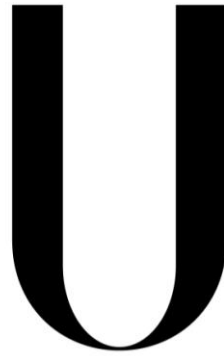


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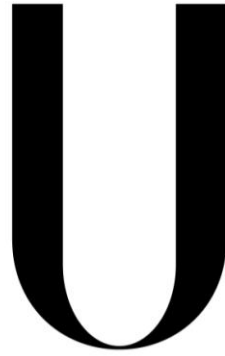
**Acidification effects in the behavioural responses
of temperate reef fish larvae**

Joana Maria Pereira de Paula e Castro

Dissertação
Mestrado em Ecologia Marinha

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Orientadores:
Prof. Doutor Henrique Cabral (Faculdade de Ciências da Universidade de
Lisboa) e Doutora Ana Faria (Instituto Superior de Psicologia Aplicada)

2014

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Resumo

Desde o século passado que, devido essencialmente à queima de combustíveis fósseis, agricultura e desflorestação, as concentrações de CO₂ têm vindo a aumentar significativamente na atmosfera terrestre, levando a sérios impactes ambientais. Os níveis de CO₂ atmosférico aumentaram de cerca de 280 ppM (partes por milhão) para cerca de 390 ppM em 2010 e prevê-se que continuem a subir, podendo atingir valores acima dos 1000 ppM em 2100. Como a atmosfera e os oceanos funcionam como sistemas interligados, as alterações que ocorrem num vão ter consequências no outro. Assim, com o aumento do CO₂ na atmosfera, a pressão parcial do CO₂ ($p\text{CO}_2$) da água do mar também aumenta, o que, por sua vez, conduz a uma alteração química do carbonato, resultando na acidificação da água (redução do pH).

Atualmente, o pH médio das águas dos oceanos é cerca de 8,2, uma décima abaixo dos valores pré-industriais. Tendo como base os cenários realistas para as futuras emissões de CO₂, prevê-se que o pH possa diminuir ainda mais 0,3 - 0,4 unidades até 2100.

Estas variações vão ter graves consequências ecológicas no planeta Terra, podendo causar uma perda global de biodiversidade, distúrbios nos processos dos ecossistemas e redução de bens e serviços ecológicos úteis ao Homem. Nos organismos marinhos, as consequências poderão ser drásticas, como por exemplo, diminuição das taxas de calcificação, alterações de processos fisiológicos e, também, variações no comportamento de alguns peixes. Uma vez que as fases iniciais de vida são mais vulneráveis às mudanças do ambiente, o mais provável é que os efeitos da acidificação sejam detetados mais facilmente nestes estágios do ciclo de vida. É portanto, fundamental compreender as consequências de um meio mais ácido em larvas e juvenis, que, por sua vez, se podem refletir nas populações adultas.

A maioria dos peixes de recife são caracterizados por possuírem um ciclo de vida com duas fases: uma na qual o estágio larvar pelágico se desloca para águas abertas, longe do recife, e outra envolvendo o recrutamento para águas costeiras para assentar, vivendo aqui o resto da sua vida adulta. Outrora, pensava-se que a dispersão larvar era passiva e a localização do sítio de assentamento determinada totalmente pelas

correntes oceânicas. Hoje sabe-se que as larvas exibem natação ativa e orientada, podendo, deste modo, controlar a sua dispersão e assentamento.

Para se orientarem no meio pelágico, as larvas, recorrem essencialmente a pistas olfativas e auditivas. Para além da orientação, os odores, são bastante úteis na deteção e evasão a predadores. No que diz respeito aos sons dos recifes, estes podem ser tanto de origem abiótica (movimento da água, rebentamento das ondas, chuva na superfície da água e eventos geológicos) como biótica (vários organismos como peixes e invertebrados), funcionando como uma pista bastante complexa. Estes sons podem variar com a hora do dia, provocando a deslocação larvar em direção aos recifes durante a noite, com o objectivo de assentamento. Durante o dia, as larvas afastam-se dos recifes, de modo a evitarem predadores associados a estes e para se alimentarem.

Estudos recentes, onde foram utilizados peixes de recifes de coral, já demonstraram que o aumento do CO₂ e a consequente diminuição do pH modificam negativamente o comportamento e as respostas sensoriais das fases iniciais de vida destes peixes, alterando as suas respostas a pistas olfativas, auditivas e até mesmo visuais. No entanto, pouco se sabe sobre a resposta de larvas de peixes temperados à acidificação do Oceano prevista para as próximas décadas.

Assim, os objetivos deste estudo centraram-se na verificação das influências que os baixos níveis de pH podem ter no comportamento e nos sistemas sensoriais envolvidos na sobrevivência das populações larvares de peixes de recifes rochosos temperados. Mais especificamente, verificou-se se a acidificação afeta a resposta das larvas a pistas olfativas pertencentes a predadores prováveis de serem evitados aquando da procura de locais de assentamento e, também, a pistas auditivas utilizadas, possivelmente, na procura desses mesmos locais. Para esse efeito, larvas selvagens de peixe-rei (*Atherina presbyter*) e de caboz-da-areia (*Pomatoschistus pictus*) foram capturadas e expostas a dois tratamentos de pH, ~ 8,10 (tratamento controlo) e ~ 7,6 (tratamento acidificado). O tratamento controlo foi estabelecido através da difusão do ar envolvente na água dos aquários e o tratamento acidificado simulado através do bombeamento de CO₂ na água, com o auxílio de um sistema de controladores de pH eletrónicos que mantiveram o pH ao nível desejado.

Posteriormente, as larvas de ambos os tratamentos, foram submetidas a experiências onde se utilizaram odores de potenciais predadores (experiência de odores) e sons diurnos dos recifes pertencentes à mesma localização de onde as larvas foram capturadas (experiência de sons).

Para a realização das experiências de odores, foi utilizada uma câmara com dois canais de escolha que recebia água de duas fontes diferentes, tratadas com pistas olfativas diferentes. Aqui, as larvas eram capazes de nadar livremente e escolher qualquer um dos lados da câmara. No início de cada ensaio, uma larva foi colocada no centro da extremidade a jusante da câmara e, depois de 2 minutos de aclimatização, a posição da larva na câmara foi registada a cada 5 segundos, durante 2 minutos. O peixe-rei foi testado com pistas olfativas de robalo (*Dicentrarchus labrax*) e o caboz-da-areia com pistas olfativas do blenideo *Lipophrys trigloides*.

Nas experiências de sons, utilizou-se uma câmara de escolha auditiva, a qual consiste numa câmara tubular de acrílico inserida dentro de um aquário de vidro acusticamente isolado. Numa das extremidades da câmara havia uma coluna submersa que emitia os sons do recife e que foi trocada para o lado oposto, a meio de cada série de ensaios. No início de cada ensaio, uma larva foi libertada no centro da câmara e, após 1 minuto de aclimatização, a sua posição foi registada.

As larvas de peixe-rei e de caboz-da-areia responderam de modo diferente aos testes olfativos. Ao contrario do que se esperava, as larvas de peixe-rei criadas em condições controlo, não evitaram a pista odorífera do predador (robalo). Tal comportamento pode ter tido três causas diferentes: (1) as larvas de peixe-rei poderiam ainda ser *naïve* a qualquer pista produzida pelo robalo adulto, devido à ausência de contacto com este; (2) é possível que as larvas de peixe-rei sejam demasiado pequenas para pertencerem à alimentação do robalo adulto, não representando este, portanto, uma grande ameaça como predador; (3) a água do robalo veio de um aquário onde a água circula por uma sistema de tanques com outras espécies, podendo, assim, o odor do robalo estar diluído noutros odores. Quando as larvas de peixe-rei criadas em condições baixas de pH foram testadas, a resposta sensorial olfativa ao predador manteve-se semelhante.

Relativamente às larvas de caboz-da-areia, quando se testaram as que foram criadas em pH controlo, também não se verificou que tivessem evitado a pista olfativa do predador (*L. trigloides*). Esta aparente ausência de resposta está, provavelmente, relacionada com o facto do *L. trigloides* não se alimentar de caboz-da-areia. Em contrapartida, a resposta comportamental, perante a pista odorífera do predador, das larvas criadas em condições acidificadas de pH, mudou significativamente, evitando, estas, fortemente o odor do *L. trigloides* (81% do tempo). Este comportamento pode representar uma deficiência sensorial, estando as larvas a responder a um odor ao qual não deveriam reagir, porque o *L. trigloides* não representa um perigo para o caboz-da-areia. Isto sugere que os baixos níveis de pH podem causar danos no mecanismo olfativo larvar, resultando num erro onde ocorre uma confusão na deteção de pistas olfativas importantes para a sobrevivência de larvas de algumas espécies de recife.

Como foi observado nos testes olfativos, as duas espécies-alvo responderam de maneira diferente aos sons de recife emitidos. Contrariamente ao que se esperava, as larvas de peixe-rei criadas tanto no tratamento controlo, como no acidificado, não responderam aos sons de recife. Possivelmente, as suas larvas não necessitam de responder aos sons dos recifes, isto porque o peixe-rei tem um ciclo de vida particular, em que o adulto desova em águas costeiras e rasas e a larva eclode muito bem desenvolvida, permanecendo no mesmo sítio, em cardumes, para depois se afastar da costa, em adulto. Adicionalmente, esta é uma espécie de vida totalmente pelágica. Assim, o peixe-rei, não necessita de localizar um recife para se estabelecer. Outra possibilidade pode estar relacionada com o facto dos sons utilizados neste estudo estarem fora da sensibilidade auditiva deste peixe, o que não foi possível verificar devido à carência do aparelho apropriado para a realização do método *Auditory Brainstem Response (ABR)*, uma técnica eletrofisiológica que permite definir limites de audição.

As larvas de caboz-da-areia criadas em condições controlo evitaram fortemente os sons diurnos dos recifes. Tais resultados estão de acordo com estudos anteriormente realizados, onde foi demonstrado que, normalmente, durante o dia as larvas afastam-se dos recifes a fim de evitarem os predadores do recife e se alimentarem e durante a

noite movem-se em direção ao recife para assentarem. Quando se testaram as larvas criadas em tratamento pH 7,6, o seu comportamento mudou significativamente. As larvas inverteram ou perderam as suas capacidades auditivas, não apresentando qualquer resposta aos sons. Estes resultados sugerem que níveis elevados de acidificação podem comprometer comportamentos auditivos fundamentais à sobrevivência destas larvas e que as respostas a pistas auditivas não irão compensar a potencial perda de outros sentidos, tais como o olfativo.

A hipótese de adaptação dos peixes a um ambiente acidificado tem sido pouco estudada mas, não deve ser posta de lado. No entanto, é pouco provável que muitas das espécies marinhas possuam variações genéticas adaptadas a estas rápidas mudanças. Provavelmente, só as espécies com tempos de geração muito rápidos vão ser capazes de acompanhar o ritmo da mudança e adaptar-se. É importante que, de futuro, se façam experiências de longa duração (com várias gerações), isto porque pode ser que ocorra aclimatização e/ou adaptação. Com peixes, é mais difícil realizar este tipo de experiências, mas pode-se, pelo menos, fazer com a descendência de progenitores criados em ambiente acidificado, como já tem vindo a ser feito por outros investigadores.

O presente estudo forneceu evidências de que acidificação do Oceano pode afetar significativamente as respostas sensoriais (olfativas e auditivas) de larvas de alguns peixes de recife temperado, causando impactes potencialmente negativos para a sobrevivência larvar e para reposição e sustentabilidade de populações.

Este foi o primeiro estudo que verificou os efeitos resultantes do aumento das concentrações de CO₂ nos oceanos no comportamento sensorial de peixes de recifes temperados, tendo sido um trabalho de grande importância nesta temática. Não obstante, é necessária mais investigação nesta área, tanto na realização do mesmo estudo em mais espécies (porque nem todas as espécies de larvas de peixes respondem da mesma forma às alterações do pH), como no aperfeiçoamento dos métodos experimentais, com o objetivo de compreender melhor as repercussões desta ameaça. Adicionalmente, uma vez que aumento global da temperatura irá atuar

simultaneamente com a acidificação do Oceano, seria também de grande interesse estudar os efeitos da interação entre estes dois fatores.

