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SHORT COMMUNICATION

Differential glycogen utilization in shark acid- and base-regulatory gill cells

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ABSTRACT

Na^+/K^+-ATPase (NKA)- and vacuolar H^+-ATPase (VHA)-rich cells in shark gills secrete excess acid and base, respectively, to seawater to maintain blood acid–base homeostasis. Both cell types are rich in mitochondria, indicating high ATP demand; however, their metabolic fuel is unknown. Here, we report that NKA- and VHA-rich cells have large glycogen stores. Glycogen abundance in NKA-rich cells was lower in starved sharks compared with 24 h post-fed sharks, reflecting higher energy demand for acid secretion during normal activity and glycogen replenishment during the post-feeding period. Conversely, glycogen abundance in VHA-rich cells was high in starved sharks and it became depleted post-feeding. Furthermore, inactive cells with cytoplasmic VHA had large glycogen stores and active cells with basolateral VHA had depleted glycogen stores. These results indicate that glycogen is a main energy source in both NKA- and VHA-rich cells, and point to differential energy use associated with net acid and net base secretion, respectively.

KEY WORDS: pH regulation, Alkaline tide, Alkalosis, Metabolism, ATPase, Acidosis, *Triakis semifasciata*, Energy

INTRODUCTION

Acid–base regulation is crucial for all organisms because it prevents homeostatic alterations that would negatively affect essential physiological functions including metabolism, gas exchange and muscle contraction, among many others (reviewed in Roos and Boron, 1981). In elasmobranchs (sharks, rays and relatives), two populations of distinct epithelial gill cells facilitate blood acid–base regulation: acid-secreting Na^+/K^+-ATPase (NKA)-rich cells that secrete H^+ to the surrounding water and absorb HCO_3^- into the blood during blood acidosis, and base-secreting vacuolar H^+-ATPase (VHA)-rich cells that secrete HCO_3^- and absorb H^+ during blood alkalosis (Piermarini and Evans, 2001; Reilly et al., 2011; Roa and Tresguerres, 2016; Roa et al., 2014; Tresguerres et al., 2005). These cell populations are likely differentially activated during a wide range of acid–base disturbances normally experienced by sharks, including after feeding (Wood et al., 2007, 2005, 2009), during exhaustive exercise (Richards et al., 2003), and following both changes in water temperature (Heisler, 1988) and exposure to environmental hypercapnia (Heisler, 1988). Reflecting the high energetic cost of secreting acid and base, both NKA- and VHA-rich gill cells have large numbers of mitochondria (Roa et al., 2014). In theory, NKA-rich cells have increased ATP demand during blood acidosis, and VHA-rich cells during blood alkalosis. However, nothing is known about the metabolic fuel sources used by shark ion-transporting gill cells.

In animal, fungal and bacterial cells, excess glucose is polymerized into glycogen, which serves as a main energy reserve. Expectedly, cells with high metabolic activity typically have readily available access to large glycogen stores. For example, myocytes have distinct intracellular glycogen pools (Fridén et al., 1989; Marchand et al., 2002) that fuel different molecular events involved in muscle contraction (Gejl et al., 2017; Nielsen et al., 2011), and neurons are supplied by lactate derived from glycogen stored in adjacent astrocytes during periods of high energy demand (reviewed in Brown, 2004). Recently, glycogen has been identified as an energy source for NaCl regulation by gill mitochondrion-rich ionocytes in tilapia (*Oreochromis mossambicus*) (Tseng et al., 2007). Similar to astrocytes (Choi et al., 2012), glycogen is predominantly stored in glycogen-rich accessory cells adjacent to ionocytes within the gill epithelium. Because gill glycogen content declined upon transfer from freshwater to seawater, it was hypothesized that glycogen is hydrolyzed to glucose and transferred from accessory cells to ionocytes to fuel NaCl secretion (Chang et al., 2007; Tseng et al., 2007).

