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Does ammonia trigger hyperventilation in the elasmobranch, *Squalus acanthias suckleyi*?

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ABSTRACT

We examined the ventilatory response of the spiny dogfish, to elevated internal or environmental ammonia. Sharks were injected via arterial catheters with ammonia solutions or their Na salt equivalents sufficient to increase plasma total ammonia concentration $[T_{Amm}]_a$ by 3–5 fold from 145 ± 21 μM to 447 ± 150 μM using NH_4HCO_3 and a maximum of 766 ± 100 μM using $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ caused a small increase in ventilation frequency (+14%) and a large increase in amplitude (+69%), while Na_2SO_4 did not. However, CO_2 partial pressure (P_{aCO2}) also increased and arterial pH_a and plasma bicarbonate concentration ($[HCO_3^-]_a$) decreased. NH_4HCO_3 caused a smaller increase in plasma ammonia resulting in a smaller but significant, short lived increases in ventilation frequency (+6%) and amplitude (36%), together with a rise in P_{aCO2} and $[HCO_3^-]_a$. Injection with $NaHCO_3$ which increased pH_a and $[HCO_3^-]_a$ did not change ventilation. Plasma ammonia concentration correlated significantly with ventilation amplitude, while ventilation frequency showed a (negative) correlation with pH_a. Exposure to high environmental ammonia (1500 μM NH_4HCO_3) did not induce changes in ventilation until plasma $[T_{Amm}]_a$ increased and ventilation amplitude (but not frequency) increased in parallel. We conclude that internal ammonia stimulates ventilation in spiny dogfish, especially amplitude or stroke volume, while environmental ammonia only stimulates ventilation after ammonia diffuses into the bloodstream.

1. Introduction

Fish can be confronted with elevated ammonia levels through high environmental ammonia exposure, for example in densely populated aquaculture facilities or in areas subject to heavy eutrophication such as estuaries and coastal waters (e.g. Eddy, 2005). Plasma ammonia concentrations ($[T_{Amm}]_a$) can also rise as a consequence of increased endogenous metabolic ammonia production, for example after a meal, after severe exercise or during stress. It has long been known that, at least in mammals, elevated levels of plasma ammonia cause central stimulation of breathing (Wichser and Kazemi, 1974). In ammoniotelic fish, the possibility of a similar hyperventilatory response had been suggested repeatedly, and intuitively makes sense as it might facilitate branchial ammonia excretion (Wright and Wood, 2012). Whether ammonia excretion actually does improve due to increased ventilation remains to be proven (Randall and Ip, 2006). However, in most cases where plasma $[T_{Amm}]_a$ levels are elevated due to endogenous ammonia production, for example after a meal (e.g. Bucking and Wood, 2008) in parallel with the specific dynamic action (SDA), or after strenuous exercise (e.g. Wood, 1988) in parallel with the excess post-exercise O_2 consumption (EPOC), increased O_2 demands or changes in acid–base status could induce ventilatory responses as well. Reduced arterial P_{aO2} induces hyperventilation, and this is most likely regulated peripherally (for review Perry et al., 2009). Involvement of central control is disputed and so far not proven. Especially on gill arch I and II, neuroepithelial cells (NECs) stimulating ventilation respond to changes in PO_2, as well as to changes in arterial P_{aCO2} and pH_a (Gilmour, 2001; Milsom and Burleson, 2007). Whether these NECs respond to arterial hypoxaemia or environmental hypoxia, or both, seems to dependent on the species, or even the location of the NEC and its innervating fibre.

Early on, Hillaby and Randall (1979) observed that ventilation in trout was stimulated after intravascular injection of ammonia salts.

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This was examined in more detail by McKenzie et al. (1993), who also noticed hyperventilation, but could not conclude confidently whether this was due to ammonia elevation or the accompanying blood acid–base changes. This was eventually solved by Zhang and Wood (2009) who provided evidence that ammonia can stimulate ventilation in rainbow trout in the absence of changes of acid–base status or other blood gases. In this study it was ventilatory stroke volume (large changes) rather that ventilation frequency (small changes) which was primarily increased, and the authors suggested that hyperventilation was an adaptive response to facilitate ammonia excretion, as well as to help elevate oxygen uptake at times of increased oxidative demands, such as after exercise or after a meal. Later studies demonstrated that ammonia's respiratory role as a ventilatory stimulant was probably mediated both peripherally, involving branchial NECs (Zhang et al., 2011, 2014), as well as centrally through the brain as hyperventilatory responses to ammonia correlate more closely with concentrations of ammonia in the brain than in plasma or CSF (Zhang et al., 2013), and that the response appeared to be primarily to internal rather than external ammonia (Zhang et al., 2011, 2014).

So it seems clear that in ammoniotelic, bony fish, ammonia does serve as a respiratory gas and induces hyperventilation, although a possible function of hyperventilation in ammonia excretion needs to be confirmed. What about fish that do not need to excrete their metabolic nitrogen waste, but rather retain nitrogen as much as possible? Such an example can be found in elasmobranchs, who retain nitrogen by way of urea and use urea as an osmolyte to prevent water loss to the marine environment (Smith, 1936; Hazon et al., 2003). These primitive fish evolved millions of years ago and go to great length to conserve high plasma and tissue urea levels, including having relatively tight, impermeable gill epithelia with high cholesterol levels, and active urea back-transporters in both the gills (Wood et al., 1995, 2013; Part et al., 1998; Fines et al., 2001; Hill et al., 2004) and kidney (Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen et al., 1972) to help retain urea. Nevertheless, sharks also show hyperventilation after a meal as part of the SDA, without a change in arterial P_{aO2} or P_{aCO2}, the classical regulatory mechanisms for ventilation in vertebrates (Wood et al., 2005). This was accompanied by an increase in plasma $[T_{Amm}]_a$ and ammonia excretion, but unchanged or reduced urea excretion (Wood et al., 2005, 2007, 2010; Kajimura et al., 2006, 2008). Since sharks try to retain nitrogen as much as possible, why would plasma ammonia increase unless it serves as a signal? Perhaps ammonia serves as a respiratory stimulant to counteract any depression of ventilation caused by the post-prandial 'alkaline tide' in both elasmobranchs (Wood et al., 2005) and teleosts (Bucking and Wood, 2008; Cooper and Wilson, 2008; Wright and Wood, 2012)? Furthermore, plasma $[T_{Amm}]_a$ levels also increase after exhaustive exercise in dogfish sharks, another situation in which increased ventilation is needed (Richards et al., 2003).

In elasmobranchs, as in teleosts, NECs also seem to be present, and thus could play a role. In spotted dogfish (*Scyliorhinus canicula*), the O_2 receptors mediating hypoxic bradycardia have a diffuse distribution within the orobranchial cavity (Butler et al., 1977; Taylor et al., 1977), but in spiny dogfish (*Squalus acanthias suckleyi*) at least the CO_2/pH chemoreceptors seem to be exclusively branchial (McKendry et al., 2001) and respond to external rather than internal stimuli (Perry and McKendry, 2001).

