Dell’Acqua, Ombretta, Sara Ferrando, Mariachiara Chiantore, and Valentina Asnaghi. "The impact of ocean acidification on the gonads of three key Antarctic benthic macroinvertebrates." *Aquatic Toxicology* 210 (2019): 19-29.

https://doi.org/10.1016/j.aquatox.2019.02.012

The impact of ocean acidification on the gonads of three key Antarctic benthic macroinvertebrates

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Declarations of interest: none. Each of the co-authors approved the submitted version

Contributors

OD carried out the experiment, collected data, performed the analyses and wrote the paper;

SF performed the histological assay and analyses;

MC and VA planned the experimental design and revised the manuscript, approving the submitted version

Abstract

CO_2 atmospheric pressure is increasing since industrial revolution, leading to a lowering of the ocean surface water pH, a phenomenon known as ocean acidification, with several reported effects on individual species and cascading effects on marine ecosystems. Despite the great amount of literature on ocean acidification effects on calcifying organisms, the response of their reproductive system still remains poorly known. In the present study, we investigated the histopathological effects of low pH on the gonads of three key macroinvertebrates of the Terra Nova Bay (Ross Sea) littoral area: the sea urchin *Sterechinus neumayeri*, the sea star *Odontaster validus* and the scallop *Adamussium colbecki*. After 1 month of exposure at control (8.12) and reduced (7.8 and 7.6) pH levels, we dissected the gonads and performed histological analyses to detect potential differences among treatments. Results showed significant effects on reproductive conditions of *A. colbecki* and *S. neumayeri*, while *O. validus* did not show any kind of alteration. Present results reinforce the need to focus on ocean acidification effects on soft tissues,

particularly the gonads, whose damage may exert large effects on the individual fitness, with cascading effects on the population dynamic of the species.

Keywords: ocean acidification, histopathology, gonads, macrobenthos, Antarctica

1. Introduction

The rising of atmospheric CO_2 concentration, since industrial revolution, has already caused the drop of ocean surface water pH of about 0.1 unit, a process known as ocean acidification (OA, Caldeira and Wickett 2003). OA is expected to exert a large number of impacts on marine organisms and ecosystems, but pathways and the extent of these impacts are still poorly understood, since organism response varies across species and even between life stages of the same species (e.g. Ries et al. 2009; Hendriks et al. 2010; Kroeker et al. 2010).

The Antarctic marine environment is supposed to be one of the most threatened by rising atmospheric CO_2, because of the peculiar seawater physico-chemical parameters (McNeil and Matear 2008; Fabry et al. 2008). Antarctic invertebrates, in turn, are not expected to successfully cope with current climate change pattern, occurring at a faster rate than ever (Meredith and King 2005; Hofmann and Todgham 2010), because of their low metabolism (Peck 2002). Their response to climate change impacts still needs to be fully understood and an in deep investigation on polar species and ecosystems is strongly necessary.

Although shell and skeleton dissolution are the most direct (and most studied) effects of OA, several other studies have focused on different aspects, such as survival, metabolic rate, growth, etc. (e.g. Michaelidis et al. 2005; Widdicombe and Spicer 2008; Wittmann and Pörtner 2013). Yet, despite this huge increase of knowledge, one aspect still remains poorly investigated: the effect of OA on the reproductive system. In fact, while a large number of papers deals with fertilization and embryonic and larval development (e.g. Kurihara et al. 2008; Byrne 2012; Van Colen et al. 2012; Byrne and Przeslawski 2013; Barros et al. 2013), so far only three papers have investigated the effects of OA on the gonads (Kurihara et al. 2013; Uthicke et al. 2014 and Mos et al. 2016), finding alterations in coelomic fluid ion compositions, reduced energy intake (leading to a delay in gonad maturation) and a general poor condition in the gonads of specimens exposed to low pH.

In order to investigate the reproductive system, histological techniques are very useful to detect sublethal effects and histopatology falls in the middle of the relationship 'response time' *vs* 'ecological significance' (Thiéry et al. 2012), allowing to detect effects also in relatively short-term experiments (in the range of few weeks). In fact, the histological survey of gonad tissues is often used to detect the welfare of organisms both in field and in laboratory conditions (e.g. Vaschenko et al. 1997; Lehmann et al. 2007; Martinez et al. 2014; Smaoui-Damak et al. 2006; Ortiz-Zarragoitia and Cajaraville 2010). In this view, gonads are considered a 'sentinel' organ, since they are commonly the first organs to be affected by energy re-allocation in case of organism stress, when a trade-off occurs between reproduction and somatic maintenance. This may be aggravated in calcifying organisms, which reserve a high energy percentage to the repair and maintenance of hard tissues (Sokolova et al. 2012; Haag et al. 2016).

In the present study, we investigated the potential effects of OA on the gonads of three macrobenthic invertebrates, key species in the Antarctic littoral ecosystem: the sea urchin *Sterechinus neumayeri*, the sea star *Odontaster validus* and the scallop *Adamussium colbecki*. These species are endemic of High-Antarctic areas, circumpolar and locally very abundant, playing an important role in the littoral ecosystem of Terra Nova Bay (Ross Sea, Chiantore et al., 2002).

S. neumayeri and *O. validus* are the most abundant echinoderms in shallow waters around Antarctica. These two echinoderms are generalist feeders, though *S. neumayeri* preferably feeds on benthic algae and detritus, while *O. validus* is mainly a scavenger and predator (McClintock 1994; Brey et al. 1995; Gillies et al. 2012; Taboada et al. 2013). Both species are dioecious and display a gametogenic cycle of 18-24 month, at least for females, with overlapped maturation of two cohorts of oocytes (separated by a year), simultaneously present in their gonads (Pearse 1965; Pearse and Giese 1966). Because of their abundance and trophic role, *S. neumayeri* and *O. validus* are considered key species in the shallow water benthic ecosystem (McClintock et al. 1988; McClintock 1994; Brey et al. 1995) and effects of climate change on these two echinoderms could lead to dramatic community shifts.

The Antarctic scallop, *A. colbecki* represents a key link in the bentho-pelagic coupling (Chiantore et al. 1998) and a food source for higher trophic levels (Vacchi et al. 2000; Dell'Acqua et al. 2017). Despite being patchily distributed (Schiaparelli and Linse 2006), it can be locally very abundant,

such as in Terra Nova Bay (Chiantore et al. 1998), where *A. colbecki* 'beds' play a relevant role in the coastal organic carbon flux and CO_2 sequestration in the shells.

S. neumayeri fertilization, embryo and larval development have been widely investigated in regard to OA, showing an overall robustness, also when warming acts as a co-stressor (e.g. Ericson et al., 2012; Collard et al., 2013; Morley et al 2016). So far, only one study has dealt with the effects of OA on *O. validus*, investigating its larval development and has found a decline in larval survival at pH 7.6, together with a significantly decreased body width (Gonzalez-Bernat et al., 2013). The same stands for *A. colbecki*, for which only one study dealing with OA effects is available: Benedetti et al. (2016) found a slight to moderate effect of low pH, but only in concert with other stressors (metals and/or warming).

The aim of the present study is to assess the effects of OA on gonadal development and the histological features of gonadal tissue of these three key Antarctic macroinvertebrates, in order to further elucidate benthic responses of animals in the Southern Ocean where near-future ocean acidification is predicted to be particularly pronounced (Fabry et al. 2009).

2. Material and methods

2.1. Specimens and field data collection

The experiment was performed at the Italian Mario Zucchelli Station (MZS), during the 2014-2015 Italian Antarctic Expedition. Specimens of *A. colbecki*, *S. neumayeri* and *O. validus* were collected by SCUBA divers on 10th December 2014, at around 15 m depth in Tethys Bay (74° 41.407' S; 164° 06.311' E), about 2 km from MZS. Specimens were immediately transported to the aquarium facility of the base and the shells of *A. colbecki* specimens were cleaned from epibionts by gentle brushing. All the specimens were acclimated in two (*O. validus*, as predator, was kept alone) 100 L refrigerated (-0.5°C) aquaria supplied with flow-through unfiltered seawater pumped from the water intake located in front of the station (at 6 m depth).

Littoral seawater parameters (temperature, salinity, and pH) were periodically measured using a CTD probe (Ocean Seven 310 CTD - Idronaut, Brugherio, Italy; Table 1), from 10th December 2014 to 27th January 2015, always at the same time of the day (10:30 to 12:00), both at the site of the water intake and at the site where the investigated specimens were collected.

