'OCEANS AND LAKES'

INTERUNIVERSITY MASTER IN MARINE AND LACUSTRINE SCIENCE AND MANAGEMENT

Behavioural effects of Ocean Acidification in the Tropical Reef fish, *Amphiprion ocellaris*

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Abstract

Rising concentrations of atmospheric carbon dioxide (CO_2), lead to a rise of CO_2 concentration in ocean water through a process called ocean acidification. In this study the effects of these higher concentrations were tested on behavioural changes, including coupling formation and olfactory sensing, in a tropical reef fish, *Amphiprion ocellaris*, for a period of 32 days. Behavioural changes in routine behaviour and boldness, were most apparent as CO_2 concentrations increased. Coupling formation seemed to be impaired at a pH of 7.4, though not yet at a pH of 7.8 which is predicted to occur by 2100. No clear differences were found in olfactory sensing between different CO_2 concentrations, giving no proof that ocean acidification might impair the olfactory mechanism.

Introduction

Rising concentrations of atmospheric carbon dioxide (CO_2) are causing global warming and ocean acidification (Fabry *et al.*,2008; Orr *et al.*, 2005). Its levels have increased from preindustrial levels of 280 ppmv with nearly 40% to 384 ppmv in 2007 (Solomon *et al.,* 2007). During 650 000 years before the industrial revolution, atmospheric conditions varied between 180 and 300 ppmv (Siegenthaler *et al.,* 2005). However, currently levels are rising with 0.5% per year (Forster *et al.*, 2007). This is around a hundred times faster than any change during the past 650 000 years (Royal Society, 2005; Siegenthaler *et al.*, 2005). Rising atmospheric CO_2 is tempered by oceanic uptake, approximately one-third of the anthropogenic CO_2 produced in the past 200 years has been taken up by the oceans (Sabine *et al.*,2004; Sabine and Feely, 2007). Without this ocean sink, the change in atmospheric CO_2 concentration would be 55% higher than the observed change (Sabine *et al.*, 2004).

Ocean uptake of CO_2 causes changes in ocean water chemistry. Through the hydrolysis of CO_2, the hydrogen ion concentration [H+] increases, leading to pH reductions, which is why this problem is referred to as ocean acidification (Orr *et al.*, 2005; Doney *et al.*, 2009). The changes to present day values correspond with a 0.1 pH decline since preindustrial levels. Ultimately ocean CO_2 values are predicted to reach 1000 ppmv CO_2 by the year 2100 and 1900 ppmv CO_2 by 2300 which would correspond with a pH decline of up to 0.77 by the year 2300 (Caldeira and Wickett, 2003; Meehl *et al.*, 2007). According to the IPCC emission scenarios (Houghton *et al.*, 2001), the average surface ocean pH would decrease with 0.3-0.4 units compared to pre-industrial values by 2100 (Figure 1) (Caldeira and Wickett, 2005).

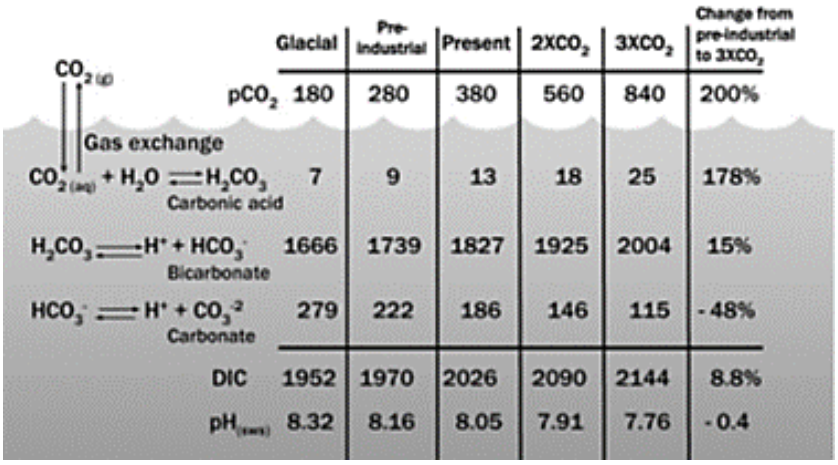

Figure 1. Concentrations of carbon species (in units of µmol kg^{-}) and pH values during glacial, pre-industrial, present day, two times pre-industrial CO_2, and three times pre-industrial CO_2. Changes in inorganic carbon system were calculated by assuming equilibrium with the atmospheric CO_2. pH is based on the seawater scale. Last column represents the changes from pre-industrial levels to three times atmospheric CO_2 (modified from Feely et al. (2004) and Kleypas et al. (2006) by Fabry et al. (2008). Further modified from Fabry et al. (2008)).

Ocean uptake of CO_2 alters the chemistry of seawater. The inorganic carbon system is one of the most important chemical equilibria in the ocean and is largely responsible for controlling the pH of seawater (Fabry *et al.*, 2008). Dissolved inorganic carbon appears in seawater in three forms; aqueous carbon dioxide ($CO_{2(aq)}$) which includes carbonic acid (H_2CO_3), carbonate ion (CO_3^{2-}) and bicarbonate (HCO_3^-). When CO_2 is dissolved in seawater, H_2CO_3 is formed. Most of this dissociates

into HCO_3^- and a hydrogen ion (H^+). This hydrogen ion in turn reacts with CO_3^{2-} and forms HCO_3^-. Thus increasing concentration of CO_2 in seawater lead to a decrease in CO_3^{2-} concentrations and an increase in H_2CO_3, HCO_3^- and H^+. It is this increase in H^+ that leads to the decrease in pH, experienced in ocean acidification (Fabry *et al.*, 2008). All these reactions are fully reversible (Figure 1) (Millero *et al.*, 2002). The projected decrease of 0.3-0.4 pH compares to an 150% increase in hydrogen ions and a 50% decrease of carbonate ion concentrations (Orr et al., 2005).

These changes in ocean CO_2 partial pressure and resulting changes in pH levels are predicted to severely impact marine organisms (Munday et al., 2012a). This can occur through decreased calcium carbonate ($CaCO_3$) saturation, which affects the calcification rates. Evidence indicates that these rates will decrease in low-latitude corals which form their reefs out of aragonite, and in phytoplankton which form their shells out of calcite (Gattuso *et al*., 1998; Kleypas *et al*., 1999; Langdon *et al*., 2003; Riebesell *et al*., 2000; Zondervan *et al*., 2001; Orr *et al*., 2005). Furthermore elevated levels of CO_2 (hypercapnia) can impact marine organisms via disturbance to acid-base physiology (Fabry *et al.*, 2008). With the increasing levels of CO_2 in seawater, dissolved CO_2 will diffuse more easily across animal surfaces and will equilibrate in both intra- and extracellular spaces. The internal levels will rise until a sufficient level is reached that restores the CO_2 excretion with the environmental level. As CO_2 and internal body fluids react, H^+ will increase and pH will decrease. Mechanisms to deal with this acidification are limited and include the following: passive buffering of intra- and extracellular fluids; the transport and exchange of relevant ions: the transport of CO_2 within the blood of those species with respiratory pigments and metabolic suppression to wait out periods of elevated CO_2 (Fabry et al., 2008).

Fish were initially believed to be safe from high CO_2 concentrations and its effects, as studies demonstrated that mortality only occurred at extremely high concentrations (higher than 10 000 ppmv.) (Ishimatsu *et al.*, 2008) These levels exceed those predicted for acidification by the IPCC, at levels from 30 000 to 50 000 ppm CO_2. Mortality reasons might be cardiac failure or nephrocalcinosis, where calcareous precipitates build up and then obstruct the kidney tubules (Lee et al., 2003; Hayashi et al., 2004; Foss et al., 2003). Additionally, fish can partially reduce the acidosis of increased CO_2, through the Cl^-/HCO_3^- exchanger in the gill epithelium. This leads to decreased Cl^- concentration and increased HCO_3^- concentration in the extracellular fluid (Jutfelt *et al.*, 2013). However, there is the chance of additional energy expenditure for osmoregulation when CO_2 levels rise. Because fish actively expel Cl^-, additional reductions in plasma Cl^- would require fish to use up additional energy which compensates for these losses (Ishimatsu *et al.*, 2008). There is also increasing evidence that suggests ocean acidification induces sublethal effects including alterations in otolith growth, alterations in olfaction and consequential detection of substrates, predators, prey and parents, differences in behavioural lateralization and impaired learning (Checkley *et al.*, 2009; Bignami *et al.*, 2013; Nilsson *et al.*, 2012; Jutfelt *et al.*, 2013; Munday *et al*., 2009; Cripps *et al.*, 2011; Dixson *et al.*, 2010; Munday *et al.*, 2010; Chivers *et al.*, 2014).

Neurosensing and behaviour

Most of the studies to date on the subject of effects of ocean acidification on behaviour concerns tropical fish species, often strongly associated with coral reef habitats. An important phase within the life cycles of most reef fish is the pelagic larval phase, where larva spend several weeks in the open ocean before settling and developing into adult form (Briffa *et al.*,2012). The survival of the larvae and the species depends on their ability to locate suitable habitats at the end of their pelagic phase (Munday *et al., 2*009). Evidence suggests that they do so through olfactory cues (Atema *et al.,*2002; Arvedlund and Takemura, 2006; Gerlach *et al.,*2007) and through reef sounds (Montgomery *et*

*al.,*2006). Disruption of the ability of larvae to detect these sounds and cues, could have far-reaching effects on the adult populations. Munday *et al.* (2009) reared orange clownfish (*Amphiprion percula*) under three different CO_2 conditions from the egg hatching until the end of the larval phase and showed that elevated CO_2 and resulting low pH disrupted the olfactory mechanism by which the clownfish discriminated between cues leading to suitable and unsuitable habitats. In fact olfactory signals that were normally avoided, were favoured instead. This could lead not only to a decline in rate of survival, but also changes in patterns of dispersal and connectivity between the populations. (Munday *et al., 2*009).

