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Estradiol shapes mutualistic behaviour of female cleaner fish (*Labroides dimidiatus* - Valenciennes, 1839): Potential implications of environmental disturbance

Murilo S. Abreu[a], João P.M. Messias[b], Ana C.V.V. Giacomini[c], Marta C. Soares[b,*]

[a] Graduate Program in Pharmacology, Federal University of Santa Maria (UFSM), Santa Maria, RS 97105-900, Brazil
[b] CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
[c] Graduate Program in Environmental Sciences, University of Passo Fundo (UPF), Passo Fundo, RS, 99052-900, Brazil

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ABSTRACT

The presence of endocrine-derived compounds in the environment occurs due to a myriad of human or industrial activity and can disrupt the endocrine system of animals, including fish. One important group of endocrine disruptors are the estrogens, such as 17-β estradiol (E_2, estradiol). Estrogens are gonadal steroid hormones, able to be influential even in small concentrations. Here, we demonstrate that E_2 is linked to female' decisions made by an important coral reef species, the cleaner fish *Labroides dimidiatus*, during interactions with other reef fishes (known as clients). E_2 treatment in natural conditions interfered directly in the cooperative relationships, by increasing cleaners' willingness to interact with clients, providing greater amounts of physical contact to their fish partners. We discuss the meaning of the observed behavioural disruption produced by E_2, which by affecting a key species (cleaners) may produce a cascade impact in the aquatic ecosystem.

1. Introduction

The majority of estrogens, such as 17-β-estradiol (E_2, estradiol), that are found in aquatic environments come from domestic effluents, and release to the ecosystems to the ecosystems higher amounts of E_2 arising from human and veterinary medicine use (Ma et al., 2016; Meijide et al., 2016). In fact, studies have reported the presence of E_2 in waste, surface waters and marine sediment of various countries, in concentrations ranging from low ng L^{-1} to μg L^{-1} levels (Fei et al., 2017; Zhang et al., 2011). The question is that E_2 drainage is severely influential even at low concentrations (Meijide et al., 2016; Nazari and Suja, 2016). Waterborne E_2 is known to cause a complete inhibition of sexual displays in male guppies (Bayley et al., 1999), as well as the disruption of male sexual behaviour in goldfish (*Carassius auratus* (Linnaeus, 1758)) (Bjerselius et al., 2001). Male goldfish exposed to dietary and waterborne E_2 for 24–28 days during the spawning period, decrease pushing and spawning behaviours (Bjerselius et al., 2001). Similarly, both waterborne and dietary exposure to E_2 is acknowledged to reduce male courtship and spawning behaviours in goldfish Carassius auratus and Japanese medaka *Oryzias latipes* (Oshima et al., 2003; Schoenfuss et al., 2002). Furthermore, the exposure of zebrafish (*Danio rerio* (Buchanan-Hamilton, 1822)) embryos and larvae to supraphysiologic concentrations of estrogens are required to achieve physiologically-relevant doses in vivo (Souder and Gorelick, 2017). This means that estrogenic exposure is able to produce short and long-term changes that may lead to serious consequences to human and aquatic population users of these resources.

Additionally, estrogens are crucial modulators of central cognitive processes, working in interaction with glucocorticoids but mostly in a clear engagement with some monoaminergic systems, more remarkably with the dopaminergic system (Prasad et al., 2015; Vahaba and Remage-Healey, 2015). For instance, estradiol seems to have both acute and chronic effects on the functional activity of dopamine (DA) release, reuptake, and some of the downstream targets of DA receptor activation (Yoest et al., 2014). Considering that the dopaminergic system is well conserved across vertebrates, with teleost fish being referred as a relevant example (Kapsimali et al., 2003), one could expect estrogenic impact on fish dopaminergic transmission to shape individual learning, reward processing, memory, and motivation (Colzato and Hommel, 2014; Vahaba and Remage-Healey, 2015). For instance, recent studies have found that the dopaminergic system is able to significantly influence cleaner fish *Labroides dimidiatus* social learning competence (Messias et al., 2016a) and the ability to make behavioural decisions (Messias et al., 2016b), mediating the motivational incentive that

* Corresponding author.
E-mail address: marta.soares@cibio.up.pt (M.C. Soares).

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cleaners assign to cues (Raihani et al., 2012), particularly if these are novel (Soares et al., 2017).

In marine ecosystems, the mutualistic relationship between fish cleaners and their clientele, comprised of dozens of other teleost species, may be compromised in the presence of estrogenic contaminants. Fish cleaners influence the activity and health of all fish species (clients) that visit them (Grutter et al., 2003; Ros et al., 2011; Soares et al., 2011). Indeed, some clients are estimated to visit cleaners at their living territories (cleaning stations) up to 5–30 times per day, with maximal estimates above 100 visits (Grutter, 1995), to have their ectoparasites, diseased or dead tissue removed. However, because these cleaners seem to aim to remove client mucus preferentially (instead of ectoparasites, referred as cheating), which is detrimental to the client, this mutualistic cleaner-client system is also notorious for the existence of conflicts of interest (Bshary and Côté, 2008). To counterbalance for cleaners' mucus preferences, they improve their cleaning service by providing a form of physical contact (known as tactile stimulation or massages) to clients, touching them with their pectoral and (especially) pelvic fins, which has stress-reduction benefits to clients (Soares et al., 2011) and it is hence used to control clients behaviour, particularly in managing potential aggression by the predatory clients towards the cleaner (Cant, 2010).

