UNIVERSIDADE DO ALGARVE

CHEMICAL COMMUNICATION IN THE MOZAMBIQUE TILAPIA:

A ROLE FOR AMINO ACIDS?

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Chemical communication in the Mozambique tilapia: a role for amino acids?

Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Assinatura_____

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Contents

Resumo

A tilápia de Moçambique (*Oreochromis mossambicus*) é um ciclídeo africano que apresenta incubação bucal maternal, e que se encontra naturalmente distribuída em cursos de água no sul de África. Os machos dominantes apresentam grandes bexigas urinárias, que têm a capacidade de armazenar uma quantidade substancialmente superior do urina do que a das fêmeas, que é libertada em contexto apropriado (cortejo ou agressividade macho-macho). Demonstrou-se que os machos são capazes de diferenciar o odor de fêmeas em processo pré-ovulatório das que estão em processo pós-ovulatório, o que provoca um aumento do processo de libertação de urina em machos; e que os machos dominantes apresentam uma urina com maior potência olfativa do que a de outros machos (subordinados). Isto sugere que a urina poderá ter um papel importante na comunicação química entre elementos desta espécie.

Pesquisas anteriores efetuadas no nosso laboratório identificaram um esteroide glicurónico na urina de machos, e que age como feromona (endócrino) em fêmeas. Este composto pode representar a maior parte da potência olfativa da parte não-polar da urina, e a concentração do mesmo poderá estar positivamente correlacionada com a dominância dos machos. No entanto, a fração polar também contém odoríferos. Porém, estão ainda por identificar os compostos responsáveis pela potência olfativa nesta fração.

Esta investigação tem como objetivo estabelecer quais poderão ser os aminoácidos responsáveis pela potência olfativa na parte polar da urina de tilápia de Moçambique. O primeiro passo realizado foi verificar a sensibilidade olfativa da tilápia de Moçambique a 20 aminoácidos, usando para tal um electro-olfactograma (EOG). O segundo passo foi identificar os aminoácidos presentes na urina nos machos de tilápia de Moçambique. Como terceiro passo, estimamos se a potência olfativa da fração polar da urina dos machos poderá ser explicada por esses aminoácidos, ou se outros odoríferos – ainda não identificados – estão também presentes; adicionalmente, um filtrado de urina e uma mistura artificial de aminoácidos de urina de tilápia do Nilo (uma espécie filogeneticamente próxima da tilápia de Moçambique) foram usadas para verificar a sua potência em *O. mossambicus*, e para comparar os resultados obtidos para as duas espécies de tilápia. Finalmente, mediu-se a concentração de aminoácidos na urina de machos

provenientes de diferentes estratos sociais, para explorar possíveis correlações entre a concentração de aminoácidos na urina e a hierarquia dos machos na sociedade.

Como resultado, os aminoácidos L-cisteína, L-glutamina e L-treonina produziram o estímulo mais potente na concentração mais alta testada (10^{-4} M), e os aminoácidos L-prolina e L-aspartato foram os que produziram o estímulo menos potente em *O. mossambicus*.

Para identificar os aminoácidos presentes na urina dos machos dominantes, as amostras de urina foram submetidas a um processo de derivatização, e a concentração de aminoácidos foi calculada através de cromatografia gasosa e espectroscopia de massa (GC-MS). O mesmo processo foi aplicado para quantificar os aminoácidos predominantes (L-arginina, L-ácido glutâmico e L-fenilalanina) na urina de machos originários de diferentes estratos sociais.

Entre os três aminoácidos mencionados acima, o mais abundante na urina de machos dominantes de tilápia de Moçambique foi a L-arginina, com a potência olfativa mais alta (10^{-4} M).

Quatro grupos de aminoácidos (*a*, *b*, *c*, *d*) foram identificados de acordo com os seus limites de deteção, e três outros grupos foram identificados pela similaridade dos seus fatores γ. Os aminoácidos de natureza básica obtiveram os limites de deteção mais baixos, e os que continham grupos imino ou grupos acídicos apresentaram menor potência. Os aminoácidos de natureza básica geralmente apresentavam também valores mais baixos de fatores γ que os aminoácidos de outras classes. Por oposição, os fatores γ dos odoríferos das classes acídicas e hidroxilo eram geralmente altas.

A potência olfativa do filtrado de urina de *O. mossambicus* foi significativamente mais alta do que a da mistura artificial de aminoácidos, preparada com base na concentração de aminoácidos previamente identificada na urina da *pool* de machos dominantes por GC-MS. O limite de deteção estimado para a mistura de urina de *O. mossambicus* foi obtido para uma diluição de $10^{-5.4}$, e para o filtrado de urina foi de $10^{-6.2}$. A comparação estatística da potência olfativa entre o filtrado de urina de tilápia de Moçambique e a mistura artificial de aminoácidos de urina de tilápia do Nilo identificaram que o limite de deteção para a mistura (diluição a $10^{-5.8}$) era

inferior ao filtrado (diluição a $10^{-5.1}$). Os fatores γ não foram estatisticamente diferentes nem para a mistura e filtrado de urina de *O*. *mossambicus*, nem para a mistura ou filtrado de urina de *O. niloticus*. O filtrado de urina de tilápia do Nilo foi menos potente para o sistema olfativo de tilápia de Moçambique que o filtrado coespecífico (Limites de diluição de $10^{-5.1}$ e $10^{-6.2}$, correspondentemente).

A concentração de L-arginina na urina foi positivamente correlacionada com o índice de dominância dos peixes (R=0.495; *P*<0.05).O aminoácido L-arginina estava presente em concentrações substancialmente mais altas na urina de machos dominantes, em relação aos grupos intermédio e subordinado. Este apresentava também uma maior variabilidade de concentrações em machos dominantes (DI>0.5), do que em machos subordinados (DI<0.5).

De maneira similar ao aminoácido L-arginina, as concentrações de L-ácido glutâmico apresentaram também uma maior variabilidade de concentrações em machos dominantes do que em machos subordinados. No entanto, não se verificou nenhuma correlação com a dominância (R=0.399; *P*=0.0657).

A concentração na urina de L-fenilalanina apresentar uma variabilidade similar nos grupos de machos dominantes e machos subordinados, e não apresenta correlação com o índice de dominância (R= -0.385, *P*=0.0769). Em todos os grupos as concentrações de L-fenilalanina na urina foram inferiores às de L-arginina e L-ácido glutâmico.

Podemos então concluír que:

1. A tilápia de Moçambique tem sensitividade olfativa aos vinte aminoácidos essenciais e não-essenciais, mas a capacidade e habilidade dos aminoácidos para provocarem uma resposta olfativa aguda através de mudanças na sua concentração são diferentes entre si.

2. A tilápia de Moçambique tem uma maior sensitividade ao filtrado de urina coespecífica, do que ao filtrado heteroespecífico de tilápia do Nilo. A potência olfativa do filtrado de *O. mossambicus* pode ser largamente, mas não completamente, explicada pela presença de determinados aminoácidos (L-arginina, L-ácido glutâmico e L-fenilalanina). No entanto, outros compostos odoríferos polares deverão também estar presentes.

3. Os aminoácidos L-arginina e L-ácido glutâmico são componentes importantes do odor da urina do macho de tilápia de Moçambique, e poderão indicar o *status* de

dominância do peixe na comunidade e definir, possivelmente, a sua identidade individual.

Palavras-chave: Tilápia de Moçambique, olfato, aminoácidos, dominância, electro-olfactograma.

Abstract

The Mozambique tilapia (*Oreochromis mossambicus*) is a maternal mouth-brooding cichlid from the southern Africa.

The olfactory sensitivity of the species to 20 amino acids was assessed using the electro-olfactogram (EOG). We estimated whether the olfactory potency of the polar fraction of male urine can be explained by the presence of identified amino acids. In addition, filtrate and amino acid mixture of the urine of Nile tilapia were used to estimate their olfactory potency for *O.mossambicus*. Finally, concentrations of the main amino acids were measured in the urine of males of different social status and the correlations between amino acid concentration and hierarchical status were explored.

L-cysteine, L-glutamine and L-threonine were the most potent stimuli at 10^{-4} M while L-proline and L-aspartate were the least potent.

Four groups of amino acids were identified according to their thresholds of detection and three groups – according to the similarity of their γ-factors.

The estimated threshold of detection for *O.mossambicus* mixture was higher than that for the filtrate. On the contrary, the threshold of detection for the mixture of Nile tilapia was lower than that for the filtrate

The concentration of L-arginine in the urine was positively correlated with fish dominance index. Both L-arginine and L-glutamic acid concentrations had much greater variability in dominant males (DI>0.5) than in subordinate males (DI<0.5). The urinary concentrations of L-phenylalanine had similar variability in dominant and subordinate groups.

The Mozambique tilapia has olfactory sensitivity to all twenty amino acids tested. The fish showed more acute sensitivity to conspecific urine filtrate than to the heterospecific. Olfactory potency of *O.mossambicus* filtrate can be largely but not fully explained by the presence of L-arginine, L-glutamic acid and L-phenylalanine. L-arginine and L-glutamic acid may indicate the dominance status of the fish and, possibly, individual identity.

Key words: Mozambique tilapia, olfaction, amino acids, dominance, electro-olfactogram.

