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Assessing the impacts of seawater acidification and emerging contaminants on different life stages of the sea urchin *Paracentrotus lividus*

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1. SUMMARY

Since pre-industrial time, because of rapid increase of atmospheric CO_2, two main phenomena at global level are generated: climate change and ocean acidification. Up to 30% of atmospheric carbon dioxide is dissolved in the oceans and is lowering the seawater pH. To date, a decrease in the average pH value of ocean surface, of approximately 0.1 units, is already observed. Further decrease of 0.3-0.5 units is predicted for the year 2100. Among future threats for coastal marine areas, the presence of pharmaceutical compounds is of great concern. The most used pharmaceuticals are commonly detected in the aquatic environment. These compounds are biologically active molecules designed to interact at cellular/molecular level in the target organism. However, they may interact also with non target organisms, affecting specific animal functions (e.g., development, growth, and reproduction) at low concentrations, such as those detected in the environment.

In order to produce more realistic data about the effects of ocean acidification on parents and their offspring, adult *P. lividus* were acclimated under control (8.1) and reduced pH (7.7 and 7.4) during gametogenesis. At the end of the acclimation period, physiological and biochemical responses were evaluated in males and females separately. Several parameters such as respiration rate, ammonia production, gonadosomatic index, some enzymatic activities and coelomocytes number and volume were affected by low pH and in some of them an opposite response in males and females was found.

In order to evaluate if long-term exposure of adults to reduced seawater pH could influence the performance of their offspring, sperm and larvae from different acclimation treatments were exposed at control and low pH. Sperm, velocity, ATP content and consumption, and oxygen consumption were analyzed at three pH values (8.1, 7.7 and 7.4). Larvae were reared at 8.1 and 7.7 pH for 20 days and their length and mortality were evaluated. Acclimation at low pH increased the ATP content in sperm and no influence on other parameters was observed. Greater somatic rod length was observed in larvae from low pH parents but, despite adult acclimation, in all larval groups, significantly lower length was found in all 7.7 pH treatments compared to control pH.

A second aim of this PhD project was to assess the effects of seawater acidification and of some pharmaceuticals on different life stages of sea urchin *Paracentrotus lividus.* Eighth pharmaceuticals belonging to different therapeutic classes and most frequently detected in the environment have been considered: clofibric acid (lipid-lowering), caffeine (metabolic stimulator

and adjuvant), diclofenac, ibuprofen (anti-inflammatory drugs) and propranolol (β-blocker), sulfadiazine, trimethoprim (antibiotics) and triclosan (antibacterial agent).

Effects of the mixture of clofibric acid, caffeine, diclofenac, and propranolol at environmentally relevant concentration (0.5 μg l^{-1}) under acidified conditions were investigated performing short-term embryoassays and long-term exposure of *P. lividus* larvae. Negative effects of mixture were highlighted only in long-term experiment at all pH values, underlying the need for longer experimental exposure to evaluate real hazard of single pollutants or their mixture in the environment. Interaction between low pH value (7.7) and each compound was also investigated through short-term embryoassays performed at two pH values (8.1 and 7.7) and five nominal concentrations (0.5, 1.0, 5.0, 10.0, 15.0 μg l^{-1}). LOEC (lowest observed effect concentration) values for all compounds at both pHs were defined analyzing early larval development and growth. In some cases, the presence of synergic effects between stressors was explained by the increased bioavailability of pharmaceuticals dissolved in seawater under low pH values. This relationship depends on the chemical characteristics of the compounds and could be useful to address substances which could be potentially more hazardous in future scenarios even if their concentration in the environment will remain the same.

2. RIASSUNTO

Dal periodo pre-industriale, a causa del rapido aumento dell'anidride carbonica in atmosfera, a livello globale sono stati generati due principali fenomeni: cambiamenti climatici e acidificazione degli oceani. Più del 30% dell'anidride carbonica atmosferica viene assorbita dagli oceani abbassando così il pH dell'acqua di mare. Fino ad oggi, è già stato registrato un calo medio del valore di pH delle acque superficiali di circa 0.1 unità ed un ulteriore abbassamento, di 0.3-0.5 unità, è previsto per l'anno 2100. Tra le future minacce per le aree marine costiere, la presenza di composti farmaceutici desta molta preoccupazione. Questi composti sono molecole biologicamente attive, progettate per interagire a livello cellulare o molecolare nell'organismo bersaglio. Tuttavia possono agire anche in organismi non bersaglio, colpendo specifiche funzioni dell'animale (es. sviluppo, crescita e riproduzione) anche a basse concentrazioni come quelle rilevate in ambiente.

Per riuscire ad ottenere risultati più realistici sugli effetti dell'acidificazione sui genitori e sulla loro progenie, in questo progetto di dottorato, individui adulti di *P. lividus* sono stati acclimatati a pH di controllo (8.1) e a pH ridotti (7.7 e 7.4) durante la gametogenesi. Alla fine del periodo

dell'acclimatazione, sono state valutate alcune risposte fisiologiche e biochimiche nei maschi e nelle femmine separatamente. Diversi parametri, come il tasso di respirazione, la produzione di ammoniaca, l'indice gonadosomatico, alcune attività enzimatiche e il numero e il volume dei celomociti, sono stati influenzati dal basso pH e per alcuni di essi è stata osservata una risposta opposta nei maschi e nelle femmine.

Per comprendere se una lunga esposizione dei genitori a ridotto pH può influire sulle performance della loro prole, spermi e larve ottenuti dai diversi trattamenti di acclimatazione sono stati esaminati al pH di controllo e a basso pH. Negli spermi, la velocità, il contenuto e il consumo di ATP e il consumo di ossigeno, sono stati valutati a tre pH (8.1, 7.7 e 7.4). Le larve sono state allevate per 20 giorni a pH 8.1 e 7.7 ed è stata valutata la loro mortalità e lunghezza. L'acclimatazione a basso pH ha portato ad un aumento del contenuto di ATP negli spermi, mentre non ha influito sugli altri parametri osservati. Nelle larve è stata osservata una maggiore lunghezza della spicola somatica quando provenivano da genitori mantenuti a pH basso, ma in tutti i gruppi, le larve mantenute a pH 7.7 hanno mostrato una lunghezza minore dei rispettivi controlli nonostante la pre-esposizione dei genitori.

Il secondo scopo di questo progetto di dottorato è la valutazione degli effetti dell'acidificazione e di alcuni farmaci sui diversi stadi vitali del riccio di mare *Paracentrotus lividus*. Sono stati considerati otto farmaci, appartenenti a classi terapeutiche diverse e più frequentemente rilevati in ambiente: acido clofibrico (ipolipemizzante), caffeina (stimolante metabolico e coadiuvante), diclofenac, ibuprofene (anti-infiammatori non steroidei), propranololo (betabloccante), sulfadiazina, trimetoprim (antibiotici) e triclosan (antibatterico).

Sulle larve di *P. lividus*, attraverso test embriologici a breve e lungo termine, è stato saggiato l'effetto della miscela di acido clofibrico, caffeina, diclofenac e propranololo, ad una concentrazione di rilevanza ambientale (0.5 µg l^{-1}) e in presenza di acidificazione. Si sono riscontrati effetti negativi della miscela solo negli esperimenti a lungo termine a tutti i pH testati, sottolineando così la necessità di utilizzare tempi di esposizione più lunghi per valutare il reale pericolo rappresentato dai singoli contaminanti e delle loro miscele in ambiente.

È stata inoltre valutata l'interazione tra pH ridotto (7.7) e ciascun farmaco, attraverso test embriologici a breve termine effettuati a due valori di pH (8.1 e 7.7) e a cinque concentrazioni nominali (0.5, 1.0, 5.0, 10.0, 15.0 µg l^{-1}). I valori delle LOEC (concentrazioni più basse a cui è stato osservato un effetto) sono stati definiti per ciascun farmaco a ciascun pH attraverso osservazioni sullo sviluppo e sulla crescita larvale. In alcuni casi, effetti sinergici tra i due stressogeni potevano

essere spiegati dalla più alta biodisponibilità dei composti disciolti in acqua di mare a basso pH. Questa relazione può essere calcolata in base alle caratteristiche chimiche dei composti e potrebbe essere utile nell'identificare sostanze potenzialmente più pericolose in uno scenario futuro, anche se le loro concentrazioni in ambiente non dovessero aumentare.

3. INTRODUCTION

3.1. Ocean acidification

Ocean acidification (OA) is a phenomenon of lowering seawater pH, mainly due to the dissolution of elevated atmospheric CO_2. Atmospheric CO_2 originates mainly from anthropogenic activities, in particular from fossil fuels' combustion (coal, petroleum and natural gas) which started extensively at the beginning of the industrial revolution (Le Quéré et al., 2009). Land-use practices, as well as deforestation, enhanced the effects of rising atmospheric CO_2 concentration (van der Werf et al., 2009; Lapola et al., 2014). Since the beginning of the industrial revolution, atmospheric partial pressure of CO_2 (pCO_2) has increased from 267 ppm to 390 ppm in 2011 (IPCC 2013). Rate of anthropogenic CO_2 emissions is accelerating. An increase of 29% was estimated in 2000-2008 (Le Quéré et al., 2009). The ocean represents a major sink for this gas and absorbs from 30 to 50% of CO_2 from the atmosphere (Sabine et al., 2004; Toseland et al., 2013). As a consequence, the carbonate chemistry of the ocean is modified with effects on pCO_2 in water, dissolved inorganic carbonate, alkalinity, calcium carbonate saturation state and the concentration of hydrogen ions. Increase of hydrogen ions originates the ocean acidification phenomenon (Feely et al., 2010; Beaufort et al., 2011). Since pre-industrial time, ocean surface pH has decreased by approximately 0.1 units (IPCC, 2013). The present average pH value for shallow and surface seawaters is 8.1; the average value predicted for the year 2100 is 7.7 pH which is already experienced in the natural environment but only for short time periods, during extremes of variability; the extremes of natural variability predicted by 2100 drop to 7.4 pH (Etheridge et al., 1996; Gattuso and Hansson, 2011). Due to reduction in carbonate ions, OA will also decrease the buffering capacity of seawater (Thomas et al., 2009).

The impact of ocean acidification is highly population and species specific. Effects detected in laboratory experiments are extremely dependent on geographic area, life-history stages or the biological responses considered in the studied species.

At latitudinal level, OA will probably act with different intensity. High latitude regions are naturally poor of carbonate ions in seawater, due to increased solubility of CO_2 at low temperature and

ocean mixing pattern. These regions present lower degree of supersaturation of carbonate minerals and consequently lower buffer capacity of seawater compared to surface waters of temperate and tropical regions. Furthermore, polar invertebrates tend to have low metabolic rates and slow growth rates, together with long generational time compared to warmer taxa. Therefore, organisms from low latitudes may have fewer opportunities to evolve effective adaptations to cope with OA (Orr et al., 2005, Bates et al., 2009; Olafsson et al., 2009).

Although ocean acidification mainly acts on surface seawater layer, even deep ocean waters are affected by this phenomenon. In deep environments, OA is occurring more slowly, but environmental stability, over long time scale, may have reduced the tolerance of deep-sea species to environmental changes, thus decreasing the potential for adaptation (Dahlhoff, 2004).

Concerning the differing life-history stages, there are evidence that even if fertilisation appears robust to future acidification of oceans, larval development is more vulnerable across major invertebrate groups, such as molluscs and echinoderms. In particular, there are evidence showing that OA can be particularly harmful to organisms which start to build calcareous structures in their larval and/or juvenile stages (Ross et al., 2011). In order to better understand which are the most vulnerable life-history stages under OA conditions, long-term multigenerational experiments are needed.

All changes in ocean water chemistry will affect a range of biological processes in marine organisms, including calcium carbonate precipitation, fixation and respiration of CO_2, regulation of internal pH and uptake of nutrients for growth. The effects of ocean acidification have been evaluated on a wide range of biological processes and species. The first challenge is to understand which physiological mechanisms will be affected and if consequences for the fitness will occur. The second question is how the effect on individual organisms will be mitigated or amplify at the ecosystem level.

Protective structures in marine biota, such as skeleton and shells, are mostly built by calcium carbonate and are present in so called marine calcifying organisms belonging to several taxonomic groups which occupy diverse ecological niches. Calcium carbonate structures are most often in the form of calcite, aragonite, high-magnesium calcite or their mixture (Politi et al., 2004). OA reduce carbonate ion concentration, along with calcium carbonate minerals saturation and may lead to reduced rates of calcification.

The best documented and widely observed biological effect is a decrease in calcification of calcium carbonate structures in many taxa of calcifers (Fabry et al., 2008b; Ries et al., 2009). Few

studies have also shown no effect or increased calcification under low seawater pH (Ries et al., 2009; Wood et al., 2008; Miller et al., 2009).

Another important biological process considered by several authors is the capacity to maintain internal pH homeostasis. In most heterotrophs internal pH is lower than in seawater (Hochachka and Somero, 2002). Inner CO_2 in these organisms is produced as byproduct of their metabolic activity. Thanks to biological membrane permeability to dissolved CO_2, acidbase balance in the organism is maintained utilizing a concentration gradient between internal and external pH. Increasing concentration of external dissolved CO_2 could affect this mechanism and consequently the homeostasis of internal pH, with negative effects on a variety of cellular functions ranging from protein synthesis to calcification (Pörtner et al., 2004). Multicellular organisms usually possess good passive buffering capacities, and most of them have specialized organs involved in acidbase balance (Melzner et al., 2009). In future acidified oceans, metabolic costs of the acclimation at high external pCO_2 may result in slow growth or decreased fitness of some organisms whereas other will be able to acclimate (Wood et al., 2008).

3.2. *Paracentrotus lividus*

3.2.1 Distribution

The sea urchin *P. lividus* is ecologically relevant, especially in the Mediterranean Sea, where it plays a dominant role as a grazer in most shallow benthic communities, and acts as keystone species controlling dynamic, structure and composition of infralittoral macroalgal assemblages (Boudouresque and Verlaque, 2001; Hereu 2006, Privitera et al., 2008).

This species is also economically relevant and exploited for its highly valued gonads. More active *P. lividus* fisheries in Europe are in Ireland, Scotland, Italy and Spain (Andrew et al., 2002; Kelly and Chamberlain, 2010; Norman et al., 2010). Due to the commercial value, some of natural populations are overexploited and there is increasing concern about their collapse (Andrew et al., 2002). Recently, research about this species is focalised on land-based culture (James, 2006), larval and juvenile feeding (George et al., 2001, 2004), artificial feed (Fernandez, 1997), larval rearing systems (Christiansen and Siikavuopio, 2007), gonad enhancement (Akiyama et al., 2001; Fernandez and Boudouresque, 2000; Shpigel et al., 2005) and wild stock enhancement (Gonzalez-Henriquez et al., 2009).

As well as in the Mediterranean, *P. lividus* is distributed in the north-eastern Atlantic, from Scotland and Ireland to southern Morocco and the Canary Islands (Boudouresque and Verlaque,

2001). It is a sub-littoral species living at depth up to 10-20 m (Gamble, 1965; Tortonese, 1965; Allain, 1975; Harmelin et al., 1980) and only some individuals occur at depths down to 80 m (Cherbonnier, 1965; Tortonese, 1965). It is mainly distributed on solid rocks, boulders and in the meadows of seagrasses *Posidonia oceanica* and *Zostera marina* (Mortensen, 1927; Tortonese, 1965; Ebling et al., 1966; Verlaque, 1987).

3.2.2. Reproduction

In Mediterranean, its reproduction period is extended throughout the year but geographical area and the habitat characteristics can lead to inter- and intra-population variations in the spawning period or in the period of maximum gonad production.

Tomšić et al., (2010) suggested one spawning mode per year for a population from central east Adriatic Sea (BristinaPristina Bay) with reproductive period from March to July and the peak of reproduction in April. In Mediterranean and in Ireland, both one (in Spring) and two (in Spring and late Autumn) spawning events were documented by other authors. Differences in time and frequency of reproductive period depend on climatic conditions, light regimes, energy sources availability and prevailing hydrodynamic conditions (Crapp and Willis, 1975; Chiantore et al., 2008). Gonad growth appear to be hindered by high summer temperatures (Byrne, 1990; Spirlet et al., 2000), so the probability to observe double spawning events is higher in eutrophic geographic areas with low temperatures (Lozano et al., 1995).

In *P. lividus,* the sexes are separated, although hermaphroditism has been observed (Drzewina and Bohn, 1924; Byrne, 1990), and the fertilization is external.

3.2.3. Sperm

In broodstock spawners, fertilisation success is enhanced by spawning aggregations and the synchronous release of gametes (Levitan, 2005)). To achieve fertilisation, sperm are subjected to intense selection, as only the least part of sperm produced is destined to fertilize an egg (Birkhead and Moller, 1998). Furthermore, in the majority of sexually reproducing species, sperm competition is present and males are selected according to high quality ejaculates (Parker, 1970).

Sperm competitiveness is influenced by sperm length, concentration (Benzie and Dixon, 1994), swimming velocity and longevity (Levitan, 2000) both in internal and external fertilisers.

Another important source of fertilisation success is gamete compatibility (Evans and Marshall, 2005). Sea urchin eggs release chemical cues, which enhance sperm movement, aid sperm to

swim in the direction of the eggs (Darszon et al., 2008) and participate in the acrosome reaction (Vacquier and Moy, 1997). Gamete recognition proteins, which drive sperm and eggs identification and fusion (Vacquier, 1998), can produce differences in male fertilisation rates, exhibiting strong affinity for sperm with certain recognition protein genotype (Palumbi, 1999).

The sperm phenotypes which could influence sperm competition in external fertilisers are still poorly understood. In external fertilisers OA may have an important role because sperm have limited buffering capacity of internal pH (Kroeker et al., 2010); in addition, a large number of steps taking place inside the sperm cell, and that are critical to fertilisation, are pH-dependent (Nishigaki et al., 2014).

In *P. lividus* male gonad, sperm cells are stored in a quiescent state at 7.2 pH. After the release into seawater, pH of the sperm increases to optimal alkaline value for dyneine-ATPase activity (7.6) and flagellar movement, along with mitochondrial respiration, begins and sperm start to swim actively (Christen et al., 1982). Rising cytosolic ADP levels stimulate mitochondrial oxidative phosphorylation, which is the only ATP-providing pathway in sea urchin spermatozoa. In sea urchin sperm, energy is almost completely obtained from fatty acid oxidation (Rothschild and Cleland, 1952; Mita and Yasumasu, 1983; Hansbrough et al.,1980). This reaction occurs in single mitochondrion placed at the base of sperm head (Baccetti and Afzelius, 1976). Dyneine-ATPase accounts for almost all ATP used in sperm (Christen et al., 1982, 1983).

In *P. lividus* spermatozoa, lipid components are represented mainly by phospholipids (72%), cholesterol (15%), cardiolipins (12%), whereas triglycerides are present in trace (Mita et al., 1994).

In *P. lividus,* sperm motility and velocity change during gonad cycle and are highest during the spawning stage therefore could be used as tools for evaluating sperm quality (Fabbroccini et al., 2016).

3.2.4. Eggs

In invertebrates, including echinoderms, beyond environmental factors (Fenaux et al., 1994; Miller and Emlet, 1999; Schiopu et al., 2006; Liu et al., 2007), larval development and general condition are strongly influenced also by nutrients stored in eggs and larvae (Vaïtilingon et al., 2001; Pechenik, 2006; Pernet et al., 2006). Favourable environmental conditions (López et al., 1998; Lamare and Barker, 1999; Liu et al., 2007) and/or good maternal provisioning of nutrients (Gallager et al., 1986; Strathmann et al., 1992; Pernet et al., 2004) could influence the duration of the vulnerable planktonic stage and offspring performance.

Among nutrients stored in echinoderm eggs, fatty acids, as a component of most lipids, appear to be very important for larval development. In particular, fatty acids are involved in larval energy supply (as in triglycerides) and in structural components (as in membrane phospholipids) and they act as precursor of bioactive molecules (Sargent et al., 2002; Tocher, 2003).

3.2.5. Life stages

P. lividus presents two life phases: an early and brief planktonic developmental phase (up to 3–4 weeks), and a benthic adult phase with a lifespan of 8–15 years (Ebert, 2007; Tomšić et al., 2010). Males and females aggregate for spawning and simultaneously release their gametes (Cherbonnier, 1954).

Planktonic phase has be estimated to be 23 to 29 days long in natural environment (Pedrotti, 1993); in laboratory, in the presence of non-limiting food conditions, it can be reduced to 14 to 19 days (George et al., 1989, Fenaux et al., 1992). Larvae from well-fed parents, have lower mortality, faster growth and metamorphose earlier compared to starved or poorly fed parents (George et al., 1989). Planktonic phase is composed by several developmental stages that could be divided in no-feeding stages (blastula, gastrula, prism) and feeding stages (4-armed echinopluteus, 6-armed echinopluteus with developement of posterodorsal arms, 8-armed echinopluteus with developement of preoral arms, competent larva with developement of pedicellars and the adult rudiment) (Fenaux, 1985). During the planktonic phase, larvae feed on microalgae. With the metamorphosis of the competent larva, the juvenile stage is reached and the benthic phase starts. Metamorphosis usually lasts less than 1 h and is followed by a postlarval endotrophic stage which lasts a few days until formation of anus and mounth is completed. This stage Is followed by the exotrophic juvenile stage and then the adult stage (Gosselin and Jangoux, 1998).

Sexual maturity, evaluated in laboratory, occurs at a diameter of 13-20 mm, and in 5 month old individuals (Cellario and Fenaux, 1990); it is supposed to occur later in natural environment, with 1 cm-variations in differing geographic areas (Ouréns et al., 2011).

3.2.6. Skeleton

Sea urchin *P. lividus* is a marine calcifier: its skeleton is mainly formed by high-magnesium calcite which is one of the most soluble form of calcite (Morse et al., 2006). It is composed of the test, the spines and the chewing apparatus, called Aristotele's lantern. In adult sea urchins, the dermis embeds the skeleton which is covered by epidermis. The calcification process is intracellular and

occurs within closed syncytia, so skeleton is never directly in contact with seawater. Sea urchin skeleton provides protection against hydrodynamic forces and predators and allows locomotion and foraging. Consequently, deterioration of skeleton may lead to negative consequences on sea urchin fitness (Dubois and Chen, 1989).

3.2.7. Coelomic fluid

Coelomic fluid (CF) is the main circulatory medium of echinoderms. It is enclosed in the main body cavity and together with the water vascular system allows gas transportation (Farmanfarmaian, 1966).

The CF of echinoderms contain circulating cells (coelomocytes) which have functions ranging from metabolite transport to immunity (Endean, 1966). In echinoderms, coelomocytes are heterogeneous in morphology and size, and cell type proportion varies with the species and the physiological conditions of an individual, as a response to environmental factors, pollutants, pathogens or accidental injuries (Matranga et al., 2000; Pinsino et al., 2007; Ramírez-Gómez et al., 2010). Three major cell types in *P. lividus* coelomic fluid are amoebocytes (13%), vibratile cells (7%), and phagocytes (80%) (Pinsino and Matranga, 2014). Amoebocytes (red and white) are mobile cells probably involved in the first phase of pathogen immobilization (Smith, 1981). Red amoebocytes contain natural red pigment (echinochrome) within cytoplasmatic vesicles, which is thought to be an anti-bacterial agent (Service andWarklaw, 1985; Smith, 1981). Rapid increase of red amoebocytes is observed in specimens collected from polluted sea water or accidentally injured (Matranga and Bonaventura, 2002; Matranga et al., 2000, 2005, 2006; Pinsino et al., 2008). Vibratile cells contain large cytoplasmic granules, recognized as primary lysosomes. Exocytosis of these granules may be linked with the clotting reaction (Smith et al., 2010). Phagocytes are the most abundant immune cell type in *P. lividus* and exhibit two morphotypes, with two major different functions: petaloid and filopodial cells are actively involved respectively in phagocytosis and triggering of clot formation (Pinsino and Matranga, 2014). Phagocytes are also implicated in encapsulation, aggregation, graft rejection, wound repair, as well as in cytolytic/cytotoxic reactions and transport of materials through vesicles (Hillier and Vacquier, 2007; Matranga et al., 2000; Smith, 1981).

It is demonstrated that in *P. lividus* female possess higher number of coelomocytes and consequently highest levels of cytotoxic and haemolytic activity. Furthermore, coelomocyte lysate

and coelomic fluid agglutinating activity is more evident in females than in males (Arizza et al., 2013).

Organic compounds in CF are mainly represented by amino acids, reduced sugars, proteins, lipids and nitrogenous wastes and CF composition is directly linked to the nutritional state of the organisms (Ferguson, 1964; Holland et al., 1967; Binyon, 1972).

3.2.8. Coelomic fluid buffer capacity

Even if echinoderms are considered osmoconformers, because ionic composition of their CF slightly differs from seawater (Bialaszewicz, 1933; Binyon, 1972). This results in much higher buffer capacity in CF than in seawater (Collip, 1920; Sarch, 1931). The pH of CF is usually 0.5 to 1.5 units lower than the pH of surrounding seawater mainly due to CO_2 retention (slow diffusion rate) and acidic metabolites accumulation (Cole, 1940; Hyman, 1955; Farmanfarmaian, 1966). In starfish and holothurians, diffusion of CO_2 through the body wall is more efficient than in sea urchins which present heavily calcified skeleton that slows this process. Moreover, the first two taxa possess specialized structures for gas exchange, papulae in starfish and respiratory trees in holothurians (Hyman, 1955; Ruppert et al., 2004). Structures specialized in gas exchange are less efficient in sea urchins and this process principally occurs at the tube feet level. Less capacity to eliminate CO_2 by diffusion through the body wall or specialized structures could explain higher concentration of CO_2 in CF of sea urchins (Collip, 1920); moreover, an higher buffer capacity of CF in this taxa is expected (Collard et al., 2013). Numerous enzymatic reactions depend on the maintenance of intracellular pH (Pörtner, 2008), so protons have to be transported in extracellular fluids. If pH of CF decreases, additional energy will be required to proton transport, from intra- to extracellular compartments, with a reduction of the energy that can be allocated to other processes such as growth or reproduction (Pörtner et al., 1998; Pörtner et al., 2000; Melzner et al., 2009). As a response to reduced seawater pH, there are growing evidence that sea urchin are able to accumulate bicarbonate ions into CF (Spicer et al., 2011; Stumpp et al., 2012).

In *P. lividus* buffering capacity of CF is primarily due to the bicarbonate buffer system of seawater (representing about 63%). It is also partly due to coelomocytes (around 8%) and to compounds present in the coelomic fluid (about 15%) which are possibly proteins or small molecules like lactate and accumulated bicarbonate ions. Coelomocytes could act by passive transfer of amino nitrogen to the CF, by adsorption of protons on the surface of the cells or by dissolution of H_2CO_3 inside the cells and consequent release of the bicarbonate ion into the CF (Delaunay, 1926;

Heisler, 1986). Molecules such as lactate, can act as proton acceptor reducing internal acidosis (Spicer et al., 2011).

3.2.9. *P. lividus* and acidification

P. lividus is also an important model species that has been used in research since the turn of century, with applications from molecular and developmental biology to ecotoxicology (Czihak 1971; D'Adamo et al., 2014; Fabbrocini et al., 2014; Kazama and Hino, 2012

Concerning the effects of acidification on sea urchins, the most studied species, , are *Strongylocentrotus droebachiensis*, *Strongylocentrotus purpuratus* and *Heliocidaris erythrogramma* (Dupont and Thorndyke, 2013).

In literature, effects of acidification on different life-history stages of *P. lividus* are also reported.

No alteration of the mechanical proprieties of the tests were observed in *P. lividus* adults and juveniles, after twelve and one month of exposure, respectively, at pH levels expected for 2100 (Collard et al., 2015; Asnaghi et al., 2014).

In adults the buffer capacity of the coelomic fluid under reduced pH was also investigated. Catarino et al (2012) showed that the same ratio Mg/Ca was maintained in coelomic fluid of *P. lividus* exposed to different pH treatments (8.0, 7.7, 7.4) and the influence of passive skeleton dissolution on extracellular fluid buffer capacity was excluded. When exposed to low pH (7.7 and 7.4), *P.lividus* increased coelomic fluid buffer capacity, but only at 7.7 pH (and not at 7.4 pH) it was able to compensate coelomic pH. In addition, CF buffer capacity in *P. lividus* increased with feeding and was not different between genders (Collard et al., 2015).

Campbell et al., (2016) found that *P. lividus* sperm exposed to 7.7 pH reduce the swimming speed by 18.8 % and motile sperm by 9.8 %, together with a significant reduction of swimming linearity and straightness. Furthermore, positive relation between swimming speed and paternity was highlighted and maintained across pH treatments, but positive relationship between sperm motility and male fitness was lost at low pH.

Only one experiment on pre-exposed parents was performed with *P. lividus*. Sperm obtained from males maintained for 6 months at pH similar to average future values (750 pCO_2), exhibit higher VCL compared to sperm from control group when measured at their respective acclimatory pCO_2 level (Graham et al., 2015).

