TITLE: The evolution of cavefish odor perception shifts fish response from avoidance to approach when exposed to alarm and death odors.

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ABSTRACT: An animal's survival is dependent on activating the correct behavioral circuit in response to a sensory stimulus. These stimuli include environmental odorants that evoke positive approach behavior and negative avoidance behavior. While stimulus perception has evolved similarly across ecological niches, we still do not know the mechanisms that drive evolution of stimulus perception across taxa. The blind Mexican cavefish provides a unique model for understanding how changes in environmental pressures impact the evolution of the vertebrate brain and behaviors. For instance, cavefish exhibit sleep loss due to life in an environment with no sunlight. Therefore, we wondered how odor perception has evolved in cave adapted populations that experience vastly different environmental conditions and odors than their surface ancestors. We found that surface fish display approach behaviors when exposed to food and social odors, while exhibiting avoidance behaviors when exposed to alarm and death odors. Conversely, we find that cave populations exhibit approach behaviors when exposed to alarm and death odors in the dark, while only females show approach behaviors when exposed to social odors. In addition, we find that surface to cave F_2 hybrids display a range of behaviors, from full avoidance to approach when exposed to social, alarm and death odors, that suggests these differences are genetically inheritable. Finally, forebrain phospho-ERK mapping revealed overlap in the activity of thalamic and preoptic subregions of the brain when cavefish were exposed to food or death odors. Taken together, we find that changes to cavefish odor perception provide a unique model for investigating the genetic and neurological mechanisms underlying olfactory evolution in vertebrates.

INTRODUCTION

Vertebrate evolution has maintained conserved sensory percepts over hundreds of millions of years due to the stability of environmental sensory signals [1, 2]. Common sensory signals are received and integrated to compute the appropriate behavioral output to find food, avoid predation, find mates and overall increase the probability of survival [1, 3]. While our general knowledge regarding sensory integration and behavioral output is quite comprehensive, less is known about how genetic diversity and environmental variability drive sensory perception [4-6]. This includes how sensory perception of specific environmental cues are changed from positive to negative valences or vice versa.

Taxis towards, or away from, chemical signals appeared very early in the history of life, predating the Bacteria-Archaea separation, and has remained a mainstay of mobile life forms since, including in humans [7, 8]. Common odors in our environment are generally characterized by our brain as being positive or negative [9, 10]. For instance, death odors are released during decomposition, providing negative cues that signal danger, area avoidance and life preservation [11-13]. In contrast, the smell of prey provides positive cues that trigger approach behaviors associated with foraging [14-16]. While these odor percepts are generally conserved across taxa, changes in environmental pressures and life history traits can influence the evolution of odor perception [3, 13]. This is increasingly relevant in the context of the anthropogenic impact on ecological niches over time, coupled to the remarkable plasticity of the olfactory system, especially in aquatic animals [3, 17]. Therefore, species adapted to extreme environments with variation in food availability [18], predation [19], and social structures [20], provide unique models for understanding the biological mechanisms underlying evolution of odor perception and the adaptability of odor stimulated behavior.

An example of this is the blind Mexican cavefish, *Astyanax mexicanus*, a teleost fish species comprised of ancestral, river dwelling surface populations and over 30 cave-adapted populations of blind cavefish, of which some have independently evolved and share convergent troglobitic features [21, 22] (Figure 1a). These include regressive traits, such as eye degeneration and loss of pigmentation, and constructive traits, including expansions of taste receptors, lateral line and olfactory epithelia [23-27]. These constructive traits are thought to compensate for eye regression, with the olfactory system of cavefish exhibiting larger sensory organs and greater sensitivity to specific amino acids that may improve survival in the dark [25, 28]. Despite insights on smell ability, less is known about how odor perception evolved in cavefish and whether common environmental odors evoke the same approach and avoidance behaviors as in ancestral surface fish.

Here, we asked whether cavefish odor perception is variable, and if so, whether these variations impact odor stimulated behaviors across surface and cave populations of *A. mexicanus.* First, we show that surface fish exhibit similar odor perception valences (negative and positive) to zebrafish. Second, we demonstrate that light influences cavefish olfactory behavior, with darkness revealing light masked changes in odor

perception. Third, we show that cavefish exhibit positive bout poses and approach behavior to odors associated with fish injury and death, suggesting a change in odor perception coding. Fourth, cavefish females and not males exhibit positive associations with social odors, suggesting that social olfaction is sexually dimorphic in cavefish. Fifth, we utilize surface to cave F_2 hybrids to show that social, alarm and death related odor perception is heritable, which provides a functional model to investigate the genetic architecture that underlies vertebrate odor perception. Finally, we employed tissue clearing and brain mapping to show that cavefish exhibit overlap between brain activity in the thalamus and preoptic region when exposed to food and death odors. Overall, this study reveals changes in cavefish odor perception that may underlie an improved foraging strategy in an environment with variable resources.

RESULTS

Surface fish approach food and social odors, but avoid odors of injury and death

The olfactory system has evolved to discriminate between different categories of odorants, including food, social, predatory and death-associated odors (Figure 1b)[12, 29-33]. To determine how odor perception has evolved in cavefish, we developed an odor discrimination assay that exposes adult fish to different odor classes dissolved in 0.5% DMSO water (Figure 1b, Table 1). DMSO was leveraged as an anchor to maintain odor position and a biological carrier to amplify the smell (Supplementary Figure S1)[34{Maes, 2012 #103]}. Behavior tanks were then split into an odor zone and a no-odor zone and odor perception was then analyzed by converting the duration of zone times into an odor index, (OR= $\frac{[zone\ 2 - zone\ 1]}{zone\ 2 + zone\ 1}$), that ranged from +1.0 to -1.0 (Figure 1b, Methods Section ‘approach and avoidance analysis’). To validate this approach, we first exposed surface fish to water shams and food-soaked water, and analyzed odor indices in 1-minute intervals (Figure 1c&d). Using this methodology, we found that surface fish exhibit no change in behavior when exposed to a water sham and exhibit positive odor indices during the first minute of exposure to food extract (Figure 1c, Supplemental Table S1-3). Overall, a 1-minute time bin produced positive values for the smell of worms (food) and bile (social) odors, and negative values for alarm (skin) and death-associated odors (decay) (Figure 1d, Supplemental Table S4-5). To better contextualize the behaviors underlying these indices, we analyzed bout movements and constructed subject and population ethograms for each odor (Figure 1e&f, Table 2). We found that surface fish exhibited swim bouts we term ‘positive’, including quick circles and biting behavior when exposed to food odors (Figure 1e-g, Supplemental Figure S2, Supplementary Videos S1-2), and a few instances of dance-like shimmy’s when exposed to social behaviors (Figure 1f&g, Supplemental Figure S2, Supplementary Videos S3). In contrast, we found that alarm and death associated odors elicited bouts we termed ‘negative’, included freezing, darting, spinning and corner digging (Figure 1f&g, Supplementary Figure S2, Supplementary Videos S4-7). These results clearly demonstrate that surface fish maintain ancestrally conserved approach behaviors via positive bouts (e.g. circling and biting; Figure 1e) and avoidance behaviors via negative bouts (e.g. freezing and digging; Figure 1e).

Cavefish exhibit positive odor indices when exposed to skin extract in the dark

Work in alarm behavior has identified several molecules released from fish skin that triggers anxiolytic responses [29, 31-33]. Previous research has shown that cave morphs of *A. mexicanus* do not perform an alarm response when exposed to skin extract from either surface or cave morphs [35]. However, those experiments did not test odor responses in different lighting conditions and previous research suggests that cavefish behaviors are influenced by the presence of light [36, 37]. To test whether cavefish react to alarm substances, we exposed surface fish and Pachon, Molino and Tinaja populations of cavefish to skin extract in three different light conditions: direct diffused white light, indirect white light and diffused infrared light (IR) (Figure 2). As previously reported, cavefish showed no change in behavior when exposed to skin extract in direct diffused white light (Figure 2b, Supplementary Table S6&7). In contrast, when we measured the odor index of cavefish in indirect low light and the dark, we found odor indices became positive in low light and robustly positive in complete darkness (Figure 2e&h, Supplementary Table S8-10), suggesting that light exposure greatly influences reactivity to odor cues. At a higher level of behavioral resolution, surface fish exhibited a decrease in immobility when going from light to dark (less freezing) and increased darting time, while cavefish exhibited an increase in odor zone circling, which shifted odor indices more positive from light conditions to dark (Figure 2c,f&I, Supplementary Table S11-13). Overall, these experiments demonstrate that cavefish odor perception is impacted by light and that cavefish exhibit approach behaviors when exposed to skin extract in darkness.