1. Introduction

Olfaction refers to the sense of smell (Hara, 2011a). Fish, as other animals, are able to smell some chemical substances, such as amino acids and bile salts, that are present in their environment. These odorants stimulate olfactory receptor neurons after binding to receptor molecules in the plasma membrane (Stacey & Sorensen, 2011). Fish olfactory neurons interact almost directly with odorants in the surrounding water and are only protected by the nasal cavity and mucous layer (Tierney *et al*., 2010). The olfactory epithelium lines the cavity floor forming a rosette-like arrangement of folds or lamellae (Hara, 1994; Hara, 2011b). The fish olfactory system has a similar structure to that of other vertebrates and consists of olfactory receptor neurons (ORNs) which express olfactory receptor molecules, olfactory bulbs, where ORNs with the same receptor types converge in glomeruli, output neurons (mitral cells) and their terminal regions in several brain regions (telencephalon, hypothalamus and others; reviewed by Stacey & Sorensen, 2006; Stacey & Sorensen, 2009; Stacey & Sorensen, 2011).

Sensing of chemosignals in the environment is critical for fish as it conveys information crucial for health, survival and reproduction (Spehr & Munge, 2009). Studies conducted with various species have shown their ability to recognize their own smells (Thünken *et al*., 2009) and discriminate conspecific and heterospecific odours (Stacey &Sorensen, 2011). This is important, for instance, for hierarchical stability in the Nile tilapia (Giaquinto & Volpato, 1997; Gonçalves-de-Freitas *et al*., 2008).

Broadly speaking, ‘a pheromone’ can be defined ad any substance (or mixture of compounds) that is released by an individual and is able to cause a specific and adaptive response in individuals of the same species and does not require learning (Stacey & Sorensen, 2011). The term ‘hormonal pheromone’ is narrower and refers to a pheromone that contains at least one hormonal compound (hormone, hormonal metabolite or precursor; Stacey & Sorensen, 2011). The role of pheromones has been studied in different aquatic animals, including fish and invertebrates. For instance, a multicomponent pheromone released by male blennies (*Salaria pavo*) was shown to be an attractant for mature females (Serrano *et al*., 2008) while the odour of pre-ovulatory carp and goldfish females is able to increase milt quality and volume in males within several hours (Stacey, 2010; Stacey & Sorensen, 2011).

Depending on the species, steroids, bile acids, amino acids and prostaglandins (including their precursors and derivatives) are substances that have been found to play pheromonal roles in fish (Hara, 1994; Stacey & Sorensen, 2006). For example, male goldfish release large quantities of androstenedione, a steroid hormone, which functions as a pheromone (Sorensen *et al*., 2005). However, the high olfactory sensitivity of fish to amino acids is not fully understood. It is believed to be involved mainly in feeding, but also migration, kin recognition and reproduction (Kawabata, 1993; Hara, 1994; Laberge & Hara, 2001; Yambe *et al*., 2006). Amino acids form an essential part of fish diet but, even so, some of them are excreted in the urine after feeding (Ng *et al*., 1996). A significant amount of amino acids is released in the urine of mature rainbow trout (Sato & Suzuki, 2001), irrespective of prior feeding. Yambe *et al*. (2006) characterized L-kynurenine, a major metabolite of L-tryptophan, as a 'non-hormonal pheromone' in female masu salmon (*Oncorhynchus masou*). Released in the urine of mature females, it functions as a male attracting-pheromone by advertising the reproductive status of the female. Therefore, it is suggested that, as an odour is rarely comprised of a single compound, amino acids may sometimes be a part of the complex urinary clue (Ache & Young, 2005, Spehr & Munge, 2009).

The Mozambique tilapia (*Oreochromis mossambicus*) is a maternal mouth-brooding cichlid (Stickney, 2000; Schultz, 2004) naturally distributed in the rivers of southern Africa (Van der Waal, 2012). Due to its high tolerance of diverse environmental conditions, it has become the second most produced tilapia species in the world (after Nile tilapia; El-Sayed, 2006; Russell, 2012). *O.mossambicus* is omnivorous and polygynous (Lovell, 2002; Pestsmart, 2012; Russell, 2012). Males dig circular nests with their mouths, forming dense breeding areas ('leks') (Russell, 2012). Dominant males adopt characteristic black coloration and defend their territories, whereas subordinate males rarely change their coloration and do not court females (Barata *et al*., 2008). Tilapia species are prolific spawners and start reproducing from 5-6 months (Lovell, 2002; Pestsmart, 2012). Fertilized eggs and consequent larvae are incubated in the female's mouth for 20-22 days (Pestsmart, 2012). Dominant males have larger urinary bladders than subordinates and store substantially more urine for release in appropriate context (Barata *et al*., 2007; Barata *et al*., 2008; Keller-Costa *et al*., 2012). Males are able to discriminate the odour of pre-ovulatory females from post-ovulatory. This, in turn, evokes an increase in urination frequency (Miranda *et al*., 2005; Barata *et al*., 2008). Dominant males

also transfer chemical information of high olfactory potency during aggression by increasing their urination rate (Barata *et al*., 2007).

Previous research in our laboratory has identified a steroid glucuronide in male urine, which has a strong pheromonal (endocrine) effect on females. This substance could account for most of the olfactory potency of the non-polar fraction. The concentration of this glucuronide in the urine is positively correlated with male dominance (data not published). Nevertheless, the polar fraction also contains potent odorants; the compounds responsible for this olfactory potency are still to be identified.

The current research consisted of four parts. Firstly, the olfactory sensitivity of Mozambique tilapia to 20 amino acids was assessed using the electro-olfactogram (EOG). Secondly, we identified amino acids present in male urine. Thirdly, we estimated whether the olfactory potency of the polar fraction of male urine can be explained by these amino acids, or whether some other – as yet unidentified - odorants are also present. In addition, urine filtrate and artificially prepared amino acid mixture of the urine of Nile tilapia (*Oreochromis niloticus*), a species closely related to the Mozambique tilapia, were used to identify their olfactory potency for *O.mossambicus* and to compare the results for the two species. Finally, concentrations of the main amino acids were measured in urine of males of different social status to explore possible correlations between amino acid concentration and hierarchical status.

2. Materials and methods

2.1. Experimental animals

Mature Mozambique tilapia (*Oreochromis mossambicus*) were raised in captivity and maintained at the University of Algarve (Portugal). Males and females were kept together in plastic tanks (119 x 155 x 100 cm; 1800 l) with sand substrate and constant aeration. The photoperiod was 12h L: 12h D, and the water was maintained at 25 - 27 °C. Fish were fed once a day with commercial cichlid pelleted feed. In total 24 animals (15 females and 9 males) were used for the EOG recording (SL = 163 ± 23 mm, BW = 70 ± 34 g, gonadosomatic index (GSI = gonad weight/body weight x 100), 4.76 ± 4.6% for females and 0.38 ± 0.16% for males). 22 males of different social status were used for the assessment of amino acid concentrations in the urine filtrate (SL = 160 ± 21 mm; BW = 133 ± 47 g).

2.2. EOG recording

Electro-olfactogram recordings were performed based on the method previously described for Mozambique tilapia (Frade *et al*., 2002; Miranda *et al*., 2005; Barata *et al*., 2007; Barata *et al*., 2008). Fish was anaesthesized in fresh aerated water containing 200 $\mathrm{mg\,l^{-1}}$ MS222 (3-aminobenzoic acid ethylester, Sigma-Aldrich, Germany) and 400 $\mathrm{mg\,l^{-1}}$ sodium bicarbonate for pH buffering. Then the animals were immobilized with an intramascular injection of gallamine thriethiodide (3 $\mathrm{mg\,ml^{-1}}$ of 0,9% NaCl solution) applied in the dosage 1 $\mathrm{ml\,kg^{-1}}$ body weight. The anaesthesized and immobilized fish was wrapped in wet paper and placed in a padded Perspex V-clamp with their gills irrigated with dechlorinated charcoal-filtered and aerated tap water containing 100 $\mathrm{mg\,l^{-1}}$ of MS222 delivered *via* a silicone tube placed in the mouth. The olfactory rosette was exposed by cutting a part of cartilage around the nostril. The epithelium was constantly irrigated during the experiment with dechlorinated and charcoal-filtered tap water running at 6 $\mathrm{ml\,min^{-1}}$. Recording and reference electrodes used to register DC voltage were made from two glass micropipettes and filled with agar gel (4% agar in 0.9% NaCl plus a few drops of food colouring). The recording electrode was placed close to the olfactory epithelium, between two lamellae. This position provided the highest response to 10^{-5} M L-serine standard solution. Reference electrode was located on the head, close to the exposed nostril, slightly pressing the skin (Figure 1).

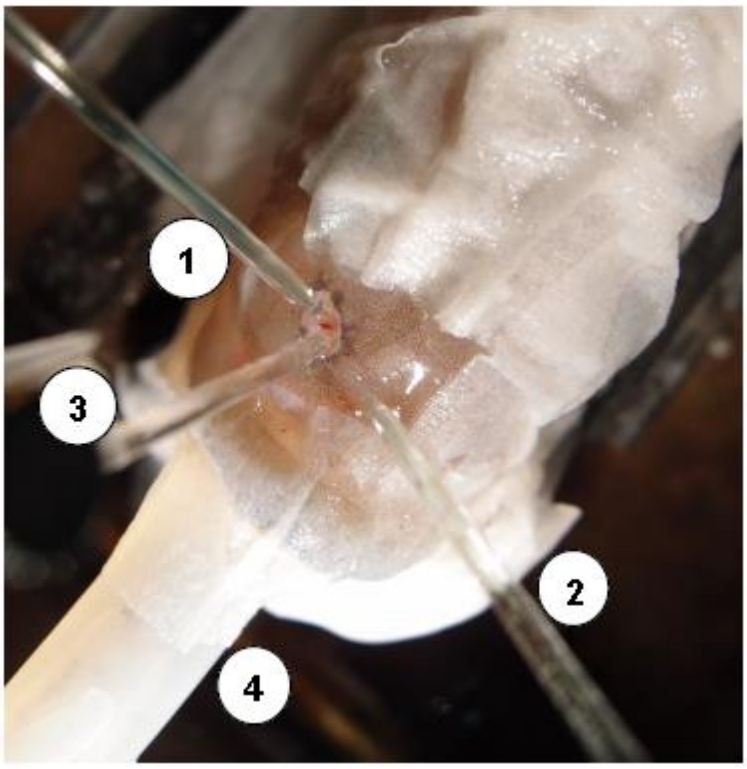


Figure 1. Setting for the EOG recording: 1 - recording electrode, placed in proximity to the olfactory epithelium; 2 - reference electrode, slightly pressing the skin near the open nostril; 3 – stimulus delivery tube; 4 – mouth tube.