The gills of marine sharks perform the vast majority (>97%) of the H^+ and HCO_3^- transport for blood acid–base regulation, and, unlike teleost and freshwater shark gills, they do not play a significant role in blood NaCl regulation (Heisler, 1988). Therefore, shark gills are an excellent model to study energy metabolism in relation to acid–base regulation. Because gill NKA- and VHA-rich cells are responsible for acid and base secretion, respectively, we hypothesize that both cell types have high glycogen abundance and show differential energy utilization depending on feeding status. Furthermore, active VHA-rich cells can be identified by the presence of VHA in the basolateral membrane, as opposed to the cytoplasmic localization observed in inactive VHA-rich cells (Roa et al., 2014; Tresguerres et al., 2005, 2010, 2007). This mechanism is known as the 'VHA translocation', and it is an essential regulatory mechanism that drives H^+ absorption into the blood and HCO_3^- secretion into the water to compensate for blood alkalosis (reviewed in Tresguerres, 2016). In contrast, NKA is always found in the basolateral membrane regardless of blood acid–base status (Tresguerres et al., 2005), indicating that the regulatory mechanisms for acid secretion do not involve the reversible insertion and removal of NKA into the basolateral membrane.

In this study, we investigated the presence of glycogen stores in leopard shark (*Triakis semifasciata* Girard 1855) gill cells, and the use of glycogen as a metabolic fuel by NKA- and VHA-rich cells during feeding-related blood acid–base disturbances. Furthermore, we specifically investigated glycogen use in active base-secreting cells identified based on their basolateral VHA localization.

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MATERIALS AND METHODS

Experimental animals

All experiments were approved by the Scripps Institute of Oceanography–University of California San Diego Animal Care Committee under protocol number S10320 in compliance with the Institutional Animal Care and Use Committee guidelines for the care and use of experimental animals. Leopard sharks were caught from La Jolla Shores, CA, USA, and housed in tanks with flowing seawater. Sharks were offered chopped squid or mackerel three times a week, which they consumed *ad libitum*.

Feeding experiments

Gill samples from starved and fed leopard sharks were obtained from a previous feeding experiment (Roa et al., 2014). Briefly, eight juvenile leopard sharks (mean body mass 145.5±8.4 g; mean length 34.3±0.83 cm; three male, five female) were randomly separated into two treatment groups (starved versus fed) with four sharks in each treatment. All eight sharks were starved for 5 days, and then four randomly selected sharks were collected as the 'starved' treatment. The remaining sharks were force-fed with blended squid (∼5% body mass) and collected 24 h later as the 'fed' treatment. All sharks were euthanized by an overdose of tricaine methanesulfonate (0.5 g l^{-1}) prior to sampling.

Gill tissue sampling

For immunohistochemistry, gill samples were fixed in 0.2 mol l^{-1} cacodylate buffer, 3.2% paraformaldehyde and 0.3% glutaraldehyde (catalog no. 29651, Electron Microscopy Sciences, Hatfield, PA, USA) for 6 h, transferred to 50% ethanol for 6 h and stored in 70% ethanol until further processing, as described previously (Roa and Tresguerres, 2016; Tresguerres et al., 2005).

Antibodies and reagents

Two polyclonal rabbit antibodies were used in this study: commercially available anti-NKA antibodies raised against the mammalian α-subunit (Santa Cruz Biotechnology, Dallas, TX, USA) and custom-made polyclonal rabbit antibodies raised against a conserved peptide in the VHA B-subunit (AREEVPGRRGFPGY). Both antibodies specifically recognize NKA and VHA in leopard shark gills (Roa and Tresguerres, 2017; Roa et al., 2014). In addition, a custom-made monoclonal mouse anti-glycogen antibody (Baba, 1993; Nakamura-Tsuruta et al., 2012) was kindly provided by Drs Otto Baba and Yung-Che Tseng, which specifically detects glycogen in fish gill cells (Chang et al., 2007; Tseng et al., 2007). Fluorescent secondary antibodies goat anti-mouse Alexa Fluor 555 and goat anti-rabbit Alexa Fluor 488 were obtained from Invitrogen (Grand Island, NY, USA).

