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Decreased retention of olfactory predator recognition in juvenile surgeon fish exposed to pesticide.

Frédéric Bertucci ^{a,b,†,*}, Hugo Jacob ^{a,c,†}, Alexandre Mignucci ^{a,†}, Camille Gache ^a, Natacha Roux ^{a,d}, Marc Besson ^{a,d}, Cécile Berthe ^a, Marc Metian ^c, David Lecchini ^{a,e}

^a EPHE, PSL Research University, UPVD-CNRS, USR3278 CRIOBE, 98729 Moorea, French Polynesia.

^b Laboratoire de Morphologie Fonctionnelle et Evolutive, Université de Liège, Campus Sart Tilman, Bât. B6c, Allée de la Chimie 3, 4000 Liège, Belgium.

^c International Atomic Energy Agency, Environment Laboratories, 4a, Quai Antoine 1er, Principality of Monaco, Monaco.

^d Observatoire Océanologique de Banyuls-sur-Mer, UMR7232; Université Pierre et Marie Curie Paris; 1 avenue du Fontaulé, 66650 Banyuls-sur-Mer, France.

^e Laboratoire d'Excellence "CORAIL", Moorea, French Polynesia.

[†] These authors contributed equally to this work

* Corresponding author: fred.bertucci@gmail.com; +32 4 366 50 33

Abstract

Dory, the animated surgeonfish created by the Pixar Animation studios, famously suffered from short-term memory loss leading to many adventures. In reality, many fishes have excellent cognitive abilities and are able to learn and retain important information such as the identity of predators. However, if and how cognition can be affected by anthropogenically altered oceanic conditions is poorly understood. Here, we examine the effect of a widely used pesticide, chlorpyrifos, on the retention of acquired predator recognition in post-larval stage of the surgeonfish *Acanthurus triostegus*. Through associative learning, post-larvae of *A. triostegus* were first observed to forage significantly less in the presence of conspecific alarm cues and alarm cues associated to a predator's odor. The retention of this anti-predator behavior was estimated to last between 2 and 5 days in the absence of pesticide. However, environmentally-relevant concentrations of chlorpyrifos ($1 \mu\text{g.L}^{-1}$) induced the loss of this acquired predator recognition. This reduced ability to recognize learned predators is discussed as it may lead to more vulnerable fish communities in coastal areas subjected to organophosphate pesticide pollution.

Keywords: Coral Reef; Conservation; organophosphate pesticides; Chemical cues; Larval recruitment.

1. Introduction

In Disney-Pixar's Finding Dory, the title character short-term memory resulted in many adventures. Could fiction meet reality, especially in a world polluted by pesticides? Coral reefs are one of the most diverse ecosystem in the world, home to between 1 and 9 million species (Reaka-Kudla, 1997). In addition to their ecological and biodiversity value, many human populations rely on coral reefs for the goods and services they provide (e.g., tourism, shoreline protection, fisheries, and cultural values; Bellwood *et al.*, 2004). However, coral reefs are threatened by numerous global and local stressors such as ocean warming, acidification, pollution, or physical disturbance (Hoegh-Guldberg, 2014). Today, 75% of world's coral reefs are directly threatened and 95% may disappear by mid-century (Hoegh-Guldberg *et al.*, 2007). Coral reefs, even the most isolated ones, are increasingly polluted by chemicals such as pesticides (Fabricius, 2005; Lavers and Bond, 2017). Despite numerous studies reporting the impact of chemical pollution on the biology, ecology, ethology and evolution of coral reef organisms (Deb and Das, 2013; Besson *et al.*, 2017), no studies have examined how chemical pollution can affect the cognitive abilities of coral reef organisms, for instance the retention of learned information i.e. memory. Memory is an essential cognitive function that permits animals to acquire, retain, and retrieve different kinds of information (Kelley and Magurran, 2007). It allows individuals to take advantage of previous experience to solve a whole array of problems that their environment confronts them with, such as how to recognize conspecifics, identify predators, find foraging places, and learn the consequences of their behavior (Kelley and Magurran, 2007). For example, in fishes, remembering prior experience with predators increases the subsequent chances of survival in a future encounter (Kelley and Magurran, 2007). Thus, any reduced capacity to recall information could have dramatic fitness consequences. In vertebrates, this 'memory loss' can arise for various reasons like bilateral hippocampal lesions (Scoville and Milner, 2000), sleep deprivation (Maquet, 2001), Alzheimer disease (Shankar *et al.*, 2008) or organophosphate pesticide exposure (Levin *et al.*, 2002) but this has mainly been studied in humans and rats, as opposed to marine fishes where literature is very scarce (Hatfield and Johansen, 1972; Besson *et al.*, 2017). Several studies have also highlighted the negative effects of pesticides on the behavior of honeybees involved in

foraging, implying that pollinator population decline could be the result of a failure of neural function of bees exposed to pesticides in agricultural landscapes (Dacher *et al.*, 2005; Williamson and Wright, 2013). Here, we looked at how exposure to an organophosphate pesticide (chlorpyrifos) often encountered in coral reefs (Cavanagh *et al.*, 1999; Leong *et al.*, 2007; Botté *et al.*, 2012) may affect the retention of memory in post-larval stage of a coral reef fish.

