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RESEARCH ARTICLE

Physiological responses to hypersalinity correspond to nursery ground usage in two inshore shark species (*Mustelus antarcticus* and *Galeorhinus galeus*)

Louise Tunnah[1], Sara R. C. MacKellar[1], David A. Barnett[2], Tyson J. MacCormack[3], Kilian M. Stehfest[4], Andrea J. Morash[1], Jayson M. Semmens[4] and Suzanne Currie[1,*]

ABSTRACT

Shark nurseries are susceptible to environmental fluctuations in salinity because of their shallow, coastal nature; however, the physiological impacts on resident elasmobranchs are largely unknown. Gummy sharks (*Mustelus antarcticus*) and school sharks (*Galeorhinus galeus*) use the same Tasmanian estuary as a nursery ground; however, each species has distinct distribution patterns that are coincident with changes in local environmental conditions, such as increases in salinity. We hypothesized that these differences were directly related to differential physiological tolerances to high salinity. To test this hypothesis, we exposed wild, juvenile school and gummy sharks to an environmentally relevant hypersaline (120% SW) event for 48 h. Metabolic rate decreased 20–35% in both species, and gill Na^+/K^+-ATPase activity was maintained in gummy sharks but decreased 37% in school sharks. We measured plasma ions (Na^+, K^+, Cl^-) and osmolytes [urea and trimethylamine oxide (TMAO)], and observed a 33% increase in plasma Na^+ in gummy sharks with hyperosmotic exposure, while school sharks displayed a typical ureosmotic increase in plasma urea (~20%). With elevated salinity, gill TMAO concentration increased by 42% in school sharks and by 30% in gummy sharks. Indicators of cellular stress (heat shock proteins HSP70, 90 and 110, and ubiquitin) significantly increased in gill and white muscle in both a species- and a tissue-specific manner. Overall, gummy sharks exhibited greater osmotic perturbation and ionic dysregulation and a larger cellular stress response compared with school sharks. Our findings provide physiological correlates to the observed distribution and movement of these shark species in their critical nursery grounds.

KEY WORDS: Hyperosmolarity, Elasmobranch, Heat shock proteins, Trimethylamine oxide, Urea, Ionic dysregulation

INTRODUCTION

Many species of sharks are born, or spend a portion of their juvenile period, in shallow, coastal nurseries (Castro, 1993; Helfman et al., 1997). These coastal environments are challenging habitats prone to fluctuations in abiotic factors like salinity, temperature and oxygen saturation (Barletta et al., 2005; Heithaus et al., 2009). Estuarine salinities are typically lower than seawater (SW; ~34‰), but when freshwater input is low, evaporation rates are high or marine access is restricted, hypersaline conditions can occur (Potter et al., 2010). Acute and chronic changes in abiotic factors are significant drivers of elasmobranch movement. Temperature influences habitat selection (Morrissey and Gruber, 1993) and migration timing in juvenile elasmobranchs (Grubbs et al., 2005; Heupel, 2007; Knip et al., 2010). However, salinity has one of the strongest influences on the distribution and abundance of inshore sharks and rays (for review, see Yates et al., 2015; Schlaff et al., 2014). Direct effects of an abiotic factor on an animal's physiology are thought to be the primary mechanism directing elasmobranch movement, yet empirical data linking spatial ecology with whole-animal physiology are lacking. Salinity-induced elasmobranch movement may occur to achieve optimal physiological homeostasis and/or because a critical environmental limit has been reached.

Salinity has significant effects on physiology, and for elasmobranchs it may be particularly challenging because of their unique osmoregulatory strategy as ionoregulating osmoconformers. Marine elasmobranchs usually maintain an internal osmotic pressure either isosmotic or slightly hyperosmotic to the surrounding SW. Osmoconformation is achieved using osmolytes, of which urea and trimethylamine oxide (TMAO) are the most common (Smith, 1931; Withers et al., 1994; Treberg et al., 2006). The challenge for elasmobranchs in hypersaline conditions is to maintain the directionality of osmotic gradients. Collectively, the osmoregulatory processes of ion regulation, osmolyte synthesis and osmolyte retention in normal SW constitute approximately 10–15% of an elasmobranch's standard metabolic rate (Kirschner, 1993). To our knowledge, there are no data on the energetics of elasmobranch osmoregulation in response to hypersalinity. As increased salinity can increase metabolic rate in teleost fishes (Fry, 1971), metabolic rate in elasmobranchs exposed to hypersalinity may also be higher than that in normal SW (Cramp et al., 2015). Thus, for young sharks in nurseries, where salinity changes are often rapid and ephemeral, metabolic, ionic and osmotic perturbations caused by acute hypersalinity may result in physiological stress.

Cellular stress is another potential consequence of increased salinity. Hypersalinity causes water efflux across the cell membrane, increases molecular crowding and DNA damage, and can result in apoptosis if cellular homeostasis cannot be re-established (Alfieri and Petronini, 2007; Burg et al., 2007). The most ubiquitous stress response across taxa is the induction of heat shock proteins (HSPs). These proteins are upregulated in response to a variety of stressors, including osmotic perturbations, and act to maintain the structure and function of other cellular proteins (Feder and Hofmann, 1999). The induction of HSPs in fishes has been well described, especially in response to increases in temperature (for review, see Currie and

[1]Department of Biology, Mount Allison University, Sackville, NB, Canada E4L 1G7. [2]Atlantic Cancer Research Institute, Moncton, NB, Canada E1C 8X3. [3]Department of Chemistry and Biochemistry, Mount Allison University, Sackville, NB, Canada E4L 1G8. [4]Fisheries and Aquaculture Centre, Institute for Marine and Antarctic Studies, University of Tasmania, Taroona, TAS 7053, Australia.

*Author for correspondence (scurrie@mta.ca)

S.C., 0000-0002-7734-7200

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Schulte, 2014). Less is known about the cellular response to osmotic stress in fishes, and most of what we know comes from studies on teleosts. For example, in Mozambique tilapia (*Oreochromis mossambicus*), gill HSP70 and HSP90 were significantly induced following transfer from freshwater to 20‰ hypertonicity (Tang and Lee, 2013). MacLellan et al. (2015) reported a significant induction of HSP70 in an elasmobranch, the spiny dogfish shark (*Squalus acanthias*), following a 48 h exposure to 70% SW, which appeared to result from osmotically induced cell damage in the gills. Thus, HSPs may also have a protective role in response to salinity stress in elasmobranchs.

Communal shark nurseries have been defined as areas consistently used by multiple shark species, across years, and for weeks to months at a time (Heupel et al., 2007). The Pittwater Estuary (depth ∼4 m) in Hobart, Tasmania, is a model example of a shark nursery (Stevens and West, 1997; McAllister et al., 2015). In the summer months, extended warm, dry periods increase evaporative water loss, causing hypersaline conditions (∼40–47‰; Marine Culture Pty Ltd). School sharks (*Galeorhinus galeus*) and gummy sharks (*Mustelus antarcticus*) are just two inshore species that commonly use this area. Adult school shark females visit transiently to pup before moving back to offshore waters, while juveniles remain (1–2 years) and develop in the nursery without parental care (Olsen, 1954; McAllister et al., 2015). Less is known about the nursery ground usage of gummy sharks, but both pups and juveniles are found in the Pittwater region, although less consistently and less abundantly than school sharks (Stevens and West, 1997). Notably, acoustic monitoring of electronically tagged school and gummy sharks in the Pittwater Estuary between January 2012 and May 2013 showed that school sharks did not leave the estuary during the summer period. In contrast, gummy sharks regularly moved between Pittwater Estuary and the adjoining Frederick Henry Bay (depth ∼15 m) during the same period (J. D. McAllister, A. Barnett, K. Abrantes and J.M.S., in review). Hypersaline events (>40‰) in the Pittwater Estuary occurred on 84 out of 134 days in the summer of 2013 (i.e. 63% of the time; Marine Culture Pty Ltd.), whereas in Frederick Henry Bay, salinity is lower and relatively stable (mean 33.9±0.2‰ from 1991 to 1994; Crawford and Mitchell, 1999). School and gummy sharks are in the family Triakidae but have distinct life histories. Gummy sharks are relatively short lived, fecund and localized mostly to the southern coast of Australia, whereas school sharks are highly migratory, long lived and slow to mature (Last and Stevens, 2009). Notably, school sharks are listed as a vulnerable species and as one in decline by the International Union for the Conservation of Nature (IUCN). Remarkably, little is known about the physiology of either species, with no information on how either school or gummy sharks cope with changes in salinity, particularly as juveniles inhabiting nurseries.

