed α-level of 0.05, and an effect size d of about 1.52 (derived from Van Havre and FitzGerald, 1988, and FitzGerald and Morrissette, 1992)] is greater than 99% (Cohen, 1988). We had therefore enough power (> 80%; i.e., the convention suggested by Cohen, 1988) to find a significant preference if it existed in our sample.

Effect of the pike

Half of the sticklebacks were tested with additional supply of pike odor because the stimulus of a predator could be expected to affect the stickleback's preference for siblings. With pike odor the sticklebacks stayed in the front area a significantly shorter time than when tested with control water (Figure 3). However, pike odor did not significantly influence the test fish's choice for siblings (two-way ANOVA: effect of pike: $F = 0.27$, df = 1, $p = .60$, effect of sib group: $F = 2.27$, df = 10, $p = .02$, interaction: $F = 1.59$, df = 10, $p = .12$). Moreover, the test fish did not significantly prefer the sibling or the non-kin side when it was in the front area (Figure 3).

Effect of sib group and weight of stimulus shoal

The amount of time a stickleback spent either on the sibling side or the non-kin side depended significantly on the sib group the test fish originated from (see previous paragraph). The relative weight of the non-kin stimulus group could partly explain these effects because the test fish preferred the side of the heavier stimulus group when no pike odor was present, while the opposite effect seemed to occur when pike odor was present (Figure 4). However, when analyzed separately, the weight of the non-kin stimulus group had only a significant effect when pike odor was absent ($F = 5.89$, df = 4, $p = .001$) than when it was present ($F = 0.80$, df = 4, $p = .53$). The fish that were tested with control water preferred significantly heavier non-kin shoals (Figure 4). This result remained the same when the exponent of the length–weight correlation was changed from 1.4 to either 1.2 or 1.6.

DISCUSSION

The sticklebacks in our experiment showed no preference for their siblings when they were tested singly in a standard fluviarium (Höglund, 1961) where odor of nonfamiliar siblings and odor from nonfamiliar nonsiblings was offered simultaneously on two sides in the current. Because both groups consisted of unfamiliar fish, we can exclude any preference based on familiarity (Brown and Smith, 1994; Griffiths, 1997; Griffiths and Magurran, 1997; Van Havre and FitzGerald, 1988). A power analysis revealed that we had a 99% chance of finding an effect of preference for siblings of the effect size found earlier (FitzGerald and Morisette, 1992; Van Havre and FitzGerald, 1988), which is sufficient to accept the null hypothesis (threshold 80%, see Cohen, 1988).

If our procedure did not allow the test fish to appreciate the odor of conspecifics, our experiment would not be deci-