Additionally to finding suitable habitats, there is also a decrease in survivability should larvae not be able to detect and avoid olfactory cues from predators. Dixson *et al.* (2010) tested the effects of elevated CO_2 concentrations on the ability of clownfish larvae to discriminate between odours of predators and non-predator fishes. Both eggs and larvae were exposed to large concentrations of CO_2, and then larvae were tested immediately after hatching and at settlement stage. Whereas the post-hatchlings were capable of detecting the proper cues, and avoiding predators, settlement stage larvae were incapable of doing so (Dixson *et al.,* 2010). Similar results were found by Munday *et al.* (2010) which showed that *A. percula* larvae reared at 850ppm were completely impaired in their ability to sense predators. Effects were even seen from elevated levels of 700ppm, though some individuals showed no difference in behaviour with the control group at this level, whereas others became attracted to predator cues instead. Similar results were found for Ward's damselfish larvae. Considering this 700ppm could be the threshold for impairment of the olfactory sensing (Munday *et al.*,2010; Briffa *et al.,*2012).

However the rate of survival would also be depended on the ability of the predator to detect and catch its prey. The brown dottyback*, Pseudochromis fuscus,* was tested on its ability to detect chemical cues produced by its prey when exposed to elevated CO_2. It was shown that after CO_2 treatment the predators spend 20% less time in a water stream with the smell of an injured prey than the control group (Cripps *et al.,* 2011). Ferrari *et al.* (2011) showed that with predators *P. fuscus* and juveniles of four different damselfish species exposed to high concentrations of CO_2 the predation rate was double that of the control group. How the dynamics of predator-prey interactions will be affected depends on how the interacting species are affected by the elevated CO_2 conditions (Allan *et al.*, 2013).

Effects of elevated CO_2 on reef fish are not limited to impairments in the olfactory system, but also in the visual and auditory responses. In juvenile damselfish, *Pomacentrus amboinensis*, a weaker anti-predator reaction was found at the sight of an adult spiny chromis (*Acanthochromis polyacanthus*) when exposed to 850 ppm CO_2 (Ferrari *et al.,*2012). Auditory choices were tested in juvenile *A. percula* after being exposed to concentrations of 390 ppm and enriched situations leading up to 900 ppm. Juveniles from the 390 ppm conditions avoided predator-rich noise, whilst juveniles with higher concentration treatments did not. It was suggested that the enriched situations caused a decrease in otoliths, as it did in the white sea bass (*Atractoscion nobilis) (*Checkley Jr. et al., 2009). However, no changes in growth were found in *A. percula* indicating that the impairment in auditory detection must have another cause (Simpson *et al.*, 2011; Munday *et al.*, 2011).

Beyond these sensory effects, there have been changes found in the behavioural lateralization (Domenici *et a*l., 2014; Jutfelt *et a*l., 2013). Behavioural lateralization is the tendency to favour the left or right side during behavioural activities, which is an expression of brain functional asymmetries (Domenici *et al.*, 2012). In *P. wardi* it was shown that elevated CO_2 at 935 ppm reversed the lateralization bias from right to left (Domenici *et al.*, 2014). The three-spined stickleback, *Gasterosteus aculeatus*, no longer showed any preference for right or left after a twenty-day

treatment of 991 ppm CO_2. This effect remained after a forty-day treatment of elevated CO_2 as well. *G. aculeatus* showed no signs of acclimatization (Jutfelt *et a*l., 2013). The same loss of preference was shown in the yellowtail demoiselle, *Neopomacentrus azyrson*, after a four-day treatment of 900 ppm CO_2 (Nilsson *et al.*, 2012). Finally learning ability was impaired in *P. amboinensis* after exposure to elevated CO_2 levels of 850 ppm. Regardless of learning method, the exposed damselfish failed to respond appropriately to their predator *P. fuscus* (Ferarri *et al.*, 2012).

The diversity of these effects in both behaviour and sensory systems point to an effect in brain function and recognition of elevated CO_2. Nilsson *et al.* (2012) proposed these alterations in behaviour can be traced back to the neurotransmitter gamma-aminobutyric acid type A or GABA-A. This is a major inhibitory neurotransmitter in the vertebrate brains, with high conductivity for Cl^- and HCO_3^-. In normal circumstances, the opening of GABA-A receptors leads to the hyperpolarization and inhibition of the neuron, due to an influx of Cl^- ions from the extracellular to the intracellular space (Nilsson *et al.*, 2012; Hamilton *et al.*, 2013).

When exposed to high CO_2-level conditions, fish regulate acid-base ions, primarily bicarbonate and chloride to maintain blood and tissue pH and avoid acidosis. They accumulate HCO_3^- with compensatory reduction in Cl^-, with the primary site of this ion exchange being the gills in juvenile and adult fish. The resulting altered concentrations of Cl^- and HCO_3^- between cells and plasma are thought to turn the GABA-A receptors from hyperpolarizing and inhibitory to depolarizing and excitatory because of an outflow of these anions (Nilsson *et al.*, 2012; Hamilton *et al.*, 2013; Chivers *et al.*, 2014; Lai *et al.*, 2015; Chung *et al.*, 2014; Regan *et al.*, 2016; Munday *et al.*, 2015).

This was tested on juvenile clownfish and damselfish which had been exposed to elevated CO_2 and then treated with gabazine, a GABA-A receptor antagonist. This restored the olfactory responses and behavioural lateralization in these fishes (Nilsson *et al.*,2012). The involvement of GABA-A has been reinforced in an article by Chivers *et al.* (2014) where fish were taught to recognize a predator in the presence of gabazine, could not react to this cue until the CO_2 effects had ceased, indicating that during the CO_2 exposure the sensory information could not be processed. Research done by Lai *et al.* (2015) found the same results in *Gasterosteus aculeatus*, a temperate fish, where lateralization of fish kept in elevated CO_2 of 992 ppm for forty days, was completely restored. Research on *Pangasianodon hypophthalmus*, a fish native to hypercapnic waters, found the same behavioural changes when these fish were exposed to normocapnia levels, as when reef fish are exposed to high-CO_2 environments. Gabazine treatment reduced these abnormalities in their behaviour (Regan *et al.*, 2016).

All of this indicates that there is an involvement of the GABA-A receptor and that elevated CO_2 can affect critical sensory processes in marine fish. Still as the research on *P. hypophthalmus* suggests, and assuming that this fish or its ancestors were at one point native to normocapnic environments, fish are capable of adapting to increases in environmental CO_2 and ocean acidification, though it could be that the present rate of increase of CO_2 is too high to allow for adaptation through natural selection (Regan *et al.*, 2016).

There is also a certain tolerance of marine fishes' notable in several studies. In juvenile damselfish *P. amboinensis*, there was no effect of CO_2 on sensory assessments if the CO_2 concentration was below 850 ppm. Treatments of 550 ppm and 700 ppm gained no results regarding behavioural impairments (Ferrari *et al.*, 2012). *Pomacentrus wardi* showed individual variation in tolerance towards elevated CO_2 at 700 ppm. About half of the treated larvae from *P. wardi* remained unaffected after the elevated CO_2 treatment. In fact there were no behavioural differences between this half and the control group. If such variation in individual tolerance is heritable, it could lead to a selection of CO_2

tolerant phenotypes in nature, capable of resisting the effects of ocean acidification (Munday *et al.*, 2012b). The same individual tolerance was found in larvae of *A. percula* reared in 700 ppm CO_2, with half of the individuals being unaffected by the elevated CO_2 treatment (Munday e*t al.*, 2010). If this individual variation is based in genetic variation, then a rapid selection of tolerant genotypes might follow, though not much is known whether they will then be capable of coping with higher CO_2 levels than 700 ppm (Munday *et al.*, 2012a).

Social behaviour

Anemonefish, of the genus *Amphiprion*, are monogamous protandrous hermaphrodites. This means females are the largest and dominant members of a social group and control the production of other females by aggressiveness towards males (Fricke and Fricke, 1977; Iwata *et al.*, 2008). Second-rank individuals become males and others will remain as unreproductive individuals. If a female is taken from a social unit, a male will change its sex to female and the largest of the unreproductive individuals becomes male (Moyer and Nakazono, 1978; Ross, 1978; Fricke and Fricke, 1977). If a group of juveniles is raised together in captivity, the largest individual will become female and the next largest a male, whereas the rest will remain as unreproductive and sexually immature individuals (Goldstein, 1989). Pair formation has often been regarded as a random event (Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Kuwamura and Nakashima, 1988). However, Hattori (2004) showed that while in the field, settlement of juveniles might be random, the recruitment is not and could be affected by the number and body size of the residents, as well as the host size. The sex change however does not happen only after a certain body size is attained, but takes place when the male attains the highest social rank at the disappearance or death of the previous female. Some females may thus be smaller than a male of the same species at another host (Moyer and Nakazono, 1978).

Study aim

The aim of this study was to investigate and verify if CO_2 has an effect on individual behaviour, including boldness and olfactory sensing. Expected was that boldness would increase alongside a change in routine behaviour over time. Olfactory senses were expected to be disrupted, leading to a decrease in success rate in a T-maze set-up. Finally, it was tested if enriched CO_2 conditions had an effect on the social behaviour and pair formation of the fishes.

Material and Methods

Study species

Amphiprion ocellaris, or false clownfish, were bought from Reef Company in Belgium, whom acquire their clownfishes from Sustainable Aquatics in Jefferson City, America where the fishes were hatchery bred. At the moment of acquisition fishes were in between eight and ten months old. The fishes were kept in a 140L tank at a salinity of 32ppt to acclimatize for a week at a pH of 8.0 at normoxic levels and fed daily with Dr. Bassleer Biofish Food. Synthetic seawater was made manually from deionized water to which salt (Hw Marine-mix professional) was added until the desired salinity of 32ppt was reached. After acclimatization fishes were divided into three groups of twelve fish each into 50L tanks. All animals were exposed to a constant temperature of 23°C within a climate chamber and fed at the end of each day or after experiments had been concluded. They were under a 14h light, 10h dark regime with the light turning on at 8 in the morning. Water quality was checked each day with Tetra kits for nitrate and ammonia after which the water was changed if the concentration was deemed too high at 0.25mg/L ammonia and 0.3 mg/L nitrate. Half of the water in the tank was removed after which the tank was refilled to 50L.

CO_2 treatments

The three groups were submitted to different CO_2 treatments to simulate ocean acidification. One group was kept at a control concentration of pH 8.0, another at a treatment of pH 7.8 and the last at pH 7.4. Electronic pH probes (WTW 3310) were attached to the aquaria in which the desired pH should reach a concentration of 7.8 and 7.4 units. These probes were connected to a computer which controlled the valve system connected to a CO_2 cylinder. The pH probes were freshly calibrated each day with pH buffers (Merck) kept at 23°C. Water in the aquaria was monitored through the program 24 hours a day, being checked every five minutes and adjusted automatically by the system if there was a deviation from the desired pH concentrations by opening up the valves and injecting CO_2 within the water. The pH level was maintained within ± 0.05 units. Aquaria were aerated to maintain proper air saturation. Different tests took place after multiple treatment days (Table 1). The total treatment of CO_2 lasted for 32 days. In the first nine days locomotor and olfactory sensing test were done by assessing 3D individual behaviour and with a T-maze. The other 23 days were utilised to assess social behaviour.

