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RESEARCH ARTICLE

Reproductive and metabolic state differences in olfactory responses to amino acids in a mouth brooding African cichlid fish

Alexandre A. Nikonov, Julie M. Butler, Karen E. Field, John Caprio and Karen P. Maruska*

ABSTRACT

Olfaction mediates many crucial life-history behaviors such as prey detection, predator avoidance, migration and reproduction. Olfactory function can also be modulated by an animal's internal physiological and metabolic states. While this is relatively well studied in mammals, little is known about how internal state impacts olfaction in fishes, the largest and most diverse group of vertebrates. Here we apply electro-olfactograms (EOGs) in the African cichlid fish *Astatotilapia burtoni* to test the hypothesis that olfactory responses to food-related cues (i.e. L-amino acids; alanine and arginine) vary with metabolic, social and reproductive state. Dominant males (reproductively active, reduced feeding) had greater EOG magnitudes in response to amino acids at the same tested concentration than subordinate males (reproductively suppressed, greater feeding and growth rates). Mouth brooding females, which are in a period of starvation while they brood fry in their mouths, had greater EOG magnitudes in response to amino acids at the same tested concentration than both recovering and gravid females that are feeding. Discriminant function analysis on EOG magnitudes also grouped the male (subordinate) and female (recovering, gravid) phenotypes with higher food intake together and distinguished them from brooding females and dominant males. The slope of the initial negative phase of the EOG also showed intra-sexual differences in both sexes. Our results demonstrate that the relationship between olfaction and metabolic state observed in other taxa is conserved to fishes. For the first time, we provide evidence for intra-sexual plasticity in the olfactory response to amino acids that is influenced by fish reproductive, social and metabolic state.

KEY WORDS: *Astatotilapia burtoni*, Electro-olfactogram, EOG, Feeding, Olfaction, Teleost

INTRODUCTION

Soluble chemical compounds in the aquatic environment provide relevant cues or signals to mediate crucial behaviors such as feeding, reproduction, predator avoidance and navigation/migration. These environmental stimuli are detected by the chemosensory systems of olfaction and taste (gustation), which represent phylogenetically old senses. The olfactory system of vertebrates responds when odorants bind to molecular olfactory receptors located on olfactory receptor neurons (ORNs) within the sensory portion of the olfactory epithelium. In fishes, the olfactory system plays a vital role in many life-history behaviors such as reproduction (Lastein et al., 2015), kin recognition (Hinz et al., 2013), parental care (Wisenden et al., 2014), larval settlement (Atema et al., 2002; Leis et al., 2011), homing and migration (Stabell, 1992; Li et al., 1995; Bett and Hinch, 2016), and prey and predator detection (Hara, 1993; Wisenden, 2000; McCormick and Manassa, 2008). Although much is known about how the olfactory system of fishes responds to different odorant classes (e.g. amino acids, bile salts, steroids, nucleotides, prostaglandins) (Caprio, 1978; Sorensen et al., 1988; Nikonov and Caprio, 2004, 2007; Cole and Stacey, 2006; Rolen and Caprio, 2007; Tricas et al., 2009; Meredith et al., 2012; Buchinger et al., 2014), little is known about how olfactory responses may be altered within a species that undergoes plasticity in olfactory-mediated behaviors, such as feeding and reproduction.

Amino acids are common components of natural prey, and feeding behaviors in fishes can be triggered by olfactory reception of even single amino acids (Lindstedt, 1971; Valentincic and Caprio, 1994a,b; Hara, 2006). In many taxa [e.g. *C. elegans* (Colbert and Bargmann, 1997), axolotls (Mousley et al., 2006), *Drosophila* (Root et al., 2011) and mammals (Julliard et al., 2007; Prud'homme et al., 2009)], feeding state and the resulting regulatory metabolic factors (e.g. hunger and satiety peptides, glucose, lipids, amino acids) can modulate olfactory responses such that fasting increases and satiety decreases olfactory responses. These changes in olfactory responses function to maintain nutritional homeostasis and can motivate hungry animals to seek food and fed animals to divert attention to other non-food related tasks. The mechanisms responsible for this plasticity are not well understood, but some evidence exists for the modulation of ORN responses within the olfactory epithelium itself. For example, in both rodents and the axolotl, neuropeptide Y (a feeding stimulant) increases the amplitude of the electro-olfactogram (EOG) response to food-related odorants, but only in fasted individuals (Mousley et al., 2006; Negroni et al., 2012; Palouzier-Paulignan et al., 2012). Thus, the response of the peripheral olfactory system is influenced by nutritional status to mediate olfactory-driven behaviors that vary throughout the life history of the animal.

Reproductive and hormonal status can also influence olfactory responses, but it is primarily examined in the context of changes in detection thresholds to reproductively relevant compounds (i.e. pheromones). In humans, for example, olfactory sensitivity to male social odors (e.g. androstadienone, androsterone) is higher in fertile compared with non-fertile women (Lundstrom et al., 2006; Renfro and Hoffmann, 2013), and olfactory perception in mammals can vary across the menstrual and estrous cycle (Kumar and Archunan, 1999; Derntl et al., 2013; Kanageswaran et al., 2016). In fishes, steroid hormones also influence olfactory responses to pheromonal compounds (Cardwell et al., 1995; Belanger et al., 2010; Ghosal and Sorensen, 2016). These studies suggest that changes in olfactory responsiveness can be odor type-dependent and may also be related to internal hormonal state. While gender differences in olfactory capabilities are evident from fishes to

Department of Biological Sciences, Louisiana State University, 202 Life Sciences Building, Baton Rouge, LA 70803, USA.

*Author for correspondence (kmaruska@lsu.edu)

K.P.M., 0000-0003-2425-872X

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mammals (Irvine and Sorensen, 1993; Michel and Lubomudrov, 1995; Brand and Millot, 2001), there is a paucity of information on olfactory plasticity within each sex. In addition to pheromones, such as steroids, bile acids and prostaglandins, some evidence exists in fishes that amino acids are used as social signals among conspecifics (Yambe et al., 2006; Ward et al., 2011; Kleinhappel et al., 2016; Kutsyna et al., 2016). However, little is known in any taxa about how olfactory responses to amino acids or food-related cues may vary with reproductive or social status.

The African cichlid fish *Astatotilapia burtoni* (Günther 1894) is a species well suited to examining the influence of metabolic and reproductive state on olfactory responses because both males and females cycle between states that differ dramatically in energy investment and reproductive capacity (reviewed in Fernald, 2002; Maruska and Fernald, 2014) (Fig. 1). How might the trade-offs between resource allocation and reproductive capacity in both male and female *A. burtoni* influence their olfactory responses to food-related odorants? While there are links between metabolic state, food intake and olfaction in many animals from insects to humans (Palouzier-Paulignan et al., 2012), this relationship has not yet been examined in fishes, the largest and most diverse group of vertebrates with over 30,000 species. The goal of the present study, therefore, was to test the hypothesis that olfactory responses to food-related cues (i.e. amino acids) vary with fish metabolic, social and reproductive state.

MATERIALS AND METHODS

Experimental animals

Adult laboratory-bred African cichlid fish *A. burtoni* were derived from a wild-caught population collected from Lake Tanganyika, Africa, in the 1970s (Fernald and Hirata, 1977). Fish were maintained in mixed-sex groups in flow-through aquaria under environmental conditions similar to those of Lake Tanganyika (pH 8.0, 28–30°C, 300–50 µS cm^{-1}, 12 h:12 h light:dark cycle, constant aeration). Fish were fed cichlid flakes daily (Aquadine, Healdsburg, CA, USA), and supplemented with frozen brine shrimp two to three times per week. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA.

The *in situ* EOG technique was used to test the olfactory responses of dominant (standard length, SL=54.1±4.3 mm s.d.; body mass, BM=3.9±1.2 g s.d.; gonadosomatic index, GSI=0.99±0.35 s.d.) and subordinate adult males (SL=45.3±5.5 mm;