To address the question whether elasmobranchs would respond to ammonia as a respiratory gas, and whether the response would be elicited by internal and/or external responses, we exposed spiny dogfish (*S. acanthias suckleyi*) to both internally elevated ammonia levels and environmentally increased ammonia levels. We injected either an isotonic NaCl solution (control), an isotonic ammonia solution of NH_4HCO_3 or $(NH_4)_2SO_4$ aimed at increasing plasma ammonia concentrations to 500 μM, or their Na salt equivalents $NaHCO_3$ and Na_2SO_4. Arterial pH_a, P_{aO2}, P_{aCO2}, and plasma $[T_{Amm}]_a$ and $[HCO_3^-]_a$ were monitored before and at 2 and 30 min after injection and ventilation frequency and amplitude were recorded immediately before and at 2, 10 and 30 min after injection. Additionally, dogfish were exposed to high environmental ammonia (1500 μM NH_4HCO_3) in the external water, and the same measurements were taken for up to 24 h after the onset of exposure.

2. Material and methods

2.1. Experimental set-up

Pacific spiny dogfish (*Squalus acanthias suckleyi*) were caught by angling in the vicinity of Bamfield, British Columbia, Canada in the summers of 2009 and 2011 and subsequently kept in a large concrete indoor tank (151,000 L) served with running aerated Bamfield Marine Station seawater (12–14 °C, salinity 30‰). Dogfish were kept at least 1 week before experiments began. Fish were fed twice a week with commercially purchased frozen hake (*Merluccius productus*, a marine teleost) which were first thawed and deheaded.

In total 20 adult male dogfish with an average weight of 1.87 ± 0.07 kg were used. They were caught from the tank, and immediately anaesthetised in a 100 mg L^{-1} MS-222 seawater solution neutralised with $NaHCO_3$ for surgery. Dogfish were placed on a V-shaped operating table, and their gills were constantly irrigated with anaesthetic throughout surgery. A small incision was made, approximately 5 cm anterior to the caudal fin, to the vertebrae, exposing the cartilaginous haemal canal. This canal was punctured with a #22 needle creating a small hole for a PE50 polyethylene cannula, filled with heparinised 50 i.u./ml dogfish saline (6 mM $NaHCO_3$, 257 mM NaCl, 7 mM $NaSO_4$, 0.1 mM NaH_2PO_4, 4.1 mM KCl, 3 mM $MgSO_4$, 5 mM glucose, 2 mM $CaCl_2$, 350 mM urea, 15 mM TMAO). The cannula was inserted in the artery, which was verified by the presence of sufficient blood pressure to create a spontaneous blood flow even when the catheter was held above the heart. When the first cannula was accidently inserted in the vein, as seen by the lack of sufficient blood pressure and the dark colour of the blood, this cannula was left in place to avoid excessive bleeding, closed with a short pin, and a second cannula was inserted in the artery. The catheter(s) was held in place by a sleeve of PE160 secured with two sutures to the skin. Additionally, a flared PE160 cannula was inserted in the branchial chamber between the 1st and 2nd gill slit. It was held in place by a second short piece of flared PE240 which was glued to the PE160 cannula on the outside of the fish. The PE160 cannula was positioned in a 90° angle on the skin to allow the dogfish to breathe freely and was returned with a large loop towards the back of the dogfish where it was held in place by two sutures to the skin just before the dorsal fin. Dogfish were revived by artificial ventilation with anaesthetic-free seawater and were left to recover overnight in covered black Plexiglas fish boxes. The boxes were 98 cm in length, 20 cm in width and 25 cm in height, and contained 47.5 l Bamfield Marine Station seawater (14 °C, 30‰) with a flow-through of 0.75l min^{-1} and were served with perimeter aeration.

In injection experiments, we injected 1.1 ml of various isotonic 500 mM solutions per kg body weight over 5 min. At this injection rate, no behavioural response or irritation was observed when injecting with saline. Ventilation was measured immediately before and at 2, 10 and 30 min after the 5 min injection period in all treatments. Immediately following the pre-injection ventilation measurements and at 2 and 30 min after the end of the injection period, 0.6 ml blood was sampled using a 1 ml gastight Hamilton syringe, replaced with 0.6 ml saline and analysed as described below. Since injection with 500 mM $(NH4)_2SO_4$ overshot our aim to increase plasma ($[T_{Amm}]_a$) to 500 μM, we decided

to keep the 500 mM concentration for NH_4HCO_3 as well, resulting in the desired increase in plasma ammonia (see Section 3).

In the experiments where dogfish were exposed to high environmental ammonia (1500 μM), flow-through was stopped and 50 ml of a concentrated stock solution of NH_4HCO_3 was added along the length of the fish box. Measured concentrations were 1472 ± 62 μM (N = 6). This did not change the pH of the sea water (8.0–8.1).

2.2. Analytical procedures

For ventilation measurements, the branchial cannula was filled with sea water and connected to a pressure recording system which consisted of a pressure transducer (Statham P23BB, Statham Instruments, Oxnard, CA, USA), a transducer amplifier (Harvard Apparatus, Holliston, MA, USA), and an oscillograph (Harvard Apparatus, Holliston, MA, USA). The pressure transducer was calibrated directly by connection to a column containing different heights of water. This system allowed recording of ventilation frequency (breaths min^{-1}) and the ventilation pressure amplitude (mmHg), as an index of stroke volume. Ventilation frequency was calculated as the frequency of breaths in 1 min at the designated time. Ventilation amplitude was calculated as the average value of ten measurements of amplitude (randomly selected from periods of normal breathing, not using episodes of coughing or disturbance) at the designated time.

Blood samples of fish fitted with an arterial cannula were immediately analysed for arterial pH and arterial oxygen tension (P_{aO2}). The whole blood pH_a and P_{aO2} was measured in 14 °C thermostated chambers perfused with Bamfield Marine Station seawater using a Radiometer GK2401 C glass combination electrode coupled to a PHM82 standard pH meter (Radiometer Ltd., Copenhagen, Denmark), and a polarographic oxygen electrode coupled to a polarographic amplifier (Model 1900, A-M System, Everett, WA, USA), respectively. The remainder of the blood sample was immediately centrifuged (5 min at 10,000 × g), and sealed plasma sub-samples were taken and kept at 5 °C for determination of the levels of total CO_2 concentration later that day, or frozen in liquid nitrogen and kept in the freezer (−80 °C) until later determination of plasma $[T_{Amm}]_a$. Plasma total CO_2 was measured in duplicate on 50 μl samples using a Corning model 965 CO_2 analyzer (Lowell, MA, USA). $[T_{Amm}]_a$ was measured using a commercial kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method and assayed using a 4054 UV/visible spectrophotometer (LKB-Biochrom, Cambridge, England). P_{aCO2} was calculated using the solubility of carbon dioxide (α_{CO2}) and the apparent pK (pK_{app}) for dogfish plasma according to Boutilier et al. (1984): $P_{aCO2} = Cco_2/(\alpha_{CO2}\ (10^{pH-pKapp} + 1))$ with Cco_2 being total plasma CO_2 concentration. Plasma $[HCO_3^-]_a$ concentration was calculated as the difference between total plasma CO_2 and $\alpha_{CO2} \cdot P_{aCO2}$. Ammonia in water samples was determined using the salicylate-hypochlorite method (Verdouw et al., 1978).