Table 1: Experiments and the treatment day on which they took place.

Experiment	Treatment day
3D individual behaviour, T-maze	0
3D individual behaviour, T-maze	4
3D individual behaviour, T-maze	9
Social (day 0)	10
Social (day 4)	13
Social (day 7)	16
Social (day 14)	23
Social (day 22)	32

Locomotor and Olfactory sensing tests

For the tests, a total of 36 fish divided over three treatment groups: control (pH= 8.0H), lower pH (pH= 7.8) and lowest pH (pH= 7.4). The experiments were conducted in a series of identical transparent glass 5L tank filled with stock water of the same salinity and temperature as the treatment tanks. Whilst this does mean *A. ocellaris* was not tested in water of the same Pco_2, previous research has shown that it takes several days (Lai et al, 2015) before CO_2 effects upon behaviour cease to be. Individual fish were netted randomly from the treatment tanks and instantly placed within the experimental tanks. The water within these tanks was refreshed each time a new fish was introduced. Six fish of each group were randomly netted and used in the experiment. After use, they were temporarily kept separated from the original acclimation tank so as not to confuse between fishes already monitored and remaining unused fish. Six from another treatment were then randomly netted, then six from the last treatment, before the remaining six fish from the first treatment were used and so on. This was done to remove any possible variation that might occur with timing differences throughout the day.

Locomotor activity (or routine activity) for each individual fish was monitored with Zebracube Video-Tracking system (ZebraLab, ViewPoint Life Sciences, France). Using a high-speed infrared camera the fish were tracked for eight minutes after an acclimatization of minutes. Individual fish were kept in the 5L transparent glass experimental tanks when being moved into the Zebracube. Within this part of the experiment the movement of the fish throughout the tanks is tracked, monitoring whether it remains within one body length to the edges and corners, or if it moves towards and across the centre. Inactivity stands for swimming speed under a certain threshold, both the inactivity distance (inadist [cm]) and duration of this inactivity (inadur [s]) were measured. Higher swimming speeds were measured with small movement distance (smldist [cm]) and duration (smldur[s]).

After eight minutes a plastic opaque barrier was introduced into the tank, keeping the fish from seeing the introduction of a novel object into the tank. The object was a brown glass cylindrical bottle, filled with water so it could not float, of 6cm diameter and 11cm high. It was monitored how quickly and how close the fish came towards the novel object once the opaque barrier was removed to measure the boldness of the fish. Proximity was measured with body lengths. Closest to the object meant the fish moved within one body length of the object, nearby being two body lengths and far-off when it did not fall underneath these parameters. The body length was kept constant for all fish. The monitoring of fish with the novel object lasted for eight minutes, after which the object was removed from the tank and the fish were moved into the next set-up of the experiment. The variables tracked within this experiment consisted out of the number of times the fish entered a certain zone and the duration within that zone.

A T-maze (Figure 2) was used to assess the ability of *A. ocellaris* to discriminate between water containing different odour stimuli. The T-maze measured (64cm on 58cm) and was filled with seven cm water with the same salinity and temperature as the water within the acclimation and experimental tanks. At one end of the T-maze a paper tea dispenser (t-sac, chlorine freeNAME) containing Dr. Bassleer Biofish food was hung, whereas at the other end there was an empty tea dispenser to avoid the fish focussing upon the tea dispenser form instead of the food stimulus. At the start of each trial the fish coming from the Zebrafish Cube was introduced into the experimental arena into a small area (15/13cm) kept separately with an opaque barrier and left to acclimatize for two minutes. The barrier was then removed upon which the fish swam until it entered one of the

runways of the T-maze. Turning into a runway was determined by crossing over the threshold of 5cm into the runway of 22cm. Each choice was recorded manually by direct visual observation, though all choices were also recorded by camera (Casio EX-F1). The fish were then gently ushered back into the smaller area and kept behind the barrier for another two minutes, before they were released again. Success and failure of turning towards the food was recorded three times for each fish. At the third attempt for each fish, the tea dispenser containing the food was switched to the other end of the T-maze and replaced by the empty one at the other end.

These experiments ran over a total of ten days, including a 'Day 0' on which the treatment hadn't started yet. Overall the CO_2 treatment had lasted for nine days at the conclusion of these experiments.

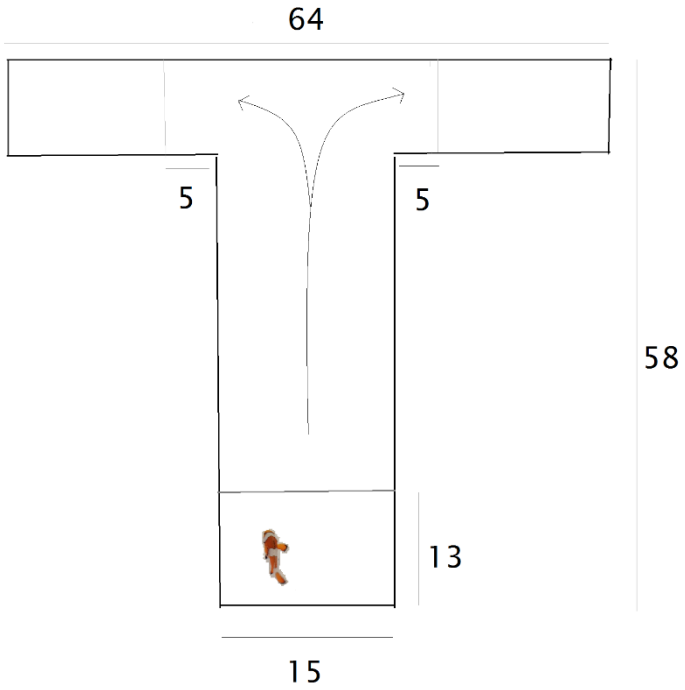

Figure 2: Diagram (top view) of the T-maze used for the olfactory sensing tests. Measurements are in cm. Not to scale.

Social behaviour

Fishes from each treatment and the control tank were separated in pairs. Each individual was placed with a larger or smaller individual until all 12 fish within each tank were paired. They were placed per pair in separate permeable two litres breeding nets (JBL NBox, L:17 x B:12.5 x H:13.5) which were in turn placed in the larger 50L tanks all fish had been in previously, thus ensuring that all fish within a treatment were in the same water.

The test used a total of 36 fish divided over three treatment groups in 17 pairs, meaning six pairs for both the control group and the 7.8 treatment, whereas the 7.4 treatment only had five pairs due to mortality of one fish previous to the social experiment. The experiment was conducted in a series of identical transparent glass five litre tank filled with stock water of the same salinity and temperature as the treatment tanks, similar to the previous experiments.

After an hour acclimatization within these tanks, pairs were placed in the Zebracube and after five minutes acclimatization were measured for eight minutes for inter-individual distance (iid) between fish and polarization of the fish (meaning how often they swam in the same direction). Afterwards fish were placed back into their breeding net within the treatment tank.

These experiments took place over a total of 22 days, from the 10th day of CO_2 treatment until the 32nd. Upon each day of the experiment the same order of pairs was followed, with the overall experiment, including acclimatization, lasting around 3 hours. First two pairs of the Control group were measured, followed by the first two pairs of the 7.8 treatment, then the first two of the 7.4 treatment, followed by the next two pairs of the Control group and so on.

Blood acidosis

At the end of the 32nd treatment day, after the last social experiment, all clownfish were euthanized. With MSC222 fish were put to sleep after which blood was taken with 0.5ml syringes treated with Heparin to avoid blood clotting, through a caudal puncture. Blood pH was immediately taken with a pH probe and noted down. Individuals were then weighted before being placed in 4% formaldehyde for 24 hours, after which they were placed in 70% ethanol to start the fixation process.

Statistical analysis

Files from the Zebracube for the routine, novel object and social behaviour were loaded into Excel. Artefacts that might occur after wave movement or movement of small particles were removed from the dataset. Excel was used to calculate the average inadur, inadist, smldur and smldist for the routine behaviour, whereas the duration and count of times within a zone were calculated for the novel object behaviour, and the average iid and polarization were calculated for the social behaviour. Success percentages for each group on each experiment day were calculated for the T-maze experiment.

Routine and novel object behaviour were analysed in Rstudio. One -way ANOVAs were used to analyse the results of different treatment days within groups, whereas two-way ANOVAs were used with factors of day and treatment. Social behaviour was analysed in the same manner, with one-way ANOVAs for the differences in between treatment days within groups, and two-way ANOVAs with different CO_2 treatment and social day as difference. Post hoc Tukey tests were used to test for differences between groups after the two-way ANOVAs. Possible correlation between blood pH and different treatment group was analysed with Spearman correlation tests.

Results

Individual behaviour

Fish within the control treatment group showed the largest difference over time in average inactivity duration and distance, as well as in average small movement duration and distance, with p-values of 0.041, 0.032, 0.029, 3.01E-06 respectively (Table 2). The average inactivity decreased over the period of CO_2 treatment, with the highest inactivity being on Day 0 of the treatment (Figure 2, A). The same is true for average inactivity distance, the highest being at the beginning of the treatment and decreasing over time (Figure 2, B). Both decreased with over 50% compared to Day 0 of the treatment. Average small movement duration and distance however increased over time, with over 50%, the highest duration and distance measured at the end of the nine day experiment (Figure 2, C and D).

Fish exposed to CO_2 resulting in a pH of 7.8 only show significant difference over time in the average small movement distance (p-value: 0.027). Average inactivity duration and distance remain stable during the experiment, though average smldur increases slightly, whereas the smldist increases significantly towards the end (Figure 2). Fish of the 7.4 pH treatment showed no significant difference in any of the variables over the nine days of the first experiment (All $p>0.05$, Table 2). Overall average inadur, inadist and smldur decreased over time, whereas average smldist increased (Figure 2).

All variables differed significantly between the control group and the CO_2 treatment groups, though there was no significant difference in any variable when comparing the fish from the 7.8 and 7.4 treatment group (Table 2). For all groups the average small movement duration and distance are much larger than the inactivity duration and distance.

