Within- and trans-generational responses to combined global changes are highly divergent in two congeneric species of marine annelids

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Abstract

Trans-generational plasticity (TGP) represents a primary mechanism for guaranteeing species persistence under rapid global changes. To date, no study on TGP responses of marine organisms to global change scenarios in the ocean has been conducted on phylogenetically closely-related species, and we thus lack a true appreciation for TGP inter-species variation. Consequently, we examined the tolerance and TGP of life-history and physiological traits in two annelid species within the genus *Ophryotrocha*: one rare (*O. robusta*) and one common (*O. japonica*). Both species were exposed over two generations to ocean acidification (OA) and warming (OW) in isolation and in combination (OAW). Warming scenarios led to a decrease in energy production together with an increase in energy requirements, which was lethal for *O. robusta* before viable offspring could be produced by the first generation. Under OA conditions, *O. robusta* was able to reach the second generation, despite showing lower survival and reproductive performance when compared to control conditions. This was accompanied by a marked increase in fecundity and egg volume in F2 females, suggesting high capacity for TGP under OA. In contrast, *O. japonica* thrived under all scenarios across both generations, maintaining its fitness levels *via* adjusting its metabolomic profile. Overall, the two species investigated show a great deal of difference in their ability to tolerate and respond *via* TGP to future global changes. We emphasize the potential implications this can have for the determination of extinction risk, and consequently, the conservation of phylogenetically closely-related species.

Key words: life history, metabolomics, ocean warming, ocean acidification, tolerance, phenotypic plasticity.

Introduction

Phenotypic plasticity, referred to here as the ability of an individual to adjust its phenotype in response to environmental changes experienced within and between generations (Ghalambor et al. 2007; Fox et al. 2019), has received increasing attention in the last two decades, particularly within the context of species' responses to ongoing global changes (Chevin et al. 2013; Reusch 2014). Phenotypic plasticity is recognized as a mechanism enabling organisms to buffer negative impacts when exposed to rapid environmental changes (Ghalambor et al. 2007), along with favouring rapid adaptive responses in the new set of environmental conditions (Pigliucci et al. 2006, Ghalambor et al. 2015). This said, phenotypic plastic responses have been historically investigated within a single generation (Pigliucci 2001), and only in the last two decades has the interest in trans-generational effects generated considerable advancements in our understanding of parental (Mousseau and Fox 1998) and epigenetics effects (Duncan et al. 2014). However, marine organisms have been largely understudied within this context (c.f. Marshall 2008; Hofmann 2017; Eirin-Lopez and Putnam 2019). Only recently, the investigation of trans-generational (i.e. across two generations) life-history and physiological responses of marine species is being conducted in both invertebrate and vertebrate marine species within the context of global changes (e.g. Parker et al. 2012; Salinas and Munch 2012; Charkravarti et al. 2016; Donelson et al. 2016; Shama et al. 2016; Jarrold et al. 2019).

Species' plastic responses are recognised as highly diversified during the ontogeny of marine organisms (e.g. Walther et al. 2010, 2011; Small 2013) and across generations (Gibbin et al. 2017a). Most importantly, phenotypic plasticity across multiple life stages and generations may represent a primary mechanism for guaranteeing individuals' survival and ultimately species' persistence in a changing ocean (Munday et al. 2012; Sunday et al. 2014). Increasing our current critical understanding of species' ability for trans-generational plasticity (TGP) is thus essential to evaluate species' extinction risks and evolutionary potential within the context of global changes (Van Oppen et al. 2015; Fox et al. 2019). This is most relevant when considering that global changes entail the co-occurrence of multiple physical and chemical alterations of the ocean (Pörtner 2008; IPCC 2014), where the role of TGP within the context of species' ability to cope with multiple global change drivers is largely unknown (c.f. Chakravarti et al. 2016, Gibbin et al. 2017a, b; Jarrold et al. 2019).

The increase in atmospheric CO_2 concentration, due to anthropogenic emissions, is driving an unprecedented warming of the planet's oceans (IPCC 2014). In addition, high CO_2 levels lead to higher surface seawater pCO_2, causing a progressive decrease in seawater carbonate concentration and pH, as well as an increase in bicarbonate ion concentration: a phenomenon defined as ocean acidification (OA) (Caldeira and Wickett 2003). Ocean warming (OW) and OA are occurring simultaneously, multiplying the potential threat to species persistence. On the one hand, the fundamental impacts of elevated temperature on biological systems are well understood (Angiletta 2009), and our comprehension of the biological implications of OA for marine organisms is on the rise (Melzner et al. 2009). On the other hand, our understanding of the combined impacts of multiple drivers is still limited. This is in part due to the complexity of documenting the responses of biological systems to multiple stressors in general (Gibson et al. 2012; Kroeker et al. 2013; Côté

et al. 2016), and more so for TGP. What little understanding we have is based on a small number of single species studies conducted in different phyla: e.g. annelids, arthropods and echinoderms (Vehmaa et al. 2012; Chakravarti et al. 2016; Koenigstein et al. 2016; Gibbin et al. 2017b; Griffith and Gobler 2017; Hoshijima and Hofmann 2019; Karelitz et al. 2019; Jarrold et al. 2019). Consequently, we do not have a true appreciation of the potential variation in TGP responses to future combined global changes in species belonging to the same taxonomical group.

Our aim is to determine whether phylogenetically closely-related species show fundamentally different trans-generational responses to the combined exposure to levels of OW and OA predicted to occur by the end of the 21st century. We compared the within- (F1) and between- (F1 and F2) generation life history responses (growth, reproductive success and egg volume) to OW and OA, in isolation and in combination, of two annelid species from the genus *Ophryotrocha* (Annelida). In order to assess the energetic costs associated with species' plastic responses, we characterised their metabolomic profiles (Viant 2007). These two species present a similar ecology but a contrasting biogeography (Simonini et al. 2009, 2010). *Ophryotrocha japonica* is a species of Pacific origin that has colonised the Mediterranean and Atlantic coasts of Europe, thus occurring in a wide range of environmental conditions (Simonini et al. 2002, 2009). Conversely, *O. robusta* is a rare species endemic to the Mediterranean Sea, with a well-known distribution limited to three close locations in Southern Italy (Simonini et al. 2009). Previous studies using another widespread congeneric species, *O. labronica*, provided evidence for within and across generation, isolated and combined effects of OW and OA on life history and physiology (Rodriguez-Romero et al. 2015; Chakravarti et al. 2016; Gibbin et al. 2017a, b). In these studies, temperature played a primary role in defining the biological responses of *Ophryotrocha* species (Prevedelli et al. 2005; Massamba-N'Siala et al. 2012; Gibbin et al. 2017a, b), while OA acted as an additional physiological challenge (Rodriguez-Romero et al. 2015; Chakravarti et al. 2016). Based on these experimental observations, as well as on expected differences in the thermal niches (*sensu* Hutchinson 1978) due to the different extent of their distribution of *O. japonica* and *O robusta*, we hypothesized that these two species differ substantially in their sensitivity and capacity for within- and trans-generational plastic responses to OA and OW in isolation, and even more so when combined. Despite being congeneric, we expect *O. robusta* to (*i*) be less tolerant to environmental stressors, (*ii*) show reduced capacity for TGP, as well as (*iii*) show higher metabolic costs under global change scenarios, when compared to its relative *O. japonica.*

Materials and Methods

Study species

Ophryotrocha japonica and *O. robusta* are both subtidal, interstitial species (4-5 mm in length), that colonise fouling communities commonly found in organically-enriched coastal waters (Prevedelli et al. 2005; Simonini et al. 2009, 2010; Thornhill et al. 2009). Both species are gonochoric and reproduce semi-continuously during an extended reproductive period, laying eggs in tubular masses that hatch with direct development (Prevedelli et al. 2006; Paxton and Åkesson 2010). A newly hatched individual generates its first

brood in a relatively short amount of time (approx. 47 d at 24 °C), and parental care is provided for the whole duration of the development of the eggs (Paxton and Åkesson 2010; Prevedelli et al. 2005). The worms used in our study came from laboratory strains maintained at control laboratory conditions (salinity: 35; temperature: 24 ° C; pH_{NBS} = 8.1; photoperiod L: D of 12: 12 h) for approx. 20-25 generations and originated from approx. 40 indiv. collected in 2008 in the harbour of Porto Empedocle (Sicily, Italy; 37°17'4''N, 13°31'3''E) for *O. robusta*, and from 100 indiv. collected in 2010 in the harbour of La Spezia (Liguria, Italy, 44° 6' 24" N, 9° 49' 45" E) for *O. japonica*.

Experimental design

Breeding pairs (48 for each species) were assembled from the laboratory strains (F0 generation) and isolated in separate wells of eight culture plates (six well, Costar, VWR, Radnor, PA, USA) for reproduction (see Appendix S1, Electronic Supplementary Material). Once a sufficient offspring (F1 generation) was obtained, 20 F1 indiv. were randomly taken from each of the 12 broods, 21 d after hatching, and transferred to one of four temperature-pH treatments: 24 ± 1 °C and pH 8.2 ± 0.1 (corresponding to 400 μatm pCO_2 – control (C)), 24 ± 1 °C and pH 7.7 ± 0.1 (1500 μatm pCO_2 (OA)), 28 ± 1 °C and pH 8.2 ± 0.1 (400 μatm pCO_2 (OW)), and 28 ± 1 °C and pH 7.7 ± 0.1 (1500 μatm pCO_2 (OAW)). The control conditions chosen guaranteed high reproductive performances in both species (Massamba-N'Siala, *pers. comm.*), while the elevated temperature (+ 4 °C) and pH decrease (-0.4) represented predicted change scenarios for the end of 21st the century in the Mediterranean Sea (RCP 8.5 scenario, IPCC 2014). The elevated temperature occurs already (albeit infrequently) where the species were collected, and it is expected to occur with greater frequency as warming progresses and heat waves intensify (IPCC, 2014).

Experimental system and procedures

The four experimental scenarios were maintained using a carbon dioxide (CO_2) and temperature manipulation experimental system (Gibbin et al. 2017b). This system was composed of two large tanks (60 cm x 30 cm x 15 cm, vol. 13 L) for each temperature condition, half-filled with tap water. These tanks acted as water baths and were thus equipped with an aquarium heater (Theo 11702, Hydor, Sacramento, CA USA), a submersible water pump to ensuring homogenous heat distribution (Koralia nano 900, Hydor, Sacramento, CA, USA), and a Perspex sheet to limit heat dissipation through evaporation. Each tank contained four airtight experimental containers (Sterilite, 26 cm x 18 cm x 17 cm, vol. 4.5 L); two perfused with CO_2-enriched air to generate the elevated pCO_2 conditions (verified with a CO_2 analyser - LI-840A, Li-Cor, Lincoln, NE, USA), and two with ambient air to maintain control pH-pCO_2 conditions. Air (ambient or CO_2-enriched) was supplied continuously to each container by an air pump (Mistral 4000, Aqua Medic, Bissendorf, Germany). Each experimental container housed four culture plates; two for each species. Each plate housed three F1 or F2 breeding pairs, and their corresponding broods (n = 3 pairs/brood *per* plate, 12 replicate pairs/brood total *per* scenario*species*generation). Plates were filled with artificial sea water (salinity 35) made by dissolving artificial sea salt (Reef Crystals, Instant Ocean, Blacksburg, Virginia, USA)

in distilled water. They were covered with a breathable sealing film (Aeraseal, Alpha Laboratories Ltd, Eastleigh, UK), which allowed gas exchanges whilst limiting evaporation, avoiding large fluctuations in salinity and temperature.

F1 indiv. were transferred into the elevated temperature treatment through a gradual pre-exposure of 1 °C h^{-1} from the control temperature (Massamba-N'Siala et al. 2012).

Once F1 individuals reached sexual maturity, 12 breeding pairs were formed *per* species and scenario by pairing females and males taken from different broods, in order to prevent inbreeding (Massamba N'Siala et al. 2011). Each pair was isolated in one well of a six-well plate (as described above) and their first egg mass was used to obtain the next generation (F2). Pairs were maintained in their treatments beyond the first spawning for a total experimental period of 60 d for each generation, which encompassed two spawning events. The first egg masses laid by F1 individuals were brought to hatch in order to establish the next generation, while the second egg masses of both generations were used to determine female fecundity, as F1 egg masses could not be manipulated for measuring this trait. For comparability with F1, secondary egg masses were used to determine fecundity in F2. F2 individuals were reared until sexual maturity and then paired as described above for F1 pairs, maintaining them at the same conditions as their parents throughout the entire experimental period (see Appendix S1, Electronic Supplementary Material). Sea water in the wells was changed every 2 d and worms were fed on the same day by adding ~ 1 mL of spinach minced in sea water (300 g L^{-1}) (Rodriguez-Romero et al. 2015).

