Neuro-molecular characterization of fish cleaning interactions

Ramírez-Calero[1], S., Paula, J. R. [2], Otjacques, E. [2,3], Rosa, R. [2], Ravasi, T. [4,5], Schunter C. [1]*

[1]*Swire Institute of Marine Science, Division for Ecology and Biodiversity, School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong SAR*

[2]*MARE – Marine and Environmental Sciences Centre, Faculdade de Ciências da Universidade de Lisboa, Laboratório Marítimo da Guia, Av. Nossa Senhora do Cabo, 939, 2750-374, Cascais, Portugal.*

[3]*Pacific Biosciences Research Center, Kewalo Marine Laboratory, University of Hawai'i at Manoa, Honolulu, HI, USA*

[4]*Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, 1919–1 Tancha, Onna-son, Okinawa 904–0495, Japan*

[5]*Australian Research Council Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, 4811, Australia*

**Correspondence to: Celia Schunter (celiaschunter@gmail.com)*

ABSTRACT

Coral reef fish exhibit a large variety of behaviours crucial for fitness and survival. The cleaner wrasse *Labroides dimidiatus* displays cognitive abilities during interspecific interactions by providing services of ectoparasite cleaning, thus serving as a good model to understand the processes of complex social behaviour. However, little is known about the molecular underpinnings of cooperative behaviour between *L. dimidiatus* and a potential client fish (*Acanthurus leucosternon*). Therefore, we investigated the molecular mechanisms in three regions of the brain (fore-, mid-, and hindbrain) during the interaction of these fishes. Here we show, using transcriptomics, that most of the transcriptional response in both species was regulated in the hindbrain and forebrain regions and that the interacting behaviour responses of *L. dimidiatus* involved immediate early gene alteration, dopaminergic and glutamatergic pathways, the expression of neurohormones (such as isotocin) and steroids (e.g. progesterone and estrogen), as well as social decision-making genes. In contrast, in the client, fewer molecular alterations were found, mostly involving pituitary hormone responses. The particular pathways found suggested learning and memory processes in the cleaner wrasse, while the client indicated stress relief and a reduction in aggression.

Keywords: Social Behaviour, Molecular Pathways, Transcriptomics, Species Interaction, Learning and Memory

1. Background

Social behaviour allows species to establish biological relations through intra- and interspecific interactions. These relationships prompt species to generate social mechanisms to survive (*e.g.* detect predators), reproduce (*e.g.* courtship) and thrive in nature (*e.g.* territoriality, living in groups; [1]). Indeed, social behaviour is an ability that promotes responses in organisms to react to specific situations, including biotic factors (*i.e.* competition for shelter or food) and also to respond to their physical environment [2,3]. This ability to respond to stimuli can be regulated to optimize their relationships with conspecifics and other species, allowing them to perform more effectively in nature [1]. At present, the study of social behaviour and its mechanisms have been centred on understanding the capacity to regulate and change social relationships (social plasticity) that can enhance and promote survival [4,5].

Many studies have focused on understanding the genetic, epigenetic, endocrine and neural mechanisms underlying social behavioural responses [1,6,7]. For example, model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* have been used to study the genetic systems that underlie neural and sensory processes [1,8]. In addition, considerable advancements have been made using mammalian species such as *Mus musculus* and *Microtus ochrogaster*, in particular to understand social brain circuits, reproduction, aggression and social bonds [9–14]. Finally, other animals such as apes, humans, fish, molluscs and even social insects have been studied to understand social behavioural traits and mechanisms such as cognitive capacity, brain size, interspecific cooperation, and gene expression [8,15–21].

To understand the underlying mechanisms of social interactions, the use of fish, with well-developed social systems and behaviours is valuable as it offers a unique opportunity to understand how these processes are shaped and have evolved [1,4,6,22,23]. In particular, the coral reef cleaner wrasse *Labroides dimidiatus* is a widely studied species known for its cognitive abilities and for its important role in cleaning ectoparasites from hosts, and in turn enhancing fish biodiversity in local communities [24,25]. Studies on this species as a model for behaviour, have focused on neural physiological responses in its interaction with other species and how different social situations such as the establishment of social bonds modulate their behaviour [26–29]. In fact, dopamine and serotonin levels influence the motivation of cleaners to engage in interactions [30] and are induced during social stress [38]. Furthermore, it was suggested that monoamines (dopamine and serotonin) play a role in how cleaners modulate the service of cleaning as well as their willingness to interact, thus showing their capacity to react rapidly to new social scenarios (*i.e.* presence of new clients [5,32]). However, even though these studies have been conducted at behavioural and neurobiological scales, the lack of molecular implications is evident, which is crucial to explain the functional basis of behavioural processes.

In this study, we identified the molecular signatures of the interaction behaviour of *L. dimidiatus* with its client fish *Acanthurus leucosternon* (figure 1). Whole-genome differential gene expression patterns were analysed by comparing both cleaner and client individuals after social cleaning interactions against individuals that did not interact (control). We investigate these molecular signatures across the three main regions of the brain: forebrain (FB), midbrain (MB) and hindbrain (HB). Consequently, identifying the underlying molecular processes and neural pathways altered in the brain of these two fish species during their social interaction is key to contribute to the understanding of mutualistic cleaning behaviour at lower levels of biological organization. The cleaner wrasse is a promising

system to exhibit underlying mechanisms driving social plasticity and allows us to identify the functions used in the processes of sensory information in the brain in vertebrate species interactions.

