1 **Profiles of cooperative brains: A discriminant analysis of cleaner and**
2 **client fish monoaminergic responses to different social contexts**

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25 **Abstract**

26 Vertebrate cognitive function requires a dynamic coordination of multiple
 27 specialized areas of the brain. The challenge here is to understand how these brain areas
 28 respond in dependence to the neurophysiological mechanisms in place, as to enable the
 29 successful processing of information. For instance, social and cooperative behaviour has
 30 been linked to the activation of some specific brain areas, mostly associated with reward
 31 processing. Here we evaluated a classic model system of cooperation between species
 32 of fish and compared datasets of brain monoaminergic response. We analysed by using
 33 multivariate discriminant analysis the exposure of cleaners, *Labroides dimidiatus*, to
 34 several social-related conditions, as well as the response of one client species, *Naso*
 35 *elegans*, to similar contexts. We demonstrate that the variable appraisal of each social
 36 challenge contributes to brain dopaminergic and serotonergic changes, in cleaners and
 37 clients, with both showing the diencephalon and optic tectum as main areas of
 38 metabolite response. The role of the serotonergic system activation was mostly
 39 demonstrated at the diencephalon and cerebellum of cleaners, a response that was
 40 driven by mutualistic interaction, contact with client. Our current evidence is the first to
 41 jointly demonstrate the level of selective similarity in brain monoaminergic mechanisms
 42 that underlie fish mutualistic and social engagement, for both sides of these
 43 partnerships.

44

45 **Key-words:** Cooperation, monoaminergic mechanisms, brains, cleaners, clients

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47

1. Introduction

The neural substrate of cooperative behaviour is gaining increasing attention to the recognition that cooperation is widespread in nature [Dugatkin 1997], especially on fish species [Soares et al. 2018]. Cleaner wrasses (*Labroides dimidiatus*) are mutualistic fish that engage in cooperative behaviour, inspecting the surface, gills and sometimes the mouth of visiting ‘client’ reef fish, eating ectoparasites, mucus, scales and dead or infected tissue [Bshary and Côté 2008; Soares 2017]. Cleaners display flexibility in adjusting their cleaning services in accordance with clients, varying between “cooperating” (removing ectoparasites) or “defecting” (eating fish mucus) [Bshary and Côté 2008]□.

There is limited evidence for a role of monoamines on cooperative behaviour. In healthy humans, tryptophan depletion reduced the level of cooperation shown by participants in the first day of an iterated Prisoner’s dilemma game [Wood et al. 2006]. In cleaner wrasses, fluoxetine and the serotonin (5-HT) 1A receptor agonist 8-OH-DPAT increased cooperative behaviour (raising engage in cleaning behaviour and the frequency of tactile stimulation), while blocking serotonin synthesis (by pCPA or antagonizing the 5-HT_{1A} receptor (WAY 100,635)) decreased cleaner’s cheating levels [Paula et al. 2015]□. The blocking dopamine (DA) D1 receptors also increases cooperative behaviour (raising tactile stimulation and induces cleaners to initiate more interactions with clients) [Messias et al. 2016]□. These effects appear to be mediated by the degree of familiarity with the client, with D1 antagonists increasing tactile stimulation only when interacting with novel clients [Soares et al. 2017]□. It has been suggested [Soares et al. 2018] that DA modules predicted outcomes, with reductions in DA signalling contributing to an omission of reward (when the outcome is worse than predicted, e.g., lower probability of getting food during an interaction, or higher

73 likelihood of being punished), suggesting that 5-HT and DA have opponent roles in
74 tracking outcomes.

