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RAPID COMMUNICATION

Shoaling behaviour is differentially altered by ethanol and dopamine D1 receptor modulators in tropical marine forage fish

Trevor J. Hamilton, David I. Kline, and Martin Tresguerres

Abstract: Anchovies are filter-feeding fish that inhabit nearshore environments worldwide. With increasing human pharmaceutical use, drugs that alter neurological functioning are becoming more prevalent in aquatic ecosystems via wastewater effluent, creating the need for tests that can reliably determine sublethal effects of these drugs on coastal fish populations. In this study, we used Caribbean anchovies (*Anchoa* spp.) as a tropical marine fish model to test drug-induced alterations of locomotion and shoaling behaviour with a video-based analysis system. Consistent with its anxiolytic effects in zebrafish (*Danio rerio*), ethanol decreased shoal cohesion in anchovies. We also characterized the effects of drugs known to modulate the dopaminergic system in zebrafish and rodents. A D1 receptor agonist (SKF 38393) and a D1 receptor antagonist (SCH 23390) increased the time anchovy spent in the center of the arena, but neither drug had an impact on shoal cohesion. Finally, the D1 receptor agonist caused significantly lower meandering compared with fish treated with the D1 receptor antagonist and ethanol. This study suggests that anchovy is a suitable Caribbean marine model for toxicology studies.

Résumé : Les anchois sont des poissons filtreurs qui résident dans des milieux littoraux partout sur terre. L'usage croissant de produits pharmaceutiques destinés à l'usage humain fait en sorte que des drogues qui modifient la fonction neurologique, transportées par les effluents d'eaux usées, sont de plus en plus répandues dans les écosystèmes aquatiques, nécessitant des tests pouvant établir de manière fiable les effets sublétaux de ces drogues sur les populations de poissons côtiers. Nous utilisons des anchois des Caraïbes (*Anchoa* spp.) comme modèle de poisson marin tropical pour examiner les modifications induites par des drogues des comportements locomoteur et de rassemblement en banc à l'aide d'un système d'analyse basé sur la vidéo. À l'instar de ses effets anxiolytiques chez les poissons zèbres (*Danio rerio*), l'éthanol réduit la cohésion des bancs d'anchois. Nous caractérisons également les effets de drogues qui modulent le système dopaminergique chez les poissons zèbres et les rongeurs. Un agoniste du récepteur D1 (SKF 38393) et un antagoniste du récepteur D1 (SCH 23390) accroissent le temps passé par les anchois au centre de l'arène, mais ni l'un ni l'autre n'a d'effet sur la cohésion du banc. Enfin, les poissons traités à l'agoniste du récepteur D1 se déplacent significativement moins en méandres que les poissons traités à l'antagoniste du récepteur D1 et à l'éthanol. L'étude porte à croire que l'anchois constitue un modèle de poisson marin des Caraïbes qui se prête aux études toxicologiques. [Traduit par la Rédaction]

Introduction

Human mental health issues are being treated with psychiatric drugs more than ever before (Levinthal and Hamilton 2015), resulting in increased contamination of freshwater and marine environments (Brodin et al. 2014). Some of those drugs have been designed to modify dopamine neuron (dopaminergic) signaling to treat diverse diseases such as Parkinson's, schizophrenia, attention deficit hyperactivity disorder, anxiety disorders, and depression (Levinthal and Hamilton 2015). Ultimately, the goal is to alter neural functioning and treat the symptoms of these conditions to stabilize aberrant human behaviour. However, psychiatric drugs are excreted in urine and eventually end up in wastewater where they can enter aquatic environments and alter the behaviour of aquatic organisms by acting on analogous neural circuitry. It is therefore essential to understand whether basic behavioural tests can be used in candidate species before examining the potential impact of these psychiatric drugs on aquatic organisms' behaviour and ecology.

