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Correspondence and requests for materials should be addressed to C.H. (cornelia.hinz@uni-oldenburg.de)

Olfactory imprinting is triggered by MHC peptide ligands

Cornelia Hinz[1], ri Namekawa[2], Jasminca Behrmann-Godel[3], Claus Oppelt[3], Aaron Jaeschke[1], Anke Müller[1], Rainer W. Friedrich[2,4] & Gabriele Gerlach[1]

[1]Department of Biology and Environmental Sciences, Carl von Ossietzky University Oldenburg, Carl von Ossietzky Str. 9-11, 26111 Oldenburg, Germany, [2]Friedrich Miescher Institute for Biomedical Research, Maulbeerstraße 66, P.O. Box 2543, 4058 Basel, [3]Limnological Institute at the University of Konstanz, Mainaustraße 252, 78457 Konstanz, Germany, [4]University of Basel, 4003 Basel, Switzerland.

Olfactory imprinting on environmental, population- and kin-specific cues is a specific form of life-long memory promoting homing of salmon to their natal rivers and the return of coral reef fish to natal sites. Despite its ecological significance, natural chemicals for olfactory imprinting have not been identified yet. Here, we show that MHC peptides function as chemical signals for olfactory imprinting in zebrafish. We found that MHC peptides consisting of nine amino acids elicit olfactory imprinting and subsequent kin recognition depending on the MHC genotype of the fish. *In vivo* calcium imaging shows that some olfactory bulb neurons are highly sensitive to MHC peptides with a detection threshold at 1 pM or lower, indicating that MHC peptides are potent olfactory stimuli. Responses to MHC peptides overlapped spatially with responses to kin odour but not food odour, consistent with the hypothesis that MHC peptides are natural signals for olfactory imprinting.

Olfactory imprinting during early development influences future environmental[1], social[2] and dietary preferences[3,4] in a wide range of species from invertebrates to humans. In an ecological context, olfactory imprinting is known to guide salmon to their natal rivers when they return from the sea to mate and spawn[5]. The underlying chemical cues may be pheromones of their own population[6] and/or environmental cues encountered during the downstream migration towards the ocean[7,8]. Olfactory imprinting may also play a major role in the orientation of more marine species which disperse at larval stages but return and settle at natal habitats[1]. Homing based on chemical cues appears to be a common strategy to find locations that have proven successful for reproduction in the past.

In addition, juveniles of many animal species use chemical cues to identify related conspecifics for shoaling[1]. This preference for kin groups appears to be beneficial for survival at juvenile stages and can turn into kin avoidance in adults in order to prevent inbreeding[9]. Recognizing and differentiating kin from non-kin can be based on a phenotype-matching consisting of a two-step process[9]: (1) an imprinting phase early in life when a larva learns a template of kin; and (2) the recognition process later in life when an animal matches sensory cues of unfamiliar individuals to this template to differentiate between kin and non-kin. While other studies have focused on the recognition process involved in mate choice[10,11], sexual behaviour and pregnancy block[12,13] we focused on the imprinting phase, i.e. its behavioural, genetic and neuronal background as well as the chemical signals involved.

We used zebrafish (*Danio rerio*, Hamilton 1822) as a model for studying olfactory imprinting. Zebrafish imprint on an olfactory template of kin during a narrow (24 hrs) time window at day 6 post fertilization (6 dpf) combined with a visual input from kin; zebrafish do not imprint on the odour or visual appearance of unrelated individuals during this sensitive period[14,15]. This suggests a genetic predisposition to kin odour. Gerlach et al.[14] suggested that this predisposition could be based on genes of the major histocompatibility complex (MHC). In the vertebrate immune system, MHC class I and II genes play a fundamental role in discriminating 'self' and 'non-self' by presenting pathogen-derived peptides to lymphocytes. The MHC is characterized by its high polymorphism, making MHC similarity between individuals a good indicator for their relatedness. Studies on different species have shown that individuals can match their own MHC genotype with the genotypes of conspecifics[11,14,16–18] but little is known about the underlying mechanisms. The olfactory signals may be peptides ("MHC peptides") that are bound by MHC proteins and occur in bodily fluids including urine. Because the identity of the peptides that are bound directly reflects the structure of the polymorphic peptide binding region of

the MHC protein, MHC peptides may act as chemical signals that convey information about the MHC genotype of an individual[17,19]. Consistent with this hypothesis, MHC peptides were reported to influence mate choice decisions in sticklebacks (*Gasterosteus aculeatus*)[10] and pregnancy block in mice[12], but their influence on kin recognition and olfactory imprinting had not been investigated yet. We found that in a specific strain of zebrafish a specific set of MHC peptides evokes imprinting on kin, and that MHC peptides are potent odours that activate scatted populations of neurons in the olfactory bulb. These results indicate that MHC peptides are chemical signals underlying olfactory imprinting and long lasting memory of kin.

Results

MHC peptides can evoke imprinting. To test the hypothesis that MHC peptides are chemical signals relevant for olfactory imprinting we exposed individual zebrafish larvae to a mixture of 5 different MHC peptides at 6 dpf (MHC_{Mix}; see Methods). After raising larvae for another 1–3 days, their preference for water from their kin versus water from a non-kin population was tested in a two-channel Atema choice flume[9] (Fig. S1, Supplementary Information). Olfactory imprinting is reflected by a preference for kin water in this assay. Larvae could be imprinted on kin by exposure to MHC_{Mix} at 6 dpf (Fig. 1, E1 and Table 1).

