Please cite this article in press as: Mathuru et al., Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish, Current Biology (2012), doi:10.1016/j.cub.2012.01.061

Current Biology 22, 1–7, March 20, 2012 ©2012 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2012.01.061

Report

Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish

Ajay S. Mathuru,[1] Caroline Kibat,[1] Wei Fun Cheong,[2] Guanghou Shui,[2,4] Markus R. Wenk,[2,5] Rainer W. Friedrich,[6,7] and Suresh Jesuthasan[1,3,8,*]

[1]Neuroscience Research Partnership, Biomedical Sciences Institutes, 61 Biopolis Drive, Singapore 138673, Singapore
[2]Department of Biochemistry
[3]Department of Physiology
National University of Singapore, 2 Medical Drive, Singapore 117597, Singapore
[4]Neurobiology Program, Life Science Institute, National University of Singapore, 8 Medical Drive, Singapore 117456, Singapore
[5]Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore
[6]Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland
[7]University of Basel, 4003 Basel, Switzerland
[8]Neuroscience and Behavioral Disorders Program, Duke-NUS Graduate Medical School, 8 College Road, Singapore 169857, Singapore

Summary

The ability to detect and avoid predators is essential to survival. Various animals, from sea urchins to damselfly larvae, use injury of conspecifics to infer the presence of predators [1–7]. In many fish [1, 8, 9], skin damage causes the release of chemicals that elicit escape and fear in members of the shoal. The chemical nature of the alarm substance ("Schreckstoff" in German) [1], the neural circuits mediating the complex response, and the evolutionary origins of a signal with little obvious benefit to the sender, are unresolved. To address these questions, we use biochemical fractionation to molecularly characterize Schreckstoff. Although hypoxanthine-3 N-oxide has been proposed to be the alarm substance [10, 11], it has not been reliably detected in the skin [12] and there may be other active components [13, 14]. We show that the alarm substance is a mixture that includes the glycosaminoglycan (GAG) chondroitin. Purified chondroitins trigger fear responses. Like skin extract, chondroitins activate the medial dorsal posterior olfactory bulb, a region innervated by crypt neurons [15] that has a unique projection to the habenula [16]. These findings establish GAGs as a new class of odorants in fish, which trigger alarm behavior possibly via a specialized circuit.

Results and Discussion

We used video tracking to quantify alarm behavior of individual, naive zebrafish by measuring swimming speed and vertical position (Figures 1A–1C). Although isolation is not natural for zebrafish, the behavior of individual fish is reminiscent of that in a school, if only more dramatic (see Movies S1 and S2 available online), reflecting either increased anxiety and/or adoption of an alternate defense strategy (i.e., freezing versus shoaling). Skin extract has been reported to affect behavior in a concentration-dependent manner [17]. We defined 1 unit as the amount of skin extract required to trigger both darting and subsequent slowing down (Figures 1D–1G). The time spent in the lower third of the tank (bottom dwelling) increased significantly from 11.7% to 74.4% (Figure 1H) with 1 unit of the extract. Hypoxanthine-3 N-oxide (H3NO) caused a mild increase in the amount of darting (Figure 1F), but not of slow swim episodes (Figure 1G), even at high concentrations (10 ug/ml). Moreover, fish did not move to the bottom third of the tank (Figure 1H). Hence, in our assay, skin extract but not H3NO elicited all features of the alarm response.

To characterize the alarm cue, we fractionated skin extract and tested the activity of individual fractions in the behavioral assay. Pilot studies using hydrophilic columns (Figure S1A) revealed that the active components are highly polar. Using anion-exchange chromatography followed by high-resolution gel-filtration (Figures S1B and S1C) we found two fractions, a high (HMW; ~30 kD by protein standard) and a low molecular weight fraction (LMW; ~1 kD), that elicited clear behavioral responses. HMW substances evoked mainly slow swimming and descent to the bottom of the tank without initial darting, whereas LMW substances increased darting but caused little slow swimming (Figures 1D–1H). This finding suggests that the alarm pheromone in zebrafish is a mixture of compounds, echoing findings of Levedeva et al. in minnows [13].

We carried out a series of tests on crude skin extract to classify the alarm substance. Pronase or peptidase treatment did not reduce activity of the extract, indicating that proteins are unlikely to be critical components. Biological activity partitioned to the aqueous and aqueous-methanol phase with Folch's extraction, suggesting that most lipids are also unlikely to be active constituents. Substances with the ability to induce slow swimming (similar to HMW) could be bound and eluted from a column containing wheat germ agglutinin (WGA), a lectin that binds to glycans, establishing that the alarm pheromone contains glycans (Figures 2A–2E). When the HMW fraction from the size separation column was itself run through the WGA column, the eluate emerged as a series of peaks (Figure 2F), indicating that a component of HMW is bound by WGA column and is sensitive to mechanical shearing. This suggests that HMW substances may be made of long polymers, possibly polysaccharides. Mass spectrometric analysis of HMW and LMW fractions failed to yield any obvious candidates, however, because too many peaks were detected.

