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The stress physiology of extended duration tonic immobility in the juvenile lemon shark, *Negaprion brevirostris* (Poey 1868)

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ABSTRACT

Tonic immobility (TI) is a reversible coma-like stasis inherent to a variety of terrestrial and aquatic taxa, including elasmobranchs, yet virtually nothing is known about its underlying neurological and physiological processes in any taxa. The purpose of this research was to investigate the physiological effects of TI on the juvenile lemon shark (*Negaprion brevirostris*). Eight juvenile lemon sharks were subjected to four, three-hour treatments during which blood was sampled at 0, 30, 90 and 180 min, over a 6 week period. Treatments were differentiated by the method of maintaining the shark, either in TI, or allowed to swim freely between blood samples and the presence or absence of a pre-treatment exercise period designed to simulate the capture induced exhaustion that usually precedes the use TI in the field. The results suggest that TI is an inherently stressful experience, which magnifies the degree of perturbation observed in a number of blood chemistry parameters. It is thought that TI induced a short term reduction in ventilatory efficiency, which appeared to be countered by a series of compensatory mechanisms that include increased ventilation rates, and maintenance of the primary stress response. TI remains one of the most enigmatic areas of biology for all taxa and further research into its underlying psychological, physiological and neurological processes is recommended.

1. Introduction

Tonic immobility (TI) is an unlearned, reversible, coma-like stasis displayed by a large number of taxa (Gallup, 1974). In general, TI is thought to be the final stage of a 'defensive cascade' of behaviours initiated in response to the presence of a predator (Ratner, 1967). This cascade, which begins with a period of voluntary immobility intended to decrease the probability of detection and heighten responsiveness, then transitions through the 'flight or fight' response, and if escape is unsuccessful resulting in capture and restraint (i.e. by a predator), terminates with the onset of TI (Marx et al., 2008). TI is characterised by a catatonic motionless posture and a profound but reversible physical immobility which, in terrestrial vertebrates, is caused by muscle rigidity and unresponsiveness to painful stimulation (Marx et al., 2008; Ratner, 1967).

Nearly all research into TI has focused on terrestrial vertebrates such as lizards (e.g. Edson and Gallup, 1972), chickens (e.g. Gallup et al., 1976), guinea pigs (e.g. Bis Vieira et al., 2011) and humans (e.g. Marx et al., 2008). However, this phenomenon is also exhibited by a large number of elasmobranchs (Henningsen, 1994; Watsky and Gruber, 1990; Whitman et al., 1986). Like many terrestrial vertebrates, TI in sharks is characterised by a state of immobility (Henningsen, 1994; Watsky and Gruber, 1990), yet in contrast to their terrestrial counterparts, sharks exhibit relaxed muscle tone (the "limp" response; Whitman et al., 1986). In addition, for species with the ability to self ventilate via buccal pumping, individuals in TI exhibit deep rhythmical ventilations (Watsky and Gruber, 1990). In sharks, TI is typically induced by rapid dorsoventral inversion (Watsky and Gruber, 1990; Whitman et al., 1986) and its onset is relatively rapid (<1 min—Henningsen, 1994; Whitman et al., 1986), lasting for less than a minute to several hours in unrestrained individuals (Henningsen, 1994; Watsky and Gruber, 1990).

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TI is commonly used to safely restrain and handle sharks following capture for both scientific research (e.g. Brooks et al., 2011b; Holland et al., 1999; Murchie et al., 2009) and aquarium husbandry (e.g. Gruber, 1980; Henningsen, 1994). However, at present, the effects of TI on the physiological homeostasis of elasmobranchs is unknown, especially when coupled with the exhaustive anaerobic exercise and acute physiological disruption associated with most elasmobranch capture events (e.g. Mandelman and Skomal, 2009; Skomal, 2007). The little research that has been conducted to date suggests that TI is relatively benign, given the limp muscle tone and deep rhythmical ventilations exhibited (Watsky and Gruber, 1990). In addition, heart rate and blood pressure have been found to remain stable in blacktip reef sharks (*Carcharhinus melanopterus*) maintained in TI and provided with branchial irrigation (Davie et al., 1993).

The purpose of this project was to investigate the physiological and behavioural effects of extended duration tonic immobility in juvenile lemon sharks. Lemon sharks are members of the largest of the shark families, the carcharhinids, and are widely distributed throughout the tropical and sub-tropical western Atlantic and Caribbean (Compagno, 1984). Juvenile lemon sharks are easily captured and maintained in captivity (Dallas et al., 2010; Gruber, 1980), and are commonly found in the mangrove creeks surrounding Cape Eleuthera, making it an ideal subject animal for this study. To the authors' knowledge, this study represents the first investigation into the physiological effects of TI in any species to date.

2. Methods

This study was conducted between June 9th and October 1st 2009, at the Cape Eleuthera Institute (CEI), Eleuthera, The Bahamas (24.54° N 76.12° W). All research was carried out under research permits MAF/FIS/17 and MAF/FIS/34 issued by the Bahamian Department of Marine Resources and in accordance with CEI animal care protocols developed within the guidelines of the Association for the Study of Animal Behaviour and the Animal Behaviour Society (Rollin and Kessel, 1998).

2.1. Animal collection, transport and husbandry

Juvenile lemon sharks were collected from local mangrove creeks using conventional hook-and-line angling gear that consisted of a standard spinning rod, a steel leader and a 9/0 circle hook. Upon capture, lemon sharks were transferred to a 200 l cooler of seawater, the hook was removed, the total length (cm) measured and the sex identified. The cooler was transferred to a boat for the journey back to the CEI laboratory, typically taking between 8 and 16 min. To ensure adequate oxygenation during transport, ~50% of the water in the cooler was exchanged with fresh seawater every 5 min during the journey. Upon arrival at the laboratory, sharks were housed individually in 13,000 l (3.7 m diameter × 1.25 m depth) circular tanks continuously supplied with fresh seawater at a rate of approximately 120 l h^{-1} (Dallas et al., 2010). Individual sharks remained in captivity for 4–6 weeks during the experimental period prior to being released back into the creek from which they were caught. Sharks were offered food daily in the form of chunks of bonito tuna (*Euthynnus alletteratus*) or Spanish sardines (*Sardinella aurita*) (Gruber, 1980). Dissolved oxygen, temperature and salinity were measured twice daily with a YSI 85 oxygen, salinity and temperature probe (YSI Inc, Yellow Springs, Ohio, USA). Between June and October 2009, four male and four female juvenile lemon sharks (x Total Length = 679 mm, ±21.8 S.E.) were captured and maintained according to these protocols. During the course of the experiments the ambient water temperature ranged from 19.2 to 37.1 °C (x = 25.8, ±0.15 SE), dissolved oxygen ranged from 5.12 to 9.46 mg l^{-1} (x = 6.58, ±0.02 SE) and salinity 34.1–39.8 ppt (x = 36.6, ±0.19 SE).