Out of seven tested families, only the family line 6 (OL6) of E1 showed responses to MHC_{Mix}. Larvae of lines 1, 2, 3, 4, 5 and 7 showed no preference for MHC_{Mix}, indicating that the preference for MHC_{Mix} depends on the genetic background (Fig. 1, E2 and Table 1). OL6 larvae imprinted on MHC_{Mix} also preferred water conditioned with MHC_{Mix} over untreated water, while non-imprinted OL6-fish (without prior exposure to kin odour or MHC_{Mix} during the sensitive phase) did not prefer the MHC_{Mix} (Fig. 1, E1 and Table 1). Larvae that had been raised with kin odour compared to MHC_{Mix} expressed a significantly higher preference for kin (Mann-Whitney-U (MWU): $n = 61$; $z = -2.727$; $p = 0.006$) and for the MHC_{Mix} (MWU: $n = 61$; $z = -2.876$; $p = 0.004$). We conclude that MHC peptides can function as chemical signals for imprinting in zebrafish, but might represent not all components of natural kin odour.

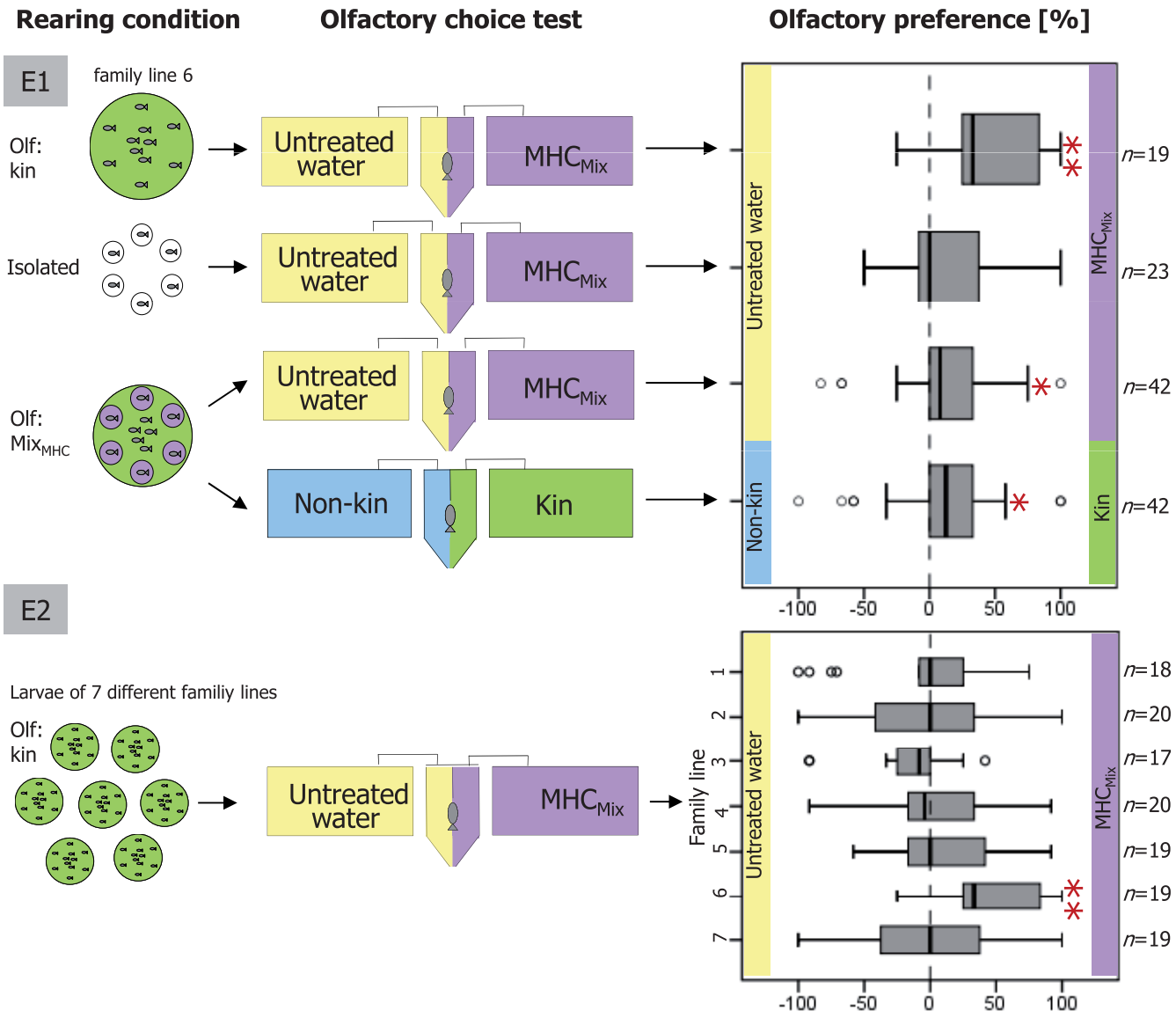

Figure 1 | E1 MHC peptides can trigger kin recognition. Larvae of family 6 = OL6 preferred MHC_{Mix} over untreated water when raised with full siblings. Larvae without exposure to kin odour during the sensitive phase did not develop a peptide-preference. Single raised larvae from the peptide responsive strain that were visually exposed to kin and olfactory exposed to MHC_{Mix} on day 6 pf significantly preferred MHC_{Mix} over untreated water and they also preferred kin over non-kin. E2 Reponses to MHC_{Mix} of different family lines.

