



## Investigating the Effect of Familiarity on Kin Recognition of Three-Spined Stickleback (*Gasterosteus aculeatus*)

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Received: December 18, 2012  
Initial acceptance: February 10, 2013  
Final acceptance: March 27, 2013  
(J. Wright)

doi: 10.1111/eth.12091

### Abstract

The ability to discriminate between related and unrelated individuals has been demonstrated in many species. The mechanisms behind this ability might be manifold and depend on the ecological context in which the species lives. In brood-caring species, both familiarity and phenotype matching are known to be used in kin recognition. However, results of studies disentangling these two phenomena have proved contradictory. We aimed to broaden our knowledge about the mechanisms of kin recognition using shoaling preferences of three-spined stickleback (*Gasterosteus aculeatus*) as a model behavior. In our first experiment, focal fish had the choice to shoal either with kin or unfamiliar non-kin. In half of the trials, kin groups were composed of familiar individuals, while they were unfamiliar in the other half. Focal fish significantly preferred kin as shoaling partner, a result which was not reinforced by familiarity. In our second experiment, focal fish were given the choice between a shoal of familiar kin and a shoal of unfamiliar kin. Here, focal fish did not show any significant preference. These results indicate that familiarity does not impact stickleback's ability to recognize kin. Furthermore, they show that familiarity does not overrule recognition based on phenotype matching or innate recognition, underlining the importance of these mechanisms. Finally, our results lead to the assumption that individual recognition might play a minor role also in non-kin-based preferences for familiars.

The ability to discriminate between individuals differing in their relatedness to a focal subject has been demonstrated for a variety of taxa, ranging from microbes (Mehdiabadi et al. 2006) and plants (Biedrzycki & Bais 2010) to chimpanzees (*Pan troglodytes*) (Vokey et al. 2004). Although the benefits of differentiating between related and unrelated conspecifics are, at least theoretically, well understood (e.g., Hamilton 1964; Pusey & Wolf 1996), the mechanisms underlying kin-based discrimination are still debated and may vary between animal species and contexts (e.g., Hepper 1986; Waldman 1987; Tang-Martinez 2001; Mateo 2004; Penn & Frommen 2010). When siblings remain together for part of their lives, familiarity is a

reliable measure of kinship. Under these circumstances, an individual has the possibility to learn phenotypic cues of its close relatives and to use such familiarity or individual recognition to identify its relatives later in life. When, on the other hand, individuals do not continuously live in family groups, it is important to recognize unfamiliar kin as well. Such recognition may be based on self-reference (Dudley & File 2007; Villinger & Waldman 2008) or on innate recognition, for example, through recognition alleles (e.g., Queller et al. 2003). Furthermore, kin recognition has often been shown to arise through indirect familiarity or 'phenotype matching' (Holmes & Sherman 1982; Fletcher 1987), in which an individual first

builds a template based on cues from familiar, related conspecifics, and later uses this template to evaluate the relatedness of unknown conspecifics (e.g., Heth et al. 1998; Gerlach & Lysiak 2006; Le Vin et al. 2010). The different mechanisms of kin recognition may play a role also sequentially within a species, depending on the developmental state and the context (e.g., Mateo 2004; Schausberger 2007).

While some authors argue that phenotype matching and familiarity are categorically distinct mechanisms (e.g., Holmes & Sherman 1983; Blaustein et al. 1987), others state that they are variations of the same ontological process (e.g., Porter 1988; Tang-Martinez 2001). Familiarity is sometimes thought to be the more reliable way of recognizing kin, as phenotype matching might be more prone to recognition errors due to an excess overlap in cues (Penn & Frommen 2010). Furthermore, benefits of decisions based on familiarity (see below) might overrule the benefits of decisions based on kinship (e.g., Edenbrow & Croft 2012). On the other hand, the phenotypes of familiar and unfamiliar kin should be similar, as they share a common genetic background. Thus, in species in which phenotype matching plays a crucial role, individuals might no longer be able to discern familiar and unfamiliar individuals due to their similar phenotypes, thus making a choice between familiar and unfamiliar relatives impossible. Studies investigating the interplay between familiarity and phenotype matching in kin recognition have produced mixed results: While some found familiarity having a strong impact on kin recognition (e.g., Ligout & Porter 2003; Edenbrow & Croft 2012), others found phenotype matching to be the dominant effect (e.g., Thünken et al. 2007b; Le Vin et al. 2010). Furthermore, both mechanisms might work additively (Lizé et al. 2012) or have no effect at all (e.g., Ruf et al. 2010).