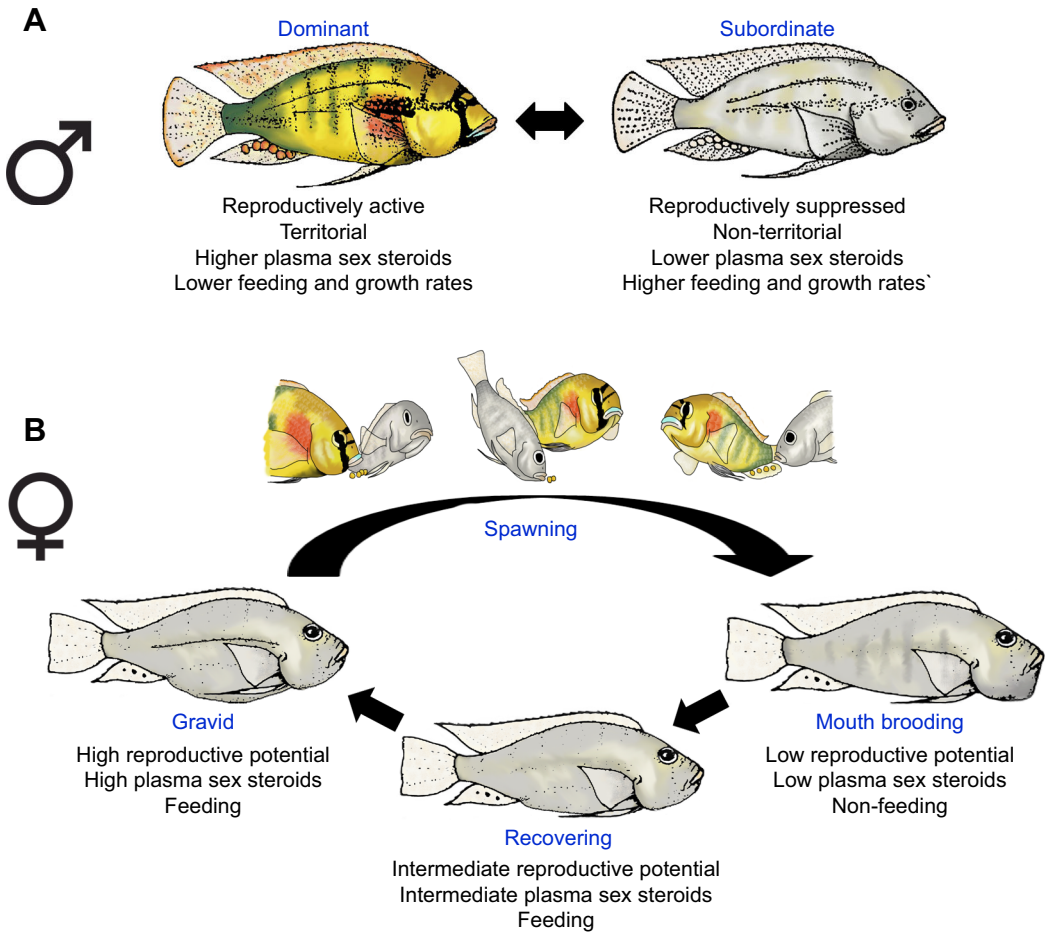

Fig. 1. Characteristics of male and female *Astatotilapia burtoni* phenotypes. (A) Males are either dominant and territory-holding or subordinate and non-territorial; these phenotypes differ in coloration, reproductive, hormonal and metabolic states. Males can also reversibly switch between these social states depending on the social environment. (B) Females also differ in reproductive, hormonal and metabolic states. Sexually receptive gravid females will spawn with dominant males and then carry the developing fry in their mouths for ~2 weeks (mouth brooding), which is a period of starvation, reduced ovarian growth and lower levels of circulating steroids. After releasing free-swimming fry, females undergo a recovery period characterized by ovarian recrudescence and increased feeding until they become gravid again and ready to spawn.

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BM=2.1±0.75 g; GSI=0.33±0.16), and gravid (SL=45.1±3.1 mm; BM=2.2±0.62 g; GSI=8.1±1.8), mouth brooding (SL=40.4±3.2 mm; BM=1.4±0.46 g; GSI=0.50±0.20) and recovering females (SL=44.3±3.1 mm; BM=2.0±0.55 g; GSI=1.2±0.80). These different phenotypes within each sex represent different reproductive, metabolic and social states (Fig. 1). Male *A. burtoni* exist in a social hierarchy that includes two reversible phenotypes, dominant and subordinate. Dominant males hold territories, are brightly colored and reproductively active, and spend the majority of their time performing reproductive and territorial defense behaviors. Because dominant males are more focused on territory defense and reproduction, they are often less attentive to feeding during their typical 4–5 week territory-holding tenure. Subordinate males are more drably colored and reproductively suppressed, do not hold territories, and spend their time shoaling with females. Without territories to defend, subordinate males also typically spend more time feeding and show higher growth rates than dominant males (Fernald and Hirata, 1977; Hofmann et al., 1999). For the EOG experiments described here, dominant and subordinate males were classified based on coloration, stereotypical behaviors performed by each social status, and GSI, as done previously (Maruska and Fernald, 2013, 2014). Females spawn with males when they are gravid (full of eggs) and sexually receptive. Following spawning, females care for developing fry inside their mouths for ∼2 weeks, and then recover from mouth brooding back to a gravid state, which is characterized as a period of feeding and ovarian recrudescence. During the mouth brooding period, females typically do not feed and show decreased body mass and delayed ovarian cycles (Renn et al., 2009; Grone et al., 2012). Gravid females were selected for EOG experiments based on distended abdomens prior to feeding, and GSI of ≥6.0 (indicative of carrying large eggs prior to spawning) was later verified (ovulation status was not known). Experimental mouth brooding females contained developing fry in their buccal cavity that were late-brood (days 10–14 of 2 week brood period), while recovering females were in a period of recrudescence and were neither gravid nor mouth brooding with a GSI range of 0.39–2.64.

Experimental setup

Fish were quickly netted from community tanks prior to feeding to ensure that all individuals were in a similar short-term feeding state (e.g. all were fed ∼24 h earlier). Our goal was to compare EOG responses from groups of fish that differed in long-term (days-weeks) metabolic state associated with reproduction, rather than short-term deprivation or satiety. Fish were then anesthetized in ice-cold cichlid-system water [reverse osmosis (RO) water supplemented with Tanganyika buffer (Seachem, Madison, GA, USA) to pH 8.0 and Cichlid Lake Salt (Seachem) to 300–400 µS cm^{-1}] and immobilized with an intramuscular injection of the paralytic agent pancuronium bromide (2.5 µg g^{-1} BM in 0.9% NaCl). Additional injections were applied as needed during the experiment. The fish were positioned in a Plexiglas container, stabilized by clamps, and kept moist by wet Kimwipes®. Ventilation was provided during the experiments by a gravity-fed tube inserted into the mouth supplying a constant flow of aerated cichlid-system water over the gills. Access to the olfactory epithelium was achieved by careful removal of a small amount of skin, connective tissue and cartilage surrounding the single naris opening on the left side of the fish.

EOG recordings

The EOG is a negative electrical potential recorded in the water immediately above the fish's olfactory epithelium in response to odorant stimuli and represents the summated generator potential of the ORNs (Silver et al., 1976; Scott and Scott-Johnson, 2002). The recording and reference electrodes were Ag/AgCl pellet electrodes (World Precision Instruments, Sarasota, FL, USA) fitted with a saline-agar-filled glass capillary tube (tip diameter 100 µm, 0.15 mol l^{-1} NaCl, 0.5% agar). The recording electrode was positioned immediately above the circular olfactory epithelium, which typically contains seven to nine lamellae in this species (Fig. 2), whereas the reference electrode was placed on the skin between the eyes; the fish was grounded via a syringe needle in the tail musculature. The EOG responses were DC amplified (Grass P-18, Astro-Med, West Warwick, RI, USA), digitized on a CED Micro 1401 A-D converter running Spike 2 software (Cambridge Electronics Design, Cambridge, UK) and stored on computer for later analysis. At the end of each experiment, the fish was measured for SL, weighed for BM and euthanized by rapid cervical transection. Gonads were removed and weighed (gonad mass, GM) to calculate GSI [GSI=(GM/BM)×100] as a measure of reproductive investment.

Odorant stimuli and delivery

Olfactory stimuli consisted of L-alanine (Ala; neutral amino acid with a short side chain) and L-arginine (Arg; basic amino acid) at concentrations of 10^{-6}, 10^{-5} and 10^{-4} mol l^{-1}. Stimuli $<10^{-6}$ mol l^{-1} were not used in this investigation as preliminary tests indicated that responses to lower concentrations were often within control levels, making any determination of threshold

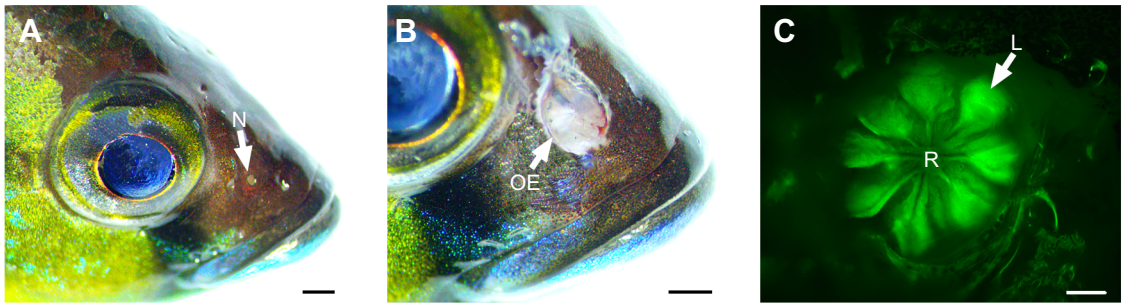

Fig. 2. Location and morphology of the naris and olfactory epithelium in *Astatotilapia burtoni*. (A) Photograph of a dominant *A. burtoni* male illustrating the location of the right olfactory naris (N) opening in front of the eye. *Astatotilapia burtoni* has a single naris opening on each side of the head. (B) Photograph of the same fish shown in A with the exposed olfactory epithelium (OE) located beneath the naris opening. (C) Photograph of an exposed olfactory epithelium in a dominant male stained with the vital dye DASPEI showing eight lamellae (L) around the central raphe (R) [image in C is from our previous study (Butler et al., 2016)]. Scale bars, 1 mm (A,B) and 500 µm (C).