MHC class II allele similarity correlates with imprinting on kin. To investigate whether olfactory imprinting in zebrafish is based on MHC allele similarity, we used eggs from 10 different zebrafish pairs that were obtained from different sources and most likely carried different alleles of MHC and other genes. From each pair, siblings were raised in pairs for 8–12 days, tested behaviourally for kin recognition and genotyped for MHC class I and MHC class II alleles. Using SSCP gel electrophoresis, we analysed the amplified exon 3 of MHC class I genes and exon 2 of genes DAA and DAB, which are assumed to represent the only functional MHC class II genes in zebrafish[16]. We compared the relationship of the MHC class I and II allele similarity (by band matching) between sibling pairs and their olfactory preference for

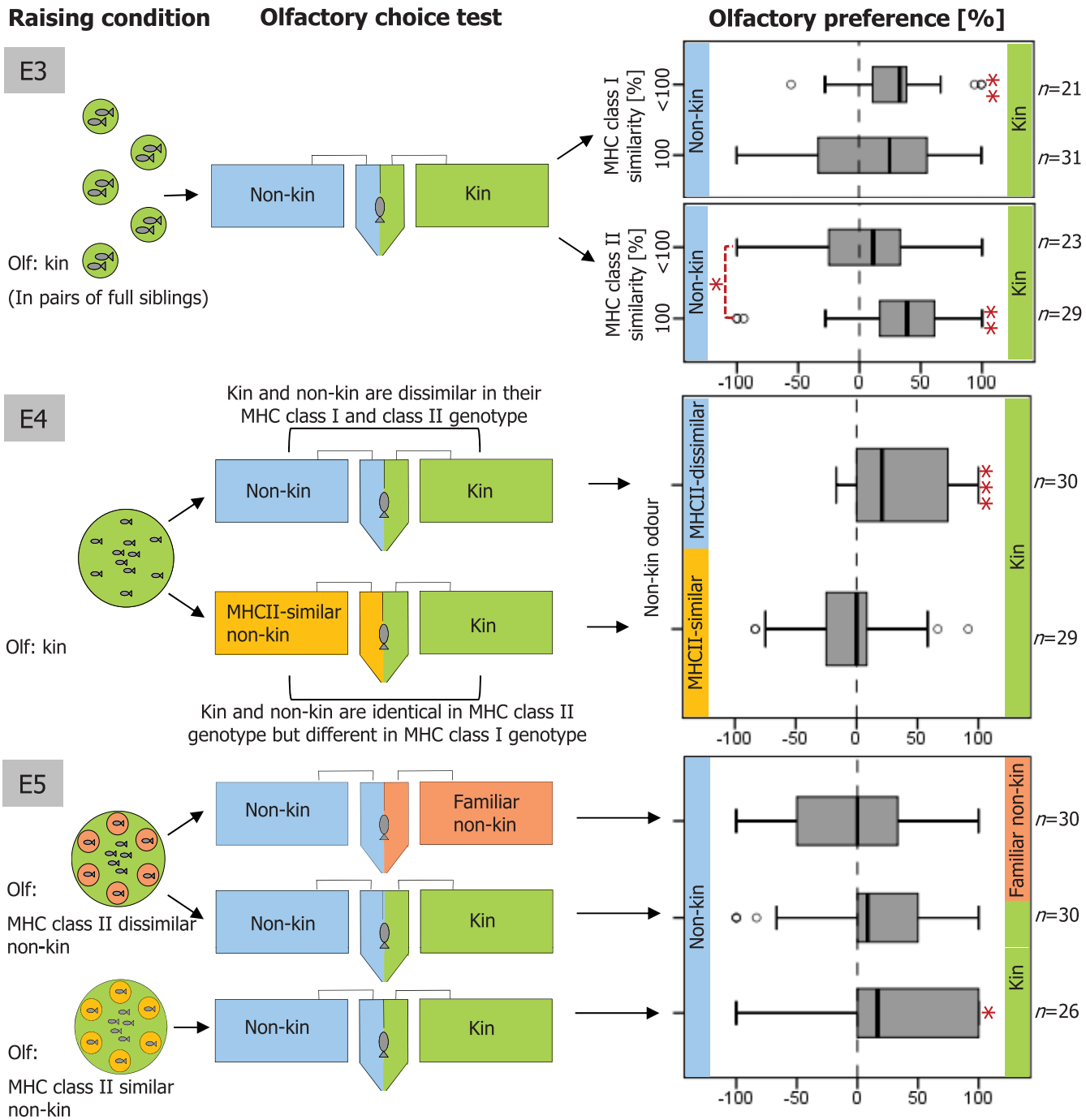

Figure 2 | E3 Imprinting on kin is correlated with MHC class II allele similarity but not MHC class I similarity. Larvae were raised in pairs of two full siblings which differed in MHC alleles. For MHC class II we found a significant difference in kin preference between larvae that were 100% identical in MHC class II genes DAA and DAB and larvae with lower MHC class II similarity. For MHC class I similarity, the difference in preference between those two groups was statistically not significant. E4 MHC class II similarity determines kin recognition. Larvae did not differentiate between kin and MHC class II identical non-kin larvae while they differentiated kin from a MHC class II dissimilar non-kin family. E5 Larvae can imprint on odour cues of unrelated but MHC class II similar larvae. Larvae that were exposed to the olfactory cues of MHC class II similar non-kin on 6 dpf preferred kin odour over non-kin odour in a flume choice test, while larvae that were exposed to the olfactory cues of MHC class II dissimilar non-kin neither preferred the familiar non-kin over unfamiliar non-kin nor kin over non-kin. Box plots show median, upper and lower quartile and whiskers with maximum 1.5 interquartile range; * indicates statistical significance $p<0.05$, ** $p < 0.01$ and *** $p < 0.001$.

kin (Fig. 2, E3 and Table 1). Larvae raised with a 100% MHC class II similar sibling expressed significantly higher olfactory preference for kin versus non-kin odour than larvae raised with a sibling of lower MHC class II similarity (MWU: $n = 52$, $z = -2.224$, $p = 0.026$, Fig. 2, E3 and Table 1). Larvae that were raised with a 100% MHC class II identical sibling expressed an olfactory preference for kin versus non-kin odour while a lower MHC class II similarity did not result in kin recognition (Fig. 2, E3 and Table 1).