Table 2: p-values of average inactivity duration (inadur), inactivity distance (inadist), small movement duration (smldur) and small movement distance (smldist) for each treatment group compared over different treatment days, and compared between different treatment groups. Significant differences are represented with an asterix (*).

	Average inadur	Average inadist	Average smldur	Average smldist
Control	0.04126728*	0.03207101*	0.02825891*	3.01E-06*
7.8	0.467594	0.4777996	0.7814459	0.02668321*
7.4	0.1510086	0.1505083	0.1152424	0.1275948
Control -7.8	0.0003985*	0.0002893*	0.0013937*	0.0332464*
Control - 7.4	0.0013249*	0.0011114*	0.0061515*	0.0110045*
7.8 - 7.4	0.9463163	0.9338538	0.8999031	0.9061178

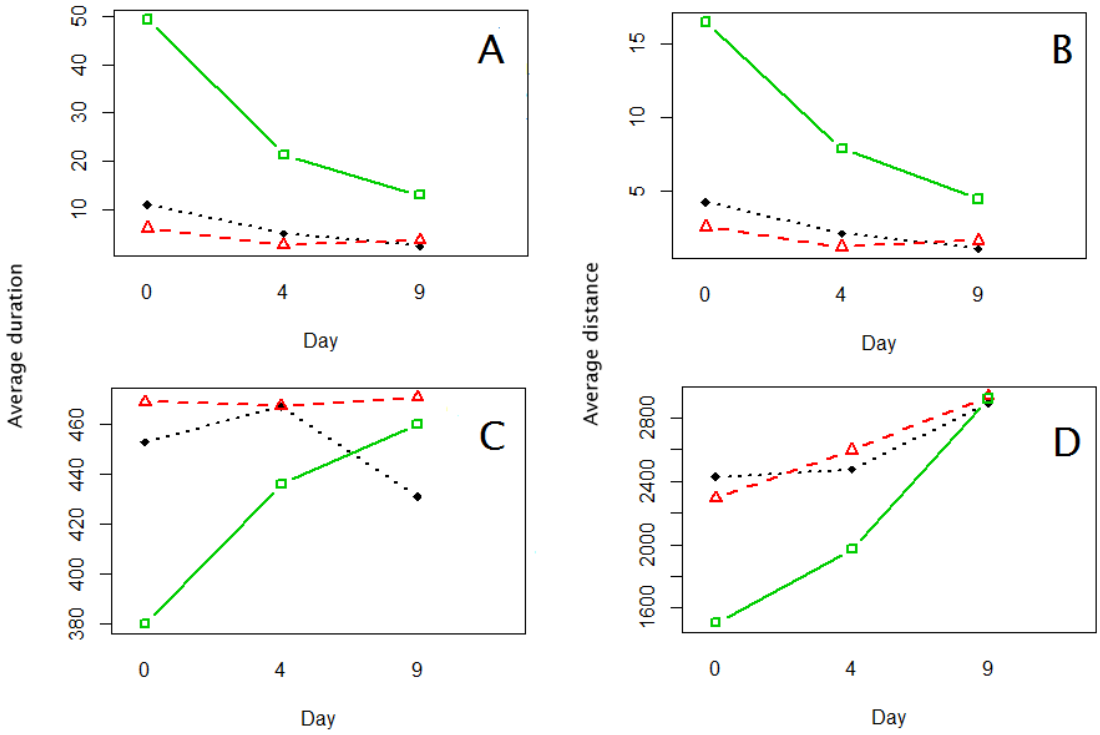

Figure 2: A: Average Inactivity duration (s), B: inactivity distance (cm), C: small movement duration (s) and D: small movement distance (cm) of each group. The control group is represented by full green line with square symbols. The CO_2 treatment groups of pH= 7.8 and pH = 7.4 are represented by the dotted red line with triangles and dotted black line with diamonds respectively. Days stand for the different CO_2 treatment days.

Fish from all groups spent most of their time (69-73%) more than two body lengths away from the brown glass bottle introduced as the novel object, spending only 0.5-0.8% of their time closest to the object. There are however no significant difference in between groups, or when comparing the different treatment days within a group. The number of times fish from a group came closest to the

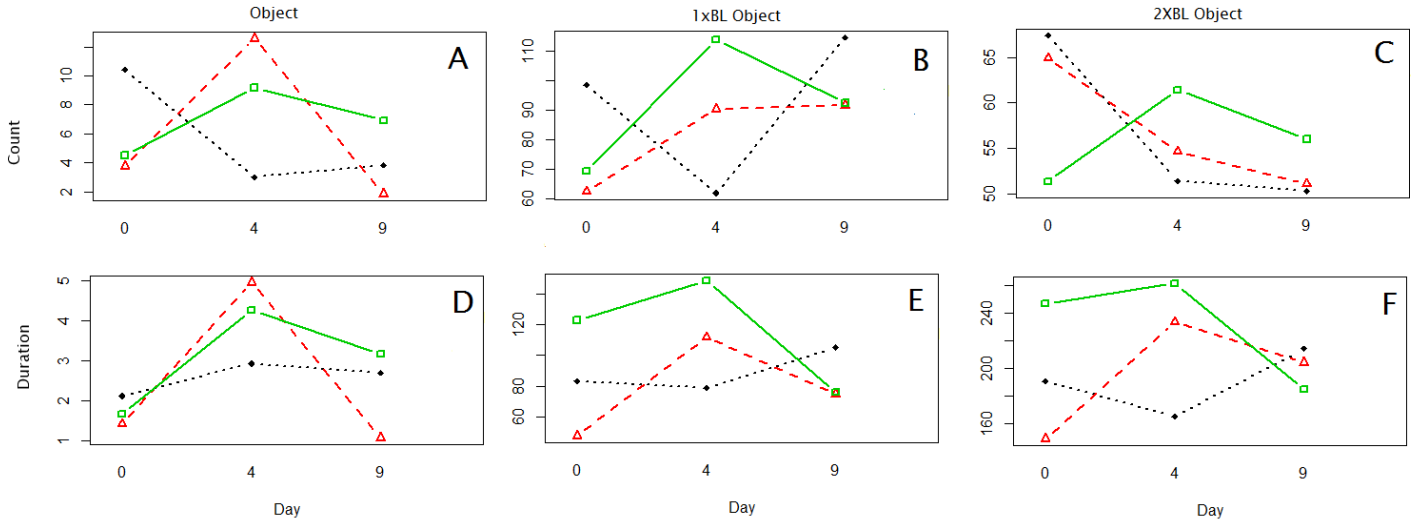

Figure 3: A: Average count that fish entered the zone closest to the object, B: average count that fish entered the zone one body length away from the object, C: average count that fish remained two body lengths away from the object, D: average duration that fish spend in the zone closest to the object, E: average duration that fish spend in the zone one body length away from the object, F: average duration that fish spend two body lengths away from the object. Control group is represented by full green line with square symbols. pH= 7.8 is represented by the dotted red line with triangles. The 7.4 treatment group is represented by the dotted black line with diamond symbols. X-axis represents the different treatment day on which the experiment took place.

object first increased for both the control and 7.8 treatment group. For the control group it led to an overall increase of the count, whereas the number dropped for the 7.8 group after the initial increase. The count for the 7.4 treatment decreased over time. This is mirrored in the duration spent closest to the object, where the duration for the control and 7.8 treatment increases. However the duration for the 7.4 treatment increases as well. Duration within the zone closest to the object was lowest of all durations spend when compared to the other zones further from the object (Figure 3).

The number of times fish enter the zone one body length away from the object, increases for both the control and 7.8 treatment group. For the control group it increases highly from day 0 to the fourth day, after which it decreases again, though the final number of counts on day 9 remains higher than the initial count on day 0. The group of treatment pH =7.4 shows the opposite, where it decreases highly between day 0 and 4, but then increases for day 9. The last count here being slightly higher than the initial count at day 0. Duration within this zone first increased then decreased for the control group, dropping to the same value as the final value of the 7.8 treatment, which is lower than the initial duration on day 0. Similar the duration for the 7.8 treatment within this zone first increased then decreased again on the ninth day, though the final duration is higher than the initial. The 7.4 treatment duration shows a decrease first on the fourth day, after which its increases, leaving the final measurement of duration lower than the initial one (Figure 3).

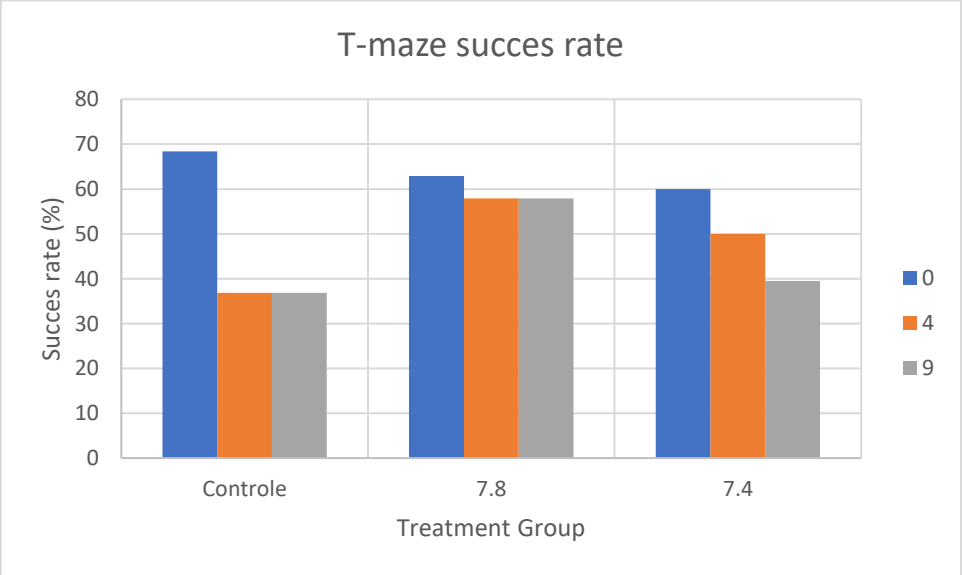

Figure 4: Success rate, expressed in percentage, of turning towards the food in the T-maze experiment for each treatment group. Different columns represent the different treatment days on which the experiment took place. There were no significant differences between groups or within groups when comparing treatment days.