Palavras-chave: acidificação oceânica; respostas auditivas; respostas olfativas; comportamento sensorial; larvas de peixes de recife

Abstract

Due to anthropogenic activity, atmospheric CO₂ levels have been increasing since the last century. Consequently, the average levels of surface pH in the ocean is drastically dropping, affecting marine life, including changes in fish behaviour. In many coastal marine fish the selection of the adult habitat occurs in the pelagic larval phase, relying on hearing and olfaction for orientation as well as for predator avoidance and communication. In the present study, the effects of ocean acidification in the ability of fish larvae to detect olfactory cues from potential predators and auditory cues from adult habitats (reefs) were tested. Larvae of sand-smelt (*Atherina presbyter*) and painted goby (*Pomatoschistus pictus*) were reared in a control CO₂ treatment (pH~8.10) and in a high CO₂ treatment (pH~7.6). Later, fishes were subjected to odour experiments in a two channel choice flume and to sound experiments in an auditory choice chamber. Sand-smelt larvae reared in both control and acidified treatment did not show any response to olfactory cue and to reef sounds. Larval painted goby reared in high CO₂ treatment and exposed to olfactory cue, strongly avoided the cue, what did not occur in larvae reared in control CO₂ treatment. Regarding to sound tests, painted goby larvae from control treatment discriminated reef noises, as expected, however this behavior was absent in larvae reared in an acidified treatment. This study provided evidence that ocean acidification might affect the sensorial responses (olfactory and auditory) of larvae in some temperate reef fish, with potentially injurious impacts on their survival.

Key Words: ocean acidification; auditory response; olfactory response; sensory behaviour; reef fish larvae

1. Introduction

Over the last century, the concentrations of carbon dioxide (CO₂) and other greenhouse gases, resultant mainly from combustion of fossil fuels, agriculture and deforestation, have been significantly increasing in Earth's atmosphere, leading to serious environmental impacts (Raven *et al.*, 2005; IPCC, 2007). Levels of atmospheric CO₂ have steadily raised since pre-industrial times (about 265 years ago), from about 280 ppm (parts per million) to nearly 390 ppm in 2010 (Solomon *et al.*, 2007; Tans & Keeling, 2014) (figure 1). It is expected that these levels will continue increasing at least until the next century, and unless emissions are substantially reduced, it can reach levels above 1000 ppm in 2100 (Raven *et al.*, 2005).

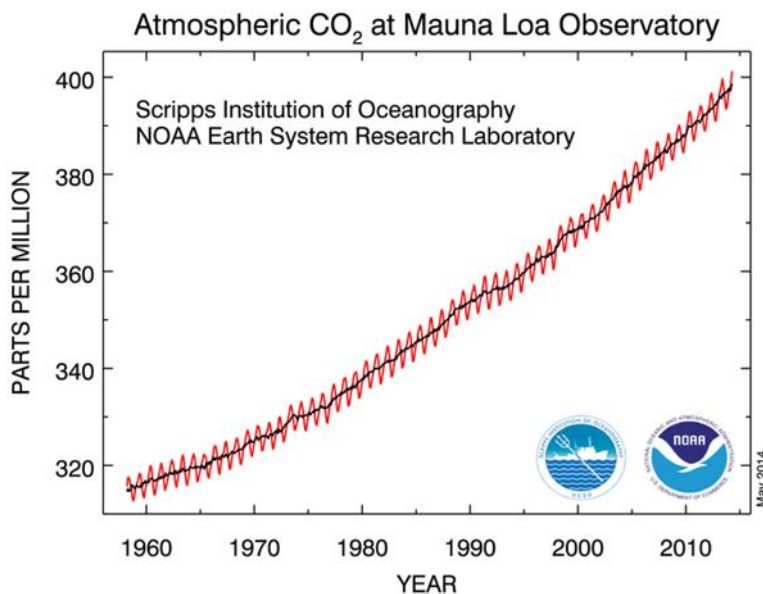
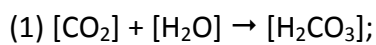


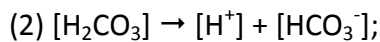
Figure 1 Keeling curve, variations in CO₂ concentration (ppm) in Earth's atmosphere (Tans & Keeling, 2014).

Oceans have a fundamental role in the main processes that occur on Earth, and represent around 70% of its surface. They encompass a wide variety of habitats and ecosystems, contributing greatly to planet's biodiversity and providing livelihood for humanity (Raven *et al.*, 2005). The atmosphere and the oceans act as a linked systems, where changes that occur in one part of the system have consequences on the other (Hoegh-Guldberg *et al.*, 2007). Thus, with the increasing of the atmospheric CO₂ the

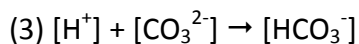
partial pressure of CO₂ ($p\text{CO}_2$) in seawater also increases. When in contact with the oceans, CO₂ is absorbed, leading to a chemical change of the carbonate of seawater (Gattuso & Buddemeier, 2000; Zeeb-Gladrow & Wolf, 2001). This causes ocean acidification (decrease in pH), which is occurring at a much faster rate than at any time in the past 650,000 years, representing a serious threat to marine ecosystems. In the atmosphere, CO₂ is a chemically unreactive gas but, when dissolved in seawater, becomes more reactive getting involved on several chemical, physical and biological reactions (Raven *et al.*, 2005). CO₂ is dissolved in water and reacts with its molecules (H₂O) forming carbonic acid (H₂CO₃):



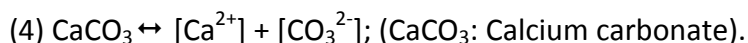
Carbonic acid dissociates into H⁺ (hydrogen ion) and HCO₃⁻ (bicarbonate ion):



Due to the increase of H⁺, CO₃²⁻ (carbonate ion) reacts with H⁺ forming HCO₃⁻:



Thus, the dissolution of CO₂ in seawater leads to an increase in concentrations of H⁺ (acidification), H₂CO₃ and HCO₃⁻ and to a decrease of CO₃²⁻ concentrations. This decrease in CO₃²⁻ prevents the formation of carbonate minerals and promotes its dissolution, by influencing the equilibrium point of the following reaction:



In summary, the rising level of CO₂ in seawater increases the concentration of H₂CO₃, HCO₃⁻ and H⁺ and decreases the concentration of CO₃²⁻ and pH (Raven *et al.*, 2005) (figure 2).

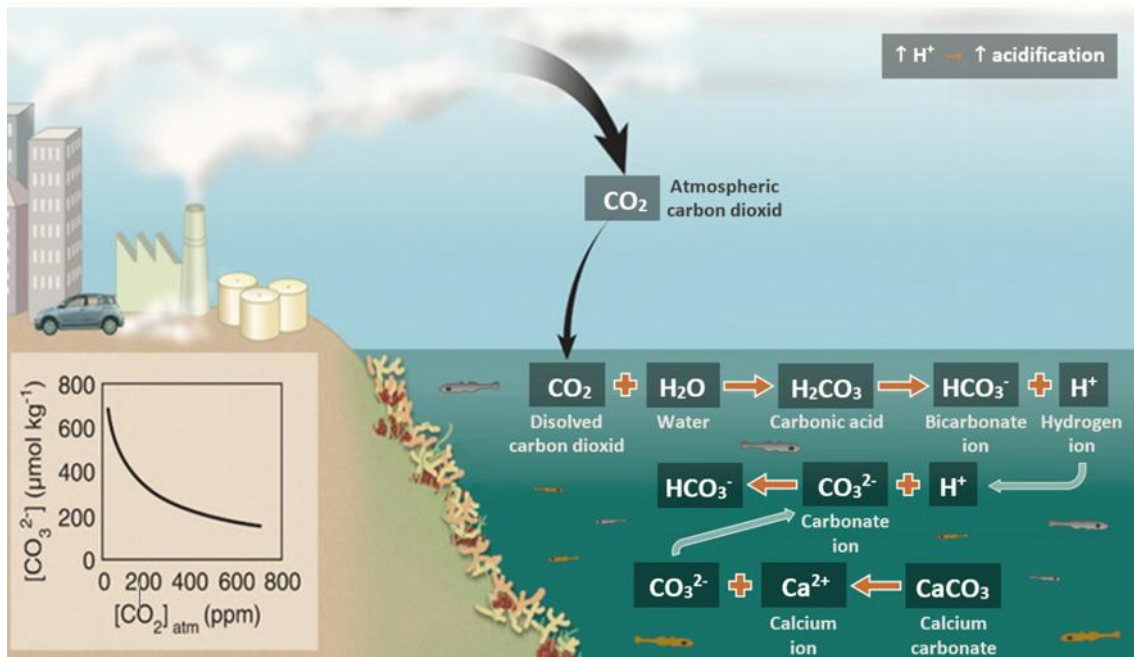


Figure 2 Diagram of CO₂ cycle between the atmosphere and the ocean. Atmospheric carbon dioxide is absorbed by the ocean where it bonds with water generating carbonic acid. This acid then releases a bicarbonate ion and a hydrogen ion (increasing ocean acidification). Also, this hydrogen ion combines with free carbonate ion in the water, producing another bicarbonate ion. The free carbonate ion would otherwise be available to marine animals make calcium carbonate shells and skeletons. (Diagram adapted from Hoegh-Guldberg *et al.*, 2007).

It is estimated that nearly half of the additional CO₂ produced by humans over the past 250 years has been absorbed by the ocean. The actual seawater pH is on average 8.2, 0.1 units lower than the pre-industrial values (Raven *et al.*, 2005). Based on realistic scenarios for future emissions of CO₂, it is expected that the oceanic pH may decrease even more 0.3 - 0.4 units by the year 2100 (Caldeira & Wickett, 2003) (figure 3), which would result in acidity values never reached in the last 400 000 years (Feely *et al.*, 2004).

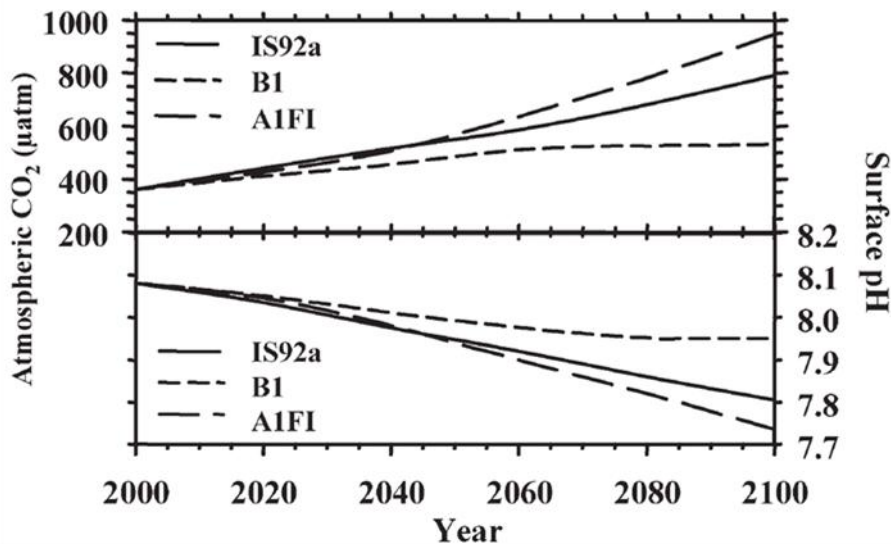


Figure 3 Atmospheric CO₂ concentration and global average surface seawater pH projected under three scenarios: the “business-as-usual” IS92a CO₂ emissions scenario (IS92a), the most (B1) and the least (A1FI) conservative SRES scenario (adapted from Meehl *et al.*, 2007 in Fabry *et al.*, 2008).