Serendipitously, we noted that zebrafish exposed to extract obtained by vigorous shaking of fish without injury (sloughing, which releases mucous [18]) also displayed mild alarm behavior characterized by increased darting (Figure 3). Heating the slough at 95°C for 2 hr enhanced its activity (Figure 3). This treatment can cause breakdown and release of glycosaminoglycans (GAGs) [19], which are a major component of mucous, suggesting that GAGs are a likely component of

*Correspondence: suresh.jesuthasan@nrp.a-star.edu.sg

Please cite this article in press as: Mathuru et al., Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish, Current Biology (2012), doi:10.1016/j.cub.2012.01.061

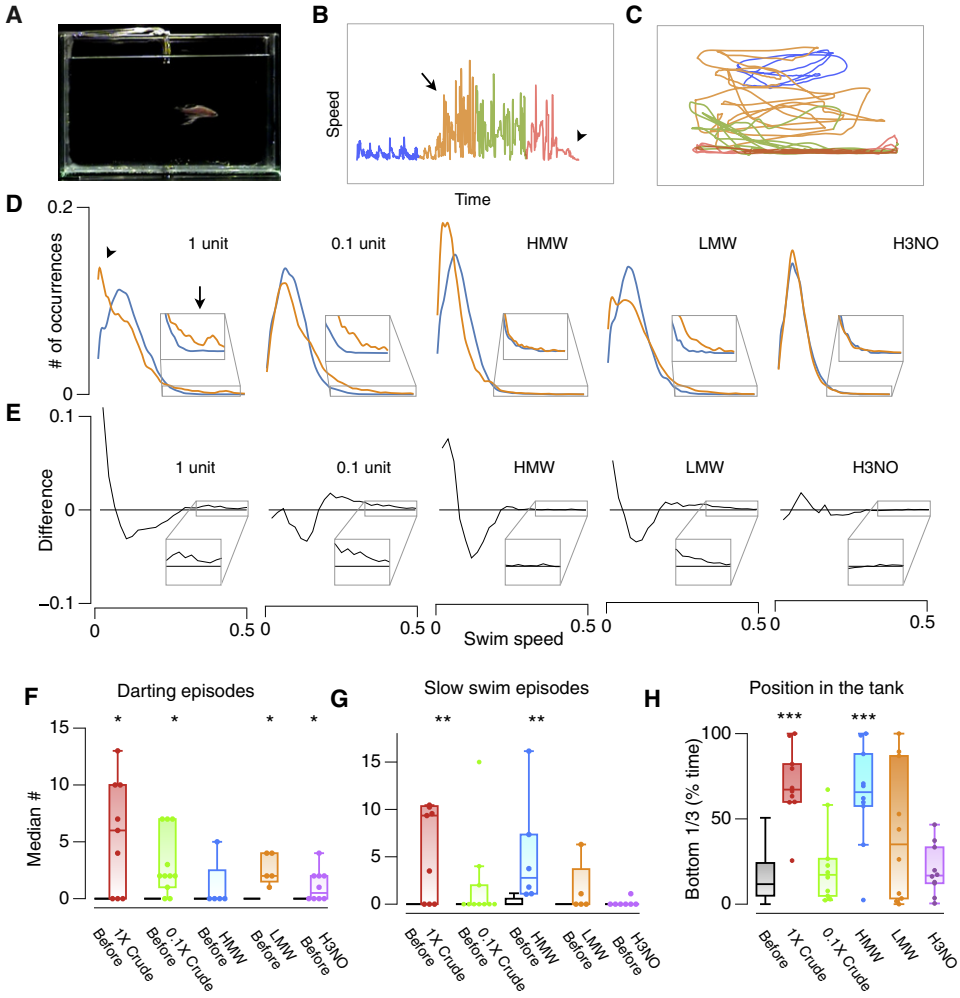


Figure 1. The Response of Zebrafish to Crude and Fractionated Skin Extract

(A) During behavioral experiments, substances are delivered to individual fish via the tube at the top of the tank (side view).

(B) Swim speed of one fish before (blue) and after (orange) addition of crude skin extract. The extract elicited an increase in speed (arrow), followed by reduction (arrowhead).

(C) Trajectory of the fish before (blue) and after (orange, green, red; each color represents 20 s) addition of crude skin extract. Note that the fish moved to the bottom of the tank (side view). See Movie S2.

(D–H) Behavioral response of all individuals (n = 10) exposed to a particular substance.

(D) Distribution of speed (mm/25 ms) during a 1 min period, before (blue) and after (orange) addition of test substance. Slowing down is reflected by an increase in the number of occurrences of low speed swimming, whereas darting is reflected by an increase in the occurrences of higher speed swimming, which can be more clearly seen in the insets.

(E) Difference between speed distribution before and after substance addition.

(F) Median number of darts before (black) or after addition of test substance. Boxes show 25th and 75th percentiles, and whiskers represent 1.5 IQR (interquartile range).

(G) Median number of slow swim episodes before or after test substance.

(H) The percentage of time spent by fish in the lower 1/3 of the tank after the addition of different substances (color code: red, 1 unit crude skin extract; green, 0.1 unit crude skin extract; blue, HMW; orange, LMW; purple, H3NO). In (B) and (D), the arrow indicates faster swimming, whereas the arrowhead indicates slow swimming. *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ (Wilcoxon signed-rank test). See Figure S1.

the alarm substance. To test this hypothesis, we first checked for their presence in skin extract, using chemical assays capable of detecting GAGs such as phenol-sulphuric acid, alcian blue, and Elson-Morgan assays. All tests were positive, indicating that these glycans are present in skin extract. The concentration of GAGs in fractions from the ion exchange column, which was measured using alcian blue absorbance at 620 nm, correlated with behavioral activity (Figure 3F), consistent with GAGs being active components of the alarm substance.

