Case study: Anoxia tolerance in the Pacific hagfish (*Eptatretus stoutii*)

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Key points

- Pacific hagfish possess an exceptionally low metabolic rate, allowing them to subsist through anaerobic metabolism for many hours.
- Their systemic heart has low ATP demands that lie within its glycolytic capacity.
- During anoxia the heart rate decreases by 50% while the stroke volume doubles, cardiac output drops by 33% and its energy needs drop by 25%.
- Suppression of metabolic rate by 50% during anoxia extends energy reserves.
- Hagfish can buffer against acidosis by exchanging blood Cl^- for HCO_{3^-} across their gills.
- Ionoconformation gives them a higher concentration of Cl^- than other vertebrates allowing for a strong buffering capacity to tolerate acidosis during anoxia.

Glossary

Extant A presently living taxonomic group. The opposite of extinct.

Normoxia The presence of oxygen at saturating concentrations in an environment.

Hypoxia Low environmental oxygen. Less than 30% of the maximum oxygen for the given conditions.

Anoxia No environmental oxygen present.

Osmoconformer An organism that maintains an internal osmolarity that is isotonic to their environment. This minimizes the movement of water into and out of the cells.

Ionoconformer An organism that does not regulate the concentration of ions within its body. Therefore, the concentration of ions is in equilibrium with the environment.

Oxidative phosphorylation (OXPHOS) A series of redox reactions involving the flow of electrons along transporter complexes in the inner membrane of the mitochondria to create a proton gradient, with oxygen as the final electron acceptor. Coupled to the generation of adenosine triphosphate (ATP).

Proton motive force (pmf) The potential energy stored by the concentration of protons within the intermembrane space of the mitochondria. This energy consists of both the concentration difference across the membrane (ΔpH) and the charge difference ($\Delta\Psi$). Used to phosphorylate ADP to ATP.

Lactate fermentation An anaerobic alternative pathway of glycolysis where pyruvate is converted to lactate rather than feeding into the Krebs cycle.

Apoptosis Controlled cell death where cellular components are packaged into vesicles for later disposal.

Necrosis Uncontrolled cell death where the cell lyses, releasing potentially toxic cellular components into the extracellular space.

Acidosis Lowered pH of blood or other bodily fluids.

Exaptation A character that evolved for one purpose that is later able to serve a different purpose.

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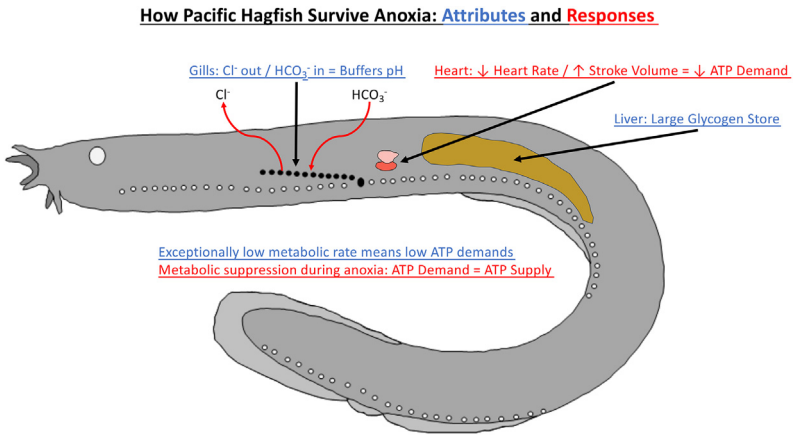
Reoxygenation The return of oxygen following a period of anoxia.
Reactive Oxygen Species (ROS) A family of reactive chemical species derived from O_2. Many ROS are radicals (containing one or more unpaired electrons).