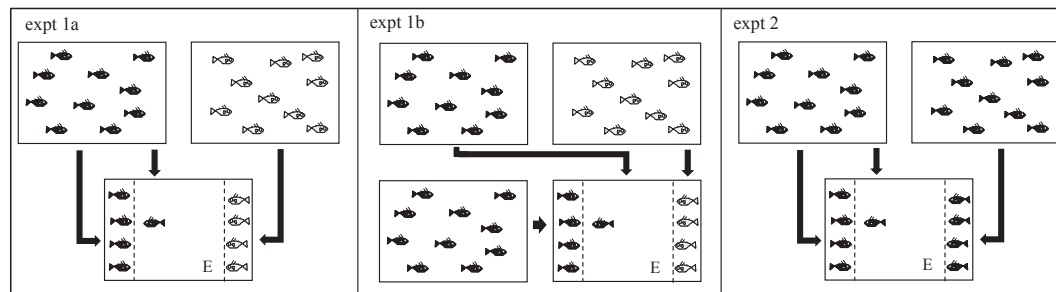
### Living in Shoals

Many animal species live at least for part of their life in groups, which are usually not composed randomly (Pitcher & Parrish 1993; Krause & Ruxton 2002). A well-documented factor influencing shoaling decisions of many fish species is familiarity (see Ward & Hart 2003 for a review). Shoaling with familiars is known to be beneficial in terms of reduced aggressive behavior (Höjesjö et al. 1998; Gómez-Laplaza 2005), improved foraging efficiency (Griffiths et al. 2004), or as a prerequisite for the development of cooperative interactions (Utne-Palm & Hart 2000). Relatedness is another factor influencing shoaling decisions in several fishes (e.g., Arnold 2000; Behrmann-Godel et al.

2006; Gerlach & Lysiak 2006; Piyapong et al. 2011; Hesse et al. 2012). Shoaling with kin may provide indirect benefits by increasing the individual's inclusive fitness (Hamilton 1964) through increasing the reproductive success of its relatives. This can be facilitated, for example, through altruistic behavior or the reduction in competition (e.g., Brown & Brown 1993; Gerlach et al. 2007). Additionally, individuals that join kin may increase their inclusive fitness if larger shoals lead to lower individual predation threat and thus higher survival for all group members (Ward & Hart 2003). Finally, group-defending behavior is expected to be more pronounced in groups of related individuals (Waldman 1988; Wu et al. 2010).

### Three-Spined Stickleback

Three-spined stickleback (*Gasterosteus aculeatus*) are known to recognize both familiar and unfamiliar kin and use this ability in many different contexts. During mate choice, they prefer to mate with unrelated conspecifics (Frommen & Bakker 2006; Mehliis et al. 2008), thus avoiding the negative effects of inbreeding (Frommen et al. 2008). Brood-caring males are able to assess their amount of paternity in the brood and adjust their cannibalistic behavior accordingly (Mehliis et al. 2010). The mechanism of kin recognition in stickleback has most thoroughly been investigated in shoaling decisions. Outside the breeding season, juvenile and adult stickleback form loose shoals (Wootton 1976). Here, familiarity and relatedness are known to influence shoal choice. Juvenile as well as adult fish prefer familiar kin and familiar non-kin over unfamiliar non-kin as shoaling partners (FitzGerald & Morrissette 1992; Barber & Ruxton 2000; Frommen & Bakker 2004; Frommen et al. 2007a,c). Furthermore, they prefer unfamiliar kin over unfamiliar non-kin (van Havre & FitzGerald 1988; FitzGerald & Morrissette 1992; Frommen et al. 2007c). The preference for familiar non-kin is reversed under food-limited conditions (Frommen et al. 2007b). This finding can be interpreted as individuals avoiding competition with assumed relatives underlining the importance of familiarity as a proximate factor for kin recognition. Finally, fish originating from the same population as used in the current experiments did not show any preferences when given the choice between familiar kin and familiar non-kin (Frommen et al. 2007a), leading to the assumption that phenotype matching is the main mechanisms triggering kin recognition in this species, while innate recognition via recognition alleles or self-reference only play a minor role.