Seawater temperature, pH, salinity, and dissolved inorganic carbon (DIC) were measured every 2 d during the experimental period. All wells were sampled on the first day, while on subsequent sampling days only two wells *per* plate were sampled. Temperature was measured with a high accuracy J/K input thermocouple thermometer (HH802U, OMEGA, Laval, QC, Canada, ± 0.1 °C), salinity with a portable refractometer (DD H2Ocean, MOPS aquarium supplies, Hamilton, ON, Canada ± 1.0), and pH_{NBS} with a portable pH meter (Seven Compact, Metler Toledo, Columbus, OH, USA, ± 0.01) which was calibrated once a week using a three point calibration with standard NBS scale buffers (BDH® VWR Analytical, Radnor, PA, USA. pH buffers at 25 °C: 4.00 ± 0.01, 7.00 ± 0.01, 9.18 ± 0.01). Water samples (5 mL) were taken from the wells for future determination of DIC (Dissolved Inorganic Carbon) concentration based on the procedure described by Dickson et al. (2007). These water samples were conserved in 5 mL vials devoid of headspace, poisoned with a drop of saturated mercuric chloride solution (0.02 % concentration), and stored in the dark until analysis. DIC concentration was measured with the method described by Hall and Aller (1992) with an ionic chromatography system (IC5-1000. Dionex, USA). Water samples were injected into a fluid reagent stream (chloric acid, HCl) in which the stable phase of interest is gaseous (CO_2). The stream carrier went through a gas transfer cell with a gas permeable hydrophobic membrane. On the other side of this membrane there was a receptor reactive stream (potassium hydroxyde, KOH) in which the gaseous phase is not stable. The receptor stream went through a detector, which measured solute quantity transferred (Ruzicka and Hansen 1988). Transferred solute effect on receptor stream conductivity was used to calculate DIC concentration. Additional carbonate system parameters (CO_2 partial pressure (pCO_2), total alkalinity (TA), bicarbonate and carbonate ion concentration (HCO_3^- and CO_3^{2-}), and calcite and aragonite (Ω_{cal} and Ω_{ara}) were

calculated using the CO_2SYS program (Lewis and Wallace 1998) with constants from Mehrbach et al. (1973) corrected by Dickson and Millero (1987), and KSO_4 constants from Dickson (1990). These measurements are present in Table 1.

Determination of life-history traits

All adult life-history measurements were conducted on females because their contribution to population demography is more relevant than that of males, and generally, maternal environment is more influential on offspring responses than the paternal environment (Stearns, 1992; Shama et al., 2014). Survival rate for F1 and F2 females was calculated as the percentage of individuals remaining after 49 d of exposure, which corresponded to the number of days at which 100% mortality occurred in *O. robus*ta F1 females under the OW scenario. Female size at each generation was measured following each spawning event and used to calculate female growth rates, defined as the number of chaetigers (segments bearing bristles) added daily from the moment they hatch until they reach their maximum size recorded during the duration of the experiment. Degeneration events, defined as the loss of chaetigers during an individual development, were also recorded.

The percentage of pairs producing viable offspring in each generation was used as a proxy for female reproductive success. Egg masses were photographed under medium (x 40) and high (x100) magnification 24 h after deposition using a light microscope (Laborlux S, Leitz, Oberkochen, Germany) equipped with a digital camera (OMAX A-3530U, Kent, WA, USA). Medium magnification pictures were used to count the number of eggs as a proxy for fecundity. High magnification pictures were analysed to determine the egg volume, a proxy for egg quality and thus parental investment. The longest and shortest axes of 10 eggs *per* mass were measured using imageJ (Schneider et al. 2012), and egg volume calculated using the formula:

(Eq. 1) $$Egg\ mass\ volume = \frac{4}{3} * \pi * A^2 * B$$

where *A* is the shortest radius and *B* the longest radius (Simonini and Prevedelli 2003).

Determination of metabolomic profiles

Energy metabolism and fatty acid composition were determined by characterising the metabolomic profiles in both males and females of each F1 and F2 pair, targeting specific metabolites that have key roles in energy metabolism and cellular function. Individuals were flash frozen in a 1.5 mL centrifuge tube at the end of the exposure period of each generation, and maintained at -80 °C. Since no *O. robusta* F1 pairs survived exposure to OW and OAW scenarios, metabolomics analyses were performed on the few individuals remaining from the brood of origin after pairing (approx. 20 indiv.). These individuals were kept in OW and OAW conditions for the same amount of time as the breeding pairs. The characterisation of metabolomic profiles was carried out using the liquid chromatography-tandem mass spectrometry (LCMS) method

described by Lu et al. (2006). The technique was modified in order to be applied to small marine organisms such as interstitial annelids (*pers. comm.* Fanny Vermandele, Mathieu Babin, Peter Thor and Piero Calosi), particularly to prevent the formation of salt adducts when injecting marine organism samples. This involved a fast "cold quenching, salt-eliminating" extraction using ammonium carbonate as an extraction solution. Briefly, 4.8 g of ammonium carbonate (trace metals-grade 99.999 %; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 L of Nanopure water (18.0 Ω, Barnstead infinity system, Lake Balboa, CA, USA) to create a 50 mM ammonium carbonate solution. Then, 0.4 mL of this solution was added to 1.6 mL of Nanopure water and 8 mL of methanol in order to produce a final 8:2 methanol: water-10 mM ammonium extraction solution. In order to ensure sensitive detection of the targeted metabolites, the method was by developed using different mix of standards. The amino acid standard was obtained from Phenomenex (Torrance, CA, USA) and the free fatty-acid standard was created by hydrolyzing a FAME 37 standard (Sigma-Aldrich, St. Louis, MO, USA). To do so, 250 µL of FAME 37 was evaporated under nitrogen in a 1.5 mL centrifugal tube. 50 µL of KOH 6.25 % (w/v) in Nanopure water was then added and the tube was heated for 30 min at 60 °C. 950 µL of the extraction solution was then added, and the tube was centrifuged at 8 000 rpm. The supernatant was then transferred to a HPLC amber vial and was stored in -80 °C. For all the other metabolites, standards were obtained from Sigma-Aldrich (St. Louis, MO, USA), and individual metabolite solutions were created by precisely weighing each standard into clear HPLC vials to produce 1 mg mL^{-1} solution in the extraction solution. A final working solution, containing all targeted metabolites, was created by pooling 1000 µL of the amino acid standard, 2000 µL of the free fatty acid standard, 500 µL of glucose and 50 µL of each other individual metabolite solution. Extraction solution was added to the mix to reach a final volume of 10 mL. From this final working solution, a serial dilution 1:1 in the extraction solvent was conducted to create 10 different metabolite concentration solutions for the calibration curve. Once the method was developed and tested for the targeted metabolites, analyses were carried out on individual specimens. Each centrifuge tube containing a single frozen individual was bathed in liquid nitrogen to avoid thawing and metabolite degradation during the manipulations. 250 µL of the extraction solution, kept continuously at -80 °C, was added to the tube and the sample was crushed with a potter pestle (blue pre-sterilized, Axygen, Tewksbury, MA, USA). The sample was then sonicated for 3 s (Sonication bath, model Symphony, VWR, West Chester, PA, USA) and centrifuged at 11 000 rpm for 3 min at 4 °C (centrifuge 5430R, Eppendorf, Hamburg, Germany). Then, 225 µL of the supernatant was transferred to an amber HPLC vial with insert (Wheaton, New Jersey, USA) and injected in a liquid chromatography system (Accela, Thermo Electron Corporation, San Jose, CA, USA) equipped with a 150 mm X 2 mm Luna C5 guard column for Phenomenex (Torrance, CA, USA). The system was adjusted with the following parameters: autosampler temperature set at 4 °C, a column temperature set at 20 °C, an injection volume of 25 µL and a solvent flow rate of 200 µL min^{-1}. LCMS-grade acetonitrile (OmniSolv, EMD Chemical, Gibbstown, NJ, USA) obtained from VWR International (West Chester, PA, USA) was used for the mobile phases. 50 mM of acetonitrile (ACN) in carbonate water (90:10) was used for mobile phase A, whilst mobile phase B was composed of ACN: 5 mM ammonium carbonate water solution. The gradient program started at 2 % of mobile phase A over 2 min and reached 98 % of mobile phase A at 6 min, maintained until 15 min. The initial conditions of 2 % of mobile

phase A was re-established at 17 min and was followed by a conditioning of 3 min for a total run time of 20 min. The identification of metabolites previously separated was then achieved on an Orbitrap LTQ Discovery high-resolution mass spectrometer (HRMS) (Thermo Electron Corporation, San Jose, CA, USA), sequentially in a positive and negative mode. The electrospray ionization spray voltage was of 5000 V in positive mode and of 3200 V in negative mode. Nitrogen was used as sheath gas at 55 arbitrary units with a capillary temperature of 325 °C and the scan range was from 60 to 1000 *m*/z (mass to charge ratio) for both modes. HRMS data were then analysed on Xcalibur 2.0 software (Thermo Electron Corporation, San Jose, CA, USA) using a 10 ppm mass tolerance. For each targeted metabolite, a calibration curve was created (see above) and the best linear, linear log-log or quadratic log-log relationship was chosen to build the curve. Metabolite concentration for all the samples where then assessed from the area of the working standard solution by extract ion integration.

Statistical analysis

Mixed effect linear models were used to test for the effects of the fixed factors 'species', 'scenario', 'generation' and their interactions on growth rate, fecundity and egg volume. Since fecundity and egg volume are usually linked to female size (Massamba-N'Siala et al., 2011, Marshall and Keough 2008), body size was included in the models as a covariate.

In preliminary analyses, 'tank' and 'container' were set as random factors to control for any pseudoreplication effect. As the factor 'tank' was not found to be significant in all analyses and 'container' had a significant effect only on fecundity (minimum $F_{5, 39} = 4.17$; $P = 0.01$), and considering that models including and excluding these terms did not differ statistically from each other, random factors were considered marginal and removed from the analyses.

As *O. robusta* F1 pairs did not reproduce under both high temperature scenarios, we analyzed the life-history traits responses in three different steps: (1) *O. robusta versus O. japonica* within-generational responses (F1) to all environmental change drivers, using 'scenario' (C, OA, OW, OAW) and 'species' as factors; (2) *O. robusta versus O. japonica* trans-generational responses (F2) to OA, using 'generation', 'scenario' (C, OA) and 'species' as factors; (3) trans-generational responses of the *O. japonica* to all environmental change drivers, using 'generation' and 'scenario' (C, OA, OW, OAW) as factors. Percentage of survival, number of degeneration events and percentage of pairs producing viable offspring were tested according to step 1) and 2) using a χ^2 test.

All data were tested for assumptions of normality and heteroscedasticity using Shapiro-Wilk test and Leven test, respectively. Some data did not meet these assumptions even after transformation. However, the high level of replication and the experimental design used allowed us to consider our analyses robust (Underwood 1996; Melatunan et al 2013). Pairwise comparisons within scenarios, species or generations were performed whenever a significant interaction or main effects were found, using the 95% confidence interval test calculated for estimated marginal means.

To explore patterns of metabolites variability among scenarios (C, OA, OW, OAW), and between species and generations, principal component analyses (PCA), followed by hierarchical ascendant classification on the PCA output, were run using the package FactoMineR (Husson et al. 2017) and multivariate variance analyses (MANOVA) were performed.

Statistical analyses were conducted using R (version 3.2.2) and the graphic interface R commander using a significant threshold of $\alpha = 0.05$ (RStudio Team 2015).

Results

Mean values of each life-history traits, statistical outputs of the chi-square test (χ^2) tests and pair-wise comparisons are summarised, respectively, in Appendices S2, S3 and S4 of the Electronic Supplementary Material. Statistical outputs of the MANOVA test are shown in Appendix S5. All values shown in the text are mean ± SE.

Ophryotrocha robusta *and* O. japonica *within-generational responses (F1) to ocean global change scenarios*

Survival. Under ocean warming (OW) and ocean acidification*warming (OAW) scenarios, *O. robusta* reached 100 % mortality after 49 and 37 d of exposure respectively (Fig. 1a; Appendix S6, Electronic Supplementary Material). However, mortality was only 33 % under ocean acidification (OA) and significantly higher than that measured under control (C) scenario ($\chi^2 = 10.56$; $P = 0.01$). All females of *O. japonica* survived the exposure period in all four scenarios (Fig. 1a).