Behavioural neuroscience tests have been used to examine pharmacological and toxicological impacts of substances in fish, with zebrafish (*Danio rerio*) being the most well-studied experimental species (Tierney 2011). The effects of anthropogenic substances on fish neural activity has been demonstrated through tests of boldness, aggression, activity, feeding, reproduction, and sociality (McCallum et al. 2017). A common measure of sociality prominently used in zebrafish is shoaling behaviour. This test is based on the natural propensity of prey fish to swim in groups as an antipredatory defense and measures the tightness of a shoal as an indication of anxiety-like behaviour (Maaswinkel et al. 2013). In zebrafish, this has been validated with pharmacological compounds like ethanol that acts as an anxiety-reducing drug (anxiolytic), decreasing shoal tightness (Miller et al. 2013). Drugs that alter dopaminergic receptors, like the D1 antagonist SCH 23390, also decrease shoaling in zebrafish (Scerbina et al. 2012). Recently, shoaling behaviour was also used to test anxiety levels in a marine fish exposed to elevated CO_2 levels (Kwan et al. 2017).

Anchovies (*Anchoa* spp.) are filter-feeding forage fishes of the Engraulidae family. They are found in nearshore environments

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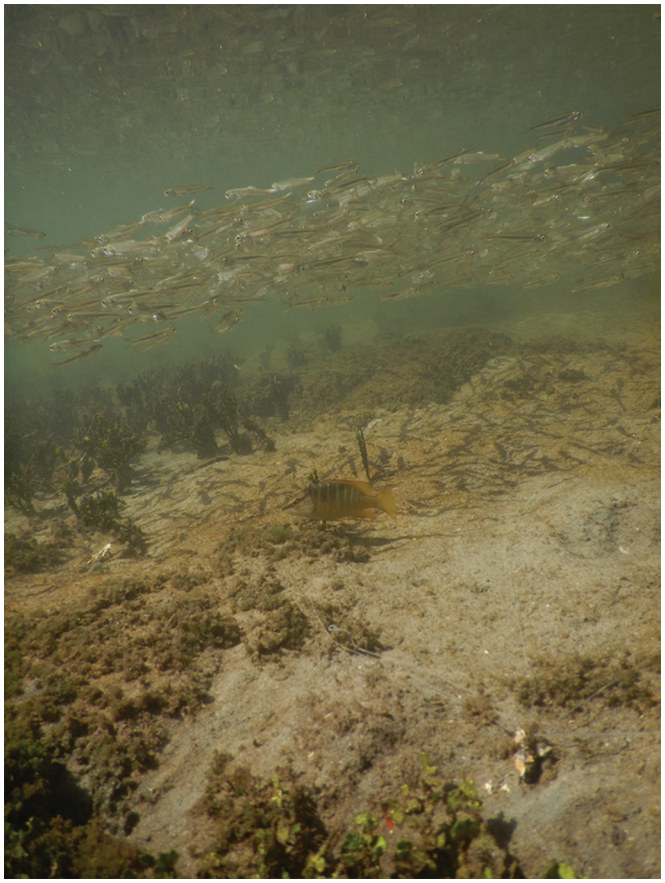
Fig. 1. School of wild anchovies from the dock of the Smithsonian Tropical Research Institute (STRI) where fish were caught for this study. The fish at the bottom is a juvenile schoolmaster snapper (*Lutjanus apodus*), a natural anchovy predator (Rooker 1995).



