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Kin recognition in zebrafish, *Danio rerio*, is based on imprinting on olfactory and visual stimuli

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The evolution of cooperative behaviour requires mechanisms to avoid investing in conspecifics that are not increasing an individual's direct or indirect fitness. This suggests that selection should favour the capability of recognizing kin. One mechanism to discriminate between kin and nonkin is based on phenotype matching, when an individual learns a template of itself or of its kin and can later use this template to recognize even unfamiliar kin.

In this study, we found that in zebrafish olfactory kin recognition depended on an imprinting process that required the two-step learning process of olfactory as well as visual cues of kin. Larvae that were exposed to either visual or olfactory cues or to both cues of nonkin did not show imprinting. This capability of imprinting on kin but not on nonkin cues can be explained by genetic predisposition or self-referencing. Through this combined imprinting process larvae can avoid false imprinting on unrelated individuals.
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Kin recognition and differential treatment of kin and nonkin can be a fundamental process in the evolution of social behaviour. Cooperative behaviour can be an evolutionarily stable strategy when benefits allocated to individuals outweigh costs (Hamilton 1964a, b). The higher the genetic relatedness, the better is the balance of benefits to costs; but this process is susceptible to cheating when unrelated organisms are not recognized as such and steal benefits. This might have favoured the evolution of better recognition processes, enabling organisms to differentiate between kin and nonkin (Waldman 1987; Hepper 1991; Tang-Martinez 2001; Mateo 2004).

Several mechanisms of kin recognition have been identified (reviewed in Tang-Martinez 2001; Mateo 2004); here, we refer to phenotype matching, which allows for recognition of even unfamiliar kin because individuals establish an olfactory, visual or acoustic template for their kin during early development and compare this template to cues from unfamiliar individuals later in life. In the aquatic environment, phenotype matching has been shown in several species, for instance in tadpoles of the Ferguson's toad, *Bufo scaber* (Gramapurohit et al. 2006), several fishes such as the cooperatively breeding African cichlid *Neolamprologus pulcher* (Le Vin et al. 2010) or the monogamous cichlid *Pelvicachromis taeniatus* (Hesse et al. 2012), guppies, *Poecilia reticulata* (Hain & Neff 2007) and bluegill sunfish, *Lepomis macrochirus* (Hain & Neff 2006). Observed genetic compositions of wild populations of fishes indicate that this mechanism may be used to form kin-structured groups such as in Atlantic cod, *Gadus morhua* (Herbinger et al. 1997), coho salmon, *Oncorhynchus kisutch* (Quinn & Busack 1985), common shiners, *Notropis cornutus* (Ferguson & Noakes 1981), Eurasian perch, *Perca fluviatilis* (Gerlach et al. 2001) and three-spined sticklebacks, *Gasterosteus aculeatus* (FitzGerald & Morrissette 1992), although Peuhkuri & Seppä (1998) did not find high relatedness among individuals within stickleback schools. However, very little is known about the underlying learning process by which animals achieve the ability to differentiate even unfamiliar kin from nonkin.

In the wild, very little is known about zebrafish behaviour (Spence et al. 2007), including whether they associate with kin. However, in laboratory experiments the zebrafish has been an ideal model organism to study these processes: larvae can recognize kin

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by olfactory cues and thereafter prefer to associate with kin (Mann et al. 2003) by using a phenotype-matching process (Gerlach & Lysiak 2006). Larvae imprint on an olfactory template of their kin during a 24 h time window on day 6 post fertilization (Gerlach et al. 2008) and can later use this template to recognize even unfamiliar kin. At 25 °C larvae hatch on day 4 post fertilization and start moving freely at about 7 days post fertilization. Individuals in kin groups grew 15% more than individuals in nonkin groups (Gerlach et al. 2007). Surprisingly, larvae do not imprint on the olfactory cues of kin when kept in isolation. Therefore, the aim of this study was to understand whether and which additional cues (visual or tactile) are involved in imprinting.

METHODS

Experimental Animals

Wild-type zebrafish originated from two different aquarium zebrafish suppliers in Germany. Fish were kept in our zebrafish facility in 10-litre breeding tanks connected to a recirculating water filtration system (ZF0601 Zebrafish Stand-Alone 'Aquatic Habitats', U.S.A., http://aquatichabitats.com). The adult fish were maintained at 25 ± 1 °C under a 13:11 h light:dark cycle and were fed twice daily with commercial flake food and live brine shrimp, *Artemia salina*. For breeding, each female was housed with one male in a 3-litre tank. Egg dishes were placed in the tanks in the afternoon and collected the following morning. All eggs and larvae were placed in glass dishes in an incubator (SANYO MIR 553) and maintained at 25 ± 0.5 °C. The dishes were cleaned and a quarter of the water was replaced with fresh water daily. After hatching, which occurred between the 3rd and 4th day post fertilization, larvae were fed with live *Paramecium caudatum*. Descendants of the same adult pair were termed 'family'.

Experimental Design

We studied the influence of olfactory and visual cues on imprinting under varying conditions.