75 A relevant question focussing on the role of monoamines in this mutualistic
76 system regards the importance of physical contact. Monoaminergic signalling has been
77 proposed to mediate social behaviour in fish, increasing dopamine levels after visual
78 contact with conspecifics [Saif et al. 2013]. In addition, in cleaning interactions,
79 physical contact (known as massages) occurs frequently as it is provided by cleaners,
80 and functions to benefit clients in terms of stress reduction [Soares et al. 2011]. Thus,
81 the monoamines could act at various points of the cooperative and mutualistic
82 interactions (e.g., client evaluation, motivation to approach the client, maintenance of
83 cleaning engagement after initial contact, and during inspection [Soares 2017]).
84 Moreover, DA is associated with the social behaviour network as to reinforce responses
85 to salient social stimuli [O'Connell and Hofmann 2011]. Here we follow on recent
86 published work of [Abreu et al. 2018b] and use this dataset to infer, only this time, by
87 using multivariate discriminant analysis, on monoamine mediation on the motivation to
88 approach and engage in social and mutualistic interactions by cleaners *L. dimidiatus*
89 when introduced to a client or to another conspecific (in visual or physical access).
90 Furthermore, we also used the previous published dataset of [Abreu et al. 2018a] to
91 associate monoamines with the clients (*Naso elegans*) drive to being cleaned (when
92 introduced to a cleaner) or to engage in social interactions (when introduced to another
93 conspecific). We thus examined the level of selective similarity in brain monoaminergic
94 mechanisms, by measuring brain monoamines DA and 5-HT and metabolites DOPAC
95 (3,4-dihydroxyphenylacetic acid) and 5-HIAA (5-hydroxy indole acetic acid), at various
96 brain areas (forebrain (FB - which included the olfactory bulbs and the telencephalon),

diencephalon (DL), optic tectum (OT), cerebellum (CB) and brain stem (BS)), that may underlie fish mutualistic and social engagement, for both sides of these partnerships.

Material and Methods

2.1. Experimental conditions

We analysed data from experiments conducted at the fish housing facilities of the Oceanário de Lisboa (Lisbon, Portugal). In these experiments were used 53 Indo-Pacific bluestreak cleaner wrasses, *Labroides dimidiatus* and 18 adults blond naso tang *Naso elegans* (family Acanthuridae; referred to as “clients”). For more information about the experimental conditions see [Abreu et al. 2018b; Abreu et al. 2018a]. The quantification of monoamines in fish occurred by high performance liquid chromatography with electrochemical detection (HPLC-EC). In brains were analyzed the DA and 5-HT, as well as the metabolites DOPAC (3,4-dihydroxyphenylacetic acid) and the 5-HIAA (5-hydroxy indole acetic acid), as described by [Øverli et al. 1999]. The experiments were carried out in accordance to the approved guidelines by the Oceanário de Lisboa and the Portuguese Veterinary Office (Direccao Geral de Veterinaria, license # 0420/000/000/2009).

2.1.1. Cleaners perspective

Here, we used data from [Abreu et al. 2018b]. Cleaners were exposed to five different contextual treatments. Indeed, on each experimental day, one of the five following treatments was randomly allocated to each subject cleaner wrasse aquarium: a) conspecific (*L. dimidiatus*, n = 10), b) client (*N. elegans*, n = 11), c) conspecific inside another smaller aquarium (n = 12), d) client inside another smaller aquarium (n = 10) and e) ball (white ball, about 5 cm in diameter, which stayed at the bottom, completely sessile, n = 10), see Fig 1. Cleaners were exposed to treatments for the next

122 60 minutes. At the end of experiments, each subject cleaner wrasse was captured and
123 immediately sacrificed with an overdose of tricaine solution (MS222, Pharmaq; 500–
124 1000 mg/L) and the spinal cord was sectioned. The brain was immediately dissected
125 under a stereoscope (Zeiss; Stemi 2000) into five macro-areas: forebrain (FB - which
126 included the olfactory bulbs and the telencephalon), diencephalon (DL), optic tectum
127 (OT), cerebellum (CB) and brain stem (BS) (for a drawing of the major brain areas,
128 please see [Abreu et al. 2018b; Abreu et al. 2018a]. Major brain areas were frozen with
129 dry ice and the stored at - 80°C.

130 2.1.2. Clients perspective

131 We used data from [Abreu et al. 2018a], which included two of the treatments,
132 and added a third context (ball group), similarly to use in cleaner perspective (see
133 above). Three different contextual treatments were allocated to our subject clients (*N.*
134 *elegans*): a) conspecific (*N. elegans*, $n=7$), b) a cleaner (*L. dimidiatus*, $n=7$) and c) ball
135 (same as above, $n=4$) see Fig 1. Cleaners were exposed to treatments for the next 60
136 minutes. At the end of experiments, each subject client was captured and immediately
137 sacrificed and brain micro-dissected as explained above.

