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Jellyfish blooms impacts on Mediterranean aquaculture: a multidisciplinary approach

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El azar afortunado suele ser casi siempre el premio del esfuerzo perseverante

(Santiago Ramón y Cajal)

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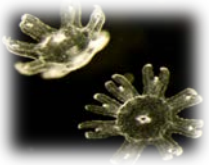

General Abstract

The blooms of jellyfish have repeatedly affected highly productive aquaculture operations worldwide and are currently recognized as a factor that negatively impacts marine fish farming. Nevertheless, information in the literature about the interactions between jellyfish and caged fish remains limited, both in the number of incidents reported and in the experiments conducted to better understand the effects of the cnidarians on fish. The present work was conceived as an attempt to fill this lack of awareness.

A perception survey (20 questions) was performed in 21 Mediterranean offshore aquaculture facilities from Italy, Spain, Malta and Tunisia (*chapter 1*). The interview was organized in 3 sets of questions: general knowledge on jellyfish and their blooms, JB qualitative impacts on farm's activity and JB quantitative impacts. The main results obtained from the surveys showed that fish farmers believed that the frequency of jellyfish blooms has increased during the last 10 years, and recognised significant impact of these organisms on different marine human activities, mainly tourism but also aquaculture. Moreover, high percentage of interviewees agreed on the significant economic impact that jellyfish may have on their own sector. However, just 20% of them recognised to have had problems with jellyfish in their facilities. The information gathered allowed us to identify the first records of fish mortality events due to jellyfish blooms in Mediterranean fish farms.

Chapter 2 of the thesis describes spatial and temporal distribution of jellyfish in southwestern Mediterranean marine aquaculture facilities, and investigates the role of gelatinous zooplankton on fish gill disorders and mortalities. Monitoring of zooplankton, phytoplankton and histological screening of fish gills was performed biweekly in two Spanish aquaculture facilities (located in the Alboran Sea, near to Almería and Málaga cities) where sea bass mortalities without a known causative agent were previously

recorded. Analyses showed significant and positive relation between cnidarians and recorded fish mortality, but not relationships of these mortalities with other gelatinous zooplankton groups or phytoplankton identified species. Histological analyses on fish gills were also positive correlated with cnidarian densities, reinforcing previous results.

Concurrently to this monitoring, fouling monitoring (*chapter 3*) with net panels was carried out near to juvenile cages, since fish affected by mortalities were 15-70 g weight. The study was divided in 2 periods (6 months each), in order to describe the cages biofouling community during the complete immersion period of fry cage and spanning 1 year of samplings. All fouling organisms were identified and divided in 7 different phyla to obtain biomass as dry weight ($g \cdot cm^{-2}$). Seven hydrozoan species were recorded during the complete monitoring, with *Pennaria disticha* being the predominant one during first period and *Ectopleura larynx* during the second. For both hydroids mature reproductive structures were identified (at different stages) and reproductive periods were identified through identification of eumedusoids and actinulae larvae in zooplankton samples. Also growth rate was calculated, *P. disticha* being the species with faster growth (0.29 mm d^{-1}).

In the literature I found just two experimental works about the direct effects that jellyfish blooms might have on caged fish health. Aforementioned studies were focused on the morphological impact (at gill damage level) that this interaction may have on fish (*Salmo salar*), but no more information about effects on gill integrity or fish metabolism on salmonids or other commercial species exists. Thus, we performed 3 different experiments (chapters 4, 5 & 6) to explore the effects of this interaction on sea bass and sea bream health using histological, biochemical and physiological approaches. *Chapter 4* aimed to evaluate gill injuries suffered by *Sparus aurata* individuals after short exposure to 3 different *Pelagia noctiluca* jellyfish densities. Our data revealed that extent and intensity of damage increased with time and also with jellyfish densities. Fish contacted with medium jellyfish densities presented a recovery of gill epithelium after 3 weeks from the exposure, while those individual belonged to the highest jellyfish density group presented immediately significant damage and after 2 weeks fish mortalities started. In *chapter 5* we evaluated physiological fish (*Dicentrarchus labrax*) response to abiotic

(temperature and hypoxia) and biotic (jellyfish stinging) stressors, through resprimetric measurements of oxygen rate (M_{O_2}) and critical oxygen pressure (P_{O_2crit}). Results demonstrated an increase in oxygen uptake and P_{O_2crit} in treated fish, showing synergistically action of temperature rise and jellyfish contact, increasing fish sensitivity to decreased oxygen concentration in water column.

Last contact experiment corresponded with *chapter 6*. The study of neurotransmitters as stress indicators showed that after contact between jellyfish and sea bass, expression of different proteins and hormones was decreased and then recovered after 1 week from the exposure to jellyfish. This information agreed with histological analyses of gill tissue and demonstrated a significant effect in the central nervous system affecting cholinergic and serotonergic systems, as well as dopamine pathways.

General Introduction

Gelatinous zooplankton

This group includes the polyphyletic assemblage made by cnidarian medusae and siphonophore colonies, ctenophores, larvaceans, doliolids, chaetognaths, polychaetes and other non-crustacean soft-bodied planktonic organisms (Haddock 2004). The common characteristic of this polyphyletic group is its watery constitution with a low carbon/water content ratio and low energy densities (Lucas et al. 2011). The word "gelatinous" refers to the overall consistency of these animals: their body is mostly made of extracellular matrix (*mesoglea*). The *mesoglea* is present in all animals, but in gelatinous zooplankton organisms it represents the largest portion of the whole body (Boero 2013). Throughout the thesis we will considerer as *gelationous zooplankton* all the aforementioned groups, and as *jellyfish* just organisms belong to Cnidaria phylum.

The main morphological synapomorphy of Cnidaria gelatinous zooplankton is the presence of cnidocytes (specialized stinging cells). Their life cycle is often complex, and typically involves two basic body forms, the medusa (pelagic stage) and the polyp (benthic stage). Polyps usually form colonies and increase their number through asexual reproduction; they can withstand unfavorable environmental conditions by forming resistant cyst. Of all the cnidarian groups, however, hydrozoans have the greatest variation in life cycles and the polyp or medusa stages are entirely lacking for some groups (Collins 2002; Dumont 2009).

Jellyfish blooms and the ecosystem

Interactions between jellyfish and the ecosystem are varied. Jellyfish act as key predators, preying on crustacean zooplankton, phytoplankton, protist and fish larvae (Purcell 1989), having significant impact on community structure. As consumers of primary and secondary production, jellyfish can play a key role in energy influx and

carbon sequestration, through the accumulation of jellyfish carcasses at the seabed, being used by bacterioplankton for respiration (Lebrato et al. 2012).

Jellyfish can also provide shelter and food to many fish (Doyle et al. 2014). The most common associations are among juvenile fish and scyphomedusae, as for example between *Cotylorhiza tuberculata* and *Trachurus* spp., or *Rhizostoma pulmo* and *Merlangius merlangus* (Purcell and Arai 2001).

Due to their high water content, jellyfish are often presumed to be a poor food source and a trophic dead end (Sommer et al. 2002), however, in addition to the vertebrate predators that extensively consume gelatinous species (as the leatherback sea turtle *Dermochelys coriacea*), exists an increasing list that includes 124 species of fish which are reported as feeding occasionally or predominately on jellyfish (Arai 2005; Pauly et al. 2009; Milisenda et al. 2014).

Jellyfish role in ecosystem service is not negligible, since they have been source of important compounds for science, as for example with the discovery and subsequent development of the green fluorescent protein (GFP) (Zimmer 2009), which is usually used for protein labelling, allowing to observe protein expression localization and translocation. In addition, jellyfish are traditional food in many Asian countries since hundreds of years. At least 10 species of jellyfish (all Rhizostomeae) are commercially harvested, being *Rhopilema esculentum* the most important species. Not only fisheries but also jellyfish aquaculture has been developed during recent decades in Asian countries (Purcell et al. 2013).

Causes of jellyfish blooms increase

Although dense jellyfish aggregations are a natural feature of healthy pelagic ecosystems (Graham et al. 2001), and periodic fluctuations in occurrence and abundances have been demonstrated for some species, during recent years more severe and frequent outbreaks of jellyfish have been observed in different areas including the Mediterranean Sea (Goy et al. 1989; Brotz et al. 2012; Condon et al. 2013).

Several causes have been hypothesized as drivers of jellyfish blooms (Fig. 1). Climatic cycles and global warming have been considered forefather of jellyfish distribution expansion, allowing tropical species move toward sub-tropical and temperate latitudes. Water warming may also accelerate medusa growth and ephyrae production (Purcell et al. 2007; Richardson et al. 2009). However, anthropogenic stress has been hypothesized as main cause of jellyfish blooms increase around the world, being overfishing, eutrophication and habitat translocation the stressors that most contribute to facilitate jellyfish blooms formation.

- *Overfishing*: many fish are at the same time competitors and predators of jellyfish, so the action of overfishing eliminating or reducing abundances of these species may open up ecological space for jellyfish.
- *Eutrophication*: eutrophication generates high N:P ratios, altering the plankton community structures with shifts from large diatoms to small flagellates, allowing short trophic chains with small zooplankton that may favor jellyfish feeding over fish (Purcell et al. 2007). Water turbidity could also benefit jellyfish since they are tactile predators compared with fish which are visual predators (Purcell 2012)
- *Habitat translocation*: the transport between locations is mostly via ballast water and fouling biota attached on ship hulls.

Multiple factors could also act simultaneously and synergistically to increase jellyfish blooms. For example, in the *Mar Menor* (Spanish coastal lagoon) jellyfish blooms followed high eutrophication, construction and extensive habitat modification that changed the ecosystem through the replacement of sea grasses by the invader algae *Caulerpa prolifera*, the introduction of oysters that acted as additional substrate for jellyfish polyps, turbidity increased and bottom waters became hypoxic, producing accelerated declined of fish populations in the lagoon (Pagés 2001).

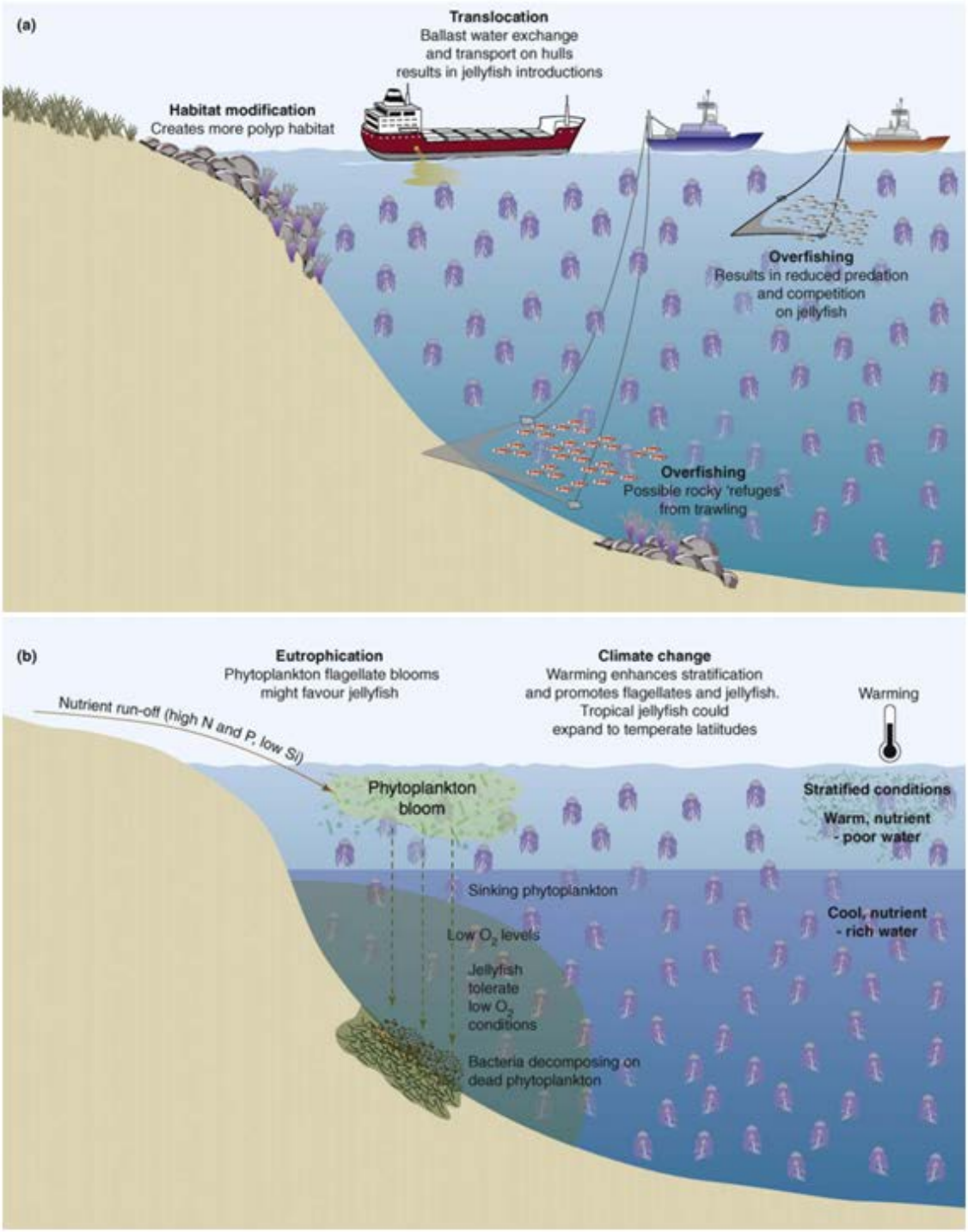

Figure 1. Probable mechanisms promoting jellyfish outbreaks: (a) Summary of the impacts of habitat modification, translocations and overfishing on jellyfish outbreaks; (b) Summary of the impacts of eutrophication and climate change on jellyfish outbreaks. Jellyfish symbols represent jellyfish blooms (Richardson et al. 2009).

Consequences of jellyfish blooms increase

Controversially, being anthropogenic stress the main responsible of jellyfish bloom rise, marine human activities suffer many consequences of this gelatinous zooplankton increase. The most affected sectors are tourism, fishing and aquaculture (Fig. 2). The negative effects of jellyfish on coastal tourism (beach closures and stings) and fishing operations are well-known, while the negative interactions between jellyfish and farmed fish are an increasing problem through the intensification of aquaculture operations in many coastal areas worldwide, but are usually underestimated leaving a huge lack of knowledge about the negative impacts of this interaction (Baxter et al. 2011a; Purcell et al. 2013).

- *Tourism*: stings from jellyfish cause discomfort and sometimes medical emergencies for swimmers. When pelagic cnidarians occur in great abundance, stinging can occur at epidemic levels having important consequences at economic and human health levels (Purcell et al. 2007; Kontogianni and Emmanouilides 2014).
- *Fishing*: Interference with fishing operations is the most frequently reported problem occurring with great abundances of jellyfish. Large catches of jellyfish can split the fishing nets and ruin the quality of the catch (Purcell et al. 2007; Graham et al. 2014).

Figure 2. Jellyfish blooms impacts on marine human activities: a) *Rhizostoma pulmo* catch in fisherman nets; b) *Pelagia noctiluca* bloom in Canterias beach (Gran Canaria, Spain).

Jellyfish blooms impacts on marine finfish aquaculture

Aquaculture is an important source of economic income for local societies and sustains over 40 % of global fish production, and specifically mariculture supports nearly 30 % (US $ 23.5 billion) of the total economical value of farmed finfish species (FAO 2014). Interaction between jellyfish and marine caged fish has been recorded in several occasions in the last years, leading to severe episodes of fish mass mortality (Rodger et al. 2011; Purcell et al. 2013). Jellyfish can enter fish cages either intact or broken up into tentacles and other body fragments pushed by currents and waves washing in through the net cages. Several species of cnidarian jellyfish have been reported to affect marine farmed fish of inducing skin lesions and gill damage caused by nematocyst discharge and venom injection usually leading to local inflammatory response, cell toxicity and histopathology (Fig. 3). Prolonged nematocyst discharges in fish tissues may often lead to secondary bacterial infections and associated systemic reactions, including respiratory and osmoregulatory distress, altered behaviour, and death (Helmholz et al. 2010; Baxter et al. 2011a). Impacts of low to medium jellyfish density are usually neglected or underestimated, and low incidence of unspecific pathologies or mortalities are generally labeled as unknown "water borne irritant damage", being noticed just mass mortalities caused by conspicuous jellyfish species (Marcos-López et al. 2014). Some of the most important jellyfish species involved in farmed fish mortalities are the scyphozoans *Pelagia noctiluca* and *Aurelia aurita*, the hydromedusae *Solmaris corona* and *Phialella quadrata*, and the siphonophore *Muggiaea atlantica* (Table I).

Figure 3. Farmed fish poisoned by *Pelagia noctiluca* jellyfish: a) *Sparus aurata* after contact with *Pelagia noctiluca* swarm in Tunisian fish farm (photo Dr. Raouf Dhaouadi, 2009); b) gills of farmed Atlantic salmon (*Salmo salar*) exhibiting patch of necrotic tissue (ringed) (Rodger et al. 2011).

Pelagia noctiluca is the most abundant stinging scyphozoan jellyfish in the Mediterranean Sea. It has direct development, so its cycle does not include a benthic polyp stage (Fig. 4). This characteristic allows *P. noctiluca* populations to inhabit oceanic as well as coastal ecosystems and may explain its biogeography. This species has also a wide vertical distribution, as it has been found commonly between 150 m depth and the water surface (Franqueville 1971; Malej 1989).

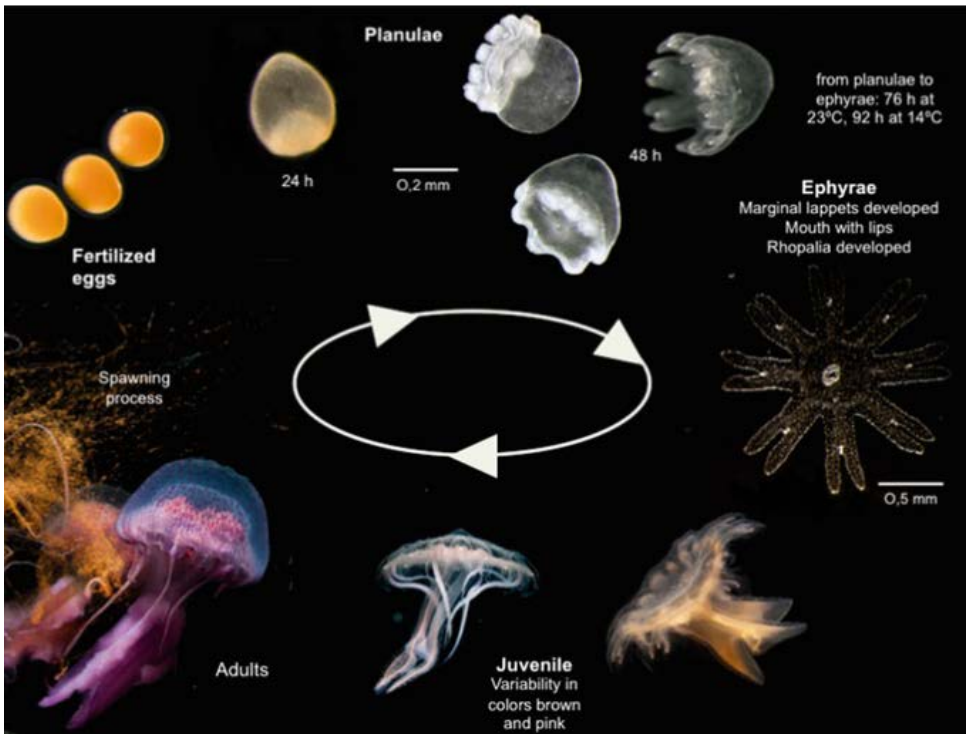

Figure 4. *Pelagia noctiluca* life cycle (Canepa et al. 2014)

Muggiaea atlantica is a calycophoran siphonophore. Diphyid calycophores, which represent the majority of siphonophore species, have two different forms: the polygastric colonies, where the distal extremity of the stolon fragments to release

monogastric sexual colonies or eudoxids (the second form) (Fig. 5). The eudoxids live autonomously, themselves budding several successive gonophores. Once each gonophore has liberated its gametes, it is detached, and it degenerates while a new one develops (Carre and Carre 1991). Siphonophores colonies are very fragile and only the swimming nectophores or reproductive gonophores, and sometimes the bracts, are usually found in plankton samples. They arrive to fish farm facilities through currents, as well as *P. noctiluca* or *S. corona* (pelagic narcomedusae). Periodic seasonal and inter-annual fluctuations of *M. atlantica* and *M. kochi* have been used as indicators of water mass movements (Mackie et al. 1988; Carre and Carre 1991).

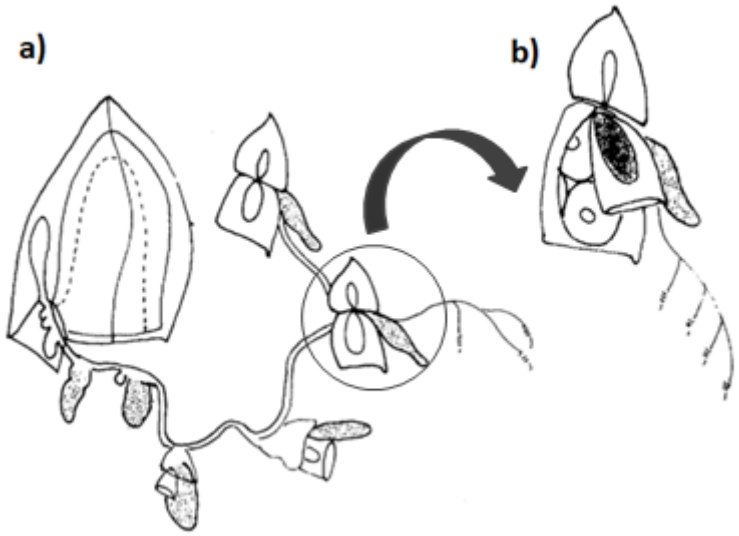

Figure 5. Calycophoran siphonophore: a) polygrastric phase; b) Eudoxid phase with developed gonophores (Carre and Carre 1991).

There are also hydrozoans such as the Leptomedusae *P. quadrata* or *Obelia dichotoma* with dimorphic life cycles (including polyp and medusa stages); but although this is the classical described life cycle for hydrozoans (with the exception of siphonophores), several modifications may be present, being the most important one the suppression of medusa stage. *Ectopleura larynx* is a common fouling species in northern Europe aquaculture facilities (Guenther et al. 2010) and its distribution includes also the Mediterranean Sea. Its life cycle includes polyp and actinula larvae stages, which will

attach to the substrate and will develop new colonies. Laboratory experiments demonstrated its potential harmful action on caged fish (Baxter et al. 2012) and well as its capacity to rapidly grow after the net cages washing process (Carl et al. 2010) (look at Chapter 3 for *E. larynx* life cycle). All these species are usually forming part of cage biofouling community in marine aquaculture facilities and when environmental conditions are optimal, thousands of tiny jellyfish/larvae can be released inside the cages.

The most important bibliographic references about the interaction between jellyfish blooms and farmed fish include the work of Rodger et al. 2011, a paper describing the clinical sign of fish pathology exposed to jellyfish, as well as a review on farmed fish mortalities in aquaculture facilities around the world (update by Purcell et al. 2013, Table I). Laboratory experiments performed by E. Baxter were focused on the evaluation of farmed salmonids gill damage after exposure to the scyphozoan *Aurelia aurita* and the aforementioned hydroid *Ectopleura larynx* (Baxter et al. 2011b; Baxter et al. 2012). Moreover, Baxter carried out monitoring of gelatinous zooplankton in two Irish fish farms and investigated the role of hydrozoans in farmed fish gill disorders (Baxter et al. 2011a). Marcos-López et al. 2014 described in detail the gill injuries that *Pelagia noctiluca* jellyfish could cause in caged salmon. The majority of publish papers are focused on the negative effects of jellyfish on *Salmo salar* in northern Europe marine facilities, while no information exists about jellyfish interaction with Mediterranean aquaculture or its commercial species and neither about the effects of jellyfish stinging on fish metabolism.

Table I. Published records of jellyfish interfering with aquaculture operations around the world (Purcell et al. 2013).

Jellyfish species	Dates	Locations	Type of damages	Reference
Asia				
Aurelia aurita s.l.	Jul–Sep 1950	Lake Hachirogata, Akita Prefecture	Mass mortality of fish and bivalves	Yasuda, 1988
*Porpita porpita**	Aug–Oct 2000	Kyoto, Fukui Prefectures	Mortality of penned fish	Yasuda, 2003
*Pelagia noctiluca**	Apr 2004	Ehime Prefecture	Mortality of penned fish	Uye (pers. info. from local fisherman)
Australia/Indo-Pacific				
Aurelia sp.	Summer 1998–2001	Tasmania	Atlantic salmon	Tasmanian Aquaculture and Fisheries Institute, 2003
Unidentified	<1995	India	Giant tiger prawns	Rajagopal *et al.* 1989
Rhizostome scyphozoan	2006	Goa, India	Shrimp	RA Sreepada (pers. com.)
Europe				
Aurelia aurita	June 2010	Northwest Ireland	Acute gill lesions and mortality in Atlantic salmon	Mitchell *et al.*, 2011
*Pelagia noctiluca**	1994	Brittany, France	Salmon and trout	Merceron *et al.*, 1995
*Pelagia noctiluca**	Nov 2007	Western Ireland	250000 salmon killed	Doyle *et al.*, 2008
Cyanea capillata	1996	Loch Fyne, Scottland	Thousands of salmon killed, £250000 loss	Anon, 1996
*Solmaris corona** *Muggiaea atlantica**	Aug–Nov 2009	Western Ireland	Severe gill damage and potential mortalities in Atlantic salmon	Baxter *et al.*, 2011a
*Solmaris corona**	Summer 1997	Shetland Isles	Salmon killed	Anon, 1997a
*Solmaris corona**, *Phialidium* sp., *Leuckartiara octona*, *Catablema vesicarium*	Aug 2001–2002	Isle of Lewis in the Outer Hebrides, Scotland	2747680 salmon killed in 11 incidents, £5 mil loss	Johnson, 2002
*Apolemia uvaria**, *Aurelia aurita*, *Cyanea capillata*, *Bolinopsis infundibulum**	Nov 1997–Feb 1998 and 2003; 1994 and 1995; May–June 1986	West coast of Norway and Sweden	Mass mortalities of Atlantic salmon	Båmstedt *et al.*, 1998; Heckmann, 2004
Phialella quadrata	Aug 1984	Shetland Isles, Scotland	Killed 1500 salmon and severe gill damage	Bruno and Ellis, 1985
*Muggiaea atlantica**	Aug 2002	Norway	Killed > 100000 salmon	Fosså *et al.*, 2003
North America				
Moerisia lyonsi	1970s; May–Oct 1994–1997	Mesocosms, Louisiana and Maryland USA	Killed decapods; ≤ 13.6 med. L^{-1}	Sandlifer *et al.*, 1974; Purcell *et al.*, 1999

Source: Updated from Purcell *et al.*, 2007.
*Holoplanktonic, all others have a benthic stage.

My work is an attempt to answer all these questions using an integrated and multidisciplinary approach. The thesis was divided in 3 blocks formed by 6 chapters. First block (*Knowledge of jellyfish*) corresponds with *chapter 1*, which is a social perception study focuses on fish farmers perception about jellyfish blooms. Second block (*Case studies*) is formed by *chapter 2 and 3*, and includes different monitoring of gelatinous zooplankton and biofouling community of cages in Mediterranean aquaculture facilities. Third thesis block (*Experimental evidences*) is formed by *chapters 4, 5 and 6*, which are three different laboratory experiments to investigate the impact on fish gill integrity and metabolism after exposure to jellyfish stinging.

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Aims of the thesis

General objective:

To deeply investigate about the consequences that jellyfish blooms may have on farmed fish health and consequently on marine aquaculture sector, especially in the Mediterranean Sea.

Specific objectives:

- To understand Mediterranean fish farmers perception about jellyfish blooms and their impact on aquaculture sector.

- To investigate the role of hydrozoans jellyfish in gill disorders of *Dicentrachus labrax* in Mediterranean aquaculture.

- To characterize the hydroid assemblage on Mediterranean fish cages: composition, growth and reproductive periods.

- To evaluate gill injures that jellyfish may caused on farmed *Sparus aurata*.

- To evaluate physiological fish (*Dicentrarchus labrax*) response to abiotic (temperature and hypoxia) and biotic (jellyfish poisoning) stressors.

- To study the role of neurotransmitters as biochemical stress indicators after contact between jellyfish and *Dicentrarchus labrax* individuals.

Chapter 1

Jellyfish blooms perception in Mediterranean finfish aquaculture

Bosch-Belmar M., Azzurro E., Pulis K., Milisenda G., Fuentes V., Kéfi-Daly Yahia O., Micallef A., Deidun A., Piraino S. **(In prep. for Environmental Science and Policy)**

Introduction

In spite of some controversy when trying to find global trends (Condon et al. 2013), in some locations jellyfish blooms are increasing in frequency and severity (Purcell et al. 2007; Brotz and Pauly 2012). Assessing the ecological and societal consequences of these events is one of the pressing challenges for marine researchers (Pitt and Lucas 2014; Graham et al. 2014). Some anthropogenic stressors have been suggested as potential causes of increasing jellyfish mass occurrence, including ocean warming, eutrophication, overfishing, and the increase of artificial hard substrates (Purcell 2007; Richardson et al. 2009).

Even when scientists still have not confirmed the presumed global increase on the frequency of jellyfish blooms (hereafter referred as JB), the problems involving these proliferations have broad and far-reaching social consequences on many human activities (Purcell et al. 2007). Tourism and recreation may be negatively affected because of jellyfish harmful stings which in some cases cause the closure of beaches during summer period (CIESM 2001), fisheries may be negatively affected by net

clogging and deterioration (Richardson et al. 2009), aquaculture facilities due to significant injuries on caged fish and mortality episodes (Purcell et al. 2013), and some industrial activities by clogging desalination filters or cooling water intake pipes for industries and power plants (Ghermandi et al. 2015).

Aquaculture represents an important source of food production in the Mediterranean (FAO 2014). In the last decade, this activity has experienced important economic losses attributable to jellyfish, mainly due to chronic problems of gill damages and fish mortality events (Rodger et al. 2011; Baxter et al. 2011b). Although the information about JB impact on aquaculture is scarce, its negative consequences on caged fish had been recorded and documented several times in the North Sea where in 2007, 2013 and 2014 different JB caused farmed fish mortality events (Doyle et al. 2008; Raffaele 2013; Berwald 2014; FIS 2014). Jellyfish can enter fish cages either intact or broken up into tentacles and other body fragments pushed by currents and waves washing in through the net cages (Baxter et al. 2011b, Mitchell et al. 2012).

Several species of cnidarian jellyfish have been reported to affect marine farmed fish of inducing skin lesions and gill damage caused by nematocyst discharge and venom injection, usually leading to local inflammatory response, cell toxicity and histopathology (Baxter et al. 2011c, Helmholz et al. 2010, Rodger et al. 2011).

Information about the extent of the damage caused by jellyfish is rarely available, and limited knowledge exists on the effects of these interactions. Gill disorders have become one of the most serious causes of mortality in aquaculture facilities in Northern Europe, with average losses of 12 % per year (Baxter et al. 2011b). Unfortunately, impacts of low-medium jellyfish density are usually neglected or underestimated, and these occurrences are generally labelled as "unknown water borne irritant damage" (Marcos-López et al. 2014). Noteworthy, even if fish survive after the envenomation, growth problems could occur afterwards (Baxter et al. 2011a).

Recent studies highlight the negative consequences of JB on Mediterranean tourism (Kontogianni and Emmanouilides 2014; Ghermandi et al. 2015) and fisheries (Palmieri et al. 2014), but to our best knowledge no information exists for the aquaculture sector. For

this reason, the main objective of this work was to investigate the perception of fish farmers about jellyfish and the potential impacts of their blooms on aquaculture activities, as well as the current consequences of the interaction between JB and marine fish farms activities in the Mediterranean Sea.

Materials and Methods

Study area

Interviews with fish farmers were carried out between February 2014 and February 2015, over four different Mediterranean countries: Italy, Spain, Tunisia and Malta. Facilities were all represented by grow-out offshore floating cages. Gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) were the main farmed species in Italy, Spain and Tunisia, while in Malta these species represented 21 % of aquaculture grown, Bluefin tuna (*Thunnus thynnus*) being the chief farmed species (79 %).

Survey structure and data collection

We contacted a total of 42 finfish aquaculture facilities obtaining the collaboration of 21 of them. Surveys were performed face-to-face or by telephone, depending on the availability of fish farms. Workers were interviewed individually to minimise 'group effect' bias. Interviews were performed in the native or official languages of each country.

People were interviewed on the basis of a structured questionnaire (appendix I) which included 19 questions organised in 3 different sections: *I) general knowledge on jellyfish and their blooms* (e.g. which jellyfish sp. the interviewees recognised and which are the most frequently sighted, the frequency of jellyfish blooms, etc.); *II) JB qualitative impacts on farm's activity* (i.e. on structures and material, health of workers and farmed fish); *III) JB quantitative impacts* (categorical estimation of potential impact on aquaculture economy). Answers were structured in a dichotomous format (yes/no), with the exception of the economic impact valuation where an increasing number scale from 0 to 5 was presented (0 = mean none effect of JB on aquaculture activity, 1-2 = low effect, 3 =

medium economic effect and 4-5 = high economic impact). The answers, where an explaining response was required, were afterwards categorised to perform the data analysis. Fish farmers were also invited to provide any further comment they deemed useful to clarify their answers. To facilitate species identification, jellyfish pictures of the most commonly blooming taxa were shown to the respondents (appendix I).

Statistical analysis

The jellyfish species mentioned in each interview were used to build a presence/ absence dataset in which each survey was considered as an independent sample with the species as variables. This dataset was explored with multivariate analyses to test for possible relationships between the recorded jellyfish species and both social and geographic factors. To test differences for the factors "location" (fixed with 4 levels) and "professional profile" of workers (fixed with 6 levels and orthogonal with "location") a two-way PERMANOVA analysis was performed. The same statistical analysis was used to test for differences between "location" factor and "years of experience" in the sector (fixed with 5 levels and orthogonal to "location") and between "location" and "farmed fish species" in the involved facilities (fixed with 2 levels and nested in "location"). We also used a one-way PERMANOVA for testing the factor "season" (4 levels).

Answers to perception questions about the impact of JB on anthropogenic activities were equally organised in a matrix and two-way PERMANOVA analysis was carried out with the same experimental design previously used for the jellyfish matrix. In addition, one-way PERMANOVA analysis (for location factor) was performed to test different respondents' answers about the potential economic impact of JB on aquaculture.

Subsequently, post hoc Pair-wise t-test and SIMPER analysis were performed. Statistical analyses were performed with PRIMER6 & PERMANOVA+ software package (Plymouth Marine Laboratory, UK).

Results

Characteristics of the respondents

A total of 51 fish farmers were interviewed (9 from Italy; 11 from Spain; 7 from Tunisia and 24 from Malta) corresponding to 21 different fish farms (6 from Italy; 5 from Spain; 4 from Tunisia and 6 from Malta). Interviewed people had a number of years of aquaculture sector experience ranging from 3 to 50, with 43% of them working in this activity for more than 10 years. The interviewed professional profiles varied from field technicians, divers or skippers to fish farm directors, veterinarians, administrators and technical, production, operations and quality managers. The average of spend hours at sea by field workers was 6 per day during all the year.

I) General knowledge on jellyfish and their blooms

General knowledge of jellyfish varied much among countries. The jellyfish species that interviewees were able to identify were significantly different among places (F_3= 6.67, p= 0.001), except for Italy and Spain (t= 1.58, p= 0.057), but in all cases, *Pelagia noctiluca* was the most well-known jellyfish species with a contribution to similarity higher than 45% according to SIMPER analysis. Differences among countries were independent from the farmed fish species and the interviewees professional profile (F_1= 1.759, p= 0.124 and F_5= 0.739, p= 0.729, respectively). However, the number of years of experience in the sector presented significant differences about the jellyfish species that farmers were able to identify (F_4= 1.995, p= 0.016) but it was not related with location factor (non significant interaction: F_6= 1.277, p= 0.198). Significant differences were found between respondents with1-5 years experience and those with 10-20 years and more than 20 years.

In Malta and Tunisia the interest on these gelatinous organisms was relatively low and more than 70% of interviewed people affirmed 'to do not know anything about jellyfish'. In Spain and Italy, 91% and 67% of respondents shared with us information about jellyfish and their proliferation (Table I). Data provided by respondents was mainly focused on climate change (with the increasing temperature of the oceans), overfishing and loss of jellyfish predators, and the consequent increase of jellyfish blooms and distribution areas. Most of this information (65%) was obtained from media such as

television (news and scientific outreach programs, etc), and in some cases came from prior knowledge about the biology and ecology of cnidarians and scientific literature.

Country	Yes (%)	No (%)
Spain	90.9	9.1
Italy	66.7	33.3
Malta	12.5	87.5
Tunisia	28.6	71.4

Table I. Percentages of interviewees with previous general knowledge on jellyfish and their blooms in all 4 involved countries.

All of the interviewees observed jellyfish in the areas where the fish farms were located (beaches and harbours) and described jellyfish blooms as occurring each year, mainly in summer ($p < 0.005$ in all pair-wise comparisons) (Fig. 1). More than half of the respondents complained about the increase of the density and frequency of jellyfish blooms in the last 10 years; one third stated that these events were constant over time and a very low percentage mentioned a decrease in jellyfish density and frequency (Fig. 2). Significant differences among countries were also found regarding the JB sightings in the aquaculture facilities. Species recorded by Maltese interviewees were different from all the other countries ($p = 0.001$ in all pair-wise comparisons). These differences were attributable to the *comb jellies*, being the third most sighted species in Malta after *Pelagia noctiluca* and *Cotylorhiza tuberculata*. Italy, Spain and Tunisia did not show significant differences among them ($p > 0.05$) (Fig. 3). *P. noctiluca* blooms were recorded in all four countries by more than 90 % of interviewers, followed by *Cotylorhiza tuberculata, Rhizostoma pulmo,* and *Velella velella* (Fig. 4). Respondents affirmed to have seen these species in different areas (harbour, beaches, open sea and near to the aquaculture cages).

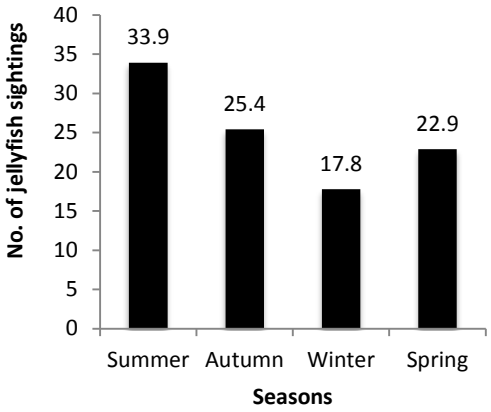

Figure 1. Occurrence of jellyfish blooms by season according to interviewees (represented by percentages).

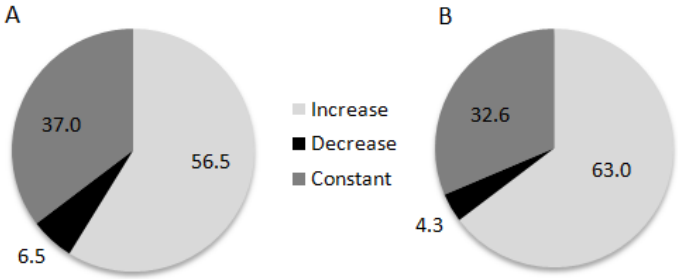

Figure 2. Fish farmers perception on jellyfish blooms frequency (A) and jellyfish density (B) variations in the last 10 years (represented by percentages).

