Transcriptome sequencing of olfactory related genes in olfactory transduction of large yellow croaker (*Larimichthy crocea*) in response to bile salts

Jiabao Hu [1, 2, 3] , **Yajun Wang** [Corresp., 1, 2, 3] , **Qijun Le** [1, 2, 3, 4] , **Na Yu** [1, 2, 3] , **Xiaohuan Cao** [1, 2, 3] , **Siwen Kuang** [1, 2, 3] , **Man Zhang** [1, 2, 3] , **Weiwei Gu** [1, 2, 3] , **Yibo Sun** [1, 2, 3] , **Yang Yang** [1, 2, 3] , **Xiaojun Yan** [Corresp. 1, 2, 3]

[1] Key Laboratory of Applied Marine Biotechnology, Ningbo University, Ministry of Education, Ningbo, China

[2] Key Laboratory of Marine Biotechnology of Zhejiang Province, Ningbo University, Ningbo, China

[3] College of Marine Sciences, Ningbo University, Ningbo, China

[4] Ningbo Entry-Exit Inspection and Quarantine Bureau Technical Centre, Ningbo, China

Corresponding Authors: Yajun Wang, Xiaojun Yan
Email address: wangyajun@nbu.edu.cn, yanxiaojun@nbu.edu.cn

Fish produce and release bile salts as chemical signalling substances that act as sensitive olfactory stimuli. To investigate how bile salts affect olfactory signal transduction in large yellow croaker (*Larimichthy crocea*), deep sequencing of olfactory epithelium was conducted to analyse olfactory-related genes in olfactory transduction. Sodium cholates (SAS) have typical bile salt chemical structures, hence we used four different concentrations of SAS to stimulate *L. crocea*, and the fish displayed a significant behavioural preference for 0.30% SAS. We then sequenced olfactory epithelium tissue, and identified 9938 unigenes that were significantly differentially expressed between SAS-stimulated and control groups, including 9055 up-regulated and 883 down-regulated unigenes. Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses found eight categories linked to olfactory transduction pathways that were highly enriched with some differentially expressed genes (DEGs), including Olfactory receptor (*OR*), Adenylate cyclase type 3 (*ADCY3*) and Calmodulin (*CALM*). Genes in these categories were analysed by RT-qPCR, which revealed aspects of the pathway transformation between odor detection, and recovery and adaptation. The results provide new insight into the effects of bile salt stimulation in olfactory molecular mechanisms in fishes, and expands our knowledge of olfactory transduction, and signal generation and decline.

1   **Transcriptome sequencing of olfactory related genes in olfactory transduction**

2   **of large yellow croaker (*Larimichthy crocea*) in response to bile salts**

4   Jiabao Hu [1,2,3], Yajun Wang [1,2,3] *, Qijun Le [1,2,3,4], Na Yu [1,2,3], Xiaohuan Cao [1,2,3], Siwen Kuang [1,2,3], Man

5   Zhang [1,2,3], Weiwei Gu [1,2,3], Yibo Sun [1,2,3], Yang Yang [1,2,3], Xiaojun Yan [1,2,3] *

7   [1] Key Laboratory of Applied Marine Biotechnology, Ningbo University, Ministry of Education, Ningbo, China

8   [2] Key Laboratory of Marine Biotechnology of Zhejiang Province, Ningbo University, Ningbo, China

9   [3] College of Marine Sciences, Ningbo University, Ningbo, China

10   [4] Ningbo Entry-Exit Inspection and Quarantine Bureau Technical Centre, Ningbo, China

12   Correspondence:

13   Dr Yajun Wang,

14   wangyajun@nbu.edu.cn;

15   Dr Xiaojun Yan,

16   yanxiaojun@nbu.edu.cn

27   **Abstract**

28   Fish produce and release bile salts as chemical signalling substances that act as sensitive olfactory stimuli.

29   To investigate how bile salts affect olfactory signal transduction in large yellow croaker (*Larimichthy crocea*),

30   deep sequencing of olfactory epithelium was conducted to analyse olfactory-related genes in olfactory

31   transduction. Sodium cholates (SAS) have typical bile salt chemical structures, hence we used four different

32   concentrations of SAS to stimulate *L. crocea*, and the fish displayed a significant behavioural preference for

33   0.30% SAS. We then sequenced olfactory epithelium tissue, and identified 9938 unigenes that were

34   significantly differentially expressed between SAS-stimulated and control groups, including 9055 up-regulated

35   and 883 down-regulated unigenes. Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and

36   Genomes (KEGG) analyses found eight categories linked to olfactory transduction pathways that were highly

37   enriched with some differentially expressed genes (DEGs), including Olfactory receptor (*OR*), Adenylate

38   cyclase type 3 (*ADCY3*) and Calmodulin (*CALM*). Genes in these categories were analysed by RT-qPCR,

39   which revealed aspects of the pathway transformation between odor detection, and recovery and adaptation.

40   The results provide new insight into the effects of bile salt stimulation in olfactory molecular mechanisms in

41   fishes, and expands our knowledge of olfactory transduction, and signal generation and decline.

42

Introduction

44   Bile salts are highly structurally variable in vertebrates, and can be classified into three types; C (27) bile

45   alcohols, C (27) bile acids, and C (24) bile acids, with default hydroxylation at C-3 and C-7 (*Hofmann et al.,*

46   *2010*). They are biliary constituents derived from cholesterol that are synthesised in the liver and stored in the

47   gall bladder (*Haslewood, 1967*). Regulated by different transport proteins, these salts are released into the

48   intestinal lumen through enterohepatic circulation (*Trauner and Boyer, 2003*). Because bile salts can help

49   intestines to digest and absorb dietary lipids and fat-soluble vitamins (*Haslewood, 1967*; *Fuentes et al., 2018*),

50   they are included in the diet of fishes to improve growth and digestive enzyme activities (*Deshimaru et al.,*

51   *1982*; *Alam et al., 2015*). Moreover, many studies on behaviour and physiology have reported that bile salts are

52   important chemical signalling substances as well as effective olfactory stimuli in fishes, which have distinct

53   sensitivity to different components (*Zhang et al., 2001*; *Døving et al., 1980*), but molecular studies have been

54   limited.

55   During olfactory activity, odourant molecules released into the environment bind to olfactory related

56   receptors (*Kaupp, 2010*). To date, three types of receptor genes have been identified in fishes, namely olfactory

57   receptors (ORs) (*Freitag et al., 1998*), vomeronasal receptors (VRs) (*Freitag et al., 1995*), and trace amine-

58   associated receptors (TAARs) (*Eisthen, 2004*). Among them, OR genes play an essential role in many odor

59   detecting activities (*Hu et al., 2017*; *Yabuki et al., 2016*; *Bird et al., 2018*).   ORs encoding G protein-coupled

60   receptors expressed in ciliated sensory neurons were previously identified in *Rattus norvegicus* (*Buck and Axel,*

61   *1991*). According to function, OR genes can be divided into two classes; class I (α, β, γ, δ, ε and ζ) and class II

62   (η, θ, κ and λ) (*Niimura and Nei, 2005*; *Hoover, 2013*). In fishes, OR genes mainly belong to class I, which are

63   believed to recognize water-soluble odours (*Freitag et al., 1998*; *Zhou et al., 2011*). ORs are member of a

64   multigene family of G protein-coupled receptors and seven transmembrane domain proteins (*Buck and Axel, 1991*),

65   and $G_{\alpha olf}$, one subunit of the G protein-coupled to OR, activates adenylyl cyclase in olfactory sensory cells (*Hansen*

66   *et al., 2004*; *Schild and Restrepo, 1998*; *Gonçalves et al., 2016*). Olfactory signals are eventually transmitted to

67   the brain via regulation of distinct factors in olfactory transduction (*Meredith et al., 2012*).

