Elsevier Editorial System(tm) for Science of the Total Environment

Manuscript Draft

Manuscript Number: STOTEN-D-19-01035

Title: Ocean acidification impact on marine invertebrate spermatozoa: new evidence for stress resilience

Article Type: Review Article

Keywords: ascidian; microcosm; ocean acidification; offspring quality; reproduction; sperm quality

Corresponding Author: Dr. Elisabetta Tosti, Ph.D

Corresponding Author's Institution: Stazione Zoologica

First Author: Alessandra Gallo

Order of Authors: Alessandra Gallo; Raffaele Boni; Maria Cristina Buia; Vincenzo Monfrecola; Maria Consiglia Esposito; Elisabetta Tosti, Ph.D

Abstract: Rising atmospheric CO2 is causing a progressive decrease of seawater pH, termed ocean acidification. Predicting its impact on marine invertebrate reproduction is essential to anticipate the consequences of future climate change on species fitness and survival. Ocean acidification may affect reproductive fitness either in terms of gamete or progeny quality threating species survival. Despite an increasing number of studies focusing on the effects of ocean acidification on the early life history of marine organisms, very few have investigated the effects on marine invertebrate gamete quality. In this study, we set up two experimental approaches simulating the ocean conditions predicted for the end of this century, in situ transplant experiments at a naturally acidified volcanic vent area along the Ischia island coast and microcosm experiments, to evaluate the short-term effects of the predicted near-future levels of ocean acidification on sperm quality of the ascidian Ciona robusta after parental exposure. In the first days of exposure to acidified conditions, we detected alteration of sperm motility, morphology and physiology followed by a rapid recovery of physiological conditions that provides a new evidence of resilience of ascidian spermatozoa in response to ocean acidification. Overall, the toleration of adverse conditions opens a new scenario on the endangered marine species capacity to continue to reproduce and persist in changing oceans.

Suggested Reviewers: Harvey Florman
University of Massachusetts Medical School
harvey.florman@umassmed.edu
Expertise on sperm physiology

Jason Hall-Spencer
University of Plymouth
jason.hall-spencer@plymouth.ac.uk
expertise on ocean acidification

Noa Shenkarn
Tel-Aviv University

shenkarn@post.tau.ac.il
expertise on ascidians as biological indicators of marine environments

Jean-Pierre Gattuso
Laboratoire d'Océanographie de Villefranche
gattuso@obs-vlfr.fr
expertise on the response of marine organisms to ocean acidification

Remi Dumollard
Laboratoire de Biologie du Développement de Villefranche sur mer
remi.dumollard@obs-vlfr.fr
Expertise on reproductive biology in ascidian

Opposed Reviewers:

To the Editor of Environment International

Dear Prof. Adrian Covaci,

Please find attached the research article "Ocean acidification impact on marine invertebrate spermatozoa: new evidence for stress resilience" by Alessandra Gallo, Raffaele Boni, Maria Cristina Buia, Vincenzo Monfrecola, Maria Consiglia Esposito and myself, for consideration in Environment International.

Ocean acidification is a matter of increasing concern and this paper represents an absolute novelty in the climate change scenario. In fact, in order to predict the impact of a future acidified marine environment on marine invertebrate gamete quality that underlies reproductive success, we set up two different experimental approaches: microcosm and *in situ* transplant. In the latter, we took the unique opportunity to utilize a naturally acidified site that is among the few sites all over the world with these characteristics. The present study is the first that evaluates the impact of acidified seawater on that male reproductive physiology of marine invertebrate demonstrating resilience in spermatozoa that has never been described to date. Furthermore, we evidenced that, differently from many classes of environmental stressors, seawater acidification impact on sperm quality is not based on a mechanism of action involving oxidative stress.

For these reasons, we believe that this paper represents a significant advance in the context of the interface between biological systems and global changes.

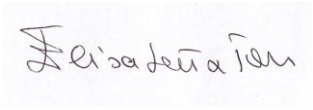
The MS has not been previously published and has not been submitted for publication elsewhere while under consideration.

On the behalf of all Authors, I declare no conflict of interest.

Thank you in advance for your attention to our manuscript.

Sincerely yours,

Elisabetta Tosti



Ocean acidification impact on marine invertebrate spermatozoa: new evidence for stress resilience

Alessandra Gallo[1], Raffaele Boni[2], Maria Cristina Buia[3], Vincenzo Monfrecola[1], Maria Consiglia Esposito[1], Elisabetta Tosti*[1]

[1] Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Napoli, Italy

[2] Department of Sciences, University of Basilicata, 85100, Potenza, Italy

[3] Center of Villa Dohrn Ischia – Benthic Ecology, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, P.ta S. Pietro, Ischia, Naples, Italy

Correspondence to:

*Elisabetta Tosti

Department of Biology and Evolution of Marine Organisms

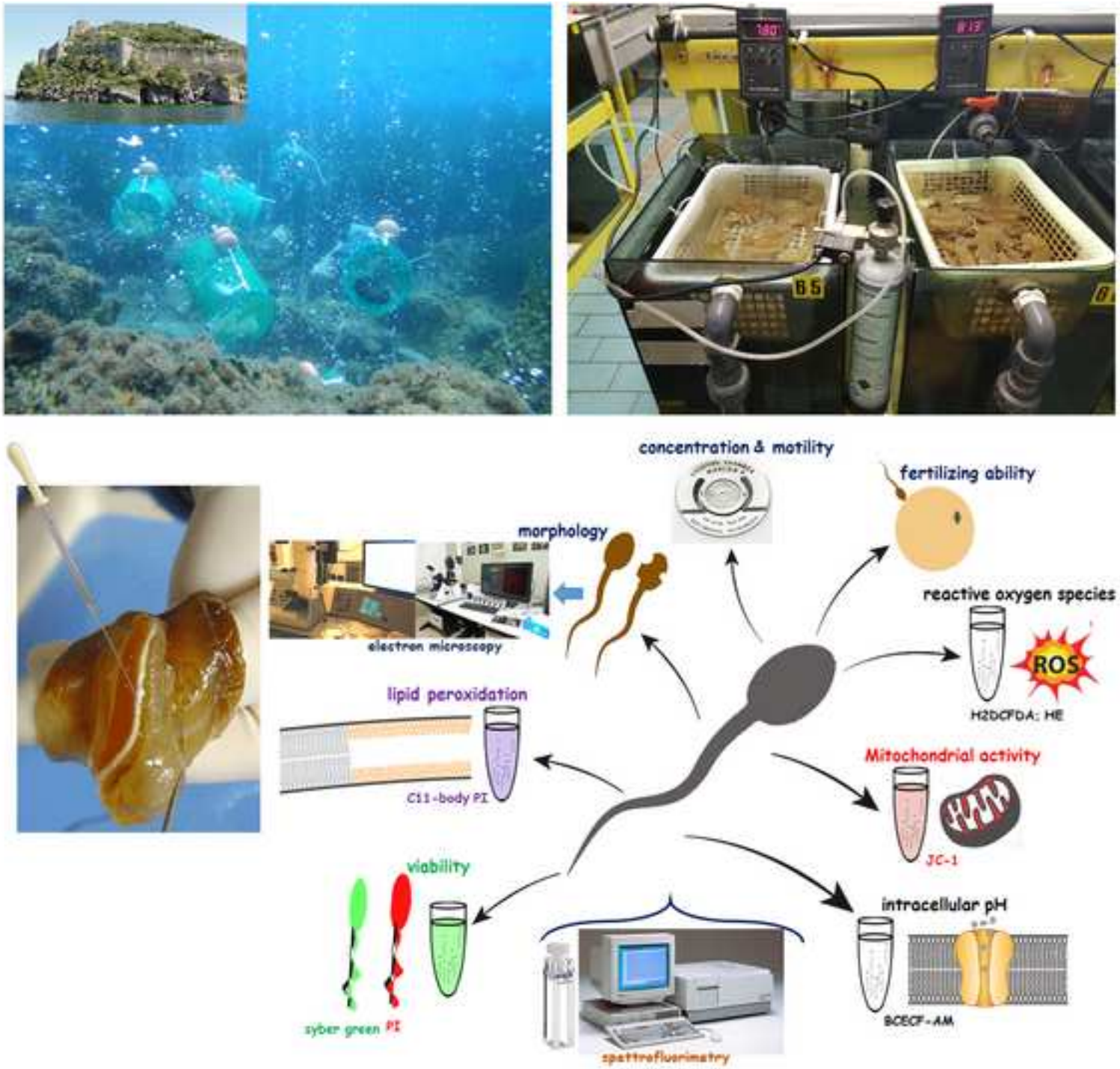
Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Naples, Italy.

Phone +390815833288; Fax +390817641355

email: tosti@szn.it

Keywords: ascidian, CO_2 vents, fertilization, microcosm, ocean acidification, offspring quality, spermatozoon, sperm quality

graphical abstract.tif



1. Impact of ocean acidification on sperm quality of the ascidian *Ciona robusta* was investigated.
2. Two experimental approaches were set up to simulate the ocean conditions predicted for the end of this century.
3. Alteration of sperm motility, morphology and physiology was detected in short-term exposure.
4. A rapid recovery of physiological conditions was observed within one week.
5. New evidence of resilience in ascidian spermatozoa in response to ocean acidification.

Click here to view linked References

Ocean acidification impact on marine invertebrate spermatozoa: new evidence for stress resilience

Alessandra Gallo[1], Raffaele Boni[2], Maria Cristina Buia[3], Vincenzo Monfrecola[1], Maria Consiglia Esposito[1], Elisabetta Tosti*[1]

[1] Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Napoli, Italy

[2] Department of Sciences, University of Basilicata, 85100, Potenza, Italy

[3] Center of Villa Dohrn Ischia – Benthic Ecology, Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, P.ta S. Pietro, Ischia, Naples, Italy

Correspondence to:

*Elisabetta Tosti

Department of Biology and Evolution of Marine Organisms

Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Naples, Italy.

Phone +390815833288; Fax +390817641355

email: tosti@szn.it

Declarations of interest: none.

ABSTRACT

Rising atmospheric CO_2 is causing a progressive decrease of seawater pH, termed ocean acidification. Predicting its impact on marine invertebrate reproduction is essential to anticipate the consequences of future climate change on species fitness and survival. Ocean acidification may affect reproductive fitness either in terms of gamete or progeny quality threating species survival. Despite an increasing number of studies focusing on the effects of ocean acidification on the early life history of marine organisms, very few have investigated the effects on marine invertebrate gamete quality. In this study, we set up two experimental approaches simulating the ocean conditions predicted for the end of this century, *in situ* transplant experiments at a naturally acidified volcanic vent area along the Ischia island coast and microcosm experiments, to evaluate the short-term effects of the predicted near-future levels of ocean acidification on sperm quality of the ascidian *Ciona robusta* after parental exposure. In the first days of exposure to acidified conditions, we detected alteration of sperm motility, morphology and physiology followed by a rapid recovery of physiological conditions that provides a new evidence of resilience of ascidian spermatozoa in response to ocean acidification. Overall, the toleration of adverse conditions opens a new scenario on the endangered marine species capacity to continue to reproduce and persist in changing oceans.