Growth rates and degeneration events. *Ophryotrocha robusta* grew significantly faster (0.17 ± 0.02 chaetigers d^{-1}) than *O. japonica* (0.14 ± 0.003 chaetiger d^{-1}) ('species' effect, Table 2a), and neither 'scenario' in isolation nor its interaction with 'species' affected growth rates. Conversely, degeneration events occurred under all scenarios, but showed higher incidence in *O. robusta* ($\chi^2 = 11.28$, $P = 0.01$; Fig. 1b). On the one hand, 25 % of *O. robusta* females exposed to OA and OW lost chaetigers, and this percentage doubled under OAW scenario. On the other hand, only 8 % of *O. japonica* females exposed to OW and OAW showed degeneration events and at a rate comparable to that observed under the C scenario (Fig. 1b).

Reproductive success. In *O. japonica*, 91.67 and 83.33 % of pairs produced viable offspring under OW and OAW, respectively, whilst none of *O. robusta* pairs reproduced in both scenarios (Fig. 2). Under OA, all *O. japonica* pairs produced viable offspring, while 75 % of *O. robusta* pairs did ($\chi^2 = 121.97$, $P < 0.001$; Fig. 2). Fecundity and egg volume changed in the two species depending to the scenario of exposure, as indicated by the presence of a significant 'species'*'scenario' interaction (Table 2a). *Ophryotrocha robusta* had failed to reproduce under OW and OAW, while *O. japonica* showed a 10 and 20 % decrease in fecundity and a 35 and 18 % increase in egg volume under OW and OAW, respectively, compared to the C scenario. Under OA, fecundity decreased by 33 and 75 % in *O. robusta* and *O. japonica*, respectively (Table 2a; Fig 3a), while in both species egg volume did not change when compared to the C scenario (Table 2a; Fig 3b).

Ophryotrocha robusta *and* O. japonica *trans-generational responses (F1-F2) to ocean acidification*

Survival. All *O. japonica* females survived the exposure to C and OA at both generations. *Ophryotrocha robusta*, on the contrary, showed no mortality events under the C scenario at F1, but it suffered a 9.09 % mortality at F2 (Fig. 1a). Under OA, *O. robusta* survival was higher for F1 females (58 %) than for F2 females (43 %) (χ^2 = 9.58; *P* = 0.01; Fig. 1a).

Growth rates and degeneration events. Ophryotrocha robusta grew significantly faster (0.21 chaetigers d^{-1}) than *O. japonica* (0.16 chaetigers d^{-1}) ('species' effect, Table 2b) and both species had significantly higher growth rates at F1 (0.24 chaetigers d^{-1}) when compared to F2 (0.20 chaetigers d^{-1}), as indicated by the term 'generation' being significant (Table 2b). Degeneration events occurred in 8 % of *O. robusta* females at F1, while they were not recorded at F2 in both species (Fig. 1b).

Reproductive success. Under OA, all F2 pairs of *O. japonica* produced viable offspring, while only 58 % of *O. robusta* pairs reproduced (Fig. 2). However, these differences were not detected as being significant (χ^2 = 7.59; *P* = 0.06). In both species, fecundity measured at F1 under OA was lower than in the C scenario. However, in F2 whilst *O. japonica* maintained the same fecundity as in F1, *O. robusta* increased fecundity by 187 % compared to the C scenario, as indicated by the presence of a significant three-way interaction between the terms 'species', 'scenario' and 'generation' (Table 2b; Fig 3a). A significant interaction between 'species', 'scenario' and 'generation' was also found for egg volume (Table 2b; Fig 3b). While in both species the volume of the eggs laid did not change between scenarios at F1, at F2 it increased under OA in *O. robusta* to values that were significantly higher than those recorded under the C scenario (+ 33.33 %). Similarly, but with a reverse trend, egg volume decreased under OA in *O. japonica* to values that were significantly lower than those recorded under the C scenario (- 30 %) (Fig. 3b).

Ophryotrocha japonica *trans-generational responses (F1-F2) to all ocean global change scenarios*

Growth rates and degeneration events. Ophryotrocha japonica growth rates were significantly higher at F2 than F1, as indicated by the term 'generation' being significant (Tab. 2c), but they did not differ among scenarios (Table 2c). In F2, degeneration events were detected only under OAW in 16.67 % of females (Fig. 1b).

Reproductive success. All *O. japonica* pairs produced viable offspring in both generation and all scenarios tested (Fig. 2). F2 females were significantly more fecund than F1 ones, as indicated by the term 'generation' being significant (Table 2c), but their eggs were on average smaller than those produced during F1. This trend changed depending on the scenarios, as indicated by the presence of a significant 'scenario*generation' interaction for egg volume (Table 2c; Fig 4). In more detail, F1 eggs were characterised by a larger volume when exposed to OW and OAW compared to the C scenario, whilst no significant difference was found between OA and C scenarios. This trend changed in F2, where egg volume was negatively affected by the exposure to OA and OW (Fig 4a, b), but it did not change under OAW (Fig 4c).

Metabolomics profiles comparison

Metabolomic profiles were compared among the four scenarios in *O. robusta* individuals from F1 (Fig. 5a). The two axes of the PCA explained 65 % of the total variation, and a significant 'scenario' effect was found ($F_{1,10}$ = 2.90, P = 0.01). The metabolomic profile of individuals exposed to C and OA conditions clustered separately from the scenarios involving elevated temperature (i.e. OW and OAW). The separation of these two groups was mainly driven by a reduction in the concentration of molecules involved in the energy metabolism (Fig. 5b). Specifically [ATP], [ADP], [glutamate], [malate], [fumarate] and [aspartate] were lower under OW and OAW, compared to C and OA conditions.

A second PCA documented in *O. robusta* and *O. japonica* significant differences in the metabolomic profile after F1 exposure to OW and OAW ($F_{3, 12}$ = 20.30, P = 4.64 e^{-05}; Fig. 6a). The first axis explained 50.3 % of the variation mainly driven by differences in fatty acid composition, while the second axis accounted for 21.4 % of the variation, correlated to metabolites involved in the energy metabolism (Fig. 6b). Specifically, the stearic acid C18:0 was more present in *O. robusta*, while [ATP], [NAD], [glutamate], [glucose] and [aspartate] were in higher concentration in *O. japonica*.

Finally, a third PCA compared the metabolomic profiles from F1 and F2 females of *O. japonica* (Fig. 7). Significant 'generation' and 'scenario' effects ($F_{3, 32}$ = 1.76, P = 0.017) differentiated the metabolomic profile of the two generations, with the first two axes explaining 69.44 % of the variation (Fig. 7a). During F1 exposure, the metabolite composition in C conditions differed from that in OA, OW and OAW, a distinction driven by differences in fatty acid reserves. In control conditions, females showed higher levels of fatty acid reserve (C16:3, C18:2, C18:1, C20:1, C18:3), [fumarate], [malate] and [AMP] (DM. 1 = 52.61 %). Conversely, no difference between control and global change scenarios was observed for F2. The F2 generation differed from F1, as it showed lower concentrations for molecules involved in the cell energy metabolism, i.e. [ATP], [ADP], [NAD], [aspartate], [glutamate] and [glucose] (DIM. 2 = 16.83 %) (Fig. 7b).

Discussion

Our study provides, for the first time, empirical evidence supporting a significant level of divergence in across-generation impacts of combined ocean warming (OW) and ocean acidification (OA) on life-history traits in two congeneric marine species. In addition, we offer a mechanistic underpinning for the observed patterns of sensitivity *via* metabolomic profiling. We show that the annelid *Ophryotrocha robusta* is significantly less tolerant to OW and OA, both in isolation and combined (OAW), when compared to its congeneric *O. japonica*. Overall, chronic exposure to elevated temperature represents the main factor negatively affecting *O. robusta* fitness, as elevated temperature levels tested are lethal before the species is able to produce a viable progeny. As expected, the combined effect between OW and OA causes a greater negative impact on *O. robusta* life history performances. Within- and trans-generational responses vary between traits allowing for life-history and physiological adjustments that differ greatly between the two species. Altogether, the common species, *O. japonica*, thrives across all scenarios, whilst the endemic species, *O. robusta*, shows tolerance levels well below the magnitude of temperature and pH change expected for the

end of the century (IPCC 2014). In this sense, whilst our work cannot be considered to provide ultimate evidence for whether extant endemic or rare species show greater vulnerability to combined environmental change drivers, our results are consistent with the hypothesis that rare species may be more sensitive to global changes compared to their more widespread congeners (e.g. Bozinovic et al. 2011; Calosi et al. 2008; Calosi et al. 2010).

The severe effect of ocean warming on O. robusta

Future OW and OAW scenarios represent sub-optimal conditions that will be detrimental to the measured functions of *O. robusta*. Individuals of this species do not survive past 49 d of chronic exposure to OW conditions, despite being already sporadically exposed in the field to the test temperature, and most importantly, they do not produce any viable progeny. In addition, 100 % mortality is reached under OAW conditions within 37 d, indicating the presence of an interactive effect between OW and OA. Growth patterns are also negatively affected, as confirmed by a marked increase in degeneration events. Temperature increases are known to accelerate the metabolic rates up to a maximum level at optimal temperature, beyond which the thermal stability and function of structural proteins and enzymes are compromised. This is followed by physiological failure, by mean of accumulation of thermal-related damages, which sets the upper thermal tolerance limits of marine ectotherms (Hochachka and Somero 2002). The metabolomics profiles of *O. robusta* under OW and OAW support this idea of a physiological breakdown. Individuals exposed to these scenarios experience in fact a decrease in energy availability, as indicated by the reduction in [ATP], a key metabolite in cellular energetics, and [NAD], a cofactor implicated in the redox reactions of electron transport, together with a decrease in a number of molecules also involved in the Krebs's cycle, when compared to the profiles of individuals exposed to C and OA scenarios. Under a sustained chronic exposure to warming scenarios, these changes in the energy metabolism could cause the shutdown of *O. robusta* most sensitive and demanding functions such as reproduction and growth, while gradually leading worms toward a phase of irreversible physiological damage and, ultimately, to death (Pörtner and Gutt 2016). It is important to mention that in our experiment, the physiological impairment we report was accompanied by the cessation of feeding activities, evidenced by the lack of visible food in the worms' intestines, and the gradual deterioration of the worms' physical conditions in individuals kept under elevated temperature. This was highlighted by the abnormal increase in the volume of the coelomic cavity of certain individuals. In contrast, all individuals of *O. japonica* survive and more than 80 % produce viable progeny under OW and OAW. They also lay larger eggs under these conditions, suggesting an increase in parental investment.

Differences in metabolomic responses of *O. robusta* and *O. japonica* help explain observed trends in life-history traits, as contrary to what was observed for *O. robusta, O. japonica* increases its energy production in F1 when exposed to global change scenarios: as shown by an increase in [ATP], [NAD] and [aspartate]. This increase is, however, accompanied by a decrease in lipid content. These changes suggest that energy metabolism is likely enhanced in *O. japonica*, enabling it to maximise important functions at a higher level of biological organisation. The decrease in lipid content we report suggests the existence of potential long-term

costs, which may translate in the multigenerational fitness costs as observed in other species (i.e. Shama and Wegner 2014; Gibbin et al. 2017a; Jarrold et al. 2019), and may explain the reduction in egg volume reported for *O. japonica* after two generations of exposure to the OW scenario. However, in the F2 of *O. japonica*, lipid content is comparable between C and all global change scenarios, suggesting the capacity of this species to reach a complete metabolic recovery after two generations of exposure. This said, Gibbin et al. (2017a) showed that physiological impairment caused by global change drivers may be detectable in terms of fitness again or only after multiple generations.

Trans-generational plasticity can help species to cope with global changes, but it is no silver bullet

Ocean acidification is the only global change scenario tested in this study where *O. robusta* produces a viable progeny. However, even under this scenario, this species shows a higher sensitivity compared to *O. japonica* across both generations. After 49 d of exposure in F1 *O. robusta* shows 40 % mortality, and around 25 and 40 % of surviving females do not reproduce at F1 and F2, respectively. In contrast, no mortality or reproductive failure is detected in *O. japonica* in both generations.

In our study, the two species show similar patterns of trans-generational responses for growth rates to OA, this scenario causing a reduction in growth at F2 when compared to control conditions. It has been widely documented that OA can negatively impact growth through non-beneficial plastic responses, as the increase in energetic requirements needed to maintain homeostasis affects organisms' ability to maintain and repair their cellular systems (Melzner et al. 2009; Stumpp et al. 2012). The effect of OA on growth rates has been observed within a generation when exposure included early life stages (Chakravarti et al. 2016). This said, evidence also exists for the partial or total restoration of growth performances after two generations of exposure to OA (Parker et al. 2012; Miller et al. 2013; Chakravarti et al. 2016).