68   Many recent studies have focused on the identification and expression of olfactory related receptor genes

69   in fishes (*Zhu et al., 2017*; *Fatsini et al., 2016*; *Cui et al., 2017*). In the present study, to increase our

70   knowledge of gene expression in the whole olfactory transduction system in fishes following stimulation by

71   bile salts, we identified the optimal concentration for stimulation in *L. crocea*, and performed deep sequencing

72   of olfactory epithelium tissue using an Illumina HiSeq 2500 platform. Subsequent GO and KEGG pathway

73   analyses identified significantly differentially expressed genes (DEGs) enriched in eight categories related to

74   olfactory transduction, and gene expression levels were confirmed for selected genes by RT-qPCR. The results

75   indicate that bile salts have attractant effects on *L. crocea*. The findings provide new insight into effects of bile

76   salt stimulation on olfactory molecular mechanisms in fishes, and expand our knowledge of olfactory

77   transduction and olfactory signalling.

78

79

80   **Methodology**

81   **Fish and bile salt stimulation treatments**

82   The large yellow croakers (mean weight = 20 ± 1.2 g) used in the study were commercially reared at 25–

83   27°C in Xiangshan Bay, Zhejiang, China. All fish experiments were conducted in accordance with the

84   recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The

85   Animal Care and Use Committee of Ningbo University approved the protocols.

86   Sodium cholates (SAS) with typical bile salt chemical structures were chosen for stimulation treatments

87   (*Haslewood, 1967*). Four different concentrations of SAS diluted in distilled water (0.20%, 0.30%, 0.40% and

88   0.50%) were applied to SAS groups, while the control group (Control) was treated with by distilled water

89   alone. SAS was released into cultured water slowly using an air stone tube (submerged in the center of the

90   pond) equipped with a 20 mL syringe (100 individuals per group, three independent biological replications).

91   The behavioural responses of each fish were classified as two types: biting the air stone (a positive feeding

92   response), and swimming close to the air stone without biting (a positive movement response). We replaced the

93   tested fish with another new fish for subsequent replications at all concentrations. Culture water was changed

94   after every test, and each test was performed at 24 h intervals. Behaviours were recorded with a camera for 5

95   min, and the number of each type of response was recorded and analysed statistically by one-way analysis of

96   variance (ANOVA) and Tukey's multiple comparison tests (SPSS, version 16.0).

97   The concentration that produced the highest number of behavioural responses was used for subsequent

98   stimulation experiments, which were performed as described as above. After stimulation, we captured control

99   group fish, and fish from SAS groups exhibiting significantly positive feeding responses, and immediately

100   extracted olfactory epithelium tissues by cutting the nostrils. Olfactory epithelium tissues from 15 randomly

101   selected fish were extracted and pooled into three 1.5 mL RNAase-free tubes (three independent biological

102   replicates for each group) and stored in liquid nitrogen for RNA-seq and RT-qPCR experiments.

103

RNA isolation, library construction and Illumina sequencing

105   Total RNAs were extracted using TRIzol reagent (Invitrogen, CA, USA). RNA was monitored on 1%

106   agarose gels, RNA purity was checked using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA),

107   RNA concentration was measured using a Qubit RNA Assay Kit with a Qubit 2.0 Fluorimeter (Life

108   Technologies, CA, USA), and RNA integrity was assessed using an RNA Nano 6000 Assay Kit with a

109   Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

110   Sequencing libraries were generated using an NEBNext Ultra RNA Library Prep Kit for Illumina (NEB,

111   CA, USA) and barcodes were added to attribute sequences to each sample. Clustering of the barcoded samples

112   was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina).

113   After cluster generation, library preparations were sequenced on an Illumina HiSeq 2500 platform and paired-

114   end reads were generated.

115

Assembly of sequencing data and gene annotation

117   Raw data were firstly processed through in-house perl scripts, and clean data were obtained by removing

118   reads containing adapters or poly-N sequences, and reads of low quality. Q20, Q30 and GC values were

119   calculated, and all downstream analyses were based on high-quality clean data.

120   The reference genome of the large yellow croaker was downloaded from the National Center of Genome

121   Research website (https://www.ncbi.nlm.nih.gov/genome/?term=JPYK-00000000) (*Ao et al., 2015*), and data

122   were mapped using TopHat (version 2.0.12) and Bowtie2 (*Trapnell et al., 2009*;*Langmead et al., 2009*).

123   Unigenes were searched using BLASTX against the National Center for Biotechnology Information (NCBI)

124   non-redundant protein sequence (NR) database, the NCBI non-redundant nucleotide sequence (NT) database,

125   and Gene Ontology (GO), KEGG Orthology (KO) and SwissProt databases with an E-value threshold of 10^{-5}.

126

Identification of differentially expressed genes (DEGs) and functional analysis

128   Differential expression analysis was performed using the DEGSeq R package (1.20.0) and Reads per

129   Kilobase Millon Mapped Reads (RPKM) values (*Mortazavi et al., 2008*). The resulting *p*-values were adjusted

130   using the Benjamini and Hochberg's approach for controlling the false discovery rate. DEGs were selected

131   with the criteria adjusted *p*-value < 0.05 and |log2fold-change| >1.

132   GO enrichment analysis of DEGs was implemented by the GOseq R package, and KEGG enrichment was

133   used to identify putative functions and pathways of DEGs (http://www.genome.jp/kegg/).

134

Real-time quantitative PCR (RT-qPCR) analysis

136   Total RNA was reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China).

137   Primers were designed using Primer 5.0 software (Table 1). β-actin served as an internal normalisation control for

138   RT-qPCR analysis, and reactions contained 2 μl cDNA, 1 μl forward and reverse primers, 10 μl SYBR Green I

139   Master Mix (TaKaRa), and 6 μl water. RT-qPCR was performed on an Eppendorf PCR machine (Mastercycler ep

140   Realplex, Hamburg, Germany) with one cycle at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 58°C for 15

141   s, and 72°C for 20 s. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method, and statistical analysis was

142   performed using independent sample t-tests (SPSS, version 16.0).

143

144   **Results**

145   **Selecting the optimal concentration of bile salts and assessing fish responses**

146   For bile salt stimulation treatments, SAS was diluted four different concentrations, added slowly to water,

147   and *L. crocea* responses were monitored (Fig. 1). For feeding responses, the fish reacted most obviously to

148   0.30% SAS (17.67 ± 0.58 fish responded in 5 min). Meanwhile, for movement responses, they exhibited

149   optimal attraction responses to 0.30% SAS (64.33 ± 3.51 fish responded in 5 min) and 0.40% (48.33 ± 3.51

150   fish responded in 5 min). Thus, we chose 0.30% SAS for subsequent RNA-seq and RT-qPCR experiments.

151

152   **Results and analysis of transcriptome sequencing data**

153   cDNA libraries were constructed from control and SAS groups, resulting in 39,805,502 and 39,116,990

154   raw reads, and more than 81% raw reads were filtered to yield clean reads. In total, 25,684,902 and 25,830,011

155   clean reads were mapped to the reference genome of *L. crocea* for control and SAS groups, respectively, and

156   the Q30 value was >95% for libraries (Table 2).

157

158   **Identification and functional annotation of DEGs**

159   Transcriptome data from olfactory epithelium tissue of control and SAS groups were compared, and 19,197

160   unigenes were annotated, of which 9938 DEGs met the criteria (|log2Foldchange| >1 and *p* < 0.05). Of these,

161   9055 were up-regulated and 883 were down-regulated (Fig. 2). Three types of olfactory-related receptor genes

162 were found to be differentially expressed in our data (all up-regulated), comprising 59 *ORs*, two *VRs* and 17

163 *TAARs*.