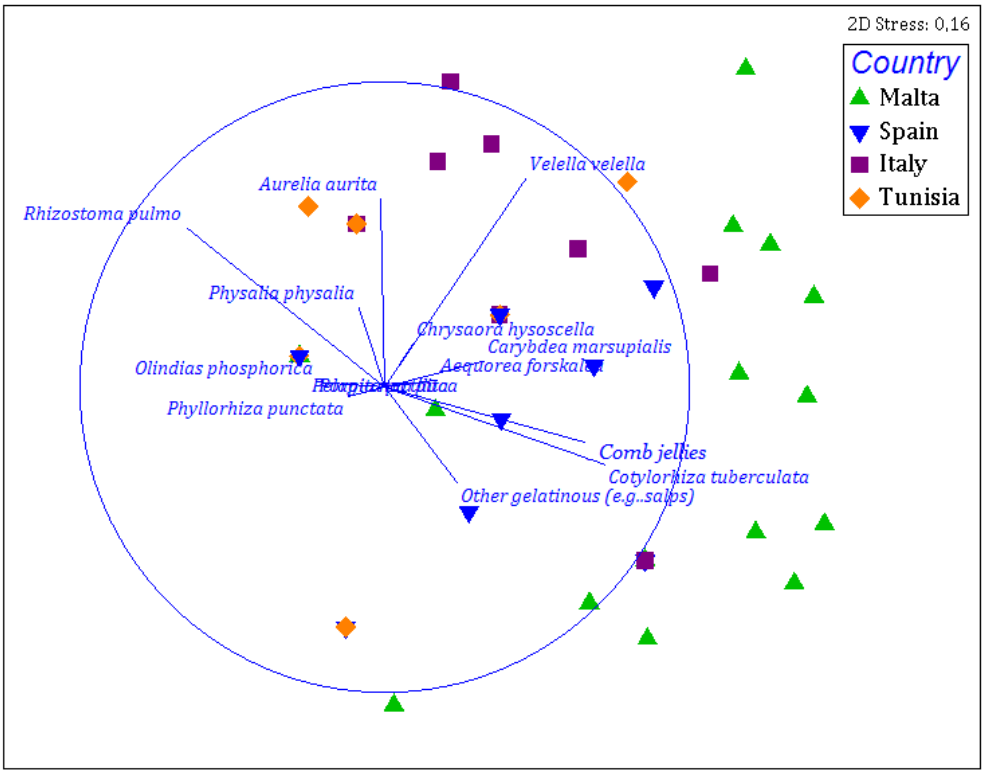

Figure 3. Non-metric multidimensional scaling (nMDS) representing differences in jellyfish sightings among countries and the main species responsible of these differences.

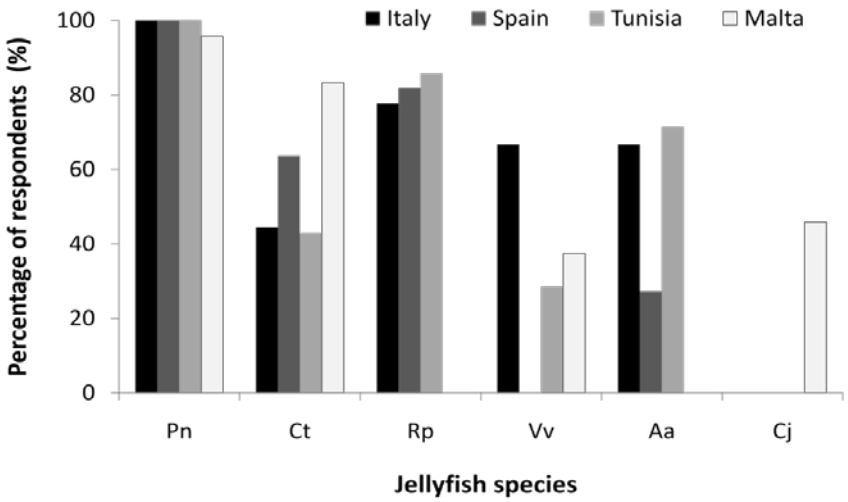

Figure 4. Most sighted jellyfish species by country; where Pn means: *Pelagia noctiluca*, Ct: *Cotylorhiza tuberculata*, Rp: *Rhizostoma pulmo*, Vv: *Velella velella*, Aa: *Aurelia aurita*, Cj: Comb jellies.

II) JB qualitative impacts on farm's activity

Differences among countries were observed regarding the impact of JB on marine anthropic activities and ecosystem (F_3= 4.280, p= 0.001). SIMPER analysis showed that according to farmer's perceptions, tourism was the most affected sector by jellyfish blooms in all countries, followed by aquaculture and fisheries, except for Tunisia, where aquaculture was perceived as the most injured activity. Italian and Spanish respondents complained about the negative effect of JB on fisheries, while Maltese and Tunisian fish farmers did not considere this interaction important. The impact of these gelatinous organisms on the ecosystem was considered of low importance for the majority of interviewees from all 4 involved countries.

Whereas, perception about the impact of JB on the aquaculture sector was significantly different among countries (F_3= 7.706, p= 0.001). In Italy, Spain and Tunisia 78%, 91% and 86% respectively of fish farmers considered the proliferations of gelatinous organisms as a factor that negatively affects aquaculture activities (Table II). Around the 77% of these positive answers affirmed that the biggest impact on aquaculture would be due to the jellyfish stings on divers working at the facility, and 86% considered that this phenomenon could have a negative impact on the health of cultured fishes. Otherwise,

just 30% of Maltese respondents opined that JB may have a real practical effect on aquaculture activities (Table II). Pair-wise analysis showed that Malta was significantly different from the other three countries, which were similar among themselves (Table III). The perceptions of fish farmers about the impact of JB on aquaculture were not significantly affected by the workers professional profile (F_6= 0.993, p= 0.46) and years of experience factors (F_6= 0.813, p= 0.608), as well as their interaction with location (F_6=0.9926, p= 0.46 and F_6= 0.8132, p= 0.608, respectively). Nevertheless, farmed species was an important factor related with aquaculture perception of respondents (F_1=12.063, p= 0.001).

Table II. Jellyfish blooms impact on marine human activities and ecosystem presented as percentage of positive interviewees answers.

	Malta	Italy	Spain	Tunisia
	Yes (%)			
Tourism	95.8	100.0	100.0	71.4
Ecosystem	25.0	22.2	63.6	28.6
Fisheries	29.2	55.6	90.9	28.6
Aquaculture	29.2	77.8	90.9	85.7
Structures	28.6	0.0	20.0	16.7
Human health	100.0	71.4	100.0	50.0
Fish health	28.6	100.0	100.0	50.0

Table III. Pair-wise comparisons of fish farmers answers about jellyfish blooms impact on aquaculture activities.

Groups	t	P(perm)
Malta - Spain	40.215	0.002
Malta - Italy	27.131	0.025
Malta - Tunisia	29.396	0.014
Spain - Italy	0.790	0.563
Spain - Tunisia	0.323	1
Italy - Tunisia	0.379	1

Biofouling was also identified as problematic issue for aquaculture facilities from Italy (100% of affirmative answers), Spain (90.9%) and Tunisia (87.5%), being of minor importance for Maltese facilities (25%). Problematic organisms were the Mediterranean oyster and common barnacle for Maltese fish farms, and different species of bivalves,

algae and hydroids in the other 3 countries. Just Spanish and Italian fish farmers (56% and 50% respectively) named hydroids as annoying fouling organism, specifically the species *Ectopleura larynx* and *Pennaria disticha*, which affected facilities by clogging the net cages and field technicians through painful stings.

Overall, 20% of the respondents recognised serious problems with jellyfish in their facilities. These related to harmful stings to divers, fish mortalities, clogging nets or occlusion of boat engines. Because of the performed interviews, it was possible to document 3 different fish mortality events in Mediterranean aquaculture facilities due to jellyfish contact (Table IV).

Table IV. Reported problems with *Pelagia noctiluca* in different Mediterranean mariculture facilities; where ED means: External damage; GD: Gill damage; RS: Respiratory distress; FM: Fish Mortality. ***** Adult jellyfish observations were made by scuba diving, while density of juveniles was calculated after sampling with zooplankton net.

Country	Date	Jellyfish sp.	Fish sp.	Jellyfish (ind · m^{-3})*	Bloom duration	Fish damage	Problem resolution	Impact
Tunisia	2008	*P. noctiluca* (juveniles)	*D. labrax* *S. aurata*	8000	10 h	ED	Nothing	FM (150T/18T)
Tunisia	Mar-May 2014	*P. noctiluca* (juveniles)	*D. labrax*	100-150	--	RS	Nothing	FM
Spain	Apr-Oct 2011-2014	*P. noctiluca*	*D. labrax*	7-10	Days	--	Net change	Structural damages
Spain	2011	*P. noctiluca*	*D. labrax*	--	48 h	RS, GD	Formaldehyde	FM (10T)

Moreover, 36% of fish farmers claimed to have had mortality fish events where a causative agent was not identified by veterinarians, and gill injuries and respiratory distress were the main pathological signs. They also recognised to have never considered jellyfish or any planktonic organism as a possible harmful force.

III) JB quantitative impacts

Significant differences about the potential economic impact that JB could have on aquaculture sector were found among locations (F_3= 18.604, p=0.001). Post-hoc pair-wise analysis showed that just Malta differed from all the other countries (p< 0.01 in all comparisons). According to the majority (70%) of fish farmers from Italy, Spain and Tunisia, JB could have a medium to high economic impact on the aquaculture sector (Fig. 5); and according to more than 80% of these respondents, the occurrence of dense jellyfish blooms should be an important variable to be considered to identify appropriate locations for marine aquaculture facilities. In Malta, 42% of the respondents thought that jellyfish blooms could have potentially significant economic impact on their activity, and less that the 30% agree that jellyfish blooms should be considered as a relevant factor in the facilities location's decision.

Remarkable examples of JB impacts were recorded in the course of interviews. Fish mortality events due to jellyfish happened in Spanish aquaculture facility in 2011 had serious economic consequences, with losses of approximately 50,000 € for the company. In addition, every time that a net cage is changed the estimated costs are 4,000 € and 3,000 € for a formaldehyde treatment. In 2008, fish mortalities in Tunisian facilities supposed dramatic economic losses for the company, leading to near bankruptcy.

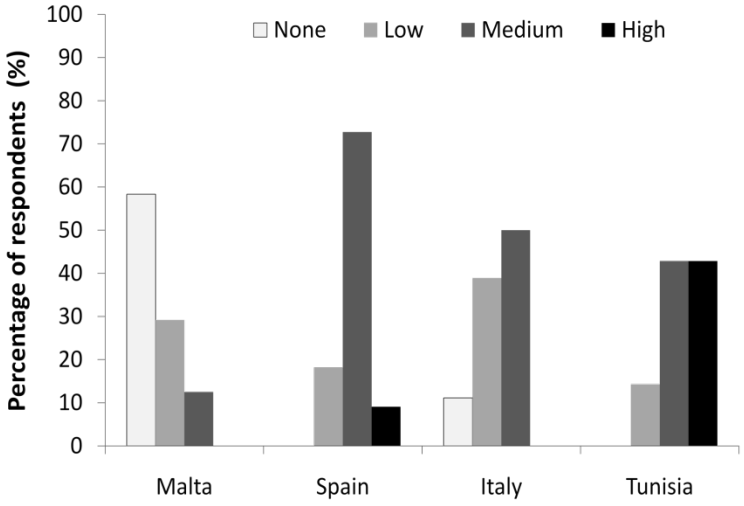

Figure 5. Potential economic impact of jellyfish blooms on aquaculture by country

Discussion

Our findings highlight that JB may seriously interfere with Mediterranean off-shore aquaculture, affecting culture facilities, and fish health as well as underwater technicians during their daily work. The majority of interviewed farmers were well aware of the risks associated to JB on aquaculture sector and to recognize some of the most common bloomer jellyfish species in the Mediterranean Sea.

According to the farmers' knowledge, *P. noctiluca* was the most frequent species and the primary cause of mortality events in caged fishes. This species is one of the most common stinging jellyfish across the Eastern Atlantic and the Mediterranean Sea and similar caged fish mortality events have been reported in Northern Europe (Rodger et al. 2011). It has been demonstrated that *P. noctiluca* has the potential to reproduce all year long in some areas of the Mediterranean (Rottini Sandrini and Avian 1991; Milisenda et al. 2016), generating large blooms that interfere with different marine human activities (Canepa et al. 2014).

Also cnidarian fouling species have been identified as annoying organisms for Mediterranean fish farms. *Ectopleura larynx* and *Pennaria disticha*, are two common

hydrozoans species in the Mediterranean Sea of which colonies have a rapid growth and reproduction rates (Carl et al. 2010). Interviewed fish farmers affirmed that these species caused harmful stings to field technicians when manipulating the cages nets. Nevertheless, few studies about the impact of cnidarian fouling species on human health (Tezcan and Sarp 2013) and farmed fish stocks (Baxter et al. 2012) exist in the literature. Consequences for fish welfare are still poorly understood. In the literature there are few studies focused on the impact of JB on fish health (Baxter et al. 2011c; Baxter et al. 2011b; Baxter et al. 2012; Marcos-López et al. 2014); however, small colonial hydroids may release hundreds of small medusae during reproductive periods and blooms of tiny pelagic hydrozoans that may arrive at the marine facilities and penetrate into the fish cage injuring fish, are inconspicuous and usually neglected and their impacts may be labelled as "unknown water borne irritant damage" (Marcos-López et al. 2014).

The majority of interviewees expressed their concern about the increasing frequency of jellyfish blooms in the last decade. The degree of awareness on the issue of JB showed significant differences among countries. For example, in comparison to Tunisian and Maltese respondents, Italian and Spanish farmers showed a better knowledge about JB and generally a greater availability to provide information on this subject.

The perception about the impact of JB on aquaculture differed among countries and also among facilities with different farmed fish species. More than 65% of Maltese respondents opined that JB do not have significant effects on aquaculture activities. Moreover, the most majority of interviewed Maltese facilities growth exclusively bluefin tuna, while all the other Mediterranean facilities cultivated sea bass and sea bream. Tuna fish seems do not suffer severe consequences due to jellyfish stings. This is probably due to the large size of these animals but also to the fact that both cages and mesh size in tuna facilities are bigger with respect to the other cultured species with a low probability of net clogging. Probably, due to invisible impact of JB on farmed tuna, Maltese farmers consider no effects of jellyfish outbreaks on farmed fish health and no potential economic impact of these gelatinous organisms on aquaculture facilities.

The present work provides the first information regarding the perception of Mediterranean fish farmers on JB. We documented some impacts on aquaculture and

new fish mortality events caused by jellyfish. Considering the lack of technological solutions to mitigate the impacts of JB on fish farms (Rodger 2007), increasing our knowledge on the spatial distribution and temporal trends of these events is of primary importance to limit or prevent economical losses (Doyle et al. 2008).

In the reported cases at the present study, Tunisian fish farmers did not take any countermeasures and one of the facilities was nearly bankrupt due to the mortality of almost all of the caged fish stocks. Spanish facilities tried to avoid the unexpected mortality by using a formaldehyde treatment (a common treatment against ectoparasites) in the absence of an adequate action protocol and several time from 2011 to 2014 when huge swarm of *P. noctiluca* surrounded sea bass cages, they changed the net assuming high economic costs.

Together with the increasing growth of aquaculture sector (FAO 2014), the interaction of mariculture activities with jellyfish outbreaks should receive more attention. Due to the potential severe consequences for caged fish health and the companies' economy, the evidence exposed in this study and elsewhere in the literature, the negative impacts of JB on aquaculture activities deserve further consideration. The development of participative monitoring programs and actively involving fish farmers in tracking the occurrence of JB can be promising for a better understanding of this phenomenon. Certainly the cooperation between fish farmers and research institutions is advisable to estimate and evaluate the consequences of JB on Mediterranean aquaculture. Outreach and training programs to fish farms staff would help to raise awareness on this emerging issue and to evaluate the feasibility of action plans, with measures for prevention, mitigation and adaptation.

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Appendix I

Jellyfish identification guide used during surveys performed in Chapter 1.

Aquaculture survey

A) Initial data

Survey nº: _____
Interviewer: _____
Date: _____

B) Personal data

Name: _____ Age: _____
Sea working time (years): _____
Activity at the company: _____
h/day spend at sea: _____

Company name: _____ City: _____
Farm species: _____
Night surveillance in the installation: Yes □ No □

I) General knowledge on jellyfish and their blooms

1- Have you ever observed jellyfish in the area where your facility is located?
Yes □ No □

2- What season(s) can you detect jellyfish?
□ Spring □ Summer □ Autumn □ Winter

Especially in months: 1 2 3 4 5 6 7 8 9 10 11 12
(circle one or more)

3- In your opinion, in the last 10 years the **frequency** of jellyfish blooms:
□ increased □ decreased □ remained constant

4- In your opinion, in the last 10 years the **abundance** of jellyfish:
□ increased □ decreased □ remained constant

5- Jellyfish can be observed in:
□ harbour □ beaches □ open sea □ near to the fish farm

6- Can you estimate approximate densities of jellyfish swarms?

☐ ≤ 1-2 individuals/m³ ☐ 5-10 individuals/m³ ☐ >10 individuals/m³

7- What species of jellyfish? (See the guide)

☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ ___ ☐ ___ ☐ ___

8- Did you see one or more of the following jellyfish species? (Look at the guide)

☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ ___ ☐ ___ ☐ __

9- Do you know any information about jellyfish in Mediterranean Sea? ☐ Yes ☐ No

If yes, what do you know? _____

_____

10- In your opinion, jellyfish blooms may produce impact on:

tourism	☐ Yes	☐ No
ecosystem	☐ Yes	☐ No
fisheries	☐ Yes	☐ No
aquaculture	☐ Yes	☐ No

II) JB qualitative impacts on farm's activity

11- If jellyfish are observed in the vicinity of the facility where you work, you can see them:

☐ inside the cages ☐ around the cages ☐ away from the cages

12- In the case of jellyfish impact on aquaculture facilities, problems will arise as:

Damage to structures	☐ Yes	☐ No
Human health (Stings, envenomation)	☐ Yes	☐ No
Fish health	☐ Yes	☐ No

☐ Others _____

13- Did your facility experience any problem with jellyfish? ☐ Yes ☐ No

If not:

What do you believe is the reason why you are not having problems with these organisms?

_____

If yes:

13.1. When have these events taken place?					
13.2. What jellyfish species was/were?					
13.3. What density?					
13.4. How long has this event lasted?					
13.5. Have fishes suffered any health problems? What kind of problems?					
13.6. Have structural damage been produced?					
13.7. How have you solved the problem?					
13.8. What economic impact has it had on your company?					

14- Fish mortalities with an unknown origin have been registered?

☐ Yes ☐ No ☐ DK/NA/REF

If yes, brief description of the pathology: _____

_____

15- Do you know about any unusual phenomenon occurring in the proximity of the aquaculture facility? (e.g. "colored" water, thermal anomalies)

☐ Yes ☐ No ☐ DK/NA/REF

If yes, brief description of the phenomenon:

_____

_____

16- In the facility where you work, are there problems with biofouling (fouling organisms on submerged structures in the facility)?

☐ Yes ☐ No

What are the species which cause the most problems, and why?

_____

_____

III) JB quantitative impacts

17- In your opinion, what would be the economic damage that a proliferation of jellyfish could generate in the activity that you develop?
(0 = None) 0 1 2 3 4 5 (5= High)

18- Do you think that the records of dense jellyfish blooms should be a study factor in deciding the location of an marine aquaculture facility? ☐ Yes ☐ No

19- Do you know other facilities elsewhere in Italy and/or the world, in which jellyfish blooms produced a significant impact? ☐ Yes ☐ No

Where? What kind of problems? _____

Chapter 2

The role of hydrozoan jellyfish in European sea bass (*Dicentrachus labrax*) gill disorders in Mediterranean aquaculture

Bosch-Belmar M., Milisenda G., Girons A., Totti C., Piraino S., Fuentes V. **(In prep. for MEPS)**

Introduction

Aquaculture activity represents an important source of economic income for local societies and has undergone a rapid expansion around the world (FAO 2014). Proliferations of jellyfish are currently recognized as a factor that negatively impacts marine fish farming (Rodger et al. 2011a; Baxter et al. 2011; Purcell et al. 2013). Jellyfish have affected highly productive aquaculture operations worldwide. Fish farm facilities from Asia, Australia, North and South America have reported fish mortalities due to the interaction with blooms of different jellyfish species (Palma et al. 2007; Willcox et al. 2008; Doyle et al. 2008; Purcell et al. 2013). In northern Europe, *Pelagia noctiluca* and *Aurelia aurita* were involved in several fish mortality events in aquaculture facilities. High mortalities of more than 400,000 fish were recorded in different aquaculture facilities from Ireland and Scotland when large swarms of *P. noctiluca* surrounded the salmon cages in autumn 2007, 2013 and 2014 (Doyle et al. 2008; Raffaele 2013; FIS 2014).

Likewise, *Aurelia aurita* medusae were responsible for a significant salmon mortality in an Irish facility during the summer 2010 (Mitchell et al. 2013). Most of these events have been related to large scyphozoan species; nevertheless, some studies note the impact of small hydrozoans on aquaculture. The most well documented episodes involved the siphonophore *Muggiaea atlantica* and the hydromedusae *Solmaris corona* and *Phialella quadrata* as causing fish mortalities in different Irish and Scottish fish farms (Baxter et al. 2011; Purcell et al. 2013; Fitridge and Keough 2013). Of all the cnidarian groups, hydrozoans have the greatest variation in life cycles and the polyp or medusa stages are entirely lacking for some groups (Collins 2002; Dumont 2009). Planktonic hydrozoans will arrive to fish farms through currents, and are small enough to pass through the net cages. Benthic species will be forming part of net cages fouling community and when environmental condition will be optimal, will release hundred of stinging small jellyfish or larvae inside the cages (Baxter et al. 2011). These tiny and almost transparent jellyfish and siphonophores often go unnoticed, but can form very high density blooms and be inhaled by fish, inflicting severe damage due to nematocyst discharge (Fosså et al. 2003). The kind and intensity of morphological injuries caused by these contacts were described by different authors (Baxter et al. 2011; Bosch-Belmar et al. 2014; Marcos-López et al. 2014). These studies agree that just few hours of contact with jellyfish stinging cells may severely damage fish gill tissue, having potential consequences at different levels. Gill disorders are considered to be a rising problem for the aquaculture sector, caused by jellyfish phytoplankton, parasites, bacteria and viruses (Rodger 2007).

In Northern Europe, different monitoring programs for plankton in fish farms have been performed, but in the Mediterranean Sea, little or no monitoring of plankton in marine aquaculture facilities exists. In 2012, a monitoring program for gelatinous zooplankton, phytoplankton, and fish pathogens started at two off-shore fish farms located in southern Spain. Both facilities suffered several sea bass (*Dicentrachus labrax*) mortality events during the preceding years when no causative agent was identified; specifically, bacterial and known viral diseases, parasites or hypoxic events in the fish cages were dismissed. The weights of injured fish ranged between 15 to 70 g and the clinical signs they presented were similar to those described by Rodger et al. (2011) in farmed fish exposed to a jellyfish bloom: lethargic behaviour, swimming close to the surface, and

visible respiratory stress. Sometimes the fish stopped feeding and sudden increases of mortalities or moribund individuals was observed in each episode. The main objectives of the present research were I] to document the spatial and temporal distribution of the planktonic hydrozoan community at both aquaculture facilities, and II] to determine their possible roles as agents of gill disorders. In order to assess the presence of potential causative agents of gill disorders and mortality in farmed fish, samples of phytoplankton were taken and histological analysis of fish gill tissues was performed.

Materials and Methods

Study sites

Aquaculture facilities were located in Málaga Bay and the Almería Gulf (Alboran Sea), which are 206 km apart (Fig. 1). The Alboran Sea is the transitional area connecting the Atlantic Ocean to the Mediterranean Sea. This area has a very high hydrodynamism, where upwelling of cold, nutrient-rich subsurface waters, either wind-induced or due to north–south excursions of the Atlantic jet (Sarhan et al. 2000), allows the plankton communities to bloom (Sanchez-Vidal et al. 2004).

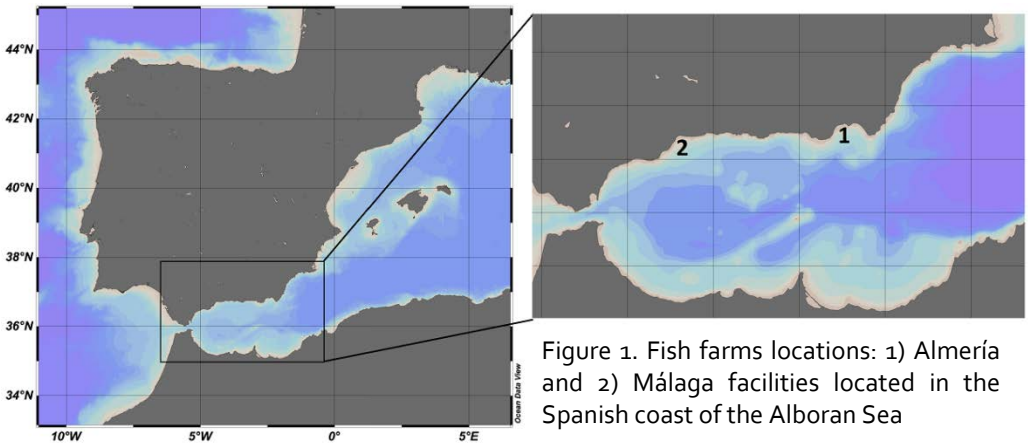

Figure 1. Fish farms locations: 1) Almería and 2) Málaga facilities located in the Spanish coast of the Alboran Sea

Both fish farms are off-shore facilities that grow European sea bass (*Dicentrarchus labrax*) and Gilthead sea bream (*Sparus aurata*) from 15 g fry to commercial size fish in floating cages. Cages were 25 m in diameter by 10 meters long where maximum depth of water column varied from 30 to 50 m.

Sampling and sample processing

Monitoring was performed from January 2012 to June 2014 for the Almería facility and from June 2013 to June 2014 for the Málaga fish farm. Temperature and fish mortality data were provided by the facilities staff. Temperature was recorded daily at 5 m depth by a sensor (Oxyguard) located in the cages and fish mortality was reported weekly.

Zooplankton samples were collected biweekly, using a net of 200-µm mesh with a filtering cod-end and a digital flow meter (Hydrobios, model 438110) was incorporated to determine the volume of filtered water. Three vertical net hauls were performed at each sampling sites: two immediately outside the cages and two external sites (where fish farms perimetral buoys were located, approximately 250 m far from cages north and south) to characterize the gelatinous zooplankton community inside and outside the facility. A preliminary study demonstrated that there were no significant differences in gelatinous zooplankton densities inside and outside the fish cages. Monthly zooplankton samplings inside and outside Almería fish farm cages were performed during 8 months previous to our monitoring started. Analyzed results showed that there were not significant differences in gelatinous zooplankton densities inside or outside the cages (F_1= 1.90, p= 0.07). Thus, we decided to sample immediately outside the cages, so as not to stress the farmed fish by sampling. All plankton samples were preserved in a 4% neutral buffered formalin solution. In the laboratory, gelatinous zooplankton was quantified (individuals·m^{-3}) and identified in 5 taxa (Cnidaria, Polychaeta, Larvacea, Thaliacea, Chaetognatha). Cnidarian zooplankton was identified to genus or species level in most cases.

Phytoplankton samples were collected at the same time and sites as zooplankton, by use of a Lund tube (a weighted polyethylene tube 2 cm in diameter) of 5 m length (Lund and Talling 1957). From the total sample, a subsample of 500 ml was taken after homogenization, and was preserved by adding 1 ml of neutral Lugol's Iodine solution. Samples were analyzed at the Dipartimento di Scienze della Vita e dell'Ambiente (Università Politecnica delle Marche (Ancona, Italy). A variable subsample volume (25–63 ml depending on the abundance observed) was settled in an Utermöhl chamber (Edler and Elbrächter 2010). Species identification and counting were performed using an inverted microscope (Zeiss Axiovert 135) equipped with phase contrast at 400x magnification, in 10-30 random fields, in order to obtain a significant cell number. For each sample, abundances were expressed as number of cells per liter (cells·l^{-1}). Results were screened for species potentially harmful for farmed fish.

Five to ten fish were randomly sampled for gill analysis from monitoring cages once a month and every time that mortality in cages was recorded. Fish were caught using a hand net and moribund fish were avoided at each sampling time to ensure that the fish sampled were representative of the population as a whole. Sampled individuals were anesthetized using clove oil and immediately were killed by cold shock treatment. Gill samples were taken from the second gill arch and were fixed in 10% neutral-buffered formalin. Samples were embedded in paraffin and 4-µm sections were stained with haematoxylin and eosin standard protocol. Slides were examined microscopically at 50x, 100x and 400x magnifications.

The gill score protocol created by Mitchell et al. (2012) was used to quantify gill damage. The index criteria for gill histopathology were lamellar hyperplasia, lamellar fusion, circular anomalies (necrosis or sloughing), and lamellar oedema. A score from 0 to 3 was assigned for each parameter depending on injury extent (1: <10% affected surface, 2: 10-50% and 3: >50% of the gill epithelium injured). Ancillary criteria, such as hypertrophy, haemorrhage and the presence of specific pathogens, were assigned a score of 0 or 1. Pathogens affecting *Dicentrarchus labrax* included the parasites *Cryptocaryon* sp. and the monogenea *Diplectanum* sp. The injury scores were summed and total scores between 1

and 3 were considered to be typical of gills regularly observed in marine-farmed fish (A. Girons pers. obs.)

Statistical analyses

Multiple analyses of variance - (Anderson 2001) PRIMER software - were performed in order to test for differences in the gelatinous zooplankton assemblage among covariates (time, farm, sampling site). The experimental design was composed by three factors: 1) "Time", fixed with 17 levels; 2) "location", fixed and orthogonal with 2 levels; and 3) "sampling site", fixed and orthogonal with 2 levels. Moreover, the species that contributed most to the similarity in each Location were characterized using the SIMPER routine (Clarke 1993).

An ordination of the zooplankton community based on the density of each group was obtained with a principal component analysis (PCA) in order to test for organisms of the zooplankton associated with the mortality events or changes in temperature in the Almería facility. The PCA analysis was performed with the R-language function Princomp, which is available in the Vegan library (Oksanen et al. 2005) of the R software platform.

Generalized linear models (GLM) with a binomial distribution and a logit link were used to describe the probability of a mortality event at different densities of gelatinous zooplankton and phytoplankton.

Bivariate linear regression was applied to test for a relationship between the total gill score and cnidarian density, after checking for the normality and homoscedastic condition. At last, GLM model setting a Poisson error family and a logit link has been employed to describe the variation in density of cnidarians harmful species among different temperature's values. These analyses were performed using the free statistical software R, version 3.2.3 (http://cran.r-project.org).

Results

The densities of gelatinous zooplankton were tested for differences over the length of sampling, between fish farms, and between sampling sites (cages or external sites) at each farm. A total number of 608 zooplankton samples were analyzed, and 32 taxa of cnidarians were identified (24 hydromedusae, 7 siphonophores and 1 scyphozoan). Differences in total gelatinous zooplankton densities between cages and external sampling sites were not significant at the monitoring facilities ($p > 0.05$); however, the gelatinous zooplankton community changed significantly between facilities and over time (Table I).

Table I. Multiple analyses of variance (PERMANOVA) of the gelatinous zooplankton community comparing the Almería and Málaga aquaculture facilities (location), sampling sites (cage, external point) and sampling dates (time). "Lo"= location, 2 levels (Almería, Málaga); "Ti" = time, 17 levels; "Po"= site, 2 levels (cage, external point). $P < 0.05$ was considered significant.

Source	df	SS	MS	Pseudo-F	P(perm)	perms
Lo	1	1157.8	1157.8	4.9129	0.005	999
Ti	16	49927	3120.4	13.242	0.001	999
Po	1	202.6	202.6	0.85972	0.476	997
Lo x Ti	13	8186.4	629.73	2.6722	0.001	998
LoxPo	1	138.96	138.96	0.58968	0.649	999
Ti x Po	12	4785.8	398.82	1.6924	0.017	998
Lo x Ti x Po	6	1981.7	330.28	1.4015	0.148	997
Res	173	40768	235.66			
Total	223	$1.305 \cdot 10^5$				

Four cnidarians species previously implicated in mass mortality events of farmed salmonids were identified in the Almería and Málaga fish farms; the siphonophore *Muggiaea atlantica*, the hydromedusae *Phialella quadrata* and *Solmaris corona*, and the ephyrae of *Pelagia noctiluca*. *M. atlantica* presented high densities in both facilities, with peaks in March 2012 and 2014 in Almería and November - December 2013 and March 2014 in the Málaga fish farm. A highly reproductive population was observed over these

periods (spring and autumn) with high abundances of both polygastric colonies and eudoxid stages recorded.

P. quadrata and *S. corona* presented low densities with discrete abundance peaks during autumn; *P. noctiluca* ephyrae were observed several times during monitoring, but always at low-medium densities in both facilities. In addition, some characteristic species from open waters were recorded at low densities (i.e. *Solmundella bitentaculata* hydromedusae and the siphonophores *Chelophyes appendiculata* and *Abylopsis tetragona*).

Almería fish farm

To test relationships between fish mortalities recorded in the facility, different gelatinous zooplankton groups and temperature, a Principal component analysis (PCA) model was performed (Fig. 2). The only group significantly and positively correlated with mortality in the fish farm were cnidarians (Z_1= 4.039, p= 0.00005) (Fig. 3). SIMPER analysis (Table II) showed that the most representative species at the facility were *Hydractinia carica*, *Aglaura hemistoma* and *Obelia dichotoma*, while the most abundant species were *O. dichotoma*, *M. atlantica* and *M. kochi*.

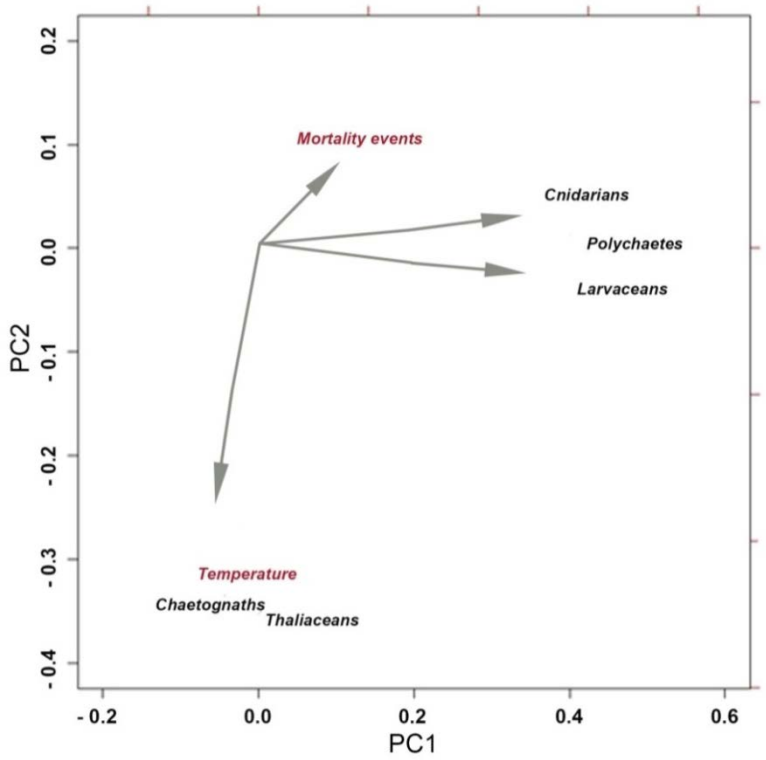

Figure 2. Principal component analysis (PCA) of gelatinous zooplankton (cnidarians, polychaete larvae, larvaceans, chaetognaths, and thaliaceans), fish mortality and temperature at

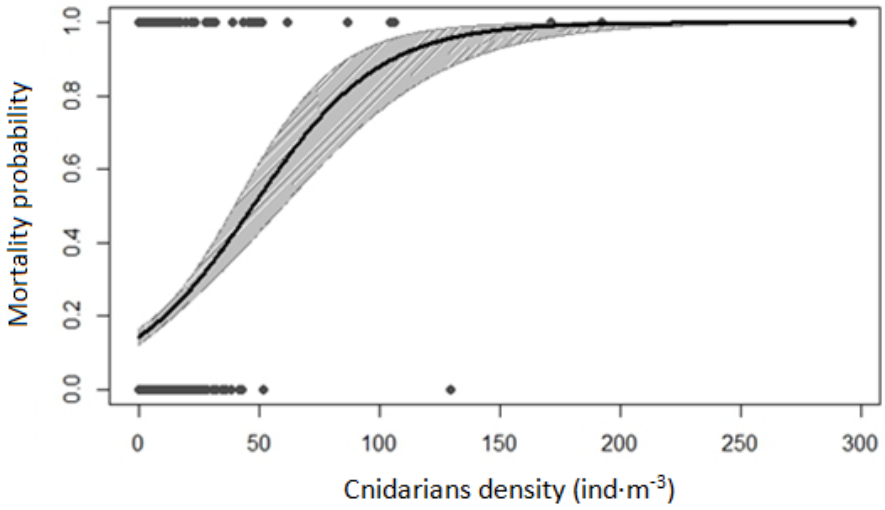

Figure 3. Probability of a fish mortality event at different densities of cnidarians in the Almería facility. The dots at the top and bottom axes represent the mortality presence/absence over the monitoring.

Table II. Cnidarian species SIMPER analysis at both facilities

Almería fish farm
Average similarity: 14.32

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Hydractinia carica	0.44	1.74	0.24	12.13	12.13
Aglaura hemistoma	0.38	1.69	0.25	11.83	23.96
Obelia dichotoma	0.42	1.56	0.25	10.92	34.89
Abylosis eschscholtzi	0.31	1.13	0.21	7.86	42.75
Muggiaea kochi	0.30	1.07	0.22	7.45	50.20
Muggiaea atlantica	0.40	1.05	0.21	7.35	57.55
Rhopalonema velatum	0.18	0.52	0.21	3.64	61.19

Málaga fish farm
Average similarity: 14.23

Species	Av.Abund	Av.Sim	Sim/SD	Contrib%	Cum.%
Muggiaea atlantica	0.64	2.33	0.36	16.39	16.39
Hydractinia carica	0.56	2.05	0.32	14.43	30.82
Eucheilota paradoxica	0.48	1.87	0.25	13.11	43.93
Abylosis eschscholtzi	0.34	1.59	0.25	11.17	55.10
Stauridiosarsia gemmifera	0.69	1.42	0.21	10.00	65.10

Among all identified cnidarian species, only 3 were correlated with fish mortalities: the siphonophores *M. kochi* (Z_1=3.350, p= 0.02) and *M. atlantica* (Z_1= 2.547, p= 0.010), and *Ectopleura larynx* actinulae larvae (Z_1= 2.462, p= 0.014) (Fig. 4). By contrast, temperature was significantly and negatively related with mortality (Z_1= -5.694, p= $1.24 \cdot 10^{-8}$) and significantly and positively related with the above cnidarian species (F_1= 31.258, p=$4.609 \cdot 10^{-8}$) (Fig. 5).