2.3. Statistics

All data have been reported as means ± 1 SEM (N = number of fish). Relationships were assessed by non-parametric Kruskal–Wallis followed Dunn's multiple comparisons test and correlations were calculated using Pearson's correlation coefficient (GraphPad Prism 6.00, GraphPad Software Inc.). A significance level of $P < 0.05$ was used throughout.

3. Results

Control plasma $[T_{Amm}]_a$ levels before injections were on average 145 ± 21 μM. Injecting the ammonia salt $(NH_4)_2SO_4$ into the bloodstream of the dogfish increased plasma ammonia levels to 766 ± 100 μM ($P < 0.001$) within 2 min which was above the intended 500 μM (Fig. 1C). Half an hour after injection levels had only slightly decreased to 632 ± 195 μM ($P < 0.01$) showing considerable variation in the extent of this decline. Arterial injections of NH_4HCO_3 increased plasma $[T_{Amm}]_a$ levels closer to the intended 500 μM to 447 ± 150 μM after 2 min ($P < 0.05$). At 30 min after injection, the ammonia concentrations had levelled off to 374 ± 190 μM, again showing a large variation (Fig. 2C). No significant changes in plasma $[T_{Amm}]_a$ occurred when dogfish were injected with any of the sodium salts, NaCl, Na_2SO_4 or $NaHCO_3$ (Figs. 3C, 4C and 5C).

Dogfish were injected with NaCl as a control (Fig. 3). This did not lead to any changes in ventilation, neither in frequency nor amplitude (Fig. 3A and B). Dogfish took about 36 breaths min^{-1}, each with an amplitude between 0.8 and 1.0 mmHg. Since ventilation did not change, arterial blood oxygen P_{aO2} did not change (Fig. 3D) and also pH_a remained stable (Fig. 3F). However, both arterial bicarbonate $[HCO_3^-]_a$ (Fig. 3G) and P_{aCO2} increased (Fig. 3E). For $[HCO_3^-]_a$ this was significant 2 min after injection with an increase from 3.84 ± 0.40 mM to 4.63 ± 0.28 mM ($P < 0.05$) and for P_{aCO2} 30 min after injection with an increase from 0.81 ± 0.09 Torr to 1.22 ± 0.11 Torr ($P < 0.05$).

In contrast, injections with ammonium salts caused increases in both ventilation amplitude and frequency, with larger relative changes in the former. Injection of $(NH_4)_2SO_4$ caused a whole range of changes (Fig. 1). Ventilation frequency immediately increased from 36 ± 2 breaths min^{-1} to 39 ± 1 breaths min^{-1}, after 2 min injection ($P < 0.01$), which further increased to 41 ± 1 breaths min^{-1} after 10 min ($P < 0.001$) and 40 ± 1 breaths min^{-1} after half an hour (Fig. 1B). Amplitude peaked 2 min after injection from 1.0 ± 0.1 mmHg to 1.6 ± 0.2 mmHg ($P < 0.001$), after which it slowly started to recover to 1.3 ± 0.2 mmHg after 10 min ($P < 0.05$) and returned to normal after half an hour (Fig. 1A). Nevertheless, P_{aO2} did not increase significantly (Fig. 1D). $(NH_4)_2SO_4$ injections decreased pH_a significantly from 7.94 ± 0.05 to 7.36 ± 0.04 after 2 min ($P < 0.001$) with only a partial recovery to 7.65 ± 0.04 after 30 min ($P < 0.001$) (Fig. 1F). $[HCO_3^-]_a$ and P_{aCO2} were seriously affected. $[HCO_3^-]_a$ levels dropped from 4.46 ± 0.33 mM to 1.44 ± 0.24 mM after 2 min ($P < 001$) and were still reduced to 3.25 ± 0.30 mM after 30 min ($P < 0.001$) (Fig. 1G). P_{aCO2} significantly increased after 30 min from 1.14 ± 0.12 to 1.68 ± 0.08 Torr ($P < 0.05$) (Fig. 1E). Injections of the corresponding control solution Na_2SO_4 caused no changes in acid–base status, or in ventilation, and indeed all parameters remained stable (Fig. 4).

Arterial injections of NH_4HCO_3 caused a more transient increase in ventilation frequency and amplitude (Fig. 2A and B). Frequency increased from 36 ± 1 to 38 ± 1 breaths min^{-1} ($P < 0.05$ after 2 min) (Fig. 2B), and amplitude increased from 1.1 ± 0.1 to 1.4 ± 0.2 mmHg after 2 min ($P < 0.01$) after which it slowly returned to control level (Fig. 2A). P_{aO2} (Fig. 2D) and pH_a (Fig. 2F) remained stable, but both $[HCO_3^-]_a$ and P_{aCO2} levels were elevated 2 min after the injection. $[HCO_3^-]_a$ increased from 4.80 ± 0.44 to 6.11 ± 0.41 mM ($P < 0.01$) (Fig. 2G) and P_{aCO2} from 1.23 ± 0.09 to 1.63 ± 0.13 Torr ($P < 0.05$) (Fig. 2E). By 30 min, both parameters had returned to normal values. Injections of the matching control solution $NaHCO_3$ did not induce any changes in ventilation (Fig. 5A and B), although $[HCO_3^-]_a$ did increase from 4.92 ± 0.40 to 8.55 ± 0.98 mM ($P < 0.01$) (Fig. 5G). Increases in P_{aCO2} were highly variable and therefore not significant but average values increased from 1.28 ± 0.10 Torr up to 1.62 ± 0.51 Torr (Fig. 5E).

Exposure of dogfish to high environmental ammonia (1500 μM NH_4HCO_3), resulted in elevations of ventilatory amplitude (Fig. 6A), but not frequency (Fig. 6B). Amplitude remained stable through the first 2 h, but progressively increased thereafter, attaining a significant elevation at 4 h ($P < 0.05$), 12 h ($P < 0.01$) and 24 h ($P < 0.01$) to 1.9 ± 0.2, 2.2 ± 0.2 and 2.1 ± 0.2 mmHg respectively.