It is expected that higher CO₂ levels will result on serious impacts for Earth’s ecological systems, causing an overall loss of biodiversity, disruptions to ecosystems processes and a reduction in the ecological goods and services provided to human societies (Thomas *et al.*, 2004; Lovejoy & Hannah, 2005). High CO₂ levels can cause harmful effects in some calcifying marine organisms by decreasing the saturation of calcium carbonate and changing the calcification processes used on the production of shells, plates and skeletons (on corals and molluscs, for example) (Gattuso *et al.*, 1999; Leclercq *et al.*, 2000; Raven *et al.*, 2005; Kleypas *et al.*, 2006). Photosynthetic and non-photosynthetic micro-organisms, the base of ocean food web, are other affected by ocean acidification (Riebesell *et al.*, 2000; Huesemann *et al.*, 2002; Feely *et al.*, 2004; Raven *et al.*, 2005). For juvenile and adult fish it is unlikely that the skeletal growth will be affected, since these life stages have precise control over their internal ionic environment (Munday *et al.*, 2008). Yet, some variations can happen, such as variations on skeletal or otolith (fish ear bone) development during their embryonic or larval stages (Munday *et al.*, 2008). In some species, physiological processes can also be affected due to a decrease in pH levels of cellular fluids (Feely *et al.*, 2007).

Research in the field of ocean pH effects are becoming increasingly frequent. However, there is a major gap on such studies regarding marine fish and their behavior (Ishimatsu *et al.*, 2008). In general, early life history stages are more vulnerable to changes in the surrounding environment, therefore, effects of acidification are most likely to be detected on this phase of the life cycle. It is critical to understand the consequences of an acidic environment in larval and juvenile survival, behavior and recruitment, which may have significant consequences in adult populations.

Reef fish cycle

The life cycle of fishes commonly includes four phases: embryo, larva, juvenile and adult. Some individuals manage to survive longer, reaching a fifth stage, the senescence phase (Fuiman, 2002).

The majority of reef fish are characterized by having a pelagic, generally larval, stage resulting in a bipartite life cycle. These two phases (pelagic and benthic) vary in morphology, size, habitat, food and behavior (Leis, 1991). The spawning can be either broadcast or demersal, and is followed by the pelagic larval phase that recruit to nearshore waters. Here they metamorphose and, in the case of fish with demersal adult life, settle to spend the rest of their adult phase (Leis, 1991; Leis & McCormick, 2002; Leis, 2006). Larvae may be subject to dispersal over long distances, from days to weeks (depending on the species), growing and developing in open waters (Fuiman, 2002). In practice, a larva becomes juvenile when metamorphosis occurs and the external appearance of the individual changes (development of fins, sense organs, skeleton, external pigmentation, etc.). However, for many species with this bipartite life cycle, the settlement of larvae, where they become associated with a substrate (coral, rock, or bivalve reef or vegetation), is sometimes used as a synonym for metamorphosis (Fuiman, 2002).

Reef fish Larval Behaviour

During the development of reef fish larvae before metamorphosis, when some morphological and physiological systems develop, two different stages can occur (drawing from invertebrate terminology; Cowen, 1991): a pre-competent stage, where larva undergoes a fast development and growth; and a competent stage, in which occurs a body maintenance and a reduced growth (Leis & McCormick, 2002). As reef fish larvae have to find a settlement site, the timing of metamorphosis is probably related not only to developmental stage, but also to better success in finding suitable settlement habitat (Leis, 2006).

In the past, it has been assumed that pre-settlement stages of reef fishes were poor swimmers, not controlling their trajectories and passively drifting with currents. According to this view, all larvae behaved in the same way, regardless of species, ontogenetic stage or location (Leis, 2006). However, in the past 20 years, several studies seem to indicate the opposite. Studies in larval distribution have demonstrated that larvae of different species but originated from the same habitats show different distributions patterns (Reviews in Leis, 1991; Boehlert, 1996; Cowen & Sponaugle, 1997). This could not happen if larvae were passive swimmers, otherwise species would all have equal distributions. There are also some evidences showing some degree of self-recruitment on populations. Larvae and juveniles are able to settle on their natal reefs, representing in some cases, locally spawned larvae, 60% of all recruits (Swearer *et al.*, 1999; Jones *et al.*, 1999, 2005; Taylor & Hellberg, 2003; Almany *et al.*, 2007; Planes *et al.*, 2009). In addition, some species of perciform larvae, approaching settlement, show remarkable swimming abilities (Stobutzki & Bellwood, 1994, 1997; Leis *et al.*, 1996; Leis & Carson-Ewart, 1997; Fisher *et al.*, 2000; Fisher & Bellwood, 2002), which allow them to control its dispersion and settlement. Thus, reef fish larvae can act as active particles (Stobutzki & Bellwood, 1997; Dudley *et al.*, 2000; Fisher *et al.*, 2000; Leis & Carson-Ewart, 2003), having great skills of movement that can be even better than those of newly settled individuals (Stobutzki & Bellwood, 1994; Leis & Carson-Ewart, 1997; Stobutzki, 1998).

Fish larvae also seem to show oriented swimming (Leis & Carson-Ewart, 1999, 2000, 2003; Leis *et al.*, 2006a, b, 2007) in response to reefs, from distances of at least 1 km (Leis *et al.*, 1996; Stobutzki & Bellwood, 1998). There are indications that this orientation behavior differs between night and day, with larvae moving towards reefs at night to settle (Stobutzki & Bellwood, 1998) and offshore during the day to avoid reef-based predators and to feed (Leis & Carson-Ewart, 1998; Stobutzki & Bellwood, 1998; Kingsford *et al.*, 2002). However, it remains unclear what cues are used for dispersion and orientation in the pelagic environment. In nature, there are many cues used for orientation and navigation, which may contain visual, auditory, olfactory, magnetic, and kinetic information. A cue can be more or less valuable depending on the environment where it is propagated, on the utility of the information that it carries and on the ability that the receiver has to detect and interpret it. Consequently, multiple cues will work through different distances and with different degrees of accuracy, being used to solve spatial challenges, according to their hierarchies (Kingsford *et al.*, 2002). For example, large migratory fish (salmon and tuna) use olfactory and magnetic cues and sun orientation for their orientated swimming in the open water (Ogden & Quinn, 1984). Juveniles and adult migratory fish of coral reefs use the compass orientation (although these cues are not very clear) (Ogden & Quinn, 1984; Quinn & Ogden, 1984).

Regarding fish larvae orientation abilities, little is known. However, some cues are suggested as being very useful for larvae on the selection of their settlement sites over long distances. During the orientation in the sea, vision can only be used over tens of meters, due to the attenuation of light in water. Since cues have to be detected from far, visual cues may be removed from this range, as well as the lateral line. However, for vertical distribution and settlement behavior, vision might be very useful (Myrberg & Fuiman, 2002; Kingsford *et al.*, 2002).

On the other hand, sound (Tolimieri *et al.*, 2002; Simpson *et al.*, 2004; Montgomery *et al.*, 2006) and olfactory (Sweatman, 1988; Atema *et al.*, 2002; Arvedlund & Takemura, 2006; Gerlach *et al.*, 2007) cues emanating from reefs are likely to be the most potentially cues used by pelagic reef fish larvae to recognize and locate a suitable and

'healthy' reef to settle. However, it is still unclear what can be detected, at what development stages it can be identified and over what spatial scales orientation can operate (Leis, 2006). These two kinds of cues can carry information on the direction and the type of habitat from which they come from (Kingsford *et al.*, 2002; Montgomery *et al.*, 2006) acting over kilometer-scales.

Larvae of many reef fishes have well-developed olfactory organs (Arvedlund *et al.*, 2007; Lara, 2008), supporting the hypothesis that they use olfactory signals to guide them toward to a settlement site. This assumption has been demonstrated in some studies. It was showed that fish larvae in a current, ignored downstream sites and had preference for upstream sites to settle (Elliott *et al.*, 1995; Lecchini *et al.*, 2005a). Settling larvae of some species could detect differences between the smell of their home reef and other reefs, preferring their home reef for settlement (Gerlach *et al.*, 2007). Apogonids larvae were able to distinguish and preferred olfactory cues from lagoon waters comparing with ocean waters (Atema *et al.*, 2002). Finally, Elliott *et al.* (1995) showed that during settlement, juvenile anemonefishes (*Amphiprion melanopus*) use olfactory cues to detect and select the host anemones (also verified by Arvedlund *et al.*, 1999 and Dixon *et al.*, 2008). Settling larvae also responded to olfactory cues from resident conspecific adults (Sweatman, 1988; Døving *et al.*, 2006). However, odours have a disadvantage as an orientation cue, as they are current-dependent and diluted with distance from the source, being their components possibly degraded (Montgomery *et al.*, 2001; Leis & McCormick, 2002). The olfactory cues thus act over small distances and can only be detected when larvae is near to odour source. These cues are normally involved in the avoidance of predators during the settlement process (Munday *et al.*, 2009; Dixon *et al.*, 2010). Although vision and mechanoreception are also used by larval reef fishes to identify and avoid predators (Chivers *et al.*, 2001) olfaction is probably far more significant during the settlement stage, since normally larvae settle at night when visual predator cues detection is much lower (Dixon *et al.*, 2010). Being this stage a period of an enormous predation risk (Caley *et al.*, 1996; Almany & Webster, 2006), the capacity to recognize such cues is very important to ensure larval survival.

Underwater sound has a huge potential to act as an orientation cue. Sound travels fast (4.4 times faster in water than in air) over long distances (hundreds to thousands of meters) through water with low attenuation, being highly directional (Urlick, 1983; Rogers & Cox, 1988; Richardson *et al.*, 1995; Popper & Carlson, 1998) and current-independent (because it is not dispersed by currents) (Armsworth, 2000). It can carry biological information about reefs (Myrberg, 1978; Hawkins & Myrberg, 1983; Stobutzki & Bellwood, 1998; Montgomery *et al.*, 2001; Kingsford *et al.*, 2002), varying the noise with the local fauna (Cato, 1980; McCauley & Cato, 2000; Radford *et al.*, 2008a, b). Auditory cues from reefs are a complex signal originated either from abiotic and/or biotic sources. Abiotic sounds can consist of physical processes acting along the reef such as the movement of water, geologic events, waves breaking on the coast and raindrops on the water surface (Popper & Hastings, 2009). Biotic sounds might be produced by several organisms such as fishes and invertebrates (snapping shrimp and urchins). These sounds can be produced with different purposes, such as communication over short and long distances with mates, offspring or conspecifics, or to catch prey (Popper & Hastings, 2009). Reef sounds may vary with time (season, moon phase and time of day) and the biological chorus has cyclical patterns in intensity, having a maximum during summer evenings around the new moon period (Cato, 1978; Radford *et al.*, 2008b).

Broadly speaking, the frequency hearing range of fishes lies between 30 and 1000 Hz, however there is great variation in hearing sensitivity among species. There are species with special adaptations that can detect sounds up to 3000 - 5000 Hz and other that are sensitive to infrasound or ultrasound (Slabbekoorn *et al.*, 2010).

Regarding to fish larvae it is somehow difficult to determine their auditory skills. In the past, it was believed that their hearing capabilities were quite limited (Myrberg & Fuiman, 2002). Nowadays, still little is known about what sounds (frequencies and intensities) larvae can hear and use, and over what scales (for navigation, for example), as well as the ontogenic development of these capabilities. The work of Egner & Mann (2005) and Simpson *et al.* (2005a) suggests that coral reef fish early embryonic stages can hear and that the response to noise probably changes with fishes' ontogeny.

Kenyon (1996) also shows that juveniles of two pomacentrid damselfish species, *Stegastes partitus* and *Stegastes variabilis* exhibited relatively poor auditory thresholds in comparison to conspecific adults. Apparently, larval fishes can hear between 100 and 1000 Hz (Leis *et al.*, 2011).

Several field studies have demonstrated evidences that reef sounds are potentially useful cue for settlement stage larvae to locate a suitable settlement habitat (Tolimieri *et al.*, 2000). By using light traps and underwater loudspeakers in order to determine whether fish larvae were attracted to sound emanating from reef, it was demonstrated for the first time that sound is a potential navigational cue in the aquatic environment. In this study the authors verified that temperate water triplefin larvae were more attracted to the light traps with underwater speakers broadcasting reef noise than to the silent light traps. For some settlement-stages coral reef larvae it was likewise verified (Leis *et al.*, 2003; Simpson *et al.*, 2004). Also, by using patch reefs from dead coral rubble playing reef recordings, Simpson *et al.* (2005b) registered a higher natural settlement rate when compared to silent control patches. Further, some larvae are able of directional hearing, localizing a sound source (Tolimieri *et al.*, 2004; Leis & Lockett, 2005) and can differentiate between artificial (pure tones) and natural sounds (reef noises) (Leis *et al.*, 2002). This response of coral reef fish larvae to auditory cues has been also physiologically demonstrated (Wright *et al.*, 2005).