2.2. Experimental set up

After a two-week period to allow the individuals to adjust to the laboratory conditions, the experiment started using a flow through system, in order to avoid O_2 depletion and uncontrolled

123 pH reduction due to respiration of the animals. Seawater was first pumped to a 100 L aquarium,
124 where the water was cooled to controlled -0.7°C, and afterwards to three 50 L header tanks, two
125 of which were set as treatment and one served as a control (unmanipulated water). Two nominal
126 pH treatment levels, 7.8 and 7.6, were chosen according to literature (Cao et al., 2007; Gonzalez-
127 Bernat et al., 2013) and were reached by bubbling pure CO_2 in two of the three header tanks. The
128 addition of CO_2 was made through two independent electronic valves, in connection with two pH
129 electrodes set on 7.8 and 7.6 and regulated by a continuous pH-stat system (IKS Aquastar,
130 Karlsbad, Germany). A third pH electrode was mounted on the pipe that fed the control header
131 tank in order to monitor the natural littoral seawater pH.
132 Eight specimens per species were subjected to each pH level: in order to control for a
133 potential 'tank effect', the individuals of each species were equally divided between two tanks for
134 each pH level. Consequently, we set up six tanks (experimental units or replicates; 20 x 25 x 20 cm)
135 for each pH level (2 for each species). Each tank contained 4 specimens for a total of 24 individuals
136 per species across the different tanks. The specimens for experimental exposure were randomly
137 selected, with the only restriction of the size range in order to perform the experiment on adult
138 specimens only. For each species, means and standard deviations of the 24 individuals were
139 calculated, resulting in 76 ± 3 mm shell height (SH, the distance from the umbo to the opposite
140 side of the shell) for *A. colbecki*, 37 ± 3 mm test diameter (without spines) for *S. neumayeri* and 43
141 ± 3 mm in mean distance from center of disc to arm tip (R) for *O. validus*.
142 Each experimental unit was directly fed, by gravity, from one of the header tanks with an
143 individual pipe, regulated manually by PVC valves at a rate of 150 ml/min, which assured a water
144 renewal rate of 60%/h in each experimental unit. After filling the experimental units, the water
145 was discharged through a relief hole. All the tanks were covered with transparent lids to avoid gas
146 exchange and specimen escape. The cover was only removed every 2-3 days for about 5 minutes,
147 in order to clean the tank bottom by siphoning. The organisms were not fed, but, since unfiltered
148 seawater was taken in, diatoms and other detrital material were available in the water. This
149 system, of course, could supply food for the filter feeding *A. colbecki* and, partially, the generalist
150 *S. neumayeri*, but could not meet the food demand of *O. validus*. Yet, both the sea urchin and the
151 sea star can stand long periods of time without food with no apparently ill effects (Brockington et
152 al. 2001; Agüera et al. 2016).
153 The pH electrodes of the pH-stat system were intercalibrated every 3-4 days on total scale
154 (pH_T) using TRIS buffer solutions with a salinity of 35 psu (Dickson et al., 2007) and cross checked

against the pH_T values measured with two different multiprobes that were previously calibrated on Antarctic littoral waters: Ocean Seven 310 CTD and C6 Muti-sensor Platform (Turner Design, San Jose, CA, USA).Temperature, salinity and pH were recorded from each header tank with multiprobes throughout the experimental period, while triplicate seawater samples for total alkalinity (TA) measurements were collected once a week, poisoned with $HgCl_2$ and stored at +4°C. In Italy, TA was determined at the Polytechnic University of Ancona (Italy) using an open cell potentiomentric titration according to Dickson et al. (2007) procedures and standards. Temperature, salinity, pH_T and TA were used to calculate pCO_2 of the three pH levels with SWCO2_V2 (http://http://neon-old.otago.ac.nz/research/kah/software/swco2/index.html) software, using the equilibrium constants of Millero et al. (2006), since the lowest value of their temperature range (0 – 40°C) is close to Antarctic waters. In addition, further duplicate seawater samples were periodically taken downstream from each header in order to measure nutrient concentration. The experiment lasted from 26th December 2014 to 28th January 2015 (34 days). At the end of the experiment, all specimens were measured, weighed and dissected. Gonads were dissected, weighed and fixed separately for each specimen in Bouin solution, then rinsed in ethanol 70% and stored at +4°C in order to return the samples to Italy. The gonado-somatic index (GSI) was assessed as follows:

GSI = gonad ww (g) * 100/body ww (g)

ww = wet weight.

2.3. Sample processing and statistical analyses

Once in Italy, the gonads were further dissected for the analysis. For *S. neumayeri* and *O. validus*, whose gonads are constituted of five portions, each of them was split into 2 halves; 5 out of the 10 halves for each specimen (24 sea urchins and 24 sea stars) were used for histology. In total, we analyzed 120 gonad portions for each species. For *A. colbecki*, we used three uncontiguous pieces from the middle of the gonad of each individual specimen (24 individuals), for a total of 72 gonad slices for this species. The selected portions were embedded in paraffin and cut with a microtome in order to obtain 6 μm thick sections on microscope slides. Afterwards, Hematoxylin-Eosin staining (Bio Optica Spa, Milan, Italy) was performed, mounting the cover slip with Eukitt (Kindler GmbH, Freiburg, Germany) and letting the slides dry for 24 hours. Since, preliminarily, a large mucus production in the gonoduct epithelium of *A. colbecki* specimens was noticed, additional slides were prepared (only for this species) to be stained with Alcian Blue-P.A.S

reaction for mucopolysaccharides (Bio Optica Spa, Milan, Italy). Sections were examined with a Leica DMRB light microscope and images were acquired with a Leica CCD camera DFC420C. Pictures were acquired at 10x and 20x magnification, covering at least 80% of the surface. Such high coverage of the gonad histological survey allowed for a reliable estimation of the potential damages. As far as the mucus production analysis, pictures of the Alcian Blue – P.A.S. stained slides were acquired, covering at least 80% of the total duct epithelium.

The gametogenic stage (GS) of the dissected specimens was assessed following Pearse and Giese (1966) for *S. neumayeri*, Pearse (1965) for *O. validus* and Berkman et al. (1991) for *A. colbecki*. Since during summer the three investigated species are at the end of the gametogenesis (*S. neumayeri*, Pearse and Giese 1966) or in maturation (*O. validus* and *A. colbecki*, Pearse 1965; Chiantore et al. 2001), no gonads in recovery stage were observed. The assigned stages were: early maturation = 1, advanced maturation = 2, ripe = 3, initial spawning = 4, advanced spawning/partially spent = 5, spent = 6.

Given the fundamental lack of reported knowledge on specifically OA effects on gonads, we decided to compare our histological evidences with considered published baseline of 'well-state' gonads of sea urchins, scallops and sea stars (e.g. Belkhedim et al. 2014; Zheng et al. 2014; Baeta et al. 2016), including the species here investigated (Pearse 1965; Pearse and Giese 1966; Berkman et al. 1991). Thanks to literature on altered gonads because of other stressors, such as hydrocharbons (Schäfer and Köhler, 2009) and temperature (Delorme and Sewell, 2016), we were able to ascribe gonads (or parts of) to 'good' or 'poor' conditions. We recorded any sign of 'anomalies' in our specimens and we computed the ratio of the total altered area on the total photographed area (anomaly ratio). For mucus analysis, we computed the ratio between the length of the membrane with mucus overproduction and the total photographed membrane length. This ratio also allowed to pool females and males in the statistical analysis to provide statistical robustness.

The Kruskal-Wallis test (hereafter KWt), performed using the R software package PMCMR (Pohlert, 2014; R Core Team, 2013), was applied to assess differences between treatments. Unfortunately, KWt does not allow for nested design, but it was selected because of its robustness for such small sample size. The KWt test was applied on the tank level and not on the pH treatment level, according to Cornwall and Hurd (2016). In case of significance, the Nemenyi-Damico-Wolf-Dunn test for post-hoc comparisons among tanks was performed. The tested response variables were: GSI, GS and anomaly ratio.

3. Results

3.1. Field and aquaria saeawater variables

The experimental set up, including the acidification system, provided stable seawater parameters for the whole experimental duration. Range of littoral seawater variables, at 6 (water intake) and 15 m (collection site) are displayed in Table 1. The cooling system in the aquarium provided an experimental temperature range in agreement with the natural variability at the collection site of the treated specimens (Tab. 2). Both temperature and pH values in the littoral waters changed over the season (Tab. 1), so that the experimental treatment 7.8 actually fell in the range of the natural field variability. The trend of the nutrient concentration along the experimental period is displayed in Fig. 1. The flow-through system assured the nutrients and cathbolites to be maintained under a potential toxic threshold (Suckling et al., 2015). A seasonal trend for nutrient concentrations was observed, as expected for the period, concurrent with the algal bloom: on 10th January all the nutrients reached the lowest levels, few days after the pack-ice break (pers. obs.).

Tab. 1. Field seawater variables. Means and standard deviations of the littoral seawater variables recorded at the water intake site (6 m) and at the specimen collection site (15 m) during the experimental period.