Success rate (%) decreased for all groups over the period of the T-maze experiment (Figure 4). The control group start on Day 0 with the highest success rate of 68%, but decreased the most, dropping to 36% on the fourth and ninth day of the experiment, a success rate lower than those of either CO_2 treatment group. The fish of the 7.8 treatment group decreased from a success rate of 62% to 57% on day 4 and 9. The pH= 7.4 group showed a decrease each day, starting from 60% to 50% on the fourth day, to 39% on the ninth day. None of the decreases between success rate within groups were significantly different (all $p>0.05$), nor were there any significant differences to be found when comparing the different treatment groups.

Social behaviour

Table 3: p-values of average inter-individual distance (iid) and polarization for each treatment group compared over different treatment days, and compared between different treatment groups. Significant differences are represented with an asterix (*).

	Average iID	Average polarization
Control	5.79e-06 *	9.823e-09 *
7.8	0.1704	0.001932*
7.4	0.737	0.4433
Control -7.8	0.6849628	0.9351481
Control - 7.4	0.8048783	0.6226048
7.8 - 7.4	0.9853240	0.8215773

Fish paired together from the control group showed significant difference in average inter-individual difference (iid) and polarization, with p-values of 5.79E-06 and 9.823E-09 respectively (Table 3). The average iid decreased over the 21 day lasting experiment with over 70%. The same is true for the average polarization, which decreased with over 60% (Figure 5). Fish of the pH= 7.8 treatment only show significant difference in average polarization (p-value: 0.0019) but not in average iid. Both the average iid and polarization decrease as the experiment furthers. Average iid decrease slightly from day 0 to day 13, but then decreases dramatically towards day 21. Fish of the CO_2 treatment resulting in pH=7.4 show no significant difference in either average iid or polarization. Both of these variables first decrease on the third day and then increase again on the sixth day, after which they both decrease again to a value lower than the initial values measured on day 0. There are no significant differences for either variable when comparing the different treatment groups.

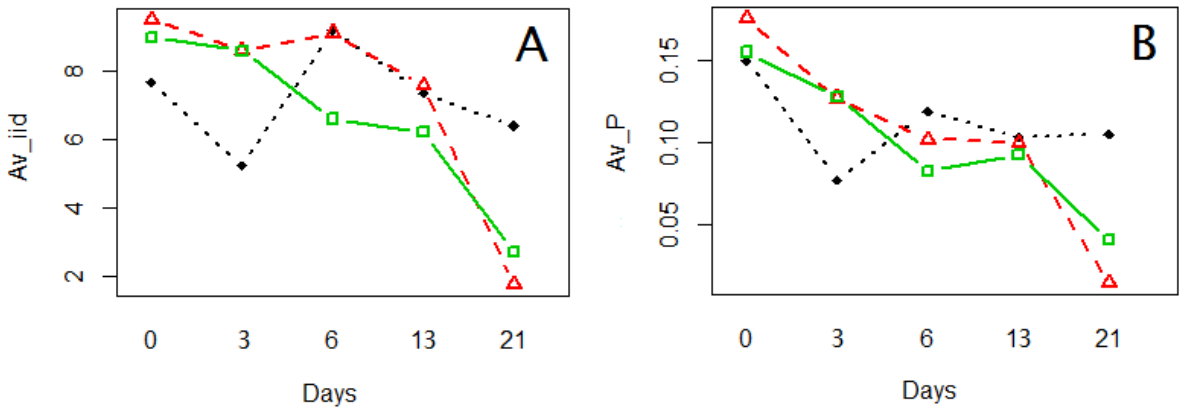

Figure 5: A: average inter-individual distance (iid) of fish within each treatment group. B: Average polarization (P) of fish within each treatment groups. X-axis represent the days of the social experiment. The control group is represented by the full green line with square symbols. The 7.8 pH treatment is represented by the dotted red line with triangles. The pH = 7.4 treatment is represented by the dotted black line with diamond symbols.

There was no significant difference in blood pH for fish in between groups when measured after the last social experiment. All ranged between pH= 7.02 and pH= 7.54. During the experiment four fish died in the control group on the 18th, 26th, 27th and 29th day of the total treatment. In the pH= 7.8 treatment 5 fish died on the 23rd, 31st and 33rd treatment day. Three fish more died in the pH = 7.4 treatment on the 10th, 25th and 31st treatment day.

Discussion

Individual behaviour

Results show that elevated CO_2 and the consequential reduced pH of the seawater that could occur in the following 100 years could alter several aspects of behaviour of false clownfish. In the control group, which remained in a pH of 8.0 to simulate current conditions of seawater, there were changes in inactivity duration and distance, showing that over time fish within this group became more active during the experiment. This rise in activity was also shown in the increasing small movement duration and distance. Eventually the activity in the control group peaks at the end of the experiment. Possibly fish in the control group got accustomed to the experiment, leading to less anxiousness followed by the increase seen in activity. Considering the novel object experiment, an increase in boldness is similarly seen for the control group. Overall they came closer to the object more often and for a longer duration as the experiment period advanced. This can be seen in the increasing count of times fish came closest to the object and one body length away from the object.

Fish from the 7.8 treatment show only a significant difference in the small movement distance, but not in duration, nor is there any significant difference in the average inactivity duration and distance, in fact these parameters remain stable throughout the experiment. Activity levels therefore remain consistent through the experiment period as well. Considering the control group had increasing levels of activity, it could be that the increasing CO_2 treatment impaired the ability of the fish in getting used to the experiment, which could be interpreted as a decrease in learning ability . Therefore, they lack the increased activity as in the control group and showed consistent levels of activity.

What needs to be considered however, are the large differences in values for day 0 when comparing all treatments. As day 0 was done before the CO_2 treatment started, this day can be considered as a control for each treatment. For instance the initial value for inactivity duration is 5 times lower for the 7.8 and 7.4 treatment when comparing with the control group. Therefore, comparing the values of the treatment groups with the control group should be treated with caution.

The 7.4 treatment led to an overall decrease in average inactivity duration and distance, as well as an overall decrease in small movement duration, though the average small movement distance increased. No significant differences could be found however, indicating that just as in the 7.8 treatment group the activity levels did not significantly change and remained consistent throughout the experiment period. The remark remains that the initial values of day 0 for individual behaviour are different when compared with the control group, though they are similar with those of the 7.8 treatment group. Mostly the pattern for individual behaviour of the 7.4 and 7.8 treatment group remain similar over time.

This consistency can also be found in the novel object experiment. Though the count that the fish came closest to the object does initially increase for the 7.8 treatment it then drops to a lower value than that of day 0 of the experiment, though there is no significant difference to be found. Therefore it cannot be stated that the fish from the pH= 7.8 treatment get more bold throughout the experiment as the control group did. This is also shown in the consistency of the duration spend closest to the object by the 7.8 treatment group. Though the count does not change significantly for the 7.4 treatment group, it does decrease with 80% if taking day 0 as 100%. Duration of the time spend closest to the object however remained consistent, with no significant difference, indicating that overall the boldness of the fish within the 7.4 treatment decreased.

Methods for individual behaviour were similar to those used by Regan *et al.* (2016) on fish native to a hypercapnic environment, where a routine behaviour trial lasted for five minutes, followed by a novel object trial to measure boldness for five minutes. Similarly, fish of the low-CO_2 were more active than those of high CO_2 treatments. Other research (Hamilton *et al.*,2013) found that anxiety behaviour increased in high CO_2 treated fish instead. Jutfelt *et al.* (2013) found that fish exposed to CO_2 examined a novel object, five times less long that the control group, indicating that the CO_2 exposed fish were less bold and/or less curious than the control fish, which echoes the results found within this experiment where overall the control fish spend more time more often closer to the object.

Considering the T-maze experiment all groups had a decrease in success of finding the food source. The largest decrease in success rate could be found in the control group and the smallest in the 7.8 treatment group, though no significant differences could be found in any of the groups. It therefore seems that none of the groups underwent any positive learning experience and in fact lost the ability the detect the food, though this effect seemed largest in the control group.

Research done on chemosensory are mostly done by experimenting on the reaction to predator olfactory cues (Munday *et al.*,2010; Briffa *et al.,*2012; Dixson *et al.*,2010; Nilsson *et al.*,2012) or to suitable habitat, or parental cues (Munday *et al.*, 2009). In all cases higher CO_2 concentrations and the resulting lower pH values lead to impairment of the fishes' ability to respond properly to cues. However within this experiment, results are inconclusive and mostly unreliable due to the large difference in the control group. Whilst previous research used flume chambers (Munday *et al.*,2010; Briffa *et al.,*2012; Dixson *et al.*,2010; Gerlach *et al.*, 2007) to keep water sources and their odour separate, it is possible that the use of a simple T-maze caused the odour of the food to become present all through the water, instead of the odour pinpointing of single source. To avoid this water was switched regularly, but even so it could have led to distorted results and given the wrong impression.

Social behaviour

This experiment was set up to measure possible coupling behaviour between two fishes of the same treatment group. Expected was that they would swim closer to each other, and move into the same direction more often as the experiment continued, thus leading to a decrease in iid and an increase in polarization. A decrease is indeed found for couples from the control and 7.8 treatment groups, with a significant difference found for the couples from the control group. This indicates that these fish kept closer to each other as the experiment period continued. For the fish from the control group this effect seemed to happen gradually over the 21-day period of the experiment, whereas the idd decreased slowly the first 13 days for the 7.8 treatment group and decreased tremendously thereafter. The fish from the 7.8 treatment thus spend a longer time swimming further away from each other when compared with the control group, but eventually ended up staying closer to each other than the control group on the 21th day. It therefore seems that the coupling behaviour for the 7.8 treatment group was initially tampered if assuming that the control group follows the normal pattern, but after the 13th day it overshot the control group and formed closer couples.

The 7.4 treatment group shows a pattern that decreases, rises and decreases again without a clear pattern. Thus, it seems that the higher concentration of CO_2 used in this treatment impaired the ability of the fish to form couples. Judging from these results it is clear that higher concentrations of CO_2 and the resulting decrease in seawater pH have an impact on the coupling behaviour of the fish.

The same patterns can be found for the polarization, where it decreases for both the control and 7.8 treatment groups, but decreases and rises for the 7.4 treatment group. The higher values for polarization in the beginning of the experiment show that the fish were more akin to turn and swim in the same directions at this time, than they were at the end of the experiment with lower values. This means that though the fish of the control and 7.8 treatment groups swam closer to each other at the end of the experiment, they also turned into different directions more often. The 7.4 treatment group once again shows no clear pattern, indicating that the higher CO_2 concentration has an influence on the social behaviour as was similarly shown with the pattern for average iid.