Keywords: ascidian, fertilization, microcosm, ocean acidification, offspring quality, sperm quality

1. Introduction

The increasing atmospheric concentration of carbon dioxide (CO_2), resulting mainly from human activities, are concurrently driving fundamental changes in ocean chemistry and a lower of seawater pH, known as ocean acidification (OA) (Guinotte and Fabry, 2008). Over the last two centuries, the surface water pH has decreased of 0.1 unit and estimates based on the Intergovernmental Panel on Climate Change (IPCC) suggest that it will drop from a pre-industrial value of about 8.2 to about 7.8 by the end of this century (Caldeira and Wickett, 2003). This is creating critical concern in the scientific community due to the possible negative impact exerted by OA on the organisms living and reproducing into marine environment (Gallo and Tosti, 2016). The most well-known impact of a lower pH on marine biota arises from reduced carbonate ion concentration in seawater, which affects the production of calcareous structures, as exo- or endo-skeletons, in marine organisms from plankton to benthic molluscs, echinoderms, and corals (Beaufort et al., 2011; Bolton et al., 2016; Fabry, 2008; Findlay et al., 2009; Koch et al., 2013; Martin et al., 2008; Monteiro et al., 2016; Orr et al., 2005; Porzio et al., 2018). Recently, it has also demonstrated that OA alter the anchoring ability of mitilids, by weakening the proteinaceous byssus, potentially affecting community structure and the sensory perception of different marine organisms threating their reciprocal communication, behavior and survival (Ashur et al., 2017; Li et al., 2017; O'Donnell et al., 2013; Zupo et al., 2016).

Some organisms living in extreme habitats or exposed to multiple stressors develop an adaptive capacity to live and reproduce in such negative conditions. Species maintenance, fitness and survival depend on reproductive success. Reproduction is the highly specialized process of cell interaction that generates a new individual of the same species through different events such as recognition, binding and fusion of gametes. Reproductive success strongly depends on gamete quality. In free spawning marine species, gametes are released in

seawater where fertilization and embryo development occur; consequently, gametes and embryo may be exposed to chemo-physical stressors present in the seawater that, altering their quality and physiology, may affect fertilization success, embryo development, larval viability and, subsequently, species fitness and survival.

Climate change including warming and acidification together with chemical pollution are threating marine organisms exerting reprotoxic effects on invertebrates that appear to be highly vulnerable to environmental stressors (Boni et al., 2016; Byrne, 2012; Gallo, 2018; Gallo et al., 2016; Gallo et al., 2018b; Gallo et al., 2011; Gallo and Tosti, 2013; Gallo and Tosti, 2015b; Hall-Spencer et al., 2008). To date, it has been demonstrated that parental exposure of diverse marine invertebrate species to OA had a direct negative impact on reproductive capability affecting embryo and larval development, larval settlement success, and offspring quality (Pansch et al., 2018). Despite an increasing number of studies focusing on the effects of OA on the early life history of marine organisms, very few have investigated OA impact on marine invertebrate gamete quality. Sperm quality is defined as the ability of spermatozoa to fertilize an oocyte and allow the development of normal embryo and can be quantitatively estimated by evaluating several parameters such as motility, morphology, mitochondrial activity, intracellular pH and oxidative status (Bobe and Labbé, 2010). To date, the OA impact on marine invertebrate sperm quality has been assessed by evaluating only two parameters, such as motility and fertilization capability, in few marine invertebrate species. In particular, in different sea urchin species it was demonstrated that these two parameters were negatively impacted by acidified seawater (Schlegel et al., 2012). To our knowledge, at present the impact of OA on sperm quality and physiology in ascidian has not yet been investigated. In this line, the present study aimed to evaluate the OA effects on a set of sperm quality parameters underlying fertilization competence in the ascidian *Ciona robusta*. This (previously *Ciona intestinalis* type A, (Brunetti et al., 2015)) is a marine invertebrate

broadcast spawner, whose reproductive physiology is well known (Satoh, 1994). The sensitivity of *C. robusta* gametes to different environmental stressors has been investigated showing adverse effects on some events which are at the basis of reproductive mechanisms (Gallo, 2018; Gallo et al., 2011; Gallo and Tosti, 2013; Gallo and Tosti, 2015b; Gallo and Tosti, 2016). All together, these data led also us to propose *C. intestinalis* as an emerging model to study the impact of chemo-physical stressors in marine environment (Gallo and Tosti, 2015a).

In this study, sperm sensitivity of *Ciona robusta* to short-term acidified seawater exposure was evaluated by using two approaches, *in situ* transplant and microcosm experiments, aiming to simulate the ocean conditions predicted for the end of this century. Animals were exposed in a naturally acidified site due to CO_2 vent emissions along the Ischia island (Naples) coast in the first and in artificially acidified seawater by CO_2 bubbling in the second one. Several sperm quality parameters underling fertilization competence and offspring quality were evaluated as toxicity endpoints after paternal exposure to acidified conditions for one week.

2. Material and Methods

2.1 Animal collection

Adults of *Ciona robusta* (formerly known as *C. intestinalis* type A) were collected from Gulf of Naples (Italy) and homogeneously divided in two sub groups for *in situ* transplant and microcosm experiments. The experiment were replicated two years monthly in January and February of 2017 and 2018.

2.2 *In situ* transplant experiment

Field studies were conducted at Castello Aragonese (Island of Ischia, Gulf of Naples, Tyrrhenian Sea, Italy; 40°43.866′N, 13°57.806′E) where volcanic emissions vents occur in shallow waters emitting 1.4×10^{6} l d^{-1} of gas comprising 90–95% CO_2 and other traces gases

(no sulfur) (Hall-Spencer et al., 2008). Into this stretch of coastal water, a natural pCO_2 gradients is well established with natural temporal fluctuations, allowing for an examination of the effects of decreasing pH conditions, such as those predicted at the close of this century. Fieldwork was carried out at two stations along this gradient situated at approximately 3 m depth: the reference station (control pH 8.1) situated 400 m from the gas vent area and the acidified station (pH 7.8) adjacent to the vents.

Ascidians were randomly allocated into 9 cages that initially have been placed at reference station to acclimatize. After 7 days, one cage was collected and animals carried to the laboratory (0 day); 4 cages were left in the control station while the remaining 4 cages were moved to the 7.8 pH station. Over a timespan of a week, ascidians were collected from both stations at different intervals (1, 2, 3 and 7 days) and carry to the laboratory.

2.3 Microcosm experiments

After collection, ascidian were transported to Stazione Zoologica Unit of Marine Resources for Research laboratory, where they were acclimated in aquaria with running seawater at 18 °C for 7 days. The experimental facility consisted of a control tank, in duplicates, supplied with natural seawater (pH 8.1 ± 0.1 and 37 ± 0.5 ppm salinity) and tank, in duplicates, containing seawater acidified at pH 7.8 ± 0.1. Seawater was acidified by means of a bubbling system suppling CO2 gas injected trough the water as very fine bubbles allowing the gas to rapidly dissolve. The CO_2 system is plugged with a pH controller adjusted at pH 7.8; once the required pH is achieved, the supply of CO_2 is halted via an automated feedback relay system. After 7 days acclimatization period, ascidians were randomly distributed into control and acidified thanks (1 ascidian/L). Animals were collected at the start of experiments (0 day) and from experimental tanks after 1, 2, 3 and 7 days of exposure.

2.4 Sperm collection

Ascidians from either *in situ* transplant and microcosm experiments were anesthetized on ice and spermatozoa were collected from dissected sperm ducts and held at 4 °C. All experiments were conducted within the same day of animal collection and two pools were prepared, respectively, from three different animals.

2.5 Sperm concentration and motility

Sperm concentration was evaluated by a Makler counting chamber. Sperm motility analysis was performed by using the free Computer Assisted Sperm Analyzer software that is a plugin open source for the software ImageJ.

2.6 Sperm viability

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Life technologies, Milan, Italy). This kit consists of SYBR-14 stock solution (1mM in DMSO) and propidium iodide solution (PI; 2.4 mM solution in water). SYBR-14 permeates cells with preserved membrane integrity and emits green fluorescence. Contrastingly, PI permeates only spermatozoa that lost membrane integrity. Fluorescent staining was carried out as previously reported (Gallo et al., 2018a). Briefly, an aliquot of spermatozoa diluted at 5 x 10^6 cell/ml were stained by adding SYBR-14 (final concentration 100 nM) and incubated in the dark for 15 minutes; then PI (final concentration 12 µM) was added and incubated an additional 15 minutes. Samples were washed with FNSW by centrifuged at 800g for 10 min and then resuspended in FSWN and analysed by spectrofluorometer (Shimadzu RF-5301PC, Japan) using a quartz microtube (10x4 mm, high precision, Hellma Analytics, Mullheim, Germany). SYBR-14 and PI fluorescence were measured at 500-530 nm (live spermatozoa) and at 570-650 nm (dead spermatozoa), respectively. Sperm viability was calculated as a ratio between live spermatozoa (SYBR-14 stained) and the sum of dead (PI-stained) and live spermatozoa.

2.7 Mitochondrial membrane potential (MMP)

To determine the MMP, the vital mitochondrial dye JC-1 was used. This dye is freely permeable to cells and is a useful tool for investigating mitochondrial activity since it undergoes reversible transformation as MMP changes from aggregate to monomer forms shifting the fluorescence emission from red (~595 nm) to green (~535 nm). Consequently, MMP value can be determined by red/green ratio. Aliquots of spermatozoa diluted at 1 x 10^6 cell/mL were stained by adding 5 µM JC-1, diluted from a 7.7mM stock solution in DMSO, and incubating for 30 min at 18 °C. After staining, spermatozoa were centrifuged for 15 min at 800g, the pellet was resuspended in FNSW and transferred to quartz cuvette for spectrofluorometric analysis using 488 nm excitation and 500-650 nm emission wavelengths. The ratio between red and green fluorescence emission peak values indicates the MMP. Controls were prepared by exposing JC-1 loaded spermatozoa to the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 5 µM) that disrupts MMP resulting in a shift from red to green fluorescence.

2.8 Intracellular pH (pH_i) and external pH (pH_e)

The pH sensitive fluorescent chromophore 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), which freely diffuse through the plasma membrane, was used to evaluate pH_i. In the cell, this dye is hydrolyzed by esterases releasing the intracellularly trapped indicator, BCECF, retained within the cytoplasm and its fluorescence intensity is dependent upon the pH. The ester form of BCECF (5 mM BCECF-AM) was added to the sperm suspensions (1 x 10^6 cell/mL) and incubated in the dark at 18 °C for 30 min. Then, the suspensions were centrifuged for 15 min at 800g, the sperm pellet was resuspended in FNSW and transferred to the quartz cuvette for spectrofluorometric analysis. The pH_i value was measured by alternately exciting BCECF at 440 nm and 490 nm and recording the fluorescence emission peak values at 535 nm. Subsequently, the ratio between the two obtained emission peaks was converted to its respective pH_i by using a calibration

curve. This was constructed on each experiment by incubating sperm suspensions in a calibration buffer solution (135mM KCl, 5mM HEPES, 290 mOsm) at pH 6.5, 7.0, 7.5 in presence of 5 µM nigericin, which acts as a potassium ionophore promoting K^+/H^+ exchange.