In *O. robusta*, the decrease in growth rate following the trans-generational exposure to OA is accompanied by a marked increase in fecundity levels compared to F1 and by an increase in parental investment (Rodriguez-Romero et al. 2016; Bouquet et al. 2018). Even the fecundity of *O. japonica* benefits from trans-generational exposure to OA, as the reduction in eggs output observed at F1 is fully recovered in the following generation albeit at the expense of egg volume. The re-allocation of the energy among different functions through life-history trade-offs appears to provide the mechanism underpinning the observed trans-generational plastic responses. In *O. robusta*, trade-offs occurring under OA scenario may account for energy being diverted from growth to reproduction, as documented for other marine species when exposed to increasingly challenging environmental conditions (Pistevos et al. 2011).

No trade-offs between life-history traits are detected in *O. japonica*, this species showing higher tolerance to all global change scenarios tested across both generations when compared to *O. robusta*. In *O. japonica*, trans-generational adjustments are observed for survival, growth rates and fecundity, but are not determined by the scenarios tested. Eggs' volume is the only trait positively affected by acute exposure to OW and OAW, as described previously, but this beneficial plastic response observed in F1 disappears

following two generations of exposure to OA and OAW and is even reversed under OW. Interestingly, this pattern is accompanied by an energy trade-off between fatty acid reserves and energy production across generations: the former decreasing and the latter increasing under global change scenarios when compared to C conditions. In F2, differences in metabolite concentrations between global changes and C scenarios are no longer evident, suggesting that individuals adjust their metabolism to cope with elevated temperatures and/or pCO_2 conditions by the second generation of exposure. Our results differ with those of other studies performed, for example, in marine sticklebacks (e.g. Shama and Wegner 2014; Shama et al. 2014, 2016), juvenile damselfish (Donelson et al. 2012) and even the congeneric annelid *Ophryotrocha labronica* (Chakravarti et al. 2016). In fact, these studies showed negative effects of high temperature on aerobic metabolism, hatching success or growth rate following acute exposure, after which a complete compensation was observed following trans-generational exposure.

Altogether our results confirm that within and trans-gen erational plasticity in both life-history and physiological performances may help some ectothermic species to cope with global change drivers. However, the presence of trade-offs and energy costs associated to plastic responses points to the fact that these mechanisms may not always be beneficial (Shama and Wegner 2014, Chakravarti et al. 2016, Jarrold et al. 2019), or be comparable across and among species, as shown in this study by divergent trans-generational effects even between phylogenetically-closely related species. This said, we cannot completely disregard the idea that other mechanisms contributed in determining the trans-generational responses we observed, such as the random sampling of different genotypes across generations or different levels of (long-term) acclimation regimes: F1 being more acute than F2, as F2 individuals complete their entire life cycle under the experimental conditions.

In particular, the inability to distinguish between the contribution of selection from that of plasticity in defining trans-generational changes is an intrinsic limitation of global change trans-generational experiments, particularly in sexually reproducing metazoans for which non-breeding designs cannot be easily employed (Gibbin et al. 2017; Donelson et al. 2019). This said, several studies have provided evidences for the occurrence of rapid evolutionary changes across two generations whenever the high selective pressure of the new environment causes a significant increase in mortality levels (> 50 % *per* generation) (Vidal and Horne 2009; Christie et al. 2012; Thor and Dupont 2015), and/or marked variations in reproductive success (Donelson et al. 2012). Accordingly, the high mortality levels and low reproductive success observed in *O. robusta* under OA in the F1 could have selected for more tolerant genotypes, whose offspring (F2) showed higher fecundity. Conversely, we assume selection to be of marginal importance compared to TGP in *O. japonica* under OW and OAW, given we report 100 % survival and high reproductive success (> 80 %) in both scenarios in the F1. Therefore, in this species, it is plausible that the changes observed in the F2 are mainly due to TGP.

Conclusions

Our results provide additional insights into the trait- and species-specific effects of combined global changes on marine organisms across successive generations. We confirm that both within- and trans-generational plasticity may represent an important mechanism to help species in coping with rapid environmental changes (Ghalambor et al 2007; Calosi et al 2016; Donelson et al. 2018). However, global changes may be happening too quickly for some species to 'outrun' them through plastic responses (Quintero and Wiens 2013; Welch et al. 2014), and the magnitude of abiotic changes may overcome species' sensitivity levels, as reported in our study. Embracing the diversity and complexity of species' responses to rapid environmental changes is challenging, but it is needed, if we want to reliably predict the impact of global changes. This said, we do not have the time or resources to test all known species, which however represent only a small fraction of existing species, before the negative effects of global changes on extant biodiversity levels are detected at the regional and global scale (Calosi et al. 2016). It is therefore imperative we set out to test broad eco-evolutionary questions using the most appropriate taxa, in order to define general principles with which to guide a critical understanding of marine biodiversity responses to complex global change scenarios. These will be key to guiding adaptive management of natural resources and biodiversity conservation (Calosi et al. 2016). Our results do not allow us to conclude that the eco-evolutionary forces at play define the differences in within and trans-generational responses reported for the two congeneric annelids species with different biogeography investigated here (see Garland and Adolph 1994). Nevertheless, our results, together with evidence from both the paleo and modern records (Calosi et al. 2019), enable us to generate the hypothesis that differences in species' physiological niches may represent a plausible explanation for the existence of differences in the pattern of sensitivity of phylogenetically closely-related species. We suggest that endemic and rare species may be at greater risk of decline under global changes, when compared to widespread and common species, even when considering their ability for TGP responses. As such, our work has potential important implications for the level and distribution of biodiversity locally and globally in the future ocean, this ultimately determining community compositions and ecosystems' functioning (Lyons et al. 2005; Mouillot et al. 2013; Violle et al. 2017). The idea that rare species will be more at risk under global change is increasingly supported (Calosi et al. 2008; Mouillot et al. 2013), but requires further testing on a broader phylogenetic scale within the context of global changes (Calosi et al. 2019). Ultimately, our study provides a rational to investigate TGP through combined future global change drivers among species with different biogeography.

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

559 All applicable international, national and/or institutional guidelines for sampling, care and experimental use of
560 organisms for the study have been followed and all necessary approvals have been obtained.

561 *Data availability*

562 The datasets generated during and/or analysed during the current study are available from the corresponding
563 author upon reasonable request and are available on the PANGAEA data library.

564

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Table 1: Mean ± SE of the seawater physico-chemical parameters measured [salinity, temperature, pH_{NBS}, Dissolve Inorganic Carbon (DIC)] and calculated [CO_2 partial pressure (pCO_2), Total Alkalinity (TA), bicarbonate and carbonate ions concentrations (HCO_3^- and CO_3^{2-}), calcite and aragonite saturation (Ω_{cal} and Ω_{ara})] for each scenario tested (C = control, OW = ocean warming, OA = ocean acidification, OAW = ocean acidification and warming). Capital letters indicate significant differences between treatments.

Scenario	Salinity	pH_{NBS}	Temperature (°C)	DIC (µmol kg^{-1})	TA[a] (µmol kg^{-1})	pCO_2 (µatm)	$[HCO_3^-]$[a] (µmol kg^{-1})	$[CO_3^{2-}]$[a] (µmol kg^{-1})	Ω_{cal}[a]	Ω_{ara}[a]
C	34.48 ^{A} ± 0.85 n = 376	8.16 ^{A} ± 0.14 n = 683	24.29 ^{A} ± 1.10 n = 705	2326.80 ^{A} ± 6.84 n = 32	2681.60 ^{A} ± 8.76 n = 32	436.08 ^{A} ± 2.24 n = 32	2048.28 ^{A} ±5.35 n = 32	266.28 ^{A} ± 1.91 n = 32	6.48 ^{A} ± 0.05 n = 32	4.21 ^{A} ± 0.03 n = 32
OW	35.38 ^{A} ± 3.36 n = 379	8.10 ^{A} ± .17 n = 675	28.20 ^{B} ± 0.89 n = 681	2208.01 ^{C} ± 16.09 n = 20	2583.56 ^{A} ± 17.07 n = 20	433.61 ^{A} ± 7.96 n = 20	1921.10 ^{A} ± 14.98 n = 20	275.60 ^{A} ± 3.13 n = 20	6.70 ^{A} ± 0.08 n = 20	4.43 ^{A} ± 0.05 n = 20
OA	33.43 ^{B} ± 0.23 n = 391	7.67 ^{B} ± 0.13 n = 698	24.37 ^{A} ± 1.02 n = 704	2595.39 ^{D} ± 8.57 n = 31	2686.93 ^{A} ± 8.61 n = 31	1645.61 ^{B} ± 10.58 n = 31	2449.12 ^{B} ± 8.15 n = 31	100.13 ^{B} ± 0.59 n = 31	2.44 ^{B} ± 0.02 n = 31	1.60 ^{B} ± 0.01 n = 31
OAW	35.20 ^{B} ± 2.53 n = 390	7.69 ^{B} ± 0.13 n = 697	28.18 ^{B} ± 1.14 n = 686	2525.08 ^{B} ± 17.92 n = 20	2568.85 ^{A} ± 17.76 n = 20	2141.30 ^{B} ±65.04 n = 20	2371.09 ^{B} ± 16.43 n = 20	82.99 ^{B} ± 1.61 n = 20	2.04 ^{B} ± 0.04 n = 20	1.38 ^{B} ± 0.12 n = 20

[a] Calculated by CO_2SYS program (Lewis and Wallace 1998) with constants from Mehrbach et al. (1973) and corrected by Dickson and Millero (1987) and KSO_4 constants from Dickson (1990).

Table 2: Effects of within- (F1) and trans-generational (F1-F2) exposure to control (C), ocean acidification (OA), ocean warming (OW) and combined ocean acidification and ocean warming (OAW) on *Ophryotrocha robusta* and *O. labronica*'s growth rate, fecundity and egg volume. Degrees of freedom (df), *F*-value (*F*) and probability level (*P*). Bold numbers represent significant *P*-values (α = 0.05).

		Growth rate			**Fecundity**			**Egg Volume**		
		df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
a) F1; *O. robusta* vs *O. japonica*; C, OA, OW and OAW	Size				1	57.91	> **0.001**	1	6.47	**0.01**
	Species	2	4.58	**0.01**	1	4.20	**0.04**	1	23.25	> **0.001**
	Scenario	3	0.29	0.83	3	20.25	> **0.001**	3	32.56	> **0.001**
	Species*Scenario	3	0.12	0.95	3	8.13	> **0.001**	3	8.36	> **0.001**
b) F1-F2; *O. robusta* vs *O. japonica*; C and OA	Size				1	2.90	**0.003**	1	5.11	**0.02**
	Species	1	4.7	**0.01**	1	4.61	**0.04**	1	14.16	> **0.001**
	Scenario	1	1.64	0.20	1	0.30	0.59	1	4.95	**0.03**
	Generation	1	5.45	**0.02**	1	0.01	0.91	1	204.13	> **0.001**
	Species*Scenario	1	1.66	0.20	1	0.06	0.81	1	1.46	0.23
	Species*Generation	1	2.59	0.11	1	1.62	0.21	1	0.05	0.83
	Scenario*Generation	1	1.43	0.23	1	14.94	> **0.001**	1	6.08	**0.01**
	Species*Scenario*Generation	1	0.90	0.34	1	7.41	**0.01**	1	5.70	**0.02**
c) F1-F2; *O. japonica*; C, OA, OW and OAW	Size				1	32.89	> **0.001**	1	5.17	> **0.001**
	Scenario	3	0.40	0.86	3	1.58	0.20	3	10.82	> **0.001**
	Generation	2	5.08	**0.01**	1	17.82	> **0.001**	1	205.84	> **0.001**
	Scenario*Generation	3	1.53	0.20	3	2.21	0.09	3	26.84	> **0.001**