**Fig. 1:** Experimental set-up. In experiment 1, stimulus fish were either familiar (expt 1a) or unfamiliar (expt 1b) full-sibs or unfamiliar non-sibs. In experiment 2, stimulus fish were either familiar full-sibs or unfamiliar full-sibs. The three experimental tanks [E] show the respective trial combinations during the experiments. The other tanks show the different rearing conditions. Different colors show different grades of relatedness; black equals kin, white equals non-kin.

While familiarity as well as kinship is known to influence shoaling behavior in stickleback, the interplay of these two mechanisms is still unclear. Different scenarios are imaginable: On a proximate level, familiarity might enhance an individual's preference for related individuals, for example, because individual recognition might reinforce other kin recognition mechanisms. Furthermore, direct benefits of shoaling with familiar individuals and indirect benefits of shoaling with relatives might be additive. On the other hand, familiarity might have a disruptive effect, overruling a possible preference for relatives. This outcome should be expected if direct benefits of shoaling with familiars would be larger than the rather indirect benefits of shoaling with kin. Yet, another possibility is that relatedness assessed by phenotype matching might elicit the strongest response. This scenario might be most likely when direct benefits of shoaling with familiar individuals are small compared with the benefits gained when shoaling with kin. If this scenario was true, there would be no reason to differentiate between familiar and unfamiliar kin, leading to no significant differences in the focal fish's choice. To disentangle the effects of these different mechanisms, we conducted two experiments using a classical two choice design. In experiment 1, the focal fish had the choice between a group consisting of full-sibs and a group of unfamiliar non-sibs. In half of the trials, the full-sibs were familiar to the focal fish (Fig. 1, expt 1a), while in the other half, they were unfamiliar (Fig. 1, expt 1b). The unrelated shoal was always unfamiliar to the test fish; thus, the only difference between treatment 1a and 1b was familiarity toward the kin group. This experiment allowed us to test whether familiarity increases the shoaling preference for kin in stickleback. In experiment 2, the focal fish had the choice between a group of familiar kin and a group of unfamiliar kin (Fig. 1, expt 2). A

preference for the familiar fish in this experiment would underline the importance of familiarity in shoaling decisions, while the lack of any preference would emphasize the significance of phenotype matching.

## Methods

### Experimental Subjects

Three-spined stickleback used in the experiments were laboratory-bred offspring of anadromous fish, which had been caught during their spring migration in April 2010 from a large (Kemper 1995), genetically heterogeneous population (Heckel et al. 2002) on the island of Texel, the Netherlands. The F1 generation was obtained by mating randomly chosen wild-caught males and females in June and July 2010. Parental fish were only used once. Clutches were taken out of the nests two hours after fertilization to exclude paternal effects. Each clutch was divided into two subgroups, which were reared apart from each other. Thus, we created familiar and unfamiliar kin for each of the sib groups. One week after hatching, group sizes were reduced to 20 individuals to minimize differences in growth rate of the hatchlings due to different fish density. Fish were kept in an air-conditioned room under standardized winter light regime (day length 8L:16D, temperature  $17 \pm 1^\circ\text{C}$ ) to prevent them from reaching reproductive state. Rearing tanks contained 45 l of tap water and were separated from each other by green opaque partitions. Thus, fish in each group were familiar to each other but had no opportunity to have contact with other groups. Water in the tanks was cleaned and aerated through an internal filter, and a third of the water volume was replenished with tap water several times a week. Water quality was checked regularly. Fish were fed

daily in excess with defrosted *Chironomus* larvae. To minimize potential biases due to differences in intrinsic state (van Havre & FitzGerald 1988; Frommen et al. 2012), we only used fish showing no signs of developing breeding coloration or egg production. As high nitrite levels are known to affect development, outer appearance and swimming behavior of fish (Wang et al. 2006; Rodrigues et al. 2007; Simmons et al. 2012), we took care not to take fish from aquaria where high nitrite levels ( $>0.1$  mg/l) were measured within three days prior to the experiments. Focal individuals and most of the stimulus fish were used only once to avoid effects of multiple testing. Six stimulus fish were used twice, but never in the same experiment or in the same group combination.