Stimuli solutions were delivered *via* a computer-controlled three-way solenoid valve. Successive stimuli were introduced after an interval of at least 1 minute. Standard solution of 10^{-5} L-serine and blank (dechlorinated, charcoal-filtered tap water not containing any odorant) were run before and after a series of concentrations for each stimulus. Response signals were amplified (x 10^{3} - for signals below 7 mV and x10^{2} - for signals above 7 mV). The recording was done using computer software (Axoscope version 9.1, Molecular Devices, Sunnyvale, CA, USA). The amplitude of the first peak was measured (Figure 2). The obtained value was blank-subtracted and normalized to 10^{-5} M L-serine (the values of standard and blank preceding the concentration range of a given stimulus were used).

At the end of the experiment the fish was killed with a sharp blow on the head and standard length (SL), body weight (BW) and gonad weight (GW) were measured and gonadosomatic index (GSI) was calculated.

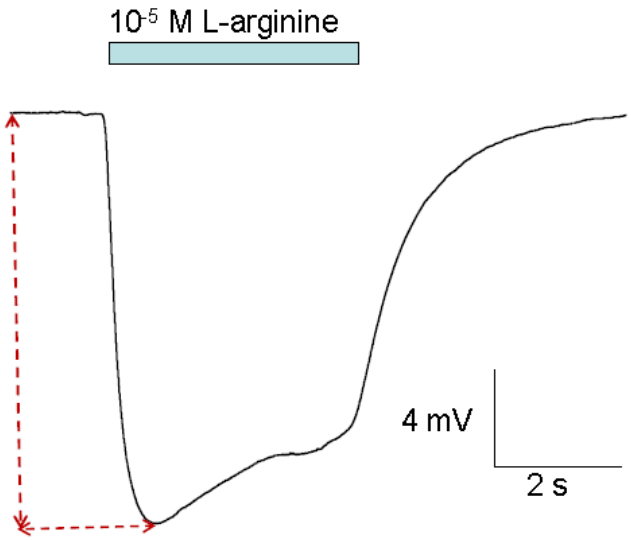


Figure 2. Typical electroolfactogram (EOG) for 10^{-5} M L-arginine and measurement of the amplitude of the first peak.

2.3. Stimuli preparation

2.3.1. Amino acids

20 amino acids were assessed for their olfactory potency in Mozambique tilapia (Table 1). Only L-isomers were used since they constitute the major part of naturally occurring amino acids. Moreover, L-isomers generally have greater olfactory potency than D-isomers (Hara, 2011c). A range of concentrations from 10^{-4} M to 10^{-9} M was prepared for each amino acid using dechlorinated charcoal-filtered tap water and frozen stock aliquots with the initial amino acid concentration 10^{-2} M or 10^{-3} M. All stimuli solutions were prepared on the day of experiment.

Table 1. Amino acids used for olfactory potency assessment by EOG

Essential	**Nonessential**
L-arginine	L-alanine
L-histidine	L-asparagine
L-isoleucine	L-aspartic acid
L-leucine	L-cysteine
L-lysine	L-glutamic acid
L-methionine	L-glutamine
L-phenylalanine	Glycine
L-threonine	L-proline
L-tryptophan	L-serine
L-valine	L-tyrosine

2.3.2. Amino acid mixtures

Stock solutions of amino acid mixtures were prepared based on the concentrations of predominant amino acids in the filtrates of pooled urine from dominant Mozambique and Nile tilapia (Table 2). Frozen aliquots of amino acid mixtures were used as stocks for the series of stimuli with different dilutions (1:100; 1:1000; 1:10000; 1:100000; 1:1000000). All dilutions were prepared on the day of experiment using dechlorinated charcoal-filtered tap water.

Table 2. Concentrations of amino acids in the urine of *O.mossambicus* and *O.niloticus*

Amino acid	***O.mossambicus* mixture (amount in mM)**	***O.niloticus mixture* (amount in mM)**
L-arginine	1.95	2.07
L-glutamic acid	0.71	0.3
L-phenylalanine	0.51	N/A
L-asparagine	N/A	1.88
L-tyrosine	N/A	1.03
L-histidine	N/A	0.29
L-lysine	N/A	0.40
L-alanine	N/A	0.27
L-leucine	N/A	0.18

2.3.3. C18 solid phase extraction of polar fraction (filtrate) from tilapia urine samples

Urine samples from individual dominant, intermediate and subordinate males diluted 1:2 v/v in ultra-pure water were passed through solid phase extraction cartridges (500 mg C18 sorbent, 6 ml glass reservoir, Isolute®, Biotage). The polar aqueous phase (filtrate) was collected and stored at -20°C for further analysis. Non-polar compounds retained by the cartridge were eluted with methanol afterwards.

2.4. Identification and quantification of amino acids in urine

2.4.1. Preparation and derivatization of urine samples

Urine samples were diluted 1:2 in ultra-pure (Milli-Q) water and then passed through C 18 solid phase extraction cartridges. The resultant filtrates were derivatized with with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Supelco, Sigma Aldrich, Portugal) plus trimethylchlorosilane (TMCS; Merck, Sigma Aldrich, Portugal) following the procedure described by Deng *et al*.(2005).

Briefly, samples (300 µL) and the internal standard (L-Norvaline (Sigma Aldrich, Portugal), 5µg mL^{-1}) were placed into a 1.5mL screw-cap vial and lyophilized. When completely dry, 100µL of acetonitrile and 100µL of BSTFA + TMCS (98:2, v/v) were

added. The reaction was performed under microwave irradiation at a power of 750W for 60s. After cooling to room temperature, 1µL of each sample was injected into the GC-MS. Each sample was prepared in triplicate.

2.4.2. Gas chromatography and mass spectrometry (GC-MS)

The GC-MS system employed was a Varian 450-GC, 240 MS ion trap detector (Emilio de Azevedo Campos, SA, Portugal). The capillary column used for simple chromatography was a 30m x 0.25 mm I.D.; 0.25µm film thickness (Bruker, BR-5MS, Bruker, Portugal). Helium at a flow rate of 1mL min^{-1} was used as carrier gas. The initial temperature of the oven was set at 60°C during 5 min followed by an increase to 80°C at 20°C min^{-1} and then to 150°C at 5°C min followed by an increase to 280°C at 15C min^{-1} and this final temperature was maintained for 5 min. The temperature of the injection port was set at 250°C and that of the transfer line and the trap at 220°C and 250°C respectively. Splitless mode was elected; the mass-selective detector was operated in electron impact (EI) mode at 70eV of activation energy. To confirm the mass fragment of the derivatives, data were obtained in the full scan mode from m/z 70 to 250. The examples of a chromatogram and mass spectra are represented in Figures 3 and 4.

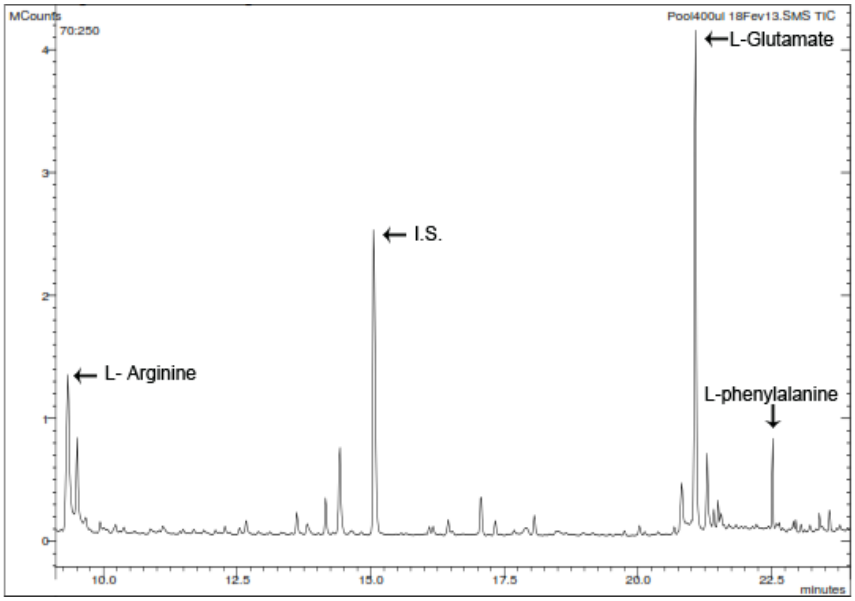
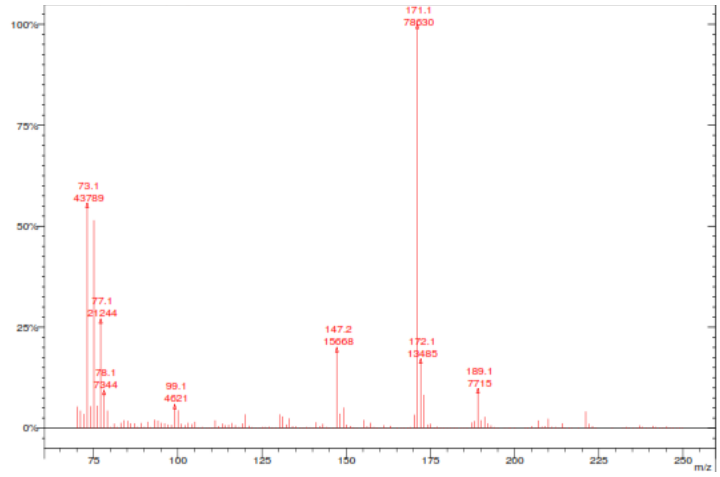
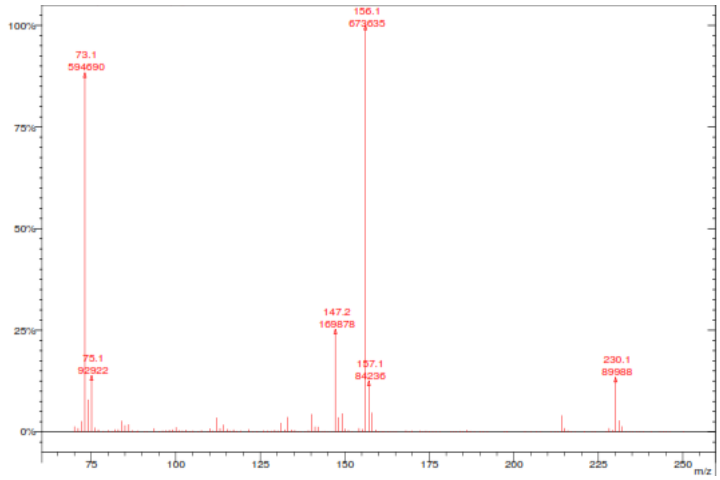