138 2.2. Statistical analysis

139 We used multivariate discriminant analysis (MDA) to test for differences in the
140 pattern of metabolite expression among the 5 social contexts for cleaners perspective, or
141 the 3 social contexts for clients perspective. One set of MDA uses as data the
142 concentrations of each monoamine (DA and 5-HT) and metabolite (DOPAC and 5-
143 HIAA) at each brain area. This allows us to find the combination of metabolites and
144 brain areas that discriminate the most the different social contexts. Another set of MDA
145 uses as data the ratios of DOPAC/DA and 5-HT/5-HIAA as these will enable us to
146 interpret the relation between monoaminergic production and its subsequent metabolic

147 use. We interpret the two principal discriminant functions (DF1 and DF2) of each MDA
148 based on trait loadings higher than 0.40 [Hair et al. 2010]. We assessed the significance
149 of the discrimination within the entire set of social contexts using Wilk's Lambda, and
150 we used ANOVAs with post-hoc Tukey HSD tests to find which pairs of social context
151 differ significantly in DF1 or DF2. All values were standardized using script available
152 [Coghlan 2013] and normality was checked through q-q plots. All analyses were run
153 with R (version 3.3.3; R Core Team, 2017) package car (version 2.1-4; [Fox and
154 Weisberg 2011] and MASS (version 7.3-47; [Venables and Ripley 2010]).

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156 Open science practices

157 Data for this manuscript can be found at our GitHub repository.

158 (<https://github.com/lanecunifesspa/cleaners>).

159

160 3. Results

161 3.1. Cleaners perspective

162 Overall, social contexts differed in the levels of metabolite expression (Wilk's
163 lambda = 0.03, $p = 0.001$; Fig.2A) and in metabolite ratios (Wilk's lambda = 0.23, $p =$
164 0.02; Fig.2 B) across the brain tissues of cleaners. For metabolite levels, DF1 and DF2
165 explained 83.9% of the variation between all the contexts. DF1 differed significantly
166 between most pairs of contexts, except for the comparison between client vs.
167 conspecific inside another aquarium, which instead were found to differ in DF2 (Fig.
168 2A, Table 1). DF1 was characterized mostly by strong negative loadings of DOPAC at
169 the brain stem, and of DA, 5-HIAA and 5-HT at the diencephalon of cleaners (loadings
170 < -0.40 ; all other loadings $< |0.29|$; Fig. 2C and Table 2). DF2 was characterized mostly

171 by a strong positive loading of DA at the optic tectum (loading = 0.58; all other loadings
172 < |0.36|; Fig. 2C and Table 2).

173 When using instead ratios of metabolite expression in the brain tissues of
174 cleaners, DF1 and DF2 together explained 75% of the variation among contexts. DF1
175 differed significantly between cleaners that were prevented to interact with clients
176 (inside a smaller aquarium) from those that could engage freely with clients (Fig 2B;
177 Table 1). DF1 also differed between fully interactive contexts (i.e. interaction with
178 clients) and either controls or interaction with conspecifics, and between clients in
179 separate aquaria and conspecifics in separate aquaria (Fig 2B; Table 1). DF2 differed
180 only between interaction with clients and either conspecifics in separate aquaria or
181 conspecifics (Fig 2B; Table 1). DF1 was characterized mostly by loadings of 5-HIAA/5-
182 HT ratios at the diencephalon and at the cerebellum, although in different directions
183 (loadings = -0.51 and 0.45, respectively; all other loadings < |0.33|; Fig. 2D and Table 3).
184 DF2 was characterized mostly by a strong negative loading of 5-HIAA/5-HT ratio at the
185 diencephalon and cerebellum (loading = -0.50 and -0.41, respectively; all other loadings
186 < |0.27|; Fig. 2D and Table 3).