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suspect. All chemicals were reagent grade (Sigma-Aldrich, St Louis, MO, USA) and RO-water (pH 8.1) served as the rinse solution and solvent for the stimuli. Amino acid analyses were conducted at the LSU AgCenter Biotechnology Laboratory (Dionex ICS-3000 system consisting of a GS50 Gradient Pump, an AS50 Autosampler, and an ED50 electrochemical detector) to determine the free amino acid concentrations of the recirculating cichlid-system water where fish were housed and the solvent solution used for odorant application. Both RO-water and cichlid-system water contained Ala and Arg levels that were below the detection level of the analysis system (<20 picomole). Stock solutions (10 mmol l^{-1}) were prepared weekly and test solutions were prepared daily.

A constant flow (50 µl s^{-1}) of solvent control water at 22°C bathed the olfactory organ for several minutes, followed by five to eight 4-s applications of Ala and Arg at three different test concentrations (10^{-6}, 10^{-5} and 10^{-4} mol l^{-1}) presented randomly. This randomized delivery was verified not to influence EOG responses when odorants of different type or concentration were delivered sequentially (Fig. 3). A stimulus duration of 4 s was chosen because preliminary experiments testing different durations revealed that the EOG response magnitude showed minimal variance and plateaued at ∼3–4 s, indicating that longer durations were not necessary and shorter durations were too variable. The amino acid stimuli were delivered by an eight-channel controlled gravity perfusion system with the same pressure across all individuals tested (VC3-8PG, ALA Scientific Instruments, Farmingdale, NY, USA). Stimulus solutions were delivered through separate tubes (MLF-8 millimanifold, ALA Scientific) and inter-stimulus intervals were 90–120 s. Control RO-water was also tested intermittently throughout each recording period. A total of ∼50–0 stimulus presentations occurred for each fish tested.

Data and statistical analyses

The peak amplitude (mV) of the EOG response was measured for each 4 s odorant application. Peak amplitude values were then averaged across the repeated five to eight presentations of each odorant at the same concentration within each fish. To characterize the shape of the odorant-evoked EOG waveform, the following parameters were measured in a subset of animals from each of the five fish groups: (1) the slope of the initial negative phase measured at 70% of peak amplitude in mV s^{-1}, and (2) the time period at 50% of peak amplitude. Each of these two measures was calculated from three representative waveforms evoked to each amino acid at each test concentration per fish (N=3–5 fish analyzed per group).

Peak amplitudes, slopes and 50% times of the EOG responses were compared using generalized linear mixed models (GLMMs). GLMMs account for data with non-normal distributions or heterogeneous variances within the effects of the model, allowing for rigorous, but more biologically realistic, comparisons (Venables and Dichmont, 2004; Bolker et al., 2008). Amino acid was used as the within-subjects repeated measures variable, and concentration was nested under amino acid. Sex, reproductive state, amino acid and concentration were assigned as fixed factors and individual fish subjects as a random factor. Body size was used as a covariate in the statistical models because there were differences in standard length and body mass among fish groups (ANOVA, $F_{4,33}$=12.90, P<001), and some positive correlations between body size and EOG response amplitudes (Pearson correlations, r=0.37-0.43, P<0.05). Thus, using body size as a covariate in the analysis removes any effects of this confounding variable on the EOG responses. Pairwise comparisons with least significant difference (LSD) adjustments were used to determine differences between sexes, reproductive states, amino acids and test concentrations. Bonferroni corrections or other similar procedures were not used because they reduce statistical power and increase the chance of type II errors, especially in small sample sizes. While these correction tests do reduce type I errors, their unacceptable effects on statistical power can mask potential biologically relevant results (Nakagawa, 2004). Discriminant function analysis was used to group animals based on EOG responses (amplitude, slopes) using within-groups co-variances and all groups were considered equal. Missing values [i.e. responses with peripheral waves (PWs), oscillations in the recorded EOG] were replaced with the group mean.

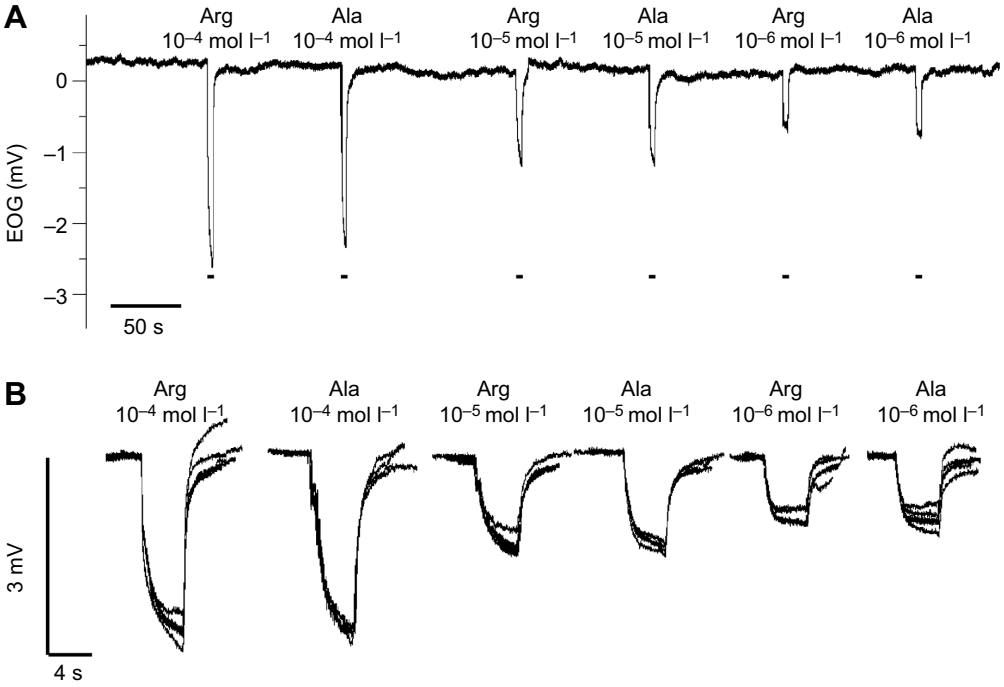

Fig. 3. Example of sequential randomized stimulus application and electro-olfactogram (EOG) response traces in the cichlid *Astatotilapia burtoni*. (A) Representative EOG traces from a single fish (recovering female) to show randomized stimulus delivery of Ala and Arg at three different test concentrations during a single experiment. Lines beneath each trace represents the duration of the 4 s stimulus application. (B) Example of five overlaid EOG traces from a single recovering female to illustrate the stability and repeatability (shape, amplitude) of the response to five repeated stimulus applications of each amino acid at each test concentration.

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Chi-square tests were also used to test for differences in the presence of PWs among fish groups within each sex. Statistical comparisons were made in SPSS 24 (IBM, Armonk, NY, USA) or SigmaPlot 12.3 (Systat, San Jose, CA, USA).

RESULTS

EOG responses

EOG recordings were obtained from 37 *A. burtoni* fish. EOG responses to amino acids showed an initial phasic negative deflection that reached 80% peak amplitude within 0.5–2.0 s (depending on concentration, with the response to higher stimulus concentrations reaching peak amplitude at the shorter time) followed by a slower recovery back to pre-stimulus levels (Figs 3–5). The shape and amplitude of the EOG responses to both Ala and Arg were consistent and repeatable, respectively, across the different tested concentrations (Fig. 3).

Reproductive, social and metabolic state differences

The magnitude of the EOG in response to each amino acid at the three tested concentrations showed similar dose–response relationships across female reproductive states (brooding, recovering, gravid) (Fig. 4B,D) and male social status (dominant, subordinate) (Fig. 5B,D). There was no overall effect of sex ($F_{1,172}$=0.006, P=0.936) or amino acid type ($F_{1,172}$=0.661, P=0.417) on magnitude of the EOG. However, there was an overall effect of concentration ($F_{2,172}$=160.081, P<0.001) and reproductive state ($F_{4,172}$=26.500, P=0.035) on the magnitude of the EOG responses within each sex, but no interaction between reproductive state and concentration (P=0.348). Mouth brooding females had a greater magnitude of EOG responses at the higher tested concentrations of amino acids (Ala 10^{-4} mol l^{-1}: P=0.002; Ala 10^{-5} mol l^{-1}: P=0.015; Arg 10^{-4} mol l^{-1}: P<0.001) than recovering and gravid females (Fig. 4B,D). However, EOG

