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Adaptations of a deep sea scavenger: High ammonia tolerance and active NH_4^+ excretion by the Pacific hagfish (*Eptatretus stoutii*)

Alexander M. Clifford [a,b,*], Greg G. Goss [a,b], Michael P. Wilkie [b,c]

[a] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada
[b] Bamfield Marine Sciences Centre, Bamfield, British Columbia, V0R 1B0, Canada
[c] Biology Department, Wilfrid Laurier University, Waterloo, Ontario, N2L 3C5, Canada

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ABSTRACT

The Pacific hagfish (*Eptatretus stoutii*) has an exceptional ability to both withstand and recover from exposure to high external ammonia (HEA). This tolerance is likely due to the feeding behavior of this scavenger, which feeds on intermittent food falls of carrion (e.g. fish, large marine mammals) during which time it may be exposed to high concentrations of total ammonia ($T_{Amm} = NH_3 + NH_4^+$) while burrowed inside the decomposing carcass. Here we exposed hagfish to 20 mmol L^{-1} T_{Amm} for periods of up to 48 h and then let animals recover in ammonia-free seawater. During the 48 h HEA exposure period, plasma T_{Amm} increased 100-fold to over 5000 μmol L^{-1} while ammonia excretion (J_{amm}) was transiently inhibited. This increase in plasma T_{Amm} resulted from NH_3 influx down massive inwardly directed ΔP_{NH3} gradients, which also led to a short-lived metabolic alkalosis. Plasma [T_{Amm}] stabilized after 24–48 h, possibly through a reduction in NH_3 permeability across the body surface, which lowered NH_3 influx. Ammonia balance was subsequently maintained through the re-establishment of J_{amm} against an inwardly directed ΔP_{NH3}. Calculations of the Nernst potential for ammonia strongly indicated that J_{amm} was also taking place against a large inwardly directed NH_4^+ electrochemical gradient. Recovery from HEA in ammonia-free water was characterized by a large ammonia washout, and the restoration of plasma T_{Amm} concentrations to near control concentrations. Ammonia clearance was also accompanied by a residual metabolic acidosis, which likely offset the ammonia-induced metabolic alkalosis seen in the early stages of HEA exposure. We conclude that restoration of J_{amm} by the Pacific hagfish during ammonia exposure likely involves secondary active transport of NH_4^+, possibly mediated by Na^+/NH_4^+ (H^+) exchange.

1. Introduction

It has been long known that internal buildups of ammonia in vertebrates lead to ammonia toxicity, which is characterized by over-excitation of the nervous system due to N-methyl-D-aspartate overactivation, the generation of reactive oxygen species, apoptosis or necrosis of neurons and glial cells in the brain, ATP depletion, and potentially fatal encephalopathy caused by ionic disturbances and increases in brain water content (see Ip et al., 2001b; Felipo and Butterworth, 2002; Walsh et al., 2007 for reviews). In mammals, ammonia toxicity can arise from liver damage, which results in a failure to convert ammonia to less toxic urea via the ornithine urea cycle (Felipo and Butterworth, 2002). However, for fish living in aquatic environments, there is no need to convert ammonia to urea because the high water solubility and diffusability of ammonia allows it to be readily excreted across the gills in most aquatic environments (see Wright, 1995; Wilkie, 1997; for comprehensive reviews). For this reason, the major nitrogenous waste product in most teleost fishes is ammonia while urea predominates in marine elasmobranchs and coelacanths, which retain urea as an osmolyte in seawater (Griffith, 1991; Ballantyne, 2001). However, some teleosts either live in or are transiently exposed to environments that do not favor ammonia excretion such as the Lake Magadi tilapia (*Oreocrhomis alcalicus* graham) which lives in highly alkaline (~pH 10) water (Randall et al., 1989), and certain air breathing fishes including the lungfishes (Smith, 1930; Janssens and Cohen, 1968; Wood et al., 2005). Strategies for detoxification include increases in glutamine production to detoxify ammonia as employed by the swamp eel *Monopterus albus* (Ip et al., 2004), ammonia volatilization as used by mangrove killifish (*Kryptolebias marmoratus*) during air exposure (Frick and Wright, 2002), and even active NH_4^+ transport, as proposed for the mudskipper (*Periophthalmodon schlosseri*) and the

Abbreviations: ΔH^+_m, cumulative metabolic acid load; β, Buffering capacity; ANOVA, analysis of variance; ATP, adenosine tri-phosphate; EDTA, ethylenediaminetetraacetic acid; E_{NH4+}, Nernst potential for NH_4^+; GS, glutamine synthetase; Hct, hematocrit; HEA, high environmental ammonia; J_{amm}, ammonia flux; J_{Urea}, urea flux; NH_3, ammonia; NH_4^+, ammonium; NKA, sodium potassium ATPase; PAGE, poly-acrylamide gel electrophoresis; ΔP_{NH3}, NH_3 partial pressure gradient; PCA, perchloric acid; TBS, tris-buffered saline; TBST, tris-buffered saline containing 0.1% triton X-100; T_{Amm}, total ammonia; TEP, transepithelial potential; TMS, tricaine methylsulfonate.