Histological analyses revealed severe damage in the fish gills, including generalized inflammation of gill epithelium, lamellar hyperplasia and fusion, oedema and in many cases necrotic patches with advanced bacterial infection (Fig. 6). Gill scoring analysis showed different peaks of severe gill damage in the sampled fish. The relation between histological scores and total cnidarian densities was significant and positive (F_1= 12.56, p= 0.0006 and r= 0.33) (Fig. 7a). Highest farmed fish mortalities were recorded in April – May 2012, January 2013, and March 2014, but also elevated scores were observed in November 2012 and April 2014 (Fig. 8a).

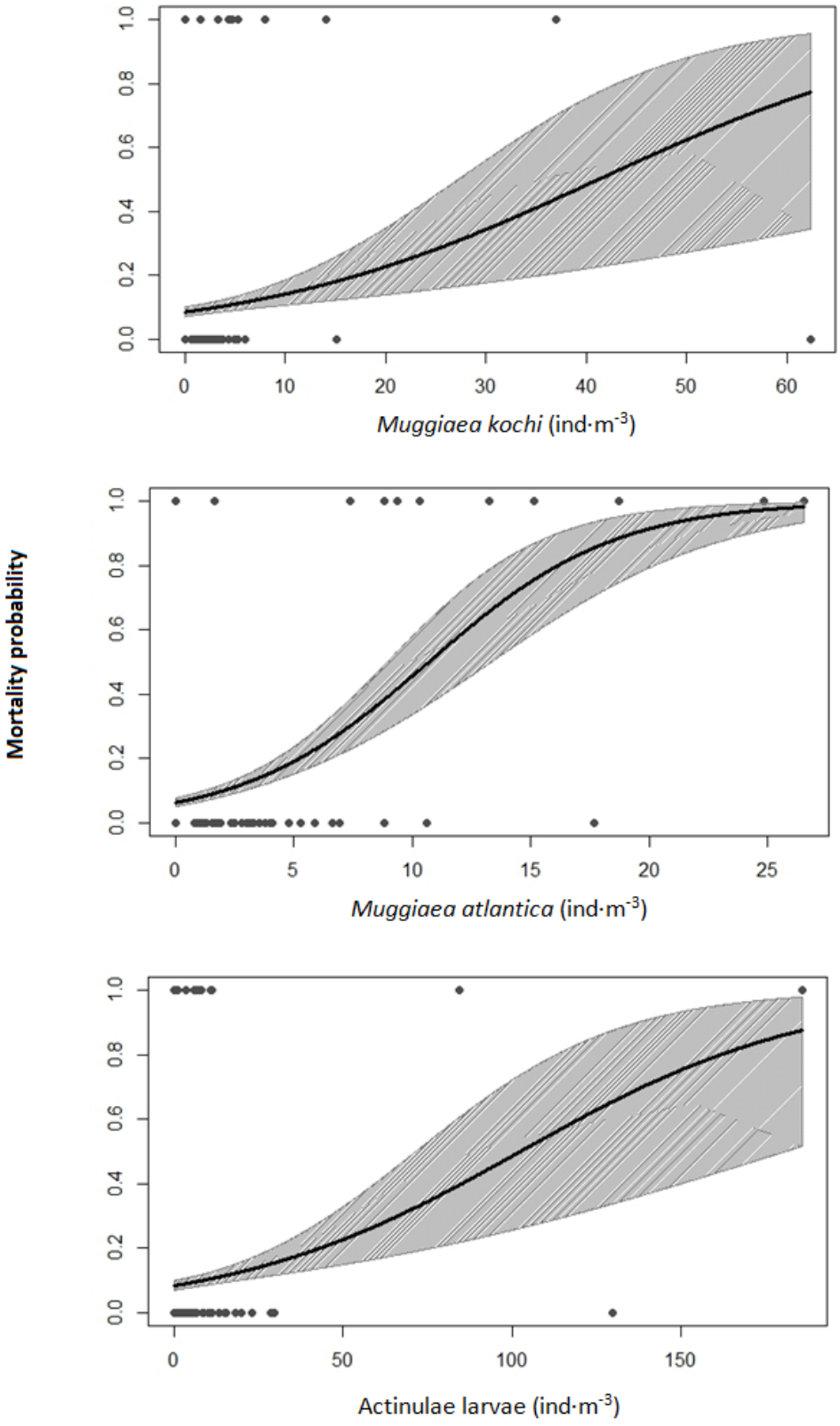

Figure 4. Probabilities of fish mortality event at different densities of identified harmful cnidarian species (*Muggiaea kochi*, *Muggiaea atlantica* and *Ectopleura larynx* actinula larvae). The dots at the top and bottom axes represent the mortality presence/absence over the monitoring.

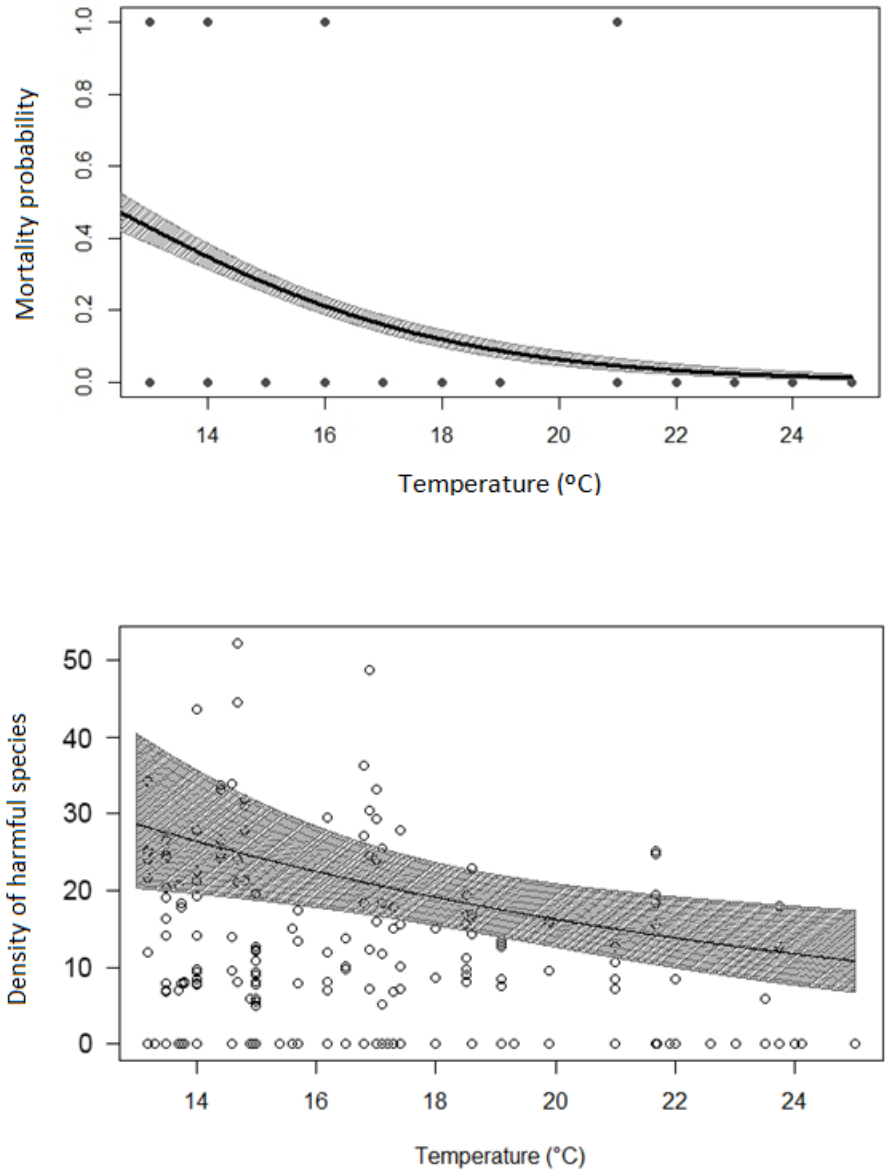

Figure 5. Probability of a fish mortality event at different water temperatures (above) and sampled densities (total no. m^{-3}) of harmful cnidarian species by water temperature (below).

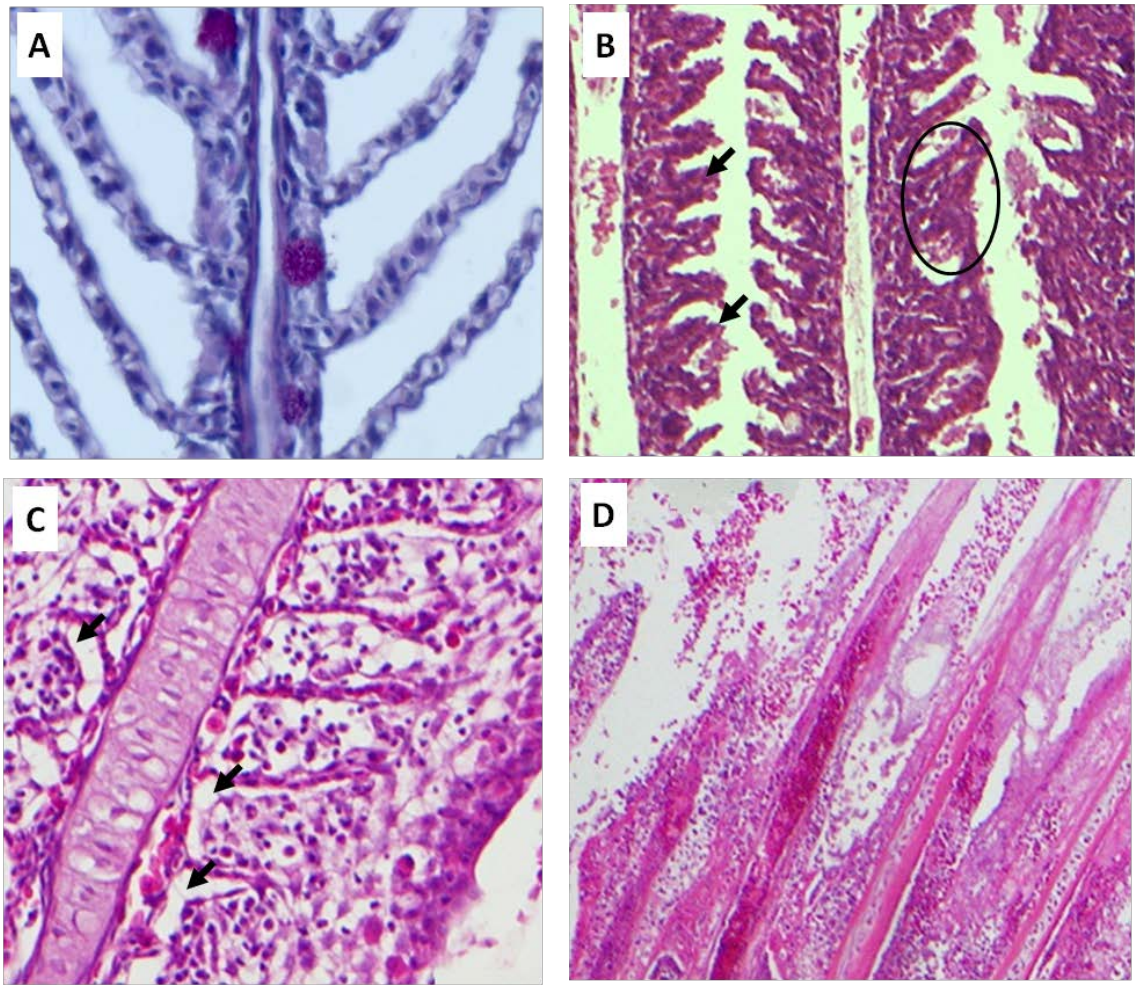

Figure 6. A. Healthy gills with undamaged primary lamellae and presence of mucous cells (400x); B-D. Pathological features in gills from fish sampled *Dicentrarchus labrax* at aquaculture facilities in Spain (400x): B. Hyperplasia of primary lamellae (black arrows) and lamellar fusion (black circle); C. Lamellar oedema (black arrows); D. Cellular degeneration with necrotic patches.

The cnidarian assemblage differed between facilities, with an average dissimilarity of 88%. SIMPER analysis (Table II) showed that the most representative species in the Málaga fish farm were *Muggiaea atlantica*, *Hydractinia carica* and *Eucheilota paradoxica*. These species were also the most abundant, together with *Stauridiosarsia gemmifera*. The siphonophore *M. atlantica* was the most frequent and abundant cnidarian species in the facility. High abundances of *M. atlantica* and *M. kochi* reproductive stages also were recorded in the facility.

Significant and positive relation between gill scores and cnidarian densities was observed (F_1= 14.707, p=0.0003 and r= 0.43) (Fig. 7b). The only fish mortality event recorded in this facility was at the end of November and continued in December 2014, but cnidarian density peaks associated with high gill damage scores were observed also in March and May 2014 (Fig. 8b).

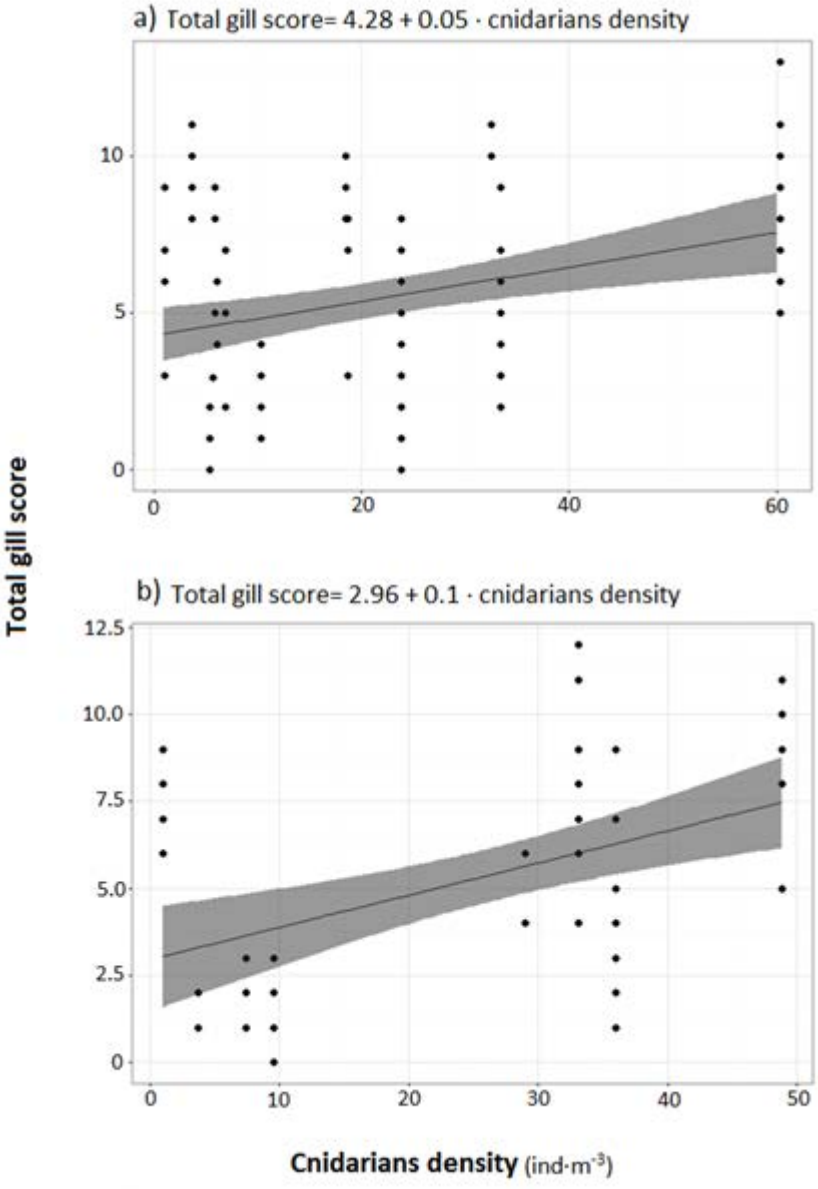

Figure 7. Bivariate linear regression between total gill score and cnidarian density for the Almería facility (a) and the Málaga fish farm (b).

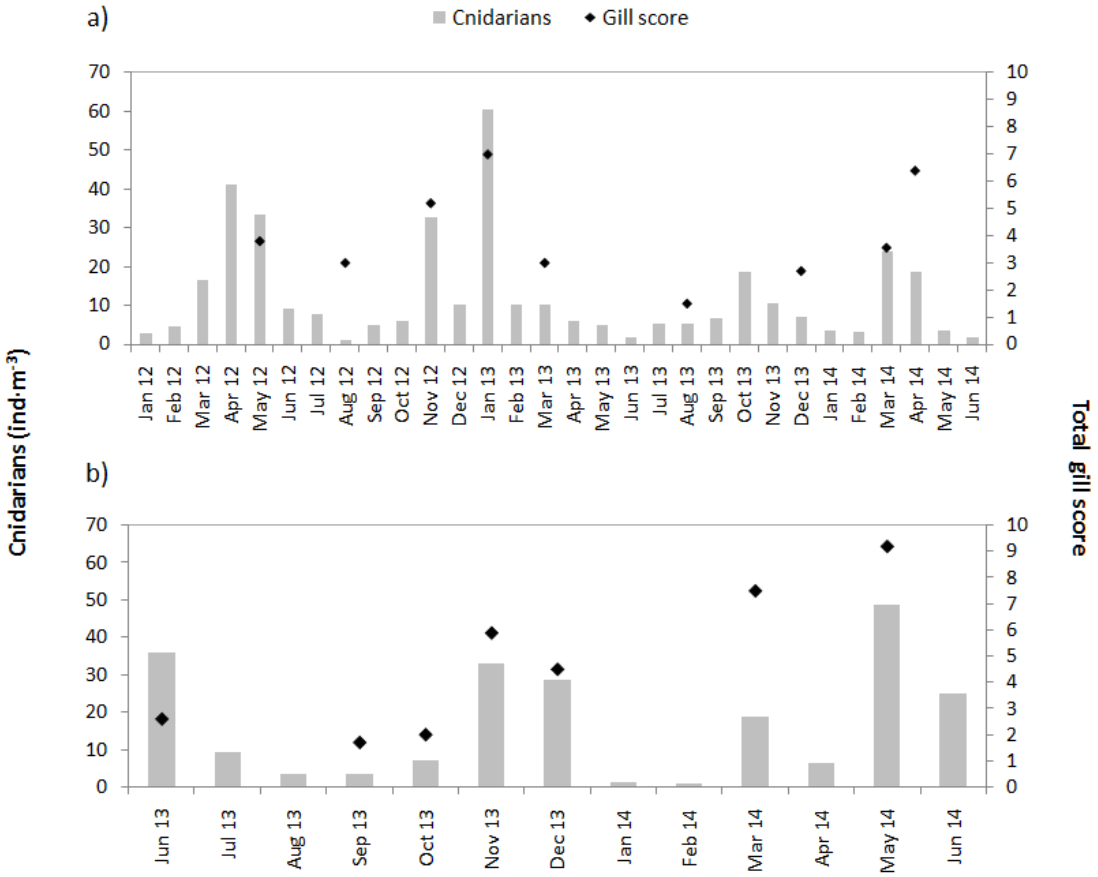

Figure 8. Cnidarian densities and gill scores over time for the Almería (a) and the Málaga fish farms.

More than 70 genera or species of phytoplankton were identified in collected samples from both facilities. Several microalgae genera potentially harmful for fish were recorded at high densities in both fish farms. Among diatoms, a number of *Chaetoceros* species (i.e. *C. lorenzianus*, *C. laciniosus* and *C. didymus*) occurred several times throughout the monitoring period in both facilities, with a density peak of $8.43 \cdot 10^5$ cells·l^{-1} in April 2012 in Almería fish farms, when fish mortality was recorded. The silicoflagellate *Dictyocha speculum* was found at high densities in Málaga installation ($2.6 \cdot 10^3$ cells·l^{-1}) and *Pseudo-nitzschia* spp. occurred during 2012, with an abundance peak in August ($3.1 \cdot 10^5$ cells l^{-1}), but the presence of both species did not coincide with fish mortality events.

Discussion

Information about jellyfish bloom impacts on marine finfish aquaculture is scarce and almost absent in the Mediterranean Sea. To our knowledge this is the first monitoring program of gelatinous zooplankton performed in Mediterranean aquaculture facilities to investigate its temporal and spatial distribution, as well as its role on farmed fish gill disorders. Results showed that fish mortalities were correlated with low temperatures and the presence of 3 hydrozoan species, the siphonophores *M. atlantica* and *M. kochi* and actinula larvae of *E. larynx* hydroids.

When mortalities were recorded, the average temperature was 14.69 ± 0.55 ºC (mean ± SE). Total cnidarian densities also were correlated with low temperatures, with very low densities during summer period, especially those species with a benthic stage in their life cycle. This agrees with previous observations for the Mediterranean Sea where benthic suspension feeders experience summer dormancy due to summer impoverishment, leaving only dormant basal stolons (González-Duarte et al. 2013) (Bavestrello et al. 2006).

In the analyzed samples, some species occurred that are characteristic from open waters, such as *Solmundella bitentaculata* hydromedusae and the siphonophores *Chelophyes appendiculata* and *Abylopsis tetragona* (Mills et al. 1996). The occurrence of these species could mean a water inflow of Atlantic waters to coastal zones in the Alboran Sea. The central and southern Alboran Sea are areas strongly influenced by inflowing Atlantic waters and considered a 'key point' for new entries of Atlantic hydrozoan species into the Mediterranean (Medel and López-González 1998; Boero et al. 2003); in contrast, the northern coast of the Alboran Sea is influenced by Mediterranean waters coming from the Catalan Sea (Bouzinac et al. 2003).

The siphonophore *Muggiaea atlantica* was previously identified as a potentially harmful species for marine aquaculture in northern Europe, together with *S. corona* and *P. quadrata* (Baxter et al. 2011). Those species were also identified in collected samples but statistical analyses showed non-significant relation of both hydromedusae densities and

fish mortalities (Z_1= -0.006, p= 0.995 and Z_1= -0.002, p= 0.998 for *S. corona* and *P. quadrata* respectively). High abundances of *Muggiaea* spp. eudoxid stages were recorded several times in both fish farms. Each calycophoran siphonophore polygastric stage asexually produces several eudoxids, a sexually-reproductive stage that feeds with stinging tentacles, as is the polygastric stage. High abundances of eudoxids could be detrimental for farmed fish health because hundreds of cnidocytes are present in the tentacles and could damage fish tissues. Mortality recorded in November-December 2014 in the Málaga facility coincided with a *Muggiaea* spp. reproductive event, reaching densities of 30.2 ± 5.33 polygastrics m^{-3} and hundreds of eudoxids in the water column. *Pelagia noctiluca* is the most abundant and one of the most painful stinging scyphozoan jellyfish in the Mediterranean Sea (Russell 1970), with the potential to reproduce all year long in some areas, such as the Strait of Messina (Milisenda et al. 2016). The Almería and Málaga facilities endured big swarms of *P. noctiluca* adult jellyfish several times, with associated economic consequences for the fish farms (unpublished data, thesis chapter 1). In zooplankton samples, *P. noctiluca* ephyrae were observed at densities up to15 ind m^{-3} several times in both facilities (mainly in February, June and October).

Ectopleura larynx actinula larvae also were correlated with fish kill events in the Almería facility. The actinulae were presented when temperatures were low, from January to May and from November to December in both facilities. Over the course of monitoring, several reproductive periods were observed, with the released actinula larvae reaching very high densities in the water during January 2013 (> 200 ind · m^{-3}) when thousands of fish died. This hydroid usually forms part of cage fouling community in the North Sea (Carl et al. 2010; Guenther et al. 2010; Baxter et al. 2012) and in the Mediterranean Sea aquaculture facilities (pers. obs) and has been a severe problem in the North Sea farms. Guenther et al. (2010) demonstrated that after washing to clean the nets, this species regrows and occludes the net apertures rapidly. Underwater cleaning of the net cages resulted in higher numbers of *E. larynx* actinulae, juveniles and polyps in the water column (Carl et al. 2010). This species could significantly affect caged fish health by injuring gill tissue after contact (Baxter et al. 2012).

Obelia dichotoma is a common hydroid species in Mediterranean coastal areas (Bouillon et al. 2004; González-Duarte et al. 2015). Its small hydromedusae were found at high densities in the Almería facility (maximum of 197.35 ind m^{-3}) coinciding in some cases with fish mortalities. This species was observed throughout the year, both hydroids and medusae, forming part of biofouling and zooplankton communities in the Almería and Málaga facilities. Despite its high concentrations, relationship between *O. dichotoma* and recorded fish kill events were not significant (F_1= -1.55, p= 0.121). Nevertheless, it should be considered as a potentially harmful species for caged fish.

Harmful algal blooms are often associated to fish kills episodes worldwide (Treasurer et al. 2003; Burridge et al. 2010). Fish toxic species are included in most groups including dinoflagellates, diatoms, silicoflagellates and prymnesiophytes (Granéli and Turner 2006). In this study, some phytoplankton taxa previously related with farmed fish kill events (Cembella et al. 2002; Treasurer et al. 2003), were recorded at high densities in both fish farms. *Chaetoceros* is a colonial diatom genus characterized by long setae, which either can clogging gills causing asphyxia or can penetrate the gill tissues causing histological damages (Smayda 2006). Fish kills attributed to *Chaetoceros* occurred in Canada and USA (Rensel 1992). In literature *Chaetoceros* abundances of 10^5 cells·l^{-1} were associated to fish kill events (Treasurer et al. 2003). In analyzed samples we recorded abundances of the same order of magnitude, therefore *Chaetoceros* should be taken into account as phytoplankton species that may had contributed to fish mortality events even if statistical analyses did not show significant relation between this genera and fish kill events.

Gill scoring was significant and positively correlated with cnidarian densities, and demonstrated the existence of severe gill disorders even when fish mortalities were not recorded. For example, in April 2014 in the Almería facility and May 2014 in the Málaga fish farm, gill scores were 6 ± 1.1 and 9.2 ± 0.6, respectively and jellyfish occurred in high densities (19.71 ± 3.07 ind·m^{-3} in Almería and 48.80 ± 10.79 ind·m^{-3} in Málaga facility). Experimental studies with the scyphozoan *Aurelia aurita* showed gill epithelium recovery

required 2 weeks after even brief (10 h) contact between jellyfish and salmonids (Baxter et al. 2011b). Equally, laboratory experiments from chapter 4, demonstrated partial recovery of sea bream gill tissue after 3 weeks from fish exposure to medium densities of *P. noctiluca*. Repeated contact with jellyfish could be responsible not only for fish mortality but also for poor growth and performance in farmed fish (Rodger et al. 2011a; Rodger et al. 2011b).

Due to the growth of the aquaculture sector and the increased frequency of jellyfish blooms in some coastal waters, the negative interactions of stinging jellyfish on caged finfish is expected to become a substantial issue producing highly relevant economic losses (Purcell et al. 2013). Gelatinous zooplankton monitoring will be vital to obtain site-specific information about jellyfish populations, including their seasonal occurrence and densities, and it is essential to better understand roles of jellyfish in fish gill disorders and mortalities.

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Chapter 3

Hydroid assemblages on Mediterranean fish cages: composition, growth and reproductive periods

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Introduction

Biofouling is an important problem and costly factor in marine finfish aquaculture worldwide. The accumulation of biofouling organism on fish nets can reduce water flow and affect oxygen supply and the susceptibility of farmed fish to diseases (Braithwaite and McEvoy 2004; Baxter et al. 2011). The occlusion and increased weight of the net can also cause structural stress as well as a reduction in cage buoyancy and increased net deformation (Bloecher et al. 2013).

The succession patterns and composition of biofouling on floating cages may differ from those described for hard substrates or seabed communities, because net cage material differs from natural substrates and could affect fouling community characteristics (Greene and Grizzle 2007). The most common macrofouling found on aquaculture structures are from the planktonic propagules of algae, and the larvae of invertebrates such as hydroids, ascidians, sponges, bryozoans, barnacles, bivalves, and polychaetes (Fitridge et al. 2012; Fitridge and Keough 2013).

Most of the time biofouling is deleterious to shellfish stocks and farmed fish cultures by acting as reservoirs of pathogens or clogging nets and reducing water exchange in the cages. Organisms such as polychaete worms excavate the shells of shellfish, affecting their development and increasing their vulnerability to predators and parasites. Some tunicates compete with cultivated mussels for food. Hydroids also are considered problematic organisms for aquaculture, due to their effects on the cage structure and farmed species health (Fitridge et al. 2012). Fitridge and Keough (2013) observed that *Ectopleura crocea* fed on mussels larvae and fouled the shells of cultivated mussels, causing significant reduction in length and weight. Baxter et al. (2012) simulated the in situ net cleaning process used in aquaculture cages; after cleaning, small pieces of *Ectopleura larynx* (Ellis and Solander, 1786) hydroid colonies remained suspended in the water column and were inhaled by experimental fish, causing severe gill injuries. Over the last decade, *Ectopleura larynx* has become one of the most common fouling organisms in northern Europe aquaculture, causing increasing problems for fish farmers (Guenther et al. 2010). In the Mediterranean Sea, this species together with *Pennaria disticha* (Goldfuss, 1820) have been identified as problematic for marine fish farms (thesis chapter 1). Although hydroid fouling on nets is cost- and labour-intensive for fish farming operations, there is a profound lack of knowledge on detailed community composition.

Similar gill injures to those caused by *E. larynx* in the laboratory were observed in Irish fish farms, where salmon mortalities were correlated with high densities of cnidarian zooplankton in the facility (Baxter et al. 2011). The same phenomenon was observed in Spanish aquaculture facilities, where mortalities of juvenile European sea bass (maximum weight 66 ± 5.3 g) were correlated with high abundances of small planktonic hydrozoans in the installations (Bosch-Belmar et al. 2015b). The limited information on the effects of jellyfish in aquaculture and the potential impact of hydroids on fish farms and farmed species health led to the main objectives of the present work: (I) to investigate about the hydroid composition on aquaculture cages and (II) to study the seasonal variability, growth and sexual reproductive periods of dominant species.

Materials and Methods

Study site

Fouling was monitored in a Spanish fish farm located in the eastern part of the Alboran Sea (37º 24' 37.82'' N, 1º 32' 6.06'' W). Fish farm was off-shore and grew European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) in floating cages. Cultivated stocks ranged from 15 g fish to commercial size. Fish stocks between 15 and 70 g are introduced in cages designed for fry fish (mesh size of 1 cm, cage diameter of 25 m and depth of 6 m). When they reach optimal weights, the net is changed to an adult pen net (mesh size of 2 cm and depth of 15 m). Fry fish are introduced to marine cages twice a year, usually in April – May and September – October, depending on fry production rhythms on shore.

Experimental design and field methods

In order to cover a complete production year, two monitoring periods were established, from May to November 2013 (I) and from November 2013 to June 2014 (II), simulating the immersion periods of juveniles' cages (maximum 6 months).

At each period, four rectangular metallic structures (120 x 80 cm), each with 6 panels (24 panels), were positioned in the northwest part of the facility, near the juveniles' cages. Each experimental panel was constructed with a single 40 x 40 cm piece of cage net. The mesh dimensions and the antifouling treatment were the same as used in the juveniles' cage (100 mm and NI5 - Netchem antifouling) (Fig. 1). The large frame was attached vertically to the side of one of fry cages at 5 m depth. The first sample set (3 panels at each sampling time) was collected 1 month after the panels' immersion, and subsequent sets 2-3 week intervals, depending on weather conditions.

Panels were carefully collected by SCUBA divers and immediately fixed in formaldehyde 10% solution. In addition, at each sampling time, 3 zooplankton samples were collected. Vertical net hauls were performed using a 200-µm mesh net with a filtering cod end and

a digital flow meter to calculate the volume of filtered water (Hydrobios, model 438 110). Samples were preserved in 4% neutral buffered formalin solution.

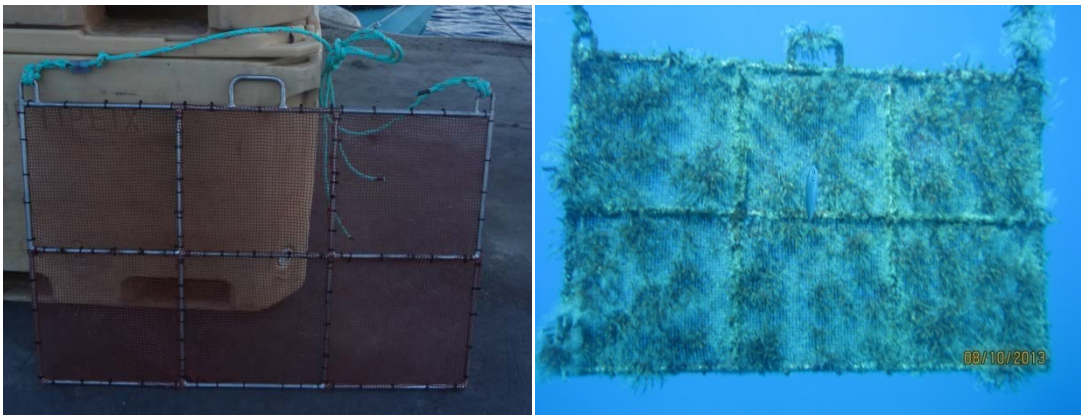

Figure 1. Net panels used for biofouling monitoring: new and clean panels (left) and panels with fouling after few weeks from the start of the monitoring (right)

Laboratory methods

In the laboratory, all fouling organisms were separated and identified to the lowest taxonomic level: suborder for crustaceans, species for hydroids, and genus for the remaining taxa. Mobile animals, such as platyhelminthes or polychaetes, living on the net but theoretically able to move short distances between nets were included in the analysis because they are essential parts of the net fauna. A 1-cm wide margin along the border of the panels was excluded to avoid potential edge effects.

Experimental panels were inspected with a stereomicroscope at 10x and 20x power and organisms carefully removed with forceps and again preserved in 4% formalin solution. Then the panels were brushed to collect all algae attached to the nylon and placed in the formalin.

Richness was calculated for every panel as the total number of taxa present. For biomass ($g \cdot m^{-2}$) analysis, most organisms were grouped by phylum or subphylum (Crustacea, Annelida, Nemertina, Mollusca, Echinodermata, Bryozoa, Platyhelminthes, Nematoda); all algae were considered in a single group. Cnidarians were separated by class (Hydrozoa and Anthozoa). Collated fouling was dried at 60 °C to a constant weight (~ 48 hours) to

determine dry weights. Density (ind·m^{-2}) was calculated for each group except for colonial cnidarians, bryozoans and algae, since was not possible identified single individuals.

Growth rates and reproductive stage of hydroids colonies were determined for the most abundant species (*Ectopleura larynx*, *Pennaria disticha* and *Obelia dichotoma*). The length of colonies at each sampling time was measured on all three panels on 30 randomly selected hydrocauli - main stem of a fixed, erect hydroid colony - for each species, and averages were calculated. Reproductive stage of the colonies was evaluated using the descriptions of Schuchert 2006 for *P. disticha,* and Allman 1872 and Schuchert 2010 for *E. larynx* (Schuchert 2006; Schuchert 2010) Categorical classifications were established according to the different development phases of gonophores described by those authors (Table I).

Data analysis

Two-way analysis of variance (ANOVA) was applied to test for differences in richness, total biofouling biomass, and hydroid biomass between Periods I and II (fixed factor, 2 levels), and among sampling times within each period (fixed factor, 8 levels). Differences in hydroid colony maturation were tested using one-way ANOVA for each species with sampling time as fixed factor.

A Multiple Regression Linear Model was used to test for differences in hydroid growth (expressed as hydrocaulus length) by time (expressed as day of the experiments, as a continuous explanatory variable) and species (categorical explanatory variable: *O. dichotoma* vs. *P. disticha* during Period I, and *O. dichotoma* vs. *E. larynx* during Period II). For each period, we tested two different models, one testing interactions between species and time factors (M1: hydrocaulus length = species · time) and another without interaction (M2: hydrocaulus length = species + time). All models were fitted and compared with each other using the corrected Akaike's Information Criterion (AIC) (Burnham and Anderson 2002). All analyses were carried out using the statistical software R (R Core Team 2015, v.3.2.2).

Table I. Descriptions of different maturation stages of the hydroids *Ectopleura larynx* and *Pennaria disticha* reproductive structures. Table based on Schuchert 2006, 2010 and Allman 1872.

Maturation stage	***Ectopleura larynx***	***Pennaria disticha***
0	No reproductive structures	No reproductive structures
1	Small gonophores with no distinct structures fixed as sporosacs above hydranth tentacles	Small gonophores oblong medusoids arising on short pedicels just above whorl of long filiform tentacles
2	Gonophores oval to spherical. Female gonophores with red spadix that can protrude out of sporosac opening (opening is terminal)	Developed eumedusoid with four radial canals and four marginal bulbs, with small velum, without ocelli, tentacles normally absent
3	Mature female gonophores with four tentacle-like tubercles around opening at distal end. Female sporosacs filled with numerous small cells forming an egg-like mass	
4	Form of processes very variable, occasionally reduced or absent, but usually increase in size with the enlarging gonophores. Visible developed actinula inside the sporosac.	

Results

Taxa Richness

A total of 29 and 25 taxa belonged to 12 different phyla were identified during monitoring Periods I and II, respectively (48 experimental panels total). The most frequent macrofouling organisms were algae and different crustaceans (orders Amphipoda and Tanaidacea), which were the most abundant taxon during both monitoring periods, followed by anthozoans, which reached more than 10^3 ind·m^{-2} from June to November and molluscs (*Mytilus galloprovincialis*) (maximum densities of 100 ind·m^{-2}).

Richness differed significantly between monitoring periods (F_1= 7.392, p= 0.001) and within each period (F_7= 13.14, p= 0.001 (I) and F_7= 4.289, p= 0.001 (II)) (Fig. 2). Richness between May and November was highest during the end of August 2013 (21 taxa). From November to June, the highest number of species recorded was in June 2014 with 15 identified taxa.

Biomass and community composition

Total biofouling biomass (g·m^{-2}) was significantly different between periods (F_1= 8.418, p= 0.001), being higher during Period II than Period I (305.79 ± 0.83 g m^{-2}, 224.10 ± 0.94 g m^{-2}, respectively). Significant differences among sampling times were also observed (F_7= 37.224, p= 0.001, Period I- and - F_7= 18.187, p= 0.001, Period II) (Fig. 2). The groups that most contributed to biomass were crustaceans and algae, followed by cnidarians (hydroids and anthozoids) for the first period and cnidarians and molluscs for the second (Fig. 3).