MHC class I allele identity did not correlate with kin recognition at later stages: siblings that were raised with a 100% MHC class I similar sibling showed no recognition while MHC class I dissimilar siblings did (Fig. 2 E3 and Table 1). But the difference in preference between those two groups was statistically not significant (MWU: $n = 52$; $z = -0.168$; $p = 0.867$). This result suggests that MHC class II genes are involved in olfactory imprinting but likely not MHC class I genes.

We next examined whether MHC class II similarity is sufficient for recognition. If so, a larva should be unable to differentiate between odour from kin and from unrelated larvae with similar MHC class II genes. To test this hypothesis, we first used two males and two females that were identical in their SSCP band patterns for MHC class II genes but different for class I genes. For all parental fish we verified that similar SSCP band patterns represent similar alleles by sequencing all bands of MHC class I and class II (see Methods and Supplementary Information). A BLASTn search of NCBI Genbank showed that we had successfully amplified the MHC class II genes DAA and DAB and zebrafish MHC class I genes. In olfactory choice tests, larvae did not differentiate between kin and non-kin larvae with similar MHC class II while they preferred kin over a third non-kin family with dissimilar MHC class II (Fig. 2, E4 and Table 1).

The hypothesis that MHC class II similarity is sufficient to generate imprinting also predicts that unrelated fish should imprint on each other when they share the same MHC class II alleles. We therefore raised larvae of both breeding pairs individually in beakers but surrounded by full siblings which provided the necessary visual signal. At 6 dpf they received olfactory cues of MHC class II dissimilar non-kin or MHC class II similar non-kin. When stimulus water came from randomly selected non-kin families, larvae failed to imprint and showed no preference for water from kin or familiar non-kin (Fig. 2, E5 and Table 1). However, when larvae were exposed to water from MHC class II-similar non-kin, they developed a significant preference for kin odour (Fig. 2, E5 and Table 1). Based on these results, we conclude that imprinting and kin recognition is based on MHC class II similarity. We found no evidence that MHC class I alleles influenced the olfactory choice. However, because we could not amplify all MHC class I genes, we cannot exclude this possibility entirely.

Olfactory detection of MHC peptides and kin odour. Our behavioural experiments (Fig. 1 E1, E2 and Table 1) suggest that zebrafish perceive MHC peptides as odorants. We tested this hypothesis by multiphoton imaging of odour-evoked calcium signals in the olfactory bulb using a transgenic zebrafish line[20] that expresses the genetically encoded calcium indicator GCaMP2[21] under the control of the pan-neuronal HuC promoter[22]. Experiments were performed in this transgenic line (n = 8) or in fish obtained by crossing the GCaMP2-expressing line to the OL6 background for one or two generations (n = 9). In each fish, responses were measured in 6–9 focal planes spaced at 10 µm in z to cover the entire OB on one side of the brain.

The HuC promoter drove expression of the calcium indicator in most, if not all, neurons in the larval olfactory bulb. As observed previously[23], basal indicator fluorescence was higher in the principal