187

188 3.2. Clients perspective

189 Regarding clients, the exposure to different social contextual treatments was
190 solely found to differ significantly in the levels of metabolite expression (Wilk's lambda
191 = 0.01, $p = 0.02$), showing a clear separation between the individuals exposed to
192 different contexts (Fig. 3A) but not for metabolite ratios (Wilk's lambda = 0.42, $p =$
193 0.51, Fig 3B).

194 For monoaminergic metabolite production, DF1 and DF2 explained 99% of the
195 variation and differ significantly among all pairs of contexts, except for the context of a

cleaner with a client, which was not significant for DF2 (Table 4). DF1 seems to be explained by two areas of client fish *N. elegans*: the diencephalon (varying in terms of DOPAC and 5-HIAA, in opposite directions) and the optic tectum (solely for DOPAC; Fig. 3C, Table 2), while DF2 seems to be explained by the DA on the diencephalon (Fig. 3C, Table 2). Surprisingly, no differences were found in the overall ratio patterns (monoaminergic ratios DOPAC/DA and 5-HIAA/5-HT; Wilk's lambda = 0.42, $p = 0.51$).

4. Discussion

Discriminant analysis is a powerful statistical tool that enabled us to determine the contribution of social and mutualistic behaviour to the modulation of brain monoaminergic levels of cleaners and clients, and specifically those linked to the activation of some localized brain areas. We found that brain monoaminergic response to social challenges shared interesting similarities for cleaners and clients, with most relevant alterations occurring at the diencephalon and optic tectum, in reference to DAergic and 5-HTergic change. While client fish clearly responded to differences posed by conspecifics compared to heterospecifics, we discovered that it was cleaners' 5-HT system that mostly contributed to separate mutualistic activities from all others. This interaction is changed crucially whenever these individuals were given the possibility to physically engage in cleaning (occurring at the cerebellum) or were prevented to interact (occurring at the diencephalon) [Abreu et al. 2018b].

Broad analysis of cleaners' monoaminergic response in respect to all different treatments suggests the distinction of 3 groups: a first with inaccessible clients and ball group, a second composed by inaccessible conspecifics and a third that included conspecifics and heterospecifics that were fully accessible. Specifically, the DF1 axis

221 represented most of the variation observed, being mostly associated with DA, 5-HT and
 222 5-HIAA at the diencephalon and DOPAC at the brain stem; while the DF2 mostly
 223 distinguished those stimuli that cleaners could physically access from those they could
 224 not, being these last group associated with DA levels at the optic tectum. It is usually
 225 considered that monoaminergic increase (DA or 5-HT) means an increment in
 226 production while the elevation of metabolite levels (DOPAC or 5-HIAA) signals for the
 227 actual system activation and monoamine usage (see [Saif et al. 2013]). One potential
 228 explanation for the individualized response of the DAergic system at the optic tectum,
 229 which corresponds with the ball group, is cleaners' visual stimulation, in the absence of
 230 any other stimulation; while the DOPAC at the brain stem should be related to an
 231 increase of motor activity, as cleaners moved around the smaller client-aquaria.
 232 Moreover, the remaining relevant 5-HIAA varied at the diencephalon and all in response
 233 to the inability to interact with clients. While there was a clear distinction between the
 234 cleaners that could freely contact with stimuli and those that were unable (DA
 235 stimulation at the optic tectum), it was solely by looking at the
 236 metabolite/neurotransmitter ratios that the differences between being able to clean or
 237 not, were finally disclosed: the DF2 axis distinguishing between mutualistic and non-
 238 mutualistic activities and the DF1 discriminating between the actual physical contact, of
 239 those that were able to fully inspect a client (stronger association with 5-HIAA/5-HT at
 240 the cerebellum) and those that could not engage in cleaning (stronger association with
 241 5-HIAA/5-HT at the diencephalon). These points for an extra turnover of 5-HTergic
 242 metabolites at the diencephalon whenever cleaners fail to achieve their needs and an
 243 increase of neurotransmission at the cerebellum during mutualistic activities.