Consequences for larvae in a future acidified ocean

The increasing of CO₂ and pH reduction in seawater have been found to adversely modify the behavior and sensory responses of early life history stages of reef fishes. Previous studies have found changes in larvae natural responses to odours and sounds. For example, Munday *et al.* (2009) reported that clownfish larvae (*Amphiprion percula*) reared in pH 7.8 became highly attracted to odours that avoided in ambient pH conditions (similar results observed by Dixon *et al.*, 2010 and Munday *et al.*, 2010) and larvae reared in pH 7.6 showed no response to those olfactory cues. The authors suggest that such disruptions were due to a damage in the transfer of chemosensory signals within the neurosensory system caused by the alteration of pH and ρCO_2 (not

affecting this change the development of the external sensory apparatus of larvae). Munday *et al.* (2010) showed that larvae exposed to a high CO₂ concentration had a 5-9 fold higher mortality rates due to predation, with many individuals becoming attracted to the predator's odour or losing the ability to sense it. The loss of aptitude to respond appropriately to odours when exposed to elevated CO₂ conditions was also verified for pre-settlement damselfish (*Pomacentrus amboinensis*), as well as the inefficiency in their learning (Ferrari *et al.*, 2012a).

Regarding to the auditory response, other experiment showed that juvenile clownfish (*Amphiprion percula*) reared in ambient CO₂ conditions avoided predator-rich daytime reef noises. In its turn juveniles reared in higher CO₂ conditions were not able to respond appropriately to these sounds. (Simpson *et al.*, 2011).

Altogether, these results suggest that the ocean acidification, caused by the additional CO₂ absorption by the ocean surface can have remarkable consequences on the recruitment success of adult populations, disturbing the functioning of marine ecosystems.

It becomes to essential predict the effects of ocean acidification in different areas, including temperate zones, for instance the Portuguese coast.

Objectives

Despite the recent advances in the understanding of the impacts of ocean acidification on coral reef fish larvae, little is known on the response of temperate fish larvae to the increasing levels of CO₂ expected in the coming decades. This study aims to provide essential data on the influence of those oceanic conditions on the behavior and sensory systems involved in the survival of larval populations of temperate reef fish, as well as their resilience. Specifically, the objectives of this study are:

(1) Assess if CO₂-induced seawater acidification scenarios affect the ability of temperate rocky reef fish larvae to respond to predator **olfactory cues**, likely to be avoided when searching for reefs and settlement sites;

(2) Assess if CO₂-induced seawater acidification scenarios affect the ability of temperate rocky reef fish larvae to respond to **auditory cues**, likely to be used when searching for reefs and settlement sites;

For this purpose, wild-caught larvae of sand-smelt (*Atherina presbyter*) and painted goby (*Pomatoschistus pictus*) were exposed to two pH levels, ~ 8.10 (control CO₂ treatment) and ~ 7.6 (high CO₂ treatment).

2. Materials and methods

2.1. Experimental species

For this research, larvae of two species of teleost fishes were used, *Atherina presbyter* (Cuvier, 1829) and *Pomatoschistus pictus* (Malm, 1865).

A. presbyter (sand-smelt) is a small pelagic fish species that inhabits coastal areas and estuaries, near surface. Its distribution ranges from the British Isles and southern North Sea to the Canary Islands, Mauritania and Cape Verde Islands (Quignard & Pras, 1986), having also been reported in the Azores archipelago (Santos *et al.*, 1997). It belongs to the Atherinidae family that is represented in the central-eastern and north-eastern Atlantic by a single genus, *Atherina*, and three species (*A. hepsetus*, *A. boyeri* and *A. presbyter*) (Fischer *et al.*, 1981; Quignard & Pras, 1986). *A. presbyter* is the commonest species of this family in the north-eastern Atlantic (Creech, 1990).

This species reproduces in spring and summer (Billard, 1997), spawning inshore in very shallow waters (Bamber *et al.*, 1985). Their large eggs are often associated with seaweed (Bracken & Kennedy, 1967; Bamber *et al.*, 1985), and larvae hatch after 15-16 days, with a total length of 6.7-7.5 mm (Bamber *et al.*, 1985). Hatched larvae are well developed and remain inshore in shoals, amongst shelter, and near the water surface (Bamber *et al.*, 1985). Larvae are active predators on visually selected animals in the water column (feeding strategy similar to adult), feeding mainly zooplankton (Bamber *et al.*, 1985). These shoals include a range of ages (sizes) and can be found in shallow water near the shore on the coast or in rock pools (Holt, 1898, 1899; Ehrenbaum, 1905; Miller, 1962; Bracken & Kennedy, 1967; Palmer, 1979; Bamber *et al.*, 1985), which allows them to escape predation by adult shoals (Bamber *et al.*, 1985). The postlarval development (figure 4) is divided into 5 stages (related with swimbladder, melanophores, fins and scales development), until juvenile shows all the external characteristics of the adult fish (at their total length (TL) 23 - 24 mm) (Bamber *et al.*, 1985). When juvenile's fins are fully developed (when fish reaches ~18 mm TL), they tend to start moving away from the shallow waters edge to live their adult life (probably swimming becomes so efficient that replaces sheltering as an escape strategy) (Bamber *et al.*, 1985).

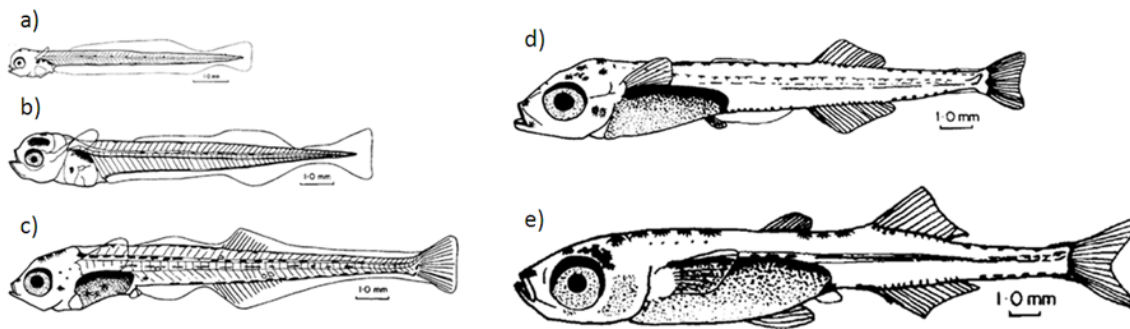


Figure 4 Postlarval development of *Atherina presbyter*: a) newly hatched postlarva, total length 7.0 mm, b) 10.7 mm TL specimen, c) 13.4 mm TL specimen, d) 15.4 mm TL specimen, e) 19.0 mm TL specimen (Palmer & Culley, 1984).

P. pictus (painted goby) is a small benthic-coastal fish species that preferably inhabits on shallow gravelly or sandy environment (1-55 m in depth) (Miller, 1986). Its distribution occurs along the east coast of the Atlantic Ocean and the Mediterranean (Miller, 1986). It is one of the eleven species of *Pomatoschistus* genus in the Gobiidae family (Miller, 1986). This family comprises the greatest number of known fish species and are quite successful (Nelson, 1994).

Its reproductive period depends on its geographic region, it may start in February or March and lasts until June or July (Miller, 1986). In Portugal, it goes at least from March to July (Pedro, 2006). During this period, male builds the nest under empty bivalve shells laying on sand (Miller, 1986). The protection of the nests that are used as spawning sites are ensured by males that also provide parental care of the eggs (Miller, 1986). Larvae hatch with 2.7-3.0 mm of total length (Russel, 1976) after 11-12 days (Miller, 1986), developing in planktonic environment (Lebour, 1920). Its young can be found in tide pools (Miller, 1986). In Figure 5 it can be seen some stages of the postlarval development.

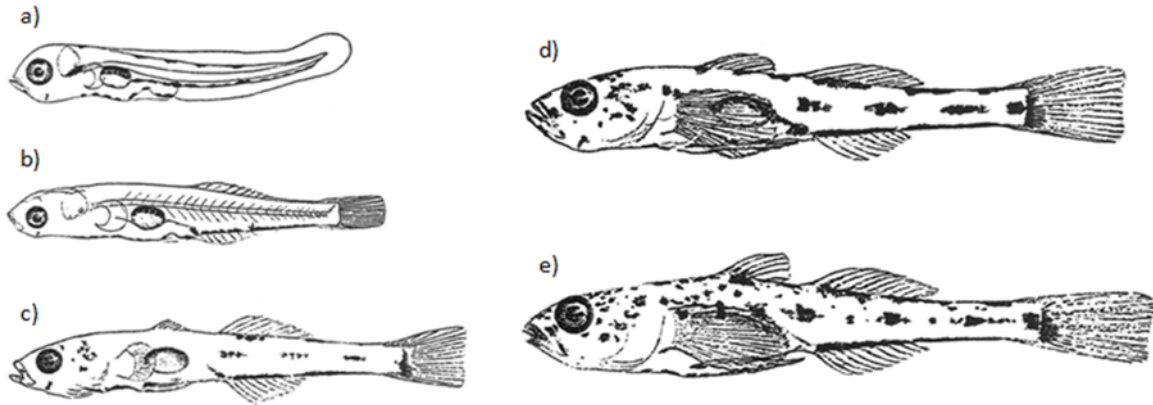


Figure 5 Postlarval development of painted goby: a) newly hatched postlarva, total length 3.0 mm, b) 7.0 mm TL specimen, c) 15.0 mm TL specimen, d) 19.0 mm TL specimen, e) 22.0 mm TL specimen (Petersen, 1919).

These species were chosen because they are good model species to answer to behavioral ecology questions, as they are easily captured during the larval stage, easily reared in captivity, and abundant in the Portuguese coast.

2.2. Collection of larvae

Larvae of sand-smelt, *Atherina presbyter*, and painted goby, *Pomatoschistus pictus*, were collected in the very nearshore of Alpertuche beach (38° 28' N; 8° 59' W), located at the Arrábida Marine Park (Portugal), using a 1 mm mesh dip net, during the month of July 2013. Sand-smelt schools were mainly collected at the surface, while painted goby larvae were collected close to the bottom.

Collected larvae were placed in a 30L bucket in ambient seawater, with gentle aeration and immediately transported to the laboratory. Larvae from each species were then transferred to separate 35 L aquariums, with fresh seawater, and left for one day to recover from transport and handling stress. Following this period, larvae were randomly assigned to a pH treatment, according to the experiment. Olfaction and hearing experiments were carried out at two pH levels, of ~ 8.10 (control CO₂) and

~ 7.6 (high CO₂). These pH values were both representative of the present-day pH conditions and the expectations for the future.

Larvae were fed twice a day with *Artemia* sp. *nauplii* and a summer light cycle (14:10 h light : dark cycle) was simulated using fluorescent lights.

The time (in days) that larvae of each experiment were exposed to the pH treatments prior to sensory tests is summarized in table 1. Due to logistical reasons, the minimum period of time that larvae were exposed to pH treatment was 2 days (sand-smelt of control CO₂ treatment in hearing experiments). In comparison with the other periods may be considered short. However, it seems to be an acceptable period of time, since it is in the pH control treatment, in which there is no influence of pH decrease. In addition, similar periods have been used with larvae and juvenile fish in other studies (Tolimieri *et al.*, 2004; Lecchini *et al.*, 2005b).

Table 1 Summary table with the range of time (in days) spent by larvae in each treatment prior to sensory tests (for both olfaction and hearing experiments).