The mucous of fish skin has been shown to contain the GAGs chondroitin sulfate and hyaluronic acid [20]. Chondroitins are linear, heterogeneous polymers, made of disaccharides that are variably sulfated, whereas hyaluronic acid is a homogenous chain of nonsulfated disaccharides. Mass spectroscopy confirmed the presence of either nonsulfated chondroitin or hyaluronic acid, as well as some forms of sulfated chondroitin, in the active fractions (Figure S2A; Table S1). Using fluorescence-assisted carbohydrate gel electrophoresis (FACE) to monitor disaccharide composition,

Please cite this article in press as: Mathuru et al., Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish, Current Biology (2012), doi:10.1016/j.cub.2012.01.061

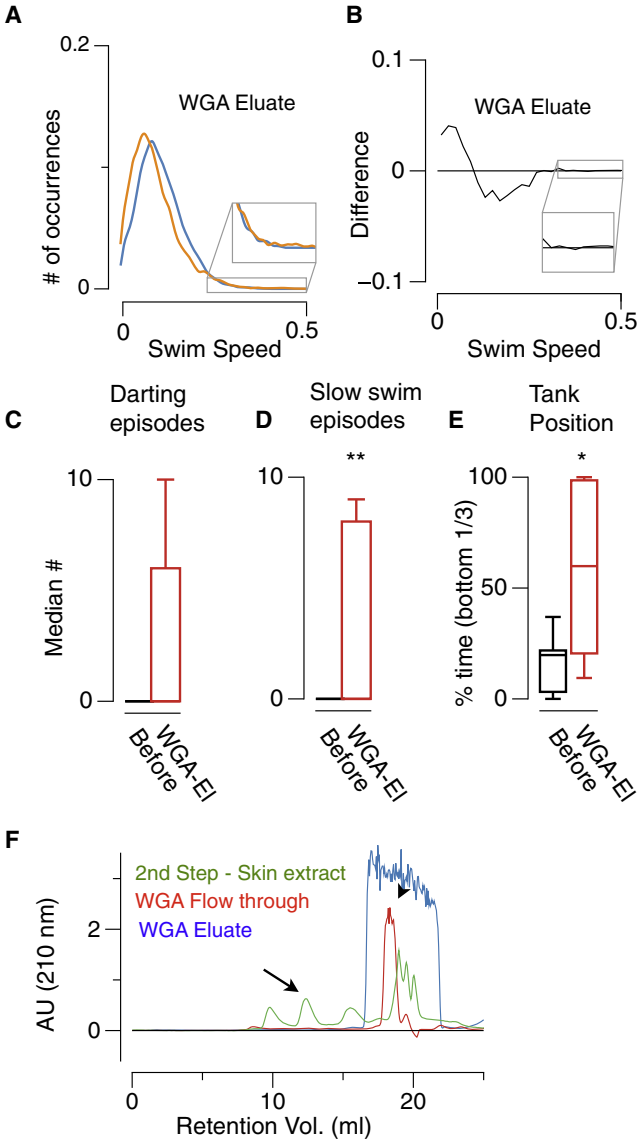


Figure 2. The Zebrafish Alarm Substance Contains a Glycan

(A) Distribution of speed over a 1 min period, before (blue) and after (orange) addition of WGA eluate.

(B) Difference between speed distribution before and after WGA eluate addition. Insets magnify the tail that shows darting episodes.

(C) Median number of darting episodes before (black) or after WGA eluate (red) addition.

(D) Median number of slow swim episodes before (black) or after (red) WGA eluate addition.

(E) Percentage of time spent in the lower 1/3 of the tank after addition of WGA eluate.

(F) Fast protein liquid chromatography profiles of eluate (blue) or flow-through (red) from a WGA column loaded with HMW. The green line shows HMW (arrow) and LMW (arrowhead) obtained in the second step of skin extract fractionation on the gel-filtration column Superdex-75. The WGA eluate and flow-through, which are derived from HMW, run at a smaller size than HMW. *p < 0.05; **p < 0.01 (Wilcoxon signed-rank test).

three different disaccharides, nonsulfated chondroitin (C0S), chondroitin-4-sulfate (C4S or CS-A), and chondroitin-6-sulfate (C6S or CS-C) (in 1:1:1 ratio, Figure 3G), could be detected; hyaluronic acid was below detection threshold in HMW (Figure S2C). Consistent with this, the HMW fraction was sensitive to chondroitinase ABC, which cleaves most forms of chondroitin (Figure 3H), but not to hyaluronidase from *Streptomyces hyalurolyticus*, which is specific to hyaluronic acid (Figure S2D). When chondroitin was immunodepleted from slough using the monoclonal antibody CS-56 [21], behavioral responses were reduced (Figures 3A–3E). Together, these data indicate that the alarm substance includes chondroitin.

We examined how enzymes that specifically cleave chondroitin altered the behavioral effect of slough, to test whether release of chondroitin fragments triggers the alarm response. Moreover, different glycosidases, which have different digestion patterns and substrate preference, can shed light on the nature of the alarm signal. Because slough elicits weaker response than 1X unit skin extract (Figures 1 and 3A–3E), this was used as the substrate. Twenty-minute treatment with chondroitinase ABC, which is expected to yield chondroitin sulfate oligosaccharides of different sizes [22], increased darting, slow swimming, and movement to the lower third of the tank (Figure 4). In contrast, 24 hr digestion with chondroitinase ABC or short digestion with chondroitinase AC II, both of which yield disaccharides, did not elicit increase in darting or slow swim episodes over responses to slough. Treatment with chondroitinase B, which does not digest C4S or C6S, also showed minimal increase. This suggests that the active components include chondroitin oligosaccharides, with a minimal size of a tetrasaccharide (~1,000 Daltons), and argues against the active component being a molecule associated with chondroitin.