Site	Date	Temperature (°C)	Salinity (psu)	pH
Water intake (6 m)	10th Dec 2014	-1.7 ± 0.04	34.55 ±0.01	7.78±0.00
	15th Dec 2014	-1.42 ± 0.01	34.72± 0.00	7.88± 0.00
	18th Dec 2014	-0.59 ± 0.01	34.58 ± 0.00	7.99 ± 0.00
	20th Dec 2014	-0.54 ± 0.01	34.60 ± 0.01	8.00± 0.01
	29th Dec 2014	-0.81 ± 0.03	34.41 ± 0.01	8.05 ± 0.01
	2nd Jan 2015	-0.18 ± 0.01	34.52 ± 0.01	8.05 ± 0.00
	23rd Jan 2015	0.10 ± 0.25	33.30 ± 0.30	8.04 ± 0.02
	24th Jan 2015	0.34 ± 0.00	33.00 ± 0.00	8.06 ± 0.00
	27th Jan 2015	0.42 ± 0.05	33.66 ± 0.04	8.02 ± 0.01
Collection site (15 m)	10th Dec 2014	-1.75 ± 0.00	34.71 ± 0.00	7.78 ± 0.00
	15th Dec 2014	-1.46 ± 0.00	34.74 ± 0.00	7.86 ± 0.00
	19th Dec 2014	-0.58 ± 0.01	34.64 ± 0.00	7.98 ± 0.00

	20th Dec 2014	-0.62 ± 0.01	34.63 ± 0.00	7.95 ± 0.00
	29th Dec 2014	-0.64 ± 0.02	34.54 ± 0.01	7.99 ± 0.00
	2nd Jan 2015	-0.23 ± 0.02	34.59 ± 0.00	8.00 ± 0.00
	23rd Jan 2015	-0.25 ± 0.00	33.69 ± 0.00	8.01 ± 0.00
	24th Jan 2015	-0.30 ± 0.04	34.28 ± 0.03	8.02 ± 0.00
	27th Jan 2015	-0.16 ± 0.04	34.17 ± 0.02	8.01 ± 0.00

237

238 **Tab. 2. Experimental seawater variables.** Means and standard deviations of the seawater
239 variables recorded in the three treatments throughout the experiment, from 26th December 2014
240 to 28th January 2015. pCO_2 are calculated with SWCO2 software, using equilibrium constants from
241 Millero et al. (2006).

Nominal treatment	**Temperature (°C)**	**Salinity (psu)**	**pH**	**TA (µmol/kg_{SW})**	**pCO2 (ppm)**
control pH	-0.395 ± 0.12	33.06± 0.02	8.12 ± 0.05	2208.5± 75.7	381.7 ± 31.2
7.8	-0.362 ± 0.09	33.05± 0.01	7.814 ± 0.02	2238.3± 118.2	730.3 ± 35.5
7.6	-0.369 ± 0.12	33.09 ± 0.02	7.625 ± 0.02	2254.6± 89.2	1085.4 ± 55.9

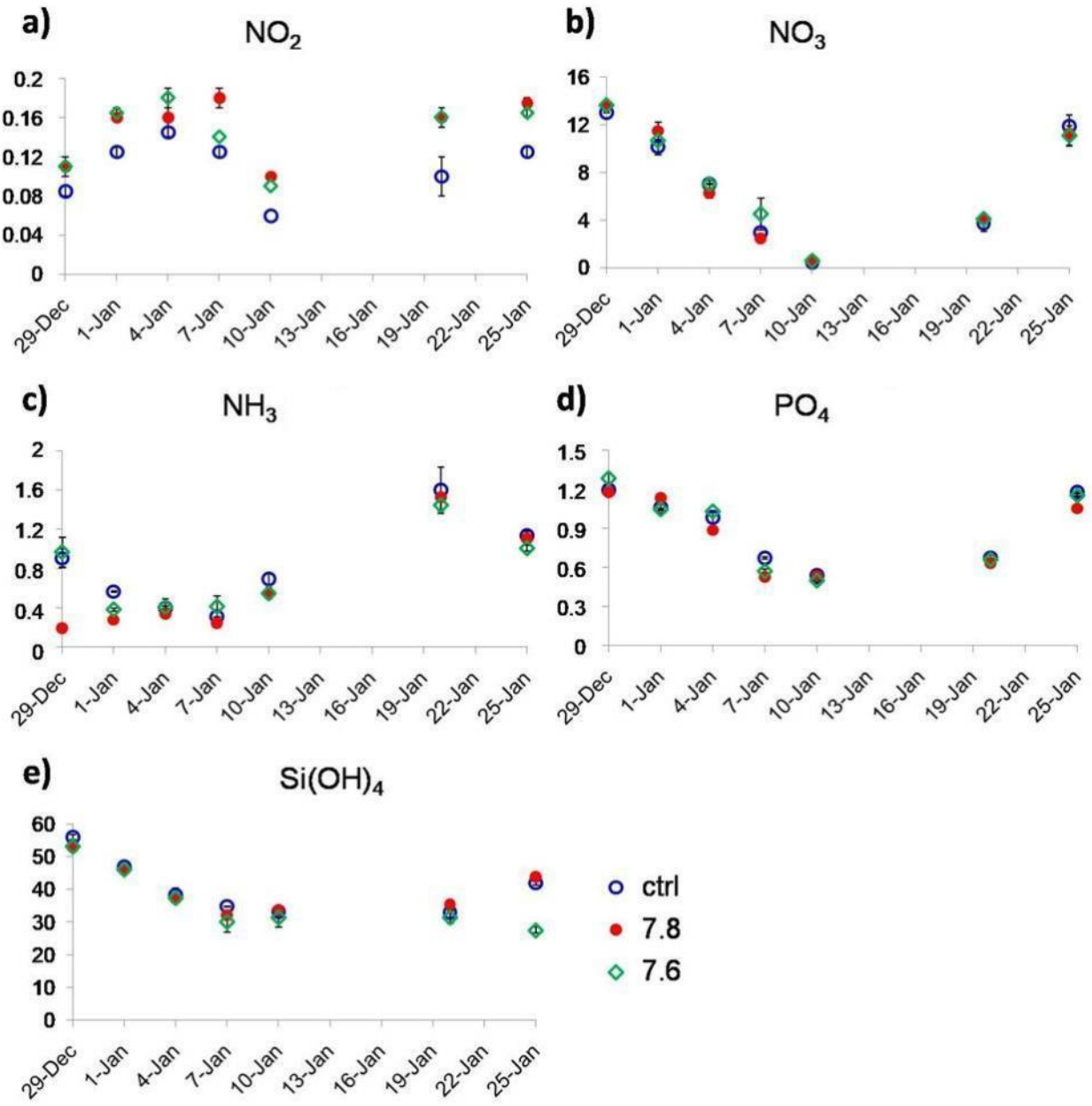


Fig. 1. Nutrient trends during the experiment. Trends of nutrient concentrations in the three treatments along the experimental period. Empty circles indicate control treatment, filled circles represent pH 7.8 and diamonds pH 7.6. Sampling date is reported on *x*-axis and nutrient concentration (µmol/l) on *y*-axis. (this figure is 1.5-column fitting image and it should be in color in the online version only)

3.2. Histological assay

Overall anomalies found in the experimental specimens with respect to treatment are displayed in Tab. 3. Single species response to OA treatments are described in the following sections.

252 **Tab. 3. List of histological anomalies.** Observed anomalies in the gonads associated to the
253 nominal treatment and number of individuals in which the given type of anomaly was detected.
254 Some individuals displayed more than a single type of anomaly. Percentage of individuals is
255 calculated, for each pH, as number of specimens displaying anomalies on a total of 8 individuals.

Species	pH	Alteration	% ind
A. colbecki	Ctrl	Leakage of germinal tissue outside the acinus	12.5
	7.6	Disruption of the acinus basal membrane, with concurrent leakage of germinal tissue outside the acinus	75
		Fibrosis	25
		Disruption of the trabeculae and connective tissue	12.5
		Hemocyte infiltration	50
		Neoplasia (overgrowth of undifferentiated germinal cells)	12.5
S. neumayeri	Ctrl	Spermatozoa detached flagella (head missing)	12.5
	7.8	Spermatozoa detached flagella (head missing)	12.5
		Intense desquamation of ovary sac tissue, including the germinal tissue, and concurrent fibrosis	62.5
		Hermaphroditism	12.5
		Atretic oocytes	25
		Neoplasia (overgrowth of undifferentiated germinal cells)	12.5
	7.6	Spermatozoa detached flagella (head missing)	12.5
		Intense desquamation of ovary tissue, including~~ed~~ the germinal tissue, and concurrent fibrosis	25
		Hermaphroditism	12.5
		Neoplasia (overgrowth of undifferentiated germinal cells)	25
		Disruption of acinus basal membrane, with concurrent disorganization of the germinal tissue (that lost the radial architecture) and fibrosis	37.5
		Diffuse lipofuscin-like pigments (LPP, Vaschenko et al. 2012)	12.5

O. validus	7.8	Desquamation of follicle tissue with concomitant hemocytes infiltration	12.5

3.2.1. Adamussium colbecki

Kruskal-Wallis test for GSI provided significant results (p = 0.043) and Dunn Test for post-hoc comparisons indicates significant higher values for the pH 7.6 tanks compared to all the other treatments, except ctrlb, that significantly differs from ctrla (Fig. 2). Both ctrl and pH 7.8 tanks accounted for a gonad stage ranging from 1 (early maturation) to 3 (ripe gonads, only two specimens), while pH 7.6 showed a higher mean value (4, initial spawning), as 6 specimens on 8 were in spawning and the others accounted for ripe gonads. KWt and Dunn Test confirmed these observations, being p-value = 0.015 due to the tanks in pH 7.6.