Immunohistochemistry

Fixed gills were dehydrated, embedded in paraffin and sectioned at 7 µm thickness as previously described (Roa and Tresguerres, 2016, 2017; Roa et al., 2014). Rehydrated sections were incubated in blocking buffer (PBS, 2% normal goat serum and 0.02% keyhole limpet hemocyanin, pH 7.7) for 1 h and then in a solution of rabbit anti-NKA (12 µg ml^{-1}) or rabbit anti-VHA (6 µg ml^{-1}) antibodies combined with mouse anti-glycogen antibody (1:20) overnight at 4°C. Slides were washed in PBS (3×), incubated in a mixture of goat anti-rabbit and anti-mouse secondary antibodies (1:500 each) at room temperature for 1 h, incubated with the nuclear stain Hoechst 33342 (Invitrogen; 5 µg ml^{-1}) for 5 min, washed in PBS (3×), and permanently mounted in FluoroGel with Tris buffer (Electron Microscopy Sciences). Sections incubated with anti-glycogen antibodies and excess glycogen (25 µg $µl^{-1}$) served as controls for anti-glycogen immunoreactivity. Immunofluorescence was detected using an epifluorescence microscope (Zeiss AxioObserver Z1) connected to a metal halide lamp and appropriate filters. Zeiss Axiovision software and Adobe Photoshop were used to adjust digital images for brightness and contrast only.

Glycogen content in gill cells from starved and fed leopard sharks

ImageJ image analysis was used to determine glycogen content in NKA- and VHA-rich gill cells in gill tissue sections from starved (n=4) and fed (n=4) sharks. Glycogen content was estimated by measuring the area of individual NKA-rich cells (480 total, 240 starved, 240 fed) and VHA-rich cells (480 total, 240 starved, 240 fed), and reported as glycogen fluorescence/cell area. To minimize confounding effects resulting from analyzing cells cut at different depths during gill sectioning, glycogen quantification was performed on cells of roughly the same size and with a visible nucleus.

Statistical analyses

t-tests were used to analyze differences in glycogen content in NKA-rich cells from starved versus fed sharks, VHA-rich cells from starved versus fed sharks, and VHA-rich cells with cytoplasmic versus membrane VHA localization in fed sharks. All data are given as means±s.e.m. and statistical significance was set at P<0.001.

RESULTS AND DISCUSSION

In free-feeding sharks, the anti-glycogen monoclonal antibody specifically labeled a subpopulation of cells along the gill interlamellar region (Fig. 1A). Because the anti-glycogen antibody was used at a lower dilution (1:20) compared with previous studies on tilapia (1:200 and 1:300; Chang et al., 2007; Tseng et al., 2007), immunostaining was repeated on other sections using a 1:300 dilution and obtained qualitatively identical results (not shown). In addition, the immunofluorescence signal obtained with the 1:20 dilution was completely abolished when the antibody was preabsorbed with 25 µg $µl^{-1}$ glycogen (Fig. 1B), which is the same glycogen concentration used in preabsorption controls in Tseng et al. (2007). Altogether, these results indicate that the glycogen immunostaining protocol used in the present study specifically labeled glycogen stores in shark gill cells.

Further immunostaining with anti-NKA or anti-VHA antibodies revealed those glycogen-rich cells were either acid-secreting NKA-rich cells (Fig. 1C,D) or base-secreting VHA-rich cells (Fig. 1E,F). Notably, we observed no differences in the glycogen content of NKA- and VHA-rich cells, likely because of fluctuations in the energetic demands of NKA- and VHA-rich cells as the animals dynamically switch between states of blood acidosis and alkalosis as a result of changes in gastric inputs and digestion during pre- and post-feeding periods (Papastamatiou and Lowe, 2004, 2005; Tresguerres et al., 2007; Wood et al., 2005).