Zebrafish skin contains three different chondroitin disaccharides (Figure 3G). Because signaling properties of chondroitin are dependent on sulfation [23], in addition to length, we tested the contribution of differently sulfated forms of chondroitin. Chondroitin polymers from natural sources can be enriched in one disaccharide but are usually not exclusive for that disaccharide. Commercially available chondroitin sulfate derived from shark cartilage (Sigma C4384), which mainly contains C6S and C4S, elicited all features of alarm behavior (slow swimming, darting, and bottom dwelling; Figure 4; see also Movie S3). Chondroitin from sturgeon notochord (Seikagaku 400658), which predominantly contains C4S, elicited weaker responses than shark CS in all three parameters. Because C0S oligosaccharides were not available commercially, we prepared and tested a GAG extract from *C. elegans*, which makes only nonsulfated chondroitin [24]. This caused an increase in slow swimming episodes and in bottom dwelling but not darting (Figure 4). These observations suggest that C6S, or a mix of C6S and C4S, and to a lesser extent C0S or C4S, can trigger fear responses. When compared to the natural skin extract of zebrafish (Figures 1F–1H), however, the absolute value of each behavioral parameter is lower.

The alarm substance is detected by the olfactory system in fish [25–27]. Chondroitin also acts via the olfactory system, because blocking the naris prevents a response (Figure S3). To compare the pattern of olfactory bulb (OB) activation by chondroitin with that of skin extract, we used a transgenic zebrafish line with broad expression of the calcium indicator GCaMP2 [28] (Tα1:GCaMP2) to detect odor-evoked activity in vivo by wide-field fluorescence microscopy. Four-dimensional imaging was carried out mainly on 3-week-old larvae, whose transparency and small size enable the entire bulb to be imaged at each time point in intact fish. Partially purified

Please cite this article in press as: Mathuru et al., Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish, Current Biology (2012), doi:10.1016/j.cub.2012.01.061

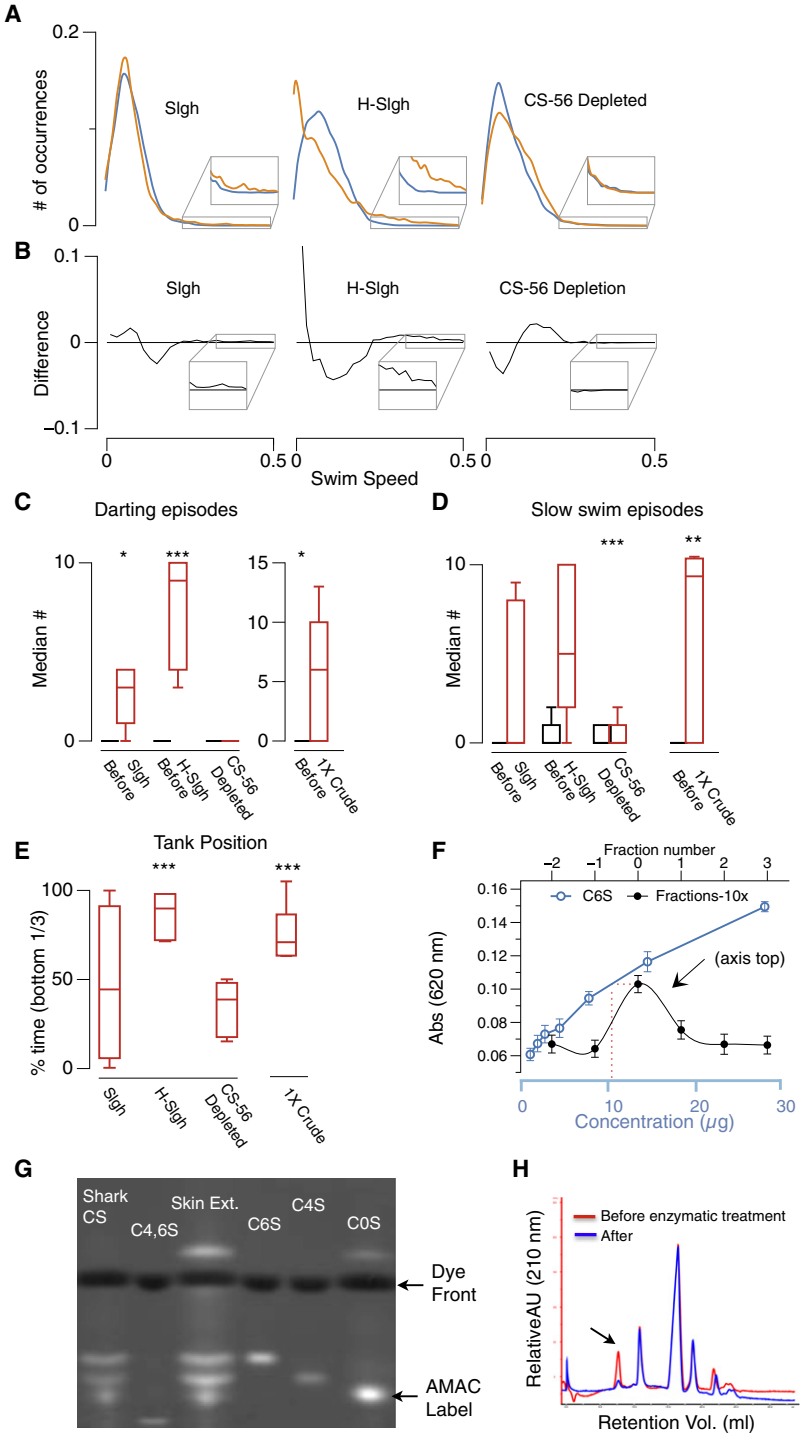


Figure 3. Glycosaminoglycans, a Component of Slough, May Be a Constituent of Schreckstoff

(A) Distribution of speed over a 1 min period, before (blue) and after (orange) addition of slough (slgh), heated slough (h-slgh), or immunodepleted slough (CS-56 depleted).