Once again results should be considered with caution, as previous results within this research might not be completely reliable. It is thus important to realize that as all experiments were done with the same fishes and same CO_2 set-up, these results might too show a distortion and could be unreliable. Additionally in a study done by Iwata *et al.* (2008) on *A. ocellaris*, sexual dimorphism within tissue wasn't found after 1 year and 2 months after hatching, as the fishes used within this experiment were younger than a year, it is possible that they were not ready to form couples yet, though in many sex-changing fishes, sex differentiation in the brain begins earlier than it can be seen in the tissues (Semsar and Godwin, 2003).

Blood acidosis

Similar to Regan *et al.* (2016) there was no significant difference to be found in the blood pH between fish exposed to high CO_2 and those to low CO_2. However high-CO_2 fish did have a higher P_{CO2} and [HCO_3^+] which could not be calculated within this experiment, due to the low volume of blood sampled from each fish. It should be noted however that Regan *et al.* (2016) used fish native to a hypercapnic environment, which further avoided the impairments in behaviour shown in other studies. Blood acidity was expected to change however as CO_2 diffuses across the fish gills and acidifies blood and other tissues (Ishimatsu *et al.*, 2005, Pörtner *et al.*, 2005). Reduced pH of seawater is also expected to acidify blood and tissue but is expected to act more slowly (Claiborne *et al.*, 2012). The reason that no differences are found in blood pH within this experiment, is most likely due to that fact blood samples were taken after the last social tests, meaning the fish had been removed from the CO_2- exposed water for several hours, which could have been long enough to regulate their internal pH by acid-base regulation (Nilsson *et al.,* 2012).

Mortality

Mortality of the fishes only started at the onset of social experiment, the first individual dying on the first day of the couple forming in the 7.4 treatment group. It could be argued that it took until the tenth day of the CO_2 treatment for lethal effects to take place, however considering that fish also died in the control group in the same period (and with one mortality more than the 7.4 treatment, and one less than the 7.8 treatment) this seems unlikely. In previous research (Ishimatsu *et al.*, 2008) it has also been stated that mortality only occurs at much higher CO_2 concentrations than used in this experiment, at levels that even exceed those predicted for acidification by the IPCC, at levels from 30 000 to 50 000 ppm CO_2.

More likely is that the stress of the repeated experiment lead to the eventual mortality. It also important to note that sometimes the bigger fishes in the couple, became very aggressive towards the smaller one, going as far as actively attacking them and causing biting wounds. Several of the

mortalities were noted to have missing pieces on their fins and tails most likely due to the aggressiveness of their larger companion. It is very possible that the stress of the experiment and of the aggression towards the smaller fish, are what caused the mortalities in the later days of the experiment period instead of the CO_2 treatment.

Conclusion

It can be concluded that high concentrations of CO_2 had an impact on the behaviour of *A. ocellaris*. Activity levels and boldness became less as CO_2 levels increased, with the control having the highest activity and being the boldest towards the novel object. Acidification however showed no clear signs of disrupting the olfactory mechanism when comparing different treatment groups, though upon considering day 0 a control day, success rate at locating the food decreased. As locating food is needed for survival, disruption to this would have significant consequences.

Further, results showed that ocean acidification had an effect on couple forming behaviour. Whereas the 7.8 treatment group follows the pattern of the control group, this is not so for the fish of the 7.4 treatment group with higher CO_2 concentrations, in fact fish from this group showed no significant distance in inter-individual distance or polarization which were used to indicate couple forming, showing that they were incapable of forming couples with the experiment period.

As mentioned before though, all results should be interpreted with caution, as large differences were found within controls which might lead to unreliability of the results. Most likely stress and aggressiveness had a large impact upon results, especially in the later part of the experimental period, which most likely also had the largest impact on the mortality rates found.

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Research proposal

Name of the applicant: Natacha Van Malder

Affiliation: University of Antwerp

Project title:

The effects of ocean acidification on a tropical, temperate and cold-water fish: a behavioural and genetical approach.

Layman's summary

Write a short summary explaining your research proposal in a way that is understandable to non-biologists.

Due to rising concentrations of carbon dioxide (CO_2) in the atmosphere, the concentration CO_2 within the ocean rises as well. Due to these higher concentrations, the acidity of the ocean's waters increases, which is called ocean acidification. One of the effects on fishes found due to these higher concentrations are differences in behaviour when comparing with fish from normal conditions. However not much is known about how fishes react over long-term exposure to such conditions, nor is there much known about fishes from different climate zones as most research until now has been done on tropical species. Further there hasn't been research yet towards the genetics and how certain genes might explain differences in behaviour. The aim of this study is to find answers to these questions.

Project outline

Describe your research proposal in detail. Start with sketching the state of the art in the field of concern. Explain which experiments or observations incited your research questions ('Background'). Then specify the aims and objectives of your study ('Aims and objectives'). Finally, describe the methods you intend to use to achieve your research goals.

Background

Atmospheric levels of carbon dioxide have increased by nearly 40%, over the last 250 years, from pre-industrial levels of approximately 280 ppm (parts per million) to nearly 384 ppm in 2007 (Solomon *et al*., 2007). Rising atmospheric CO_2 is tempered by oceanic uptake, approximately one-third of the anthropogenic CO_2 produced in the past 200 years has been taken up by the oceans (Sabine *et al*.,2004; Sabine and Feely, 2007). Ocean uptake of CO_2 causes changes in ocean water chemistry. Through the hydrolysis of CO_2, the hydrogen ion concentration $[H^+]$ increases, leading to pH reductions, which is why this problem is referred to as ocean acidification (Orr *et a*l., 2005; Doney *et al*., 2009). Ultimately ocean CO_2 values are predicted to reach 1000 ppm CO_2 by the year 2100 and 1900 ppm CO_2 by 2300 which would correspond with a pH decline of up to 0.77 by the year 2300 (Caldeira and Wickett, 2003; Meehl *et al*., 2007).

These changes in ocean CO_2 partial pressure and resulting changes in pH levels are predicted to severely impact marine organisms (Munday *et al.,* 2012a). Fish were initially believed to be safe from high CO_2 concentrations and its effects, as studies demonstrated that mortality only occurred at extremely high concentrations (higher than 10 000 µatm.) (Ishimatsu *et al*., 2008), levels which far exceed the predictions made by the IPCC (Houghton *et al*., 2001). However there is increasing evidence that suggest ocean acidification effects induce sublethal effects including alterations in otolith growth, alterations in olfaction and consequential detection of substrates, predators, prey and parents, differences in behavioural lateralization and impaired learning (Checkley *et a*l., 2009; Bignami *et al*., 2013; Nilsson *et al*., 2012; Jutfelt *et al*., 2013; Munday *et al*., 2009; Cripps *et al*., 2011; Dixson *et al*., 2010; Munday *et al*., 2010; Chivers *et a*l., 2014).

The diversity of these effects in both behaviour and sensory systems point to an effect on brain function and recognition of elevated CO_2. Nilsson *et al*. (2012) proposed these alterations in behaviour can be traced to the neurotransmitter gamma-aminobutyric acid type A or GABA-A. This is a major inhibitory neurotransmitter in the vertebrate brains, with high conductivity for Cl^- and HCO_3^-. In normal circumstances, the opening of GABA-A receptors leads to the hyperpolarization and inhibition of the neuron, due to an influx of Cl^- ions from the extracellular to the intracellular space (Nilsson *et al*., 2012; Hamilton *et al*., 2013). This was tested on fishes exposed to elevated CO_2 by treating them with gabazine, a GABA-A antagonist. This restored the functions impaired by the CO_2 treatment (Nilsson *et al*.,2012; Chivers *et al*., 2014).

All of this indicates that there is an involvement of the GABA-A receptor and that elevated CO_2 can affect critical sensory processes in marine fish. Still there is research that shows fish are capable of adapting to increases in environmental CO_2 and ocean acidification, though it could be that the present rate of increase of CO_2 is too high to allow for adaptation through natural selection (Regan *et al.*, 2016). There is also a certain tolerance of marine fishes' notable in several studies, where fishes showed individual variation in tolerance towards elevated CO_2 (Munday *et al*., 2010; Munday *et al*., 2012b).

It is not yet clear which specific processes and genes, if any, are involved. Nor is there any information on why effects of acidification might differ in between species, or even in between

individuals within the same species. As such it becomes clear that more research is needed to fully comprehend the possible effects of ocean acidification upon marine fishes.

Aims & objectives

This study would be separated into several objects, though all would be centred around ocean acidification and the effects this has on the behaviour of marine fishes.

The first objective would encompass the global study and would be to compare the difference in behaviour of fishes living in different environments. Until now most studies of behavioural effects on fishes had been done on tropical fish, often strongly associated with coral reef habitats. Only a couple studies have looked at temperate fish (Jutfelt *et al*., 2013; Lai *et al*., 2015). The idea would be to do the same tests on tropical, temperate and cold-water fish, to see how they differ in reaction to the elevated CO_2, if in fact differences can be found.

The second objective would be to see if the effects found would be long-lasting. Most studies done so far are focussed on short-term effects, as it has been proven effects of CO_2 treatment start four days after the CO_2 treatment starts (Nilsson *et al*., 2012). The longest studies done so far range between a month to forty days (Lai *et al*., 2015). Interesting would be to see if fish would adapt to the higher CO_2 concentrations and possibly if the behavioural impairments would lessen or cease.

As there has been no research done on the genetics that could influence the change in behavioural differences found in certain individuals, it would be interesting to perform a DNA extraction and see which genes could be linked to behavioural impairments.

Methods

To stimulate ocean acidification the concentration of treatment water will be elevated to 1000ppm CO_2, which corresponds with a pH around 7.6. This is the value predicted to occur by 2100. A second treatment would be of a pH = 7.3 expected to be reached by 2300.

Fish from these treatments and a control group would be put to identical experiments on behavioural lateralization and these will be carried out in the same environmental conditions as during the exposure. Fish will be individually introduced into a double T-chamber (according to Domenici *et al.,*2012 and Jutfelt *et al.,*2013) and encouraged to move until a choice towards the left

or right is made, which will be recorded by visual observation. This will be repeated five times for each fish randomly netted from the treatment or control tank. As the effects of elevated CO_2 have been proven to start after a four-day exposure (Nilsson *et al*., 2012), individuals will be closely followed for those first days, the experiment being done on day 0, day 2 and day 4 after which the experiment done will lessen to twice a week for a total of three months. Relative and absolute lateralization indexes will be calculated according to Domenici *et al.* (2012).