Experiment	Treatment	Time in treatment prior to sensory tests	Cue
Sand-smelt: <u>Olfaction</u>	Control CO ₂	11 - 18 days	Predator vs. blank
	High CO ₂	11 - 18 days	
Painted goby: <u>Olfaction</u>	Control CO ₂	7 days	Predator vs. blank
	High CO ₂	7 - 21 days	
Sand-smelt: <u>Hearing</u>	Control CO ₂	2 - 17 days	Daytime reef sounds vs. silence
	High CO ₂	15 - 16 days	
Painted goby: <u>Hearing</u>	Control CO ₂	16 - 36 days	Daytime reef sounds vs. silence
	High CO ₂	6 - 14 days	

2.3. Experimental set-up and seawater manipulations

The experimental set-up consisted of 2 x 200 L recirculating seawater systems that were maintained at different conditions of pH and each supplied seawater to 6 replicate 35-L aquariums. Each 200 L recirculating system was equipped with biological, mechanical, chemical and ultraviolet filtration. Temperature and salinity were kept constant during experiments (values shown in Table 2). The artificial seawater was obtained in the laboratory by blending a commercial salt mixture (Tropic Marin®) with filtered freshwater. To ensure the maintenance of the pH treatments where larvae were reared, CO₂ was dosed to a set pH_{NBS}. The control treatment was established by the diffusion of ambient air in the seawater. The acidification condition was simulated by pumping the seawater with CO₂, through a system of an electronic pH-controllers (Tunze Aquarientechnik, Germany) that maintained pH at the desired level. The pH controller was attached to a sump. Rearing tanks were sealed on top with a clear glass lid to limit CO₂ exchange with the atmosphere. Temperature, salinity and pH of each aquarium were independently validated twice daily, using a portable meter (SevenGo DuoPro, SG23) calibrated weekly with fresh buffers (Mettler Toledo). pH was measured on the National Bureau of Standards (NBS) scale. Oxygen levels were maintained above 90% saturation by the mixing action of the diffusion pumps in the sumps.

Samples for total alkalinity (TA) determinations were collected once a week, from each treatment, placed in air-tight containers without air space, stabilized by mercuric chloride poisoning (Dickson *et al.*, 2007) and kept at +4°C until further analysis. Analysis of TA were performed using the spectrophotometric method described by Sarazin *et al.* (1999). Average seawater ρCO_2 was calculated using these parameters in CO2SYS, with the constants of Mehrbach *et al.* (1973) refit by Dickson & Millero (1987). Estimated seawater parameters are shown in Table 2.

Biological activity in the recirculating system and occasional water exchanges caused fluctuations in pH and total alkalinity of seawater during the experiment.

Table 2 Summary of mean seawater parameters in control and acidified treatments.

Treatment	pH _{NBS} ± SD	T(°C) ± SD	Salinity ± SD	TA μmol Kg ⁻¹ SW ± SD	ρCO ₂ μatm ± SD
Control CO ₂	8.10 ± 0.05	15.91 ± 0.65	33.85 ± 0.71	2545 ± 231.39	537.15 ± 55.98
High CO ₂	7.61 ± 0.02	15.98 ± 0.42	33.69 ± 0.22	2876.67 ± 148.99	2080.56 ± 99.37

Although pH values were within an expected range, ρCO₂ values were higher both in control and high treatments than expected, indicating that a higher amount of CO₂ was required to acidify water in this experiment. The use of a commercial salt mixture is most likely responsible for these values. Nevertheless, these values were representative of the present-day conditions in many coastal areas (corresponding to ~400 μatm atmospheric ρCO₂; Solomon *et al.*, 2007; Keeling *et al.*, 2009) and of what was predicted by the Intergovernmental Panel for Climate Change for the year 2300 (~2000 μatm ρCO₂; Caldeira & Wickett, 2005; IPCC 2007). Total alkalinity of Tropic Marin® seawater was slightly higher in all treatments than values reported from the natural seawater (~2300-2500 μmol kg⁻¹ seawater) (Zeebe & Wolf-Gladrow, 2001; Riebesell *et al.*, 2010), as is typical for artificial sea salt formulations. Thus, the estimates of the effects of ocean acidification obtained in the present study need to take this in consideration, as the CO₂-induced changes in pH and carbonate chemistry might be stronger in the natural seawater with lower alkalinity.

2.4. Odour experiment

2.4.1. Olfactory cues

To test the response (preference or avoidance) of larvae reared on different pH treatments to olfactory cues, a two channel choice flume was used (figure 6), adapted from Gerlach *et al.* (2007). Larvae were able to freely swim to either side of the chamber. Water from two different sources, treated with different olfactory cues, flowed through plastic tubes to the chamber (see figure 6). The flume allows a laminar flow separation of the two water sources. A constant gravity-driven flow of 100 mL min⁻¹ per channel, which was measured with a flow meter, was maintained throughout all experiment. To ensure that the flow was separate and parallel in the two channels,

with no turbulence or eddies, dye tests were performed before the experiments. At the beginning of each trial, a single larva was released into the center of the downstream end of the chamber. After 2 min of acclimation to the two water choices, the position of larva on each side of the chamber was recorded every 5 sec, over 2 min. This procedure was followed by 1 min of rest, during which the water sources were switched in order to outwit any side preference that was not related with the water source. After this minute, the entire test was repeated, including the 2 min acclimation period.

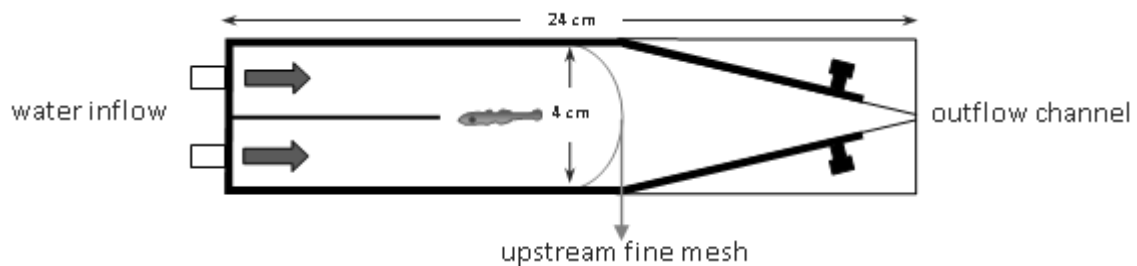


Figure 6 Two channel choice flume (design adapted from Gerlach *et al.*, 2007).

In each trial, a larva was given a choice in the flume chamber between a water source treated with a specific olfactory cue and an identical water source without that cue. Artificial seawater was used in the olfactory tests because it was assumed to contain no biological cues that the larvae may have become accustomed to during rearing.

Specific olfactory tests included:

- (1) Untreated seawater compared with untreated seawater (blank1 vs. blank2), this was used as a blank control;
- (2) Seawater treated with the olfactory cue produced by predators compared with untreated seawater.

To test the response of larvae to olfactory cues from a predator, water from a tank of adult sea bass, *Dicentrarchus labrax*, was used to test *Atherina presbyter* larvae. Sea

bass was chosen as it is known to prey on small pelagic fishes, such as atherinids (Spitz *et al.*, 2013). For logistical reasons, the same odour source could not be used to test olfactory discrimination of *Pomatoschistus pictus*. For this species, water from a tank of adult blenny *Lipophrys trigloides* was used. As the natural range of concentrations of specific olfactory cues in the environment is unknown, this protocol was designed simply to test for a reaction to the odor at an arbitrary concentration.

This protocol was performed for the two pH treatments, control CO₂ (8.1) and high CO₂ (7.6).

After the test, fish were killed with the anaesthetic MS222 and fixed in 96% ethanol. Larvae were latter photographed under a dissecting microscope for further measurements.

2.4.2. Statistical analyses

Due to the high variance and non-normal distribution of most data, it was not possible to use parametric statistics. Instead non-parametric statistical tests were used.

To test an effect of time in treatment in the response of larvae to cues, the Kolmogorov-Smirnov test was used. When no differences were found, data were treated together.

To determine if larvae exhibited a preference or avoidance for the tested cue, the mean proportion of time spent in the stream of water containing the predator cue (or, in blank1 vs. blank2, the designated) was compared against the null expectation of 0.5 for no preference using the one-sample Wilcoxon signed rank test.

The Kolmogorov-Smirnov test was again used to verify if the response of larvae was similar in the presence and absence (control situation) of the predator cue, as well as to compare the larvae response to that cue when reared in different pH levels.

For all tests, the significance level was $\alpha=0.05$.

2.5. Sound experiment

▪ Details of daytime reef recording

The daytime reef recording was conducted at the Arrábida Marine Park, at the very nearshore, and a depth of 8 meters (25/05/2013, 1245 hrs.), under calm conditions. These diurnal sounds were taken with an omnidirectional hydrophone (HiTech HTI-96-MIN with inbuilt preamplifier, High Tech Inc., Gulfport MS) connected to a digital Sony PCM-M10 recorder (96 kHz 24-bit Recording). The recording had a total of 2 min. Later, the recording acoustic data was analyzed using the software Avisoft SASLab Pro (Avisoft Bioacoustics, Berlin, Germany) to obtain the spectral level (see figure 7). The total sound intensity [rms (rootmeansquare) expressed as dB re 1 μ Pa] of this recording was 122.6 dB re 1 μ Pa.

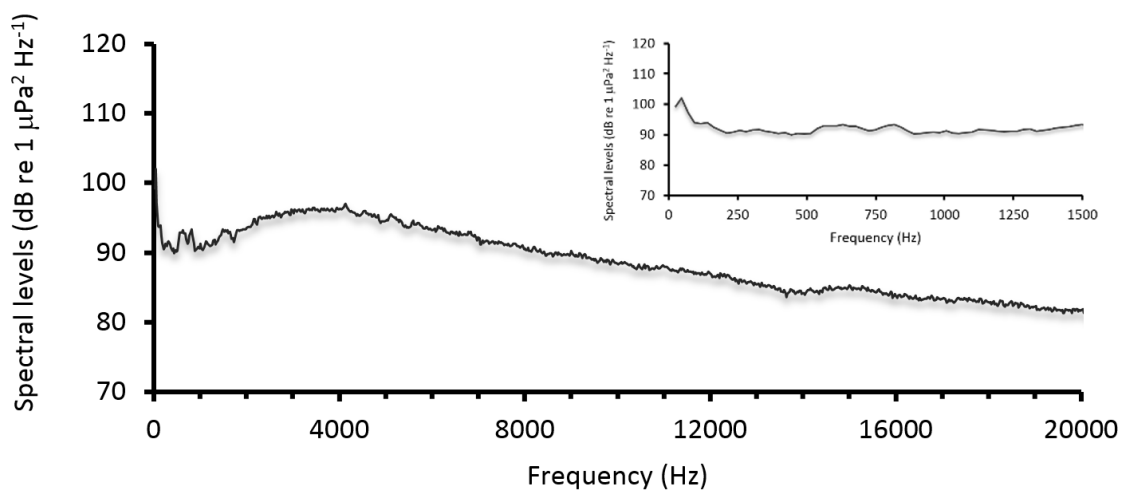
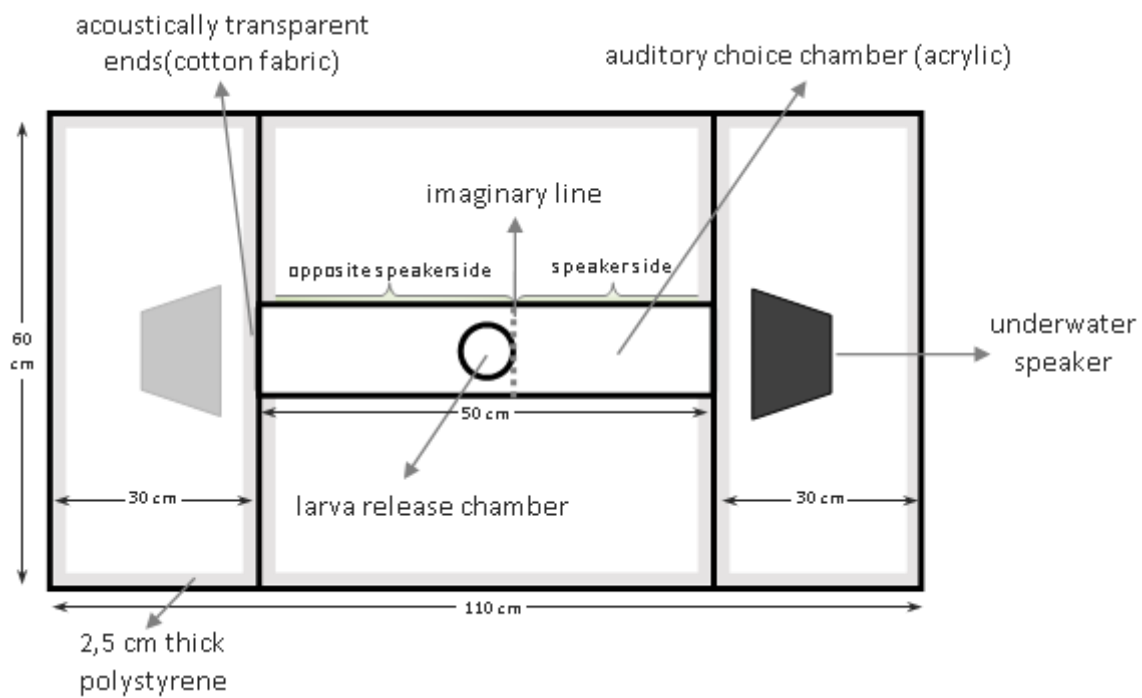


Figure 7 Spectral level analysis of the recorded sound used on this experiment.