2.2. Experimental design

Juvenile lemon sharks were subjected to a series of four treatments with a minimum rest period of four days between trials. In all cases the subject animal resumed feeding the same day, or the day following a trial. Prior to a trial the water level in the tank was lowered to a depth of approximately 60 cm to facilitate the capture and handling of the animals. During previous laboratory experiments it was observed that shark swimming speed was temporarily elevated immediately following a change in water level; as such, a 24 h acclimation period was established between the lowering of the water level and the trial. All eight sharks were subjected to four treatments in a random order and each shark was subjected to an individual treatment only once.

During all treatments, blood samples were taken 0, 30, 90 and 180 min from the commencement of the trial. Treatments were differentiated by the method of maintaining the subject animal during the course of the three hours, either held in TI by a field assistant, or allowed to swim freely between blood samples, both coupled with the presence or absence of an initial exercise period. Exhaustive exercise, in the form of three minutes of chasing and tail grabbing, was incorporated into the study design to simulate the physiological stress that typically precedes the use of TI. Chasing and tail grabbing have been shown to produce physiological responses similar to those imposed by angling (Kieffer, 2000; Suski et al., 2006, 2007; Wood, 1991). The four treatments consisted of all possible interactions of these two variables, consisting of two treatments whereby a subject animal was maintained in TI for three hours, one of which had an initial exercise period and one which did not, and two treatments where the subject animal was allowed to swim freely between blood samples, one of which had an initial exercise period and one which did not. Across all treatments, sharks were netted in under 25 s (x = 11.6, ± 2.3 SE), inverted until the onset of tonic immobility, which took less than 100 s (x = 60.2, ±4.8 SE), and immediately blood sampled. The time elapsed from netting to blood draw was typically less than 120 s (x = 104.7, ±7.4 SE).

2.3. Behavioural observations

Lemon sharks supplement ram ventilation by buccal pumping, which allows them to remain stationary on the sea bed for long periods of time (Kessel et al., 2009). Depending on the treatment, one of two behaviours important for respiratory regulation was quantified. For treatments that required the maintenance of animals in TI for an extended period, preventing the use of ram ventilation, ventilation rates were determined by counting the number of contractions of the buccal chamber in one minute (v m^{-1}; Barreto and Volpato, 2004; Chapman et al., 2010; Shultz et al., 2011). Ventilation rates were measured six times over the course of a 15 min observation period immediately after blood sampling at 0, 30, 90 and 180 min. Swimming speed, which is an important behaviour for respiratory regulation in ram ventilating sharks (Parsons and Carlson, 1998), was quantified for treatments where the subject animal was allowed to swim freely between blood samples. Relative swimming speed was determined by counting the number of tail beat cycles per minute (tbc m^{-1}), defined by the complete movement of the tail through the cycle returning to the start position (Graham et al., 1990). This measure was not designed to give absolute swimming speeds, but rather to identify relative changes in swimming speed both within and between treatments. Relative swimming speed was quantified on a similar schedule to ventilation rates, measured six times over the course of a 15 min observation period immediately after blood sampling at 0, 30, 90 and 180 min. Control values for ventilation rates and tail beat frequencies were taken during the 4–6 day rest periods between treatments, but not within 24 h of the completion of a treatment. Sharks were observed for a minimum of five, 15 min observation periods during which either ventilation rates, or tail beat frequencies

were quantified every third minute depending on the behaviour of the shark at the time. Behavioural observations were only conducted on four of the eight sharks (M = 2, F = 2; x Total Length = 680 mm, ±21.7 S.E.).

2.4. Blood sampling and analyses

Blood (~1 ml) was drawn by caudal venipuncture using a 38 mm, 20 gauge needle and a 3 ml syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). All syringes were washed with the anticoagulant sodium heparin prior to drawing blood. A portion of the blood sample was immediately transferred from the syringe to a 0.5 ml microcentrifuge vial. From this vial, approximately 100 μl of whole blood was immediately transferred from the syringe into an iStat point of care device (Heska Corporation, Fort Collins, CO, USA) for immediate analysis of pCO_2, pH, and lactate (Brooks et al., 2011a; Gallagher et al., 2010; Mandelman and Farrington, 2007; Mandelman and Skomal, 2009). The time from blood draw to the insertion of the cartridge into the iStat was <4 min. To confirm there was no significant change in blood chemistry parameters during this period a series of six samples were run in duplicate, one using the standard protocol and one using blood transferred directly from the syringe to the iStat cartridge <1 min post blood draw. A pooled t-test showed no significant differences between the standard protocols and the immediate analysis of the blood for pH ($p = 0.885$), pCO_2 ($p = 0.759$), HCO_3 ($p = 0.676$) and lactate ($p = 0.964$). Glucose was measured by adding 10 μl of whole blood to an Accu-Chek glucose metre (Roche Diagnostics, Basel, Switzerland) which has previously been validated for use on fish (Cooke et al., 2008). The balance of the sample was transferred to a 3 ml vacutainer containing lithium heparin (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and spun in a centrifuge (Clay Adams Compact II Centrifuge) for 5 min at 10,000 g to separate plasma from red blood cells. Plasma was transferred to a microcentrifuge tube using a pipette and stored at −20 °C.

Plasma samples were subsequently transported in liquid nitrogen to a laboratory at the University of Illinois where they were stored in an ultra-cold freezer (−80 °C) prior to analysis of ions and urea concentrations. Plasma sodium and potassium levels were quantified using a flame photometer (Cole-Parmer Single-Channel Digital Flame Photometer, model WU-02655-00, Vernon Hills, IL, USA). Plasma urea, chloride, magnesium, and calcium levels were quantified using commercially available kits (BioAssay Systems, Hayward, CA, USA—Urea DIUR-500, Chloride DICL-250, Magnesium DIMG-250; Calcium DICA-500).