Figure 3. Example of a urine sample chromatogram with the peaks of the three amino acids of interest and the internal standard (I.S.) L-norvaline.

a)



b)



c)

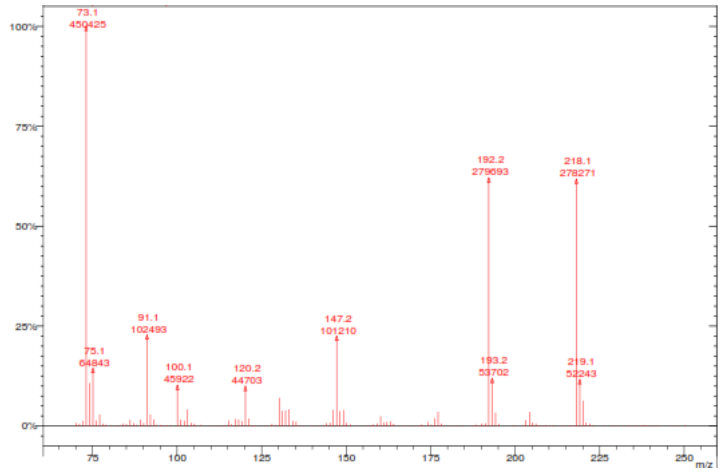


Figure 4. Mass spectra of L-arginine (a), L-glutamic acid (b) and L-phenylalanine (c) in a urine sample.

2.4.3. Quantification of amino acids

To construct the calibration curves, amino acids (L-glutamic acid, L-arginine and L-phenylalanine) were prepared at various concentrations (5, 12.5, 25, 37.5 and 50 µg ml^{-1}) and treated with the same derivatization and GC-MS analytical procedure as for the samples. The calibration curves were obtained by plotting the peak area ratio between the derivatives of amino acids and that of L-norvaline (internal standard). To measure the limits of quantification and detection, standards were serially diluted and processed according to the procedure described above.

2.5. Assessment of male social status and calculation of dominance index (DI).

Five males and five females of similar standard length and body weight (coefficient of variation of body weight less than 5%) were assigned to each social group. Males were marked with coloured plastic tags (T-bar anchor FD94, Floy Tag Inc., Seattle, WA, USA) attached to the muscle near the dorsal fin. Regular focal observations of each male started from day four after the group establishment and were performed following the procedure described by Keller-Costa *et al*. (2012).

Dominance index (DI) was calculated for each male each day as a division of the sum of all dominant behaviours by the sum of all dominant and subordinate behaviours and ranged from zero to one (Barata *et al*., 2007; Keller-Costa *et al*., 2012). Finally, the mean DI was calculated for each male after 5 days of observation. The individuals with DI below 0.16 were selected as subordinates while those with the DI above 0.8 were assigned as dominant males. The males with DI in the range from 0.2 to 0.5 were selected as intermediates. After the daily observations, urine samples were collected from each male by a gentle squeeze of the area immediately above and anterior to the urogenital papilla. These samples were stored at -20°C for further analysis.

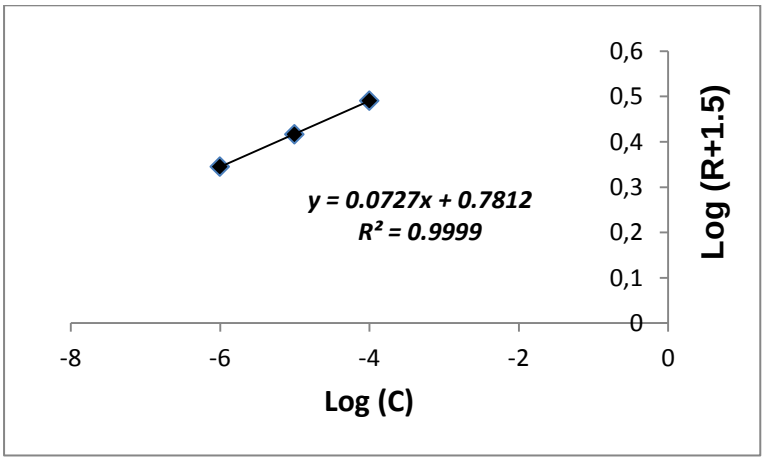
2.6. Data analysis

The normalized EOG responses for the tested stimuli (amino acids, filtrates and mixtures) were used for statistical analysis. For each odorant only concentrations that elicited responses significantly greater than zero were chosen. The data were log-transformed as previously described (Hubbard *et al*., 2003; Hubbard *et al*., 2011).

To estimate thresholds of detection, linear regression lines were built for each repetition of every odorant examined using the formulas: y = log (N+1.5), where N is a normalized response, and x = log (C or D), where either a concentration (C) or a dilution (D) was utilized depending on the odorant. Threshold values were estimated from the equation of the regression line, y = a*Log (C) + b, where a and b are constant values, and y = 0.1761 (the value calculated from the equation
y = log (N+1.5), when N = 0), (Figure 5a).

Factor γ (gamma), which represents a number of log units of concentration change per one log unit of change in the response (Hubbard *et al*., 2011), was employed to infer about differences in slopes of the regression lines for the stimuli tested. In this case linear regression lines were plotted as log (C or D) vs log (R), where R is a response to a given concentration of an odorant. Based on the equation of the linear regression line obtained (y = ax + b), factor γ was taken as a value equal to constant a of the equation (Figure 5b).

a)



b)

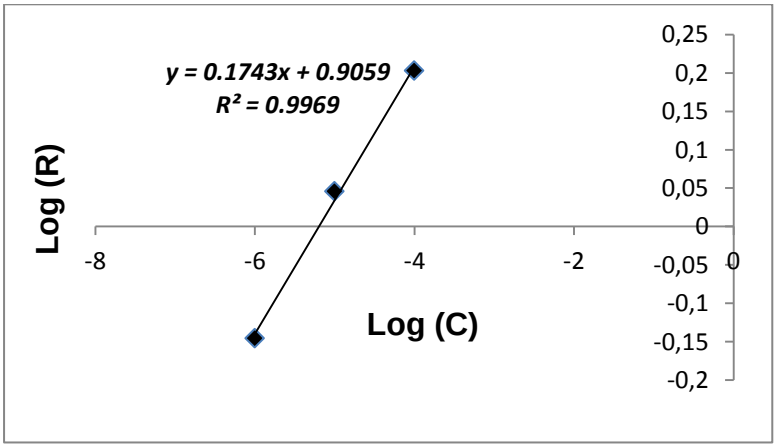


Figure 5. Examples of linear regression lines used for L-arginine:
a) Calculation of threshold of detection (constant a = 0.0727; constant b = 0.7812; y = 0.1761). Therefore, Log [C] = (0.1761-0.7812)/0.0727; C =$10^{-8.3}$.
b) Estimation of *γ*-factor (*γ* = 0.1743).

2.7. Statistical data treatment

Statistical analysis was performed using SigmaStat statistical software. Kruskal-Wallis One Way Analysis of Variance on Ranks followed by All Pairwise Multiple Comparison Procedures (Dunn's Method) were employed for the analysis of threshold data for the amino acids studied. One-way ANOVA followed by All Pairwise Multiple Comparison Procedures (Tukey Test) were used to compare *γ*-factors for the amino acids. A Paired Two-Sample t-Test for Means was applied to compare threshold and gamma values between filtrates and mixtures for *O.mossambicus* and *O.niloticus* and also between the two species.