Given the distinct ecospatial dynamics of school and gummy sharks in the Pittwater Estuary, our goal was to identify the physiological and cellular effects of environmentally relevant hypersalinity on these species. While we cannot rule out other environmental drivers of shark movement, there is a large and compelling body of evidence demonstrating the importance of salinity on elasmobranch spatial ecology (Heupel and Simpfendorfer, 2008; Simpfendorfer et al., 2011; Yates et al., 2015). If distinct gummy and school shark nursery distribution patterns occur because a physiological limit to hypersalinity has been reached, then we predicted that school and gummy sharks would show physiological and cellular stress in response to increased salinity in a lab setting. We further predicted that gummy sharks would experience enhanced physiological stress and osmotic perturbation compared with school sharks when exposed to a salinity challenge, corresponding to their diminished nursery usage during hypersaline conditions. To test our hypothesis, we exposed wild-caught school and gummy sharks from the Pittwater Estuary to a 120% SW stress (41‰) for 48 h and measured aerobic metabolic rate, plasma ions, osmolytes and indicators of cellular stress in the tissues after hypersaline exposure, and following recovery at 100% SW. Overall, both juvenile school and gummy sharks showed signs of stress with hypersalinity; however, the osmoregulatory and cellular effects were more pronounced in gummy sharks.

MATERIALS AND METHODS

Animal collection and care

One year old gummy (*M. antarcticus* Günther 1870; *N*=18, 52.7± 18.4 cm) and young-of-the-year school [*G. galeus* (Linnaeus 1758); *N*=15, 45.8± 8.9 cm] sharks were caught using long lines in Pittwater Estuary and Frederick Henry Bay (42.79°S, 147.54°E) outside Hobart, Tasmania, in March 2014. Sharks were transported to the Institute for Marine and Antarctic Studies (IMAS) in Hobart and fitted with numbered fin tags to allow individuals to be identified. Gummy sharks were held for 1–5 days and school sharks were held for 3–11 days in outdoor 2×2×1 m holding tanks before use in experiments. Tank ammonium and nitrite levels were monitored daily. Holding temperatures and salinity were 16.8±1.0° C and 34.6±0.3‰, respectively. Sharks were fed frozen squid daily, but were fasted 24 h prior to, and during, experiments to regulate feeding status. All study protocols were approved by the University of Tasmania Animal Ethics Committee (no. A13796).

Experimental treatment and sampling

Gummy and school sharks were exposed to a hypersaline experimental condition (120% SW; representative of summer nursery conditions). Holding tank salinity was raised from 34.6 ±0.29‰ to 41.3±0.08‰ using Instant Ocean sea salt (Spectrum Brands, Blacksburg, VA, USA). By raising the salinity in each holding tank, all individuals within each species experienced the salinity challenge simultaneously, therefore standardizing the experimental exposure. This 20% increase over 24 h (0.28‰ h^{-1}) was monitored using an HQ40d portable meter and CDC401 conductivity probe (Hach, CO, USA) and was subsequently verified by measuring tank water osmolality using a Wescor Vapro 5520 Vapour Pressure Osmometer (Logan, UT, USA). Sharks were held at this increased salinity for 48 h before tank water was lowered back to 34‰ over 3 h (7.3‰ h^{-1}), where they remained for a further 21 h. Blood samples (0.5 ml) were drawn from unanaesthetized, restrained fish via caudal puncture using a 22-gauge needle and syringe washed with heparinized shark saline (in mmol l^{-1}: 280 NaCl, 6 KCl, 3 $MgCl_2$, 0.5 Na_2SO_4, 1 Na_2HPO_4, 4 $NaHCO_3$, 360 urea, 5 $CaCl_2$, 72 TMAO and 5 glucose, with 50 units ml^{-1} heparin, pH 7.8; modified from Villalobos and Renfro, 2007). Blood sampling, including netting, bleeding and return to the tank, was always <2 min in duration. Samples were taken before the salinity was increased as an internal fish control (t=0 h), 24 h later once the experimental salinity was reached (t=24 h), 24 and 48 h into the osmotic stress (t=48 h, t=72 h), and at recovery after 21 h in 100% SW (t=96 h).

We performed a control experiment to account for any handling or holding stressors (Table S1). Fish were held at 34‰ for 72 h and repeated blood samples were taken as described above at t=0, 24, 48 and 72 h. We measured haematological markers generally associated

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with the stress status of the animal – haemoglobin (Hb), haematocrit (Hct) and mean cell haemoglobin concentration (MCHC) – and observed no statistically significant changes over time. Because of limited access to wild fish in the nursery, school sharks used in the control experiment were also used in the hypersaline condition after a 5 day recovery at 100% SW. Thus, school sharks were in captivity for longer than gummy sharks. Our control data (Table S1) indicate minimal, if any, effects of handling; thus, we are confident there was no cross-tolerance between handling and salinity exposure. To ensure that animals had fully recovered from control sampling, we measured whole-blood glucose and lactate and MCHC and compared t=0 h samples from control and experimental animals. The MCHC of control fish at t=0 h (176±3.2 g l^{-1}) was significantly higher (P<0.05) than that of experimental fish at t=0 h (135 ±5.0 g l^{-1}), suggesting that captive holding was having some haematological impact on these animals. However, whole-blood glucose, another indicator of overall fish health, was not significantly different between control and experimental groups (7.9±0.6 versus 8.8±0.19 mmol l^{-1}, P=0.21), and whole-blood lactate remained less than 0.01 mmol l^{-1}. Thus, overall we are confident that a 5 day recovery period was sufficient.

Both gummy and school sharks were terminally sampled after pithing the brain and transecting the spinal cord. Samples of gill and white muscle were excised, flash frozen in liquid nitrogen and stored at −80°C for subsequent analysis. In the experimental group, tissues were taken after 48 h in hypersaline water (gummy N=5, school N=4) and after 21 h recovery at 100% SW (gummy N=4, school N=3). In the 100% SW control group, sharks were sampled after 48 h (gummy N=3) and 72 h (gummy N=2, school N=3).

Metabolic rate

Routine oxygen consumption was measured in a separate group of school (N=6; ∼450 g) and gummy (N=5; ∼750 g) sharks under both control and hyperosmotic conditions as per the time course outlined above. The rate of oxygen consumption ($\dot{M}_{O_2}$) of individual sharks was calculated from the decline in oxygen over time in a 14.4 l respirometry chamber submerged within the holding tank. Background oxygen consumption in the empty chamber was measured daily, and all reported $\dot{M}_{O_2}$ values are appropriately corrected. The sharks were guided gently into the chamber, without netting, and habituated (1 h) with constant water flow. A preliminary experiment (N=3), where O_2 consumption was monitored at 100% SW for 3 h, determined rapid stabilization of $\dot{M}_{O_2}$ after only 1 h, probably due to the minimal fish disturbance with this approach. The chamber was sealed (15 min) and the decline in O_2 concentration in the chamber was then measured using a Fibox O_2 probe (PreSens, Regensburg, Germany). Between measurement periods, the chambers were flushed until O_2 saturation returned to 100% (10 min) using a submersible pump and a three-way valve, and then resealed. Two slopes of oxygen depletion over time were averaged to calculate routine mass-specific $\dot{M}_{O_2}$ (mg O_2 kg^{-1} h^{-1}), taking into account background O_2 consumption rate (measured as oxygen depletion in the empty respirometer), the volume of the chamber, shark mass, temperature and daily barometric pressures.