At pH values close to levels expected for 2100, negative effects on fertilisation rate were observed, together with slower cleavage rate and larval growth during a 48h exposure period (Cohen-Rengifo et al., 2013; Moulin et al., 2011; Martin et al., 2011).

When exposed at low pH for 35 days, *P. lividus* larvae showed higher mortality rate at pH 7.4 respect to controls (8.1 pH), but not at pH 7.7. At 7.7 pH, larval development speed increased and larval morphology at a given stage did not differ respect to controls. Nevertheless, settlement at 7.7 pH was delayed but the test diameter of metamorphosed larvae was larger compared to controls. At 7.4 pH, delay in development and no settlement were observed (Garcia et al., 2015).

The distribution of *P. lividus* and *Arbacia lixula* around carbon vents at Vulcano, Italy, was studied in relation to the pH gradient present in this area. Significant negative relationship between abundance and increased seawater pCO_2 was observed around carbon vents by Hall-Spencer et al. (2008) and Calosi et al. (2013). Echinoderms along with molluscs exhibit poorest acidbase regulation ability amongst the phyla, thus being amongst the most vulnerable to elevate pCO_2 (Widdicombe et al., 2009; Hale et al., 2011; Christen et al., 2012). Calosi et al. (2013) explain with this low homeostatic ability decreases in abundance of echinoids and bivalves in the presence of increased seawater pCO_2, as observed around the carbon vents at Vulcano. However, in the same area, Kroeker et al. (2013) did not found differences between *P. lividus* abundance or size in natural and low pH zones, even though they reported a significantly lower number of urchin grazing halos in low pH zones. This difference was explained with reduced grazing rates due to physiological and behavioural impacts on sea urchins and/or increased growth rates or nutrient quality of algae in response to increased pCO_2.

3.3. Pharmaceuticals in marine environment

Interest regarding the presence of pharmaceuticals and their metabolites in aquatic environments, began three decades ago (Richardson et al., 1985; Aherne and Briggs, 1989). In the last few years, with improvement of analytical capacity and the possibility to quantify concentrations in low range (ng l^{-1}), there is an increasing attention to define pharmaceutical concentrations in natural waters (Lolić et al., 2015). High amounts of drugs, belonging to the most common therapeutic classes, such as antibiotics, are used in quantities similar to those of many agrochemicals or other micropollutants (Jones et al., 2001). In England, 25 of most common pharmaceuticals are used in amounts of over 10 tons per year and, in particular, over 100 tons

per year are estimated for consumption of Paracetamol, Metformin hydrochloride and Ibuprofen (Jones et al., 2002).

After administration, some drugs can be degraded in the body and become completely inactive, but others, like those excreted renally or not fully absorbed from the gut, can be excreted in their active forms (Ares, 1999). Because of so high consumption, sewage system, and then waste water treatment plants (WWTPs), receive daily tons of these compounds. The removal efficiency of pharmaceuticals in WWTPs is extremely variable, with compounds which pass these plants almost intact and others presenting close to 100% removal efficiency (Kunkel and Radke, 2012). Due to incomplete removal, the presence of these compounds is actually documented in all aquatic compartments: surface waters, ground waters, seawater and even tap waters (Loos et al., 2010, Loos et al 2013a,b). Discharge of drugs in seawater occurs mainly from municipal effluent discharge and from marine aquaculture, but also from agriculture and aquatic activity (Grigorakis et al., 2011). Coastal seawaters may be more susceptible to present higher pharmaceutical concentrations than offshore seawater because of high population density and many economic activities in coastal areas (Martínez et al., 2007). More than one hundred pharmaceuticals and their metabolites have been detected in marine coastal waters with a wide range of concentrations, from picograms to micrograms per liter (Gaw et al., 2014). Most frequently detected compounds are antibiotics, non-steroidal anti-inflammatories and analgesics. In European coastal areas, most frequently detected antibiotics are erytromycin, sulfamethoxazole, metronidazone and trimethoprime ranging from 0.2 to 870 ng l^{-1} (Hughes et al., 2012; , Sekovski et al., 2012;, Crain et al., 2009; Love et al., 2011; Le et al., 2005, Minh et al., 2009, Kookana et al., 2014; Metcalfe et al., 2011). In American and Asian coastal areas, high environmental concentrations are detected for doxycyline, nofroxacin, ofloxacin and sulfamethoxazole, ranging from 3 to 6.8 µg l^{-1} (Daughton, 2003; Richardson et al., 2005; Zhao et al., 2010).

Among non-steroidal anti-inflammatories and analgesics, diclofenac, ibuprofen, naproxen, ketoprofen, salicylic acid, acetaminophen and codeine are the most detected in North, Mediterranean and Adriatic Sea and even in Pacific and Indian Ocean at concentration up to few hundred ng l^{-1} (Rodriguez-Navas et al., 2013; Loos et al., 2013b; Vidal-Dorsch et al., 2012; Gros et al., 2012; Wille et al., 2010).

Contrary to other compounds, pharmaceuticals are biologically active molecules designed to interact with their biological targets, in human and veterinary use as well. Therefore, they have the potential to affect also non target species with detrimental effects on specific functions and

processes (e.g., development, growth, and reproduction) even at low concentrations such as those detected in the environment (Franzellitti et al., 2015). Due to high consumption, and low removal efficiency, there is a continuous input of pharmaceuticals into the environment, and most of them are also persistent because they are designed to retain their chemical structure long enough to do their therapeutic work, so many of them may remain in the environment for long time (Ternes, 2000).

Presently, even if there is increasing concern toward the risks for the potential adverse effects on marine organisms exposed to these compounds, Environmental Risk Assessment procedures for pharmaceuticals in the marine ecosystems is still lacking (Mezzelani et al., 2016).

Recently, European Union established regulatory guidance to assess the presence of pharmaceuticals in the aquatic environment (Directive 2013/39/EU amending Directives 2000/60/EC and 2008/ 105/EC) (European Commission, 2013) as regards priority substances in the field of water policy. A watch list of pharmaceuticals was created including the sex hormones 17alpha-ethinylestradiol and 17beta-estradiol, and the NSAID diclofenac. Regarding marine waters, monitoring programmes for continuous evaluation of their environmental status should be established according to the Marine Strategy Framework Directive (European Commission, 2008), including the evaluation of the impact of hazardous substances as pharmaceuticals.

3.3.1. Clofibric acid (CA)

CA is a blood lipid regulator used in human medical care. It was first marketed in the US in 1967 and its annual production is estimated in the low kiloton range (IARC, 1996). Therapeutic doses are usually relatively high (1-2 g day^{-1} per person) and therapy is extended for long, sometimes life-long, period (Buser et al., 1998). CA is an acidic pharmaceutically active compound with 3.2 pKa value (Avdeef et al., 2000; Gao and Deshusses, 2011). It is regarded as highly mobile and persistent in the environment with an estimated lifespan in the environment of 21 years (Buser et al., 1998; Zuccato et al., 2000; Winkler et al., 2001; Tixier et al., 2003) and its occurrence in the aquatic environment is documented since forty years ago in different compartments. The highest concentrations of CA in European marine coastal areas were reported by Loos and colleagues (2013) in the Adriatic Sea in Italy (0-186 ng l^{-1}) and by Thomas and colleagues (2004) in five estuarine areas in United Kingdom (98.4-111 ng l^{-1}). In the North Sea concentrations ranged from 0.01 to 18.6 ng l^{-1} (Buser et al., 1998; Spongberg et al., 2011; Weige et al., 2005) and the same maximum concentration was found in Elba estuary in Germany (Weigel et al., 2002). In extra-

European seawaters, in the northern coast of Taiwan in particular, concentration ranging from 1.5 to 30 ng l^{-1} were reported (Fang et al., 2012).

3.3.2. Caffeine (CAF)

CAF is a potent stimulant of the central nervous system and is frequently used as coadjuvant in prescription and non-prescription drugs including stimulant tablets, headache and cold remedies, tablets for the relief of menstrual pain, weight control aids and diuretics in concentrations from 15 to 200 mg/tablet or capsule. About 80-90% of caffeine extracted from green coffee is used in the beverage industry and the remainder and synthetic caffeine are used in pharmaceutical applications (IARC, 1991). Even if it is efficiently removed in well-functioning wastewater treatment plants, due to its extensive use, caffeine has a widespread distribution and is present in all waters influenced by human domestic emissions to such a point to be proposed by several authors as a tracer for domestic sewage (Seiler et al., 1999; Siegener and Chen, 2002; Buerge et al., 2003). CAF is a weak base with pKa of 14 (Zylber-Katz et al., 1984). Half-live for this compound in estuarine and coastal marine samples vary between 3.5 to >100 days (Benotti and Brownawell, 2009). In literature relatively high concentrations of caffeine (82–367 ng l^{-1}) were reported for the northern Adriatic Sea and for the Boston Harbour (140-1600 ng l-1) (Loos et al., 2013b; Siegener and Chen, 2008). In sampling sites located in different areas, variable concentrations were detected: 2-16 ng l^{-1} in the open North Sea (Weigel et al., 2002), 30-74 ng l^{-1} close to Stockholm in Sweden (Magnér et al., 2010), 7–87 ng l^{-1} close to Tromsø in Norway (Weigel et al., 2004), 5–71 ng l^{-1} in MassachusettBay seawater (Siegener and Chen, 2008) and 5–149 ng l^{-1} on the west coast of Vancouver Island, British Columbia, Canada (Verenitch and Mazumder 2008).

3.3.3. Diclofenac (DCF)

DCF is the most widely prescribed nonsteroidal anti-inflammatory drug worldwide (McGettigan and Henry, 2013) with anti-inflammatory, analgesic, and antipyretic properties used as active ingredient in many oral and topic formulations (Memmert et al., 2013). It was introduced in 1973 (Lesney, 2004) and its estimated worldwide annual consumption in human and veterinary medicine is >1000 tons (Zhang et al., 2008). In last decades it has been regularly detected in surface waters also because of its low biodegradability and the low capacity of activated sludge to adsorb this compound in sewage treatment plants (Zhang et al., 2008; Pal et al., 2010). Diclofenac is an acidic compound with pKa=4 (Carter et al., 2014).

In European coastal and estuary areas, higher concentrations of DCF were found in Ireland and United Kingdom ranging from 60 to 550 and from 57 to 195 ng l^{-1}, respectively (McEneff et al., 2014; Thomas and Hilton, 2004). Lower concentrations were detected in coastal areas of other countries of Europe: 7.7-63.4 ng l^{-1} in Seine Estuary, in France (Togola and Budzinski, 2007), 6.2 ng l^{-1} in Elbe Estuary, in Germany (Weigel et al., 2002) and 2 ng l^{-1} near Stockholm in Sweden (Wahlberg et al., 2011). In extra-European seawaters, highest values were observed in Yangtze Estuary and coastal zone, in China (283-843 ng l^{-1}) and in the northern coast of Taiwan (2.5-53.6 ng l^{-1}) (Fang et al., 2012; Yang et al., 2011).

3.3.4 Ibuprofen (IBU)

IBU is one of the most important and widely used human non-prescription medicine. It is a nonsteroidal anti-inflammatory drug, used in treatment of rheumatic disorders, pain and fever (Davies and Avery, 1971) with a relatively high therapeutic dose (600-1200 mg day^{-1}). Ibuprofen's physicochemical properties together with many published analytical chemistry data suggest rather high mobility in aquatic environment and low persistence compared with other pharmaceuticals (Buser et al., 1999). It is an acidic pharmaceutical with pKa=4.91 (Jones et al., 2002). Estimated half-life in the field is 32 days (Tixier et la., 2003). Highest concentrations in marine environment were reported in United Kingdom in Tyne River Estuary and Five Estuary regions ranging from 144 to 2370 and from 124 to 928 ng l^{-1} respectively (Roberts and Thomas, 2006, Thomas and Hilton, 2004).

3.3.5. Propranolol (PR)

PR is a beta-adrenergic receptor blocker. This group of drugs is used in cardiovascular diseases and represents one of the most important groups among prescription drugs. Annual consumption, estimated in Germany, reach 100-250 tons (Schwabe and Paffrath, 2004). It is a weak base with pKa=9.5 (Carter et al., 2014). The highest concentrations were detected in Europe in Tyne River Estuary in United Kingdom (35-107 ng l^{-1}) and in extra-Europe regions in Yangtze Estuary in China (RANGE)(Yang et al., 2011).

3.3.6. Sulfadiazine (SD) and trimethoprim (TMP)

SD and TMP are antibiotics and they are efficient against Gram-positive and Gram-negative bacteria, the first by inhibiting the tetrahydrofolat synthesis and the second by inhibiting dihydrofol acid reductase (Rang and Dale, 1987). They are among the most frequently prescribed antibiotics for human and veterinary use (Managaki et al., 2007) since 1969 (Kasanen et al., 1978). TMP has basic nature with pKa 7.3 whereas SD has acidic nature and pKa 6.43 (ElShaer et al., 2012; Lin et al., 1997). Data about environmental concentrations in marine coastal areas for SD highlight significant concentrations in China ranging from 0.1 to 71.8 ng l^{-1}. Presence of TMP is reported in many European and extra-European marine coastal areas. In Europe the highest concentrations were detected in Ireland (60-870 ng l^{-1}) and United Kingdom (11-569 ng l^{-1}) (McEneff et al., 2014; Thomas and Hilton, 2004).

3.3.7. Triclosan (TRC)

TRC also known as Irgasan, is an antibacterial agent widely used in soaps, toothpastes, skin creams, first-aid products and plastics (Jones et al. 2000; Orvos et al. 2002). It presents low water solubility (12 mg l^{-1}), high lipophilicity (log Kow of 4.8) and a pKa value of 8.1 (Reiss et al. 2002). Triclosan is incompletely removed in wastewaters treatment plants and remains in effluent water as well as in sewage sludge biosolids (Stasinakis et al. 2008; Higgins et al. 2011). Its concentrations ranged from 0.01 to 6.9 ng l^{-1} in seawater samples from the German Bight (Xie et al., 2008) and it was also detected in seawater from Tai Po and Victoria Harbours in Hong Kong at concentrations from 15 to 110 ng l^{-1} (Wu et al., 2007).

3.4. Interaction between stressors

Coastal ecosystems are characterized by high variability in chemico-physical features of seawater, due to both natural environmental conditions (e.g., freshwater input, upwelling, atmospheric deposition), and human activities (e.g., organic matter and nutrient inputs, pollution by toxic organic compounds and metals, acid rains, climate change effects and over fishing.).

More than in other marine environments, in coastal areas ocean acidification is acting together with many other rapid changes that may proceed synergistically and modify the evolutionary rules and shape of marine ecosystems (Hall-Spencer et al., 2015).

Pollution, together with overexploitation, habitat loss and invasive species, is considered the most widespread and pressing threat among anthropogenic threats in coastal marine environments (Crain et al., 2009). Furthermore, the presence of emerging contaminants, such as

pharmaceuticals, in coastal marine areas is expected to increase, mainly due to increasing population inhabiting these areas and increasing pressure by some human activities, such as aquaculture (Burridge et al., 2010).

Due to the presence of extremely high numbers of chemicals in all environments, several national and international institutions have highlighted the need to evaluate the risks of exposure to pollutant mixtures and multiple stressors (NRC, 1994; PCCRARM, 1997; Mileson et al., 1999; US EPA, 2000, 2003; ATSDR, 2004; WHO, 2009; European Scientific Committees, 2011).

In the environment the organisms are rarely exposed to a single stressor, but they mainly experience a combination of different stressors and probably traditional chemical risk assessment, focalized on single compounds is not realistic enough to evaluate a real threat for living organisms (Callahan and Sexton, 2007).

In addition to interactions between chemicals, there are possible interactions with other factors, such as climate variables (Schlink et al., 2002; Leitte et al., 2009), which need to be addressed.

In particular, changes in pH values influence the chemical properties of most pharmaceuticals (Banni et al., 2015; Nichols et al., 2015). The degree of ionization of pharmaceuticals depends on pH of the medium and chemical properties of compounds, such as the ionisation constant (pKa).

For example, unionized and more lipophilic form of the b-blocker propranolol and the antidepressant fluoxetine increase with increasing pH (Owen et al., 2009; Brooks et al., 2003); consequently, they are expected to be more effectively adsorbed onto particulate matter or bioaccumulated by the organisms.

Compounds whose lipophilic and more toxic forms increase under reduced seawater pH could represent in future higher harmfulness even if their nominal concentrations in the environment remain the same.

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4. RESULTS

4.1. RESEARCH ARTICLE I: **Acclimation to low pH during gametogenesis in *Paracentrotus lividus***

4.1.1. INTRODUCTION

Ocean uptake of anthropogenic CO_2 is inducing ocean acidification (OA) and is changing seawater carbonate chemistry. This phenomenon represents a challenge for several marine organisms. Long-term exposure and transgenerational studies are needed to produce data for accurate future predictions about the effects of ocean acidification. Studies on acclimation of adults to altered conditions during gametogenesis could provide information about species' ability to cope with changing environment and about phenotypic plasticity of both parents and their offspring. To date, all acidification studies on *P. lividus* have been focused on the skeletal mechanical properties and acid-base balance of adults, performance of sperm and early development of larvae (Asnaghi et al., 2014; Collard et al., 2013, 2015; Catarino et al., 2012; Cohen-Rengifo et al., 2013; Campbell et al., 2016; Martin et al., 2011; Moulin et al., 2011). Only one study cover the entire larval development (Garcia et al., 2015) and only one study was made on sperm performance using pre-conditioned males (Graham et al., 2015). Furthermore, in no previous study differences in the response of males and females have been investigated, partially due to the difficulty of determining sex non-destructively. There are evidence that OA can differentially affect physiology, reproduction, biochemistry and survival in males and females of marine invertebrates (Ellis et al., 2014; Lane et al., 2015; McClellan-Green et al., 2007). When exposed to OA, organisms may change their resource energy allocation and due to higher production cost of eggs compared to sperm, females could be consider more vulnerable to this stressor. Only in 3.77% of 511 acidification studies (published between January 2008 and May 2016) on fish, crustaceans, echinoderms and molluscs, sex-based differences were assessed. When tested, sex significantly influenced the response to OA suggesting that sex have to be consider in order to correctly evaluate the impact at population level (Ellis et al., 2017).

Here, adult *P. lividus* were exposed at three pH values (8.1, present average value for shallow and superficial sea waters; 7.7, value predicted for the year 2100; 7.4, extreme predicted value by 2100) for two months (Etheridge et al., 1996; Gattuso and Hansson, 2011). Physiological, biochemical, immunological and reproductive responses were investigated separately in males and females.

4.1.2. MATERIALS AND METHODS

Species collection

About 140 adult specimens of *P. lividus*, with live weight of 36.7±11.4 g and test diameter of 4.5±0.5 mm, were collected by SCUBA divers at *ca*. 5 m depth in the vicinity of the Hydrobiological Station "Umberto d'Ancona" in the south basin of the Venice Lagoon (NW Adriatic Sea, Italy) between February and March 2017. In order to obtain individuals at the same stage of gametogenesis and to recognize male and female sea urchins, during the first week of April 2017, all individuals were induced to spawn, by injecting 0.5 ml of 0.5 M KCl solution into the coelom, through the peristome membrane. After that, animals were allowed to recover and acclimate to indoor conditions, at 20 °C temperature , 33 PSU salinity and at normal pH (8.1), for a week.

Adult sea urchin culture system

After acclimation period, post-spawning specimens of *P. lividus* were cultured under three pH values: 8.1 (8.06±0.06), 7.7 (7.67±0.05) and 7.4 (7.40±0.06). Three 60 l tanks, each with at least six males and nine females, were used for each experimental condition. Each tank was filled independently with filtered sea water (5 µm) at a flow rate of 300 ml/min and was equipped with an aerator. In low pH (7.7 and 7.4) replicates, the pH value was maintained constant, by bubbling CO_2 thanks to pH electrodes (ACQ310N-PH by Aquatronica) placed in each tank and connected to electronic pH controlling device (ACQ110 Aquarium Controller Evolution by Aquatronica).

In order to promote the gonadal maturation, sea urchins were fed *ad libitum* with fresh macroalgae, *Ulva rigida*, and maintained at 18 °C temperature (Grosjean et al., 1998) and 9hL:15hD photoperiod (Spirlet et al., 2000).

At each experimental condition, adult responses were evaluated through measurements of respiration rate (after 7, 14, 21 and 40 days), ammonia production (after 7, 14 and 21 days) and assimilation rate (after 7 and 14 days) on four males and four females for experimental condition. After 40 days, righting time was measured on six males and six females. After two months of exposure, six males and six females (two per replicate tank) were used to evaluate gonadosomatic index, superoxide dismutase (SOD) and catalase (CAT) activity in gonads and digestive system, coelomocytes number and volume (TCC and CV respectively) and lysozyme activity in haemolymph and coelomocytes.

Respiration rate

To measure oxygen consumption, a sea urchin was placed in 0.8 l glass respirometry chamber with a magnetic stirring rod placed under a perforated bottom. Chambers were filled with 0.45 µm filtered seawater at each pH studied and held in an incubator at 20 °C. Oxygen concentration was measured after 30, 60 and 90 minutes using an optical oxygen meter (Fiber-Optic Oxygen Meter - Piccolo2, Pyro Science GmbH, Aachen, Germany). Oxygen saturation never fell below 70 % during the trial.

Oxygen uptake ($\mu molO_2\ min^{-1}\ g^{-1}$) was calculated by multiplying the slope of the oxygen depletion curve by the volume of seawater inside the chamber and dividing by the live sea urchin weight. The volume of water was determined by subtracting the volume of each sea urchin from the total volume in the chamber.

Ammonia excretion

Ammonia excretion ($\mu molN\text{-}NH_3\ min^{-1}\ g^{-1}$) was measured on water samples collected from each respirometry chamber after 90 minutes and was calculated from the difference in ammonia concentration between the chambers with and without animals and referred to sea urchin live weight.

Assimilation efficiency

To measure assimilation efficiency, sea urchins were placed individually in 2.5 l beakers filled with filtered seawater (0.45 µm) for 24h at 20 °C and during this period they were not fed. Faeces from each backer were vacuum-collected and filtered on weighed filters and rinsed with distilled water to remove salt. Filters were dried for 24h at 60°C and dry weight was registered before ashing in muffle furnace at 450 °C for 4h (Conover, 1966; Reid et al., 2010). Then, filters were reweighed and ash weight was determined. The same process was applied in triplicate on diet samples. Organic content (OC) was calculated as ash-free dry weight represented by the difference between dry and ash weight of faeces or algae. Absorption efficiency (AE) was calculated according to Conover (1966): $AE = [(Diet_{OC} – Faeces_{OC})/(1\text{-}Faeces_{OC})xDiet_{OC}]x100$.

Righting time

Each animal was tested 3 times in water coming from its tank. At the beginning of the trial, the sea urchin was placed on its aboral surface and the time for the animal to right itself completely

was recorded. The test was carried out using a 5 l plastic rectangular container with smooth surface and large enough to avoid contact between vertical walls and the animal. After the first experimental trial, water was completely removed from the container in order to detach the sea urchin without disturbing it. Each individual sea urchin was maintained in its experimental tank for 1h before the repetition of the measurement.

Gonadosomatic index (GSI)

Live sea urchins were weighed and dissected to obtain the gonads. Dissected gonads were weighed and GSI was calculated as percentage of fresh weight of gonads respect to total fresh weight.

Superoxide dismutase (SOD) and catalase (CAT) activity

Gonads and digestive tract were dissected from each sea urchin and aliquots of the tissues were placed in tubes and immediately frozen at -80 °C until analysis. The gonads and digestive tract were thawed on ice and homogenised (1:4, w:v) in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.15 MKCl, 0.5 Msucrose, 1 mMEDTA, 1 mMDithiothreitol (DTT, Sigma) and 40 µg/ml Aprotinin (Sigma). They were centrifuged at 12,000g for 45 min at 4 °C and supernatants (SN) were collected for assays.

Total SOD activity was measured in the gonads and digestive tract with the xanthine oxidase/cytochrome C method in accordance with Crapo et al. (1978). Tissues were homogenised as described above, and the cytochrome C reduction by superoxide anion generated by xanthine oxidase/hypoxanthine reaction was detected spectrophotometrically at 550 nm at room temperature (20°C). Enzyme activity was expressed as U/mg of proteins with one unit of SOD being defined as the amount of sample producing 50% inhibition in the assay conditions. The reaction mixture contained 46.5 µM KH2PO4/K2HPO4 (pH 8.6), 0.1 mM EDTA, 195 µM hypoxanthine, 16 µM cytochrome c, and 2.5 µU xanthine oxidase. For SOD assay, as well as for the other biochemical assays, tissue protein concentrations were quantified in accordance with Bradford (1976).

Gonad and digestive tract CAT activity was measured following the method described in Aebi (1984). Decreases in absorbance of a 50 mM H2O2 solution (Ɛ = -0.0436 mM-1 cm-1) in 50 mM phosphate buffer (pH 7.8) and 10 µl of S12 were continuously recorded at 240 nm and at 10-s

intervals for 1 min. The results were expressed in U/mg of proteins with one unit of CAT being defined as the amount of enzyme that catalysed the dismutation of 1 µmol of H2O2/min.

Haemolymph collection

2 ml of haemolymph were collected from the peristomial membrane of each animal, with a plastic syringe, and stored in ice. 1 ml of haemolymph was used to determine the total coelomocyte count (TCC) and coelomocyte volume (CV) and 1 ml was used to measure both lysozyme-like activity and total protein concentration in coelomocyte lysate (CL) and cell free haemolymph (CFH).

Total coelomocyte count and coelomocyte volume

A Coulter counter (Z2 mod., Beckman Coulter) was used to determine TCC and CV after adding 1 ml of haemolymph to 20 ml of filtered seawater (0.45 µm). TCC results were expressed as the number of coelomocytes ($x10^6$) ml $haemolymph^{-1}$. The haemocyte volume was expressed in picolitres (pl).

Lysozyme activity

Lysozyme activity was quantified in both CFH and CL. Haemolymph from each sea urchin was centrifuged at 780 g for 10 min. The supernatant, corresponding to CFH, was collected, whereas the haemocytes were resuspended in distilled water and sonicated at 4°C for 1 min. CL and CFH were frozen and stored at -80°C before analyses. 50 µl of CL and CFH were added to 950 µl of a 0.15% suspension of *Micrococcus lysodeikticus* (Sigma) in 66 mM phosphate buffer, pH 6.2, and the decrease in absorbance (ΔA min-1) was continuously recorded at 450 nm for 5 min at room temperature. Standard solutions containing 1, 2.5, 5 and 10 µg lysozyme per ml of 66 mM phosphate buffer, pH 6.2, were prepared from crystalline hen egg white lysozyme (Sigma). The average decrease in absorbance per minute was determined for each enzyme solution, and a standard curve of enzyme concentration versus ΔA min^{-1} was drawn.

One unit of lysozyme was defined as the amount of enzyme producing activity equivalent to 1 µg of lysozyme, in the conditions described above. Results were expressed as µg lysozyme mg $protein^{-1}$.

Statistical analysis

Differences in oxygen uptake, ammonia excretion and assimilation efficiency were tested by 3-way ANOVA followed by multiple comparisons with Tukey HSD method. For all the other parameters considered, differences between treatments were tested by Mixed model analysis followed by Multiple comparisons of means with p values adjusted by Holm method. All statistical analyses were performed using R software (R Core Team 2013).

4.1.3. RESULTS

Respiration rate

Respiration rate (Fig. 1) was significantly affected by pH value and sex, whereas no effect of the experimental duration was found. Throughout the experiment, respiration rate was lower in sea urchins kept at 7.7 pH compared to control condition, and in females when compared to males. In particular, males maintained at 8.1 pH presented higher respiration rate than males maintained at 7.7 pH and females at 8.1 and 7.7 pH (Table 1).

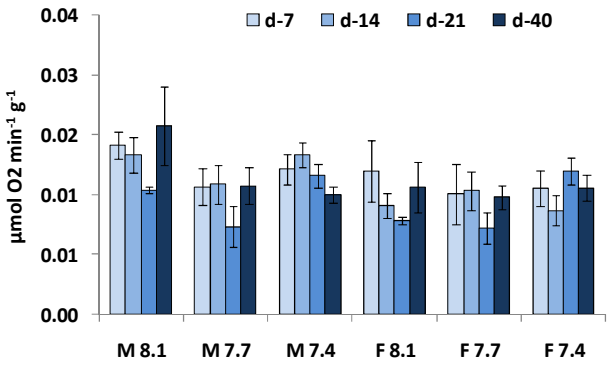

Fig. 1 Respiration rate of *P. lividus* males and females after 7, 14, 21 and 40 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.032 *
sex	0.006 **
time	0.084
pH:sex	0.151
pH:time	0.187
sex:time	0.609
pH:sex:time	0.800

pH	p adj.
8.1 : 7.7	0.031 *
8.1 : 7.4	0.774
7.7 : 7.4	0.143

sex	p adj.
M : F	0.006 **

pH : sex

	M 8.1	M 7.7	M 7.4	F 8.1	F 7.7
M 7.7	0.025 *				
M 7.4	0.679	0.539			
F 8.1	0.025 *	1.000	0.530		
F 7.7	0.006 **	0.996	0.250	0.996	
F 7.4	0.093	0.996	0.836	0.996	0.915

Table 1 3-way ANOVA: respiration rate values results.