### Experimental Design and Procedure

We performed experiments between January 28 and February 25, 2011. To avoid potential sequential effects, experiments were performed in blocks of three trials, with each block containing one trial of each stimulus shoal combination (expt 1a: familiar kin vs. unfamiliar non-kin, expt 1b: unfamiliar kin vs. unfamiliar non-kin, expt 2: familiar kin vs. unfamiliar kin); hence, the experimental design was balanced. As differences in hunger levels are known to influence shoaling preferences of stickleback (Krause 1993; Frommen et al. 2007b), we fed fish in excess with *Chironomus* larvae thirty minutes before starting a trial.

The experiments were conducted in a glass tank ( $80 \times 35 \times 40$  cm,  $l \times w \times h$ ), which was divided into three compartments using perforated clear Plexiglas partitions, allowing visual contact and exchange of water and chemical signals, but keeping the fish in their designated area. The two outer compartments (stimulus compartments) measured  $20 \times 35 \times 40$  cm, while the middle compartment (test compartment) measured  $40 \times 35 \times 40$  cm. A fluorescent light placed above the tank provided lighting. Disturbance of the fish was prevented by green Styrofoam placed around all sides of the tank and a black curtain hung in front of the set-up. Behavior of the focal fish was recorded from above using a web camera connected to a computer. Before the start of each trial, we filled the tank with aged and aerated tap water to a level of 20 cm. As kin recognition in stickleback is mediated by olfactory cues (Mehlis et al. 2008), we completely changed the water after each trial to remove confounding odors of prior trials. Stimulus groups contained four individuals, which were size-matched between the two groups in a given trial. The average standard length of the respective stimulus

groups did not differ significantly in any of the experiments (Related-samples Wilcoxon matched-pairs signed-ranks test; experiment 1:  $N = 45$ ,  $Z = 464.5$ ,  $p = 0.66$ ; experiment 2:  $N = 22$ ,  $Z = 133$ ,  $p = 0.54$ ). All fish were caught from their rearing tank immediately before the start of each trial. The stimulus fish were placed into the stimulus compartments, and the focal fish was placed into a transparent cylinder (12 cm in diameter) in the center of the test compartment. We avoided side biases by alternating the side with related stimulus fish between trials. After two minutes, video recording was started, the cylinder was lifted using a pulley system, and the fish were left alone in the experimental room. Thirty minutes later, the camera was stopped, all fish were removed from their chambers, and their standard length was measured to the nearest mm. Afterward, fish were marked by clipping the tip of one of their dorsal spines and put back into their rearing tank.

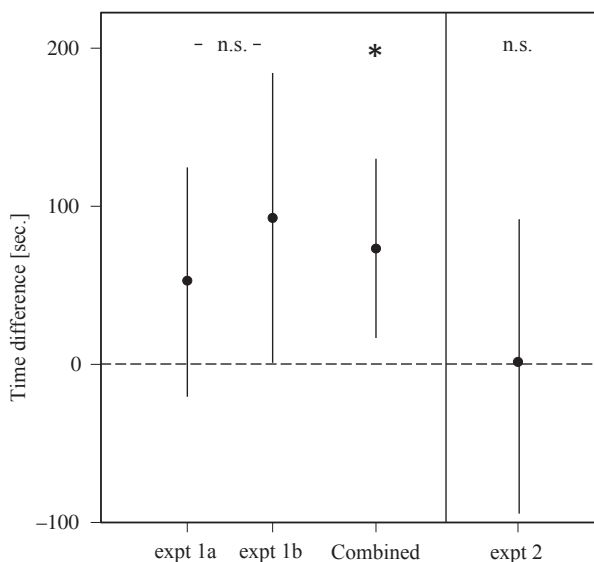
### Data Analyses

To analyze the videos, we marked choice zones on the computer monitor in front of each stimulus compartment measuring 10 cm, equaling approximately three body lengths of the stickleback used in the experiment, which is a standard measure for shoaling behavior (Pitcher & Parrish 1993). Additionally, we marked a line dividing the middle compartment into two equal-sized halves. Focal fish were allowed to become familiar with the test tank during the first 15 min. Within this time, we recorded whether the focal fish crossed the middle line at least once. In two trials of experiment 1 (kin vs. non-kin) and in two trials of experiment 2 (familiar kin vs. unfamiliar kin), the focal fish did not visit both sides of the experimental tank, so we excluded these replicates from the analyses. We excluded one further trial from experiment 1 as the kin group and the focal fish were mistakenly taken from a tank containing water with a high nitrite level ( $>0.5$  mg/l). This resulted in a sample size of 45 in experiment 1, in which in 22 cases, the kin group was familiar, while in 23 cases, the kin group was unfamiliar, and a sample size of 22 in experiment 2. Some trials were mistakenly stopped prematurely. Consequently, to use the same time frame in each trial, we analyzed 12 minutes of video recordings that were available for all trials instead, starting at minute 15. Videos were watched naively with respect to the type of the experiment and the sides that were occupied by familiar kin, unfamiliar kin or non-kin. We measured the time focal fish spent in the choice zones using Observer XT 7.0©. The mea-