164   To investigate the functions of DEGs, 9245 unigenes (8424 up-regulated and 821 down-regulated) were assessed

165 in terms of the three main GO classifications, namely biological processes (BP), cellular component (CC), and

166 molecular function (MF; Fig. 3). According to the criteria (p-value < 0.001), single-organism process

167 (GO:0044699), intrinsic to membrane (GO:0031224) and substrate-specific channel activity (GO:0022838)

168 were highly represented, and potentially play an important role in olfactory responses to bile salts.

169   To identify KEGG pathways between control and SAS groups, 3140 DEGs were mapped to 321 pathways,

170 and 20 pathways were highly enriched according to q-value <0.05 (Table 3). Among these pathways, olfactory

171 transduction (map04740) was mainly involved in olfactory responses to bile salts. In this pathway, 73

172 differentially expressed olfactory-related genes were enriched among eight categories including calmodulin

173 (CALM, k02183), adenylate cyclase 3 (ADCY3, k08043), guanine nucleotide-binding protein G (olf) subunit

174 alpha (GNAL, k04633), calcium/calmodulin-dependent protein kinase (CaM kinase) II (CAMK2, k04515),

175 olfactory receptor (OLFR, k04257), cyclic nucleotide gated channel beta 1 (CNGB1, k04952), cyclic

176 nucleotide gated channel alpha 3 (CNGA3, k04950) and protein kinase A (PKA, k04345; Fig. 4). Significantly

177 differentially expressed olfactory-related genes in these categories were subsequently analysed RT-qPCR

178 (Table 4).

179   In the olfactory transduction pathway (Fig. 4), olfactory stimulation could be divided into odor detection,

180 and recovery and adaptation. During odor detection, odour-activated OLFR stimulates G protein release

181 protein GNAL, and ADCY3, which is positively regulated by GNAL, stimulates cAMP release; cAMP is then

182 transported out of the olfactory cell, and Na^+ and Ca^{2+} are exchanged by CNGB1. This process is an example

183 of signal production and amplification. During recovery and adaptation, an increase in cAMP activates PKA,

184 which phosphorylates OLFR; meanwhile, CALM represses CNGB1 and activated CAMK2 to suppress

185 ADCY3 by phosphorylation. This process represents an example of signal suppression.

186

187 **RT-qPCR analysis of eight categories related to olfactory transduction**

188   The expression levels of nine DEGs related to the olfactory transduction pathway were validated by RT-qPCR.

189 These genes were all significantly expressed in the olfactory epithelium (* p <0.05 and ** p <0.01), especially

190 *CAMK2*, *ADCY3*, *OR 2D3* and *CNGB1*, confirming the reliability of the transcriptome sequencing data (Fig. 5).

191 Furthermore, *CNGB1 a* and *CNGB1 b* both belonging to *CNGB1*, and *CNGB1 a* (up-regulated) displayed more

192 significant differential expression than *CNGB1 b* (down-regulated).

193

194 **Discussion**

195 **Bile salts act as effective olfactory stimuli in fishes**

196   Fish can display different olfactory responses to different levels of odours. Using electroolfactograms

197 (EOGs), many fish species have been shown to possess specific thresholds to different levels of cholic acid

198 (CA) (*Meredith et al., 2012*; *Døving et al., 1980*). In the present study, we found that *L. crocea* acted differently

199 to different concentrations of bile salts based on behavioural analysis, and responded optimally to 0.3% SAS rather

200 than to higher levels. We believe that fishes have limited olfactory related receptors, which leads to limited

201 olfactory ability, explaining why they do not exhibit significantly more intense behaviour with increased levels

202 of odours. This suggests that fishes may possess a maximum detection peak for concentrations of certain

203 stimuli, and a similar phenomenon has been observed in other studies (*Zhao, 2007*; *Hu et al., 2017*).

204   The olfactory sensitivity of fishes can also be measured using EOGs. However, unlike EOG analysis, our

205 behavioural experiments revealed fish response to stimuli directly (approach and avoidance). By imitating

206 conditions in which fishes detect bile salts in natural environments, we found that *L. crocea* performed feeding

207 movements upon exposure to SAS, which suggests that the fish had a particular preference toward SAS. Indeed,

208 many studies have demonstrated that some bile salts could be good phagostimulants for fish feed (*Hu et al.,*

209 *2017*; *Rolen and Caprio, 2008*; *Yamashita et al., 2010*), suggesting that they may act on both olfactory and

210 taste pathways in fish, and might be good attractants.

211

212 **Analysis of olfactory related receptor genes in the odorant transduction cascade**

213   In *L. crocea*, OR gene family is the largest one of three receptor genes families (*Ao et al., 2015*; *Zhou et*

214 *al., 2011*). In our current study, 59 OR genes were found to be all up-regulated after the fish were stimulated,

215 many more than two other two types of receptors, consistent with previous studies (*Saraiva and Korsching,*

216   *2007*; *Hashiguchi and Nishida, 2006*; *Hu et al., 2017*). Thus, ORs appear to be the major receptors responding

217   to bile salts in *L. crocea*. Fish produce and release bile salts as sex pheromones to communicate with other

218   individuals (*Zhang et al., 2001*). However, regarding pheromone receptors in the epithelium (*Muramoto et al.,*

219   *2011*), only two VR genes were differentially expressed (up-regulated) in the present study. We speculate that

220   the fish used in our study might be juveniles, hence VRs were not sensitive to sex pheromones at this stage of

221   the life cycle. Moreover, 17 TAAR genes were found to be all up-regulated following stimulation by SAS in

222   our study. Interestingly, TAARs could only be activated by amines at trace level in a previous study (*Borowsky*

223   *et al., 2001*), and SAS is not an amine, suggesting that the fish might release some amines substances to

224   communicate with each other in response to SAS. Our study reveals that ORs might be the main bile salt

225   receptors in the olfactory epithelium during different developmental stages in fish species.

226

227   **Signal transduction and regulation components**

228   During signal transduction, ORs bind to their corresponding G proteins, among which Gα is one of most

229   important subunits (*Jones and Reed, 1989*). In the present study, $G_{\alpha olf}$ was released in the olfactory

230   transduction pathway after ORs were activated by SAS, and two $G_{\alpha olf}$ genes were up-regulated alongside high

231   expression of *ORs*, which suggests that olfactory receptors bind to G protein possessing the $G_{\alpha olf}$ subunit. Some

232   studies on olfactory sensory neurons also have confirmed similar binding relationships of them (*Jones and*

233   *Reed, 1989*; *Ronnett and Moon, 2002*). However, only three $G_{\alpha olf}$ genes were identified in *L. crocea*, indicating

234   that they may be a small gene family in this fish species.

235   In the present study, the $G_{\alpha olf}$ subunits activated ADCY3, which led to a rise in cAMP levels during

236   olfactory transduction, which is of clear relevance to signal transduction (*Jones and Reed, 1989*; *Dhallan et al.,*

237   *1990*; *Menco et al., 1992*). We also found that ADCY3 was enriched among up-regulated genes in the

238   pathway, which suggests that this factor could act positively on signal transduction, and play a key role in

239   regulating transformation of the pathway via the cAMP levels.   Moreover, ADCY3 was the first factor in

240   secondary signal transduction (Fig. 4), and some other studies have reported that signal transduction can be

241   disrupted if ADCY3 genes are mutated (*Brunet et al., 1996*; *Hacker, 2000*). Thus, our results indicate that

242   ADCY3 is one of most important factors mediating signal transduction between primary and secondary signal

243   transduction.