2.5. Conversions

The iStat point of care device is designed for use on endothermic animals, and, as such, is thermostated to 37 °C. To accurately quantify in vivo blood gas and pH values for exothermic species, it is necessary to correct for temperature (Reeves, 1977). Each pH and pCO_2 value generated was therefore adjusted using in tank seawater temperature taken at the beginning of each treatment (Brooks et al., 2011a; Gallagher et al., 2010; Mandelman and Skomal, 2009). Recent research has suggested that species-specific conversions are required to generate absolute blood gas and pH values from raw iStat data (Gallagher et al., 2010). However, given the present lack of conversion values specifically for the lemon shark, established protocols described by Mandelman and Skomal (2009) were used, which provide relative differences in pH and pCO_2 whilst taking into account variation in water temperature over the course of the study. Bicarbonate (HCO_3^-) was calculated via the Henderson–Hasselbalch equation using temperature corrected pCO_2 and pH values (Brooks et al., 2011a; Mandelman and Skomal, 2009).

2.6. Data analysis

All analyses were performed using JMP 7.0.1 (SAS Institute, Cary, NC, USA) and the level of significance (α) for all tests was 0.05. Data were analysed using a repeated measures multivariate analysis of variance (RM-MANOVA) (O'Brien and Kaiser, 1985), which incorporated the presence and absence of TI (TI/NoTI) and exercise (Ex/NoEx) and the interaction between the two (TI/NoTI * Ex/NoEx) as fixed effects. In addition, individual sharks were incorporated into models as fixed effects to account for individual variation both within and between treatments. Where RM-MANOVAs indicated significance between treatment groups, post-hoc pooled t-tests were used to compare means at specific sampling points. The threshold of significance (α) for the post-hoc pooled t-tests was not subjected to Bonferroni corrections when performing multiple comparisons. The use of Bonferroni corrections has been strongly contested as it reduces the probability of Type I error at the cost of inflating the probability of the equally deleterious Type II error (Nakagawa, 2004; Perneger, 1998; Rothman, 1990).

3. Results

Independent of each other, both TI (TI/NoTI) and exhaustive exercise (Ex/NoEx) had significant effects on a number of blood parameters, as did individual variation between subject animals. However, the interaction between TI and exercise (TI/NoTI * Ex/NoEx) had no statistical effect on any blood chemistry parameter and are not discussed further. Furthermore, given the lack of a combined effect, the effects of TI and exercise are presented separately to facilitate visualisation of the data.

Across all treatments, lemon sharks responded to the stress of capture and restraint with significant disruption to their physiological homeostasis over time (Tables 1 and 2). Whole blood pH was significantly depressed at the 30 min sampling point but recovered to a less perturbed state through the 90 and 180 min sampling points (Figs. 1a and 3a). Carbon dioxide followed a pattern directly inverse to that of pH, increasing between the 0 and 30 min, but became significantly depressed after 180 min (Figs. 1b and 3b). There was a significant increase in lactate values through the 0, 30 and 90 min sampling points, however there was no significant rise between 90 and 180 min (Figs. 1c and 3c). Variation in bicarbonate concentration mirrored that of pH, becoming significantly depressed at the 30 min sampling point when compared to both the 0 and 180 min sampling points (Figs. 1d and 3d). Glucose concentrations were significantly higher at the 30, 90 and 180 min sampling point compared to the 0 min sampling point (Figs. 1e and 3e). Plasma magnesium (Figs. 2a and 4a) and sodium (Figs. 2b and 4b) became significantly elevated by the 180 min sampling point compared to the 0 min point, and there was a significant decrease in plasma potassium concentrations across sampling points (Figs. 2c and 4c). Plasma chloride, calcium and urea did not vary across sampling points (Figs. 2d–f and 4d–f).

3.1. The effects of tonic immobility

Tonic immobility significantly affected a number of blood chemistry parameters (Tables 1 and 2). The magnitude of the blood acidosis observed in animals maintained in TI between the 0 and 30 min sampling points was double that for those sharks that were allowed to swim freely between blood samples (x Δ pH TI = 0.22; x Δ pH NoTI = 0.11) (Fig. 1a). This acidosis was mirrored by a significant increase in carbon dioxide at the 30 min sampling point for animals maintained in TI, mean values of which were over double those of animals allowed to swim freely between blood samples (Fig. 1b). Neither lactate nor bicarbonate concentrations were significantly affected by tonic immobility despite significant perturbation following capture and restraint (Fig. 1c–d). Animals maintained in TI were significantly hyperglycemic compared to those that were not (Fig. 1e). Furthermore, glucose concentrations became significantly elevated between the 0 and 90 min sampling points for animals maintained in TI, however, there was no significant variation across sampling points for animals allowed to swim freely between blood samples (Fig. 1e). Animals maintained in TI presented significantly greater disruption to their electrolyte

Table 1
RM-MANOVA results for effects of tonic immobility and exercise on whole blood chemistry in the juvenile lemon shark (*Negaprion brevirostris*). Bold font indicates significance.

Factor	pH	Carbon dioxide	Lactate	Bicarbonate	Glucose
Time	**$p = <0.001$, $F_{3,16} = 60.03$**	**$p = <0.001$, $F_{3,18} = 37.33$**	**$p = <0.001$, $F_{3,17} = 88.89$**	**$p = <0.001$, $F_{3,18} = 41.63$**	**$p = <0.001$, $F_{3,17} = 27.86$**
TI/NoTI	**$p = 0.018$, $F_{1,18} = 6.75$**	$p = 0.124$, $F_{1,20} = 2.58$	$p = 0.389$, $F_{1,19} = 1.09$	$p = 0.353$, $F_{1,20} = 0.9$	**$p = 0.011$, $F_{1,19} = 8.05$**
TI/NoTI * Time	$p = 0.091$, $F_{3,16} = 2.57$	**$p = 0.049$, $F_{3,18} = 3.12$**	$p = 0.634$, $F_{3,17} = 0.58$	$p = 0.238$, $F_{3,18} = 1.54$	**$p = 0.027$, $F_{3,17} = 3.91$**
Ex/NoEx	**$p = <0.001$, $F_{1,18} = 19.76$**	**$p = 0.024$, $F_{1,20} = 5.99$**	**$p = 0.003$, $F_{1,19} = 11.76$**	**$p = 0.002$, $F_{1,20} = 12.08$**	$p = 0.866$, $F_{1,19} = 0.03$
Ex/NoEx * Time	$p = 0.071$, $F_{3,16} = 2.8337$	$p = 0.222$, $F_{3,18} = 1.61$	**$p = 0.036$, $F_{3,17} = 3.89$**	**$p = 0.006$, $F_{3,18} = 5.85$**	$p = 0.569$, $F_{3,17} = 0.69$
TI/NoTI * Ex/NoEx	$p = 0.117$, $F_{1,18} = 2.71$	$p = 0.101$, $F_{1,20} = 2.96$	$p = 0.559$, $F_{1,19} = 0.354$	$p = 0.858$, $F_{1,20} = 0.03$	$p = 0.489$, $F_{1,19} = 0.49$
TI/NoTI * Ex/NoEx * Time	$p = 0.754$, $F_{3,16} = 0.4$	$p = 0.591$, $F_{3,18} = 0.65$	$p = 0.205$, $F_{3,17} = 1.69$	$p = 0.451$, $F_{3,18} = 0.92$	$p = 0.053$, $F_{3,17} = 0.312$
Individual sharks	**$p = 0.026$, $F_{7,18} = 3.07$**	**$p = 0.026$, $F_{7,20} = 2.98$**	**$p = 0.021$, $F_{7,19} = 3.2$**	**$p = 0.032$, $F_{7,20} = 2.84$**	**$p = <0.001$, $F_{7,19} = 6.54$**