Ammonia excretion

Significant effects of time and interaction between pH and sex were observed on ammonia excretion (Fig. 2). The rate progressively decreased with time, in particular in males maintained at 7.4 pH (Table 2).

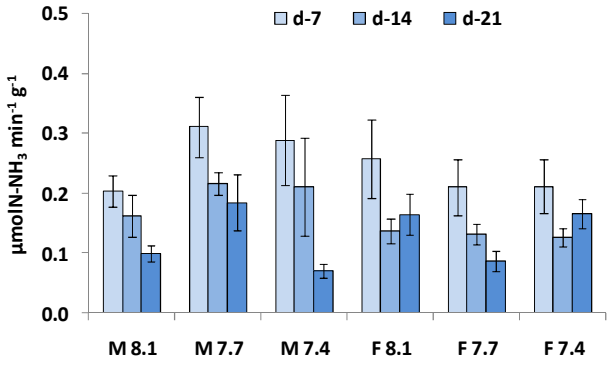

Fig. 2 Ammonia excretion of *P. lividus* males and females after 7, 14 and 21 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.724
sex	0.157
time	$3e^{-5}$ ***
pH:sex	0.037 *
pH:time	0.977
sex:time	0.195
pH:sex:time	0.417

pH	p adj.
8.1 : 7.7	0.703
8.1 : 7.4	0.938
7.7 : 7.4	0.89

sex	p adj.
M : F	0.157

day	p adj.
7 : 14	0.003 ***
7 : 21	0.000 ***
14 : 21	0.313

sex:pH:time	p adj.
M 7.4 d-7 : M 7.4 d-21	0.047 *

Table 2 3-way ANOVA: ammonia excretion values results.

Assimilation efficiency

No effects of the experimental parameters on assimilation efficiency were detected.

Righting time

Although not statistically significant, a decrease in righting time was shown in females under reduced pH, but not in males (Fig. 3).

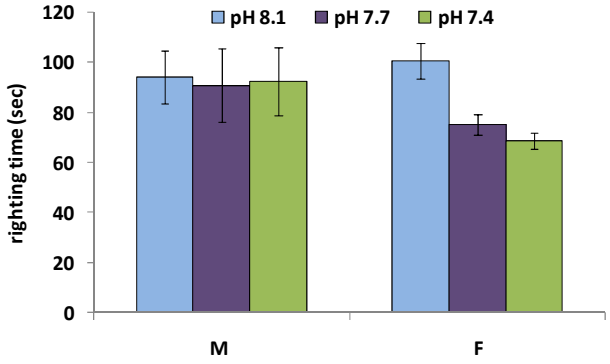

Fig. 3 Righting time of *P. lividus* males and females after 40 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

Gonadosomatic index

GSI values were significantly influenced by pH/sex interaction (Table 3); with decreasing pH, they showed an opposite trend in the two sexes, slightly increasing in males and markedly decreasing in females (Fig. 4). Females maintained at 7.4 pH showed a significant lower GSI compared to females from control (8.1 pH) condition (Table 3).

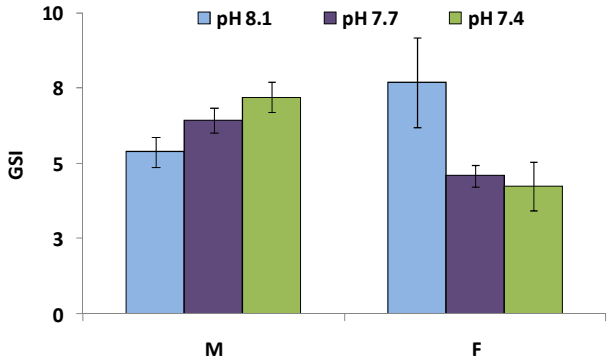

Fig. 4 Gonadosomatic index of *P. lividus* males and females after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.439
sex	0.241
pH:sex	0.006 **

	M 8.1	M 7.7	M 7.4	F 8.1	F 7.7
M 7.7	1.000				
M 7.4	0.908	1.000			
F 8.1	0.417	1.000	1.000		
F 7.7	1.000	0.987	0.301	0.105	
F 7.4	1.000	0.567	0.120	0.035 *	1.000

Table 3 Linear mixed model: GSI values results.

Superoxide dismutase and catalase activity

In both gonads and digestive tract, SOD activity did not vary significantly due to either pH or sex. CAT activity in gonads (Fig. 5) was significantly affected by sex (Table 4) and showed higher values in females than in males. In addition, CAT activity in digestive tract (Fig. 6) showed a significant effect of sex and pH. A significant increase of CAT activity was observed at both 7.7 and 7.4 pH in females (Table 5).

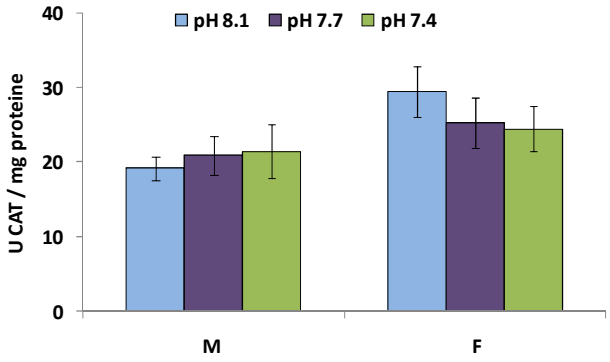

Fig. 5 CAT activity in *P. lividus* male and female gonads after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.888
sex	0.024 *
pH:sex	0.450

Table 4 Linear mixed model: results of CAT activity values in gonads.

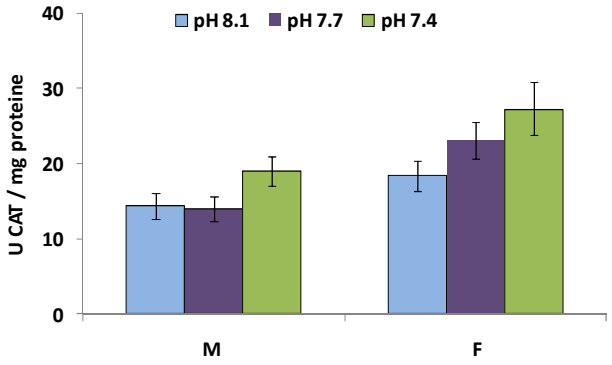

Fig. 6 CAT activity in *P. lividus* male and femaledigestive tract after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.011 *
sex	0.000 ***
pH:sex	0.453

	M 8.1	M 7.7	M 7.4	F 8.1	F 7.7
M 7.7	1.000				
M 7.4	0.900	0.826			
F 8.1	0.900	0.900	1.000		
F 7.7	0.035 *	0.03 *	0.900	0.900	
F 7.4	0.000 ***	0.000 ***	0.058	0.034 *	0.900

Table 5 Linear mixed model: results of CAT activity values in digestive tract.

Total coelomocyte count and coelomocyte volume

TCC (Fig. 7) and CV (Fig. 8) were significantly affected by pH (Table 6 and 7 respectively), showing the highest values at pH 7.4 in both males and females. In females, the number of coelomocytes at 7.4 pH significantly increased when compared to that recorded at 7.7 pH (Table 6).

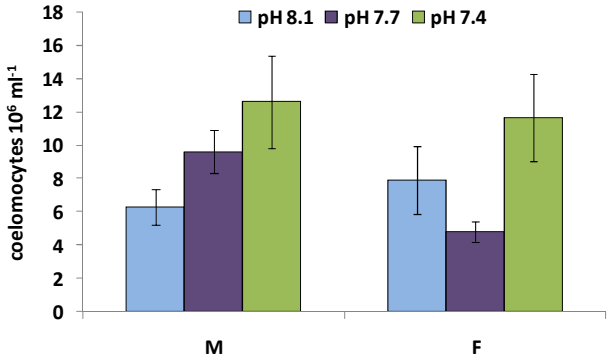

Fig. 7 TCC in *P. lividus* males and females after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.014 *
sex	0.361
pH:sex	0.219

	M 8.1	M 7.7	M 7.4	F 8.1	F 7.7
M 7.7	1.000				
M 7.4	0.173	1.000			
F 8.1	1.000	1.000	0.663		
F 7.7	1.000	0.652	0.034 *	1.000	
F 7.4	0.418	1.000	1.000	1.000	0.099

Table 6 Linear mixed model: results of TCC values.

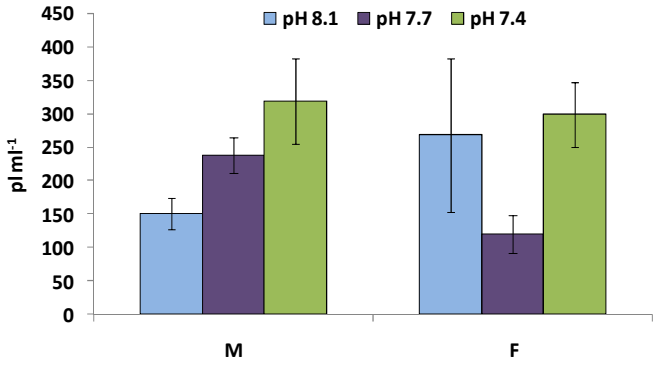

Fig. 8 CV in *P. lividus* males and females after 60 day exposure at 8.1, 7.7, 7.4 pH. Mean ± se.

	pR(>F)
pH	0.031 *
sex	0.360
pH:sex	0.098

Table 7 Linear mixed model: results of CV values.

Lysozyme activity

Lysozyme activity showed the same pattern of variation in cell-free haemolymph (Fig. 9) and coelomocytes (Fig. 10). In both,pH and sex did not significantly affected lysozyme activity and, even though a significant pH/sex interaction was found (Table 8 and 9 respectively). Females showed significantly higher lysozyme activity than males at control condition in CFH. In males at 7.7 pH, a significant increase of this parameter respect to controls was observed (Table 8).

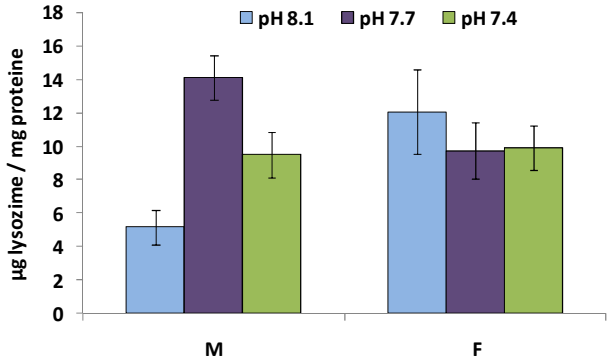

Fig. 9 Lysozyme activity in CFH of male and female *P. lividus* after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.127
sex	0.465
pH:sex	0.006 **

	M 8.1	M 7.7	M 7.4	F 8.1	F 7.7
M 7.7	0.001 **				
M 7.4	0.543	0.510			
F 8.1	0.034 *	1.000	1.000		
F 7.7	0.510	0.543	1.000	1.000	
F 7.4	0.486	0.543	1.000	1.000	1.000

Table 8 Linear mixed model: results of lysozyme activity in CFH values.

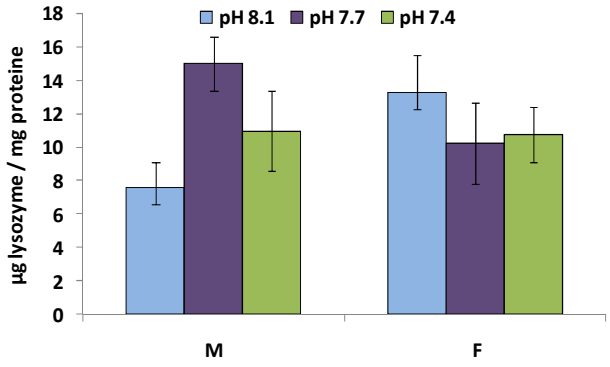

Fig. 10 Lysozyme activity in CL of male and female *P. lividus* after 60 day exposure at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.493
sex	0.880
pH:sex	0.040 *

Table 9 Linear mixed model: results of lysozyme activity in CL values.

4.1.4. DISCUSSION

In this study, a general higher respiration rate was observed in males compared to females, and lower at 7.7 but not at 7.4 pH, respect to 8.1 pH values. After six weeks of acclimation to seawater acidification, decreased respiration rate was found also in *Echinometra mathaei* (Uthicke et al., 2012). In this experiment, ammonia production was reduced with time. In males and females, an opposite response at low pH was observed. Males presented an increase of ammonia excretion as observed in *Strongylocentrotus droebachiensis* in response to 45-days exposure to ocean acidification (Stumpp et al., 2012). Similar results were obtained in marine bivalves (Lindinger et al., 1984; Michaelidis et al., 2005; Thomsen and Melzner, 2010) and sipunculid worm (Langenbuch and Pörtner, 2002). Ammonia production is an indicator of protein metabolism and it was hypothesized that its secretion could act as an additional acid extrusion mechanism in mussels (Thomsen and Melzner, 2010), worms (Langenbuch and Pörtner, 2002) and sea urchins (Stumpp et al., 2012).

In the two sexes, an opposite effect of low pH was observed also in GSI, with a significant decrease at 7.4 pH in females and an increasing trend at low pH values in males. Gonads are very plastic organs in sea urchins that can be used as energy storage. Gonads can be filled or depleted depending on animal conditions. In females, reduction in GSI under low pH could be indicative of detrimental effects and greater energy request to maintain homeostasis, with consequent reduction of energy to invest in reproduction.

SOD and CAT are considered the primary antioxidant enzymes. They prevent oxidative damageremoving reactive oxygen species (ROS) produced during normal metabolism and after

oxidative injury. In particular, SOD dismutates two molecules of superoxide anion(O_2^-) to hydrogen peroxide(H_2O_2) and O_2 and CAT is the most important H_2O_2 scavenger in cells and reduces H_2O_2 to water and O_2. SOD and CAT activities were found to be very high in *P. lividus* gonads suggesting an important role of this organ in sea urchin antioxidant defence. In particular, CAT activity resulted higher than in other invertebrate species highlighting a probable high H_2O_2 production (Perez-Trigo et al., 1995).

In this experiment, SOD activity did not vary significantly due to either pH or sex but CAT activity was significantly different between sexes, with higher activity in females in both gonads and digestive tract. In digestive tissues, significant effect of pH was also observed with significant increases of enzymatic activity at low pH values. A general increased CAT activity was observedalso in two bivalves, the clam *Chamelea gallina* and the mussel *M. galloprovincialis*, in response to low pH and high temperature values (Matozzo et al., 2013).

No differences between sexes were found for TCC and CV. After 60-day exposure, these parameters were affected by pH and showed an increase under lower pH value.

At control condition, higher lysozyme activity was found in females. This confirm findings of other authors which demonstrated that in *P. lividus* females possess higher number of coelomocytes and consequently highest levels of cytotoxic and haemolytic activity. Furthermore, coelomocyte lysate and coelomic fluid agglutinating activity was found more evident in females than in males (Arizza et al., 2013).

In CFH lysozyme activity resulted significantly increased at 7.7 pH in males, whereas in females, despite a decreasing tendency of lysozyme activity with decreasing pH, no significant effect of the experimental conditions was observed. These results could be relevant if considered that coelomocytes have functions ranging from metabolite transport to immunity (Endean, 1966) and that their morphology, size and cell type proportion vary with physiological conditions of individuals as a response to environmental factors, pollutants, pathogens or accidental injuries (Matranga et al., 2000; Pinsino et al., 2007; Ramírez-Gómez et al., 2010). The success of immunity response depends not only on coelomocytes total number, but also on cellular types. Three major cell types in *P. lividus* coelomic fluid are red and white amoebocytes (13%), vibratile cells (7%), and phagocytes (80%) (Pinsino and Matranga, 2014). Lysozyme production is mainly supported by vibratile cells (Smith et al., 2010) and red amoebocytes (Coles and Pipe, 1994) while phagocytes are most abundant immune cell type in *P. lividus* (Pinsino and Matranga, 2014). It was

demonstrated that when exposed to the chemical pollutant lindane, total number of *P. lividus* coelomocytes decrease but number of red amoebocytes increases (Stabili and Pagliara, 2015).

In our experiment an increasing trend of TCC and CV at low pH values was observed only in males, while in females the variations of these parameters were not so linear, making suppose different modulation of coelomocyte number and type when exposed to low pH. Further studies could be useful to ascertain whether effects on cell type proportions can result from exposure to reduce pH, as already observed after pollutant exposure (Stabili and Pagliara, 2015).

In our experiment, significant responses to long-term low pH exposure were observed in all parameters evaluated, except for assimilation efficiency, righting time and SOD activity.

Some responses exhibited the same trend in males and females (respiration rate, CAT activity, TCC) but significant effects were not always present in both sexes. In others parameters, an opposite trend was displayed in two sexes (ammonia excretion, GSI, lysozyme activity). These results underline the importance to investigate further male and female responses separately in order to avoid losing significant effects and to infer more realistic and relevant information at population level.

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4.2. RESEARCH ARTICLE II: **Influence of parent acclimation to low pH during gametogenesis on offspring performance in *Paracentrotus lividus***

4.2.1. INTRODUCTION

Anthropogenic atmospheric carbon dioxide is increasing since the onset of the industrial revolution. Up to a third of CO_2 is absorbed by the oceans leading to 0.1 unit lowered values in current pH of surface seawaters. A reduction of 0.3-0.5 units is predicted by the year 2100 (Houghton et al. 2001; Caldeira & Wickett 2003, 2005; Royal Society 2005; Canadell et al. 2007). Together with pH, increased dissolution of carbon dioxide is reducing calcium carbonate saturation, which may have important consequences for marine organisms, calcifers in particular (Doney et al., 2009). Sea urchin adults and larvae have been considered in many ocean acidification studies. In most studies, each life stage was considered separately and then also the data concerning sea urchin larval development under ocean acidification are mostly produced from adults maintained under ambient pH. Each life stage could have differing degree of sensitivity to environmental stressors, and disturbance in one stage can carry over into the following stage. When exposed to an environmental stressor, females could increase energetic investment in gametes with beneficial effects for offspring but this increased energy expenditure can also induce negative carry-over effects that persist into later stages (Podolsky and Moran, 2006). In marine invertebrates, both positive and negative effects were reported. Oysters pre-exposed to low pH for 5 weeks during gametogenesis showed increased maternal energetic investment in offspring with positive effects on larval growth (Parker et al., 2012). On the other hand, exposure of intertidal barnacles to stressful conditions during the pelagic phase reduced juvenile performance (Emlet and Sandro, 2006).

Long-term exposure of parents to reduced seawater pH during gametogenesis could influence the performance of their offspring, by altering the quality of gametes but also affecting phenotypic plasticity and possibly improving capability of gametes and larvae to cope with the new environmental condition. Transgenerational studies, with adult acclimation to altered conditions, are producing contrasting results respect to short-term exposures acting within the same generation (Donelson et al., 2012; Miller et al., 2012; Form and Riebesell, 2012; Dupont et al., 2013; Calosi et al., 2013). In order to evaluate potential occurrence of adult acclimation effects on offspring performance, in this work, we used gametes obtained from pre-spawned *P. lividus* maintained under different pH conditions (8.1 control, 7.7 and 7.4 pH) for two months. In sperm

from males kept at each experimental pH, sperm velocity, ATP consumption, and oxygen consumption were evaluated at three pH values (8.1; 7.7 and 7.4). Larvae from each parental group were reared at 8.1 and 7.7 pH for 20 days and larval mortality and growth were assessed.

4.2.2. MATERIALS AND METHODS

Sperm collection

Adults were collected, prepared and exposed in sea urchin culture system at three pH values (8.1, 7.7 and 7.4) as described in previous ART III.

After two months of exposure, spawning was induced in male sea urchins by injecting 1 ml of 0.5 M KCl solution into the coelom, through the peristome membrane. Sperm from each male was collected dry using a micropipette and stored in small tubes at 4 °C. In sperm of 5 males from each pH treatment (S1 = sperm from males kept at pH 8.1, S2 = sperm from males kept at pH 7.7, S3 = sperm from males kept at pH 7.4), velocity (VCL = curvilinear velocity), respiration rate and ATP content were measured at three pH values (8.1, 7.7, 7.4).

Sperm velocity

Subsamples of dry sperm from each male were diluted with 0.45 µm filtered seawater (FSW) at 8.1, 7.7 and 7.4 pH, at a concentration of 5 x 10^6 sperm ml^{-1}. Diluted sperm suspensions were maintained at 20 °C of temperature in 24 ml glass vials. Sperm curvilinear velocity (VCL) was measured immediately after activation and then after 5, 15, 30 and 60 minutes. Sperm movement was recorded using Pinnacle Studio 15 (Corel Corporation) software and Leica DM 750 microscope. Video analysis were made with sperm tracker CEROS (Hamilton Thorne Research, Beverly, MA, USA). A minimum of 200 sperm was tracked per time point.

Sperm respiration rate

Subsamples of dry sperm from each single male were diluted in FSW at 8.1, 7.7 and 7.4 pH, at a concentration of 5 x 10^6 sperm ml^{-1}. For each pH, three replicates were set up in 24 ml glass respirometry chambers provided with a magnetic stirring. Chambers were held in an incubator at 20 °C. Oxygen concentration was measured after 0, 5, 15, 30 and 60 minutes using an optical oxygen meter (Fiber-Optic Oxygen Meter - Piccolo2) and during the trial, oxygen saturation never fell below 70 %. Sperm respiration rate was calculated as slope of the oxygen depletion curve

multiplied by the volume of seawater in the respirometry chamber and divided by the number of cells. Results were expressed as $\mu molO_2\ min^{-1}\ 10^9 cell^{-1}$.

Sperm ATP content and consumption

Subsamples of dry sperm from each male were diluted in FSW at 8.1, 7.7 and 7.4 pH, at a concentration of 5×10^6 sperm ml^{-1}. 24 ml glass vials were used and held in an incubator at 20 °C. At each time point (0, 5, 15, 30, 60 minutes), 500 µl of diluted sperm were placed in Eppendorf tubes and immediately frozen at -80 °C until analysis. Endogenous ATP concentrations were measured using a luciferase-based kit (ATPlite) from Perkin Elmer (Boston, USA). Luminescence was measured by a multi-mode microplate spectrofluorometer (Perkin Elmer Envision) and ATP was quantified according to a standard curve. ATP concentration measured immediately after sperm activation (0 minutes) were used to detect differences in ATP content of sperm from different adult treatments (S1, S2 and S3 sperm from males maintained at 8.1, 7.7 and 7.4 pH respectively during gonad maturation). Whole dataset was used to calculate ATP consumption rate, expressed as slope of the ATP depletion curve and defined as $nmolATP\ min^{-1}\ 10^9 cell^{-1}$.

ATP consumption / Oxygen consumption

The ratio between oxygen and ATP consumption was calculated and expressed as $nmolATP/\ nmolO_2$.

ATP consumption/average VCL

Data obtained from VCL analyses were used to calculate average VCL of sperm from different male treatments (S1, S2, S3) when exposed at different pH (8.1, 7.7 and 7.4). ATP consumption rate was divided by average VCL and this value was expressed as $nmolATP\ 10^{15} cell^{-1} \mu m^{-1}$.

Sea urchin larvae culture system

After two months of adult exposure, spawning was induced in 3 male and 5 female sea urchins per experimental pH by injection of 1 ml of 0.5 M KCl solution into the coelom, through the peristome membrane. Sperm from each male was collected dry using micropipettes and stored in small tubes at 4 °C pending fertilization. The eggs from each female were gathered in 250 ml beakers filled with 0.45 µm filtered sea water (FSW). In order to remove spine and algae fragments, the eggs were filtered with 200 µm mesh nylon filter and then retained using 20 µm

mesh nylon filter and suspended in FSW. The density of eggs was evaluated by counting subsamples under binocular microscope and the density of sperm was determined by a Coulter Counter (Beckman Coulter, Mod. Z2) after 1:20000 dilution in FSW. Equal number of eggs from each female were placed in 250 ml beaker filled with FSW. In each beaker with eggs, equal number of sperm from each male was added, maintaining standard sperm:egg ratio (1250:1) according to the recommendations of Dinnel *et al*., 1987. Fertilization success was checked after 15 minutes, ensuring the presence of fertilization membrane in at least 90 % of eggs. After fertilization, embryos (F1= embryos from parents kept at pH 8.1, F2= embryos from parents kept at pH 7.7, F3= embryos from parents kept at pH 7.4), were distributed in tanks at two pH values (8.1 and 7.7) and larvae were harvested for 20 days. Two 50 l tanks per experimental condition (12 cultures in all) were performed at a density of 5 embryo ml^{-1}. Each tank was equipped with an aerator placed on the bottom of the tank in order to gently maintain suspended sea urchin larvae. Throughout the 20-day experiment, 30 % of filtered seawater (2 µm) was replaced in each tank every three days. Larvae were fed every three days with *Isochrysis galbana*, at a density of 30000 cells ml^{-1}in the experimental tanks. Concentration of algae in tanks was verified and suitable quantity of algae was added to maintain constant concentration of food. In low pH (7.7) replicates, the pH value was maintained constant by bubbling CO_2 thanks to pH electrodes (ACQ310N-PH by Aquatronica) placed in each tank and connected to an electronic pH controlling device (ACQ110 Aquarium Controller Evolution by Aquatronica).

Larval mortality

Larval mortality was monitored every 5 days in each tank. 3 l subsample from the tank was concentrated 12 times by vacuum-removing the water. From the remaining 250 ml, three 1 ml aliquots were collected and larval density was evaluated under binocular microscope.

Larval growth

In each tank, larval growth was monitored after 5, 10 and 15 days and lengths of somatic rod (SL) and post-oral rod (POL) were measured on 95, 75 and 43 larvae, respectively.

Statistical analyses

Differences in VCL and larval growth were tested by 3-way ANOVA followed by multiple comparisons with Tukey HSD method. Differences in all other parameters were tested by Mixed

model followed by Multiple comparisons of means with p values adjusted by Holm method. All statistical analyses were performed using R software (R Core Team 2013).

4.2.3. RESULTS

Sperm velocity

Sperm of males maintained at different pH (S1, S2 and S3) did not show significantly different VCL when subject to different post-activation pH (8.1, 7.7, 7.4) (Fig. 1A, B, C). However, velocity of S3

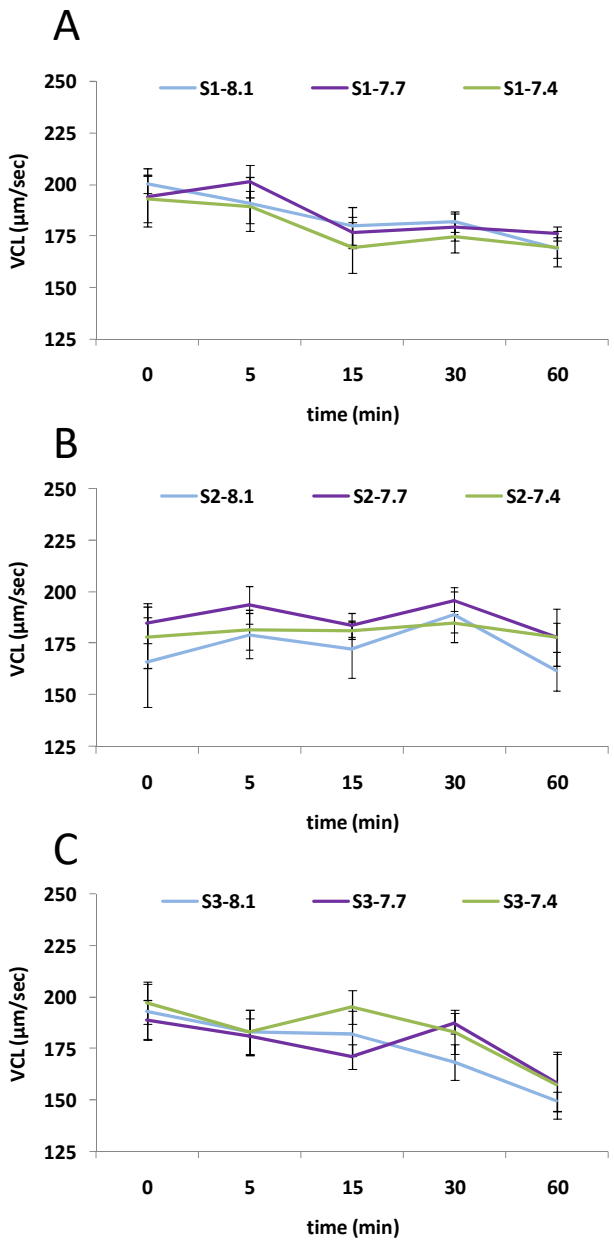

Fig. 1 VCL of sperm from *P. lividus* males maintained at 8.1 (A, S1), 7.7 (B, S2) and 7.4 (C, S3) pH during gametogenesis. Sperm were activated and VCL was measured after 0, 5, 15, 30 and 60 minutes at the three pH values. Mean ± se.

	pR(>F)
pH	0.281
S	0.534
time	$3e^{-5}$ ***
pH:S	0.357
pH:time	0.919
S:time	0.039 *
pH:S:time	0.995

pH	p adj.
8.1 : 7.7	0.252
8.1 : 7.4	0.613
7.7 : 7.4	0.792

S	p adj.
S1 : S2	0.808
S1 : S3	0.504
S2 : S3	0.873

time	p adj.
0 : 5	0.981
0 : 15	0.184
0 : 30	0.612
0 : 60	0.000 ***
5 : 15	0.468
5 : 30	0.906
5 : 60	0.000 ***
15 : 30	0.937
15 : 60	0.079
30 : 60	0.009 **

S:time

	S1-0	S1-5	S1-15	S1-30
S1-5	0.999			
S1-15	0.188	0.611		
S1-30	0.468	0.894	1.000	
S1-60	0.064	0.321	1.000	0.999

	S2-0	S2-5	S2-15	S2-30
S2-5	0.999			
S2-15	1	0.999		
S2-30	0.959	0.999	0.995	
S2-60	1	0.983	0.999	0.781

	S3-0	S3-5	S3-15	S3-30
S3-5	0.991			
S3-15	0.997	1		
S3-30	0.959	1.000	1.000	
S3-60	0.001 **	0.099	0.081	0.209

Table 1 3-way ANOVA: results of VCL values.

sperm (Fig. 1C) significantly decreased throughout 60 min post-activation, whereas in S1 (Fig. 1A) and S2 sperm (Fig. 1B) no significant variation was found (Table 1).