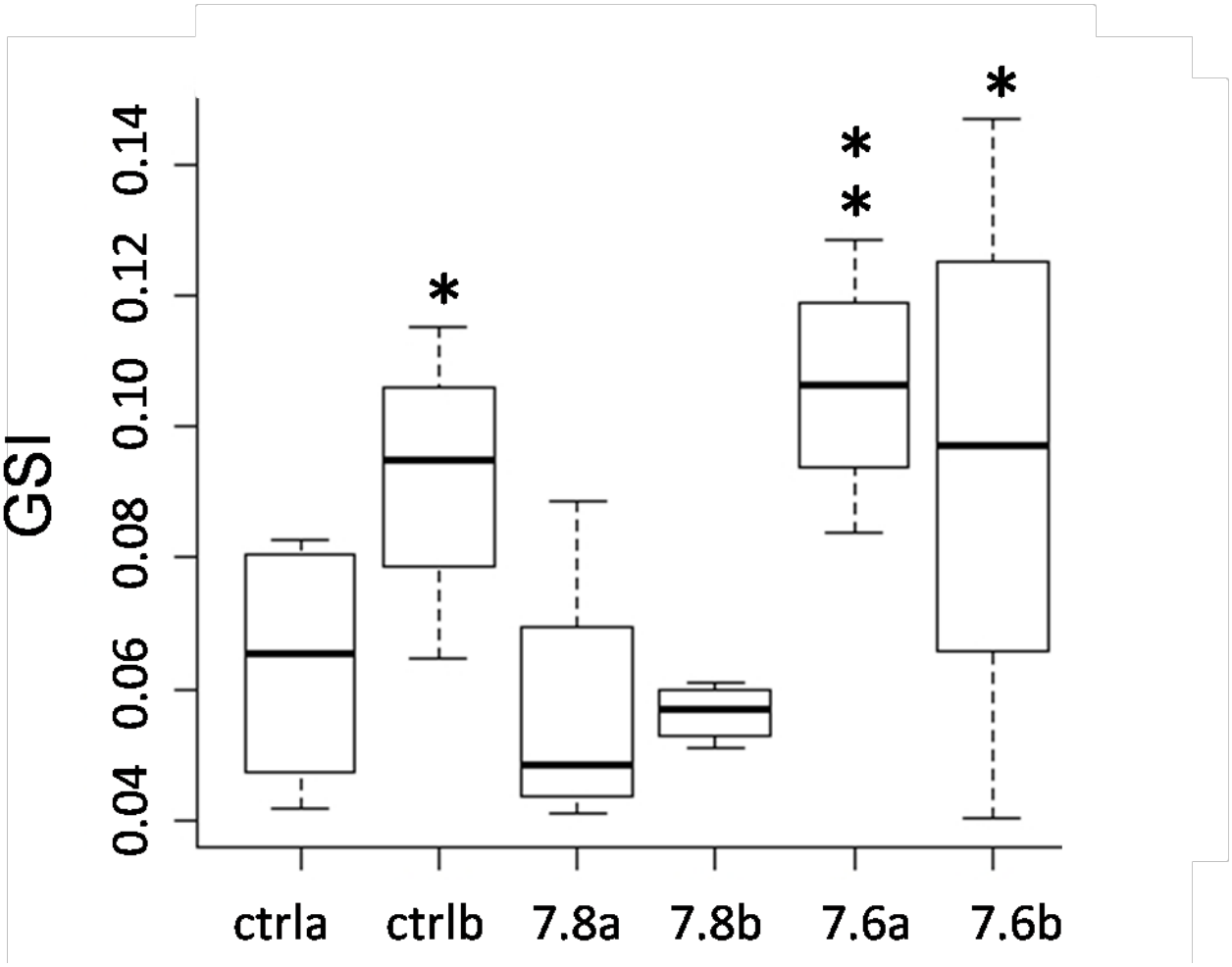


Fig. 2. *Adamussium colbecki* GSI *vs* tank level. Boxplot showing the difference, among tanks, in the gonado-somatic index (GSI) of *A. colbecki*. Middle line: median; boxes: 25-75 percentiles; whiskers: lowest and highest data points. *A. colbecki* GSI values on *y*-axis; tank level on *x*-axis: the

nominal pH is indicated by ‘ctrl’, ‘7.8’, ‘7.6’, while the letters ‘a’ or ‘b’ refer to the tank replicates. Boxes with the same number of asterisks are statistically not different. N = 4 per individual tank. (this figure is a single column fitting image)

The mucus presence in the gonoducts epithelium did not show significant differences among tanks (p = 0.650), as well as the occurrence of anomalies ratio (p = 0.060). Despite the lack of significance, we found different types of anomaly in some specimens at pH 7.6, indicating that some kind of stress occurred, although with a large inter-individual variability. Herein, we describe the most representative anomalies, those occurred in 50% or more specimens.

Fig. 3 shows gonads of female and male specimens maintained under control conditions, compared with the ones at pH 7.6, where anomalies were found. Fig. 3a and 3b show gonads in the control specimens that are in very good conditions, either with ripe ovaries or mature acini. Conversely, specimens from pH 7.6 display some level of anomalies: massive hemocyte infiltration (Fig. 3c and 3d, female and male, respectively) and disruption of male follicle basal membrane with leakage of germinal cells (Fig. 3e).

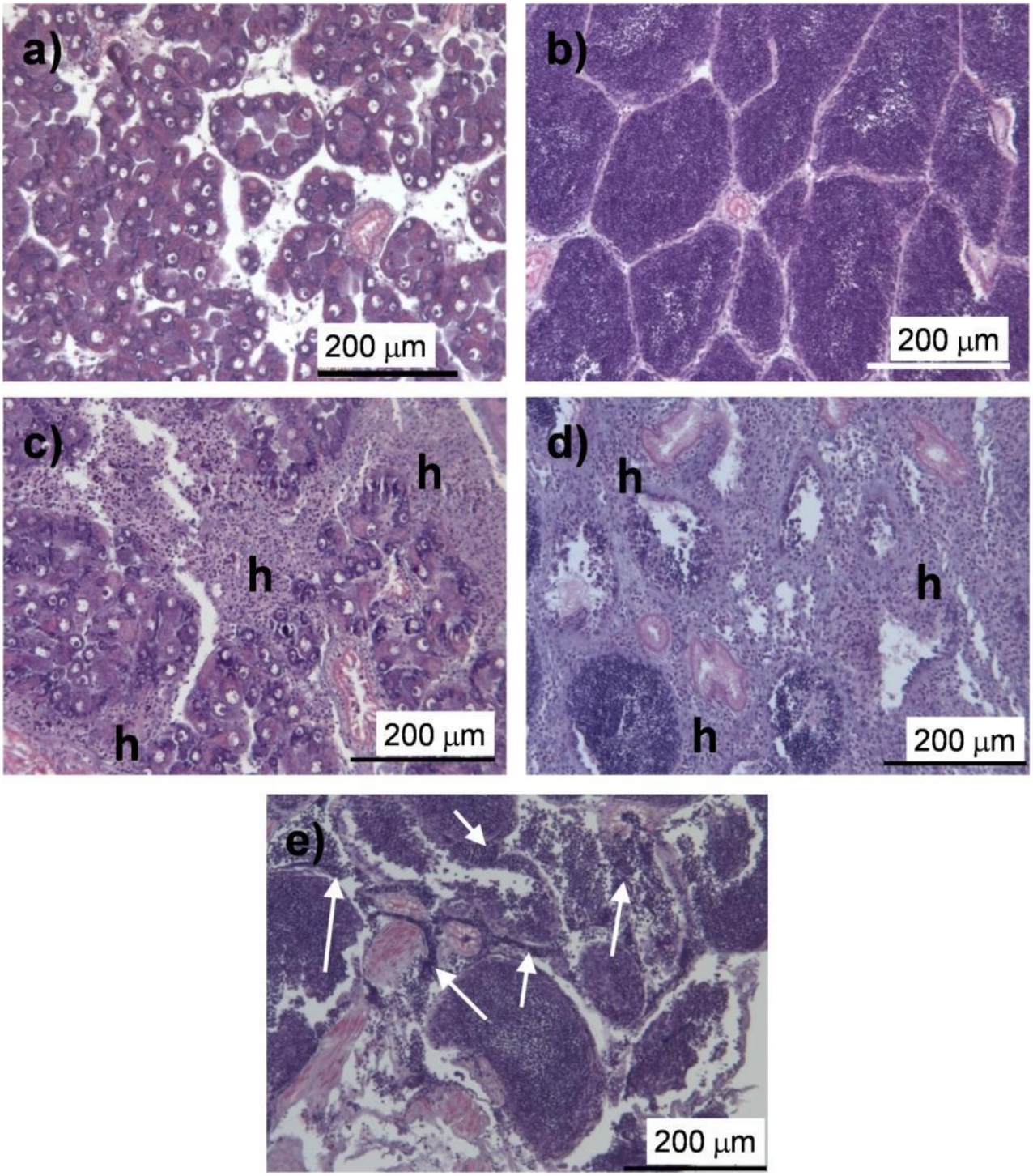


Fig. 3. *Adamussium colbecki* gonad histology. Representative gonad tissue of *A. colbecki* maintained at control and pH 7.6. (a, b) Normal histology of a female and a male gonad from control pH, with ovaries and testis in advanced maturation. (c, d) Female and male gonads from pH 7.6, showing a massive hemocyte intrusion in the follicles (indicated with 'h'). (e) Gonad of a male specimen from pH 7.6, showing disruption of acinus epithelium and leakage of germinal cells (white arrows) between acini. (this figure is 2-column fitting image and it should be in color in the online version only)

Fig. 4 shows two male gonads stained with Alcian Blue-PAS. Mucous components are highlighted by a more intense blue along the gonoduct epithelium. Around half of the specimens maintained at pH 7.6 showed larger mucus production compared to the control specimens, but the difference was not statistically significant