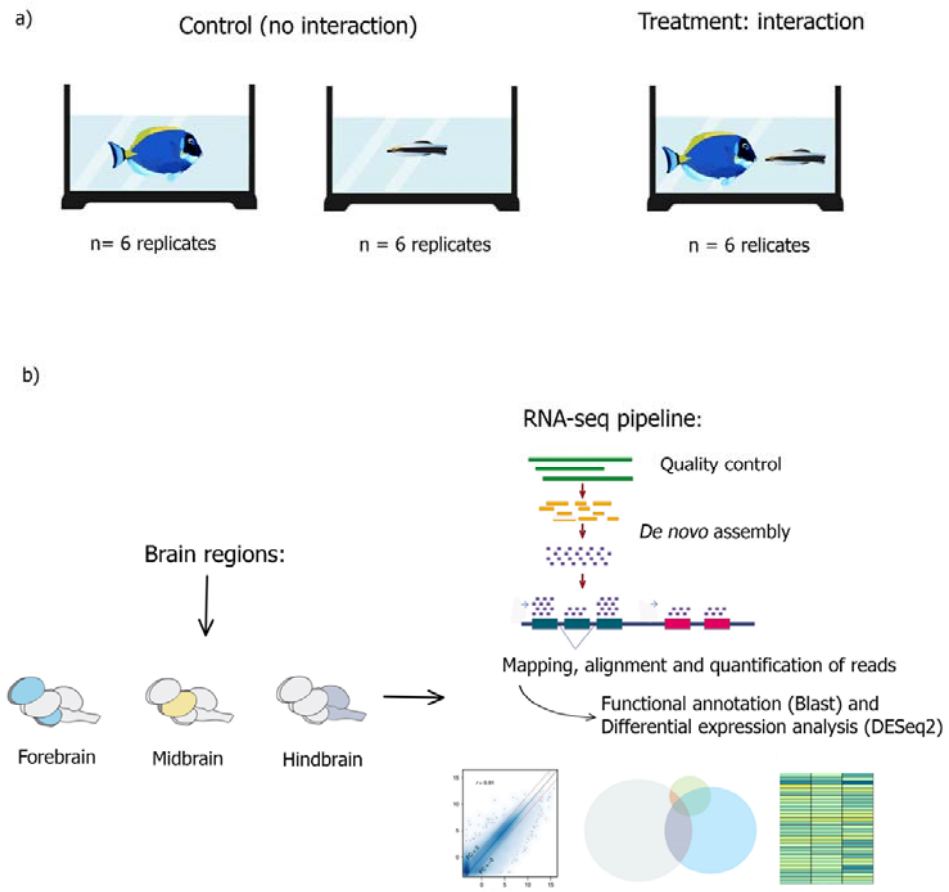

Figure 1. a) Experimental design in which *Labroides dimidiatus* and *Acanthurus leucosternon* were kept separately (control: no interaction) or allowed to interact (treatment: interaction) in the observation tanks. 40min of video were recorded. b) Brain regions dissections (forebrain, midbrain and hindbrain) for each species, and RNA-seq pipeline including *de novo* transcriptome assembly, differential gene expression and functional analysis

2. Methods

(a) Experimental setup

To test for the molecular mechanisms involved in the interaction between two fish species, twelve adult individuals of *L. dimidiatus* and *A. leucosternon* were collected from the wild in the Maldive Islands and transported by TMC-Iberia to the aquatic facilities of Laboratório Marítimo da Guia in Cascais, Portugal (figure 1). Fishes were habituated for 28 days to laboratory conditions. Water parameters were monitored daily and automatically controlled using an aquarium computer (Profilux 3.1N GHL, Germany). Seawater conditions were kept similar to their capture site (salinity = 35 ± 0.5, temperature 29 ± 1°C, pH 8.10 ± 0.05). Each cleaner fish was kept separately in individual tanks (20L) to avoid aggression as they are highly territorial animals. In contrast, surgeonfish (*A. leucosternon*) were held in groups of three individuals in 20L tanks. Fish were fed *ad libitum* once per day. Each

experimental tank had a flow-through aquatic system in which levels of alkalinity, dissolved carbon and pH were strictly maintained. Natural seawater was pumped from the sea to a storage tank of 5m3 and then filtered and UV-irradiated with a Vecton V2 300 Sterilizer before reaching the experimental tanks. Experimental tanks were kept under a photoperiod of 12 h/12 h (light/dark cycle). Ammonia and nitrate levels were checked daily using colorimetric tests and always kept below detectable levels (Salifert Profi Test, Netherlands). Seawater temperature was regulated using chillers (Frimar, Fernando Ribeiro Lda, Portugal) and underwater heaters 300 W, (TMC-Iberia, Portugal). Salinity was daily monitored with a V2 refractometer (TMC-Iberia, Portugal), and pH and temperature with a VWR pH 1100H pH meter (Avantor, US).

Behavioural tests started after the 28 days of laboratory acclimation. The tests were performed in observation tanks (40 L) set in an isolated observation room. The fish were either placed into a tank with i) no-interaction (control) or ii) interaction (cleaner-client pair). In the no-interaction treatment, cleaners or clients were kept alone in the observation tank (control), while for the interaction treatment, pairs composed of 1 cleaner and 1 client were kept together in the observation tank, allowing them to have close contact (figure 1). Their interactions were filmed for 40 minutes. At the end of the observation period, each fish was immediately euthanized by severing the spine, body length was measured, and three separated regions of the brain were immediately dissected out: forebrain, midbrain, and hindbrain [33]. Tissues were placed in a tube, snap-frozen and kept at -80° C for further processing.

(b) Behavioural Video Analyses

Behavioural analysis was performed in both treatments. For no-interaction individuals (control), we looked for abnormal behaviour and stress such as erratic movement, secession of swimming or aggressive postures. No abnormal or stress like events were found. For the interaction treatment individuals, we analysed cleaning behaviour according to Paula et al, 2019. Cleaning behaviour was grouped into two categories, (i) cleaning interactions & motivation and, (ii) interaction quality [30]. To characterise cleaning interactions & motivation, we measured the number of interactions (cleaning interactions, *i.e.* close body inspection and removal of damaged tissue or scales including inspection allowing posture of the client), the number of interactions initiated by cleaners and client posing displays. Interaction quality was determined by the duration of interactions, the number of client jolts (conspicuous signals that indicate cheating [34]), the number of client chases and the number and duration of tactile stimulation events (with pectoral fins, which reduces stress levels and can prolong interaction duration [35,36]). All behavioural videos were analysed using the event-logging software “Boris” [37].

(c) RNA extraction and transcriptome assembly

For RNA extractions, the frozen tissue was removed from the freezer, and 300μl of RTL Buffer added with several sterile silicon beads for tissue homogenization in a Tissuelyzer (Qiagen) for 30 seconds at maximum speed to then follow the RNAeasy Mini Kit protocol (Qiagen). DNA contamination was removed by DNase I treatment (Qiagen) according to the manufacturer’s protocol. The resulting RNA was tested for quality and quantity in a Qubit fluorometer, and RNA Integrity Number (RIN) was obtained using an Agilent Bioanalyzer, and samples with a RIN > 8 were retained. mRNA-focused sequencing libraries were designed with Illumina TruSeq v3 kits and sequenced for paired-end reads of 150bp length on

an Ilumina Hiseq4000 at the King Abdullah University of Science and Technology corelab facility [38]

In order to assess the molecular basis of species interactions, the obtained sequences were processed following a bioinformatic pipeline. Raw read quality was examined using FastQC v. 0.11.9 (Andrews S. 2010), then reads were trimmed and adapters removed to avoid the presence of poor-quality sequences in our *de novo* assembly and downstream analysis. For this, we used Trimmomatic v.0.36 (Bolger, A. *et al*., 2014) with parameters as follows: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:4 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:40. For both species, there is currently no genome reference available, hence a *de novo* transcriptome assembly was constructed for both species separately using the reads from five samples per species with the trinity software v. 2.8.5 [39,40]) using the following parameters*: --seqType* for fastqc data, a *--max_memory* 20G, *--CPU* 24 *--full_cleanup*, *--SS_lib_type*, and *--left –right*. Consequently, the assembly with the most complete and longest transcripts and the highest percentage of protein recovery was chosen (>95%, Supplementary table S4). The statistics of the assemblies were obtained using trinity script *TrinityStats.pl*.

To assess the quality of the assembly, we investigated the read representation against our *de novo* assembly using the aligner Bowtie2 v. 2.3.4.1 [41], with the *--very-sensitive* parameter. To reduce the transcript redundancy and to obtained only coding transcripts, we used the software transdecoder v. 5.5.0 [40] to identify the candidate coding regions ORF (open reading frame), keeping the option *-single_best_only*. From these results, we retrieved the output file with the final candidate ORF regions of more than 100bp and then conducted a BLAST analysis using the Swissprot/Uniprot and Zebrafish databases obtained from www.uniprot.org (Swiss-Prot: release November, 2019) and NCBI (*Danio rerio,* number of accession: txid7955, release Apr 2018), respectively. Moreover, we explored the completeness of our assembly using BUSCO v3 [42,43] to obtain the number of conserved ortholog content from our results represented in the dataset *Actinopterygiiodb9*. Finally, we computed the Nx statistics to estimate the approximate length of our transcripts (N50) using the trinity script *trinity.stats.pl.* The N50 statistic allows us to know that at least half (50%) of the total transcripts have a specific length.