2.9 Intracellular reactive oxygen species (ROS) determination

Intracellular ROS levels were determined through two different oxidation-sensitive fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE), used to analyze the intracellular content of hydrogen peroxides (H_2O_2) and superoxide anions (O_2^-), respectively.

H_2DCF-DA is freely permeable across cell membranes and is incorporated into hydrophobic regions of the cell where its acetate moiety is cleaved by esterase producing the impermeant non-fluorescent 2',7'-dichlorodihydrofluorescein (H_2DCF). The H_2DCF is oxidized by H_2O_2 to the highly fluorescent 2',7'-dichlorofluorescein (DCF) which emits fluorescence at ~530 nm in response to 488 nm excitation wavelengths. Sperm aliquots diluted at 5 x 10^6 cell/mL were stained by adding H_2DCF-DA to final concentration of 10 µM and incubated for 30 min in the dark at 18 °C. Then, sperm suspensions were centrifuged for 15 min at 800g, the pellet was resuspended in FNSW and incubated for additional 30 min. Subsequently, sperm suspensions were centrifuged, the pellet was resuspended in FNSW and transferred to the quartz cuvette for spectrofluorometric analysis. A positive control was prepared by incubating the sperm suspension with hydrogen peroxide. Fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelengths of 500-560 nm.

The hydroethidine (HE; Life techonologies, Milan, Italy) is used to estimate the intracellular O_2^- level. HE is the sodium borohydride-reduced form of ethidium. It is freely permeable to cells and exhibits blue fluorescence in the cytoplasm until oxidized; when oxidized by O2, it generates two red fluorescent products the 2-hydroxyethidium (2-OH-E^+) and the ethidium (E^+). The latter binds to DNA emitting at 610 nm in response to 488-nm excitation. The 2-

222 $OH\text{-}E^+$ is a highly specific red fluorescent product, which was unique to superoxide, other
223 oxidants in fact did not form the same product upon reaction with HE. Consequently, to
224 selectively detect the superoxide-specific product ($2\text{-}OH\text{-}E^+$) the excitation light was set at
225 350 nm (Robinson et al., 2006). Aliquots of spermatozoa diluted at 30 x 10^7 spermatozoa/ml
226 were stained by adding HE to final concentration of 20 µM and incubated for 30 min in the
227 dark at 18 °C. Following, sperm suspensions were diluted by adding NFSW and transferred to
228 the quartz cuvette for spectrofluorometric analysis. A positive control was prepared by
229 incubating the sperm suspension with menadione. Fluorescence intensity was measured at an
230 excitation wavelength of 350 nm and emission wavelengths of 500-700 nm.

231 **2.10 Lipid peroxidation**

232 Lipid peroxidation was evaluated using the fluorescent membrane probe C11-$BODIPY^{581/591}$.
233 This probe is an oxidation-sensitive fluorescent fatty acid analog, which is easily incorporated
234 into membranes and sensitive to oxidation with an emission spectra shifting from red (~595
235 nm) to green (~520 nm) fluorescence upon oxidation. Sperm suspensions ($2x10^7$
236 spermatozoa/mL) were incubated for 30 min in the dark at 18 C° with 5 µM C11-
237 $BODIPY^{581/591}$ in DMSO. After staining, spermatozoa were centrifuged, the pellet
238 resuspended in FNSW and transferred to quartz cuvette for spectrofluorimetric analysis. A
239 positive control was prepared by incubating the samples with two peroxidation promoters
240 (150 µM ferrous sulfate and 750 µM vitamin C) and FNSW, respectively. The fluorescence
241 intensity was measured at 488 nm excitation and 500–650 nm emission wavelengths. Then, a
242 ratiometric analysis was performed by relating fluorescence emission peak value at ~520 nm
243 to the sum of fluorescence emission peak values at ~520 and ~590 nm.

244 **2.11 Ultrastructural analyses: scanning and transmission electron microscopy**

Following exposure to acidified conditions both *in situ* transplant and microcosm experiments, spermatozoa were fixed for 1 h at RT in 2.5% glutaraldehyde solution in 0.2 M sodium cacodylate buffer and 20% FNSW. Samples were washed for 10 min once in sodium cacodylate buffer and twice in distilled water and, then, postfixed for 1 h at RT in 1% osmium tetroxide in distilled water. For scanning electron microscopy (SEM), after dehydration in an ascending ethanol series (30, 50, 70, 90, and 100%), samples were mounted on studs, then coated with palladium and examined under a JEOL JSM 6700F microscope. For transmission electron microscopy (TEM), samples were treated for 15 min in propylene oxide, infiltrated in 1:1 propylene oxide/Epon 812 overnight and then embedded in fresh resin at 60 °C for 48 h. Ultrathin sections were cut on Leica Ultracut ultramicrotome, stained with 4% uranyl acetate for 30 min and 3% lead citrate for 30 sec, collected on 200 mesh thin bar copper grids, and observed with a LEO 912AB microscope (Zeiss, Göttingen, Germany). Three true replicates for either control or treatment groups were performed; ten sections for each replicate were analyzed.

2.12 Fertilizing capability

The effects of seawater acidification on sperm fertilizing capability was assessed by evaluating fertilization rate and the induction of transmissible damages to the offspring. Aliquots of spermatozoa ($1x10^6$/ml), collected by animals exposed to acidified conditions either *in situ* transplant or microcosm experiments were added to 10 ml of FNSW containing approximately 200 oocytes randomly collected from ascidians not exposed to acidified seawater nor used for sperm collection. As control, sperm aliquots collected from not exposed animals was used for fertilization. The fertilization dishes were incubated in a culture chamber at 18°C; after 50 min, fertilization occurrence was assessed by the formation of the first cleavage. To evaluate the offspring quality, the dishes were incubated up to 24 h and larvae percentage with normal morphology was evaluated.

2.13 Statistical analysis

Data were reported as the mean ± standard deviation (SD) and tested for normal distribution by the Shapiro-Wilk test and for variance homogeneity by Leven's test. Since the two assumptions were accepted, the one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was performed. For values expressed as percentages, data were analyzed after arcsine transformation to achieve normality. For the pH, whose values are not characterized by a continuous distribution, a transformation in H^+ concentration was applied. The significance level was set at p=0.05.

3. RESULTS

3.1 Sperm concentration

Sperm concentration, expressed as 10^9 spermatozoa/ml, was not affected by OA. In fact, the concentration of spermatozoa collected from adults exposed to acidified seawater in both *in situ* transplant and microcosm experiments did not significant differ from those recorded in control ascidians (Figure 1).

3.2 Sperm motility

Motility, expressed as percentage of motile spermatozoa on the whole sperm population, in ascidians exposed to acidified seawater significantly decreased in comparison to those recorded in control ascidians at day 2 and 3 of experiment. On day 7 after exposure, sperm motility percentage from ascidians exposed in acidified seawater did not significant differ from those assessed in control ascidians (Figure 2).

3.3 Sperm viability

On day 2 and 3 after exposure, the viability of spermatozoa collected from ascidian exposed to acidified seawater in *in situ* transplant experiment was significantly lower than that evaluated in control ascidian (50.58±4.86 *vs* 35.47±2.76 at day 2; 50.34±4.53 *vs* 37.49±1.52

294 at day 3; P< 0.05) (Figure 3). On day 7 after exposure, sperm viability from ascidians exposed
295 in acidified seawater did not significant differ from those assessed in control ascidians.
296 In microcosm experiments, the sperm viability assessed in ascidian exposed to acidified
297 seawater did not significant differ from those recorded in control ascidian (Figure 3).

298 **3.4 MMP**

299 In *in situ* transplant experiment, after 2 and 3 days of exposure a significant decrease of
300 MMP values was observed in spermatozoa collected from ascidian exposed to acidified
301 condition compared to control ascidian (35.00 ± 2.76 *vs* 22.35±3.52 at day 2 and 28.88 ±
302 1.95 *vs* 12.77 ± 1.31 at day 3; P< 0.05). On day 7, MMP assessed in spermatozoa from
303 ascidians exposed in acidified seawater did not significant differ from those of control
304 ascidians (Figure 4).
305 In microcosm experiments, on day 1 and 2 of exposure, the MMP measured in
306 spermatozoa from ascidians exposed to acidified seawater were not significant different
307 from those recorded in spermatozoa collected from control ascidians. At day 2 of
308 exposure, the MMP detected in spermatozoa collected from ascidians exposed to acidified
309 seawater significantly decreased in comparison to MMP recorded in spermatozoa of
310 control ascidians (40.84 ± 2.65 *vs* 27.54 ± 1.3; P< 0.05) (Figure 4).

311 **3.5 Extracellular and intracellular pH**

312 External pH was not significant affected by OA; in fact, pH_e values measured in seminal
313 plasma collect from ascidian exposed to acidified seawater either in *in situ* transplant or in
314 microcosm experiments did not significant differ from those measured in seminal plasma
315 collected from ascidian not exposed (Figure 5).
316 In *in situ* transplant experiments, on day 1 the pH_i of spermatozoa collected from
317 ascidians exposed to acidified seawater high significantly decreased compared to pH_i
318 recorded in spermatozoa collected from control ascidians (9.40±0.08 *vs* 7.79±0.09; P<

319 0.01). In the following days, the sperm pH_i recorded in ascidians exposed to acidified
320 seawater did not significant differ from those assessed in control ascidians.
321 Similarly, in microcosm experiment, sperm pH_i in ascidian exposed to acidified seawater
322 significantly decreased in comparison to that recorded in spermatozoa collected from
323 control ascidians (9.30 ± 0.07 *vs* 9.00 ± 0.07; $P< 0.05$). In the following days, the sperm
324 pH_i recorded in ascidians exposed to acidified seawater did not significant change compared
325 to those measured in control ascidian (Figure 5).

326 **3.6 Intracellular reactive oxygen species (ROS) level**

327 ***3.6.1 Hydrogen peroxides (H_2O_2)***

328 The intracellular level of H_2O_2 recorded in spermatozoa exposed to acidified seawater did
329 not significantly vary compared to those recorded in spermatozoa collected from not
330 exposed ascidians (Figure 6).

331 ***3.6.2 Superoxide anions (O_2^-)***

332 The intracellular level of O_2^- recorded in spermatozoa exposed to acidified seawater in both
333 *in situ* transplant and microcosm experiments did not significantly differ in comparison to
334 those recorded in spermatozoa collected from control ascidians (Figure 6).

335 **3.7 Lipid peroxidation**

336 Ocean acidification did not affect plasma membrane lipid peroxidation; in fact, the level
337 of lipid peroxidation observed in spermatozoa collected from ascidians exposed to
338 acidified seawater in both *in situ* transplant and microcosm experiments did not
339 significantly differ from those recorded in spermatozoa collected from control ascidians
340 (Figure 7).

341 **3.8 Fertilization capability**

Acidified condition did not affect sperm fertilizing capability in ascidian *C. robusta*. All over the exposure period, fertilization rate did not significant change, furthermore transmissible damages to the offspring were not observed (data not shown).