The sound from the daytime reef recording seems to have quite activity according to its range of frequencies and intensities. This activity can be caused by distant shipping industrial or seismic-survey noise, wind and wave noise, and biological noise (the three primary sources in most shallow waters regions) (Greene, 1995). The noise of wind and waves produces frequencies between 10 and 1000 Hz, whereas biological noises can cover a very wide range of frequencies (Knudsen *et al.* 1948; Wenz 1962).

▪ The auditory choice chamber

The response to the reef sound was tested in an auditory choice chamber (figure 8), adapted from Simpson *et al.* (2011). The auditory choice chamber consisted of an acrylic tubular chamber inside a glass aquarium coated with polystyrene foam. Both ends of the tubular chamber were covered with cotton fabric (acoustically transparent ends). At one of the ends it was placed an underwater speaker, which was moved to the opposite side of the aquarium in the middle of every series of tests, in order to outwit any side preference that was not related with the sound source. The underwater speaker broadcasted the desired sound towards the chamber. At the center of this chamber there was an observation window where larvae were released – the larva release chamber.



- 25 cm deep glass aquarium

Figure 8 Auditory choice chamber (adapted from Simpson *et al.*, 2011).

In order to reduce the low frequency ambient background noise in the auditory choice chamber and influence of the building's vibrations, the following adaptations were made:

- The choice aquarium was coated with polystyrene foam and laying in layers of rockwool and roofmate, on the top of a table;
- Each leg of the table was placed inside a vase with sand and rockwool, in order to avoid direct contact of the table with the floor.

▪ **Adjust of the broadcast sound level in the auditory choice chamber**

The recorded sounds were played back in the auditory choice chamber using a sound system that consisted of a Mp3 player, an amplifier module and an underwater speaker (Electrovoice UW-30, Lubell Labs Inc., Columbus, OH, USA).

Initially it was intended to use a similar intensity to that recorded in the rocky reef during the recording of sounds (similar overall spectral composition) but due to technical difficulties this was not possible. Thus, the criteria used for choosing the sound level was to have a louder sound than the background noise and the existence of a gradient of sound inside the auditory chamber, to assure larvae could choose which way to go, avoiding or going in the direction of sound. The original recording of the reef sounds was played back inside the chamber, and with the aid of the hydrophone, the emitted sound was recorded during 2 min at the near end (near the speaker) and at the far end of the chamber (opposite side of the speaker). This procedure was performed until the existence of a gradient of sound along the chamber was verified, using the Avisoft SASLab Pro to analyze the sound. The spectral level characteristics of the broadcast sound at the beginning and at the end of the tubular chamber can be seen in figure 9, which also shows the gradient.

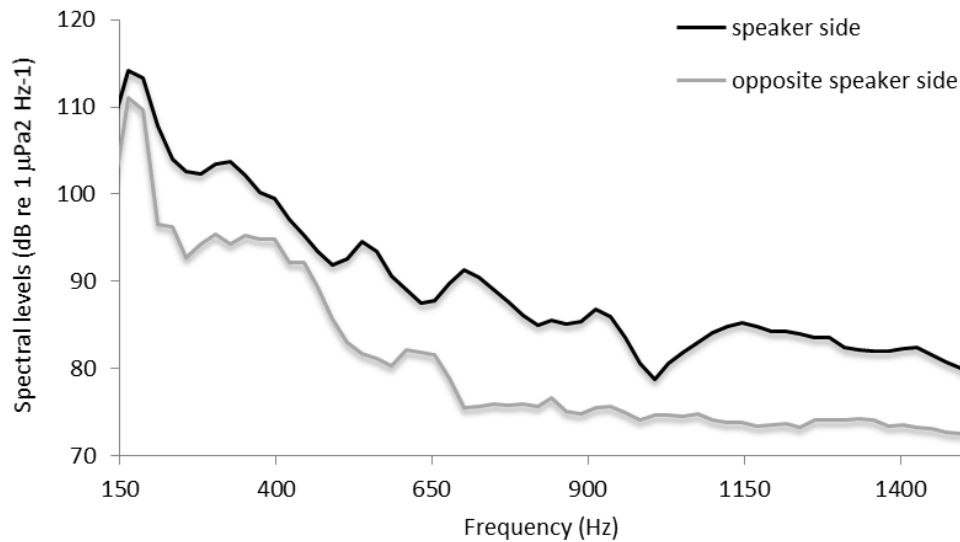


Figure 9 Spectral level analysis of the broadcast sound at the beginning and at the end of the tubular chamber. It can be seen that the sound level is higher at the speaker side than at the opposite speaker side.

In order to analyze the ambient background sound existing in the experimental laboratory, silence recordings (no replayed sound) were also made. It was then verified that the ambient background noise would not affect much this study, since the broadcast noise levels were higher than the ambient background noise.

It was not possible to verify if the sound intensity was within the hearing thresholds of the tested species, since we did not have the necessary equipment to investigate this. To determine thresholds of hearing, one would have to proceed an electrophysiological technique, the Auditory Brainstem Response (ABR), as described in Kenyon *et al.* (1998) and Simpson *et al.* (2011). The ABR measures in the eighth cranial nerve and brainstem auditory nuclei, the electrical potential produced in response to sound. This is considered to be the ideal method to use in the hearing abilities of settlement-stages fish larvae research, since it allows a quick measurement of the auditory capacity (Higgs *et al.*, 2002).

2.5.1. Auditory cues

To perform the acoustic experiment, the method used by Simpson *et al.* (2011) was adapted. At the beginning of each trial, one larva was placed in the release chamber, in

the middle of the auditory choice chamber. After 1 min of acclimation, the reef sounds were played (2 min recording in loop), and after 1 min, the larva was released in the tubular chamber, and its position recorded every 5 sec, over 2 min. The position of the larva could either be “speaker side” or “opposite speaker side” - the tubular chamber was divided with an imaginary line, and the area under larval release chamber was considered as opposite speaker side (see Figure 8.). Between each series of trials, the underwater speaker was switched to the opposite end of the auditory choice chamber. In addition, the tubular chamber was constantly flushed to avoid any olfactory gradient which could influence larva’s choice and frequently renewed to maintain its temperature constant.

The entire procedure described above was also performed in silence (no replayed sound), in order to test the potential for extraneous cues which could bias directional swimming behavior of larvae.

This protocol was performed for the two pH treatments, control CO₂ (8.1) and high CO₂ (7.6).

After the test, fish were killed with the anaesthetic MS222 and fixed in 96% ethanol. Larvae were latter photographed under a dissecting microscope for further measurements.

2.5.2. Statistical analysis

Because of the high variance and the non-normal distribution of data, it was not possible to use parametric statistics. Instead, non-parametric statistical tests were used.

To test an effect of time in treatment in the response of larvae to cues, the Kolmogorov-Smirnov test was used. When no differences were found, data were treated together.

To determine if larvae exhibited a preference or avoidance for a side of the auditory choice chamber the mean proportion of time spent in the opposite side was compared

against the null expectation of 0.5 for no preference using the one-sample Wilcoxon signed rank test. This test was performed for both silence and sound experiments.

The Kolmogorov-Smirnov test was again used to verify if the response of larvae was similar with the reef sounds and with silence (control situation), and to test the effect of CO₂ treatment on the response of larvae between treatment groups.

For all tests, the significance level was $\alpha=0.05$.

3. Results

3.1. Olfactory cues

Regarding *A. presbyter* larvae response to predator cues, no statistically significant differences were found after spending 12 and 18 days in each pH treatments (Kolmogorov-Smirnov test: $p > 0.10$). Thus, data were grouped per treatment.

In the control test, where neither stream of seawater in the flume contained an olfactory cue, larvae of both pH treatments showed random movement, spending approximately equal amounts of time on each side of the chamber (figure 10). Larvae reared in control CO₂ treatment (8.1) did not show any response in the presence of olfactory predator cue (One-sample Wilcoxon signed rank test, $n=23$, $p > 0.60$). Larvae reared in high CO₂ treatment (7.6) showed a slight preference for the predator cue, spending 63% of their time in that stream of water (One-sample Wilcoxon signed rank test, $n=23$, $p < 0.05$), but this apparent preference was not statistically significant. Lastly, larvae did not exhibit any significant differences in their behavior response to predator, when comparing between the pH treatments where they were reared (Kolmogorov-Smirnov test: $p > 0.10$).

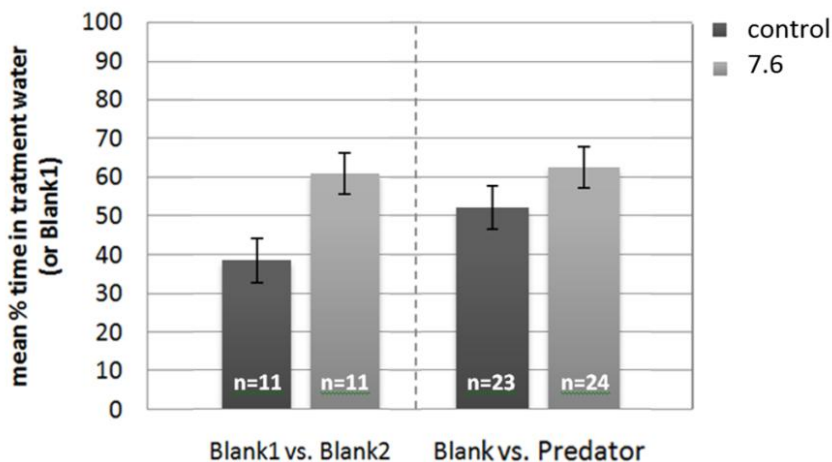


Figure 10 *A. presbyter* larval response (mean \pm s.e.m) to olfactory cue from a predator when reared at different pH levels (control and high CO₂). The first pair of columns represents the mean percentage of time that larvae spent on one side (blank1) of a two channel choice flume when both stream of water contained untreated seawater (blank2 represents the other side of the choice flume). The following columns represent the mean percentage of time that larvae spent in the stream of water containing seawater treated with the chemical cue when one stream contained the cue (predator) and the other stream did not (blank). The sample size of each test is given on bars.

For *P. pictus* larvae, no statistically significant differences were also found in the response to predator cues after spending 7 and 21 days in low pH treatments (Kolmogorov-Smirnov test: $p > 0.10$). Consequently, data were grouped for this treatment.

Larvae of this species showed a similar response as *A. presbyter* when tested in the control situation (blank 1 vs. blank 2), spending approximately equal amounts of time on each side of the chamber (figure 11). However, when larvae reared in high CO_2 treatment were exposed to olfactory cue, there was a strong avoidance for this cue (One-sample Wilcoxon signed rank test, $n=17$, $p < 0.001$) that did not occur in larvae reared in control CO_2 treatment (One-sample Wilcoxon signed rank test, $n=8$, $p > 0.60$). A significant effect of CO_2 treatment was detected, with the response of the high CO_2 treatment being significantly different from the control treatment (Kolmogorov-Smirnov test, $p < 0.005$).