The life cycle of most coral reef fish species includes a planktonic larval phase which usually lasts from one to sixty weeks, followed by a sedentary reef-associated juvenile & adult phase (Leis and McCormick, 2002). After the pelagic phase, larvae return to the reef to continue their development into juveniles, and adults (i.e., recruitment phase) (Barth *et al.*, 2015). During recruitment, post-larval fishes (i.e., larvae undergoing metamorphosis) (Holzer *et al.*, 2017) are subjected to strong selective pressures to choose suitable reef habitat (larval fishes experience 90% mortality during recruitment due to predation) (Doherty, 2002; Lecchini *et al.*, 2007). Many fish species show therefore specific habitat preferences based on the relative abundance of predators and competitors for food and space (Leis and McCormick, 2002; Lecchini *et al.*, 2014; Barth *et al.*, 2015). Hence, survival at this life stage is often mediated by anti-predator behaviors (Brown and Chivers, 2007; Lecchini *et al.*, 2014). These anti-predator mechanisms include the ability of fish post-larvae to rapidly learn and retain the identity of predators to survive until the adult stage. However, due to anthropogenic perturbations, post-larval perception and ability to learn may be impaired (Hatfield and Johansen, 1972; Barth *et al.*, 2015; Besson *et al.*, 2017; Lecchini *et al.*, 2017). For example, some studies have found that ocean acidification (Chivers *et al.*, 2014) and habitat degradation (McCormick and Lönnstedt, 2016) can reduce the capacity of early life stages of fishes to chemically learn predator odor. However, no study has yet explored the potential impact of waterborne pesticide on the olfactory-based information retention in marine fishes.

To better understand the potential effects of pesticides on the vulnerable early life stage of coral reef fishes, we examined how organophosphate pesticides affected information retention of the predator *Rhinecanthus aculeatus* in the convict surgeonfish *Acanthurus triostegus*. We used chlorpyrifos (CPF), a broad spectrum organophosphate insecticide, which is one of the most widely used insecticide in the

world (Cavanagh *et al.*, 1999; Leong *et al.*, 2007; Botté *et al.*, 2012; Zhang *et al.*, 2017; Sastre *et al.*, 2018) and that is often encountered in tropical or coral reefs waters (Kapernick *et al.*, 2006; Leong *et al.*, 2007; Salvat *et al.*, 2012). Specifically, we assessed the anti-predator response of *A. triostegus* post-larvae by video recording their foraging behavior (a reduced foraging activity is known to be a common anti-predator response in fishes) (Kelley and Magurran, 2003; Holzer *et al.*, 2017) in the presence of different stimuli in order to identify the behavioral response to predation (Exp. 1), if this response is learnt (Exp. 2) and for how long in the presence or absence of pesticide (Exp. 3).

2. Materials and Methods

2.1. Experimental design

This study was conducted at the CRILOBE Research Station, Moorea Island, French Polynesia from March to August 2017. The focal species, the convict surgeonfish, *Acanthurus triostegus*, is a common Indo-Pacific species that is highly abundant in French Polynesia and feeds on turf algae (Siu *et al.*, 2017). Post-larvae of *A. triostegus* (2.1 ± 0.5 cm, mean \pm SE) were collected using crest nets at Moorea Island (Besson *et al.* 2017; Holzer *et al.*, 2017). The fish used in the different experiments were at the same developmental stage. Indeed, Holzer *et al.* (2017) showed the absence of variability in the ontogenetic stage of *A. triostegus* post-larvae when they recruit on the reef at Moorea. Following capture, fish were brought back to CRILOBE and transferred to 12 L aquaria filled with UV-sterilized and filtered (10 μ m filter) seawater prior to being tested. Post-larvae were tested the day of their capture (after a period of at least 8h in order to recover from the stress of capture) and over the 5 subsequent days (Holzer *et al.*, 2017).