Blood parameters and plasma ions

Hct was measured in duplicate using a SpinCrit Microhaematocrit centrifuge (SpinCrit, IN, USA). Hb was measured using a HemoCue® Hb 201+ system (Ängelholm, Sweden) and corrected for fish blood as per Clark et al. (2008). Whole-blood glucose and lactate concentrations (∼5 µl blood each) were determined using OneTouch Ultra2 (LifeScan, Milpitas, CA, USA) and Lactate Pro™ (Arkray Global Business, Inc., Kyoto, Japan) handheld meters. Such point-of-care field devices have been used in elasmobranchs (e.g. Awruch et al., 2011; French et al., 2015) and have been validated for lactate in school (Awruch et al., 2011) and shortfin mako shark (French et al., 2015). However, these instruments are not precise and we used values for relative comparisons; absolute values should be interpreted with caution (see Stoot et al., 2014). The remaining blood sample was then spun at 17,949 ***g*** for 4 min at 4°C. The buffy coat was discarded and the plasma supernatant and red blood cell pellet were flash frozen separately in liquid nitrogen and stored at −80°C.

Plasma osmolality (mmol kg^{-1}) was determined using a Wescor Vapro 5520 Vapour Pressure Osmometer, chloride ion concentration (mmol l^{-1}) with a Chloride Analyzer 925 (Nelson Jameson Inc., Marshfield, WI, USA), and plasma sodium and potassium concentrations (mmol l^{-1}) using a SpectraAA 220 Atomic Absorption Spectrometer (Varian, Mulgrave, VIC, Australia), all according to the manufacturers' directions.

Protein extraction and HSP immunoblotting

Soluble protein was extracted from frozen gill and white muscle and prepared for SDS-PAGE as per Fowler et al. (2009), with minor modifications. As indicators of cellular stress, we measured several HSPs (HSP70, 90, 110) in gill and white muscle (5–15 µg). Gel electrophoresis was performed using Bolt™ 4–12% Bis-Tris mini gels (ThermoFisher Scientific, Burlington, ON, Canada) and MOPS SDS running buffer (ThermoFisher Scientific) at 200 V. Separated proteins were transferred (20 V) to a polyvinylidene difluoride (PVDF) membrane using the Bolt™ Mini Blot module and transfer buffer (25 mmol l^{-1} bicine, 25 mmol l^{-1} Bis-Tris, 1 mmol l^{-1} EDTA; pH 7.2). All membranes were then blocked for 1 h at room temperature, or overnight at 4°C, in 5% milk powder dissolved in Tris-buffered saline with Tween 20 (TBS-T; 25 mmol l^{-1} Tris, 138 mmol l^{-1} NaCl, 0.1% Tween 20, pH 7.6). All antibody incubations were 1 h in duration at room temperature and both primary (HSP70: 1:5000 polyclonal rabbit affinity purified HSP70 antibody; AS05 083A, Agrisera, Vännäs, Sweden; HSP90: 1:2500 monoclonal mouse HSP90; SMC-107, StressMarq Biosciences, Victoria, BC, Canada; HSP110: 1:2500 polyclonal rabbit HSP110 antibody; SPC-195, StressMarq Biosciences) and secondary (HSP70: 1:10,000 goat anti-rabbit IgG-HRP; SAB 300, Enzo Life Sciences, Farmingdale, NY, USA; HSP90: 1:5000 goat anti-mouse IgG-HRP; ab5870, AbCam Inc., Toronto, ON, Canada; HSP110: 1:5000 goat anti-rabbit IgG-HRP; SAB 300, Enzo Life Sciences) antibodies were diluted in 1% milk powder TBS-T solution. Chemiluminescent detection of protein bands was performed using Lumigen ECL Ultra (Southfield, MI, USA). Blots were imaged and analysed using a VersaDoc™ MP 4000 Molecular Imager (Bio-Rad, Mississauga, ON, Canada) and Image Lab® software (see Fig. S1 for representative images). Equal protein was loaded on each gel and verified and visually assessed by Coomassie staining of PVDF membranes after immunodetection, as described by Welinder and Ekblad (2011). HSP70 and HSP90 were quantified relative to standard curves run on each gel (HSP70: 3.75–75 ng recombinant rat HSP70/72 protein; SPP-758; HSP90: 3.75–75 ng native human HSP90 protein standard; SPP-770, Enzo Life Sciences). For HSP110, 12.5 µg of white muscle protein from one school shark served as an internal standard. All band densities were expressed relative to this sample. A molecular weight ladder (Magic Mark™ XP, ThermoFisher Scientific) was also run on each gel to verify the molecular weight of the target band.

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Protein damage

Ubiquitin in gill and white muscle tissues was measured as an indirect indicator of protein damage, using dot blots. Sample protein (0.25 µg µl^{-1}) and ubiquitin standard (0.05 µg µl^{-1}; sc-111402, Santa Cruz Biotechnology, Dallas, TX, USA) were loaded onto a nitrocellulose membrane (Bio-Rad) and blocked in 5% BSA/TBS-T. A mouse primary antibody (BML-PW8805-0500, Enzo Life Sciences; 1:2500 in 5% BSA/TBS-T), which detects polyubiquitinylated but not monoubiquitinylated proteins or free ubiquitin, was used. The secondary antibody was a goat anti-mouse IgM (ab97230, AbCam Inc.; 1:20,000 in 0.1% BSA/TBS-T). Levels of ubiquitin in each sample were imaged and expressed relative to the dot intensity of the ubiquitin standard.

Osmolytes

Urea and TMAO (mmol l^{-1}) were measured in plasma, gill and white muscle samples. For urea analysis, deproteinized (perchloric acid precipitated) samples were assayed in quartz cuvettes as per Rahmatullah and Boyde (1980). Liquid Chromatography Mass Spectrometry (LC-MS) was used to measure TMAO concentration. As in MacLellan et al. (2015), we verified TMAO concentrations obtained from LC-MS by assaying a subset of samples using the procedure outlined in Wekell and Barnett (1991) with quartz cuvettes. Plasma was deproteinized 1:4 in ice-cold acetone, and gill and muscle with 5% trichloroacetic acid (TCA). Samples were spiked with deuterated (d9) TMAO and diluted in a water/methanol/formic acid solution. All standards and samples (5 µl) were injected onto a SeQuant®, ZIC®-HILIC column (3 µm, 100 Å, 2.1 mm×15 cm; EMD Millipore, Etobicoke, ON, Canada) and eluted under isocratic solvent conditions (50/50 acetonitrile/water containing 0.2 mmol l^{-1} ammonium acetate; flow rate of 100 µl min^{-1}). The LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated as in MacLellan et al. (2015). A linear calibration curve was used to calculate the concentration of TMAO (mmol l^{-1}) in each sample, plotted as the ratio of endogenous to labelled TMAO peak heights. Standards (0.0005–0.1 mmol l^{-1}) were spiked with d9-TMAO. Our TMAO concentrations are from tissue homogenates and, as detailed in MacLellan et al. (2015), absolute concentrations are dependent on the volume of extracellular fluid perfusing the tissue.

Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity (NKA; µmol mg^{-1} total protein h^{-1}) was measured as an indicator of tissue function in response to hypersaline stress. The protocol was modified (MacLellan et al., 2015) from that outlined in McCormick (1993).