244 Clients brain monoaminergic levels was also tested in respect to 3 main social
 245 challenges, revealing interesting similarities but also some relevant dissimilarities with

246 cleaners' overall response; for instance, all 3 groups clearly discriminated, with the DF1
247 axis again distinguishing the majority of the variation observed, being strongly
248 associated with 5-HIAA at the diencephalon and DOPAC at the optic tectum but also
249 with DOPAC at the diencephalon, in an opposite direction; while the DF2 mostly
250 selecting living from non-living stimuli, being this distinction mostly related with DA
251 levels at the diencephalon, in the presence of non-living stimuli. Thus, in the control
252 treatment, clients seem to experience a general stimulation of the diencephalic DAergic
253 system at the diencephalon contrasting with the overall stimulation at the optic tectum
254 in cleaners.

255 Overall, teleost fish (and other vertebrate groups) cognitive function requires a
256 dynamic coordination of multiple specialized areas of the brain. We demonstrate that the
257 variable appraisal of each social challenge contributes to brain dopaminergic and
258 serotonergic changes, in cleaners and clients, with both showing the diencephalon and
259 optic tectum as main areas of monoaminergic response. However, the role of the
260 serotonergic system activation was mostly demonstrated at the diencephalon and
261 cerebellum of cleaners, a response that was driven by the exposure to mutualistic
262 stimuli. Our current results are the first to jointly demonstrate the level of selective
263 similarity in brain monoaminergic mechanisms that underlie fish mutualistic and social
264 engagement, for both sides of these partnerships.

265

266 **Author contributions**

267 M.C.S. conceived the study. A.C.R.G. performed statistical analysis. All authors
268 participated in writing and provided comments and criticism and gave final approval for
269 publication. The authors declare no competing interests.

270

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329 **Figures captions**

330 Figure 1. Experimental schematics. (A) cleaner and (B) client perspective.

331

332 Figure 2. Cleaner's discriminant analysis plot of DF1 versus DF2 to visualize how the
 333 discriminant functions discriminate between contextual treatments, for both
 334 monoaminergic metabolites and monoaminergic ratios, synthesized at each brain area.
 335 Bigger symbols with dotted drop line in the linear discriminant plot indicate centroid of
 336 each group. DF1 and DF2 explained variation is, respectively, for monoaminergic
 337 metabolites analysis (A) 63.8% and 20.1% and for monoaminergic ratios (B), 50.8%
 338 and 25.1%. (C) and (D), Trait loadings of each variable on DF1 and DF2; the most
 339 relevant metabolites to interpret DF1 or DF2 (trait loadings > 0.40) are shown in green.
 340 See the methods section for acronyms.

341

342 Figure 3. Clients' discriminant analysis plot of DF1 versus DF2 to visualize how the
 343 discriminant functions discriminate between contextual treatments, for both
 344 monoaminergic metabolites and monoaminergic ratios, synthesized at each brain area.
 345 Bigger symbols with dotted drop line in the linear discriminant plot indicate centroid of
 346 each group. DF1 and DF2 explained variation is, respectively, for monoaminergic
 347 metabolites analysis (A), 87.4% and 12.6% and for monoaminergic ratios (B), 70.2%
 348 and 29.8%. In (C) and (D), relevant metabolites (correlational coefficient > 0.40) are
 349 shown in green. See the methods section for acronyms.

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353 Tables

354 Table 1. Pairwise correlations of contextual groups' differences that are significantly

355 explained by each of the discriminant functions, for cleaners. * significant relationships

356 ($p < 0.05$)

	Monoamine		Monoamine ratio	
	DF1	DF2	DF1	DF2
Conspecific vs. Control	0.02*	6.85E-06*	1.00	0.75
Conspecific inside aquarium vs. Control	5.07E-11*	0.98	0.63	0.34
Heterospecific vs. Control	2.92E-07*	<0.01*	<0.01*	0.42
Heterospecific inside aquarium vs. Control	0.02*	0.37	0.11	0.51
Conspecific inside aquarium vs. Conspecific	2.47E-05*	5.55E-07*	0.58	0.99
Heterospecific vs. Conspecific	0.03*	0.65	<0.01*	0.05*
Heterospecific inside aquarium vs. Conspecific	1.48E-06*	<0.01*	0.21	0.07
Heterospecific vs. Conspecific inside aquarium	0.16	2.23E-05*	0.08	<0.01*
Heterospecific inside aquarium vs. Conspecific inside aquarium	2.49E-13*	0.12	<0.01*	0.01
Heterospecific inside	7.95E-12*	0.05	8.49E-07*	1.00