For experiments, olfactory predator cues were taken from the Picasso triggerfish (*Rhinecanthus aculeatus*), an abundant species that predate on fish post-larvae. Heterospecific, non-predator cues were taken from the Manybar goatfish (*Parupeneus multifasciatus*). Several *R. aculeatus* and *P. multifasciatus* at adult stages were collected with gill nets from fringing reefs and kept individually in 250 L flow-

through plastic tanks for 48 h with no food before being used to produce olfactory cues. For each replicate of each experiment, new *R. aculeatus* and *P. multifasciatus* were used.

2.2. Experiment 1: Response of *A. triostegus* to chemical cues associated with predation risk

To test baseline responses to odor cues, post-larvae were exposed to five different odor treatments: 1) control seawater, consisting of UV-sterilized and filtered (10 µm filter) seawater; 2) heterospecific odor, consisting of 30 mL of water extracted from the 1.5 L tank that had contained one goatfish for 1 h ; 3) Predator odor, consisting of 30 mL of water extracted from a 1.5 L tank that had contained one triggerfish for 1 h ; 4) 30 mL of alarm cue; and 5) conditioning stimuli, consisting of 15mL of predator cue and 15mL of alarm cue. To produce each 30mL batch of alarm cue, one *A. triostegus* was euthanized by cold shock. Following the method of McCormick and Lönnstedt (2016) to get alarm cues in coral reef fish, five superficial cuts were made with a clean scalpel on each side of the fish. Fish with cuts were then dipped in a tube filled with 30 mL of seawater which was then collected (McCormick and Lönnstedt, 2016). For each test, 30 mL of cues were used within 10 min following its synthesis.

For each condition, 3 *A. triostegus* post-larvae were placed in a 7 L tank, filled with control seawater and with coral rubble (30 cm³) covered with turf algae (Holzer *et al.*, 2017). The coral rubbles with turf algae were collected close to the crest net site (i.e. Temae site on north-east coast of Moorea) and was characterized by a strong current, avoiding a high sedimentation on the rubbles. All coral rubbles were covered with a large quantity of turf algae and collected at the same site. Selection of the rubbles was mainly based on the sufficient quantity of turf algae cover in order to avoid any artefact due to low quantity of food for fish post-larvae. After an acclimation period of 10 min, one of the five stimuli (30 mL) was added in the tank by using an eyedropper. The experimenter stayed invisible to the fish thanks to black plastic covers placed on the outer walls of the tank in order to avoid stress the fish. Trials were video recorded (video set up 50 cm above the tank - Takara GoPros in 1080 p/30 fps) and the total number of bites per fish made on turf algae was counted during three randomly selected sections of 2 min

over 30 min. However, we did not select the 2 min sections in the first 10 min period to avoid a potential stress of fish.

Each fish was only tested once. Following each trial, all water and stimuli were removed and tank was refilled with control seawater and new coral rubble. Trials were replicated 6 times per stimuli with a new batch of fish stimuli (i.e. 18 fish per stimuli – 90 post-larvae in total).

2.3. Experiment 2: Retention of olfactory memory of *A. triostegus*

To determine whether post-larvae of *A. triostegus* could use chemical alarm cues to learn to associate the odor of a novel predator with risk, the paired alarm cue and predator odor were used to induce an associative learning of that predator, following the method of Brown and Chivers (2007).

After reef capture, 3 *A. triostegus* post-larvae were conditioned with both alarm cue and predatory cue for 1 h (same protocol as in Exp. 1). Then, fish were transferred to a 7 L tank filled with control seawater and remained there for up to 5 days for repeated testing. Fish were not fed during this period and the aquarium only contained PVC tubes as shelter. Trials were performed at 4 separate intervals (1 h, 1, 2, and 5 days after initial conditioning – tests conducted at the same time of day). Three conditioned fish per replicate were tested to the predator cue according to the same sampling protocol as in Exp. 1. As control, we used a group of 3 fish post-larvae ‘conditioned’ to control seawater. The fish in control group and in conditioned group were captured and tested at the same time. Thus, this experiment was replicated with a new batch of fish 6 times for each stimuli at each time intervals (i.e., fish conditioned by alarm/predator cues vs. fish conditioned with control seawater at 1 h, 1, 2, and 5 days). A total of 144 fish were used in this experiment.