Next, we investigated the effects of acid–base stress on glycogen utilization in 5-day starved and 24 h post-fed sharks. Because starved sharks secrete net acid across the gills and fed sharks excrete net base (Wood et al., 2005, 2007, 2009), we hypothesized increased glycogen utilization by NKA-rich cells in starved sharks and by VHA-rich cells in fed sharks. Indeed, NKA-rich cells from starved sharks had very little glycogen (Fig. 2A–C), but the glycogen content in each cell became replenished in the post-feeding period, when sharks are actively secreting base and NKA-rich cells are inactive (Fig. 2D–F). On average, the glycogen content

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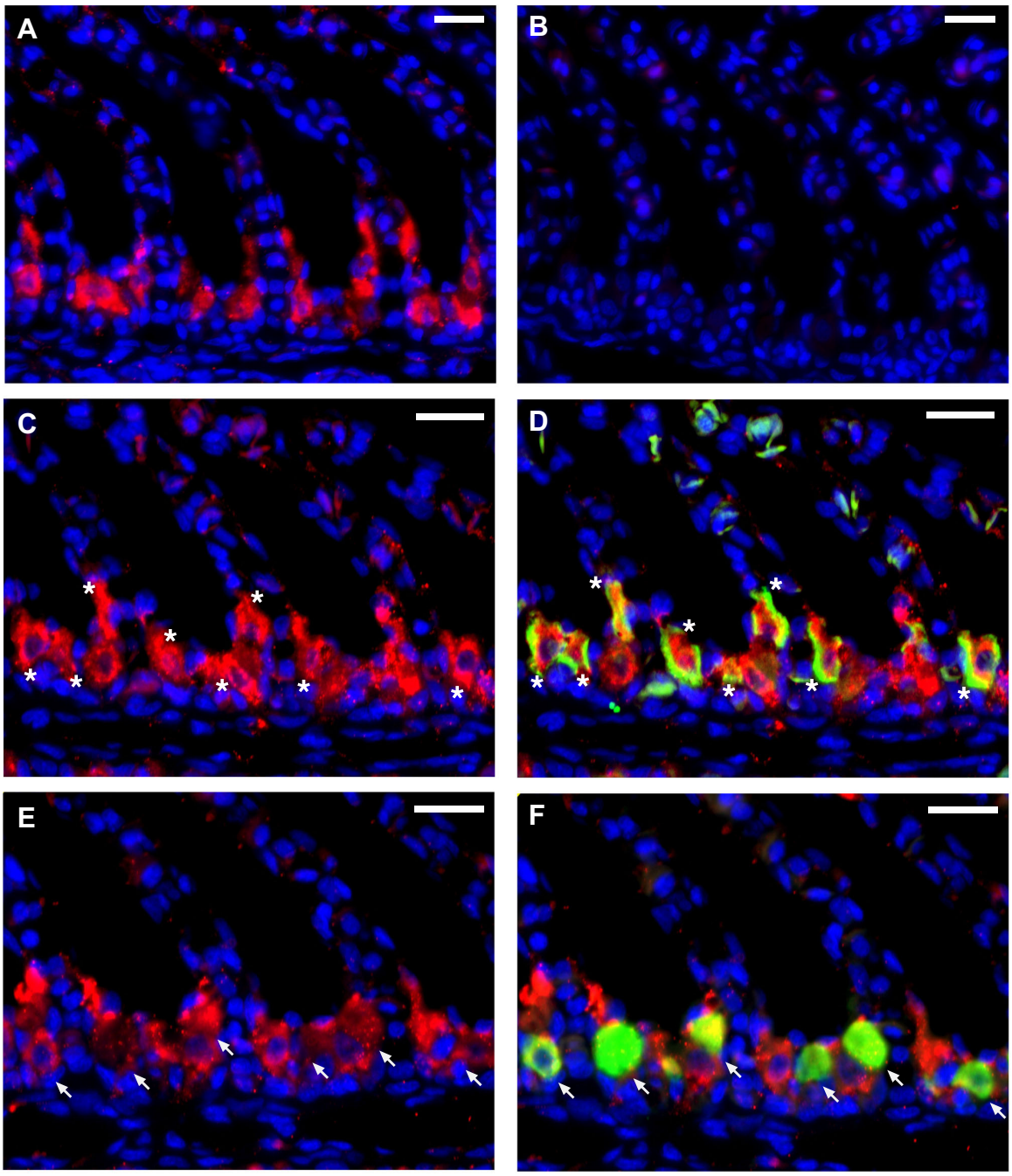