* Corresponding author at: Department of Biological Sciences, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada. Tel.: +1 780 729 8399.
E-mail address: alex.clifford@ualberta.ca (A.M. Clifford).

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climbing perch (*Anabas testudineus*) during exposure to air or exposure to high environmental ammonia (Randall et al., 1999; Wilson et al., 2000; Tay et al., 2006; Chew et al., 2007; Ip et al., 2012). The swamp eel and weatherloach (*Misgurnus anguillacaudatus*) are also able to withstand millimolar increases in plasma ammonia (Chew et al., 2001; Tsui et al., 2002; Ip et al., 2004). While a wealth of knowledge on ammonia excretion by teleosts and elasmobranchs has been generated in the last 20 years (see Weihrauch et al., 2009 for review), we know relatively little about how nitrogenous wastes are excreted by the agnathan fishes, as represented by the lampreys (Petromyzontidae) and the hagfishes (Myxinidae), which are the earliest extant representatives of the vertebrate lineage (Bardack, 1991). A better understanding of how these jawless fishes handle and excrete nitrogenous wastes should provide valuable insight into the patterns of nitrogenous waste production and excretion and how they evolved in the vertebrates (Weihrauch et al., 2009).

As opportunistic scavengers, hagfishes are among the first marine organisms to begin devouring "food drops," defined as carrion that sink to the ocean floor from the upper depths or water surface (Collins et al., 1999). Carrion may simply be fish, including by-catch from commercial fishing (Collins et al., 1999), or extremely large marine mammals (Smith and Baco, 2003). As the hagfish devour the carrion they may burrow into the decomposing carcass via orifices in the body, or beneath the operculum in the case of fishes (Collins et al., 1999).

In terrestrial environments, the early stages of decomposition mainly involve the breakdown of cells and tissues by anaerobic bacteria, leading to the generation of "putrefactive gases" such as hydrogen sulfide and ammonia, which can lead to the deposition of very high concentrations of nitrogen compounds in the surrounding environment including $(NH_4^+)_2SO_4$, which can exceed 525 μg g^{-1} (~29 mmol L^{-1}; Carter et al., 2007). There is little data on the biochemical process of putrefaction in aquatic environments, but since many of the anaerobic bacteria involved in the putrefactive process arise from the gut (Carter et al., 2007), it seems reasonable to assume that decomposing fish or marine mammal carcasses would generate large amounts of ammonia. Thus, high ammonia tolerance is likely a pre-requisite for the scavenging life-style of hagfishes, which are also likely to encounter hypoxic and acidic conditions due to the generation of lactic acid and propionic acid in the early stages of decomposition, and perhaps high CO_2 concentrations in later stages (Carter et al., 2007). Indeed, the ability of the hagfish to withstand and recover from acidic, hypoxic and hypercapnic conditions has been reported in recent years (Parks et al., 2007; Tresguerres et al., 2007; Cox et al., 2011; Clifford et al., 2014). However, less is known about hagfish nitrogenous waste metabolism and excretion. The main nitrogenous waste product for hagfishes is ammonia, with little reliance on urea excretion under routine conditions (Walsh et al., 2001). Hagfish can also withstand short-term exposure to high environmental ammonia (HEA; Braun and Perry, 2010), but the underlying mechanisms of their ammonia tolerance have not yet been identified. Immunohistochemical evidence suggests that the Pacific hagfish (*Eptatretus stoutii*) relies on Rhesus glycoprotein mediated-ammonia excretion under such conditions (Braun and Perry, 2010), but it is not known if the hagfish can withstand longer periods of HEA, or how acid–base balance, and ammonia balance are maintained under such extreme conditions.

