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Kin recognition in zebrafish: a 24-hour window for olfactory imprinting

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Distinguishing kin from non-kin profoundly impacts the evolution of social behaviour. Individuals able to assess the genetic relatedness of conspecifics can preferentially allocate resources towards related individuals and avoid inbreeding. We have addressed the question of how animals acquire the ability to recognize kin by studying the development of olfactory kin preference in zebrafish (*Danio rerio*). Previously, we showed that zebrafish use an olfactory template to recognize even unfamiliar kin through phenotype matching. Here, we show for the first time that this phenotype matching is based on a learned olfactory imprinting process in which exposure to kin individuals on day 6 post fertilization (pf) is necessary and sufficient for imprinting. Larvae that were exposed to kin before or after but not *on* day 6 pf did not recognize kin. Larvae isolated from all contact with conspecifics did not imprint on their own chemical cues; therefore, we see no evidence for kin recognition through self-matching in this species. Surprisingly, exposure to non-kin odour during the sensitive phase of development did not result in imprinting on the odour cues of unrelated individuals, suggesting a genetic predisposition to kin odour. Urine-born peptides expressed by genes of the immune system (MHC) are important messengers carrying information about 'self' and 'other'. We suggest that phenotype matching is acquired through a time-sensitive learning process that, in zebrafish, includes a genetic predisposition potentially involving MHC genes expressed in the olfactory receptor neurons.

Keywords: kin recognition; olfactory imprinting; zebrafish

1. INTRODUCTION

Several mechanisms have been proposed by which individuals may discriminate kin. One way to identify possible relatives is to treat any conspecific that shares a particular location or degree of familiarity as kin. Kin recognition requires more stringent criteria when proximity and familiarity with conspecifics are not sufficiently reliable to detect true genetic relatedness. In a more specific method of kin recognition known as phenotype matching, an individual learns a template of its own phenotype (Mateo & Johnston 2000) and/or that of its familiar kin (Sherman *et al.* 1997), and later compares the phenotypes of unfamiliar animals with this template (Tang-Martinez 2001). Such phenotype matching depends on a consistent correlation between phenotypic and genotypic similarity, so that detectable traits are more alike among close relatives than among more distantly related or unrelated individuals (Holmes & Sherman 1983). Templates may consist of visual (Cooke *et al.* 1972; Hauber 2000), auditory (Beecher 1982; Gottlieb 1982) or chemical cues (Hepper 1986).

We suggest that recognition by direct familiarity and that by phenotype matching are based on different learning and memory systems. The kin template for phenotype matching should be either innate or acquired early in the development and should remain inflexible to change over the individual's lifetime. Familiarity with specific individuals occurs throughout a lifetime and may involve a more flexible process of learning and forgetting based on continuing experience. Because both mechanisms may be used by the same individual, the genetic and sensory basis of the two can be difficult to disentangle. The ease of experiential manipulation of fertilized fish eggs and developing larvae has enabled us to study pure phenotype matching and the constraints of template acquisition through isolation and cross-fostering experiments in zebrafish (*Danio rerio*).

In a previous study, we showed that juvenile zebrafish use phenotype matching based on the olfactory cues to differentiate between unfamiliar kin and non-kin, preferring the odour of unfamiliar full siblings to unfamiliar unrelated individuals (Gerlach & Lysiak 2006). However, increasing familiarity also increased olfactory preference for kin, showing that zebrafish are capable of recognizing kin through past experience as well as by phenotype matching. We also demonstrated that kin recognition in zebrafish has an immediate selective advantage as juvenile zebrafish housed in kin groups grew significantly faster than those in groups of unrelated individuals (Gerlach *et al.* 2007*b*). Accelerated growth in fish frequently correlates with greater survival and earlier fertility, as in wild Atlantic salmon (*Salmo salar* L.: Garant *et al.* 2003).

In other teleost species, both mechanisms of kin recognition have been shown as follows: kin recognition by phenotype matching; e.g. in coho salmon (*Oncorhynchus kisutch*: Quinn & Busack 1985), Arctic charr (*Salvelinus alpinus* L.: Olsén 1989; Winberg & Olsen 1992) and

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rainbow trout (*Oncorhynchus mykiss*: Brown *et al.* 1993) and kin recognition by familiarity, e.g. in guppies (*Poecilia reticulata*: Griffiths & Magurran 1999), bluegill sunfish (*Lepomis macrochirus*: Hain & Neff 2006) and sticklebacks (*Gasterosteus aculeatus*: Frommen *et al.* 2007).

Here, we report the results of behavioural experiments, documenting the developmental time window in which zebrafish larvae learn the olfactory template for kin later used in phenotype matching.