Statistical analyses

All statistical analyses were performed in SPSS (IBM Inc., Markham, ON, Canada) with an α-critical level of 0.05. Homogeneity of variance was assessed using Levene's test, and residuals for normality (Kolmogorov–Smirnov). When required, data were transformed (log or square root) to satisfy assumptions; however, because we were using wild fish and had small, unequal sample sizes in some groups, transformations were not always successful. Non-parametric tests were unsuitable for our repeated experimental design; thus, consulting Glass et al. (1972), who stated that many parametric tests are not affected by violation of assumptions, we proceeded with parametric methods. For this reason, *P*-values close to 0.05 should be interpreted with caution.

Blood, plasma and aerobic metabolic rate data were analysed using a split-plot ANOVA to determine the effects of fish nested within species, and of time on each dependent variable. When an overall significant effect of time was determined, a Tukey's *post hoc* test was used to identify time points where means significantly differed. When a significant interaction between species and time was determined, data were always split to allow the effect of time to be compared in each species individually using a 1-way randomized block ANOVA with Tukey's *post hoc* to compare main effects.

Gummy shark control tissues sampled at *t*=48 h (*N*=3) and *t*=72 h (*N*=2) were statistically compared using a non-paired two-tailed *t*-test. Samples were not significantly different for any endpoint and so the data were pooled. Pooling was performed because of the small sample size at *t*=72 h, which precluded presentation of means±s.e.m. for these data, and also to allow easier comparison with school shark control tissues, which were only sampled at one time point (*t*=72 h, *N*=3). Subsequently, all tissue data were analysed using a 2-way fixed-factor ANOVA (species and time) with Tukey's *post hoc* tests for time. When a significant interaction between species and time was determined, as above, data were always split to analyse the effect over time within each species separately.

RESULTS

Rate of O_2 consumption

Both school and gummy sharks exhibited a significant decrease in aerobic oxygen consumption after 48 h in 120% SW (*t*=72 h; Fig. 1). However, a statistically significant species×time interaction indicates that the two species were responding uniquely to hypersaline exposure (*P*=0.047). Because of this interaction, we did not directly compare metabolic rate between species. Gummy sharks showed a 20% decrease in metabolic rate (*t*=0 h to *t*=72 h), which remained at this level after a 21 h recovery period at 100% SW. In contrast, school sharks exhibited a 35% decrease in metabolic rate at *t*=72 h but fully recovered upon return to 100% SW (*t*=96 h).

Ion and osmolyte concentrations

As predicted, both species significantly increased the osmolality of their plasma (*P*<0.001) in response to a 20% increase in tank

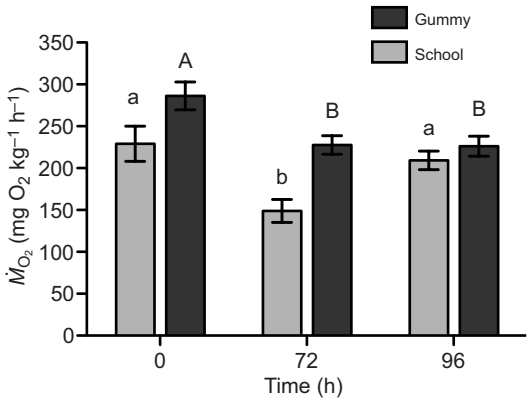

Fig. 1. Rate of routine oxygen consumption ($\dot{M}_{O_2}$) in gummy sharks (*Mustelus antarcticus*) and school sharks (*Galeorhinus galeus*) exposed to hypersaline conditions. Sample sizes are *N*=6 school sharks and *N*=5 gummy sharks for all sampling time points (means±s.e.m.). There was a significant species×time interaction (*P*=0.047) so data were split to analyse the response over time in each species individually. Different capital letters indicate significant changes in gummy shark metabolic rate over time (1-way randomized block ANOVA, *P*=0.006). Different lowercase letters indicate significant changes in school shark metabolic rate over time (1-way randomized block ANOVA, *P*=0.002).

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salinity. At all time points, gummy shark plasma osmolality was significantly higher than that of school sharks (P<0.001). Before salinity manipulation, gummy and school shark plasma osmolality was 1001±2.4 and 984±3.1 mmol kg^{-1}, respectively. The osmolality of both species increased (16%) by t=24 h (gummy sharks 1189±4.1 mmol kg^{-1}, school sharks 1169±7.0 mmol kg^{-1}) and this increase was maintained for the 48 h hypersaline exposure. Within 21 h of return to 100% SW, the plasma osmolality of both species returned to control levels (P=0.74).

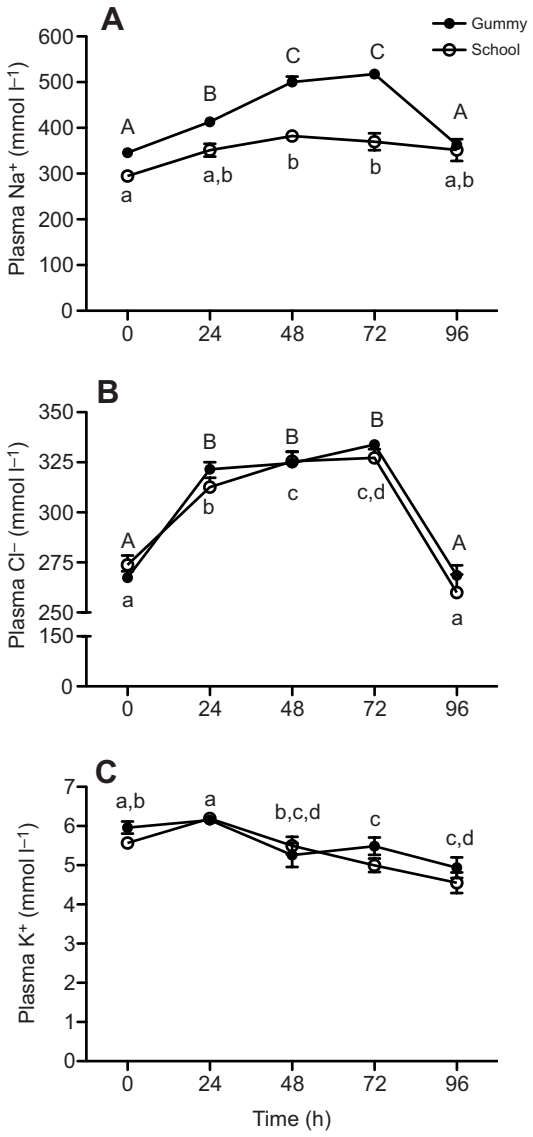

Fig. 2. Plasma ion concentrations in gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to hypersaline conditions. Blood was sampled before the salinity increase at time t=0 h at 100% seawater (SW; N=7 school sharks, N=9 gummy sharks), 24 h later (t=24 h) once the experimental salinity was reached at 120% SW (N=8 school sharks, N=9 gummy sharks), 24 h (t=48 h) and 48 h (t=72 h) into the hypersaline event (N=8 school sharks, N=9 gummy sharks), and after a 21 h recovery at 100% SW [t=96 h; N=3 school sharks, N=4 gummy sharks], means±s.e.m. (A) Sodium ions. Because of a significant species×time interaction (split-plot ANOVA, P<0.001), data were split to analyse the change over time in each species separately [1-way repeated measures (RM) randomized block ANOVA]. Different capital letters indicate significant changes in gummy shark plasma (P<0.001); different lowercase letters indicate significant changes in school shark plasma (P=0.005). (B) Chloride ions. Data were split and analysed by 1-way RM randomized block ANOVA because of a significant (P=0.041) species×time interaction. Different capital letters indicate significant changes in gummy shark plasma (P<0.001); different lowercase letters indicate significant changes in school shark plasma (P<0.001). (C) Potassium ions. Different lowercase letters indicate significant changes over time for the two species combined (split-plot ANOVA, P<0.001).