We annotated the transcripts of the final *de novo* transcriptome assemblies for each species using BLAST+ 2.10.0: December 16, 2019, and the databases Swissprot/Uniprot protein database (release November 29, 2019), Zebrafish (*Danio rerio,* release Apr 2018) for both species, and the Ballan wrasse (*Labrus bergylta*, release March 2020) for *L. dimidiatus* only, obtained from Ensembl (number of accession: GCA_900080235.1). We used *L. bergylta* because it is the closest species to *L. dimidiatus* with an available genome annotation. Finally, we used Omicsbox v. 1.3 [44] to functionally annotate the transcripts with Gene Ontology (GO terms) and KEGG pathways.

(d) Differential Expression Analyses

To perform differential expression analyses, we quantified transcript abundance for each species using the trinity script *align_and_estimate_abundance.pl* as follows: i) reference was built by selecting *--est_method* RSEM [45] *--aln_method* bowtie2 *--trinity_mode* *--prep_reference* and ii) quantification was run by indicating only RSEM v1.3.3 as quantification method and Bowtie2 [41] as mapping tool in the same way as for i) reference. With the final output files, we built a gene expression matrix using the script

abundance_estimates_to_matrix.pl [40], and we filtered out transcripts with no expression by using the script *filter_low_expr_transcripts.pl* while retaining the most highly expressed isoforms for each gene using the command *--highest_iso_only*.

To statistically evaluate differential gene expression, we used DESeq2-package v. 1.26.0 [46] in R with a Wald test statistic with a *design = ~ treatment* for each region of the brain separately. We used an FDR p-adjusted significance value of 0.05 and an absolute log2fold change threshold of 0.3 as a cutoff. We compared the individuals from control vs interaction to determine their significant differential gene expression for the three regions of the brain forebrain (FB), midbrain (MB) and hindbrain (HB) to retrieve the molecular response specific to these areas for both species, but separately for each species. Once statistically differentially expressed genes (DEGs) were obtained, functional enrichments were performed using Fisher's exact test by testing the DEG subsets against the whole *de novo* transcriptome with a significance cutoff of FDR 0.05 in Omicsbox v. 1.3. Each fish species was analysed separately to capture the full breadth of differential gene expression per species due to the fact that the molecular reactions vary across organisms and the two species are not closely related.

3. Results

Behavioural analysis

In the interaction treatment every pair of *L. dimidiatus* and *A. leucosternon* engaged in cleaning interactions. On average, each pair engaged in 43 ± 17.5 interactions, with an average duration of 13 ± 3.6 seconds, corresponding to a proportion of time spent interacting of 13 ± 6.6 % out of the 40 minutes of behavioural trials. Of these interactions, on average, 75 ± 13.7 % were initiated by the cleaner. The cleaner fish' dishonesty was answered with an average of 3 ± 2.8 client jolts and 2 ± 1.9 chases. Lastly, cleaners used tactile stimulation for reconciliation on average 3 ± 3.4 times. In the control, both *L. dimidiatus* and *A. leucosternon* were swimming around the tank without any display of abnormal behaviour or stress. All behavioural data can be found in Supplemental table S1.

De novo transcriptome assembly

The obtained final *de novo* transcriptome assemblies are the first references for both *L. dimidiatus* and *A. leucosternon* and resulted in 114,687 and 123,839 coding transcripts, respectively. Values of N50 indicated that at least half of the transcripts had a length of 1,813 and 2,827 bases, respectively. For *L. dimidiatus* and *A. leucosternon*, a total of 26,380 and 30,770 transcripts were annotated using the Swissprot database, respectively (BioProject: PRJNA726349). In addition, a total of 8,379 and 6,438 transcripts were annotated using the *Danio rerio* reference, and 28,988 transcripts were annotated only for *L. dimidiatus* using *Labrus bergylta* database. Further metrics and assembly steps can be found in supplementary table S2 and S3.

Gene expression analyses

Labroides dimidiatus

We evaluated the gene expression differences between individuals from control, which had no interaction, against individuals from the interaction treatment in each of the

three brain regions. 46 commonly differentially expressed genes (DEGs) across all brain regions were found exhibiting 45 enriched functions such as dendrite cytoplasm, mRNA binding, sodium ion transmembrane, transporter activity, ATP binding, positive regulation of dendritic spine morphogenesis (figure 2c; Supplementary table S4). When analysing differential gene expression for each brain region separately, we found that the hindbrain (HB) exhibited the highest number of differential gene expression among the brain regions (2,728 DEGs, figure 2a), followed by the forebrain (FB; 1,414 DEGs) and the midbrain (MB; 421 DEGs). In the HB, 1370 significantly enriched functions were found, including negative regulation of neuron apoptotic process, synaptic cleft, AMPA glutamate receptor activity, long-term memory, sensory perception of sound, and a total of 14 enriched functions related to behaviour such as motor behaviour, grooming behaviour, behavioural fear response (Supplementary table S7 and figure S3). In the FB, 985 biological processes were related to behaviour such as signal transduction, calmodulin binding, social behaviour, locomotory exploration behaviour, vocalization behaviour, among others (Supplementary table S5 and figure S1). Finally, for the MB, functional enrichment only resulted in 357 significant functions, including nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, neuronal action potential and sodium ion transmembrane transport (Supplementary table S6 and figure S2). Biological functions regarding behaviour in this brain region were related with behavioural response to pain, locomotory behaviour, male courtship behaviour, maternal behaviour and behavioural defence response (Supplementary table S6).

Acanthurus leucosternon

For *A. leucosternon*, the total number of DEGs was lower in comparison with *L. dimidiatus*. Only three common differentially expressed genes were found across the three brain regions, which are RNA/RNP complex-1-interacting phosphatase (DUS11), ATP-dependent DNA helicase PIF1 (PIF1) and a third gene for which no annotation was obtained. However, when analysing differential gene expression in each brain region separately, we found 139 DEGs in the HB and nine enriched in functions such as DNA metabolic process, DNA repair, biological regulation and protein binding (Supplementary table S10). For the FB, 52 DEGs were detected with six enriched molecular functions in signalling receptor activator activity, receptor regulator activity, hormone activity, and two biological functions: gas and oxygen transport (Supplementary table S8). Finally, 41 DEGs were found in the MB, and only Rab protein signal transduction was significantly enriched (Supplementary table S9). Like *L. dimidiatus*, the HB exhibited the highest number of DEGs in the comparison of control vs interaction (figure 2a & b).