(B) Difference between speed distribution before and after substance addition. Insets magnify the tail that shows darting episodes.

(C) Median number of darting episodes before (black) or after test substance (red) addition.

(D) Median number of slow swim episodes before (black) or after (red) test substance addition.

(E) Percentage of time spent in the lower 1/3 of the tank after substance addition. The response to crude skin extract is shown for comparison.

(F) Quantitation of GAGs in fractions (concentrated 10-fold) from the ion-exchange column, determined by alcian blue binding. Fraction number 0 is the most active fraction. The blue lines show absorbance of different concentrations of chondroitin-6-sulfate (C6S), serving as a standard.

(G) FACE analysis of disaccharides in skin extract.

(H) Effect of chondroitinase ABC on skin extract, shown here eluting on a size fraction column. The arrow indicates the HMW peak that is lost. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Wilcoxon signed-rank test). See also Figure S2.

skin extracts reproducibly activated the olfactory bulb in three distinct loci (Figures 5A–5E), which appear to be in the anterior plexus, the lateral chain, and the mediodorsal posterior bulb [29]. Similar loci were detected in fish aged 4–5 weeks (Figure S3), which have a clear behavioral response [30], and also in adults (data not shown). LMW activated the mediodorsal and the lateral regions in 19/21 fish. Both HMW and WGA eluate consistently activated the mediodorsal locus (Figures 5F–5K; 15/16 and 5/6, respectively). The lateral and anterior loci were activated by HMW in 10/16 fish, whereas eluate from the WGA column failed to trigger a consistent response (1/6). This suggests that LMW, HMW, and WGA eluate share a common factor that triggers response in the mediodorsal glomerular region.

Purified shark chondroitin sulfate, like WGA eluate, caused a calcium increase only in the mediodorsal locus (Figure 5M; 18/22 fish). Co-localization analysis indicates that there is substantial overlap in the activity triggered by chondroitin and skin extract (Figure S3; Table S2). H3NO did not trigger any significant activity at the mediodorsal locus but caused a response in the lateral and anterior loci in 4/11 fish (Figures 5N and 5R). Bile acids showed a strong signal more anteriorly, as has been reported previously [31], and a small signal in the mediodorsal locus (Figures 5O and 5S), whereas another class of odorant, amino acids, activated the ventrolateral bulb only [31] (data not shown). Hence, chondroitin and skin extract activate a common locus of the bulb, which is the mediodorsal posterior region.

In crucian carp, electrophysiological recordings of mediodorsal posterior region of the olfactory bulb show responses to skin extract [27], indicating that this region may mediate innate fear in other fish also. The mediodorsal posterior bulb in zebrafish is innervated by crypt cells [15], a subset of olfactory sensory neurons with no previous known function. Mitral cells from this region of the bulb have a projection to the right habenula [16], in addition to targets in the ventral telencephalon [15], suggesting that the GAG

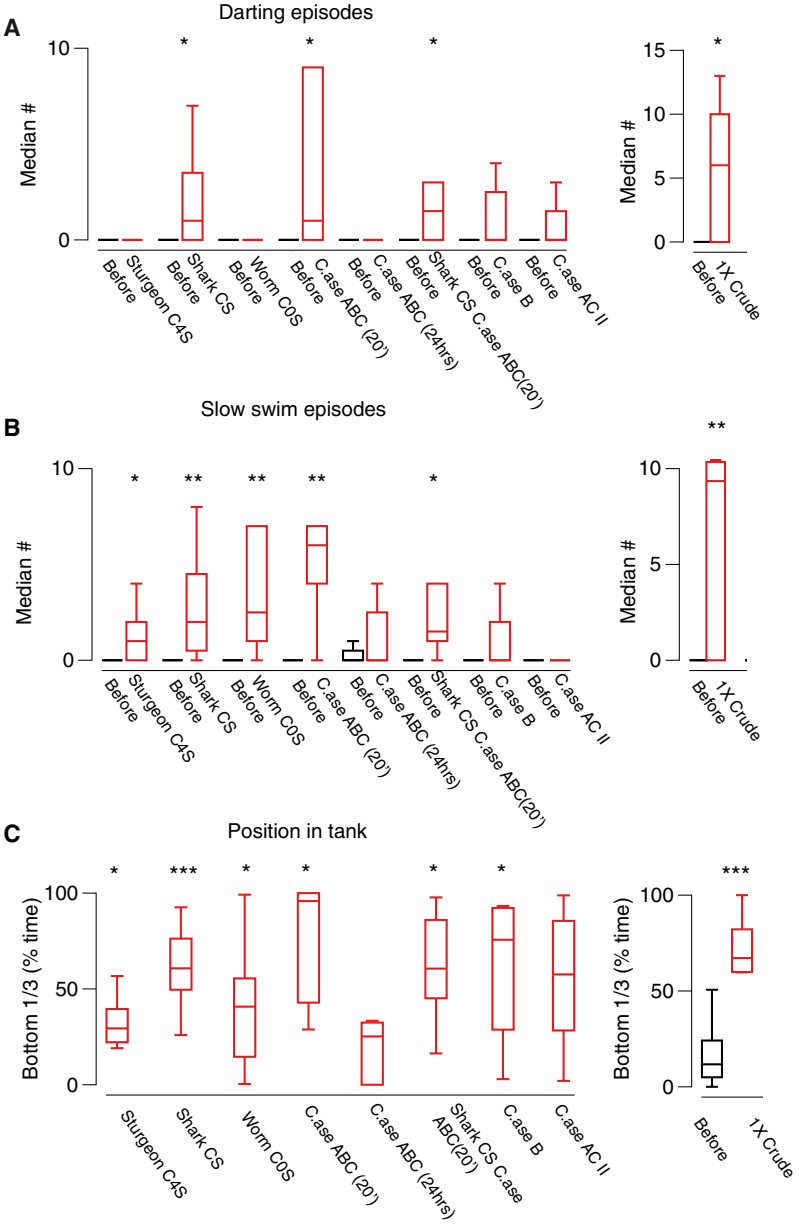


Figure 4. Chondroitin Oligosaccharides Are an Active Component of the Alarm Substance

(A) Median number of darts before (black) or after test substance addition (red).