This will then be repeated for the tropical, temperate and cold-water species, so that these results can eventually be compared.

DNA extraction:

After euthanization with MSC222 and sampling of blood with a heparinized syringe, the brain will be excised and frozen in liquid nitrogen. Blood pH, P_{CO2} and HCO_3^- will be measured. Brain intracellular pH will be measured using the methods of Pörtner *et al.* (1990), where tissue is ground to a fine powder under liquid nitrogen. Approximately 0.1g of the powder will be transferred to a 1.5ml centrifuge tube with 0.8ml of metabolic inhibitor comprising 150mmol^{-1} potassium fluoride and 6mmol^{-1} nitrilotriacetic sodium for 45 seconds and the resulting supernatant represents the cytosol of the tissue sample. pH measurements will be made on this supernatant (Regan *et al.*, 2016).

Expected output

Explain if you think that the study will lead to scientific publications, indicate in which journals you hope to publish. If you think your study will lead to practical applications or will help achieve biological conservation objectives, please indicate which and how. Also point out how you intend to dissipate your results to the general public

Hopefully this study will provide insight in the complications of ocean acidification and thus climate change on life within the oceans. While it is already known that ocean acidification will have its effects upon calcifying organisms, the effects upon fishes are lesser known but therefore not less interesting. Fishes do not only provide as an important food source for humans, but are also important key stones within the ecosystems and losing them could lead to changes or even collapsing of ecosystems. This study could provide insight in how fishes react to long-term effects as would occur in nature and show how severe this possibly could be. It would provide knowledge towards where and why fishes (or more in general the climate) would have to be protected, and what the consequences would be if steps are not taken. Insight into genetics could help show if any

genes provide an advantage or disadvantage towards ocean acidification and how populations will change based on this.

Time schedule

Work out a time plan for the proposed study, preferably on a monthly or quarterly basis. (max. 300 words)

Quartile 1	Literature research, set-up aquaria to host fish
Quartile 2	acclimatization fishes, further experimental set-up, start of experiment with first species
Quartile 3	completion experiment with first species, start of dissections
Quartile 4	preparations of set-up for second species, acclimatization of fishes, start of tissue analysing and DNA extractions
Quartile 5	Start and completion of experiment second species, further DNA extractions of species 1
Quartile 6	preparations of set-up for third species, acclimatization of fishes, start of dissections of species 2, continuing DNA extractions
Quartile 7	Start and completion of experiment with third species, further DNA extractions of species 1 and 2
Quartile 8	cleaning up set-up, start dissections of species, continuing DNA extractions
Quartile 9	finishing DNA extractions
Quartile 10	reserved time for redoing or behindhand experiments, start of data management
Quartile 11 & 12	Data management and processing from individual behaviour tests and DNA extractions. Studying species seperately and comparing them
Quartile 13 & 14	Discussion of results, writing of papers
Quartile 15 & 16	publishing of papers, sending in for assessments, peer reviews

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Provide a list of the books, papers and websites you have cited throughout the proposal.

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Ocean acidification and its effect on behaviour of marine fish: literature review

By Natacha Van Malder

Introduction

Atmospheric levels of carbon dioxide have increased by nearly 40%, over the last 250 years, from pre-industrial levels of approximately 280 ppm (parts per million) to nearly 384 ppm in 2007 (Solomon *et al*., 2007). The present CO_2 concentration is rising at a rate of around 0.5% per year, due to human activity (Fabry *et al.*, 2008). This rate of increase is faster than has occurred for millions of years (Doney *et al.*, 2009).

This rising concentration of atmospheric CO_2 is causing global warming and ocean acidification (Fabry *et al.,*2008). Rising atmospheric CO_2 is tempered by ocean uptake which acts as a sink and account for nearly a third of the anthropogenic CO_2 added to the atmosphere. Without this ocean sink, the change in atmospheric CO_2 concentration would be 55% higher than the observed change (Sabine *et al.*, 2004). Elevated atmospheric CO_2 leads to an increase in CO_2 absorption by the oceans, causing changes in seawater carbonate chemistry (Briffa *et al.*, 2012).

Hydrolysis of CO_2 in seawater increases the hydrogen ion concentration [H^+] (Orr *et al.,*2005). This causes pH reductions and is therefore referred to as ocean acidification (Fabry *et al.*, 2008; Briffa *et al.*, 2012). In addition to this, aqueous concentration of CO_2 will increase and carbonate ion concentration (CO_3^{2-}) will decrease, making it more difficult for calcifying organisms to form calcium carbonate for their skeletons (Orr *et al.*, 2005).

In detail the ocean acidification process is as follows: normally dissolved inorganic carbon (DIC) appears within seawater in three forms: bicarbonate ion (HCO_3^-), aqueous carbon dioxide ($CO_{2\,(aq)}$) and carbonate ion (CO_3^{2-}). When CO_2 dissolves in water, H_2CO_3 is formed. Most of this then dissociates into a hydrogen ion (H^+) and HCO_3^-. The hydrogen ion can then react with CO_3^{2-} to form bicarbonate. Therefore adding CO_2 to the ocean increases concentration of H_2CO_3, HCO_3^- and H^+, whilst it decreases the concentration of CO_3^{2-} and lowers pH (Fabry *et al.*, 2008).

CO_2 emissions could exceed 900 ppm by the year 2100, according to predictions of the Intergovernmental Panel on Climate Change (IPCC), which could cause a reduction of ocean pH by 0.3-0.4 units (Royal Society, 2005). This projected decrease of 0.3-0.4 pH compares to an 150% increase in hydrogen ions and a 50% decrease of carbonate ion concentrations (Orr *et al.*, 2005).

Such changes in seawater chemistry are a form of anthropogenic pollution of marine environments. This pollution has the potential to affect animal behaviour (Briffa *et al.*, 2012). Hypercapnia, the elevated partial pressure of CO_2 (pCO_2) in seawater, leads to more readily diffusion of dissolved CO_2 across animal surfaces and equilibrates in

both intra- and extracellular spaces. As in sea water CO_2 will react with internal fluids causing hydrogen ions to increase and thus the pH to decrease. Mechanisms to deal with this acidification are limited and include the following: passive buffering of intra- and extracellular fluids; the transport and exchange of relevant ions: the transport of CO_2 within the blood of those species with respiratory pigments and metabolic suppression to wait out periods of elevated CO_2 (Fabry *et al.*, 2008).

Fish can partially reduce the acidosis of increased CO_2, through the Cl^-/HCO_3^- exchanger in the gill epithelium. This leads to decreased Cl^- concentration and increased HCO_3^- concentration in the extracellular fluid (Jutfelt *et al.*, 2013). Still very high levels of elevated CO_2 can kill fish, though these levels exceed those predicted for acidification by the IPCC, at levels from 30 000 to 50 000 ppm CO_2. Mortality reasons might be cardiac failure or nephrocalcinosis, where calcareous precipitates build up and then obstruct the kidney tubules (Lee *et al*., 2003; *Hayashi et al., 2004*; Foss *et al.,* 2003). Further there is the chance of additional energy expenditure for osmoregulation when CO_2 levels rise. Because fish actively expel Cl^-, additional reductions in plasma Cl^- would require for fish to use up additional energy to compensate these losses (Ishimatsu *et al.*, 2008).

Effects on behaviour

There are three ways in which elevated CO_2 might influence the behaviour of fishes. First through the disruption of proximate causal mechanisms, such as metabolic processes, which determine the rates at which behaviour can be performed. Second is the disruption of the ability to gather information and correctly asses this information to make decisions. The third way is that animals could be capable of detecting and avoiding polluted locations, which would alter their normal movement patterns and their distribution (Briffa *et al.*, 2012).

Studies have revealed several behavioural disturbances, such as the disturbance of olfactory senses. A study (Devine *et al.*, 2012) on adult five-lined cardinalfish, *Cheilodipterus quinquelineatus*, exposed the fish to levels of 550, 700 and 950 ppm for four days. All showed an impaired ability to distinguish between odours from home- versus foreign-sites, which reduced the homing success up to 31% when released at 200m from home sites. Fish from the 700 and 950 ppm treatment also appeared to be less cautious, spending more time away from their shelter. The same bold behaviour was found in *Pomacentrus wardi*, the Ward's Damsel Fish. Half of the individuals treated at 700 ppm were more active and move a greater distance away from their shelter (Munday *et al.*, 2012a). Disruption to the settlement and homing process could cause changes in the patterns of dispersal and connectivity in these populations. It might significantly impact the ability of species to evolve in rapidly changing environments. These alterations might suggest impairment of the general cognitive function (Munday *et al.*, 2009*a*; Devine *et al.*, 2012).

Impairment of olfactory discrimination was also found in orange clownfish larvae (*Amphiprion percula*) reared in seawater at 7.8 pH (estimated at 1,000 ppm). They could no longer discriminate between cues that might be useful for settling and those

leading to unsuitable settlement sites. In fact they were attracted to olfactory cues that normally were avoided (Munday *et al.*, 2009*a*). Larvae of *A. percula* further use olfactory cues to distinguish between predatory and non-predatory species. After their rearing in seawater of 7.8 pH, the larvae became attracted to the smell of predators. As the ability to detect predators is an important mechanism of survival, such a change in behaviour could lead to increasing mortality, which could lead to a decrease in population (Dixson *et al.*, 2010).

Effects were found not only in prey fish, but in predator species as well. Cripps *et al.* (2011) tested the ability of *Pseudochromis fuscus,* the Brown Dottyback*,* to detect chemical cues produced by its prey when exposed to elevated CO_2. It was shown that after CO_2 treatment the predators spend 20% less time in a water stream with the smell of an injured prey than the control group. This predator of other reef fish also showed lower capture success when exposed to 880 ppm CO_2, though a species of its prey, *Pomacentrus amboinensis*, was found to have shorted escape distances and longer reaction distances, as well as impaired reaction to chemical and visual alarm cues when encountered with a predator (Allan *et al.*, 2013; Lönnstedt *et al.*, 2013). Further research on this predator and four of its prey species found that predation rates were higher in circumstances of elevated CO_2, though the predators lost its prey species preference and started consuming all four species of prey equally, where previously it had preferred only two species. So how the dynamics of predator-prey interactions will be affected depends on how the interacting species are affected by the elevated CO_2 conditions (Allan *et al.*, 2013; Ferrari *et al.*, 2011).