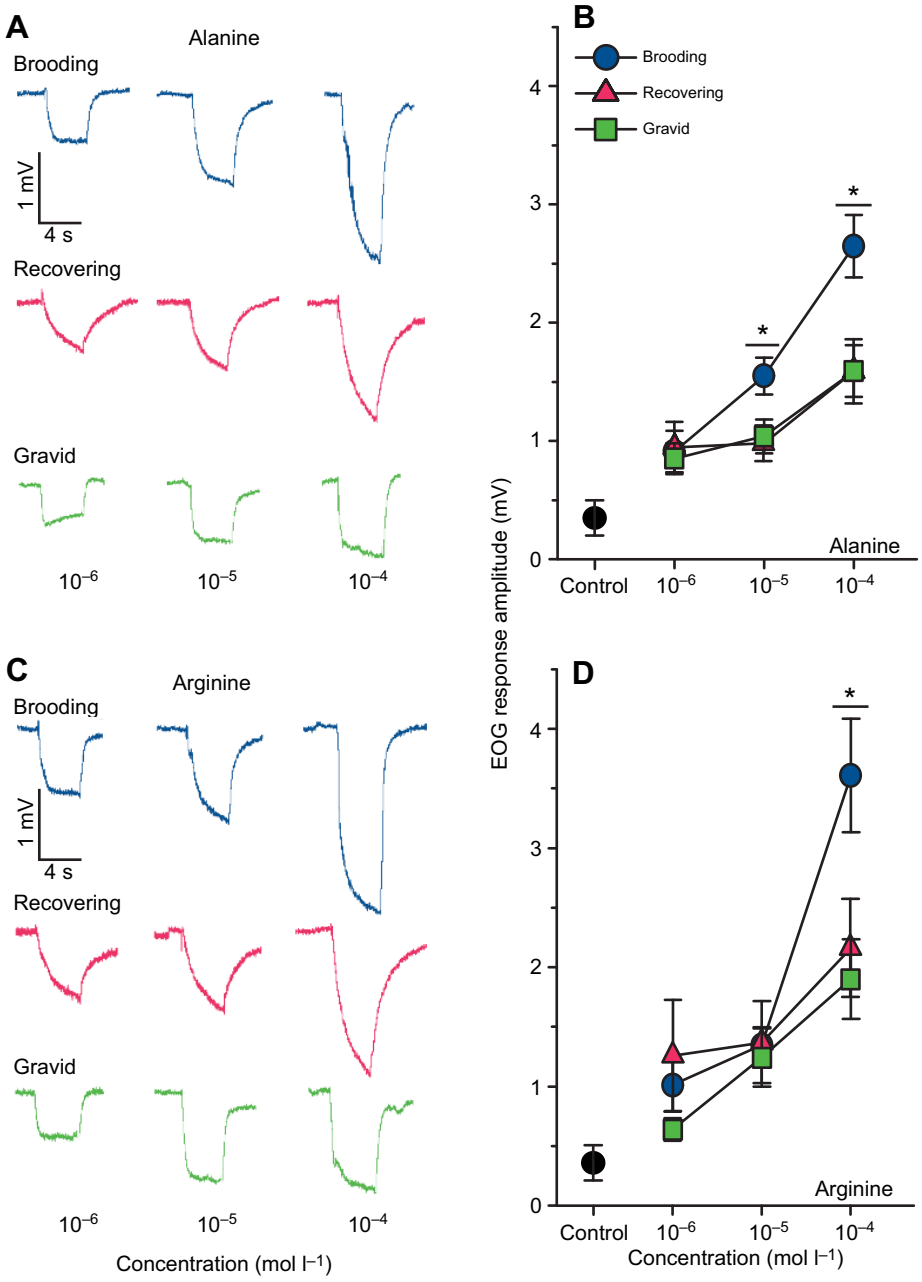

Fig. 4. Peak EOG amplitudes in response to amino acid stimulation differ among females of different metabolic and reproductive states in *Astatotilapia burtoni*. (A) Example EOG traces to alanine at each test concentration from representative brooding [gonadosomatic index (GSI)=0.63], recovering (GSI=2.02) and gravid (GSI=8.43) females. (B) Dose responses for EOGs of brooding, recovering and gravid females to alanine at three different test concentrations. EOG response amplitude is greater for brooding females at 10^{-5} and 10^{-4} mol l^{-1} concentrations compared with gravid and recovering females. (C) Example EOG traces to arginine at each test concentration from the same representative brooding, recovering and gravid females shown in A. (D) Dose responses for EOGs of brooding, recovering and gravid females to arginine at three different test concentrations. EOG response amplitude is greater for brooding females at the 10^{-4} mol l^{-1} concentration compared with gravid and recovering females. Data in B and D are plotted as means±s.e.m., N=4–8 animals per group. Response amplitudes to water control applications are plotted for reference. Lines with asterisks indicate statistical differences (P<0.05) among fish groups at each test concentration from the generalized linear mixed model (GLMM) comparisons using body size as a covariate (see Results for details).

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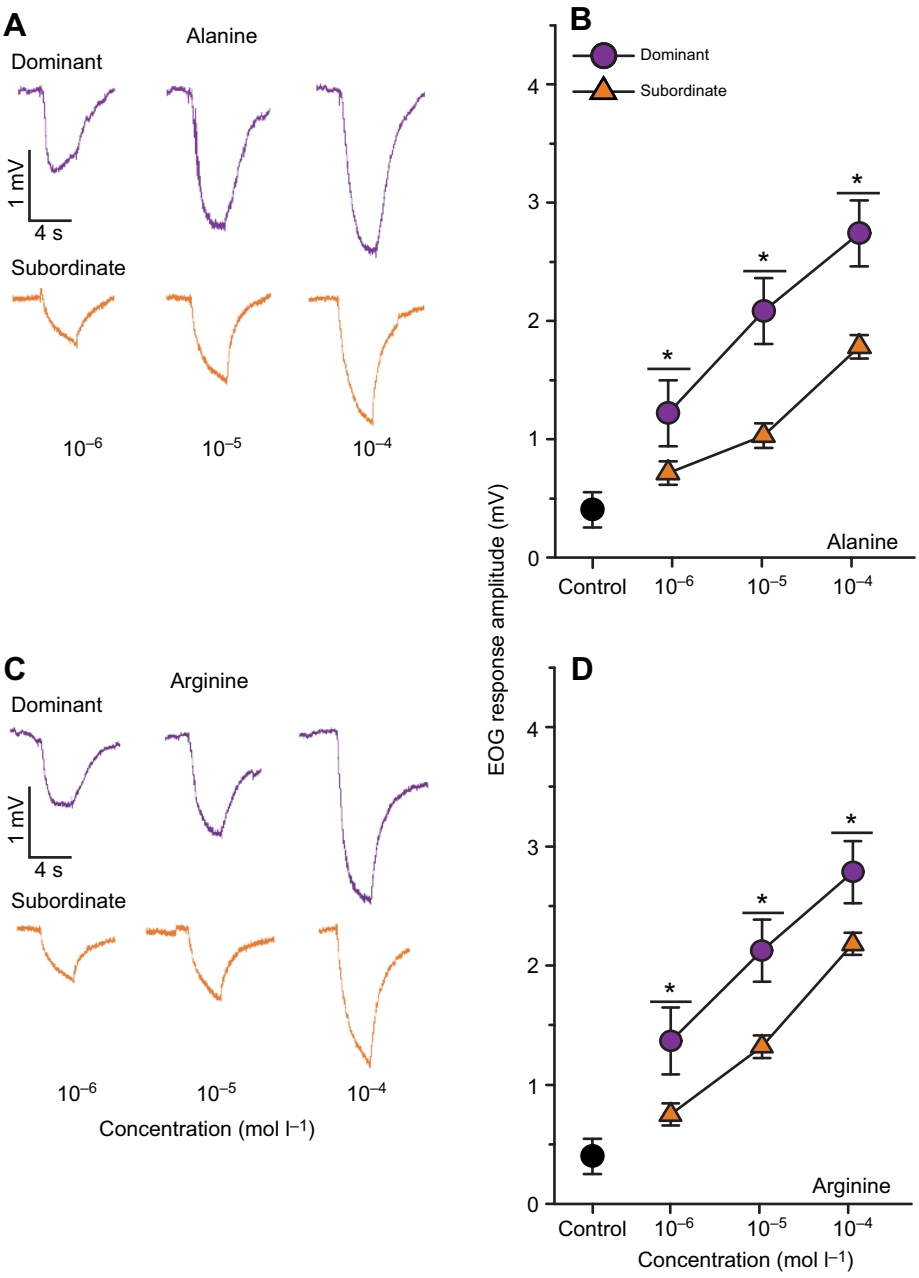

Fig. 5. Peak EOG amplitudes in response to amino acid stimulation differ between dominant and subordinate male *Astatotilapia burtoni*. (A) Example EOG traces to alanine at each test concentration from representative dominant (GSI=0.68) and subordinate (GSI=0.21) males. (B) Dose responses for EOGs of dominant and subordinate males to alanine at three different test concentrations. EOG response amplitude is greater for dominant males across all test concentrations. (C) Example EOG traces to arginine at each test concentration from the same representative dominant and subordinate males shown in A. (D) Dose responses for EOGs of dominant and subordinate males to arginine at three different test concentrations. EOG response amplitude is greater for dominant males across all test concentrations. Data in B and D are plotted as means±s.e.m., *N*=4–8 animals per group. Response amplitudes to water control applications are plotted for reference. Lines with asterisks indicate statistical differences ($P<0.05$) among fish groups at each concentration from the GLMM comparisons using body size as a covariate (see Results for details).

response magnitude did not differ among female states at lower concentrations (10^{-6} mol l^{-1} for Ala; 10^{-6} and 10^{-5} mol l^{-1} for Arg). Gravid and recovering females had a similar magnitude of EOG responses to both amino acids (Ala: $P=0.965$; Arg: $P=0.950$). In males, dominant individuals had greater EOG magnitudes compared with subordinate individuals for both Ala ($P=0.022$) and Arg ($P=0.050$) at all tested concentrations (Fig. 5B,D).