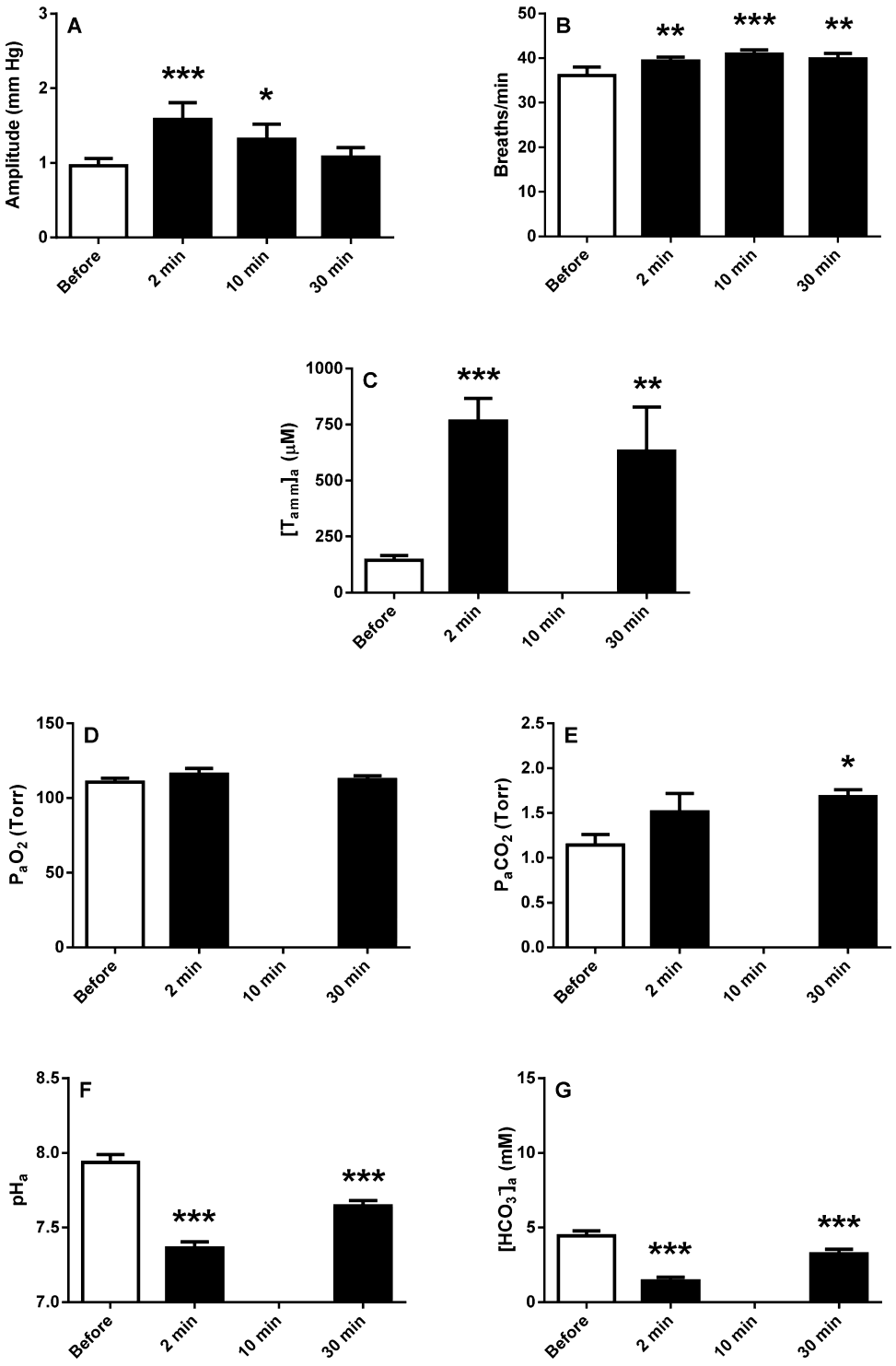

Fig. 1. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, injected with 500 mM $(NH_4)_2SO_4$. Mean ± SEM, $N = 11$, *significant difference with value before injection ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Notably, this did not happen until plasma $[T_{Amm}]_a$ rose, with increases to 805 ± 127 μM ($P < 0.01$), 983 ± 107 μM ($P < 0.001$) and 1371 ± 219 μM ($P < 0.001$) at these time points respectively (Fig. 6C). At 2 h of high environmental ammonia, plasma ammonia was 457 ± 82 μM, but amplitude (1.7 ± 0.2 mmHg) was not yet significantly increased. It is also notable that these slow but large increases in ventilatory amplitude in response to high environmental ammonia occurred in the absence of any changes in pHa (Fig. 6F), $[HCO_3^-]_a$ (Fig. 6G), P_{aCO2} (Fig. 6E), or P_{aO2} (Fig. 6D), pointing to a direct effect of elevated $[T_{Amm}]_a$.

Correlation analysis (Graphs provided in electronic Supplementary material) confirmed that ventilation amplitude ($P < 0.0001$), but not frequency, correlated with plasma ammonia. Even though the correlation between ventilation amplitude and plasma ammonia proved to be highly significant, correlation coefficient $r = 0.49$, indicating that other control mechanisms might have played a role