surements started as soon as the focal fish's head entered a given choice zone and stopped as soon as the head left the choice zone. From these time values, we calculated a preference difference by subtracting the time spent near the unrelated shoal from the time spent near the kin shoal (expt 1) or subtracting the time spent near the unfamiliar kin shoal from the time spent near the familiar kin shoal (expt 2). A positive value thus indicates a preference for related fish (expt 1) or familiar fish (expt 2). Additionally, to be able to control for among-individual variation in activity and in the drive to shoal, we calculated the overall time spent shoaling by adding up the time spent in both choice zones. To compare shoaling preferences in experiment 1, we ran a linear mixed-effects model (LMM) with preference difference being the dependent variable, total time spent shoaling as a covariate and familiarity (that is whether the related shoal was familiar or not) as a fixed factor. A significant effect of the familiarity term would indicate that preferences in experiment 1a and 1b differed and, thus, that familiarity influences test fish's choice for the related shoal. Furthermore, a significant intercept would indicate an overall preference for either the related or unrelated shoal. As some families provided up to three focal fish, we included family of origin as a random factor to account for non-independence in the data and to avoid pseudoreplication. To analyze data of experiment 2, we ran a LMM with preference difference as the dependent variable, total time spent shoaling as a covariate and family of origin as a random factor. We applied a backward removal of variables with  $p > 0.05$  to avoid problems potentially arising from the inclusion of non-significant effects (Engqvist 2005) and obtained relevant statistics by re-entering removed variables one by one to the final model. In all cases, we used two-tailed testing. Statistics were calculated using R (Version 2.6.0); LMMs were implemented using the 'lme' function of the 'nlme' package.

## Results

In experiment 1, familiarity had no effect on the time test fish spent near the related shoal (familiarity:  $F_{1,16} = 0.49$ ,  $p = 0.49$ ). The same was true for the total time spent shoaling ( $F_{1,16} = 2.39$ ,  $p = 0.13$ ). However, focal fish preferred kin over non-kin as shoaling partners as indicated by a significant intercept ( $F_{1,28} = 6.79$ ,  $p = 0.015$ ; Fig. 2). In experiment 2, we observed no preference for familiar or non-familiar kin ( $F_{1,15} = 0.01$ ,  $p = 0.94$ ; Fig. 2). Total time spent shoaling ( $F_{1,16} = 1.08$ ,  $p = 0.34$ ) was not related to the strength of preferences either.



**Fig. 2:** Differences in time spent in front of the respective stimulus shoals. A value close to zero indicates no preference for one of the stimulus shoals. Experiment 1: Time spent next to familiar kin (expt 1a) or unfamiliar kin (expt 1b) minus time spent next to non-kin. Familiarity does not significantly influence focal fish's preferences for the related group. Overall, focal fish preferred kin over non-kin as shoaling partners. Experiment 2: Time spent next to familiar kin minus next to unfamiliar kin. Focal fish did show no significant preference for familiar or unfamiliar kin. Given are means and 95% confidence intervals. \*:  $p < 0.05$ , n.s.:  $p > 0.1$ .