The shape (slope of the initial negative phase) of the EOG in response to amino acid stimuli also differed among fish of different metabolic and reproductive states in both females and males (Figs 6 and 7). Overall, there was no significant effect of sex or amino acid on the slope of the EOG response. However, there were main effects of reproductive state ($F_{4,16}=4.791$, $P=0.016$) and concentration ($F_{2,32}=47.237$, $P<0.001$) and an interaction between reproductive state, amino acid and concentration ($F_{16,20}=2.717$, $P=0.019$; Figs 6 and 7). Gravid females had a steeper slope than mouth brooding females ($P=0.042$) while recovering females were intermediate between the two (Fig. 6A,B). Dominant males also had a steeper slope than subordinate males ($P=0.012$; Fig. 7A,B). In general, higher amino acid concentrations resulted in steeper slopes of the EOG response. Application of 10^{-4} mol l^{-1} stimuli resulted in an EOG response with a greater slope than those tested at 10^{-5} and 10^{-6} mol l^{-1} ($P<0.001$ for both). Further, EOG responses evoked by the 10^{-5} mol l^{-1} test stimuli had steeper slopes than those evoked by 10^{-6} mol l^{-1} test stimuli ($P=0.004$). The significant interaction between reproductive state, amino acid and concentration revealed that response differences in reproductive state were dependent on concentration, which indicates that the highest concentration (10^{-4} mol l^{-1}) resulted in the greatest differences owing to reproductive state while the lowest concentration resulted in the smallest. In addition, the reproductive state differences in slope are slightly greater in response to Arg compared with Ala. Overlays of representative EOG traces from three different fish responding to Ala (10^{-4} mol l^{-1}) and Arg (10^{-4} mol l^{-1}) illustrate the

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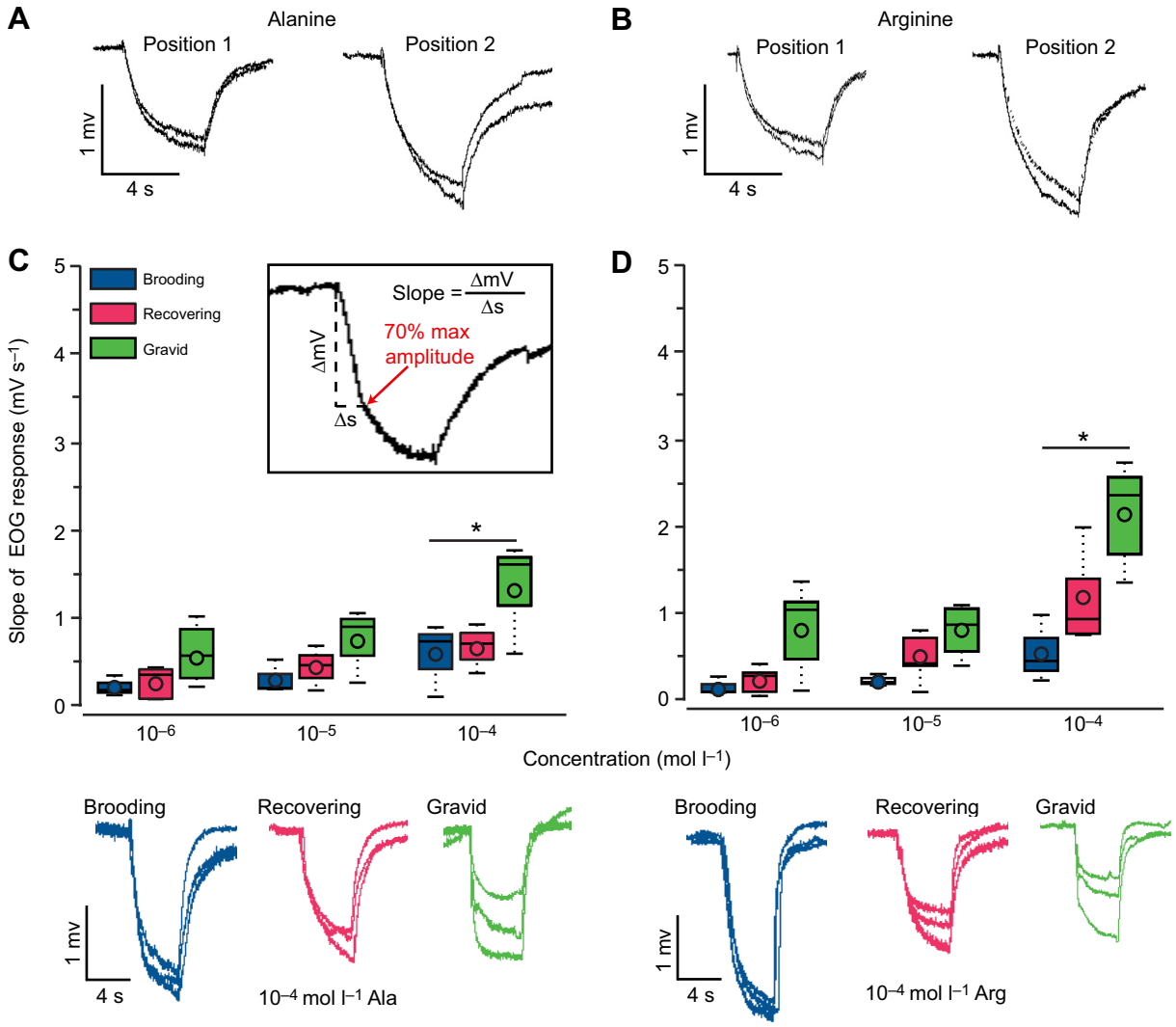

Fig. 6. The slope of the initial negative phase of the amino acid-evoked EOG response differs among females of different metabolic and reproductive states in *Astatotilapia burtoni*. (A,B) Representative EOG recordings in response to application of 10^{-4} mol l^{-1} Ala and 10^{-4} mol l^{-1} Arg at two different electrode positions (1 and 2) in a single recovering female. The two traces at each position are repeated applications of the same 10^{-4} mol l^{-1} Ala or Arg solutions. (C,D) Slope of the EOG in response to three tested concentrations of Ala (C) and Arg (D) in brooding, recovering and gravid females. The slope is steeper in gravid females compared with recovering and brooding females but was only significant at the highest concentration. Tukey's box plots were used to plot the data: median is represented by a line and mean by an open circle within the box, the box extends to the furthest data points within the 25th and 75th percentile, and whiskers extend to the furthest data points not considered outliers. *N*=3–5 animals per group; response slopes were corrected for fish body size. Lines with asterisks indicate statistical differences ($P<0.05$) among fish groups at each test concentration (see Results for details). Inset in C shows how the slope of the initial negative phase of each EOG response was measured as the change in amplitude (ΔmV) divided by the change in time in seconds (Δs). Records beneath each graph show representative EOG traces in response to 10^{-4} mol l^{-1} Ala and Arg overlaid from three different individuals within each fish group.

reproducibility of the slopes across individuals within their respective fish group (Figs 6 and 7). In contrast to the observed slope differences, the time period at 50% amplitude of the EOG response did not differ with sex, reproductive state, amino acid or concentration ($P>0.05$ for all factors).

Discriminant function analysis (DFA) combines all input variables into a single composite score and identifies which variables contribute to differentiation between the five animal groups. Three different DFAs were run to determine which variables best distinguished the different fish groups: (1) EOG peak amplitude alone, (2) EOG slope alone and (3) EOG amplitude and slope combined (Fig. 8). The DFA for EOG amplitude alone extracted two significant functions ($P<0.001$) that explained more than 95% of the data variance. Function 1 explained 54.6% of the variance, was loaded most strongly by the highest concentrations (10^{-4} mol l^{-1}) of Arg, and separated mouth brooding females from other fish (Fig. 8A). Function 2 explained an additional 40.7% of the variance, was loaded most strongly by the highest concentration (10^{-4} mol l^{-1}) of Ala and separated dominant males and, to a lesser extent, mouth brooding females from subordinate males, gravid females and recovering females. Based on EOG response amplitudes, the DFA accurately classified 100% of males (both dominant and subordinate) and 86% of mouth brooding females, but only 50% of recovering and 37.5% of gravid females. Interestingly, recovering and gravid females were commonly misclassified as each other or as subordinate males, indicating a similarity in EOG response patterns between recovering females, gravid females and subordinate males.