(B) Median number of slow swim episodes, before (black) or after test substance (red).

(C) Percentage of time spent in the lower 1/3 of the tank after substance addition. Test substances are chondroitin purified from other sources, at a concentration of 1 μg/ml, or zebrafish slough digested as indicated. For comparison, the response to crude skin extract is shown on the right. *p < 0.05; **p < 0.01; ***p < 0.001 (Wilcoxon signed-rank test).

component of the alarm substance may engage a unique circuit.

The data presented here support the interpretation that a mixture elicits alarm behavior and that chondroitin is an active ingredient of the alarm cue in zebrafish. Purified chondroitin, at a concentration equivalent to that found in behaviorally active fractions, elicits components of alarm behavior in a laboratory setting—darting, slow swimming, and bottom dwelling. However, chondroitins could not trigger fear responses to the same extent as crude skin extract. Also, chondroitins, like WGA eluate, did not elicit calcium increase in the lateral and anterior glomeruli that were activated by behaviorally relevant skin extract fractions. This suggests the presence of additional active components in skin extract. This additional substance(s) may include compounds with a nitrogen oxide group such as that present in H3NO, a motif suggested to be important in other species [11]. This could account for the activity in the lateral and anterior glomerular loci in imaging experiments and mild darting observed in behavioral assays with H3NO both here and in other studies [32]. We were unable to determine the identity of these additional components, however, despite careful analysis of mass spec data from different fractions.

Epidermal club cells, which have been associated with Schreckstoff on the basis of comparative histology [9], contain GAGs [33] and may therefore be a source of chondroitin that acts as an alarm substance. It is also possible that these cells, upon rupturing, release enzymes that cleave chondroitin from proteoglycans or mucous, thus necessitating physical injury for Schreckstoff release. The heterogeneity of sulfation, even within a single chondroitin chain, provides one possible basis for the varying cross-species recognition of the alarm cue among fishes [8, 9]. The evolution of Schreckstoff has caused debate [34], because there appeared to be no direct benefit to the sender. Our results support the idea that the alarm cue precursors are maintained in the sender for functions unrelated to their ability to trigger fear [35]; in this case, one function may be as a component of mucous. Reliable release and degradation of chondroitin, specifically during predation, may have driven the evolution of their detection as alarm cues in the receivers consequentially.