balance compared to those allowed to swim freely between blood samples. Plasma magnesium, sodium and calcium were significantly elevated, and plasma potassium significantly depressed for animals maintained in TI (Fig. 2 a–d). The time scales over which these perturbations were presented varied, with magnesium becoming maximally elevated at the 30 min sampling point (Fig. 2a), in contrast to plasma sodium and calcium, which did not reach maximum perturbation until the 180 min sampling point (Fig. 2b and 2d). The presence or absence of TI had no significant effect on plasma chloride or urea.

3.2. The effects of exhaustive exercise

Exhaustive exercise increased the magnitude of homeostatic disruption in a number of blood chemistry parameters (Tables 1 and 2). Whole blood pH was significantly more depressed in animals subjected to exhaustive exercise when, compared to those that were not (Fig. 3a). This acidosis was accompanied by significantly elevated levels of carbon dioxide (Fig. 3b), lactate (Fig. 3c), and significantly depressed levels of bicarbonate (Fig. 3d) in exercised animals. Exhaustive exercise did not affect the hyperglycemic response (Fig. 3e), nor did it have any significant effect on any blood plasma constituents (Fig. 4a–f).

3.3. Behavioural observations

For treatments involving extended durations of TI, ventilation rates were significantly elevated when compared to control values at all sampling points for both treatments, and increased significantly from the 0 and 30 min sampling points through to the 180 min sampling point (Table 3, Fig. 5a). Exhaustive exercise caused a small but significant increase in ventilation rates (Fig. 5a). For treatments not involving extended durations of TI, tail-beat-cycles were significantly elevated when compared to control values at all sampling points, particularly at the 0 min sampling point (Table 2, Fig. 5b). Exhaustive exercise had no significant effect on tail-beat-cycles, however, there was a significant interaction between exercise and sampling point; further analysis indicated fewer tail-beat-cycles at the 30 min sampling point in animals subjected to exhaustive exercise (Fig. 5b).

3.4. Individual variation in the stress response

Lemon sharks exhibited considerable individual variation in the stress response (Tables 1–3). Across all treatments, significant differences in blood chemistry between individual sharks were established for ten of the eleven parameters analysed including whole blood pH, carbon dioxide, lactate, bicarbonate, glucose, magnesium, sodium, chloride, calcium and urea. There was no significant variation in plasma potassium between sharks. Furthermore, there was significant variation in both ventilation rates, and tail beat cycles between individual sharks.

4. Discussion

The purpose of this study was to investigate the physiological and behavioural effects of TI on juvenile lemon sharks. The results suggest that TI is an inherently stressful experience, which magnifies the degree of perturbation observed in a number of blood chemistry parameters.

Tonic immobility appears to disrupt the short term ventilation efficiency of juvenile lemon sharks as indicated by significant elevation of carbon dioxide at the 30 min sampling point, which is the likely cause of the concomitant drop in blood pH at the same point (Mandelman and Skomal, 2009). This is potentially due to the restriction of lemon sharks to the apparently less efficient ventilation method of buccal pumping, in contrast to the ram ventilation conducted during free swimming treatments. By the end of both TI and non-TI treatments all animals had reduced carbon dioxide concentrations to a similar point suggesting that, in response to this reduction in ventilatory efficiency, lemon sharks initiated a number of compensatory mechanisms that successfully improved gas exchange. One such compensatory mechanism is suggested by the significant increase in ventilation rates compared to control values observed during TI treatments. Increases in ventilation rates are known to increase the capacity for gas exchange by increasing the volume of water passing over the gill lamellae (Butler and Metcalfe, 1989; Carlson and Parsons, 2003; Hawkins et al., 2004). Furthermore, it is postulated that physical adaptations to the gills and circulatory system typically associated with the primary and secondary stress response (McDonald and Milligan, 1997), were sustained during TI, which, in combination with elevated ventilation rates, facilitated the decline of carbon dioxide concentrations.

The primary stress response is characterised by a hormonal cascade of catecholamines and corticosteroids which in turn trigger a number of physiological and physical adaptations designed to promote the capacity for 'flight or fight', and promote survivorship in the short term (Busch and Hayward, 2009; Romero, 2004; Skomal and Bernal, 2010). Some of these adaptations (e.g. the recruitment of additional gill lamellae and the

Table 2
RM-MANOVA results for effects of tonic immobility and exercise on blood plasma ions and urea in the juvenile lemon shark (*Negaprion brevirostris*). Bold font indicates significance.