In the present study, Pacific hagfish were exposed to nominal total ammonia concentrations (T_{Amm} equals the sum of $NH_3 + NH_4^+$) of 20 mmol L^{-1} for 48 h to determine how HEA affected blood T_{Amm} and other nitrogenous waste product concentrations (urea, glutamine). In addition, measurements were made to quantify how HEA influenced acid–base balance, to describe how ammonia and urea excretion (J_{amm}, J_{Urea}) patterns across the body surface were affected by HEA, and to determine how these processes take place. We hypothesized that the Pacific hagfish would be able to tolerate long-term HEA exposure by converting it to less toxic urea and/or glutamine, and that the animals would be able to prevent ammonia from reaching toxic concentrations by excreting ammonia against large inwardly directed NH_3 and/or NH_4^+ electrochemical gradients, as previously suggested by Braun and Perry (2010).

2. Materials and methods

2.1. Experimental animals and holding

Pacific hagfish (*Eptatretus stoutii*; $N = 24$; average mass = 118 ± 10 g, range 65–270 g) were captured using bottom-dwelling traps, baited with strips of Pacific hake (*Merluccius productus*), from Trevor Channel, off the southwest coast of Vancouver Island, BC, Canada. Traps were checked daily and captured hagfish were transported to the Bamfield Marine Sciences Centre where they were housed in aerated, darkened 20 m^3 tanks continuously receiving seawater. The hagfish were used for experiments within 3 weeks, and were fed bi-weekly with strips of hake, but fasted for at least 1 week prior to experimentation. All animals were collected with Department of Fisheries and Oceans Canada authorization (scientific collection permit no. XR-214-2011), and experiments were approved by the Bamfield Marine Science Centre Animal Care Committee (protocol number BMSC RS-11-26) and followed Canadian Council of Animal Care guidelines.

2.2. Experimental protocol

2.2.1. Exposure to high external ammonia

One day prior to experiments, Pacific hagfish ($N = 18$) were transferred to 10 L buckets receiving continuously flowing seawater. The next morning, they were exposed to a nominal NH_4Cl concentration of 20 mmol L^{-1} in 5 L of aerated seawater, and held under static conditions for 24–48 h. Representative water samples (15 mL) were collected daily to verify that the water total ammonia concentrations ($T_{Amm} = [NH_3] + [NH_4^+]$) matched target values.

2.2.2. Quantification of ammonia and urea excretion rates

Rates of ammonia excretion (J_{amm}) and urea excretion (J_{Urea}) were measured in a sub-set of animals ($N = 6$) under control (seawater only) conditions (mean $[T_{Amm}] = 2.2 \pm 1.0$ μmol L^{-1} (SD); $N = 6$ measurements), during exposure to HEA (mean $[T_{Amm}] = 21.6 \pm 2.0$ mmol L^{-1}; $N = 72$), and during a post-ammonia exposure recovery period in nominally ammonia-free sea water (mean $[T_{Amm}] = 6.3 \pm 2.2$_μmol L^{-1}; $N = 12$). Under control conditions, water flow to the buckets was cut-off and the water volume lowered to exactly 1.0 L. The very low basal J_{amm} and J_{Urea} rates of Pacific hagfish, determined in preliminary experiments (see Discussion), necessitated the low water volume used to measure control excretion rates. Water samples (15 mL) were then collected at the beginning of the measurement period (0 h) and after 4 h, and then frozen at −20 °C, until analyzed for water T_{Amm} and urea concentration. During the 48 h HEA exposure, water volume was adjusted to 3 L, and water samples (15 mL) were collected every 4 h, over three 8 h flux intervals (0–8 h, 8–16 h, 16–24 h) and one 12 h flux interval (36–48 h). Following each flux interval, 2/3 of the water in the 3.0 L bucket was replaced with fresh, aerated seawater containing a nominal concentration of 20 mmol L^{-1} NH_4Cl. All water samples were frozen at −20 °C until analyzed.

Following the 48 h HEA exposure, the animals were allowed to recover in nominally ammonia-free seawater for 24 h. Due to the rapid accumulation of water T_{Amm} that was anticipated, the flux measurement periods were of shorter duration than during HEA (1–4 h rather than 8 h), and the volume of water in the buckets was adjusted to 5.0 L instead of 3.0 L to prevent increases in T_{Amm} that could subsequently impair J_{amm}. Accordingly, post-HEA J_{amm} and J_{Urea} were measured from 0 to 1 h, 1 to 2 h, and 2 to 4 h, at which point the water in the buckets was replaced with nominally ammonia-free seawater and another flux initiated from 4 to 8 h. Water flow was then re-established to the containers for 12 h, followed by a final J_{amm} and