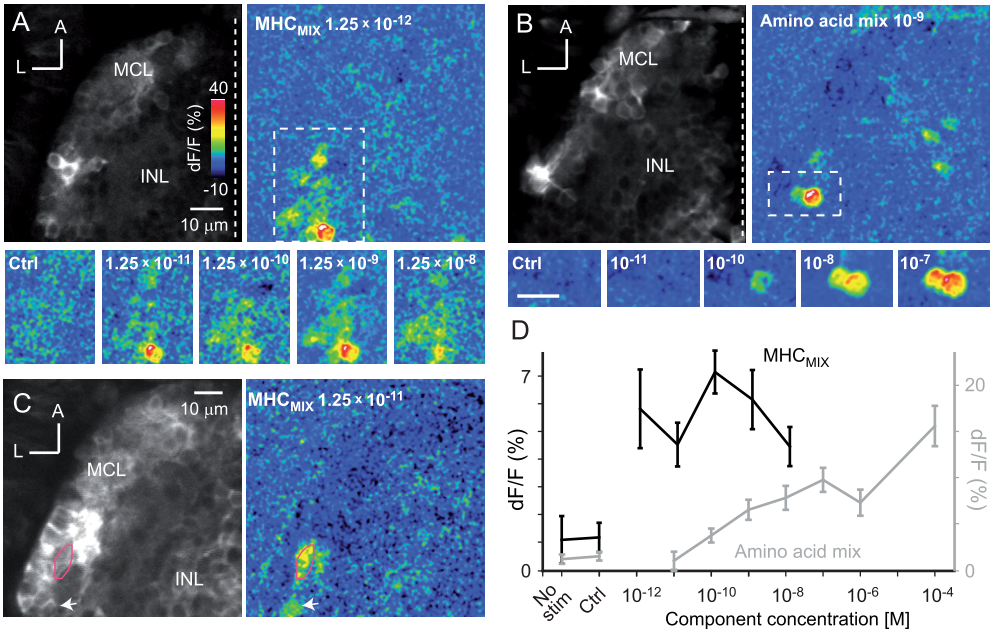

Figure 3 | Responses of olfactory bulb neurons in zebrafish larvae to MHC peptides and amino acids. (A): top left: HuC:GCaMP2 expression (single optical section taken by multiphoton microscopy *in vivo*). Dorsal view; anterior (A) is to the top, lateral (L) is to the left, dashed line indicates midline. Top right: colour-code of relative fluorescence change (dF/F) evoked by the MHC peptide mix (1.25×10^{-12} M) in the same field of view. Bottom: calcium signals evoked by pure medium (Ctrl) and fish water containing different concentrations of MHC peptides in the rectangular region outlined by the dashed rectangle above. HuC:GCaMP2 transgenic line was crossed to OL6. (B): HuC:GCaMP2 expression and responses to a mixture of seven amino acids at different concentrations in another larva (same orientation). Concentration refers to the concentration of each amino acid in the mixture. (C): HuC:GCaMP2 expression and responses to MHC_{Mix} (1.25×10^{-11} M) in a HuC:GCaMP2 fish without OL6 background. MHC_{Mix} evoked a neuropil response (red outline) and a soma response (arrow). (D): Mean dF/F evoked by MHC_{mix} and the amino acid mixture as a function of concentration, averaged over all neurons (MHC_{mix}: n = 42 neurons in 6 fish; amino acid mix: n = 40 neurons in 11 fish). No stim: no stimulus; Ctrl: application of medium without odours. MCL: mitral cell layer. INL: interneuron layer.

neuron (mitral cell) layer than in deeper (interneuron) layers (Fig. 3A). Upon stimulation with MHC_{Mix}, fluorescence changes were observed in small, scattered populations of neurons and neuropil regions in the mitral cell and interneuron layers (Fig. 3A). No response was evoked by pure zebrafish medium (Fig. 3, "Ctrl"), while a mixture of seven amino acids, which are natural (food) odorants for aquatic animals, evoked strong responses (Fig. 3B). Responses to MHC_{Mix} were observed in fish with (Fig. 3A) or without (Fig. 3C) the OL6 background. The number of responding neurons in fish with OL6 background (4.11 ± 0.92 somata per OB; mean ± s.e.m.; $n = 9$ fish) was not significantly different from the number of responding neurons in fish that were not crossed to OL6 (2.75 ± 1.19 somata per OB; $n = 8$; $p = 0.45$, unpaired two-tailed t-test).

We next examined responses to MHC peptides at concentrations between 1.25×10^{-12} M and 1.25×10^{-8} M (n = 42 neurons in 6 fish including 3 with OL6 background). The upper limit of this concentration range corresponds to 10 times the concentration of MHC peptides in serum and urine of mammals[10]. Responses were observed throughout this concentration range and, on average, did not increase with concentration (Fig. 3D). This might, in part, be due to long-lasting adaptation because we usually applied the lowest concentration first. Thresholds for MHC peptides are therefore around 10^{-12} M or even in the sub-picomolar range. Responses to amino acids, in contrast, had substantially higher thresholds (10^{-10}–10^{-9} M) and increased with concentration (Fig. 3D; n = 40 neurons in 11 fish).

If MHC peptides are olfactory signals involved in imprinting, responses to MHC peptides should overlap with responses to kin water. Indeed, a subset of peptide-responsive neurons was also activated by kin water (Fig. 4A; total of 31 optical sections in three fish). In addition, kin water stimulated also other neurons, presumably because it contains a variety of different compounds. The overlap between responses to MHC peptides and food extract, in contrast was low, even though food extract evoked strong and widespread activity throughout the olfactory bulb (Fig. 4B; total of 24 optical sections in six larvae)[23]. MHC responsive neurons were found mainly in the ventro-lateral region of the olfactory bulb (Fig. 3), which is well established as an amino acid responsive area in larvae[23] and adult zebrafish[24]. This area is innervated by olfactory sensory neurons with microvilli[25] expressing V2r-family receptor proteins and transient receptor potential channel C2 (TRPC2)[26]. Consistent with this observation, at least some MHC peptides are detected by V2r-family receptors of vomeronasal sensory neurons in mice[13].

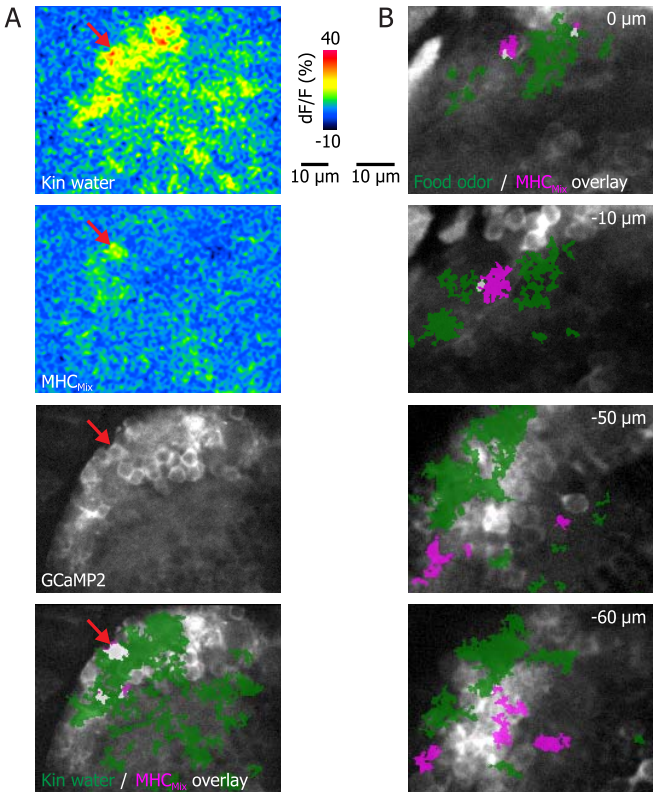

Figure 4 | Responses in the olfactory bulb to MHC peptides overlap with responses to kin water. (A): response patterns evoked by kin water and MHC peptides, and expression of HuC:GCaMP2 in the same field of view. Bottom image shows an overlay of the areas activated by kin water (green) and MHC peptides (magenta), superimposed on the HuC:GCaMP2 expression pattern. Areas activated by both stimuli appear white. Response areas were extracted by thresholding each response pattern at 2.5 SDs over the mean baseline fluctuations (see Methods). Red arrow depicts a soma that responded to kin water and MHC peptides. (B): Overlay of thresholded response patterns evoked by food extract (green) and MHC peptides (magenta) at different depth in the olfactory bulb. The response to food extract was widespread but distinct from the response to MHC peptides.