Sperm respiration rate

Only pH (Table 2) significantly affected sperm oxygen consumption (Fig. 2). This parameter was not affected by the condition of sire maintenance (S) and no interaction between factors was observed. Higher oxygen consumption was found at 7.7 pH in all groups of sperm.

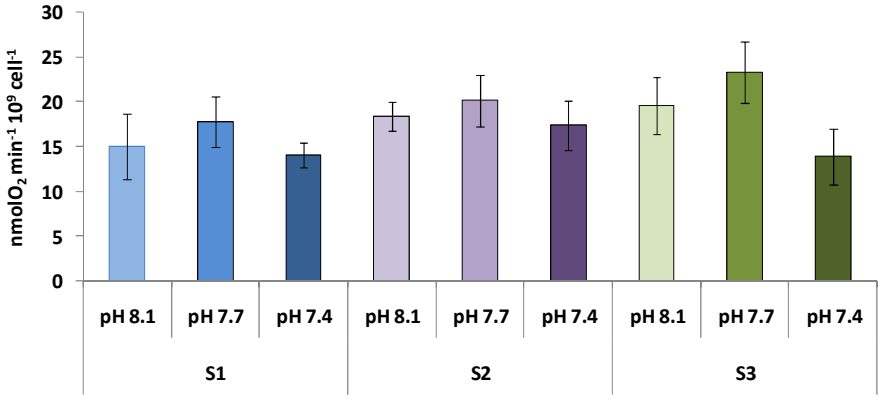

Fig. 2 Respiration rate of S1, S2 and S3 *P. lividus* sperm, measured at the three pH values (8.1, 7.7 and 7.4). Mean ± se.

	pR(>F)
pH	0.045 *
S	0.469
pH:S	0.667

Table 2 Linear mixed model: results of sperm respiration rate.

Sperm ATP content and consumption

Sperm ATP content (Fig. 3) was significantly influenced by pH of sire maintenance. In S3 sperm significantly higher ATP values were detected compared to S1 sperm (Table 3).

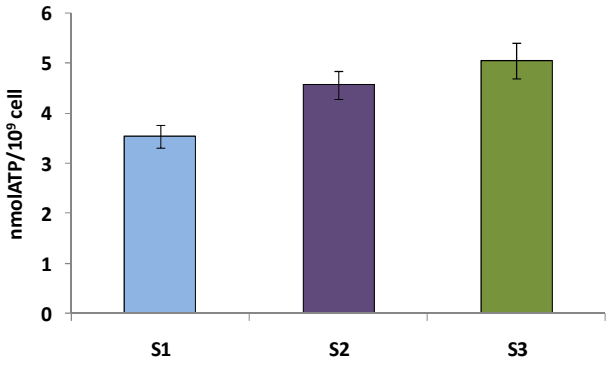

Fig. 3 ATP content in S1, S2 and S3 *P. lividus* sperm. Mean ± se.

	p
S1:S2	0.0553
S1:S3	0.003 **
S2:S3	0.511

Table 3 Linear mixed model: results of sperm ATP content.

pH significantly influenced ATP consumption rate (Fig. 4) and significant interaction between pH and sperm maturation condition (S) was also found (Table 4). Higher values were present at 8.1 pH in all sperm, in particular S2 sperm. When exposed at 8.1 pH, S2 sperm exhibited higher consumption than when exposed at both 7.7 and 7.4 pH and also when compared to S1 and S3 at 8.1 pH. The lowest ATP consumption rate was found in S3 sperm when exposed at 7.4 pH and this value was significantly lower than all values measured at 8.1 pH (Table 4).

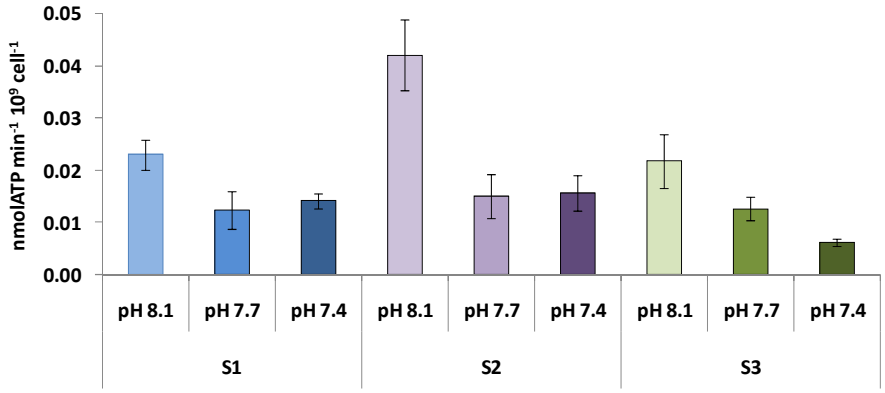

Fig. 4ATP consumption rate of S1, S2 and S3 sperm maintained at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	9*e-8***
S	0.074
pH:S	0.01 **

	S1-8.1	S1-7.7	S1-7.4	S2-8.1	S2-7.7	S2-7.4	S3-8.1	S3-7.7
S1-7.7	0.184							
S1-7.4	0.610	1.000						
S2-8.1	0.011 *	1e-6 ***	2e-6 ***					
S2-7.7	1.000	1.000	1.000	2e-10 ***				
S2-7.4	1.000	1.000	1.000	7e-10 ***	1.000			
S3-8.1	1.000	1.000	1.000	0.005 **	1.000	1.000		
S3-7.7	1.000	1.000	1.000	1e-6 ***	1.000	1.000	0.486	
S3-7.4	0.048 *	1.000	1.000	9e-10 ***	1.000	1.000	0.002 **	1

Table 4 Linear mixed model: results of ATP consumption rate.

ATP consumption/oxygen consumption

ATP consumption/oxygen consumption ratio (Fig. 5) was significantly affected by pH (Table 5) and exhibited higher values at 8.1 pH. This difference from other pH conditions is significant only in S2 sperm when compared to those kept at 7.7 pH.

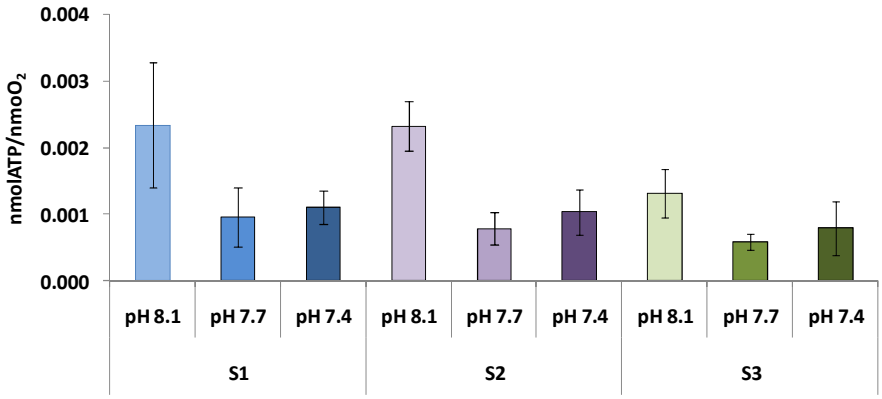

Fig. 5 ATP consumption/oxygen consumption ratio in S1, S2 and S3 sperm maintained at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	0.000 ***
S	0.511
pH:S	0.646

	S1-8.1	S1-7.7	S1-7.4	S2-8.1	S2-7.7	S2-7.4	S3-8.1	S3-7.7
S1-7.7	0.066							
S1-7.4	0.182	1.000						
S2-8.1	1.000	0.796	1.000					
S2-7.7	0.411	1.000	1.000	0.021 *				
S2-7.4	0.990	1.000	1.000	0.130	1.000			
S3-8.1	1.000	1.000	1.000	1.000	1.000	1.000		
S3-7.7	0.182	1.000	1.000	0.184	1.000	1.000	1.000	
S3-7.4	0.411	1.000	1.000	0.421	1.000	1.000	1.000	1.000

Table 5 Linear mixed model: results of oxygen consumption : ATP consumption ratio.

ATP consumption/average VCL

The ratio ATP consumption rate/average VCL confirmed results above reported for ATP, highlighting highest values in S2 sperm at 8.1 pH and lowest values in S3 sperm at 7.4 pH.

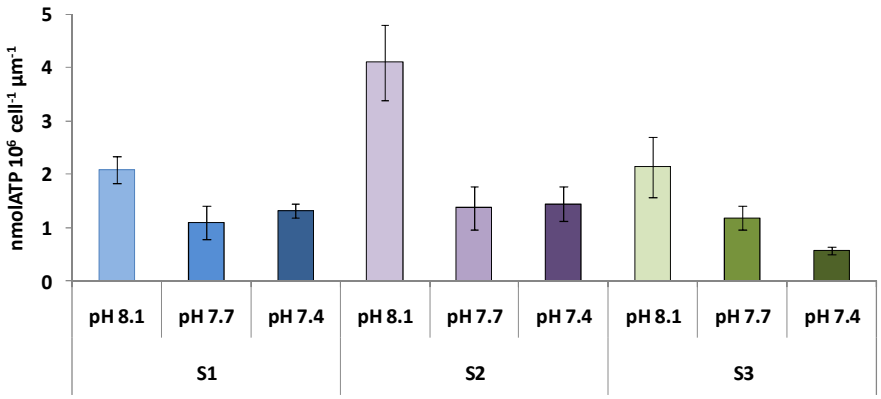

Fig. 6ATP consumption :average VCL ratio of S1, S2 and S3 sperm maintained at 8.1, 7.7 and 7.4 pH. Mean ± se.

	pR(>F)
pH	5e-8 ***
S	0.069
pH:S	0.003 **

	S1-8.1	S1-7.7	S1-7.4	S2-8.1	S2-7.7	S2-7.4	S3-8.1	S3-7.7
S1-7.7	0.232							
S1-7.4	0.966	1.000						
S2-8.1	0.003 ***	2e-7 ***	2e-6 ***					
S2-7.7	1.000	1.000	1.000	1e-11 ***				
S2-7.4	1.000	1.000	1.000	7e-11 ***	1.000			
S3-8.1	1.000	1.000	1.000	0.003 **	1.000	1.000		
S3-7.7	1.000	1.000	1.000	4e-7 ***	1.000	1.000	0.403	
S3-7.4	0.069	1.000	1.000	1e-10 ***	1.000	1.000	0.001 **	1.000

Table 6 Linear mixed model: results of ATP consumption : average VCL ratio.

Larval mortality

In larvae from each parental condition mortality was not significantly different at the two experimental pH (8.1 and 7.7). Mortality was affected by the condition of parent maintenance (F) with higher mortality in F2 and F3 larvae.

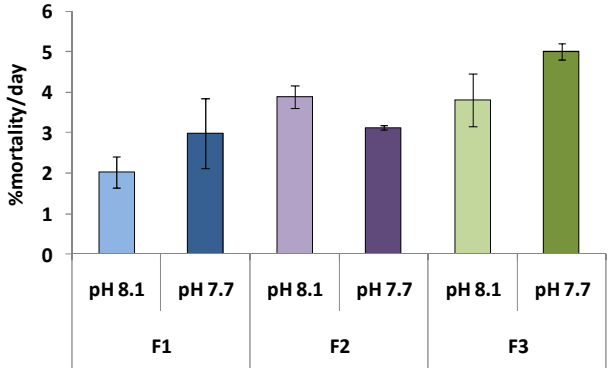

Fig.7 Mortality rate of F1, F2 and F3 *P. lividus* larvae maintained at 8.1 and 7.7 pH for 20 days. Mean ± se.

	pR(>F)
pH	0.293
F	0.023 *
pH:S	0.171

	F1-8.1	F1-7.7	F2-8.1	F2-7.7	F3-8.1
F1-7.7	0.731				
F2-8.1	0.203	0.770			
F2-7.7	0.630	0.999	0.859		
F3-8.1	0.232	0.826	0.999	0.904	
F3-7.7	0.036 *	0.162	0.628	0.202	0.567

Table 7 Linear mixed model: results of larval mortality rate.

Larval growth

Larval growth, expressed as length of the somatic rod (Fig. 8), was significantly affected by all the experimental variables, i.e., pH of parent maintenance (F), pH of larval maintenance (pH) and time (Table 8). F1 and F2 larvae showed significantly smaller SL at 7.7 pH at all sampling times, while F3 larvae did not significantly differ under different pH values at day 15 post-fertilization. When exposed at 8.1 pH larvae from different parent conditions were almost always different with

higher length in F2 and F3. When exposed at 7.7 pH F3 larvae were significantly longer than F1 and F2.

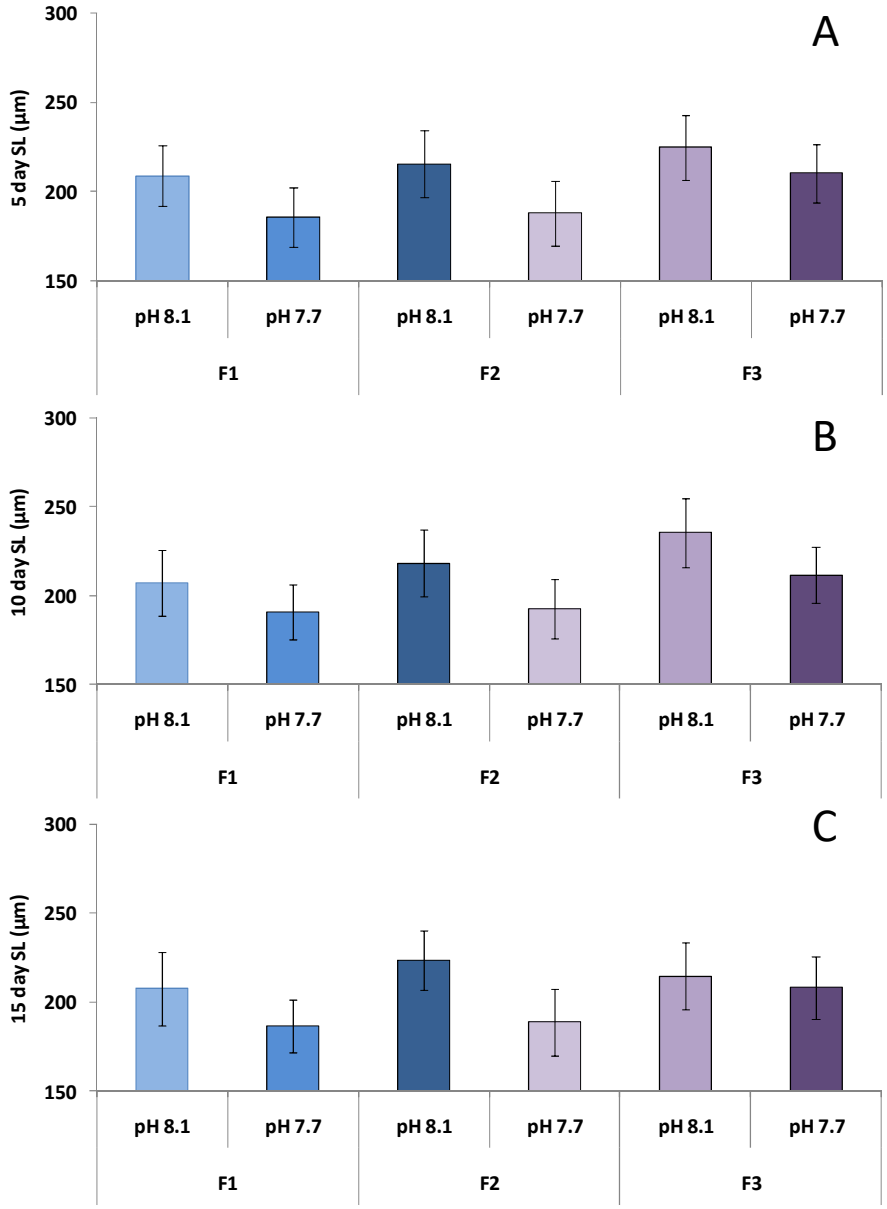

Fig. 8 Somatic rod length of F1, F2 and F3 *P. lividus* larvae exposed at 8.1 and 7.7 pH for 5 (A), 10 (B) and 15 (B) days. Mean ± se.

	pR(>F)
pH	***
F	***
time	***
pH:F	***
pH:time	0.918
F:time	**
pH:F:time	***

SL	d5	d10	d15
F1-8.1 : F1-7.7	***	***	***
F2-8.1 : F2-7.7	***	***	***
F3-8.1 : F3-7.7	***	***	ns

	d5	d10	d15
F1-8.1 : F2-8.1	ns	*	**
F2-8.1 : F3-8.1	*	***	ns
F1-8.1 : F3-8.1	***	***	ns

	d5	d10	d15
F1-7.7 : F2-7.7	ns	ns	ns
F2-7.7 : F3-7.7	***	***	***
F1-7.7 : F3-7.7	***	***	***

Table 8 3-way ANOVA: results of somatic rod length values.

Similar to somatic rod length, also the measure of the post-oral rod (Fig. 9), was significantly influenced by all the variables and their interactions (Table 9). However, at the various sampling times, in larvae from the same parents, significant differences between pH values were not maintained in the post-oral rods as steadily as in the somatic rods. Contrary to what observed for SL, post-oral length of F3 larvae at 7.7 pH presented lower values compared to F1 and F2 at same pH.

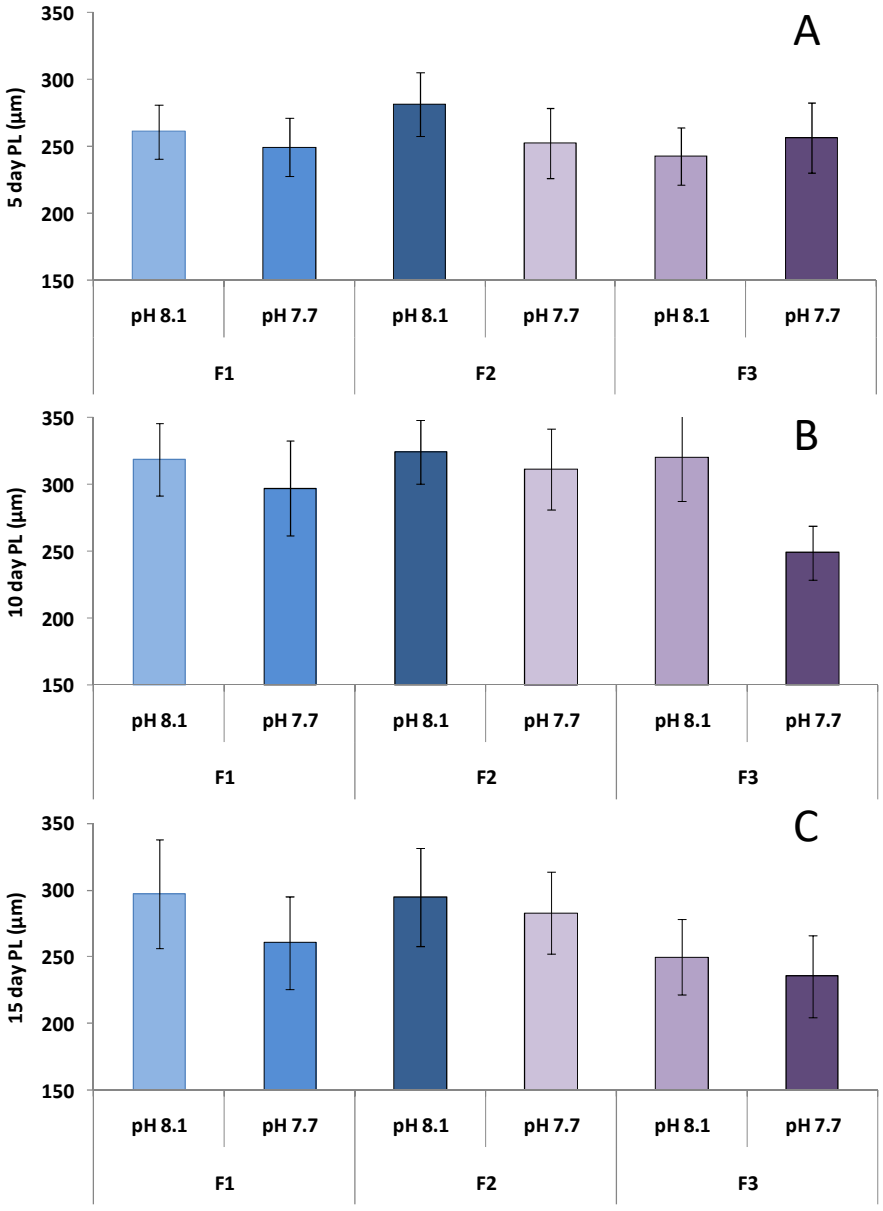

Fig. 9 Post-oral rod length of F1, F2 and F3 *P. lividus* larvae exposed at 8.1 and 7.7 pH for 5 (A), 10 (B) and 15 (B) days. Mean ± se.

	pR(>F)
pH	***
F	***
time	***
pH:F	***
pH:time	***
F:time	***
pH:F:time	***

POL	d5	d10	d15
F1-8.1 : F1-7.7	ns	***	***
F2-8.1 : F2-7.7	***	ns	ns
F3-8.1 : F3-7.7	ns	***	ns

	d5	d10	d15
F1-8.1 : F2-8.1	***	ns	ns
F2-8.1 : F3-8.1	***	ns	***
F1-8.1 : F3-8.1	ns	ns	***

	d5	d10	d15
F1-7.7 : F2-7.7	ns	ns	*
F2-7.7 : F3-7.7	ns	***	***
F1-7.7 : F3-7.7	ns	***	**

Table 9 3-way ANOVA: results of post-oral rod length values.

4.2.4. DISCUSSION

In this study, exposure of parents to low pH during gametogenesis influenced energy content and consumption in sperm and mortality and growth in larvae when exposed at different pH values.

ATP content ranged from 2.42 to 7.85 nmol/10^9cell which is consistent with data reported by other authors (Mita et al., 1994). Significant higher ATP content in S3 group was present showing greater investment in sperm energy by males maintained at 7.4 pH.

VCL was affected by time with significant reduction, at the end of the observation time, only in group S3. Contrary to what observed by other authors (Graham et al., 2015; Campbell et al., 2016),

At each time of sperm VCL measurement, sperm from whichever sire never significantly differed in VCL values recorded under the three post-activation pH tested.

Changes in *P. lividus* sperm motility and velocity, during gonad cycle, was demonstrated with highest values during the spawning stage therefore this parameter is considered a tool for evaluating sperm quality (Fabbroccini et al., 2016). In our results, VCL was not different between groups (S) despite higher ATP content in sperm developed under 7.4 pH (S3). Possibly, sperm ATP content could be used as an indicator of parental investment in gametogenesis, but it is not directly linked to sperm VCL. In *Crassostrea gigas*, higher ATP concentration was found in spermatozoa from tetraploid males than in spermatozoa from diploid males, but the percentage of motile spermatozoa was lower in tetraploid males, with shorter movement duration, suggesting that initial ATP level is not the fundamental parameter to define sperm vitality in time (Suquet et al., 2010).

A large number of steps taking place inside the sperm cell are pH-dependent and are critical to fertilisation (Nishigaki et al., 2014). In *P. lividus* male gonad, sperm cells are stored in a quiescent state at 7.2 pH. After the release into seawater, pH of the sperm increases to optimal alkaline

value for dyneine-ATPase activity (7.6), and flagellar movement, along with mitochondrial respiration, begins and sperm start to swim actively (Christen et al., 1982). Rising cytosolic ADP levels stimulate mitochondrial oxidative phosphorylation, which is the only ATP-providing pathway in sea urchin spermatozoa. In sea urchin sperm, energy is almost completely obtained from fatty acid oxidation (Rothschild and Cleland, 1952; Mita and Yasumasu, 1983; Hansbrough et al.,1980). This reaction occurs in single mitochondrion placed at the base of sperm head (Baccetti and Afzelius, 1976). Dyneine-ATPase accounts for almost all ATP used in sperm (Christen et al., 1982, 1983).

In this experiment pH affected oxygen consumption with general higher values at 7.7 pH and lower at 7.4 pH in all groups of sperm. Significant lower ATP consumption rate was present in S2 and S3 groups but not in control one (S1). Studies about ionic regulation in sea urchin sperm showed that when external seawater pH value is below 7.0, respiration in cells is extremely low, and dyneine-ATPase is inhibited. At pH values ranging from 7.0 to 7.4 respiration rate increases and is balanced with ATP consumption, while at higher pH, ATP production reaches highest rate and exceeds ATP production (Christen et al., 1986). Our results confirmed the decreasing trend in ATP consumption with deacreasing seawater pH only in group of sperm developed at lowered pH conditions (S2 and S3).

ATP consumption rate and oxygen consumption rate related to oxygen consumption rate (ATP/O_2) and average VCL (ATP/VCL) exhibited significant effects of pH with lower values at low pH. If we consider the oxygen consumption as an index of ATP production, lower values in ATP/O_2 and ATP/VCL could suggest more balanced energy turnover and lower energy consumed for movement, respectively. Unlike S2 and S3 sperm groups, significant differences between pH were not found in ATP/O_2 and ATP/VCL ratios in control group of sperm (S1).

These results suggest that sperm performances (VCL, ATP content and ATP consumption) of control group (S1) are not affected by low pH values and consequently by future ocean acidification conditions. Furthermore, when gametogenesis occurs in acidified conditions, sperm activated at low pH could have longer vitality at low pH then at 8.1 pH because it can maintain the same VCL but with significant lower energy consumption.

Considering that sperm competitiveness is influenced by sperm length concentration (Benzie and Dixon, 1994), swimming velocity and longevity (Levitan, 2000), this represent an advantage for fertilization success in this species under future acidified conditions. In order to highlight effects

of pH on fertilization success further and longer observation of sperm velocity, longevity and fertilization capacity is necessary.

Larvae from parents maintained at 7.7 (F2) and 7.4 pH (F3) exhibited similar or higher somatic rod length when compared to F1 larvae. Fewer differences were found between larval groups when post-oral length was considered. In particular, after 10 days of exposure at 7.7 pH, larvae obtained from parents acclimated at 7.4 pH (F3) showed lower POL compared to the other groups.

Stukling et al. (2015) found that, when exposed for 16 months at low pH, sea urchin *Sterechinus neumayeri* produced larger eggs compared to control. GSI but not egg size were considered in females used in this study. GSI in females maintained at 7.4 pH resulted lower than in control and this result could be due to the production of lower number of larger eggs and could also explain higher SL of F2 and F3 larvae.

Differences between short acclimation period (4-6 months)and long acclimation period (14-16 months) were found in sea urchins *S. neumayeri* and *Strongylocentrotus droebachiensis.* Unlike the short acclimation, after long acclimation, in both studies, larval performance was not significantly different across treatments (Stukling et al., 2015; Dupont et al., 2013).

Even if adults used in this study had spawned before exposure at low pH, and entire gametogenesis occurred under experimental conditions, significant negative effects of low pH on larval growth were observed. Longer parent exposure have to be performed, in order to understand if *P. lividus* larvae are able to overcome negative effects during early larval development, after adult acclimation.

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4.3. RESEARCH ARTICLE III: **Combined effects of ocean acidification and a pharmaceutical mixture on *Paracentrotus lividus* larvae subject to short-term and long-term exposures**

4.3.1. INTRODUCTION

Accumulation of pharmaceutical compounds in coastal ecosystems, mostly due to release via riverine and municipal effluent discharges, is rapidly increasing as a consequence of worldwide increased population living within coastal limits (Martínez et al., 2007), rising human consumption pharmaceuticals, and enhancement of aquaculture production (Grigorakis et al., 2011).