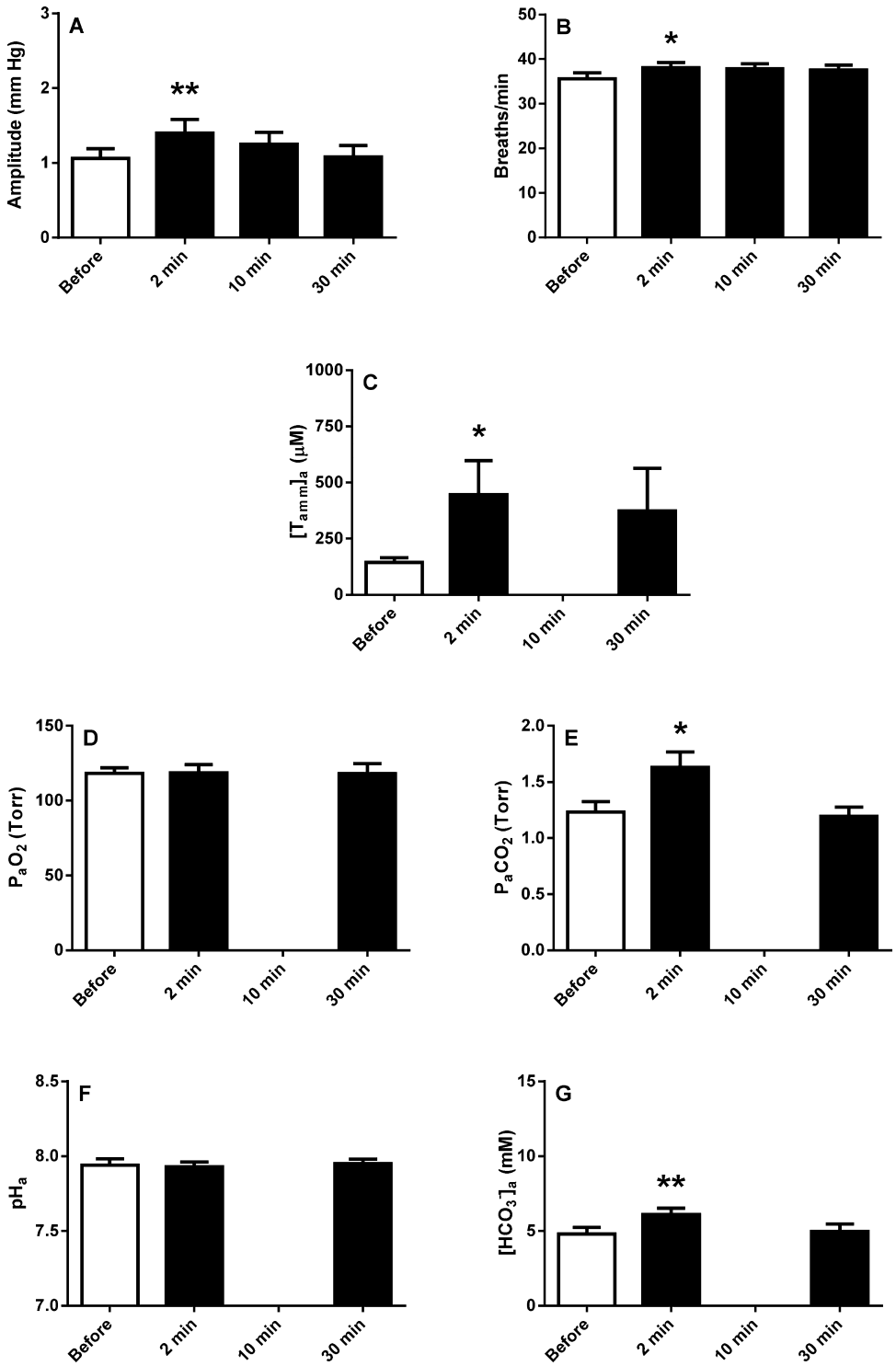

Fig. 2. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, injected with 500 mM NH_4HCO_3. Mean ± SEM, $N = 11$, *significant difference with value before injection (*$P < 0.05$, **$P < 0.01$).

as well. Neither ventilation amplitude nor frequency showed a significant correlation with P_{aO2} or P_{aCO2}, but ventilation frequency showed a weak negative correlation with arterial pHa ($P < 0.01$, $r = -0.19$). Ventilation amplitude was not correlated with pH_a.

4. Discussion

From our results, it is obvious that none of the injected Na salts caused a change in ventilation frequency or amplitude. Na_2SO_4 did not induce changes in any of the measured parameters. Both NaCl and $NaHCO_3$ caused a transient increase in plasma $[HCO_3^-]_a$, but this did not lead to any modification in ventilation frequency or amplitude. This is in contrast to the increases in ventilation seen earlier in the teleost rainbow trout after injections with $NaHCO_3$ (McKenzie et al., 1993; Zhang and Wood, 2009). Clearly, changes in $[HCO_3^-]_a$ could be expected when injecting $NaHCO_3$. The fact that NaCl also induced an increase in plasma $[HCO_3^-]_a$ could be explained by the chloride shift with increased activities of the

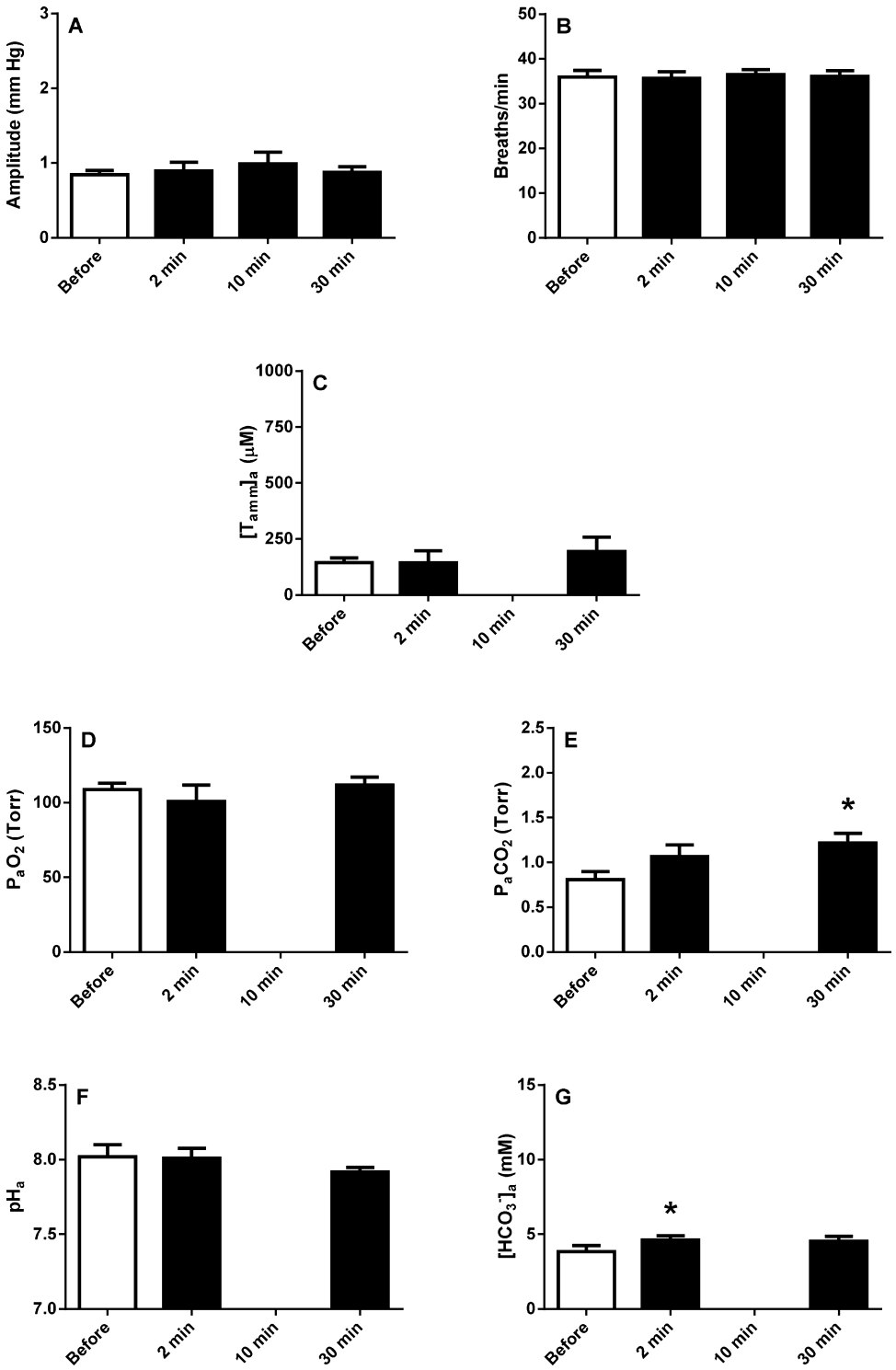

Fig. 3. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, injected with 500 mM NaCl. Mean ± SEM, $N = 11$, *significant difference with value before injection ($P < 0.05$).