aquarium vs.				
Heterospecific				

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360 Table 2. Coefficients of each monoaminergic metabolites, synthesized at each brain
361 area, for both clients and cleaners. DF1 and DF2 explained variation is, respectively,
362 87.4% and 12.6% for clients, and 63.8% and 20.1% for cleaners. * important
363 metabolites (correlational coefficient > |0.40|).

	Cleaners		Clients	
	DF1	DF2	DF1	DF2
BS_DOPAC	*-0.419	0.137	-0.321	0.220
BS_5-HIAA	0.185	-0.110	-0.147	0.011
BS_DA	0.196	0.364	-0.219	0.116
BS_5-HT	0.233	0.102	0.053	0.180
DL_DOPAC	-0.087	-0.257	*0.427	0.062
DL_5-HIAA	*-0.536	0.116	*-0.401	0.085
DL_DA	*-0.503	0.022	-0.214	*0.503
DL_5-HT	*-0.588	0.167	-0.154	0.396
FB_DOPAC	-0.153	-0.305	-0.363	-0.107
FB_5-HIAA	0.167	0.212	-0.228	-0.329
FB_DA	-0.068	-0.272	-0.267	-0.081
FB_5-HT	-0.276	0.190	0.166	-0.302
OT_DOPAC	0.045	-0.260	*-0.444	0.117
OT_5-HIAA	0.187	-0.012	-0.041	0.007
OT_DA	-0.165	*0.582	-0.214	0.051
OT_5-HT	0.075	0.233	0.011	-0.211
CB_DOPAC	0.067	-0.316	-	-
CB_5-HIAA	-0.201	-0.235	-	-

CB_DA	0.050	-0.151	-	-
CB_5-HT	-0.292	-0.183	-	-

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Table 3. Coefficients of each monoaminergic ratios, synthesized at each brain area, for both clients and cleaners. DF1 and DF2 explained variation is, respectively, 70.2% and 29.8% for clients, and 50.8% and 25.1% for cleaners. * important metabolites (correlational coefficient > |0.40|).

	Cleaners		Clients	
	DF1	DF2	DF1	DF2
BS_DOPAC/DA	-0.332	-0.276	-0.081	-0.033
BS_5-HIAA/5-HT	-0.182	-0.252	-0.341	0.310
DL_DOPAC/DA	0.324	-0.166	0.710	-0.269
DL_5-HIAA/5-HT	*-0.514	*-0.495	-0.138	-0.514
FB_DOPAC/DA	-0.165	0.265	0.136	-0.357
FB_5-HIAA/5-HT	0.227	0.233	-0.551	-0.012
OT_DOPAC/DA	0.077	0.220	-0.278	0.380
OT_5-HIAA/5-HT	0.252	-0.156	0.309	0.526
CB_DOPAC/DA	0.309	-0.210	-	-
CB_5-HIAA/5-HT	*0.446	*-0.413	-	-

382 Table 4. Pairwise correlations of contextual groups' differences that are significantly
383 explained by each of the discriminant functions, for clients. * significant relationships
384 ($p < 0.05$)

	Monoamine		Monoamine ratio
	DF1	DF2	DF1 and DF2
Heterospecific vs. Control	4.67E-11 *	6.26E-06*	-
Conspecific vs. Control	2.96E-05*	3.38E-07*	-
Conspecific vs. Heterospecific	4.72E-14*	0.29	-

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A*Labroides dimidiatus***Conspecific** *Naso elegans***Client** **Conspecific inside another smaller aquarium** **Client inside another smaller aquarium** **Ball** **B***Naso elegans***Conspecific** *Labroides dimidiatus***Cleaner** **Ball**

Monoamines

Monoamines ratio

DF1

Monoamines

Monoamines ratio