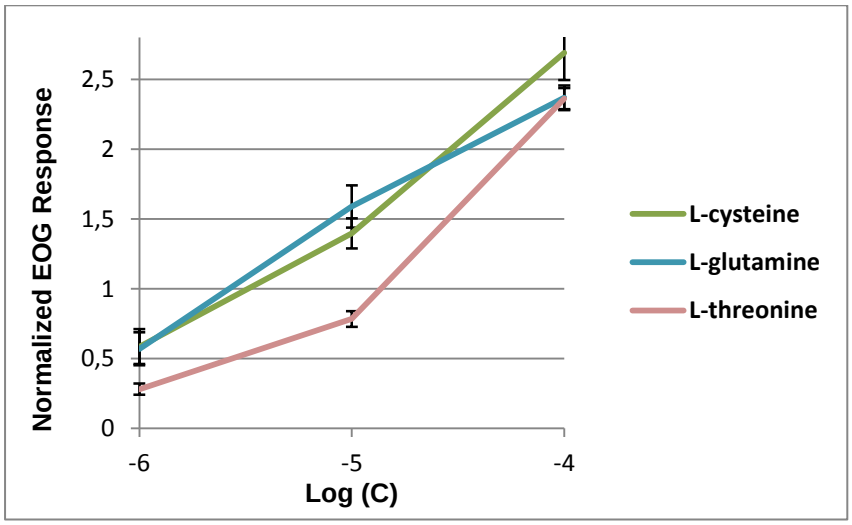
To characterize the relationship between the hierarchical status of Mozambique tilapia males and the quantities of amino acids released in the urine, the amounts of L-arginine, L-glutamic acid and L-phenylalanine calculated in the urine samples using GC-MS method were correlated with fish dominance index, standard length and body weight. Numerical values and the signs of the correlation coefficients (positive or negative) as well as p-values for each correlation were used to make an inference about the relationship between the variables. At all stages of data analysis $P<0.05$ was considered statistically significant.

3. Results

3.1. Olfactory sensitivity to amino acids

Mozambique tilapia has olfactory sensitivity to all twenty amino acids tested. However, semi-logarithmic plots of normalized EOG responses to different concentrations of the stimuli versus log (C) were not identical in shape for different amino acids (Figure 6). L-cysteine, L-glutamine and L-threonine were the most potent stimuli at the highest concentration tested (10^{-4} M); the normalized EOG responses were 2.689, 2.368 and 2.362 respectively. On the other hand, L-proline and L-aspartate were the least potent for *O.mossambicus* at 10^{-4} M with normalized EOG responses of 0.796 and 0.586. Among the three amino acids most abundant in the urine of dominant male Mozambique tilapia, L-arginine had the highest olfactory potency at 10^{-4} M, though, compared to other amino acids tested, its normalized EOG response (1.766) was just above the average (1.638). L-glutamic acid and L-phenylalanine had similar normalized EOG responses 1.156 and 1.145 respectively); lower than those for L-arginine and the average for the twenty amino acids.

a)



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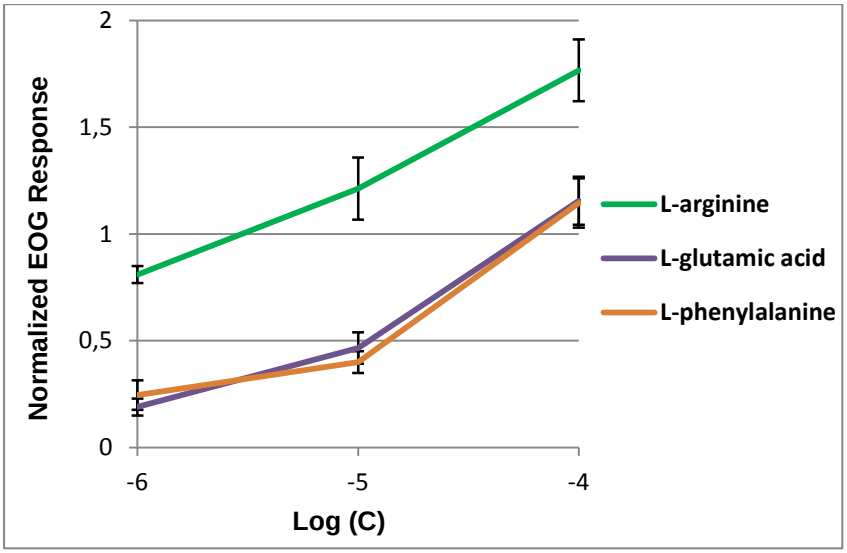


c)

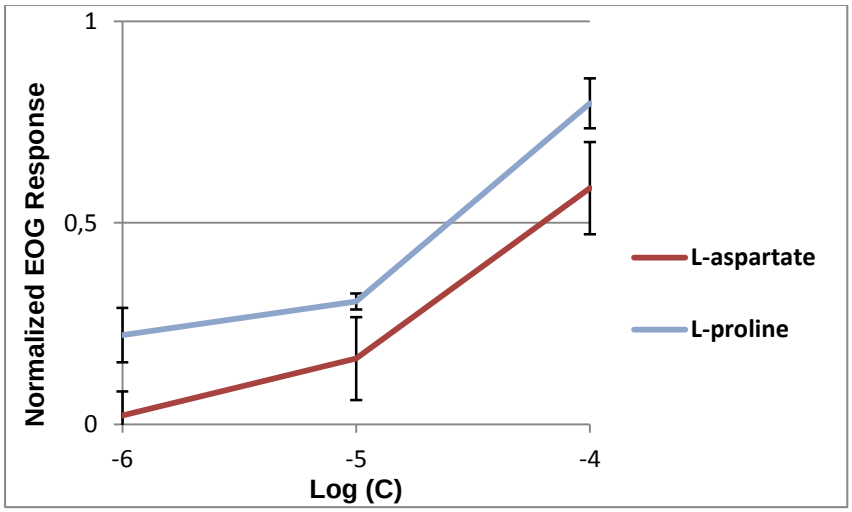


Figure 6. Normalized EOG responses to eight representative amino acids at different concentrations (the bars represent ± SEM to the mean, n = 6, 7 or 8).

The estimated median values of the lowest concentrations of the amino acids detected (thresholds of detection) were statistically different (*P*=<0,001). The *post-hoc* analysis of data (All Pairwise Multiple Comparison Procedures, Dunn's Method) allowed the identification of 4 groups of amino acids (*a*, *b*, *c*, *d*) accordingly (Figure 7).

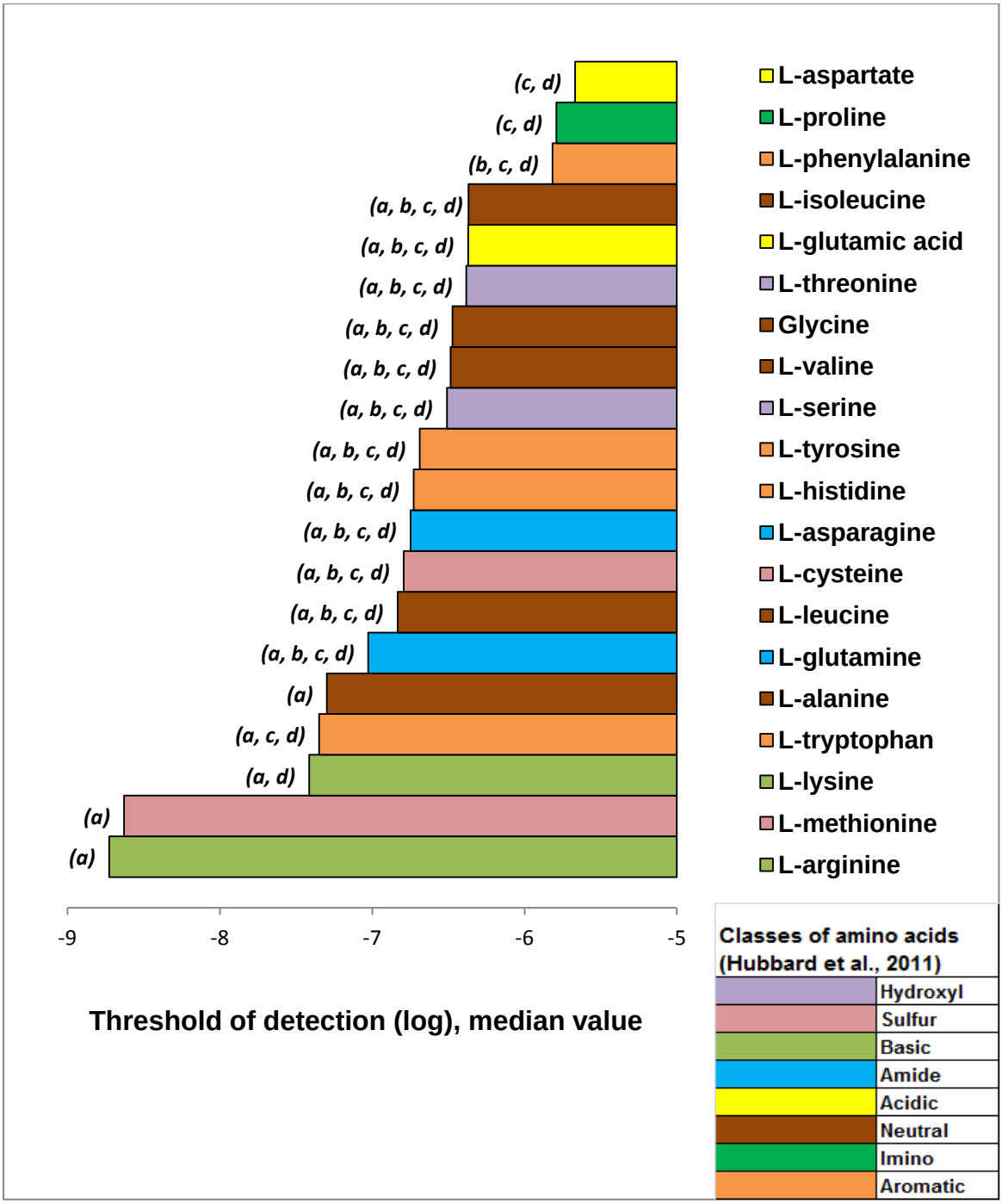


Figure 7. Grouping of amino acids according to the statistical similarity of their thresholds of detection.