3.9 Sperm ultrastructural morphology

Sperm morphology was affected by seawater acidification. After 3 days of parental exposure to acidified conditions in both *in situ* transplant and microcosm experiments, ascidian spermatozoa observed at the SEM exhibited a range of structural modifications at the level of the plasma membrane. These modifications included the interruption of plasma membrane continuity, an increased fracture at the point of intersection between the head and the overlying mitochondrion, the detachment and sliding of the mitochondrion, up to the loss of the morphology of the head and the mitochondrion (Figure 8).

TEM confirms these data showing the progressive separation of the mitochondrion from the sperm head two structures and the loss of sperm normal morphology with fragmentation, rounding and sliding of the mitochondrion (Figure 9).

4. DISCUSSION

In this study, we have shown for the first time a short-term impact of OA on the sperm quality of the marine invertebrate *Ciona robusta* by using two different approaches, *in situ* transplant and microcosm experiments, and analyzing a set of sperm quality parameters underling fertilization competence. Recently, the importance of short-term sperm exposure has been highlighted; it helps to explain species-specific differences in response to OA (Campbell et al., 2017). In particular, short-term exposure (7-days) has been performed to test species reaction and acclimation ability to environmental conditions (Truebano et al., 2018).

Several studies investigated the effects of OA on reproduction in marine invertebrates focusing on fertilization success. Some of these reported a negative impact of OA on fertilization in different marine species across broad taxonomic groups (Albright et al., 2016;

Barros et al., 2013; Gonzalez-Bernat et al., 2013). However, other authors demonstrated that species with external fertilization are tolerant to ocean acidification (Chua et al., 2013; Havenhand and Schlegel, 2009; Ho et al., 2013; Martin et al., 2011). Nonetheless, at present, studies focusing on the influence of OA on gamete quality are scarce (Caldwell et al., 2011; Campbell et al., 2016; Foo and Byrne, 2017; Foo et al., 2018; Frommel et al., 2010; Havenhand and Schlegel, 2009; Morita et al., 2010). Fertilization success is strictly correlated to gamete quality defined as the ability of the gametes to fertilize and to be fertilized. In literature, a negative impact of OA on oocyte quality of different marine species was reported (Foo and Byrne, 2017; Foo et al., 2018), while data on the OA impact on sperm quality are scarce and few parameters of sperm quality, such as motility, swimming and fertilizing capability, have been assessed in response to acidified seawater. To our knowledge, no any study has previously investigated the short-term impact of OA after parental exposure on a set of ascidian sperm quality parameters.

Motility is a sperm quality parameter largely used to assess the effects of several environmental stressors since it is simple and rapid to analyze. The OA influence on sperm motility was evaluated in different marine species with contrasting results. In fact, a decrease of sperm motility under acidified conditions was reported and this alteration was related to a low probability of gamete interaction and fertilization success (Havenhand et al., 2008; Morita et al., 2010; Nakamura and Morita, 2012), while others have found no effects of OA on sperm motility (Frommel et al., 2010; Havenhand and Schlegel, 2009). In contrast, only one study showed an improvement in swimming speed under acidified conditions (Caldwell et al., 2011). Here, we observed a negative impact of OA on ascidian sperm motility since the percentage of motile spermatozoa decreased after exposure. The energy required for motility is provided by mitochondrial activity (Kasai et al., 2002), which can be assessed by evaluating changes in MMP that in active spermatozoa is higher than in non-active ones.

MMP has been demonstrated to be affected by several chemo-physical stressors (Boni et al., 2016; Gallo et al., 2016; Gallo et al., 2018b). In this study, we demonstrated that OA affects also mitochondrial activity, in fact the MMP of ascidian spermatozoa decreased after two days of exposure and reached a peak on day 3 to slowly recover the physiological conditions. These results suggest that mitochondrial activity impairment may be the cause of sperm motility decrease under OA. On the other hand, it has been shown that OA affects sperm motility by modulating ion channel activity (Nishigaki et al., 2014). The activation of marine spermatozoa occurs almost instantly upon release into seawater due to an influx of external Na^+ into the spermatozoa triggering the release of H^+ ions within the cell elevating pH_i, which activates dynein ATPase (Tosti, 1994; Tosti and Ménézo, 2016). In this study, the pH_i of spermatozoa collected from ascidian exposed to acidified condition undergoes a pH sharp decrease that can explain the observed reduction in sperm motility. In fact, the enzymes regulating this process have a different optimal pH, so their activity is inhibited under lowered pH. Furthermore, the low pH may negatively affect the efficiency of the mitochondrial electron transport chain, which has been recognized to be one of the major cellular generators of ROS (Agarwal et al., 2014). ROS are produced in spermatozoa under normal physiological conditions and, at low and controlled concentrations, are required for crucial processes such as capacitation, acrosome reaction, and sperm-oocyte fusion (de Lamirande et al., 1997; Guerriero et al., 2018; Takeshima et al., 2018). At high concentrations or in the presence of reduced antioxidant defenses, which lead to ROS quenching, ROS may induce oxidative damage to DNA, proteins and lipids. Sperm plasma membrane, in particular, is highly susceptible to oxidative stress due to the high content of structurally unstable polyunsaturated fatty acids that confer the necessary fluidity for membrane fusion occurring at fertilization (Tosti and Ménézo, 2016). The oxidant attack on plasma membrane results in lipid peroxidation compromising plasma membrane fluidity (Aitken, 2017). Some studies suggest

417 that the mechanism of toxic action of seawater acidification goes through oxidative stress
418 (Gomiero et al., 2018; Jeeva Priya et al., 2017). Here, we demonstrated that OA did not affect
419 the oxidative status of spermatozoa; in fact, we did not observe any increase of intracellular
420 level of two different ROS species nor lipid peroxidation in spermatozoa collected from
421 animal after short-time exposure to acidified condition. The occurrence of fertilization, here
422 observed, is corroborated by the integrity of sperm plasma membrane after OA exposure,
423 which is a fundamental requisite for gamete recognition, binding and fusion.
424 In our study, besides the mitochondrial dysfunction, seawater acidification also induces
425 mitochondrial ultrastructural damages. Ascidian sperm head is surrounded by a single large
426 mitochondrion, which undergo swelling and migration, and finally is lost at fertilization
427 (Satoh, 1994). Under acidified condition, electron microscopy analysis reveals a detachment
428 and sliding of the mitochondrion from its typical position up to the loss of the normal
429 morphology of both sperm head and the mitochondrion itself.
430 We also demonstrate that acidified seawater impairs the vitality of ascidian spermatozoa,
431 which is a key determinant of sperm quality. In marine animals, sperm viability has been
432 assessed after exposure to different environmental stressors but never after OA exposure. The
433 fluorometric staining combination of SYBR-14 and PI is a rapid and reliable assay for
434 determining the percentage of live and dead spermatozoa in marine invertebrates (Gallo et al.,
435 2018a) proposed as a novel foresight predictor of sperm quality in the ecotoxicological
436 assessment of marine water status (Harlıoğlu et al., 2018). By using this method, we found
437 that after 2 and 3 days of exposure to acidified conditions the percentage of live spermatozoa
438 decreased in *in situ* transplant experiments while it was unaffected in microcosm experiments.
439 Microcosm is an artificial and simplified ecosystem used to simulate and predict the effect of
440 controlled environmental conditions on marine biota; on the contrary, field surveys cannot
441 easily link cause and effect due to the biological complexity of natural ecosystems in which

biota is affected by several factors. In this study, comparing *in situ* transplant and microcosm experiments, we demonstrated that short-term seawater acidification parental exposure affects sperm morphology, motility and functionality inducing mitochondrial dysfunction and pH_i decrease. The difference in the sperm viability obtained in the two experimental approaches suggests that under field conditions other stressors may act synergistically to seawater acidification. On the contrary, acidified condition did not affect sperm concentration suggesting that short-term exposure to seawater acidification does not influence the spermatogenetic process.

Sperm quality parameters are associated with fertilization success. In this study, acidified conditions does not affect fertilization rate nor offspring quality. This seems to be in contrast with the sperm morphological and functional alterations observed after OA exposure. Possibly, the low percentage of normal spermatozoa is sufficient to induce oocyte fertilization; moreover, recent evidences show a scarce relevance of abnormal morphology on fertilization success (Kovac et al., 2017).

Resilience is defined as the ability to overcome environmental disturbances. Natural communities as coral reefs have been shown to be resilient to climate change, however anthropic impact is highly decreasing this natural adaptation capacity. Yet, studies aimed to increase ecosystem and species resilience play a fundamental role in the management of the climate disturbance impact (Côté and Darling, 2010). Recently, it has demonstrated that two different ascidian species were tolerant to seawater acidification (Brown et al., 2018). Here, acidified seawater causes ascidian sperm physiology alterations in the first days of exposure followed by a fully recovery to physiological conditions within one week providing a new evidence of ascidian spermatozoa resilience to OA. Resilience, due to the capacity of spermatozoa to recover from OA induced disturbances, may facilitate ascidian reproduction and persistence in a future ocean climate change.

Acknowledgements

We wish to acknowledge Dr. F. Formisano for art graphic of figures. We thank Mr. G.L. Zazo and Mr. M. Cannavacciuolo for providing ascidian; the Captain V. Rando for his assistance in field work and Mr. A. Macina for technical support in animal maintenance. Thanks are also due to Mr. G. Gragnaniello for electron microscopy advices and Mr. F. Iamunno, Dr. R. Graziano, and Mr. G. Lanzotti for scanning and transmission electron microscopy assistance. A.G. was supported by SZN postdoc fellowship. This paper reports the results of the graduation thesis by Dr. M.C. Esposito.

Funding sources

AG has been supported by a Stazione Zoologica Anton Dohrn post-doc fellowship; ET and MCB received Stazione Zoologica Anton Dohrn institutional funding; RB received funding from University of Basilicata.

Author Contributions

AG, RB, MCB and ET designed methodology; AG, RB, MCB, VM, MCE performed the experiments; AG, RB collected and analyzed the data; ET supervised the project; AG and ET wrote the paper with input from all authors that gave final approval for publication.

References

Agarwal, A., Virk, G., Ong, C., du Plessis, S.S., 2014. Effect of oxidative stress on male reproduction. The world journal of men's health 32, 1-17.

Aitken, R.J., 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Molecular reproduction and development 84, 1039-1052.

Albright, R., Caldeira, L., Hosfelt, J., Kwiatkowski, L., Maclaren, J.K., Mason, B.M., Nebuchina, Y., Ninokawa, A., Pongratz, J., Ricke, K.L., 2016. Reversal of ocean acidification enhances net coral reef calcification. Nature 531, 362.

Ashur, M.M., Johnston, N.K., Dixson, D.L., 2017. Impacts of ocean acidification on sensory function in marine organisms. Integrative and comparative biology 57, 63-80.

Barros, P., Sobral, P., Range, P., Chícharo, L., Matias, D., 2013. Effects of sea-water acidification on fertilization and larval development of the oyster Crassostrea gigas. Journal of Experimental Marine Biology and Ecology 440, 200-206.

Beaufort, L., Probert, I., De Garidel-Thoron, T., Bendif, E.M., Ruiz-Pino, D., Metzl, N., Goyet, C., Buchet, N., Coupel, P., Grelaud, M., 2011. Sensitivity of coccolithophores to carbonate chemistry and ocean acidification. Nature 476, 80.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. General and comparative endocrinology 165, 535-548.