The degradation in the body and the removal efficiency in wastewater treatment plant are extremely variable among pharmaceuticals (Ares, 1999; Kunkel and Radke, 2012). In the last few years, improvement of analytical capacity allowed to detect these compounds in natural waters even when present in low range concentrations (ng l^{-1}) (Lolić et al., 2015). The concentrations of the pharmaceuticals measured in coastal waters range from 0.01 to 6800 ng l^{-1}, and the most frequently detected are antibiotics, non-steroidal anti-inflammatories and analgesics (Gaw et al., 2014). Pharmaceutical compounds are designed to be persistent and biologically active molecules even at low concentrations (Franzellitti et al., 2015; Ternes, 2000) and their effect on the marine biota is of increasing concern.

In the environment, the organisms are rarely exposed to single compounds but more generally to a complex mixture of stressors. Several national and international institutions have highlighted the need to evaluate risks from mixtures and multiple stressors (NRC, 1994; PCCRARM, 1997; Mileson et al., 1999; US EPA, 2000, 2003; ATSDR, 2004; WHO, 2009; European Scientific Committees, 2011). There is a need to focalize on more realistic chemical risk assessment methods which consider combined effects of the most common stressors present in the environment (Callahan and Sexton, 2007). In addition to interactions between chemicals, there are possible interactions with other factors, such as climate variables (Schlink et al., 2002; Leitte et al., 2009), which are threatening marine coastal areas and need to be addressed.

As a consequence of increasing atmospheric CO_2, at global level two main phenomena are generated: global warming and ocean acidification. Ocean acidification (OA) is a process of lowering sea water pH, mainly due to increased dissolution of CO_2 in seawater. Since pre-industrial time, ocean surface pH has decreased by approximately 0.1 units (IPCC, 2013) and current average pH value for surface and shallow sea waters is 8.1. Average pH value predicted for the year 2100, is 7.7 and the extreme predicted value is 7.4 (Etheridge et al., 1996; Gattuso and

Hansson, 2011). Low pH value could act detrimentally *per se*, but it could also show synergic or negative effects when combined with chemical compounds, influencing their chemical properties (Banni et al., 2015; Nichols et al., 2015).

When present in literature, the lowest observed effect concentrations (LOECs) of most pharmaceuticals in aquatic organisms are usually several times higher than their environmental concentrations. This apparently high tolerance to pharmaceutical compounds, even in marine species (Gaw et al., 2014), could be due to relatively short laboratory tests performed with single compounds. Considering that in the wild, organisms are exposed to mixtures of various chemicals, for long time, it is important to investigate chronic effects of mixtures of these compounds at environmentally relevant concentrations. One of the most commonly used approach to assess chronic effects of contaminants or other environmental stressors is represented by toxicity/vulnerability testing in early-life stages of aquatic organisms (McKim, 1985).

In these experiments four pharmaceuticals, belonging to different therapeutic classes, were used: clofibric acid (lipid-lowering), caffeine (metabolic stimulator and adjuvant), diclofenac (anti-inflammatory drug) and propranolol (β-blocker). These pharmaceuticals, commonly detected in worldwide coastal areas (Gaw et al., 2014), were tested on *P. lividus* embryos, alone and in mixture at a concentration of 0.5 μg l^{-1}, which can be considered environmentally relevant (Table 1). In several assays, performed on sea urchin early life stages, the exposure to the experimental conditions tested starts after the formation of the fertilization membrane or after the first cellular divisions. In order to evaluate also the effects of the exposure starting prior to fertilization, in short-term experiment two fertilization approach were carried out i.e. eggs were fertilized in control conditions and in treatment conditions. The effects of the mixture of pharmaceuticals were evaluated performing short-term (24-48h) and long-term (24 days) experiments.

CA

Environment	Country	Sampling Location	ng l^{-1}	Reference
Coastal	Europe	North Sea	0.01 - 18.6	Spongberg et al., 2011
Estuary	Germany	Elbe Estuary	18.6	Weigel et al., 2002
Estuary	United Kingdom	Five Estuaries	98.4 - 111	Thomas and Hilton 2004
Coastal	Taiwan	Northern coast of Taiwan	1.5 - 30	Fang et al., 2012

CAF

Offshore	Italy	North Adriatic Sea	82-367	Loose et al., 2013
Estuary	Norway	Tromsø Sound	7-87	Weigel et al., 2004
Coastal	Sweden	Stockholm	30-74	Magnér et al., 2010
Offshore	Germany	Elbe Estuary	2-16	Weigel et al., 2002
Coastal	United States of America	Boston Harbour	140-1600	Siegener and Chen, 2002
Coastal	Canada	Vancouver Island	5-149	Verenitch and Mazumder 2008
Coastal	United States of America	Massachusetts Bay	5-71	Siegener and Chen, 2002

DCF

Coastal	Frace	Mediterranean Sea	1500	Togola and Buzinski, 2008
Coastal	Ireland	Ireland	60 - 550	McEneff et al., 2014
Estuary	United Kingdom	Five Estuaries	57 - 195	Thomas and Hilton 2004
Estuary	France	Honfleur, Seine Estuary	7.7 - 63.4	Togola and Budzinski 2007
Estuary	Portugal	Arade Estuary	31	Gonzalez-Rey et al., 2015
Estuary	Germany	Elbe Estuary	6.2	Weigel et al., 2002
Coastal	Spain	Mediterranean Sea	4	Gros et al., 2012
Coastal	Sweden	Stockholm	2	Wahlberg et al., 2011
Estuary and coastal	People's Republic of China	Yangtze Estuary and coastal zone	283- 843	Yang et al., 2011
Coastal	Taiwan	Northern coast of Taiwan	2.5 - 53.6	Fang et al., 2012
Coastal	Singapore	Marina Bay	4 - 38	Wu et al., 2010
Coastal	Singapore	Singapore	<1.5 - 11.6	Bayen et al., 2013
Estuary	Canada	Halifax Coastal watershed	2 - 6	Comeau et al., 2008
Coastal	United States of America	Southern California,	0.6	Vidal-Dorsch et al., 2012

PR

Environment	Country	Sampling Location	ng l^{-1}	Reference
Estuary	United Kingdom	Tyne River Estuary	35 to 107	Roberts and Thomas, 2006
Estuary	United Kingdom	Five Estuaries	10 - 56	Thomas and Hilton 2004
Coastal	Belgium	Ostend, Nieuwpoort, Zeebrugge Harbours	1 - 24	Wille et al., 2010
Estuary	Portugal	Douro River Estuary	3.2	Madureira et al., 2010
Estuary and coastal	People's Republic of China	Yangtze Estuary and coastal zone	0.3 - 142	Yang et al., 2011

Table 1 - Concentrations of clofibric acid (CA), caffeine (CAF, diclofenac (DCF) and propranolol (PR), detected in seawater (ng l^{-1}).

4.3.2. MATERIALS AND METHODS

Chemicals

All test compounds were obtained from Sigma–Aldrich. Stock solutions in absolute ethanol at a concentration of 10 mg ml^{-1} were set for all pharmaceuticals tested and were used to obtain a working solution at a concentration of 10 mg l^{-1} in distilled water.

pH was adjusted to experimental values by bubbling CO_2 and the values were controlled with a pHmeter (CRISON mod. BASIC20).

Species and embryo collection

Adult specimens of *P. lividus* were collected by SCUBA divers at *ca*. 5 m depth in the vicinity of the Hydrobiological Station "Umberto d'Ancona" in the south basin of the Venice Lagoon (NW Adriatic Sea, Italy) in March 2016, at the beginning of the gonadal maturity period in this species for the Adriatic sea (Tomšič *et al*., 2010). Sea urchins were maintained for one week in flowing filtered water (20 µm) at 20°C temperature and 33 PSU salinity and were fed with *Ulva rigida*.

Gametes were obtained by injecting 1 ml of 0.5 M KCl solution into the coelom, through the peristome membrane. Sperm from each male was collected dry by using a micropipette, and stored in small tubes at 4 °C pending fertilization. The eggs from each female were gathered in 250 ml beakers filled with 0.45 µm filtered sea water (FSW). In order to remove spine and algae fragments, the eggs were filtered with 200 µm mesh nylon filter and then concentrated with 20 µm mesh nylon filter and suspended in FSW. The density of the eggs was evaluated by counting subsamples under optical microscope (Leica DM 750) and the density of sperm was determined by a Coulter Counter mod. Z2 after 1:20000 dilution in FSW.

In each experiment, fertilization was performed using gametes from three males and three females.

Short-term exposure

In short-term exposure experiment A, sea urchin embryos were exposed to each compound and to the relative mixture at the of 0.5 µg l^{-1}. In short-term exposure experiment B, combined effects of the mixture under different pH values (control pH, 8.1 and low pH, 7.7) were investigated. In both the experiments, two different approaches were used, i.e., eggs were fertilized in control conditions and in treatment conditions.

For fertilization in control conditions, equal number of eggs from each female was placed in 500 ml beaker filled with FSW. In each beaker with eggs, equal number of sperm from each male was added, maintaining standard sperm:egg ratio (1250:1), according to the recommendations of Dinnel *et al*., 1987. For fertilization in treatment conditions, eggs and sperm were fertilized with same procedure but in FSW previously adjusted at the various experimental conditions. Fertilization success was checked after 15 minutes, ensuring the presence of fertilization membrane in 90% of eggs at least. Fertilized eggs were washed to remove the sperm and distributed in 250 ml glass vessels filled with FSW at the experimental conditions.

Six 250-ml replicates per experimental condition were performed, each with approximately 50 eggs ml^{-1} ,and were maintained at 23 °C and 33 PSU. Three replicates were fixed with 10% neutralized formalin after 24 hours, and other three were fixed 48 hours after fertilization. In each replicate 200 individuals were observed to determine the frequency of the developmental stages and growth anomalies at 24h and 48h after fertilization. Development index was calculated, by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by the number of individuals. Length of the somatic rod of 60 four-armed echinoplutei was measured in all replicates fixed after 48h from fertilization.

Long-term exposure

In this experiment five individuals per sex were used to obtain gametes. Collection of the gametes was performed as described above and the fertilization was carried out in FSW at 20 °C of temperature and 33 PSU of salinity

Fertilized eggs were exposed at three pH values (8.1, 7.7 and 7.4), in the presence and in the absence of the mixture of clofibric acid, caffeine, diclofenac and propranolol, each at 0.5 µg l^{-1}. Thirty 2.5 l glass vessels (five for experimental condition) were set up, at a density of 5 eggs ml^{-1}. Throughout 24-days experiment, 80 % of 2 µm- filtered seawater was replaced in each vessel every two days. Larvae were fed daily with *Isochrysis galbana*, at a density of 2000 cells ml^{-1}. The concentration of the algae in the vessels was checked daily and a suitable quantity of algae was added to maintain constant concentration of food.

Mortality was checked every two days in each replicate. Three 1-ml aliquots from five times concentrated replicates were analyzed to count larvae at the optical microscope. After 10 and 20 days, in three randomly selected replicates, 20 and 30 larvae respectively were photographed (Leica DM 750 microscope connected to Leica DFC 295 camera) and the somatic rod length was measured. At the same time points and in the same replicates, 50 larvae were observed to determine the frequency of the various developmental stages and that of growth anomalies .

Statistical analysis

Differences in larval rod length and development indices were evaluated by Mixed model analysis followed by Multiple comparisons of means with p values adjusted by Holm method. The R software (R Core Team 2013) was used.

Differences in mortality, percentage of abnormal larvae and frequency of larval stages, were assessed by PERMANOVA and Pair-Wise test using PRIMER software (PRIMER-E Ltd, Luton, UK)

4.3.3. RESULTS

Short-term exposure - experiment A

After 24h, the frequency of the larval stages showed a small delay in development due to fertilization in treatment conditions (Fig. 1B) respect to fertilization in control condition (Fig. 1A): lower percentage of prism stage (63 and 71%, respectively) and higher percentage of pre-hatching stages (33 and 24%, respectively). Indeed, significant negative effect of exposure start (just before or after fertilization) was observed only on larval development index after 24h (Fig. 2; Table 2).

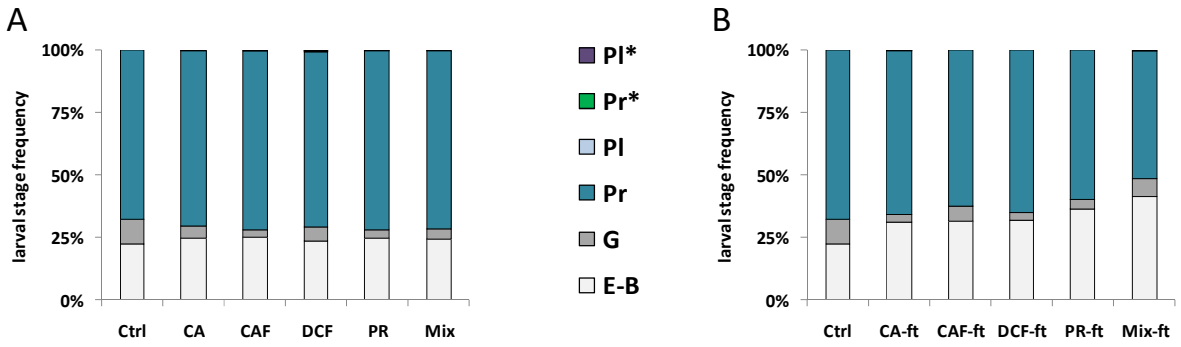

Fig. 1 Short-term experiment A: frequency of larval stages 24h after fertilization in control conditions (A) and treatment conditions (B) and exposure at 0.5 µg l^{-1} of clofibric acid (CA), caffeine (CAF), diclofenac (DCF), propranolol (PR) and their mixture. E, egg; B, blastula; G, gastrula; Pr, prism; Pl, four-armed pluteus; Pr*, abnormal prism; Pl*, abnormal four-armed pluteus).

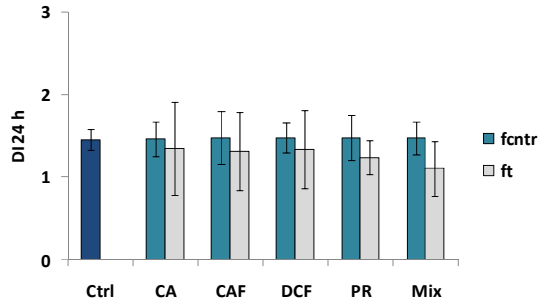

Fig. 2 Short-term experiment A: 24h development index (DI) calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Mean ± sd.

	NumDF	DenDF	F.value	Pr(>F)
pharm	5	22	0.4912	0.779
fert	1	22	6.8576	0.016 *
pharm:fert	5	22	0.6123	0.691

Table 2 Short-term experiment A: DI 24h, Mixed model results.

After 48h, the same tendency was observed, mainly due to lower percentage of four-armed echinopluteus: 73% in samples with fertilization in treatment conditions (Fig. 3B), 81% in samples fertilized in control condition (Fig. 3A), but no effect of exposure start on developement index was observed (Fig. 4; Table 3).

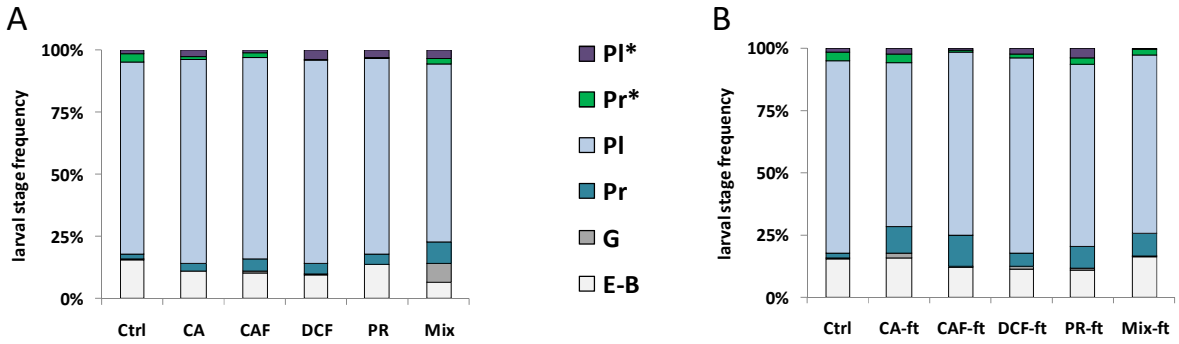

Fig. 3 Short-term experiment A: frequency of larval stages 48h after fertilization in control conditions (A) and treatment conditions (B) and exposure at 0.5 µg l^{-1} of clofibric acid (CA), caffeine (CAF), diclofenac (DCF), propranolol (PR) and their mixture. (E, egg; B, blastula; G, gastrula; Pr, prism; Pl, four-armed pluteus; Pr*, abnormal prism; Pl*, abnormal four-armed pluteus).

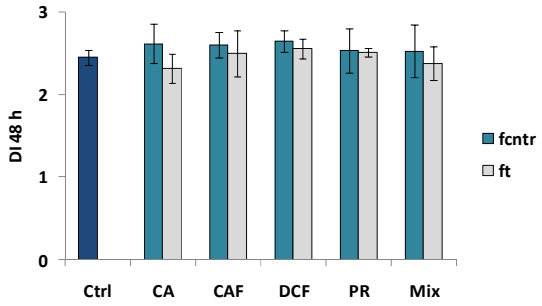

Fig. 4 Short-term experiment A: 48h development index (DI) calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Mean ± sd.

	NumDF	DenDF	F.value	Pr(>F)
pharm	5	22	0.7429	0.59970
fert	1	22	3.5972	0.07108 .
pharm:fert	5	22	0.5770	0.71705

Table 3 Short-term experiment A: DI 48h, Mixed model results.

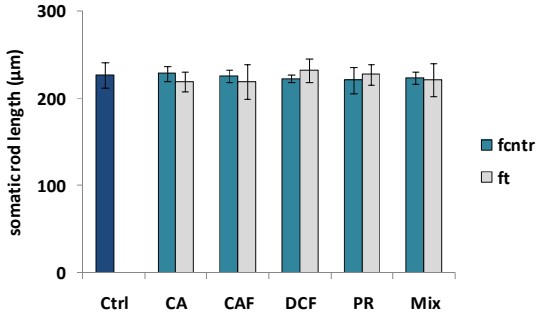

Fig. 5 Short-term experiment A: somatic rod length of 48h old echinopluteus exposed to 0.5 µg l^{-1} of clofibric acid (CA), caffeine (CAF), diclofenac (DCF), propranolol (PR) and their mixture. Mean ± sd.

	NumDF	DenDF	F.value	Pr(>F)
pharm	5	24	0.160	0.9747
fert	1	24	0.009	0.9238
pharm:trat	5	24	0.460	0.8017

Table 4 Short-term experiment A: somatic rod length after 48h, Mixed model results.

No significant effect of pharmaceuticals or their mixture was observed on larval development index after 24 (Fig. 2; Table 2) and 48h (Fig. 4; Table 3) and on growth after 48h (Fig. 5; Table 4).

Short-term exposure - experiment B

24h after fertilization, a reduction of prism stage and increase of pre-hatching stages were observed at 7.7 pH, irrespective of the fertilization method used (Fig. 6). At 8.1 pH, the presence of the mixture reduced the percentage of prisms and increased the percentage of pre-hatching stages in samples fertilized in treatment conditions (51 and 41 % respectively) (Fig. 6B), but not in samples fertilized in control conditions (71 and 24 % respectively)(Fig. 6A). Nevertheless, development index was negatively affected only by 7.7 pH (Fig. 7; Table 5).

After 48h, an increase of abnormal four-armed plutei in samples fertilized in treatment conditions and exposed at 7.7 pH was observed, both in the presence and absence of mixture (Fig. 8 B). Nevertheless no effect of pH, pharmaceutical mixture or exposure start was observed on development index (Fig. 9; Table 6) even if a decrease of four-armed pluteus stage (Fig. 8A,B) was present at 7.7 pH. Larval growth was negatively affected by 7.7 pH only (Fig. 10; Table 7).

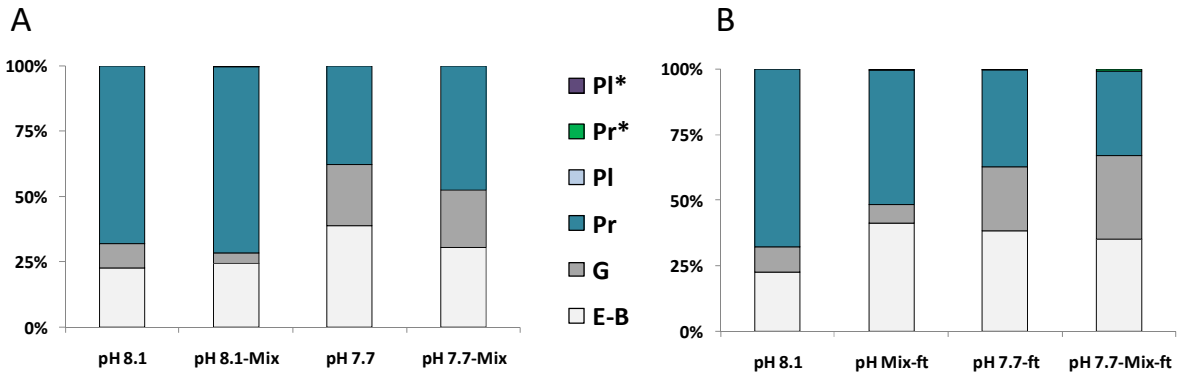

Fig. 6 Short-term experiment B: frequency of larval stages 24h after fertilization in control conditions (A) and treatment conditions (B) and exposure at 8.1 and 7.7 pH and at 0.5 µg l^{-1} mixture of clofibric acid (CA), caffeine (CAF), diclofenac (DCF) and propranolol (PR). E, egg; B, blastula; G, gastrula; Pr, prism; Pl, four-armed pluteus; Pr*, abnormal prism; Pl*, abnormal four-armed pluteus).

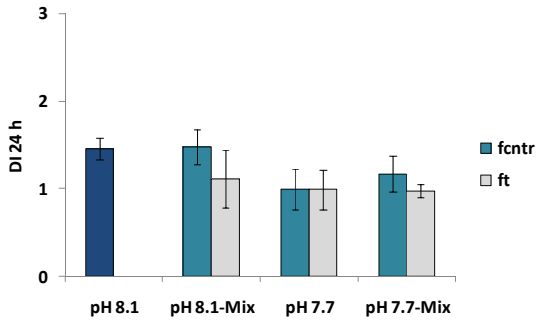

Fig. 7 Short-term experiment B: 24h development index (DI) calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Mean ± sd.

	NumDF	DenDF	F.value	r(>F)	
pH	1	14	23.79	0.00024	***
pharm	1	14	0.32	0.57576	
fert	1	14	4.06	0.06344	.
pharm:pH	1	14	3.12	0.09912	.
pharm:fec	1	14	3.99	0.06553	.
fert:pH	1	14	0.37	0.55255	
phar:fer:pH	1	14	0.39	0.54114	

	8.1 mix	7.7	7.7 mix
8.1	0.14	***	***
8.1 mix		**	*
7.7			0.453

Table 5 Short-term experiment B: DI 24h, Mixed model results.

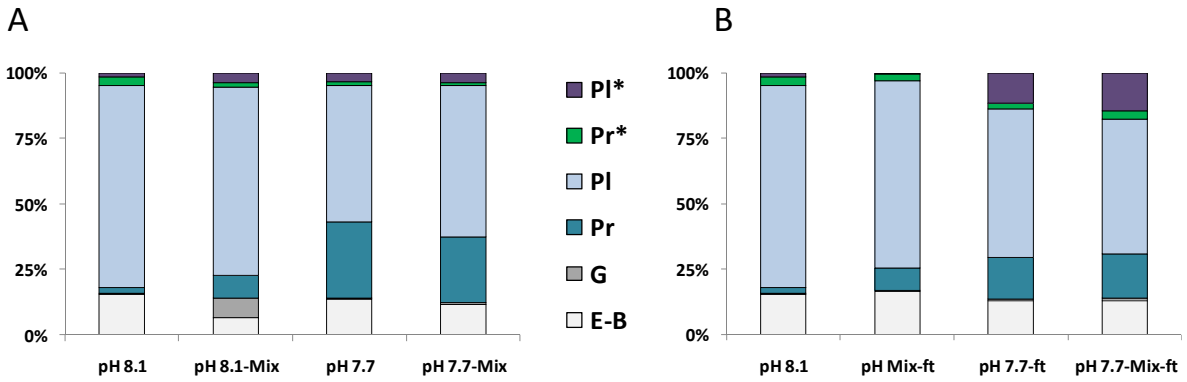

Fig. 8 Short-term experiment B: frequency of larval stages 48h after fertilization in control conditions (A) and treatment conditions (B) after exposure at 8.1 and 7.7 pH and 0.5 µg l^{-1} mixture of clofibric acid (CA), caffeine (CAF), diclofenac (DCF) and propranolol (PR). E, egg; B, blastula; G, gastrula; Pr, prism; Pl, four-armed pluteus; Pr*, abnormal prism; Pl*, abnormal four-armed pluteus).

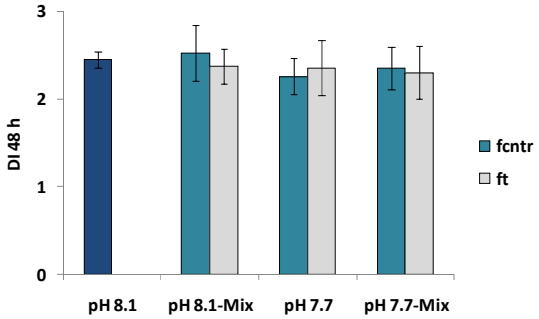

Fig. 9 Short-term experiment B: 48h development index (DI) calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Mean ± sd.

	NumDF	DenDF	F.value	Pr(>F)
pharm	1	14	0.018	0.8956
fert	1	14	0.117	0.7374
pH	1	14	2.833	0.1145
pharm:fert	1	14	0.925	0.3525
pharm:pH	1	14	0.021	0.8873
fert:pH	1	14	0.398	0.5385
fert:pharm:pH	1	14	0.000	0.9916

Table 6 Short-term experiment B: DI 248h, Mixed model results.

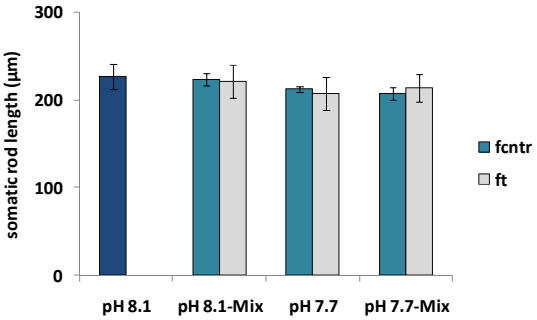

Fig. 10 Short-term experiment A: somatic rod length of 48h old echinopluteus exposed to 0.5 µg l^{-1} of clofibric acid (CA), caffeine (CAF), diclofenac (DCF), propranolol (PR) and their mixture. Mean ± sd.

	NumDF	DenDF	F.value	Pr(>F)
pH	1	18.003	18.7672	0.0004013 ***
cond	1	18.003	0.2590	0.6170126
pH:cond	1	18.003	2.4613	0.1340931

	8.1 mix	7.7	7.7 mix
8.1	0.239	***	***
8.1 mix		***	***
7.7			0.99

Table 7 Short-term experiment B: somatic rod length after 48h, Mixed model results.

Long-term exposure

Mortality, observed during long-term experiment, was rather high in all experimental treatments ranging from 50% at second day to more than 80% at the end of the experiment (Fig. 11). Significantly higher mortality was observed at 7.4 pH respect to both 8.1 and 7.7 pH, whereas no difference was present between 8.1 and 7.7 pH. At all pH values tested, the mixture showed a significant negative effect (Table 8).

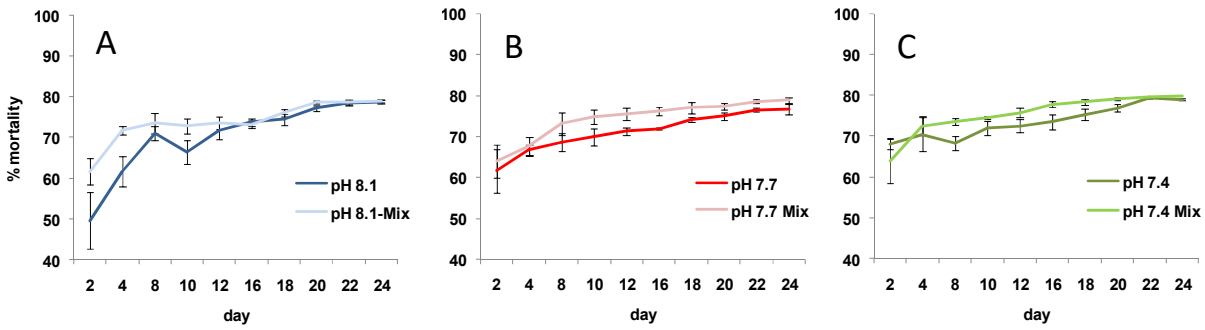

Fig. 11 % of mortality evaluated on *P. lividus* larvae exposed at mixture of pharmaceuticals (CA, CAF, DCF, PR at 0.5 µg l^{-1}) and at 3 pH values (8.1, A; 7.7, B; 7.4, C) during 24 days exposure. Mortality was evaluated every two days in five replicate vassels. Mean ± sd.