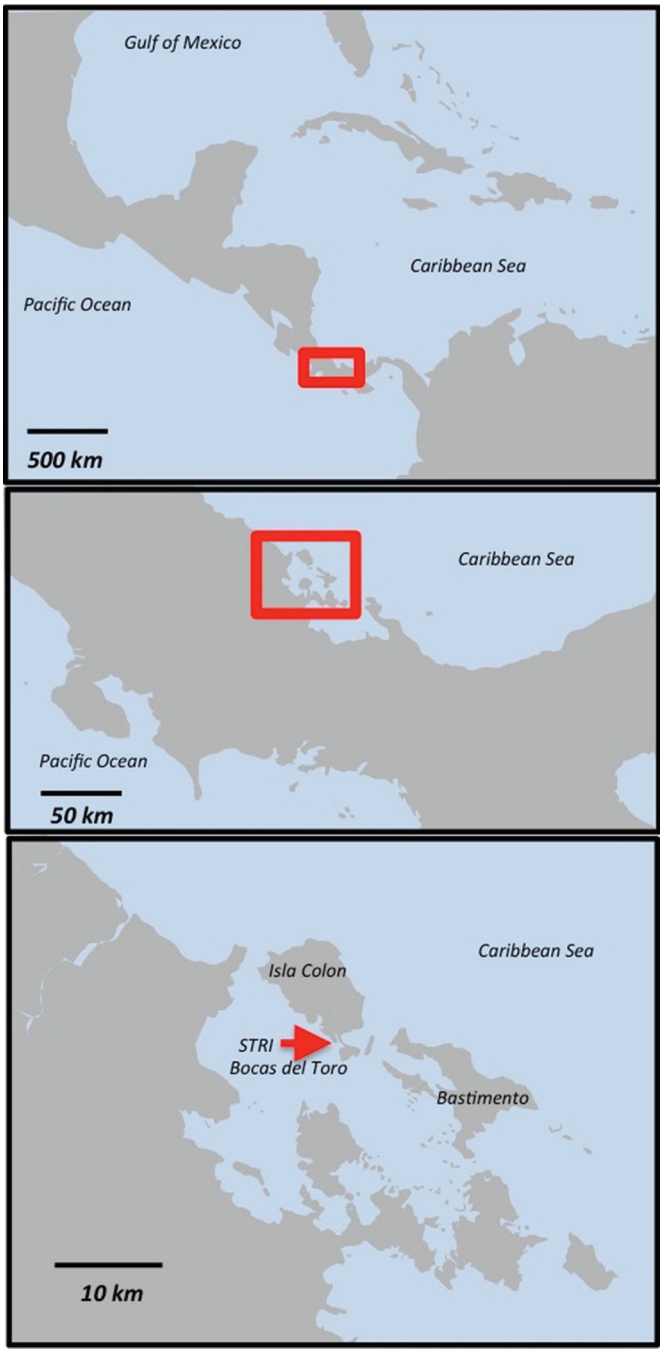
worldwide (Bloom and Lovejoy 2012), including the Caribbean. They are economically important fishes (Checkley et al. 2017) and a vital prey source for seabirds, fish, and other animals. Anchovies are social fishes that swim together in shoals as a defense mechanism against predators (Fig. 1), which in combination with their nearshore distribution, make them an ideal marine model species to study pollution and toxicology; yet, there have been no behavioural or pharmacological studies on these fishes to date. In this study, we tested shoaling behaviour in Caribbean anchovies and their response to ethanol and dopamine D1 receptor agonists and antagonists to examine social behaviour in these fishes.

Methods

Animals and housing

Wild anchovies (n = 208, 5–6 cm in length) were caught using hand nets off the dock of the Smithsonian Tropical Research Institute (STRI; Bocas del Toro, Panama) (Fig. 2). Fish were housed in groups (~50 fish per tank) in circular plastic tanks (150 L) with flow-through seawater (29.5 ± 0.5 °C) that was constantly aerated. Fish were habituated for 24–48 h in the aquarium before experimental testing. The outdoor experimental aquarium system at STRI has natural light, and the fish maintained their natural ~12 h light : 12 h dark cycle. Fish were fed sinking mini fish pellets (Omega One sinking mini-pellets, OmegaSea Ltd., USA) once daily. On experimental trial days, fish were fed after behavioural testing. Once behavioural testing was completed, approximately 60 fish were fixed in 3% paraformaldehyde and transported to Scripps

Fig. 2. Location of the Smithsonian Tropical Research Institute (STRI) (Bocas del Toro, Panama), where fish were caught and the experiments were performed.



Institution of Oceanography (University of California, San Diego) and were identified as *Anchoa lyolepsis* and *Anchoa lamprotaenia*, with an equal prevalence.

Shoaling test procedure

Behavioural tests took place during sunlight hours between 0900 and 1700. Fish were netted from the housing tank and placed in dosage tanks in groups of four, then transferred to the adjacent testing room. Dosage tanks were 20 cm long, 13 cm wide, and 14 cm tall, filled with 1 L of seawater, and placed away from auditory or visual stimuli to alleviate additional stress on the fish. Fish remained in the dosage tanks for 30 min and then were netted

and placed into the shoaling arena. The shoaling arena was a white plastic circular arena (diameter 27.5 cm with 11 cm tall walls) filled with fresh seawater prior to every trial to a height of 5.5 cm. Shoaling trials were 10 min in duration and recorded with a Basler Pylon x64 high-resolution black and white camera secured 1 m above the experimental arena and connected to a laptop running Ethovision XT (version 10.0; Noldus, Virginia, USA) motion-tracking software. Trials began immediately after fish were placed into the arena. The arena was divided into a center and outer zone in Ethovision, with a centered virtual circle with a diameter of 8.9 cm, corresponding to ~1.5 body lengths of the average fish tested. Interindividual distance (IID) and nearest neighbor distance (NND) was calculated as commonly done in shoaling studies (Maaswinkel et al. 2013). IID is the mean of all distances between each anchovy and its three neighbors for the shoal of four fish. NND is the mean of the distance between each fish and its closest neighbor for the shoal of four fish. Meandering (turn angle divided by distance moved) and distance moved were also quantified using Ethovision XT.