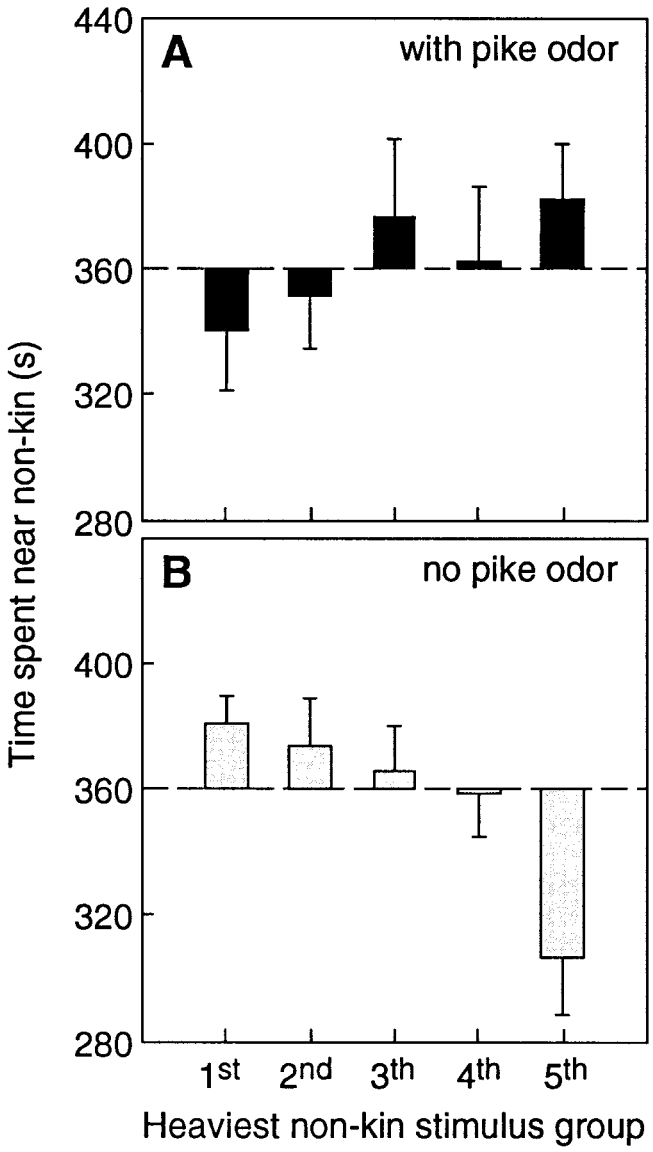

Figure 4
The time spent near non-kin (deviation from the null expectation) compared to the relative weight of the five non-kin stimulus groups per sib group and pike treatment (means and SE) (A) when pike odor was added and (B) when control water with no pike odor was added. Two-way repeated-measures ANOVA with 11 sib groups as independent replicates: effect of pike: $F = 0.17$, df = 1, $p = .69$; effect of non-kin group weight order: $F = 0.69$, df = 4, $p = .61$; interaction: $F = 3.78$, df = 4, $p = .01$.

sive. The test fish showed, however, a significant preference for the heavier of the two shoals (total calculated weight of whole shoal) from which the water with the odor had been taken. This result proves that our test fish could appreciate odor cues from conspecifics. It makes sense functionally, if a heavier shoal usually consists of more members conferring both a larger dilution and confusion effect (Milinski, 1977; Ohguchi, 1981; see Pitcher and Parrish, 1993, for a review). Given a simultaneous choice of two equidistant shoals of conspecifics that differed in membership size, three-spined sticklebacks preferred the larger shoal (Keenleyside, 1955; Ranta et al., 1992), especially under predation risk (Krause et al., 1998). Because this odor-based preference for the heavier shoal shows that our sticklebacks were able to choose a side depending on odor cue from conspecifics, our experimental procedure should have revealed a sibling odor preference if it existed. Therefore, and because we used a blind protocol with strict randomization of fish and procedures, our finding of no sibling odor preference in juvenile three-spined sticklebacks comes close to a proof of the null hypothesis, at least for our study population. We do not know (but cannot exclude the possibility) of any other context in which juvenile sticklebacks might express kin discrimination.

Our findings are in agreement with predictions from theoretical studies of the effects of genetic relatedness on the predicted size of social groups under free entry (Giraldeau and Caraco, 1993; Higashi and Yamamura, 1993); they are, however, at variance with earlier studies on kin recognition and choice of shoal mates in three-spined sticklebacks (FitzGerald and Morisette, 1992; Van Havre and FitzGerald, 1988). Recently Peuhkuri and Seppä (1998) studied the kin structure in 24 natural schools of juvenile three-spined sticklebacks using allozymes as genetic markers. Their results suggest that, on average, schools are random samples from the genetic pool of their Finish study population, which agrees with our findings (see also Mitchell et al., 1995).

We found a strong reaction of our test fish when water from a tank with a pike that had digested three-spined sticklebacks was added to the current: the test fish avoided the upstream part of the test chamber. This result is in agreement with previous findings (Gelowitz et al., 1993). However, addition of the odor of pike did not significantly increase a potential preference for the test fish's siblings, which does not agree with the hypothesis of an antipredator function of preferring shoals with siblings (FitzGerald and Morrisette, 1992). Although we cannot rule out that the avoidance of pike odor outweighed any other potential preference (e.g., for siblings or the larger shoal), this results corroborates our main finding that our sticklebacks did not prefer to shoal with their siblings.

We thank R. Eggler, J. Rauch and N. Treichel for technical help, the referees for helpful comments, and the Swiss National Science Foundation for support.

REFERENCES

Avise JC, Shapiro DY, 1986. Evaluating kinship of newly settled juveniles within social groups of the coral reef fish *Anthias Squampinnis*. Evolution 40:1051–1059.

Barnard CJ, 1990. Kin recognition: problems, prospects, and the evolution of discrimination systems. Adv Study Behav 19:29–81.

Blaustein AR, Bekoff M, Byers JA, Daniels TJ, 1991. Kin recognition in vertebrates: what do we really know about adaptive value? Anim Behav 41:1079–1083.

Brown GE, Brown JA, Crosbie AM, 1993. Phenotype matching in juvenile rainbow trout. Anim Behav 46:1223–1225.

Brown GE, Smith RJF, 1994. Fathead minnows use chemical cues to discriminate natural shoalmates from unfamiliar conspecifics. J Chem Ecol 20:3051–3061.

Chivers DP, Brown GE, Smith RJF, 1995. Familiarity and shoal cohesion in fathead minnows (*Pimephales promelas*): implications for antipredator behaviour. Can J Zool 73:955–960.

Cohen J, 1988. Statistical power analysis for the behavioral sciences. Hillsdale, NJ: Lawrence Erlbaum Associates.

FitzGerald GJ, Morrissette J, 1992. Kin recognition and choice of shoal mates by threespine sticklebacks. Ethol Ecol Evol 4:273–283.