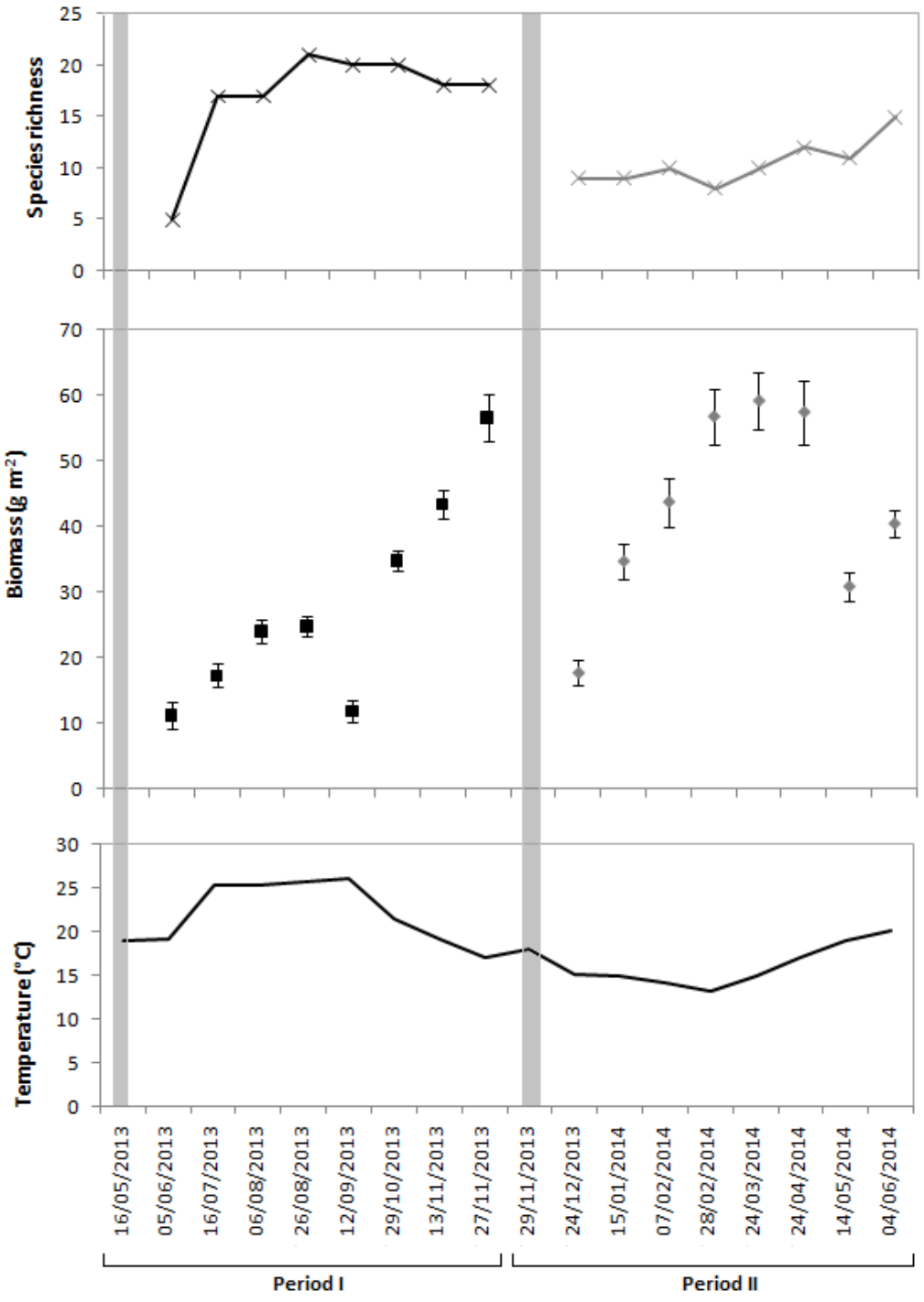

Figure 2. Richness, total fouling biomass and temperature for both monitoring periods. Period I: May to November 2013; Period II: November 2013 to June 2014) at an aquaculture facility on the southern coast of Spain. Grey vertical bars on the figure indicate the start of monitoring for both periods.

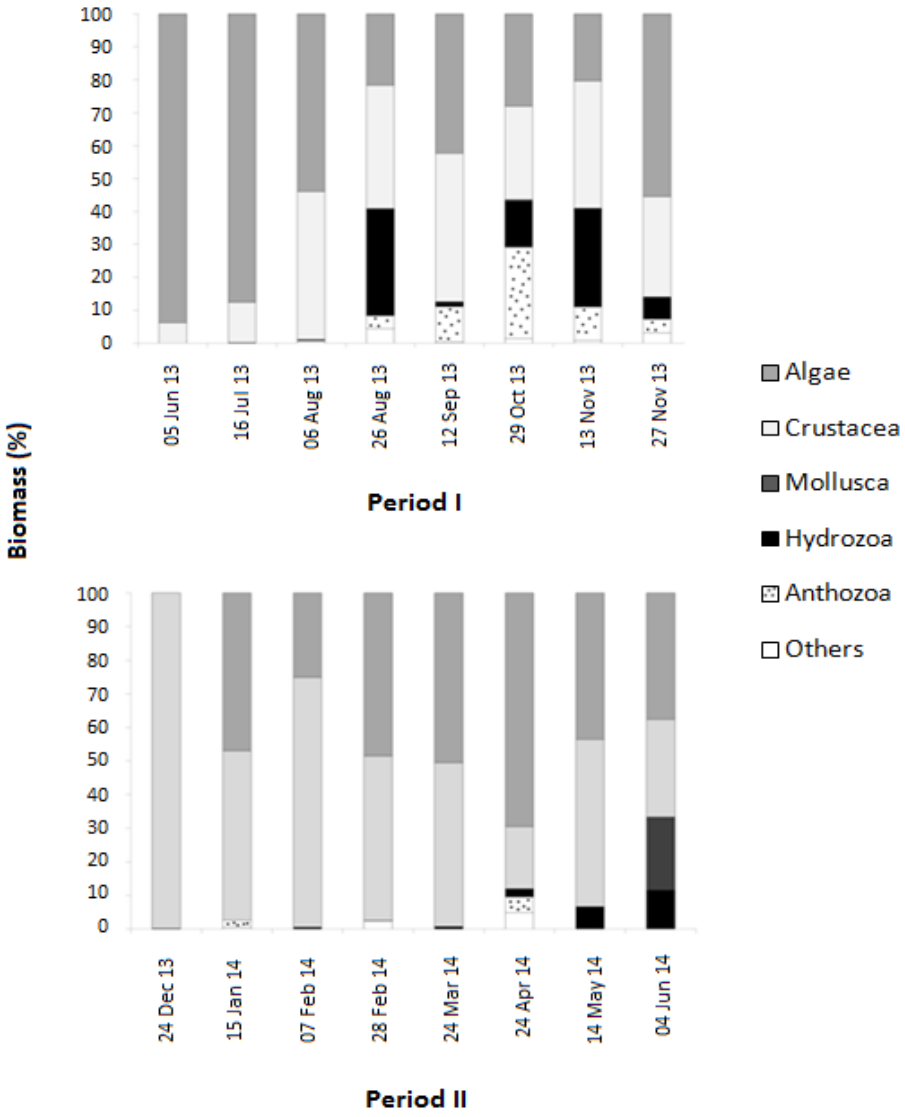

Figure 3. Biomass percentage by groups at at an aquaculture facility on the southern coast of Spain. Period I: May to November 2013; Period II: November 2013 to June 2014)

Biofouling community composition was significantly different between the two periods (F_1= 24.981, p= 0.001) (Fig. 4). Taxa that most contributed to that difference were anthozoans, nemertines and polychaetes, which were almost absent during Period II. During both periods, the first colonizers of cage net panels were microalgae and crustaceans. From May to November, polychaetes, nematodes and nemertines represented small quantities of biomass; by July, marked macroalgae colonization had occurred, together with molluscs and anthozoids. Hydroids were the latest colonizers, appearing in late August, and remaining as one of the predominant groups until the end of the sampling time. Nevertheless, in June 2013 a few small colonies of *Obelia dichotoma* and presumably *P. disticha* hydrorhiza were observed. During Period II, different hydroid species were observed among the earliest macrofouling settlers and during the complete study period, being a high percentage of biofouling cover, but representing just 3% of total biomass. Period II was characterised by the low representation of most previously identified groups (polychaetes, nemertines and anthozoids) (Table II). Molluscs were most abundant during the first period, being one of the most frequent groups, but did not obtain high biomass due to their small dimensions. In contrast, during the second period, numbers of this group were small, but with a high biomass.

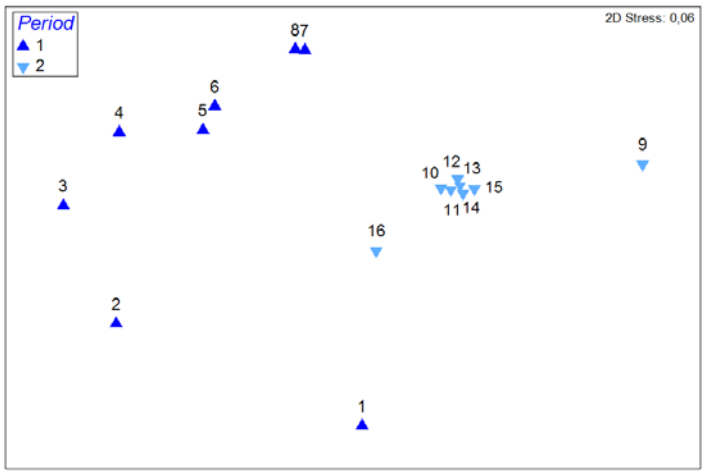

Figure 4. Non metric multi-dimensional scaling representing biofouling community composition at an aquaculture facility on the southern coast of Spain. Period I: May to November 2013; Period II: November 2013 to June 2014). Numbers on the figure represent time: 1- 8 period I; 9-16 period II).

Hydroid species

Seven species of colonial hydroids were identified on the experimental panels over the duration of the monitoring, 4 Anthothecata and 3 Leptothecata species described for the Mediterranean fauna (Bouillon et al. 2004) (Table II). Recorded species were *Pennaria disticha*, *Obelia dichotoma* and *Halecium pusillum* (Period I) and *Ectopleura larynx*, *Obelia dichotoma*, *Coryne prolifera*, *Sertularella ellisii* and *Eudendrium racemosum* (Period II). Colonial hydroid biomass differed significantly between periods ($F_1 = 28.818$, $p = 0.001$). Although the highest hydroid biomass was observed during the first period of monitoring, the highest species richness was recorded during the second. *Ectopleura larynx* and *P. disticha* were the most abundant hydroids in sampled panels.

Table II. Presence/absence of fouling organisms on finfish aquaculture netting throughout monitoring (Period I: May to November 2013, t1-t8; Period II: November 2013 to June 2014, t9- t16) on the southern coast of Spain.

	Period I								Period II							
	t 1	t 2	t 3	t 4	t 5	t 6	t 7	t 8	t 9	t 10	t 11	t 12	t 13	t 14	t 15	t 16
Crustacea																
S.O. Caprellida	X	X	X			X	X	X	X	X	X	X	X	X	X	X
S.O. Gammaridea	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
S.O. Tanaidomorpha		X	X	X	X	X	X	X								
Polychaeta																
Fam. Nereidae	X	X	X	X	X	X	X	X								
Fam. Syllidae		X				X										
Nematoda																
Non- identified		X	X	X	X	X	X	X	X	X	X			X	X	
Nemertina (n.i.)																
Non- identified		X	X	X	X	X	X	X		X						
Planaria (n.i.)																
Non- identified				X	X	X										
Mollusca																
Mytilus galloprovincialis		X	X	X	X	X		X							X	X
Musculus sp.			X	X	X											X
Arca sp.				X												
Irus sp.				X												
Doto sp.					X	X	X	X								
Fam. Hiatellidae																X
Chlamys sp.																X
Briozoa																
Or. Cyclostomatida		X	X													
Anthozoa																
Or. Actiniaria			X	X	X	X	X	X		X	X	X				
Hydrozoa																
Pennaria disticha				X	X	X	X	X	X							
Halecium pusillum					X	X	X	X								
Obelia dichotoma			X		X		X	X		X	X	X	X	X	X	X
Ectopleura larynx									X	X	X	X	X	X	X	X
Coryne eximia													X	X	X	X
Sertularella ellisii																X
Eudendrium racemosum																X
Echinodermata																
Arbacea sp.					X											
Algae																
Ceramium sp.		X	X	X	X	X	X									
Antithamnionella sp.		X	X	X	X	X	X	X								
Polysiphonia sp.		X	X	X	X	X					X	X	X	X	X	X
Giraudia sp.		X														
Hincksia sp.		X	X	X	X	X	X									
Jania sp.				X	X	X	X	X								
Laurencia sp.								X								
Trichleocarpa sp.							X	X			X	X				
Bryopsis sp. (2)							X	X					X	X	X	X
Chaetomorpha sp.													X	X	X	X
Cladophora sp.															X	X

Results for hydroid growth rate models are represented in Table III.

Table III. Hydroids growth rate models. M1: with factors interaction; M2: without interaction, at two experimental periods (Period I: May to November 2013; Period II: November 2013 to June 2014) at an aquaculture facility on the southern coast of Spain.

Period	Model	AIC_c	P	R^2
I	M1	420	$2.2 \cdot 10^{-16}$	0.86
I	M2	469	$2.2 \cdot 10^{-16}$	0.72
II	M1	473	$2.2 \cdot 10^{-16}$	0.92
II	M2	561	$2.2 \cdot 10^{-16}$	0.81

M1, with interaction between factors, was better than M2 without interaction in both experimental periods. A posteriori analysis of validation, checking for homogeneity, normality and independence of the selected model was carried out. Growth equations for the first period were:

O. dichotoma hydrocaulus length= 2.7 + 0.04 d

P. disticha hydrocaulus length = 7 + 0.29 d

As shown in Table IV, growth rates for both species were significantly different in Period I (F_1= 69.834, p= 4.932^{-12}); specifically, *P. disticha* growth was 0.29 mm d^{-1}, while *O. dichotoma* was 0.04 mm d^{-1} (Fig. 5a).

Growth equations for the second period were:

O. dichotoma hydrocaulus length= 1.7 + 0.04 d

E. larynx hydrocaulus length= 1.7 + 0.12 d

Growth rates were significantly different for the two species during the second period (F_1= 135.55, p=2.2^{-16}; Table IV). *E. larynx* growth was 0.12 mm d^{-1}, while *O. dichotoma* growth rate was 0.04 mm d^{-1} during the second period, which was the same rate as in Period I (Fig. 5b).

Table IV. Multiple Regression Linear Model to test differences in hydroid growth over time and by species (*Obelia dichotoma* vs. *Pennaria disticha* during Period I, and *Obelia dichotoma* vs. *Ectopleura larynx* during during Period II). Period I: May to November 2013; Period II: November 2013 to June 2014) at an aquaculture facility on the southern coast of Spain.

Period I	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Species	1	3099.9	3099.9	167.787	$< 2.2^{-16}$
Time	1	4001.7	4001.7	216.600	$< 2.2^{-16}$
Species · time	1	1290.2	1290.2	69.834	4.932^{-12}
Residuals	68	1256.3	18.5		
Period II					
Species	1	1894.25	1894.25	427.25	$< 2.2^{-16}$
Time	1	2706.73	2706.73	610.51	$< 2.2^{-16}$
Species · time	1	600.98	600.98	135.55	$< 2.2^{-16}$
Residuals	104	461.09	4.43		

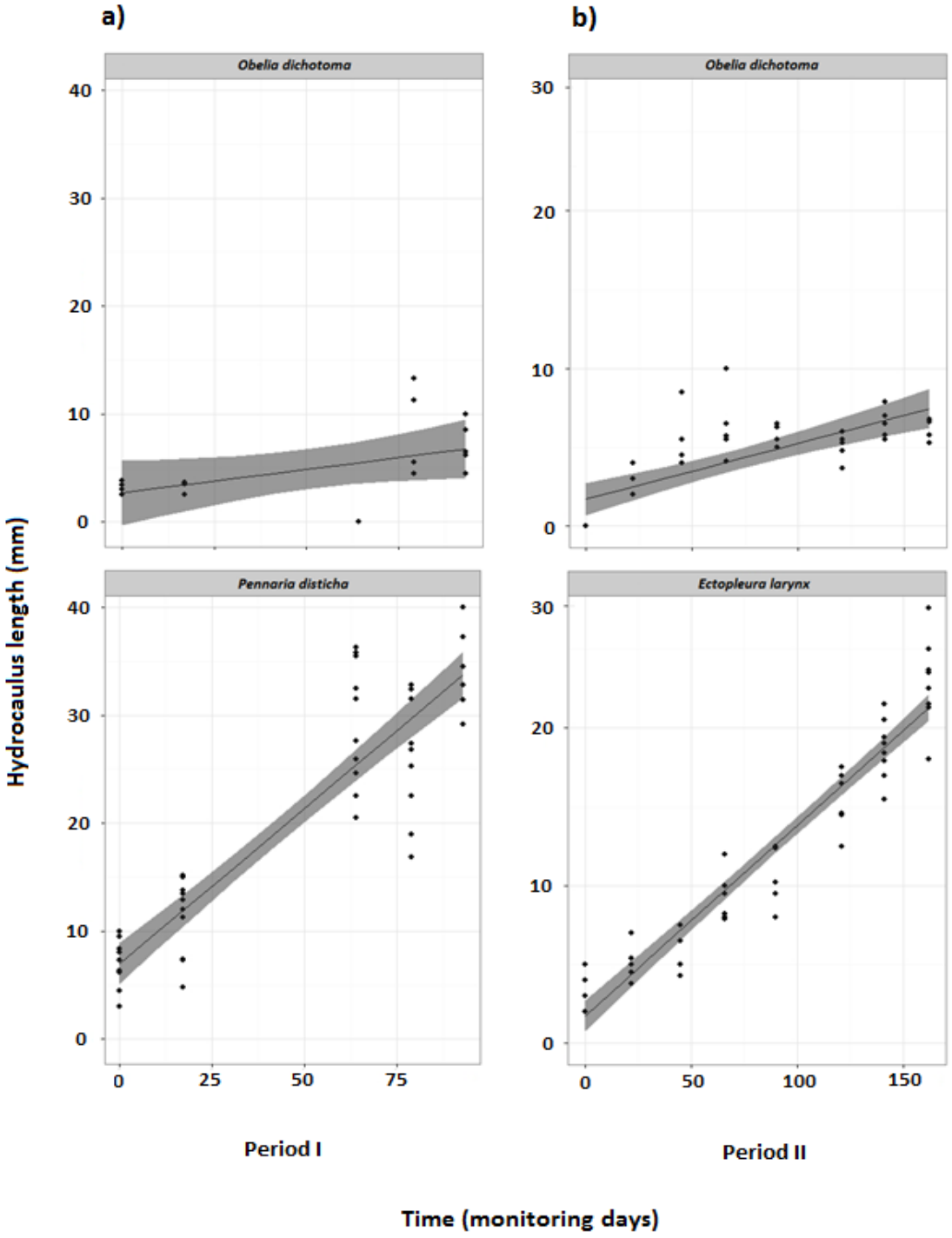

Figure 5. Variance of hydroid growth (as hydrocaulus length) over time (monitoring days) for *Obelia dichotoma* and *Pennaria disticha* during the first period (a) and *Obelia dichotoma* and *Ectopleura larynx* during the second period (b) at an aquaculture facility on the southern coast of Spain (Period I: May to November 2013; Period II: November 2013 to June 2014)

Reproductive stages of *P. disticha* were observed from September to November (Fig. 6). Significant differences among maturation stages in time were observed (F_3= 5.059, p= 0.01). Even if mature eumedusoids were observed attached to hydranths, their density in zooplankton samples was low (1.6 ± 0.4 ind·m^{-3} in October 2013) (Fig. 7a). Mature gonophores of *Ectopleura larynx* were recorded from March to May 2014. Significant differences in maturation were observed over time (F_2= 5.115, p= 0.009), although maturation stages from 0 to 4 were observed at all sampling times (Fig. 8). Zooplankton samples from March to June contained several free actinulae larva of *E. Larynx* (Fig. 7b). Likewise, hydromedusae of *O. dichotoma* were recorded during the entire monitoring period at low densities, but no developed gonothecae were observed in sampled colonies.

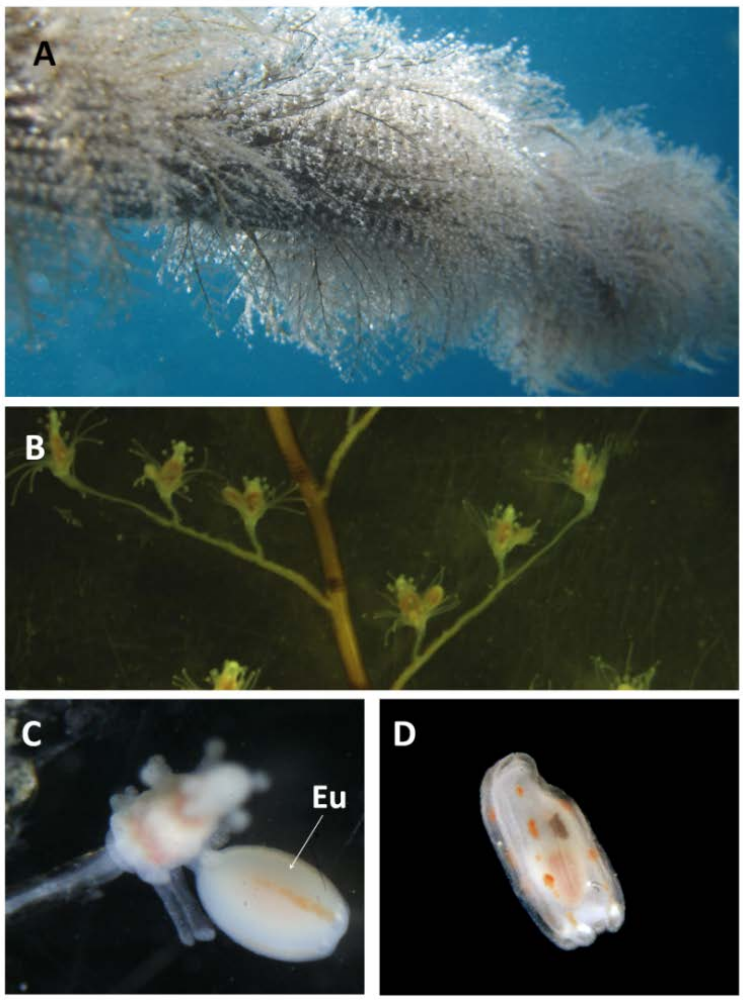

Figure 6. Growth and development of *Pennaria disticha* hydroid at an aquaculture facility on the southern coast of Spain: A) *P. disticha* colonies on a cage rope; B) Developing polyps of sampled panels; C) Hydranth with growing eumedusoid (Eu); D) Free eumedusoid after release.

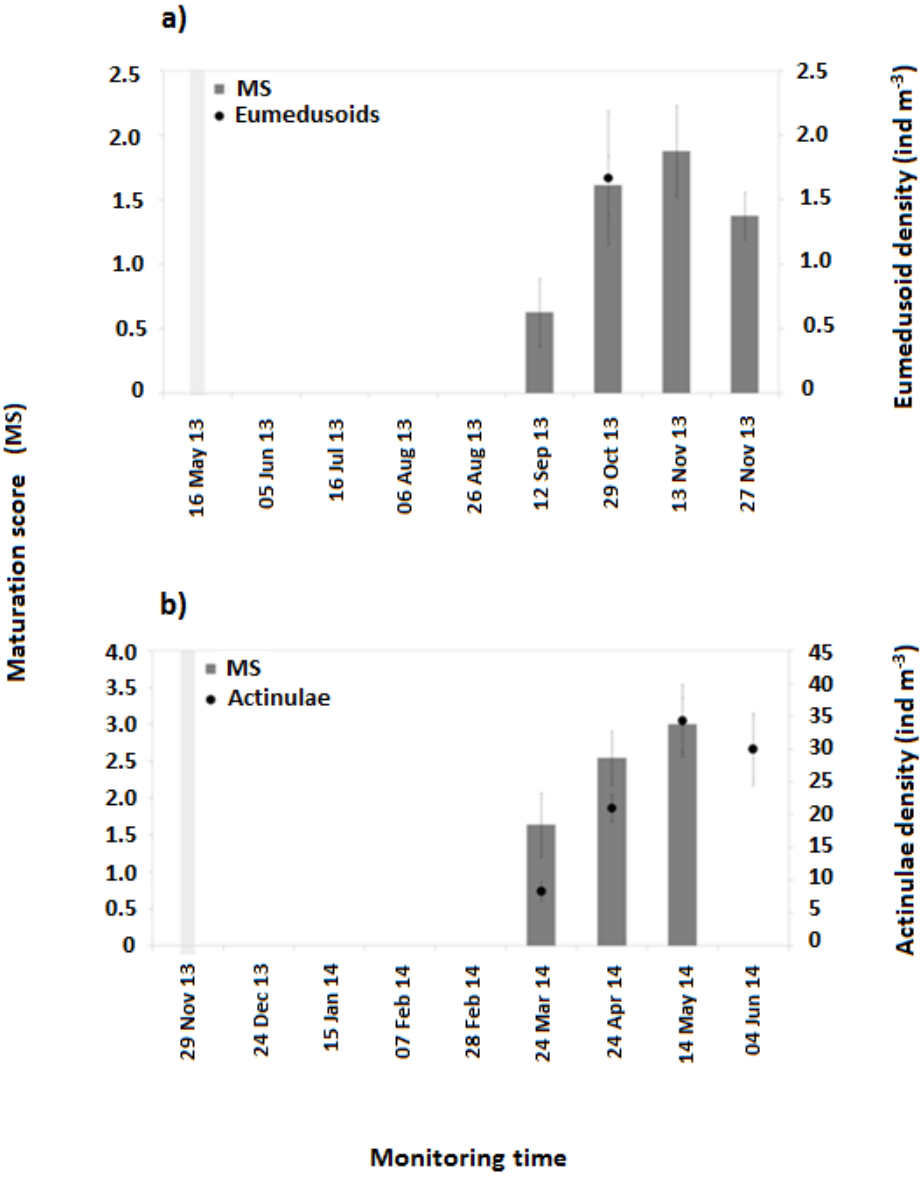

Figure 7. Maturation stage of hydroid colonies sexual reproductive structures and density of released *P. disticha* eumedusoids (a) and *E. larynx* actinulae larvae (b).

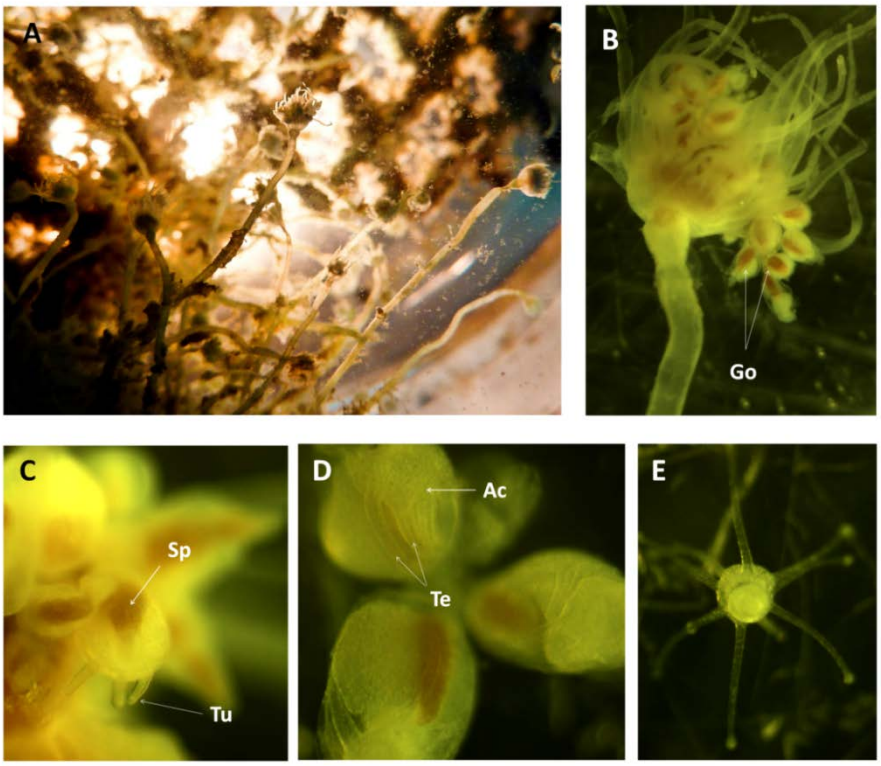

Figure 8. Growth and development of Ectopleura larynx hydroid: A) polyp colonies settled in monitoring panels; B) *E. larynx* mature polyp with gonophores (Go); C) Developing gonophore with visible spadix (Sp) and tubercules (Tu); D) Actinulae larvae (Ac) inside gonophore with developed tentacles (Te); E) Actinulae larvae after relase from gonophore.

Discussion

Biomass and community composition of biofouling on aquaculture cages changed with time and between study periods, showing intense seasonality in fouling colonization. The biofouling community was mainly dominated by algae and benthic crustaceans. Fry cages did not have serious problems with mollusc settlement, due to small individual size (low biomass), even when high densities of *Mytilus galloprovincialis* were observed.

Hydroids were present over the complete monitoring period. *O. dichotoma* (both stages, polyp and medusa) was the only hydroid species recorded over the time, while the other 6 species showed marked seasonality. The lack of hydroid colonizers at the beginning of the first period could be attributed to a "summer impoverishment", which is a typical seasonal pattern in the Mediterranean coasts during the summer period, when

hydrozoans disappear, leaving only the dormant basal stolon (Bavestrello et al. 2006; González-Duarte et al. 2013).

Negative consequences of interactions between gelatinous zooplankton and caged fish have been documented several times in the North Sea where blooms of various species caused farmed fish mortality events in the last ten years (Doyle et al. 2008; Purcell et al. 2013). These events are usually attributed to large scyphozoans, such as *Pelagia noctiluca*, but also small hydrozoans (*Phialella quadrata* and *Muggiaea atlantica*) have been responsible of mass fish mortalities in different mariculture facilities (Mitchell and Rodger 2011; Baxter et al. 2011), and hydroids, including *E. larynx* and *P. disticha* have been identified as potentially harmful fouling species to farmed fish. Usually, gelatinous zooplankton is not considered as possible harmful agent for aquaculture, and low levels of mortality and unspecific gill pathology with unknown cause, are generally attributed to waterborne irritant damage (Marcos-López et al. 2014). The misinformation of aquaculture facilities together with the inconspicuous character of these organisms, lead to underrate the potential damage that jellyfish could inflict on aquaculture facilities.

Ectopleura larynx and *P. disticha* were the most abundant hydroids in sampled panels. *P. disticha* represented significant biomass contribution to total fouling biomass. Colony growth was 0.29 mm·day^{-1}, reaching 40 mm in length at the end of monitoring. *P. disticha* growth rates were similar to growth rates of another benthonic organism in aquaculture as the Asian green mussel *Perna viridis,* which grows 0.23 mm d^{-1} (Rajagopal et al. 1998). *E. larynx* showed also quick growth, increasing length in 0.12 mm per d^{-1}, and *O. dichotoma* maintained equal growth rates during both periods (0.04 mm·d^{-1}).

Pennaria disticha and *E. larynx* have been previously related with injuries to human and fish health, through harmful stings to divers and serious skin and gill damage to caged fish (Baxter et al. 2012; Tezcan and Sarp 2013). Moreover, actinulae larvae of *E. larynx* were identified in zooplankton samples at high densities in Period II. According to Carl et al. 2010, species from the genus *Ectopleura* have high reproduction rates and under stress conditions can release higher number of actinulae, even if the polyp has been detached after net cleaning. In contrast, *P. disticha* effects on farmed fish health have not been

studied even though its colonies become large when settled on aquaculture structures with specific environmental conditions.

To our knowledge, the present study is the first to provide an analysis focused on hydroid community composition in Mediterranean aquaculture cages, as well as their reproductive periods. April – May and October – November are periods in which fingerlings of 15-20 g start growth process in sea cages. These periods coincide with seasonal plankton blooms and with hydroid reproductive periods. Consequently, identification of potentially harmful species and understanding their growth and reproduction is fundamental to look for efficient solutions to hydrozoan biofouling colonization, creating new protocols such as new periods for fry introduction in sea cages or different times to clean the nets of fouling organisms.

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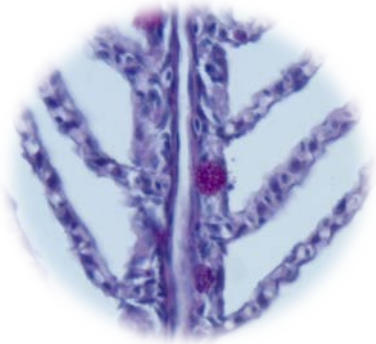

Chapter 4

Jellyfish stings trigger gill disorders and increased mortality in farmed *Sparus aurata* (Linnaeus, 1758) in the Mediterranean Sea

Bosch-Belmar M., M'Rabet C., Dhaouadi R., Chalghaf M., Daly Yahia M. N., Fuentes V., Piraino S., Kéfi-Daly Yahia O. **(2016, PlosOne 11 (4): e0154239)**

Introduction

In recent years, negative interactions between jellyfish blooms (JB) and marine finfish aquaculture have been increasingly reported including mass fish mortalities with severe economic impacts on aquaculture companies (Purcell et al. 2007; Rodger et al. 2011). Jellyfish can enter fish cages either intact or broken up into tentacles and other body fragments pushed by currents and waves washing in through the net cages (Baxter et al. 2011b; Mitchell et al. 2012). Several species of cnidarian jellyfish have been reported to affect marine farmed fish of inducing skin lesions and gill damage caused by nematocyst discharge and venom injection usually leading to local inflammatory response, cell toxicity and histopathology (Helmholz et al. 2010; Rodger et al. 2011; Baxter et al. 2011b). Prolonged nematocyst discharges in fish tissues may often lead to secondary bacterial infections and associated systemic reactions, including respiratory and osmoregulatory distress, altered behaviour, and death (Bruno and Ellis 1985; Seaton

1989; Rodger et al. 2011; Baxter et al. 2011c). In particular, gills have vital roles, being the main site of gas exchange, osmoregulation, acid-base balance, and excretion of nitrogen compounds (Marques dos Santos et al. 2012). Gill disorders have become one of the most serious causes of mortality in marine farmed salmon in Northern Europe, with average losses of 12 % per year (Baxter et al. 2011c).

The scyphomedusa *Pelagia noctiluca* (Forsskål, 1775) is one of the most common stinging jellyfish species across the Eastern Atlantic and the Mediterranean Sea, producing major outbreaks with subsequently highly negative impacts on human activities, including caged finfish aquaculture (CIESM 2001; Canepa et al. 2014). On the Mediterranean Spanish coast, *P. noctiluca* is responsible for gill damage on the marine farmed fish *Dicentrarchus labrax,* leading to reduction of fish growth rate and even death (Baxter et al. 2011a). Additional fish mortality events related to *P. noctiluca* abundance were also recorded in Tunisian facilities (unpublished data). Massive outbreaks of mauve stingers were documented in the Eastern Atlantic (Irish Sea) to kill several hundred thousands of Atlantic salmons in 2007 (Doyle et al. 2008), 2013 (Raffaele 2013; Marcos-López et al. 2014) and again in 2014 (Berwald 2014; FIS 2014). In the same region, a bloom of moon jellyfish *Aurelia aurita* was responsible for a significant salmon mortality in summer 2010 (Mitchell et al. 2013; Purcell et al. 2013). Also tiny jellyfish were identified as potentially harmful species for aquaculture facilities, such as the hydromedusae *Solmaris corona* and *Phialella quadrata* (Baxter et al. 2011b), and the siphonophore *Muggiaea atlantica* that caused the death of > 100,000 farmed fish in Norway (Fosså et al. 2003).

Previous studies demonstrated also that some jellyfish species - such as *P. quadrata* and *P. noctiluca* - can act as vectors of *Tenacibaculum maritimum*, the causative agent of tenacibaculosis, a major bacterial disease affecting fish mariculture worldwide, which heavily exacerbates the impacts of jellyfish sting envenomations (Toranzo et al. 2005; Avendaño-Herrera et al. 2006; Ferguson et al. 2010; Delannoy et al. 2011).

However, information on jellyfish impacts on fish aquaculture is mainly restricted to severe killing events. Also, research on mechanisms and patterns of jellyfish impacts on fish aquaculture is still limited. As a result, impacts of low to medium jellyfish density are usually neglected or underestimated, whereas low incidence of unspecific pathologies or

mortalities are generally labelled as unknown "water borne irritant damage" (Marcos-López et al. 2014). Likewise, apart from trophic interactions between jellyfish and fish larvae and eggs, the impact of jellyfish envenomation on wild fish health is still unknown. Data from laboratory experiments may therefore provide insights on the potential, so far neglected consequences of high density jellyfish blooms in natural fish populations.

Due to its high adaptability to intensive rearing conditions, the gilthead sea bream *Sparus aurata* (Linnaeus, 1758) represents one of the most suitable species for cultivation in ponds and marine cages, leading to the most important fish production in the Mediterranean Sea, reaching near 160.000 tonnes in 2012 (Colloca and Cerasi 2015). In parallel, overproduction led to cutbacks in market price, calling for further reduction of production costs. To increase knowledge on impacts of gelatinous plankton blooms on Mediterranean caged fish species and support early monitoring of risks for aquaculture production, an experimental assay was set up to assess [I] the potential histopathological damage that *P. noctiluca* jellyfish shreds produce on gills of cultured *S. aurata*, [II] the impacts of different jellyfish densities on cultured fish health, and [III] the histological evolution of gill lesions over time following initial jellyfish sting treatment.

Materials and methods

This study was performed in accordance with the European Commission Directive 2010/63/EU. The experimental protocol was designed to comply with the European policy of the "3 Rs" (Reduce, Refine, and Replace) in aquatic animal experimentation and was approved by the Institut Supérieur de Pêche et d'Aquaculture de Bizerte (Research unit 05/ur/11-15), which is under the double supervision of the Tunisians Ministry for Agriculture and the Hydraulic resources, and of the Ministry for Higher education and the Scientific Research and Technology.

The maintenance of animals during the experiment as well as the euthanasia procedure was monitored and carried out by trained and competent staff, in order to minimise animals' suffering.

Animals' maintenance and experimental setup

A total number of 136 *Sparus aurata* adult fish (mean weight of 200 ± 19.23 g) were obtained from "Tunisian Teboulba Fish" aquaculture facility and transported to the Institut Supérieur de Péche et d'Aquaculture de Bizerte, Tunisia (ISPA). Fish were homogeneously distributed in 8 circular tanks of 300-L each (fish stocking density of around 9 kg m^{-3}) and allowed to acclimate for one week before starting the experiment. All tanks were supplied by a continuous flow (renewal rate of 23 l h^{-1}) of double-filtered (5-µm, 1-µm mesh) seawater (FSW). The water circulation flow was kept at natural sea temperature of 15.5 ± 1.0 °C and 36.8 ± 0.3 salinity) with aeration to keep dissolved oxygen at 100 % saturation. Throughout the experiment, the fish were daily fed with standard commercial pellets (Skretting S.A.) and maintained under a natural photoperiod (12 h light, 12 h dark).

Jellyfish (4.5 ± 0.9 cm bell diameter) were collected by hand net the day before the start of the experiment from the Channel of Bizerte (Tunisia) and maintained in 25 litres buckets with FSW and at low density for one day. *Pelagia noctiluca* jellyfish is not an endangered or protected species. Specimens from Bizerte gulf were collected without the need of a permit because sampling was never conducted in a restricted marine area. To simulate a realistic encounter between jellyfish that had been pressed by currents against aquaculture cages and cultured fish, jellyfish were chopped into small (≥ 1 cm) pieces immediately prior to the start of the jellyfish exposure. The four treatment groups consisted of two control tanks (without jellyfish) and six tanks with chopped *P. noctiluca* at low (LJ), medium (MJ), and high jellyfish densities (HJ): 10, 25 and 50 jellyfish m^{-3} (approximately equivalent to 350 g, 875 g and 1750 g jellyfish biomass, respectively). These densities were predetermined to reproduce a range of different jellyfish concentrations observed during *P. noctiluca* bloom periods in Tunisian waters and Sicily Channel (unpublished observations). A 1-mm stainless steel mesh was placed at the outlet of each tank preventing jellyfish pieces to spill out the experimental tanks.

The experiment began when jellyfish pieces were placed simultaneously in all treatment tanks with fish. The maximum fish-jellyfish interaction lasted 8 h; after that, all jellyfish

pieces were removed using a 200-µm mesh hand net. The exposure time to jellyfish tissue of 8 h was used to represent the minimum night time with *P. noctiluca* jellyfish in surface waters, following sunset and the diel vertical migration of their crustacean prey (Franqueville 1971; Axiak 1984; Ferraris et al. 2012).