2. MATERIAL AND METHODS

(a) *Study animals*

Adult zebrafish were maintained in mixed sex groups on a recirculating system (AHAB Aquatic Habitats) under a 14 L : 10 D cycle at 26°C. Adults were fed with dry fish pellets and live *Artemia* daily. Mating pairs were selected from a mixed wild-type group originating from a variety of pet shops and breeding centres in USA. Eggs were collected from pairwise crosses of adult wild-type fish and reared in full sibling groups. Two hours after the initiation of spawning, eggs were removed from the mating tank and placed in Petri dishes for maintenance during rearing. We split each egg batch and raised two kin groups in separate Petri dishes (diameter 10 cm). We used one of these kin groups to create stimulus water for olfactory choice tests (see below); we tested the other group for kin preference as a control to determine the sibling group's baseline degree of kin preference. Experimental larvae isolated from the same parental cross were raised as single individuals in smaller Petri dishes (diameter 4 cm). Experimental larvae were isolated from kin groups or recombined with other individuals from the same condition at 9.30 hours. Daily, remaining food was removed and 50% of the water was renewed in all Petri dishes.

Zebrafish larvae hatched at 4–5 days post fertilization (dpf) at 26°C under the same light regime as the adults; they were fed rotifers enriched with powdered *Spirulina* algae (Salt Creek, Inc.) and Hatchfry Encapsulon (Argent Laboratories) from hatching until 10 dpf, at which point live *Artemia* were added to their diet.

(b) *Time window for learning the olfactory template for kin recognition*

To determine when zebrafish larvae learn the olfactory cues for kin recognition, we isolated larvae from kin groups at progressive days of development and compared their preference for kin odour with that of the control individuals raised in groups of full siblings. The size of kin groups varied between 30 and 50 individuals. Isolation at 0 dpf refers to the period directly pf: an individual isolated at 0 dpf never experienced sibling odour. To determine more precisely the time window for learning the olfactory template of kin, we isolated larvae at 0 dpf, then combined previously isolated larvae of the same family on specific days, i.e. 6 or 7 dpf, and then reisolated the larvae until testing for olfactory preference at age 21 to 30 dpf. Larvae in this experiment originated from 35 different mating pairs ('families').

(c) *Cross-foster experiment*

To determine the specificity of kin odour imprinting, we tested whether cross-fostered larvae learn the olfactory cues of non-kin odour and later develop a preference for the odour of foster full siblings. This experiment required the identification of the foster larva among non-kin larvae. Identifying individual larvae after they had been combined as eggs was for all practical purposes impossible. Therefore, we exposed larvae to non-kin odour using a six-well tissue culture plate (12.7×8.2 cm) in which the bottom of each well (1.4 cm diameter) was replaced by a net (0.5 mm mesh size). This plate was placed into a dish (20×15 cm) filled with water (depth 4 cm), which allowed the plate to float. This method was allowed for odour exchange while keeping eggs and larvae physically separate. Approximately 30 eggs from the same parents (full siblings) were introduced into the outer dish ('background fish') immediately after fertilization (0 dpf) and kept there until testing. In each of the six wells of the floating plate, we placed a single egg. These six eggs originated from five different families plus one egg from the same family used as background fish. Because the fine netting over the wells potentially limited water and odour flow, the wells were flushed four times a day by a (gloved) experimenter who gently moved the plate up and down without totally emptying the wells of water. Experimental larvae therefore had access to olfactory (and perhaps visual) cues through the net but no physical contact with the background larvae. At 20–30 dpf, we tested the isolated larvae for their olfactory preference for the odour cues of the foster background family versus an unrelated unfamiliar group. We used larvae from nine different mating pairs to create 'background families'.

(d) *Odour choice tests*

Olfactory preference tests were conducted in a two-channel choice flume with a steady driven flow generated by a peristaltic pump (40 ml min per channel; approx. 3 cm s^{-1} at the water surface measured with dye; see Gerlach & Lysiak (2006) and Gerlach *et al.* (2007*a*) and electronic supplementary material). Regular dye tests ensured that the flume maintained two distinct parallel-flowing water masses (A and B), which remained entirely separated up to the downstream mesh screen. Water masses A and B allowed no neutral area in the flume. Single fish were placed into the flume with both water sources (with their inherent odour stimuli) running and were given 3 min to acclimate and experience both sides of the flume. Fish could swim freely between water masses. We recorded the position of the fish's head and nose in one or the other water flow every 10 s during two 3-min periods separated by a 1-min transition period to switch water sources as a control for possible (non-olfactory) side bias of the fish. If the larvae swam directly at the centreline between both water masses, the location would be recorded as 'unclear' and excluded from the analysis.