While the majority of the odorants belonged to all four groups with no significant differences among the odorants within each group (P<0.05), two amino acids belonged only to three groups (*a*, *c*, *d* – for L-tryptophan that had high olfactory potency, and *b*, *c*, *d* – for L-phenylalanine, one of the least potent amino acids). L-lysine, with one of the lowest thresholds of detection, was assigned to groups *a* and *d*. L-alanine, L-methionine and L-asparagine were also among the most potent amino acids and were the only ones belonging to group *a* only, with L-arginine showing the lowest threshold of detection of all amino acids ($10^{-8.7}$ M). L-aspartate and L-proline were both referred to groups *c* and *d* only and, conversely had the highest thresholds of detection ($10^{-5.7}$ M and $10^{-5.8}$ M correspondingly) among all amino acids examined.

Statistical analysis of ɣ-factors found differences (P<0.001) among the tested amino acids. The *post-hoc* All Pairwise Multiple Comparison Procedures (Tukey's Test) revealed three distinct groups of amino acids according to similarity of their *ɣ*-factors (Figure 8). This similarity means that the stimuli in each group have statistically identical slopes of their log (C) vs log (R) regression lines that could be indicative of similar olfactory sensitivity of the Mozambique tilapia to changes of concentration.

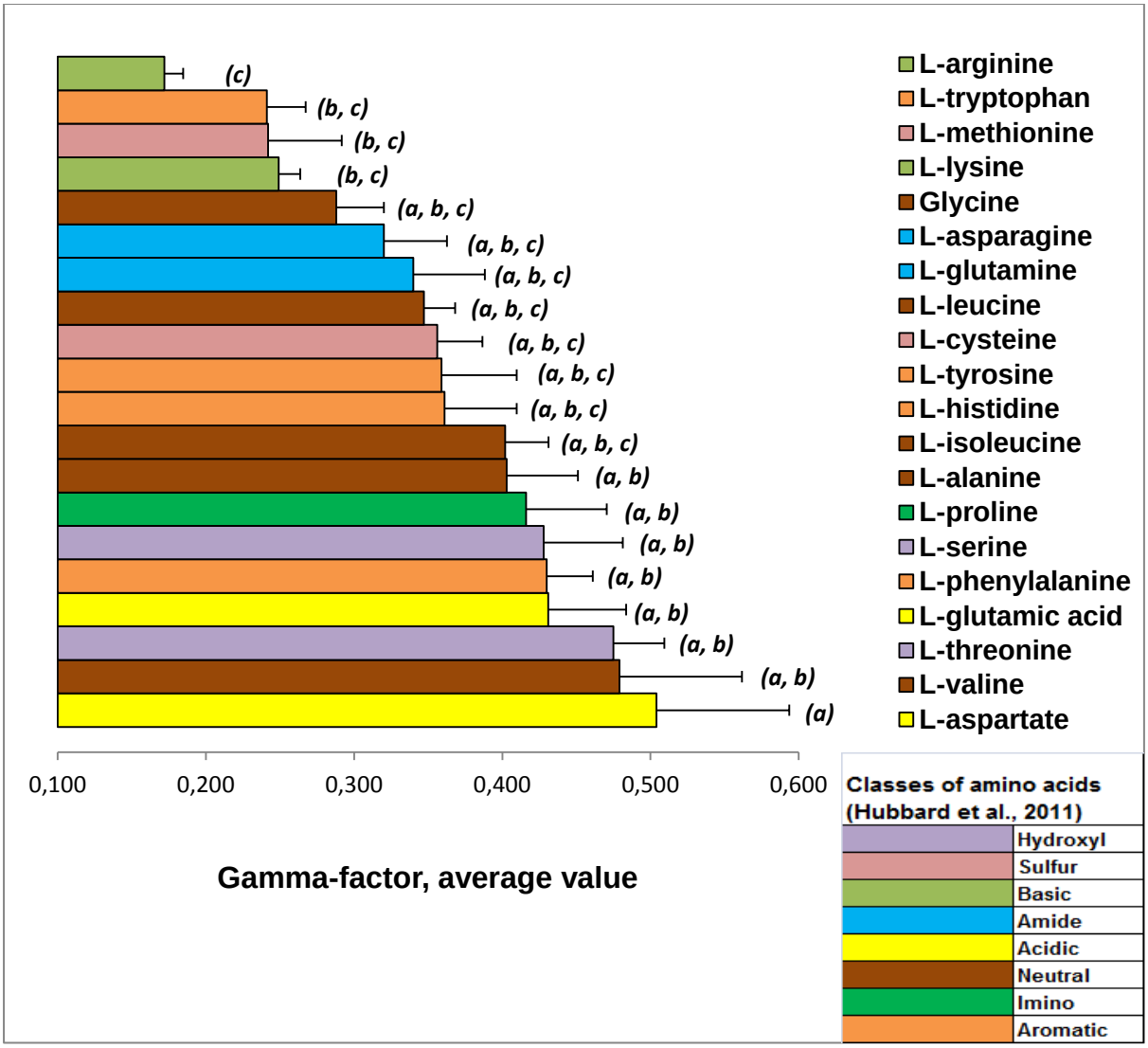


Figure 8. Grouping of amino acids according to the statistical similarity between the values of *γ*-factors.

Here only eight amino acids were found to belong to all three groups (*a*, *b* and *c*). The tilapia was most sensitive to changes in the concentration of L-aspartate (*γ*=0.504), and this was the only stimulus included just in group *a*. On the contrary, the fish has low sensitivity to concentration changes in L-arginine (*γ*=0.172); this was a unique amino acid in group *c* only. Three more odorants following L-aginine in the order of increasing *γ*-factor (L-tryptophan (0.241), L-methionine (0.242) and L-lysine (0.249)) belonged to both *b* and *c* groups while six amino acids, L-valine (0.479), L-threoine (0.475), L-glutamic acid (0.431), L-phenylalanine (0.430), L-proline (0.416) and L-alanine (0.403), with sequentially increasing *γ*-factors and located above L-aspartate on the scale, were placed together to both *a* and *b* groups.

Figures 7 and 8 also show the differences between the classes of amino acids based on their structure and chemical characteristics. Basic amino acids had the lowest thresholds of detection whereas those from imino and acidic groups were the least potent. The sensitivity of tilapia olfactory system to other amino acid classes was variable.

In addition, basic amino acids generally had lower ɣ-factors than amino acids from other classes. On the contrary, *ɣ*-factors of the odorants from acidic and hydroxyl classes were generally high.

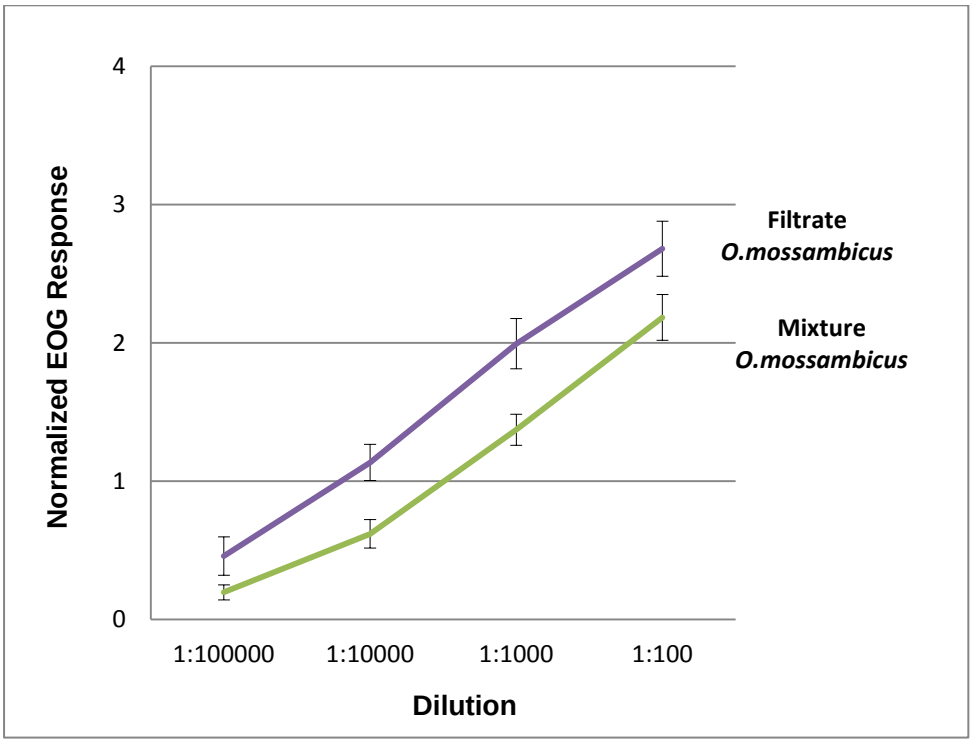
3.2. Urine filtrates and amino acid mixtures

Urine filtrates and amino acid mixtures for *O.mossambicus* and *O.niloticus* were found to have different olfactory potency for Mozambique tilapia. The graphs of normalized EOG responses versus dilution (Figure 9) indicate that at minimum dilution (1:100) filtrates of *O.niloticus* and *O.mossambicus* elicited larger responses than those for respective mixtures (3.638 and 2.680 for Nile tilapia and Mozambique tilapia filtrates versus 2.491 and 2.183 for corresponding mixtures).