Cavefish maintain ancestral odor perceptions to avoid caustic odorants and approach food odorants

To test odor perception more broadly, we performed the same odor index assay using several food, social, alarm and death-associated odors that elicit ancestrally derived reactions in other fish taxa [12, 29-33]. First, we tested water shams as a neutral odor and found that neither surface or cavefish populations reacted to a small jet of water (Figure 3a&b, Supplemental Figure S3, Supplemental Table S14). Next, we tested a well-studied negative odor that activates nociceptors in fish, mustard oil (allyl isothiocyanate), and found that like zebrafish, both surface fish and cavefish exhibited negative odor indices (Figure 3a&b, yellow label; Supplemental Figure S3, Supplemental Table S15). After establishing a negative odor, we tested three odors derived from food sources, shrimp[38], worms[39] and bat guano[40], that are naturally found in Mexican caves and rivers. Again, both surface fish and cavefish exhibited positive odor indices for all food odors tested (Figure 3a, green labels; Supplementary Table S4, Supplemental Table S16-19). These experiments show that cavefish maintain an ancestral odor perception of aversion to painful odors and approach movements towards food odors.

Only female cavefish exhibit approach behaviors when exposed to common social odors

Previous work in *A. mexicanus* suggests that blind cavefish lost most social behaviors, including shoaling and schooling, and are considered “asocial” [41-44]. Thus, we wondered whether an asocial organism would respond to common social odorants. To answer whether cavefish maintain ancestral social odor perception, we tested our fish using urea, ammonia, bile, and prostaglandin $F_{2\alpha}$ that have been shown to elicit kin recognition and breeding in other fish [29-31]. We found that surface fish exhibit positive odor indices for ammonia, bile and urea, which suggests these odors are ancestrally positive (Figure 3a-c, purple label; Supplementary Figure S5, Supplemental Table S20-27). Conversely, we found that cavefish exhibited no variation in behavior when exposed to social odors, suggesting a loss of social odor perception (Figure 3a-c, purple labels; Supplementary Figure S5, Supplemental Table S20-26). In addition, we found that prostaglandin $F_{2\alpha}$ produced no change in behavior across all populations (Supplementary Figure S5, Supplemental Table S26). However, when we examined populations by sex, we found that cavefish exhibit sexual dimorphism, with females exhibiting positive odor indices and males showing neutral indices (Supplementary Figure S6, Supplemental Table S27&28). These experiments suggest that social odor perception has evolved in cavefish to be sexually dimorphic, with only females exhibiting positive odor indices in response to social odors.

Cavefish exhibit approach behaviors when exposed to alarm and death associated odors

Finally, alarm and death-associated odors elicit strong alarm responses in zebrafish [11, 12, 28, 29, 31-33, 35]. For instance, zebrafish exhibit a range of behaviors when exposed to these odors, including extreme stimuli avoidance towards decay odorants, such as putrescine and cadaverine. Similarly, we found that surface fish on average exhibited negative odor indices when exposed to alarm substances, such as spermine and commensal skin derived bacteria *Staphylococcus saprophyticus*, and death odors, such as putrescine and cadaverine (Figure 3a&b, red labels; Supplementary Figure S7, Supplemental Table S29-37). However, ethograms provide a more complex picture, with surface fish showing a range of erratic bottom dwelling, freezing and corner digging (Figure 3e&f, red labels). Conversely, cavefish exhibited neutral to positive indices, that are characterized primarily by circling behavior (Figure 3e, red labels; Supplemental Table S29-37). Furthermore, all cavefish populations exhibited positive odor indices and increased positive bout types across all death associated odors, including an increase in circling and biting behavior (Figure3e&f; Supplementary Figure S8, Supplemental Table S38-45). Taken together, these results suggest that cavefish exhibit approach behavior with positive behavioral bouts when exposed to environmentally derived alarm and death-associated odors.

The range of surface to cavefish F_2 hybrid odor indices suggests that social, alarm and death odor perceptions are genetically inherited

Our odor perception results suggest that cavefish have evolved novel odorant perceptions in response to social, alarm and decay associated odorants. Our next

question was whether these neurobehavioral changes are genetically encoded. Although many cave populations are physically separated from surface dwelling populations, surface and cave adults can be bred to produce fertile hybrid offspring [21, 45]. Hybridization provides a functional genetic tool to determine the heritability of a trait and facilitate the mapping of genetic variants to biological traits. To test whether variation in odor perception is genetically encoded in cavefish, we crossed surface fish to Tinaja cavefish and backcrossed the offspring to test surface to cave F_2 hybrids for odor perception behavior (Figure 4a). We tested each individual F_2 hybrid for social (ammonia), alarm (skin extract) and death (decay extract) odor perception and then assessed correlations with biological features, such as eyes, sex and behavior-to-behavior relationships (Figure 4b-i). First, we found that each odorant class elicited a range of odor indices across the hybrid population, suggesting that these behaviors are genetically encoded (Figure 4b, Supplemental Table S46). Next, while we did not find any associations between anatomical features and behavior (Figure 4c&d, Supplemental Table S47-53), we did discover that hybrid females exhibited positive odor indices when exposed to social odors, but did not find sexual dimorphism when exposed to skin or decay odors (Figure 4e&f, Supplemental Table S54-56). Finally, we found that hybrid populations exhibit expected behavior-to-behavior correlations, such as a negative association between social and alarm behaviors, and a strong positive association between alarm and death associated behaviors (Figure 4g-i, Supplemental Tables S57-59). The observed hybrid odor perception range and associations between behaviors suggests that odor perception differences in surface fish and cavefish are genetically encoded and that similar brain regions may encode variation across similar odor subtypes.

Brain activity mapping finds overlap in activity of the thalamus and preoptic region of food and decay odor exposed cavefish

While cavefish exhibit positive odor indices and swimming bouts when exposed to a range of odor classes, we wondered whether these different classes of odors elicit similarities in neural activity across the forebrain. The protein total-ERK (t-ERK) becomes phosphorylated (p-ERK) following an action potential and p-ERK expression in active neurons peaks within 3 minutes of firing [46]. Therefore, this phosphorylation event provides a minute-scale biomarker for neural activity that is perfect for mapping responses to sensory stimuli. To map odorant stimulated neural activity, we first exposed individual Pachon cavefish to one odorant for 10 minutes before whole-brain protein staining and tissue clearing (Figure 5a). Then, to analyze across samples, we generated an autofluorescence template and reference brain to register all brain images into the same 3D space (Figure 5a-c). Finally, we used conspicuous features of the tERK expression pattern (e.g. structural) and food exposed p-ERK expression patterns (e.g. physiological) to segment identifiable subregions that are likely to correlate with odor attraction and appetite (Figure 5c&d). We therefore focused our analysis on subregions of the dorsal pallium (amygdala-like), thalamus and preoptic region (Figure 5d). We found that food exposed, and decay exposed fish exhibited similar increases in p-ERK levels of the thalamus and preoptic region, while skin and decay exposed fish showed similar increases of p-ERK levels of the dorsal pallium (Figure 5e&f,

Supplemental Tables S60-60-67). Of these four regions, the thalamus exhibited the largest numerical and most visibly discernable variation across conditions, with bilateral neuropil regions exhibiting the same increase in pERK across food exposed and death exposed odorants (Figure 5e; flanked by yellow asterisks). While our sample sizes are low, these activity patterns suggest that decay and food scents elicit similar signatures within regions known to contribute to appetite and odor perception.

DISCUSSION

Odor perception and odor induced behaviors allow organisms to survive by pursuing food and mates, while avoiding predation and disease. The odor perception of most organisms is conserved across ecological niches, with positive valences and approach toward food and social odors, and negative valences and avoidance towards threats of predation and death [7, 8, 47, 48]. Our odor perception screen suggests that cavefish olfactory behavior has changed from avoidant and negative to approach and positive in relation to signals of predation and death. The frequency and magnitude of these changes likely reflect the change in evolutionary pressures experienced in cave pool environments, such as a reduction in nutritional availability and decreased predation. This initial behavioral study provides a new neurogenetic model for uncovering the genes and neurons that functionally control odor perception in vertebrates.