To obtain stimulus water for each experiment, we placed nine larvae from each kin group (stimulus fish) overnight in separate 9-l aquaria. Test and stimulus zebrafish were of the same age (±3 days). By using both equal numbers and size-matched fish to create kin and non-kin stimulus water, we assumed that stimulus water contained equivalent concentrations of odour cues. To ensure that each family actually possessed the ability to recognize unfamiliar full siblings, we tested the olfactory preference of a second group of individuals that were raised with kin. To ensure that familiarity did not influence their olfactory preference, we used for olfactory choice tests larvae that were raised separately from the kin group larvae that were used to create stimulus water. In the second experiment, we generated

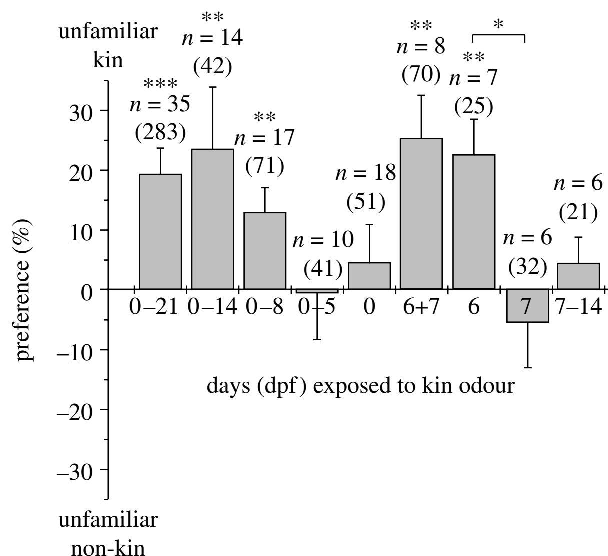

Figure 1. Olfactory preference for kin versus non-kin odour. Odour preference in larvae that were isolated on progressive days of development, or at specific dpf: 0 = larvae never exposed to kin; 0–21 = larvae raised together with kin until testing at 21–30 dpf. Preference is expressed as the per cent difference in a number of observations between the sides with kin versus non-kin odour (±s.e.). Positive bars indicate preference for kin odour; negative bars indicate preference for non-kin odour. *n*, no. of families tested (number of larvae tested in parentheses); *statistical significance $p<0.05$, **$p<0.01$, ***$p<0.001$.

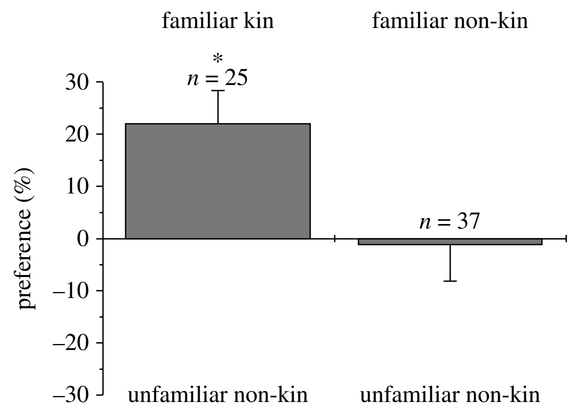

Figure 2. Imprinting on non-kin odour. Larvae from the same (familiar kin) and a different family (familiar non-kin) unlike the background fish were isolated in wells. 'Familiar kin' larvae were tested for olfactory preference of their own family familiar kin odour (= background fish) versus 'unfamiliar non-kin' odour. Familiar non-kin larvae were tested for preference of 'familiar non-kin' odour (= background fish) versus 'unfamiliar non-kin' odour.

stimulus water from unfamiliar background fish for comparison with water from animals who were not related to either background or test fish.

(e) *Data analysis*

To balance for larval mortality, more than one larva per family was raised under either of the described conditions. One to ten larvae per family were tested per exposure category. To avoid pseudo-replication, we calculated the mean value of olfactory preference for kin odour by family. We calculated the difference between the numbers of observations in which a test fish was observed swimming in kin stimulus water versus non-kin stimulus water. Olfactory preference is expressed as the percentage of observations spent in kin odour (±s.e.). A random distribution across water masses (zero difference) is expected if a fish did not express a preference for one of the odour stimuli; a negative value indicates a preference for non-kin odour, and a positive value for kin odour. We tested whether mean preference values per family were significantly different from zero using a Wilcoxon signed rank test (two-tailed) in the program JMP v. 5.0 (SAS Institute, Inc. 1995). We used the same program to conduct the non-parametric van der Waerden tests to compare olfactory preference between groups of different exposure periods.