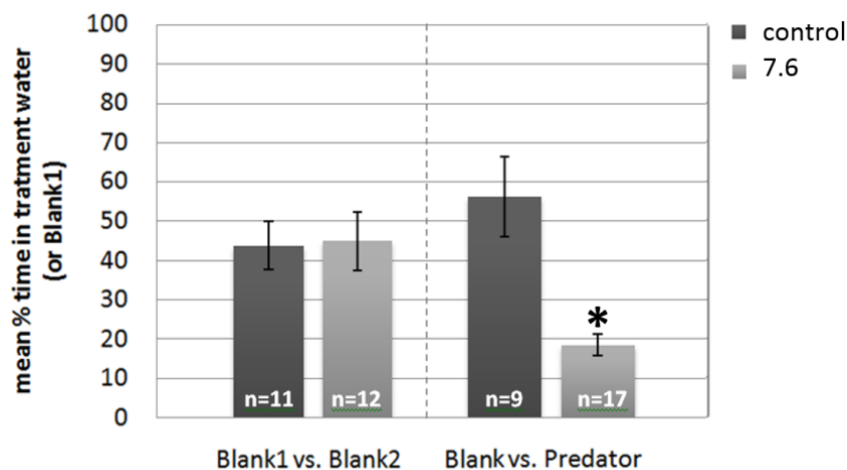


Figure 11 *P. pictus* larval response (mean \pm s.e.m) to olfactory cue from a predator when reared at different pH treatments (control and high CO_2). The first pair of columns represents the mean percentage of time that larvae spent on one side (blank1) of a two channel choice flume when both stream of water contained untreated seawater (blank2 represents the other side of the choice flume). The following columns represents the mean percentage of time that larvae spent in the stream of water containing seawater treated with the olfactory cue when one stream contained the cue (predator) and the other stream did not (blank). The sample size of each test is given on bars. * $p < 0.005$.

3.2. Auditory cues

Regarding control tests, during silent experiments, when neither side of the auditory choice chamber had replayed sound, *A. presbyter* larvae reared in both pH treatments showed no significant preferences for a side of the chamber (control CO₂: One-sample Wilcoxon signed rank test, n=13, p>0.60; high CO₂: One-sample Wilcoxon signed rank test, n=15, p>0.30), with larvae spending between 41 to 54 % at the opposite speaker side of the chamber (figure 12). When presented with reef sounds, larvae also showed no preference or avoidance for playback sounds, spending approximately equal amounts of time in the in the speaker side of the chamber, that contained the auditory cue and in the opposite speaker side (control CO₂: One-sample Wilcoxon signed rank test, n=15, p>0.30; high CO₂: One-sample Wilcoxon signed rank test, n=15, p>0.20). Overall, there were no significant differences in the behavior of larvae reared in the different pH treatments (Kolmogorov-Smirnov test, p>0.10).

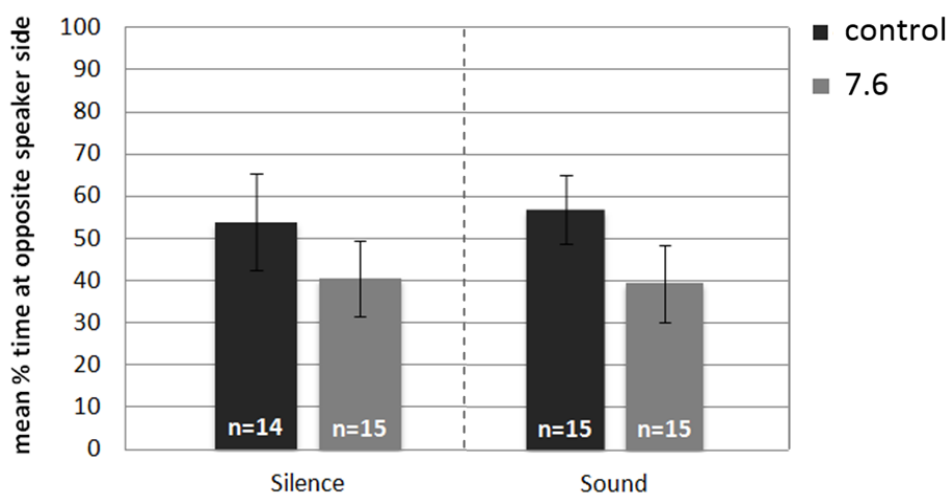


Figure 12 *A. presbyter* larval response (mean \pm s.e.m) to acoustic playback of daytime reef noise when reared at different pH treatments (control and high CO₂). The first pair of columns represents the mean percentage of time that larvae spent on the opposite end of an auditory choice chamber, when in silence. The following columns represent the same but with the reef noise. The sample size of each test is given on bars.

For *P. pictus* larvae, there were no statistically significant differences in the response to the reef noises after spending 16 and 36 days in control CO₂ treatment, and 7 and 14

days in high CO₂ treatment (Kolmogorov-Smirnov test, $p>0.10$). Consequently, data were grouped per treatment.

Larvae from control CO₂-conditions significantly avoided the daytime reef recordings (One-sample Wilcoxon signed rank test, $n=30$, $p<0.004$), spending, on average, 73% per cent of the time in the half of the chamber away from the speaker (figure 13). By contrast, no avoidance behaviour was detected in larvae reared in CO₂-enriched conditions (One-sample Wilcoxon signed rank test, $n=27$, $p>0.80$).

A significant effect of CO₂ treatment was detected, with the response of the high CO₂ treatment being significantly different from the control treatment (Kolmogorov-Smirnov test, $p<0.03$). No deviation from random movement was observed in the trials without playback sound: mean percentage of time spent by each group towards the speaker ranged from 46 to 55 % (One-sample Wilcoxon signed rank test, $n=15$, $p>0.50$ throughout).

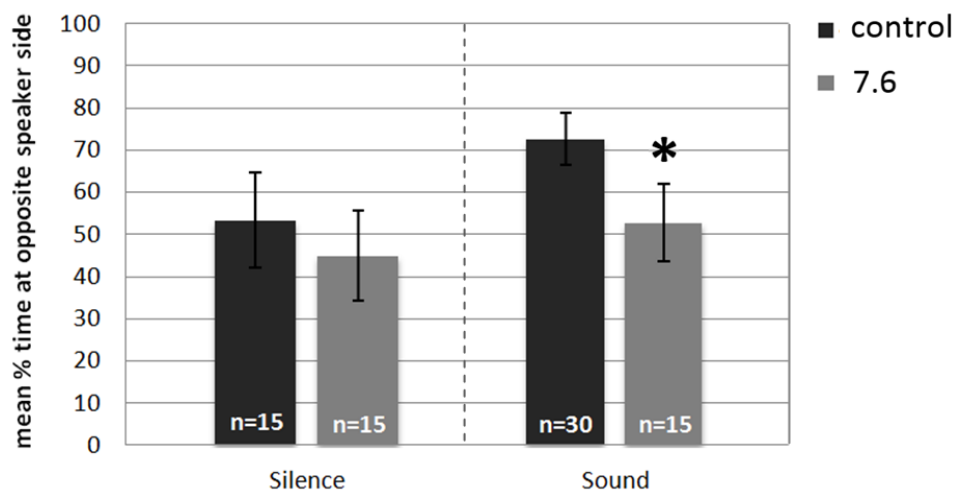


Figure 13 *P. pictus* larval response (mean \pm s.e.m) to acoustic playback of daytime reef noise when reared at different pH treatments (control and high CO₂). The first pair of columns represents the mean percentage of time that larvae spent on the opposite end of an auditory choice chamber, when in silence. The following columns represent the same but with the reef noise. The sample size of each test is given on bars. * $p<0.03$.

4. Discussion

Recent studies on tropical reef fishes have already showed that low pH values (*i.e.* high ρCO_2 levels) affect sensorial behavior in early life stages of these fishes, by modifying their responses to olfactory (Munday *et al.*, 2009, 2010; Dixon *et al.*, 2010; Ferrari *et al.*, 2012a), auditory (Simpson *et al.*, 2011) and even visual cues (Ferrari *et al.*, 2012b; Chung *et al.*, 2014). The present study, suggest that such pH conditions might also significantly affect the sensorial responses (to olfactory and auditory cues) of larvae in some temperate reef fishes. Such behavioral impairment in reef larvae may have remarkable consequences in population replenishment and sustainability (Munday *et al.*, 2010).

4.1. Olfactory cues

Sand-smelt and painted goby larvae responded differently to the tested olfactory cues. Opposite to what was expected, sand-smelt larvae reared in control conditions did not avoid the predator cue when sea bass odours were presented in the choice flume. This lack of anti-predator response was surprising since sea bass is known to be a predator of this species. There might be three possible hypotheses for these results. It is known that learning has an important role in the development of anti-predator response of many fish (Kieffer & Colgan, 1992; Kelley & Magurran, 2003; Ferrari *et al.*, 2010) and it may provide a mean by which naïve prey will recognize a novel predator and react appropriately to this threat (Brown, 2003), increasing the chances of individuals to survive an encounter. This knowledge can be achieve by pairing alarm cues [olfactory cue released from the damaged epidermis of a prey that was attacked or captured by a predator, providing thus a reliable indicator of predation threat to conspecifics and some heterospecifics (Chivers & Smith, 1998)] with a predator cue (visual, odour or sound) (Smith, 1999; Suboski, 1990; Ferrari *et al.*, 2010; Larson & McCormick, 2005; Holmes & McCormick, 2010). Perhaps sand-smelt larvae were still naïve to any cue produced by adult sea bass due to the absence of contact with it. The behavioural response to predation risk might have great energetic costs, which commonly involves

reducing the amount of time spent on other fitness-promoting activities such as foraging and growth (Sih, 1980; Milinski, 1993; Sogard, 1997; Fievet *et al.*, 2008). It is thus an advantage that larvae have the flexibility to discriminate between different degrees of predation threat, in order to decrease the costs of reacting to a false alarm [threat-sensitive predator avoidance hypothesis suggested by Helfman (1989), “which predicts that prey individuals will trade-off predator avoidance against other activities by altering their avoidance responses in a manner that reflects the magnitude of the predatory threat”].

Another possibility is related to the fact that prey response, or absence of it, to potential predators odours may vary, among others, depending on a predator’s body size (Helfamn, 1989; Kusch *et al.*, 2004). Perhaps sand-smelt larvae are too small and do not enter in the size selection ranges of the adult sea bass, and therefore do not represent a major predator threat. As far as it is known, the larval stages do not belong to the feeding preferences of the adult sea bass (Spitz *et al.*, 2013).

The other hypothesis for the apparent lack of anti-predator response by sand-smelt larvae reared in control conditions might be related to the intensity of predator odours. Water from a sea bass tank was collected at a public aquarium (Aquário Vasco da Gama), where water circulates through a network of tanks replenished with other species. The sea bass odours, might therefore, be diluted in other odors. In the future, a more appropriate procedure to obtain a most reliable predator's odor would be as follows (adapted from Holmes & McCormick, 2010): place the predators into acclimation aquariums for 36 h, such that all fecal matter passes through the system and prevent contamination of the final cue. After this, place individual fish into separate aquariums with fresh seawater and constant aeration for four days, without changing the water or feeding the fish (to prevent fecal matter from contaminating the final odour). Then draw the water from the aquarium and freeze it in portions until needed in the experiments.

When sand-smelt larvae reared in low pH conditions were tested, there was a slight change in the chemosensory response to the predator, with larvae spending approximately 63% of the time in the predator cue. However, this preference was not

significantly different from larvae reared in control conditions. In order to clarify this result it is suggested carry out the experiment with a greater N.

Regarding painted goby larvae, there was not also an avoiding behavior of larvae reared in control pH in presence of *L. trigloides* odor cue. This apparent lack of response might be related to the fact that this blenny feeds on mussels, other benthic invertebrates and algae (Zander, 1986), and it is not known to feed of fish larvae, not being therefore a known painted goby predator. To test the relation between these species, in laboratory conditions, *P. pictus* larvae were present to adult *L. trigloides* and there were no predator-prey response. Therefore, this species was probably not the most appropriate choice to test a predator response. When larvae reared in low pH conditions were tested, their behavioural response significantly changed, strongly avoiding *L. trigloides* odour (81% of the time). Although *L. trigloides* is not a painted goby predator, such behaviour may represent a sensorial impairment, responding larvae to an odor they should not react. Such predator-prey response, in natural conditions, would require energy consumption which otherwise could be used in growth and development. This result suggests that seawater acidification can cause damage in the larval olfactory mechanism, resulting in a confusion error in the detection of important olfactory cues for larval survival of some reef species. Examples of these cues are those used in the recognition of a predator or in the selection of a suitable habitat (cues that could help them locate and navigate towards reef habitat and select an appropriate settlement site). Thus, larvae will not adequately respond to odours that they may be subjected in their environment. Previous studies have also verified changes in chemosensory behavioural response of fish larvae reared in acidified pH. Munday *et al.* (2009) for example, verified that clownfish (*Amphiprion percula*) larvae reared in lower pH conditions than the current-day, loosed their olfactory ability, showing preference for odours of inappropriate habitats and failing to avoid predator cues. Dixon *et al.* (2010) also verified in their work a reduction in olfactory predator detection in clownfish larvae reared in acidified water. Larvae became strongly attracted to the smell of predators (also reported by Munday *et al.*, 2010 and Nilsson *et al.*, 2012).