Drug administration

Ethanol (Sigma-Aldrich, St. Louis, Missouri, USA) was administered at 1.0% (*v*/*v*) in 1 L of seawater water for 30 min. Concentrated stock solutions of D1 receptor agonist SKF 38393 hydrochloride and D1 receptor antagonist SCH 23390 hydrochloride (abcam, Cambridge, UK) were prepared in distilled water (and dimethylsulfoxide (DMSO) for SKF 38393), and mixed with 1 L of seawater prior to experimentation for final concentrations of 1 mg·L^{-1} for SCH 23390 and 10 mg·L^{-1} for SKF 38393. A subset of control experiments was performed with the same volume of DMSO (500 μL in 1 L) in the dosage tank. There were no significant differences in any variable between control and control + DMSO groups, so these were combined. Drug concentrations were determined from previous research on zebrafish (Scerbina et al. 2012; Tran et al. 2015; Naderi et al. 2016; Johnson and Hamilton 2017) and the teleost cichlid fish (Mok and Munro 1998). All drug and control groups were interspersed throughout the testing days to control for any potential time of day or circadian effects.

Ethics statement

All experiments were approved by the Institutional Animal Care and Use Committee at the Smithsonian Tropical Research Institute (protocol 2016-1020-2019) in compliance with the IACUC guidelines for the care and use of experimental animals.

Statistical analysis

Normality was tested with D'Agostino and Pearson omnibus normality test, and all data sets fit the normal distribution. Shoals of four fish were considered a single replicate (n = 1), so the sample size represents shoals of fish, not individual fish. One-way ANOVAs with Tukey's multiple comparison post hoc test were used to compare control with experimental groups. Data were analyzed using Graphpad Prism 6.0 (California, USA).

Results

Shoaling behaviour

Ethanol decreased shoal cohesion measured by an increased IID (10.2 ± 0.8 cm, n = 8) compared with controls (6.7 ± 0.2 cm, n = 16; $F_{[3,48]}$ = 20.03, P < 0.0001, one way ANOVA; Fig. 3*a*). However, the D1 agonist (6.1 ± 0.2 cm, n = 14) and the D1 antagonist (7.1 ± 0.4 cm, n = 14) did not induce any effects on IID compared with controls. Similarly, ethanol significantly increased NND (10.0 ± 0.7 cm, n = 8) compared with controls (6.5 ± 0.2 cm, n = 16; $F_{[3,48]}$ = 21.16, P < 0.0001, one-way ANOVA), and neither the D1 agonist (5.9 ± 0.2 cm, n = 14) nor the D1 antagonist (6.8 ± 0.3 cm) were different from controls (Fig. 3*b*). However, there were significant differences in the time the shoal occupied the center zone ($F_{[3,48]}$ = 23.90, P < 0.0001, one way ANOVA). Ethanol significantly decreased the time spent in the center zone (65.3 ± 7.9 s, n = 8) compared with the control group (102.5 ± 6.6 s, n = 16), whereas the D1 agonist (138.0 ± 7.6 s, n = 14) and D1 antagonist (158.0 ± 7.9 s, n = 14) increased it (102.5 ± 6.6 s, n = 16) compared with controls (Fig. 3*c*).

Fig. 3. The effect of D1 receptor agonist SKF 38393 (D1a; 10 mg·L^{-1}), D1 receptor antagonist SCH 23390 (D1ant; 1 mg·L^{-1}), and ethanol (Eth; 1% *v*/*v*) on anchovy social behaviour: (*a*) interindividual distance (IID), (*b*) nearest neighbor distance (NND), and (*c*) time in the center of the arena. Letters indicate a significant difference between the groups (P < 0.05, one-way ANOVA; the same letter indicates a lack of a statistically significant difference). All data are mean ± standard error.