Gelowitz CM, Mathis A, Smith RJF, 1993. Chemosensory recognition of northern pike (*esox lucius*) by brook stickleback (*culaea inconstans*): Population differences and the influence of predator diet. Behaviour 127:105–117.

Giraldeau L-A, Caracao T, 1993. Genetic relatedness and group size in an aggregation economy. Evol Ecol 7:429–438.

Grafen A, 1990. Do animals really recognize kin? Anim Behav 39:42–54.

Grafen A, 1992. Of mice and the MHC. Nature 360:530.
Griffiths SW, 1997. Preferences for familiar fish do not vary with predation risk in the European minnow. J Fish Biol 51:489–495.
Griffiths SW, Magurran AE, 1997. Familiarity in schooling fish: how long does it take to acquire? Anim Behav 53:945–949.
Griffiths SW, Magurran AE, 1999. Schooling decisions in guppies (*Poecilia reticulata*) are based on familiarity rather than kin recognition by phenotype matching. Behav Ecol Sociobiol 45:437–443.
Higashi M, Yamamura N, 1993. What determines animal group size? Insider-outsider conflict and its resolution. Am Nat 142:553–563.
Höglund LB, 1961. The reaction of fish in concentration gradients. Rep Inst Freshw Res Drottingholm 43:1–147.
Keenleyside MHA, 1955. Some aspects of the schooling behaviour of fish. Behaviour 8:183–248.
Krause J, Godin J-GJ, Rubenstein D, 1998. Group choice as a function of group size differences and assessment time in fish: the influence of species vulnerability to predation. Ethology 104:68–74.
Milinski M, 1977. Do all members of a swarm suffer the same predation? Z Tierpsychol 45:373–388.
Milinski M, Bakker TCM, 1992. Costs influence sequential mate choice in sticklebacks, *Gasterosteus aculeatus*. Proc R Soc Lond B 250:229–233.
Mitchell SE, De Meester L, Weider LJ, Carvalho GR, 1995. No evidence for kin-preferential swarming in a *Daphnia magna* population coexisting with fish. J Anim Ecol 64:777–779.
Naish K-A, Carvalho GR, Pitcher TJ, 1993. The genetic structure and microdistribution of shoals of *Phoxinus phoxinus,* the European minnow. J Fish Biol 43:75–89.
Ohguchi O, 1981. Prey density and selection against oddity by threespined sticklebacks. Z Tierpsychol 23:1–79.
Olsén KH, Winberg S, 1996. Learning and sibling odor preference in juvenile Arctic char, *Salvelinus alpinus* (L.). J Chem Ecol 22:773–786.
Penn D, Potts W, 1998. How do major histocompatibility complex genes influence odor and mating preferences? Adv Immunol 69: 411–436.
Penn D, Potts W, 1999. The evolution of mating preferences and major histocompatibility complex genes. Am Nat 153:145–164.
Peuhkuri N, Ranta E, Seppä P, 1997. Size-assortative schooling in free-ranging sticklebacks. Ethology 103:318–324.
Peuhkuri N, Seppä P, 1998. Do three-spined sticklebacks group with kin? Ann Zool Fenn 35:21–27.
Pitcher TJ, Parrish J, 1993. The function of shoaling behaviour. In: The behaviour of teleost fishes, 2nd ed (Pitcher TJ, ed). London: Chapmann and Hall; 363–439.
Rannala BH, Brown CR, 1994. Relatedness and conflict over optimal group size. Trends Ecol Evol 9:117–119.
Ranta E, Lindström K, Peuhkuri N, 1992. Size matters when three-spined sticklebacks go to school. Anim Behav 43:160–162.
Sato A, Figueroa F, O'h Uigin C, Steck N, Klein J, 1998. Cloning of major histocompability complex genes (Mhc) from threespine stickleback, *Gasterosteus aculeatus*. Mol Mar Biol Biotechnol 7:221–231.
Singer AG, Beauchamp GK, Yamazaki K, 1997. Volatile signals of the major histocompatibility complex in mouse urine. Proc Natl Acad Sci USA 94:2210–2214.
Systat, 1992. Statistics, version 5.2. Evanston, Illinois: SYSTAT.
Van Havre N, FitzGerald GJ, 1988. Shoaling and kin recognition in the threespine stickleback, *Gasterosteus aculeatus*. Biol Behav 13: 190–201.
Waldman B, Frumhoff PC, Sherman PW, 1988. Problems of kin recognition. Trends Ecol Evol 3:8–13.
Yamazaki K, Beauchamp GK, Ecorov IK, Bard J, Thomas L, Boyse EA, 1983. Sensory distinction between H-2^b and H-2^{bml} mutant mice. Proc Natl Acad Sci USA 80:5685–5688.