Recent studies concerning the extent of neuroendocrine mediation in cleaners' behaviour have shed light on how cleaners' physiological state may influence their behavioural output, being seemingly dependent on their social environment (Soares, 2017). However, the implications of the putative influence of the estrogenic system on their complex conspecific and cooperative interactions are yet to be established, considering the current impact of estrogenic disruption to the aquatic ecosystems.

2. Materials and methods

2.1. Field methods

The overall methods section was developed in accordance with previous studies (see (Messias et al., 2016b; Paula et al., 2015; Soares et al., 2014). The experiments were carried out on reefs around Lizard Island (Lizard Island Research Station, Australia, 14°40'S, 145°28'E) on 20 female cleaner wrasses, that ranged from 6.1 cm to 8.3 cm in total length (TL) and with body weight being estimated on site using a length-weight regression (for similar experimental protocols please see (Messias et al., 2016b; Paula et al., 2015; Soares et al., 2014)). Experimental manipulations tests took place in September, before the spawning period (Grutter, 2012; Waldie et al., 2011), which should occur between October and December at Lizard Island. Recent studies demonstrated that cleaners were not affected by this variable (Messias et al., 2016b; Soares et al., 2014). Moreover, all cleaners were sampled from fringing reefs, which ensured that these inhabited ecologically similar contexts, while cleaning stations varied in depth (between 2 and 10 m). The treatments were randomized, in which each focal cleaner was administered, via intramuscular injection, only with one of two compounds: saline solution for control (0.9% NaCl) or 17β-Estradiol, dosage 2 µg per gram of body mass (Geary and Asarian, 1999) (gbw, E8875 Sigma). Estradiol was first dissolved in 50 µl of ethanol and only then were the solutions made with saline (and left overnight to complete ethanol evaporation, (Soares et al., 2014)). The control solution used (saline) was also prepared with an equivalent amount of ethanol as in the treatment group. Injection volumes ranged from 20 to 50 µl, and never exceeded 3 min of handling (Soares et al., 2014). Cleaners were videotaped for the next 45 min, using video cameras in waterproof cases (Sony HDR-XR155). Because this study was done exclusively in field conditions with limitations of the number of fish used (permit allowance), and knowing that the removal of blood would mean fish death, the dosage chosen was based on previous studies. Ethical clearance to work at Lizard Island Research Station (Australian Museum), which involved animal manipulation, was obtained from University of Queensland Animal Ethics Committee (Messias et al., 2016b; Paula et al., 2015; Soares et al., 2014). The use of animals and data collection complied with the laws of Australia.

2.2. Behavioural analysis

During each video analysis, we recorded the following measures: a) the number of client' species found visiting the cleaning station, b) the number and duration (in seconds) of cleaner's inspection toward each client c) the frequency and duration of tactile stimulation provided (where a cleaner touches, with ventral body and fins, the body of the client (Bshary et al., 2001); and d) the number of jolts by clients (cleaners sometimes take bites to which the clients respond with a short body jolt (Bshary and Grutter, 2002; Soares et al., 2008).

2.3. Statistical analysis

Measures of interspecific cleaner wrasse behaviour were divided into three categories (similarly to Messias et al., 2016b): a) measures of engaging in cleaning behaviour (motivation to interact); b) measures of interactive investment (provision of tactile stimulation); and c) a measure of cleaner wrasse cheating levels (client jolt rate). All interactions with clientele were measured by: 1) the proportion of clients inspected (calculated as the total number of clients inspected/ total number of visits), and 2) the mean duration of inspection (total time of interaction/total number of interactions). Measures of investment were calculated as: 1) the proportion of interactions in which tactile stimulation (frequency of clients inspected with tactile stimulation/ total number of interactions), and 2) the proportion of time cleaners spent providing tactile stimulation to clients (total tactile stimulation duration/total interaction duration). Finally, the measurement of cleaners' cheating levels was calculated using the frequency of jolts per 100 s of inspection. Data were analysed using non-parametric tests because the assumptions for parametric testing were not met. We compared each estradiol treatment to control group using Mann–Whitney *U* tests, as is recommended for a small set of planned comparisons (Ruxton and Beauchamp, 2008).

3. Results

3.1. Estradiol effects on cleaners' likelihood to engage in cleaning

We found that cleaners under the influence of estradiol inspected more clients ($U = 8$, $n1 = 10$, $n2 = 10$, $p = 0.0007$; Fig. 1a).

3.2. Estradiol effects on cleaners' provision of tactile stimulation to clients

Cleaners under the influence of estradiol increased the proportion of interaction with tactile stimulation ($U = 18.5$, $n1 = 10$, $n2 = 10$, $p = 0.0153$, Fig. 1c), and the proportion of time spent providing tactile stimulation ($U = 15.5$, $n1 = 10$, $n2 = 10$, $p = 0.0071$, Fig. 1d). The influence of estradiol did not alter the average inspection duration (Fig. 1b).

3.3. Estradiol effects on cleaners' wrasses cheating levels

Estradiol treatment did not alter cleaners' cheating levels, according to the frequency client jolts/100 s (Fig. 1e).

3.4. Conspecific-directed behaviour

Estradiol treatment did not alter female cleaners' conspecific behaviour (Fig. 2).