244   In odor detection of olfactory transduction, high cAMP levels produced by ADCY3 activated CNGB1,

245   leading to the entrance of Na^+ and Ca^{2+} into olfactory sensory cells. Other studies have reported similar results

246   (*Michalakis et al., 2006*; *Kaupp and Seifert, 2002*). Thus, we speculate that an increase in these two ions by

247   CNGB1 might appeared to suppress the expression of *CNGA3*, another same functional channel protein-

248   encoding genes, due to competition effects. In recovery and adaptation of the pathway, activated CALM

249   resulting from increased Ca^{2+} regulated by CAMK2 suppresses CNGB1, leading to a drop in cAMP level

250   indirectly, consistent with observations in previous studies (*Cheung, 1980*; *Lynch and Barry, 1989*; *Menini et*

251   *al., 1995*). Thus, a series of interactions may cause Ca^{2+} levels to decline, reducing the intracellular and

252   extracellular charge difference. In addition, *CNGB1a* was expressed at higher levels than *CNGB1b* based on

253   RT-qPCR, which indicates that signal transduction in fish might transform odor detection into recovery and

254   adaptation. These findings reveal that *CNGB1a* and *CNGB1b* may be involved in signal production and decline

255   in the pathway, respectively.

256   PKA can help ORs to bind to G proteins (*Daaka et al., 1997*; *Zamah et al., 2002*) in a mechanism

257   mediated by cAMP (*Chang et al., 2006*), and our results showed that *PKA* (up-regulated) was enriched during

258   the recovery and adaptation aspect of olfactory transduction. Thus, PKA might suppress the initial signal level

259   by hindering the separation between receptor and G protein by phosphorylation. Combined with the results of

260   a previous study (*Taiwo et al., 1989*), our findings indicate that the inhibitory action of PKA may be activated

261   through a change in cAMP concentration due to binding between ORs and G proteins, and indirectly by

262   suppression of ADCY3. These factors might alter the electric charge in olfactory sensory cells by meditating

263   the ion concentration, which might lead to changes in electric signalling between olfactory receptor cells and

264   olfactory sensory neurons, consistent with some previous reports (*Menini et al., 1995*; *Lynch and Barry, 1989*).

265   Our results therefore indicate that odor detection in fish may occur quite rapidly, or a long time after,

266   stimulation by bile salts, and recovery and adaptation may occur once fish become familiar to this stimulation.

267

268   **Conclusion**

269   In the present study, *L. crocea* displayed a significant behavioural preference for 0.3% SAS, which could

270   be a good attractant in fishes. We performed transcriptome sequencing of olfactory epithelium tissue to

271   identify olfactory-related genes involved in the olfactory transduction pathway, and eight categories were

272   found to be highly enriched with DEGs in related DEGs, especially *CAMK2*, *ADCY3*, *OR 2D3* and *CNGB1*. The

273   pathway could be divided into two processes: odor detection, and recovery and adaptation, and involves DEGs

274   such as *CAMK2*, *CALM*, *CNGB1* and *PKA* that may regulate conversion between the two processes. Our results

275   provide new insight into the effects of bile salt stimulation on olfactory molecular mechanisms in fishes, and

276   expand our knowledge of olfactory transduction and signal production and decline.

277

Acknowledgements

279   We thank Shunshun Tao of the Xiangshan Harbor Aquaculture and Larva Limited Company and Dr. Bao

280   of Wanli College of Zhejing.

281

References

283   Ao, J., Mu, Y., Xiang, L., Fan, D., Feng, M., Zhang, S., Shi, Q., Zhu, L., Li, T., and Ding, Y. (2015). Genome

284   Sequencing of the Perciform Fish Larimichthys crocea Provides Insights into Molecular and Genetic

285   Mechanisms of Stress Adaptation. *Plos genetics* 11, 8-8. DOI: 10.1371/journal.pgen.1005118

286   Alam, M. S., Teshima, S., Ishikawa, M., and Koshio, S. (2015). Effects of ursodeoxycholic acid on growth and

287   digestive enzyme activities of Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel).

288   *Aquaculture Research* 32, 235-243. DOI: 10.1046/j.1355-557x.2001.00020.x

289   BIRD, D. J., MURPHY, W. J., FOXROSALES, L., HAMID, I., EAGLE, R. A. & VAN, B. V. 2018. Olfaction

290   written in bone: cribriform plate size parallels olfactory receptor gene repertoires in Mammalia.

291   *Proceedings of the Royal Society B Biological Sciences,* 285**,** 20180100. DOI:

292   10.1098/rspb.2018.0100

293   Brunet, L. J., Gold, G. H., and Ngai, J. (1996). General Anosmia Caused by a Targeted Disruption of the

294   Mouse Olfactory Cyclic Nucleotide–Gated Cation Channel. *Neuron* 17, 681-693. DOI: 10.1016/S0896-

295   6273(00)80200-7

296 Borowsky, B., Adham, N., Jones, K. A., Raddatz, R., Artymyshyn, R., Ogozalek, K. L., Pathirana, S. (2001).
297   Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proceedings of the*
298   *National Academy of Sciences of the United States of America,* 98(16), 8966-8971. doi:
299   10.1073/pnas.151105198

300 Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for
301   odor recognition. *Cell* 65, 175–187.   DOI: 10.1016/0092-8674(91)90418-X

302 Chang, L. Y., Yu-Ming, X. U., and Zhang, S. M. (2006). cAMP-PKA signal pathway mediates the promotive
303   effect of NGF on axon regeneration in cerebral ischemia-reperfusion rats. *Chinese Journal of Geriatric*
304   *Heart Brain & Vessel Diseases.*

305 Cheung, W. Y. (1980). Calmodulin plays a pivotal role in cellular regulation. *Science* 207, 19-27.
306   DOI: 10.1126/science.6243188

307 CUI, R., DELCLOS, P. J., SCHUMER, M. & ROSENTHAL, G. G. 2017. Early social learning triggers
308   neurogenomic expression changes in a swordtail fish. *Proceedings of the Royal Society B Biological*
309   *Sciences.* DOI: 10.1098/rspb.2017.0701

310 Døving, K. B., Selset, R., and Thommesen, G. (1980). Olfactory sensitivity to bile acids in salmonid fishes.
311   *Acta Physiologica Scandinavica* 108, 123–131. DOI:10.1111/j.1748-1716.1980.tb06509.x

312 Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997). Switching of the coupling of the beta2-adrenergic
313   receptor to different G proteins by protein kinase A. *Nature* 390, 88-91. DOI: 10.1038/36362

314 Deshimaru, O., Kuroki, K., and Yone, Y. (1982). Suitable levels of lipids and ursodesoxycholic acid in diet for
315   yellowtail [Fish]. *Bulletin of the Japanese Society of Scientific Fisheries.* DOI: 10.2331/suisan.48.1265

316 Dhallan, R. S., Yau, K. W., Schrader, K. A., and Reed, R. R. (1990). Primary structure and functional
317   expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* 347, 184-7.
318   DOI: 10.1038/347184a0

319 Eisthen, H. L. (2004). The goldfish knows: olfactory receptor cell morphology predicts receptor gene
320   expression. *Journal of Comparative Neurology* 477, 341–346. DOI: 10.1002/cne.20258

321   FATSINI, E., BAUTISTA, R., MANCHADO, M. & DUNCAN, N. J. 2016. Transcriptomic profiles of the
322   upper olfactory rosette in cultured and wild Senegalese sole (Solea senegalensis) males. *Comp*
323   *Biochem Physiol Part D Genomics Proteomics*, 20, 125-135. DOI: 10.1016/j.cbd.2016.09.001