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
day	**8**	**6823.2**	**852.9**	**42.244**	**0.001**	**999**	**0.001**
pH	**2**	**226.62**	**113.31**	**5.6122**	**0.004**	**997**	**0.007**
mix	**1**	**498.85**	**498.85**	**24.708**	**0.001**	**996**	**0.001**
dayxpH	**16**	**534.48**	**33.405**	**1.6545**	**0.066**	**997**	**0.048**
dayxmi	8	101.14	12.642	0.62616	0.79	998	0.782
pHxmi	2	21.422	10.711	0.53052	0.625	998	0.606
dayxpHxmi	16	392.51	24.532	1.215	0.247	997	0.277

PAIR-WISE TESTS Term 'pH'

Groups	t	P(perm)	Unique perms	P(MC)
8.1, 7.7	0.99239	0.316	999	0.297
8.1, 7.4	**3.3041**	**0.003**	**995**	**0.002**
7.7, 7.4	**2.3947**	**0.015**	**999**	**0.02**

PAIR-WISE TESTS Term 'mix'

Groups	t	P(perm)	Unique perms	P(MC)
0, 1	**4.9707**	**0.001**	**996**	**0.001**

Table 8 PERMANOVA and PIRE-WISE TEST: results from mortality values in *P. lividus* larvae exposed at mixture of pharmaceuticals (CA, CAF, DCF, PR at 0.5 µg l^{-1}) and at 3 pH values (8.1; 7.7; 7.4) for 24 days.

In both 10-days and 20-days old larvae, pH but not the mixture significantly affected larval growth. After 10 days, multiple comparisons of the means showed significant growth reduction in 7.4 pH treatments respect to the corresponding 7.7 pH treatment and in 7.4 pH with mixture respect to 7.7 pH without mixture (Fig. 12A; Table 9). After 20 days, larvae kept at 7.4 pH were significantly smaller respect to those at both 7.7 and 8.1 pH (Fig. 12B; Table 10).

After 10 days of exposure, larval development was negatively affected by both pH and mixture. Significant higher percentage of abnormal plutei was present at 7.7 and 7.4 pH respect to control 8.1 pH and in the presence of mixture (Fig. 13A; Table 11). After 20 days, the significant negative effect of both pH and mixture on larval development was maintained. No differences were

observed between 7.7 and 8.1 pH, whereas 7.4 pH was different from both 7.7 and 8.1 pH (Table 12). Interestingly, at 7.7 pH a faster larval development (increased presence of 6- and 8-armed plutei) was observed even though with increased frequency of larval anomalies (Fig. 13B). At 7.4 pH, larval development was strongly delayed, with the lowest frequency of 6-armed plutei; the highest number of 4-armed anomalous plutei was also observed. Under mixture exposure, increases in growth delay and presence of anomalies were found at each pH value tested.

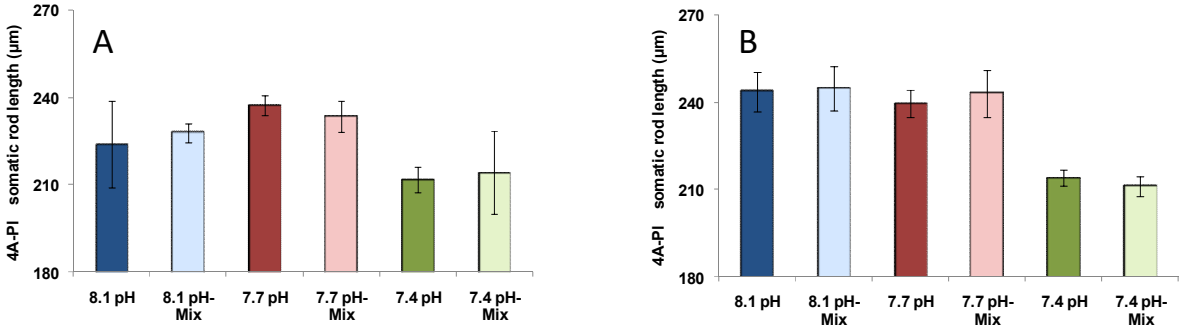

Fig. 12 Somatic rod length evaluated after 10 (A) and 20 (B) days on *P. lividus* larvae exposed at mixture of pharmaceuticals (CA, CAF, DCF, PR at 0.5 µg l^{-1}) and at 3 pH values (8.1; 7.7; 7.4). Mean ± sd.

Response: length

	Chisq	Df	Pr(>Chisq)
ph	8.6369	2	8.975e-05 ***
mix	0.0389	1	0.8436
ph:mix	0.6296	2	0.7299

	8.1	**8.1 mix**	**7.7**	**7.7 mix**	**7.4**
8.1 mix	ns				
7.7	ns	ns			
7.7 mix	ns	ns	ns		
7.4	ns	ns	**	*	
7.4 mix	ns	ns	*	ns	ns

Table 9 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae after 10 days exposure at mixture of pharmaceuticals.

Response: length

	Chisq	Df	Pr(>Chisq)
ph	104.3485	2	<2e-16 ***
mix	0.0388	1	0.8439
ph:mix	0.9047	2	0.6361

	8.1	**8.1 mix**	**7.7**	**7.7 mix**	**7.4**
8.1 mix	ns				
7.7	ns	ns			
7.7 mix	ns	ns	ns		
7.4	***	***	***	***	
7.4 mix	***	***	***	***	ns

Table 10 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae after 10 days exposure at mixture of pharmaceuticals.

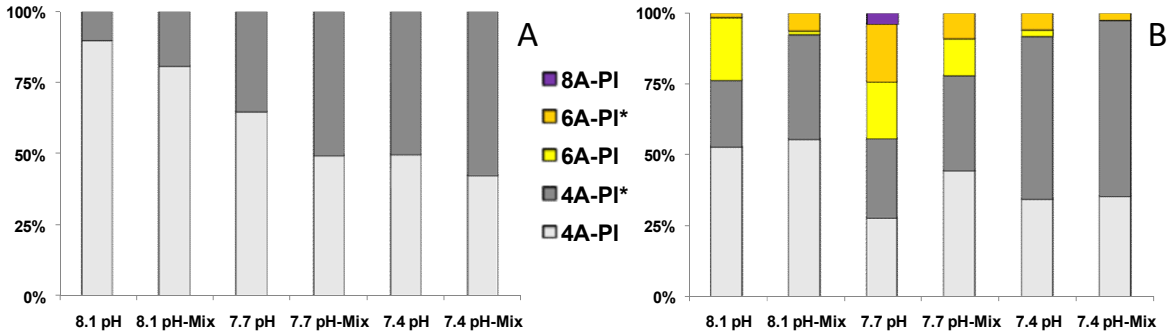

Fig. 13 frequency of larval stages 10 days (A) and 20 days (B) after exposure at 8.1, 7.7 and 7.4 pH and 0.5 µg l^{-1} mixture of clofibric acid (CA), caffeine (CAF), diclofenac (DCF) and propranolol (PR). 4A-Pl, four-armed pluteus; 4A-Pl*, abnormal four-armed pluteus; 6A-Pl, six-armed pluteus; 6A-Pl*, abnormal six-armed pluteus; 8A-Pl, eight-armed pluteus).

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
pH	**2**	**9793.6**	**4896.8**	**23.732**	**0.001**	**999**	**0.001**
mi	**1**	**1002.8**	**1002.8**	**4.86**	**0.05**	**992**	**0.047**
pHxmi	2	106.89	53.444	0.25902	0.782	998	0.767

PAIR-WISE TESTS Term 'pH'

Groups	t	P(perm)	Unique perms	P(MC)
8.1, 7.7	**5.9565**	**0.002**	**969**	**0.001**
8.1, 7.4	**6.3168**	**0.006**	**979**	**0.001**
7.7, 7.4	1.6901	0.132	960	0.112

PAIR-WISE TESTS Term 'mix'

Groups	t	P(perm)	Unique perms	P(MC)
0, 1	2.2045	0.049	996	0.058

Table 11 PERMANOVA and PIRE-WISE TEST: results from frequency of larval stages in *P. lividus* larvae exposed at mixture of pharmaceuticals (CA, CAF, DCF, PR at 0.5 µg l^{-1}) and at 3 pH values (8.1; 7.7; 7.4) for 10 days.

PERMANOVA table of results

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
pH	**2**	**6049.1**	**3024.6**	**9.7601**	**0.001**	**998**	**0.001**
mi	**1**	**980.89**	**980.89**	**3.1653**	**0.037**	**998**	**0.045**
pHxmi	2	753.11	376.56	1.2151	0.321	998	0.306

PAIR-WISE TESTS Term 'pH'

Groups	t	P(perm)	Unique perms	P(MC)
8.1, 7.7	1.8535	0.022	985	0.051
8.1, 7.4	**3.1721**	**0.009**	**984**	**0.005**
7.7, 7.4	**5.922**	**0.002**	**977**	**0.001**

PAIR-WISE TESTS Term 'mix'

Groups	t	P(perm)	Unique perms	P(MC)
0, 1	**1.7791**	**0.031**	**998**	**0.046**

Table 12 PERMANOVA and PIRE-WISE TEST: results from frequency of larval stages in *P. lividus* larvae exposed at mixture of pharmaceuticals (CA, CAF, DCF, PR at 0.5 µg l^{-1}) and at 3 pH values (8.1; 7.7; 7.4) for 20 days.

4.3.4 DISCUSSION

Negative effect of fertilization in treatment conditions was detected only in 24h development index, in short-term experiment A. Absence of this effect after 48h demonstrated that delay during early phases of development was rapidly overcome.

In short-term experiments, no effects of single pharmaceuticals were detected at the concentration tested (0.5 µg l^{-1}). Except for CAF, what we observed in this study is consistent with data present in literature for the tested compounds and their effects on *P. lividus* larvae.

Indeed, in experiments performed by Aguirre-Martinez et al. (2015) CAF showed negative effects on larval development at very low concentration (0.01 µg l^{-1}) and fertilization was affected only at concentration ≥ 10'000 mg l^{-1}. In another recent study, DCF was found to delay larval growth and

increase the percentage of abnormal *P. lividus* larvae at concentrations higher than 12.5 μg l^{-1}, whereas PR reduced the larval growth at the same concentration, but increased the percentage of abnormal larvae at 5 μg l^{-1} concentration (Ribeiro et al., 2015). No data about effects of CA on *P. lividus* are present in literature but some authors reported very high NOEC values in other organisms. Gonzales-Ortegon et al. (2015) observed no effect of CA and DCF on larval survival, development or growth of the coastal marine shrimp *Palaemon serratus* at 77 and 720 μg l^{-1} and at 42 and 720 μg l^{-1}, respectively. CA did not significantly affect survival of estuarine species such as *Palaemonetespugio* and *Fundulusheteroclitus* at concentrations ≤ 1000 mg l^{-1}.

DCF was found to induce tissue-specific biomarker responses in adults of *Mytilusgalloprovincialis* after 7 day exposure at concentration of 0.25 μg l^{-1} (Gonzalz-Rey and Bebianno, 2014).

Acute toxicity tests performed on *Daphnia magna*, *Desmodesmussubspicatus* and *Lemna minor*, with CA, DCF and PR, showed heterogeneous toxicity for these substances. PR seems to be more toxic for aquatic environment with EC50s below 10 mg l-1, whereas EC50s found for CA and DCF were between 10 and 100 mg l^{-1}.

Considering most of these data and environmental concentrations of the compounds, acute effects of such compounds in environment are totally unlikely, but harmful effects of the single compounds, after long-term exposure, cannot be excluded.

Indeed in short-term experiments, no effects of pharmaceutical mixture were detected at the concentration tested (0.5 μg l^{-1}). Negative effects of the mixture were highlighted only during long-term exposure with higher mortality, slower development and higher percentage of abnormal larvae in the presence of the mixture at all pH tested.

When pharmaceuticals are combined, their toxicity could increase strongly. In mixture of substances which act similarly, effects due to concentration additionis are usually expected, even if synergic effect cannot be excluded independently of similarity of compound mode of action.

In *Daphnia* testfor anti-inflammatory drugs diclofenac and ibuprofen, stronger combined effects than predicted, considering individual NOECs, were observed while lipid lowering clofibric acid combined with anti-epileptic carbamazepine showed unexpected concentration addition effect (Cleuvers et al., 2003)

In these experiments, we used four compounds belonging to different therapeutic classes.Their mode of action is well known only for humans and some laboratory mammals and a diverse mode of action on our model species larvae could be only supposed. Negative effects observed in long

term experiments could be due not only to concentration addition of pharmaceuticals but also to combined synergic effect.

Contrasting results from short-term and long-term experiments about the effects of the 7.7 pH were also detected. 7.7 pH delayed larval development after 24h and larval growth after 48h. After 10 and 20 day exposure, larval size was no longer different in larvae kept at 7.7 pH respect to those kept at control conditions; moreover, larval development seemed to be faster after 20 days at 7.7 pH. Mortality was not negatively affected by 7.7 pH during the experiment. However an increase of abnormal larvae was present at 7.7 pH, both after 10 and 20 days. *P. lividus* larvae exhibited high sensitivity at 7.4 pH, with negative effects on mortality, growth and development. Similar results on mortality and faster development of *P. lividus* larvae were reported by Garcia et al., (2015) after 35 days of exposure to low pH, together with delay in settlement at 7.7 pH and absence of settlement at 7.4 pH.

As demonstrated in our experiments, further studies at lower concentrations, but with longer exposure time should be performed to evaluate toxic potential of most common pharmaceuticals in the environment. *P. lividus* larvae survival and development seems to be strongly endangered by extreme pH value expected by 2100 (7.4), but exhibit a certain resistance to future average value expected for the end of the century (7.7). Our results highlighted that the sensitivity of the sea urchin larvae could considerably increase in presence of pollutants in marine coastal areas.

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4.4. RESEARCH ARTICLE IV: **Influence of ocean acidification on the toxicity of pharmaceuticals to sea urchin early-life stages**

4.4.1. INTRODUCTION

Since the beginning of the industrial revolution, atmospheric CO_2 is continuously increasing, mainly due to anthropic activities, in particular fossil fuel consumption (IPCC 2013). From 30 to 50% of this gas is absorbed by the ocean (Sabine et al., 2004; Toseland et al., 2013), mitigating the effects at the atmospheric level, first of all, the increase of the temperature at global level, process known as global warming. As a consequence of CO_2 absorption, the carbonate chemistry of the seawater is modified with effects on partial pressure of CO_2, dissolved inorganic carbonate, alkalinity, calcium carbonate saturation state and the concentration of hydrogen ions. Increaseing hydrogen ions originates the ocean acidification phenomenon (Feely et al., 2010; Beaufort et al., 2011). Today's average pH in surface sea waters is 8.1, which is 0.1 units lower respect to pre-industrial levels. Predicted average for the year 2100 is 7.7 pH, which is already present in the environment, but only as extreme value of natural pH variability (Etheridge et al., 1996; Gattuso and Hansson, 2011).

Pollution, together with overexploitation, habitat loss and invasive species, is considered the most widespread and pressing anthropogenic threat in coastal marine environment (Crain et al., 2009). In the last few years, improvement of analytical capacity and the possibility to quantify concentrations in low range (ng l^{-1}) brought to evidence about the presence of so called emerging contaminants in aquatic environments (Lolić et al., 2015). Emerging contaminants are represented by pharmaceuticals, antimicrobials, sunscreen agents, preservatives, nanoparticles and illicit drugs (Petrie et al., 2015). Fate and biological impacts of these compounds are poorly understood and most of them are currently not included in regulatory monitoring programs (Paredes et al., 2014). Pharmaceuticals are biologically active molecules designed to interact at cellular/molecular level in the target organism, however they may affect also non target organisms, affecting specific animal functions (e.g., development, growth, and reproduction) at low concentrations such as concentrations detected in the environment (Franzellitti et al., 2015). Furthermore, the presence of pharmaceuticals in coastal marine areas is expected to increase, mainly due to their increasing consumption, increasing population inhabiting these areas and increasing pressure by some human activities such as aquaculture, together with their low removal efficiency in wastewater treatment plants (Burridge et al., 2010; Kunkel and Radke, 2012). More than 200 pharmaceuticals

have been detected in river waters globally, with concentrations up to 6.5 mg l^{-1} (Hughes et al., 2013). More than one hundred pharmaceuticals and their metabolites have been detected in marine coastal waters at concentrations ranging from 0.01 ng l^{-1} to 6.8 µg l^{-1}. Most frequently the detected compounds are antibiotics, non-steroidal anti-inflammatories and analgesics (Gaw et al., 2014).

Single compound acute toxicity tests conducted in various organisms, such as crustaceans, algae and bacteria, under controlled laboratory conditions have produced median effective concentrations for a number of pharmaceuticals, with values < 1 mg l^{-1} (Bruce and Versteeg, 1992; Holten Lu¨ tzhøft et al., 1999; Halling-Sørensen, 2000; Brooks et al., 2003; Andreozzi et al., 2004; Brain et al., 2004; Eguchi et al., 2004; Cleuvers, 2005; Isidori et al., 2005a,b; DellaGreca et al., 2007; Isidori et al., 2007; DeLorenzo and Fleming, 2008; Terasaki et al., 2009; Giudice and Young, 2010). Compounds with this effect concentration, according to the EU-Directive 93/67/ EEC (Commission of the European Communities, 1996; Cleuvers, 2003), are classified as potentially very toxic to aquatic organisms.

In order to investigate the future combined effects between ocean acidification and pharmaceuticals, short-term embryoassays with *P. lividus* was performed. Eighth pharmaceuticals, belonging to different therapeutic classes were considered: clofibric acid (lipid-lowering), caffeine (metabolic stimulator and adjuvant), diclofenac, ibuprofen (anti-inflammatory drugs) propranolol (β-blocker), sulfadiazine, trimethoprim (antibiotics) and triclosan (antibacterial agent). For each compound, five nominal concentrations (0.5, 1.0, 5.0, 10.0, 15.0 µg l^{-1}) and two pH values (8.1 and 7.7) were considered. Pharmaceuticals have been selected among the most commonly detected in the marine environment (Table 1). Combined effects between stressors could be addictive, synergic or negative and LOEC (lowest observed effect concentration) observed for each chemical compound, when evaluated at the present pH (8.1), could be different from that detectable under future pH (7.7). Nevertheless, pH of aqueous media could also influence the bioavailability of dissolved chemical compounds. Indeed, when dissolved in aqueous media, chemical compounds may occur in lipophilic or in hydrophilic form. The first one is considered more toxic, because more efficiently absorbed through biological membranes. Compounds whose lipophilic form increases under reduced pH value could present lower LOEC at 7.7 pH and thus justify the addictive or synergic effects between the compound and low pH. Chemical properties of compounds, such as the ionisation constant (pKa), are useful to describe how the toxicity of pollutants could change in acidified coastal areas. Hendersen-Hesselbalch equation was used to

describe how the percentage of lipophilic form in each compound change in environmentally relevant ranges of pH.

CA

Environment	Country	Sampling Location	ng l^{-1}	Reference
Coastal	Europe	North Sea	0.01 - 18.6	Spongberg et al., 2011
Estuary	Germany	Elbe Estuary	18.6	Weigel et al., 2002
Estuary	United Kingdom	Five Estuaries	98.4 - 111	Thomas and Hilton 2004
Coastal	Taiwan	Northern coast of Taiwan	1.5 - 30	Fang et al., 2012

CAF

Offshore	Italy	North Adriatic Sea	82-367	Loose et al., 2013
Estuary	Norway	Tromsø Sound	7-87	Weigel et al., 2004
Coastal	Sweden	Stockholm	30-74	Magnér et al., 2010
Offshore	Germany	Elbe Estuary	2-16	Weigel et al., 2002
Coastal	United States of America	Boston Harbour	140-1600	Siegener and Chen, 2008
Coastal	Canada	Vancouver Island	5-149	Verenitch and Mazumder 2008
Coastal	United States of America	Massachusetts Bay	5-71	Siegener and Chen, 2008

DCF

Coastal	Frace	Mediterranean Sea	1500	Torgola and Buzinski, 2008
Coastal	Ireland	Ireland	60 - 550	McEneff et al., 2014
Estuary	United Kingdom	Five Estuaries	57 - 195	Thomas and Hilton 2004
Estuary	France	Honfleur, Seine Estuary	7.7 - 63.4	Togolaand Budzinski 2007
Estuary	Portugal	Arade Estuary	31	Gonzalez-Rey et al., 2015
Estuary	Germany	Elbe Estuary	6.2	Weigel et al., 2002
Coastal	Spain	Mediterranean Sea	4	Gros et al., 2012
Coastal	Sweden	Stockholm	2	Wahlberg et al., 2011
Estuary and coastal	People's Republic of China	Yangtze Estuary and coastal zone	283- 843	Yang et al., 2011
Coastal	Taiwan	Northern coast of Taiwan	2.5 - 53.6	Fang et al., 2012
Coastal	Singapore	Marina Bay	4 - 38	Wu et al., 2010
Coastal	Singapore	Singapore	<1.5 - 11.6	Bayen et al., 2013
Estuary	Canada	Halifax Coastal watershed	2 - 6	Comeau et al., 2008
Coastal	United States of America	Southern California,	0.6	Vidal-Dorsch et al., 2012

IBU

Estuary	United Kingdom	Tyne River Estuary	144 - 2370	Roberts and Thomas, 2006
Coastal	Spain	Mediterranean Sea	1500	Togolaand Budzinski 2007
Estuary	United Kingdom	Five Estuaries	124 - 928	Thomasand Hilton, 2004
Lagoon	Portugal	Aveiro Lagoon	242	Paiga et al., 2013
Estuary	Portugal	Arade Estuary	28	Gonzalez-Rey et al., 2015
Estuary	France	Honfleur, Seine Estuary	3 - 21.6	Togolaand Budzinski 2007
Coastal	Spain	Mediterranean Sea	16	Gros et al., 2012
Salt marsh	Spain	Cadiz Bay	10	Pintado-Herrera et al., 2013
Coastal	Sweden	Stockholm	1.9	Wahlberg et al., 2011
Estuary	Germany	Elbe Estuary	0.6	Weigel et al., 2002
Estuary	Norway	Tromsø Sound	0.01 - 0.7	Weigel et al., 2004
Estuary	Canada	Halifax Coastal watershed	6 - 230	Comeau et al., 2008
Coastal	Singapore	Marina Bay	41 - 121	Wu et al., 2010
Estuary	Japan	Tamagawa Estuary	2.4- 59.7	Nakadaet al., 2008
Coastal	Taiwan	Northern coast of Taiwan	2.4- 57	Fang et al., 2012
Coastal	Costa Rica	Costa Rica coastal waters	9 - 40	Spongberg et al., 2011
Coastal	United States of America	San Francisco Bay	37.9	Klosterhaus et al., 2013
Coastal	United States of America	Southern California,	30	Vidal-Dorsch et al., 2012
Estuary	Canada	Pictou Coastal Watershed	3 - 22	Comeau et al., 2008
Coastal	Taiwan	Southwestern coast of Taiwan	12.1	Jianget al., 2014
Estuary	United States of America	Charleston Harbor, South Carolina	5.9 - 11.7	Hedgespeth et al., 2012
Coastal	Singapore	Singapore	<2.2 - 9.1	Bayen et al., 2013

PR

Environment	Country	Sampling Location	ng l^{-1}	Reference
Estuary	United Kingdom	Tyne River Estuary	35 to 107	Roberts and Thomas, 2006
Estuary	United Kingdom	Five Estuaries	10 - 56	Thomas and Hilton 2004
Coastal	Belgium	Ostend, Nieuwpoort, Zeebrugge Harbours	1 - 24	Willeet al., 2010
Estuary	Portugal	Douro River Estuary	3.2	Madureira et al., 2010
Estuary and coastal	People's Republic of China	Yangtze Estuary and coastal zone	0.3 - 142	Yang et al., 2011

SD

Estuary	People's Republic of China	Yangtze Estuary	0.6 - 71.8	Yan et al., 2013
Coastal	People's Republic of China	Bohai Bay	4 - 41	Zou et al., 2011
Estuary	People's Republic of China	Jiulong River Estuary	0.4- 25.4	Zheng et al., 2011
Estuary	People's Republic of China	Pearl River Estuary	1.3 - 18	Liang et al., 2013
Coastal	People's Republic of China	Liaodong Bay	0.6- 9.1	Jia et al., 2011
Coastal	People's Republic of China	Beibu Bay	3.4	Zheng et al., 2012
Coastal	People's Republic of China	Dalian	2.1	Na et al., 2013
Coastal	People's Republic of China	Laizhou Bay	0.4	Zhang et al., 2012
Offshore	People's Republic of China	Bohai Sea and Yellow Sea	0.1 - 0.4	Zhang et al., 2013
Coastal	People's Republic of China	Jiaozhou Bay and Yantai Bays	0.2	Zhang et al., 2013

TMP

Environment	Country	Sampling Location	ng l^{-1}	Reference
Coastal	Ireland	Ireland	60 - 870	McEneff et al., 2014
Estuary	United Kingdom	Five Estuaries	11 - 569	Thomas and Hilton, 2004
Coastal	Belgium	Ostend, Nieuwpoort, Zeebrugge Harbours	13 - 29	Wille et al., 2010
Estuary	United Kingdom	Tyne River Estuary	4 - 19	Roberts and Thomas , 2006
Estuary	Portugal	Douro River Estuary	3.9 - 17.5	Madureira et al., 2010
Coastal	Poland	Southern Baltic Sea	0.6 - 3.4	Borecka et al., 2013
Coastal	Sweden	Stockholm	2	Wahlberg et al., 2011
Coastal	Spain	Spanish marine fish farm	0.2	Muñoz et al., 2010
Coastal	People's Republic of China	Laizhou Bay	1.3 - 330	Zhang et al., 2012
Coastal	Hong Kong	Victoria Harbour	1.4 - 216	Minh et al., 2009
Coastal	People's Republic of China	Bohai Bay	18 - 120	Zou et al., 2011
Estuary	United States of America	Jamaica Bay, New York	2.9- 72.2	Benotti et al., 2007
Coastal	Hong Kong	Hong Kong coastal waters	2.3 - 21.8	Gulkowska et al., 2007
Coastal	People's Republic of China	Liaodong Bay	1.4 - 18.2	Jia et al., 2011
Offshore	People's Republic of China	Bohai Sea and Yellow Sea	0.1 -16.6	Zhang et al., 2013
Coastal	People's Republic of China	Jiaozhou Bay and Yantai Bays	0.4 - 14	Zhang et al., 2013
Coastal	United States of America	San Francisco Bay	4.1	Klosterhaus et al., 2013
Coastal	People's Republic of China	Beibu Bay	3.8	Zheng et al., 2012
Coastal	United States of America	Southern California,	2	Vidal-Dorsch et al., 2012
Estuary	United States of America	Chesapeake Bay	1.4	Pait et al., 2006

TR

Environment	Country	Sampling Location	ng l^{-1}	Reference
Coastal	Germany	North Sea	0.01-6.9	Xie et al., 2008
Coastal	People's Republic of China	Tai Po and Victoria Harbors	15-110	Wu et al., 2007

Table 1 Concentrations of clofibric acid (CA), caffeine (CAF, diclofenac (DCF), ibuprofen (IBU), propranolol (PR), sulfadiazine (SD), trimethoprim (TMP) and triclosan (TR) detected in seawater (ng l^{-1}).

4.4.2. MATERIALS AND METHODS

Chemicals

Test compounds were all obtained from Sigma–Aldrich. Stock solution in absolute ethanol at concentration of 10 mg ml^{-1} was set for all pharmaceuticals tested and was used to obtain a second working solution at a concentration of 10 mg l^{-1} in distilled water.

pH was adjusted to experimental values by bubbling CO_2 and the values were controlled with a pHmeter CRISON mod. BASIC20.

Species and embryo collection

Adult specimens of *P. lividus* were collected by SCUBA divers at *ca*. 5 m depth in the vicinity of the Hydrobiological Station "Umberto d'Ancona" in the south basin of the Venice Lagoon (NW Adriatic Sea, Italy) in March 2016, at the beginning of the gonadal maturity period in this species for the Adriatic sea (Tomšič *et al*., 2010). Sea urchins were maintained for one week in flowing filtered water (20 µm) at 20°C temperature and 33 PSU salinity and were fed with *Ulva rigida*.