Fig. 1. Glycogen in acid-secreting Na^+/K^+-ATPase (NKA)-rich and base-secreting vacuolar H^+-ATPase (VHA)-rich leopard shark gill cells. (A) Cells with large stores of glycogen (red) were present in cells along the interlamellar gill region; (B) anti-glycogen immunoreactivity was abolished following antibody preabsorption with 25 mg ml^{-1} glycogen. (C–F) Further analyses revealed glycogen-rich (red) cells are either NKA-rich (green) acid-secreting cells (C,D; indicated with asterisks) or VHA-rich (green) base-secreting cells (E,F; indicated with arrows). Nuclei stained in blue. Scale bars: 20 μm.

in NKA-rich cells from fed sharks was ∼3-fold higher than in starved sharks (Fig. 2G). As previously shown (Tresguerres et al., 2005), NKA always had a basolateral location regardless of acid–base status.

Glycogen stores followed the opposite dynamics in VHA-rich cells. In starved sharks, the majority of VHA-rich cells that were analyzed (237/240 cells) had VHA in the cytoplasm (Fig. 3A) and were therefore inactive. Accordingly, those cells had large glycogen stores that filled the cytoplasm (Fig. 3B,C). However, blood alkalosis in the post-feeding period induced the translocation of VHA to the basolateral membrane (201/240 cells) (Fig. 3D), which activates compensatory H^+ absorption and HCO_3^- excretion (Tresguerres et al., 2005, 2007, 2010, 2006). This mechanism has previously been described in detail in post-fed dogfish (Tresguerres et al., 2007) and leopard sharks (Roa et al., 2014) (reviewed in Tresguerres, 2016). Reflecting increased energy consumption, glycogen stores in cells with basolateral VHA became depleted (Fig. 3D–F). On average, the glycogen content in all VHA-rich cells from fed sharks was ∼3-fold lower than in starved sharks (Fig. 3G). The quasi-binary pattern (∼6000 arbitrary units in cells with large