324   Freitag, J., Krieger, J., Strotmann, J., and Breer, H. (1995). Two classes of olfactory receptors in xenopus
325   laevis. *Neuron* 15, 1383-92. DOI: 10.1016/0896-6273(95)90016-0

326   Freitag, J., Ludwig, G., Andreini, I., Rössler, P., and Breer, H. (1998). Olfactory receptors in aquatic and
327   terrestrial vertebrates. *Journal of Comparative Physiology A* 183, 635-650. DOI: 10.1007/s003590050287

328   FUENTES, J., RIBEIRO, L. & ARAG O, C. 2018. Bile salts regulate ion transport in the intestine of
329   Senegalese sole. *Aquaculture*, 495, 842-848. DOI: 10.1016/j.aquaculture.2018.06.050

330   GON ALVES, I., HUBBARD, P. C., TOM S, J., QUINTELA, T., TAVARES, G., CARIA, S., BARREIROS,
331   D. & SANTOS, C. R. 2016a. 'Smelling'the cerebrospinal fluid: olfactory signaling molecules are
332   expressed in and mediate chemosensory signaling from the choroid plexus. The FEBS journal, 283, 1748-
333   1766. DOI: 10.1111/febs.13700

334   Hacker, B. (2000). Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia
335   in transgenic mice. *Neuron* 27, 487-97. DOI: 10.1016/S0896-6273(00)00060-X

336   Hansen, A., Anderson, K. T., and Finger, T. E. (2004). Differential distribution of olfactory receptor neurons
337   in goldfish: structural and molecular correlates. *Journal of Comparative Neurology* 477, 347.
338   DOI: 10.1002/cne.20202

339   Hashiguchi, Y., and Nishida, M. (2006). Evolution and origin of vomeronasal-type odorant receptor gene
340   repertoire in fishes. *BMC Evolutionary Biology* 6, 1-13. DOI: 10.1186/1471-2148-6-76

341   Haslewood, G. A. (1967). Bile salt evolution. *Journal of Lipid Research* 8, 535-550.

342   Hofmann, A. F., Hagey, L. R., and Krasowski, M. D. (2010). Bile salts of vertebrates: structural variation and
343   possible evolutionary significance. *Journal of Lipid Research* 51, 226-46. DOI: 10.1194/jlr.R000042

344   Hoover, K. C. (2013). "Evolution of Olfactory Receptors," *Humana Press*. DOI: 10.1007/978-1-62703-377-
345   0_18

346   HU, J., WANG, Y., LE, Q., NA, Y., CAO, X., ZHENG, H., KUANG, S., MAN, Z., ZHENG, J. & WU, X.
347   (2017). Transcriptomic analysis reveals olfactory-related genes expression in large yellow croaker

348   (*Larimichthys crocea*) regulated by taurine: May be a good phagostimulant for all-plant protein diets.

349   *Aquaculture Research*, 49. DOI: 10.1111/are.13559

350 Jones, D. T., and Reed, R. R. (1989). Golf: an olfactory neuron specific-G protein involved in odorant signal

351   transduction. *Science* 244, 790-795. DOI: 10.1126/science.2499043

352 Kapiloff, M. S., Mathis, J. M., Nelson, C. A., Lin, C. R., and Rosenfeld, M. G. (1991). Calcium/calmodulin-

353   dependent protein kinase mediates a pathway for transcriptional regulation. *Proceedings of the National*

354   *Academy of Sciences of the United States of America* 88, 3710-4.

355 KAUPP, U. B. 2010. Olfactory signalling in vertebrates and insects: differences and commonalities. *Nature*

356   *Reviews Neuroscience,* 11**,** 188-200.

357 Kaupp, U. B., and Seifert, R. (2002). Cyclic nucleotide-gated ion channels. *Physiological Reviews* 82, 769.

358   DOI: 10.1152/physrev.00008.2002

359 Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of

360   short DNA sequences to the human genome. *Genome Biology* 10, 1-10.

361 Lynch, J. W., and Barry, P. H. (1989). Action potentials initiated by single channels opening in a small neuron

362   (rat olfactory receptor). *Biophysical Journal* **55**, 755-768. DOI: 10.1016/S0006-3495(89)82874-7

363 Menco, B. P. M., Bruch, R. C., Dau, B., Danho, and Waleed (1992). Ultrastructural localization of olfactory

364   transduction components: the G protein subunit Gand type III adenylyl cyclase. *Neuron* 8, 441.

365   DOI: 10.1016/0896-6273(92)90272-F

366 Meredith, T. L., Caprio, J., and Kajiura, S. M. (2012). Sensitivity and specificity of the olfactory epithelia of

367   two elasmobranch species to bile salts. *Journal of Experimental Biology* 215, 2660-2667.

368   DOI: 10.1242/jeb.066241

369 Menini, A., Picco, C., and Firestein, S. (1995). Quantal-like current fluctuations induced by odorants in

370   olfactory receptor cells. Nature 373, 435-437. DOI: 10.1038/373435a0

371 Michalakis, S., Reisert, J., Geiger, H., Wetzel, C., Zong, X., Bradley, J., Spehr, M., Hüttl, S., Gerstner, A., and

372   Pfeifer, A. (2006). Loss of CNGB1 Protein Leads to Olfactory Dysfunction and Subciliary Cyclic

373   Nucleotide-gated Channel Trapping. *The Jounal of biological chemistry* 281, 35156. DOI:

374   10.1074/jbc.M606409200

375   Mortazavi, A., Williams, B. A., Mccue, K., Schaeffer, L., and Wold, B. (2008). Mapping and quantifying

376   mammalian transcriptomes by RNA-Seq. *Nature Methods* 5, 621-628. DOI: 10.1038/nmeth.1226

377   Muramoto, K., Hagino-Yamagishi, K., Tonosaki, K., and Kaba, H. (2011). Accessory olfactory bulb neurons

378   are required for maintenance but not induction of V2R vomeronasal receptor gene expression in vitro.

379   *Neuroscience Letters* 500, 6-9. DOI: 10.1016/j.neulet.2011.05.232

380   Niimura, Y., and Nei, M. (2005). Evolutionary dynamics of olfactory receptor genes in fishes and tetrapods.

381   *Proceedings of the National Academy of Sciences* 102, 6039-44. DOI: 10.1073/pnas.0501922102

382   ROLEN, S. H. & CAPRIO, J., . 2008. Bile salts are effective taste stimuli in channel catfish. *Journal of*

383   *Experimental Biology*, 211, 2786-91. DOI: 10.1242/jeb.018648

384   Ronnett, G. V., and Moon, C. (2002). G proteins and olfactory signal transduction. *Annual Review of*

385   *Physiology* 64, 189-222. DOI: 10.1146/annurev.physiol.64.082701.102219

386   Saraiva, L. R., and Korsching, S. I. (2007). A novel olfactory receptor gene family in teleost fish. *Genome*

387   *Research* 17, 1448-57. DOI: 10.1101/gr.6553207

388   Schild, D., and Restrepo, D. (1998). Transduction mechanisms in vertebrate olfactory receptor cells.

389   *Physiological Reviews* 78, 429-466. DOI: 10.1152/physrev.1998.78.2.429

390   Taiwo, Y. O., Bjerknes, L. K., Goetzl, E. J., and Levine, J. D. (1989). Mediation of primary afferent peripheral

391   hyperalgesia by the cAMP second messenger system. *Neuroscience* 32, 577-80. DOI: 10.1016/0306-

392   4522(89)90280-7

393   Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq.