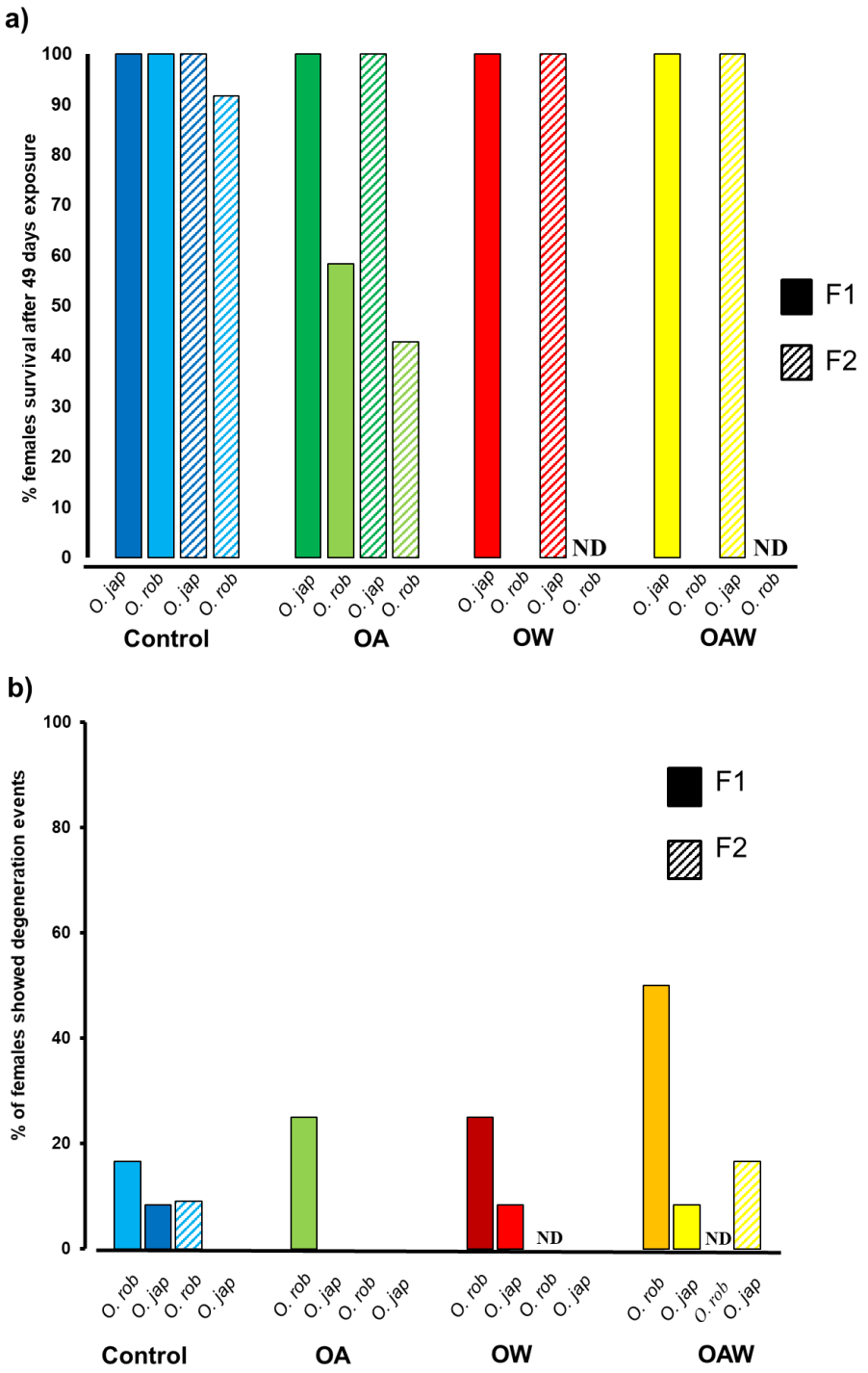

Fig. 1: a) Percentage of females surviving 49 days of exposure to control (C, blue), ocean acidification (OA, green) and ocean warming (OW, red) scenarios, in isolation and combined (OAW, yellow), and b) number of degeneration events occurring under each scenario at F1 (solid fill) and F2 (striped fill) for *Ophryotrocha robusta* (*O. rob*) and *O. japonica* (*O. jap*). ND = not determined.

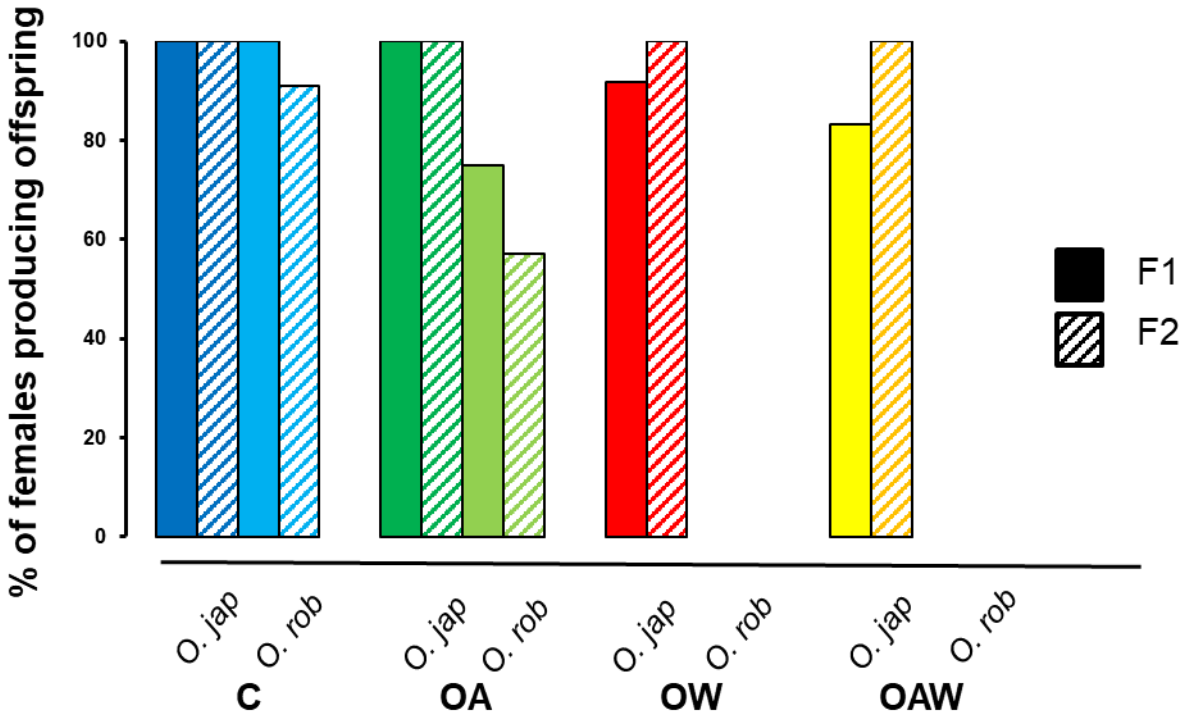

Fig. 2: Percentage of *O. robusta* (*O. rob*, light colours) and *O. japonica* (*O. jap*, bold colours) breeding pairs that produced viable offspring under control (C, blue), ocean acidification (OA, green) and ocean warming (OW, red) scenarios, in isolation and combined (OAW, yellow) during F1 (solid fill) and F2 (striped fill).

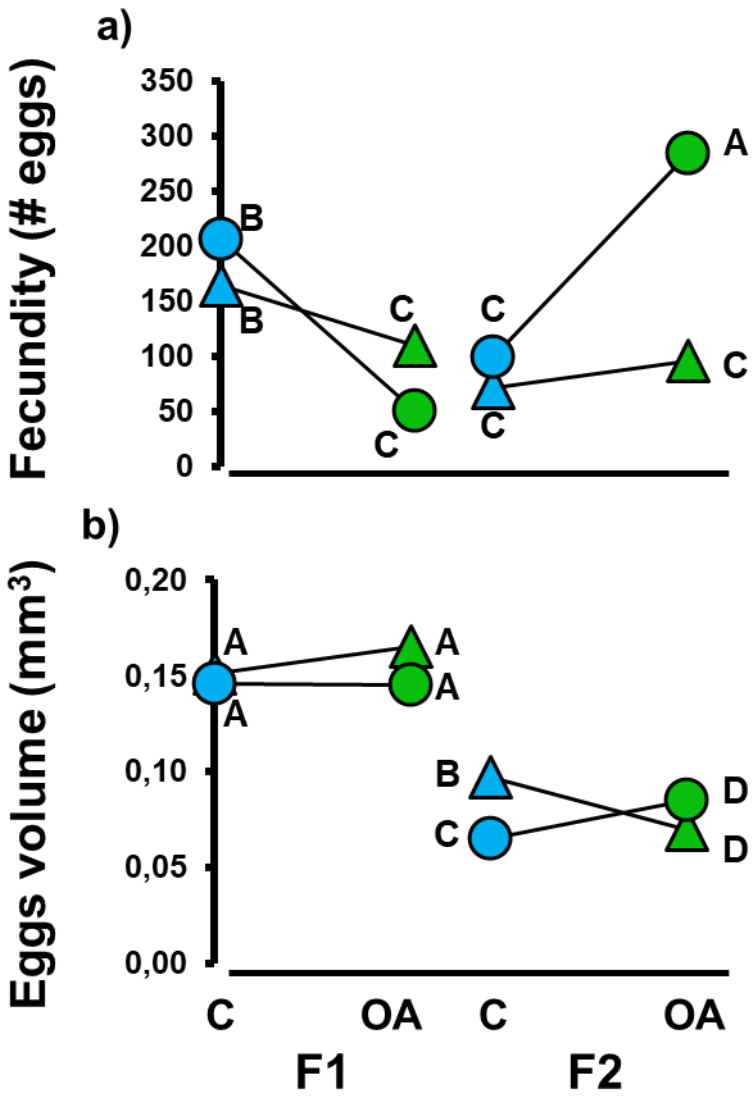

Fig. 3: Trans-generational effects of ocean acidification (OA, green) and control conditions (C, blue) on mean a) fecundity and b) egg volume in *O. japonica* (triangle) and *O. robusta* (circle). Significant differences among scenarios, species and generations are showed by different capital letters.

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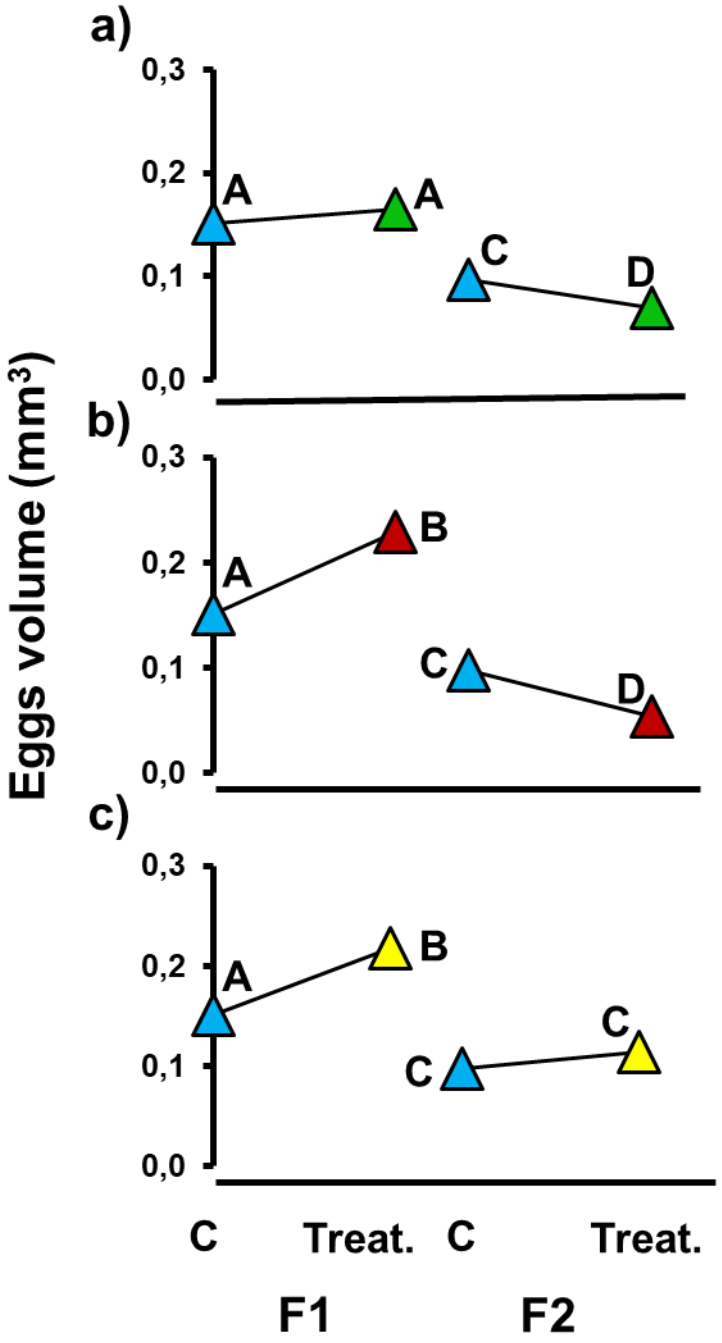

914

915 **Fig. 4:** Trans-generational effect of a) ocean acidification (green), b) ocean warming (red) in isolation and c)
916 combined (yellow) compared to control scenario (blue) in *O. japonica*. Significant differences among
917 generations are shown by different capital letters.