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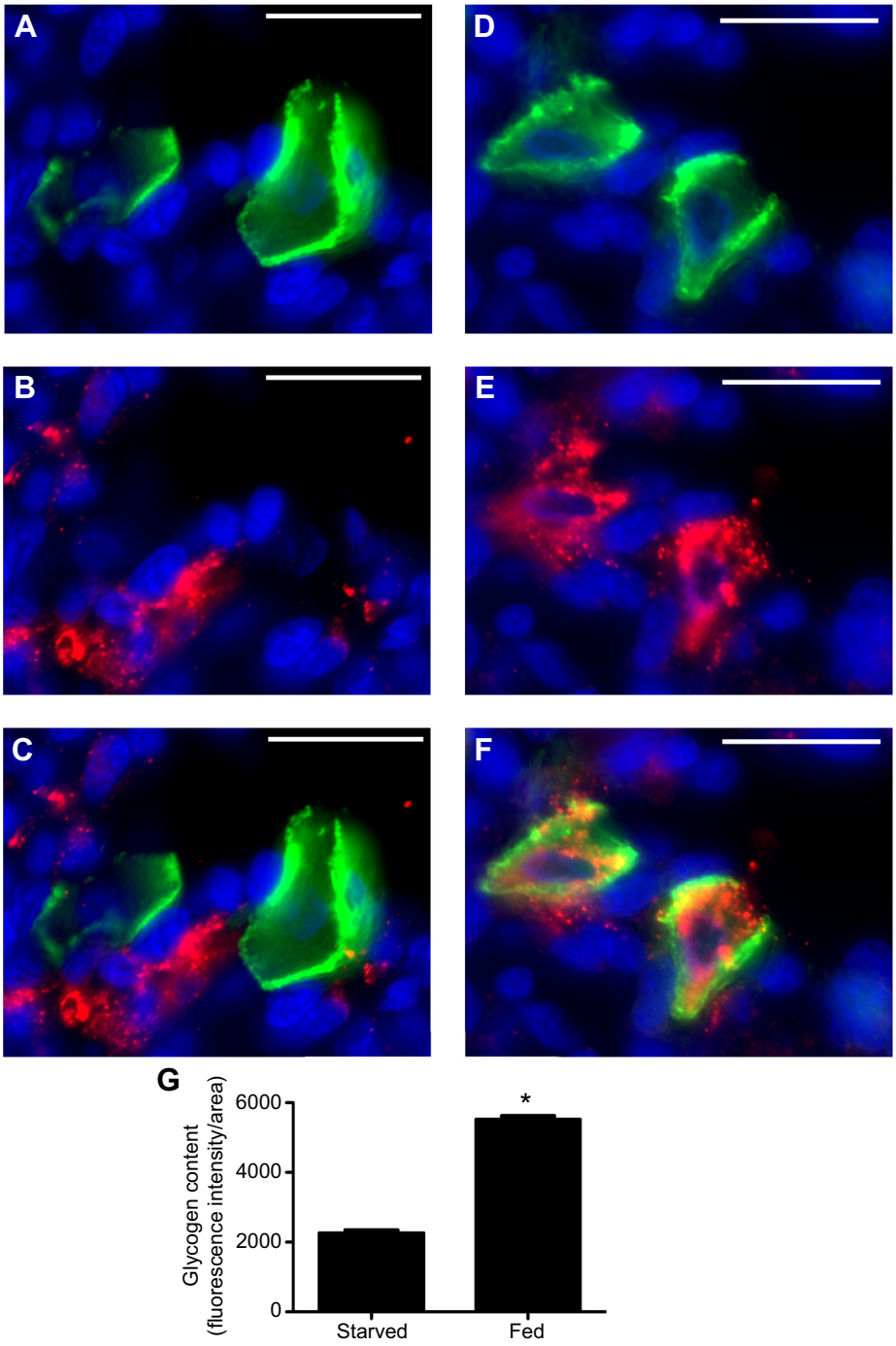

Fig. 2. Glycogen stores in acid-secreting NKA-rich gill cells from starved and fed leopard sharks. (A–F) Representative images of NKA (green) and glycogen (red) immunoreactivity in gills from starved (A–C) and fed (D–F) leopard sharks. (G) Summary of glycogen content in NKA-rich cells from four starved (n=240 cells) and four fed (n=240 cells) leopard sharks. Glycogen content was ∼3-fold significantly higher in NKA-rich cells from fed leopard sharks (*P<0.001). Nuclei stained in blue. Scale bars: 20 μm.

glycogen stores and ∼2000 arbitrary units in cells with depleted glycogen stores regardless of cell type) resulted from analyzing cells of roughly the same size, and from studying two feeding states that greatly activate or inactivate acid and base secretion in a reciprocal manner.

However, glycogen utilization was regulated based on the level of activation of each VHA-rich cell, as glycogen stores were large in inactive cells with cytoplasmic VHA localization and depleted in active cells with basolateral membrane localization regardless of the feeding status. This was clearly observed in VHA-rich cells from fed sharks (39 cells with cytoplasmic VHA and large glycogen stores, 201 cells with basolateral VHA and depleted glycogen stores; Fig. 3H). In some cases, those two glycogen abundance patterns were even observed in adjacent VHA-rich cells (Fig. 3F). In starved sharks, all three VHA-rich cells with basolateral VHA had depleted glycogen stores. However, VHA protein abundance in shark gills does not change during the alkaline tide taken place in the post-feeding period (Tresguerres et al., 2007). Instead, upregulation of HCO_3^- secretion and H^+ absorption is mediated by post-translational regulation of pre-existing proteins, namely translocation of VHA to the basolateral membrane and of the anion exchanger pendrin to the apical membrane (Tresguerres et al., 2007; Roa et al., 2014).