Fish health was monitored nine times during the experiment: shortly before jellyfish incorporation to the fish tanks (0 h), during fish-jellyfish contact (3h), one hour after the removal of the jellyfish (9h), and six later times, 24 and 48h; 1, 2, 3 and 4wk, respectively before the end of the experiment at 4 weeks. At the highest jellyfish density sampling was not carried out at 24 h and 4 weeks because of the shortage of experimental fish. At each sampling time, 4 fish were randomly sampled from each treatment group (two per tank) , anesthetised and then killed according to the current animal care rules using a lethal dose of UNICAINE 2% (lidocaine-HCl 500 ppm) (Park et al. 2011). Immediately after death, which occurred within 2-3 minutes of anaesthetic application, fish were weighed and measured, and their skin and gills visually examined for gross pathology, such as scale loss, excess mucus, pale gill filaments, swelling, necrosis and the presence of macro-parasites (Mitchell and Rodger 2011). Two gill arches were excised from each fish and immediately preserved in 10% neutral buffered formalin for histological analysis. Tissues then were embedded in paraffin, cut by microtome into 2-5 µm sections and stained following a standard haematoxylin-eosin protocol. For each gill arch, several sections were examined microscopically at 100X and 400X magnifications.

Gill score protocol

Interpretation of the gill damage was based on a recently developed gill histopathology scoring system (Baxter et al. 2011a; Mitchell et al. 2012), rating the potential damage on each gill sample by a total score ranging from 0 to 24, obtained by summation of partial scores assigned to different primary and secondary criteria. Primary parameters were related to 3 specific pathologies: epithelial hyperplasia (increased cell production), lamellar fusion, and cellular anomalies (degeneration, necrosis and sloughing). According to the presence, extent and severity of those pathologies, primary scores ranged from 0

to 3. In addition, a 0 or 1 score was attributed to the absence or presence of each of the following secondary parameters: hypertrophy, oedema, eosinophilic granular cells, inflammation, circulatory damage, congestion, bacterial pathogens and parasitic pathogens. The total score assigned for primary and secondary parameters, allowed classification of fish gill damage according to four cumulative score ranges: 0–3 = no significant pathology, 4–6 = mild gill pathology of minor clinical significance, 7–9 = moderate gill pathology of clinical significance, ≥ 10 = severe gill pathology of high clinical significance.

Statistical analysis

A Shapiro-Wilk test indicated that the assumptions of normality were violated ($p < 0.05$, SPSS v. 20.0); therefore, differences among treatments and among sampling weeks were tested using the non-parametric one-way Kruskal-Wallis test (SPSS v.20.0). Significant results were further tested by pairwise post-hoc comparisons (Mann-Whitney U test, SPSS v. 20.0), adjusted for type I error, and Similarity percentages analysis, SIMPER (PRIMER 6).

Results

Gills from the control fish group without jellyfish retained a normal morphology throughout the experiment. Each gill arch supported many distinct and regular filaments arranged perpendicularly in two rows and without significant lesions. In contrast, gross pathology in fish exposed to jellyfish pieces was observed throughout the experiment (Fig. 1), with the extent and intensity of gill damage increasing with time and jellyfish density (Fig. 2).

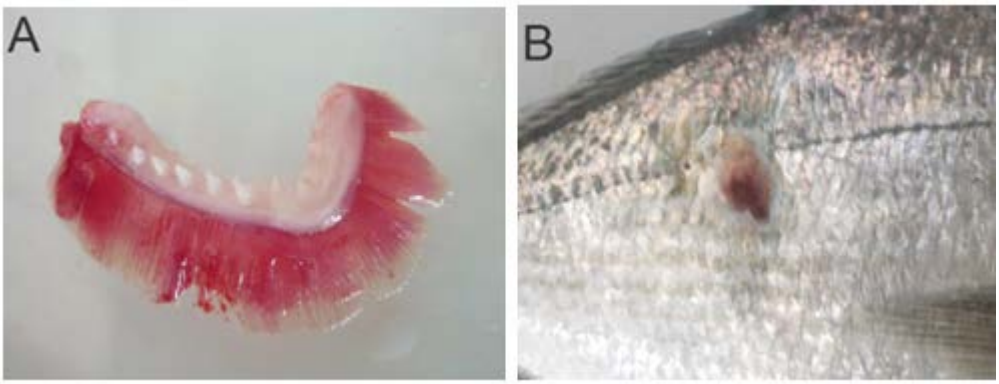

Figure 1. External lesions on *Sparus aurata* due to *Pelagia noctiluca* jellyfish exposure: A. abrasion, haemorrhage, depigmentation and increased thickness of lamellar filaments of a fish from the high jellyfish density group 24 h after exposure to jellyfish; B. wound with necrotic tissue on the flank of *Sparus aurata* fish from the medium density group 2 weeks after exposure to jellyfish.

At 3 h after initial contact with jellyfish pieces, fish gills already showed abrasion of lamellar filaments (Fig. 1A). After 24 h from the exposure to jellyfish, depigmentation, increasing thickness of lamellar filaments and haemorrhage in gill tissue were also recorded. Mild epitheliocystis (Hoffman et al. 1969; Nowak and LaPatra 2006) was observed in control and treated fish through the identification of spherical cysts that were circumscribed by an eosinophilic hyaline capsule. One day before the start of the experiment (24 h after the exposure to jellyfish), snout irritation, scale loss on the flanks and damage in the caudal and dorsal fins and operculum were also observed in fish in the medium and high jellyfish density groups (Fig. 1B). Respiratory distress, jumping and swimming near the water surface were also observed for some treated fish throughout

the exposure period to jellyfish at different jellyfish densities. A slight trend of weight reduction was observed in treated fish, possibly due to the ceased feeding behaviour observed through the experiment, but not significant statistical differences were found in weight or length analysis.

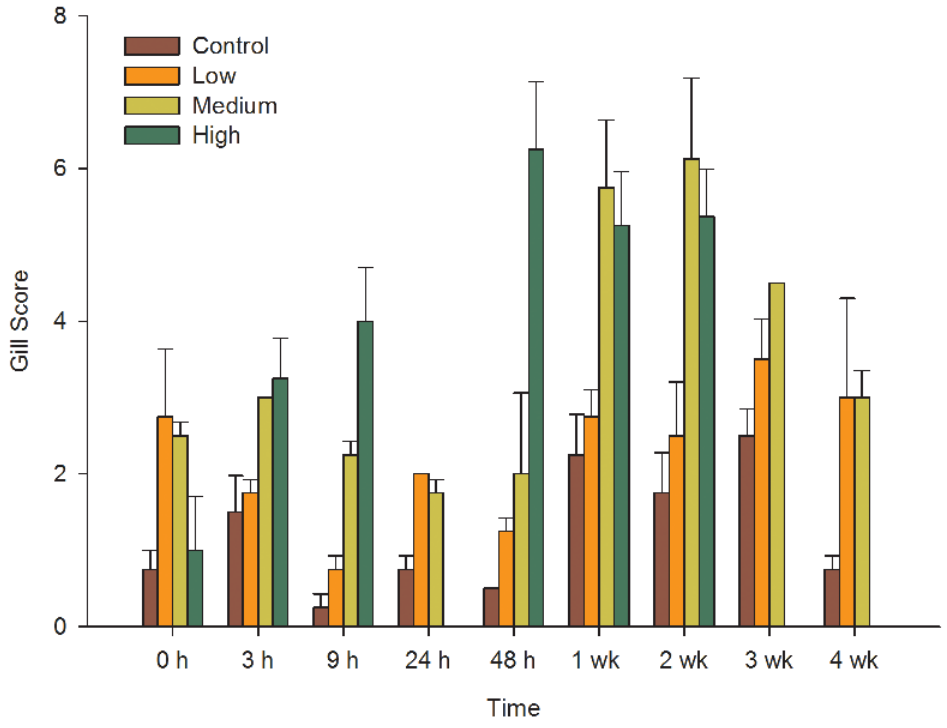

Figure 2. Average gill scores of treatment groups (control and low, medium and high *Pelagia noctiluca* jellyfish densities) before (0 h) and at different times after *Sparus aurata* exposure to jellyfish (vertical bars denote standard error).

The histopathological analysis showed that the lowest gill damage score was in the control group, characterised by low levels of lamellar hyperplasia and occasional fusion, a background level of pathology typical of marine-farmed fish (Baxter et al. 2011c). Gill scores from the control group were significantly different (lower) than all the groups with jellyfish (U_1= 25.267, p= 0.001). Gill scores also differed significantly among the groups treated with jellyfish (U_2= 7.050, p= 0.029). The gill scores in the LJ density group showed no significant differences throughout the experiment (U_8= 12.604, p = 0.126), with average scores of 2.25 ± 0.9 (SE). For the MJ density group, significant gill lesions were

observed 1 week after the start of the experiment (U_1= 4.86, p-value 0.027), with scores peaking after 2 weeks (gill score 6 ± 1.5 SE). Significant gill damage was observed immediately in the HJ density group, only 3h after the exposure to jellyfish began (U_1= 4.513, p-value= 0.034). Those high scores continued over time with a peak after 48h (6 ± 1.3 SE) (Fig.2)

Fish mortalities began during the second week of sampling in the HJ density group, after the peak in gill damage scores, with a total of 6 dead fish at the end of the experiment. Fish showed excessive mucus production and pale gills, hyperplasia in more than 50% of the tissue, severe lamellar fusion, desquamation, necrotic patches, lamellar congestion and lamellar oedema in some areas of the gills. In the MJ density group, gill scores decreased during the third and fourth week of sampling, mainly because of reduction in the percentages of hyperplasia and cellular anomalies. By contrast, fish from the LJ density group presented mild damage during the experiment, principally represented by hyperplasia and lamellar fusion (Fig. 3 and Table I).

The gill scores for the experimental treatment groups ranged from 1 to 9 over the entire experiment, with most fish displaying moderate lesions considered to be of clinical significance. The SIMPER analysis showed that lamellar fusion and hyperplasia were the most common lesions in all treated groups (Fig. 4). Also, a severe inflammatory response was noted beginning at 9h after the exposure to jellyfish. The severity of gill damages was directly proportional to jellyfish density, with increasing cellular anomalies over time.

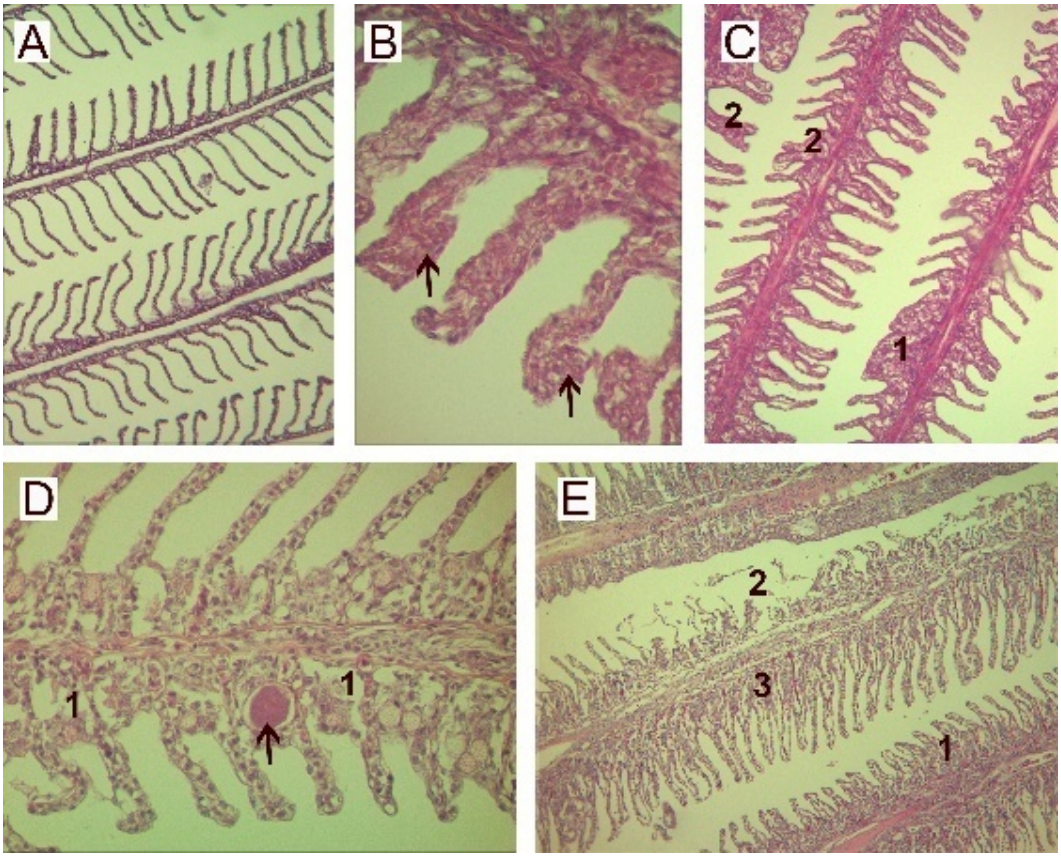

Figure 3. A. Healthy fish gill from the control group (0h) (100x); B-E. pathology in fish gills from the treatment groups: B. black arrows indicate lamellar hyperplasia on fish gill from the low jellyfish density group at 9h (400x); C. lamellar hyperplasia (1) and fusion (2) from the medium jellyfish density group after 1 week (100x); D. epitheliocystis (black arrow) and lamellar oedema (1) from the medium jellyfish density group after 3 weeks (400x); E. hyperplasia of the epithelium of the primary lamellae (1), necrosis focal of secondary lamellae (2) and circulatory disturbances (3) from the high jellyfish density group after 48h (100x).

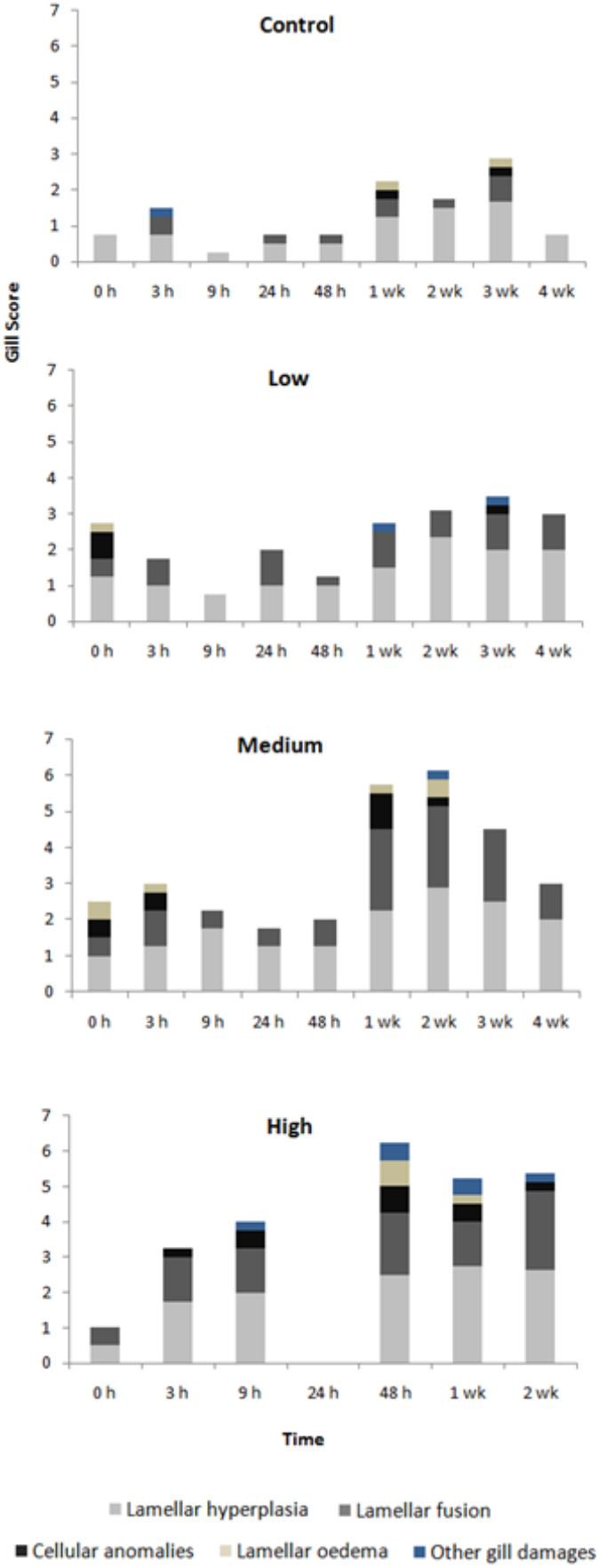

Figure 4. Kind of gill injuries observed in poisoning fish at each treatment group (control, low, medium and high jellyfish densities) over experimental time.

Table I. Histopatholical gill damage of experimental groups (control and low, medium and high jellyfish density groups) over time. MLH: Mild lamellar hyperplasia; MLF: Mild lamellar fusion; MoLH: Moderate lamellar hyperplasia; MoLF: Moderate lamellar fusion; MCA: Mild cellular anomalies; MoCA: Moderate cellular anomalies; SLH: Severe lamellar hyperplasia; MCO: Mild cellular oedema; FM: Fish mortality; (NA): data not available; (---): Non significant gill damage. Colours indicate the severity of gill damage. ☐: mild injuries; ☐: medium level of injuries; ☐ and ☐: high level of gill damage.

Treatment	p-value	0 h	3 h	9 h	24 h	48h	1 week	2 weeks	3 weeks	4 weeks
Control	$p > 0.05$	MLH	MLH	(---)	(---)	(---)	MLH	MLH	MLH MLF	MLH
Low	$p > 0.05$	MLH	MLH MLF	MLH	MLH MLF	MLH	MLH MLF	MoLH MLF	MoLH MLF	MoLH MLF
Medium	0.043*	MH	MLH MLF	MoLH	MLH	MLH MLF	SLH MoLF MCA	SLH MoLF	MoLH MoLF	MoLH MLF
High	0.041*	(---)	MoLH MLF	MoLH MLF MCA	(NA)	SLH MoLF MCO MCA	SLH MoLF MoCA	SLH MoLF FM	FM	

Discussion

Frequency of occurrence and abundance of *P.noctiluca* vary across the Mediterranean, but dense populations can be recorded most of the year at several coastal localities, such as the channel of Bizerte (Tunisia) and the Strait of Messina (Italy) (Rosa et al. 2013; Canepa et al. 2014; Milisenda et al. 2014). Our laboratory experiments simulated the potential consequences of blooms of the scyphomedusa *P. noctiluca* on finfish aquaculture farms, showing that jellyfish stings can severely affect on caged *S. aurata* fish by significant gill damage shortly after contact with jellyfish tissues and subsequent deterioration on fish health.

Comparable gill damage was observed previously in farmed salmon (*Salmo salar*) during blooms of *P. noctiluca* and *Aurelia aurita* scyphomedusae in northern Europe (Baxter et al. 2011c; Purcell et al. 2013). The first experimental challenge trial between fish in culture and jellyfish by exposed juvenile *S. salar* to realistic *A. aurita* jellyfish bloom densities showed significant and increasing gill damage starting 24 h after the initial contact (Baxter et al. 2011c).

Here we investigated the intensity of gill damage on cultured sea bream at increasing *P. noctiluca* densities. At low jellyfish density (up to 10 jellyfish m^{-3}) mild damages on fish gills were observed. Conversely, at higher jellyfish concentrations (≥ 25 jellyfish m^{-3}) impacts ranged from moderate damage, leading to potential effects on the fish metabolism, to more severe consequences including death, due to high levels of lesions and respiratory distress (Rodger et al. 2011).

Three weeks after the initial exposure to jellyfish, fish from the medium density group showed early signs of tissue repair in the gills. Recovery was characterized by significant decreases in the percentages of lamellar hyperplasia and fusion, in observed inflammatory reactions, and disappearance of cellular anomalies. At last, recovery of tissue integrity was observed in fish in the MJ density group, whereas fish from HJ density died 2 - 3 weeks after exposure to jellyfish. Exposure to HJ density led to intense and increasing gill damage, eventually impairing homeostatic mechanisms and adaptive

physiological responses (Ingerslev et al. 2010). Non bacterial infection of *Tenacibaculum* sp. was confirmed, due to the absence of filamentous bacterial mats on the necrotic patches (Powell et al. 2005). Overall, these results indicate that even short exposure to jellyfish can result in significant gill damage in marine-farmed fish, with potential increase in extent and severity of damage even when jellyfish are no longer present.

Jellyfish potential effects on marine wild fish populations might not be negligible as well. Previous research on fish-jellyfish interactions are mostly focused on jellyfish predation on fish or, conversely, the use of jellyfish biomasses by medusivorous fish as temporary or exclusive food source (Ates 1988; Purcell and Arai 2001; Milisenda et al. 2014; D'Ambra et al. 2015). The outcome of jellyfish interactions with fish populations depends on several factors affecting the probability of encounters, including water temperature, dissolved oxygen, and the size and density of predators and prey (He et al. 2015). For several jellyfish species, bloom density may reach extremely high values. *Pelagia noctiluca* in the Mediterranean Sea occurs in large swarms reaching densities over 100 medusae m^{-3} for prolonged periods (up to weeks), with temporary aggregations caused by wind, currents, coastal geomorphology and jellyfish behaviour containing up to 600 medusae m^{-3} (Zavodnik 1987; Malej 1989). These values largely exceed the experimental density values used in our fish-jellyfish interaction experiments (10, 25, 50 medusae m^{-3}). Furthermore, shortly after sexual reproduction - in springtime - large swarms of ephyrae and juvenile jellyfish are regularly encountered in the Southern Thyrrenian Sea (Aeolian islands), with much higher densities, up to several thousands of individuals m^{-3}; (Piraino, pers. observation; see also https://www.youtube.com/watch?v=D9Htbe4LGnU). Temporary paramount densities may therefore represent a key threat affecting the physiological integrity and health of fish living in sheltered areas where extremely high jellyfish aggregations occur, such as bays or fjords (with records up to 1000 *Periphylla periphylla* medusae m^{-3} (Youngbluth and Båmstedt 2001; Sornes et al. 2007)).

Thus, our experiments may provide ground to hypothesise that in an enclosed area, where jellyfish dense outbreaks occur the chance of contact between fish and jellyfish is highly increased and may cause severe skin or gills injuries which eventually develop into secondary bacterial infection and fish health issues. In parallel, increased gelatinous

biomass in enclosed or semi-enclosed areas may lead to local episodes of hypoxia, with additional severe consequences on fish health, or may affect fish distribution by displacement in areas with low jellyfish aggregations. Overall, we can speculate that the rise of both temperature and jellyfish numbers in a global change scenario may exacerbate negative impacts not only on farmed fish, but also on wild fish populations. Further investigations are required to clarify the impact of jellyfish blooms on fish populations and cultured stocks around the world.

The consequences of episodes of jellyfish proliferation can be of high importance for aquaculture, considering they could affect not only fish health, but also the growth and quality of caged fish (Mitchell and Rodger 2011; Rodger et al. 2011). The sudden and unpredictable nature of jellyfish blooms hinders the implementation of preventive measures against their negative effects in aquaculture. Because of this, the development and implementation of swift mitigation procedures are crucial and must be rooted in knowledge of the type and extent of physical damage caused by jellyfish. Even a low density of *P. noctiluca* jellyfish could be detrimental to the health of caged fish, causing minor but significant gill lesions, which may progress over time and be worsened by bacterial infections. Investigation of the different effects of *P. noctiluca* blooms will enable estimation of the response time required by aquaculture facilities to undertake appropriate countermeasures that could differ in magnitude according to the damage level. Due to the dramatic recent and projected future growth of the aquaculture sector and the increased frequency of jellyfish blooms in coastal waters, negative interactions between stinging jellyfish and caged finfish is expected to become a substantial problem with high economic losses (Purcell et al. 2013).

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Chapter 5

Concurrent environmental stressors and jellyfish stings impair caged European sea bass (*Dicentrarchus labrax*) physiological performances

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Introduction

Human activities are transforming coastal and marine ecosystems at local, regional, and global scales, exposing both individual organisms and biological communities to dramatic environmental changes by a complex array of interacting stressors (Sanderson et al. 2002; McBryan et al. 2013). The current trend of induced anthropogenic environmental changes includes increasing sea water temperatures, frequencies of hypoxia episodes, and ocean acidification (Urbina et al. 2012; Byrne and Przeslawski 2013). Concurrently, zooplankton communities respond to anthropogenic- and climate-induced changes by strong variations in their spatial distribution, structure and function (Molinero et al. 2008; Chiba et al. 2009). Jellyfish represent one of the components of plankton that seem to be responding positively to the ongoing changes. They are likely to affect the food web structure by their high consumption rates, fast growth and reproduction rates, and wide tolerance to ecosystem changes (Purcell et al. 2007; Boero et al. 2008; Gibbons and Richardson 2013). Recent analyses of jellyfish population

dynamics in Mediterranean coastal zones suggested increasing abundance and frequency of bloom formation (Kogovšek et al. 2010; Licandro et al. 2010; Brotz et al. 2012; Condon et al. 2013). Global changes such as overfishing, eutrophication and ocean warming have been proposed as mechanisms leading to jellyfish increases in many coastal waters worldwide, including the Mediterranean Sea (Mills 2001; Parsons and Lalli 2002; Purcell et al. 2007; Licandro et al. 2010; Canepa et al. 2014). These factors are causing severe negative impacts on human economic activities, such as tourism, fisheries, and aquaculture (CIESM 2001; Purcell et al. 2007; Boero 2013; Kontogianni and Emmanouilides 2014).

Aquaculture is an important source of income for local societies and sustains over 40% of global fish production and mariculture supports nearly 30 % (US $ 23.5 billion) of the total value of farmed finfish species (FAO 2014). Interactions between jellyfish and marine caged fish have been recorded on several occasions in recent years, leading to severe fish mass mortality (Rodger et al. 2011). Jellyfish can enter fish cages either intact or fragmented, as tentacles and other body parts (e.g. oral arms), washed by currents and waves against the mesh of cage nets (Rodger et al. 2011; Baxter et al. 2011b; Baxter et al. 2011a). Overall, more than 400,000 salmon were killed in Irish marine aquaculture facilities in recurrent blooms of the scyphomedusa *Pelagia noctiluca* in 2007, 2013 and 2014 (Doyle et al. 2008; FIS 2014; Marcos-López et al. 2014). The moon jellyfish *Aurelia aurita*, and the hydrozoans *Muggiaea atlantica* and *Phialella quadrata* were also involved in different farmed fish mortalities, and together with *P. noctiluca*,, were identified as potentially harmful species for aquaculture facilities (Baxter et al. 2011a). In addition, jellyfish can act as vectors of the bacterium *Tenacibaculum maritimum*, exacerbating fish gill injuries (Avendaño-Herrera et al. 2006). Beyond these few studies, limited information is available on how jellyfish affect fish health, the biological mechanisms underlying fish mortalities, or estimates of potential economic losses (Rodger et al. 2011). Only a few studies described significant fish injuries caused by the discharge of cnidocytes (specialized cnidarian stinging cells) in fish tissues (skin, gills) leading to envenomation and cellular damage (Baxter et al. 2011b; Bosch-Belmar et al. 2014).

Temperature and dissolved oxygen concentration in the water column are crucial for the development and performance of aquatic organisms through direct effects on their metabolic rates (Nilsson 2010; Sarà et al. 2014). Most fish adapt their physiological responses to sustain their metabolic rates when exposed to temperature changes or decreased dissolved oxygen levels (Pörtner 2010; Urbina et al. 2012); however, additional external factors may impair acclimation processes.

In this framework, we investigated the sensitivity of fish to the co-occurrence of environmental stressors (increased temperature) and jellyfish stings to understand the impact of jellyfish blooms on caged finfish in a global warming scenario. Experiments were designed to test the combined effects of temperature ("temperature treatment") and prolonged jellyfish contact ("jellyfish treatment") on metabolic performances (MO_2 and critical PO_2) and tissue damage on fish gills over the time. We used the jellyfish *Pelagia noctiluca* (Forsskål, 1775), the strongest stinging and most abundant scyphozoan species in the Mediterranean Sea and Eastern North Atlantic, and juveniles of *Dicentrarchus labrax* (Linnaeus, 1758), one of the most common fish species in Mediterranean marine aquaculture. This study presents important eco-physiological data to the overall fish mariculture sector in jellyfish-affected coastal areas and also for the scientific community working on the global change susceptibility of wild fish populations.

Materials and Methods

Ethical statement

The study was performed in accordance with the EU Directive 2010/63 and Italian DL 2014/26; the experimental protocol was approved by the University of Palermo. Maintenance and handling of animals during the experiment, as well as the euthanasia procedure, were monitored and carried out by authorized staff to minimise the animals' suffering.

Animal collection and maintenance

200 juvenile *Dicentrarchus labrax* (19.49 ± 5.49 g, means ± S.E.) were obtained from an aquaculture facility near Licata (Sicily, Italy). The choice of juveniles was related to severe mortality events caused by jellyfish in different Mediterranean aquaculture facilities where the most affected fish class ranged 15-60 g in weight (Bosch-Belmar et al. 2014). The fish were kept in tanks with seawater from a closed recirculated seawater system at controlled salinity, temperature and photoperiod (means ± S.E., 37.8 ± 0.08 salinity, 19.4 ± 0.4 °C, 12 h: 12 h light-dark regime). Acclimation at the experimental temperatures (21 °C and 27 °C) was gradually achieved during the week before the start of the experiments. The fish were fed daily with 2.5 % of their body mass of commercial fish feed during the acclimation period. For the duration of the experiments, the fish stock density was maintained between 12.5 and 14 kg m^{-3}, as used in *D. labrax* aquaculture cages (9-15 kg m^{-3}).

Jellyfish were collected by hand net from the port of Messina (Sicily, Italy) the day before the experiments and were maintained in 25 L buckets with filtered seawater and at low density (5 jellyfish per bucket) for one day.

Experimental setup

The experiment was carried out at two temperatures, 21 °C and 27 °C. A total of 128 fish (64 treated, 64 controls) were subject to metabolic measurements. Fish were transferred to the treatment tanks 24 h prior to the start of the experiment and maintained unfed to reduce possible anomalous metabolic responses due to residual specific dynamic action. Sixteen 7-L treatment tanks were used for the 8-h contact period, each of them containing five fish to maintain the experimental stock density. The contact duration corresponds to a realistic night time period of high jellyfish concentration in surface waters (Malej 1989; Canepa et al. 2014). To simulate a realistic encounter between caged fish and jellyfish pressed by currents through aquaculture cages, jellyfish tissues were manually cut in small pieces (≥ 1 cm) immediately prior to the start of the jellyfish exposure. The jellyfish density used was 25 medusae m^{-3} (Baxter et al. 2011b). Tanks

were supplied with air to keep dissolved oxygen at maximum levels and ensure contact between jellyfish pieces and fish. The treatment started when jellyfish tissues were randomly placed in 8 of 16 tanks with fish, whereas the other 8 tanks served as controls without jellyfish.

Immediately after the exposure period at each temperature, all replicate fish groups were pooled into two 60-L tanks, according to their experimental status (treated or control) to maximise randomisation of subsequent fish sampling for metabolic measurements. At each of four different sampling times (3, 24, 48 and 72 h after the end of the contact period), 8 fish (4 control and 4 treated) were individually transferred into the respirometric chambers for acclimation.

Respirometry and determination of critical oxygen pressure (PO_{2crit})

At each temperature (21and 27 ºC), eight independent 2-L closed respirometers supplied with filtered sea water (Millipore GF/C 0.45 µm) were used to measure the oxygen consumption rate of individual fish. Chambers were covered with an opaque plastic material to avoid visual stresses to fish throughout measurements. An agitator and small magnets were used to maintain homogeneous water mixing inside the experimental chambers. At each sampling time (3, 24, 48 and 72 h after the end of jellyfish exposure), four treated and four control fish were randomly sampled and placed in individual respirometers. Fish were left undisturbed in the respirometers for 3 hours with supplemented air to keep dissolved oxygen at the saturation level. Then the aerators were removed and chambers were carefully refilled of water and hermetically closed. Fibre-optic oxygen meters calibrated according to instructions by Pyro Science (Aachen, Germany) were used to record water oxygen levels. Fish were maintained in the respirometric chambers until the slope of the oxygen concentration curve changed suddenly. In most cases, that change occurred at 5 to 30 % of the initial oxygen concentration. At the lowest oxygen concentration fish status was surely affected but no mortalities were recorded over the complete duration of the experiment. Fish were then removed from the respirometers, marked by a small cut in the caudal pin and returned to

the original tank in order to maintain the initial density. All fish recovered well after hypoxic exposure.

Oxygen consumption rates to approximate routine metabolism (MO_2) were calculated from the decrease in oxygen content in the respirometers over time and expressed as mmol min^{-1} g^{-1}. Those times were standardized to 45 min at 21 °C and 15 min at 27 °C within the range during which the fish were able to oxyregulate. The critical oxygen pressure (PO_{2crit}), which represents the transition from oxyregulation to oxyconformation during the progressive decline of environmental oxygen tension (Nilsson 2010), was calculated as the break-point of the graph depicting the PO_2 - MO_2 relationship. PO_{2crit} was expressed in mm Hg.

Histological analysis of gill tissue

The experiments were performed in full compliance with the national rules (D.Lgs 116/92 and subsequent amendments) and the European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). After the last sampling time (72 h), 16 experimental fish (8 controls and 8 treated) were anaesthetised with 0.05 % w/v MS222 (3-aminobenzoic acid ethyl ester) and then killed according to the current animal care rules using a lethal dose of MS-222 (0.1 % w/v). Two gill arches were excised from each fish and immediately preserved in 10 % neutral buffered formalin for 48 h and transferred to 70 % ethanol for histological analysis. After dehydration, tissues were embedded in paraplast (Bio-Optica), cut by microtome into 5 µm sections and stained using hematoxylin-eosin as "routine-staining" to reveal the underlying tissue structures and conditions. Moreover, the Periodic Acid Schiff (PAS) technique was used to identify the goblet cells. The localisation and the number of chloride cells was determined by immunocytochemical techniques by using a primary antibody that recognised sodium, potassium and chloride cotransporters (Na+ /K+ /Cl – cotransporters NKCC1-T4 1:200) revealed by a second antibody Donkey Anti-Rabbit IgG (H+L) Alexa Fluor 488® (AF488) Conjugate (Southern Biotech), 1:200. For each gill arch, 9 randomly selected tissue areas,

between 25 and 34 μm^2 were screened at 200X and 400X to count the number of chloride cells.

Gill damage score

Interpretation of the gill damage was based on a recently developed gill histopathology scoring system (GHS index, Mandich et al. in prep.) that rates the damage on each gill sample by a total score obtained by summation of partial scores assigned to 12 different criteria. The evaluation of gill damage was performed as follows: for each gill sample a total of 9 sections (photographed fields), each with 10 secondary lamellae were evaluated for all 12 histopathological criteria. For each criterion, the score ranged from 0 to 6 depending on the extent and intensity of injuries (0: no significant damage, 1: damage in 1-2 of 10 lamellae; 3: damage in 3-5 of 10 lamellae; 6: 6-10 of 10 lamellae damaged).

Gill damage could be of different grades of severity, and advanced gill damage could mask previous mild injuries. Therefore, the GHS index was supplemented by a secondary classification system to separate different stages in the progression of tissue damage (according to Santos et al. 2011(Santos et al. 2011)). All histopathological criteria were split into 3 groups. The first group (first-stage lesions) was composed of hyperplasia of the lamina and the secondary lamellae, lamellar fusion, reduction of the lamellae, lamellar oedema, and cellular hypertrophy. The second-stage lesions included circulatory disturbances of the lamina such as telangiectasia and grave cellular anomalies (presence and extension of lamellar lifting); these more severe injuries lead to effects on tissue functions; the third -stage lesions included the appearance of haemorrhage, high granulocyte concentrations, and cellular degeneration of the respiratory epithelium or necrosis, which represent irreparable damages. The score assigned for each criterion was multiplied according to the severity group (x 1: mild damage group; x 10: moderate injury group; and x 100: the most severe gill damage group). The goblet and chloride cells were visually counted in each section and analysed separately from the other histopathological criteria.

Statistical analysis

To obtain critical oxygen pressures (PO_{2crit}) and approximate the break-point in the respiration curve, a Piecewise linear regression function was used (SigmaPlot v.11). Normality of respiration data was confirmed with a Shapiro-Wilk test. To test the statistical significance among treatments for MO_2 and for PO_{2crit}, ANCOVA analyses were used, considering MO_2 and PO_{2crit} as the *response* variables, time after the exposure period as a *continuous* explanatory variable, and temperatures and jellyfish treatments as *categorical* explanatory variables.

A Shapiro-Wilk test showed that the assumptions of normality were not encountered for the histopathological data ($p< 0.05$). One-way Kruskal-Wallis test was performed to test the statistical significance among jellyfish-exposed and control fish at each temperature. Significant results were further tested by pairwise post-hoc comparisons (Wilcoxon Test) adjusted for type I error. Differences in goblet cells and chloride cells were analysed using one-way analysis of variance (ANOVA). The statistical software R (R Core Team 2015, v.3.2.2) was used to perform all analyses. Package ("coin") was used to perform the Wilcoxon test (Hothorn et al. 2006).

Results

Histological analysis

The treatment groups showed obvious gill tissue injuries, most fish displaying lesions of clinical significance (Fig. 1). The most frequently observed cellular damages were hyperplasia and lamellar fusion, lamellar oedema and lifting, and cellular hypertrophy and degeneration especially in fish exposed to jellyfish at 27 ºC.

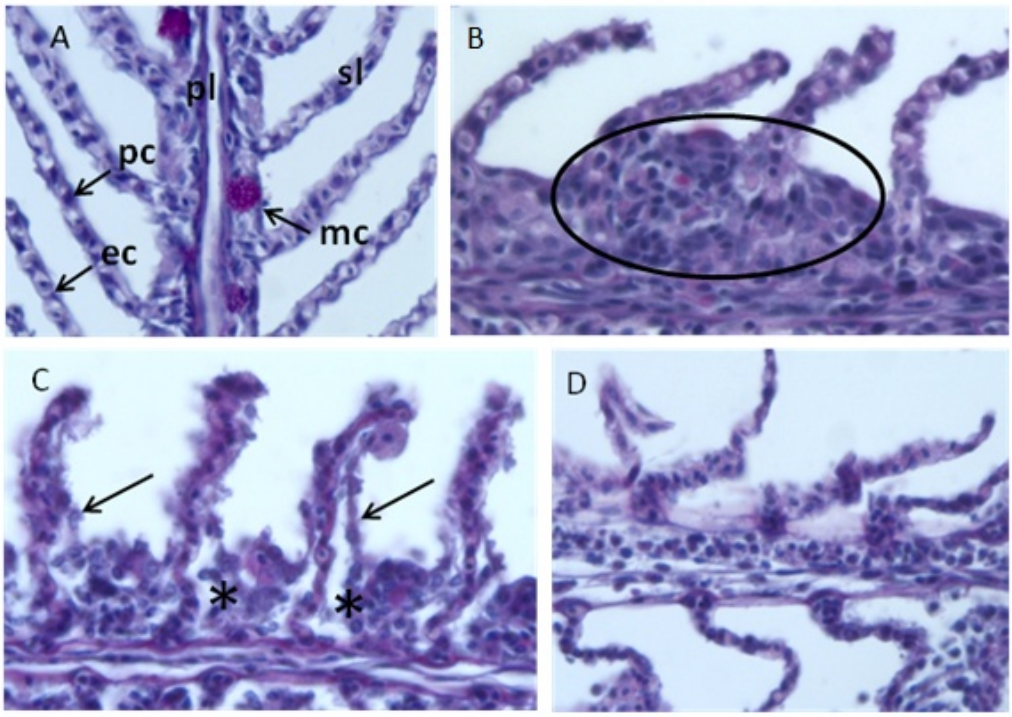

Figure 1. Gill lesions in fish exposed to *Pelagia noctiluca*. A. Control fish gills with unaltered primary lamellae (pl) with mucous cells (mc) and elongated secondary lamellae (sl) with flat epithelial cells (ep) and pillar cells (pc), (400x); B-E. Pathological features in gills from exposure to jellyfish (400x): B. Hyperplasia of primary lamella; C. Moderate lifting of epithelial cells (*) and cellular degeneration (arrows); D. Absence of respiratory epithelium and loss of structure.