Cl^-/HCO_3^- exchanger at the red blood cells, or by branchial/renal excretion of the excess Cl^- by a comparable anion exchanger after the treatment. We did observe some changes in P_{aCO2} when injecting dogfish with $(NH_4)_2SO_4$ or NH_4HCO_3, and in both cases this coincided with an increased ventilation frequency and/or amplitude. Branchial CO_2/pH chemoreceptors are expected to respond to increased CO_2 levels, however current evidence suggests that they respond to external rather than internal stimuli in Pacific spiny dogfish (McKendry et al., 2001; Perry and McKendry, 2001). This is not contradicted by our results. When injecting the fish with NaCl, the observed increases in P_{aCO2} levels did not lead to any change in ventilation, and we did not find any correlation between P_{aCO2} and either ventilation amplitude or frequency, making it unlikely that arterial P_{aCO2} was the cause of the observed hyperventilation in the former cases. Similarly, after injection with $NaHCO_3$, P_{aCO2} reached similar values (1.62 ± 0.51 Torr) as when injecting with $(NH_4)_2SO_4$ (1.68 ± 0.08 Torr) or NH_4HCO_3 (1.63 ± 0.13 Torr) but this did not lead to a change in ventilation. Actually, excretion of CO_2

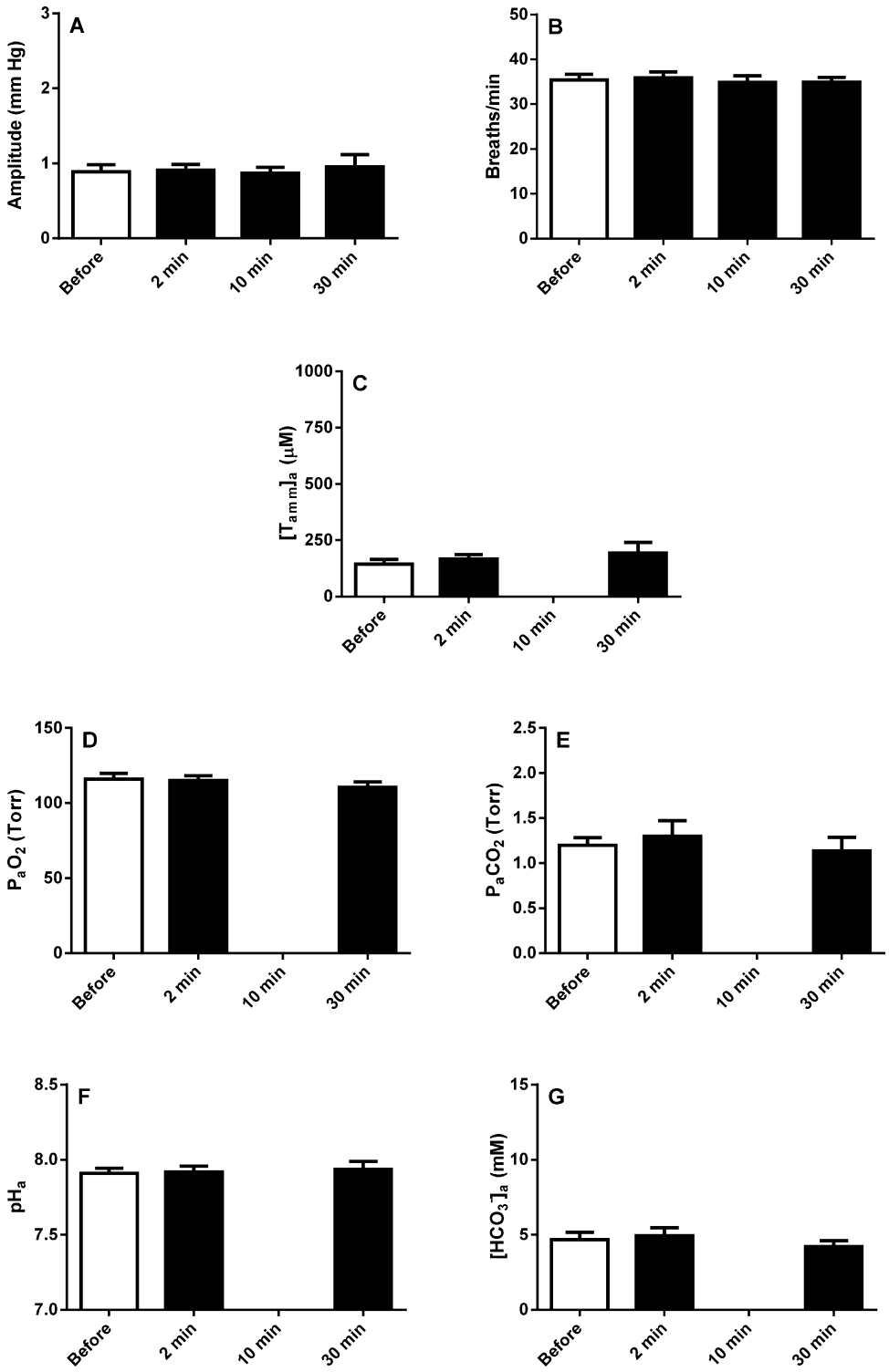

Fig. 4. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, injected with 500 mM Na_2SO_4. Mean ± SEM, $N = 11$, no significant differences with values before injection were detected.

is extremely efficient in water breathing animals, as it dissolves to a greater extent in water compared to O_2, leading to a continuous over-ventilation for CO_2 excretion. This is especially true in dogfish, which have extracellular carbonic anhydrase present on the endothelial surfaces and in the blood plasma (Gilmour and Perry, 2010 for review). The presence of extracellular carbonic anhydrase results in an extremely efficient HCO_3^-/CO_2 conversion, making it virtually impossible for dogfish to retain much excess CO_2. Therefore, it seems unlikely that the levels of P_{aCO2} seen in the present study were causing the observed hyperventilation. No changes in environmental PO_2 or arterial P_{aO2} occurred during our experiments, excluding these as possible causes of the measured hyperventilation.

Some of the injections did cause changes in arterial pH_a, which could trigger hyperventilation. Injecting $NaHCO_3$ caused a rise in pH_a with 0.2 units but did not alter ventilation. In elasmobranchs, ventilatory control of acid–base status is minimal since it is regulated by ion rather than acidic/basic equivalent exchange (Heisler,