Approaching dead and dying fish may improve resource allocation in a food variable environment

This study motivates a straightforward question: why do cavefish exhibit approach behaviors when exposed to odor sources that could be dangerous or deadly? We suggest that strong selective pressures via reduced predation or lack of resources caused a change in odor perception from odor avoidance to odor approach. Indeed, we know that cavefish have been found to be opportunistic omnivores, with gut contents ranging from crustaceans to other cavefish, and some mostly consisting of bat guano [40, 49]. For instance, cave fry from the Pachon cavefish feed mostly on crustaceans, while adult guts contain mostly detritus [49, 50]. However, we do not have enough information about the seasonality and abundance of these food sources across different cave localities. Therefore, a rewiring of odor perception for ancestrally negative odors would provide a new foraging strategy for acquiring any possible food source. Indeed, most carrion obligate avians use decay odors to find carcasses, but omnivorous predators may also scavenge using the same odors when resources are dire [51, 52]. Furthermore, our finding that cavefish exhibit more positive behavior towards odors of decay versus alarm associated odors, may suggest that an injured but reactive fish is less attractive than a lifeless decaying fish, which would not present an immediate threat (albeit a possible immunological risk by spreading disease [53]).

De novo mutations or genetic assimilation may underlie the switch from avoidance to approach for alarm and death odors

Our results also present a fundamental question of evolution: how does a new phenotype come to exist? Work across many taxa suggests that phenotypes can arise due to both *de novo* genetic mutations or plasticity, when environmental factors favor phenotypes from existing or ancestral alleles [54]. For instance, cavefish researchers have previously shown that cave-like phenotypes can be induced by manipulating phenotypic buffering. In this example researchers inhibited heat shock protein-90 (hsp-90) and then compared regressive traits such as eye size between wildtype and hsp-90 inhibited surface fish [55]. Inhibition of hsp-90 buffering caused wide variation in surface fish eye size development that was then tested for transgenerational inheritance by backcrossing surface fish with small eyes. This set of experiments revealed a phenotypic buffering paradigm that masked ancestral alleles for smaller eyes and proved that those traits could be inherited. More recently phenotypic plasticity underlying behavior was investigated in closely related genera of spadefoot tadpoles to determine what role genes and environment played in the emergence of cannibalism as a phenotype. In that study, researchers compared cannibalistic versus non-cannibalistic genera and found that changes in the environment, space and resource allocation, produced the emergence of cannibalism in the non-cannibalistic genera. Additionally, they found that genetic variation played a role in the emergence of cannibalistic behavior, wherein ancestral alleles provide phenotypic plasticity for reducing competition in a crowded environment [56]. In contrast, *De novo* mutations in several cavefish populations have been shown to influence novel phenotypes, such as eye and pigment loss [57, 58]. In regards to our study, selective pressures in the cave environment, such as availability of food, could select upon *de novo* genetic variants that contribute to a change in odor perception. [54]. In continuing work, we will test these hypotheses by (1) performing a QTL analysis to identify genetic contributions and determine whether they are ancestral or *de novo* alleles, and (2) by manipulating environmental conditions in surface fish and performing odor perception assays to identify any ancestral capacity for odor perception flexibility. Finally, convergent evolution of odor perception across several cave populations provides an ideal model for identifying genetic causes, possibly through *de novo* mutations in some populations and plasticity of ancestral alleles in others.

Sexual dimorphism in relation to social cues suggests a need for females to find males

Social cues can provide fitness advantages for individuals within a population through social coherence and mating [59]. Surprisingly we found that only female cavefish exhibit positive odor indices when exposed to social odors that are positive for both male and female surface fish. Previous fish studies have described sexual dimorphism in relation to olfactory ability, including other Characids [60], and variation in olfactory organs anatomy and physiology, such as in deep-sea fish [61, 62]. Larger nostrils, olfactory lamellae and enhanced physiology was associated with males of these species, suggesting that males needed larger and more sensitive olfactory organs for finding female mates in sparsely populated areas of the deep ocean [62]. Therefore, cavefish females may have retained approach to social cues to find males for mating in cave habitats. Indeed, we found convergence across cave populations and a strong

social odor approach phenotype in F_2 hybrid females, suggesting that this trait could be sex-linked via sex chromosomes.

Hybrid range of odor indices suggests that cavefish odor perception is a convergent cave adapted trait that arises from standing genetic variation

All three cave adapted populations of *A. mexicanus* tested here exhibited the same changes in odor perception, including similar bout types and bout frequency, when exposed to our four death-associated odors. The range of behaviors and bout types for both surface and cave fish was then recapitulated across the F_2 hybrid population. These experiments suggest that changes in odor perception are genetically encoded, which could include genes impacting the nervous system [63, 64], immune system or metabolism [3, 65, 66]. For instance, research on scavengers suggests that behavioral evolution towards searching for carrion includes expansions of olfactory receptors, or expression/coding of digestive enzymes and immunological pathway proteins [51, 52, 65]. While we have yet to perform a QTL mapping experiment, previous cavefish trait associations have found that scavenger linked genes, such as the immune cell regulator *bcl6a* and the cell adhesion molecule *epcam*, have been found to be under selection in different lineages of cavefish [67]. Our future studies will utilize QTL mapping to identify genes of interest for better understanding the genetic mechanisms driving the evolution of olfactory perception in cavefish.

Food and decay derived odors elicit similar brain activity signatures that suggest may underlie similarity in behavioral repertoires

Our adult forebrain activity mapping found that brains exposed to food and decay extract had similar activity profiles in the thalamus and preoptic region. This is especially true for large bilateral neuropil regions of the thalamus (Figure 5c; starred panel), where we did not observe large increases in activity when cavefish were exposed to DMSO or skin extract. Previous work in humans and rodents have identified the mediodorsal thalamic nucleus (MDT) as a sensory integrator that plays important roles in odor discrimination and perception [68, 69]. For instance, ischemic damage to the medial-dorsal thalamus can influence perception, resulting in parosmia like deficits in which the pleasant smell of food becomes nauseating [70, 71]. Similarly, researchers have shown that the MDT plays an important role in discriminating different odor mixtures, with deactivation of the region causing animals to reduce consumption for preferred odor-taste mixtures [72]. In addition, we also see similar activity patterns in subregions of the preoptic region, which may represent a novel role for odor perception outside of the preoptic regions well known role for processing social odor cues [73, 74]. While our dataset is small and spatially biased, the overlap of activity between food and decay exposed brains suggests that future work will allow us to map olfactory perception to specific regions and cellular subtypes.

In conclusion, this study describes a convergent change in cavefish odor perception that causes cavefish to respond positively to ancestrally negative odor cues. These

behaviors were shown to be convergent across all cave populations tested, hybrid experiments suggest these behaviors are genetically encoded and neurological correlates suggest multiple brain regions likely drive perception differences based on odorant class. Overall, our work provides a novel model for investigating the evolution of complex phenotypes and the mechanisms driving odor perception in vertebrates.

METHODS

Animal care and husbandry

All populations are kept in housing tanks in the *A. mexicanus* fish facility located in the Department of Biological Sciences at St. Johns University. *Astyanax mexicanus* stocks are maintained and cared for according to the St. John's University IACUC approved protocol #2073. Adult fish are maintained on a recirculating z-hub system (Pentair Inc., Minneapolis, MN, USA) and custom-built recirculating racks with the following parameters; temperature 22-23 °C, conductivity 200-900 µS and pH 6.8-7.8. Fish are maintained on a 14:10 ZT light:dark schedule. Fish stocks include wildtype: Surface fish Rio Choy, Pachon cavefish, Molino cavefish, Tinaja cavefish and hybrids: Surface/Tinaja F_1 and F_2 hybrids.

Odorant preparations

Commercially purchased odors were measured and dissolved in system water with 0.5% DMSO (Table 1). DMSO was added to each solution as a molecular weight for delaying odorant dispersal across odor zones (Supplementary Figure S5). All solutions were dispensed as 50 µL aliquots into 9-L behavioral tanks.