Factor	Plasma magnesium	Plasma sodium	Plasma potassium	Plasma calcium	Plasma chloride	Plasma urea
Time	**$p = <0.001$, $F_{3,18} = 16.98$**	**$p = 0.045$, $F_{3,16} = 3.37$**	**$p = 0.048$, $F_{3,17} = 3.25$**	$p = 0.226$, $F_{3,14} = 1.61$	$p = 0.226$, $F_{3,16} = 1.61$	$p = 0.099$, $F_{3,18} = 2.42$
TI/NoTI	**$p = 0.004$, $F_{1,20} = 10.37$**	**$p = 0.01$, $F_{1,18} = 8.24$**	**$p = 0.010$, $F_{1,19} = 8.09$**	**$p = 0.036$, $F_{1,16} = 5.25$**	$p = 0.406$, $F_{1,18} = 0.72$	$p = 0.132$, $F_{1,20} = 2.47$
TI/NoTI * Time	$p = 0.247$, $F_{3,18} = 2.15$	**$p = 0.025$, $F_{3,16} = 4.07$**	$p = 0.515$, $F_{3,17} = 0.79$	$p = 0.383$, $F_{3,14} = 1.09$	$p = 0.383$, $F_{3,16} = 1.09$	$p = 0.636$, $F_{3,18} = 0.58$
Ex/NoEx	$p = 0.888$, $F_{1,20} = 0.02$	$p = 0.226$, $F_{1,18} = 1.57$	$p = 0.312$, $F_{1,19} = 1.08$	$p = 0.824$, $F_{1,16} = 0.05$	$p = 0.505$, $F_{1,18} = 0.46$	$p = 0.483$, $F_{1,20} = 0.51$
Ex/NoEx * Time	$p = 0.686$, $F_{3,18} = 0.5$	$p = 0.457$, $F_{3,16} = 0.91$	$p = 0.878$, $F_{3,17} = 0.22$	$p = 0.532$, $F_{3,14} = 0.76$	$p = 0.769$, $F_{3,16} = 0.379$	$p = 0.91$, $F_{3,18} = 0.18$
TI/NoTI * Ex/NoEx	$p = 0.381$, $F_{1,20} = 0.8$	$p = 0.861$, $F_{1,18} = 0.03$	$p = 0.697$, $F_{1,19} = 0.16$	$p = 0.364$, $F_{1,16} = 0.88$	$p = 0.371$, $F_{1,18} = 0.84$	$p = 0.837$, $F_{1,20} = 0.04$
TI/NoTI * Ex/NoEx * Time	$p = 0.129$, $F_{3,18} = 2.15$	$p = 0.245$, $F_{3,16} = 1.53$	$p = 0.930$, $F_{3,17} = 0.15$	$p = 0.519$, $F_{3,14} = 0.79$	$p = 0.769$, $F_{3,16} = 0.38$	$p = 0.486$, $F_{3,18} = 0.85$
Individual sharks	**$p = <0.001$, $F_{7,20} = 6.13$**	**$p = <0.001$, $F_{7,18} = 10.89$**	$p = 0.109$, $F_{7,19} = 1.2$	**$p = <0.001$, $F_{7,16} = 6.83$**	**$p = 0.025$, $F_{7,18} = 3.09$**	**$p = 0.002$, $F_{7,20} = 5.27$**

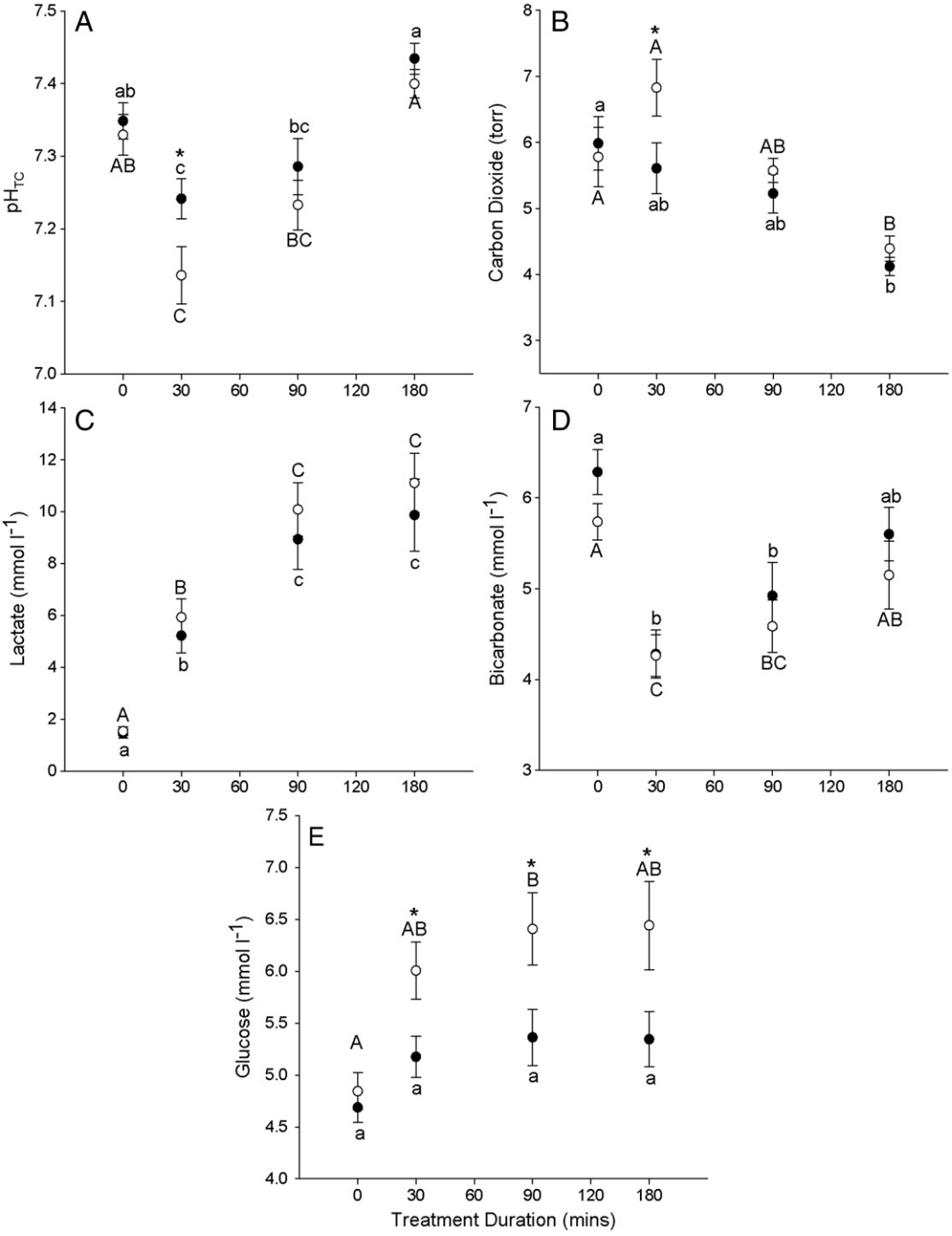

Fig. 1. Variation in blood gas and metabolite concentrations of juvenile lemon sharks (*Negaprion brevirostris*) in response to capture and restraint. Filled points (●) represent animals allowed to free swim between blood samples and clear points (○) represent animals maintained in tonic immobility. Data represent mean blood chemistry values of whole blood (A) pH_{TC}, (B) carbon dioxide, (C) lactate, (D) bicarbonate and (E) glucose (±1 SE). Asterisks (*) indicate a statistically significant difference between TI and non-TI treatments at a given sampling point (Pooled *t*-test, $\alpha = <0.05$). Dissimilar capital letters indicate statistically significant differences between sampling points for treatments involving extended TI, and dissimilar small letters for those treatments that did not involve TI (ANOVA, $\alpha = <0.05$).