Bolton, C.T., Hernández-Sánchez, M.T., Fuertes, M.-A., González-Lemos, S., Abrevaya, L., Mendez-Vicente, A., Flores, J.-A., Probert, I., Giosan, L., Johnson, J., 2016. Decrease in

525 coccolithophore calcification and CO 2 since the middle Miocene. Nature communications 7,
526 10284.

527

528 Boni, R., Gallo, A., Montanino, M., Macina, A., Tosti, E., 2016. Dynamic changes in the
529 sperm quality of Mytilus galloprovincialis under continuous thermal stress. Molecular
530 reproduction and development 83, 162-173.

531

532 Brown, N.E., Milazzo, M., Rastrick, S.P., Hall-Spencer, J.M., Therriault, T.W., Harley, C.D.,
533 2018. Natural acidification changes the timing and rate of succession, alters community
534 structure, and increases homogeneity in marine biofouling communities. Global change
535 biology 24, e112-e127.

536

537 Brunetti, R., Gissi, C., Pennati, R., Caicci, F., Gasparini, F., Manni, L., 2015. Morphological
538 evidence that the molecularly determined Ciona intestinalis type A and type B are different
539 species: Ciona robusta and Ciona intestinalis. Journal of Zoological Systematics and
540 Evolutionary Research 53, 186-193.

541

542 Byrne, M., 2012. Global change ecotoxicology: identification of early life history bottlenecks
543 in marine invertebrates, variable species responses and variable experimental approaches.
544 Marine environmental research 76, 3-15.

545

546 Caldeira, K., Wickett, M.E., 2003. Oceanography: anthropogenic carbon and ocean pH.
547 Nature 425, 365.

548

549 Caldwell, G.S., Fitzer, S., Gillespie, C.S., Pickavance, G., Turnbull, E., Bentley, M.G., 2011.
550 Ocean acidification takes sperm back in time. Invertebrate Reproduction & Development 55,
551 217-221.

552

553 Campbell, A.L., Ellis, R.P., Urbina, M.A., Mourabit, S., Galloway, T.S., Lewis, C., 2017.
554 Impacts of ocean acidification on sperm develop with exposure time for a polychaete with
555 long lived sperm. Marine environmental research 129, 268-276.

556

557 Campbell, A.L., Levitan, D.R., Hosken, D.J., Lewis, C., 2016. Ocean acidification changes
558 the male fitness landscape. Scientific reports 6, 31250.

559

560 Chua, C.-M., Leggat, W., Moya, A., Baird, A.H., 2013. Near-future reductions in pH will
561 have no consistent ecological effects on the early life-history stages of reef corals. Marine
562 Ecology Progress Series 486, 143-151.

563

564 Côté, I.M., Darling, E.S., 2010. Rethinking ecosystem resilience in the face of climate
565 change. PLoS biology 8, e1000438.

566

567 de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxygen
568 species and sperm physiology. Reviews of reproduction 2, 48-54.

569

570 Fabry, V.J., 2008. Marine Calcifiers in a High-CO_2 Ocean. Science, 1020-1022.

571

572 Findlay, H., Wood, H., Kendall, M., Spicer, J., Twitchett, R., Widdicombe, S., 2009.
573 Calcification, a physiological process to be considered in the context of the whole organism.
574 Biogeosciences Discussions 6, 2267-2284.

575

576 Foo, S.A., Byrne, M., 2017. Marine gametes in a changing ocean: Impacts of climate change
577 stressors on fecundity and the egg. Marine environmental research 128, 12-24.

578

579 Foo, S.A., Byrne, M., Gambi, M.C., 2018. Residing at low pH matters, resilience of the egg
580 jelly coat of sea urchins living at a CO 2 vent site. Marine Biology 165, 97.

581

582 Frommel, A., Stiebens, V., Clemmesen, C., Havenhand, J., 2010. Effect of ocean acidification
583 on marine fish sperm (Baltic cod: Gadus morhua). Biogeosciences (BG) 7, 3915-3919.

584

585 Gallo, A., 2018. Toxicity of marine pollutants on the ascidian oocyte physiology: an
586 electrophysiological approach. Zygote 26, 14-23.

587

588 Gallo, A., Boni, R., Buttino, I., Tosti, E., 2016. Spermiotoxicity of nickel nanoparticles in the
589 marine invertebrate Ciona intestinalis (ascidians). Nanotoxicology 10, 1096-1104.

590

591 Gallo, A., Boni, R., Tosti, E., 2018a. Sperm viability assessment in marine invertebrates by
592 fluorescent staining and spectrofluorimetry: A promising tool for assessing marine pollution
593 impact. Ecotoxicology and environmental safety 147, 407-412.

594

595 Gallo, A., Manfra, L., Boni, R., Rotini, A., Migliore, L., Tosti, E., 2018b. Cytotoxicity and
596 genotoxicity of CuO nanoparticles in sea urchin spermatozoa through oxidative stress.
597 Environment international 118, 325-333.

598

599 Gallo, A., Silvestre, F., Cuomo, A., Papoff, F., Tosti, E., 2011. The impact of metals on the
600 reproductive mechanisms of the ascidian Ciona intestinalis. Marine Ecology-an Evolutionary
601 Perspective 32, 222-231.

602

603 Gallo, A., Tosti, E., 2013. Adverse Effect of Antifouling Compounds on the Reproductive
604 Mechanisms of the Ascidian Ciona intestinalis. Marine Drugs 11, 3554-3568.

605

606 Gallo, A., Tosti, E., 2015a. The ascidian Ciona intestinalis as model organism for
607 ecotoxicological bioassays. Journal of Marine Science: Research and Development 5, e138.

608

609 Gallo, A., Tosti, E., 2015b. Reprotoxicity of the Antifoulant Chlorothalonil in Ascidians: An
610 Ecological Risk Assessment. PLoS One 10.

611

612 Gallo, A., Tosti, E., 2016. Adverse Effect of Ocean Acidification on Marine Organisms.
613 Journal of Marine Science: Research and Development 6, e139.

614

615 Gomiero, A., Bellerby, R., Zeichen, M.M., Babbini, L., Viarengo, A., 2018. Biological
616 responses of two marine organisms of ecological relevance to on-going ocean acidification
617 and global warming. Environmental Pollution 236, 60-70.

618

619 Gonzalez-Bernat, M.J., Lamare, M., Barker, M., 2013. Effects of reduced seawater pH on
620 fertilisation, embryogenesis and larval development in the Antarctic seastar Odontaster
621 validus. Polar Biology 36, 235-247.

622

623 Guerriero, G., D'Errico, G., Di Giaimo, R., Rabbito, D., Olanrewaju, O.S., Ciarcia, G., 2018.
624 Reactive oxygen species and glutathione antioxidants in the testis of the soil biosentinel
625 Podarcis sicula (Rafinesque 1810). Environmental Science and Pollution Research 25, 18286-
626 18296.

627

628 Guinotte, J.M., Fabry, V.J., 2008. Ocean acidification and its potential effects on marine
629 ecosystems. Annals of the New York Academy of Sciences 1134, 320-342.

630

631 Hall-Spencer, J.M., Rodolfo-Metalpa, R., Martin, S., Ransome, E., Fine, M., Turner, S.M.,
632 Rowley, S.J., Tedesco, D., Buia, M.-C., 2008. Volcanic carbon dioxide vents show ecosystem
633 effects of ocean acidification. Nature 454, 96.

634

635 Harlıoğlu, M.M., Farhadi, A., Gür, S., 2018. Determination of sperm quality in decapod
636 crustaceans. Aquaculture 490, 185-193.

637

638 Havenhand, J., Schlegel, P., 2009. Near-future levels of ocean acidification do not affect
639 sperm motility and fertilization kinetics in the oyster Crassostrea gigas. Biogeosciences 6,
640 3009-3015.

641

642 Havenhand, J.N., Buttler, F.-R., Thorndyke, M.C., Williamson, J.E., 2008. Near-future levels
643 of ocean acidification reduce fertilization success in a sea urchin. Current Biology 18, R651-
644 R652.

645

646 Ho, M., Price, C., King, C., Virtue, P., Byrne, M., 2013. Effects of ocean warming and
647 acidification on fertilization in the Antarctic echinoid Sterechinus neumayeri across a range of
648 sperm concentrations. Marine environmental research 90, 136-141.

649

650 Jeeva Priya, R., Anand, M., Maruthupandy, M., Hameedha Beevi, A., 2017. Biomarker
651 response of climate change-induced ocean acidification and hypercapnia studies on
652 brachyurian crab Portunus pelagicus. Global Journal of Environmental Science and
653 Management 3, 165-176.

654

655 Kasai, T., Ogawa, K., Mizuno, K., Nagai, S., Uchida, Y., Ohta, S., Fujie, M., Suzuki, K.,
656 Hirata, S., Hoshi, K., 2002. Relationship between sperm mitochondrial membrane potential,
657 sperm motility, and fertility potential. Asian journal of andrology 4, 97-104.

658

659 Koch, M., Bowes, G., Ross, C., Zhang, X.H., 2013. Climate change and ocean acidification
660 effects on seagrasses and marine macroalgae. Global change biology 19, 103-132.

661

662 Kovac, J.R., Smith, R.P., Cajipe, M., Lamb, D.J., Lipshultz, L.I., 2017. Men with a complete
663 absence of normal sperm morphology exhibit high rates of success without assisted
664 reproduction. Asian journal of andrology 19, 39.

665

Li, S., Liu, C., Zhan, A., Xie, L., Zhang, R., 2017. Influencing Mechanism of Ocean Acidification on Byssus Performance in the Pearl Oyster Pinctada fucata. Environmental science & technology 51, 7696-7706.

Martin, S., Richier, S., Pedrotti, M.-L., Dupont, S., Castejon, C., Gerakis, Y., Kerros, M.-E., Oberhänsli, F., Teyssié, J.-L., Jeffree, R., 2011. Early development and molecular plasticity in the Mediterranean sea urchin Paracentrotus lividus exposed to CO2-driven acidification. Journal of Experimental Biology 214, 1357-1368.

Martin, S., Rodolfo-Metalpa, R., Ransome, E., Rowley, S., Buia, M.-C., Gattuso, J.-P., Hall-Spencer, J., 2008. Effects of naturally acidified seawater on seagrass calcareous epibionts. Biology letters 4, 689-692.

Monteiro, F.M., Bach, L.T., Brownlee, C., Bown, P., Rickaby, R.E., Poulton, A.J., Tyrrell, T., Beaufort, L., Dutkiewicz, S., Gibbs, S., 2016. Why marine phytoplankton calcify. Science advances 2, e1501822.

Morita, M., Suwa, R., Iguchi, A., Nakamura, M., Shimada, K., Sakai, K., Suzuki, A., 2010. Ocean acidification reduces sperm flagellar motility in broadcast spawning reef invertebrates. Zygote 18, 103-107.

Nakamura, M., Morita, M., 2012. Sperm motility of the scleractinian coral Acropora digitifera under preindustrial, current, and predicted ocean acidification regimes. Aquatic Biology 15, 299-302.