The DFA on EOG slope alone extracted one significant function ($P=0.022$) that explained 70.2% of the variance

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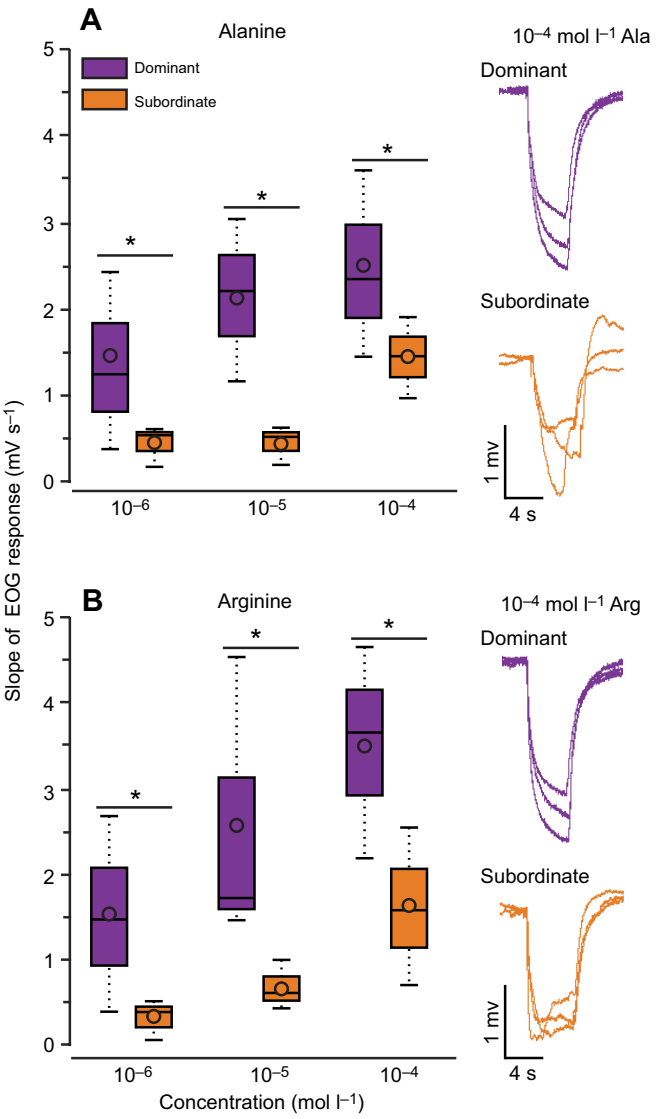

Fig. 7. The slope of the initial negative phase of the amino acid-evoked EOG response differs between males of different metabolic, social and reproductive states in *Astatotilapia burtoni*. Slope of the EOG in response to three tested concentrations of Ala (A) and Arg (B) in dominant and subordinate males. The slope of the EOG in response to both amino acids is steeper in dominant males compared with subordinate males at all tested concentrations. Tukey's box plots were used to plot the data: median is represented by a line and mean by an open circle within the box, the box extends to the furthest data points within the 25th and 75th percentile, and whiskers extend to the furthest data points not considered outliers. *N*=3–5 animals per group; response slopes were corrected for fish body size. Lines with asterisks indicate statistical differences ($P<0.05$) among fish groups at each test concentration (see Results for details). Records adjacent to each graph show representative EOG traces in response to 10^{-4} mol l^{-1} Ala and Arg overlaid from three different individuals within each fish group.

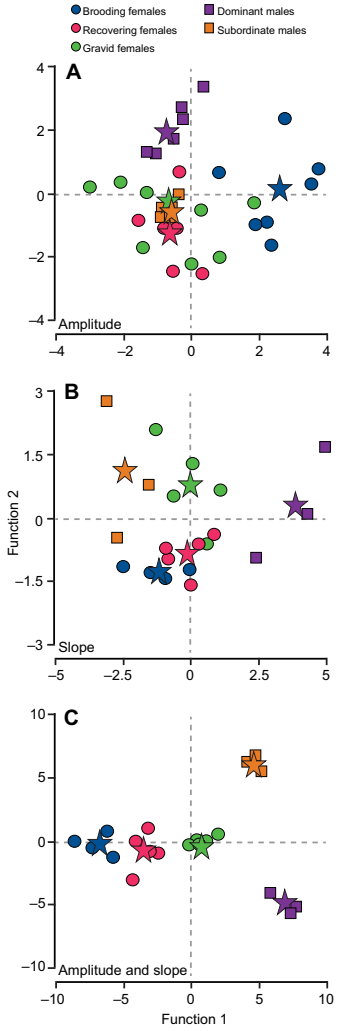

Fig. 8. Linear discriminant function analysis (DFA) of EOG amplitudes and slopes in response to alanine and arginine across male and female groups of *Astatotilapia burtoni*. (A) DFA of EOG amplitude alone identifies function 1 that discriminates brooding females from all other groups, while function 2 discriminates dominant males from other groups. Gravid females, recovering females and subordinate males had similar EOG magnitudes and grouped together. (B) DFA of EOG slope alone distinguishes dominant and subordinate males from each other along function 1, but had minimal impact on females. (C) DFA of combined EOG response amplitude and slope clearly separates and classifies 100% of animals into their respective groups. Discriminant scores are plotted for individual fish and stars represent the centroid of each fish group (*N*=5–8 fish per group in A, and 3–5 fish in B and C).

(Fig. 8B). Three other functions were also extracted, but were not statistically significant and explained the remaining ~30% of the data variance. Function 1 was loaded most strongly by the highest concentration of Arg (10^{-4} mol l^{-1}) and the middle concentration of Ala (10^{-5} mol l^{-1}). This function primarily segregated subordinate and dominant males with minimal impact on females. The second function, although not significant, explained 15.8% of the data and was loaded by the two highest concentrations of each amino acid. Together, these two functions correctly classified all dominant and subordinate males and gravid females, but misclassified recovering and mouth brooding females with each other.

The DFA using combined EOG amplitude and slope extracted three significant functions ($P<0.001$) that explained more than 99% of the data variance (only the first two are plotted in Fig. 8C). The first function explained 60.2% of the variance while functions 2 and 3 explained 28.5% and 11% of the variance, respectively. Function 1 was most strongly loaded by the slope of the response to Arg 10^{-6} mol l^{-1} and the amplitude of the response to Ala 10^{-5} mol l^{-1}, and distinguished female reproductive states and male social states. Function 2 was most strongly loaded by the slope of the response to Ala and clearly separated subordinate and dominant males, but had little impact

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on female separation. Importantly, by combining slope and amplitude of the EOG response, this DFA accurately classified 100% of animals into their respective groups.

Peripheral waves

PWs were often observed in EOG recordings from the olfactory organ of *A. burtoni* in response to 10^{-5} and 10^{-4} mol l^{-1} Ala and Arg, and exhibited dose-dependent differences (Fig. 9). PWs were not observed with 10^{-6} mol l^{-1} Ala or 10^{-6} mol l^{-1} Arg test stimuli. The oscillation frequency from the first second of the PWs was generally higher in Ala (13.5±1.0 Hz) compared with Arg (10.75± 0.5 Hz), and the range of peak-to-peak amplitudes were 4.5–14.5 mV for Ala and 3.6–10.3 mV for Arg. The greatest percentage of EOG responses exhibiting PWs in response to 10^{-4} and 10^{-5} mol l^{-1} Ala were mouth brooding females (44.4%). PWs were less evident in gravid females (36.4%) followed by recovering females (10%). However, there was no statistical difference in the percentage of EOG responses with PWs among female groups (χ^2=3.79, P=0.150). In males, there was also no statistical difference between the percentages of dominant individuals that showed PWs (35.7%) compared with subordinate individuals (27.3%) (χ^2=0.0003, P=0.986).

DISCUSSION

Our results demonstrate that the metabolic impacts on the olfactory organ previously described in other taxa also occur in fishes, the largest and most diverse group of vertebrates. Chemosensory cues are likely highly important for *A. burtoni*, which lives in shallow shore pools and riverine systems of Lake Tanganyika, Africa, where visual conditions can be turbid and dynamic. Our previous work demonstrated that chemosensory signaling is used by both males and females in inter- and intra-sexual social contexts (Maruska and Fernald, 2012; K.E.F. and K.P.M., personal observations). Here, we provide evidence for intra-sexual plasticity in the response of the olfactory organ to amino acids that varies with fish reproductive and metabolic state in both males and females. Collectively, these results highlight the importance of chemoreception for several crucial behaviors in this species, including reproduction and feeding, and demonstrate metabolic-related plasticity in the olfactory periphery for the first time in a fish species.

EOG responses

The rapid negative potential followed by a slower recovery of the EOG responses in *A. burtoni* was similar to that seen in other fishes (Shibuya, 1960; Byrd and Caprio, 1982; Evans and Hara, 1985), including other cichlids (Keller-Costa et al., 2014; Simões et al., 2015). While olfactory responses to steroidal compounds were measured previously in *A. burtoni* (Robison et al., 1998; Cole and Stacey, 2003, 2006), with some inter-sexual differences demonstrated for some steroid compounds, EOG responses to amino acids (other than the response to 10^{-5} mol l^{-1} Ala used to normalize the data) were not tested. In the present study, EOG magnitudes in response to Ala and Arg were dose-dependent and did not show evidence of saturation at the concentrations tested. We did not determine thresholds for amino acids because the relatively large responses to control water masked responses to stimulus concentrations below 10^{-6} mol l^{-1}. Therefore, the relative olfactory sensitivity of *A. burtoni* compared with other fishes is unknown. It is important to note, however, that behavioral and neural thresholds are often lower than those measured by summated recording techniques such as EOG (which contains both neural and non-neural elements) (Silver, 1982).