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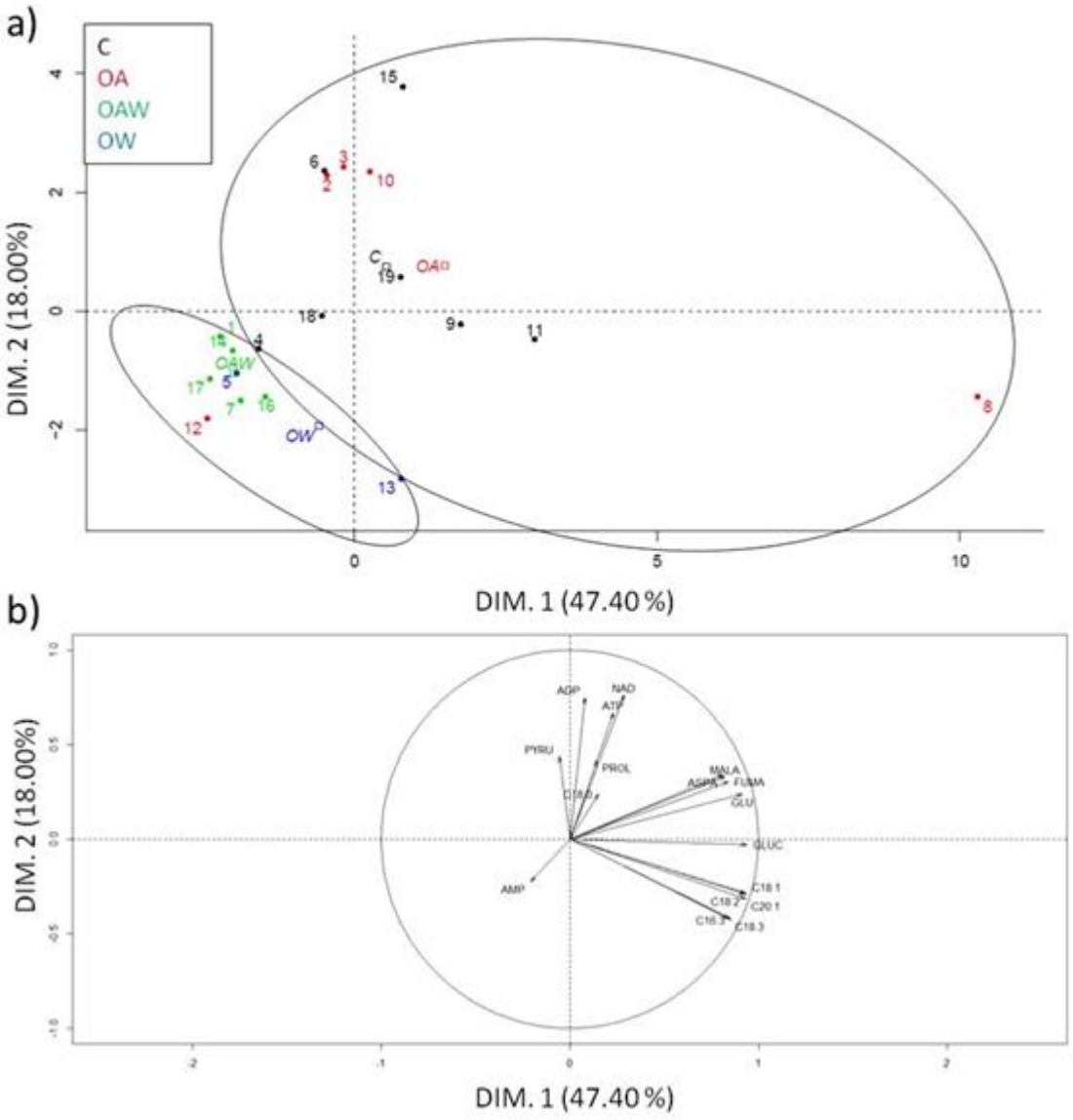
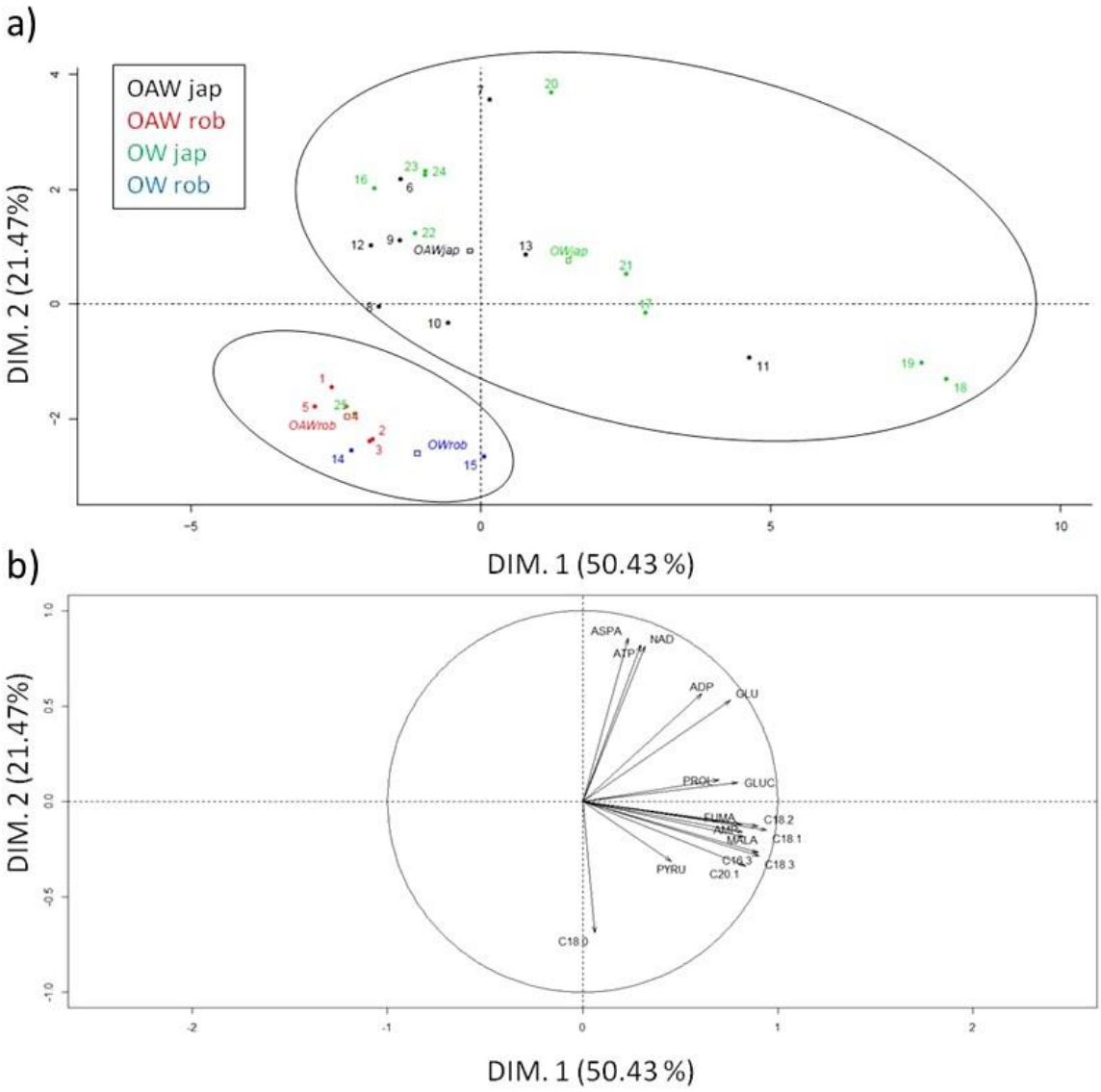


Fig. 5: a) PCA representing the variation of metabolite composition in *O. robusta* after the exposure of one generation (F1) to the control (C, black), ocean acidification (OA, red), ocean warming (OW, blue) and ocean acidification*warming (OAW, green) scenarios. Numbers represent the individual replicates. b) Correlation circle of the explanatory metabolites: aspartate (ASPA), ATP, NAD, ADP, AMP, glutamate (GLU), glucose (GLUC), proline (PROL), pyruvate (PYRU), fumarate (FUMA), malate (MALA) and a group of fatty acids (C18:0, C16:3, C18:1, C18:2, C18:3). The clustering was performed by hierarchical ascendant classification.



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928 **Fig. 6:** a) PCA representing the variation in metabolite composition of *O. japonica* (black - green) and *O.*
929 *robusta* (red - blue) after an exposure of one generation (F1) to the ocean warming (OW, blue- green) and
930 ocean acidification*warming (OAW, red - black). b) Correlation circle of the explanatory metabolites:
931 aspartate (ASPA), ATP, NAD, ADP, AMP, glutamate (GLU), glucose (GLUC), proline (PROL), pyruvate
932 (PYRU), fumarate (FUMA), malate (MALA) and a group of fatty acids (C18:0, C16:3, C18:1, C18:2, C18:3).
933 The clustering was performed by hierarchical ascendant classification.

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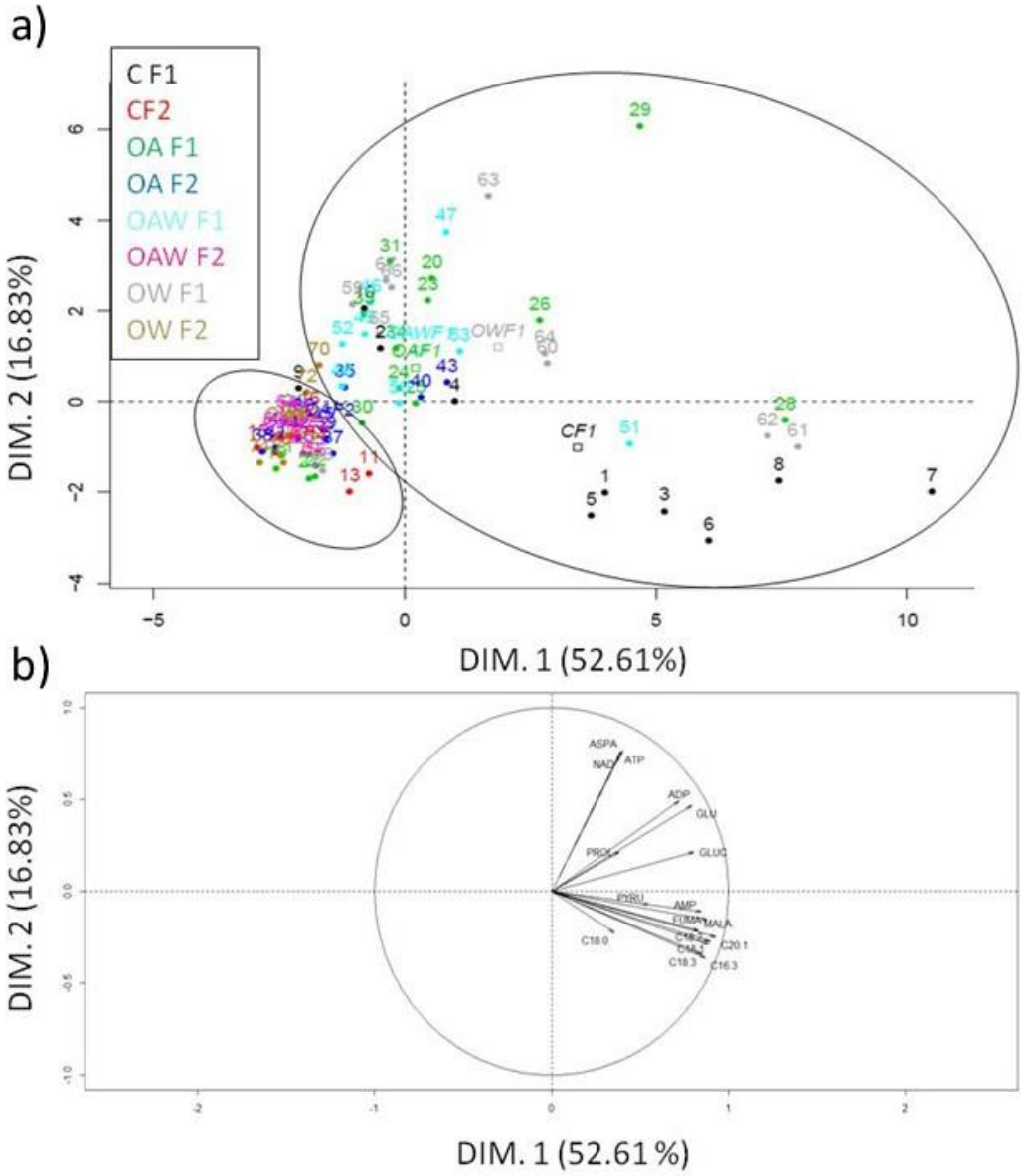