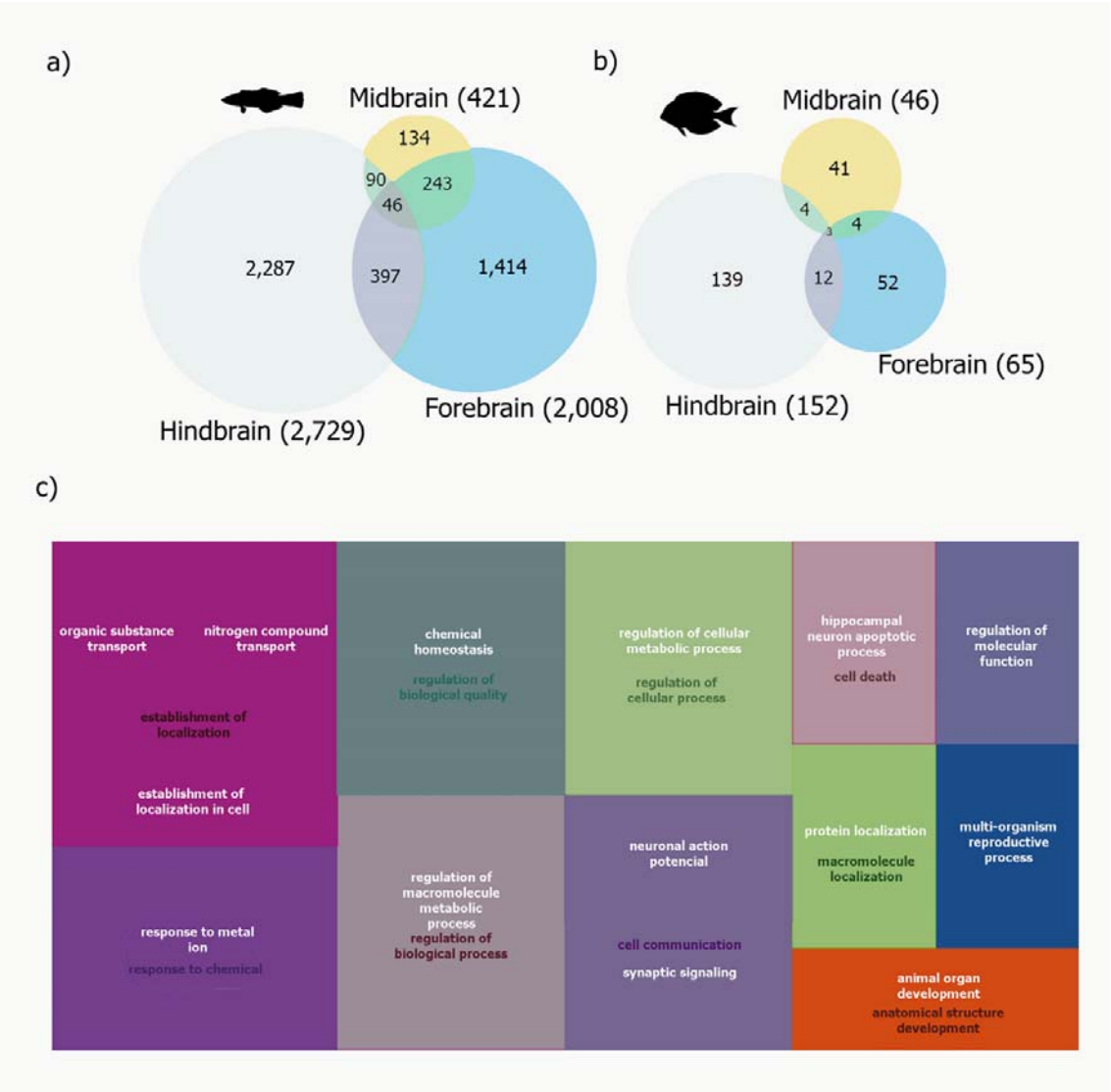

Figure 2. Number of differentially expressed genes between control and interaction for each of the three brain regions and the common overlap of (a) the cleaner fish *Labroides dimidiatus* and (b) the powder-blue surgeonfish *Acanthurus leucosternon*. Numbers in brackets represent the total differential expressed genes found at each brain region. (c) Gene Ontology treemap for *L. dimidiatus* representing the commonly enriched functions across the three brain regions when interacting with a client. Boxes with the same colour correspond to the upper-hierarchy GO-term, and its title is found in the middle of each box. For *A. leucosternon* no common enriched functions were found across the three brain regions. Description of the enriched functions for both species can be found in Supplementary table S6.

Evidence of significant differential expression of Immediate Early Genes (IEG) was found in *L. dimidiatus* with 31 differentially expressed IEGs [47] in the three brain regions (figure 3a). IEGs have been widely used to detect early neuronal responses in specific regions of the brain of vertebrates after external stimulus and are used as indicators of neural activity and adaptive plasticity in the nervous system [48]. These IEGs possibly contribute to the development of learning processes that can be useful during the social interaction of *L. dimidiatus*. However, more differentially expressed genes were found in the HB region (20) and the FB region (16) (figure 3a). Many of the functions of these genes are involved in signalling (*i.e.* RHEB, RGS6/9 and 19 [49,50]), transcription factor activation (*i.e.* KLF5/11 [51]) and plasticity (*i.e.* NPAS4L [52]), as well as binding, apoptosis, neuroprotection and

phosphorylation (MCL1, SOCS5 [53,54]). From all IEGs found, differential expression of FOS [47], JUN [47], RHEB [48], KLF11 [55] and SBK1 [56] (figure 3a; Supplementary table S11) was detected, and these genes play important roles in learning processes, memory and neural development [48]. For instance, genes FOS, SBK1 and KLF11 were differentially expressed in FB and HB, while JUN was differentially expressed in the HB only. SBK1 was downregulated in FB and HB, whereas FOS only in the HB. Finally, for *A. leucosternon*, the only IEG differentially expressed found in our dataset was JUN in the HB, hence found differentially expressed in both studied species.

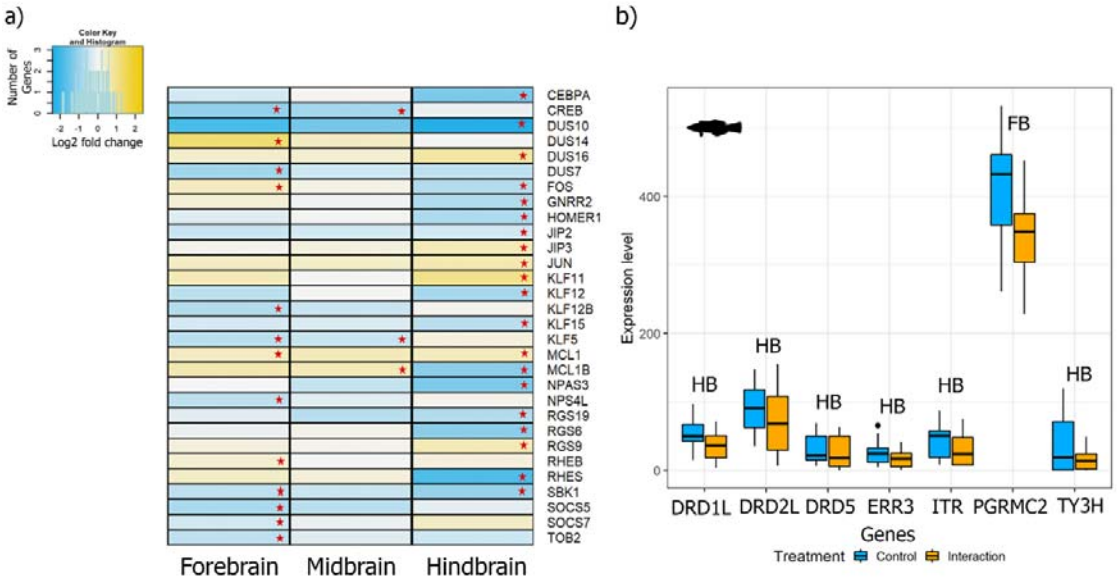

Figure 3. a) Comparative differential gene expression patterns of Immediate Early Genes between the regions of the brain of *L. dimidiatus*. Red stars represent significance. The legend indicates the reference values of log2fold changes for each DEG in the figure. b) Levels of expression based on normalized read counts of regulatory hormones genes from the Social Decision-Making Network (SDMN). These genes are significantly differentially expressed in the hindbrain (HB) and forebrain (FB) of *L. dimidiatus* between control and Interaction treatment.