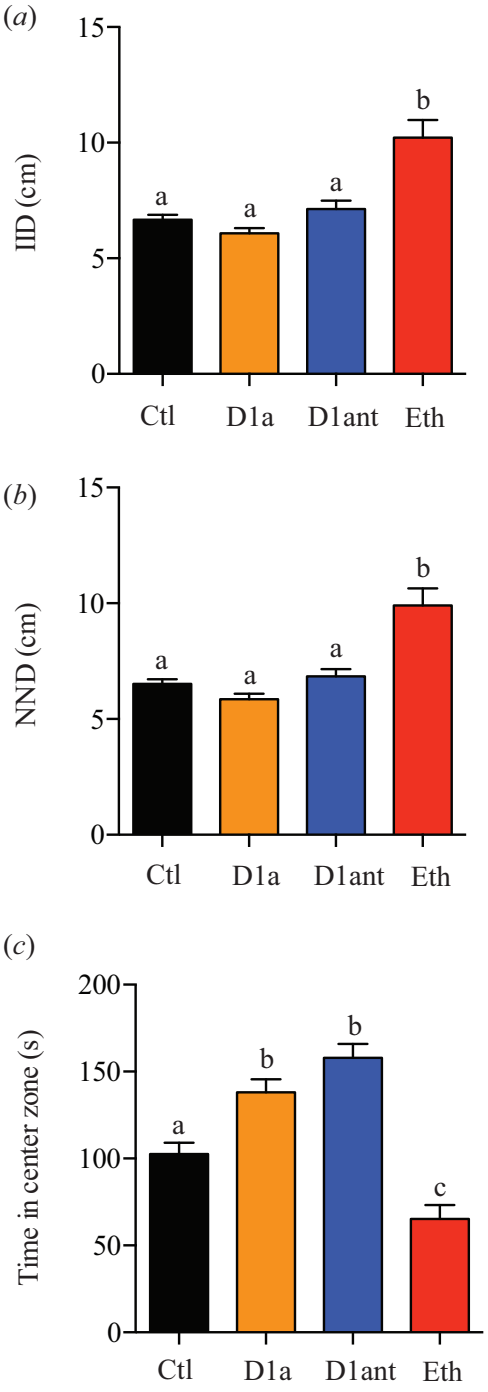
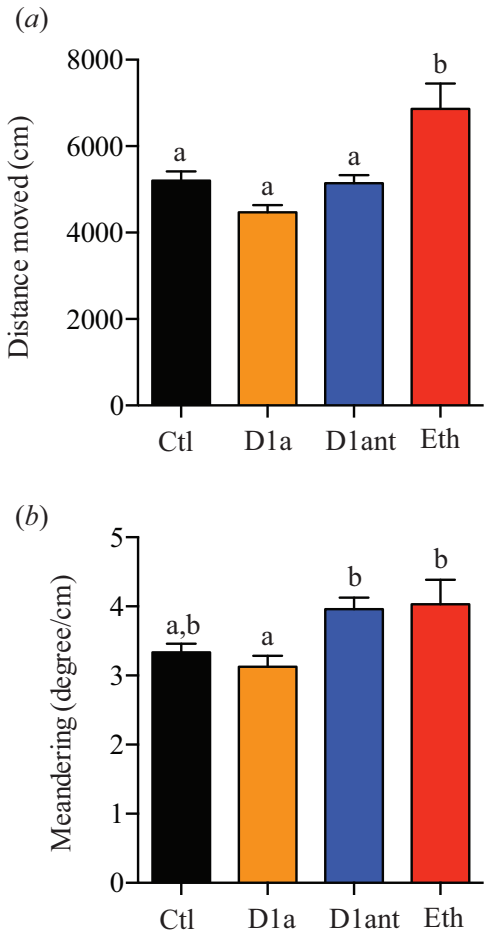


Fig. 4. The effect of the D1 receptor agonist SKF 38393 (D1a; 10 mg·L^{-1}), D1 receptor antagonist SCH 23390 (D1ant; 1 mg·L^{-1}), and ethanol (Eth; 1% *v*/*v*) on anchovy locomotion, including (*a*) distance moved over the duration of the trial, and (*b*) meandering. Letters indicate a significant difference between the groups ($P < 0.05$, one-way ANOVA; the same letter indicates a lack of a statistically significant difference). All data are mean ± standard error.