Abstract

Pacific hagfish (*Eptatretus stoutii*) are an agnathan fish capable of surviving 36 h of anoxia. This is achieved through a variety of factors. Under normoxic conditions they possess one of the lowest metabolisms recorded among vertebrates. Their systemic heart has a rate of energy consumption so low that it lies within the capacity of anaerobic metabolism. The energy needs of the heart are reduced by an anoxia-induced reduction in heart rate by 50%, partially compensated for by increased stroke volume. This reduces the energy consumption of the heart by up to a quarter during anoxia. The Pacific hagfish also utilize hypometabolism, reducing their metabolic rate by 50%, to conserve energy during anoxia. These features allow the hagfish to avoid energy depletion during anoxia. Their anoxic survival is also aided by a strong tolerance of acidosis. Pacific hagfish can survive a plasma pH of 6.8. Hagfish have a high blood Cl^- concentration due to ionoconformation with seawater. This gives the hagfish a large capacity to recover blood pH by exchanging Cl^- for HCO_3^-.

Teaching slide



Introduction

Fish are aerobic animals and therefore consume oxygen as a vital part of their biological processes. Oxygen is an essential molecule to oxidative phosphorylation, which is the primary pathway of energy metabolism for most metazoans. Indeed, only a few metazoans are known to live an entire life cycle in the absence of oxygen (Danovaro et al., 2010; Yahalomi et al., 2020). While unicellular organisms possess a greater diversity of forms of energy metabolism, for the overwhelming majority of metazoans oxygen is essential for survival. Since animals, unlike plants, do not produce oxygen themselves, they must acquire it from the environment. A significant body of research exists detailing the various morphological and physiological features that function in oxygen sensing, uptake from the environment, and transport throughout the body. The availability of oxygen in the environment plays a significant role in the form and function of respiratory and circulatory systems in fishes. For example, oxygen is more abundant in air than in water, and cold water can carry more oxygen than warm water. Therefore, a fish living in a tropical climate encounters a different oxygen environment than an Arctic fish and have different respiratory challenges. Hypoxia refers to when an environment is low in oxygen, typically when the oxygen content is below 30% of the maximum concentration for its physical conditions. Hypoxia presents a challenge that can exclude some animals from the environment while others are able to acclimate to hypoxic challenges and thrive in such environments. For reviews of how fishes are able to respond to hypoxia, please see Perry (2011), Richards (2011), Wang and Richards (2011), Rogers et al. (2016). An anoxic environment is one that is completely lacking in oxygen, which presents a far more serious challenge. No known vertebrates can live and reproduce in an anoxic environment. Indeed, for most vertebrates exposure to anoxia results in death within a matter of minutes. However, there are a few vertebrates that can tolerate anoxia for far longer than normal, surviving for hours, days, and even months. Among these are the crucian carp (*Carassius carassius*) and some species of freshwater turtles such as the red eared slider (*Trachemys scripta*) (Bundgaard et al., 2020). The ability to survive prolonged anoxia allows these species to access environments and exploit opportunities that anoxia intolerant species would be unable to. In this article we will be examining a less studied anoxia tolerant fish, the Pacific hagfish (*Eptatretus stoutii*). This species of ancient jawless fish can survive up to 36 h of anoxia (Cox et al., 2010, 2011).

Habitat and physiology of the Pacific hagfish

Hagfishes are a group of 83 species in the Class Myxini. An exclusively marine group, hagfishes have been observed in every ocean except for the polar seas. Hagfish are agnathans, meaning they lack jaws. Hagfish and their relatives in the Superclass Cyclostomi, the lampreys, are the only extant agnathan vertebrates. This makes the hagfish a very old group, with the oldest identified fossil hagfish being approximately 300 million years old (Bardack, 1998). Today, hagfish are found most commonly in cold, deep, and saline waters (Martini, 1998). While rare in shallow waters and therefore relatively unknown, hagfish are so abundant in the deep ocean that they may in fact be one of the most numerous types of fish in the world. In this article we will discuss hagfish more generally as well as focusing on the Pacific hagfish as it is one of the more well studied species. In the discussion of hagfish physiology that follows we will refer to studies on Pacific hagfish whenever possible but will sometimes have to use research with other species with the assumption that the Pacific hagfish are similar.