In addition, we found that seven out of the ten genes belonging to the Social Decision-Making Network (SDMN [57]) were differentially expressed *in L. dimidiatus*. In contrast, none of these genes were differentially expressed in *A. leucosternon* (figure 3a, Supplementary figure S4). For *L. dimidiatus*, SDMN genes were only significantly differentially expressed in the HB region, except the Progesterone receptor (PGRMC2), which was differentially expressed in the FB instead. Also, differential expression of hormones such as Estrogen (ERR3) and neurohormones like Isotocin receptor (ITR) were significant in the HB region only. Finally, as part of the SDMN, Dopamine receptors DRD1, 2, 5 and TH (Tyrosine 3-monooxygenase) were also differentially expressed in the HB region (figure 3b, Supplementary table S12). Additional dopamine genes were found differentially expressed in the HB, such as Dopa Decarboxylase (DDC), Dopamine beta-hydroxylase (DOPO) and Tyrosine 3-monooxygenase (TH). These genes were downregulated during this interaction treatment (figure 4a, Supplementary table S13), and enriched functions such as Dopaminergic synapse, Dopamine biosynthesis processes, adenylate cyclase-activating dopamine receptor signalling pathway and regulation of dopamine secretion were detected in the HB only for *L. dimidiatus* (figure 4b). Regulation of synaptic transmission, dopamine metabolic process, D1 dopamine receptor binding, and cellular response to dopamine were enriched functions only in the FB (figure 4b). Enriched functions shared in the FB and HB

were the regulation of dopamine secretion and D2 dopamine receptor binding, suggesting that both brain parts oversee the modulation and frequency of dopamine release, whereas no differential expression of these genes was found in MB.

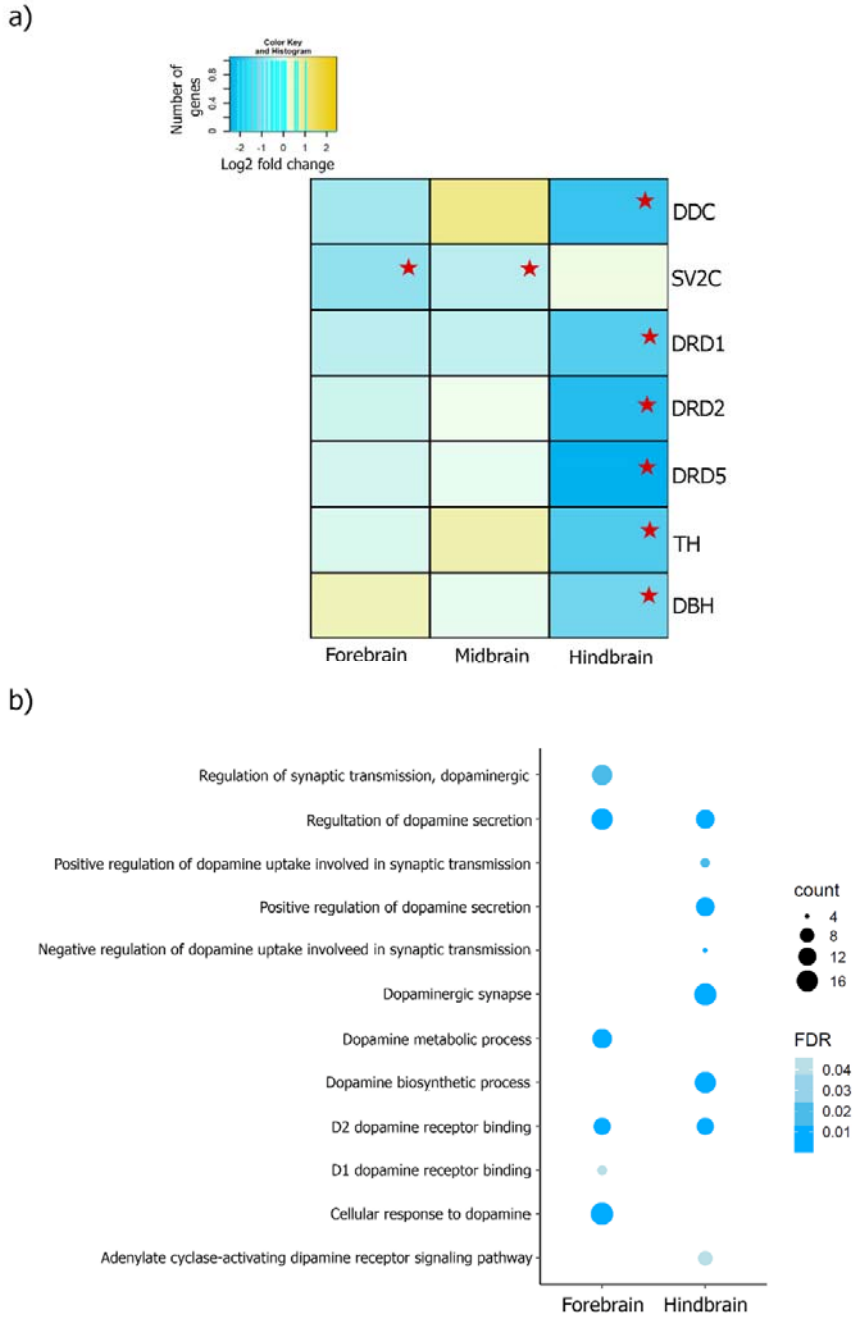

Figure 4. a) Dopaminergic synapse pathway differential gene expression in the three brain regions of *L. dimidiatus* (genes obtained from KEGG PATHWAY Database-Dopaminergic synapse, entry: map04728). The colours presented correspond to log2fold changes. The red star represents significance. The legend indicates the reference values of log2fold changes for each DEG in the figure. b) Functional enrichment of Gene Ontology (GO) terms related to Dopamine activity in *L. dimidiatus* in the fore and hindbrain. No enrichment was found for the midbrain region. The size of the circles is proportional to the number of genes observed within each GO category, and the colour of the circles is proportional to the significance (FDR value).