Nishigaki, T., José, O., González-Cota, A.L., Romero, F., Treviño, C.L., Darszon, A., 2014. Intracellular pH in sperm physiology. Biochemical and biophysical research communications 450, 1149-1158.

O'Donnell, M.J., George, M.N., Carrington, E., 2013. Mussel byssus attachment weakened by ocean acidification. Nature Climate Change 3, 587.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., 2005. Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. Nature 437, 681.

Pansch, C., Hattich, G.S., Heinrichs, M.E., Pansch, A., Zagrodzka, Z., Havenhand, J.N., 2018. Long-term exposure to acidification disrupts reproduction in a marine invertebrate. PLoS One 13, e0192036.

Porzio, L., Buia, M.C., Ferretti, V., Lorenti, M., Rossi, M., Trifuoggi, M., Vergara, A., Arena, C., 2018. Photosynthesis and mineralogy of Jania rubens at low pH/high pCO 2: A future perspective. Science of the Total Environment 628, 375-383.

Robinson, K.M., Janes, M.S., Pehar, M., Monette, J.S., Ross, M.F., Hagen, T.M., Murphy, M.P., Beckman, J.S., 2006. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proceedings of the National Academy of Sciences 103, 15038-15043.

Satoh, N. Developmental biology of ascidians. Cambridge: Cambridge University Press; 1994.

Schlegel, P., Havenhand, J.N., Gillings, M.R., Williamson, J.E., 2012. Individual variability in reproductive success determines winners and losers under ocean acidification: a case study with sea urchins. PLoS One 7, e53118.

Takeshima, T., Kuroda, S., Yumura, Y. Reactive Oxygen Species and Sperm Cells. in: Cristiana F, editor. Reactive Oxygen Species (ROS) in Living Cells. London: IntechOpen; 2018. p.48-73.

Tosti, E., 1994. Sperm activation in species with external fertilisation. Zygote 2, 359-361.

Tosti, E., Ménézo, Y., 2016. Gamete activation: basic knowledge and clinical applications. Human reproduction update 22, 420-439.

Truebano, M., Tills, O., Collins, M., Clarke, C., Shipsides, E., Wheatley, C., Spicer, J.I., 2018. Short-term acclimation in adults does not predict offspring acclimation potential to hypoxia. Scientific reports 8, 3174.

Zupo, V., Mutalipassi, M., Fink, P., Di Natale, M., 2016. Effect of Ocean Acidification on the Communications among Invertebrates Mediated by Plant-Produced Volatile Organic Compounds. Global Journal of Ecology 1, 012-018.

Figure captions

Figure 1. Seawater acidification effects on sperm concentration in *C. robusta*. Sperm concentration, expressed as the number of spermatozoa (x 10^9/ml), collected from adults of *C. robusta* after exposure to control (CTRL, pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments.

Figure 2. Seawater acidification effects on sperm motility in *C. robusta*. Percentage of motile spermatozoa collected from adult *C. robusta* after exposure to control (CTRL, pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments. * and ** indicate statistical ($P< 0.05$) and high statistical ($P< 0.01$) significant differences, respectively, with one way ANOVA.

Figure 3. Seawater acidification effects on sperm viability in *C. robusta*. Proportion of live spermatozoa collected from adult *C. robusta* after exposure to control pH (CTRL pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments. * indicates statistical differences ($P< 0.05$) with one way ANOVA.

Figure 4. Seawater acidification effects on mitochondrial activity in spermatozoa of *C. robusta*. Mitochondrial membrane potential (MMP) measured in spermatozoa of *C. robusta* after exposure to control (CTRL, pH 8.1) and acidified (AcSW, pH 7.8) seawater for 7 days in *in situ* transplant and microcosm experiments. * indicates statistical differences ($P< 0.05$) with one way ANOVA.

Figure 5. Seawater acidification effects on extra- and intra- cellular pH in spermatozoa of *C. robusta*. pH_i recorded in spermatozoa of adult *C. robusta* after exposure to normal (CTRL, pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments. * and ** indicate statistically ($P< 0.05$) and high statistically differences ($P< 0.01$), respectively, with one way ANOVA.

Figure 6. Seawater acidification effects on intracellular level of reactive oxygen species in spermatozoa of *C. robusta*. Intracellular level of hydrogen peroxide (H_2O_2) and superoxide anion (O^-_2) measured in spermatozoa of adult *C. robusta* after exposure to control (CTRL, pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments.

Figure 7. Seawater acidification effects on plasma membrane lipid peroxidation in spermatozoa of *C. robusta*. Lipid peroxidation measured in spermatozoa of adult *C. robusta* after exposure to normal (CTRL, pH 8.1) and acidified seawater (AcSW, pH 7.8) for 7 days in *in situ* transplant and microcosm experiments.

Figure 8. Seawater acidification effects on morphology of *C. robusta* spermatozoa. (A) Representative SEM image of control spermatozoa where the mitochondrion strictly adheres to the head and plasma membrane cover all the structure. The fracture indicated by the arrow delimits the area of insertion of the mitochondrial on the head. (B) spermatozoa collected from ascidians exposed to acidified seawater exhibit the enhancement of the fracture due to the

progressive focal disintegration of the head plasma membrane (arrow) and consequent detachment and sliding of the mitochondrion. In particular: C) an enhancement of the zone of insertion of the mitochondrion on the sperm head due to the initial desegregation of the plasma membrane; D) the loss of the plasma membrane regions integrity causes the detachment of the mitochondrion from the sperm head; E) a total sliding of the mitochondrion from the sperm head toward the tail, (F) a loss of mitochondrion normal oval morphology. Scale bar is 1 µm.

Figure 9. Seawater acidification effects on *C. robusta* sperm ultrastructure. Representative TEM image of longitudinal (A) and frontal (B) view of control spermatozoa in which the mitochondrion adheres to the head and plasma membrane cover all the structure. C, D, E, F) spermatozoa collected from ascidian exposed to acidified seawater show in either longitudinal and frontal views frequent interruptions and loss of plasma membrane continuity, loss of the morphology and fragmentation of the mitochondrion as indicated by arrows. Scale bar is 1µm.