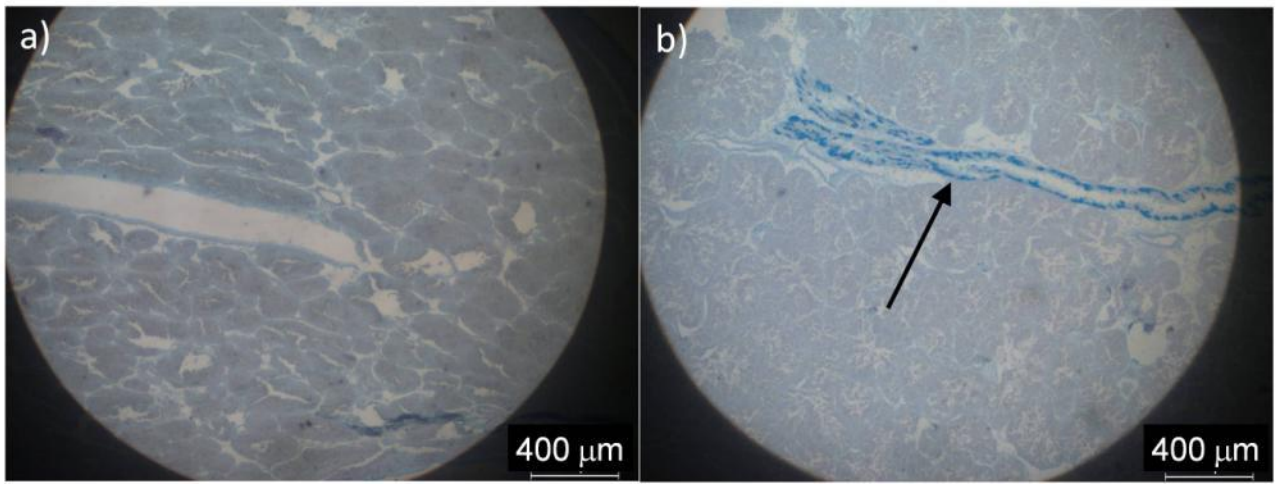


Fig. 4. *Adamussium colbecki* gonad histological evidence of mucus overproduction. Gonad tissue of *A. colbecki* maintained at control pH (a) and at pH 7.6 (b), stained with Alcian Blue-P.A.S. Mucus production is detectable along the gonoduct epithelium (see the intense blue coloration indicated by the black arrow in b), where the mucous components bind the stain (this figure is 2-column fitting image and it should be in color in the online version only)

3.2.2. Sterechinus neumayeri

GSI of *S. neumayeri* did not significantly differ among tanks (p = 0.103), with no detectable pattern among tanks either from different or same pH treatment. High individual variability was observed, as from the boxes width and the variability among tanks within the same pH (Fig. 5). All the specimens were in advanced spawning or spent stage, showing a gonad stage 5 or 6, with no differences among tanks (p = 0.409). Conversely, the anomaly ratio indicated a tank effect, which is ascribable to the treatment (p = 0.046). In fact, the post-hoc comparison shows that control tanks did not differ, but were statistically different from tanks at pH 7.8 and 7.6, that were, in turn, very similar among each other.

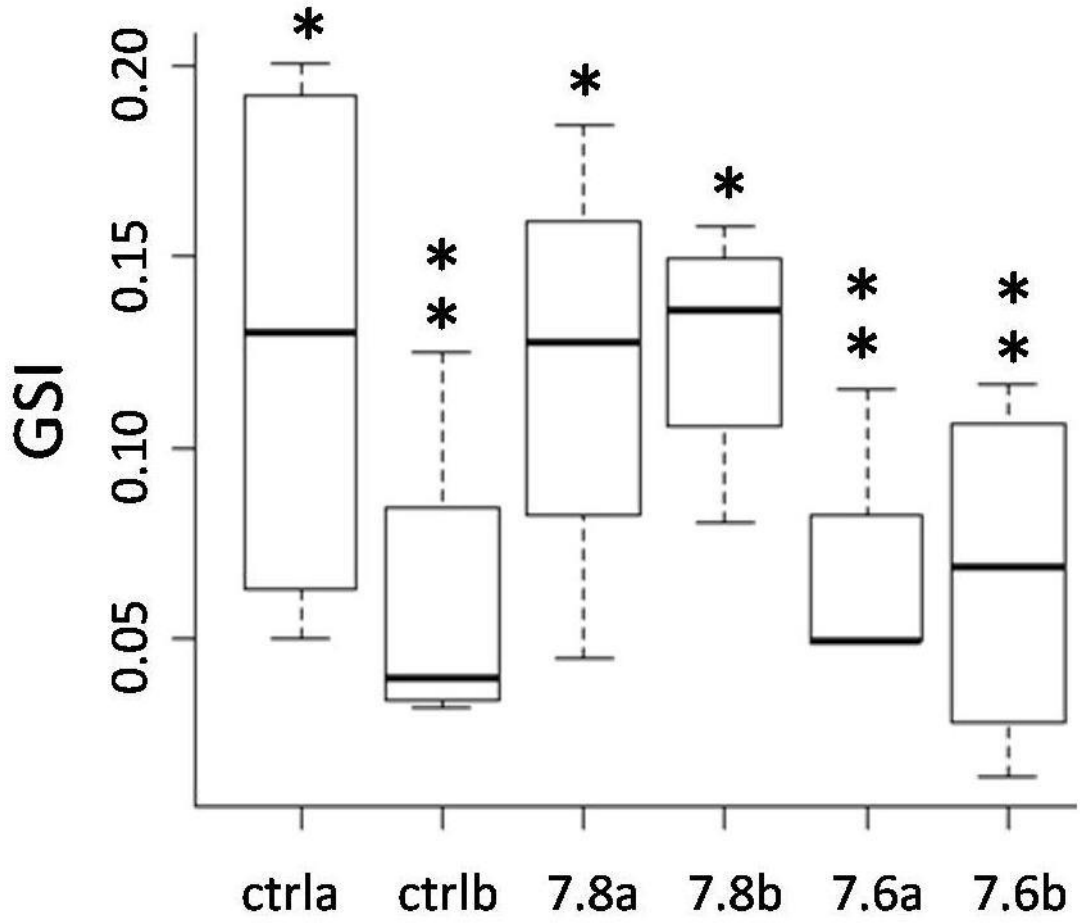
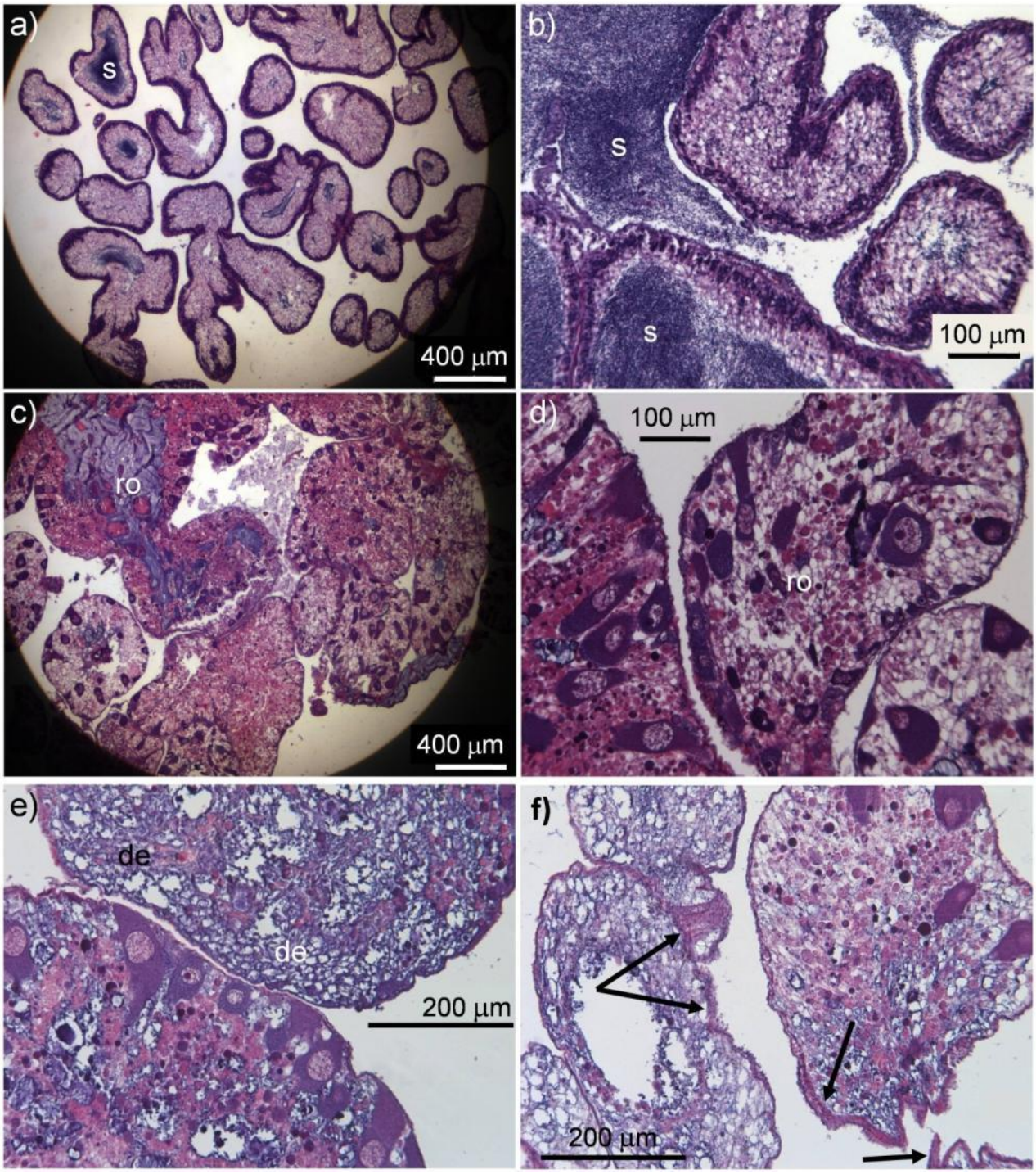