The gill damage scores in fish exposed to jellyfish were higher than in controls without jellyfish at both temperatures (21 and 27 ºC) (Table I, Fig. 2a). Wilcoxon pairwise comparisons showed significant interactions between temperature and jellyfish factors for treated fish but not for control groups (Table II, Fig. 2a). The number of goblet cells was significantly higher in fish exposed to jellyfish than in controls; also, the number of chloride cells differed significantly between control and exposed fish, but not between control fish at different temperatures (Table I, Fig. 2b-c).

Table I. Statistical results for histopathological gill damage. Kruskal-Wallis test for temperature and jellyfish factors and one-way ANOVA analyses for goblet cells and chloride cells. $p < 0.05$ was considered significant.

	Gill damage			Goblet cells			Chloride cells		
Factor	df	H value	P value	df	F value	P value	df	F value	P value
Jellyfish	1	50.651	$1.103 e^{-12}$	1	18.869	0.001	1	6.510	0.015
Temperature	1	0.010	0.919	1	3.996	0.046	1	3.580	0.063
Jelly. x Temp.		---		1	0.930	0.334	1	3.846	0.062

Table II. Pair-wise comparisons for histopathological gill damage among temperature (21and 27 °C) and jellyfish (Jelly.) treatments. The F-values (lower left) and the p-values (upper right) are reported. Because multiple comparisons were performed, the Bonferroni's correction was applied to the p-value (0.05/6 = 0.0083) and significant results are in bold.

Gill damage	Jelly. x 21°C	Controls x 21°C	Jelly. x 27°C	Controls x 27°C
Jelly. x 21°C	-	**< 0.0005**	**0.0007**	**< 0.0005**
Controls x 21°C	51.0	-	**< 0.0005**	0.1869
Jelly. x 27°C	117.0	21.0	-	**< 0.0005**
Controls x 27°C	636.0	417.5	24.0	-

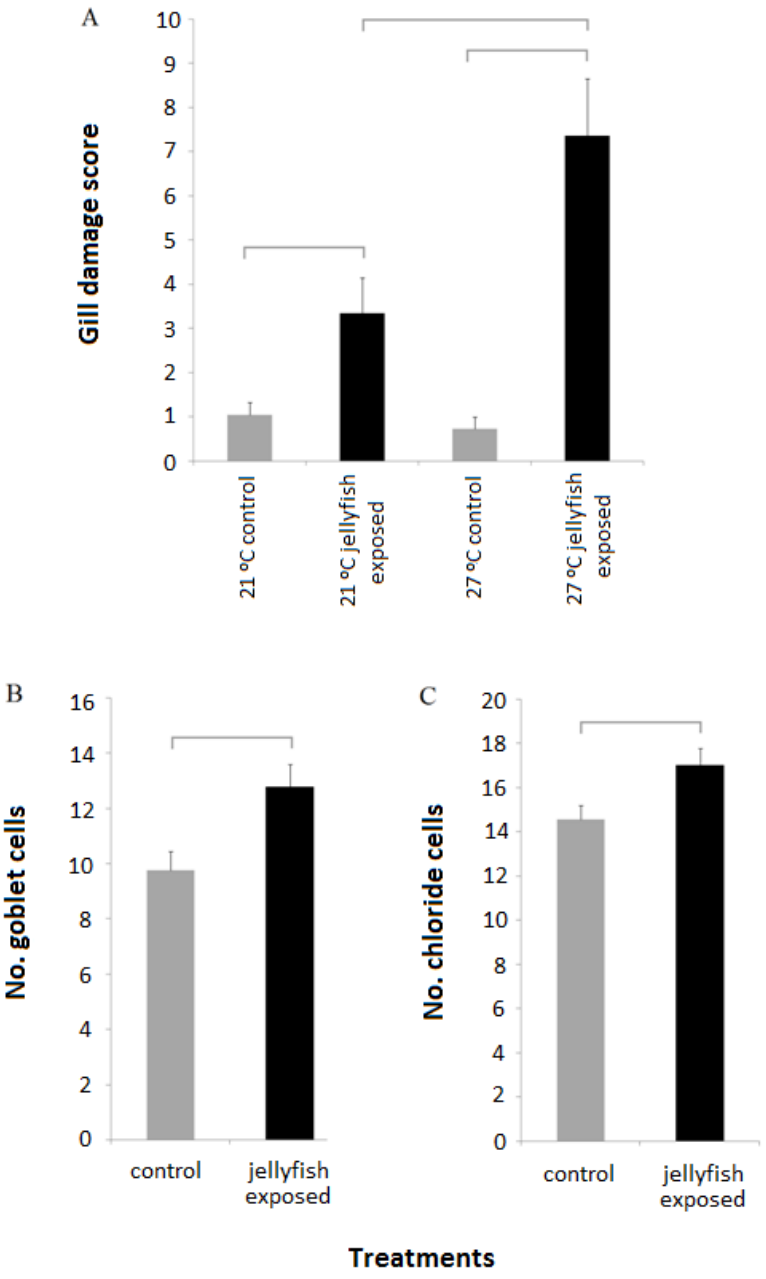

Figure 2. Gill damage scores (10-point scale) for each treatment (A), numbers of goblet cells (B) and chloride cells (C). Fish exposed to jellyfish (black bars) and control fish (grey bars). Horizontal grey lines indicate significant differences among treatments ($p < 0.05$), based on Kruskal-Wallis test for gill damage scoring and one-way ANOVAs for goblet and chloride cells.

Respirometry measurements

The oxygen consumption rate that approximates the routine metabolism (MO_2) of *D. labrax* juveniles was affected by jellyfish and temperature treatments (Table III). In addition, statistically significant differences in the critical oxygen pressure (PO_{2crit}) were found between control and jellyfish-treated fish, but not between temperatures. No significant changes were observed on fish MO_2 and PO_{2crit} over time following their exposure to *P. noctiluca* tissues (Table III).

Table III. ANCOVA statistics for oxygen consumption rates (MO_2) and critical oxygen levels ($PO_{2\ crit}$) of fish exposed to different temperatures (21 and 27 ºC) and exposed or not to jellyfish. $P < 0.05$ was considered significant.

	MO_2			**$PO_{2\ crit.}$**		
Factor	df	F value	P value	df	F value	P value
Time	1	1.357	0.246	1	2.349	0.128
Jellyfish	1	19.231	$2.46\ e^{-05}$	1	46.172	$4.13\ e^{-10}$
Temperature	1	53.849	$2.56\ e^{-11}$	1	0.173	0.678
Jelly. x Temp.	1	7.230	0.008	1	3.156	0.078

MO_2 values were significantly different at the two temperatures (Table IV, Fig. 3). Oxygen uptake was equivalent in fish exposed to jellyfish stings and control fish at 21 ºC, whereas fish exposed to jellyfish at 27 ºC had higher MO_2 than controls. PO_{2crit} at 21 ºC and 27 ºC were significantly different, with higher PO_{2crit} values in jellyfish-treated fish (averages ranged between 33-55 and 53-70 mm Hg, respectively) than in control fish (Table III, Fig. 4).

Table IV. Pair-wise comparisons for oxygen consumption rates (MO_2) among temperature (21 °C and 27 °C) and jellyfish (Jelly.) factors. The F-values (lower left) and the p-value (upper right) are reported. Because multiple comparisons were performed, the Bonferroni's correction was applied to the p-value (0.05/6 = 0.0083) and significant results are in bold.

MO2	Jelly. x 21 °C	Control x 21 °C	Jelly. x 27 °C	Control x 27 °C
Jelly. x 21 °C	-	0.0354	**<0.0005**	0.0299
Control x 21 °C	4.63	-	**<0.0005**	**<0.0005**
Jelly. x 27 °C	39.75	60.83	-	**<0.0005**
Control x 27 °C	4.95	14.38	15.03	-

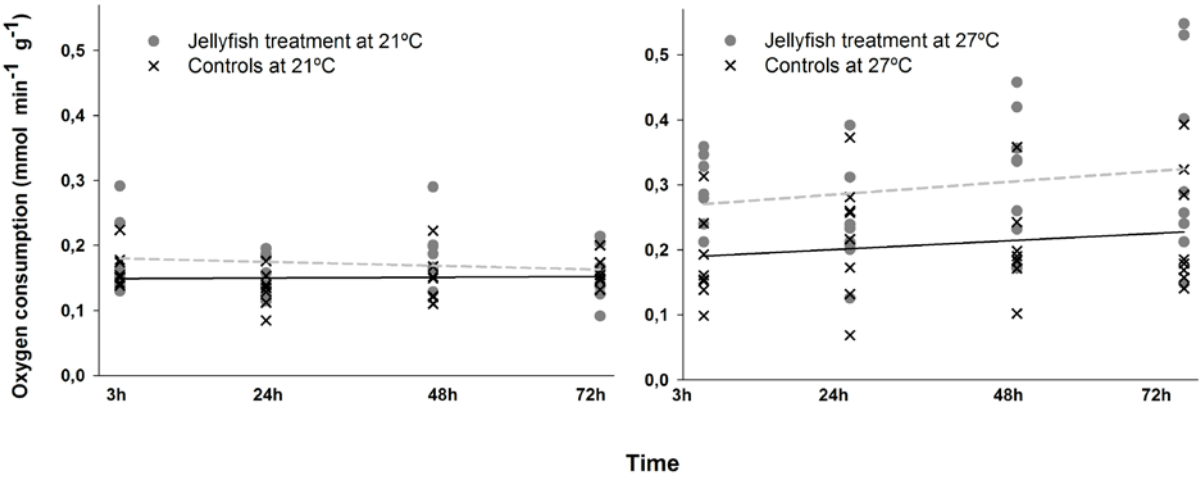

Figure 3. Oxygen consumption rates of *Dicentrarchus labrax* juveniles exposed to *Pelagia noctiluca* contact. Fish exposed to jellyfish are represented by grey dots and a dashed regression line; control fish are represented by black "x" and a continuous regression line. Experiments performed at 21°C and 27°C are shown on the left and right panels, respectively. X axes correspond to the time after the fish were exposed to jellyfish. Overall, oxygen consumption rates did not change over time: r^2 is 0.02 for treated and 0.002 for controls at 21°C (n.s.) and 0.04 for treated and 0.03 for controls at 27°C (n.s.). Regression lines have equal intercepts at 21°C; however, treated fish have higher oxygen consumption rates than controls at 27°C; see Table IV for significances.

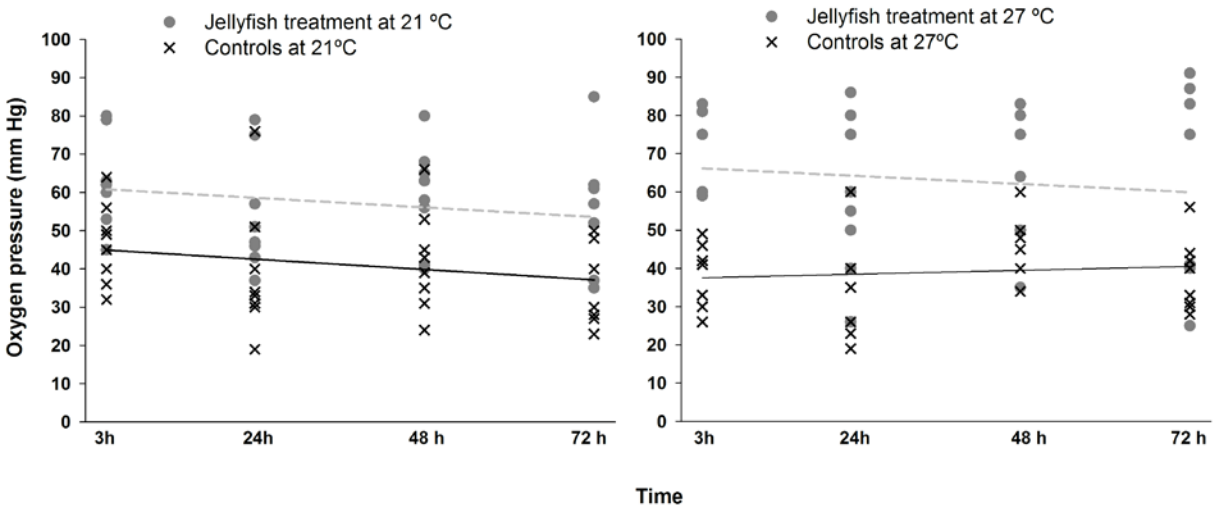

Figure 4. Critical oxygen pressures of *Dicentrarchus labrax* juveniles exposed to *Pelagia noctiluca* contact. Fish exposed to jellyfish are represented by grey dots and a dashed regression line; control fish are represented by black "x" and a continuous regression line. Experiments performed at 21°C and 27°C are shown on the left and right panels, respectively. X axes correspond to the time after the fish were exposed to jellyfish. PO_{2crit} did not change over time: r^2 is 0.04 for treated and 0.05 for controls at 21 °C (n.s.) and 0.02 for treated and 0.01 for controls at 27°C (n.s.). Regression lines have different intercepts at 21°C and 27°C showing higher PO_{2crit}s for fish exposed to jellyfish.

The observed changes of physiological responses of *D. labrax* juveniles (as MO_2 and PO_{2crit} values) related to temperature and/or exposure to jellyfish tissues were represented in a conceptual model (Fig. 5). The higher temperature led to increased fish oxygen consumption rate (a), and the jellyfish stings produced an increased PO_{2crit} (b). The combined effects of temperature and jellyfish stings caused higher oxygen uptake (c) and PO_{2crit} value (d) than the separate effect of either factor. The synergistic action of envenomation and increased temperature increased the PO_{2crit} value (i.e., anticipating the switch from the aerobic to anaerobic metabolism), thereby increasing fish sensitivity to hypoxic conditions.

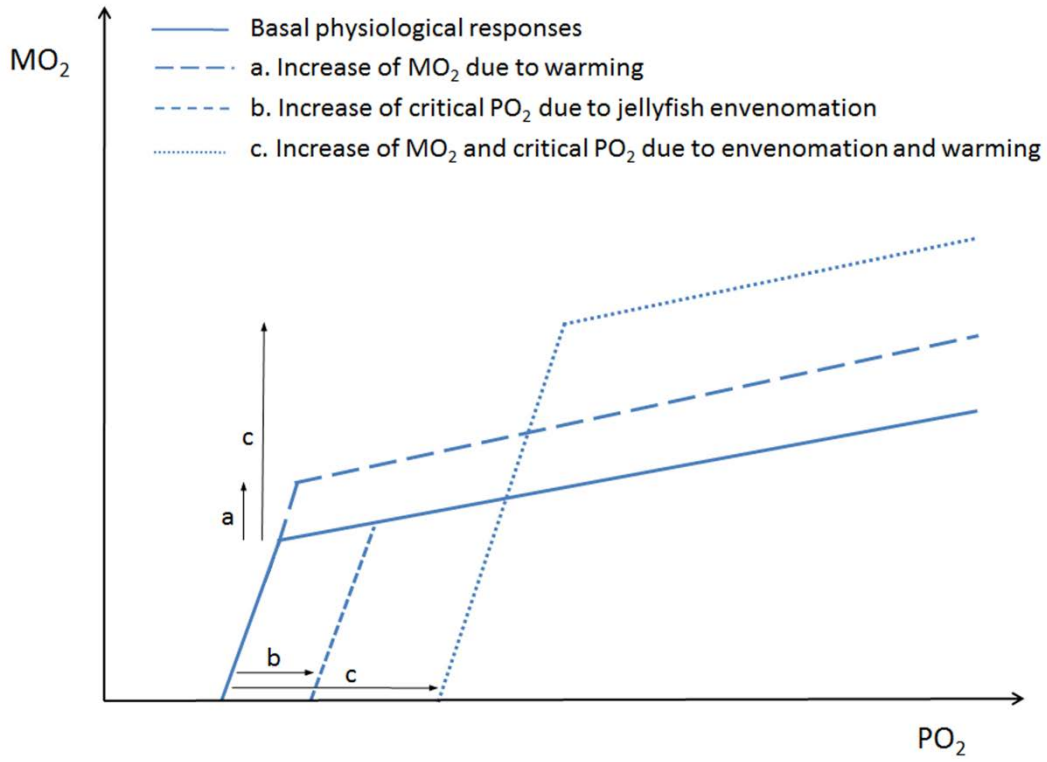

Figure 5. Unifying model of physiological responses of fish to the interaction of ocean warming and jellyfish stinging. Dashed lines represent the responses to single factors alone. Briefly, the rise of water temperature is mirrored by an increase of oxygen consumption rate (MO_2), but does not affect the sensitivity of fish to declining environmental oxygen tension (PO_2) (long dashed line); by contrast, jellyfish envenomation causes increased PO_{2crit}, which enhances the sensitivity to hypoxia (short dashed line). The dotted line represents the physiological response to the interaction of both factors and shows the enhanced vulnerability of fish.

Discussion

Previous studies on jellyfish impacts on farmed fish hypothesized that respiratory distress may impair the overall fish metabolism (Baxter et al. 2011b; Marcos-López et al. 2014). Here, for the first time, we used an integrated approach to investigate the effects of jellyfish blooms on farmed fish by the combined analysis of fish gill integrity and metabolic rates. Significant effects (increased gill damage, oxygen consumption, and critical oxygen pressure) were observed in fish at higher temperature and exposed to jellyfish.

The increased histological damage in juvenile *D. labrax* exposed to *P. noctiluca* jellyfish corroborated previous observations of adult salmon (*Salmo salar*) with severe skin and gill injuries induced by jellyfish contacts, which significantly affected fish health and survival (Baxter et al. 2011b; Marcos-López et al. 2014). Severity of gill injuries increased with factors interaction (temperature and exposure to jellyfish), which reduced gill plasticity and functioning. The observed thickening of the gill epithelium due to hyperplasia may increase the diffusion distance for gas exchange, having profound effects on the efficiency of oxygen transfer across the gill (Skidmore and Tovell 1972; Malte 1992).The increase in goblet cell numbers in fish contacted by jellyfish was paralleled by [I] increased production of mucus (data not shown), which acts as a protective barrier against microbial infections (Deplancke and Gaskins 2001; Rinaldi et al. 2005) but also forms a barrier to oxygen diffusion and contributes to hypoxia (Handy et al. 1989), and [II] an increase of chloride cells in the respiratory epithelium, which is a common response to environmental (chemical or physical) stresses, such as low-calcium and low-magnesium water, or the detection of toxicants (Perry 1997; Perry 1998).

With increasing temperature, metabolic rate and oxygen demand of ectothermic fish usually increase , but oxygen solubility declines, which exacerbates the problem caused by increased respiratory activity (McBryan et al. 2013). European sea bass increase cardiorespiratory and swimming performances in response to increased temperature (Farrell 2002)،(Claireaux et al. 2006). Similarly, higher oxygen consumption rate (Dalla Via et al. 1998; Claireaux and Lagardère 1999), growth rate, food intake and feeding efficiency (Person-Le Ruyet et al. 2004) also occur in higher temperature.

Several studies indicate that temperature and hypoxia are likely to interact synergistically on fish metabolism (Pörtner et al. 2005; Pörtner and Farrell 2008; McBryan et al. 2013). PO_{2crit} values in fish usually increase when temperature rises (Collins et al. 2013; Sørensen et al. 2014). Increased temperatures typically cause a decrease in the affinity of hemoglobin for oxygen, limiting oxygen uptake at the gills and, as a consequence, reducing fish tolerance to hypoxic conditions (Pörtner 2010; McBryan et al. 2013). By contrast, other studies suggest that increased temperature may not affect the tolerance to hypoxia in some fish species due to the intervention of homeostatic

mechanisms (e.g. the recruitment of tissue glycogen or liver lactate clearance capacity) (He et al. 2015).

An increase in PO_{2crit} values has been observed during digestion processes(Thuy et al. 2010) and may explain why hypoxic conditions might reduce appetite and growth in many fish species (Wang et al. 2009; Thuy et al. 2010). Increased PO_{2crit} values have also been observed after contamination by xenobiotics such as heavy metals, pesticides, or nanoparticles in coastal waters (Schjolden et al. 2007; Bilberg et al. 2010).

As suggested by the conceptual model (Fig. 5), our results support the hypothesis that exposure to jellyfish stings and envenomation may act synergistically with temperature, reducing fish metabolic performance, impairing their ability to withstand hypoxic conditions and, as a consequence, reducing the available energy for critical processes such as growth and reproduction (McBryan et al. 2013). Furthermore, jellyfish venoms may have hemolytic properties (Mariottini and Pane 2010) leading to exacerbation of hypoxia. In conclusion, the interaction of jellyfish envenomations with increasing temperatures may result in greater vulnerability to hypoxic conditions and in the overall reduction of fish physiological performances.

The reduction of fish homeostatic potentials due to jellyfish outbreaks in coastal waters may co-occur to produce economic losses to aquaculture facilities. Our study suggests that the interaction of direct climatic stressors (e.g. warming) with indirect effects of global change (e.g. increasing jellyfish outbreaks) may exacerbate negative impacts on fish stocks. The consequences of such interactions for human activities are numerous, but mainly affect fisheries and aquaculture. Due to the continual growth of the aquaculture sector and the increased frequency of jellyfish blooms in coastal areas, the negative interactions of stinging jellyfish on farmed fish is expected to become a substantial, recurrent issue. More research on the effects of multiple stressors on fish populations in a global change scenario is needed for a better management of living resources and the development of effective mitigation plans.

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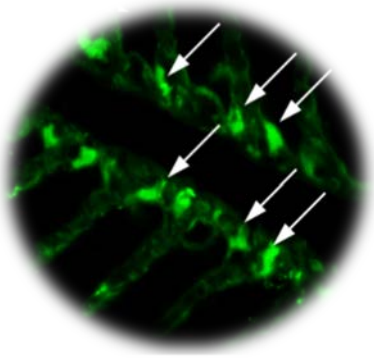

Chapter 6

Neurotoxic effects of jellyfish on farmed European sea bass

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(In prep.)

Introduction

Recent studies showed increasing trends in abundance and frequency of Mediterranean jellyfish outbreaks, leading to multiple ecological and socio-economic issues on many human activities on marine coastal zones and related economic sectors (Mills 2001; Parsons and Lalli 2002; Purcell et al. 2007; Boero et al. 2008; Richardson et al. 2009; Licandro et al. 2010; Canepa et al. 2014). Proliferations of stinging jellyfish are currently recognized as negative, indirect effects of climate change on marine fish farming and an important biotic stressor especially in coastal areas where jellyfish blooms (JB) repeatedly arrive (Purcell et al. 2007; Richardson et al. 2009; Purcell et al. 2013). Due to the growth of aquaculture sector (FAO 2014) and the increased frequency of jellyfish blooms in coastal waters (Purcell et al. 2007; Brotz and Pauly 2012; Condon et al. 2013), the negative interactions of stinging jellyfish on caged finfish is expected to become a substantial issue producing highly relevant economic losses (Purcell et al. 2013).

Impacts of low to moderate jellyfish abundances usually remain unnoticed by aquaculture farmers and low incidence of unspecific pathologies are labelled as unknown "water borne irritant damage" (Rodger et al. 2011a; Marcos-López et al. 2014). Jellyfish

can cause tissue disorders, respiratory and growth problems and even death in caged fish (Rodger 2007; Baxter et al. 2011a; Mitchell et al. 2012). Previous work was carried out on the impacts of jellies on farmed salmon aquaculture in Northern European waters (Carl et al. 2010; Baxter et al. 2011b; Baxter et al. 2011a; Baxter et al. 2012). Comparatively little or no information is available about the impacts of one of the most harmful European jellyfish species, *Pelagia noctiluca*, on the most common Mediterranean finfish aquaculture species, such as the sea bass *Dicentrarchus labrax*.

Pelagia noctiluca is the most abundant, stinging jellyfish in the Mediterranean Sea. It has been related with several farmed fish mortality events in Irish and Scottish facilities, where more than 400,000 salmonids were killed during 2007, 2013 and 2014 (Doyle et al. 2008; Purcell et al. 2013; FIS 2014; Marcos-López et al. 2014). *P. noctiluca* venom composition is well known. Different authors have studied its nematocyst morphology and functions, as well as the toxins they contain. Its venom has been described as antigenic, producing serious consequences through immunological and toxic mechanisms (Mariottini et al. 2008). It also possesses dermonecrotic and strong hemolytic properties (Mariottini et al. 2008; Mariottini and Pane 2010; Maisano et al. 2013). Previous studies have showed that contact between farmed fish and *P. noctiluca* jellyfish generated severe cellular damage in fish gills comprising haemorrhage, oedema, necrosis, lamellar epithelium sloughing and lamellar epithelium hyperplasia and fusion (Mitchell et al. 2012; Marcos-López et al. 2014). Moreover, Ayed et al. (2011) showed the cytotoxic effects of *P. noctiluca* nematocysts that include inhibition of cell proliferation, induction of heat shock proteins (Hsp 70 and 27) over-expression, and generation of intracellular Reactive oxygen species (ROS), which could induce cell lysis.

Fish gills are prime targets of toxic chemicals in the environment because they are the first organs to come in contact with environmental pollutants. Fish gills serve a variety of physiological functions, including respiratory gas exchange, osmoregulation, nitrogen excretion and acid–base regulation (Wendelaar Bonga 1997). The control of gill functioning under different physiological situations is dependent on a complex web of neural, endocrine, and paracrine signaling pathways (Evans et al. 2005). Apoptotic and proliferative activities, stress proteins as Hsp or Metallothioneins (MTs), and changes in

homeostasis regulation and neuroendocrine control mechanisms have been extensively used as biomarkers of exposure to contaminants on fish (Mauceri et al. 2002; Mauceri et al. 2005; Fasulo et al. 2010; De Domenico et al. 2011; Boix and Cauli 2012; De Domenico et al. 2013; Munari et al. 2014; Wan et al. 2015). Neurotransmitters as well as proteins or peptides implicated in their action pathways are widely used as stress biomarkers of exposure to pollutants, pesticides or heavy metals, since they play an important role in neuroendocrine regulation responses. Several studies present the effects of different pollutants and chemicals in the environment on the health of economically important fish species, showing significant damage to gill epithelium as well as neurotoxicity (e.g. Ba-Omar et al. 2011; Boyle et al. 2013).

Despite the increase of JB in the Mediterranean Sea, as well as more frequent interactions between jellyfish and marine finfish aquaculture and fishing, the neurotoxic effects of jellyfish venom as a biotic stressor in fish is still poorly understood. The purpose of this study was to evaluate the stress responses in the gills of farmed *D. labrax* after contact with stinging *P. noctiluca* tissue, particularly the function of cholinergic and serotonergic systems, as well as the dopaminergic pathways. We used histological techniques to investigate the expression of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) enzymes, serotonin (5-HT) neurotransmitter and its receptor 5-HTR_3, tyrosine hydroxylase (TyrH) protein and vasoactive intestinal peptide (VIP).

Materials and Methods

Ethics statements

The experiments were performed in full compliance with the national rules (Royal decree 53/2013) and the European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). Moreover, the experimental protocol was evaluated and approved by regional authorities and CSIC ethical committee (no. 114/2014).

Animal collection and maintenance

Fifty juvenile *Dicentrarchus labrax* (20 ± 0.75 g) were obtained from the Institute *for* Research *and* Technology *in Food and* Agriculture (IRTA) in Tarragona (Spain). The choice of juveniles was due to severe mortality events caused by jellyfish in different Mediterranean aquaculture facilities where the most affected fish class was 15-60 g in weight (Bosch-Belmar et al. 2014a). The fish were kept in tanks with a flowing seawater system (37.9 ± 0.04 salinity and 19.38 ± 0.18 °C), photoperiod 12 h: 12 h light-dark regime, and were fed daily with commercial fish feed. Fish were maintained in these conditions during 5 days and then were transferred to the treatment tanks (40 L and 3 individuals per tank) and acclimated for another 5 days before starting the experiment.

Jellyfish were collected by hand net from Barceloneta beach (Barcelona, Spain) the day before the start of the experiments and were maintained for one day in a special rectangular jellyfish aquarium (Purcell et al. 2013).

Experimental setup

To simulate a realistic encounter between jellyfish pressed by currents onto aquaculture cages and caged fish, jellyfish tissues were manually cut in small pieces (≥ 1 cm) immediately prior to the start of the jellyfish exposure. The density of jellyfish used was 25 individuals m^{-3}, as in (Bosch-Belmar et al. 2014b).

Fish exposure to jellyfish was performed in the experimental tanks for 8 h. The exposure time was chosen as a minimum night time when *P. noctiluca* jellyfish reach their highest densities in surface waters (Canepa et al. 2014). After 8 h contact, jellyfish fragments were carefully removed with a 50-µm mesh hand net. Sampling times included the initial control treatment (0 h) and then 1 h, 5 h, 9 h, 24 h, 48 h, and 1 week after the start of the experiment (T0, T1, T5, T9, T24, T48, and T1wk, respectively). The treatment started when jellyfish tissues were added to the tanks with fish. To maintain the water mixture and ensure contact between jellyfish pieces and fish, the water was gently aerated. At each time, 6 fish from 2 different experimental tanks were sampled and anesthetized

with MS-222 (0.3 g L^{-1}) through immersion and then killed according to the current animal care rules by decapitation.

Gill tissues were removed, fixed in 4% paraformaldehyde for 4 h, and then rinsed in 0.1 M phosphate buffered saline (PBS, pH 7.4). After dehydration in ethanol and embedding in paraffin, 4 mm thick histological sections were cut and stained using the hematoxylin-eosin standard protocol (H/E) to reveal the underlying tissue structures and conditions.

Immunohistochemical analysis

Samples were processed according to a standard immunofluorescence method (Mauceri et al. 1999). After deparaffinization and rehydration, samples were incubated in 5 % normal goat serum (NGS) and then overnight in a humid chamber at 4 °C with the primary antibodies listed in Table I. After a rinse in PBS, secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) conjugated to fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) were used at the working dilution of 1:100 for 2 h at room temperature. Positive controls for labeling specificity of each peptide were performed by incubating sections with antiserum pre-absorbed with the respective antigen; in addition, negative controls were performed by substitution of non-immune sera (without antibodies) for the primary antisera. All observations were made using a Zeiss Axio Imager Z1 epifluorescence microscope, equipped with an AxioCam camera (Zeiss) for the acquisition of images. The number of immunopositive cells was calculated by selected tissue sections in stained branchial sections. The count was repeated for five sections for each specimen.

Statistical analysis

Immunoreactive cells in the gill tissue were quantified using fully automatic AxioVision Release 4.5 software. Shapiro-Wilk test was used to verify that the assumptions of normality were countered for the data ($p < 0.05$). One-way ANOVA followed by Dunnetts' multiple comparisons post-hoc test was carried out to identify difference among treatment times for each biomarker, considering $p < 0.01$ as statistically significant. All analyses were performed using Prism 6.07 GraphPad software.

Table I. List of primary antibodies used to test responses of fish gill tissue to exposure to *Pelagia noctiluca* jellyfish.

Antigen	Source	Supplier	Dilution
Serotonin (5-HT)	Mouse	Dako Cytomation, Milan, IT	1:50
Serotonin receptor (5-HT_3R)	Rabbit	Sigma-Aldrich, St. Louis, MO, USA	1:100
Acetylcholine esterase (AChE)	Mouse	Chemicon International, Temecula, CA, USA	1:50
Choline Acetyltransferase (ChAT)	Rabbit	Abcam, Cambridge, UK	1:250
Tyrosine hydroxylase (TH)	Mouse	Sigma-Aldrich, St. Louis, MO, USA	1:100
VasoIntestinal Peptide (VIP)	Rabbit	Sigma-Aldrich, St. Louis, MO, USA	1:50
Anti-Rabbit IgG FITC conjugated	Goat	Sigma-Aldrich, St. Louis, MO, USA	1:100
Anti-Mouse IgG TRITC conjugated	Goat	Sigma-Aldrich, St. Louis, MO, USA	1:100

Results

Fish showed lethargic behavior and loss of appetite, but non-gross pathology was observed in sampled fish skin. Nevertheless, increased quantities of mucus were observed in treated fish gills. Gills from control fish showed a typical epithelial organization, each arch being formed by unaltered primary lamellae and elongated secondary lamellae with flat epithelial cells. Five hours after fish exposure to jellyfish tissues, lamellar filament thickening and higher erythrocyte concentrations were observed. Cellular alterations increased over time; at T9 and T24, gill epithelium presented significant lamellar hyperplasia with intense cellular hypertrophy as well as increasing presence of erythrocytes. At T48, improvements in gill tissue organization

were observed; 1 week after the start of the experiment high percentages of gill epithelium were recovered (Fig. 1).

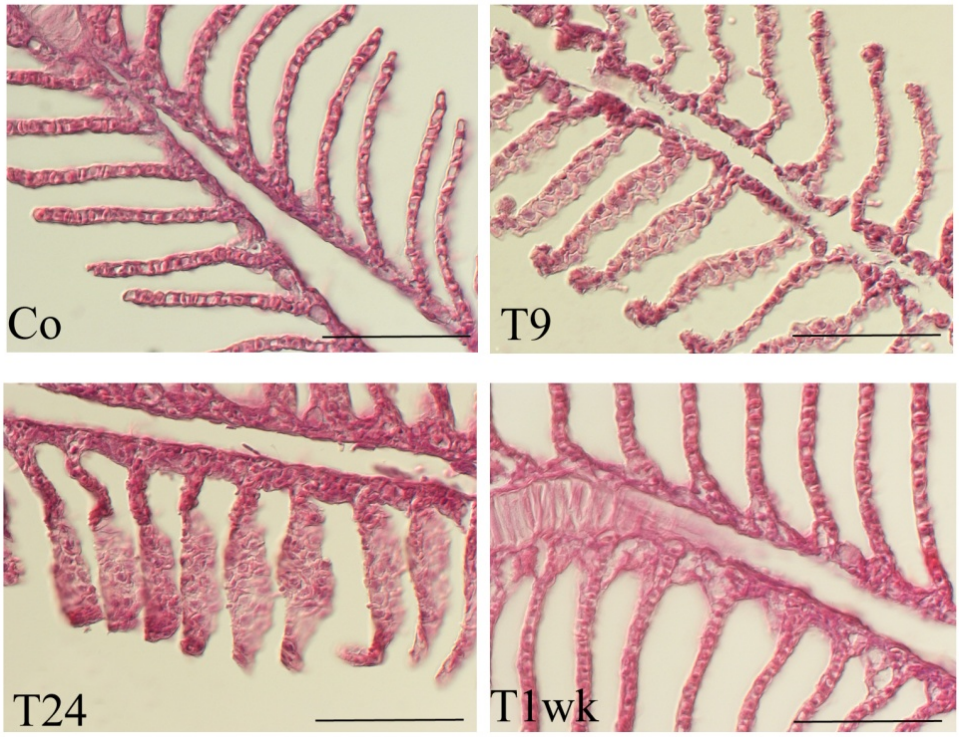

Figure 1. Gills from control fish with unaltered primary lamellae (Co); Gills from fish exposed to jellyfish after 9 h (T9), 24 h (T24) and one week (T1wk).

Results of biochemical biomarkers analyzed in fish gill epithelium showed significant immunoreactivity difference over time (Table II) and with respect to controls (Figs. 2, 3 and 4). Direct effects were observed on the cholinergic and serotonergic systems, as well as in dopamine pathways. Immunoreactivity for AchE and ChAT revealed a significant number of cells and fibers positive to both antibodies along the filament in fish from the control group. Few immunopositive cells were detected for both enzymes 1 h after the start of the experiment demonstrating a clear inhibition of immunoreactivity. At T24, an increased number of immunopositive cells indicated recovery, with initial levels reached 1 week after the start of the experiment (Fig. 2 and 3).

Table II. One-way analysis of variance for biomarkers immunoreactivity over experimental time ($p < 0.01$ was considered as significant).

Biomarkers	*F*	d.f.	*p*-value
AChE	533.5	6	< 0.0001
ChAt	184.8	6	< 0.0001
5-HT	59.11	6	< 0.0001
5-HT_3R	75.69	6	< 0.0001
VIP	29.47	6	< 0.0001
TH	45.12	6	< 0.0001

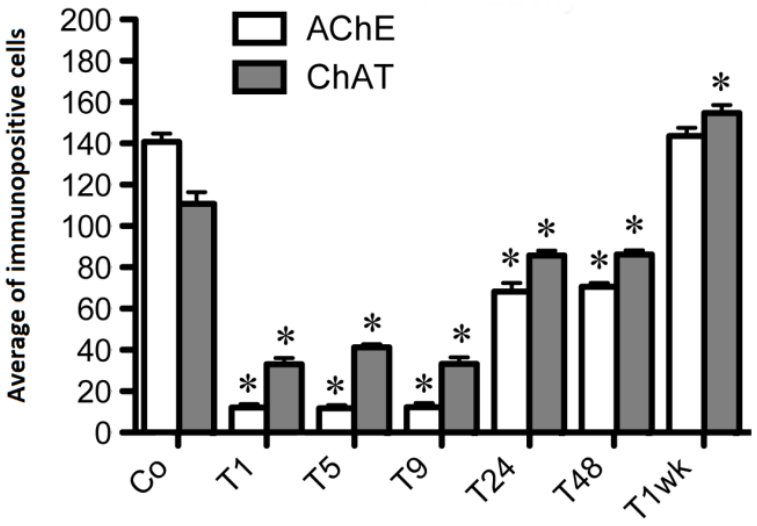

Figure 2. Average of immunopostive cells for AChE and ChAT over experimental time. Asterisks indicate significant differences between control and treated fish ($p < 0.0001$).

The serotonergic system showed the same pattern as did the cholinergic system, with an immediate decrease in immunopositive cells (T1) that was maintained during T5 and T9. The number of immunopositive cells for 5-HT_3R increased at T24 and recovered normal values, but immunoreactivity of the 5-HT neurotransmitter showed an increase at T48 followed by a decrease in positive cells at T1wk (Fig. 4).