Overall, these results demonstrate that glycogen is a major metabolic fuel for both NKA- and VHA-rich cells in shark gills. Unlike NaCl-secreting cells from marine teleost fish gills or neurons, which, respectively, rely on adjacent accessory cells and

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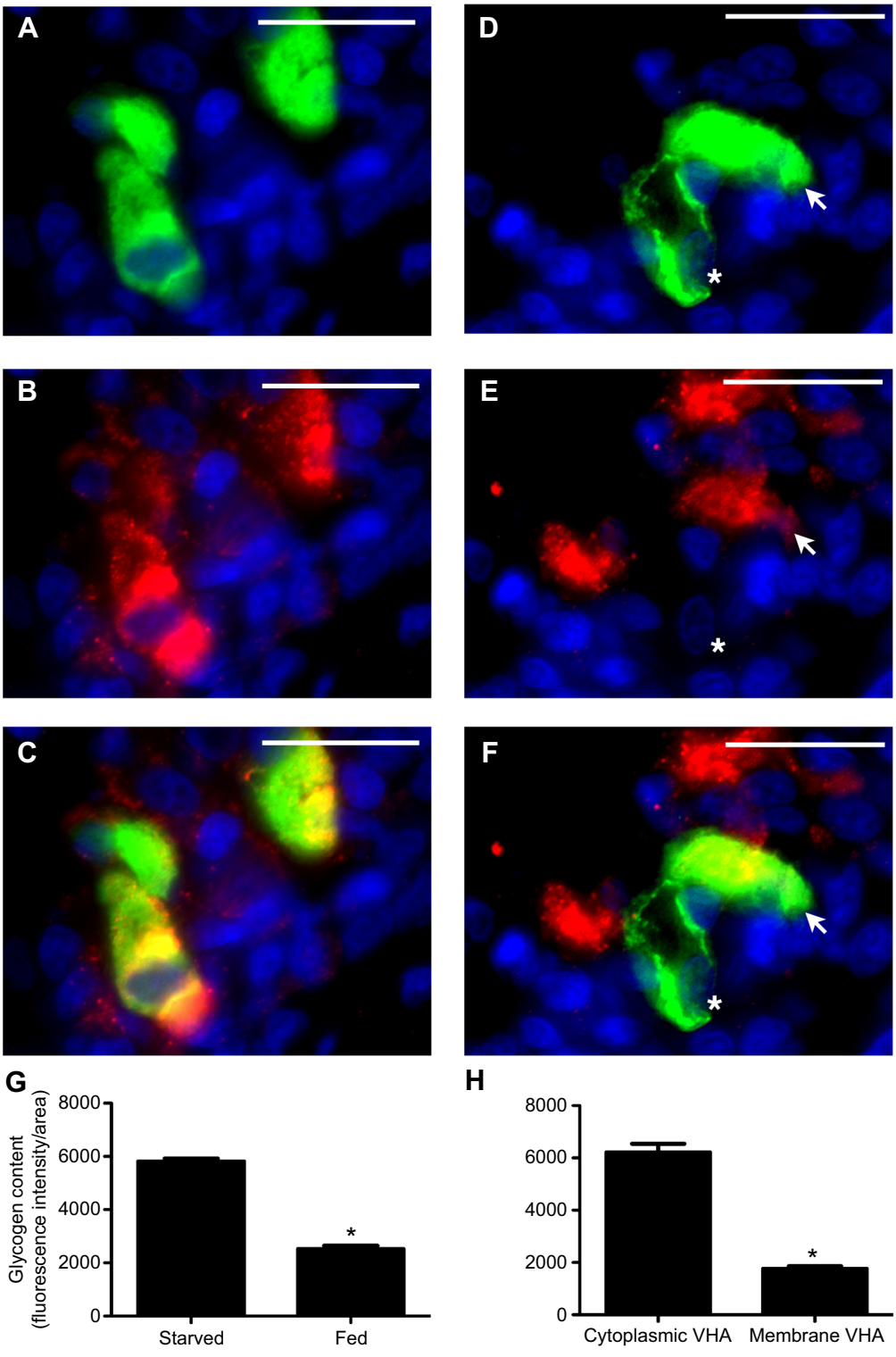