Fig. 5. *Sterechinus neumayeri* GSI at tank level. Boxplot showing the difference, among tanks, in the gonado-somatic index (GSI) of *S. neumayeri*. Middle line: median; boxes: 25-75 percentiles; whiskers: lowest and highest data points. *S. neumayeri* GSI values on *y*-axis; tank level on *x*-axis: the nominal pH is indicated by 'ctrl', '7.8' or '7.6', while letters 'a' or 'b' refer to the tank replicates. Boxes with the same number of asterisks are statistically not different. N = 4 per individual tank. (this figure is a single column fitting image)

We found a high variability of anomalies, even between tanks within the same pH. The anomalies that occurred at higher frequency are described as representative; few anomalies, occurring at lower frequency, are described in terms of their relevance associated with experimental or environmental stressors in literature. The different anomalies detected in the gonads of low pH treated *S. neumayeri*, compared with the control specimens, are displayed in Fig. 6. Panels a, b, c and d show male and female gonads in control specimens, with asynchronous ovaries and acini, either in spawning or spent stage. Panels from e to k show different types of anomalies that were found either at pH 7.8 or 7.6, sometimes in both: intense desquamation of germinal tissue and concurrent fibrosis (Fig. 6e and 6f), hermaphroditism, with either female, male

and miscellaneous follicles (Fig. 6g), neoplasia (Fig. 6h), disorganization of the germinal tissue with loss of the radial architecture (Fig. 6i), several spread anomalies (including basal membrane disruption) with the presence of lipofuscin-like pigments (LLP, Vaschenko et al. 2012) throughout the tissue (Fig. 6j), and sperm flagella detached from the head (Fig. 6k).



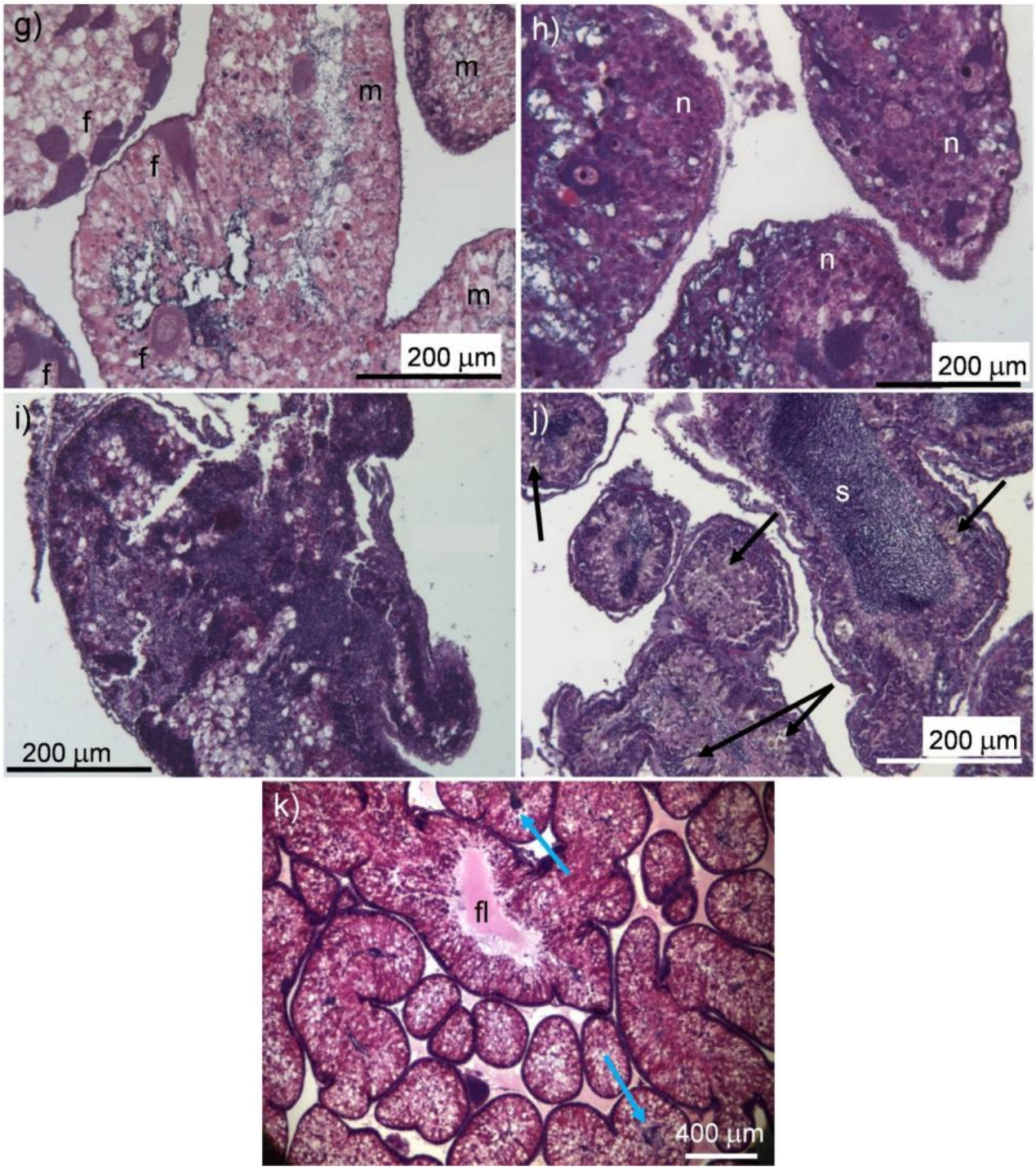


Fig. 6. *Sterechinus neumayeri* gonad histology. Gonad tissue of *S. neumayeri* maintained at control pH, pH 7.8 ~~or~~ and 7.6. (a, b) Representative histology of a male gonad showing testis either at the end of the spawning stage or spent (s = sperms). (c, d) Representative histology of a female gonad showing ovary sacs either at the end of the spawning stage or spent (ro = relict oocytes). (e, f) Representative female gonads from pH 7.8 and 7.6, respectively, showing an intense desquamation of the germinal tissue (de = desquamation) and fibrosis of the follicle membrane (black arrows). (g) Hermaphroditic specimen from pH 7.8, showing either distinct female and male

follicles, as well as miscellaneous follicles (f =female germinal tissue, m = male germinal tissue). (h) Neoplasia (indicated with n) in a female specimen from pH 7.6. (i) Male gonad of a specimen from pH 7.6. (i) Male gonad of a specimen from pH 7.6, displaying a disorganized germinal tissue that lost the radial architecture along with disruption of the acinus membrane. (j) Diffuse several anomalies in a specimen from pH 7.6, including presence of lipofuscin-like pigments (black arrow), s = sperms. (k) Detached flagella of spermatozoa, both inside lumina and between acini (fl = flagella, blue arrows =normal sperms). (this figure is 2-column fitting image and it should be in color in the online version only)

3.3.3. Odontaster validus

GSI of *O. validus* significantly differed among tanks (p = 0.038); this response seems not to be ascribable to the pH treatment, but, rather to a high variability between tanks, even within the same pH (Fig. 7). The overall GS ranged from 1 to 5, with the majority of the specimens (14 out of 24) in stage 2 (advanced maturation), but no significant difference was detected among tanks (p = 0.874). Also the anomaly ratio did not show significant differences among tanks ($p \approx 1$). The only alteration we found is displayed in Fig. 8, in a single specimen from pH 7.8