Table 1. Urea:TMAO ratio in plasma, white muscle and gill tissues across the experimental time course in school sharks (*Galeorhinus galeus*) and gummy sharks (*Mustelus antarcticus*)

	Time (h)				
	t=0	t=24	t=48	t=72	t=96
Plasma					
School	7.0:1 (7)	7.2:1 (8)	9.4:1 (8)	9.3:1 (8)	7.7:1 (3)
Gummy	3.8:1 (9)	4.0:1 (8)	4.4:1 (7)	4.0:1 (9)	4.4:1 (4)
White muscle					
School	2.2:1 (3)			2.7:1 (4)	2.1:1 (3)
Gummy	2.7:1 (5)			1.4:1 (5)	1.7:1 (4)
Gill					
School	3.2:1 (3)			2.6:1 (4)	3.2:1 (3)
Gummy	2.8:1 (5)			1.7:1 (5)	2.0:1 (4)

Samples sizes used for calculation are shown in parentheses.

School sharks and gummy sharks had distinct ionic and osmolyte responses to hypersaline exposure. After 48 h in hypersaline water, school shark plasma sodium increased by 20% (P=0.005; Fig. 2A) and plasma urea increased by 19% (P=0.002; Fig. 3A). Gummy shark plasma sodium increased by 33% (P<0.001) and plasma urea by only 12% (P<0.001). These species-specific responses are reflected by the significant species×time interaction detected for both sodium (P<0.001), and urea (P=0.038). Plasma chloride increased by 16% and 19% in school and gummy sharks, respectively (P<0.001; Fig. 2B). In contrast, plasma potassium levels significantly decreased (P<0.001; Fig. 2C), by the same magnitude in both species, across the experimental exposure and did not return to control levels at recovery, remaining depressed by ∼17%. As an indirect indicator of gill function during a hypersaline event, we measured the activity of NKA (Fig. 4). School and gummy sharks had notably different NKA activity levels and responses to hypersalinity, as indicated by a statistically significant interaction (species×time; P=0.03). Gummy shark NKA activity under control conditions was approximately one-third lower than that observed in school sharks, and these constitutively low levels were maintained for the duration of the experiment (P=0.26). In contrast, a 48 h exposure to hypersalinity depressed school shark NKA activity by 37%; however, this effect was transient as activity returned to control rates once school sharks were returned to 100% SW (P=0.03).

Plasma TMAO (Fig. 3B), an important osmolyte in elasmobranchs, did change in response to hypersalinity (P=0.039) but this effect was only significant between t=24 h and t=96 h (P=0.017), suggesting a minor role in osmoconformation. Overall, gummy sharks had significantly higher levels of TMAO compared with school sharks throughout the experiment (P<0.001). These distinct species changes in plasma TMAO and urea in response to hypersalinity resulted in changes in the urea:TMAO ratio (Table 1), particularly in school sharks. After a 48 h hypersaline exposure, the urea:TMAO ratio in school shark plasma was 9:1 (t=72 h) compared with 7:1 at t=0 h, whereas in gummy sharks, a ∼4:1 ratio was maintained throughout the experiment.

Neither TMAO (P=0.822) nor urea (P=0.595) changed significantly in response to hypersaline exposure in the white muscle of either species. Prior to salinity stress, white muscle urea was 318±18.6 mmol l^{-1} in school sharks and 307±17.4 mmol l^{-1} in

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Table 2. Haemoglobin, haematocrit, mean cell haemoglobin concentration and whole-blood glucose over time in school sharks (*G. galeus*) and gummy sharks (*M. antarcticus*) exposed to a hypersaline event

	Time (h)					*P*-value		
	t=0	*t*=24	*t*=48	*t*=72	*t*=96	Time×species	Time	Species
Hb (g l^{-1})								
School	25.4±1.4 (8)	34.9±1.3 (8)	31.4±1.4 (8)	27.2±1.9 (8)	19.5±1.2 (3)	0.019	<0.001	–
Gummy	30.8±2.5 (9)	41.5±2.7 (9)	40.6±2.9 (9)	39.1±2.2 (9)	28.6±1.6 (4)		<0.001	
Hct (%)								
School	18.9±0.8 (8)	22.8±0.8 (8)	21.0±0.8 (8)	19.5±1.6 (8)	14.7±1.3 (3)	0.054	<0.001	0.001
Gummy	24.2±2.1 (9)	32.3±2.4 (9)	31.4±1.7 (9)	29.8±1.5 (9)	20.5±0.5 (4)			
MCHC (g l^{-1})								
School	135±5 (8)	154±5.3 (8)	150±5.0 (8)	142±6.7 (8)	136±16.4 (3)	0.347	0.625	0.001
Gummy	128±3.7 (9)	129±2.1 (9)	129±4.7 (9)	131±3.6 (9)	140±8.4 (4)			
Glucose (mmol l^{-1})								
School	8.83±0.1 (8)	18.68±1.1 (8)	18.28±1.7 (8)	12.79±1.7 (8)	3.38±1.1 (3)	<0.001	0.001	–
Gummy	7.14±0.9 (9)	7.46±1.8 (9)	5.48±1.4 (9)	3.85±1.1 (9)	3.28±0.6 (4)		0.001	

Hb, haemoglobin; Hct, haematocrit; MCHC, mean cell haemoglobin concentration. Data are means±s.e.m.; sample size is shown in parentheses. MCHC was calculated as 100×(Hb/Hct).

gummy sharks (data not shown). At the same time point, TMAO in white muscle was 148±10.7 mmol l^{-1} in school sharks and 114±24.7 mmol l^{-1} in gummy sharks (data not shown). The white muscle urea:TMAO ratio in both species remained ~2:1 (Table 1) over the experiment, although the ratio was slightly lower (1.4:1 and 1.7:1) in hypersaline-exposed gummy sharks at *t*=72 h and *t*=96 h, respectively. In contrast to muscle, gill tissue TMAO was significantly affected by hypersalinity (*P*=0.018; Fig. 5A). After 48 h in hypersaline conditions (*t*=72 h), gill TMAO increased 42% in school sharks and 30% in gummy sharks, returning to control concentrations in both species at recovery (*t*=96 h). As seen with plasma TMAO, the concentration of TMAO in gill was significantly higher (*P*=0.037) overall in gummy sharks. In contrast, gill urea was not significantly affected by hypersalinity in either species (*P*=0.661, Fig. 5B). However, there was a significant (*P*=0.023) interaction of species and time, suggesting that the two shark species are responding differently to the stress in terms of gill urea. School shark gill urea demonstrated an increasing trend while gummy shark gill urea decreased non-significantly over time (Fig. 5B).

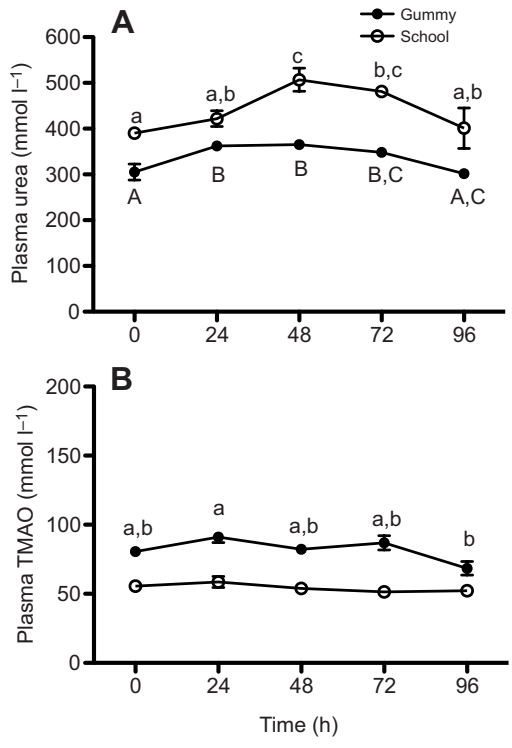

Fig. 3. Plasma osmolyte concentrations in gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to a hypersaline event. Sample sizes were: *t*=0 h(C) at 100% SW, *N*=7 school sharks, *N*=9 gummy sharks; *t*=24 h at 120% SW, *N*=8 school sharks, *N*=8 gummy sharks; *t*=48 h, *N*=8 school sharks, *N*=7 gummy sharks; *t*=72 h, *N*=8 school sharks, *N*=9 gummy sharks; and after a 21 h recovery at 100% SW [*t*=96 h(R)], *N*=3 school sharks, *N*=4 gummy sharks (means±s.e.m.). (A) Urea. Because of a significant species×time interaction (split-plot ANOVA, *P*=0.038), data were split to analyse the change over time (1-way RM randomized block ANOVA). Different capital letters indicate significant changes in gummy shark plasma (*P*<0.001); different lowercase letters indicate significant changes in school shark plasma (*P*=0.002). (B) Trimethylamine oxide (TMAO). Different lowercase letters indicate significant changes over time for both species (split-plot ANOVA, *P*<0.039); there was an overall significant difference between gummy and school sharks (*P*<0.000).