2.4. Experiment 3: Effect of chlorpyrifos on retention of risk identification in *A. triostegus*

A. triostegus were conditioned to alarm and predator cues following the protocol of Exp. 2. Following risk conditioning, one half of fish were held for 2 days in a tank filled with control seawater while the other half as held in a tank filled with water added with $1 \mu\text{g.L}^{-1}$ of CPF for 2 days. We chose $1 \mu\text{g.L}^{-1}$ as

an environmentally relevant sub-lethal dose for CPF exposure, as this dose is found in nature and had no direct significant effect on the survival and behavior of *A. triostegus* post-larvae (Holzer *et al.*, 2017). After 2 days, 3 fish reared in the tank with pesticides were placed in a 7 L tank, filled with control seawater, containing coral rubbles (30 cm³) covered with turf algae. The 3 other fish reared without pesticide were placed in a similar 7 L tank. For each treatment, the total number of bites made on turf algae was recorded during three randomly selected sections of 2 min over 30 min. This experiment was replicated with a new batch of fish 6 times for each treatment (i.e., in the presence and absence of pesticides – 36 fish in total).

2.5. Statistical Analysis

For each experiment, all statistical analyses were conducted using the *R-Cran* project free software (<http://www.rproject.org/>, *R-3.3.1*). For each test, 3 fish post-larvae were put together in order to reduce the stress of fish when kept alone in the tank. The data recorded for each trial is the total number of bites in each 2-min period for the 3 fish. Six replicates were conducted per treatment in each experiment. Comparisons of the mean number of bites per minute and per fish were performed using Mann-Whitney U-test when comparing two means (Exp. 2 and 3), and using univariate analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons (Exp. 1). Prior to ANOVA, the normality of the distribution and the homoscedasticity were assessed using Shapiro and Bartlett tests.

3. Results

To assess the anti-predator response of *A. triostegus* post-larvae, their foraging behavior (i.e., number of bites on turf algae) was examined as reduced foraging activity is known to be a common anti-predator response in fish (Deb and Das, 2013; Holzer *et al.*, 2017). Number of bites for each individual were counted from three randomly selected sections of 2 min video over 20 min (after 10min of acclimation).

3.1. Response of *A. triostegus* to chemical cues associated with predation risk

To establish baseline behavioral responses to chemical cues indicating predation risk, we compared the foraging rates of post-larval fishes presented with five different chemical stimuli: control sea water; non-predatory heterospecific odor (Manybar goatfish, *Parupeneus multifasciatus*), predator odor (Picasso triggerfish, *Rhinecanthus aculeatus*), alarm cue (olfactory cues released from the epidermis of damaged conspecifics), and conditioning odor (alarm cue presented in conjunction with predator odor). The number of bites taken within the 2-min test periods varied significantly depending on the stimuli presented (ANOVA: $F_{4,18} = 19.4$, $P = 0.0001$), ranging from 8.8 ± 0.2 bites (control sea water; mean \pm SD) to 2.7 ± 0.3 (conditioning odor). Post-larval *A. triostegus* foraged significantly less in the presence of both alarm cue odor and conditioning odor (Fig. 1) compared to control seawater, predator odor, and heterospecific odor (Tukey post-hoc tests, $P < 0.05$ for all comparisons between alarm cue or conditioning odor vs. control, predator or heterospecific odors). While non-significant, post-larval *A. triostegus* did display decreased foraging rates when exposed to predator and the heterospecific odors compared to control seawater (Tukey post-hoc tests, $P = 0.33$ for predator vs. control, $P = 0.40$ for heterospecific vs. control), which could be attributable to slight neophobia, an innate fear of any novel chemical cue (Brown and Chivers, 2007).

3.2. Retention of olfactory memory in conditioned *A. triostegus*

Post-larval *A. triostegus* were first treated with either a conditioning odor (alarm cue plus predator odor) or saltwater control for a 1 h period. Foraging rates in the presence of predator odor were then recorded at several intervals (1 h, 1, 2 and 5 days). After 1 h, conditioned *A. triostegus* exhibited significantly lower foraging rates (mean bite rate: 3.1 ± 0.5) compared to control fish (6.4 ± 0.5) (Mann-Whitney test: $P < 0.01$), suggesting conditioned fish were associating predator odor with heightened risk. A reduced feeding rate in response to predator odor was also observed at 1 and 2 days following conditioning (Mann-Whitney test: $P = 0.04$ at 1 day, $P = 0.03$ at 2 days). However, this response was no longer seen at 5 days (Mann-Whitney test: $P = 0.39$), with foraging rates now similar to control fish

(mean bite rate \pm SD: 8.9 ± 0.6 vs. 8.7 ± 0.5 respectively) (Fig. 2). These results suggest that post-larval *A. triostegus* retain olfactory cues between 2 to 5 days.