Fig. 7: a) PCA representing the variation in metabolite composition of *O. japonica* across a trans-generational exposure (F1: black-green-blue-grey; F2: red-purple-pink-gold) to the control (C, black and red), ocean acidification (OA, green and purple), ocean warming (OW, grey and gold) and ocean acidification*warming (OAW, blue and pink). b) Correlation circle of the explanatory metabolites: aspartate (ASPA), ATP, NAD, ADP, AMP, glutamate (GLU), glucose (GLUC), proline (PROL), pyruvate (PYRU), fumarate (FUMA), malate (MALA) and a group of fatty acids (C18:0, C16:3, C18:1, C18:2, C18:3). The clustering was made by hierarchical ascendant classification**.**

Figure 1

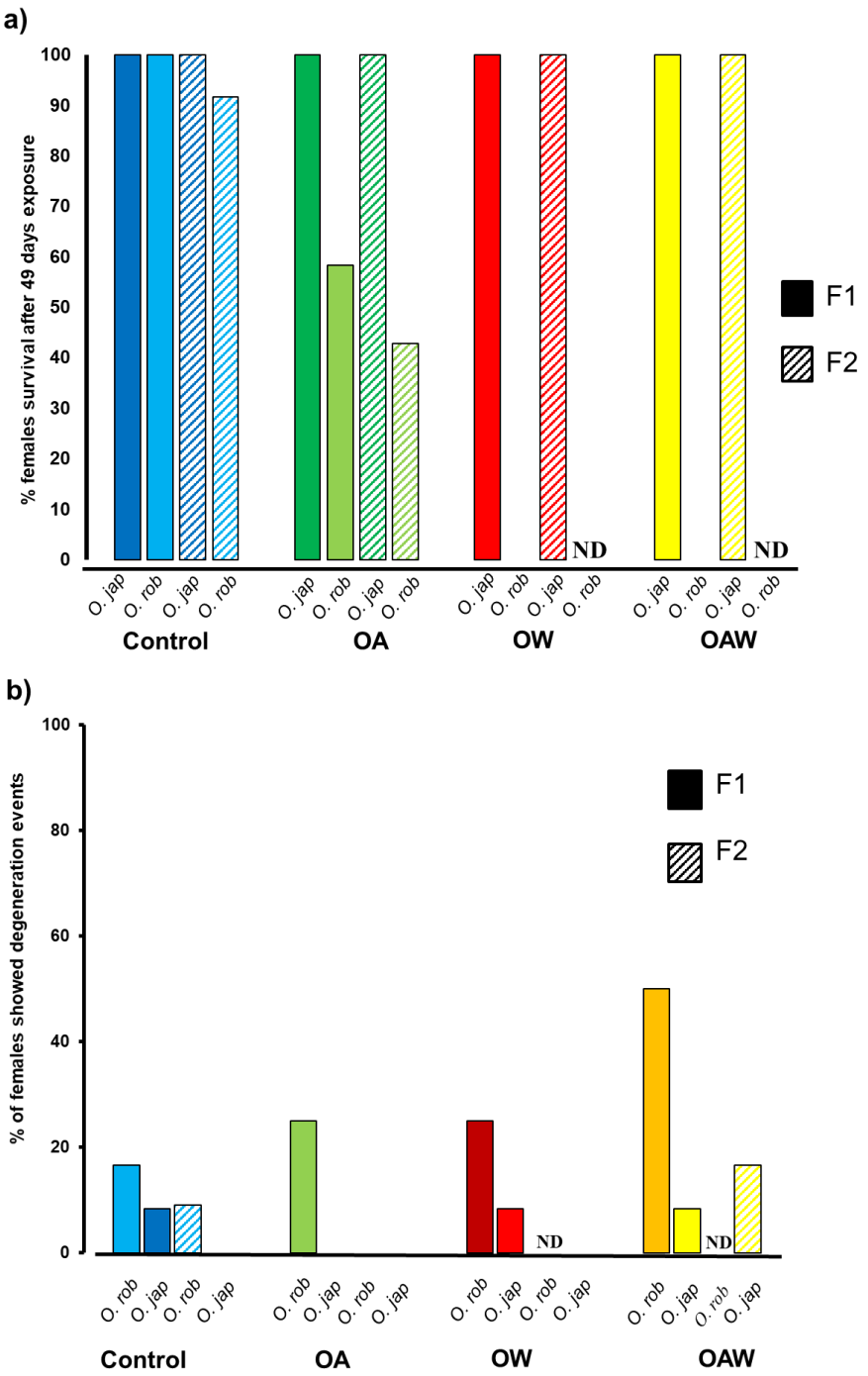

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Figure 2

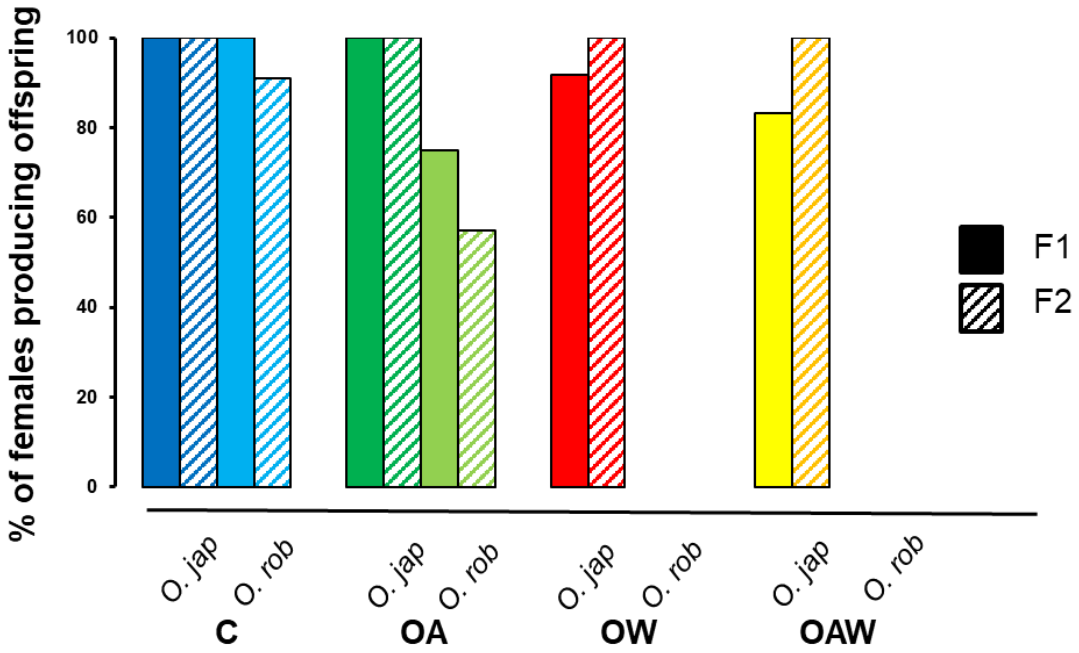

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Figure 3

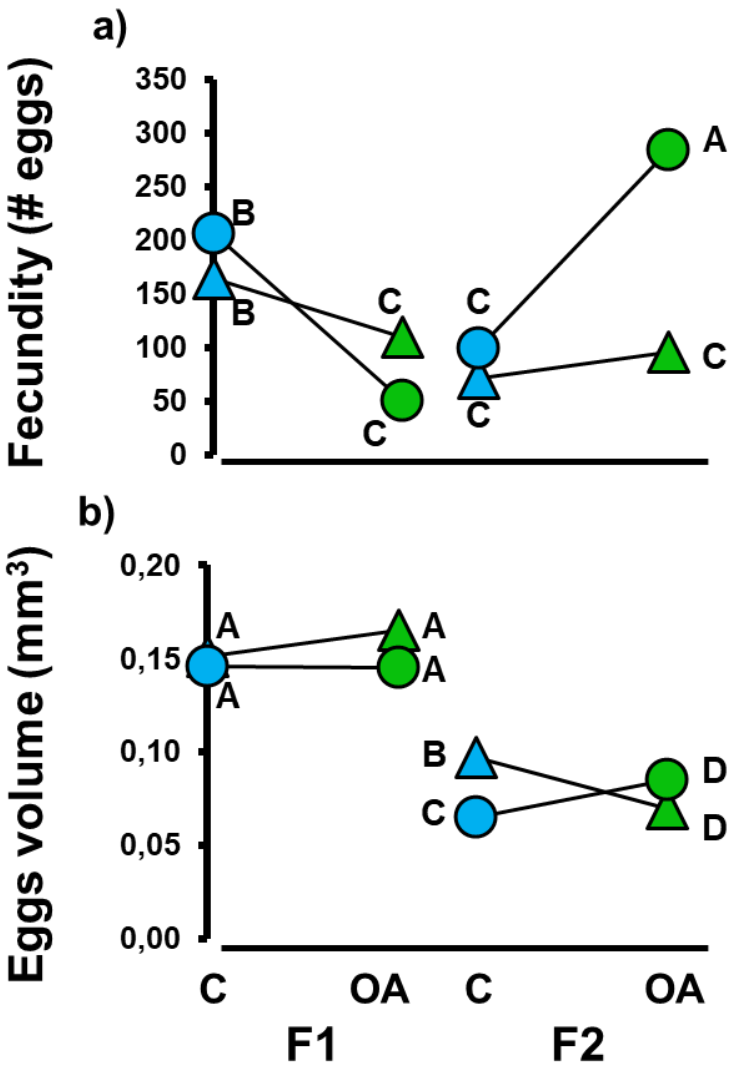

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Figure 4