Hagfish habitat

The Pacific hagfish as the name suggests can be found in the eastern Pacific Ocean from Mexico to Canada and between a depth range of 10−1000 m (Martini, 1998). The species is quite abundant in its range and is found on or near the benthos and frequently ensconced within burrows. The Pacific hagfish is commonly found below 100 m, a habitat that is characterized by low temperatures and productivity. They are known to prey upon smaller fish and invertebrates and are frequent scavengers on the corpses of large bodied animals that drift down from shallower more productive environments (Martini, 1998). As some of the most common carrion feeders in the deep ocean hagfish play an important role in the recycling of nutrients, occupying a similar role to vultures in terrestrial environments. Apart from occasional oases fueled by chemosynthesis, the deep ocean lacks any primary production as there is not sufficient sunlight for photosynthesis. Therefore, these environments are often oligotrophic, low in nutrients, and reliant on detritus and carrion that drifts down from the sunlit shallow environment. These food sources are very thinly spread, and hagfish may go months without feeding (Martini, 1998). Their environment is also a cold one with temperatures ranging from 4 to 10 °C. In addition to the scarcity of food and low temperatures, the deep ocean is frequently oxygen limited, as it is far removed from the photosynthesis and atmospheric mixing that oxygenate shallow waters. Oxygen reaches the deep ocean through downwelling currents that carry oxygenated water from the surface and circulate it through the oceans. Oligotrophy, cold temperatures, and hypoxia are all conditions that favor low metabolism and very slow-paced lifestyles. Hagfish are no exception to this and in the next paragraphs we will discuss the morphology and physiology of the hagfish and how they are adapted to their environment. These traits will in turn have a profound influence on the ability of the Pacific hagfish to survive anoxia.

Hagfish morphology

Hagfish are among the oldest extant vertebrate groups and possess many features that are not seen in more derived fish species. It is not clearly known to what extent these features represent the ancestral vertebrate condition or if they are unique to the hagfish. They are anguilliform (eel like) fish that lack scales, paired fins, jaws, and image forming eyes. The only fin they possess is a single tail fin, which they use to swim by moving their body in a sinusoidal motion. Hagfish do possess simple eyes capable of sensing light and dark that may be used to regulate their circadian rhythm or orient themselves (Collin and Lamb, 2015). Their cousins, the lamprey, have image forming eyes as adults, while the eyes of lamprey larva resemble those of hagfish. The eyes of hagfish represent an example of pedomorphosis or the retention of juvenile traits into adulthood (Collin and Lamb, 2015). Instead of relying on sight, hagfish sense food through olfaction and sensory tentacles framing their single nostril and mouth. Once they locate a corpse, they use keratin plates on their tongues to rasp at the flesh. Hagfish have a skeleton of cartilage and have secondarily lost their vertebrae, allowing them to tie themselves into knots and gain leverage on the bodies they feed on (Martini, 1998). The absence of vertebrae caused much debate over whether the hagfish could be considered vertebrates. They were previously classified as craniates due to the presence of a skull and considered an intermediate between the non-vertebrate chordates and vertebrates. The discovery of vestigial vertebral elements demonstrated that hagfish possessed vertebrae in their evolutionary history and have since lost them (Ota et al., 2011). Therefore, hagfish were reclassified as vertebrates and the craniate group is now defunct. Hagfish are best known for their ability to produce copious amounts of slime when disturbed. Lining the whole length of their body are hundreds of slime glands, the exact number of slime glands varies between different hagfish species. When stimulated these glands release threads of protein, as well as the protein mucin packaged into vesicles. These components interact with seawater to rapidly produce a large volume of slime with a similar consistency to egg whites. This slime serves as a defense mechanism, choking the mouth of their attacker and allowing the hagfish to escape (Fudge et al., 2015). While hagfish slime is of great interest to some researchers, it is a great annoyance to others, excelling at clogging experimental equipment. The final unique feature of the hagfish we will discuss is their circulatory system. The circulatory system of hagfish has been described as partially open and is hypothesized to be an intermediate form between the open circulatory systems of invertebrates and the closed systems of more derived vertebrates (Satchell, 1991). The circulatory system of hagfish operates at extremely low blood pressures and includes large blood sinuses, open spaces where blood pools rather being pushed through narrow vessels. Most notably a large subcutaneous sinus that sits under the skin and covers most of their body. Hagfish in fact have the lowest blood pressure and highest relative blood volume of any known fish species. Hagfish also possess multiple hearts, a main systemic heart that is homologous to the hearts of derived vertebrates, along with accessory hearts (Satchell, 1991). The hagfish systemic heart resembles the hearts of other fish with a single atrium and ventricle, but it lacks any