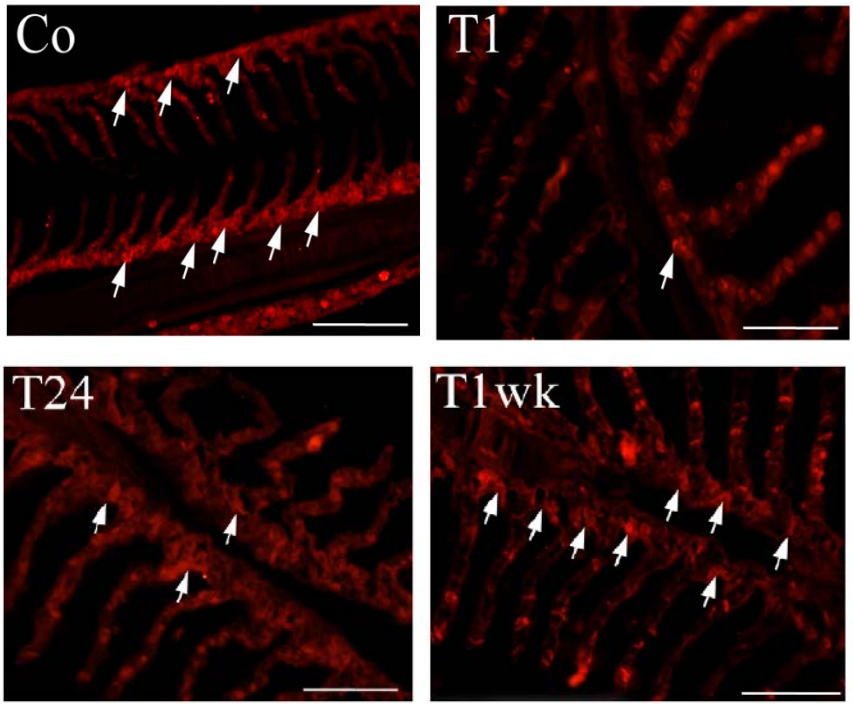

Figure 3. Example figure of immunohistochemical labeling for AChE in control and poisoning fish over the time. White arrows indicate cells immunopositive to AChE

Fish exposed to jellyfish had fewer immunopositive cells for TH and VIP, an enzyme and peptide involved in dopamine pathways and gill osmoregulation. Inhibition of TH and VIP immunoreactivity was observed at T5 and T9, but increasing at T24 until control values were reached at the end of the experiment (T1wk) (Fig. 5).

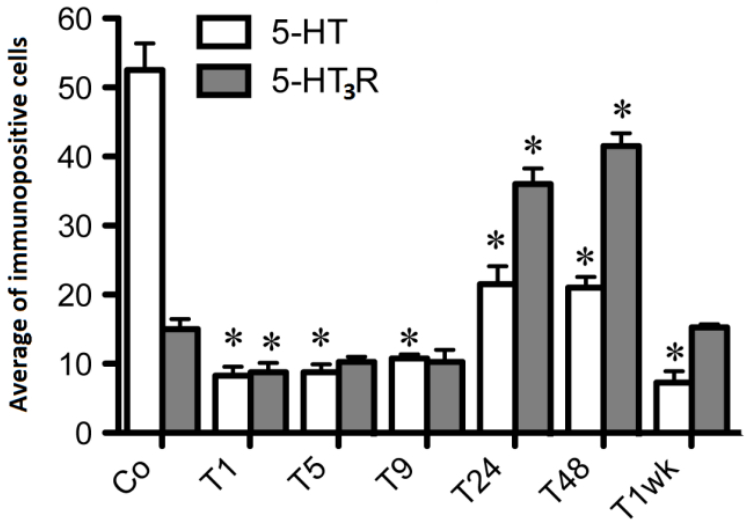

Figure 4. Average of immunopostive cells for 5-HT and its receptor 5-HT $_3$R over experimental time. Asterisks indicate significant differences between control and treated fish ($p < 0.0001$).

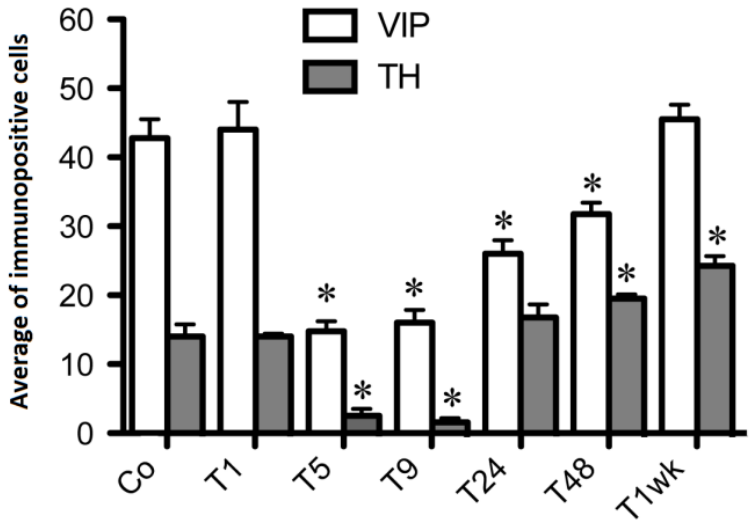

Fig. 5. Average of immunopostive cells for VIP and TH over experimental time. Asterisks indicate significant differences between control and treated fish ($p < 0.0001$).

The increases of immunoreactivity for selected biomarkers at the last sampling times agreed with recovery of gill epithelium observed 1 week after the fish were exposed to jellyfish tissue as improved tissue organization.

Discussion

The present study has demonstrated that fish exposure to jellyfish alters the function of both the nervous and endocrine systems. The damage to the gills would also affect the plasticity of the gill epithelium. The observed thickening of the gill epithelium due to hyperplasia will increase the diffusion distance for gas exchange and even small increases can have profound effects on the efficiency of oxygen transfer across the gill (Malte 1992). Moreover, the excessive mucus production observed in treated fish gills could also act as barrier to oxygen diffusion and contribute to hypoxia (Handy et al. 1989).

Immunohistochemical analyses revealed inhibition of immunoreactivity for AchE and ChAT after jellyfish contact. Both enzymes are involved in acetylcholine (ACh)

biosinthesis; ChAT synthesizes the neurotransmitter from coline and acetyl-CoA, while AchE is involved in the hydrolysis of ACh. AchE enzyme has been reported to be a specific biomarker of exposure to some pesticides, which can inhibit it (Guzmán-Guillén et al. 2015). This inhibitory action results in accumulation of ACh in the synapses of the central nervous system, neuromuscular junctions, and sympathetic and parasympathetic nerve endings, with the subsequent excessive stimulation of cholinergic nerves (Guzmán-Guillén et al. 2015; Wan et al. 2015).

Serotonin neurotransmitter is involved in the regulation of several physiological functions, from branchial and cardiovascular (Janvier et al. 1996) to gastrointestinal systems. It also powerfully modulates the immune system (Boix and Cauli 2012). In the gills, it plays different physiological roles, including regulation of basal blood vessel and cell turnover. Serotonergic neuroepithelial cells (NECs) distribution appears to reflect chemoreceptive roles related to hypoxia tolerance (Ferrando et al. 2005; Fasulo et al. 2010). Low immunoreactivity to 5-HT. together with thickening of the gill lamellae surface, could suggest the creation of a "functional hypoxia" condition (De Domenico et al. 2013).The rise in serotonin detected after 48 h could be related to remodeling of the gill epithelium, because high serotonin levels may promote apoptotic activity, allowing the removal of the interlamellar cells that proliferated after exposure to jellyfish in order to recover the normal physiology of the epithelium (Azmitia 2001; Frampton et al. 2010; De Domenico et al. 2013). The different roles played by serotonin are mediated by specific receptors. Specifically, the 5-HT_3R is an ion channel receptor which is supposed to modulate dopamine neuron activity. Its presence in gill tissue could regulate the neurotransmitter release, dopamine neuron activity, and also participate in the response to hypoxia (Barnes and Sharp 1999; Fasulo et al. 2010).

Tyrosine hydroxylase (TH) is a synthesizing enzyme involved in the dopamine biosynthesis pathway. Several studies had demonstrated that different heavy metals and pollutants could bind to TH, inhibiting catecolamine synthesis (Fernández-Dávila et al. 2012). Catecolamines have significant effects on gas transport across the gills and within the blood, thus inhibition of TH immunoreactivity in treated fish may manifest as

malfunction in gill perfusion (Brunelli et al. 2011). As well as other hormones, VIP plays a fundamental role in gill osmorregulation by rapidly stimulating chloride secretion in differentiated chloride cells (together with glucagon). VIP may also directly regulate neuroendocrine dopaminergic neuron activity (Gerhold et al. 2001). Decreases in VIP immunopositive cells after fish exposure to jellyfish may indicate imbalance in dopaminergic neurons and in ion transport mechanisms in the gill epithelium (Foskett et al. 1983).

In the natural environment, the stress response can be seen as an acute response that has evolved to enable the fish to mobilize its energy reserves as it attempts to avoid or overcome the immediate threat. Under aquaculture conditions, when the environmental stress could be chronic, the stress response can be damaging to the fishes' health by increasing susceptibility to disease or by suppressing the growth rate (Pickering 1993). Repeated contacts between caged fish and jellyfish blooms could have dramatic consequences for fish gill integrity and consequently to fish health (Rodger et al. 2011b; Mitchell et al. 2013; Marcos-López et al. 2014).

Neurotoxic effects have been demonstrated for the first time in *D. labrax* exposed to jellyfish tissues. Despite observed alterations in gill cellular structure and neuroendocrine function, treated fish showed physiological adaptation responses to stress, almost completely restoring the cellular organization of the gill epithelium and normal neuroendocrine regulation system 1 week after contact with jellyfish tissues. Nevertheless, further research is needed because longer exposures to cnidarians or higher jellyfish densities, as well as repeated encounters between farmed fish and jellyfish blooms could result in much more severe consequences for fish metabolism at various levels.

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General Discussion and Conclusions

Recent analyses on dynamics of jellyfish populations in the Mediterranean coastal zones highlighted trends of increasing abundance and frequency of bloom formation (Kogovšek et al. 2010; Licandro et al. 2010; Brotz et al. 2012; Condon et al. 2013). Interaction between jellyfish and marine finfish aquaculture has been recorded in several occasions in the last years, leading to severe episodes of fish mass mortality and severe gill disorders (Rodger et al. 2011; Purcell et al. 2013). The consequences of episodes of jellyfish proliferation can be of high importance for aquaculture, considering they could affect not only field technicians health, but also fish welfare, growth of farmed stocks and quality of commercial final product (Rodger et al. 2011; Mitchell and Rodger 2011).

The work developed in this PhD thesis represents the first contribution towards the understanding of impacts of cnidarian jellyfish blooms on the commonest Mediterranean finfish aquaculture species, the sea bass *Dicentrarchus labrax* and the sea bream *Sparus aurata*.

In spite of fish farmers awareness of jellyfish blooms and fish mortality events in several Mediterranean aquaculture facilities (Spain and Tunisia), this thesis work showed that no prevention/mitigation plan has been developed or implemented so far to face episodic events of jellyfish outbreaks.

For the first time, the occurrence, temporal distribution, and reproductive periods of planktonic cnidarians, as well as of the hydroid fouling assemblage, were investigated in Mediterranean fish farms. A significant relation between fish gill disorders, fish mortalities, cnidarian density, and low temperatures was demonstrated. In addition,

histopathological analyses led to the adaptation of a semi-quantitative index of cnidarian-induced fish gill damage, a novel tool for the early detection of gill disorders and jellyfish impacts.

The impacts of *Pelagia noctiluca*, the most abundant and harmful Mediterranean jellyfish species, on *D. labrax* and *S. aurata* were investigated from different points of view. First, we discovered that contacts with *P. noctiluca* negatively affected fish gill integrity, causing injuries of increasing severity directly related with exposure time and jellyfish density. Second, we demonstrated that basic metabolic performances of farmed fish were significantly compromised by the synergistic interaction of a) discharge of *P. noctiluca* stinging cells on fish skin and gill epithelia; b) increasing temperature; and c) decreasing oxygen conditions. Preliminary cytochemical analyses also showed that the fish neuro-endocrine system can be significantly imbalanced. As a corollary, this experiment suggested that in the current scenario of global warming and increasing trends of cnidarian blooms, the impact of jellyfish and fouling hydroids on fish aquaculture may also increase in future years. Based on these results, we speculated that wild fish populations in enclosed coastal areas (eg. shallow lagoons), exposed to a combination of increasing jellyfish blooms, rising water temperature, and oxygen depletion, might be severely affected, too. Fish exposed to jellyfish and increasing temperature showed weakened capacity to counteract episodes of hypoxia; such a reduced homeostatic potential can impair fish health and acclimation response mechanisms, leading to relevant economic losses to the aquaculture facilities.

The new information gathered from this thesis work may contribute to the development of decision tools for the management of the aquaculture farms. So far, there is no verified mitigation method to prevent jellyfish entering into finfish cages. Rodger et al. (2011) suggested several mitigations procedures (e.g. rapid installation of protective pen enclosures, bubble curtains, cage submersion, or cage towing cages outside of the jellyfish) to be developed and tested in the future, keeping in mind cost-benefit, effectiveness, and site-specific suitability.

Results obtained from field monitoring and laboratory experiments in this thesis will represent a useful background to:

- identify reproductive periods of potential jellyfish harmful species, create or adapt novel farming procedures (e.g. selection of new periods for fry introduction in sea cages, or time schedule to remove fouling organisms from fish cages). Site-specific information will be essential to identify the seasonal and inter-annual abundance and occurrence of harmful species, highlighting risk periods for each location. Therefore, routine monitoring of cnidarians should be included as part of veterinarian health plans (VHPs) in fish farms.

- investigate on impacts of different jellyfish species and densities. This information will enable estimation of the response time required by aquaculture farmers to undertake appropriate mitigation actions only when harmful jellyfish outbreaks occur nearby fish farm facilities.

The outcome of this thesis work calls for further development of efficient methods of control and mitigation of jellyfish outbreaks.

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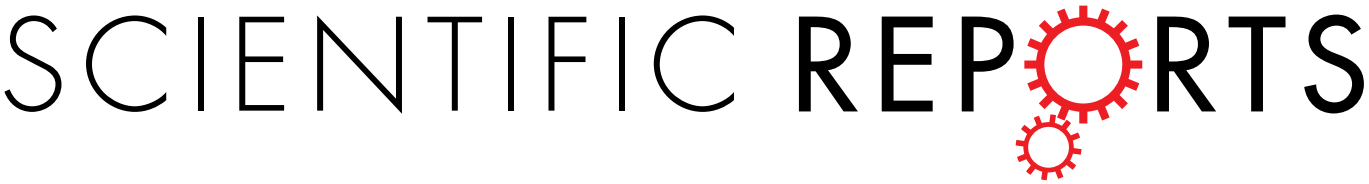

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Concurrent environmental stressors and jellyfish stings impair caged European sea bass (*Dicentrarchus labrax*) physiological performances

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The increasing frequency of jellyfish outbreaks in coastal areas has led to multiple ecological and socio-economic issues, including mass mortalities of farmed fish. We investigated the sensitivity of the European sea bass (*Dicentrarchus labrax*), a widely cultured fish in the Mediterranean Sea, to the combined stressors of temperature, hypoxia and stings from the jellyfish *Pelagia noctiluca*, through measurement of oxygen consumption rates (MO_2), critical oxygen levels (PO_{2crit}), and histological analysis of tissue damage. Higher levels of MO_2, PO_{2crit} and gill damage in treated fish demonstrated that the synergy of environmental and biotic stressors dramatically impair farmed fish metabolic performances and increase their health vulnerability. As a corollary, in the current scenario of ocean warming, these findings suggest that the combined effects of recurrent hypoxic events and jellyfish blooms in coastal areas might also threaten wild fish populations.

Human activities are transforming coastal and marine ecosystems at local, regional, and global scales, exposing both individual organisms and biological communities to dramatic environmental changes by a complex array of interacting stressors[1,2]. The current trend of induced anthropogenic environmental changes includes increasing sea water temperatures, frequencies of hypoxia episodes, and ocean acidification[3,4]. Concurrently, zooplankton communities respond to anthropogenic- and climate-induced changes by strong variations in their spatial distribution, structure and function[5,6]. Jellyfish represent one of the components of plankton that seem to be responding positively to the ongoing changes. They are likely to affect the food web structure by their high consumption rates, fast growth and reproduction rates, and wide tolerance to ecosystem changes[7–9]. Recent analyses of jellyfish population dynamics in Mediterranean coastal zones suggested increasing abundance and frequency of bloom formation[10–13]. Global changes such as overfishing, eutrophication and ocean warming have been proposed as mechanisms leading to jellyfish increases in many coastal waters worldwide, including the Mediterranean Sea[7,11,14–16]. These factors are causing severe negative impacts on human economic activities, such as tourism, fisheries, and aquaculture[7,17–19].

Aquaculture is an important source of income for local societies and sustains over 40% of global fish production; mariculture supports nearly 30% (US $23.5 billion) of the total value of farmed finfish species[20]. Interactions between jellyfish and marine caged fish have been recorded on several occasions in recent years, leading to severe fish mass mortality[21]. Jellyfish can enter fish cages either intact or fragmented, as tentacles and other body parts (e.g. oral arms), washed by currents and waves against the mesh of cage nets[21–23]. Overall, more than 400,000 salmon were killed in Irish marine aquaculture facilities in recurrent blooms of the scyphomedusa *Pelagia noctiluca* in 2007, 2013 and 2014[24–26]. The moon jellyfish *Aurelia aurita*, and the hydrozoans *Muggiaea atlantica* and *Phialella quadrata* were also involved in different farmed fish mortalities, and together with

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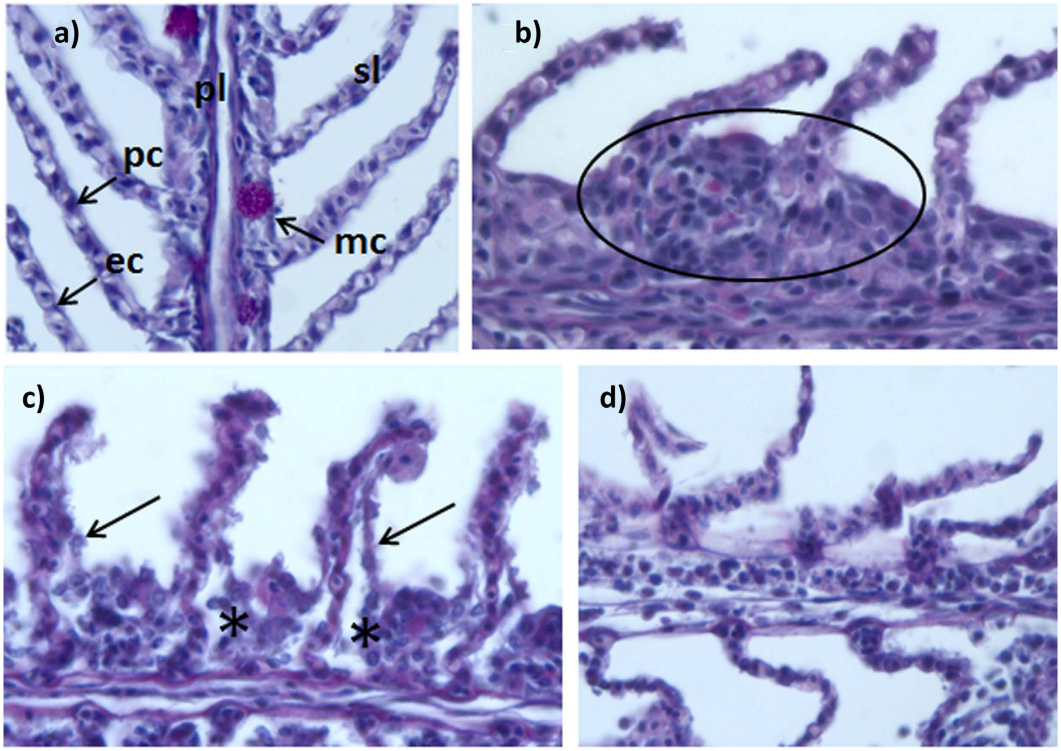

Figure 1. Gill lesions in fish exposed to *Pelagia noctiluca*. (**a**) Control fish gills with unaltered primary lamellae (pl) with mucous cells (mc) and elongated secondary lamellae (sl) with flat epithelial cells (ep) and pillar cells (pc), (400×); (**b–e**). Pathological features in fish gills from the treatment groups after 8 h exposure to jellyfish (400×): (**b**) Hyperplasia of primary lamella; (**c**) Moderate lifting of epithelial cells (*) and cellular degeneration (arrows); (**d**) Absence of respiratory epithelium and loss of structure.

P. noctiluca, were identified as potentially harmful species for aquaculture facilities[22]. In addition, jellyfish can act as vectors of the bacterium *Tenacibaculum maritimum*, exacerbating fish gill injuries[27]. Beyond these few studies, limited information is available on how jellyfish affect fish health, the biological mechanisms underlying fish mortalities, or estimates of potential economic losses[21]. Only a few studies described significant fish injuries caused by the discharge of cnidocytes (specialized cnidarian stinging cells) in fish tissues (skin, gills) leading to envenomation and cellular damage[23,28].

Temperature and dissolved oxygen concentration in the water column are crucial for the development and performance of aquatic organisms through direct effects on their metabolic rates[29,30]. Most fish adapt their physiological responses to sustain their metabolic rates when exposed to temperature changes or decreased dissolved oxygen levels[3,31]; however, additional external factors (such as pollutants or different environmental factor) may impair acclimation processes.

In this framework, we investigated the sensitivity of fish to the co-occurrence of environmental stressors (water temperature) and jellyfish stings to understand the impact of jellyfish blooms on caged finfish in a global warming scenario. Experiments were designed to test the combined effects of temperature ("temperature treatment") and prolonged jellyfish contact ("jellyfish treatment") on metabolic performances (MO_2 and critical PO_2) and tissue damage on fish gills over the time. We used the jellyfish *Pelagia noctiluca* (Forsskål, 1775), the strongest stinging and most abundant scyphozoan species in the Mediterranean Sea and Eastern North Atlantic, and juveniles of *Dicentrarchus labrax* (Linnaeus, 1758), one of the most common fish species in Mediterranean marine aquaculture. This study presents important eco-physiological data to the overall fish mariculture sector in jellyfish-affected coastal areas and also for the scientific community working on the global change susceptibility of wild fish populations.

Results

Histological analysis. The treatment groups showed obvious gill tissue injuries, most fish displaying lesions of clinical significance (Fig. 1). The most frequently observed cellular damages were hyperplasia and lamellar fusion, lamellar oedema and lifting, and cellular hypertrophy and degeneration especially in fish exposed to jellyfish at 27 °C. The gill damage scores in fish exposed to jellyfish were higher than in controls without jellyfish at both temperatures (21 and 27 °C) (Table 1, Fig. 2a). Wilcoxon pairwise comparisons showed significant interactions between temperature and jellyfish factors for treated fish but not for control groups (Table 2, Fig. 2a). The number of goblet cells was significantly higher in fish exposed to jellyfish than in controls; also, the number of chloride cells differed significantly between control and exposed fish, but not between control fish at different temperatures (Table 1, Fig. 2b,c).

Factor	Gill damage			Goblet cells			Chloride cells		
	df	H value	P value	df	F value	P value	df	F value	P value
Jellyfish	1	50.651	$1.103\ e^{-12}$	1	18.869	0.001	1	6.510	0.015
Temperature	1	0.010	0.919	1	3.996	0.046	1	3.580	0.063
Jelly. x Temp.		–		1	0.930	0.334	1	3.846	0.062

Table 1. Statistical results for histopathological gill damage. Kruskal-Wallis test for temperature and jellyfish factors and one-way ANOVA analyses for goblet cells and chloride cells. $p < 0.05$ was considered significant.

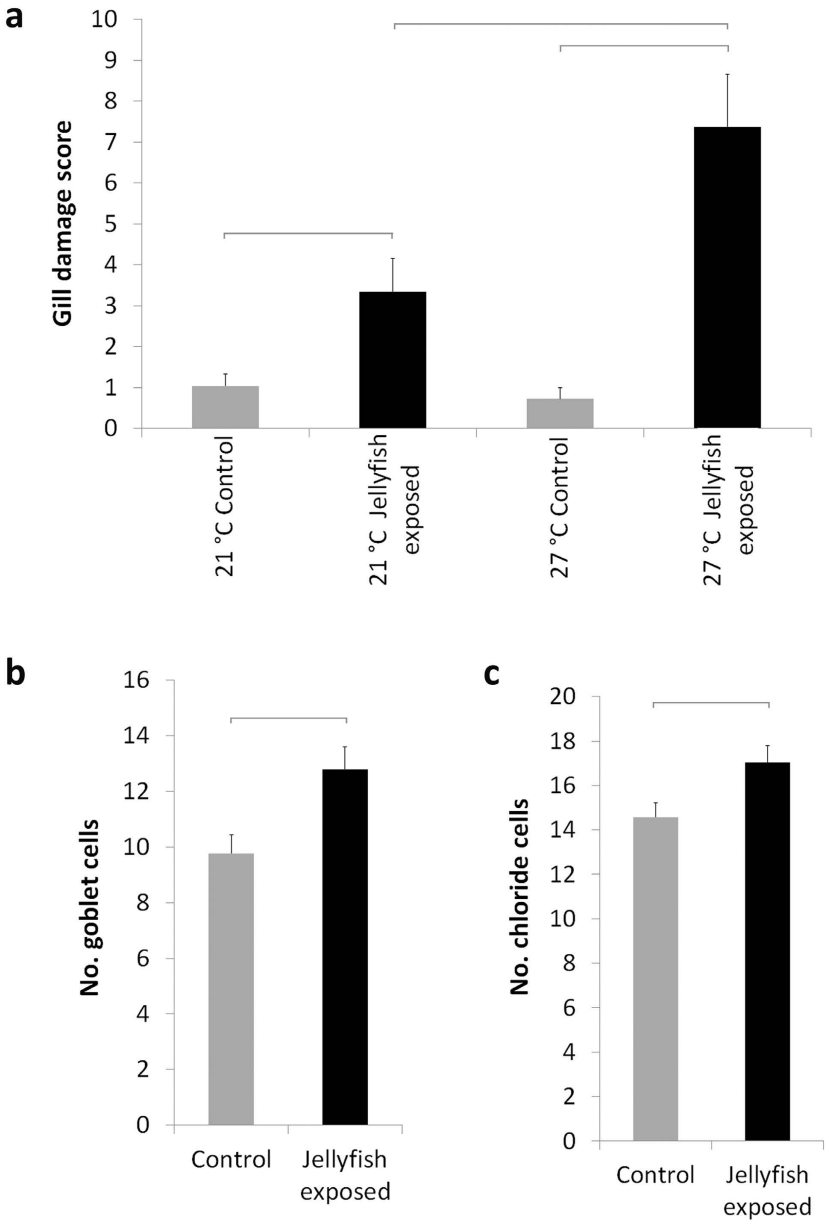

Figure 2. Gill damage scores (10-point scale) for each treatment (**a**), numbers of goblet cells (**b**) and chloride cells (**c**). Fish exposed to jellyfish (black bars) and control fish (grey bars). Horizontal grey lines indicate significant differences among treatments ($p < 0.05$), based on Kruskal-Wallis test for gill damage scoring and one-way ANOVAs for goblet and chloride cells.

Respirometry measurements. The oxygen consumption rate that approximates the routine metabolism (MO_2) of *D. labrax* juveniles was affected by jellyfish and temperature treatments (Table 3). In addition, statistically significant differences in the critical oxygen pressure (PO_{2crit}) were found between control and

Gill damage	Jelly. x 21 °C	Controls x 21 °C	Jelly. x 27 °C	Controls x 27 °C
Jelly. x 21 °C	–	**<0.0005**	**0.0007**	**<0.0005**
Controls x 21 °C	51.0	–	**<0.0005**	0.1869
Jelly. x 27 °C	117.0	21.0	–	**<0.0005**
Controls x 27 °C	636.0	417.5	24.0	–

Table 2. Pair-wise comparisons for histopathological gill damage among temperature (21 and 27 °C) and jellyfish (Jelly.) treatments. The F-values (lower left) and the p-values (upper right) are reported. Because multiple comparisons were performed, the Bonferroni's correction was applied to the p-value (0.05/6 = 0.0083) and significant results are in bold.

	MO_2			$PO_{2\ crit.}$		
Factor	df	F value	P value	df	F value	P value
Time	1	1.357	0.246	1	2.349	0.128
Jellyfish	1	19.231	$2.46\ e^{-05}$	1	46.172	$4.13\ e^{-10}$
Temperature	1	53.849	$2.56\ e^{-11}$	1	0.173	0.678
Jelly. x Temp.	1	7.230	0.008	1	3.156	0.078

Table 3. ANCOVA statistics for oxygen consumption rates (MO_2) and critical oxygen levels ($PO_{2\ crit}$) of fish exposed to different temperatures (21 and 27 °C) and exposed or not to jellyfish. $P < 0.05$ was considered significant.

MO2	Jelly. x 21 °C	Control x 21 °C	Jelly. x 27 °C	Control x 27 °C
Jelly. x 21 °C	–	0.0354	**<0.0005**	0.0299
Control x 21 °C	4.63	–	**<0.0005**	**<0.0005**
Jelly. x 27 °C	39.75	60.83	–	**<0.0005**
Control x 27 °C	4.95	14.38	15.03	–

Table 4. Pair-wise comparisons for oxygen consumption rates (MO_2) among temperature (21 °C and 27 °C) and jellyfish (Jelly.) factors. The F-values (lower left) and the p-value (upper right) are reported. Because multiple comparisons were performed, the Bonferroni's correction was applied to the p-value (0.05/6 = 0.0083) and significant results are in bold.

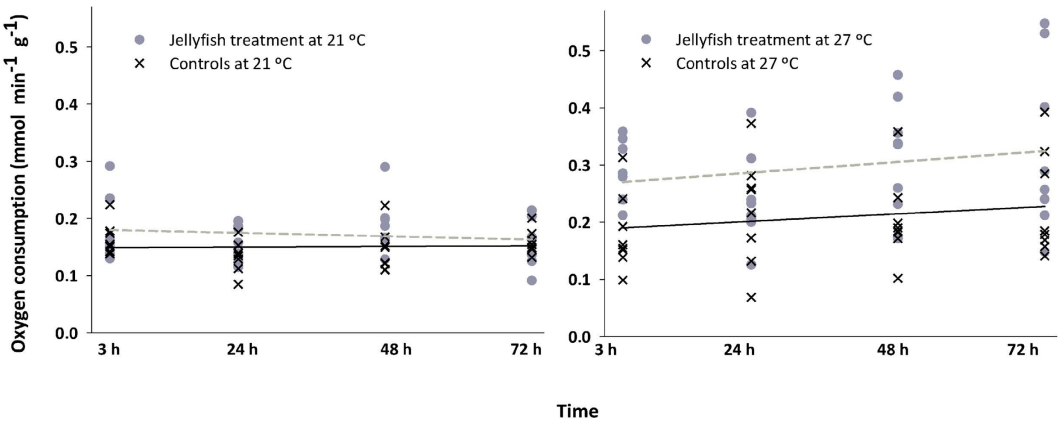

Figure 3. Oxygen consumption rates of *Dicentrarchus labrax* juveniles exposed to *Pelagia noctiluca* contact. Fish exposed to jellyfish are represented by grey dots and a dashed regression line; control fish are represented by black "x" and a continuous regression line. Experiments performed at 21 °C and 27 °C are shown on the left and right panels, respectively. X axes correspond to the time after the fish were exposed to jellyfish. Overall, oxygen consumption rates did not change over time: r^2 is 0.02 for treated and 0.002 for controls at 21 °C (n.s.) and 0.04 for treated and 0.03 for controls at 27 °C (n.s.). Regression lines have equal intercepts at 21 °C; however, treated fish have higher oxygen consumption rates than controls at 27 °C; see Table 4 for significances.

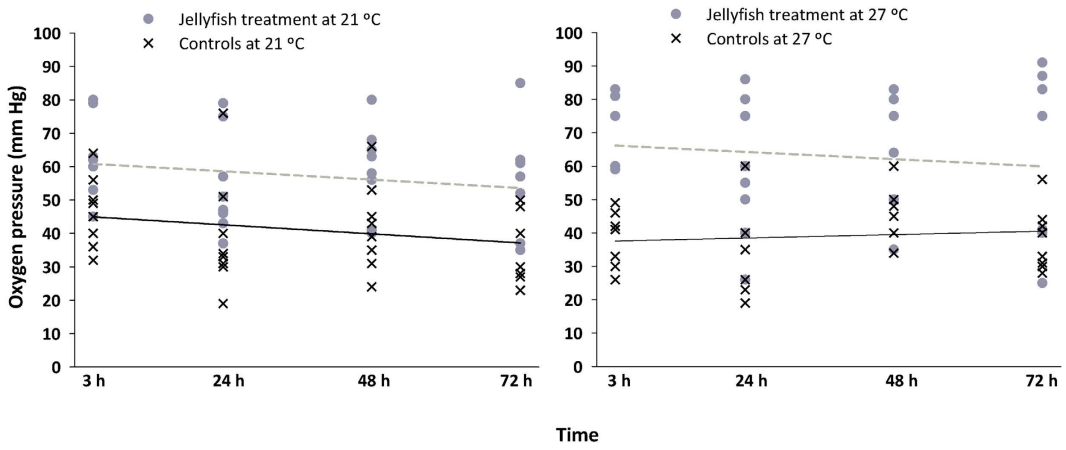

Figure 4. Critical oxygen pressures of *Dicentrarchus labrax* juveniles exposed to *Pelagia noctiluca* contact. Data from fish exposed to jellyfish are represented by grey dots and a dashed regression line; data from control fish are represented by black "x" and a continuous regression line. Experiments performed at 21 °C and 27 °C are shown on the left and right panels, respectively. X axes correspond to the time after the fish were exposed to jellyfish. PO_{2crit} did not change over time: r^2 is 0.04 for treated and 0.05 for controls at 21 °C (n.s.) and 0.02 for treated and 0.01 for controls at 27 °C (n.s.). Regression lines have different intercepts at 21 °C and 27 °C showing higher PO_{2crit}s for fish exposed to jellyfish.

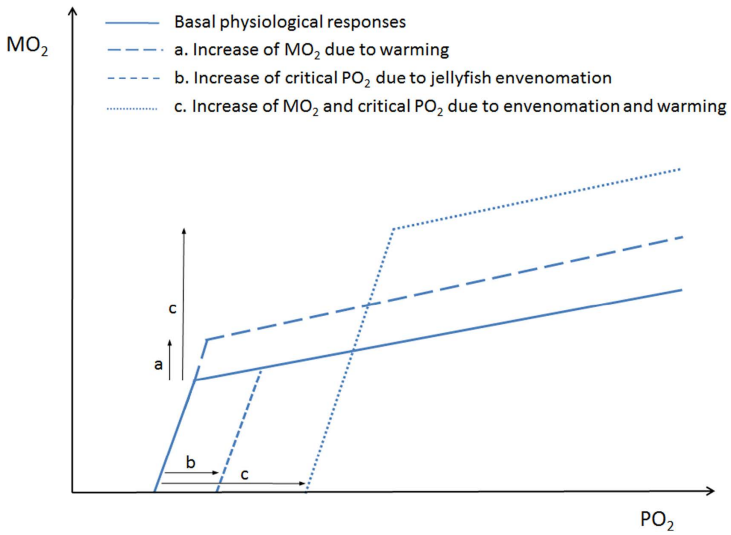

Figure 5. Unifying model of physiological responses of fish to the interaction of ocean warming and jellyfish stinging. Dashed lines represent the responses to single factors alone. Briefly, the rise of water temperature is mirrored by an increase of oxygen consumption rate (MO_2), but does not affect the sensitivity of fish to declining environmental oxygen tension (PO_2) (long dashed line); by contrast, jellyfish envenomation causes increased PO_{2crit} , which enhances the sensitivity to hypoxia (short dashed line). The dotted line represents the physiological response to the interaction of both factors and shows the enhanced vulnerability of fish.

jellyfish-treated fish, but not between temperatures. No significant changes were observed on fish MO_2 and PO_{2crit} over time following their exposure to *P. noctiluca* tissues (Table 3).

MO_2 values were significantly different at the two temperatures (Table 4, Fig. 3). Oxygen uptake was equivalent in fish exposed to jellyfish stings and control fish at 21 °C, whereas fish exposed to jellyfish at 27 °C had higher MO_2 than controls. PO_{2crit} at 21 °C and 27 °C were significantly different, with higher PO_{2crit} values in jellyfish-treated fish (averages ranged between 33–55 and 53–70 mm Hg, respectively) than in control fish (Table 3, Fig. 4). The observed changes of physiological responses of *D. labrax* juveniles (as MO_2 and PO_{2crit} values) related to temperature and/or exposure to jellyfish tissues were represented in a conceptual model (Fig. 5). The higher temperature led to increased fish oxygen consumption rate (a), and the jellyfish stings produced an increased PO_{2crit} (b). The combined effects of temperature and jellyfish stings caused higher oxygen uptake and PO_{2crit} value (c) than the separate effect of either factor. The synergistic action of envenomation and increased temperature increased the PO_{2crit} value (i.e., anticipating the switch from the aerobic to anaerobic metabolism), thereby increasing fish sensitivity to hypoxic conditions.

Discussion

Previous studies on jellyfish impacts on farmed fish hypothesized that respiratory distress may impair the overall fish metabolism[23,25]. Here, for the first time, we used an integrated approach to investigate the effects of jellyfish blooms on farmed fish by the combined analysis of fish gill integrity and metabolic rates. Significant effects (increased gill damage, oxygen consumption, and critical oxygen pressure) were observed in fish at higher temperature and exposed to jellyfish.

The increased histological damage in juvenile *D. labrax* exposed to *P. noctiluca* jellyfish corroborated previous observations of adult salmon (*Salmo salar*) with severe skin and gill injuries induced by jellyfish contacts, which significantly affected fish health and survival[23,25]. Severity of gill injuries increased with factors interaction (temperature and exposure to jellyfish), which reduced gill plasticity and functioning. The observed thickening of the gill epithelium due to hyperplasia may increase the diffusion distance for gas exchange, having profound effects on the efficiency of oxygen transfer across the gill[32,33]. The increase in goblet cell numbers in fish contacted by jellyfish was paralleled by [I] increased production of mucus (data not shown), which acts as a protective barrier against microbial infections[34,35] but also forms a barrier to oxygen diffusion and contributes to hypoxia[36], and [II] an increase of chloride cells in the respiratory epithelium, which is a common response to environmental (chemical or physical) stresses, such as low-calcium and low-magnesium water, or the detection of toxicants[37,38].

With increasing temperature, metabolic rate and oxygen demand of ectothermic fish usually increase, but oxygen solubility declines, which exacerbates the problem caused by increased respiratory activity[2]. European sea bass increases cardiorespiratory and swimming performances in response to increased temperature[39,40]. Similarly, higher oxygen consumption rate[41,42], growth rate, food intake and feeding efficiency[43] also occur in higher temperature. Several studies indicate that temperature and hypoxia are likely to interact synergistically on fish metabolism[2,44,45]. PO_{2crit} values in fish usually increase when temperature rises[46,47]. Increased temperatures typically cause a decrease in the affinity of hemoglobin for oxygen, limiting oxygen uptake at the gills and, as a consequence, reducing fish tolerance to hypoxic conditions[2,31]. By contrast, other studies suggest that increased temperature may not affect the tolerance to hypoxia in some fish species due to the intervention of homeostatic mechanisms (e.g. the recruitment of tissue glycogen or liver lactate clearance capacity)[48]. An increase in PO_{2crit} values has been observed during digestion processes[49] and may explain why hypoxic conditions might reduce appetite and growth in many fish species[49,50]. Increased PO_{2crit} values have also been observed after contamination by xenobiotics such as heavy metals, pesticides, or nanoparticles in coastal waters[51,52].

As suggested by the conceptual model (Fig. 5), our results support the hypothesis that exposure to jellyfish stings and envenomation may act synergistically with temperature, reducing fish metabolic performance, impairing their ability to withstand hypoxic conditions and, as a consequence, reducing the available energy for critical processes such as growth and reproduction[2]. Furthermore, jellyfish venoms may have hemolytic properties[53] leading to exacerbation of hypoxia. In conclusion, the interaction of jellyfish envenomations with increasing temperatures may result in greater vulnerability to hypoxic conditions and in the overall reduction of fish physiological performances.