Fig. 3. Glycogen stores in base-secreting VHA-rich gill cells from starved and fed leopard sharks. (A–F) Representative images of VHA (green) and glycogen (red) immunoreactivity in starved (A–C) and fed (D–F) leopard shark gills, with reduced glycogen content in cells with VHA localized to the basolateral membrane (D–F, asterisks) when compared with cells with VHA localized to the cytoplasm (D–F, arrows). (G) Summary of glycogen content in VHA-rich cells from four starved (n=240 cells, cytoplasmic VHA=237, membrane VHA=3) and four fed (n=240 cells, cytoplasmic VHA=39, membrane VHA=201) leopard sharks. Glycogen content was ∼3-fold significantly higher in VHA-rich cells from starved leopard sharks (*P<0.001). (H) Summary of glycogen content in inactive VHA-rich cells with cytoplasmic VHA localization (n=39) and active VHA-rich cells with basolateral membrane VHA localization (n=201) from four fed leopard sharks (*P<0.001). Nuclei stained in blue. Scale bars: 20 μm.

astrocytes for their glycogen stores, shark gill acid–base regulatory cells contain large glycogen stores themselves. Although glucose provided from postprandial intestinal absorption or from liver glycogen stores cannot be ruled out, the clear correlation between intracellular glycogen content and activity status of each individual cell (which we were able to clearly determine based on VHA subcellular localization) indicate that acid- and base-regulatory cells rely on their intracellular glycogen stores for metabolic fuel supply. Furthermore, glycogen utilization and replenishment is dynamic and differentially regulated based on acid–base challenges that determine energy requirements. Thus, shark gill acid–base regulatory cells resemble myocytes in the dynamic nature of glycogen utilization and replenishment, and both have their own intracellular glycogen stores (Graham et al., 2010; Nielsen et al., 2011; Prats et al., 2009).

To our knowledge, this is the first description of glycogen stores, utilization and replenishment in specialized epithelial acid–base regulatory cells. This study also highlights that studying processes

at the cellular level may uncover previously unappreciated complexity. For example, measuring total glycogen levels in gill homogenates would not have revealed differences between starved and fed sharks because the reciprocal changes in NKA- and VHA-rich cells would have counteracted each other.

The results from the present study open up new lines of inquiry about energy metabolism in relation to acid–base regulation. For example, what are the molecular and cellular mechanisms that regulate glycogen store utilization and replenishment? How is differential glycogen utilization by NKA- and VHA-rich cells during acid and base stress achieved? Are there any interactions between local control at the cellular level and whole-animal hormone regulation? Do liver glycogen stores supply glucose to gill acid- and base-regulatory cells, particularly during longer-term acid–base disturbances? Do acid- and base-regulatory cells use other metabolic fuels in addition to glycogen-derived glucose? Do local glycogen stores and differential utilization apply to other acid–base regulatory cells, such as those in the distal convoluted tubule and collecting duct in the mammalian kidney? What are the implications for whole-animal energy budgets during environmental acid–base stress, for example, hypercapnia and ocean acidification? The gills of marine elasmobranchs continue to be an excellent model system to elucidate novel mechanisms about acid–base regulation at the cellular level, and to generate testable hypotheses that may apply broadly to other organisms.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.N.R., M.T.; Methodology: J.N.R., M.T.; Formal analysis: J.N.R., M.T.; Investigation: J.N.R.; Resources: M.T.; Writing - original draft: J.N.R.; Writing - review & editing: J.N.R., M.T.; Visualization: J.N.R.; Supervision: M.T.; Funding acquisition: J.N.R., M.T.

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