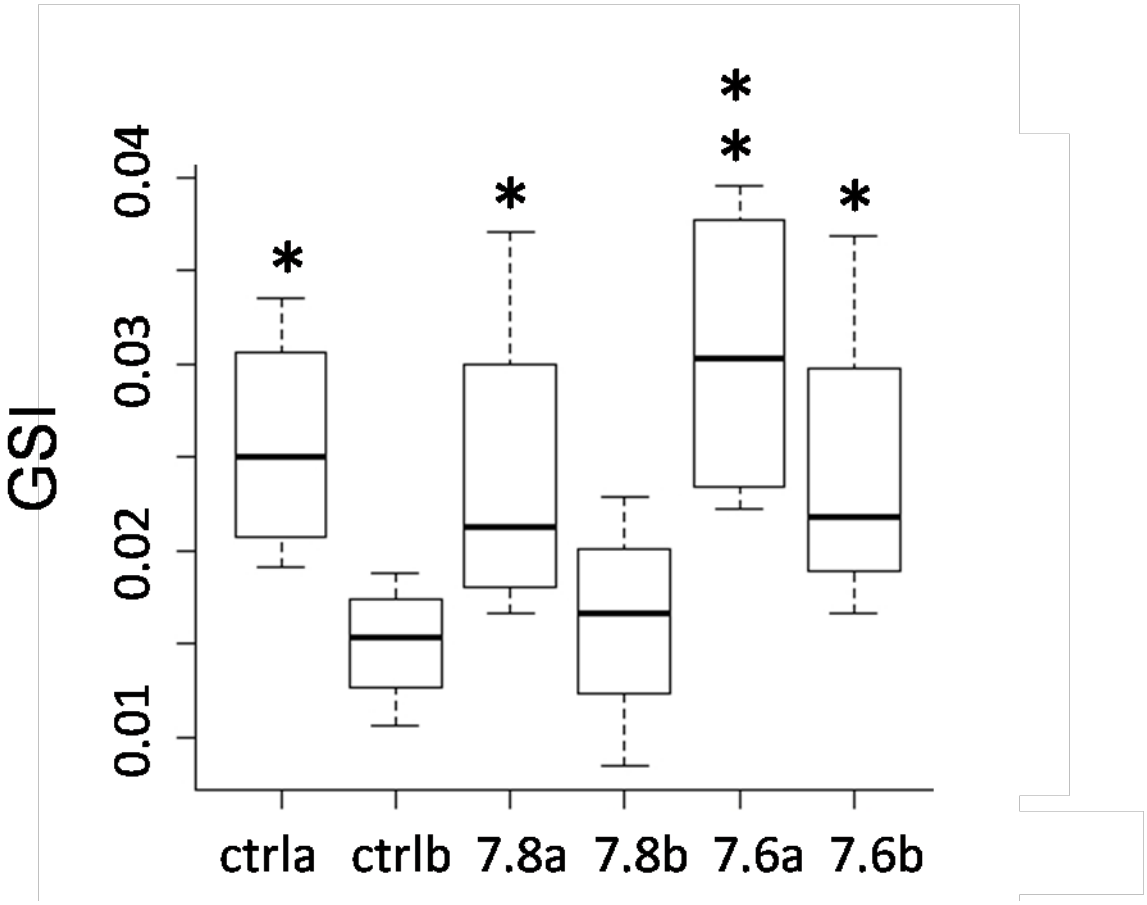


Fig. 7. *Odontaster validus* GSI at tank level. Boxplot showing the difference in the gonado-somatic index (GSI) of *O. validus* between tanks. Middle line: median; boxes: 25-75 percentiles; whiskers: lowest and highest data points. *O. validus* GSI values on *y*-axis; tank level on *x*-axis: the nominal pH is indicated by 'ctrl', '7.8' or '7.6', while letters 'a' or 'b' refer to the tank replicates. Boxes with the same number of asterisks are statistically not different. N = 4 per individual tank (this figure is a single column fitting image)

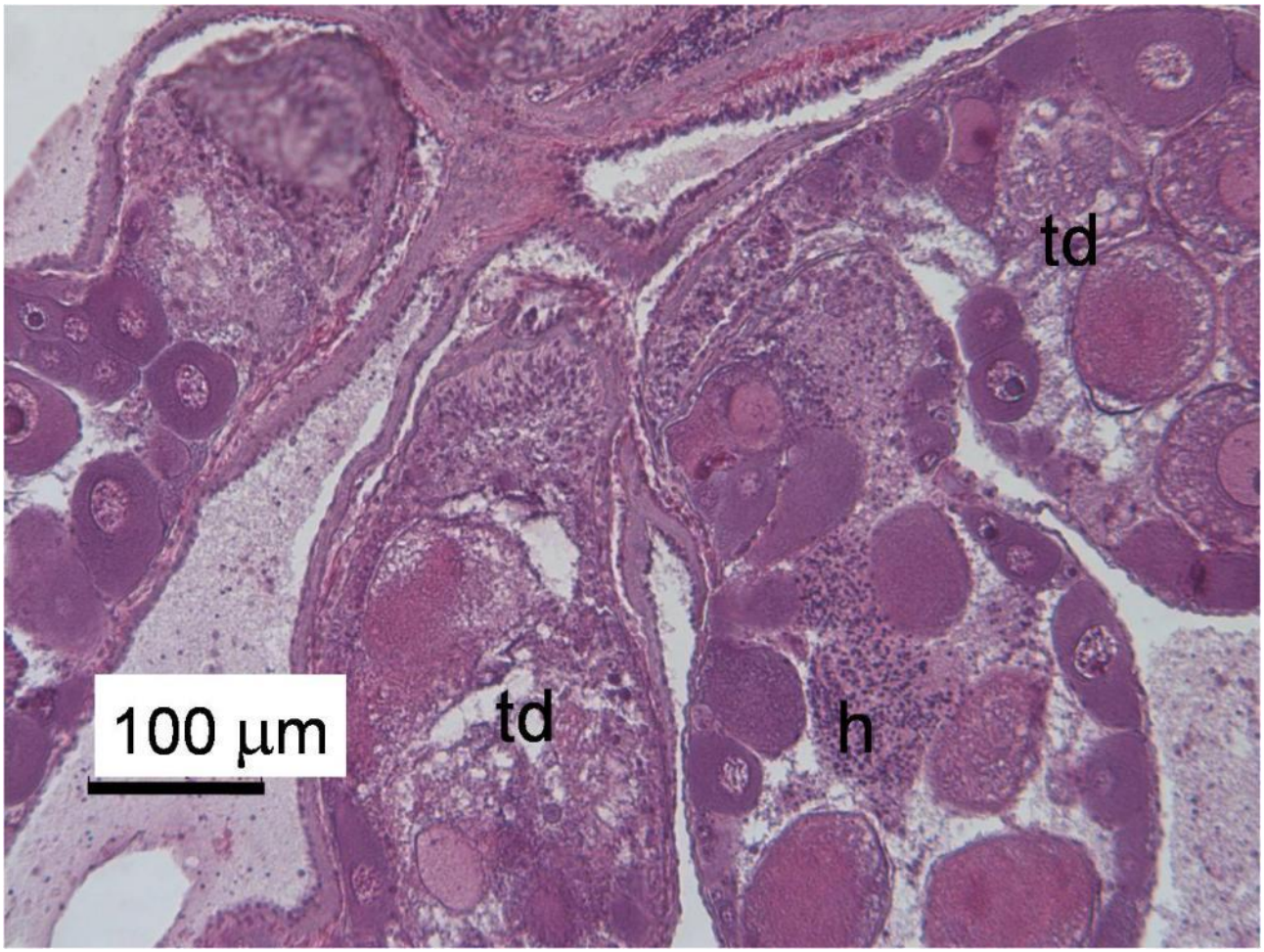


Fig. 8. *Odontaster validus* gonad histology. Gonad tissue of a female *O. validus* kept at pH 7.8, displaying tissue disruption (td) and hemocyte inflitration (h). (this figure is a single column fitting image and it should be in color in the online version only)

4. Discussion

The aim of the present study was to provide a comparable investigation on OA response in three key benthic Antarctic species from the same habitat, simultaneously exposed to the same experimental treatment in a relatively short-term experiment.

In the natural environment the pH showed a remarkable variation during the short summer season following physical and biological processes: at the beginning of the season, the lower seawater pH mirrors the high respiration processes occurring under the sea-ice and the total absence of gas exchange (Schram et al. 2015). With the progression of the summer season and the concomitant seawater temperature rise, the sea-ice bottom probably started to melt and break up, allowing partial gas exchange and the bloom of the sympagic algae. The peak of this process was reached on 10th January, but field measures close to that date were not possible because of the sea ice instability. Notably, the nominal pH 7.8 fell in the range of the littoral seawater at Terra Nova Bay during late spring. Basically, the specimens maintained at pH 7.8 experienced the advent of the summer season (phytoplankton bloom clearly visible in the aquarium water, higher temperature and lower salinity), but an unchanged surrounding pH.

Overall, in the controlled experimental conditions, a diffused effect of low pH was detected in *A. colbecki* and *S. neumayeri*, but not in *O. validus*, which did not display a clear response to OA as far as the three investigated variables: GSI, GS and anomaly ratio in gonad tissue. Although we measured and weighed the specimens at the beginning and at the end of the experiment, we did not perform any statistical analyses on body size and weight, as these species are known to grow very slowly, even less than 1 gram (*S. neumayeri*: Brey et al. 1995; Suckling et al. 2015) or 2 mm (*A. colbecki*: Heilmayer et al. 2003) per year, so that growth rate effects could not be detected over the short experimental timeframe.

4.1. Adamussium colbecki

While GS was affected by pH 7.6, nothing can be inferred about GSI. Indeed, a significant difference between the two control tanks was detected for the GSI, probably due to a high inter-individual variability that reduced the statistical power of the test. All the male specimens at pH 7.6 were in the spawning stage, while the other treatments accounted mostly for late maturation. Premature spawning in response to environmental stress has already been observed in clams and mussels naturally or experimentally exposed to pollutants (e.g. Smaoui-Damak et al. 2006; Gonzalez-Fernandez et al. 2016). Premature spawning seems to be a way to get rid of the affected gametes or to avoid gamete alteration in case of adverse environmental conditions (Gonzalez-Fernandez et al. 2016). Synchronous spawning is fundamental in broadcast invertebrates to ensure successful fertilization (Lewis and Ford, 2012) and asynchrony between males and females

404 could seriously affect the population dynamic of *A. colbecki*, known to display an intermittent
405 recruitment (Berkman et al. 1991).