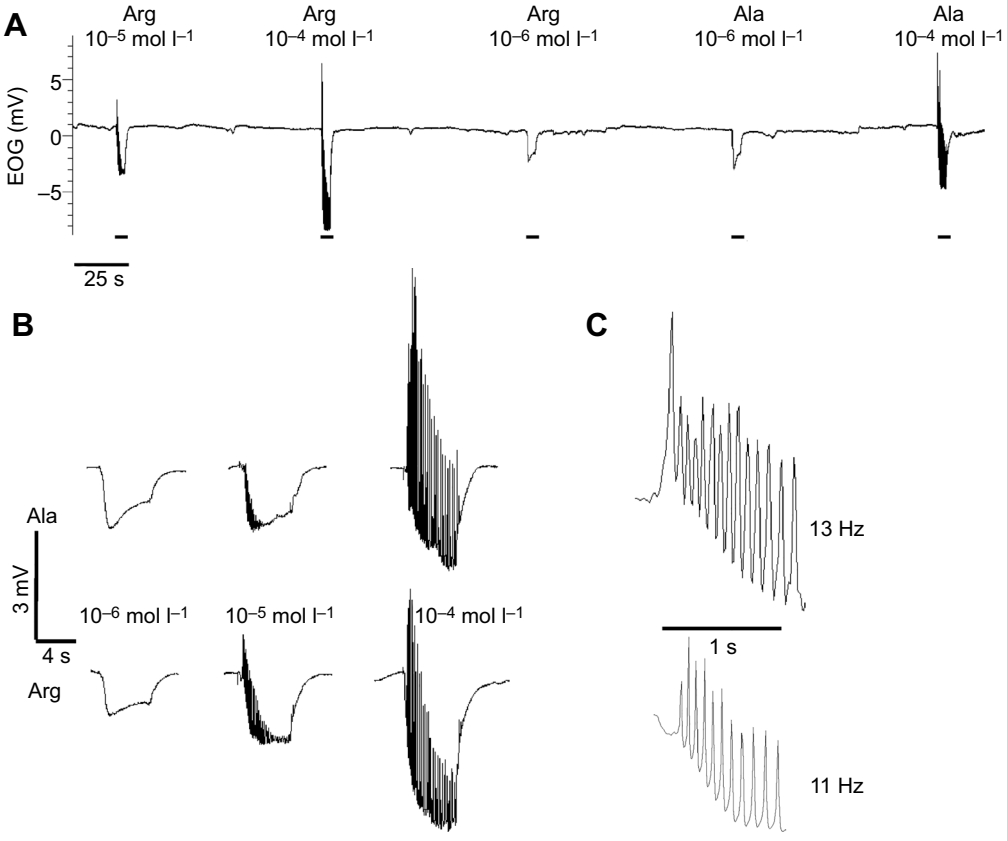

Fig. 9. Peripheral waves (PWs) in amino acid-evoked EOG recordings from a representative dominant male *Astatotilapia burtoni*. (A) Example EOG trace of sequential applications of Arg and Ala at different test concentrations shows PWs in response to Arg 10^{-5} mol l^{-1}, Arg 10^{-4} mol l^{-1} and Ala 10^{-4} mol l^{-1}, but not 10^{-6} mol l^{-1} for either amino acid. Lines beneath each trace represents the duration of the 4 s stimulus application. (B) Expanded representative EOG traces illustrate the dose-dependence of PWs in response to Ala (top traces) and Arg (bottom traces) at three different test concentrations. PWs were absent at 10^{-6} mol l^{-1}, but were more likely to occur at higher concentrations (10^{-5} and 10^{-4} mol l^{-1}) for both Ala and Arg. (C) Expanded view of the PWs from 10^{-4} mol l^{-1} shown in B for Ala (top) and Arg (bottom) illustrates differences in amplitude and oscillation frequency between the two amino acids. The GSI for this dominant male was 0.81.

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Reproductive, social and metabolic state differences in EOG responses

Here we describe differences in magnitude and slope of the EOG in response to amino acids at the same tested concentrations within both males and females that vary in reproductive, social and metabolic state. It is important to note, however, that while the amplitude of the EOG response does reflect the population of responding ORNs, the relationship between EOG magnitude and the transfer of relevant information to the olfactory bulb is unknown in any animal. Differences in EOG amplitude in response to the same tested compounds are therefore not equivalent to changes in absolute sensitivity (i.e. threshold detection levels of a stimulus) of the olfactory epithelium. There is evidence, however, that changes in EOG magnitude in response to the same tested stimuli can reflect differences in the number of responding ORNs (e.g. as during the breeding season; Nakazawa et al., 2009), or changes in ORN responsiveness modulated by molecules such as NPY and leptin that can alter the signal to noise ratio of sensory inputs transmitted to the olfactory bulbs (Mousley et al., 2006; Savigner et al., 2009). Further, studies in rodents suggest relationships between odorant-evoked EOG response magnitudes and behavioral responses in olfactory-related tasks; for example, satiety decreases EOG amplitudes, decreases olfactory sensitivity and impairs olfactory-related behavioral responses (see review by Palouzier-Paulignan et al., 2012 and references therein). Thus, while the exact significance of variation in EOG magnitude in response to the same tested stimuli has yet to be revealed, our results are interpreted in the context that this variation represents some biological relevance to the animal's olfactory processing capabilities.

Differences in EOG responses to putative pheromonal compounds (e.g. steroids, prostaglandins) among sexes and reproductive maturity are described in several fish species (Sorensen et al., 1987; Irvine and Sorensen, 1993; Moore, 1996; Murphy et al., 2001; Stacey, 2011). To our knowledge, however, the plasticity in EOG responses to amino acid stimuli is only described for the common carp, *Cyprinus carpio* (Irvine and Sorensen, 1993). In common carp, juveniles have a greater absolute EOG amplitude in response to the amino acid L-serine (the only amino acid tested) compared with sexually mature males and females (Irvine and Sorensen, 1993), which may be due to differences in energetic homeostasis between adults and juveniles. In zebrafish, EOG recordings also showed that females were more sensitive and had greater EOG amplitudes in response to the amino acid cysteine compared with males, but reproductive- or status-specific comparisons within a sex were not investigated (Michel and Lubomudrov, 1995). In fishes, olfactory receptors classified as type C G-protein coupled receptors (OlfCs) expressed in the olfactory epithelium detect amino acids and elicit feeding behaviors (Speca et al., 1999; Alioto and Ngai, 2006; Koide et al., 2009). African cichlids show a lineage-specific expansion of *OlfC* genes, which may have evolved to allow increased ability to discriminate a greater variety of amino acids and derivatives (Nikaido et al., 2013). Thus, differential expression of *OlfC* or other molecular receptor types that vary with the fish internal physiological state may contribute to intra-sexual olfactory plasticity. Examining reproductive, social and metabolic changes in olfactory responses to food-related cues, such as amino acids, is important for understanding how olfactory inputs are integrated in the brain to produce context-appropriate behaviors such as feeding and mating. Further work in a diversity of teleost fish species should provide insight on these olfactory-mediated behavior circuits.

Subordinate male *A. burtoni* showed smaller EOG magnitudes in response to Ala and Arg at the same tested concentrations compared with dominant males. This result supports the conserved relationship between long-term satiety state and reduced olfactory responses. However, reduced olfactory responses are also typically associated with lower olfactory-driven food-seeking behaviors (Palouzier-Paulignan et al., 2012). In contrast, subordinate males allocate more resources towards feeding and somatic growth in anticipation of an opportunity to acquire a territory and spawn with females (Hofmann et al., 1999). Thus, it is possible that specific metabolic or neuroendocrine signals override homeostatic long-term satiety signals in subordinate males to maintain high food intake despite reduced olfactory responses, a phenomenon similar to that observed in obese mammals (Badonnel et al., 2014). In contrast, dominant males are extremely active and spend considerable time maintaining their territories, defending them from other males, and courting and spawning with females (Fernald and Hirata, 1977; Maruska and Fernald, 2010b). This increased energy expenditure and reduced feeding behavior in dominant males suggests that greater olfactory responses may facilitate detection of food within their territorial boundaries to maximize nutrition in support of their active lifestyle during the several-week territory tenure. It cannot be ruled out, however, that differences in EOG magnitudes to amino acids between dominant and subordinate males are due to social status or reproductive state. This is especially relevant in light of the links between circulating steroid levels and olfactory responses in many animals (Doty and Cameron, 2009; Ghosal and Sorensen, 2016; Kanageswaran et al., 2016), and the fact that dominant males have higher plasma levels of estradiol, testosterone, 11-ketotestosterone and progestins compared with subordinate males (Maruska, 2015). However, because amino acid stimuli such as Ala and Arg primarily represent food-related cues, rather than social signals (but see Yambe et al., 2006; Ward et al., 2011; Kleinhappel et al., 2016; Kutsyna et al., 2016), it is more likely that differences in EOG magnitude are related to metabolic or nutritional state. This idea is also supported by our DFA of EOG amplitude, which grouped subordinate males along with gravid and recovering females, all of which show greater feeding/metabolic activity and reduced EOG magnitudes at the same tested concentrations compared with dominant males and mouth brooding females.