figure_1.tif
Click here to download high resolution image

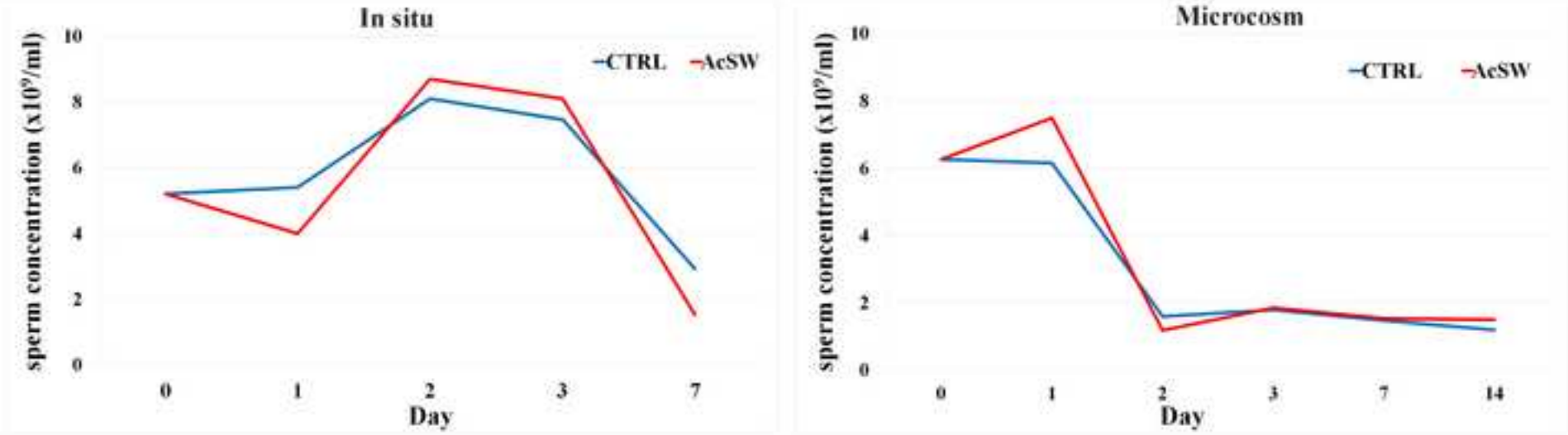


figure_2.tif
Click here to download high resolution image

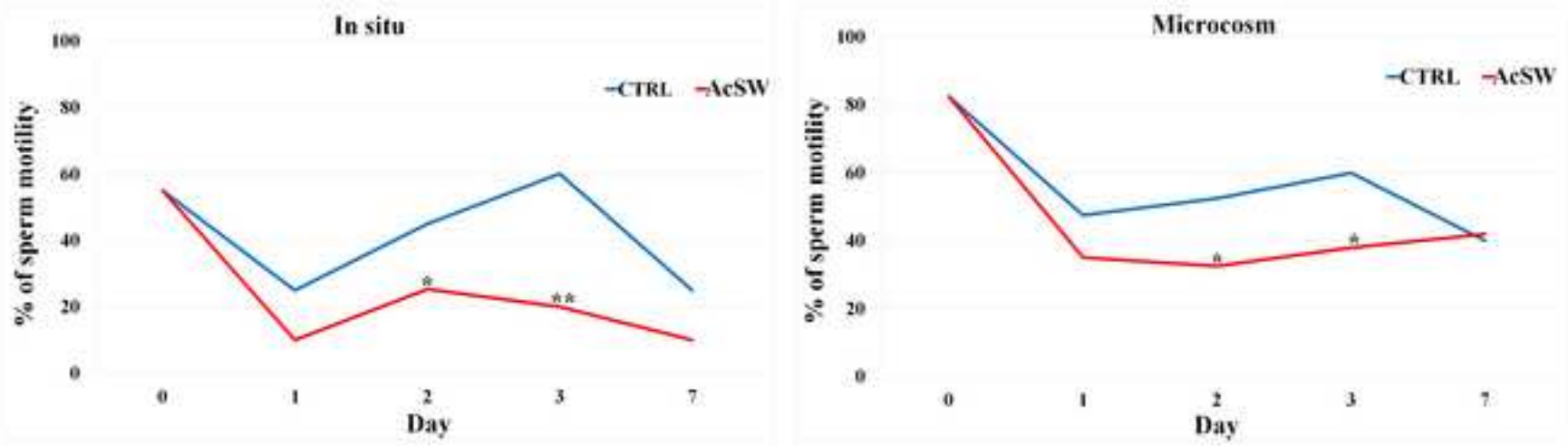


figure_3.tif
Click here to download high resolution image

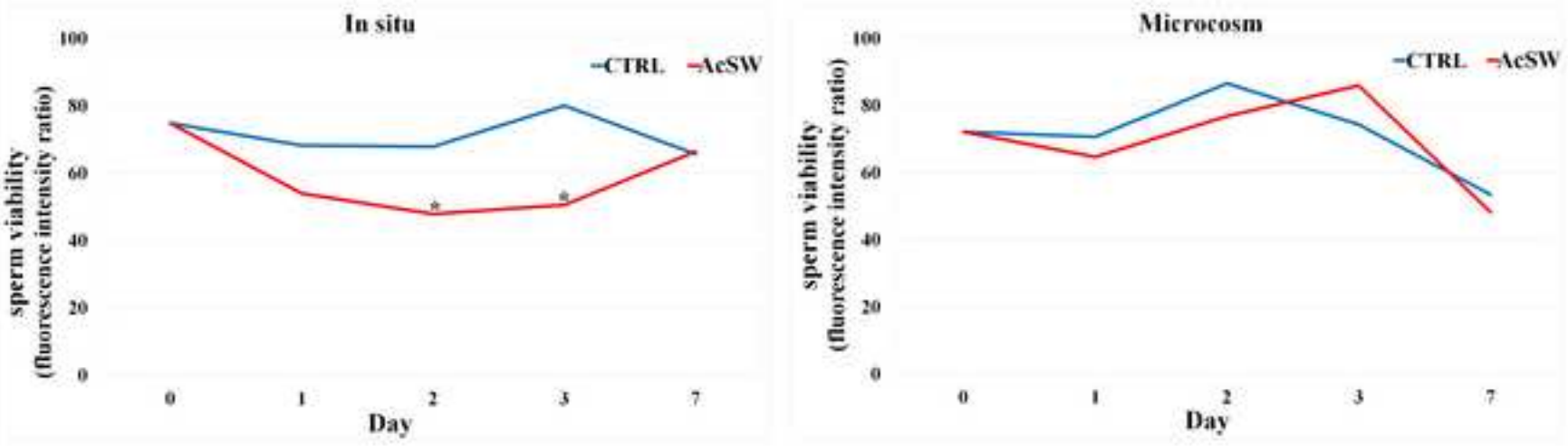


figure_4.tif
Click here to download high resolution image

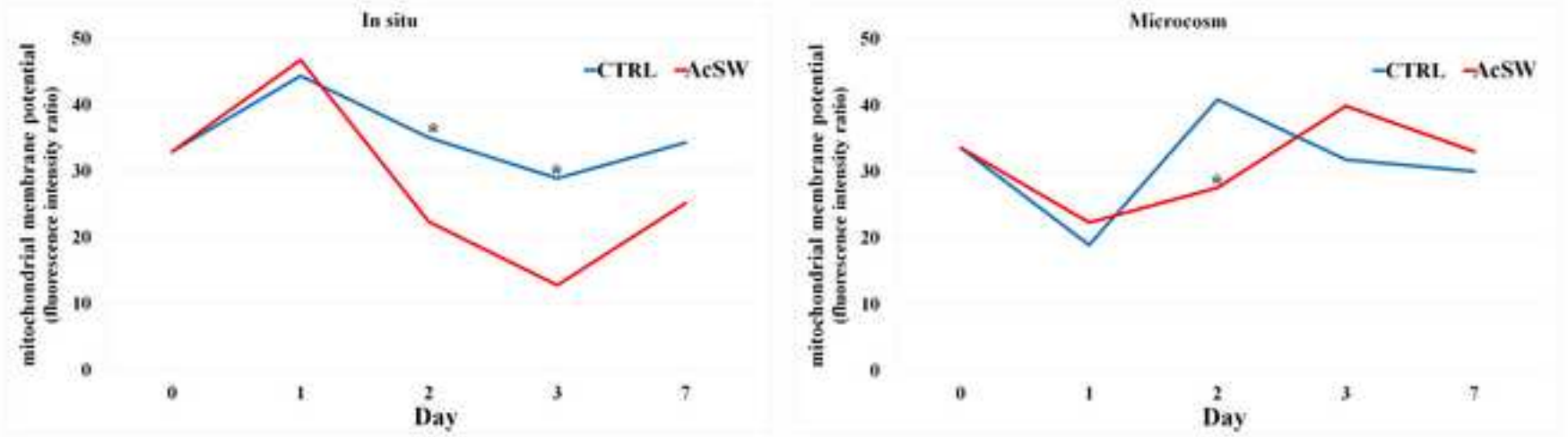


figure_5.tif
Click here to download high resolution image

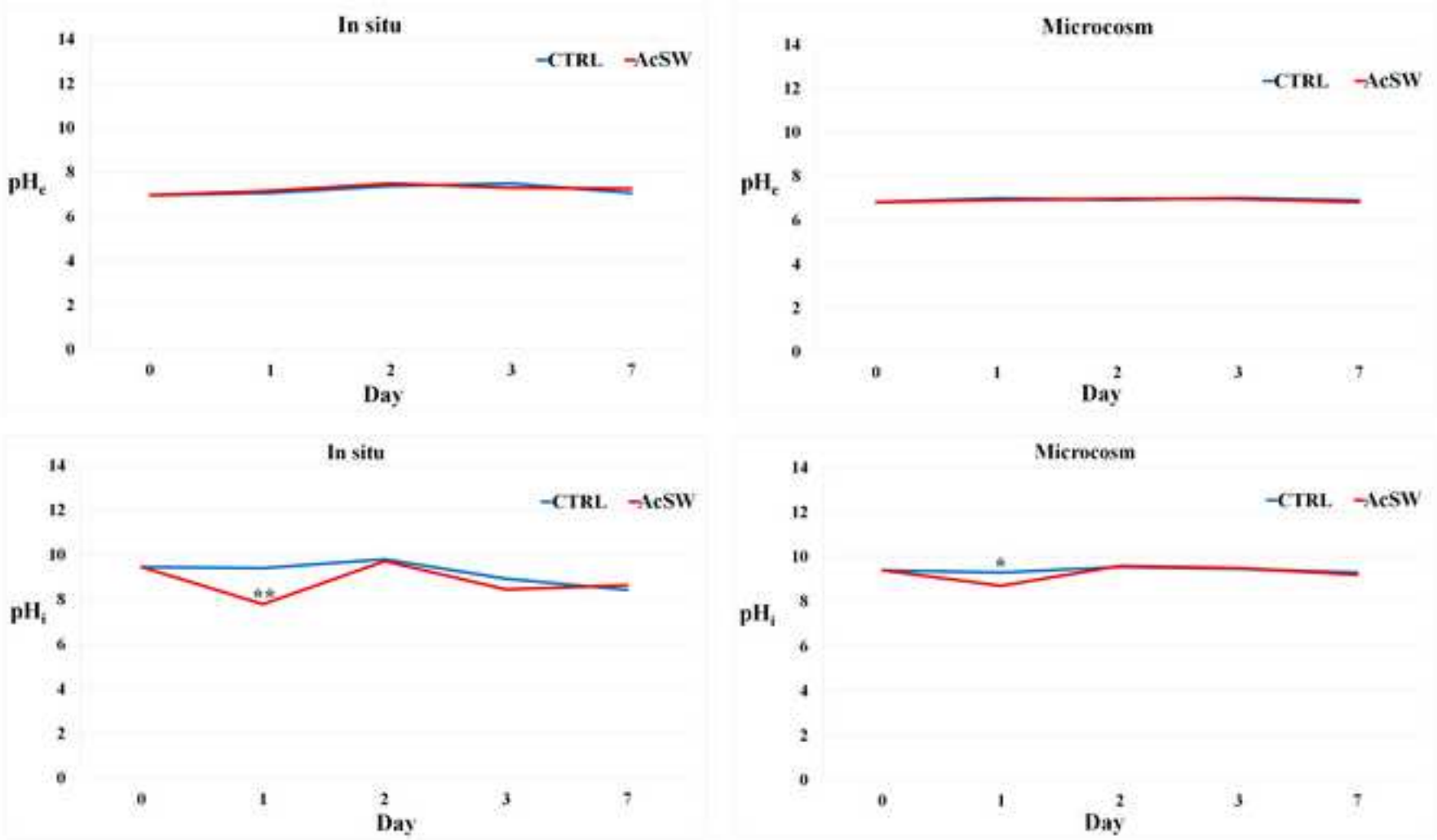


figure_6.tif
Click here to download high resolution image

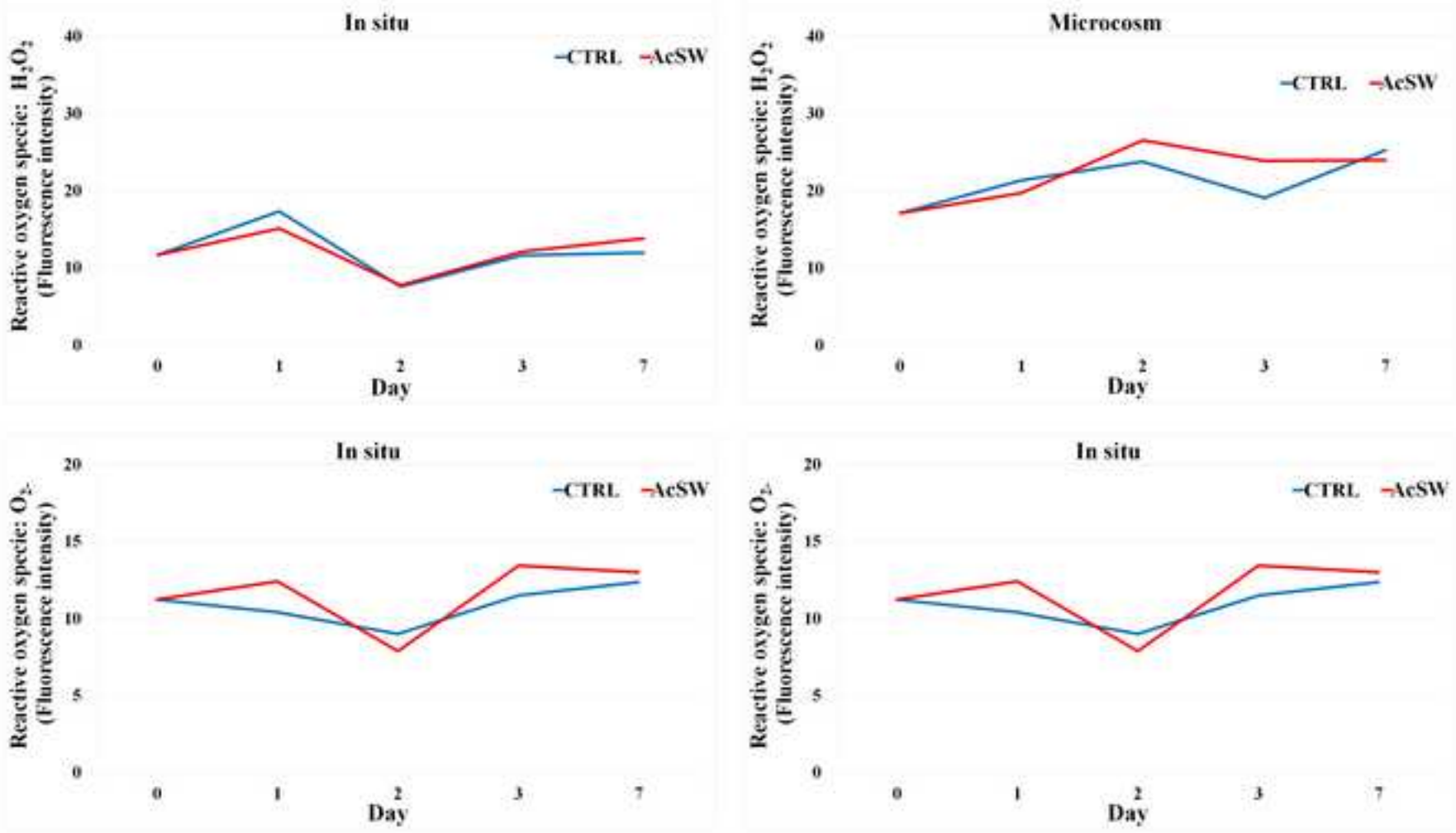


figure_7.tif
Click here to download high resolution image

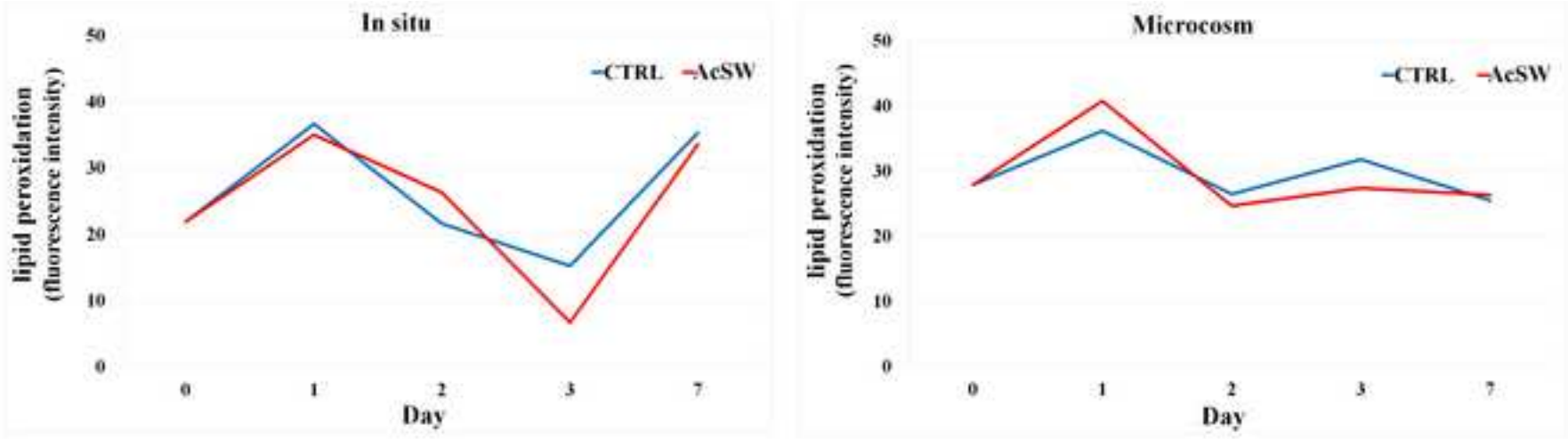


figure 8.tif
Click here to download high resolution image

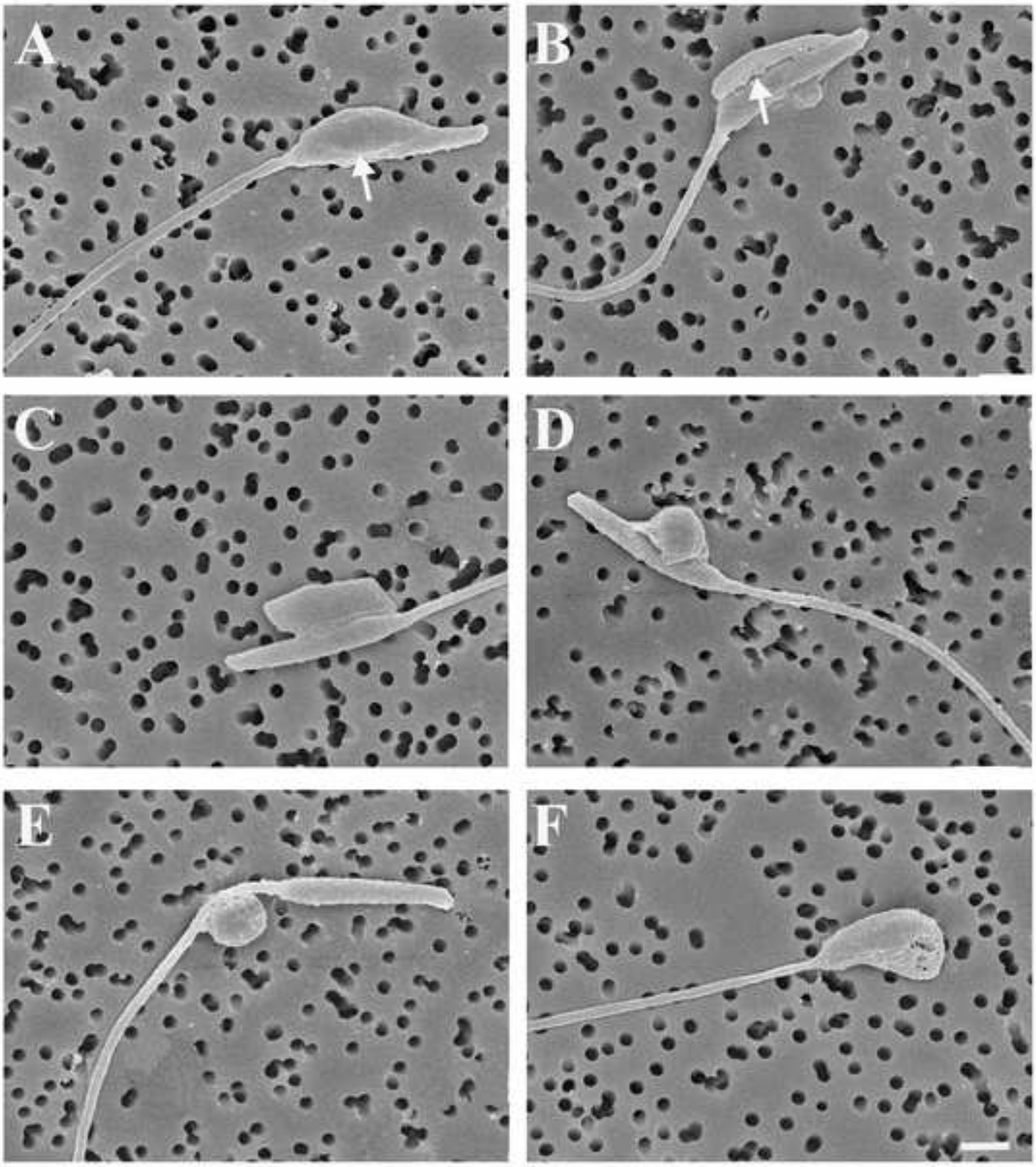
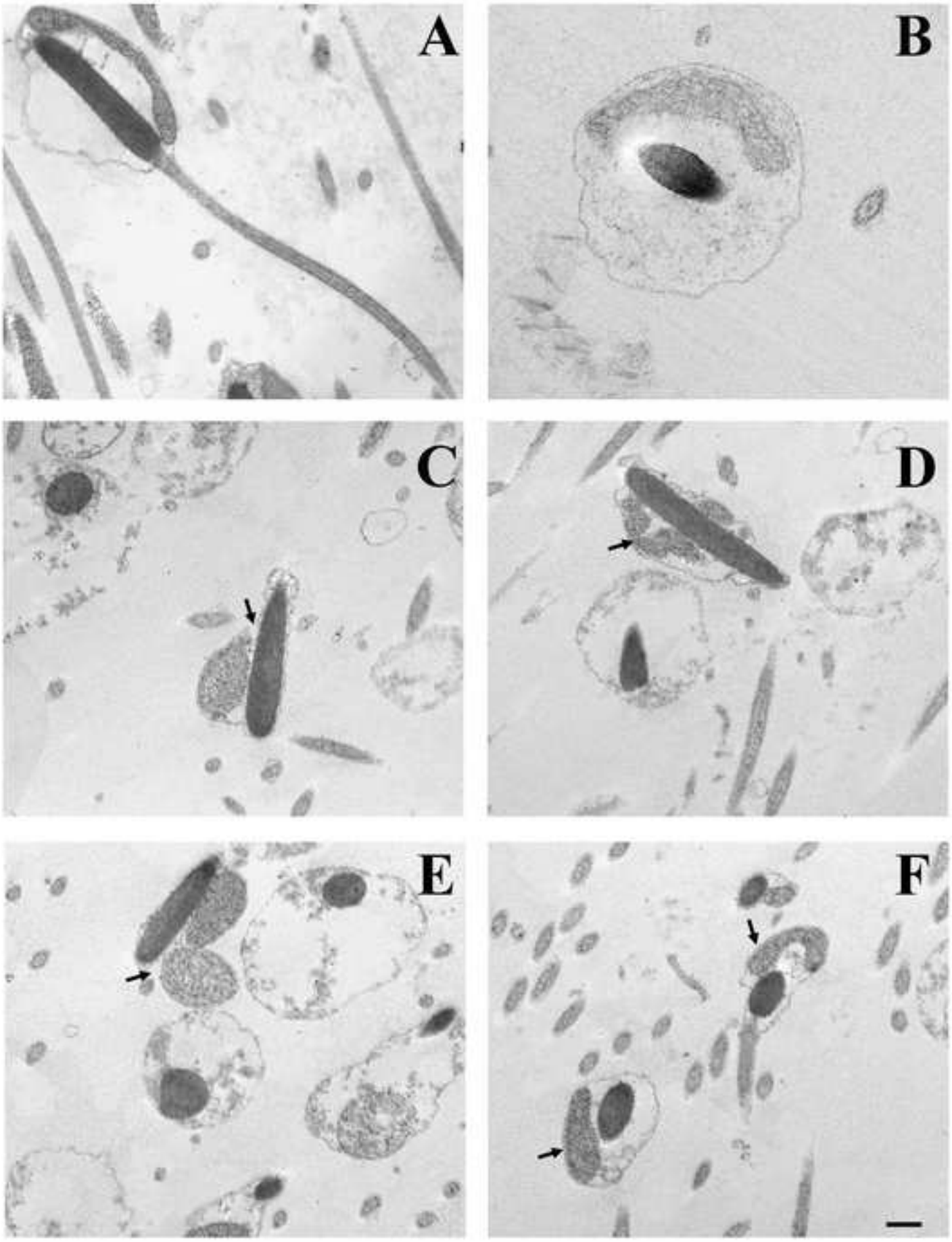


figure 9.tif
Click here to download high resolution image



Click here to download Figure: Figure Captation_Graphical Abstract.doc

Sperm sensitivity assessment in *Ciona robusta* after short-term acidified seawater exposure by setting up two experimental approaches: *in situ* transplant and microcosm. In the former, animals in cages were exposed to natural CO_2 vent emissions at Castello Aragonese (Island of Ischia, Gulf of Naples); in microcosm experiments seawater was acidified by means of a bubbling system suppling CO2 gas. After parental exposure, spermatozoa were collected from sperm duct. Different sperm quality parameters, as concentration, motility, fertilizing ability, reactive oxygen species, mitochondrial activity, intracellular pH, viability, lipid peroxidation, morphology, were evaluated by means of counting chamber, *in vitro* fertilization, spectrofluorimetry and electron microscopy.