Interaction of visual and olfactory cues for imprinting

Directly after fertilization, zebrafish eggs of each family were used in eight different experimental treatments (for details see Table 1). Larvae were raised in physical isolation in 3 cm wide beakers with 30 ml of water. Olfactory cues were provided at days 5–7 post fertilization by removing 5 ml of this water and replacing it with 5 ml of water from their kin group (kin water) or, for treatments $O_{nk}V_{nk}$ and $O_{nk}V_k$, from a nonkin group (nonkin water). Visual but no physical contact was provided by placing the beakers in glass bowls in which at least 25 other larvae were present.

Table 1
Olfactory preference of zebrafish larvae raised under different social conditions

Treatment	Mean % olfactory preference (kin−nonkin)	Confidence intervals (95%)		*N*	Wilcoxon signed-ranks test	
		Upper bound	Lower bound		*Z*	*P*
K	23.7	28.6	18.8	346	−8.522	<0.001
I	1.7	12.9	−9.4	31	−0.785	0.433
O_k	−9.1	9.2	−27.4	30	−1.444	0.149
V_k	0.3	11.6	−11.1	33	−0.320	0.749
O_kV_k	26.3	40.7	11.9	40	−3.195	0.001
$O_{nk}V_{nk}$	−5.8	10.4	−22.1	39	−0.752	0.452
O_kV_{nk}	2.6	14.0	−8.8	55	−0.409	0.683
$O_{nk}V_k$	0.0	14.8	−14.8	32	−0.155	0.876
V	8.3	35.1	−18.4	19	−0.747	0.455
V.4	20.2	46.5	−6.1	19	−1.913	0.056
V.5	43.2	59.8	26.5	28	−3.814	<0.001
V.6	18.5	37.7	−0.6	40	−1.803	0.071

For abbreviations see Figs 1 and 2.

Visual-imprinting time-sensitive period

In a previous study, we identified the sensitive period for olfactory imprinting as day 6 post fertilization (Gerlach et al. 2008). In this study we investigated whether visual imprinting occurs in the same time window as olfactory imprinting, that is, on day 6 post fertilization. To determine the timing of the critical period for visual imprinting, larvae were visually exposed to kin only at particular time points during development. Larvae were raised in isolation in semitranslucent plastic beakers (3 cm wide, 50 ml water) partially submerged in larger glass dishes. Larvae could see the silhouette of other larvae moving through the wall of the plastic beaker but could not see any pigmentation pattern of the body, as was proven by a camera placed inside a plastic beaker. Beakers did not reflect self-images. At days 5–7 post fertilization, 5 ml of water in each beaker was replaced by kin water. Larvae were kept in these semitranslucent plastic beakers until they were transferred for 1 day to glass beakers through which kin could be clearly seen. This transfer occurred on different days for different groups: one group on day 4 post fertilization (V.4), one group on day 5 post fertilization (V.5) and one group on day 6 post fertilization (V.6). A control group was kept in the milky beakers for the duration of the experiment.

Odour choice test

Larvae were tested for their olfactory preference at days 8–12 post fertilization. Stimulus water was created by placing 10 larvae into fresh water for 24 h (1 larva/litre).

Olfactory preference tests were conducted in a two-channel choice flume (Atema flume: 21 cm long × 4 cm wide, water level 2.5 cm) with a steady flow generated by a peristaltic pump (pump generator MCP Ismatec) at 42 ml/min (for details see Gerlach & Lysiak 2006; Gerlach et al. 2008). Before testing, regular dye tests ensured that the flume maintained two distinct parallel-flowing water columns (A and B). The two areas of the flume allowed a choice between two odours: water containing the scent of kin in A and water containing the scent of nonkin in B. To begin testing, single larvae were placed into the open test area of the flume. Prior to each trial, larvae were acclimated for 1 min to the flume and were able to swim freely between the two water bodies, A and B. The flume choice trials consisted of two 2 min periods, in which kin and nonkin stimulus water columns were run through the flume. After the first 2 min trial, the stimulus water columns switched sides and a 1 min acclimation time was allowed. Throughout the test, the position of the larva in the flume was recorded every 10 s. All tests were conducted blind so the observer did not know which stimulus odour was on which side.

Since we have previously shown that prior exposure increases kin recognition (Behrmann-Godel et al. 2006), we used unfamiliar kin (except for treatments $O_{nk}V_{nk}$ and $O_{nk}V_k$; see below) and nonkin to create the odour stimuli. Thus we avoided recognition by familiarity and tested only phenotype matching (see Introduction).

Larvae used in treatments K, I, O_k,V_k, O_kV_k, O_kV_{nk} and V (4–6) were tested for preference between unfamiliar kin and unfamiliar nonkin. Larvae in treatments $O_{nk}V_{nk}$ and $O_{nk}V_k$ were tested for preference between unfamiliar kin versus familiar nonkin (test larvae had been exposed to olfactory and visual cues from these nonkin larvae).

We used the Wilcoxon signed-ranks test to analyse the results of the odour preference tests. We subtracted number of observations when a test fish was on the kin side from the number of

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