Locomotion

Distance moved over the duration of the trial was significantly increased by ethanol (6865 ± 585 cm, $n = 8$) compared with controls (5201 ± 215 cm, $n = 16$; $F_{[3,48]} = 11.43$, $P < 0.0001$; Fig. 4*a*) but not by the D1 agonist (4471 ± 164 cm, $n = 14$) or the D1 antagonist (5144 ± 185 cm, $n = 14$). Fish treated with the D1 agonist meandered significantly less than fish treated with the D1 antagonist and ethanol (Fig. 4*b*; $F_{[3,48]} = 5.864$, $P = 0.0017$). However, meandering was not significantly different between the D1 agonist (3.1 ± 0.2°·cm^{-1}, $n = 14$), the D1 antagonist (3.3 ± 0.1°·cm^{-1}, $n = 14$) or ethanol (4.0 ± 0.4°·cm^{-1}, $n = 8$) compared with controls (3.3 ± 0.1°·cm^{-1}, $n = 16$; Fig. 2*b*).

Discussion

Pharmaceutical drugs are increasingly found in wastewater effluent and have the potential to alter the behaviour of aquatic organisms, potentially affecting ecosystem dynamics. We tested the effects of ethanol and dopamine D1 receptor modulators on the shoaling behaviour of anchovies, a foraging marine fish. Similar to studies on zebrafish (Echevarria et al. 2011; Gerlai et al. 2009; Maaswinkel et al. 2013; Miller et al. 2013), a model fish species that lives exclusively in fresh water, ethanol significantly reduced anchovy shoal cohesion. On the other hand, dopamine D1 receptor modulation did not change shoal cohesion or meandering but did alter anchovy location preference. These results suggest that with certain pharmacological compounds like ethanol, anchovies display disrupted social behaviour, but are more resilient to alterations in behaviour due to dopaminergic compounds at the concentrations we used. Nonetheless, examination of social behaviour with this shoaling test can be used to examine behavioural responses of various pharmaceuticals and toxicants in anchovies.

Once a drug is dissolved in water and enters the internal fluids of a fish, it can disrupt multiple physiological processes, including nervous system activity. Marine and freshwater fish have fundamentally the same nervous system; however, their different osmoregulatory physiologies (Evans and Claiborne 2005; Evans et al. 2005) may lead to differential drug effects. Freshwater fish actively take up salts across their gills and produce copious amounts of diluted urine to counteract salt loss and water gain in a hypoosmotic environment. On the contrary, marine fish constantly drink seawater, secreting excess salts across the gills, and conserve water by producing very little urine to fight dehydration in hyperosmotic seawater. Therefore, marine fish may be more likely to incorporate and retain certain chemicals present in the environment. On the other hand, rates of uptake of pentachlorophenols are greater in freshwater- compared with seawater-acclimated killifish (Tachikawa et al. 1991). To date, most studies on the effects of wastewater effluents on fish have been conducted on freshwater laboratory species such as zebrafish, goldfish (*Carassius auratus*), trout (*Oncorhynchus mykiss*), and gobies (*Neogobius melanostomus*) (Brodin et al. 2014; McCallum et al. 2017). It is therefore desirable to study the effects of pharmaceutical pollution directly on marine fish species rather than extrapolating results from freshwater species. From sociological and ecological perspectives, many countries with port cities are underdeveloped with limited to no wastewater treatment. Further research on marine fish species is needed to study the impacts of pharmaceutical pollution on nearshore tropical marine fish from developing countries with limited wastewater treatment.

Ethanol

Ethanol at a 1.0% concentration reduced social cohesion (increased IID and NND), decreased time in the center zone, and increased distance moved in anchovies. The increase in IID and NND is consistent with the established anxiolytic effect of ethanol on zebrafish in shoal-based behavioural tests (Maaswinkel et al. 2013; Kurta and Palestis 2010), which suggests a similar anxiolytic effect in anchovies. The increase in distance moved is also consistent with increased swim velocity in zebrafish exposed to 1% ethanol (Mathur and Guo 2011). We hypothesize that the ethanol-induced decrease in anchovy social cohesion reported here is due to neurochemical changes in analogous brain regions leading to anxiety reduction, as in zebrafish. Future research should involve multiple anxiety-like behaviour tests, including the light–dark test (Holcombe et al. 2013), novel approach test (Johnson and Hamilton 2017), novel tank diving test (Hamilton et al. 2017*a*), and other tests of anxiety-like behaviour (for review see Echevarria et al. 2011) to further characterize the effect of ethanol on anchovy behaviour. Cortisol measures and stressors should also be used to validate ethanol's anxiolytic actions. Taken together, this study demonstrates that IID, NND, and locomotion parameters in anchovy shoals can be used to test changes in drug-induced behavioural responses.