394   *Bioinformatics* 25, 1105-11. DOI:10.1093/bioinformatics/btp120

395   Trauner, M., and Boyer, J. L. (2003). Bile salt transporters: molecular characterization, function, and

396   regulation. *Physiological Reviews* 83, 633-71. DOI: 10.1152/physrev.00027.2002

397   Wissinger, B., ., Gamer, D., ., JaGle, H., ., Giorda, R., ., Marx, T., ., Mayer, S., ., Tippmann, S., .,

398   Broghammer, M., ., Jurklies, B., ., and Rosenberg, T., . (2001). CNGA3 mutations in hereditary cone

399   photoreceptor disorders. *American Journal of Human Genetics* 69, 722-37. DOI: 10.1086/323613

400   YABUKI, Y., KOIDE, T., MIYASAKA, N., WAKISAKA, N., MASUDA, M., OHKURA, M., NAKAI, J.,

401   TSUGE, K., TSUCHIYA, S. & SUGIMOTO, Y. 2016. Olfactory receptor for prostaglandin F2[alpha]

402   mediates male fish courtship behavior. *Nature Neuroscience,* 19**,** 897. DOI: 10.1038/nn.4314

403   YAMASHITA, S., YAMADA, T. & HARA, T. J. 2010. Gustatory responses to feeding- and non-feeding-

404   stimulant chemicals, with an emphasis on amino acids, in rainbow trout. *Journal of Fish Biology*, 68,

405   783-800. DOI: 10.1111/j.0022-1112.2006.00965.x

406   Zamah, A. M., Delahunty, M., Luttrell, L. M., and Lefkowitz, R. J. (2002). Protein kinase A-mediated

407   phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a

408   reconstituted system. *Journal of Biological Chemistry* 277, 31249-31256. DOI: 10.1074/jbc.M202753200

409   Zhang, C., Brown, S. B., and Hara, T. J. (2001). Biochemical and physiological evidence that bile acids

410   produced and released by lake char (*Salvelinus namaycush*) function as chemical signals. *Journal of*

411   *Comparative Physiology B* 171, 161. DOI: 10.1007/s003600000170

412   Zhao, H. Y. (2007). Studies on feeding stimulants for gibel carp(*Carassius auratus gibelio*). *This is for Doctor*

413   *of Science.Institute of Hydrobiology,Chinese Academy of Sciences,*Wuhan.

414   Zhou, Y., Yan, X., Xu, S., Zhu, P., He, X., and Liu, J. (2011). Family structure and phylogenetic analysis of

415   odorant receptor genes in the large yellow croaker (*Larimichthys crocea*). *BMC Evolutionary Biology* 11,

416   2546-2552. DOI: 10.1186/1471-2148-11-237

417   ZHU, G., WANG, L., TANG, W., WANG, X. & WANG, C. 2017. Identification of olfactory receptor genes

418   in the Japanese grenadier anchovy *Coilia nasus*. *Genes & Genomics*, 39, 521-532. DOI:

419   10.1007/s13258-017-0517-8

Figure 1

Fish responses to increasing concentrations of sodium cholates (SAS).

The number of fish displaying feeding (blue bars) or movement (orange bars) responses were quantified. Responses were considered significant a *$0.01 < p < 0.05$ and **$p < 0.01$ levels compared with controls. Fish exhibited optimal behavioural performance in response to 0.3% SAS.

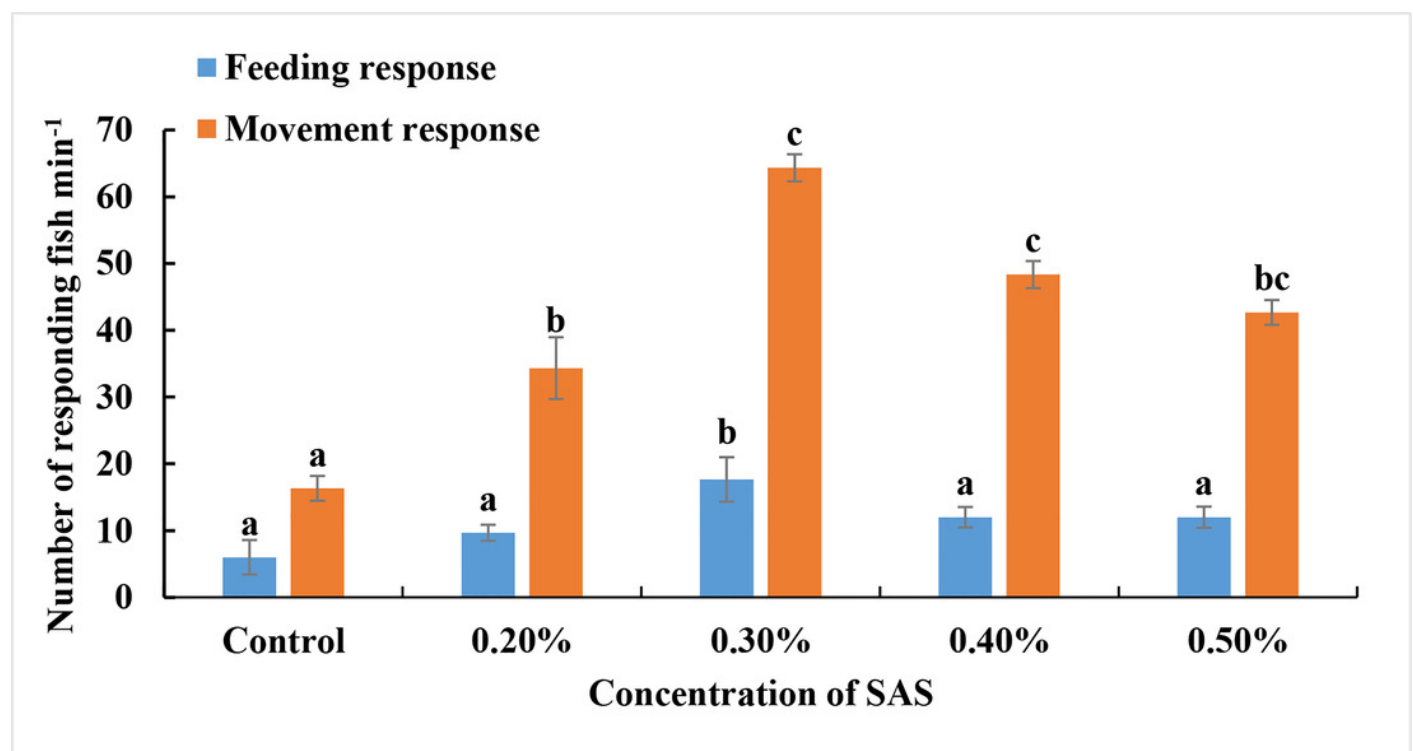



Figure 2

DEGs identified following bile salt stimulation.

Red spots represent up-regulated genes and green spots indicate down-regulated genes.
Grey spots represent genes showing no obvious change between control and SAS groups.

Figure 3

GO analysis of DEGs identified by comparing control and SAS groups. Orange represents up-regulated genes and green indicates down-regulated genes.

The height of bars is proportional to the number of DEGs.