Discussion

Our results identify MHC peptides as a chemical signal for olfactory imprinting in zebrafish. Imprinting on MHC peptides occurs during a critical period during early development, requires a match between the peptides and the MHC II genotype, and results in a persistent olfactory preference for kin at juvenile stages. This long-lasting memory is likely to mediate shoaling with genetically related juveniles[27–29]. In other species, similar olfactory imprinting mechanisms could explain the observed preference for genetically related versus foreign populations[1]. Chinook salmon (*Oncorhynchus tshawytscha*) and steelhead trout (*Oncorhynchus mykiss*), for example, establish structured groups with greater-than-average genetic relatedness[6].

Previous studies demonstrated that MHC peptides function not only in the immune system but also transmit information about genetic relationship and individuality between individuals[19], influence mate choice[10], and alter the course of pregnancy in mice[12,30]. Our results uncover an additional function of MHC peptides as a chemical signal for olfactory imprinting. In zebrafish, olfactory imprinting is specific for cues that reflect the genotype of individuals, consistent with the fact that MHC peptides represent genetic individuality.

Exposure to a defined set of MHC peptides at 6 dpf induced imprinting. MHC class II allele similarity between larvae resulted in imprinting and recognition while MHC class I allele similarity did not influence imprinting (Fig. 2 and Table 1). Larvae imprinted on kin water from non-kin fish with identical MHC class II alleles, and larvae could not distinguish between water from their own kin and water from non-kin fish with identical MHC class II genes. These results strongly suggest that MHC class II genotype critically determines the specificity of the imprinting process.

Imprinting depended on the MHC class II genotype although the MHC peptides used in this study are known to be ligands for MHC class I proteins. MHC class I and II proteins both bind peptides (usually 8–11 amino acids long) at defined anchor residues but differ in the precise arrangement of binding sites[31]. This could explain the observed dependence of imprinting induced by MHC_{Mix} on MHC class II allele similarity. An interaction between known MHC class I ligands and MHC class II proteins is further suggested by results from sticklebacks. These studies used peptides similar to those used here and found that they interacted with natural odours of males to modify mate choice depending on MHC class II allele relatedness[10].

Functional imaging in the olfactory bulb showed that MHC peptides are potent odorants for zebrafish with thresholds of 10^{-12} M or lower. Detection thresholds for MHC peptides are therefore at least 2–3 orders of magnitude below those for amino acids, which are

Table 1 | Analysis of flume choice tests (Wilcoxon Signed-Ranks Test). MOP: median olfactory preference; Olf: Olfactory stimulus presented to test larvae on 6 dpf; experiment numbers are consistent with order of boxplots in Figure 1 & 2

Raising condition	Flume test stimuli	MOP (%)	*n*	*z*	*p*	Preference
With kin	MHCMix/Untreated water	33	19	−3.348	0.001	MHCMix
Isolated	MHCMix/Untreated water	0	23	−1.593	0.111	-
Olf: MHCMix	MHCMix/Untreated water	8	42	−2.270	0.023	MHCMix
Olf: MHCMix	Kin/non-kin	12.5	42	−2.248	0.025	Kin
With kin	MHCMix/Untreated water	0	18	−0.105	0.916	-
With kin	MHCMix/Untreated water	0	20	−0.473	0.636	-
With kin	MHCMix/Untreated water	−8	17	−1.510	0.131	-
With kin	MHCMix/Untreated water	−4	20	−0.214	0.830	-
With kin	MHCMix/Untreated water	0	19	−1.281	0.200	-
With kin	MHCMix/Untreated water	33	19	−3.348	0.001	MHCMix
With kin	MHCMix/Untreated water	0	19	−0.052	0.959	-
With MHC I dissimilar kin	Kin/non-kin	33	21	−2.983	0.003	Kin
With MHC I similar kin	Kin/non-kin	28	31	−1.236	0.216	-
With MHC II dissimilar kin	Kin/non-kin	11	23	−0.504	0.614	-
With MHC II similar kin	Kin/non-kin	39	29	−2.824	0.005	Kin
With kin	Kin/non-kin	21	30	−3.643	<0.000	Kin
With kin	Kin/MHC II similar non-kin	0	30	−0.670	0.503	-
Olf: MHC II dissimilar non-kin	Familiar non-kin/nonkin	0	30	−0.701	0.483	-
Olf: MHC II dissimilar non-kin	Kin/non-kin	8	30	−1.030	0.303	-
Olf: MHC II similar non-kin	Kin/non-kin	16.7	26	−2.107	0.035	Kin

general odours for many, if not all, aquatic animals. In mice, MHC peptides also stimulate olfactory sensory neurons at very low concentrations[12,32]. Responses to MHC peptides overlapped with responses to kin water but showed little overlap with responses to food extracts, consistent with the assumption that MHC peptides are natural components of fish water.

MHC peptides evoked sparse, distributed responses in the olfactory bulb that overlapped at the single-neuron level with responses to other odorants at higher concentrations. The identity of MHC peptides may therefore be encoded by sparse patterns of activity across multiple neurons, rather than by a small set of highly selective neurons. Consistent with this observation, sensory neurons in mice respond highly selectively to multiple MHC peptides[12,13,32,33]. Responses of OB neurons to MHC_{Mix} were observed in different genetic backgrounds, indicating that individuals can detect a range of MHC peptides that is not limited to the chemical signals for imprinting. It is therefore unlikely that the specificity of the imprinting process is due to an exclusive detection of the imprinted signal by sensory neurons.