406 Hemocyte aggregation inside bivalve follicles is a regular physiological occurrence during
407 recovery stage, when hemocytes perform relict gamete resorption (Dorange and Le Pennec,
408 1989), but are normally excluded in any other gametogenic stage (Donaghy et al. 2009).
409 Anomalous hemocyte infiltration in follicles is regarded as an important biomarker of
410 inflammatory response, occurring in affected tissues (Donaghy et al. 2009; Cuevas et al. 2015), in
411 bivalves either naturally or experimentally exposed to endocrine disruptors (e.g. Ortiz-Zarragoitia
412 and Cajaraville 2010; Cuevas et al. 2015; Gonzalez-Fernandez et al. 2016).

413 Mucus production can be associated to the stress of the experimental procedures
414 (Heinonen et al. 2007), but also to an immune response to oxidative stress (Allam and Espinosa,
415 2016). Since *A. colbecki* shows signs of stress under environmental hypercapnia (Benedetti et al.
416 2016) and given the high cost of mucus production (Davies and Hawkins 1998), the low pH treated
417 scallops with a higher mucus production may have suffered from this additional energy demand.

418 *4.2. Sterechinus neumayeri*

419 In this species, no effects on GSI and GS were detected, in agreement with Suckling et al.
420 (2015), who did not detect differences even after 2 years of exposure to acidified seawater; the
421 differences recorded among tanks do not follow any pattern, but seem to be related to a high
422 inter-individual variability. The same variability can be detected in the anomaly ratio, as we found
423 different types of anomalies in different percentages, even between tanks of the same pH.

424 The most diffuse anomaly was a desquamation of gonad tissue, that occurred intensively in
425 female specimens, both at pH 7.8 and 7.6. Concurrently, atretic oocytes and disruption of part of
426 the second cohort of oocytes were found (Fig. 6e and 6f) as well as follicle membrane fibrosis (Fig.
427 6f). Schäfer and Köhler (2009) reported the same anomaly in the gonads of female *Psammechinus*
428 *miliaris* exposed to fenanthren. These anomalies, other than destroy part of the new oocytes, may
429 affect the energy budget of the sea urchin because of the effort required to repair and reconstruct
430 the ovary tissue. Alteration in gonad development can also affect the fitness of the offspring
431 (Khristoforova et al. 1984; den Besten et al. 1991), as seems to be confirmed by the findings of
432 Suckling et al. (2015). The authors reported alterations in *S. neumayeri* larvae obtained from the
433 fertilization of eggs and sperms released by adult individual maintained at low pH (7.7 and 7.5).

434 Following three peculiar anomalies are described, not because of their frequency of
435 occurrence (very low), but because they are rare in echinoids in general, and usually associated, in

literature, to endocrine disruptors. Three of our treated sea urchins had stage 2 neoplasia (Carballal et al. 2015): one female at pH 7.8, one male and the hermaphrodite (female follicle) specimen at pH 7.6. Neoplasia is the proliferation of germinal cells that prevent the normal gamete maturation (Barber, 1996; Carballal et al. 2015); so far, it has been described for only a few bivalves (Carballal et al. 2015 and references therein) and one gastropod only (Gagnaire et al. 2009) in relation to endocrine disruptors (Tay et al. 2003; Carballal et al. 2015). Two sea urchin specimens, one from pH 7.8 and one from pH 7.6 showed hermaphroditism, a trait never reported for *S. neumayeri* and very rare for echinoids in general (Boolootian and Moore, 1956; Bernard 1977 and references therein; Byrne 1990 and references therein). In bivalves, hermaphroditism can occur in case of environmental stress (Tay et al. 2003; Ortiz-Zarragoitia and Cajaraville, 2010 and references therein) and, in some cases, oocytes become relict and are substituted by sperm, because of less energy is required by male gametes (Wintermyer and Cooper, 2007; Ortiz-Zarragoitia and Cajaraville, 2010). Such process is detectable in the central follicle in Fig. 6g. One male at pH 7.6 displayed a massive presence of LLP inside follicles during the spawning stage (Fig. 6j). LLP normally occurs in the gonads during recovery stage only (Vaschenko et al. 2012; Delorme and Sewell, 2016), but is known to occur otherwise in case of oxidative stress (Schäfer et al. 2011). Finally, two male specimens (one from pH 7.8 and one from 7.6) displayed a massive presence of detached flagella, either inside the acinus lumen or between acini. We did not find any literature reference for this anomaly, but we can hypothesize that the amount of healthy sperm, able to swim and reach the oocytes, is unavoidably reduced.

Odontaster validus

The sea star *O. validus* did not display any sufferance from low pH exposure. In fact, no effects were detected for any of the investigated variables, although tank 7.6a was slightly different from the other tanks with regard to GSI (Fig. 7). Similar to *S. neumayeri*, the variability of the response seems to be due to an overall high inter-individual variability, since no pattern can be detected, either related to tanks or pH. As far as anomaly ratio, all the specimen gonads were in good conditions with only one specimen at pH 7.8 showing altered gonad tissue. Opposite to the adult resistance to OA reported in the present study, Gonzalez-Bernat et al. (2013) found reduced survival and overall morphological alterations in larvae exposed to pH 7.6, corresponding to 1129.6 μatm (similar to 1085.4 μatm, which is the pCO_2 at pH 7.6 in our experiment).

4.3. Comparison of species

The response of the three species was very variable, both in terms of effect size and typology. Indeed, while *A. colbecki* clearly displayed an advanced GS at the lowest pH, the GSI and gonad tissue did not seem to be significantly affected. Conversely *S. neumayeri* only displayed diffuse anomalies in the gonad tissue, but no altered development. A particular case is *O. validus*, which did not display any response to OA. While the experimental system was identical for the three species, the only variables that can be considered affecting the response comparison are the feeding condition and the gametogenesis stage. Food quality and availability can improve invertebrate resistance to OA effects (Asnaghi et al. 2013), providing more energy to maintain and repair tissues. Since phytoplankton was available in the aquarium water during the algal bloom, coincident with the experimental period, this could have been a source of resistance for the filter-feeding *A. colbecki*, which was in the gonad maturation stage. Probably thanks to the energy gained by feeding on the phytoplankton, the scallop was able to maintain the gonad tissue in good condition. But the perception of an unfavorable surrounding environment may have been the trigger for premature release of gametes. *S. neumayeri*, in spawning stage, was the only species that displayed a diffuse alteration of the gonad tissue. As mainly a deposit feeder, the sea urchin, although not experiencing a real starvation, was probably not able to feed as usually during the summer season. As well as other sea urchins, *S. neumayeri* can reallocate energy from gonads to other tissues in case of starvation (Russel 1995; Brockington and Clarke 2001); the specimens treated at pH 7.8 and 7.6 may have reallocated their energy, favoring, most probably, the hard tissue (Haag et al. 2016). Because the animals were in the gamete release stage, this energy reallocation would not have affected the current reproduction of the sea urchin, but, rather, the second cohort of oocytes, which require energy for the germinal cell repair. A longer-term experiment would be necessary to understand the gonad resilience of the sea urchin, following a prolonged low pH exposure. *O. validus*, being preferably a scavenging/predator, is the only species that could have been considered under starvation, yet, it is the only species that did not display any response to the treatments. The resistance of *O. validus* to the unfavorable conditions may be explained by the very low somatic maintenance costs that allow the sea star to withstand even long periods of starvation (Agüera et al. 2015). Moreover, Peck et al. (2008) showed a high resistance of *O. validus* to rising temperature; together with our results, the sea star seems to display a peculiar ability to cope with climate change features.

5. Conclusions

The response of the three Antarctic species here investigated was very variable, both in terms of effect size and typology. Histopathology with regards to OA, so far largely underestimated, needs to be further investigated in the light of its endocrine alteration effects, together with investigation on the energetic cost of their resistance/resilience to altered seawater conditions. This may be of particular interest for calcifying species, since hard tissues can demand up to 75% of the energy budget. The energy requirement is up to four times higher than the amount required for reproduction (Sokolova et al. 2012). Studies performed during the winter season are mostly required, when food scarcity potentially reduces gonad resistance and the coastal seawater is expected to act as a source of CO_2 (Schram et al. 2015). Finally, the different response of the three species to OA needs to be evaluated in the framework of species interaction and food web structure. In fact, if the predator *O. validus* will successfully cope with OA, opposite to its prey *A. colbecki,* the potential cascading effects may be larger than the expected on basis of a single species response.

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786 **Aknowledgment**

787 This study, including the experiment, the data field collection and the preparation of this
788 manuscript, has been possible thanks to the project 2013/AZ1.03: Effetti dell'acidificazione sul
789 benthos Antartico (ACAB), and also thanks to the 2015-16 project PNRA16_00105: ANTA_Biofilm,
790 both supported by the PNRA (Programma Nazionale delle Ricerche in Antartide), the Italian
791 national program for antarctic research. The authors are also thankful for all the logistic support
792 provided, in the field, by the PNRA and, in particular, by the ENEA (Agenzia nazionale per le nuove
793 tecnologie, l'energia e lo sviluppo economico sostenibile).