Furthermore, several genes differentially expressed were also found for *L. dimidiatus* related to the glutamatergic synapse pathway (figure 5; Supplementary table S14 and figure S5). Many enrichments related to this pathway were exhibited in the brain of interacting *L. dimidiatus*, such as positive regulation of synaptic transmission glutamatergic, NMDA selective glutamate receptor signalling pathway, kainate selective glutamate receptor activity and AMPA glutamate receptor activity, among others with enrichments in the three regions of the brain (figure 5b). Interestingly, more functions were shared by the FB and HB, such as AMPA glutamate receptor complex, ionotropic glutamate receptor binding, NMDA glutamate receptor activity, glutamatergic neuron differentiation, glutamate binding and NMDA selective glutamate receptor complex (figure 5b). These functions stem from a total of 72 differentially expressed genes in the brain of *L. dimidiatus*, playing a role in the glutamatergic synapse processes (Supplementary table S14). The differentially expressed genes (DEGs) within this pathway also followed our previous general pattern in which the HB region reports a higher number of significant DEGs (48), followed by the FB (46) and the MB (16; Supplementary table S14). In particular, most of the ionotropic receptors (Supplementary table S14) were differentially expressed in the HB and FB except for GRIA3 and GRIA4, which were also differentially expressed in the MB. These receptors exhibited a downregulation pattern in all three regions of the brain when interacting with another species. In contrast, metabotropic receptors were differentially expressed in HB and FB during interaction treatment (Supplementary table S14); however, main receptors GRM1, 5 and 7 showed upregulation patterns in these two brain regions, whereas GRM3 and 4 were downregulated at HB.

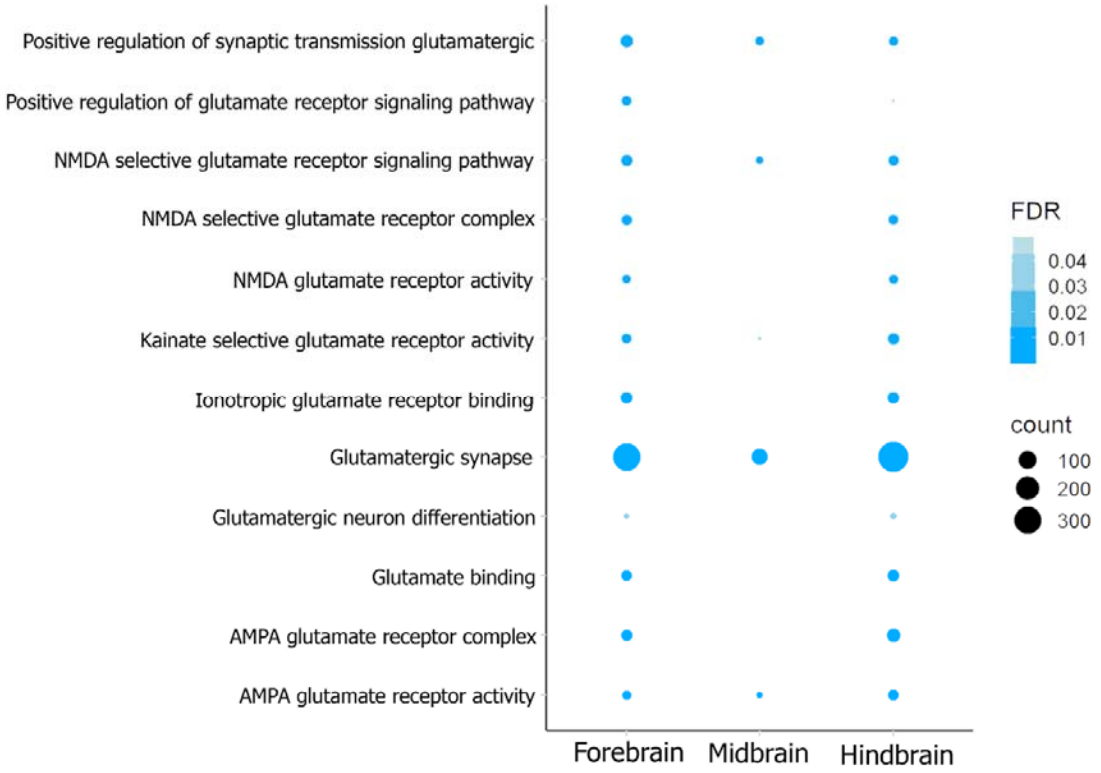

Figure 5. Functional enrichment of Gene Ontology (GO) terms related to the Glutamatergic synapse pathway in *L. dimidiatus* individuals in the fore, mid and hindbrain. The enrichments are based on differentially expressed genes of the comparison control vs interaction. The size of the circles is proportional to the number of genes observed within each GO category, and the colour of the circles is proportional to the significance (FDR value).

On the contrary, *A. leucosternon* only had two DEGs related to the glutamate pathway: Glutamate carboxylase (DCE1) and Glutamate receptor ionotropic (GRID2). However, the hormonal response was an enriched function in the brain of *A leucosternon* during the interaction treatment (Supplementary table S15). This was in concordance with the enrichment terms found in the FB region of this fish (Hormone activity, supplementary material table S15). In particular, pituitary hormones such as Somatotropin (SOMA), Prolactin (PRL), Somatolactin (SOML2), Gonadotropins (GTHB1 and 2), Thyrotropin (TSHB) and Glycoproteins (GLHA) were differentially expressed, but downregulated, during the interaction treatment. Furthermore, differentially expressed genes with functions related to calcium-binding activity, oxygen transport and signalling were upregulated during the interaction in the FB (*i.e.* CHP2, FCRL5, PTPRF, ADA1B, MICU3, Supplementary table S15 and figure S6), suggesting signalling roles in this part of the brain of *A. leucosternon* during the interaction treatment in comparison with the control.

4. Discussion

We investigated the molecular mechanisms involved in the interaction behaviour of *L. dimidiatus* with its client fish, *A. leucosternon*. We found distinct transcriptional and functional patterns for the two species when interacting with each other. For *L. dimidiatus*, the alteration of general neural processes in the brain such as dendrite cytoplasm, positive regulation of dendritic spine morphogenesis, neuronal cell body and ATP binding is found when brain regions are analysed together. However, functions related to social behaviour, behavioural fear response, locomotory exploration behaviour, motor behaviour, among others, were also observed in this fish while interacting with a client. *L. dimidiatus* is a species known for having complex mutualistic behaviour that can be adjusted upon specific cooperative signals [5,29]. We found molecular functions related to social behaviour, suggesting this fish relies on specific brain regulations to develop social mechanisms and deal with new information. This has been demonstrated at ecological and physiological levels in this species, suggesting that this fish relies on several types of behaviours such as prosocial behaviour, social recognition, social bonding, assessment of the social environment, social memory and learning [58]. These cognitive dimensions have been identified as the building blocks of cooperative behaviour in cleaner wrasses [29,58], including nonapeptides Arginine-vasotocin (AVT) and Isotocin (IT) [59], Cortisol [29,60] and Dopamine and Serotonin [30,61]. We now find molecular evidence underlying these cognitive skills by deciphering the transcriptional basis to cooperative behaviour. The molecular signatures found specify neural mechanisms used when cleaner wrasses interact with clients in social situations and environments (*i.e.* new client, social recognition, [29]). In contrast, none of these genes detected in *L. dimidiatus* were enriched in any of the brain regions of *A. leucosternon*, indicating the ability of the cleaner wrasse to modulate and adjust its behaviour. These changes occur in major areas of the brain, and therefore the exhibited underlying molecular mechanisms reveal how this behavioural modulation occurs.