vasodilatation of branchial blood vessels) are designed to promote gill perfusion and the capacity for gas exchange (Randall, 1982; Skomal and Bernal, 2010); however, these adaptations also increase the permeability of the gills to ions, resulting in disruption to the electrolyte balance of the blood (Gonzalez and McDonald, 1992; Randall, 1982). Unlike marine teleosts, marine elasmobranchs maintain themselves hyper-osmotic to their surrounding environment by retaining nitrogenous organic compounds such as urea and trimethylamine oxide (Hazon et al., 2003; Pang et al., 1977). The balance of osmolarity is derived from inorganic ions maintained at concentrations below that of the surrounding water, resulting in a continuous diffusion of ions across the gills into the blood, which is balanced by salt excretion from the rectal gland and kidneys (Shuttleworth, 1988). Lemon sharks maintained in TI presented significantly greater perturbation to their electrolyte balance than those allowed to swim freely between blood samples, suggesting that the increased permeability of the gill epithelium, and the hormonal

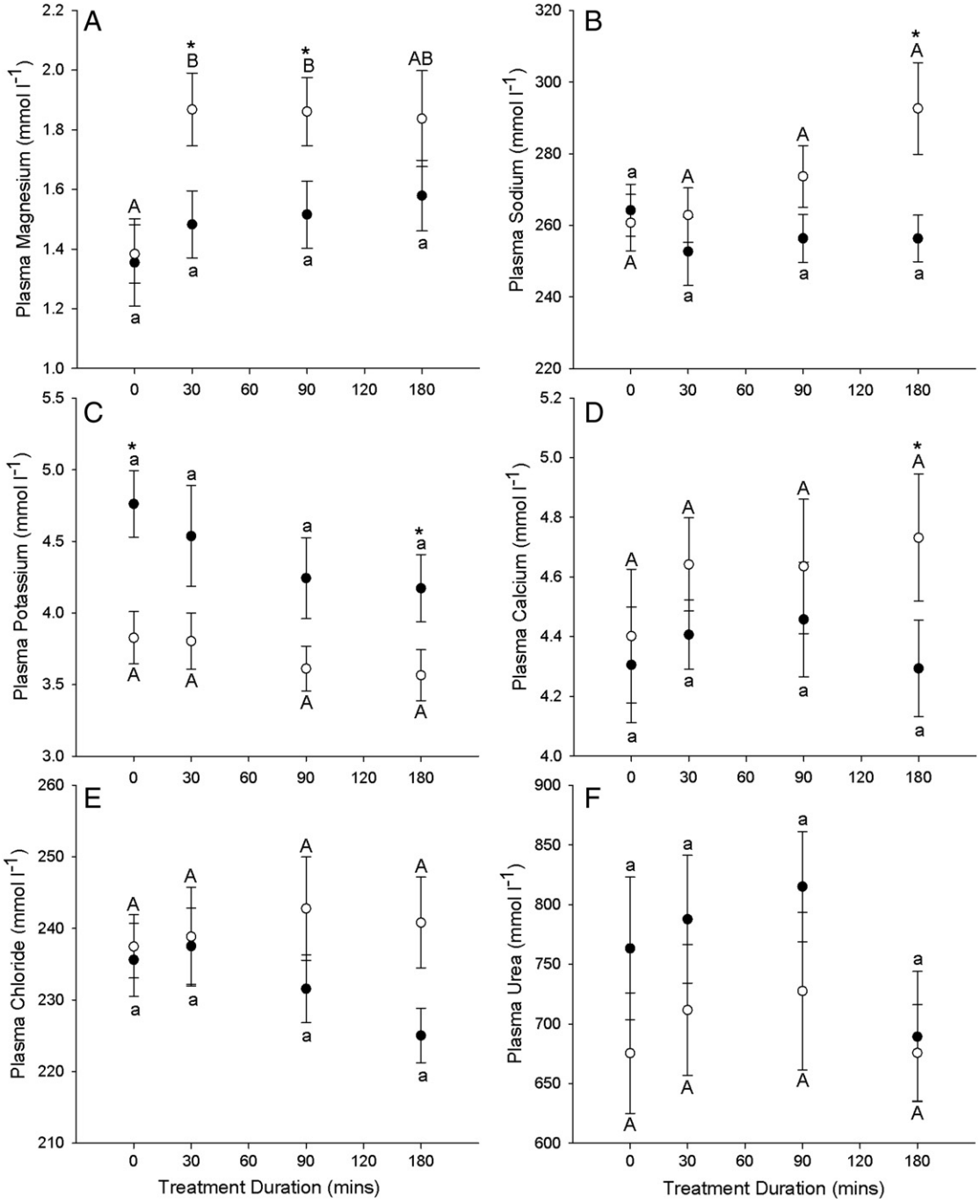

Fig. 2. Variation in plasma ion and urea concentrations of juvenile lemon sharks (*Negaprion brevirostris*) in response to capture and restraint. Filled points (●) represent animals allowed to free swim between blood samples and clear points (○) represent animals maintained in tonic immobility. Data represent mean blood chemistry values of whole blood (A) magnesium, (B) sodium, (C) potassium, (D) chloride, (E) calcium and (F) urea (±1 SE). Asterisks (*) indicate a statistically significant difference between TI and non-TI treatments at a given sampling point (Pooled *t*-test, $\alpha = <0.05$). Dissimilar capital letters indicate statistically significant differences between sampling points for treatments involving extended TI, and dissimilar small letters for those treatments that did not involve TI (ANOVA, $\alpha = <0.05$).

cascade that triggers it, was more acute during TI treatments. Further evidence of a sustained primary stress response, though not directly related to promoting gas exchange, is found in the significantly elevated glucose levels at all time points of animals maintained in TI. Glucocorticoid hormones are a key component of the primary stress response, ensuring an adequate supply of energetic substrates by controlling the degree of hepatic glycogen mobilisation (Barton, 2002; Busch and Hayward, 2009; Skomal and Bernal, 2010). The significantly elevated levels of glucose found in animals maintained in TI suggest that a chronic level of stress persisted for the duration of the experiment.