3.3. Effect of chlorpyrifos on memory retention of *A. triostegus*

Post-larval *A. triostegus* were treated with the conditioning odor for a 1 h period. Following treatment, one half of the conditioned fish were held in seawater containing $1 \mu\text{g.L}^{-1}$ of CPF for 2 days, with the other half held in untreated seawater. After 2 days, all fish were transferred to fresh seawater. After a habituation period (1 h), foraging rates in response to predator odor were recorded. Foraging rates of fish exposed to CPF was significantly higher than control *A. triostegus* (Mann-Whitney test: $P = 0.03$) (mean bite rate \pm SD: 6.1 ± 0.3 for fish exposed to CPF vs. 4.1 ± 0.5 for control) (Fig. 3), suggesting that post-larvae of *A. triostegus* lost their memory of predator odor in presence of CPF. Note a difference in bites number in condition stimulated fish after 2 days between Fig. 2 and Fig. 3. This difference could be explained by the fact that predator cue water and alarm cue water were not the same between the experiments.

4. Discussion

Dory, the famous surgeonfish might not be the only coral reef fish affected by short term memory problems. Here, we show that the olfactory memory of predator cues was estimated at between two and five days in the coral reef fish *A. triostegus* (Fig. 1, Fig. 2). Memory is a constructive process for all animals that possess a hippocampus, the part of the brain that controls the retention of information, and fish are no exception (Laland *et al.*, 2003). To the best of our knowledge, this is the first record of memory retention in the early life stage of a coral reef fish. Literature regarding memory retention following a single pairing of an alarm cue and predator cue in fishes is very scarce, but the length of time ranges from 2 days in the fathead minnows, up to 21 days in hatchery-reared rainbow trouts (Brown and Chivers, 2007). Here, in the presence of an environmentally relevant concentration ($1 \mu\text{g.L}^{-1}$) of a common pesticide (CPF), *A. triostegus* failed to retain an information usually learned in control

conditions (Fig. 3). Exposure to various pesticides was proven to impair spatial memory in rats (Yan *et al.*, 2012), impair olfactory learning and memory in honeybees (Williamson and Wright, 2013) and the ability to learn and retain a simple conditioned response in the Atlantic salmon (Hatfield and Johansen, 1972), but such an effect in an ecologically relevant context, like predator's odor memory, has never been investigated in fishes. The known inhibitory effect of CPF on acetylcholinesterase (AChE) activity in fishes and other vertebrates (Colosio *et al.*, 2003) and the role of AChE in neurotransmission in the central nervous system could likely explain the reduced olfactory memory in *A. triostegus* (Fig. 3). Sub-toxic CPF exposure during neurulation was shown to adversely affect brain development, leading to behavioral anomalies that selectively include impairment of cholinergic circuits used in learning and memory in rat juveniles (Icenogle *et al.*, 2004). Our study highlights that pesticides had a negative impact on fishes' ability to remember predators successfully via their effects on learning and memory. This result could be also partly attributed to the advanced or delayed metamorphosis induced by thyroid hormone disruption (Holzer *et al.*, 2017). Yet, as coral reef fishes are particularly vulnerable to predation at their early life stages (90% of post-larvae disappeared during the recruitment by predation) (Doherty, 2002; Lecchini *et al.*, 2007), failing to respond adequately to predation often means immediate death (Besson *et al.*, 2017). Even if anti-predation behavior seems to have an underlying genetic basis, learning plays an important role in the development of antipredator responses (Kelley and Magurran, 2003). For example, sticklebacks reared in aquariums over several generations retained their characteristic patterns of anti-predator behavior (Foster and Endler, 1999), underlying the genetic basis of the antipredator responses. On the other hand, fry of sticklebacks from a high-predation population that were protected by the father from approaching predators and subsequently returned to the nest went on to develop stronger avoidance responses towards a predator model than those that were orphaned (Tulley and Huntingford, 1987) showing the importance of learning on antipredator response. Thus, since recruitment is an essential phase in a coral reef fish's life, memory is a key tool on its way to become an adult. A loss of predator-avoidance behaviors could greatly increase predator-induced mortality rates of this sensitive life stage

with flow on impacts for adult populations of coral reef fishes, and the human communities that rely on them (Charlton *et al.*, 2016).

5. Conclusions

Our study shows that, through associative learning, the retention of olfactory memory of a predator by *A. triostegus* postlarvae lasts between 2 and 5 days (Fig. 2). However, the presence of chlorpyrifos at environmentally-relevant concentrations ($1 \mu\text{g.L}^{-1}$) induces the loss of acquired predator recognition (Fig. 3). This may lead to more vulnerable fishes in coastal areas subjected to organophosphate pesticide pollution. In addition, our approach illustrates the need to use neuro-behavioral indicators in order to assess the sub-lethal effect of pollutant on marine organisms. This will enable, as opposed to traditional ecotoxicological approach (LC 96h), to move from the effect of pollutant on individual level toward population or ecosystems level.