We observed different responses of the three brain regions, forebrain (FB), midbrain (MB) and hindbrain (HB). The HB and the FB exhibited the largest transcriptional signature with more differential gene expression during the interaction when compared to the MB in both species. This suggests that most of the stimulus development occurs in these regions, and they play essential roles in the process and maintenance of behaviour when *L. dimidiatus* is interacting with *A. leucosternon*. The HB region in teleost fishes corresponds to the cerebellum and myelencephalon (including rhombomeres, [62]), and its function has been

attributed to motor activity, autonomic responses (*i.e.* eye retraction, heart beating), spatial learning as well as learning and memory [63,64]. In addition, the FB (divided into diencephalon and secondary prosencephalon) is well known for controlling motivated behaviour, memory, instinct modulation and decision-making [62]. Several studies are in concordance with the present findings with behavioural activity detected in the FB and HB confirming that social behaviour in *L. dimidiatus* is mainly processed in these brain areas [29,65].

When analysing the specific molecular mechanisms of social behaviour, the Social Decision-Making Network (SDMN) is important in the modulation of neural mechanisms for social plasticity, specifically decision-making, learning or Long-Term Potentiation (LTP, [5,66]. These genes are also conserved across many vertebrate species, including fish, and we found 70% of the SDMN genes differential expressed in the brain of *L. dimidiatus* [57]. This result suggests that the mechanisms used to modulate the social interaction in this species were differentially expressed when encounters with *A. leucosternon* occur. For instance, the Estrogen receptor (ERR3) and Isotocin hormone receptor (ITR), modulators of neural circuits underlying behaviour that affect the perception and cooperativeness in teleost fishes [58], were differentially expressed. In particular, Isotocin is an important neurohormone in the social interactions of the cichlid fish *Neolamprologus pulcher*, as high levels of injected Isotocin modulates sensitivity of individuals depending on the social information (size of the opponent), thus inflecting the capacity of the individuals to fit their behaviour depending on the circumstances presented (social plasticity, [1,67]). Therefore, the differential expression of Isotocin and Estrogen in *L. dimidiatus* may be contributing to establishing social relationships based on the modulation of neural processes that lead to individual recognition and memory to cooperate with the client.

In addition, sex steroids (Progesterone and Estrogen) are also known to regulate learning and memory processes in teleost fish when their expression is in the hippocampus (FB), especially Progesterone [58]. Moreover, high mRNA levels of Estrogen receptor modulate dominant behaviour in female zebrafish, causing changes in social status dynamics [68]. On the one hand, we found that estrogen receptor gene expression was downregulated during the interaction of the cleaner wrasse with its client, which could suggest a reduction in dominant behaviour or possibly a submissive approach when facing a client. On the other hand, the downregulation of the progesterone receptor in the FB suggests modulation of higher brain functions such as recognition, social relationships, learning and memory when interacting with a client. It has been demonstrated that the expression of Androgen, Progesterone and Estrogen receptors in *A. burtoni* leads to the regulation of complex social behaviour, behavioural plasticity, and the evaluation of rewarding stimulus between dominant and subordinate males [69,70]. Consequently, the differential expression of sex steroids receptors (estrogen, progesterone) and neurohormone receptors (ITR) shown here in the HB and FB suggests that they might be a core behavioural mechanism regulating the modulation of the cooperative behaviour of a highly social fish such as *L. dimidiatus* when interacting with client species.

To understand how changes in gene expression mediate social cognition, Immediate Early Genes (IEGs) can provide insights into rapid shifts in behavioural states [1]. Here, we show that *L. dimidiatus* differentially expressed several IEGs in the brain during the interaction with *A. leucosternon*, whereas the client did not show any change in transcription. This indicates an important activation of transcription factors and their participation in the transduction of signals when *L. dimidiatus* is interacting with a client. For instance, the

expression of *c-fos* (FOS) has been reported in the hippocampus region (FB) of cichlid fish *A. burtoni* during social and territorial interactions of males, indicating regulation of memory, spatial processing and social recognition when interacting with non-dominant males [71]. Thus, the upregulation of *c-fos* in the FB of *L. dimidiatus* may be suggesting similar neural mechanisms from this part of the brain, but in our case, they may be activated to recognize and interact with its clients. In addition, CREB regulatory factor (CREB) is critical for the consolidation of memory (LTP, [72]) and was also found differentially expressed in the cleaner wrasse. Studies examining the molecular mechanisms of learning and memory in feeding and consolidation of new habits using mandarin fish (*Siniperca chuatsi*) have also shown a differential gene expression of this gene when fish learn to eat dead prey after a training period [73]. Therefore, the differential expression of CREB in the brain of *L. dimidiatus* may also suggest the activation of downstream processes of memory when interacting with clients in the FB and MB, which correspond to areas where LTP and associative learning occur in teleost fishes [62]. Consolidation of memory and learning is important for the cleaner wrasse as it can choose to clean clients, cheat or judge whether to provide tactile stimulation [61]. Here we observed specific molecular signatures of IEGs such as transcription factors *c-fos* and CREB, suggesting that social cognition, memory and learning processes are activated in the brain of this species during interaction with clients.

Dopamine is also one of the major molecules related to signalling in the brain of vertebrates, and it is well-known to be involved in the modulation of animal behaviour and cognition [74]. The dopamine pathway was also involved in the interaction of our cleaner wrasse with its client fish, highlighting it is one of the molecular regulators participating in the cooperative behaviour of this fish. In addition, Dopamine signals are released upon specific stimuli [75], and it has been established that it modulates the way *L. dimidiatus* consolidates interactions with the clients as well as for associative learning [61,76]. For instance, dopamine receptors D1 and D2 have been shown to be disrupted using antagonists to test whether cleaner wrasse willingness to clean remains unaffected [77]. They found that the alteration of receptors reduced levels of Dopamine transmission and increased cleaner wrasses willingness to provide more tactile stimulation which is a more “costly” behaviour. This highlights the role of this hormone in the modulation of social behaviour during interaction with clients and agrees with the pattern found in our experiment in which dopamine receptors D1 and D2 were also downregulated in the HB. Furthermore, changes in social situations can cause alteration in these receptors because animals need to re-evaluate decisions and make behavioural adjustments to new events [78], thus the downregulation of dopamine in the HB may also be due to a response of dopamine signalling upon a new stimulus in *L. dimidiatus* (having another fish in the same tank). Consequently, these molecular signatures suggest that the cleaner wrasse may be increasing its willingness to interact with the client and possibly promote major tactile stimulation and negotiation. Finally, changes in the social context produce a re-evaluation of behaviour by activating dopaminergic synapses, and this may explain the differential expression patterns of this pathway shown here. Processes of learning, social behaviour modulation and decision-making via dopamine cascades correspond to molecular signatures in the cooperative behaviour of *L. dimidiatus*.