Paired Two-Sample t-Test for Means revealed significantly higher olfactory potency of *O.mossambicus* urine filtrate in comparison to the artificial amino acid mixture for this species (t-Stat=2.489; t critical two-tail=2.364; $P<0.05$). The estimated threshold of detection for *O.mossambicus* mixture was at $10^{-5.4}$ dilution while that for the filtrate was $10^{-6.2}$. However, statistical comparison of olfactory potency of urine filtrate and amino acid mixture of Nile tilapia for the Mozambique tilapia identified that the threshold of detection for the mixture ($10^{-5.8}$ dilution) was lower than that for the filtrate ($10^{-5.1}$ dilution) (t stat= -4.465; t critical two-tail=2.365; $P<0.05$).

ɣ-factors were neither statistically different for mixture and filtrate of *O.mossambicus* (t stat=1.529; t critical two-tail=2.365; $P>0.05$) nor for mixture and filtrate of *O.niloticus* (t stat= -1.345; t critical two-tail = 2.365; $P>0.05$).

a)



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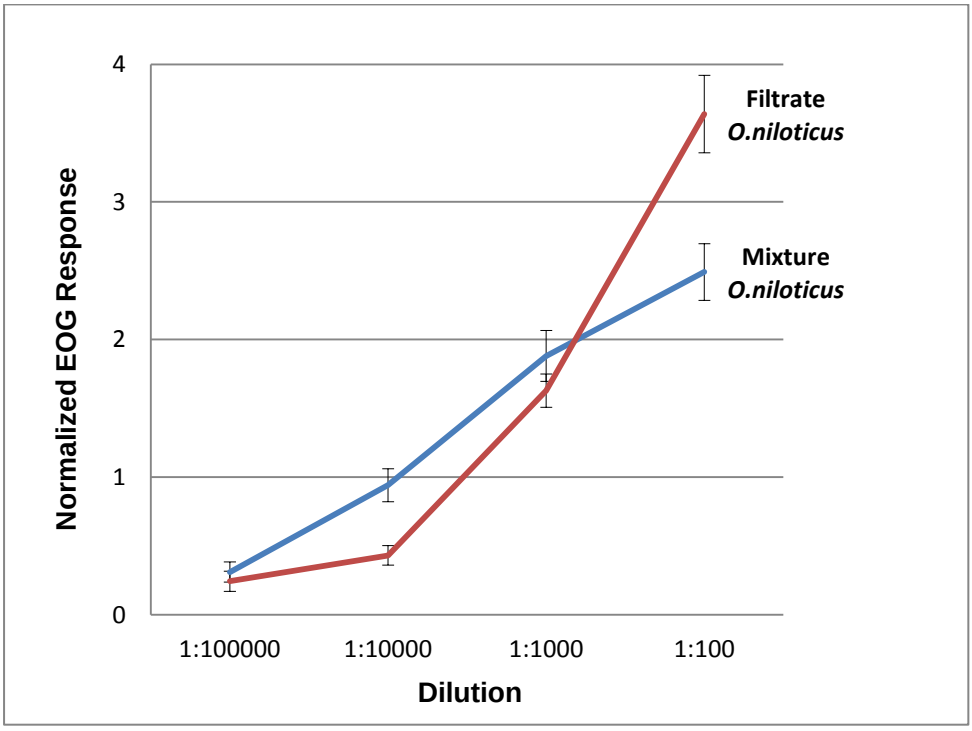


Figure 9. Normalized EOG responses of Mozambique tilapia to urine filtrates and amino acid mixtures of *O.mossambicus* (a) and *O.niloticus* (b) applied at different dilutions.

Since the predominant amino acids identified in the urine of Nile and Mozambique tilapia were different, the artificial amino acid mixtures were correspondingly different. Therefore, only thresholds of detection and *γ*-factors for urine filtrates were used for comparison between the species. Similarly to the above-mentioned results, the analysis of *γ*-factors did not demonstrate significant difference between the filtrates of *O.niloticus* and *O.mossambicus* (t stat=2.173; t critical two-tail=2.365; *P*>0.05). On the contrary, thresholds of detection for both filtrates were not the same (t-stat=3.321; t critical two-tail=2.365; p<0.05), with the urine filtrate of Nile tilapia being less potent for the olfactory system of Mozambique tilapia than the conspecific filtrate ($10^{-5.1}$ dilution and $10^{-6.2}$ dilution thresholds correspondingly).

3.3. Relationship between dominance index and concentration of amino acids in male *O.mossambicus* urine

The concentrations of L-arginine, L-glutamic acid and L-phenylalanine in the urine from males showed different patterns of correlation with dominance index (Figure 7).

The concentration of L-arginine in the urine was positively correlated with fish dominance index (R=0.495; *P*<0.05, Figure 10a). L-arginine was present at substantially higher concentrations in the urine of dominant males compared to that from intermediate and subordinate groups. F-test Two-Sample for Variances showed much greater variability in L-arginine concentration in dominant males (DI>0.5) than in subordinate males (DI<0.5; *P*<0.05, F=234.33, F crit. one-way=2.849).

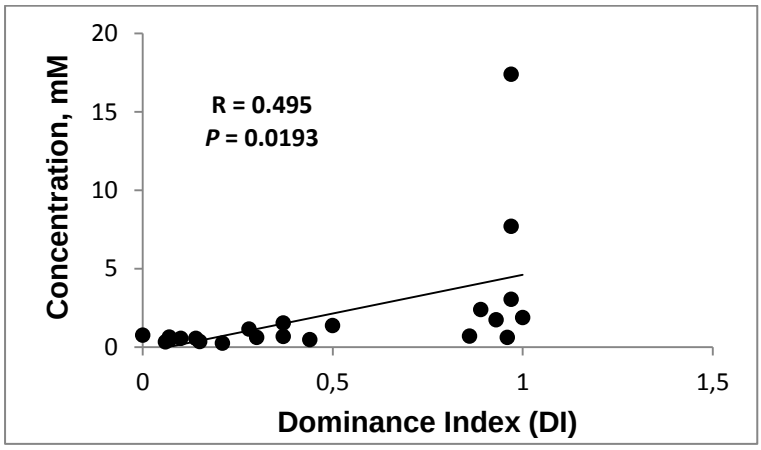
Similarly to L-arginine, the concentrations of L-glutamic acid showed higher degree of variability in dominant males than in subordinates (*P*<0.05, F=16.36, F crit. one-way=2.85). However, in this case no statistically significant correlation was seen (R=0.399; *P*=0.0657; Figure 10b).

The urinary concentrations of L-phenylalanine had similar variability in dominant and subordinate groups (*P*>0.05, F=1.37, F crit. one-way=2.85) and were not correlated with dominance index (R= -0.385, *P*=0.0769, Figure 10c). In all groups the concentrations of L-phenylalanine in the urine were negligible compared to L-arginine and L-glutamic acid.

Additionally, a strong negative correlation was discovered between the concentration of L-arginine in the urine of males of different social status and their body weight (R= -0.454, *P*=0.0337), but no significant relationship was proved

between fish body weight and dominance index (R= -0.162, p=0.471). Furthermore, no correlation was seen between urinary L-arginine and L-glutamate concentrations.

a)



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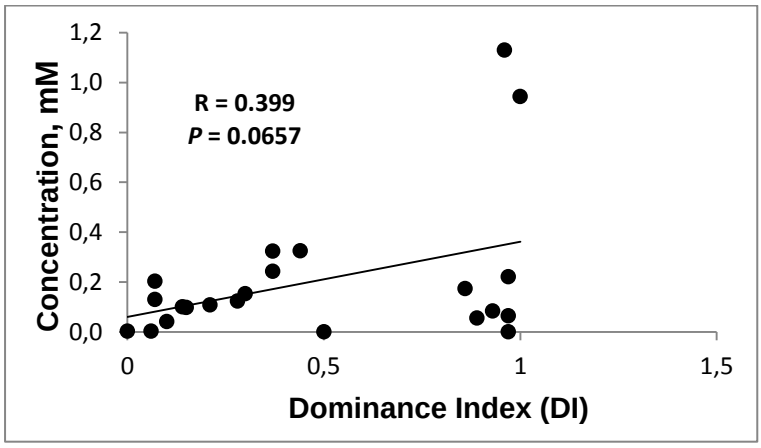

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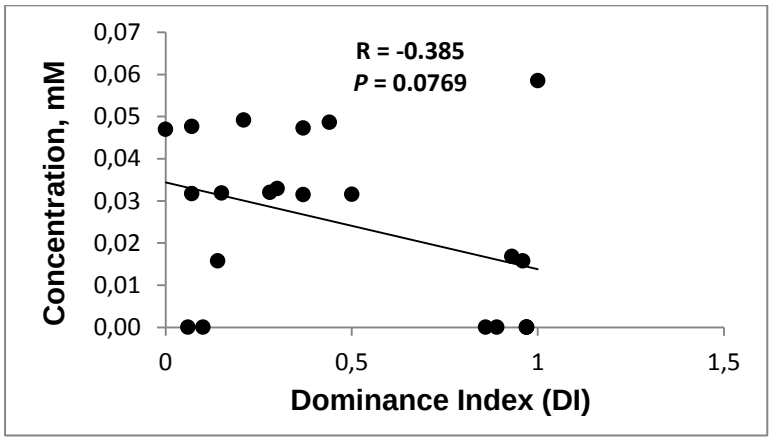

Figure 10. Correlation between dominance index (DI) and concentrations of amino acids in Mozambique tilapia urine
a) L-arginine
b) L-glutamic acid
c) L-phenylalanine

4. Discussion

4.1. Olfactory sensitivity to amino acids

The current study demonstrated the ability of *O.mossambicus* to smell amino acids in the water. L-cysteine, L-glutamine and L-threonine were the most potent at 10^{-4} M, but their thresholds of detection varied from 10^{-7} M in L-glutamine (the lowest of the three) to $10^{-6.4}$ M for L-threonine (the highest), and were not outstanding compared to other amino acids tested. This means that even if an odorant evokes a large response when concentrated, it is not necessarily detected when the concentration is low. In natural environments this may be expressed in the distance to the odorant source: while some amino acids can be sensed only in close proximity to the source, the others will be perceived from afar. However, background amino acid concentrations (that are not normally measured) may interfere. Therefore, the importance of amino acid potency and threshold of detection in odorant localization in natural environments requires further investigation.