3. RESULTS

(a) *Time window for learning the olfactory template for kin recognition*

Juvenile zebrafish that were raised in groups of full siblings during their entire development showed a significant preference for the odour of unfamiliar full siblings (kin) over unrelated individuals (non-kin) in the olfactory choice tests (figure 1). On average, when simultaneously presented with water scented by siblings versus unrelated fish of the same age, they spent 19.2% ± 4.4 ($Z=253$, $p=0.000$) more time (i.e. observations) in water containing the olfactory cues of kin than in water containing those of non-kin. Larvae isolated from all contact with conspecifics immediately after fertilization at 0 dpf did not differentiate between odour cues from kin and non-kin (4.4% ± 6.5, $Z=13$, $p=0.53$), nor did larvae whose contact with kin was restricted to the first 5 dpf (−0.6% ± 7.8, $Z=-4.5$, $p=0.65$). However, larvae that were exposed to kin for the first 8 days preferred kin odour (12.8% ± 4.3, $Z=50.0$, $p=0.008$).

Larvae exposed to full siblings only at days 6 and 7 showed a significant preference for kin (25.6% ± 7.1, $Z=18$, $p=0.008$). We determined the specific developmental window for kin odour learning by restricting kin odour exposure to 6 or 7 dpf. Exposure to full siblings for 24 hours at 6 dpf alone generated a significant preference for kin odour (22.5% ± 6.3, $Z=14$, $p=0.016$), while kin odour exposure only at 7 dpf (−5.6% ± 7.5, $Z=-1.5$, $p=0.84$) or a 7 day exposure starting at 7 to 14 dpf did not (4.3% ± 4.4, $Z=4.5$, $p=0.438$). A statistical comparison between larvae having been exposed at 6 versus 7 dpf showed a significant difference in kin odour preference (the nonparametric van der Waerden test, $\chi^2_1=9.32$, $p=0.0025$; figure 1). Combining all data, olfactory preference in larvae that were exposed to kin odour at 6 dpf showed a highly significant preference for kin odour in contrast to those that were not exposed (exposed = 16.8 ± 1.7, $n=491$; not exposed = 3.4 ± 2.7, $n=145$; non-parametric van der Waerden test, $\chi^2_1=33.5$, $p=0.0001$). Larval zebrafish therefore acquired the olfactory template for kin odour at 6 dpf in a developmental period of approximately 24 hours.

(b) *Cross-foster experiment*

Surprisingly, larvae that were exposed to a foster family through a fine mesh screen did not express preference for the olfactory cues of the foster family (−5.6% ± 6.1,

$Z=-37.5$, $p=0.492$). However, control larvae that were reared under the same conditions but originated from the foster family showed a significant preference for the cues of the foster family ($16.7\% \pm 6.96$, $Z=63$, $p=0.037$; figure 2). The odour-exposed but physically separated animals from the foster family did not differ in their preference ($21.9 \pm 6.5\%$) from their full siblings who had constant physical contact with each other ($23.2 \pm 6.5\%$; ANOVA, $F=0.023$, $p=0.87$), indicating that the mesh restricting physical contact did not prevent olfactory perception.

4. DISCUSSION

To our knowledge, this is the first investigation demonstrating a time-sensitive imprinting process related to kin recognition in fish. Imprinting takes place significantly after hatching, but while larvae still express limited mobility (Spence *et al.* 2006), perhaps coinciding with the period before larvae disperse from the spawning ground and intermingle with non-kin larvae. The neuronal processes corresponding to imprinting at 6 dpf have yet to be determined. Since hatching and development are temperature dependent, this imprinting window refers to a temperature of 26°C. Zebrafish embryos hatch with an olfactory system that appears anatomically and biochemically complete (Hansen & Zeiske 1993), but the number of olfactory neurons keeps increasing as the animal grows (Barth *et al.* 1996). Imprinting may be delayed by an inability to receive the signal until specific olfactory receptors are expressed at 6 dpf or the signal itself is not released before 6 dpf.