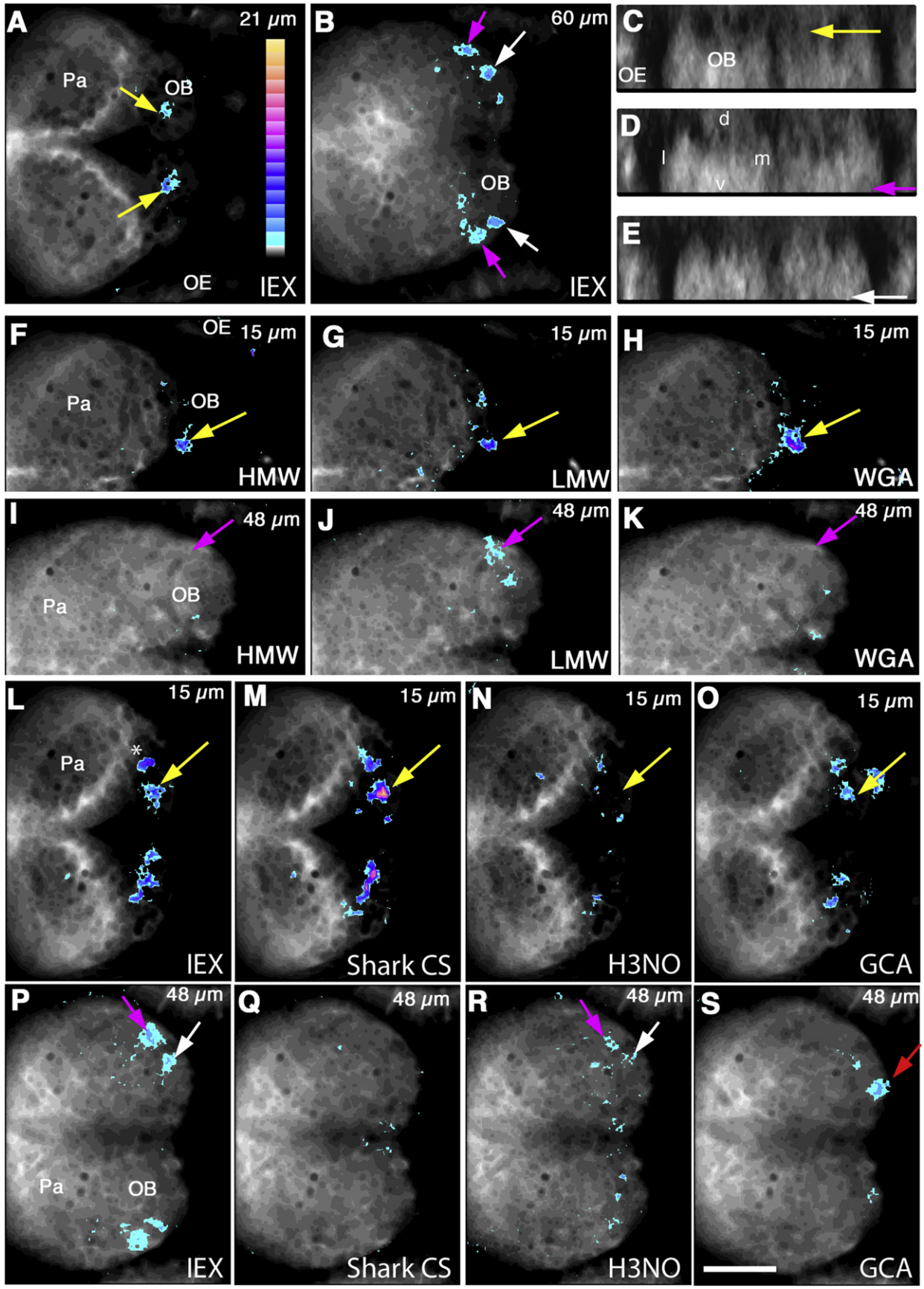
Supplemental Information

Supplemental Information includes three figures, two tables, Supplemental Experimental Procedures, and three movies and can be found with this article online at doi:10.1016/j.cub.2012.01.061.

Acknowledgments

We thank Florian Engert for the Tα1:GCaMP2 line; Patrick Gilligan, Jeffrey Yong, Ed Manser, and Daniel Hess for assistance with chromatography; Jayantha Gunaratne and Stefan Bräse for discussion on glycans; and Dale Purves, Phil Ingham, and George Augustine for comments on the manuscript. This work was funded by the Biomedical Research Council, Singapore. Pilot work was supported by the Temasek Trust. Work in the Wenk laboratory was supported by the Singapore National Research Foundation CRP Award No. 2007-04.

Please cite this article in press as: Mathuru et al., Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish, Current Biology (2012), doi:10.1016/j.cub.2012.01.061



Received: November 21, 2011
Revised: January 10, 2012
Accepted: January 30, 2012
Published online: February 23, 2012