Stress and damage indicators

MCHC was calculated to determine whether the hypersaline exposure affected haematology in either species. MCHC did not

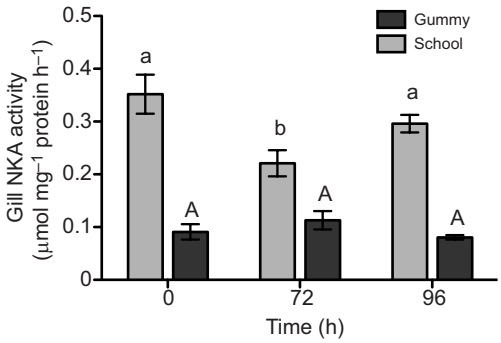

Fig. 4. Na^+/K^+-ATPase (NKA) enzyme activity in the gills of gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to a hypersaline event. Tissue samples were taken after 75 h at 100% SW [*t*=0 h(C); *N*=3 school sharks, *N*=5 gummy sharks], after 48 h at 120% SW (*t*=72 h; *N*=4 school sharks, *N*=5 gummy sharks) and after 12 h recovery at 100% SW [*t*=96 h(R); *N*=3 school sharks, *N*=4 gummy sharks] (means±s.e.m.). There was a significant species×time interaction (2-way ANOVA, *P*=0.003) so data were split to analyse responses over time in each species individually. Identical capital letters indicate no significant change in gummy shark NKA activity over time (1-way ANOVA, *P*=0.255); different lowercase letters indicate significant changes in school shark NKA activity over time (1-way ANOVA, *P*=0.033).

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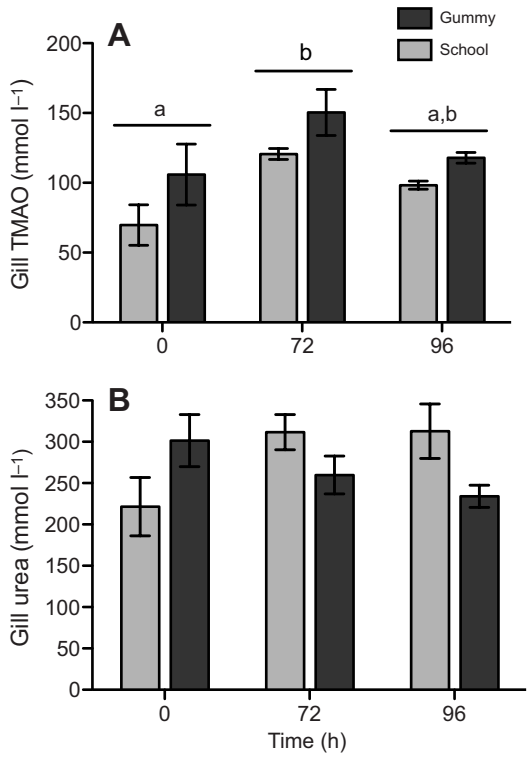

Fig. 5. Osmolyte concentrations in the gills of gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to a hypersaline event. Sample sizes and sampling procedure as outlined in Fig. 4 (means± s.e.m.). (A) TMAO. Different lowercase letters indicate significant changes in gill TMAO over time (2-way ANOVA, P=0.018); there was a significant difference between species (P=0.037). (B) Urea. Statistically significant species×time interaction (2-way ANOVA, P=0.023). No significant change in gill urea was observed over time in either species (1-way ANOVA; gummy sharks P=0.217, school sharks P=0.106).

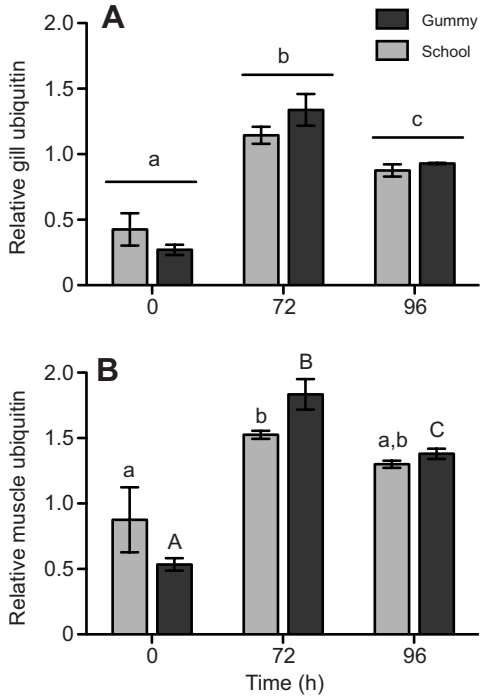

Fig. 6. Relative levels of ubiquitin in gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to a hypersaline event. Sample sizes and sampling procedure as outlined in Fig. 4 (means±s.e.m.). (A) Gill. Different lowercase letters indicate statistically significant differences over time (2-way ANOVA, P<0.001). (B) White muscle. Statistically significant species×time interaction (2-way ANOVA, P=0.014). Different letters indicate significant changes over time (1-way ANOVA) in gummy sharks (capital letters, P<0.001) and school sharks (lower case, P=0.025).

change significantly over the experimental time course in either species (P=0.625; Table 2). However, school sharks had significantly higher MCHC compared with gummy sharks (P=0.001). In response to an increase in salinity, we observed a dramatic difference in whole-blood glucose between hypersaline-exposed school and gummy sharks (P<0.001; Table 2). School shark blood glucose increased significantly (P<0.001) with hypersaline exposure, peaking at t=24 h (a 53% increase from t=0 h). At the recovery time point (t=96 h), blood glucose decreased to below control concentrations. In contrast, gummy shark blood glucose decreased significantly (P=0.01) over time, declining steadily from the onset of salinity stress (t=24 h) to recovery (t=96 h) where concentrations were lowest (Table 2).

As an indirect indicator of protein damage resulting from the hypersaline exposure, relative levels of the protein tag ubiquitin were quantified in gill and white muscle tissues. Exposure to hypersalinity significantly increased ubiquitin levels in both gill and white muscle tissues (Fig. 6). In gill, the magnitude of increase was the same in both species, more than doubling by the end of the hypersalinity exposure and remaining elevated into recovery (P<0.001; Fig. 6A). The ubiquitin response in white muscle (Fig. 6B) was species specific, as indicated by a significant species×time interaction (P=0.014). The gummy sharks (P<0.001) exhibited a 3.4-fold induction in muscle ubiquitin from control after spending 48 h in 120% SW (t=72 h). School sharks had higher constitutive levels of muscle ubiquitin compared with gummy sharks and a 1.7-fold increase in expression was observed in response to hypersaline exposure (P=0.025; Fig. 6B). A 21 h recovery period at 100% SW (t=96 h) was not sufficient for muscle ubiquitin levels to fully return to control levels in either species.