Our second novel finding regarding the constraints of kin odour imprinting was that the acquisition of a kin template requires exposure to *other* kin; zebrafish did not use their own odour cues as a reference in order to differentiate between self and non-self. Isolated individuals may not be sensitive to their own odour. The need for kin exposure for imprinting in zebrafish agrees with prior work in charr (*S. alpinus*), showing that individuals reared in isolation for 15 months cannot differentiate between kin and non-kin (Winberg & Olsen 1992). Territorial male bluegill sunfish (*L. macrochirus*) have been posited to discriminate between their own offspring and those of cuckolders using self-referencing; however, the possibility remains that males learned a kin template during their early development (Neff & Sherman 2005). Though self-referencing may function in kin recognition in other species; however, zebrafish do not use the ‘armpit effect’ (Hauber & Sherman 2000) to discriminate relatedness. A lack of self-referent kin recognition has also been shown in bluegill sunfish (*L. macrochirus*: Hain & Neff 2006), guppies (*P. reticulata*: Griffiths & Magurran 1999) and sticklebacks (*Gasterosteus aculeatus*: Frommen *et al.* 2007).

Flexible learning comes with the potential cost of learning the wrong cue. We performed one further set of experiments to investigate not just the temporal limits of the template learning process, but also its flexibility with regards to the imprinting object.

The lack of imprinting on the non-kin odour sheds light on the mechanism of phenotype matching. Apparently, the chemical signal (ligand) for the olfactory imprinting process has to closely match the receptor system of the recipient. This suggests a genetic component similar to the innate immune system where cell–cell recognition and rejection of non-self ligands are based on the similarity of MHC-derived surface proteins.

MHC genes are among the most polymorphic multigene families known and are important for the functioning of the immune system (Klein 1986). Their parallel role in the recognition of self and non-self had been studied for at least 30 years since Lewis Thomas (see Boyse *et al.* 1987) suggested that the MHC evolved due to the need for species recognition. MHC molecules are transmembrane molecules, which are shed from the cell surface and appear in body fluids such as saliva, sweat and urine (Singer *et al.* 1997). These molecules can be assessed via the olfactory system and are used as signals of genetic relatedness and health. Differences in MHC loci are well known to influence the behavioural decisions in mice (Yamazaki *et al.* 1976) and fish (Reusch *et al.* 2001; Aeschlimann *et al.* 2003).

We suggest that MHC peptides located on the cell surface of the olfactory receptors might be involved in the neural process of imprinting on kin odour. Our conclusion is supported by results of a study on juvenile Arctic charr, *S. alpinus* (Olsén *et al.* 1998). When given a choice between water scented by a full sibling whose MHC genotype was identical to their own and water scented by a full sibling whose MHC genotype was different, fish preferred water from MHC-identical siblings. The authors concluded that social learning cannot account for this result because test fish were reared in groups of siblings with variable MHC alleles. We suggest a different interpretation of their results: juvenile charr acquired their kin template in a social context by selectively learning the kin odour corresponding to their own MHC genotype and could therefore later differentiate between different MHC types. Because in our study we used kin groups of more than 20 individuals to determine the sensitive period for imprinting, the probability of any single individual experiencing the odour of at least a few kin with similar MHC alleles was high.

While odour memory and preference are encoded in brain areas such as the piriform cortex (Plailly *et al.* 2005), there is evidence that the peripheral olfactory system also undergoes changes due to conditioning and/or imprinting. The best-studied example is found in salmon, in which the peripheral olfactory system becomes physiologically tuned to home stream odours (Dittman *et al.* 1996). It has also been shown in zebrafish (*D. rerio*) that exposure to phenethyl alcohol (PEA) as juveniles resulted in specific changes in gene expression within the olfactory epithelium (Harden *et al.* 2006). Quantitative RT-PCR showed that the number of cells expressing the transcription factor, *otx2*, was upregulated in the olfactory sensory epithelia in response to PEA in 1–3 day old PEA odour-exposed fish when compared with controls (Harden *et al.* 2006). This suggests that imprinting might evoke changes in gene expression.

Our study is the first to elucidate the small temporal window for imprinting resulting in phenotype matching. The lack of flexibility in the timing of exposure or relatedness of the kin template required to develop an early odour preference for unfamiliar kin in zebrafish suggests that recognition through phenotype matching

invokes an olfactory imprinting process with a mechanism fundamentally different from the recognition based on familiarity. Because acquisition of an early odour preference is limited to related individuals, we propose that kin recognition via phenotype matching involves an interaction between genetic predisposition and individual experience, which may be mediated via the MHC. Documenting the precise developmental window during which olfactory imprinting occurs in zebrafish makes testing this hypothesis feasible and opens new possibilities for the study of the neuronal and genetic processes that have major consequences for social behaviour.

Animal care and experimental procedure were in accordance with directive of the Office of Laboratory Animal Welfare, Assurance no. A3070-01. The Animal Facility at the Marine Biological Laboratory is registered with the USDA registration no. 14-R119, and this work was approved by the Marine Biological Laboratory animal care and use protocol no. 2003–2007.

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