4.2. Auditory cues

As observed in the olfactory tests, the two target species responded differently to the broadcast reef sounds.

Contrary to what was expected sand-smelt larvae reared in both control and low pH conditions did not show any preference/avoidance to the reef sounds. In previous studies using light traps, a higher density of larvae were caught in traps broadcasting reef noises (Tolimieri *et al.*, 2000; Leis *et al.*, 2003; Simpson *et al.*, 2004). Moreover, patch reefs associated with reef recordings showed higher natural settlement rates, comparing to silent control patches (Simpson *et al.* 2005b). There may be three possible explanations for the lack of responses in the larvae of this specie. First of all, since sand-smelt is a specie with pelagic life, not having a bipartite life cycle, in other words, remain in the pelagic environment as adults, possibly these larvae do not need to show any response to reef noise. Unlike reef fish larvae, these kind of larvae do not need to locate a reef for settlement. Tolimieri *et al.* (2000) also did not verify any response of pelagic fish larvae to reef sounds emitted by an underwater loudspeaker associated to a light trap. In addition, this specie has a particular life cycle. Adult sand-smelt spawn inshore in very shallow waters, hatching a larva very well developed that remains inshore in shoals (Bamber *et al.*, 1985). Only in the adult phase, they move away from the shallow water edge (Bamber *et al.*, 1985). Again, these larvae do not require the reef sounds for guidance, and do not need to show, therefore, any response to reef noises. The third possibility is that the sounds used in the present study were out of the sand-smelt hearing sensitivity. Wright *et al.* (2010) tested, by using the Auditory Brainstem Response (ABR), an electrophysiological technique that can define thresholds of hearing, the auditory abilities of larvae of four coral reef fish species (*Lutjanus carponotatus*, *Pomacentrus amboinensis*, *Pomacentrus nagasakiensis* and *Plectropomus leopardus*) and of similar-sized individuals of two pelagic species (*Elagatis bipinnulata* and *Gnathanodon speciosus*). Five of the six species studied, detected frequencies between 100 - 2000 Hz, however, for *E. bipinnulata* the range of frequencies heard was until 800 Hz. They also verified that larvae of coral reef species had significantly more sensitive hearing at all frequencies tested than their pelagic

counterpart species. Nevertheless, the authors hypothesize that such differences may have a phylogenetic basis, instead of an adaptive basis, since the pelagic species tested were of a different family than the coral reef species. Due to the lack of proper apparatus to perform the ABR method, it was not possible to measure the hearing sensitivity of the larvae used in this study. It was therefore not possible to verify and confirm that sand smelt larvae were not able to detect the daytime reef sounds. The hypothesis of a masking effect by the background noise to justify the lack of response by sand smelt larvae to the reef sounds should not be put aside. However, once again, it was not possible to know the hearing thresholds of these larvae. In future works it would be interesting to analyze the response of sand-smelt larvae to different coastal habitats sounds. According to Radford *et al.* (2010) these habitats have significant distinct underwater sound signatures which might transmit information for a long distance about direction and habitat quality being probably extremely importance for pelagic larval stages of coastal marine organisms.

Painted goby larvae reared in control conditions strongly avoided the daytime reef noises (73% of the time spent at opposite speaker side). Although this might seem unexpected given that this is a reef dependent species, the result agree with previous field research. Heenan *et al.* (2009), used for the first time light traps broadcasting daytime reef noises and, contrary to what was expected, they verified that larval fish were not attracted to these kind of sounds. Other studies also showed that during the day, larvae move away from reefs, to avoid reef-based predators and to feed (Leis & Carson-Ewart, 1998; Stobutzki & Bellwood, 1998; Kingsford *et al.*, 2002). In turn, during the night, larvae move towards reefs to settle (Stobutzki & Bellwood, 1998; Tolimieri *et al.*, 2000; Leis & Carson-Ewart, 2003; Simpson *et al.*, 2004; Simpson *et al.*, 2005b; Simpson *et al.*, 2008b). In general, settlement-stage reef fishes are specially attracted by high-frequency sounds of reefs (mainly produced by invertebrates) (Simpson *et al.*, 2008b), contrary to adults and juveniles reef fishes that are more attracted by low-frequency sounds produced by other fishes (Simpson *et al.*, 2008a). When larvae reared in 7.6 pH treatment were tested, their behaviour was significantly different. These larvae have reversed or lost their auditory preferences, showing absence of response to sounds (53% of the time spent at opposite speaker side). Similar results

were shown in a previous study where it was found a change in the directional response of juvenile clownfish (*Amphiprion percula*) reared in acidified conditions to a reef day recording (Simpson *et al.*, 2011). This result suggests that the low acidification levels can compromise essential auditory behaviours for survival. It also suggests that for painted goby larvae, the responses to auditory cues are unlike to compensate the potential loss of other senses that acidification may cause, such as olfactory impairment.

In order to anticipate the impacts of ocean acidification on fish behaviour, it is essential to understand the mechanism behind the changes of sensory and behavioural responses. Otoliths (fish earbones) are sensory aragonite structures that help in balance, orientation and sound detection. Their size and morphology influence hearing capacity. These organs are able to detect sound due the movement of particles that sounds create in the water (Popper *et al.*, 2003; Popper *et al.*, 2005). Increasing concentrations of CO₂ expected for future may affect the growth of these structures, but little is known about how they will be affected. Results on the potential impacts of high levels of CO₂ (low pH) in otoliths of coral reef fish, seem to indicate that larvae are tolerant to moderate increases in environmental CO₂ (Munday *et al.*, 2011a, b; Simpson *et al.*, 2011). In contrast, experiments on larvae of white sea bass (*Atractoscion nobilis*) showed a higher otolith growth with elevated CO₂ concentrations (Checkley *et al.*, 2009). These opposite results could be due to different CO₂ levels used on the mentioned studies (Checkley *et al.*, 2009: ~1000 µatm and 2500 µatm; Munday *et al.*, 2011a, b; Simpson *et al.*, 2011: up to ~ 1050 µatm). It can also be assumed that there are intra- and inter-specific (even being sympatric and sharing the same ecology and life history) variations in CO₂ tolerance on fishes, where some individuals might not be affected by the changes in CO₂ concentrations (also based by Munday *et al.*, 2010; Ferrari *et al.*, 2011). Therefore, in future studies, it would be interesting to analyze otolith morphology and check if there are differences in size and/or shape depending on the pH conditions where larvae are reared (perform a similar procedure to Checkley *et al.*, 2009).

Mechanisms of behavioural impairment

Nilsson *et al.* (2012) hypothesized that the changes in the behavioural and sensory responses happen probably because the high CO₂ concentrations, and hence lower pH, interfere with the neurotransmitter function in fish. Gamma-aminobutyric acid type A (GABA-A) is the main neurotransmitter receptor in the vertebrate brain (Bormann *et al.*, 1987). Nilsson *et al.* (2012) proposed that, in acidified conditions, ionic shifts (in Cl⁻ and/or HCO₃⁻ gradient) would occur over neural membranes, which would cause a reversal of GABA-A receptor function in some neuronal circuits. This would affect behavior and originate severe modifications in sensory preferences. The authors confirmed this hypothesis by showing that the atypical behavioral effects resultants from the acidified conditions were reversed by using a treatment with a GABA-A receptor antagonist (gabazine). Hamilton *et al.* (2014) also demonstrated that acidification impairs neurophysiology of fish, affecting GABA-A receptor function. The authors verified that in juvenile rockfish (*Sebastes diploproa*), high CO₂ levels (1125 ± 100 µatm) increased their anxiety behaviour compared with control conditions (483 ± 40 µatm). Then, by applying GABA-A receptor modulators they showed that, in contrast to the antagonist gabazine, the GABA-A-receptor agonist muscimol caused a large increase in anxiety in fish. Those results are consistent with the changes in Cl⁻ ion flux through these receptors in acidified conditions. Thus, for a result analysis even more rigorous, one could also have treated the group of larvae that showed an abnormal auditory preferences with a GABA-A receptor modulator, in order to verify if the changes that happened due to the high CO₂ concentrations interfered with the neurotransmitter function in fish (perhaps with an antagonist of the GABA-A receptor likewise Nilsson *et al.* (2012) did with olfactory preferences and behavioural lateralization of larval coral reef fish). This procedure should be considered in future experiments.

The capacity for most marine organisms to adapt to the ocean acidification has been poorly studied, yet it is unlikely that many marine species have genetic variation adapted to this fast change. Probably, only species with very rapid generation times will be able to keep pace with change and adapt (Hoegh-Guldberg *et al.*, 2007). Most

of the research already performed that focuses in pH and ρCO_2 sensitivities of marine organisms is based almost entirely on short-term perturbation experiments. It will be important in the future to place long-term experiments (with several generations), since it may occur acclimation (plasticity in phenotype within and between generations) and adaptation by selection of favorable genotypes. There are already some studies that did so for some marine organisms (Burkhardt *et al.* 1999; Collins & Bell 2004), showing different adaptive responses. Since it is more difficult to perform long-term experiments with fish, one could at least perform this study with the offspring of progenitors of acidified environment, likewise Munday *et al.* (2009) and Dixson *et al.* (2010) did on their research.

The TA values of seawater in this study were higher in both control and acidified treatment (~ 2500 and $\sim 3000 \mu\text{mol Kg}^{-1}$ SW, respectively), comparing with those described from the natural seawater ($2325 \mu\text{mol Kg}^{-1}$ SW; Barry *et al.*, 2010) and with many of previous studies in ocean acidification. However, these values are within the range reported in nearshore waters of the southern Iberian Peninsula (de la Paz *et al.*, 2007, 2008). In addition, during a spring upwelling episode, Cabeçadas & Oliveira (2005) observed even higher values ($4600 \mu\text{mol kg}^{-1}$) in a location near to the sampling area of the present study. These authors also reported in their study area extreme values of ρCO_2 ($470\text{-}1770 \mu\text{atm}$) that are probably due to the transport of higher quantities of inorganic carbon from deep, colder upwelled water. The nearshore coastal regions often experience seasonal high ρCO_2 values, therefore their future ρCO_2 levels will probably exceed the $1000\text{-}1200 \mu\text{atm}$ ($\Delta\text{pH } 0.4\text{-}0.5$) projected for 2100 (Meehl *et al.*, 2007). It is of extreme importance to improve the knowledge about seawater carbonate chemistry in different oceanic systems, especially of coastal systems in order to understand better the future consequences of ocean acidification.

5. Final considerations

In conclusion, the results of this study suggest that larvae of some species are sensitive to the potential future ocean acidification scenario, caused by the uptake of additional CO₂ at the ocean surface. It may disrupt the behavioural response to both olfactory and auditory cues that may be essential for site selection at settlement in some temperate reef fish larvae and for their survival. This will reduce the recruitment success and will have consequences in long term for the sustainability of adult fish populations, affecting significantly marine biodiversity. These results strengthen the need to reduce the anthropogenic CO₂ emissions in order to prevent the intensification of ocean acidification and to ensure the protection and conservation of marine ecosystems and communities.

This was a study of great importance in the topic of prediction of the consequences resultant from increased CO₂ concentrations in the ocean. Upon our knowledge, it was the first research that studied the effects of pH changes in the sensorial behaviour, both auditory and olfactory of temperate reef fishes. More research is needed in this area, both performing this study on more species (because not all species of fish larvae respond in the same way to alterations in pH) and improving the experimental methodology, so try to understand better the repercussions of it. In addition, since global temperature rise will act simultaneous with ocean acidification (Raven *et al.*, 2005), it would be also of great importance to study the effect of the interaction between these two factors.

6. References

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