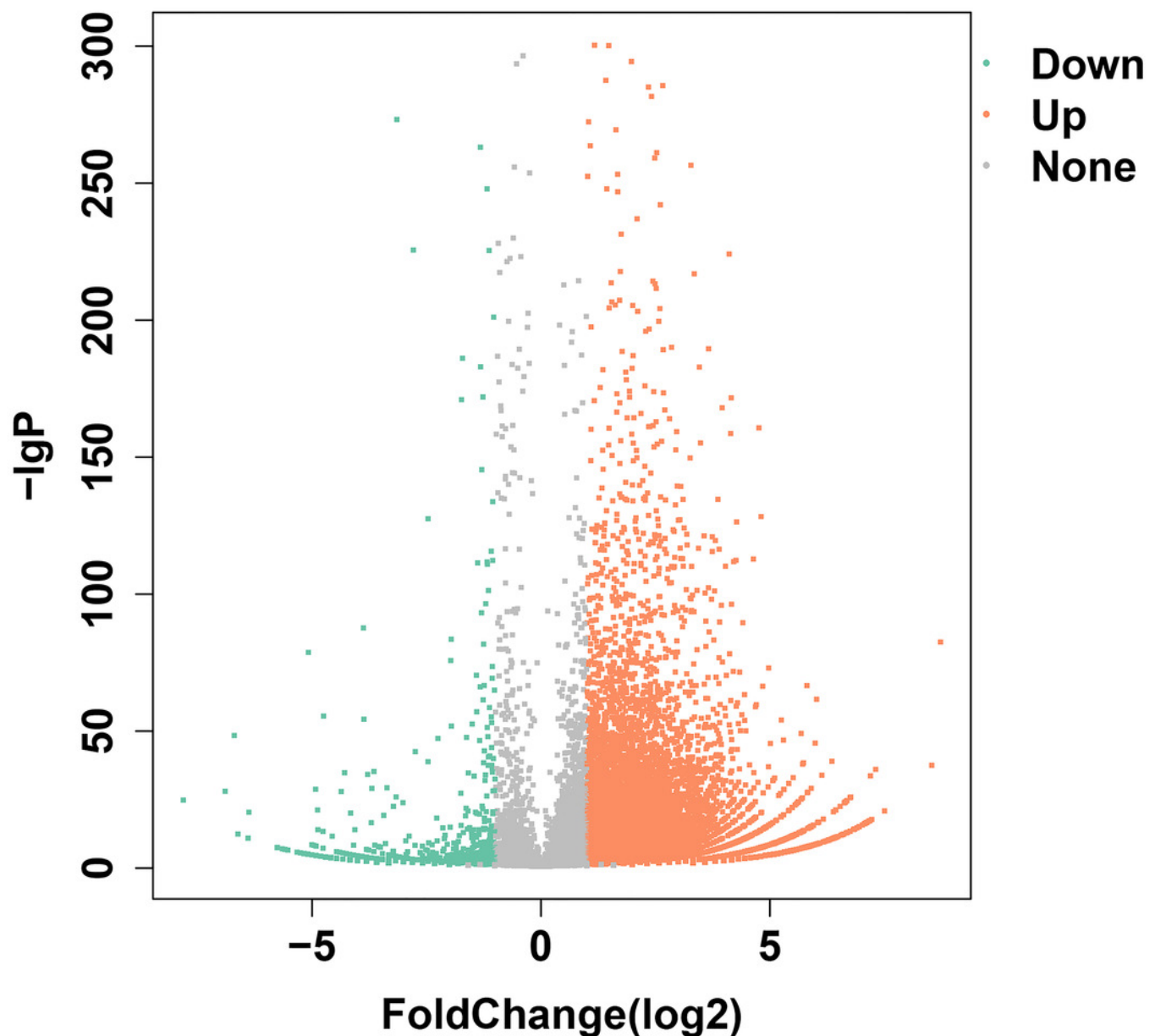



Figure 4

Significantly differentially expressed genes identified by KEGG in eight categories related to the olfactory transduction pathway.

Red indicates significantly up-regulated genes, green indicates significantly down-regulated genes, and blue indicates genes that were both up- and down-regulated. (Image credit: https://www.kegg.jp/kegg-bin/show_pathway?map04740)

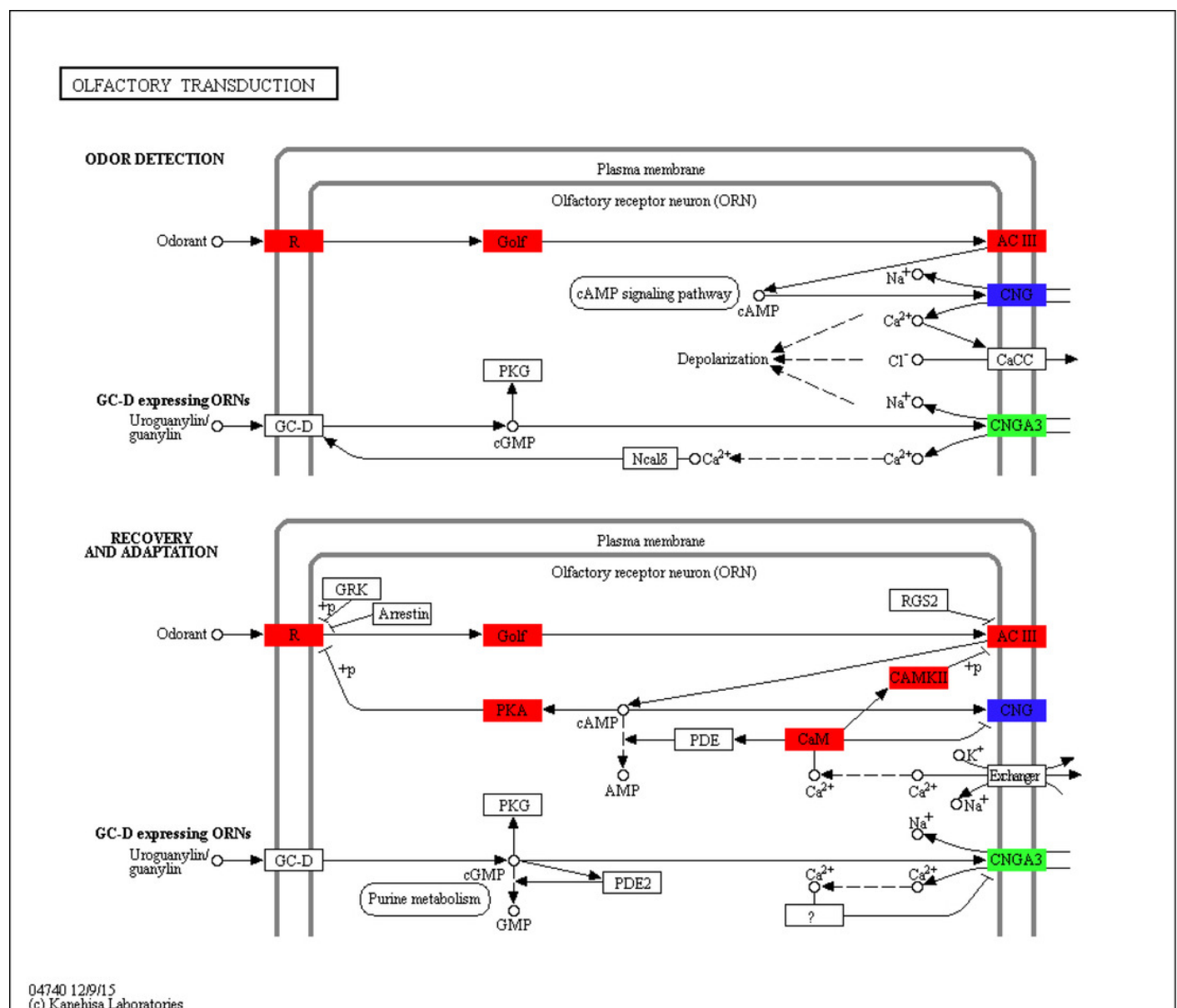



Figure 5

Relative expression levels of nine DEGs related to olfactory transduction.