To the authors' knowledge, this study represents the first investigation into the physiological response to tonic immobility of any taxa to date. Our results demonstrate that juvenile lemon sharks display physiological perturbations in response to being in tonic immobility, over and above those associated with the capture and taking of blood samples. The increased physiological perturbation associated with TI is thought to be in response to a respiratory challenge induced by confinement to buccal pumping. In response to this, lemon sharks were able to implement a number of compensatory mechanisms; which suggests that central neural processing remains intact during TI, a phenomenon

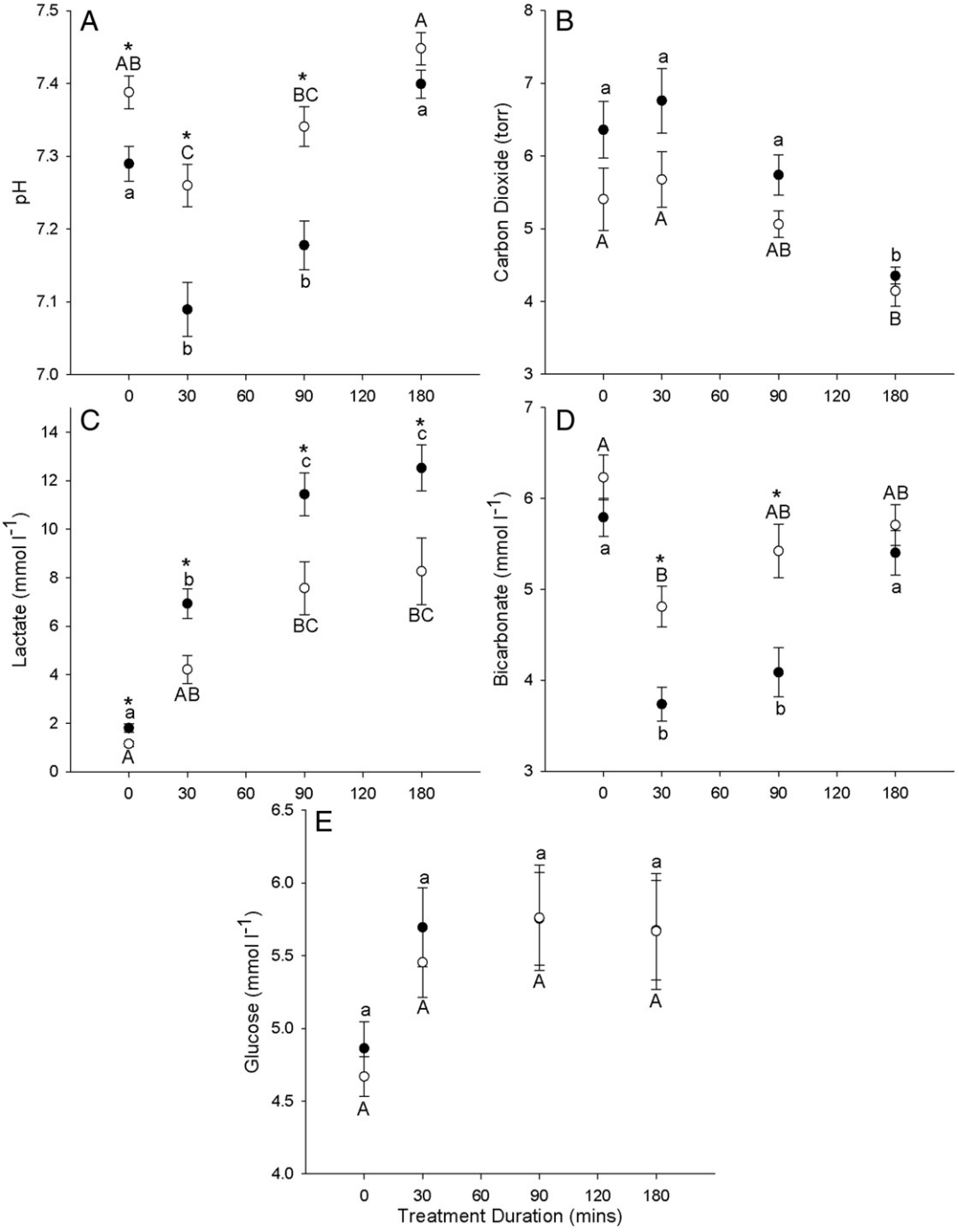

Fig. 3. Variation in blood gas and metabolite concentrations of juvenile lemon sharks (*Negaprion brevirostris*) in response to exercise. Filled points (●) represent animals subjected to exhaustive exercise and clear points (○) represent animals not exercised. Data represent mean blood chemistry values of whole blood (A) pH_{TC}, (B) carbon dioxide, (C) lactate., (D) bicarbonate and (E) glucose (±1 SE). Asterisks (*) indicate a statistically significant difference between exercise and non-exercise treatments at a given sampling point (Pooled *t*-test, $\alpha = <0.05$). Dissimilar capital letters indicate statistically significant differences between sampling points for treatments involving exercise, and dissimilar small letters for those treatments that did not involve exercise. (ANOVA, $\alpha = <0.05$).

which has been shown to exist in chickens (Gallup et al., 1980). In the short term TI increased the magnitude of physiological stress experienced by the animal, and care should be taken when using the technique for extended periods on less robust elasmobranch species, in particular those which are unable to supplement ram ventilation with buccal pumping. Furthermore, these findings have implications for all conservation physiology studies of elasmobranchs which commonly utilise TI as a means of facilitating blood sampling. It should be noted that this experiment was conducted in static water with no supplemental ventilation provided by pumps, a common practice in a number of research endeavours, and as such, further investigation into the potential mitigating effects of artificial ventilation are necessary. Despite its widespread use, TI remains one of the least studied and enigmatic areas of elasmobranch biology and further interdisciplinary research into the underlying psychological, physiological and neurological processes associated with it is highly recommended.