Based on these apparent increases in tissue protein damage, we would expect to see a corresponding induction of HSPs in the gill and white muscle of both species. In gill, gummy sharks significantly increased the expression of both HSP70 (P<0.001; Fig. 7A) and HSP110 (P=0.002; Fig. 7C) in response to hypersalinity. In contrast, we observed no statistically significant change in these HSPs (P=0.065, P=0.479) in school sharks. HSP90, in contrast, was similarly induced in both school and gummy sharks (P<0.001; Fig. 7B) with hypersaline exposure. In white muscle, hypersalinity significantly increased the expression of HSP70, HSP110 and HSP90 in both species. Gummy sharks dramatically increased muscle HSP70 (P<0.001; Fig. 7D), with the highest expression (a 5.6-fold increase from control) noted at recovery (t=96 h). School sharks had higher constitutive levels of white muscle HSP70 that only increased 1.3-fold with hypersalinity (P=0.027). White muscle HSP110 increased in both school and gummy sharks in response to hypersalinity (P<0.001; Fig. 7F) but the induction was more pronounced in school sharks (P=0.005). The induction of white muscle HSP90 in the two species (Fig. 7E) was distinct, as indicated by a significant species×time interaction (P<0.001). HSP90 levels increased significantly in school shark white muscle and remained elevated even at the end of recovery (t=96 h; P=0.001), whereas in gummy sharks, a significant induction of muscle HSP90 was observed at t=72 h (P=0.013) but levels began to decrease and were not significantly elevated from control at the end of the recovery period (P=0.084).

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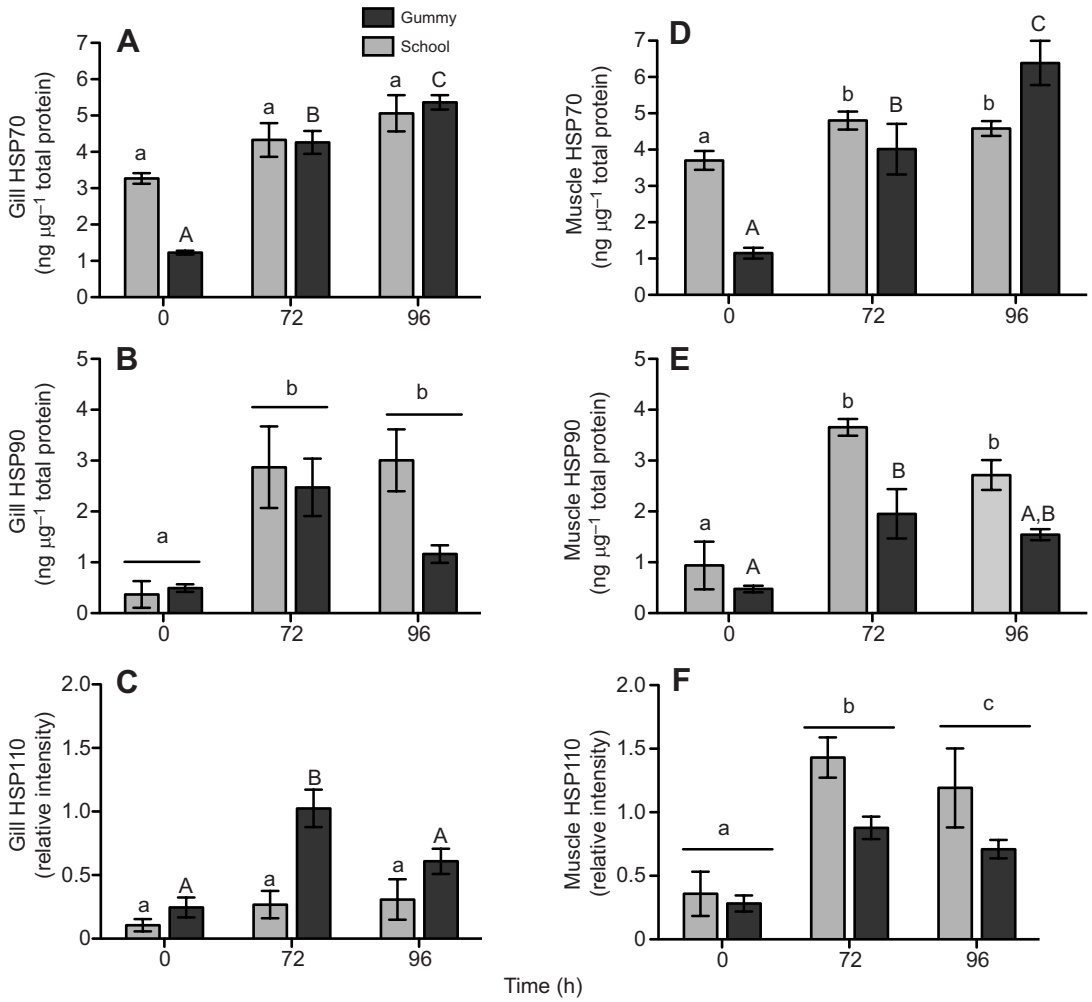

Fig. 7. Heat shock proteins (HSPs) in gummy sharks (*M. antarcticus*) and school sharks (*G. galeus*) exposed to a hypersaline event. Sample sizes and sampling procedure as outlined in Fig. 4 (means±s.e.m.). When a significant interaction was detected, data were split to analyse changes in each species separately over time. (A) Gill HSP70. Significant species×time interaction (2-way ANOVA, P=0.004). Different capital letters indicate a significant increase in gummy shark gill HSP70 over time (1-way ANOVA, P<0.000); identical lowercase letters indicate no significant change in school shark HSP70 (P=0.065). (B) Gill HSP90. Different lowercase letters show a significant increase in gill HSP90 over time in both species (2-way ANOVA, P<0.001). (C) Gill HSP110. Significant species×time interaction (2-way ANOVA, P=0.039). Different capital letters show significant changes in gummy shark gill HSP110 over time (1-way ANOVA, P=0.002); identical lowercase letters show a non-significant change in school shark HSP110 (P=0.479). (D) Muscle HSP70. Significant species×time interaction (2-way ANOVA, P<0.001). Different capital letters indicate a significant increase in gummy shark muscle HSP70 over time (1-way ANOVA, P<0.000); different lowercase letters show a significant increase in school shark HSP70 (P=0.027). (E) Muscle HSP90. Significant species×time interaction (2-way ANOVA, P<0.001). Different letters show significant changes in muscle HSP90 over time (capital letters, gummy sharks, P=0.014; lowercase letters, school sharks, P=0.001). (F) Muscle HSP110. Different letters indicate significant changes in muscle HSP110 over time in both species (2-way ANOVA, P<0.001); there was a significant difference between the two species (P=0.005).

DISCUSSION

Based on the different movement patterns of school and gummy sharks in the Pittwater nursery during the summer months when salinity is high (J. D. McAllister, A. Barnett, K. Abrantes and J.M.S., unpublished; Marine Culture Pty Ltd), we hypothesized that these species would have distinct responses to an environmentally relevant hypersaline exposure. We predicted that, compared with school sharks, gummy sharks would exhibit a greater degree of osmotic disruption and physiological stress in response to a hypersaline exposure. Indeed, gummy sharks showed plasma sodium dysregulation, a higher magnitude of HSP induction, and a failure to recover aerobic metabolic rate to control levels upon return to 100% SW, in contrast to school sharks exposed to the same conditions.