Bloodworm water was created by soaking 1 gram of frozen bloodworms in 25 mL of system water at 4 °C overnight. This solution was then centrifuge and the aqueous phased extracted as the odorant.

Shrimp water was derived by soaking 1 mL of concentrated artemia shrimp in 25 mL of system water at 4 °C overnight. This solution was then centrifuge and the aqueous phased extracted as the odorant.

Bat guano water was made by soaking 1 gram of bat guano fertilizer in 1 mL of system water and heated at 95 °C for 1 hour. This solution was then centrifuge and the aqueous phased extracted as the odorant.

Skin extract was prepared by euthanizing a surface fish or cavefish in 500mg/L Tricaine-S (MS222), scoring the skin with a scalpel and then rocking the scored fish in 40 mL of system water for two hours. Immediately following, skin extract liquid was then aliquoted into 1.5 mL tubes and heated to 95 °C for 30 minutes before being stored at -80 °C for up to 1 month. Skin extract was then diluted 1:10 in 0.5% DMSO and system water as a working stock.

Decay extract was derived using skin extract fish by adding 40 mL of system water and rocking overnight at room temperature. This decay extract water was treated the same as the skin extract (heated and spun) and stored at -80 degrees for up to 1 month.

Bacteria odor was derived by preparing commensal strain *Staphylococcus saprophyticus* ATCC 15305 extracts grown in BH infusion medium (Brain Heart Infusion Broth media BD237500; BD, Franklin Lakes, NJ). This protocol was adapted from a previous odor study in zebrafish [32]. 0.5 mL of an overnight pre-culture were used to inoculate 50 mL of fresh media. After a 3-hour incubation (30°C, 220 rpm; New Brunswick orbital shaker; Eppendorf, Hamburg, Germany) cells were harvested by centrifugation (3000g; Eppendorf 5804R centrifuge, Hamburg, Germany) and the pellets stored at -80 degrees for up to 1 month. To prepare the extract, one pellet corresponding to 25 ml of original culture was resuspended in 2.5 mL of 0.7% NaCl, and then washed twice with 2.5 ml of H_2O. The resulting resuspension was incubated for 30 minutes at 90°C in a water bath to lyse bacteria. Cell debris was removed by centrifugation while the supernatant was recovered as the odorant preparation.

Video capture and analysis

Behavior was captured using an acA1300-200um c-mount camera (Basler, inc., Exton, PA, USA), with a 16mm C Series Lens VIS-NIR (Edmund Optics, Barrington, NJ, USA) via the Pylon software (Basler, Inc.) at a frame rate of 25 fps. Each video was then initially analyzed using the Ethovision XT-14 software (Noldus, Inc, Leesburg, VA, USA). Ethovision tracking was performed using the dynamic subtraction setting set to subject lighter and darker. Tracks were then time binned and zone time cumulative distance was exported to derive the odor indices.

Olfactory behavioral experiments

Fish were transferred from the fish facility to the animal behavior room in groups of eight. They were then transferred individually to 9-liter behavioral tanks (Iwaki, Inc.; Holliston, MA, USA) and acclimated to the behavior room overnight. Tanks were then illuminated by either direct diffused light (200-220 lux), indirect white light (<0.1 lux) or direct diffused infrared light (180-240 IR lux). Experimenters used custom made cellphone night vision goggles for direct diffused infrared light experiments in the dark. DIY night-vision goggles consisted of an infrared camera (FLIR ONE Generation 3 Thermal imaging camera for Android, Teledyne, Wilsonville, OR, USA) and modified Google Cardboard (Fannego, Amazon, Inc., Seattle, WA, USA) affixed to laboratory goggles. Each odorant trial included two 5-minute videos, a pre-stimulus control video (no_stimulus) and an odor exposed video (e.g. bloodworm). To test olfactory perception, 50 µL of odorant was added to a randomized size of the tank and behavior was captured for 5 minutes. In-between odor trials, fish were removed from behavior tanks and kept in 2.5-liter zebrafish tanks while experimenters cleaned each behavior tank with 70% ethanol and hot water. Fish were then placed back in behavior tanks and given 2-hour to acclimate before subsequent odor trials. Fish were subject to no more than 2 odors per day, to reduce stress and olfactory overstimulation. Following experiments, adults were transferred back to the fish facility and returned to housing tanks.

Approach and avoidance analysis

Behavioral videos were analyzed using the Ethovision XT-16 software (Noldus, Inc., MA, USA). Ethovision tracking was performed using the dynamic subtraction setting set to subject darker, and cumulative time in zone 1 (no odor) and zone 2 (odor) were used to calculate odor indices.

Odor index = \frac{{$\Delta dt\{\backslash text\{zone_2\} - \backslash text\{zone_1\}\}$ ÷ {$\Delta dt\{\backslash$

$text\{zone_2\} + \backslash text\{zone_1\}\}$

Initially, behavioral videos for surface fish were binned at 1-minute intervals to determine peak approach for bloodworm water (significant increase in odor indices). The peak of 1-minute was then used to bin and analyze all further odorants tested in surface fish and cavefish. Numbers that are positive indicate approach (more time in zone 2; odor zone), while negative numbers indicate avoidance (more time in zone 1; no odor zone). In addition, swimming velocities were automatically calculated using the EthoVision XT software, while fish bouts and poses were called and tallied by trained investigators Ethograms were then constructed using custom python scripts and exported as pdfs.

Surface to Tinaja cavefish F_2 hybrid olfactory behavior and correlations

Individual Surface to Tinaja F_2 hybrid fish were assayed for ammonium chloride, skin extract and decay odor. Fish were behaviorally assayed as previously described and then kept in a post-assayed tank while the remaining individuals were assayed. Following our behavioral experiments, fish were anesthetized using 500mg/L of buffered Tricaine-S in system water, photographed for anatomical features, including presence and absence of eyes, eye size, presence and absence of pigment, standard length and sex (anal fin spine test and later confirmed with dissection), before fin clipping to preserve DNA samples for future studies. Finally, eye, pigment and sex comparisons were made in a group-wise fashion using odor indices, while a simple linear regression and R-squared values were used to analyze the behavioral relationship between classes of odorants. All data analysis and statistical tests were run using PRISM (GraphPad, Inc.)

Adult brain dissection, staining and tissue clearing

Pachon cavefish adults (2.6-2.9 cm SL) were added individually to 2.5 liter zebrafish with false bottoms. Fish were then transferred to the behavioral room and left to acclimate for an hour in indirect white lighting. 50 µL of odorant was then pipetted into the tank and fish were exposed to that odor for 10 minutes. Fish were then euthanized by rapid cooling in ice water for 15 minutes, followed by immediate decapitation. Heads were then fixed whole in 1 M NaOH buffered 4% PFA overnight, washed and then dehydrated in 100% MeOH. After 24 hours at -20 °C, fish brains were dissected from

the head and rehydrated with 20% steps of MeOH/PBS before being permeabilized in 1xPBS/0.3%TritonX-100/20%DMSO for 2-days at 37°C with rocking. Brains were then incubated in blocking solution (1xPBS/0.3% TritonX-100/10% BSA/10% DMSO) for 2-days, primary antibody solution (1xPBS/0.3% TritonX-100/5% BSA/10% DMSO/ 1:250 antibody) for 3-days and secondary antibody solution (1xPBS/0.3% TritonX-100/5% BSA/10% DMSO/ 1:500 antibody) for 3-days. Stained brains were then cleared using the CytoVista™ Tissue Clearing Kit (Thermo Scientific, Waltham, MA, USA).

Adult brain imaging

Stained brains were mounted to a metal stage using UV light curing resin and imaged using the Miltenyi UltraMicroscope Blaze light sheet microscope (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, GER). Image stacks were then captured using the 5x dipping objective at 2x zoom with a resolution of 0.9x0.9x2 microns. Following image capture, brain stacks were down sampled to 7.2x7.2x2 microns and hemibrains were stitched using a custom Fiji script.