Amino acids could be assigned to four groups based on their thresholds of detection. Nevertheless, these groups are not strictly distinct as many amino acids were found to belong to several (from 2 to 4) groups. Only the stimuli with the lowest thresholds of detection (L-arginine and L-methionine) could be assigned to a single group that, possibly, indicates their importance for *O.mossambicus*. Amino acids with small γ-factors generally possessed lower thresholds of detection than those with large γ-factors, and *vice versa*. This demonstrates low sensitivity of tilapia to the changes of concentration of the amino acids with higher potency. On the other hand, L-aspartate and L-proline had the highest thresholds of detection of all amino acids tested, with L-aspartate being the least potent. At the same time, L-aspartate possessed the highest γ-factor that means that this amino acid elicited more acute olfactory responses to its concentration changes than the others. Other studies also support the exceptional potency of L-arginine (Hara, 1994) and low potency of L-proline (Hara, 1994; Velez *et al*., 2007).

Nevertheless, to fully understand why some amino acids are more potent than others, we need more information. Little is known, for example, about the concentrations of amino acids in the species' natural environment. The current study attempted to explain the roles of L-arginine, L-glutamic acid and L-phenylalanine in

chemical communication between the individuals of Mozambique tilapia, but more research is required to understand the olfactory sensitivity to other amino acids.

Comparison of amino acid classes show the same relationship pattern: basic amino acids were the most potent for *O.mossambicus* and had low γ-factors while imino and acidic amino acids were sensed the least, with the acidic and hydroxyl groups having the largest values of γ-factors. Low potency of imino and acidic amino acids is consistent with other studies across different fish species and habitats (Velez *et al*., 2007; Yacoob & Browman, 2007; Hubbard *et al*., 2011).

Previous studies on olfaction in *O.mossambicus* demonstrated similar sensitivity of the species to L-serine, but the thresholds of detection were lower for L-arginine and L-threonine and higher for L-cysteine (Frade *et al*., 2002). Studies on blackspot seabream (*Pagellus bogarareo*) (Hubbard *et al*., 2011) show sulphur-containing amino acids, L-cysteine and L-methionine, to be most potent for this marine species. The threshold of detection for L-methionine was similar to that of Mozambique tilapia, but the seabream's sensitivity to L-cysteine was higher. Experiments with juvenile Atlantic halibut (*Hippoglossus hippoglossus*) also show olfactory stimulatory effectiveness of L-methionine at 10^{-3} M (Yacoob & Browman, 2007). Even though amino acid potency ranks do not exactly coincide in different species, some of the amino acids are more stimulatory than others, independently of the species studied. Also, amino acids are known to be among the most common feeding stimulants for fish (Rust, 2002). Probably, some of them may act as olfactory stimuli initiating fish arousal and food-search behaviour. In this case dietary preferences of different fish could explain why some amino acids are more potent in one species but not others.

4.2. Olfactory sensitivity of Mozambique tilapia to urine filtrates and amino acid mixtures

Comparison of *O.mossambicus* sensitivity to the polar fraction of conspecific dominant male urine (urine filtrate) and the artificial mixture of predominant amino acids showed higher olfactory potency of the former though γ-factors of the filtrate and mixture were statistically equal. This shows the same sensitivity to concentration changes in both the filtrate and the mixture; however, the lower threshold of detection of the polar urine fraction clearly indicates the presence of some odorants, other than amino acids, that are still to be identified.

Bile salts are the substances that could play a role in species recognition in Mozambique tilapia, even though they are not released in the urine. They are excreted in faeces and account for about 40% of olfactory potency in the case of the Senegalese sole (*Velez et al*., 2009).

Filtrate and mixture of *O.niloticus* also had similar *γ*-factors, but, surprisingly, the amino acid mixture was smelled by *O.mossambicus* at lower concentration than the filtrate. This is hard to interpret biologically since the filtrate comes from a different species.

Interestingly, the between-species filtrate comparison also did not reveal any difference among *γ*-factors; the sensitivity of Mozambique tilapia to concentration changes of filtrates of both species is similar. Even so, for the olfactory system for *O.mossambicus* the conspecific filtrate was more potent than that from *O.niloticus*. The similarity of *γ*-factors of filtrates for different species is not surprising considering that they belong to the same genus *Oreochromis*. Nevertheless, some mechanisms should exist to allow identification among individuals of the same species. Nile and Mozambique tilapias have different distribution ranges, but can easily inter-breed (Van der Waal, 2012). This suggests similarity of their signaling systems. However, would each species preferentially mate with conspecifics or be indiscriminate in mating? No studies have yet addressed this issue. It has been suggested that male odour is able to signal species identity (Rosenthal & Lobel, 2006). Possibly, the presence of a distinct set of amino acids in the urine of each species is a mechanism for species identification. This question requires further investigation.

4.3. Correlations between dominance index and the concentrations of predominant amino acids in Mozambique tilapia urine

The concentration of L-arginine in *O.mossambicus* urine exhibited a strong correlation with dominance index of the fish but this relationship did not exist in case of L-glutamic acid and L-phenylalanine. Since L-phenylalanine is poorly sensed by Mozambique tilapia, and its urinary concentrations were comparatively low, it is unlikely to play a significant role in male status recognition. In case of L-glutamic acid the answer is not obvious as the *P*-value approached the significance level. Additionally, similar patterns of correlation between dominance index and the concentrations of L-arginine and L-glutamic acid, with more variability between the

individuals of high social status, suggesting that further analysis may reveal correlations between the concentrations of L-glutamic acid, L-arginine and dominance index.

The concentration of L-arginine in the urine of males of different social status negatively correlated with body weight. However, no correlation was found between the body weight and the dominance index since males of similar body-weight were chosen – as part of another study – to reduce possible effects of body-size. All fish were provided with the same type of diet; therefore, this factor should not contribute to the observed variation. As L-arginine is an essential amino acid (the only source of L-arginine is the diet) then, probably, the individuals with higher growth rate incorporate more dietary amino acids into body proteins than released in urine. This may occur independently of fish dominant status and could be induced by other factors, such as genetics, health condition and stress.

However, as a strong olfactory stimulus for Mozambique tilapia, and having a significant correlation with male dominance index, L-arginine may play a role in male status recognition by conspecifics. It is well-known that a number of fish species are able to discriminate hormonal compounds (Stacey, 2010). However, hormonal pheromones originate from a homologous and evolutionarily preserved vertebrate endocrine system (Stacey & Sorensen, 2009) that can explain why the hormonal compounds sensed by related genera are similar (Stacey, 2010). Therefore, differences in ratios of hormonal and non-hormonal compounds could serve as a hallmark for species identification (Stacey, 2010). Furthermore, in the case of dominant Mozambique tilapia males, L-arginine could be a part of an individual identity message. The variations in concentrations of L-arginine and L-glutamic acid released in urine seem to indicate not only the hierarchical status of a male but also to distinguish one dominant male from the other. Previous losers of male-male encounters would therefore be able to recognize their victorious opponents, and avoid confrontation. This would then contribute to the stability of the social hierarchy. Olfaction has been found important in recognition of dominance status by American lobsters. The authors suspect that the olfactory cue transmitted by these animals bears both dominance and individual message (Karavanich & Atema, 1991).

Dominant male Mozambique tilapia store more urine than the subordinates (Barata *et al*., 2008), have more muscular urinary bladders (Keller-Costa *et al*., 2012) and should be able to better control the amount of urine released in the presence of

a female or during aggressive encounters (Barata *et al*., 2007). Additionally, some yet unknown renal mechanism is responsible for higher concentrations of L-arginine and L-glutamic acid in the urine of males with higher hierarchical status. Then these amino acids are employed to signal the status of dominant males to their rivals. In this context, it is interesting that Kawabata (1993) showed that L-arginine could induce sexual pecking behaviour and sperm release in male rose bitterling (*Rhodeus ocellatus ocellatus*).

As previously mentioned, L-arginine is an essentail amino acid, crucial for fish nutrition. Further connection between feeding and reproduction is given by the studies of swordtail fish (*Xiphophorus birchmanni*), where females demonstrated preference to the odour of well-fed males and not food-deprived (Rosenthal & Lobel, 2006).

Therefore, L-arginine and L-glutamate could contribute to the mixture of urine odorants transferring the message of social status of a male, its health and suitability for reproduction. Nevertheless, these amino acids cannot explain the full olfactory potency of Mozambique tilapia urine filtrate. Other yet unidentified polar odorants are present in dominant male urine to complement the above-mentioned amino acids and form the natural odour of male *O.mossambicus* urine.

4.4. Conclusions

1. The Mozambique tilapia has olfactory sensitivity to all twenty essential and nonessential amino acids but their potency and ability to evoke a sharp olfactory response through concentration changes are different.

2. The Mozambique tilapia has higher sensitivity to conspecific urine filtrate than to the heterospecific. Olfactory potency of *O.mossambicus* filtrate can be largely but not fully explained by the presence of amino acids (L-arginine, L-glutamic acid and L-phenylalanine). Other unknown polar compounds must be present in male urine to form the full urine odour.

3. L-arginine and L-glutamic acid are important components of the odour of male Mozambique tilapia urine which may indicate the dominance status of the fish and, possibly, individual identity.

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