Gametes were obtained by injecting 1 ml of 0.5 M KCl solution into the coelom, through the peristome membrane. Sperm from each male was collected dry by using a micropipette, and stored in small tubes at 4 °C pending fertilization. The eggs from each female were gathered in 250 ml beakers filled with 0.45 µm filtered sea water (FSW). In order to remove spine and algae fragments, the eggs were filtered with 200 µm mesh nylon filter and then concentrated with 20 µm mesh nylon filter and suspended in FSW. The density of the eggs was evaluated by counting subsamples under optical microscope (Leica DM 750) and the density of sperm was determined by a Coulter Counter mod. Z2 after 1:20000 dilution in FSW.

Equal number of eggs from each female were placed in 250 ml beakers filled with FSW. In beakers with eggs, equal numbers of sperm from each male were added, maintaining standard sperm:egg ratio (1250:1) according to the recommendations of Dinnel *et al*., 1987. Fertilization success was checked after 15 minutes, ensuring the presence of fertilization membrane in at least 90 % of eggs.

Fertilized eggs were exposed to five nominal concentrations (0.5, 1.0, 5.0, 10.0, 15.0 $\mu g l^{-1}$) of each compound and two pH values (8.1 and 7.7). Six 24-ml replicates were performed, each with approximately 50 eggs ml^{-1} and were maintained at 20 °C. Three replicates were fixed with 10% neutralized formalin after 24 h, and other three were fixed 48 h after fertilization. In each replicate 60 individuals were observed to determine the frequency of various developmental stages and growth anomalies 24 h and 48 h after fertilization. Development index was calculated, by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Length of the somatic rod of 60 four-armed echinoplutei was measured in all replicates fixed 48 h after fertilization.

Statistic analysis

Differences between values oflarval somatic rod length and between values of the development index at the various experimental conditions were tested by Mixed model analysisfollowed by multiple comparisons of the means with p values adjusted by Holm method. The R software (R Core Team 2013) was used.

4.4.3. RESULTS

Clofibric acid (CA)

With pK_a=3.2 (Gao and Deshusses, 2011), when dissolved in basic aqueous medium, CA is almost completely in ionized or hydrophilic form and its lipophilicity is expected to increase with OA: at 8.1 pH lipophilic form is represented by 0.0013% and at 7.7 pH by 0.0032% (Fig. 1A). Even if bioavailability of this compound seems to be very low in sea water, significant negative effects were observed in all parameters evaluated. After 24h from fertilization, larval development, , was delayed at concentrations ≥ 5 and ≥ 0.5 µg l^{-1} at 8.1 and 7.7 pH, respectively (Fig. 1B; Table 2), whereas after 48h the effect concentrations were higher, namely ≥ 15 and ≥ 5 µg l^{-1} at 8.1 and 7.7 pH, respectively (Fig. 1C; Table 3). 48h after fertilization, larval growth was lower at concentrations ≥ 10 µg l^{-1} at pH 8.1 and at 5 and 15 µg l^{-1} at pH 7.7 (Fig. 1D; Table 4).

Caffeine (CAF)

CAF is a weak base with pK_a=14 (Zylber-Katz et al., 1984) and when dissolved in seawater it is almost completely in hydrophilic form (Fig. 2A). Variation in lipophilicity, across seawater pH range, can be considered negligible. Significant effect of CAF was present in development index after 24h (Fig. 2B; Table 5) and in larval growth after 48h (Fig. 2D; Table 6), but multiple comparisons of the means did not highlight the presence of concentrations significantly different from concentration 0 at the same pH, so no LOEC at either 8.1 or 7.7pH was observed. Larval development after 48h showed significative effect of pH only (Fig. 2C, Table 6)

Diclofenac (DCF)

DCF is an acidic compound with pKa=4 (Carter et al., 2014). As CA and CAF, when dissolved in seawater it is mainly in hydrophilic form and its lipophilicity increases with decreasing pH of aqueous medium (0.008% at 8.1 pH; 0.020% at 7.7 pH) (Fig. 3A). Larval development index at 24h post-fertilization was negatively affected at concentrations 1 and ≥ 5 µg l^{-1} at 8.1 pH and at

concentrations ≥ 0.5 μg l^{-1} at 7.7 pH (Fig. 3B, Table 8). Significant negative effects of DCF were present also on development index measured 48h post-fertilization (fig. 3C), but no LOEC at both pH values was found (Table 9). None DCF concentrations tested influenced the larval growth at 48h post-fertilization (Fig. 3D, Table 10).

Ibuprofen (IBU)

IBU is an acidic pharmaceutical with pKa=4.91 (Jones et al., 2002). When dissolved in basic aqueous medium it is mainly in hydrophobic form and its percentage of lipophilic form is expected to increase under OA: at 8.1 pH lipophilic form is represented by 0.06% and at 7.7 pH by 0.16% (Fig. 4A). Ibuprofen affected development index after 24h (Fig. 4B, Table 11) and larval growth after 48h (Fig. 4D, Table 13). LOECs observed for these parameters at 8.1 pH were 15 and 10 μg l^{-1}, respectively, and at 7.7 pH 10 μg l^{-1} for both parameters. No significant effects of pH and IBU were found on larval development after 48h (Fig. 4C, Table 12).

Propranolol (PR)

PR is a weak base with pKa=9.5 (Carter et al., 2014). When dissolved in acidic medium it is almost completely in hydrophilic form and its percentage of lipophilic form increases with pH: at 8.1 lipophilic form is represented by 3.83% and at 7.7 pH by 1.56% (Fig. 5A). Significant effect of PR was observed on all parameters measured. Development index after 24h (Fig. 5B) was significantly lower at concentrations ≥ 10 μg l^{-1} at pH 8.1 and at 15 μg l^{-1} at 7.7 pH (Table 14). LOEC for larval development after 48h (Fig. 5C) was detected only at 8.1 pH and it was 15 μg l^{-1} (Table 15), whereas larval growth (Fig. 5D), after the same exposure time, was significantly lower at concentrations ≥ 10 μg l^{-1} at 8.1 pH, and at 15 μg l^{-1} at 7.7 pH (Table 16).

Sulfadiazine (SD)

SD has acidic nature and pK_a value is 6.43 (Lin et al., 1997). At seawater pH range, SD is mostly in hydrophilic form and the percentage of its lipophilic form is expected to increase under OA: at 8.1 lipophilic form is represented by 2.09% and at 7.7 pH by 5.10% (Fig. 6A). In this experiment, LOEC was observed only on development index after 24h, with a significant decrease at 7.7 pH at concentrations ≥ 10 μg l^{-1} (Fig. 6B; Table 17). Development index of *P. lividus* larvae after 48h showed significant effect of SD but lo LOEC values were foun (Fig. 6C, Table 18) and larval growth exhibit significant negative effect of 7.7 pH but not of SD (Fig. 6D, Table 19)

Triclosan (TRC)

TRC presents a pK_a value of 8.14 (Reiss et al. 2002). When dissolved at pH lower than 8.1, it is mostly in lipophilic form and its lipophilicity is expected to increase under OA: at 8.1 lipophilic form is represented by 52.30% and at 7.7 pH by 73.36% (Fig. 7A). TRC influenced development index after 24h (Fig. 7B) and larval growth after 48h (Fig. 7D). Development index was significantly lower at concentrations ≥ 1 μg l^{-1} at both 8.1 an 7.7 pH (Table 20) and larval growth was negatively affected by TRC only at pH 7.7 at concentrations ≥ 1 μg l^{-1} (Table 22). Negative effect of SD was observed on development index after 48h but no LOEC values were found (Fig. 7C, Table 18).

Trimethoprim (TMP)

TMP has basic nature with pK_a=7.3 (ElShaer et al., 2012). When dissolved in basic aqueous medium, TMP is almost totally in lipophilic form. The percentage of its lipophilic form is expected to decrease under OA: at 8.1 lipophilic form is represented by 86.32% and at 7.7 pH by 71.53% (Fig. 8A). In this experiment, TMP influenced the development but not the growth (Fig.8D, Table 19) of *P. lividus* larvae. After 24h (Fig. 8B) larval development index was significantly lower at concentrations ≥ 10 μg l^{-1} at 8.1 pH and at 15 μg l^{-1} at 7.7 pH (Table 23), whereas after 48h (Fig. 8C) DI was lower at concentrations ≥ 10 μg l^{-1} at 8.1 pH and ≥ 1 μg l^{-1} at 7.7 pH (Table 24).

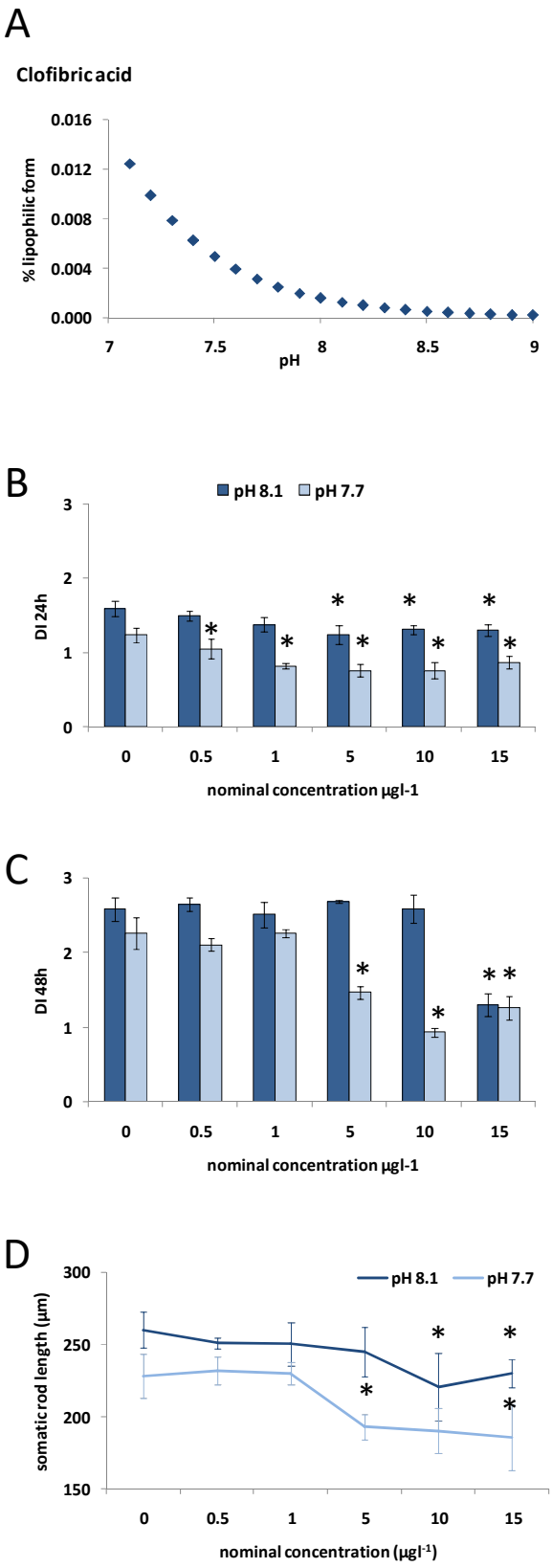

Fig. 1 Percentage of lipophilic form of clofibric acid (CA) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of CA. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are presented as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	7225	7225	1	24	226.815	9.948e-14 ***
CA	2745.8	549.2	5	24	17.24	2.9493-07 ***
pH : CA	163.1	32.6	5	24	1.024	0.4258

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	0.14	***	**	**	***	***	***	***	***	***
	0.5		1.00	*	0.34	0.29	*	***	***	***	***	***
	1			1.00	1.00	1.00	1.00	***	***	***	***	***
	5				1.00	1.00	1.00	0.29	***	***	***	***
	10					1.00	1.00	*	***	***	***	***
	15						1.00	*	***	***	***	***
pH 7.7	0							0.32	***	***	***	***
	0.5								0.07	**	**	0.34
	1									1.00	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 2 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of CA.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	14712	14711.7	1	22	290.324	3.686e-14 ***
CA	22267	4453.3	5	22	87.883	8.726e-14 ***
pH : CA	10702	2140.4	5	22	42.239	1.483e-10 ***

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	***	*	***	*	***	***	***
	0.5		1.00	1.00	1.00	***	**	***	**	***	***	***
	1			1.00	1.00	***	0.16	***	0.14	***	***	***
	5				1.00	***	***	***	***	***	***	***
	10					***	*	***	*	***	***	***
	15						***	***	***	1.00	**	1.00
pH 7.7	0							1.00	1.00	***	***	***
	0.5								1.00	***	***	***
	1									***	***	***
	5										***	0.58
	10	Adjusted p values reported -- holm method										*

Table 3 Linear mixed model and multiple comparison of means: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of CA.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	22703	22703	1	24	39.276	1.774e-06 ***
CA	33346	6669.2	5	24	11.538	9.336e-06***
pH : CA	4426	885.1	5	24	1.531	0.2176

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	***	***	0.25	*	*	***	***	***
	0.5		1.00	1.00	*	*	1.00	0.47	0.46	***	***	***
	1			1.00	*	*	1.00	0.48	0.47	***	***	***
	5				*	0.07	1.00	1.00	0.97	***	***	***
	10					1.00	1.00	1.00	1.00	1.00	1.00	1.00
	15						1.00	1.00	1.00	1.00	1.00	1.00
pH 7.7	0							1.00	1.00	*	0.06	*
	0.5								1.00	0.27	0.47	0.26
	1									0.28	0.48	0.27
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 4 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of CA.

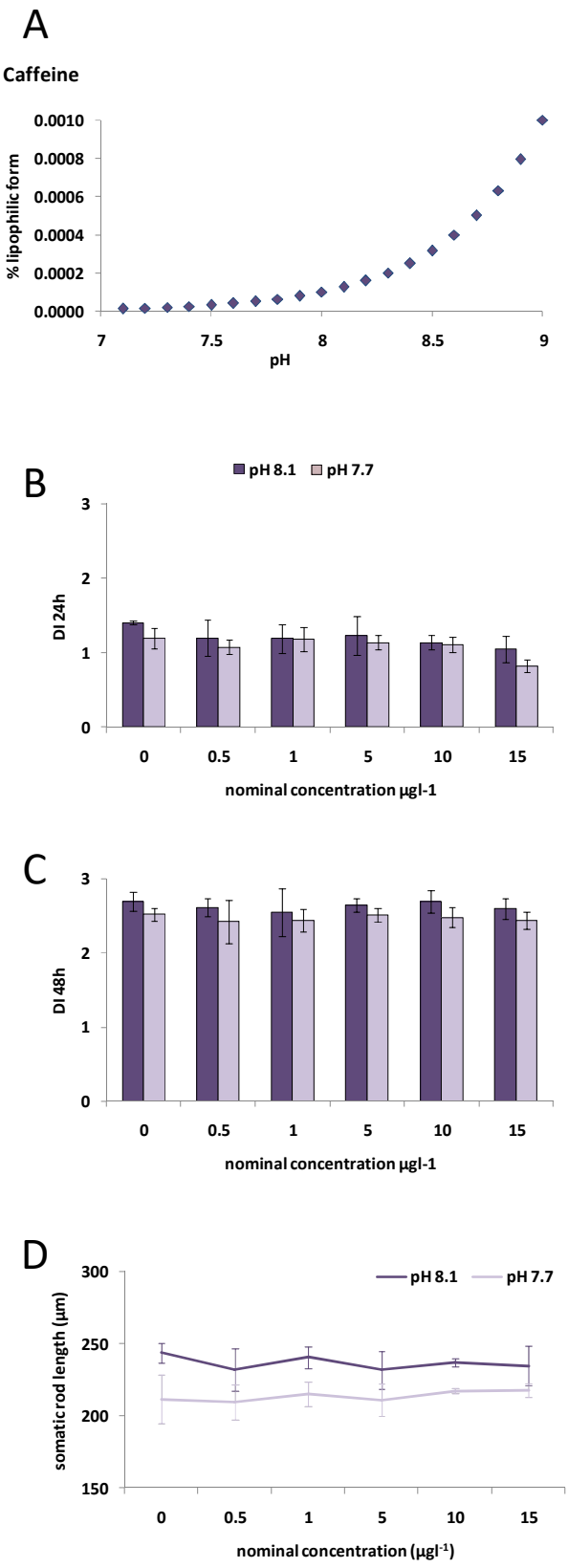

Fig. 2 Percentage of lipophilic form of caffeine (CAF) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of CAF. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	430.56	430.56	1	22	5.9393	0.023353 *
CAF	1534.65	306.93	5	22	4.2338	0.007566 **
pH : CAF	229.81	45.96	5	22	0.675929	0.675929

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	0.13	1.00	0.28	1.00	1.00	0.62	***
	0.5		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.07
	1			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.10
	5				1.00	1.00	1.00	1.00	1.00	1.00	1.00	*
	10					1.00	1.00	1.00	1.00	1.00	1.00	0.36
	15						1.00	1.00	1.00	1.00	1.00	1.00
pH 7.7	0							1.00	1.00	1.00	1.00	0.09
	0.5								1.00	1.00	1.00	1.00
	1									1.00	1.00	0.11
	5										1.00	0.36
	10	Adjusted p values reported -- holm method										0.70

Table 5 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of CAF.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	880.11	880.11	1	24	8.6562	0.007117 **
CAF	230.58	46.12	5	24	0.4536	0.806509
pH : CAF	33.14	6.63	5	24	0.0652	0.996744

Table 6 Linear mixed model: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of CAF.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	64632	64632	1	24	66.743	2.173e-08 ***
CAF	15794	3159	5	24	3.262	0.02183*
pH : CAF	6116	1223	5	24	1.263	0.31189

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	0.16	1.00	0.16	**	***	***	***	***	***
	0.5		1.00	1.00	1.00	1.00	1.00	***	0.16	*	0.41	0.47
	1			0.58	1.00	0.59	*	***	***	***	**	**
	5				1.00	1.00	1.00	*	1.00	0.40	1.00	1.00
	10					1.00	0.24	***	*	***	*	*
	15						1.00	*	1.00	0.38	1.00	1.00
pH 7.7	0							0.58	1.00	1.00	1.00	1.00
	0.5								1.00	1.00	1.00	1.00
	1									1.00	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 7 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of CAF.

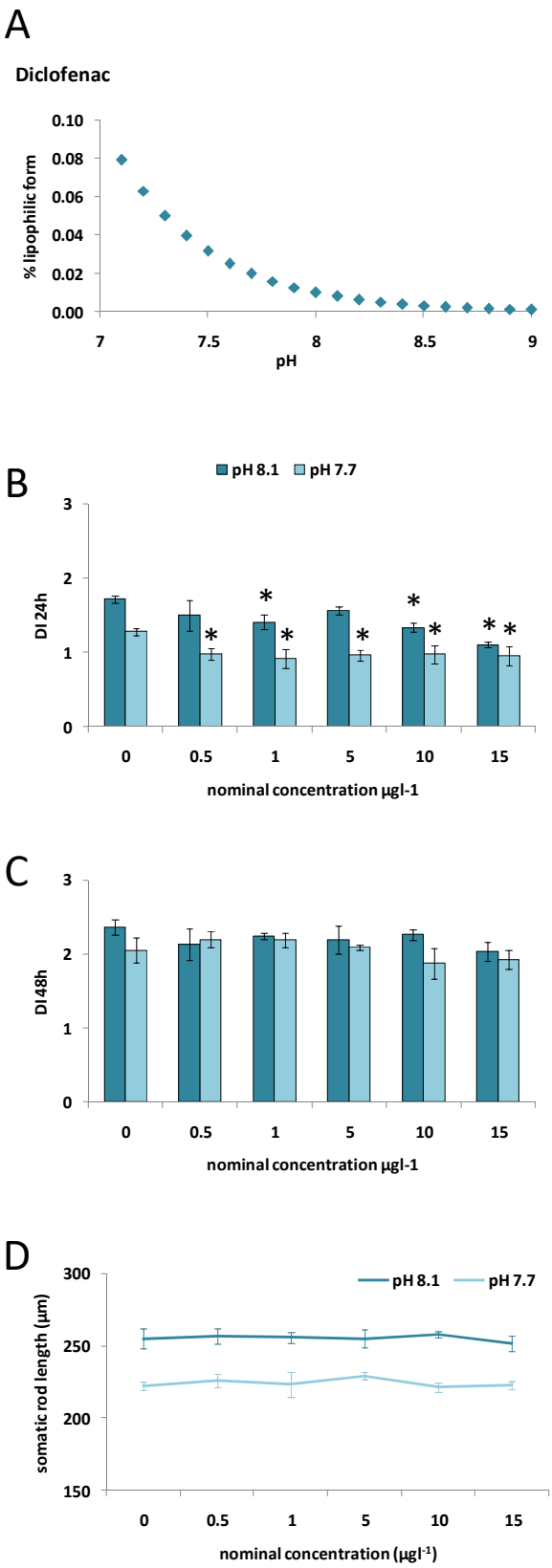

Fig. 3 Percentage of lipophilic form of diclofenac (DCF) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of DCF. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	5865	5865	1	22	176.244	5.579e-12 ***
DCF	2638.8	527.8	5	22	15.859	1.172e-06 ***
pH : DCF	677.5	135.5	5	22	4.071	0.00909 **

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	0.13	**	1.00	***	***	***	***	***	***	***	***
	0.5		1.00	1.00	0.88	***	0.14	***	***	***	***	***
	1			1.00	1.00	**	1.00	***	***	***	***	***
	5				0.09	***	**	***	***	***	***	***
	10					0.09	1.00	***	***	***	***	***
	15						0.59	1.00	0.47	1.00	1.00	1.00
pH 7.7	0							**	***	**	**	**
	0.5								1.00	1.00	1.00	1.00
	1									1.00	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 8 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of DCF.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	715.56	715.56	1	24	10.6778	0.003259 **
DCF	880.62	176.12	5	24	2.6282	0.049506 *
pH : DCF	786.23	157.25	5	24	2.3465	0.072024

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	0.17	0.26	1.00	1.00	0.75	***	**
	0.5		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	1			1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.06	0.28
	5				1.00	1.00	1.00	1.00	1.00	1.00	0.26	0.95
	10					1.00	1.00	1.00	1.00	1.00	*	0.17
	15						1.00	1.00	1.00	1.00	1.00	1.00
pH 7.7	0							1.00	1.00	1.00	1.00	1.00
	0.5								1.00	1.00	0.23	0.84
	1									1.00	0.28	0.99
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 9 Linear mixed model and multiple comparison of means: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of DCF.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	203498	203498	1	24	364.76	4.441e-16 ***
DCF	2241	448	5	24	0.8	0.5583
pH : DCF	2463	493	5	24	0.88	0.5077

Table 10 Linear mixed model results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of DCF.

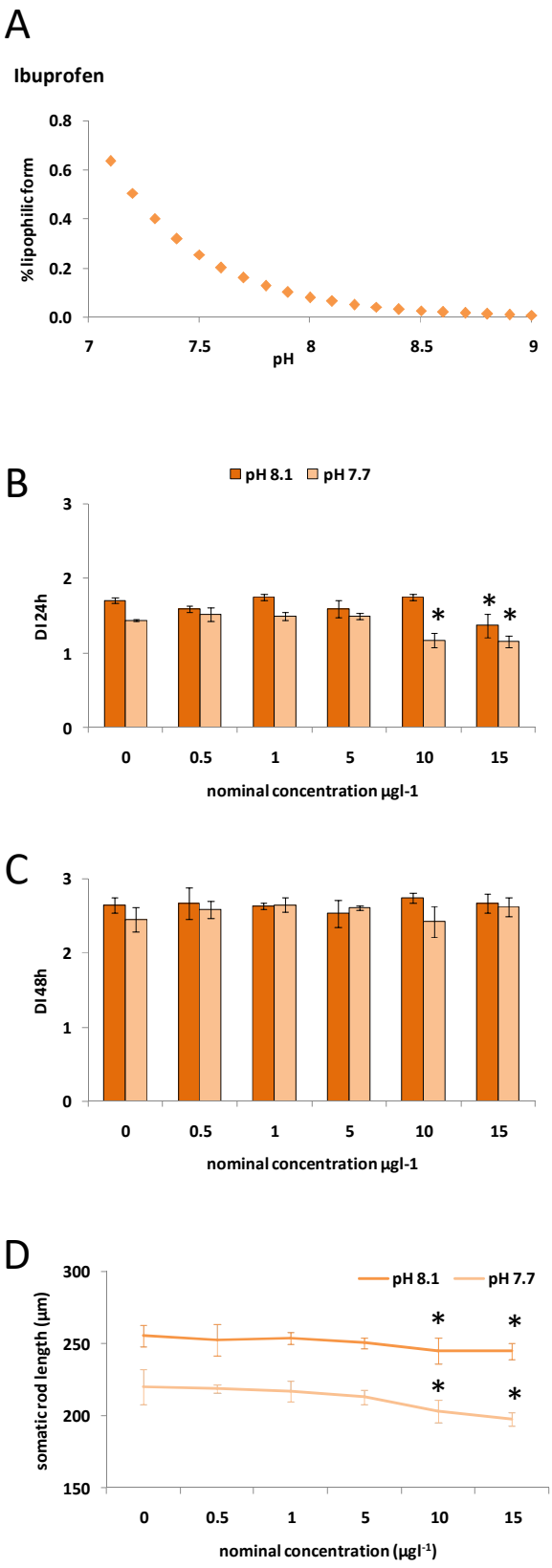

Fig. 4 Percentage of lipophilic form of ibuprofen (IBU) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of IBU. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	1980.25	1980.25	1	22	88.721	3.545e-09 ***
IBU	1775.33	355.07	5	22	15.908	1.142e-06 ***
pH : IBU	890.58	178.12	5	22	7.98	0.0002041 ***

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	***	**	0.13	0.33	*	***	***
	0.5		0.35	1.00	0.35	*	0.47	1.00	1.00	1.00	***	***
	1			0.37	1.00	***	***	*	**	**	***	***
	5				0.37	*	0.44	1.00	1.00	1.00	***	***
	10					***	***	*	**	**	***	***
	15						1.00	0.47	1.00	1.00	0.06	*
pH 7.7	0							1.00	1.00	1.00	**	***
	0.5								1.00	1.00	***	***
	1									1.00	***	***
	5										***	***
	10	Adjusted p values reported -- holm method										1.00

Table 11 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of IBU.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	266.78	266.778	1	22	2.5131	0.1272
IBU	177.22	35.444	5	22	0.33389	0.8869
pH : IBU	562.14	112.428	5	22	1.05909	0.4093

Table 12 Linear mixed model: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of IBU.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	826047	826047	1	24	1121.46	< 2e-16 ***
IBU	87066	17413	5	24	23.64	< 2e-16 ***
pH : IBU	10333	2067	5	24	2.81	0.01566 *

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	0.77	1.00	0.21	***	***	***	***	***	***	***	***
	0.5		1.00	1.00	0.17	0.15	***	***	***	***	***	***
	1			1.00	*	*	***	***	***	***	***	***
	5				0.69	0.67	***	***	***	***	***	***
	10					1.00	***	***	***	***	***	***
	15						***	***	***	***	***	***
pH 7.7	0							1.00	1.00	0.21	***	***
	0.5								1.00	0.52	***	***
	1									1.00	***	***
	5										*	***
	10	Adjusted p values reported -- holm method										0.67

Table 13 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of IBU.