Together, our results indicate that MHC peptides are chemical signals that convey information about the identity of individuals and are involved in olfactory imprinting. MHC peptides are good candidates for such chemical signals because the set of MHC peptides that is released to the external world may directly reflect the MHC genotype of an animal[17,19]. The specificity of the imprinting process for chemical cues of related kin is unlikely to arise at the level of detection but could be achieved by comparing olfactory inputs to a stored template of olfactory self-cues. Further studies of odour-evoked activity may therefore test the hypothesis that olfactory imprinting involves specific neuronal activity and plasticity in higher brain areas.

If olfactory cues from conspecifics are matched against olfactory self-representations, individuals have to distinguish between chemical signals from themselves and other individuals. Moreover, peptides used for imprinting in our OL6 zebrafish line were derived from sticklebacks[10] and from mice[34], raising the question how fish distinguish relevant chemical cues from signals released by other species. We assume that under natural conditions the peptide odour of an individual is always accompanied by other behavioural, olfactory and visual signals that provide species- and context-specificity. Indeed, imprinting of zebrafish larvae requires visual contact to kin larvae[15]. Milinski et al.[35] suggested that male three spined sticklebacks (*Gasterosteus aculeatus*) not only use MHC peptides as signals to attract females but also release a 'maleness' cue when in a reproductive state. Such cues might consist of degraded MHC class II protein components, which can be found in the urine[34] and could perhaps also serve as "species identity" cues in olfactory imprinting. Because larvae imprinted on kin water developed a stronger preference for peptide odour than fish imprinted on MHC_{Mix} this peptide mix may not represent the entire natural kin odour. Additional olfactory signals, such as those signalling species identity, might thus further enhance the imprinting process.

Methods

Experimental design. To test whether peptides can trigger kin recognition (E1) we used one mating pair (OL6) whose offspring were known to respond to a mixture of 5 different MHC-peptides (MHC_{Mix}; for details see Supplementary Information Table S2, Fig. 1, E2 and Table 1). One group of test larvae was reared with full contact to siblings; the second group was raised by separating each single individual in a glass beaker (3.5 cm diameter, water depth 4 cm). As a third group single eggs of OL6 were separated in similar small beakers which were placed in a larger glass beaker (14 cm diameter, water depth 4 cm, 7 small beakers per L large beaker) containing 20 full sibling eggs to allow visual but no physical and chemical contact between siblings. In the morning and late afternoon of day 6 pf and in the morning of day 7 pf we replaced 5 ml water of each glass beaker by the MHC_{Mix} (concentration 1.25 nMol each). Using the Atema olfactory choice flume (Fig. S1, Supplementary Information) we tested whether larvae preferred MHC_{Mix}. over untreated water and then kin over non-kin. In E2 larvae of 7 different mating pairs were tested in an odour choice test (see above) whether they were able to discriminate water conditioned with 1.25 nmol/l of each peptide from untreated water. We regarded a preference for peptide water as an indicator that the mixture of peptides might represent the natural kin odour or components of natural kin odour of the family preferring the peptides. We used a mixture of five different artificially synthesized MHC peptide ligands known from the literature: KLYEQGSNK[10], VDPDNFKLL[10], NYGVTKTDI[10], SYFPEITHI[34] and AAPDNRETF[34] (see Supplementary Information Tab. S2).

To test the influence of MHC class I and class II similarity of siblings on imprinting (E3), larvae of 11 different mating pairs were tested. Larvae were reared in small glass beakers, each containing 2 full-sibling eggs/larvae. After being tested for preference of kin versus non-kin, larvae were sacrificed and preserved in ethanol for MHC genotyping.

To test if larvae can discriminate between kin and non-kin which share the same MHC class II alleles (E4) they were reared with visual, olfactory and physical contact to siblings and tested for olfactory preference for kin versus non-kin. Two different types of non-kin were used. One group of non-kin shared the same MHC class II alleles as the test fish and the second group of non-kin were MHC class II dissimilar to the test fish. All kin and non-kin were dissimilar in their MHC class I alleles.

In E5 we tested whether larvae can be imprinted on odour cues of non-kin with identical MHC class II alleles. Since sharing the same MHC class II alleles without being related is very rare, we could use larvae from only two different breeding pairs

that shared the same MHC-genotype. Single eggs were separated in small glass beakers which were placed in a larger glass beaker containing 20 full sibling eggs to allow visual but no physical and chemical contact between siblings. In the morning and late afternoon of day 6 pf and in the morning of day 7 pf we exposed isolated larvae to holding water of non-kin which were either MHC class II similar or dissimilar. First, we tested whether test larvae differentiated between both types of non-kin: the non-kin MHC class II dissimilar line which odour they had experienced and the odour of a randomly chosen non-kin line. Then we tested whether they were imprinted indicated by odour preference of kin versus randomly chosen non-kin Secondly, we conducted the same experiment but used MHC class II similar non-kin odour for imprinting.

For a more detailed description of rearing conditions see Supplementary Information.

Larvae were tested for their olfactory preference at days 8 to12 post fertilization since preference did not differ during this age period (unpublished data). Stimulus water was created by placing 10 larvae into fresh water for 24 h (1 larva/litre).

To determine the MHC similarity between two individuals, we counted the number of bands visible on the gel of the testfish (a_{Test}) and the number of bands shared with the sibling it grew up with (a_{Shared}). We calculated the percentage of MHC similarity (MHC similarity = a_{Shared}* 100/a_{Test}). For the analysis of MHC class II genes we combined data for DAA and DAB loci, because a functional peptide binding region is formed by both proteins and we therefore considered them as one unit.