Adult brain template generation

All template construction, brain registrations and segmentation was done using Advanced Normalized Tools (ANTs) which utilizes the snap imaging tool kit (ITK) library [75]. Standard brain templates for autofluorescence was generated by applying the antsTemplateConstruction2.sh script to all whole adult brain imaging stacks using minimal user input (see custom ANTs scripts) [76]. We then used our initial template target brain as the t-ERK reference image brain to create our analysis atlas by segmenting regions that were visually identifiable by either structural (t-ERK) or physiological (p-ERK) signals in food exposed brains. In addition, updated versions of the adult zebrafish atlas (Wullimann and Mueller, 2016[77]; Kenney et al. 2021*[78]*) were used to identify each region and subregion. Segmentation was performed using the segmentation editor plugin in Fiji (ImageJ, [79]). Each brain was then registered via autofluorescence channels and inverse registered to segment the forebrain for p-ERK expression analysis. For p-ERK expression analysis, each unregistered p-ERK was normalized by dividing p-ERK signal by t-ERK signal using the image calculator function in Fiji. Normalized images were then merged with brain segmentations, before measuring average intensity for each regional segment. Normalized intensity each segment volume was then calculated by importing the normalized p-ERK and inverse registered segmentation stacks for each individual brain into Napari as separate layers [80]. Average intensity was then extracted by using the n-simpleITK (measurements) tool.

Statistical analyses

All statistical analyses were carried out using the statistical software PRISM (Graphpad, inc., USA). Individual odors were analyzed using non-parametric one-way ANOVA's, and when statistically significant, analyzed using a Tukey p-value adjusted multiple comparison test. Surface fish were treated as the control condition (i.e. ancestral

behavior) and compared to each individual cave population via the Tukey-corrected multiple comparison test.

Data Sharing

All statistical tables and custom codes can be found at olfaction_resources. All raw and transformed data will be made public upon publication.

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Figure 1. Surface fish exhibit approach to food and social odors, while avoiding alarm and death odors. **a.** Cavefish populations are found across the Sierra de El Abra mountain range of Northeast Mexico. **b.** Odors were categorized by behavioral associations in previous fish literature: food (teal), social (purple), alarm (orange) and death associated (chrimson) odors. **c**. Surface fish did not exhibit approach or avoidance to water control scent (n=23), but did show significant approach to the addition of water soaked in bloodworms (food scent; n=16). **d.** Surface fish exhibit positive approach to food and social odors (teal and purple; n=24 and n=20), while displaying negative avoidance to alarm and death odors (orange and crimson; n=27 and n=24). Representational (**e&f**) and population (**g**) ethograms for food (teal; bloodworm), social (purple; ammonia), alarm (orange; skin extract) and death (chrimson; decay extract) elicited swimming behaviors. Dotted lines denote neutral (swim, sw), negative (dart, da; dig, di; freeze, fr; spin, sp) and positive (bite, bi; circle, ci; shimmy, sh) swim bouts. Indices were compared using non-parametric ANOVA's, followed by Tukey corrected p-value multiple comparisons. p-values; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Figure 2. Light masks skin extract approach behaviors in cavefish. **a-c.** Direct white light via acrylic diffusion revealed no difference between populations when exposed to skin extract (n=9, all populations). **b.** Box plots reveal negative odor indices in surface fish and neutral odor indices in cavefish. **c.** Ethograms reveal darting (da) and freezing (fr) in surface fish, while cavefish mostly perform routine swims. **d-f** Indirect white light via low lux projections on a back wall revealed **(e)** positive odor indices in cavefish, **(f)** an increase in cavefish circling behavior and the emergence of head spinning (sp) and digging (di) in surface fish (sf&pa, n=15; mo&ti, n=12). **g-i.** Direct IR-light via acrylic diffusion resulted in **(h)** higher positive odor indices in cavefish and **(i)** revealed biting (bi) behaviors in cavefish (n=10, all populations). Indices were compared using non-parametric ANOVA's, followed by Tukey corrected p-value multiple comparisons. p-values; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Figure 3. Cavefish exhibit approach behaviors when exposed to ancestrally negative alarm and decay odors. **a.** Pairwise heatmap of odor index averages between odor and *A. mexicanus* population. Odor indices were encoded as positive (dark blue,1) to negative (dark red, -1) colors to help visualize attraction and repulsion to different odors (sample sizes in Table 3). **b.** Horizontal violin plots that exhibit positive (blue) and negative (red) odor index values for each population. Each row corresponds to the odor row and color values depicted in the heatmap. **c-f.** Representative and population ethograms for **(c&d)** social odors and **(e&f)** decay extract behaviors, where each row corresponds to a specific population. **g&h.** Quantification of **(g)** circling and **(h)** bite bouts in surface fish and cavefish populations exposed to water, bloodworms, ammonia, skin and decay extract. Odor Indices were compared using non-parametric ANOVA's, followed by Tukey corrected p-value multiple comparisons. p-values; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Figure 4. Surface to cave F_2 hybrid behavior suggests olfactory perception is genetically inherited. **a.** Surface to Tinaja F_2 hybrids were generated by crossing Surface to Tinaja F_1 siblings. **b.** Hybrid population behavior for social (purple), alarm (orange) and death (crimson) odors span the full approach to avoidance value range (n=29). **c-e.** Hybrid population comparisons binned by (**c**) eyes, (**d**) pigment and (**e**) sex (male = 17, female = 12). **f.** Representative ethograms exhibiting female circling and shimmy bouts that are indicative of mating. **g-i** Pearson correlations for (**g**) decay versus ammonia, (**h**) skin versus ammonia and (**i**) skin versus decay. Indices were compared using non-parametric ANOVA's, followed by Tukey corrected p-value multiple comparisons. p-values; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Figure 5. Brain mapping in the forebrain finds similar activity patterns in the cavefish thalamus and preoptic region when smelling food or decay extract. **a.** schematic of experimental pipeline through two weeks of dissection, staining, clearing and imaging **b.** example of adult brain registration where autofluorescence (auto subject) is used to register to our reference brain (auto ref). The final product is a reformatted brain (subject reg) in the reference space (auto ref/ subj reg). **c.** example of normalized pERK in the pallium of a skin exposed fish (arrow pointing to dorsal pallium, e.g. amygdala correlate). **d.** atlas analysis key showing the four subregions that were analyzed for activity mapping. Dm, medial pallium; PO, preoptic region; Th, thalamus. **e.** Optical sections displaying normalized p-ERK signal in Pachon cavefish exposed to DMSO (control p-ERK), bloodworm water (food p-ERK), skin extract (p-ERK) and decay extract (decay p-ERK). Each column of images is derived from a single fish for each condition, top anterior to bottom posterior positions. Flat arrows point to pallial and preoptic regions of activity, while yellow asterisks flank high p-ERK in the thalamus of food and decay exposed fish **f.** Box plots comparing regional p-ERK intensities of Pachon cavefish exposed to four conditions, control (DMSO), food (bloodworm water), skin (skin extract) and decay (decay extract). Indices were compared using non-parametric ANOVA's, followed by Tukey corrected p-value multiple comparisons. p-values; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Table 1. Odorant Table with .

Odorant	Odor	Ancestral Valence	Company	Cat #
Water	N/A	Neutral	N/A	N/A
Allyl isothiocyanate (mustard)	Pain	Negative	Sigma	W203408
Blood worm	Food	Positive	Bulk Reef	208881
Shrimp	Food	Positive	Bulk Reef	212246
Bat Guano	Food	Positive	Down-to-earth	7-3-1
Taurocholic acid (bile)	Social	Positive	Sigma	S0900000
Ammonium chloride (ammonia)	Social	Positive	Sigma	A9434
Prostaglandin $F_{2\alpha}$ (hormone)	Social	Positive	Sigma	P5069
Urea (urine)	Social	Positive	Sigma	U5378
Skin extract (alarm substance)	Alarm	Negative	N/A	N/A
Pyridine-n-oxide	Alarm	Negative	Sigma	131652
Chondroitin sulfate	Alarm	Negative	Sigma	1133570
Bacteria (*Staphylococcus saprophyticus*)	Alarm	Negative	ATCC	15305
Spermine	Alarm	Negative	Sigma	55513
Diaminohexane	Death	Negative	Sigma	613533
Decay extract (death substance)	Death	Negative	N/A	N/A
Cadaverine	Death	Negative	Sigma	D22606
Putrescine	Death	Negative	Sigma	51799