We found that the biosynthesis of dopaminergic synapses in the HB and the regulation of dopamine secretion and metabolic processes in the FB were enriched functions when *L. dimidiatus* was interacting with its client. The differential expression of Catecholamine systems such as Dopaminergic activity are strongly ligated to specific regions of the brain such as the rhombencephalon (HB) and telencephalon (FB) in teleost fishes [62]. In

particular, the FB (preoptic area, olfactory bulb, pallium, subpallium and retina) is known to behold the core of the dopaminergic reward system in vertebrates, where several dopamine neurons regulate similar processes of discrimination of sensory cues and expression of tyrosine hydroxylase (TH) as the initial step of Dopamine pathway [79]. It has been shown also that the FB is connected with the Ventral Tegmental Area (VTA) in the MB of mammals, which is in charge of evaluating environmental stimuli and regulate motivating events such as *L. dimidiatus* when interacting with clients [62,80]. Even though teleost fish lack MB dopaminergic cell groups as shown by neuron immunofluorescence in zebrafish [81], the posterior tuberculum (TPp) located in the limit between the FB and MB [64], may be accomplishing this connective role of dopaminergic signalling as a homologous part of the FB and VTA in mammals [6], and this is why we found gene expression in the MB region of several dopamine genes but none of them were differentially expressed (figure 4 a, b, Supplementary table S12). Consequently, our results highlight the potential brain areas where differential expression of dopamine occurs during the cooperative behaviour of *L. dimidiatus*.

The formation of cognitive abilities, modulation of social interactions and memory are not only determined by IEGs and Dopamine signalling. In fact, Glutamate is the primary excitatory neurotransmitter in the brain and the central nervous system of vertebrates [82]. This neurotransmitter produces glutamatergic synapses in the brain, specific sites where memory consolidation, synaptic plasticity, and storage occur. We found glutamate-related genes differentially expressed in all three brain regions of *L. dimidiatus* during its interaction with the client, indicating a key role of this pathway in the mediation of synaptic transmission, activation of neurons and synapse plasticity in the brain of this fish. Differential expression of the two major groups of glutamatergic receptors in the FB and HB regions (ionotropic and metabotropic receptors) was found. In particular, ionotropic receptors NMDA and AMPA (*gria* 1-4) suggest a contribution in the activation of Long-Term Potentiation (LTP)_processes and synaptic plasticity revealed by elevated expression in zebrafish in response to Growth Hormone (GH) induction during an "*Inhibitory avoidance test*" where electric shocks were received when entering in a dark zone [83]. We found that all ionotropic glutamatergic receptors were differentially expressed mainly in the FB and HB in *L. dimidiatus*. This expression pattern has been found in other fishes such as the electric fish (*Apteronotus leptorhynchus*; [84]) and zebrafish [85] using autoradiographic binding sites and *in situ* hybridization, respectively. Thus, our study suggests that LTP processes and synaptic plasticity may be occurring in these two brain regions in *L. dimidiatus* during an interaction. Moreover, both groups of glutamate receptors are thought to be the facilitators of synaptic plasticity upon a new stimulus, produce memory consolidation development and storage [66]. Thus, the fact that we found differential gene expression in these two brain regions, suggests that the uptake of new information for *L. dimidiatus* from the interaction with a new client may be triggering memory consolidation processes via glutamate neurotransmission. It is well-known that this fish can recognise its clients to provide services of cleaning [86], which uncover molecular signatures behind behavioural processes in the cooperative behaviour of cleaner fish.

The discovered molecular signatures of the dopaminergic pathways, immediate early gene activation and glutamate synapses were more evident in the bluestreak cleaner wrasse than in the powder-blue surgeonfish. However, we observed in *A. leucosternon* that the FB showed differential expression of pituitary hormones such as Somatotropin releasing-hormone (SOMA), Prolactin (PRL), Somatolactin (SOML2), Pro-opiomelanocortin (POMC) and Gonadotropin subunit beta-1 and 2 (GTHB1, 2). These hormones are strongly related to a great variety of functions [87]. For instance, genes SOMA and PRL play a role in locomotor

and feeding behaviour and cognitive functions [83,88]. Moreover, SOMA and SOML2 have been associated with stress and adaptation to environmental changes in the Atlantic cod *Gadus morhua* and flounder *Paralichthys oliuaceus* [87]. Even though the variability and versatility of pituitary hormones are considerable, some studies have evaluated the synergetic effects in the behaviour of rainbow trout (*Oncorhynchus mykiss*) by confronting pairs of fish after the injection of SOMA intra-peritoneally. Fish pairs containing a high expression of SOMA displayed more aggressive behaviour during the interaction between individuals [88]. In addition, the up-regulation of POMC (Pro-opiomelanocortin) in socially subordinate rainbow trout males suggested elevated cortisol levels in the Preoptic Area (POA/FB), resulting in chronic stress and food intake disruption [89]. Even though *A. leucosternon* is known to display aggressiveness during agonistic encounters by both females and males when conspecifics invade their territory [90], no studies have reported the expression of these hormones in this surgeonfish species. However, we can hypothesize that the downregulation of both POMC and SOMA in the FB of *A. leucosternon* may indicate a decrease in aggressive behaviour, as low expression of SOMA regulates this behaviour and the low expression of POMC deactivates the pituitary axis in charge of reducing cortisol levels and stress in teleost fish [91]. In addition, it is also well known that *L. dimidiatus* can provide stress relief to clients by lowering cortisol stress hormone levels through physical contact (known as tactile stimulation). This was demonstrated with a surgeonfish species (*Ctenochaetus striatus)* when allocated in aquariums with look-alike models of *L. dimidiatus* [36]. Furthermore, our behavioral trials also support this hypothesis since we found that 75% of the interactions were initiated by the cleaner. Consequently, since physical contact alone from *L. dimidiatus* can reduce stress in Acanthurid species and a downregulation of pituitary hormones SOMA and POMC was observed during the interaction treatment, our results suggest stress-relief behaviour in the client during the interaction with *L. dimidiatus* and this is evidence of the molecular responses activated in the brain of this surgeonfish species when interacting with cleaner wrasses.

In conclusion, differences in gene expression patterns in both fishes were noticeable, being *L. dimidiatus* the species with large transcriptional reprogramming and associated functions compared to *A. leucosternon*. When the two species interacted, *L. dimidiatus* activated immediate early genes, especially in the HB and FB, and an important alteration of molecular pathways associated with glutamate neurotransmission, dopamine synapse and hormones (Isotocin, Estrogen and Progesterone) was also observed. In contrast, in *A. leucosternon* the major molecular signal corresponded to decreased transcription of pituitary hormone genes, suggesting a reduction of aggressiveness during the interaction with the known capability of *L. dimidiatus* to reduce stress in its clients. Therefore, the molecular signals found in this study suggest the activation of important processes used by social species such as learning, memory, decision-making and social plasticity. Our results identify underlying molecular signatures in neural networks activated in the brain of *L. dimidiatus* and its client *A. leucosternon*. These advances to the current knowledge on cooperative behaviour allow for understanding the cleaner wrasse's remarkable cognitive abilities.

Author contributions

JRP built the experimental setup with input from RR. JRP & CS designed the project. JRP provided maintenance of aquarium setups, performed the behavioural assays and sampled the fish brains. EO and JRP analysed the behavioural videos and data. CS, with support from TR, conducted laboratory work and prepared samples for sequencing. SRC analysed data with

input from CS. SRC and CS wrote the first draft, and all author edited and approved the final manuscript.

Competing interests

We declare we have no competing interests.

Ethical note

This work was conducted under the approval of Faculdade de Ciências da Universidade de Lisboa animal welfare body (ORBEA – Statement 01/2017) and Direção-Geral de Alimentação e Veterinária (DGAV – Permit 2018-05-23-010275) following the requirements imposed by the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. A Material Transfer Agreement of biological samples (fish brains) was signed between MARE and KAUST.

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