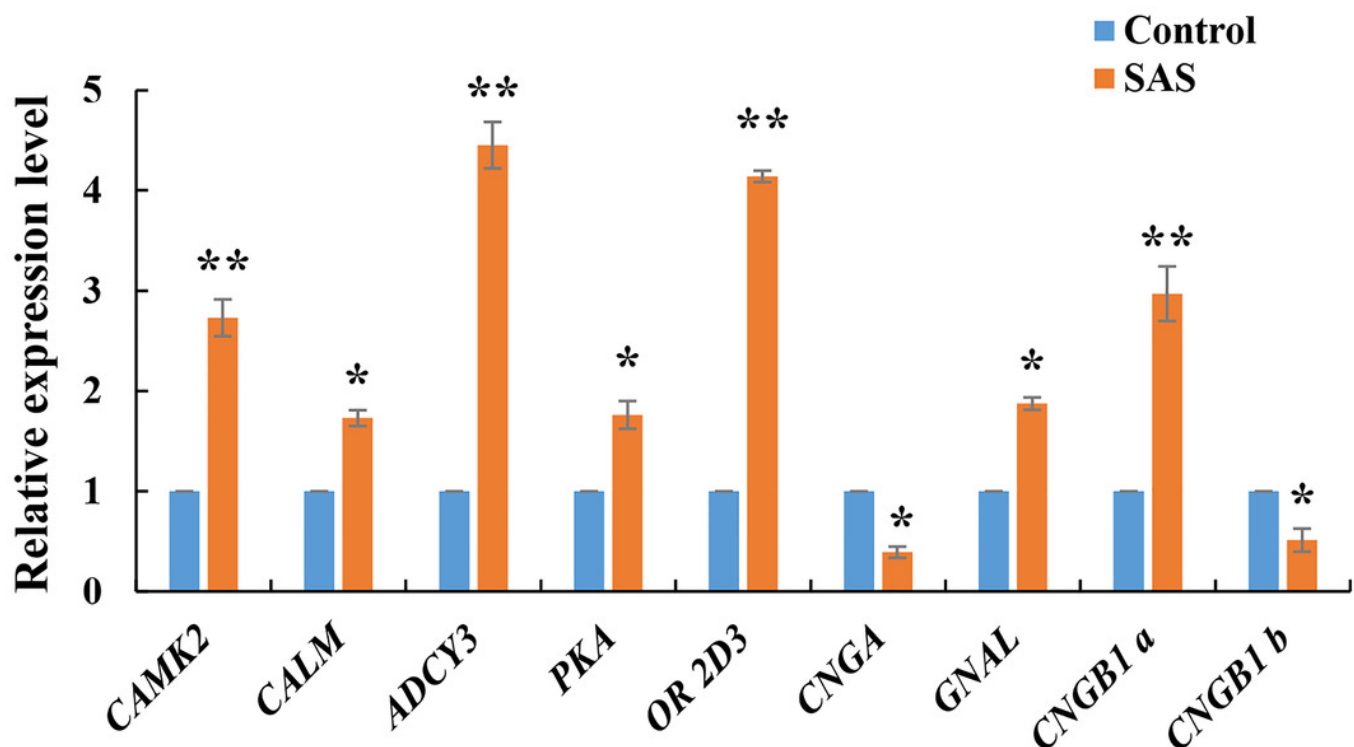
The results were calculated according to the $2^{-D\Delta CT}$ method using β-actin as an internal reference gene. *$p < 0.05$ and **$p < 0.01$.

Primers for real-time quantitative PCR (RT-qPCR).

Table 1 Primers for real-time quantitative PCR (RT-qPCR)

Gene name	Gene ID	Primer sequence (5′→ 3′)
OR 2D3	gi|734643370|	F: CTATGCCAGCACTCTCTTTC
		R: ACAAGGTGGAGGTGAGAA
CALM	gi|698455748|	F: AGGGTGTTCATTGGTGCTCG
		R: ATGTAAAGCCCACGACTCAA
ADCY3	gi|734633255|	F: AACCCATCGTTTCCTAATCC
		R: GCCGCTCTGTTTCTCCTTCT
GNAL	gi|734649985|	F: AGCATCGCTCCGCTTTC
		R: ATCCCGCTGACCTCCTACA
CAMK2	gi|734594146|	F: AATGCCACCAACGACGAG
		R: TCCACCAGGTTTCCCAGA
CNGA	gi|734644355|	F: AAGTGTTTAGCCCTGGAGATTAC
		R: CCGCTTTACTGCCCTTGATA
PKA	gi|734635100|	F: AACCCATCGTTTCCTAATCC
		R: GCCGCTCTGTTTCTCCTTCT
CNGB1 a	gi|734611524|	F: GTGTACGACGTAGCCACGAT
		R: TGAGATTCCACTGAGCGATT
CNGB1 b	gi|554826775|	F: ACTTTGTTGGTGTCTTTGCTTT
		R: TCTCGGGGGATGTTGTAGG

The sequence quality and mapping results between the SAS and Control groups

1   **Table 2 The sequence quality and mapping results between the SAS and Control groups**

Library	Control	SAS
Raw Reads Number:	39,116,990	39,805,502
Raw Reads Length (bp):	125	125
Clean Reads Number:	32,205,388	32,272,020
Clean Reads Length (bp):	125	125
Clean Reads Rate (%):	82.33	81.07
Mapped Reads:	25,684,902	25,830,011
Mapping Rate(%):	80	80
Raw Q30 Bases Rate (%):	90.57	90.23
Clean Q30 Bases Rate (%):	95.83	96.02

KEGG pathway analysis of the 20 highly enriched categories

Table 3 KEGG pathway analysis of the 20 highly enriched categories

Pathway ID	q-value	Pathway
map03010	3.77E-15	Ribosome
map05012	1.59E-08	Parkinson's disease
map03008	1.01E-07	Ribosome biogenesis in eukaryotes
map03040	3.40E-07	Spliceosome
map03050	1.29E-06	Proteasome
map03013	1.72E-06	RNA transport
map00190	1.72E-06	Oxidative phosphorylation
map03030	1.94E-06	DNA replication
map03430	4.59E-05	Mismatch repair
map00970	0.000145129	Aminoacyl-tRNA biosynthesis
map05016	0.001811227	Huntington's disease
map04740	0.004459478	Olfactory transduction
map03440	0.005177196	Homologous recombination
map03420	0.005828765	Nucleotide excision repair
map04721	0.008139829	Synaptic vesicle cycle
map04920	0.029436551	Adipocytokine signaling pathway
map04142	0.037198669	Lysosome
map05160	0.037198669	Hepatitis C
map04111	0.041314855	Cell cycle - yeast

Nine highly differentially expressed genes in 8 categories from olfactory transduction

1 **Table 4 nine highly differentially expressed genes in 8 categories from olfactory transduction**

Gene name	log$_2$FoldChange	P-value	Gene ID	Description
OR 2D3	5.388277756	1.13E-11	gi|734643370|	Olfactory receptor 2D3
CALM	2.148730614	1.35E-25	gi|698455748|	Calmodulin
ADCY3	4.803315255	4.82E-05	gi|734633255|	Adenylate cyclase type 3
GNAL	1.303754743	9.90E-96	gi|734649985|	Guanine nucleotide-binding protein G(olf) subunit alpha
CAMK2	3.964251474	6.05E-76	gi|734594146|	Calcium/calmodulin-dependent protein kinase type II subunit gamma
CNGA	-1.827161905	3.96E-06	gi|734644355|	Cyclic nucleotide-gated channel cone photoreceptor subunit alpha
PKA	2.004522347	1.89E-19	gi|734635100|	cAMP-dependent protein kinase catalytic subunit PRKX
CNGB1 a	4.165885335	1.29E-47	gi|734611524|	Cyclic nucleotide-gated cation channel beta-1
CNGB1 b	−4.004039667	0.001710637	gi|554826775|	Cyclic nucleotide-gated cation channel beta-1