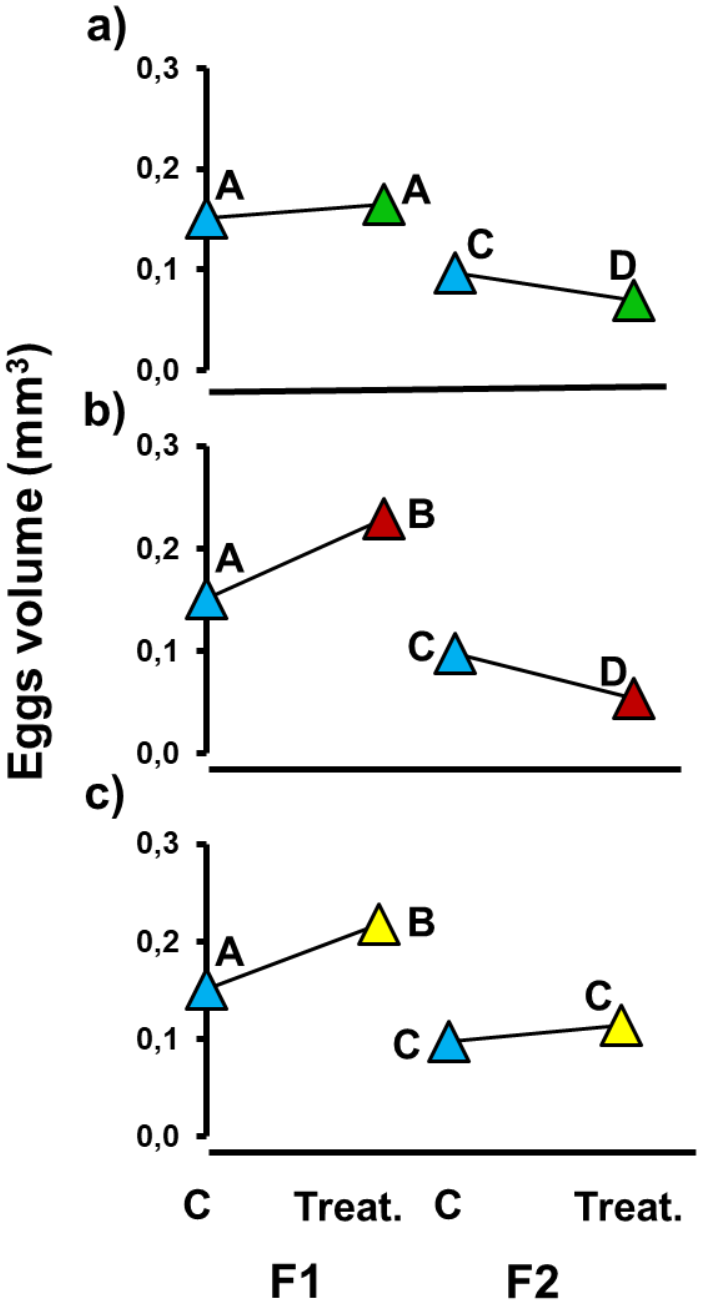

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Figure 5

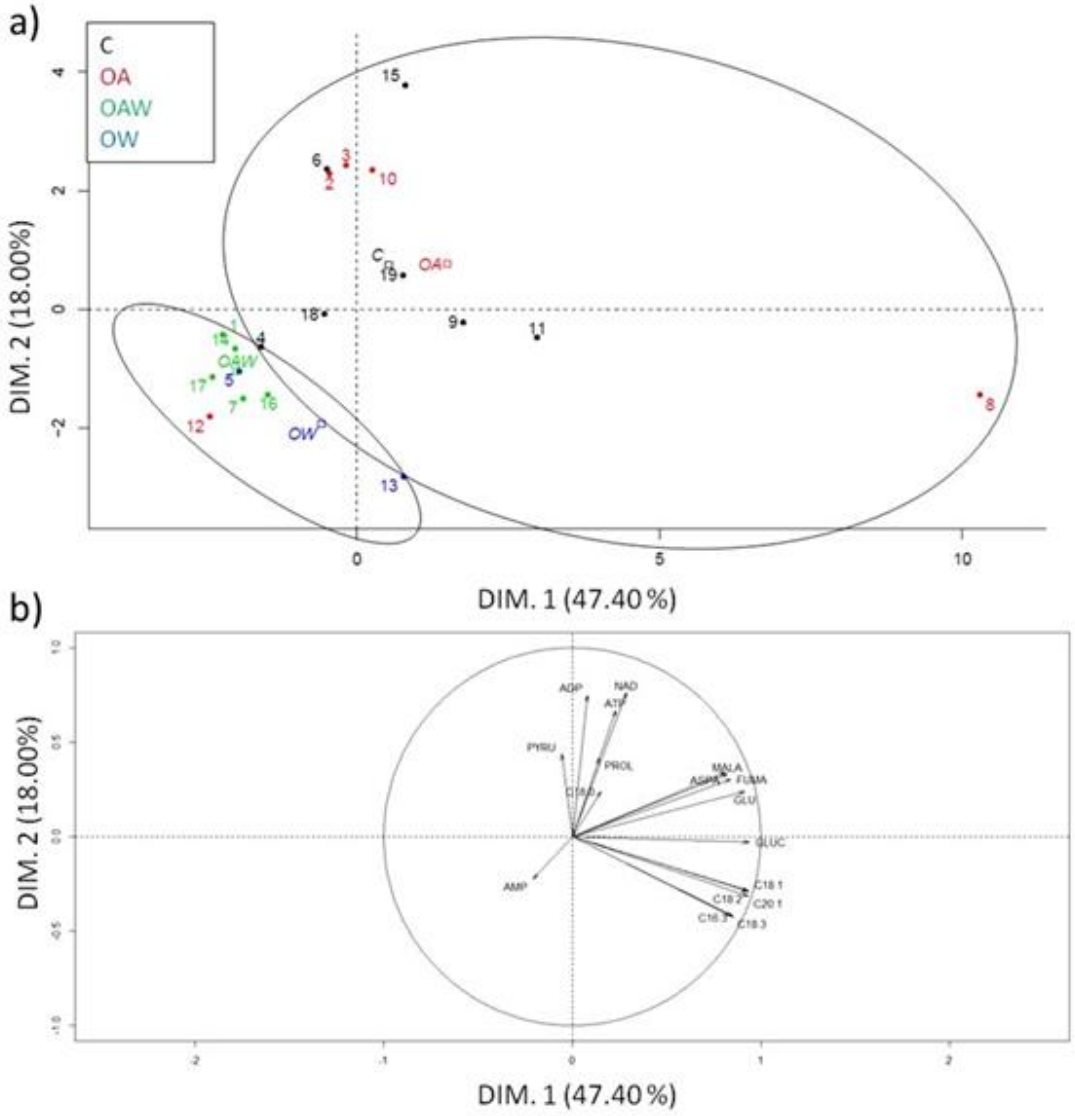

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Figure 6

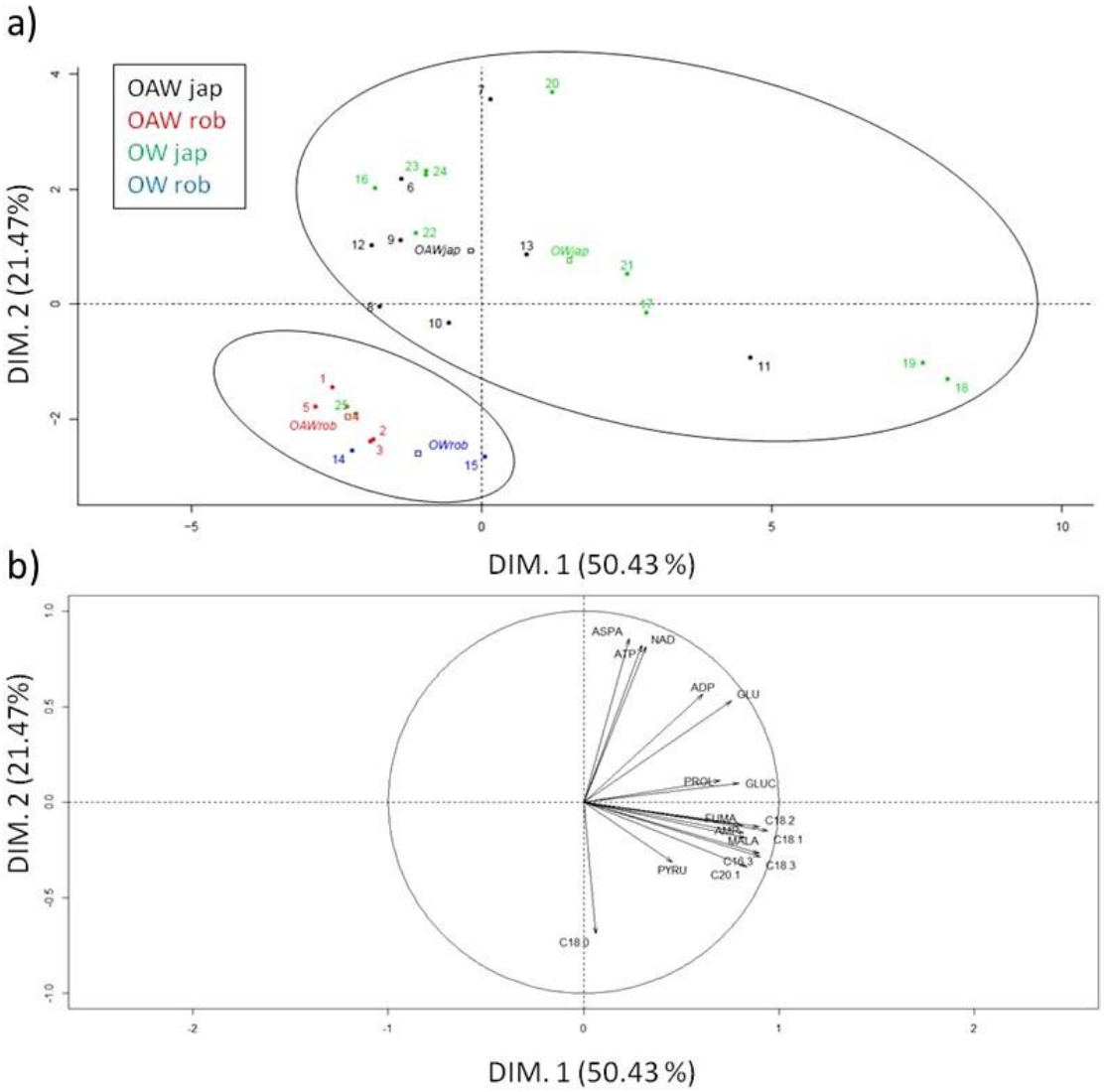
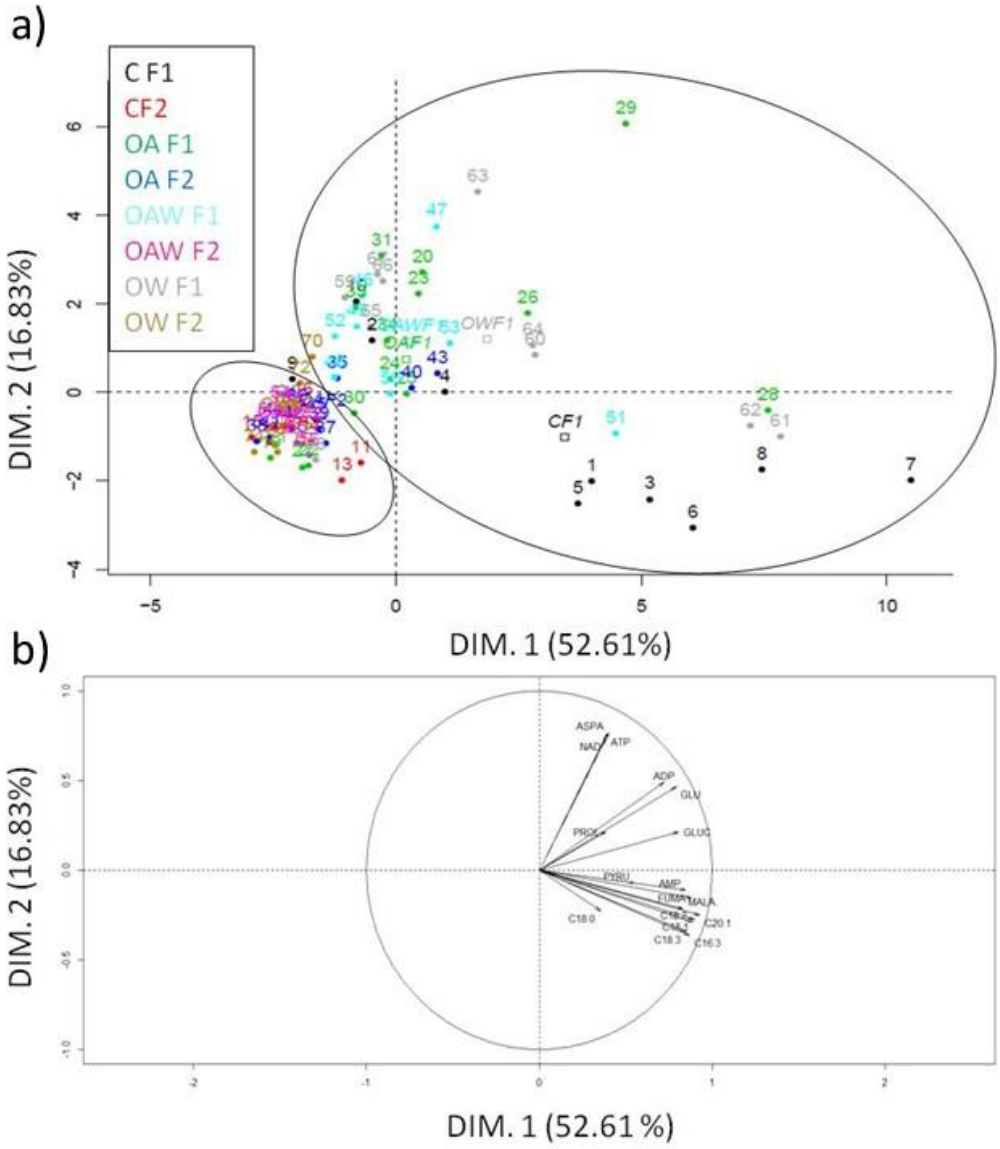


Fig. 6: a) PCA representing the variation in metabolite composition of *O. japonica* (black - green) and *O. robusta* (red - blue) after an exposure of one generation (F1) to the ocean warming (OW, blue- green) and ocean acidification*warming (OAW, red - black). b) Correlation circle of the explanatory metabolites: aspartate (ASPA), ATP, NAD, ADP, AMP, glutamate (GLU), glucose (GLUC), proline (PROL), pyruvate (PYRU), fumarate (FUMA), malate (MALA) and a group of fatty acids (C18:0, C16:3, C18:1, C18:2, C18:3). The clustering was performed by hierarchical ascendant classification.

Figure 7



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