Other disruptions of the cognitive function are shown by Ferrari *et al* (2012) who tested the visual risk assessment in juvenile damselfish, *Pomacentrus amboinensis*. They revealed that *P. amboinensis* showed a weaker anti-predator response at the sight of an adult spiny chromis (*Acanthochromis polyacanthus*) when exposed to 850ppm CO_2. They also displayed higher activity levels and foraging rate, than fish exposed to lower concentrations.

Many fishes also rely on hearing for orientation, habitat selection, predator avoidance and communication. Simpson et al (2011) tested this on *A. percula* in CO_2 conditions of 390 ppm and enriched situations leading up to 900 ppm, with an auditory choice chamber. Juveniles from the 390 ppm conditions avoided predator-rich noise, whilst juveniles with higher concentration treatments did not. It has been suggested that in enriched CO_2 conditions, the growth of otoliths (ear bones) increases in the white sea bass, *Atractoscion nobilis* (Checkley Jr. *et al.*, 2009). This wasn't found in *A. percula* however, CO_2 enriched treatments did not affect the otolith growth in any way, which means auditory disturbances must be affected in another matter (Simpson *et al.*, 2011; Munday *et al.*, 2011).

It begs the question if when one sensory system is impaired that other senses might compensate at a certain concentration of CO_2. However with a chemosensory loss as soon as 550ppm (Devine *et al.*, 2012), impaired auditory responses at 600 ppm (Simpson *et al.*, 2011) and visual risk assessment impaired at 850ppm (Ferrari *et al*, 2012), it seems that one sense cannot fully compensate for the loss of another.

Beyond sensory performance and anti-predator responses there have also been changes shown in behavioural lateralization (Domenici *et a*l., 2014; Jutfelt *et a*l., 2013). Behavioural lateralization is the tendency to favour the left or right side during behavioural activities, which is an expression of brain functional asymmetries (Domenici *et al.*, 2012). Disruption in the lateralization after enriched CO_2 treatments could be evidence to elevated CO_2 causes brain dysfunction in fishes.

In *P. wardi* it was shown that elevated CO_2 at 935 ppm reversed the lateralization bias from right to left (Domenici *et al.*, 2014). The three-spined stickleback, *Gasterosteus aculeatus*, no longer showed any preference for right or left after a twenty-day treatment of 991 ppm CO_2. This effect remained after a forty-day treatment of elevated CO_2 as well. *G. aculeatus* showed no signs of acclimatization (Jutfelt *et a*l., 2013). The same loss of preference was shown in the yellowtail demoiselle, *Neopomacentrus azyrson*, after a four-day treatment of 900 ppm CO_2 (Nilsson *et al.*, 2012).

These impairments of sensory functions, be they olfactory (Briffa *et al.*, 2012; Munday *et al*, 2014; Devine *et al.*, 2012; Dixson *et al.*, 2012; Cripps *et al.*, 2011;Munday *et al.*, 2009*a*) , visual (Ferrari *et al.*, 2012) or auditory (Simpson *et al.*, 2011; Checkley Jr. *et al.*, 2009) as well as changes in activity levels (Munday *et al.*, 2012a), increased anxiety (Hamilton *et al.*, 2014) and the effects on behavioural lateralization (Domencini *et al.*, 2011; Domencini *et al.*, 2014; Jutfelt *et al.*,2013; Nilsson *et al.*, 2012) indicate that elevated CO_2 has an effect on the nervous system function.

Nilsson *et al*. (2012) proposed these alterations in behaviour can be traced to the neurotransmitter gamma-aminobutyric acid type A or GABA-A. This is a major inhibitory neurotransmitter in the vertebrate brains, with high conductivity for Cl^- and HCO_3^-. In normal circumstances the opening of GABA-A receptors leads to the hyperpolarization and inhibition of the neuron, due to an influx of Cl^- ions from the extracellular to the intracellular space (Nilsson *et al.*, 2012; Hamilton *et al.*, 2013).

When exposed to high CO_2-level conditions, fish regulate acid-base ions, primarily bicarbonate and chloride to maintain blood and tissue pH and avoid acidosis. They accumulate HCO_3^- with compensatory reduction in Cl^-, with the primary site of this ion exchange being the gills in juvenile and adult fish. The resulting altered concentrations of Cl^- and HCO_3^- between cells and plasma are thought to turn the GABA-A receptors from hyperpolarizing and inhibitory to depolarizing and excitatory because of an outflow of these anions (Nilsson *et al.*, 2012; Hamilton *et al.*, 2013; Chivers *et al.*, 2014; Lai *et al.*, 2015; Chung *et al*., 2014; Regan *et al*., 2016; Munday *et al.*, 2015).

Evidence for this comes from the observation that gabazine, a specific GABA-A receptor antagonist, could reverse the impairments and behavioural changes. In *N. azysron* it was shown that gabazine treatment reversed the disruption in individual lateralization, which had occurred after a four-day exposure to 900ppm CO_2 (Nilsson *et al.*, 2012). The same results were found in *Gasterosteus aculeatus*, a temperate fish, where lateralization of fish kept in elevated CO_2 of 992ppm for forty days, was completely restored (Lai *et al.*, 2015). In *A. percula* reared in 900 ppm CO_2, olfactory

responses to predator odours were restored by gabazine treatment. as well as reversing the negative effect of CO_2 on the learning ability to recognize predators of juvenile damselfishes (*Pomacentrus amboinensis*) and on the retinal function in the spiny damselfish, *Acanthochromis polyacanthus* (Nilsson *et al.*, 2012; Chivers *et al.*, 2014; Chung *et al.*, 2014). Research on *Pangasianodon hypophthalmus*, a fish native to hypercapnic waters, found the same behavioural changes when these fish were exposed to normocapnia levels, as when reef fish are exposed to high-CO_2 environments. Gabazine treatment reduced these abnormalities in their behaviour (Regan *et al.*, 2016). Gabazine however caused no effect on juvenile Californian rockfish (*Sebastes diploproa*) after being exposed to 1125 ppm CO_2 for one week. Instead the GABA-A receptor antagonist muscimol, an anxiolytic which should decrease anxiety, caused a significant increase in anxiety (Hamilton *et al.*, 2013).

All of this indicates that there is an involvement of the GABA-A receptor and that elevated CO_2 can affect critical sensory processes in marine fish. Still as the research on *P. hypophthalmus* suggests, and assuming that this fish or its ancestors were at one point native to normocapnic environments, fish are capable of adapting to increases in environmental CO_2 and ocean acidification, though it could be that the present rate of increase of CO_2 is too high to allow for adaptation through natural selection (Regan *et al.*, 2016).

There is also a certain tolerance of marine fishes' notable in several studies. In juvenile damselfish *P. amboinensis*, there was no effect of CO_2 on sensory assessments if the CO_2 concentration was below 850 ppm. Treatments of 550 ppm and 700 ppm gained no results regarding behavioural impairments (Ferrari *et al.*, 2012). *Pomacentrus wardi* showed individual variation in tolerance towards elevated CO_2 at 700 ppm. About half of the treated larvae from *P. wardi* remained unaffected after the elevated CO_2 treatment. In fact there were no behavioural differences between this half and the control group. If such variation in individual tolerance is heritable, it could lead to a selection of CO_2 tolerant phenotypes in nature, capable of resisting the effects of ocean acidification (Munday *et al.*, 2012a). The same individual tolerance was found in larvae of *A. percula* reared in 700 ppm CO_2, with half of the individuals being unaffected by the elevated CO_2 treatment (Munday e*t al.*, 2010). If this individual variation is based in genetic variation, then a rapid selection of tolerant genotypes might follow, though not much is known if they will then be capable of coping with higher CO_2 levels than 700 ppm (Munday *et al.*, 2012b).

Munday and colleagues (Munday *et al*., 2015) researched effects of elevated CO_2 levels on larval yellowtail kingfish, *Seriola lalandi*, and found no growth or survival changes up to 1700 ppm CO_2. However, they did find a decline in oil globule size, indicating an energetic cost. Yet still this could indicate a tolerance of the yellowtail kingfish to elevated CO_2. As there were no behavioural changes found, it was proposed that these impairments only occur when the acid-base regulation begins during development of the gills. Research done on fish species at CO_2 seeps, showed that whilst the elevated CO_2 did lead to behavioural impairments such as in reef fish, there was no effect on metabolic rate or any difference in resting or maximum oxygen consumption between the fish from control reefs and fish from CO_2 seeps (Munday *et al*, 2014). In juvenile Atlantic cod, *Gadus morhua*, there were no

behavioural impairments as typical for reef fishes after a treatment of elevated CO_2 (1014 ppm CO_2) for 30 days. Still Atlantic cod is known to forage in deep hypercapnic waters and may therefore be tolerant to elevated CO_2 levels. This suggests that behavioural effects could be negligible in some species and that those species may prove to be robust towards ocean acidification (Jutfelt and Hedgärde, 2015).

Currently most research done on the behavioural effects of ocean acidification on fish, consists out of short term lab work, with the longest term only up to around forty days of elevated CO_2 treatment (Jutfelt *et al*, 2013), as such there is not much known about possible acclimatization of fish to elevated levels, or how this happens in nature where many other factors influence possible behavioural changes.

Because the behavioural impairments are manifested after only several days of exposure to elevated CO_2 levels and remain for several days even when returned to normocapnia level waters, it is likely that ion-regulatory changes and changes in the GABA-A receptor function involve complex long-term effects, such as altered gene expression about which little is known (Munday *et al*, 2012a; Lai *et al.*, 2015).

To conclude, rising atmospheric CO_2 conditions and the following ocean acidification, lead to several behavioural impairments, such as changes in behavioural lateralization, as well as changes in hearing, vision and olfactory senses. The neurotransmitter GABA, can lead to similar changes as observed in enriched CO_2 conditions, whilst GABA receptor antagonists can lead to a reduction of these changes indicating that GABA is most likely involved in the behaviour alterations. It is not yet clear which specific processes and genes, if any, are involved. Nor is there any information on why effects of acidification might differ in between species, or even in between individuals within the same species. As such it becomes clear that more research is needed to fully comprehend the possible effects of ocean acidification upon marine fishes.

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