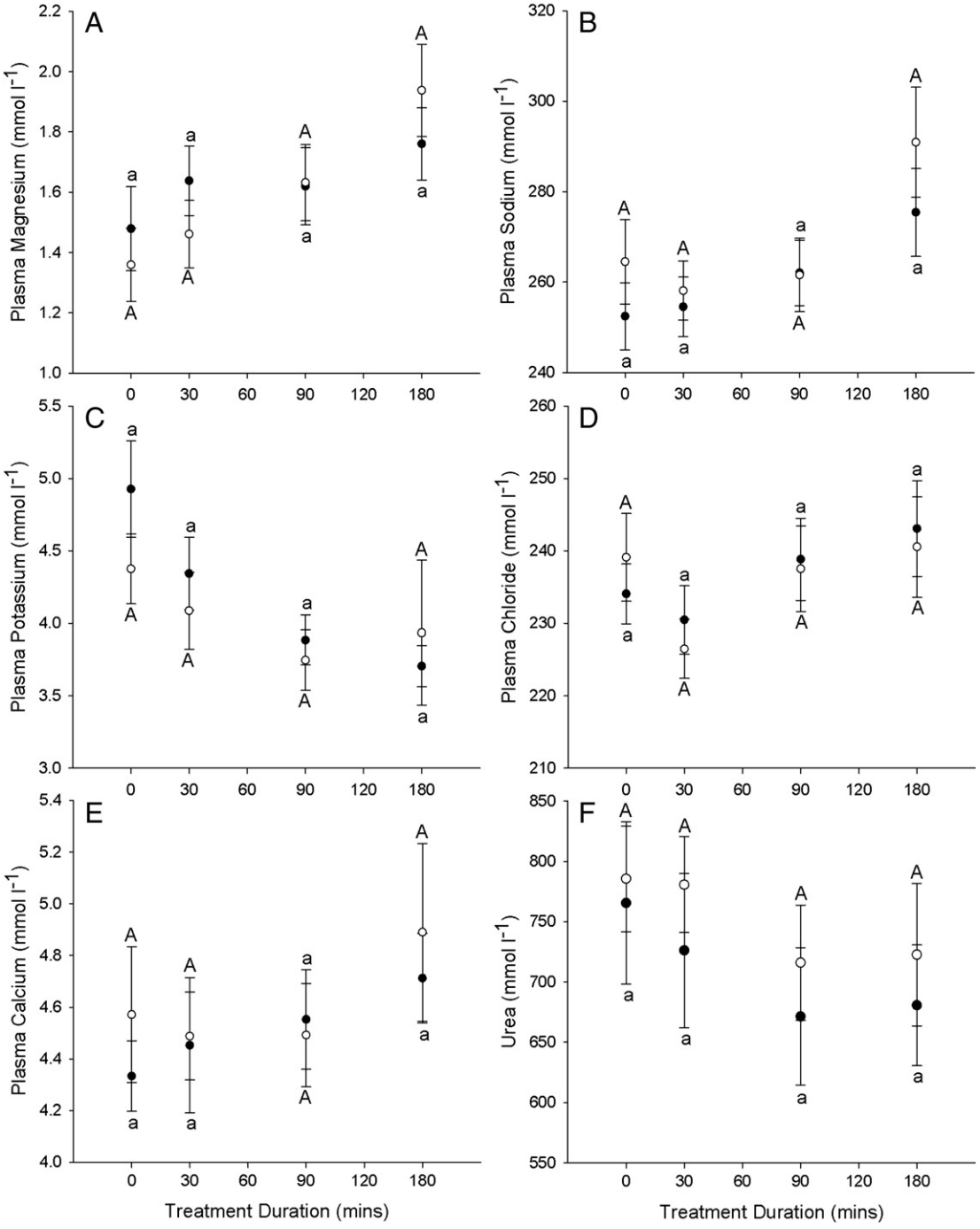

Fig. 4. Variation in plasma ion and urea concentrations of juvenile lemon sharks (*Negaprion brevirostris*) in response to exercise. Filled points (●) represent animals subjected to exhaustive exercise and clear points (○) represent animals not exercised. Data represent mean blood chemistry values of whole blood (A) magnesium, (B) sodium, (C) potassium, (D) chloride, (E) calcium and (F) urea (±1 SE). Asterisks (*) indicate a statistically significant difference between exercise and non-exercise treatments at a given sampling point (Pooled *t*-test, $\alpha = <0.05$). Dissimilar capital letters indicate statistically significant differences between sampling points for treatments involving exercise, and dissimilar small letters for those treatments that did not involve exercise (ANOVA, $\alpha = <0.05$).

Table 3
RM-MANOVA results for effects of exercise on ventilation rates (TI Treatments) and tail beat cycles (NoTI Treatments) in the juvenile lemon shark (*Negaprion brevirostris*). Bold font indicates significance.

Factor	Ventilation rate	Tail beat cycle
Time	**$p = <0.001$, $F_{4,40} = 78.95$**	**$p = <0.001$, $F_{4,37} = 29.39$**
Ex/NoEx	**$p = <0.001$, $F_{1,43} = 15.2$**	$p = 0.182$, $F_{1,40} = 1.84$
Ex/NoEx * Time	**$p = <0.001$, $F_{4,40} = 5.94$**	**$p = <0.001$, $F_{4,37} = 10.84$**
Individual sharks	**$p = <0.001$, $F_{3,43} = 42.34$**	**$p = <0.001$, $F_{3,40} = 10.84$**

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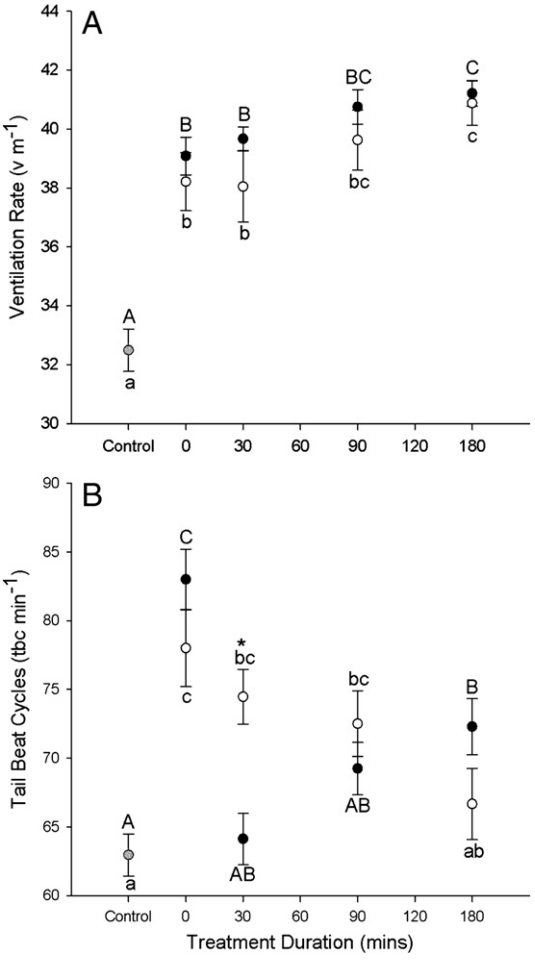

Fig. 5. Behavioural response of juvenile lemon sharks (*Negaprion brevirostris*) to exhaustive exercise, capture and restraint. Behaviour was quantified by ventilation rates for animals maintained in TI (A), and by tail-beat-cycles for those that were not (B). Filled points (●) represent animals subjected to exhaustive exercise and clear points (○) represent animals not exercised. Data represent mean values of each metric (±1 SE). Asterisks (*) denote a statistically significant difference between exercise and no-exercise treatments at a given sampling point (Pooled *t*-test, $\alpha = <0.05$). Dissimilar capital letters indicate statistically significant differences between sampling points for treatments involving exhaustive exercise, and dissimilar small letters for those treatments that did not. (ANOVA, $\alpha = <0.05$).

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