Dopamine receptor modulation

Several recent studies have investigated modulation of behavioural responses by dopamine in marine fish. Stimulation of D1 receptors with SKF 39383 increased learning in cleaner wrasse (*Labroides dimidiatus*) (Messias et al. 2016) and induced object recognition in bicolor damselfish (*Stegastes partitus*) (Hamilton et al. 2017*b*). In cichlids of the genus *Oreochromis*, apomorphine (2–

8 mg·L^{-1}), a D1 and D2 receptor agonist, increased locomotor activity, which was then blocked by the D1 antagonist SCH-23390 and also inhibited by removal of the telencephalon (Mok and Munro 1998). In the current study, we observed no significant alteration of meandering or distance moved for the D1 receptor agonist or antagonist compared with the control group. However, there was significantly more meandering induced by the D1 antagonist compared with the D1 agonist. While we cannot explain the differential effect of the dopaminergic system on the two locomotion parameters in our study compared with previous research, we speculate it could be related to testing individual fish versus shoals or the concentration and exposure of the drugs.

To our knowledge, there have been no studies with dopamine antagonists on shoaling in marine fish. However, in zebrafish the D1 receptor antagonist SCH 23390 (1 mg·L^{-1}; same as in this study) significantly decreased social attraction in the zebrafish AB strain (Scerbina et al. 2012). Interestingly, this effect was not observed in the SF strain, which was attributed to a genetic dissimilarity, as their response to ethanol was also different (Gerlai et al. 2009). In addition, locomotion was not affected by the D1 antagonist (SCH 23390) in either zebrafish strain (Scerbina et al. 2012), similar to our observations on distance moved, but not meandering in anchovy. However, in zebrafish of the AB strain, D1 antagonism produced a dose-dependent decrease in distance travelled (Tran et al. 2015). In our study, the D1 antagonist had a profound impact on the location preference of anchovies, increasing time in the center zone, despite having no effect on IID, NND, or distance moved. An increased time in the center zone, and therefore less time near the walls (thigmotaxis), can be indicative of decreased anxiety; however, we did not observe expected changes in shoal size consistent with this. The increased time in the center of the arena may suggest that "boldness" is increased. Whereas anxiety is regulated by neurotransmitters like GABA (gamma-aminobutyric acid), boldness (or exploration, which is a term more commonly used in rodent literature) is regulated by dopamine (Young et al. 2011).

We originally hypothesized that the D1 agonist and the D1 antagonist would produce opposite effects; however, both drugs induced a preference for the center of the arena. Although counterintuitive, this is not the first report of these two drugs producing a similar effect on fish behaviour; for example, both enhanced learning in zebrafish in a spatial alteration task (Naderi et al. 2016). This illustrates the complex effects of drugs on behaviour and the need for further basic studies.

Ethanol has widespread effects throughout the nervous system, by acting on specific receptors ($GABA_A$, NMDA (*N*-methyl-D-aspartate)), by altering activity of other neurotransmitters (dopamine, opioids), and nonspecifically by effecting phospholipid and membrane-bound protein composition (Levinthal and Hamilton 2015). An ethanol concentration of 1.0% induced significant changes in anchovy shoal cohesion and locomotion, suggesting that other pollutants that act via similar mechanisms could have equivalent profound effects.

Taken together, these findings indicate that not all studies on the effects of pharmaceutical compounds, like D1 agonists and antagonists, on zebrafish behaviour apply to marine fish. Another major finding is that anchovies are prone to modulation of their social behaviour by some waterborne pharmacological substances like ethanol (at concentrations far beyond environmental levels). Future studies should examine whether wastewater runoff containing compounds that alter neurochemistry could have ecological impacts on this species and other fish living in nearshore environments. Northern anchovies (*Engraulis mordax*) have been used to examine plastic pollution that can alter food webs (Savoca et al. 2017), and Caribbean anchovies are good candidates to study this and other forms of aquatic pollution. The current study validates the use anchovies as an experimental model species to investigate wastewater effluents impacts on marine fish behaviour in Caribbean nearshore environments.

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