The reduction of fish homeostatic potentials due to jellyfish outbreaks in coastal waters may co-occur to produce economic losses to aquaculture facilities. Our study suggests that the interaction of direct climatic stressors (e.g. warming) with indirect effects of global change (e.g. increasing jellyfish outbreaks) may exacerbate negative impacts on fish stocks. The consequences of such interactions for human activities are numerous, but mainly affect fisheries and aquaculture. Due to the continual growth of the aquaculture sector and the increased frequency of jellyfish blooms in coastal areas, the negative interactions of stinging jellyfish on farmed fish is expected to become a substantial, recurrent issue. More research on the effects of multiple stressors on fish populations in a global change scenario is needed for a better management of living resources and the development of effective mitigation plans.

Materials and Methods

Ethical statement. The study was performed in accordance with the EU Directive 2010/63 and Italian DL 2014/26; the experimental protocol was approved by the University of Palermo. Maintenance and handling of animals during the experiment, as well as the euthanasia procedure, were monitored and carried out by authorized staff to minimise the animals' suffering.

Animal collection and maintenance. Two hundred juvenile *Dicentrarchus labrax* (19.5 ± 5.5 g, means $\pm$ S.E.) were obtained from an aquaculture facility near Licata (Sicily, Italy). The choice of juveniles was related to severe mortality events caused by jellyfish in different Mediterranean aquaculture facilities where the most affected fish class ranged 15–60 g in weight[28].

The fish were kept in tanks with seawater from a closed recirculated seawater system at controlled salinity, temperature and photoperiod (means $\pm$ S.E., 37.8 ± 0.08 salinity, 19.4 ± 0.4 °C, 12 h: 12 h light-dark regime). Acclimation at the experimental temperatures (21 °C and 27 °C) was gradually achieved during the week before the start of the experiments. The fish were fed daily with 2.5% of their body mass of commercial fish feed during the acclimation period. For the duration of the experiments, the fish stock density was maintained between 12.5 and 14 kg m^{-3}, as used in *D. labrax* aquaculture cages (9–15 kg m^{-3}). Jellyfish were collected by hand net from the port of Messina (Sicily, Italy) the day before the experiments and were maintained in 25-L buckets with filtered seawater and at low density (5 jellyfish per bucket) for one day.

Experimental setup. The experiment was carried out at two temperatures, 21 °C and 27 °C. A total of 128 fish (64 treated, 64 controls) were subject to metabolic measurements. Fish were transferred to the treatment tanks 24 h prior to the start of the experiment and maintained unfed to reduce possible anomalous metabolic

responses due to residual specific dynamic action. Sixteen 7-L treatment tanks were used for the 8-h contact period, each of them containing five fish to maintain the experimental stock density. The contact duration corresponds to a realistic night time period of high jellyfish concentration in surface waters[16,54].

To simulate a realistic encounter between caged fish and jellyfish pressed by currents through aquaculture cages, jellyfish tissues were manually cut in small pieces ($\geq$1 cm) immediately prior to the start of the jellyfish exposure. The jellyfish density used was 25 medusae m^{-3} [23]. Tanks were supplied with air to keep dissolved oxygen at maximum levels and ensure contact between jellyfish pieces and fish. The treatment started when jellyfish tissues were randomly placed in 8 of 16 tanks with fish, whereas the other 8 tanks served as controls without jellyfish. Immediately after the exposure period at each temperature, all replicate fish groups were pooled into two 60-L tanks, according to their experimental status (treated or control) to maximise randomisation of subsequent fish sampling for metabolic measurements. At each of four different sampling times (3, 24, 48 and 72 h after the end of the contact period), 8 fish (4 control and 4 treated) were individually transferred into the respirometric chambers for acclimation. We opted to use closed respirometric chambers rather than swim tunnels to allow fish routine activity and spontaneous movement in a confined environment. We postulate this approach would fairly reflect the routine metabolic rate of cultured fish at high density and constrained living space conditions, such as in farming cage systems.

Respirometry and determination of critical oxygen pressure (PO_{2crit}). At each temperature (21 and 27 °C), eight independent 2-L closed respirometers supplied with filtered sea water (Millipore GF/C 0.45 μm) were used to measure the oxygen consumption rate of individual fish. Chambers were covered with an opaque plastic material to avoid visual stresses to fish throughout measurements. An agitator and small magnets were used to maintain homogeneous water mixing inside the experimental chambers. At each sampling time (3, 24, 48 and 72 h after the end of jellyfish exposure), four treated and four control fish were randomly sampled and placed in individual respirometers. Fish were left undisturbed in the respirometers for 3 h with supplemented air to keep dissolved oxygen at the saturation level. Then the aerators were removed and chambers were carefully refilled of water and hermetically closed. Fibre-optic oxygen meters calibrated according to instructions by Pyro Science (Aachen, Germany) were used to record water oxygen levels. Fish were maintained in the respirometric chambers until the slope of the oxygen concentration curve changed suddenly. In most cases, that change occurred at 5 to 30% of the initial oxygen concentration. At the lowest oxygen concentration fish status was surely affected but no mortalities were recorded over the complete duration of the experiment. Fish were then removed from the respirometers, marked by a small cut in the caudal pin and returned to the original tank in order to maintain the initial density. All fish recovered well after hypoxic exposure.

Oxygen consumption rates to approximate routine metabolism (MO_2) were calculated from the decrease in oxygen content in the respirometers over time and expressed as mmol min^{-1} g^{-1}. Those times were standardized to 45 min at 21 °C and 15 min at 27 °C within the range during which the fish were able to oxyregulate. The critical oxygen pressure (PO_{2crit}), which represents the transition from oxyregulation to oxyconformation during the progressive decline of environmental oxygen tension[30], was calculated as the break-point of the graph depicting the PO_2 − MO_2 relationship. PO_{2crit} was expressed in mm Hg.

Histological analysis of gill tissue. The experiments were performed in full compliance with the national rules (D.Lgs 116/92 and subsequent amendments) and the European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC). After the last sampling time (72 h), 16 experimental fish (4 controls and 4 treated at each temperature) were anaesthetised with 0.05% w/v MS222 (3-aminobenzoic acid ethyl ester) and then killed according to the current animal care rules using a lethal dose of MS-222 (0.1% w/v). Two gill arches were excised from each fish and immediately preserved in 10% neutral buffered formalin for 48 h and transferred to 70% ethanol for histological analysis. After dehydration, tissues were embedded in Paraplast (Bio-Optica), cut by microtome into 5 μm sections and stained using Hematoxylin-Eosin as "routine-staining" to reveal the underlying tissue structures and conditions. Moreover, the Periodic Acid Schiff (PAS) technique was used to identify the goblet cells. The localisation and the number of chloride cells was determined by immunocytochemical techniques by using a primary antibody that recognised sodium, potassium and chloride cotransporters ($Na^+/K^+/Cl^-$ cotransporters NKCC1-T4 1:200) revealed by a second antibody Donkey Anti-Rabbit IgG (H+L) Alexa Fluor 488 (AF488) Conjugate (Southern Biotech), 1:200. For each gill arch, 9 randomly selected tissue areas, between 25 and 34 μm^2 were screened at 200X and 400X to count the number of chloride cells.

Gill damage score. Interpretation of the gill damage was based on a recently developed gill histopathology scoring system (GHS index, Mandich *et al.* in prep.) that rates the damage on each gill sample by a total score obtained by summation of partial scores assigned to 12 different criteria. The evaluation of gill damage was performed as follows: for each gill sample a total of 9 sections (photographed fields), each with 10 secondary lamellae were evaluated for all 12 histopathological criteria. For each criterion, the score ranged from 0 to 6 depending on the extent and intensity of injuries (0: no significant damage, 1: damage in 1–2 of 10 lamellae; 3: damage in 3–5 of 10 lamellae; 6: 6–10 of 10 lamellae damaged). Gill damage could be of different grades of severity, and advanced gill damage could mask previous mild injuries. Therefore, the GHS index was supplemented by a secondary classification system to separate different stages in the progression of tissue damage (according to Santos *et al.*[55]). All histopathological criteria were split into 3 groups. The first group (first-stage lesions) was composed of hyperplasia (cell number increase) of the lamina and the secondary lamellae, lamellar fusion, reduction of the lamellae, lamellar oedema (accumulation of an excessive amount of watery fluid in the intercellular spaces), and cellular hypertrophy (cell size increase). The second-stage lesions included circulatory disturbances of the lamina such as telangiectasia (dilation of groups of capillaries) and grave cellular anomalies (presence and extension of lamellar lifting); these

more severe injuries lead to effects on tissue functions; the third -stage lesions included the appearance of haemorrhage, high granulocyte concentrations, and cellular degeneration of the respiratory epithelium or necrosis, which represent irreparable damages. The score assigned for each criterion was multiplied according to the severity group ($\times 1$: mild damage group; $\times 10$: moderate injury group; and $\times 100$: the most severe gill damage group).

The goblet and chloride cells were visually counted in each section and analysed separately from the other histopathological criteria.

Statistical analysis. To obtain critical oxygen pressures (PO_{2crit}) and approximate the break-point in the respiration curve, a Piecewise linear regression function was used (SigmaPlot v.11).

Normality of respiration data was confirmed with a Shapiro-Wilk test. To test the statistical significance among treatments for MO_2 and for PO_{2crit}, ANCOVA analyses were used, considering MO_2 and PO_{2crit} as the *response* variables, time after the exposure period as a *continuous* explanatory variable, and temperatures and jellyfish treatments as *categorical* explanatory variables.

The assumptions of normality were not encountered for the histopathological data (Shapiro-Wilk test, $p < 0.05$). One-way Kruskal-Wallis test was performed to test the statistical significance among jellyfish-exposed and control fish at each temperature. Significant results were further tested by pairwise post-hoc comparisons (Wilcoxon test) adjusted for type I error. Differences in goblet cells and chloride cells were analysed using one-way analysis of variance (ANOVA).

The statistical software R (R Core Team 2015, v.3.2.2) was used to perform all analyses. Package ("coin") was used to perform the Wilcoxon test[56].

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Author Contributions

M.B.B., V.F., S.P. and A.R. conceived and designed the experiments. M.B.B., F.G. and A.R. carried out the experiments and collected the data. M.B.B. and A.M. performed the histological analysis. M.B.B. and F.G. performed the data analysis. G.S. and S.M. provided the experimental animals, laboratory space and facilities for the experiment. M.B.B., F.G. and S.P. wrote the manuscript and all authors reviewed and edited the manuscript.

Additional Information

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PLOS ONE

RESEARCH ARTICLE

Jellyfish Stings Trigger Gill Disorders and Increased Mortality in Farmed *Sparus aurata* (Linnaeus, 1758) in the Mediterranean Sea

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Abstract

Jellyfish are of particular concern for marine finfish aquaculture. In recent years repeated mass mortality episodes of farmed fish were caused by blooms of gelatinous cnidarian stingers, as a consequence of a wide range of hemolytic, cytotoxic, and neurotoxic properties of associated cnidocytes venoms. The mauve stinger jellyfish *Pelagia noctiluca* (Scyphozoa) has been identified as direct causative agent for several documented fish mortality events both in Northern Europe and the Mediterranean Sea aquaculture farms. We investigated the effects of *P. noctiluca* envenomations on the gilthead sea bream *Sparus aurata* by *in vivo* laboratory assays. Fish were incubated for 8 hours with jellyfish at 3 different densities in 300 l experimental tanks. Gill disorders were assessed by histological analyses and histopathological scoring of samples collected at time intervals from 3 hours to 4 weeks after initial exposure. Fish gills showed different extent and severity of gill lesions according to jellyfish density and incubation time, and long after the removal of jellyfish from tanks. Jellyfish envenomation elicits local and systemic inflammation reactions, histopathology and gill cell toxicity, with severe impacts on fish health. Altogether, these results shows *P. noctiluca* swarms may represent a high risk for Mediterranean finfish aquaculture farms, generating significant gill damage after only a few hours of contact with farmed *S. aurata*. Due to the growth of the aquaculture sector and the increased frequency of jellyfish blooms in the coastal waters, negative interactions between stinging jellyfish and farmed fish are likely to increase with the potential for significant economic losses.

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Introduction

In recent years, negative interactions between jellyfish blooms (JB) and marine finfish aquaculture have been reported. Such interactions have included mass fish mortalities with severe economic impacts on the aquaculture companies [1,2]. Jellyfish can enter fish cages either intact or broken up into tentacles and other body fragments pushed by currents and waves washing in through the net cages [3,4]. Several species of cnidarian jellyfish have been reported to affect marine farmed fish of inducing skin lesions and gill damage caused by nematocyst discharge and venom injection usually leading to local inflammatory response, cell toxicity and histopathology [2,3,5]. Prolonged nematocyst discharges in fish tissues may often lead to secondary bacterial infections and associated systemic reactions, including respiratory and osmoregulatory distress, altered behaviour, and death [2,6–8]. In particular, gills have vital roles, being the main site of gas exchange, osmoregulation, acid-base balance, and excretion of nitrogen compounds [9]. Gill disorders have become one of the most serious causes of mortality in marine farmed salmon in Ireland, with average losses of 12% per year [6].

The scyphomedusa *Pelagia noctiluca* (Forsskål, 1775), also known as mauve stinger, is one of the most common stinging jellyfish species across the Eastern Atlantic and the Mediterranean Sea, producing major outbreaks with subsequently highly negative impacts on human activities, including caged finfish aquaculture [10,11]. On the Mediterranean Spanish coast, *P. noctiluca* is responsible for gill damage on the marine farmed fish *Dicentrarchus labrax*, leading to reduction of fish growth rate and even death [12]. Additional fish mortality events related to *P. noctiluca* abundance have also been recorded in Tunisian facilities (unpublished data). In 2007, a widespread occurrence of mauve stingers were documented in Irish coastal and shelf waters and caused several hundred thousand salmon mortalities [13,14]. Since then there have been several other large fish kills in UK and Irish waters [15,16]. In the same region, a bloom of moon jellyfish *Aurelia aurita* was responsible for a significant salmon mortality in summer 2010 [14,17]. Other jellyfish have also been identified as potentially harmful species for aquaculture facilities, such as the hydromedusae *Solmaris corona* and *Phialella quadrata* [3], and the siphonophore *Muggiaea atlantica* that caused the death of $>$ 100,000 farmed fish in Norway [18]. Previous studies demonstrated also that some jellyfish species—such as *P. quadrata* and *P. noctiluca*—can act as vectors of *Tenacibaculum maritimum*, the causative agent of tenacibaculosis, a major bacterial disease affecting fish mariculture worldwide, which heavily exacerbates the impacts of jellyfish sting envenomations [19–22].

Impacts of low to medium jellyfish abundances usually remain unnoticed by aquaculture farmers and low incidence of unspecific pathologies are labelled as unknown "water borne irritant damage" [15]. However, substantial gill disorders to produce low-level mortalities might be potentially correlated also to low jellyfish abundances (Baxter el al. 2011).

Much work has been carried out on the impacts of jellies on farmed salmon aquaculture in Northern European waters [3,6,23,24]. Comparatively, little or no information is available about the impacts of one of the most harmful European jellyfish species, *P. noctiluca*, on the commonest Mediterranean finfish aquaculture species, such as the sea bass *D.labrax* and the gilthead sea bream *Sparus aurata* (Linnaeus, 1758). Due to its high adaptability to intensive rearing conditions, *S. aurata* represents one of the most suitable species for cultivation in ponds and marine cages, leading to the most important fish production in the Mediterranean Sea, reaching near 160.000 tonnes in 2012 [25]. In parallel, overproduction led to cutbacks in market price, calling for further reduction of production costs.

To increase knowledge on impacts of gelatinous plankton blooms on Mediterranean caged fish species and support early monitoring of risks for aquaculture production, an experimental assay was set up to assess [I] the potential histopathological damage that *P. noctiluca* jellyfish

tissue fragments produce on gills of cultured *S. aurata*, [II] the impacts of different jellyfish densities on cultured fish health, and [III] the histological evolution of gill lesions over time following initial jellyfish sting treatment.

Material and Methods

This study was performed in accordance with the European Commission Directive 2010/63/EU. The experimental protocol was designed to comply with the European policy of the "3 Rs" (Reduce, Refine, and Replace) in aquatic animal experimentation and was approved by the Institut Supérieur de Pêche et d'Aquaculture de Bizerte (Research unit 05/ur/11-15), which is under the double supervision of the Tunisia's Ministry for Agriculture and the Hydraulic resources, and of the Ministry for Higher education and the Scientific Research and Technology.

Fish were monitored daily (early in the morning and during afternoon) over the complete experiment duration. Check-list including different humane endpoints was revised at group and also at individual level when necessary. The main established criteria were swimming behavior, skin pigmentation, frequency of opercular movements, ability of food uptake, weight loss, prostration, hyper-excitability and itching. The maintenance of animals during the experiment as well as the euthanasia procedure was monitored and carried out by trained and competent staff, in order to minimise animals' suffering.

Animals' maintenance and experimental setup

A total number of 136 *Sparus aurata* adult fish (mean weight of 200 ± 19.23 g) were obtained from "Tunisian Teboulba Fish" aquaculture facility and transported to the Institut Supérieur de Péche et d'Aquaculture de Bizerte, Tunisia (ISPA). Fish were homogeneously distributed in 8 circular tanks of 300 litres each (fish stocking density of around 9 kg m^{-3}) and allowed to acclimate for one week before starting the experiment. All tanks were supplied by a continuous flow (renewal rate of 23 l h^{-1}) of double-filtered (5-μm, 1-μm mesh) seawater (FSW). The water circulation flow was kept at natural sea temperature of 15.5 ± 1.0°C and 36.8 ± 0.3 salinity) with aeration to keep dissolved oxygen at 100% saturation. Throughout the experiment, the fish were fed daily with standard commercial pellets (Skretting S.A.) and maintained under a natural photoperiod (12 h light, 12 h dark).

Jellyfish (4.5 ± 0.9 cm bell diameter) were collected by a dip net the day before the start of the experiment from the Channel of Bizerte (Tunisia) and maintained in 25 litres buckets with FSW and at low density for one day. *Pelagia noctiluca* jellyfish is not an endangered or protected species. Specimens from Bizerte gulf were collected without the need of a permit because sampling was never conducted in a restricted marine area.

To simulate a realistic encounter between jellyfish that had been pressed by currents against aquaculture cages and cultured fish, jellyfish were chopped into small ($\geq$ 1 cm) pieces immediately prior to the start of the jellyfish exposure. The four treatment groups consisted of two control tanks (without jellyfish) and six tanks with chopped *P. noctiluca* at low (LJ), medium (MJ), and high jellyfish densities (HJ): 3, 7 and 15 jellyfish per tank with 18 experimental fish (10, 25 and 50 jellyfish m^{-3}, approximately equivalent to 350 g, 875 g and 1750 g jellyfish biomass, respectively). These densities were predetermined to reproduce a range of different jellyfish concentrations observed during *P. noctiluca* bloom periods in Tunisian waters and Sicily Channel (unpublished observations). A 1-mm stainless steel mesh was placed at the outlet of each tank preventing jellyfish pieces to spill out the experimental tanks.

The experiment began when jellyfish pieces were placed simultaneously in all treatment tanks with fish. The maximum fish-jellyfish interaction lasted 8 h; after that, all jellyfish pieces

were removed using a 200-μm mesh hand net. The exposure time to jellyfish tissue of 8 h was used to represent the minimum night time with *P. noctiluca* jellyfish in surface waters, following sunset and the diel vertical migration of their crustacean prey [26–28].

Fish health was monitored nine times during the experiment: shortly before jellyfish incorporation to the fish tanks (0 h), during fish-jellyfish contact (3h), one hour after the removal of the jellyfish (9h), and six later times, 24 and 48h; 1, 2, 3 and 4wk, respectively before the end of the experiment at 4 weeks. At the highest jellyfish density sampling was not carried out at 24 h, 3 and 4 weeks because of the shortage of experimental individuals and fish mortalities. At each sampling time, 4 fish were randomly sampled from each treatment group (two per tank), anesthetised and then killed according to the current animal care rules using a lethal dose of UNICAINE 2% (lidocaine-HCl 500 ppm) [29]. Immediately after death, which occurred within 2–3 minutes of anaesthetic application, fish were weighed and measured, and their skin and gills visually examined for gross pathology, such as scale loss, excess mucus, pale gill filaments, swelling, necrosis and the presence of macro-parasites [30]. Two gill arches were excised from each fish and immediately preserved in 10% neutral buffered formalin for histological analysis. Tissues then were embedded in paraffin, cut by microtome into 2–5 μm sections and stained following a standard haematoxylin-eosin protocol. For each gill arch, several sections were examined microscopically at 100X and 400X magnifications.

Gill score protocol

Interpretation of the gill damage was based on a recently developed gill histopathology scoring system [4,12], rating the potential damage on each gill sample by a total score ranging from 0 to 24, obtained by summation of partial scores assigned to different primary and secondary criteria. Primary parameters were related to 3 specific pathologies: epithelial hyperplasia (increased cell production), lamellar fusion, and cellular anomalies (degeneration, necrosis and sloughing). According to the presence, extent and severity of those pathologies, primary scores ranged from 0 to 3. In addition, a 0 or 1 score was attributed to the absence or presence of each of the following secondary parameters: hypertrophy, oedema, eosinophilic granular cells, inflammation, circulatory damage, congestion, bacterial pathogens and parasitic pathogens. The total score assigned for primary and secondary parameters, allowed classification of fish gill damage according to four cumulative score ranges: 0–3 = no significant pathology, 4–6 = mild gill pathology of minor clinical significance, 7–9 = moderate gill pathology of clinical significance, $\geq$ 10 = severe gill pathology of high clinical significance.

Statistical analysis

A Shapiro-Wilk test indicated that the assumptions of normality were violated ($p < 0.05$, SPSS v. 20.0); therefore, differences among treatments and among sampling weeks were tested using the non-parametric one-way Kruskal-Wallis test (SPSS v.20.0). Significant results were further tested by pairwise post-hoc comparisons (Mann-Whitney U test, SPSS v. 20.0), adjusted for type I error, and Similarity percentages analysis, SIMPER (PRIMER 6).

Results

Gills from the control fish group without jellyfish retained a normal morphology throughout the experiment. Each gill arch supported many distinct and regular filaments arranged perpendicularly in two rows and without significant lesions. In contrast, gross pathology in fish exposed to jellyfish pieces was observed throughout the experiment (Fig 1), with the extent and intensity of gill damage increasing with time and jellyfish density (Fig 2).

Fig 1. External lesions on *Sparus aurata* due to *Pelagia noctiluca* jellyfish exposure. A. Fish gill from control group; B. abrasion, haemorrhage, depigmentation and increased thickness of lamellar filaments of a fish from the high jellyfish density group 24 h after exposure to jellyfish; C. wound with necrotic tissue on the flank of *Sparus aurata* fish from the medium density group 2 weeks after exposure to jellyfish.

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At 3 h after initial contact with jellyfish pieces, fish gills already showed abrasion of lamellar filaments (Fig 1A). After 24 h from the exposure to jellyfish, depigmentation, increasing thickness of lamellar filaments and haemorrhage in gill tissue were also recorded. Mild epitheliocystis [31,32] was observed in control and treated fish through the identification of spherical cysts that were circumscribed by an eosinophilic hyaline capsule. One day before the start of the experiment (24 h after the exposure to jellyfish), snout irritation, scale loss on the flanks and damage in the caudal and dorsal fins and operculum were also observed in fish in the medium and high jellyfish density groups (Fig 1B). Respiratory distress, jumping and swimming near

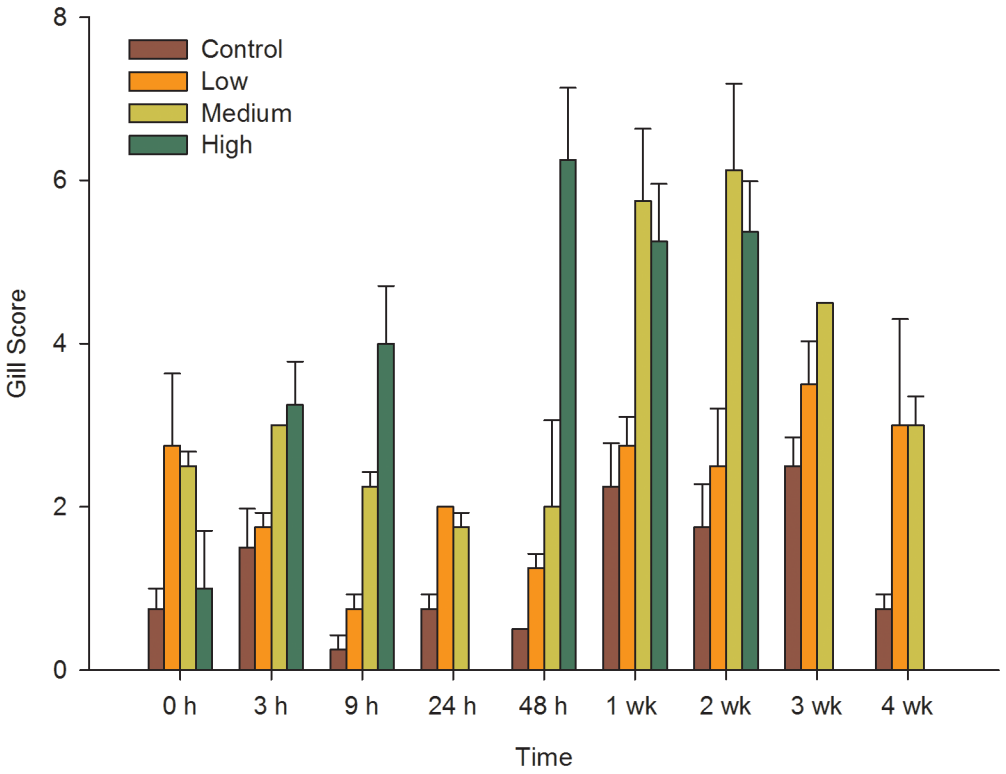

Fig 2. Average gill scores of treatment groups. Gill scores of control, low, medium and high *Pelagia noctiluca* jellyfish density groups before (0 h) and at different times after *Sparus aurata* exposure to jellyfish. Fish were not sampled from the highest jellyfish density group at 24 h, 3 and 4 weeks sampling points (vertical bars denote standard error).

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the water surface were also observed for some treated fish throughout the exposure period to jellyfish at different jellyfish densities. A slight trend of weight reduction was observed in treated fish, possibly due to the ceased feeding behaviour observed through the experiment, but no significant statistical differences were found in weight or length analysis.

The histopathological analysis showed that the lowest gill damage score was in the control group, characterised by low levels of lamellar hyperplasia and occasional fusion, a background level of pathology typical of marine-farmed fish [6]. Gill scores from the control group were significantly different (lower) than all the groups with jellyfish ($U_1 = 25.267$, $p = 0.001$). Gill scores also differed significantly among the groups treated with jellyfish ($U_2 = 7.050$, $p = 0.029$). The gill scores in the LJ density group showed no significant differences throughout the experiment ($U_8 = 12.604$, $p = 0.126$), with average scores of 2.25 ± 0.9 (SE). For the MJ density group, significant gill lesions were observed 1 week after the start of the experiment ($U_1 = 4.86$, $p = 0.027$), with scores peaking after 2 weeks (gill score 6 ± 1.5 SE). Significant gill damage was observed immediately in the HJ density group, only 3h after the exposure to jellyfish began ($U_1 = 4.513$, $p = 0.034$). Those high scores continued over time with a peak after 48h (6 ± 1.3 SE) (Fig 2)

Over the duration of experiment, 6 out of 136 experimental individuals died. Fish mortalities happened in the HJ density group during the second and third week of experiment, after the peak in gill damage scores. Gross pathology showed some slight external lesions mainly in fish flank. Fish showed excessive mucus production and pale gills, hyperplasia, severe lamellar fusion, desquamation, necrotic patches, lamellar congestion and lamellar oedema in some areas of the gills. Gill epithelium lesions are known to be responsible of respiratory problems and osmoregulation disorders, such as hydro-mineral equilibrium disturbances and alterations in the excretion of nitrogenous waste (NH_4^+). All these troubles leaded death of fish. In the MJ density group, gill scores decreased during the third and fourth week of sampling, mainly because of reduction in the percentages of hyperplasia and cellular anomalies. By contrast, fish from the LJ density group presented mild damage during the experiment, principally represented by hyperplasia and lamellar fusion (Figs 3 and 4).

The gill scores for the experimental treatment groups ranged from 1 to 9 over the entire experiment, with most fish displaying moderate lesions considered to be of clinical significance. The SIMPER analysis showed that lamellar fusion and hyperplasia were the most common lesions in all treated groups. Also, a severe inflammatory response was noted beginning at 9h after the exposure to jellyfish. The severity of gill damages was directly proportional to jellyfish density, with increasing cellular anomalies over time.

Discussion

Frequency of occurrence and abundance of *P. noctiluca* vary across the Mediterranean, but dense populations can be recorded most of the year at several coastal localities, such as the channel of Bizerte (Tunisia) and the Strait of Messina (Italy) [10,33,34]. Our laboratory experiments simulated the potential consequences of blooms of the scyphomedusa *P. noctiluca* on finfish aquaculture farms. Our results showed that jellyfish stings can severely affect caged *S. aurata* fish by causing significant gill damage shortly after contact with jellyfish tissues and subsequent deterioration on fish health.

Comparable gill damage was observed previously in farmed salmon (*Salmo salar*) during blooms of *P. noctiluca* and *Aurelia aurita* scyphomedusae in northern Europe [6,14]. This first experimental challenge trial between fish in culture and jellyfish exposed juvenile *S. salar* to realistic *A. aurita* jellyfish bloom densities showed significant and increasing gill damage starting 24 h after the initial contact [6].

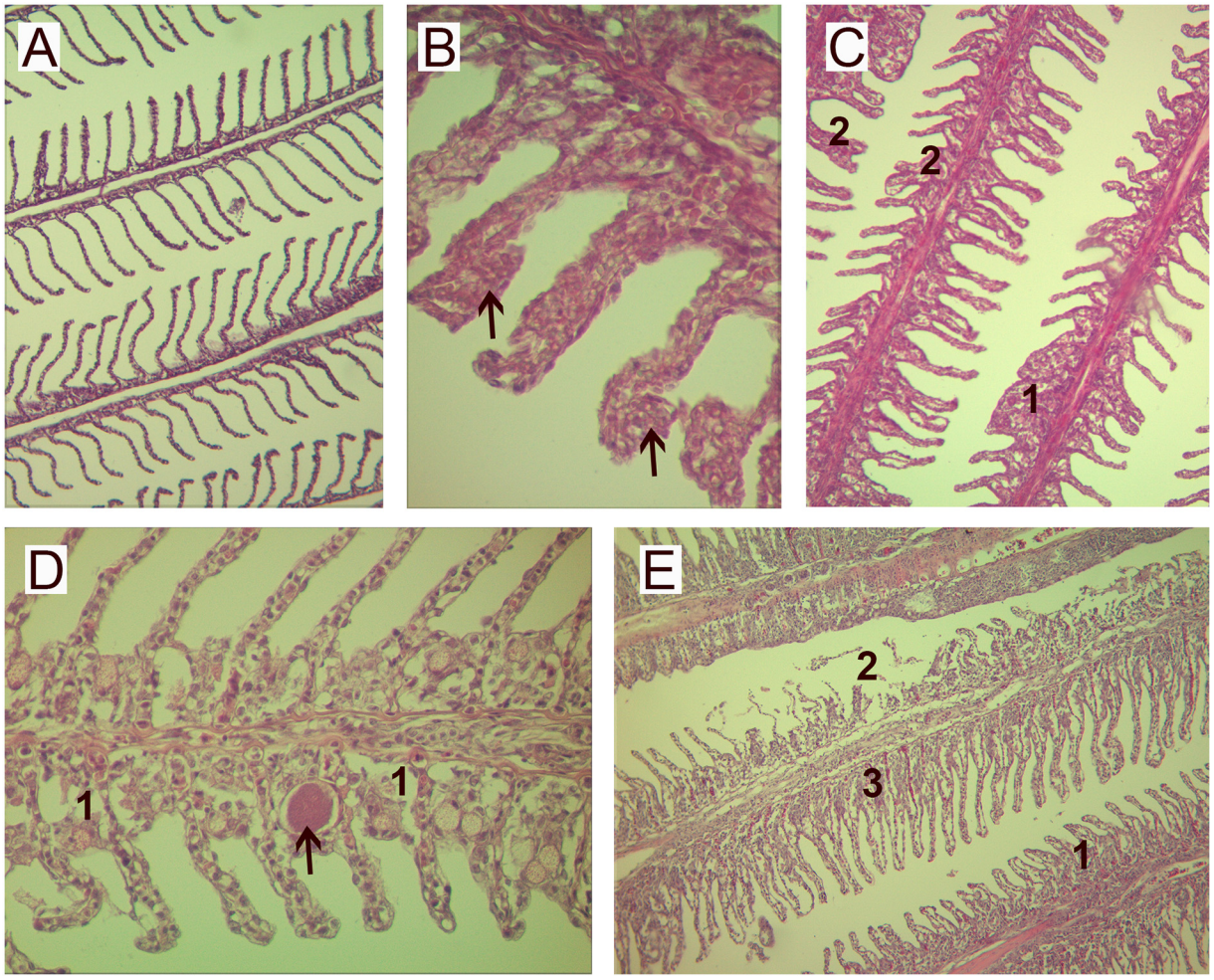

Fig 3. Gill lesions in fish exposed to *Pelagia noctiluca*. A. Healthy fish gill from the control (no jellyfish) group (0h) (100x); B-E. pathology in fish gills from the treatment groups after 8-h exposure to jellyfish: B. black arrows indicate lamellar hyperplasia on fish gill from the low jellyfish density group at 9h (400x); C. lamellar hyperplasia (1) and fusion (2) from the medium jellyfish density group after 1 week (100x); D. epitheliocystis (black arrow) and lamellar oedema (1) from the medium jellyfish density group after 3 weeks (400x); E. hyperplasia of the epithelium of the primary lamellae (1), necrosis focal of secondary lamellae (2) and circulatory disturbances (3) from the high jellyfish density group after 48h (100x).

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Treatment	p-value	0 h	3 h	9 h	24 h	48h	1 week	2 weeks	3 weeks	4 weeks
Control	p > 0.05	MLH	MLH	(---)	(---)	(---)	MLH	MLH	MLH MLF	MLH
Low	p > 0.05	MLH	MLH MLF	MLH	MLH MLF	MLH	MLH MLF	MoLH MLF	MoLH MLF	MoLH MLF
Medium	0.043*	MH	MLH MLF	MoLH	MLH	MLH MLF	SLH MoLF MCA	SLH MoLF	MoLH MoLF	MoLH MLF
High	0.041*	(---)	MoLH MLF	MoLH MLF MCA	(NA)	SLH MoLF MCO MCA	SLH MoLF MoCA	SLH MoLF FM	FM	

Fig 4. Histopatholical gill damage of experimental groups over time. MLH: Mild lamellar hyperplasia; MLF: Mild lamellar fusion; MoLH: Moderate lamellar hyperplasia; MoLF: Moderate lamellar fusion; MCA: Mild cellular anomalies; MoCA: Moderate cellular anomalies; SLH: Severe lamellar hyperplasia; MCO: Mild cellular oedema; FM: Fish mortality; (NA): data not available; (—): Non significant gill damage. Colours indicate the severity of gill damage: cream colour = mild injuries; orange = medium level of injuries; violet and purple = medium-high and high level of gill damage respectively.

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Here we investigated the intensity of gill damage on cultured sea bream at increasing *P. noctiluca* densities. At low jellyfish density (up to 10 jellyfish m^{-3}), mild damage to fish gills were observed. Conversely, at higher jellyfish concentrations ($\geq$ 25 jellyfish m^{-3}) impacts ranged from moderate damage, leading to potential effects on the fish metabolism, to more severe consequences including death, due to high levels of lesions and respiratory distress [2].

Three weeks after the initial exposure to jellyfish, fish from the medium density group showed early signs of tissue repair in the gills. Recovery was characterized by significant decreases in the percentages of lamellar hyperplasia and fusion, in observed inflammatory reactions, and disappearance of cellular anomalies. At last, recovery of tissue integrity was observed in fish in the MJ density group, whereas fish from HJ density died 2–3 weeks after exposure to jellyfish. Exposure to HJ density led to intense and increasing gill damage, eventually impairing homeostatic mechanisms and adaptive physiological responses [35]. Non bacterial infection of *Tenacibaculum* sp. was confirmed, due to the absence of filamentous bacterial mats on the necrotic patches [36]. Overall, these results indicate that even short exposure to jellyfish can result in significant gill damage in marine-farmed fish, with potential increase in extent and severity of damage even when jellyfish are no longer present.

Our results also indicate that the potential impact of jellyfish on marine wild fish populations might not be negligible. Previous research on fish-jellyfish interactions are mostly focused on jellyfish predation on fish or, conversely, the use of jellyfish biomasses by medusivorous fish as temporary or exclusive food source [34,37–39]. The outcome of jellyfish interactions with fish populations depends on several factors affecting the probability of encounters, including water temperature, dissolved oxygen, and the size and density of predators and prey [40]. For several jellyfish species, bloom density may reach extremely high values. *Pelagia noctiluca* in the Mediterranean Sea occurs in large swarms reaching densities over 100 medusae m^{-3} for prolonged periods (up to weeks), with temporary aggregations caused by wind, currents, coastal geomorphology and jellyfish behaviour containing up to 600 medusae m^{-3} [41,42]. These values largely exceed the experimental density values used in our fish-jellyfish interaction experiments (10, 25, 50 medusae m^{-3}). Furthermore, shortly after sexual reproduction—in springtime—large swarms of ephyrae and juvenile jellyfish are regularly encountered in the Southern Tyrrhenian Sea (Aeolian islands), with much higher densities, up to several thousands of individuals m^{-3}; (Piraino, pers. observation; see also https://goo.gl/G8GNl8). Temporary paramount densities may therefore represent a key threat affecting the physiological integrity and health of fish living in sheltered areas where extremely high jellyfish aggregations occur, such as bays or fjords (with records up to 1000 *Periphylla periphylla* medusae m^{-3} [43,44]).

Further investigations are required to clarify whether the potential rise of both temperature and jellyfish numbers in a global change scenario may exacerbate negative impacts not only on farmed fish, but also on wild fish populations [1,45,46].

The consequences of episodes of jellyfish proliferation can be of high importance for aquaculture, considering they could affect not only fish health, but also the growth and quality of caged fish [2,30]. The sudden and unpredictable nature of jellyfish blooms hinders the implementation of preventive measures against their negative effects in aquaculture. Because of this, the development and implementation of swift mitigation procedures are crucial and must be rooted in knowledge of the type and extent of physical damage caused by jellyfish. Even a low density of *P. noctiluca* jellyfish could be detrimental to the health of caged fish, causing minor but significant gill lesions, which may progress over time and be worsened by bacterial infections. Investigation of the different effects of *P. noctiluca* blooms will enable estimation of the response time required by aquaculture facilities to undertake appropriate countermeasures that could differ in magnitude according to the damage level. Due to the recent and projected future

growth of the aquaculture sector [47] and the increased frequency of jellyfish blooms in Mediterranean coastal waters [45,48], negative interactions between stinging jellyfish and caged finfish may turn into a substantial problem with high economic losses [14].

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Author Contributions

Conceived and designed the experiments: MBB VF. Performed the experiments: MBB CM MC. Analyzed the data: MBB CM RD SP OKDY. Contributed reagents/materials/analysis tools: MNDY MC RD. Wrote the paper: MBB CM SP VF MNDY OKDY.

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