Animal preparation for calcium imaging. A transgenic zebrafish line expressing GCaMP2.0 under the control of *HuC* promoter[20] (kindly provided by Jennifer Li, Michael Orger, Drew Robson, Alexander Schier and Florian Engert; Harvard University) and crossed to the OL6 family for most experiments. In a small number of experiments, HuC: GCamp2 fish were in-crossed. Embryos were reared in physical contact with their siblings at 25°C in a glass container filled with standard embryo medium (E3) containing (in mM): 5 NaCl, 0.17 KCl, 0.33 $CaCl_2$, and 0.33 $MgSO_4$. E3 medium used in this study never contained methylene blue or N-phenylthiourea.

Calcium imaging. Larvae were prepared for *in vivo* calcium imaging as described earlier[23]. Briefly, larvae were paralyzed in muscle relaxant mivacurim chloride for a few minutes (0.5 mg/mL, Mivacron; GlaxoSmithKline, Munich, Germany) and embedded in 2% low-melting agarose (type VII; Sigma, St. Louis, MO) in a custom-made perfusion chamber. The agarose covering the noses was removed. All animal procedures for calcium imaging were performed in accordance with official animal care guidelines and approved by the Veterinary Department of the Canton of Basel-Stadt (Switzerland). Imaging was performed using a custom-built two-photon fluorescence microscope equipped with a mode-locked Ti: sapphire laser (SpectraPhysics) and a 20× objective (NA 1.0, Zeiss) as described[36]. GCaMP2 was excited at wavelengths between 860 and 930 nm and emission was detected by an external photomultiplier-based whole-field detector through an emission filter (535 ± 25 nm). Images were acquired at 512 ms or 128 ms per frame using SCANIMAGE and EPHUS software[37,38].

Odour stimulation. Odours were delivered through Teflon tubing (inner diameter: 2 mm) that was placed near the noses (distance: 2–4 mm). Odours were introduced into a constant stream (2 mL/min) of E3 medium using a computer-controlled, custom-built electronic valve system (Lee, Westbrook, CT, USA). Odour stimuli were repeated two to six times (typically three times). All odours were dissolved in E3 medium. The five MHC peptides (Supplementary Information Tab. S2) were mixed at 12.5 nM each and then serially diluted in 10 × steps to obtain a concentration series starting from 1.25 pM.

Stimuli of different concentrations were always applied in an ascending series. The mixture of seven amino acids contained Ala, Met, His, Lys, Trp, Phe and Val. These amino acids were chosen because they have different chemical properties and collectively activate a broad range of glomeruli[23,24]. The mixture was applied at concentrations between 10^{-11} M and 10^{-4} M. Food odour was prepared by dispersing food powder based on brine shrimp (SDS100; Special Diets Services; Essex, UK) in E3 medium (about 20% w/v) and incubating the suspension overnight. The supernatant of the suspension was then diluted 1 : 1000 in E3. Kin water was prepared by incubating 20–30 siblings in a glass container filled with 11 ml of E3 medium for at least 3 h and used undiluted. All concentrations indicate the concentrations of each component in a mixture, rather than the sum of the component concentrations.

Imaging data analysis. All imaging data were analyzed using custom scripts in Python (2.6.6; http://www.python.org) using 3rd party libraries (numpy 1.6.1, scipy 0.10.0, matplotlib 1.0.0, Python Imaging Library 1.1.7, wxpython 2.8.12.1). Time-averaged response maps (dF/F) show the relative fluorescence in each pixel between a baseline period (≥1 s) before stimulus application and a response period (~2 s) following stimulation. Response maps were then spatially filtered using a 2D Gaussian kernel (sigma = 1.2 pixels) and averaged over trial repetitions. Responses of individual neurons were examined by manually drawing regions of interest (ROIs) over individual somata based on the raw fluorescence image and analyzing the time course of the fluorescence change (dF/F). Olfactory bulb neurons were classified as responding when they met two independent criteria related to response amplitude and reliability[39]. To meet the amplitude criterion, dF/F had to exceed 2.5 SDs of the mean baseline fluctuation during odour stimulation more often than expected by chance. For example, more than 3.6 frames had to exceed 2.5 SDs when the odour stimulus period was 3 s and 3 trials were done at 8 Hz image acquisition ($3 \times 3 \times 8 \times 0.05 = 3.6$). To meet the reliability criterion, at least one frame had to exceed 2.5 SDs of the mean baseline fluctuations in at least 2/3 of the trials. The threshold for significantly responding areas was defined as 3 SDs of dF/F values in the time- and trial-averaged MHC response map and was computed separately for each field of view. In a second step, pixels were removed when they were not part of a contiguous area with at least 30 pixels (corresponding to about 2×2 μm^2).

To count the number of cells responding to MHC_{mix} in an OB, we measured calcium signals in 6–9 focal planes spaced at 10 μm intervals, which covered most of the OB. In each plane, responses to 2–3 repeated applications of MHC peptides were measured and averaged. We then counted only the somata of cells that met the two response criteria. Huc:GCaMP2 fish were either in-crossed or out-crossed to nacre to obtain OL6 background free fish. Huc:GCaMP2 fish were out-crossed to OL6 at least once to obtain OL6 background fish.

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Author contributions

I.N. and C.H. wrote the first draft of the main manuscript; R.W.F. and G.G. complemented and improved the final text. I.N. prepared figures 3 & 4, C.H. all other figures and tables. Data were collected by I.N., C.H., A.M. and A.J. J.B.-G. and C.O. helped developing the screen of MHC genotypes. All authors reviewed the manuscript.

Additional information

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