innervation and has an exceptionally low heart rate (Davison, 2015). Under routine conditions the heart rate of Pacific hagfish has been measured at 10.4 ± 1.3 beats per minute (Cox et al., 2010). The accessory hearts include a portal heart, a small structure consisting of cardiac muscle tissue located on the hepatic portal vein that carries blood from the kidney to the liver. Hagfish have a relatively large liver and the additional force generated by the portal heart is likely necessary to push blood through the liver and back to the systemic heart. A caudal heart in the tail is formed from skeletal muscle and functions to push blood out of the subcutaneous sinus back into the main circulation. The caudal heart of hagfish is potentially homologous to the caudal hearts of the secondary circulation of more derived fish (Satchell, 1991). Finally, there are cardinal hearts in the head that also move blood from the subcutaneous sinus; however, these are not true hearts as they cannot generate contractile force on their own. Instead, the cardinal hearts are blood sinuses that are contracted by the movement of nearby ventilatory muscles. All these features make the circulatory system of the hagfish unique among vertebrates. With its blood sinuses and multiple hearts, it more closely resembles an invertebrate circulatory system than any other fish. These traits make the hagfish a fascinating group of animals to study. In the next paragraph we will discuss the physiological features of hagfish. Here too they prove to be unique among vertebrates.

Hagfish physiology

The physiology of the hagfish is also exceptional. As a group, the hagfish are characterized by a low energy lifestyle. While many deep-sea animals possess slow metabolisms, the hagfish are known to have among the lowest resting metabolic rates of any fish, with the New Zealand hagfish (*Eptatretus cirrhatus*) consuming oxygen at a rate of 6.93 ± 0.23 mL kg^{-1} h^{-1} at 11 °C (**Fig. 1**), this is approximately 10% of that of trout measured at 15 °C (Bushnell et al., 1984). Contributing to their low metabolic rate are both environmental and physiological characteristics. The cold temperatures of their environment lower metabolic rates through the Q10 effect. Meanwhile the limited food and oxygen in the deep ocean make conserving energy a necessity. An active fish with a high metabolism would simply be unable to acquire sustenance (or energy) fast enough to balance energy demand with energy supply. Hagfish are a quite sedentary fish. While it is unknown how far they will travel to find food in the wild, hagfish held in captivity spend most of their time immobile. Spending too much time swimming is a waste of valuable energy. By having a slow paced lifestyle, the hagfish are able to wait out long periods of starvation between rare feeding opportunities. The black hagfish (*Eptatretus deani*), which possess the lowest basal metabolic rate of any known hagfish, is estimated to be able to satisfy its energy requirements for a whole year with only 1.5 h of feeding (Smith, 1985). Hagfish are also unique in being the only vertebrates that are osmo- and ionoconformers (Clifford et al., 2015). Being osmoconformers means that the osmolarity, the solute concentration, of their tissues matches the surrounding seawater. Therefore, there is no net movement of water between their tissues and the environment. Ionoconformation means that composition of ions that make up this solute concentration are also the same as the external environment. Hagfish do regulate some ions. The monovalent ions Na^+ and Cl^- are not regulated and conform to their concentrations in seawater. While the divalent ions Ca^{2+}, Mg^{2+}, and SO_4^{2-} are regulated at levels different from the external milieu (Sardella et al., 2009). The condition of being both osmo- and ionoconformers is more common among invertebrates than vertebrates. Elasmobranchs (sharks, skates, and rays) are osmoconformers, but they achieve this with high concentrations of urea rather than matching the ion balance of their environment. Meanwhile marine teleosts are hypoosmotic, maintaining an internal solute concentration far lower than seawater. The unique osmotic strategy of the hagfish is possibly due to them being the only extant vertebrate group to never penetrate freshwater and perhaps represents the ancestral condition of vertebrates (Baker et al., 2015). Maintaining an osmotic balance different from the environment requires consuming energy to keep multiple ionic gradients far