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Data and materials availability: Data will be made fully available upon request to D.L. (lecchini@univ-perp.fr).

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Figure captions

Figure 1. Number of bites per min and per fish for *Acanthurus triostegus* post-larvae in response to chemical cues. Individuals were exposed to different chemical stimuli: Control sea water; Heterospecific odor (species: *Parupeneus multifasciatus* at adult stage); Predator odor (species: *Rhinecanthus aculeatus* at adult stage); Alarm cue (cues released from *A. triostegus* conspecific damaged skin) and Conditioning (alarm cues associated with predator odor). Values are mean \pm SD. **: $P < 0.01$; *: $P < 0.05$. Tested fish were at the same developmental stage.

Figure 2. Retention of olfactory memory in *Acanthurus triostegus* post-larvae. Individuals were initially conditioned either with both alarm cue and predatory cue (condition stimuli) or with control seawater (control stimuli), and subsequently tested at different time (1 h, 1, 2 and 5 days after) for their foraging response in the presence of the predator cue alone. Values are mean \pm SD number of bites. **: $P < 0.01$; *: $P < 0.05$; NS: Non-significant. Tested fish were at the same developmental stage.

Figure 3. Effect of chlorpyrifos on the number of bites per min and per fish for *Acanthurus triostegus* post-larvae. Individuals were initially conditioned with both alarm cue and predatory cue, and subsequently reared for 2 days either in control seawater or in seawater added with $1 \mu\text{g.L}^{-1}$ of chlorpyrifos. After 2 days, their foraging behavior was recorded in clear water added with predator cues. Values are mean \pm SD. *: $P < 0.05$. Tested fish are at the same developmental stage.