## Discussion

The present study aimed to investigate the influence of familiarity on kin recognition in three-spined stickleback, using shoaling as model behavior. The results of experiment 1 suggest that familiarity does not increase stickleback's preference for related shoal mates. Still, they support former findings showing that three-spined stickleback are able to differentiate kin from non-kin and that they prefer to shoal with their relatives (FitzGerald & Morrisette 1992; Frommen & Bakker 2004; Frommen et al. 2007c). These results are comparable to recent work on an African cichlid species. In *Pelvicachromis taeniatus*, both sexes are able to differentiate unfamiliar kin from non-kin and prefer relatives as mating partners (Thünken et al. 2007a). This preference is not affected by familiarity (Thünken et al. 2007b), underlining the crucial role of phenotype matching in kin recognition also in that species (Thünken et al. 2007b; Hesse et al. 2012). In other species, however, the two mechanisms have been shown to work additively. Lambs (*Ovis aries*), for example, show most bleats toward unfamiliar non-kin. This behavior is stepwise reduced when shown

an unfamiliar twin of a familiar pen mate, an unfamiliar twin, and a familiar pen mate (Ligout & Porter 2003). Lizé et al. (2012) described comparable results in a subsocial wasp (*Goniozus legneri*).

When both groups in the present study were of equal relatedness and differed only in familiarity (expt 2), focal fish showed no significant preference for either group. Similarly, young of the social cichlid *Neolamprologus pulcher* prefer kin as social partners, but do not show preferences when given the choice between familiar and unfamiliar kin (Le Vin et al. 2010). This finding contrasts with studies on zebrafish (Gerlach & Lysiak 2006) and mangrove killifish (Edenbrow & Croft 2012). There, focal fish preferred familiar kin over unfamiliar kin, underlining the importance of familiarity in shoaling decisions of these species. It has indeed been suggested that familiarity-based shoaling decisions may lead to lowered aggression (Höjesjö et al. 1998; Gómez-Laplaza 2005), improved foraging performance (Griffiths et al. 2004; Ward & Hart 2005), or facilitated co-operation (Utne-Palm & Hart 2000). The somewhat surprising finding that our focal fish did not prefer familiar kin over unfamiliar kin may have several non-exclusive explanations. Proximately, a mechanism comparable to phenotype matching might trigger the described preferences for familiars (Barber & Ruxton 2000; Barber & Wright 2001; Frommen et al. 2007b). Here, in contrast to 'true' individual recognition, the individual might remember cues of familiar fish, and then treat all fish bearing these cues the same (see Box 1 in Tibbetts & Dale 2007). When they later meet unfamiliar fish carrying such cues, like the unfamiliar kin in experiment 2, they are not able to differentiate between familiar and unfamiliar fish anymore. Indeed, Ward et al. (2005) demonstrated sticklebacks' preference for con- and heterospecifics bearing familiar environmental odors over those that carry unfamiliar environmental odors, which supports the idea of learning more general cues of familiarity. A reason for not using individual recognition might be that learning individual cues of several conspecifics needs a considerable amount of neural capacity (Croney & Newberry 2007), while learning more generalized familiar cues should be less demanding. In guppies, for example, the preference for familiar individuals as shoal mates has been shown to decrease as the group size in which they naturally live increases (Griffiths & Magurran 1997). Fish in our experiment were kept in groups of up to twenty individuals, which might already exceed their recognition capacity (see Bergman 2010 for a comparable argument). Furthermore, stickleback shoals are often composed of more than a

hundred individuals in nature (e.g., Poulin 1999), thus the ecological framework for the evolution of individual learning might be missing.

Whether stickleback used phenotype matching, a form of self-reference or innate recognition to recognize their relatives could not be elucidated in our experiment. However, a recent study found that three-spined stickleback treat all fish with which they grew up as kin (Frommen et al. 2007b), while they did not differentiate familiar kin from familiar non-kin (Frommen et al. 2007a). This indicates that a form of learning plays a crucial role in kin discrimination in stickleback. Thus, these studies, together with the results of the present experiments, underline the importance of phenotype matching in relatedness-based shoaling decisions of this species.

### Acknowledgements

We are grateful to Roland Sasse, Martina Krakhofer, and Eva Strasser for help with maintenance of fish and Christina Schmidl for analyzing the videos. We thank Dustin Penn, Timo Thünken, and Valentina Balzarini for discussion and comments on the manuscript and Leif Engqvist for statistical advice. Jon Wright, Alex Jordan, and three anonymous referees are acknowledged for thoughtful comments on an earlier version of the manuscript. AH was supported by the 'Lendület' programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012; www.mta.hu); SR by a Swiss National Science Foundation fellowship (PBNEP3-132801 and PBNEP3-140190). The experiments were discussed and approved by the institutional ethics committee in accordance with Good Scientific Practice guidelines and national legislation.

### Conflict of interest

The authors declare that they have no conflict of interest.

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