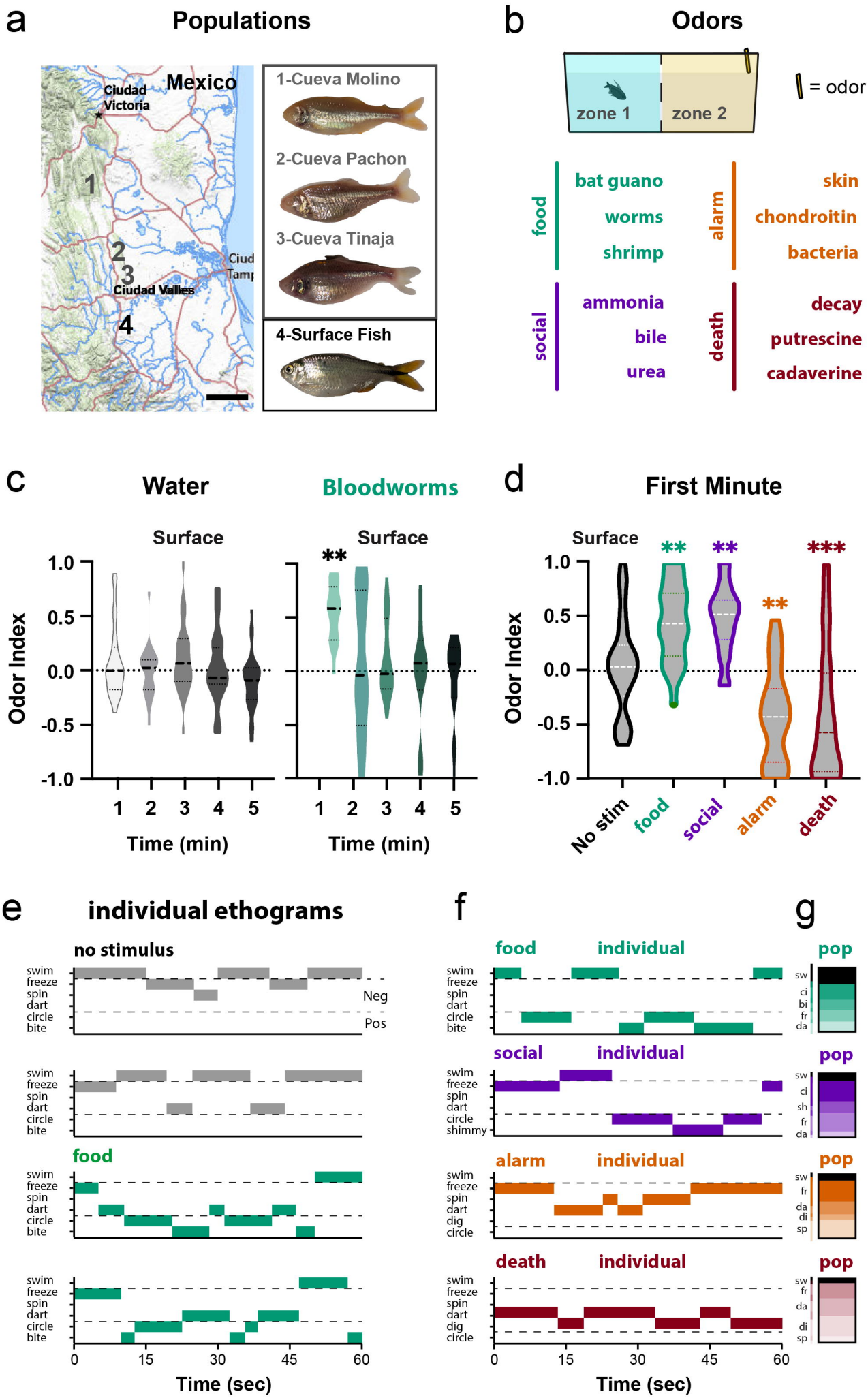
Table 2. Swim bout definitions (pictographic in Supplementary Figure S2)

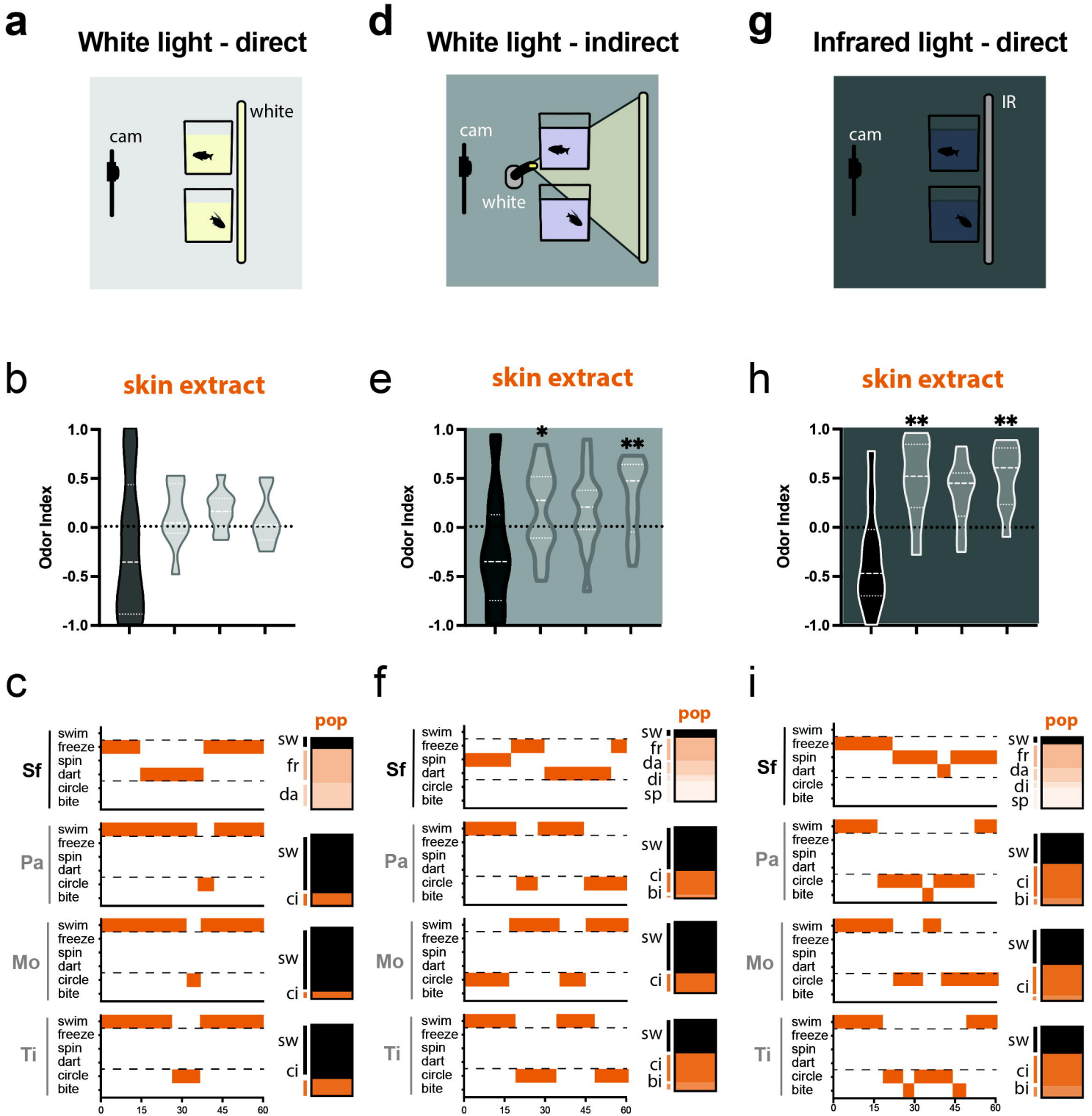
Bout Name	Definition	Tank Position	Surface Fish	Cavefish
Swim (Vid S1 &S2)	Slow swim across tank length.	column	present	present
Freeze (Vid S3)	Immobility for longer than 5 seconds.	bottom	present	absent
Spin (Vid S4)	Upside down spinning on nose, back and forth.	bottom	present	absent
Dart (Vid S5)	Fast swim sprints back and forth.	bottom	present	absent