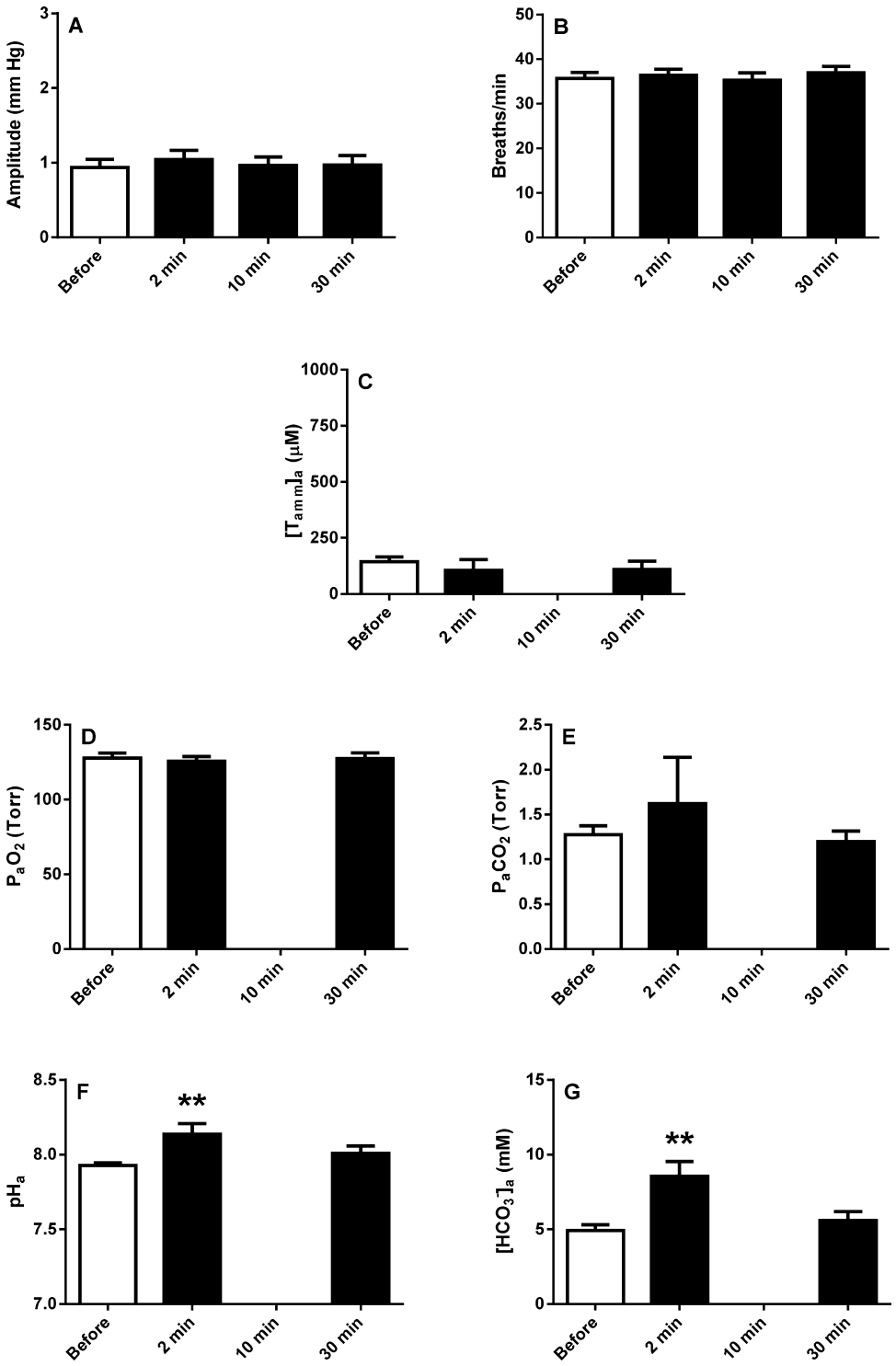

Fig. 5. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, injected with 500 mM $NaHCO_3$. Mean ± SEM, $N = 11$, **significant difference with value before injection ($P < 0.01$).

1988) and they are able to resist the alkalinizing influence of substantial basic equivalent loading rather well (Wood et al., 1995; Tresguerres et al., 2005). Injections of $(NH_4)_2SO_4$ caused a marked metabolic acidosis (decreases in pH_a and $[HCO_3^-]_a$), probably because of the rapid removal of NH_3 by ureogenesis or diffusion into tissues, leaving a strong acid (H_2SO_4) behind, similar to the situation reported earlier with NH_4Cl (leaving HCl behind, Wood et al., 1995). The hyperventilatory response to $(NH_4)_2SO_4$ was larger than with a similar injection of NH_4HCO_3 (unchanged pH_a), suggesting a direct effect of low pHa on ventilation. To a limited extent, this seems to be confirmed by the significant negative correlation between arterial pH and ventilation frequency, but there was clearly no relationship between pH_a and ventilatory amplitude. Note however, the low slope of the frequency versus pH_a relationship ($r = -0.19$), such that only small increases in frequency are associated with large decreases in pH_a and suggesting that other control mechanisms must drive the changes in ventilatory frequency as well. Several previous studies on