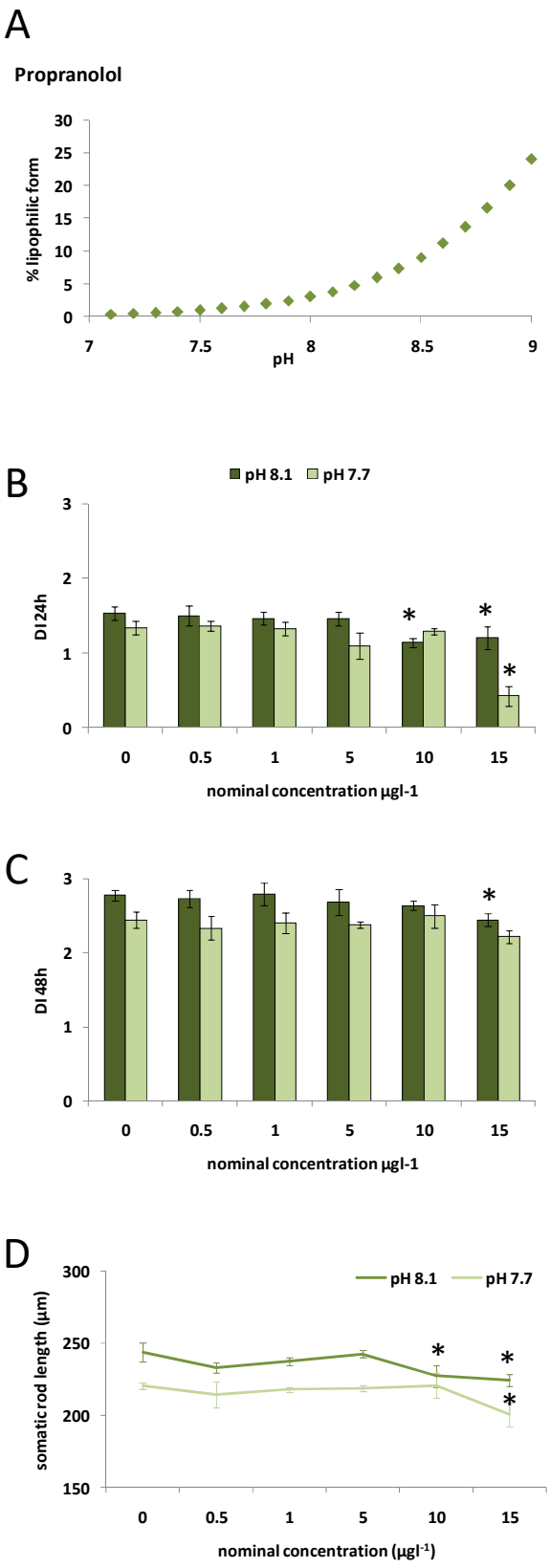

Fig. 5 Percentage of lipophilic form of propranolol (PR) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of PR. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	1936	1936	1	24	46.179	7.919e-07 ***
PR	6035.4	1207.08	5	24	28.792	5.897e-09 ***
pH : PR	2566.8	513.35	5	24	12.245	9.368e-06 ***

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	***	**	0.96	1.00	0.61	***	0.21	***
	0.5		1.00	1.00	**	*	1.00	1.00	1.00	***	0.58	***
	1			1.00	*	0.15	1.00	1.00	1.00	**	1.00	***
	5				*	0.15	1.00	1.00	1.00	**	1.00	***
	10					1.00	0.91	0.46	1.00	1.00	1.00	***
	15						1.00	1.00	1.00	1.00	1.00	***
pH 7.7	0							1.00	1.00	0.25	1.00	***
	0.5								1.00	0.11	1.00	***
	1									0.43	1.00	***
	5										1.00	***
	10	Adjusted p values reported -- holm method										***

Table 14 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of PR.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	2844.44	2844.44	1	22	64.164	5.779e-08 ***
PR	1131.14	226.23	5	22	5.103	0.002955 **
pH : PR	261.97	52.39	5	22	1.182	0.349761

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	*	*	***	**	***	0.10	***
	0.5		1.00	1.00	1.00	0.08	0.07	***	*	**	0.42	***
	1			1.00	1.00	**	**	***	***	***	0.05	***
	5				1.00	0.34	0.32	**	0.10	*	1.00	***
	10					1.00	0.94	*	0.36	0.19	1.00	***
	15						1.00	1.00	1.00	1.00	1.00	0.42
pH 7.7	0							1.00	1.00	1.00	1.00	0.43
	0.5								1.00	1.00	1.00	1.00
	1									1.00	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										0.08

Table 15 Linear mixed model and multiple comparison of means: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of PR.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	105006	105006	1	24	104.175	3.292e-10 ***
PR	47362	9472	5	24	9.398	4.617e-05* **
pH : PR	10455	2091	5	24	2.074	0.1039

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	0.64	1.00	1.00	*	**	***	***	***	***	***	***
	0.5		1.00	0.98	1.00	1.00	0.21	**	*	0.08	0.24	***
	1			1.00	0.72	0.16	**	***	**	**	*	***
	5				*	**	***	***	***	***	***	***
	10					1.00	1.00	0.17	1.00	1.00	1.00	***
	15						1.00	0.75	1.00	1.00	1.00	***
pH 7.7	0							1.00	1.00	1.00	1.00	***
	0.5								1.00	1.00	1.00	0.09
	1									1.00	1.00	**
	5										1.00	**
	10	Adjusted p values reported -- holm method										***

Table 16 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of PR.

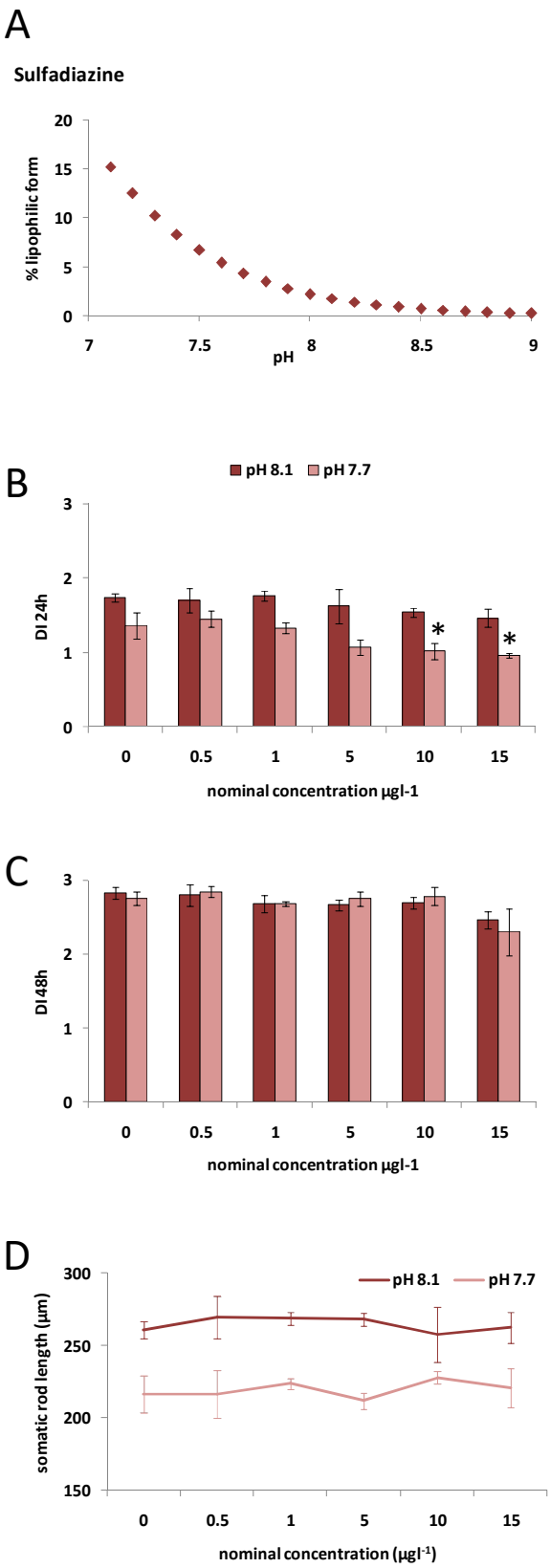

Fig. 6 Percentage of lipophilic form of sulfadiazine (SD) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of SD. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	6254.2	6254.2	1	22	134.709	7.522e-11 ***
SD	2707.4	541.5	5	22	11.663	1.362e-05 ***
pH : SD	340.7	68.1	5	22	1.468	0.2404

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	0.71	0.08	**	0.06	***	***	***	***
	0.5		1.00	1.00	1.00	0.26	***	0.19	**	***	***	***
	1			1.00	0.41	*	***	*	***	***	***	***
	5				1.00	1.00	0.13	1.00	*	***	***	***
	10					1.00	1.00	1.00	0.55	***	***	***
	15						1.00	1.00	1.00	**	***	***
pH 7.7	0							1.00	1.00	0.06	**	***
	0.5								1.00	**	***	***
	1									0.16	*	**
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 17 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of SD.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	0.17	0.17	1	24	0.0028	0.958
SD	2712	542.55	5	24	8.8589	7.148e-05 ***
pH : SD	273.95	54.79	5	24	0.8946	0.5004

Table 18 Linear mixed model: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of SD.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	85208	85208	1	24	150.847	7.716e-12 ***
SD	972	194	5	24	0.344	0.8809
pH : SD	3001	600	5	24	1.063	0.4053

Table 19 Linear mixed model: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of SD.

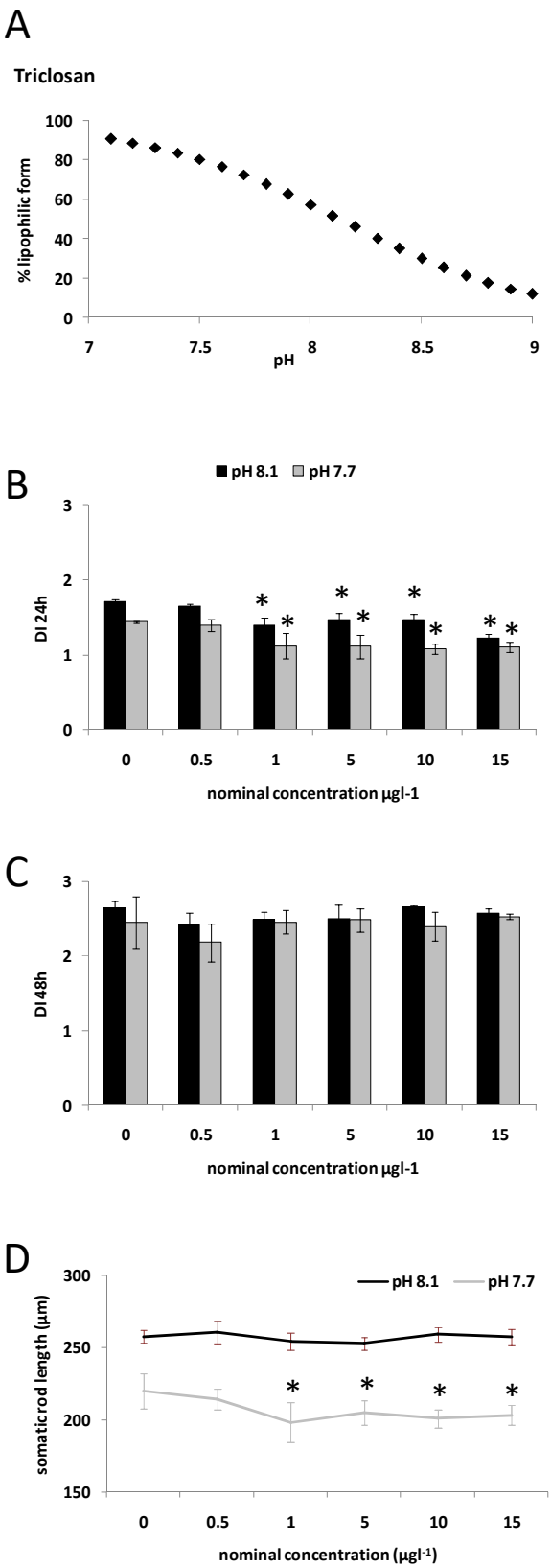

Fig. 7 Percentage of lipophilic form of triclosan (TRC) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of TRC. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	2475.06	2475.06	1	22	95.755	1.789e-09 ***
TR	2847.53	569.51	5	22	22.033	6.837e-08 ***
pH : TR	216.48	43.3	5	22	1.675	0.1825

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	***	*	*	***	**	***	***	***	***	***
	0.5		**	0.18	0.19	***	0.07	**	***	***	***	***
	1			1.00	1.00	0.41	1.00	1.00	**	**	***	***
	5				1.00	*	1.00	1.00	***	***	***	***
	10					*	1.00	1.00	***	***	***	***
	15						0.06	0.41	1.00	1.00	0.63	1.00
pH 7.7	0							1.00	***	***	***	***
	0.5								**	**	***	***
	1									1.00	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 20 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of TRC.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	556.17	556.17	1	24	5.0253	0.03449 *
TR	984.12	196.82	5	24	1.7784	0.15548
pH : TR	318.95	63.79	5	24	0.5764	0.71753

Table 21 Linear mixed model: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of TRC.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	1350745	1350745	1	24	1838.24	< 2.2e-16 ***
TR	45277	9055	5	24	12.32	8.334e-12 ***
pH : TR	26536	5307	5	24	7.22	1.024e-06 ***

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	1.00	1.00	***	***	***	***	***	***
	0.5		0.62	0.16	1.00	1.00	***	***	***	***	***	***
	1			1.00	1.00	1.00	***	***	***	***	***	***
	5				0.49	1.00	***	***	***	***	***	***
	10					1.00	***	***	***	***	***	***
	15						***	***	***	***	***	***
pH 7.7	0							0.75	***	***	***	***
	0.5								***	*	***	**
	1									0.37	1.00	1.00
	5										1.00	1.00
	10	Adjusted p values reported -- holm method										1.00

Table 22 Linear mixed model and multiple comparison of means: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of TRC.

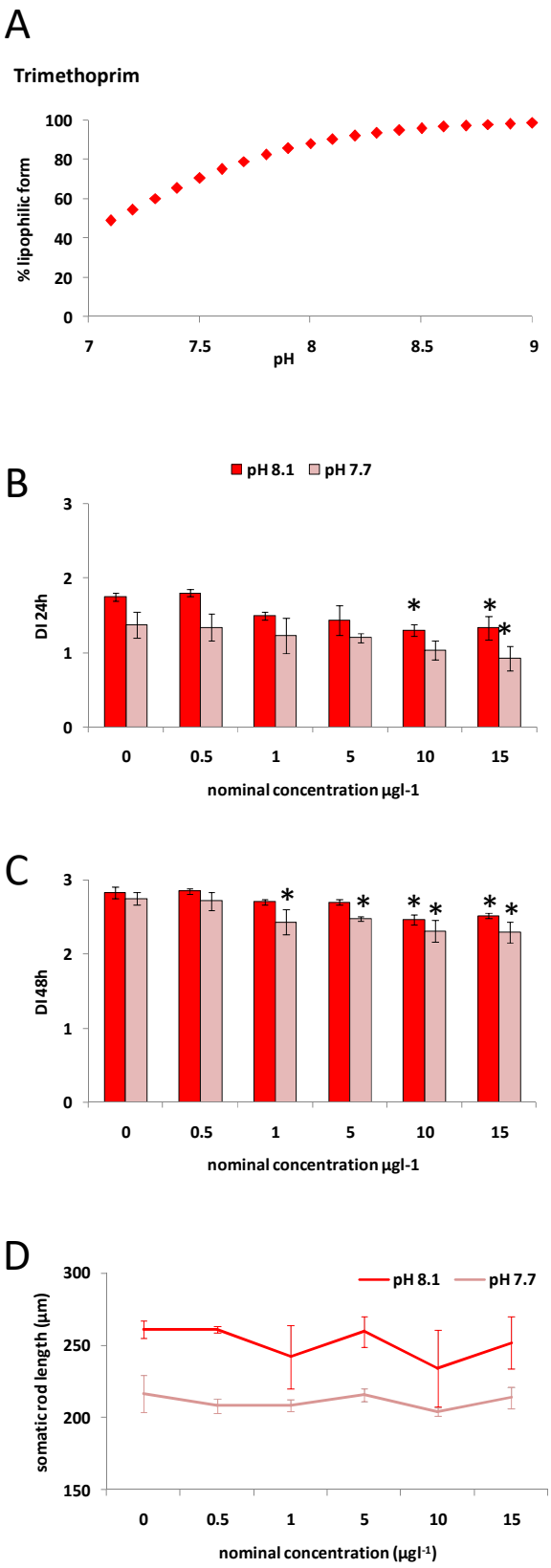

Fig. 8 Percentage of lipophilic form of trimethoprim (TMP) at differing pH values of the medium, calculated according to Hendersen-Hesselbalch equation (A). Development index (DI) after 24h (B) and 48h (C), and somatic rod length (D) of *P. lividus* larvae exposed to different concentration of TMP. DI is calculated by summing the scores assigned to each larval stage (0, egg and blastula; 1, gastrula; 2, prism; 3, four-armed pluteus; 1.5, abnormal prism; 2.5, abnormal four-armed pluteus) and dividing it by number of individuals. Data are exposed as mean ± sd (n=3). Symbol (*) indicate significant differences in comparison with concentration 0 at same pH ($p<0.05$).

DI 24h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	3680.4	3680.4	1	24	48.667	3.258e-07 ***
TMP	3823.5	764.7	5	24	10.112	2.646e-05 ***
pH : TMP	218.9	43.8	5	24	0.579	0.7157

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	0.34	**	*	0.06	*	***	***	***	***
	0.5		0.37	0.08	**	**	*	**	***	***	***	***
	1			1.00	1.00	1.00	1.00	1.00	0.73	0.42	**	***
	5				1.00	1.00	1.00	1.00	1.00	1.00	*	***
	10					1.00	1.00	1.00	1.00	1.00	0.78	0.07
	15						1.00	1.00	1.00	1.00	0.42	*
pH 7.7	0							1.00	1.00	1.00	0.18	**
	0.5								1.00	1.00	0.36	*
	1									1.00	1.00	0.42
	5										1.00	0.73
	10	Adjusted p values reported -- holm method										1.00

Table 23 Linear mixed model and multiple comparison of means: results from 24h DI values in *P. lividus* larvae exposed to different concentrations of TMP.

DI 48h	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	1056.2	1056	1	24	33.282	6.028e-06 ***
TMP	3290.6	658.13	5	24	20.738	5.298e-08 ***
pH : TMP	140.6	28.12	5	24	0.886	0.5057

		pH 8.1					pH 7.7					
		0.5	1	5	10	15	0	0.5	1	5	10	15
pH 8.1	0	1.00	1.00	1.00	***	**	1.00	1.00	***	***	***	***
	0.5		1.00	1.00	***	***	1.00	1.00	***	***	***	***
	1			1.00	0.07	0.44	1.00	1.00	*	0.11	***	***
	5				0.09	0.52	1.00	1.00	0.14	*	***	***
	10					1.00	**	*	1.00	1.00	1.00	0.61
	15						0.86	0.31	1.00	1.00	0.23	0.10
pH 7.7	0							1.00	**	*	***	***
	0.5								**	0.08	***	***
	1									1.00	1.00	1.00
	5										0.77	0.41
	10	Adjusted p values reported -- holm method										1.00

Table 24 Linear mixed model and multiple comparison of means: results from 48h DI values in *P. lividus* larvae exposed to different concentrations of TMP.

Growth	Sum Sq	Mean Sq	NumDF	DenDF	F. value	Pr(>F)
pH	96787	96787	1	24	167.968	2.499e-12 ***
TMP	4319	864	5	24	1.499	0.2273
pH : TMP	1018	204	5	24	0.353	0.875

Table 25 Linear mixed model: results from somatic rod length values in *P. lividus* larvae exposed to different concentrations of TMP.

4.4.4. DISCUSSION

A significant adverse effect of reduced pH on larval growth and development was found in all experiments. Considering all data in these experiments, after 48h exposure at 7.7 pH, reduction of somatic rod length, measured in *P. lividus* larvae, ranged from 9.5 to 17% (14% in average). For all pharmaceuticals, except for CAF, significant effect of the pharmaceutical was also present.

Considering both larval development and growth, the following LOEC (lowest observed effect concentration) values were determined:

- 5 μgl^{-1} at 8.1 pH and 1 μgl^{-1} at 7.7 pH for clofibric acid;
- 1 μgl^{-1} at 8.1 pH and 0.5 μgl^{-1} at 7.7 pH for diclofenac;
- 10 μgl^{-1} at 8.1 pH and 10 μgl^{-1} at 7.7 pH for ibuprofen;
- 10 μgl^{-1} at 8.1 pH and 15 μgl^{-1} at 7.7 pH for propranolol;
- no effect at 8.1 pH and 10 μgl^{-1} at 7.7 pH for sulfadiazine;
- 1 μgl^{-1} at 8.1 pH and 1 μgl^{-1} at 7.7 pH for triclosan;
- 10 μgl^{-1} at 8.1 pH and 1 μgl^{-1} at 7.7 pH for trimethoprim.

Lower LOEC at 7.7 pH was observed for CA, DCF, SD and TMP suggesting the presence of combined effect between stressors. On the contrary, for PR lower LOEC was observed at pH 8.1 suggesting antagonistic interaction between low pH and this pharmaceutical. For IBU and TR, the same LOECs were observed at both pHs and no effect was observed for CAF. According to Hendersen-Hesselbalch equation, lipophilic form of CA, DCF, IBU, SD and TR increase at lower pH value, whereas decrease for PR and TMP and remain rather the same for CAF at pH tested in this experiment.

If we consider expectation based on the Hendersen-Hesselbalch equation for each compound, results obtained for CA, DCF and SD are consistent with their higher bioavailability at low pH which could explain the presence of combined effect between stressors. Lower bioavailability at low pH could explain antagonistic effect between stressors observed for PR. For TMP, results are not consistent with bioavailability changes at different pH and suggest the presence of synergic or addictive effect between this compound and pH 7.7.

Although this approach in analysing data from pharmaceutical toxicity testing under different pH conditions has never been addressed in previous studies, some data from the literature can be considered for a comparison with the present findings. In *P. lividus*, Aguirre-Martinez et al., (2015) reported significant decreases in the frequencies of normal plutei at 48h after fertilization and 18 °C exposure in the presence of CAF concentrations ≥ 0.01 μgl^{-1}. In the same work, fertilization

was negatively affected at very high concentration of caffeine (10 g l^{-1}). The effect of DCF and PR were evaluated on *P. lividus* growth and development after 48h and 20 °C exposure by Ribeiro et al., (2015). These authors found significant negative effect on larval growth and development at concentrations ≥ 12.5 $\mu g l^{-1}$ for DCF, and for PR, growth was negatively affected at concentrations ≥ 12.5 $\mu g l^{-1}$ and development at concentrations ≥ 5 $\mu g\ l^{-1}$. In this work, no negative effect of DCF, were present after 48h even at the highest concentration tested (15 $\mu g\ l^{-1}$). We consider larval development also after 24h and here we found delay in development at concentration of 1 $\mu g\ l^{-1}$ of DCF. The results shown in this work on growth of larvae exposed to PR are consistent with findings in the previous work of Ribeiro et al. (2015), instead larval development after 48h, was affected only at the highest concentration considered in this experiment.

Sediment samples spiked with CAF, IBU and PR where used to evaluate the effects on *P. lividus* by Maranho et al., (2015). Environmentally relevant concentrations were considered, ranging from 0.05 to 500 ng g^{-1} for IBU e PR, and from 0.15 to 1500 ng g^{-1} for CAF, . No spermiotoxic and embryotixic effects were found for IBU, whereas for PR, negative effect was found even at the lowest concentration tested (0.05 ng g^{-1}) for both parameters. CAF showed negative effect only on larval development at all concentrations considered (LOEC 0.15 ng g^{-1}).

In conclusion, our findings highlighted that evidence of changes in bioavailability of the contaminants, alone and in mixture, under reduced pH deserves in-depth consideration in future studies. Compounds whose lipophilic and more toxic form increases under reduced pH value may exhibit a greater harmfulness in the future, even if their nominal concentrations in the environment remain the same. It would be prudent to consider changes in seawater acidity, when defining future pollution scenarios and when making decisions about the compounds that have to be included in watch list of priority pharmaceuticals.

4.4.5. REFERENCES

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5. CONCLUSIONS

Results obtained in this PhD thesis produced new information about the effects of seawater acidification in adult sea urchin *P. lividus* after long-term acclimation. For the first time physiological and biochemical responses to reduced pH values (7.7 and 7.4) were evaluated in *P. lividus* and in males and females separately. Several parameters such as respiration rate, ammonia production, assimilation efficiency, righting time, gonadosomatic index, antioxidant enzyme activities (SOD and CAT) and coelomocyte number and volume were evaluated and in almost all of them significant responses to long-term low pH exposure were found. Responses such as respiration rate, CAT activity, total coelomocyte count exhibited the same trend in males and females, but significant effects were not always present in both sexes. In ammonia excretion, gonadosomatic index and lysozyme activity, an opposite trend was displayed in the two sexes. Only in 3.77% of 511 acidification studies (published between January 2008 and May 2016) on fish, crustaceans, echinoderms and molluscs, sex-based differences were assessed. When tested, sex significantly influenced physiological, reproductive, biochemical and survival responses to ocean acidification (Ellis et al., 2017; Ellis et al., 2014; Lane et al., 2015; McClellan-Green et al., 2007). According to these results, in order to avoid losing significant effects and to infer more realistic and relevant information at population level, it is important to investigate further male and female responses separately. Under reduced pH, one of the more interesting responses observed was an opposite trend in gonadosomatic index of males and females which suggested higher investment in reproduction in males and lower in females. This result could be due to higher production cost of eggs compared to sperm, therefore females could be considered more vulnerable to stress arising from seawater acidification.

Sperm and eggs obtained from parents maintained at different pH conditions were used to evaluate if long-term exposure of adults to differing seawater pH values could affect performances of sperm, when activated at 8.1, 7.7 and 7.4 pH, and those of larvae, when reared at 8.1 and 7.7 pH.

Significantly higher values of gonadosomatic index and sperm ATP content was found in males maintained at 7.4 pH. Sperm performances (velocity, ATP content and ATP consumption) of control group (i.e., males kept at 8.1 pH) were not affected by future seawater pH conditions, but sperm from males maintained at 7.7 and 7.4 pH during gametogenesis exhibited lower ATP consumption rate at low pH than at control condition with no differences in velocity, suggesting a likely longer vitality at low pH than at 8.1 pH. Considering that sperm competitiveness is

influenced by several sperm characteristics such as length, concentration (Benzie and Dixon, 1994), swimming velocity and longevity (Levitan, 2000), possible longer vitality could represent an advantage for fertilization success of this species in future acidified conditions. Observations longer than 60 minutes on sperm velocity, longevity and fertilization capacity are necessary to better investigate this hypothesis.

When somatic and post-oral rod lengths of larvae obtained from parents maintained at different pH were compared, they resulted significantly different indicating that gametogenesis condition influenced larval growth. In particular larvae from parents maintained at low pH exhibited higher somatic rod length than larvae from parents of control group .

Stukling et al. (2015) found that, when exposed for 16 months at low pH, sea urchin *Sterechinus neumayeri* produced larger eggs compared to control. Gonadosomatic index but not egg size were considered in females used in this study. Our results showed decreased gonadosomatic index in females maintained at low pH values. This result could be due to the production of lower number of larger eggs with consequent higher length in larvae obtained from females maintained at low pH values.

Even if gametogenesis conditions influenced larval length, when larvae from each parental condition were exposed at 8.1 and 7.7 pH, significant negative effects of 7.7 pH were observed on larval growth in all larval groups. Lower size at 7.7 pH was more evident when somatic rod lengths were compared than in post-oral rod lengths. These results showed that two-month long gametogenesis under low pH had not influenced larval performance at 7.7 pH. Differences between short acclimation period (4-6 months) and long acclimation period (14-16 months) were found in sea urchins *S. neumayeri* and *Strongylocentrotus droebachiensis.* Unlike the short acclimation, after long acclimation, in both studies, larval performance was not significantly different across treatments (Stukling et al., 2015; Dupont et al., 2013). In this study entire gametogenesis occurred under experimental conditions but it is possible that longer parent exposure have to be performed, in order to understand if *P. lividus* larvae are able to overcome negative effects of reduced seawater pH during early larval development.

Ocean acidification could represent an important future threat also in combination with pollutants present in marine coastal areas, as demonstrated in experiments performed with the mixture of four pharmaceuticals (clofibric acid, caffeine, diclofenac, and propranolol) at environmentally relevant concentrations (0.5 μg l^{-1}).

In short-term experiments significant adverse effects of 7.7 pH on larval growth and development were found. After 48h exposure at 7.7 pH, somatic rod length in *P. lividus* larvae was reduced by 14%. Delay in larval development was observed after 24h, whereas it was not always present after 48h.

In long-term experiments, after 10 and 20 day exposure, larval size was no longer different in larvae kept at 7.7 pH respect to those kept at control conditions, mortality was not affected and larval development seemed to be faster after 20 days. However, an increase of abnormal larvae was present at 7.7 pH, after both 10 and 20 days. In long-term experiments *P. lividus* larvae exhibited high sensitivity at 7.4 pH, with detrimental effects on survival, growth and development. The pharmaceutical mixture did not affect larval growth and development in short-term experiments but exhibited negative effects in long-term experiments and at all pH values tested. These results suggest that, in order to evaluate toxic potential of most common pharmaceuticals in the environment or of their mixture, further studies at lower concentrations, but with longer exposure time should be performed. *P. lividus* larvae survival and development seemed to be strongly threatened by extreme pH value expected by 2100 (7.4), but exhibited a certain resistance to average pH value expected for the end of the century (7.7). Our results highlighted that the sensitivity of the sea urchin larvae could considerably increase in the presence of pollutants in marine coastal areas.

LOEC (lowest observed effect concentration) values for eighth pharmaceuticals belonging to different therapeutic classes [clofibric acid (lipid-lowering), caffeine (metabolic stimulator and adjuvant), diclofenac, ibuprofen (anti-inflammatory drugs) and propranolol (β-blocker), sulfadiazine, trimethoprim (antibiotics) and triclosan (antibacterial agent)] were evaluated at 8.1 and 7.7 pH. Synergic effects between stressors found for clofibric acid, diclofenac and sulfadiazine and negative effects found for propranolol are consistent with expectations based on the Hendersen-Hesselbalch equation for these compounds and with changes of their bioavailability when dissolved in seawater under reduced pH values. This relationship depends on the chemical characteristics of the compounds and could be useful to shed light on substances which could be potentially more hazardous in future scenarios even if their concentration in the environment will remain the same.

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