Mouth brooding *A. burtoni* females undergo a period of obligatory starvation while they care for developing young inside their mouths. These food-deprived mouth brooding females show the largest EOG magnitudes, which also supports the previously proposed hypothesis in other taxa that fasting is associated with increased olfactory responses (reviewed in Palouzier-Paulignan et al., 2012). Interestingly, mouth brooding females still show a strong motivation to feed, as they often rapidly approach food, but abruptly halt before consumption because of the fry already present in the buccal cavity. These observations suggest that the control of appetitive and consummatory feeding behaviors in females is likely regulated by different mechanisms, as shown in other invertebrate and vertebrate taxa (Sternson, 2013; Crossley et al., 2016). While the cessation of consummatory food intake is likely mediated by central mechanisms within the brain (as well as signals from egg/fry presence in the mouth), the appetitive food searching behavior is at least partially influenced by inputs from the sensory periphery (i.e. ORNs and taste receptor cells). For example, neural control of feeding and energy homeostasis is regulated primarily by hypothalamic nuclei that integrate signals from neurochemicals (e.g. neuropeptide Y, NPY; agouti-related protein, AgRP; melanocortins) as well as from peripheral metabolic factors (e.g. leptin, ghrelin, glucose, lipids) (Sohn et al., 2013; Myers et al., 2016). In *A. burtoni*, gravid females have larger NPY and AGRP (i.e. orexigenic) neurons

and smaller pro-opiomelanocortin (*pomc*) (i.e. anorexigenic) neurons in the hypothalamus compared with mouth brooding females (Porter et al., in press), which likely helps regulate feeding and metabolism. However, the plasticity in olfactory responses in females of varying metabolic states also suggests that some modulators may act at the level of the olfactory epithelium, the mechanisms of which are likely distinct from the central circuits. There is already existing evidence in vertebrates for the modulation of olfactory epithelial function by metabolic-related signaling molecules. For example, neuropeptide immunoreactive fibers from modulators such as NPY, along with several G-protein coupled receptors that have roles in regulating feeding behavior and energy homeostasis, are found in the olfactory epithelium of many vertebrates, including teleost fishes (Gaikwad et al., 2004; Negroni et al., 2012; Olender et al., 2016). Modulators such as NPY also influence olfactory responses at the epithelium level in rodents and amphibians, particularly in hungry animals (Mousley et al., 2006; Negroni et al., 2012). Collectively, these studies provide support for a physiological link between the olfactory periphery and nutritional state. Because the mouth brooders in our study were in the late brood stage, greater EOG magnitudes in response to amino acids at the same tested concentration are consistent with a potential function in preparing or priming the starved females (at perceptual and goal-directed neural circuit levels) to rapidly detect, find and consume prey/food once the brood is released. It is also possible that brooding females use amino acids and other olfactory cues to facilitate maternal care both during the brood period and after the fry are released.

We know from previous studies, however, that gravid, recovering and mouth brooding *A. burtoni* females, and dominant and subordinate males, also have different circulating sex-steroid levels (androgens and estrogens; gravid>recovering>brooding; dominant>subordinate) (Maruska and Fernald, 2010a). In fishes and amphibians, there is evidence that sex steroids can increase the sensitivity of the olfactory epithelium to specific odorants, particularly pheromones (Cardwell et al., 1995; Toyoda and Kikuyama, 2000; Belanger et al., 2010; Ghosal and Sorensen, 2016), but little is known about hormonal effects on detection of amino acid cues. In contrast, recent studies in mice show that estradiol and progesterone decrease odorant-evoked responses in the olfactory epithelium via rapid non-genomic mechanisms (Kanageswaran et al., 2016). Nevertheless, as mentioned above, the possibility that our observed differences in EOG magnitudes are due to steroid hormone modulation at the olfactory epithelium rather than metabolic state cannot be ruled out. However, if steroid hormones are involved, they likely act via different mechanisms in males and females because the relationship between plasma steroids and the EOG magnitude evoked by amino acid stimuli are opposite in females (e.g. mouth brooding females with low plasma steroids have higher EOG magnitudes at the same tested concentrations). While an obvious experiment would be to compare EOG responses in fed and starved individuals to directly test whether olfactory plasticity is mediated by metabolic or hormonal state, it is difficult to separate metabolic/feeding state from reproductive/hormonal state in *A. burtoni*. For example, even females that had their broods removed and were fed for 4 weeks had higher levels of sex steroids compared with both mouth brooding females that retained their brood (non-feeding) and females that had their broods removed and were starved for 4 weeks (Grone et al., 2012). This effect is likely due to the nutritional resources available to the fed females, which can be used for gonadal recrudescence resulting in increased sex-steroid production. Future experimental designs that carefully and independently manipulate hormonal and metabolic physiology are needed to explore the mechanisms responsible for the observed olfactory plasticity within female and male *A. burtoni*.

The slopes of the initial negative phase of the EOG in response to amino acids at the same test concentrations in *A. burtoni* also differed among fish of different metabolic and reproductive states in both females and males. The shape of odorant-evoked EOG waveforms is known to be highly variable and can be influenced by non-physiological factors such as electrode position (angle or distance from epithelium), fish size or position in the recording setup, health of the olfactory epithelium or fish, and stimulus flow dynamics within the olfactory capsule, among many others. However, because the reproductive/metabolic state differences in EOG slope in *A. burtoni* were observed consistently across all animals within a specific group with comparable recording setups, we propose that this likely represents a real physiological difference that can be used as a novel analysis tool in future EOG studies across vertebrate taxa. Our DFA of combined EOG amplitude and slope also supports this hypothesis because when both of these variables are included in the analysis, it correctly distinguished and classified 100% of the animals into their respective groups. This same distinct classification of groups was not achieved when either EOG amplitude or slope was considered alone, suggesting that combined slope and amplitude are important variables that define the typical EOG in response to the tested amino acids in each fish group. While many physiological mechanisms can contribute to differences in EOG shape, such as differences in types or abundance of ORNs or molecular receptors, ion channel properties or abundance, modulation by hormonal or metabolic factors, or variations in binding kinetics between odorant and receptor, the exact mechanism(s) responsible for the observed differences in *A. burtoni* are unknown. Whether these shape variations translate into any meaningful information sent from the epithelium to the brain requires further study.

Peripheral waves

PWs were recorded from *A. burtoni* males and females in response to both Ala and Arg. PWs were most common in mouth brooding females and dominant males, which also represent the groups within each sex that showed the greatest magnitude of the EOG in response to the tested amino acids. PWs naturally occur in every vertebrate class (Adrian, 1955; Ottoson, 1956; Nikonov et al., 2002), and previous work in the channel catfish *Ictalurus punctatus* showed that PWs enhance the magnitude of local field potentials in the olfactory bulb (Nikonov et al., 2002). This PW activity was later shown to arise from a superposition of asynchronous neural oscillators (i.e. the responding ORNs) that strengthen synaptic transfer (Diaz et al., 2007) and may be important for odorant discrimination. Thus, when fish are in a starved or reduced nutritional state, as is the case for mouth brooding females and dominant males, increased synaptic strength in the olfactory pathway may impact motivational and feeding circuits to facilitate foraging and feeding behaviors.

Conclusions

For the first time in fishes, *in situ* EOG recordings in males and females of the African cichlid fish *A. burtoni* demonstrated intra-sexual plasticity in olfactory responses to food-related amino acid cues. In males, EOG responses with greater amplitudes and steeper slopes at the same amino acid test concentrations were elicited in dominant compared with subordinate individuals. In females, EOGs

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with greater amplitudes in response to the same tested concentrations of amino acids were elicited in mouth brooding compared with both recovering and gravid individuals, while EOGs of gravid females had steeper slopes. DFA of EOG magnitude grouped animals with similar metabolic and food intake states together (subordinate males, gravid and recovering females), and separated them from both dominant males and mouth brooding females. These groupings are best explained by differences in metabolic state, supporting the conserved relationship between long-term satiety and reduced olfactory responses seen in other taxa, but may also be related to reproductive, social or hormonal status. Combined DFA with amplitude and slope of the EOG response to the tested amino acids also accurately classified all animal groups, suggesting that shape (slope) of the response possibly contains biologically relevant information. Nevertheless, we provide evidence for male and female intra-sexual plasticity in the response of the olfactory epithelium to amino acids that varies with fish reproductive, social and metabolic state. Our results provide important information for better understanding the olfactory-mediated trade-offs between resource allocation and reproductive potential in species that show behavioral and physiological plasticity related to reproduction, energy investment and species survival.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.A.N., J.M.B., K.E.F., J.C., K.P.M.; Methodology: A.A.N., J.M.B., K.E.F., J.C.; Validation: A.A.N.; Formal analysis: A.A.N., J.M.B., K.E.F.; Investigation: A.A.N., K.E.F., J.C., K.P.M.; Resources: J.C., K.P.M.; Data curation: A.A.N., K.P.M.; Writing - original draft: K.P.M.; Writing - review & editing: A.A.N., J.M.B., K.E.F., J.C.; Visualization: A.A.N., J.M.B., K.E.F., K.P.M.; Supervision: J.C., K.P.M.; Project administration: K.P.M.; Funding acquisition: K.P.M.

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