References

1. von Frisch, K. (1938). Zur Psychologie des Fisch-Schwarmes. Naturwissenschaften *26*, 601–606.
2. Eibl-Eibesfeldt, I. (1949). Über das Vorkommen von Schreckstoffen bei Erdkrötenquappen. Cell. Mol. Life Sci. *5*, 236–236.
3. Snyder, N., and Snyder, H. (1970). Alarm Response of Diadema antillarum. Science *168*, 276–278.
4. Howe, N.R., and Sheikh, Y.M. (1975). Anthopleurine: a sea anemone alarm pheromone. Science *189*, 386–388.
5. Atema, J., and Stenzler, D. (1977). Alarm substance of the marine mud snail, Nassarius obsoletus: biological characterization and possible evolution. J. Chem. Ecol. *3*, 173–187.
6. Wisenden, B.D., Chivers, D.P., and Smith, R.J.F. (1997). Learned recognition of predation risk by Enallagma damselfly larvae (Odonata, Zygoptera) on the basis of chemical cues. J. Chem. Ecol. *23*, 137–150.
7. Wudkevich, K., Wisenden, B.D., Chivers, D.P., and Smith, R.J.F. (1997). Reactions of *Gammarus lacustris* to Chemical Stimuli from Natural Predators and Injured Conspecifics. J. Chem. Ecol. *23*, 1163–1173.
8. Schutz, F. (1956). Vergleichende Untersuchungen über die Schreckreaktion bei Fischen und deren Verbreitung. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *38*, 84–135.
9. Pfeiffer, W. (1977). The distribution of fright reaction and alarm substance cells in fishes. Copeia *4*, 653–665.
10. Pfeiffer, W., Riegelbauer, G., Meier, G., and Scheibler, B. (1985). Effect of hypoxanthine-3 (N)-oxide and hypoxanthine-1 (N)-oxide on central nervous excitation of the black tetra *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. J. Chem. Ecol. *11*, 507–523.
11. Brown, G., Adrain, J.C.J., Smyth, E., Leet, H., and Brennan, S. (2000). Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides. J. Chem. Ecol. *26*, 139–154.
12. Smith, R.J.F. (1999). What good is smelly stuff in the skin? Cross function and cross taxa effects in fish "alarm substances". In Advances in Chemical Signals in Vertebrates, R. Johnston, D. Müller-Schwarze, and P.W. Sorensen, eds. (New York: Kluwer Academic/Plenum Press), pp. 475–487.
13. Lebedeva, N.Y., Malyukina, G.A., and Kasumyan, A.O. (1975). The natural repellent in the skin of cyprinids. J. Ichthyol. *15*, 472–480.
14. Kasumyan, A.O., and Lebedeva, N.Y. (1979). New data on the nature of the alarm pheromone in cyprinids. J. Ichthyol. *19*, 109–114.
15. Gayoso, J., Castro, A., Anadon, R., and Manso, M.J. (2011). Crypt cells of the zebrafish danio rerio mainly project to the dorsomedial glomerular field of the olfactory bulb (Chem Senses), in press. Published online December 13, 2011.
16. Miyasaka, N., Morimoto, K., Tsubokawa, T., Higashijima, S.-i., Okamoto, H., and Yoshihara, Y. (2009). From the olfactory bulb to higher brain centers: genetic visualization of secondary olfactory pathways in zebrafish. J. Neurosci. *29*, 4756–4767.
17. Speedie, N., and Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. *188*, 168–177.
18. Shephard, K.L. (1994). Functions for fish mucus. Rev. Fish Biol. Fish. *4*, 401–429.
19. Jeanloz, R.W., and Forchielli, E. (1950). Studies on hyaluronic acid and related substances. I. Preparation of hyaluronic acid and derivatives from human umbilical cord. J. Biol. Chem. *186*, 495–511.
20. Van De Winkel, J.G.J., Van Kuppevelt, T.H.M.S.M., Janssen, H.M.J., and Lock, R.A.C. (1986). Glycosaminoglycans in the skin mucus of rainbow trout (Salmo gairdneri). Comparative Biochemistry and Physiology Part B. Comparative Biochemistry *85*, 473–475.
21. Avnur, Z., and Geiger, B. (1984). Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell *38*, 811–822.
22. Zhang, Z., Park, Y., Kemp, M.M., Zhao, W., Im, A.R., Shaya, D., Cygler, M., Kim, Y.S., and Linhardt, R.J. (2009). Liquid chromatography-mass spectrometry to study chondroitin lyase action pattern. Anal. Biochem. *385*, 57–64.
23. Gama, C.I., Tully, S.E., Sotogaku, N., Clark, P.M., Rawat, M., Vaidehi, N., Goddard, W.A., 3rd, Nishi, A., and Hsieh-Wilson, L.C. (2006). Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. Nat. Chem. Biol. *2*, 467–473.
24. Yamada, S., Van Die, I., Van den Eijnden, D.H., Yokota, A., Kitagawa, H., and Sugahara, K. (1999). Demonstration of glycosaminoglycans in Caenorhabditis elegans. FEBS Lett. *459*, 327–331.
25. von Frisch, K. (1941). Die Bedeutung des Geruchsinnes im Leben der Fische. Naturwissenschaften *29*, 321–333.
26. Hamdani, E.H., Stabell, O.B., Alexander, G., and Døving, K.B. (2000). Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. Chem. Senses *25*, 103–109.
27. Hamdani, H., and Døving, K.B. (2003). Sensitivity and selectivity of neurons in the medial region of the olfactory bulb to skin extract from conspecifics in crucian carp, Carassius carassius. Chem. Senses *28*, 181–189.
28. Tallini, Y.N., Ohkura, M., Choi, B.-R., Ji, G., Imoto, K., Doran, R., Lee, J., Plan, P., Wilson, J., Xin, H.-B., et al. (2006). Imaging cellular signals in the heart in vivo: Cardiac expression of the high-signal Ca2+ indicator GCaMP2. Proc. Natl. Acad. Sci. USA *103*, 4753–4758.
29. Baier, H., and Korsching, S. (1994). Olfactory glomeruli in the zebrafish form an invariant pattern and are identifiable across animals. J. Neurosci. *14*, 219–230.
30. Waldman, B. (1982). Quantitative and developmental analyses of the alarm reaction in the Zebra Danio, *Brachydanio rerio*. Copeia *1982*, 1–9.
31. Li, J., Mack, J.A., Souren, M., Yaksi, E., Higashijima, S., Mione, M., Fetcho, J.R., and Friedrich, R.W. (2005). Early development of functional spatial maps in the zebrafish olfactory bulb. J. Neurosci. *25*, 5784–5795.
32. Parra, K.V., Adrian, J.C., Jr., and Gerlai, R. (2009). The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (Danio rerio). Behav. Brain Res. *205*, 336–341.
33. Ralphs, J.R., and Benjamin, M. (1992). Chondroitin and keratan sulphate in the epidermal club cells of teleosts. J. Fish Biol. *40*, 473–475.
34. Smith, R.J.F. (1992). Alarm signals in fishes. Reviews in Fish Biology and Fisheries *2*, 33–63.
35. Chivers, D.P., Wisenden, B.D., Hindman, C.J., Michalak, T.A., Kusch, R.C., Kaminskyj, S.G.W., Jack, K.L., Ferrari, M.C.O., Pollock, R.J., Halbgewachs, C.F., et al. (2007). Epidermal 'alarm substance' cells of fishes maintained by non-alarm functions: possible defence against pathogens, parasites and UVB radiation. Proc. Biol. Sci. *274*, 2611–2619.

Figure 5. Olfactory Bulb Response to Skin Extract and Purified Chondroitin Sulfate

(A–F) Increase in calcium in the mediodorsal (yellow arrow), anterior (white arrow) and lateral (magenta arrow) olfactory bulb, in response to stimulation of the olfactory epithelium with the active fraction of skin extract obtained by ion exchange chromatography.

(A and B) Dorsal view of the olfactory bulb, at two different focal planes as indicated by the numbers on the top right of each panel.

(C–E) Positions of the active loci, in frontal views generated from deconvolved z stacks at the level of arrows.

(F–K) Activity in the left olfactory bulb of a 25-day-old fish, in response to HMW, LMW, and eluate from the WGA column. The images here show a dorsal view of the left bulb, at two different focal planes.

(L–S) Response in the bulb at two different focal planes in another fish, following stimulation with partially purified skin extract (L and P), chondroitin sulfate purified from shark cartilage (M and Q), H3NO (N and R), and glycocholic acid (O and S; red arrow). The asterisk in (L) indicates a signal in the pallium. OE, olfactory epithelium; OB, olfactory bulb; Pa, pallium; IEX, active fraction from ion exchange column; d, dorsal; v, ventral; m, medial; l, lateral. Scale bar represents 50 μm; anterior is to the right in all cases. The wedge in (A) shows the look-up table used for ratio images. An intensity-modulated look-up table is used, with grays representing a ratio of 1 and yellows representing a ratio of 2.5. See also Figure S3.