Fig. 1

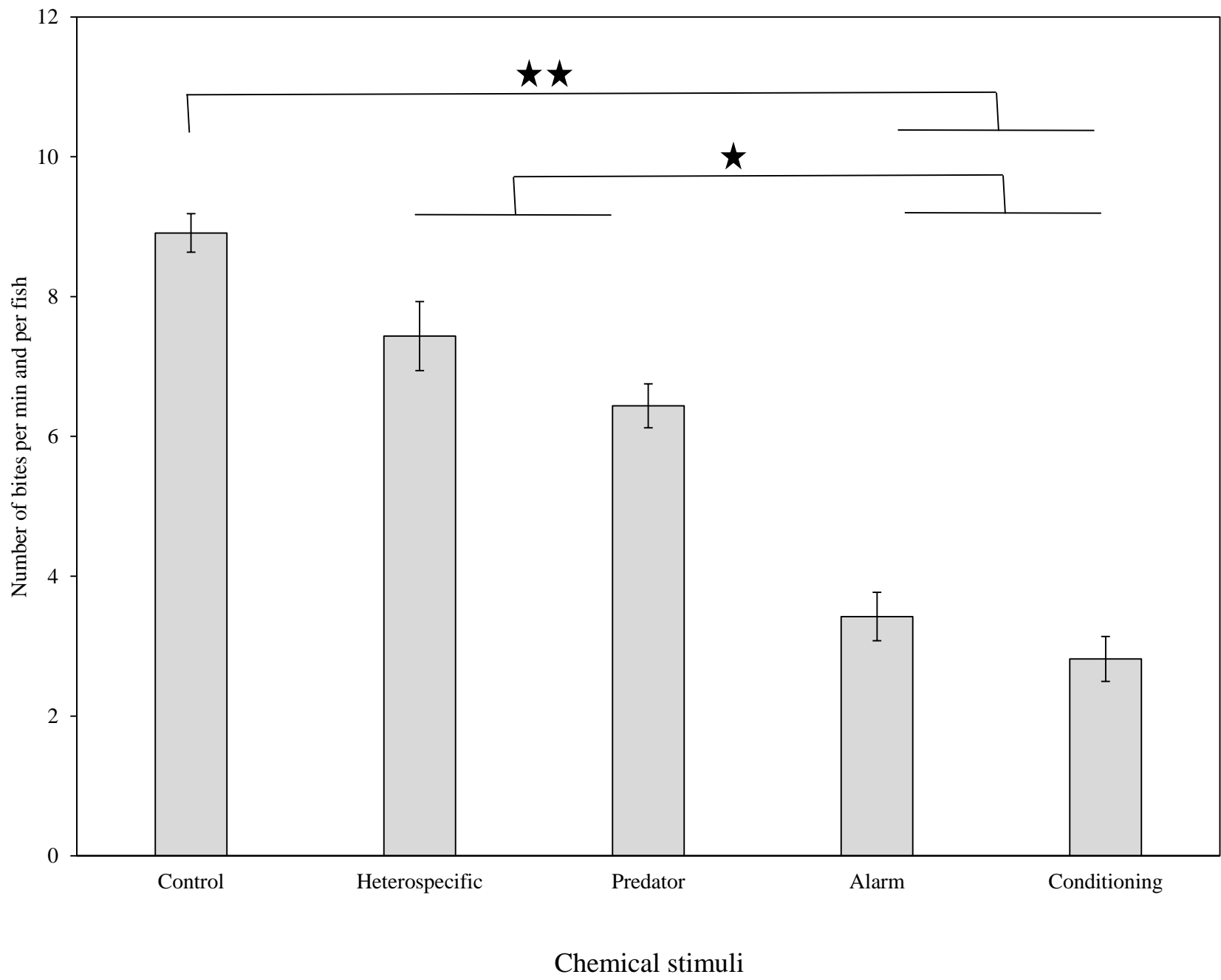


Fig. 2

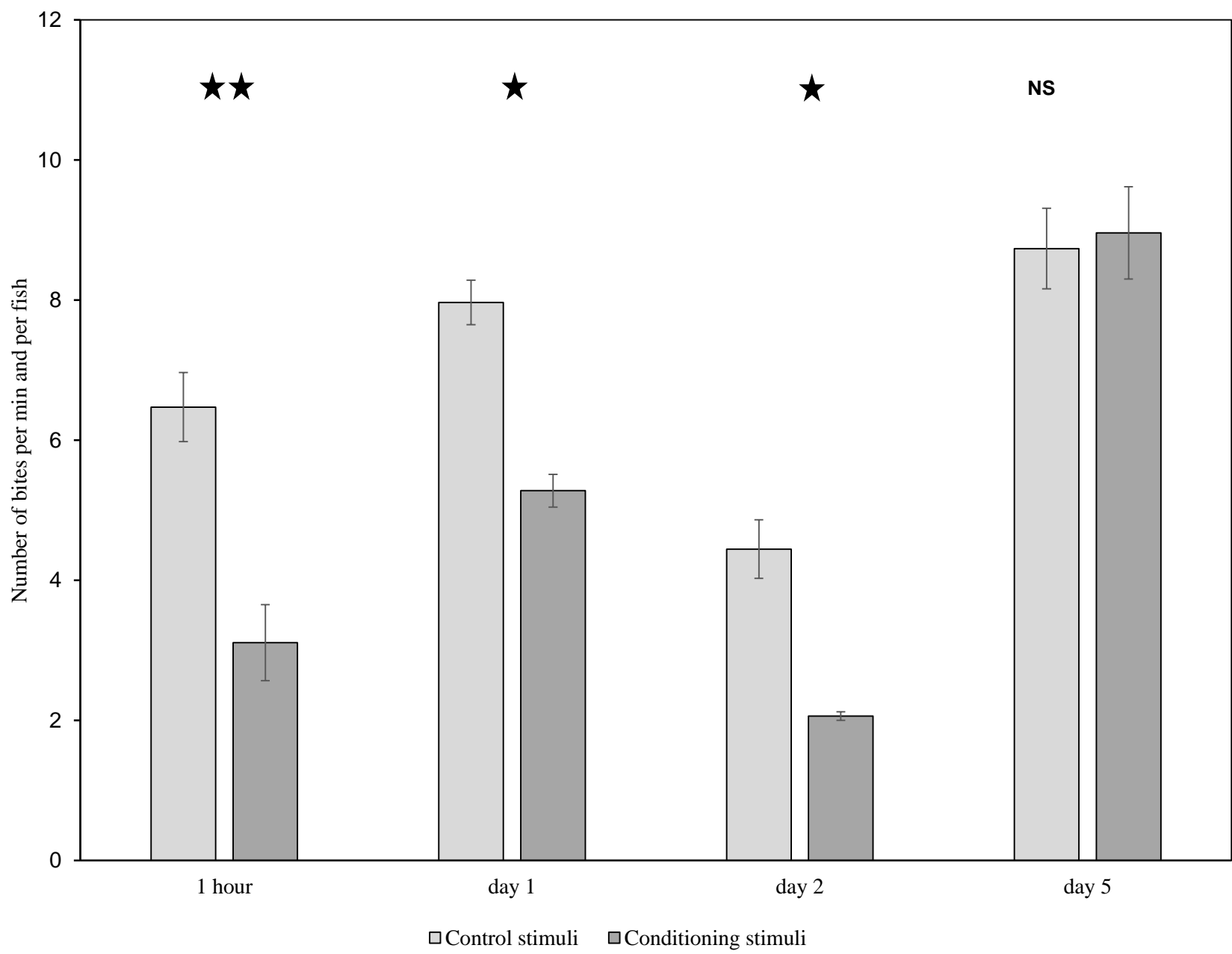
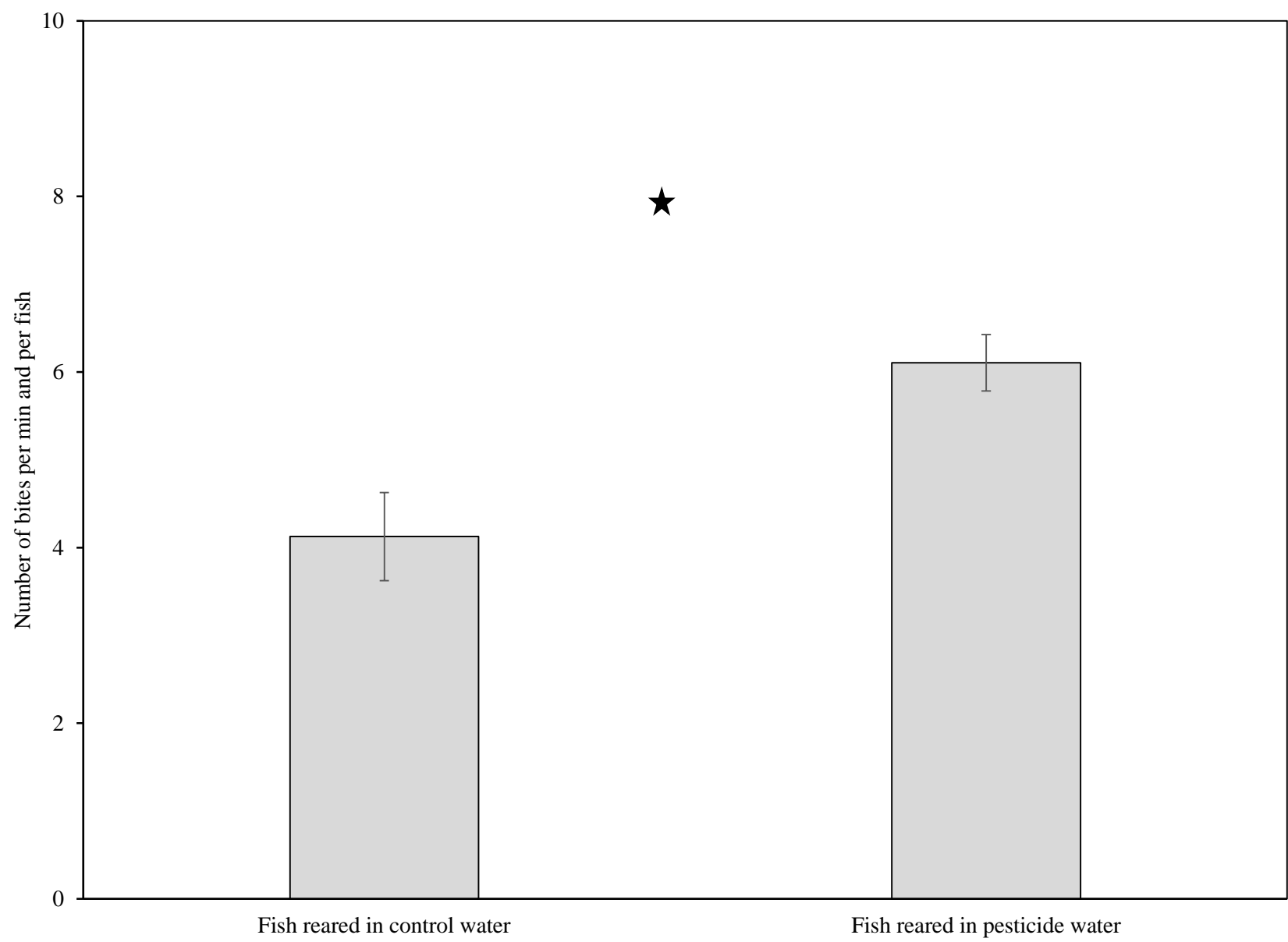


Fig. 3



- Coral reefs face many threats such as ocean warming, acidification and pollution
- No studies examined how chemicals affect the cognitive abilities of reef organisms
- This study investigated anti-predator behavior in post-larval *Acanthurus triostegus*
- This behavior was retained between 2 - 5 days in the absence of pesticide
- The pesticide Chlorpyrifos induced a loss of this crucial information