Dig (Video S6)	Driving nose into tank edge.	bottom	present	absent
Circle (Video S7&8)	No midline cross, turns back towards current zone	column	present	present
Bite (Video S9&10)	Jaw movement towards tank bottom.	bottom (bottom or column, cave)	present	present
Shimmy (Video S11&12)	Slow vertical movement with body shaking	column	present	present

Table 3. Sample sizes for odor perception screen (see Figure 3)

Odor	**Surface**	**Pachon**	**Molino**	**Tinaja**
Control (DMSO)	46	45	52	53
Mustard Oil	4	4	4	4
Bloodworm	16	16	18	16
Shrimp	8	8	8	8
Bat Guano	8	8	8	8
Taurocholic Acid	10	10	10	10
Ammonium Chloride	8	8	8	8
Prostaglandin $F_{2\alpha}$	8	8	8	8
Urea	9	9	9	9
Skin Extract	19	19	16	16
Pyridine-n-oxide	10	10	10	10
Chondroitin Sulfate	8	8	8	8
Bacteria	8	8	8	8
Decay Extract	16	15	15	14
Spermine	10	10	10	10
Cadaverine	10	10	10	10
Putrescine	10	10	10	10
Diaminohexane	10	10	10	10


a
Populations
Mexico
Ciudad Victoria
1
2
3
4
Ciudad Valles
1-Cueva Molino
2-Cueva Pachon
3-Cueva Tinaja
4-Surface Fish
b
Odors
zone 1
zone 2
= odor
food
bat guano
worms
shrimp
alarm
skin
chondroitin
bacteria
social
ammonia
bile
urea
death
decay
putrescine
cadaverine
c
Water
Bloodworms
Surface
Surface
Odor Index
1.0
0.5
0.0
-0.5
-1.0
**
1 2 3 4 5
Time (min)
d
First Minute
Surface
**
**
**
***
No stim
food
social
alarm
death
e
individual ethograms
no stimulus
swim
freeze
spin
dart
circle
bite
Neg
Pos
food
0 15 30 45 60
Time (sec)
f
food
individual
social
individual
alarm
individual
death
individual
shimmy
dig
g
pop
sw
ci
bi
fr
da
sh
di
sp


a
White light - direct
cam
white
d
White light - indirect
cam
white
g
Infrared light - direct
cam
IR
b
skin extract
Odor Index
1.0
0.5
0.0
-0.5
-1.0
e
skin extract
*
**
h
skin extract
**
**
c
f
i
pop
Sf
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Mo
Ti
swim
freeze
spin
dart
circle
bite
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da
di
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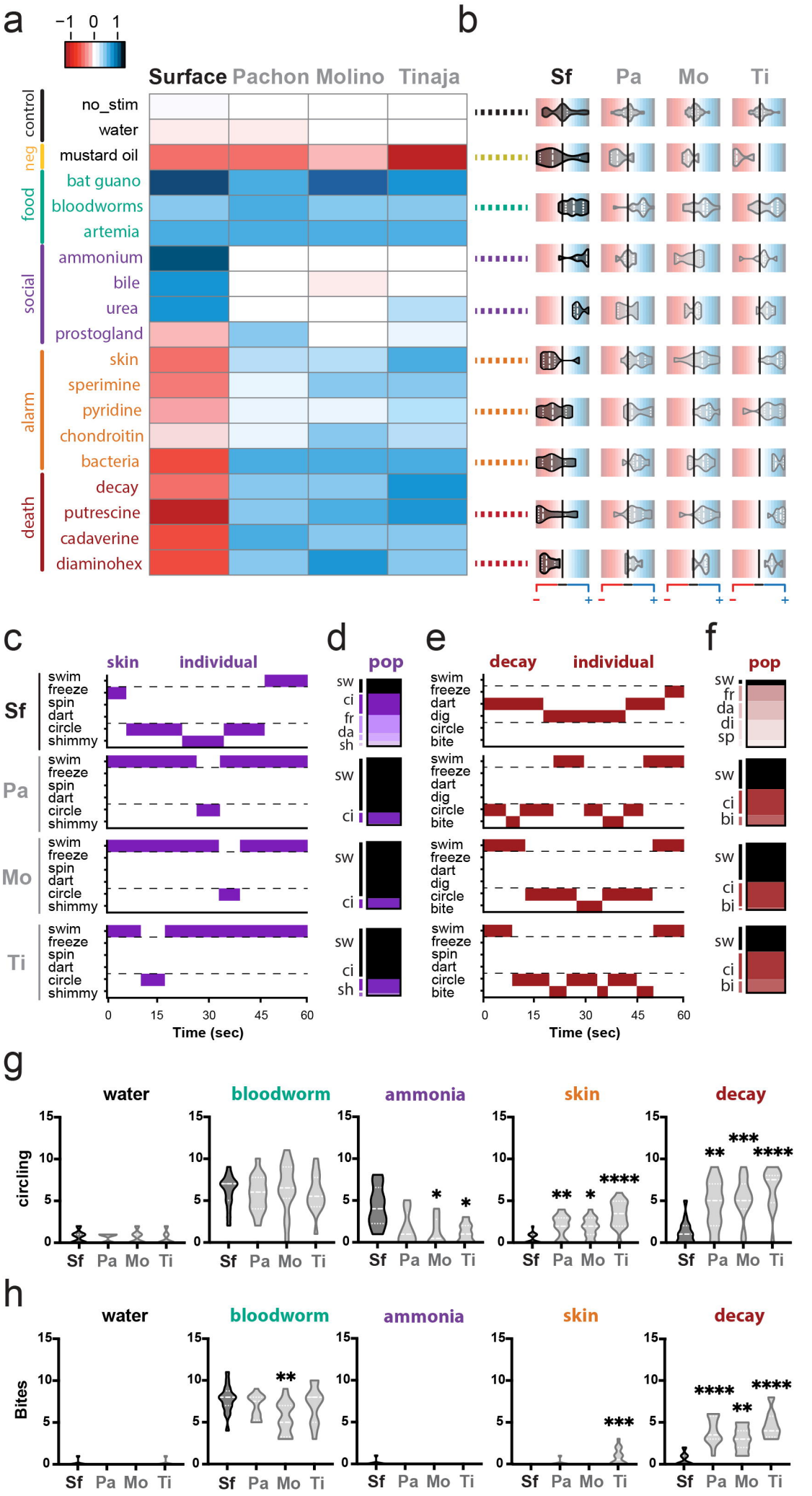
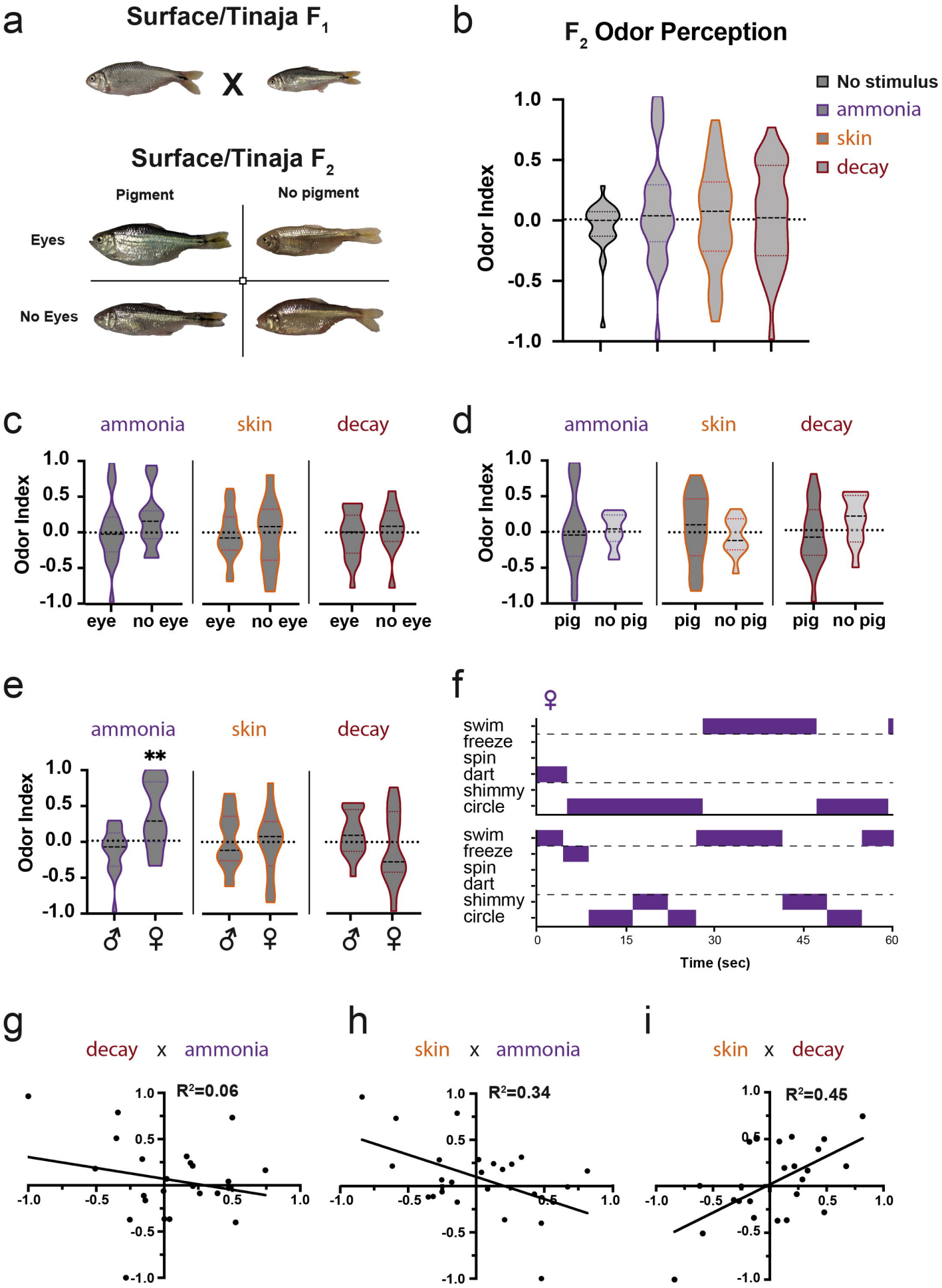
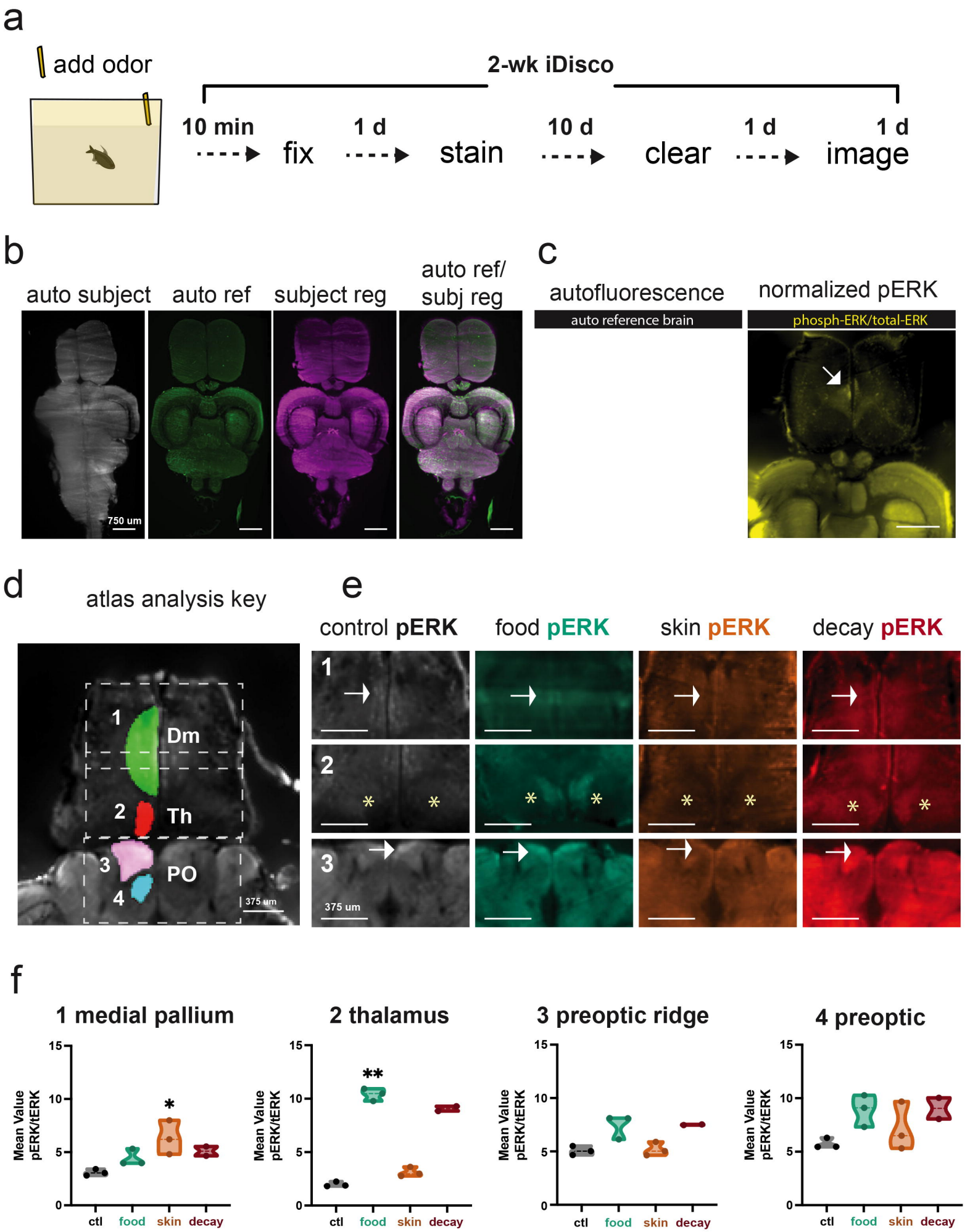
ci
bi
0
15
30
45
60