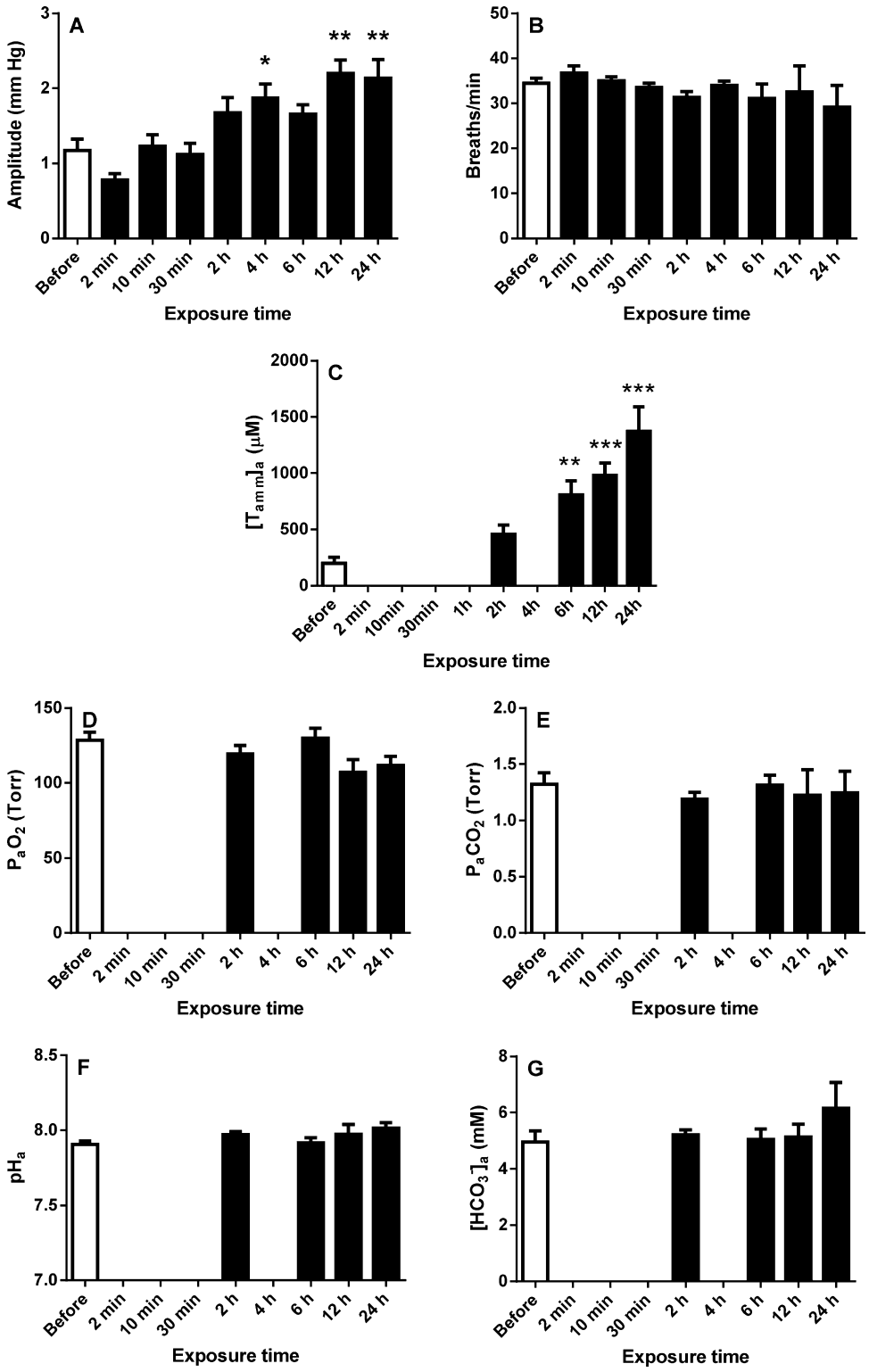

Fig. 6. Ventilation amplitude (A) and frequency (B), plasma total ammonia levels (C), arterial blood oxygen (D) and carbon dioxide tensions (E), arterial pH (F) and bicarbonate levels (G) of spiny dogfish, *Squalus acanthias suckleyi*, exposed to high environmental ammonia (1500 μM). Mean ± SEM, $N = 9$, *significant difference with value before exposure (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

elasmobranchs have concluded that there is a better correlation between changes in ventilation and changes in pH_a than in P_{aCO2} (Heisler et al., 1988; Graham et al., 1990; Wood et al., 1990).

Although we cannot exclude acidosis as at least a partial cause of the observed hyperventilation after $(NH_4)_2SO_4$ injection, especially for ventilation frequency, perhaps the most convincing results that ammonia itself serves as a respiratory gas are from the waterborne high environmental ammonia exposure where ventilation amplitude increased in parallel with plasma ammonia levels without any change in pH_a, P_{aO2}, P_{aCO2}, or $[HCO_3^-]_a$. This is supported by the highly significant correlation between ventilation amplitude and plasma ammonia using all available data from our study. These

results also indicate that ammonia sensing in dogfish is likely to be internal, in contrast with CO_2 sensing by external branchial CO_2/pH chemoreceptors (McKendry et al., 2001; Perry and McKendry, 2001), as ammonia injections elicit a response within the first 2 min while high environmental ammonia exposure had an effect only after several hours when ammonia had diffused into the bloodstream. In teleosts, the response to high environmental ammonia is faster and starts to gradually build up after 5–10 min (McKenzie et al., 1993; Zhang and Wood, 2009), but still much slower than the immediate response after injection (Zhang et al., 2011, 2013). Perhaps the tight branchial epithelium of dogfish, which has a 22-fold lower permeability to ammonia than a typical teleost (Wood et al., 1995; Part et al., 1998), causes a much slower influx of ammonia into the bloodstream in these elasmobranchs, and therefore makes the delay in response much more obvious.

The influence of internal ammonia in causing hyperventilation in trout seems to occur both peripherally involving branchial NECs (Zhang et al., 2011) as well as centrally through the brain (Zhang et al., 2013). Peripheral control in trout involves NECs and their associated afferent nerves located on the 1st and 2nd gill arch, with receptors on the 2nd gill arch detecting only internal ammonia (slow hyperventilatory response after high environmental ammonia exposure), whereas those on the 1st arch ammonia (faster response) may additionally sense external ammonia to some degree (Zhang et al., 2011, 2014). Central control of hyperventilation seemed to be dependent on brain [T_{Amm}] rather than plasma [T_{Amm}] or cerebrospinal fluid [T_{Amm}]. Also our study suggest that plasma ammonia is not the only mechanism controlling ventilation, as only 49% of the variation in amplitude can be attributed to the variation in ammonia levels, and only 19% of the variation in frequency can be attributed to changes in pH_a. Whether or not brain ammonia levels contribute to the ventilatory control remains to be examined.

A recent study shows that *in vitro*, NECs of trout are sensitive to ammonia, responding with elevations in intracellular Ca^{2+} after exposure to 1 mmol L^{-1} NH_4Cl (Zhang et al., 2011). NH_4^+ might simply enter neurons through potassium channels and depolarize neurons directly. However, while NH_4^+ can enter through K^+ channels, most values of the permeability of NH_4^+ through K^+ channels are much lower relative to K^+ (Choe et al., 2000; Randall and Ip, 2006). In the study Zhang et al. (2011), 1 mmol L^{-1} NH_4^+ challenge caused larger intracellular Ca^{2+} responses compared to the 30 mmol L^{-1} K^+ challenge. These authors suggested that ammonia can enter fish gill NECs in more efficient ways, perhaps via Rh proteins. Alternatively, ammonia could inhibit potassium channels, depolarize neurons and stimulate ventilation. Tetramethylammonium is known to effectively inhibit potassium channels but the inhibition is reliant on binding to the external binding sites of the potassium channel which in turn is dependent on the size of the molecule (while binding to the internal site is more dependent on hydrophobic properties) (Heginbotham and MacKinnon, 1992). Hille (1967) demonstrated that TEA is unique among the symmetric quaternary ammonium ions: larger and smaller derivates are not such effective inhibitors (Heginbotham and MacKinnon, 1992) which makes this mechanism less likely to play a role here. Although either of these possible explanations cannot be ruled out, this does not mean that ammonia stimulating ventilation is a pharmacological epiphenomenon based on the signaling function of potassium channels in all animals. For one reason, it occurs at ammonia levels within the normal physiological range in fish, and for another, so few other vertebrates and invertebrates have been looked, that it is impossible to draw a conclusion on this point.

Whether dogfish show peripheral and/or central control of hyperventilation due to increased ammonia remains to be examined, but at least some studies suggest a role for central CO_2/pH chemoreception in an elasmobranch (Wood et al., 1990) and the longnose gar *Lepisosteus osseus* (Wilson et al., 2000). Certainly, in higher vertebrates, the central chemoreceptors of the brain are the more important site for CO_2 detection.

5. Conclusions

In conclusion, we can say that Pacific spiny dogfish respond to increases in plasma [T_{Amm}]$_a$ with a fast hyperventilatory response, both in amplitude and frequency, with the former showing the larger changes on a relative basis. Increases in P_{aCO2} or [HCO_3^-]$_a$ did not elicit this response in our study corresponding to earlier observations that CO_2/pH sensing in dogfish is responding to external rather than internal stimuli (Perry and McKendry, 2001), but we cannot exclude the possibility that decreases in pH_a may have intensified hyperventilation. From the results with waterborne high environmental ammonia exposures, we conclude that hyperventilatory regulation seems to be controlled by internal ammonia elevation rather than external ammonia levels. This more chronic response led to an elevation in breathing amplitude rather than breathing frequency, and occurred without any change in pH_a, P_{aO2}, P_{aCO2}, or [HCO_3^-]$_a$. Whether this ventilatory control by ammonia in elasmobranchs is peripheral by branchial NECs or central by sensors responding to brain [T_{Amm}] elevation, or by both as in teleosts (Zhang et al., 2014), remains to be examined.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resp.2014.11.009.

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