The metabolic response of fishes to increases in salinity has not been widely studied, particularly in elasmobranchs. In the euryhaline cownose ray (*Rhinoptera bonasus*), environmental salinity did not affect metabolic rate (Neer et al., 2006); however, we observed a 35% decrease in $\dot{M}_{O_2}$ in school sharks and a 20% decrease in gummy sharks. Thus, an acute, 48 h exposure to a salinity of 41‰ presents a metabolic challenge for both species. Despite the more dramatic decline in $\dot{M}_{O_2}$ in school sharks, they recovered their metabolic rate to control levels upon return to 100% SW, whereas the metabolic rate of gummy sharks remained 20% lower than control values into recovery. To minimize ion gain and water loss, school and gummy sharks may be decreasing gill perfusion, and thereby O_2 uptake, resulting in decreases in aerobic metabolic rate. This possibility is reminiscent of the osmorespiratory compromise well described in teleosts (Randall et al., 1972; Gonzalez and McDonald, 1992; Sardella and Brauner, 2007) but yet to be tested in elasmobranchs. It is also possible that the observed decrease in metabolic rate affects aerobic scope and

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influences aerobic activities, and thus performance. However, in the absence of maximum metabolic rate data this remains to be determined.

We observed a 53% increase in school shark whole-blood glucose at the onset of hypersalinity and a contrasting steady decline in that of gummy sharks. This divergent glycaemic reaction suggests a robust stress response in school sharks and depletion of an energetic substrate in gummy sharks. Increases in the catecholamine adrenaline may account for the elevated whole-blood glucose in school sharks. This has been demonstrated in nursehound (*Scyliorhinus stellaris*), spiny dogfish (deRoos and deRoos, 1978) and little skate (*Raja erinacea*; Grant et al., 1969), showing that elasmobranchs have a robust adrenergic stress response (Butler et al., 1978). Both school and gummy sharks increased Hb and Hct in response to hypersaline exposure (Table 2), suggesting haemoconcentration due to water efflux. A similar Hct response was observed in response to hypersalinity in *Scyliorhinus canicula* (Good et al., 2008). In our study, MCHC did not change, but school shark values were ∼5–16% higher than that of gummy sharks, possibly providing enhanced oxygen transport.

Overall, our data indicate that school sharks respond to hypersalinity more effectively than gummy sharks. In school sharks, despite the increased ionic diffusion gradient with high salt, plasma sodium and chloride increased by only a modest 20% and 16%, respectively, suggesting effective ionoregulation. These sharks filled the remaining osmotic gap with urea, as is typical in elasmobranchs (Hazon et al., 2003). In contrast, gummy sharks showed signs of ionic dysregulation with hypersalinity in that plasma Na^+ increased substantially, whereas increases in urea were relatively modest (∼12%) compared with those of school sharks. NKA activity was also different between the two species. This active transporter is critical for osmoregulation and in elasmobranchs it functions to transport sodium from gill cells into the blood. The decreased NKA activity we observed in school sharks suggests effective ionoregulation in a high-salt environment, minimizing accumulation of plasma electrolytes. Lowered NKA activity was also observed in Atlantic stingrays (*Dasyatis sabina*) acclimated to increased salinity (Piermarini and Evans, 2000). However, in gummy sharks, where plasma electrolytes increased substantially, NKA activity, although constitutively low, did not change with high-salinity exposure. Perhaps 41‰ is beyond the osmoregulatory and ionoregulatory capacity of gummy sharks, at least as juveniles. Our study did not consider rectal gland contributions to ionoregulation. However, rectal gland NKA activity made a negligible contribution to ionoregulation in the Pacific spiny dogfish (*S. suckleyi*) exposed to hypersaline conditions (Deck et al., 2016).

We observed significant increases in plasma and gill TMAO in both species, and no change in white muscle TMAO concentration. By way of comparison, both bonnethead and bull sharks increased plasma TMAO in response to increases in environmental salinity (Mandrup-Poulsen, 1981; Pillans et al., 2005, 2006). Unfed North Pacific dogfish exposed to 130% hypersalinity for 24 h only increased TMAO in the liver and not in plasma or white muscle (Deck et al., 2016). Overall, our results indicate that TMAO does not appear to play a major role in the osmoconformation strategy of either species in response to hypersalinity. However, if school and gummy sharks obtain the majority of TMAO from their diets as in some other elasmobranchs (Goldstein et al., 1967; Treberg et al., 2006), fasting throughout experimentation may explain the only moderate increases in TMAO we observed.

We predicted that exposure to high salinity would damage elasmobranch proteins and the significant increases observed in gill and white muscle ubiquitin generally support this prediction. Given that damaged proteins are a putative trigger for induction of the heat shock response (Ananthan et al., 1986), we further predicted an induction of HSPs with hypersalinity. We have previously described HSP70 induction in spiny dogfish sharks with low salinity (MacLellan et al., 2015), and there is support in the literature for high salt concentrations initiating an increase in *hsp70* mRNA in mammalian kidney cells (Sheikh-Hamad et al., 1998), and in wild populations of black-chinned tilapia (*Sarotherodon melanotheron*; Tine et al., 2010). Yet, prior to this study, the cellular response to hypersalinity stress in elasmobranchs had not been characterized. We observed an induction of several families of HSPs (i.e. HSP70, HSP90, HSP110) with hypersalinity in both shark species, suggesting important roles for these molecular chaperones in mitigating osmotic cellular stress and damage. Notably, this is the first study to investigate HSP90 and HSP110 in any elasmobranch species. Like the HSP70 family, HSP90 and HSP110 are ATP-dependent proteins important for the refolding and degradation of stress-denatured proteins (Sontag et al., 2014) but their precise function in fishes has been little studied.

Considering the more pronounced physiological response to high-salinity exposure in gummy sharks compared with school sharks, we expected that gummy sharks would show enhanced signs of protein damage and thus HSP induction. In contrast to our prediction, the two shark species showed similar signs of protein damage (i.e. increases in ubiquitin) in gill and muscle. Despite these consistent indications of protein damage, the heat shock response was different in the two species and these differences were related to tissue type, making it difficult to conclude which species relies more heavily on HSP induction for protection. Gummy sharks significantly induced gill and muscle HSP70, 90 and 110 under hypersaline conditions whereas school sharks only induced HSP90 in gill, but all three HSPs in white muscle. Moreover, gummy sharks had an overall higher magnitude heat shock response in both gill and muscle for HSP70, but the increases in HSP90 and HSP110 were of greater magnitude in school shark muscle. Given this, one might conclude that gummy sharks have a greater heat shock response, but this argument is tenuous. It is also important to note that the school sharks were held in the lab for a few days longer than the gummy sharks. Thus, we cannot rule out other possible factors associated with length of time in the aquaria influencing our data. What is clear is that there are distinct heat shock responses between the two species despite similar signs of protein damage. This discrepancy suggests unique cellular stress signalling responses from each species in response to a common signal: protein damage. Indeed, an increase in abnormal proteins may be the putative trigger for the heat shock response (Ananthan et al., 1986); however, despite decades of research, direct demonstration of the effect of misfolded or damaged proteins on HSP induction has not been shown in eukaryotes (Wolff et al., 2014).

Shark nurseries are often subject to substantial environmental fluctuations. The observation of distinct nursery usage patterns between these two species, coincident with prevailing environmental conditions, provided an ideal model to test whether physiological tolerances could explain ecological pattern differences. Our data generally support our hypothesis and predictions indicating similar signs of cellular stress following high-salinity exposure in the two species but more negative physiological effects in gummy sharks. This offers at least one explanation for their avoidance of hypersaline environmental conditions. Despite living in similar environments as juveniles, and their close phylogenetic relationship, gummy and school sharks

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have divergent physiological and cellular responses to ecologically relevant osmotic perturbations. Our study connects the physiology of these two species to their spatial use of critical nursery grounds, environments that are increasingly susceptible to more dramatic environmental fluctuations.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Study conception and design were by S.C., A.J.M. and J.M.S. S.C., A.J.M., K.M.S. and J.M.S. conducted experiments; L.T., S.R.C.M., D.A.B., S.C. and A.J.M. analysed the data; L.T. and S.R.C.M. performed statistical analyses. All authors contributed to manuscript preparation and approved the final submission.

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Supplementary information
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