a
−1 0 1
Surface Pachon Molino Tinaja
control
no_stim
water
neg
mustard oil
food
bat guano
bloodworms
artemia
social
ammonium
bile
urea
prostogland
alarm
skin
sperimine
pyridine
chondroitin
bacteria
death
decay
putrescine
cadaverine
diaminohex
b
Sf Pa Mo Ti
c
skin
individual
Sf
Pa
Mo
Ti
swim
freeze
spin
dart
circle
shimmy
0 15 30 45 60
Time (sec)
d
pop
sw
ci
fr
da
sh
e
decay
individual
swim
freeze
dart
dig
circle
bite
0 15 30 45 60
Time (sec)
f
pop
sw
fr
da
di
sp
ci
bi
g
water
bloodworm
ammonia
skin
decay
circling
Sf Pa Mo Ti
h
water
bloodworm
ammonia
skin
decay
Bites
Sf Pa Mo Ti


a
Surface/Tinaja F1
x
Surface/Tinaja F2
Pigment
No pigment
Eyes
No Eyes
b
F2 Odor Perception
No stimulus
ammonia
skin
decay
Odor Index
1.0
0.5
0.0
-0.5
-1.0
c
ammonia
skin
decay
Odor Index
eye
no eye
d
ammonia
skin
decay
Odor Index
pig
no pig
e
ammonia
skin
decay
**
Odor Index
♂
♀
f
♀
swim
freeze
spin
dart
shimmy
circle
0
15
30
45
60
Time (sec)
g
decay x ammonia
R²=0.06
h
skin x ammonia
R²=0.34
i
skin x decay
R²=0.45


a
add odor
2-wk iDisco
10 min
fix
1 d
stain
10 d
clear
1 d
image
1 d
b
auto subject
auto ref
subject reg
auto ref/
subj reg
750 um
c
autofluorescence
normalized pERK
auto reference brain
phosph-ERK/total-ERK
d
atlas analysis key
1
Dm
2
Th
3
PO
4
375 um
e
control pERK
food pERK
skin pERK
decay pERK
1
2
3
375 um
f
1 medial pallium
2 thalamus
3 preoptic ridge
4 preoptic
Mean Value
pERK/tERK
15
10
5
0
*
**
ctl
food
skin
decay