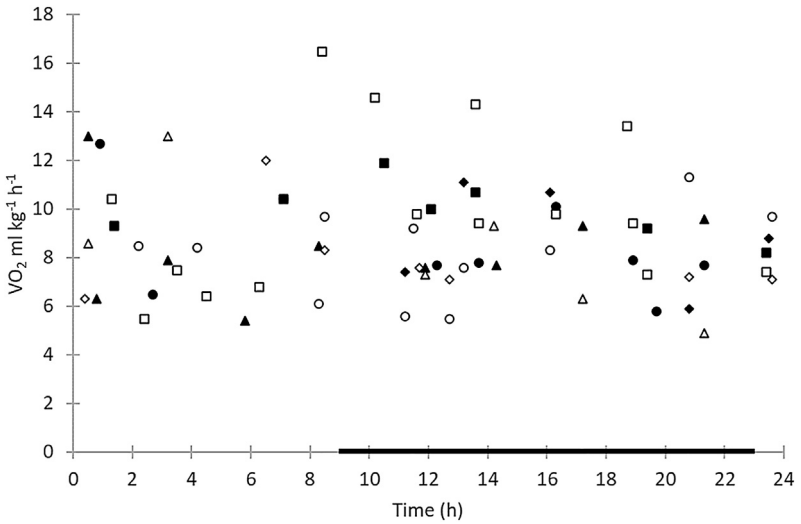


Fig. 1 Oxygen consumption of New Zealand hagfish (*Eptatretus cirrhatus*) over a 24 h period starting at 8:00 a.m. Different symbols denote different individuals. The dark shading along the x-axis indicates periods of darkness. Modified with permission from Forster (1990).

from equilibrium. Since the ions will always move down their concentration gradients toward equilibrium, they must be actively pumped back to maintain the ideal concentrations. This can make up a significant portion of an animal's energy budget. Therefore, the osmotic strategy of hagfish may help them save energy as they do not have to spend as much energy pumping ions or water across cell membranes to maintain an osmotic balance out of equilibrium with seawater. Both the low metabolic rate and osmotic strategy of hagfish will be relevant to how they are able to tolerate hours of anoxia exposure. An open question to consider during the discussion of hagfish anoxia tolerance is: Are these traits the result of the hagfish's basal position on the vertebrate evolutionary tree or are they later adaptations? If an adaptation, was the selection pressure that drove their evolution anoxia, or the conditions of the deep ocean and the need to preserve energy?

How the Pacific hagfish survive anoxia

The anoxic energy deficit

Anoxia presents a variety of challenges that animals must overcome if they are to survive longer than a few minutes. While anoxia effects the whole animal its consequences are clearly seen at the cellular level. The cells that make up an animal require a continuous input of energy to survive and perform their functions. Many biological processes require gradients to be maintained far from thermodynamic equilibrium, which in turn means that energy must be spent to keep them there. For example, the ion balance of cells is maintained far from equilibrium with the extracellular space and the membrane bound K^+ and Na^+ transporters that maintain this gradient are among the largest energy consumers in a cell. These transporters along with many enzymes in the cell rely on phosphorylation by adenosine triphosphate (ATP) to function. Therefore, a vital process in any cell is the production of ATP through a series of catabolic pathways that breakdown fuel molecules (sugars, lipids proteins) to release their chemical energy to produce ATP. In aerobic species, catabolism can be divided into two areas. The initial steps of catabolism occur within the cytosol. Pathways such as glycolysis break down molecules into pyruvate, acetyl-CoA, and NADH. In aerobic conditions these products are fed into the mitochondria, where the Krebs cycle (or tricarboxylic acid cycle) fully breaks them down into CO_2 and H^+. The reducing equivalents (NADH and $FADH_2$) produced by the Krebs cycle are then recycled by the electron transport system (ETS). The ETS is a system of protein complexes associated with the inner mitochondrial membrane. Each complex functions differently, but to summarize they accept protons and electrons from NADH and $FADH_2$ and pump the protons into the intermembrane space. Through this process they regenerate NAD^+ and FAD^+, which cycle back into the Krebs cycle. Oxidative phosphorylation creates an unequal concentration gradient (the proton motive force, pmf) across the inner mitochondrial membrane. Since moving protons against their concentration gradient requires energy, the process is made thermodynamically favorable by passing the electrons down the chain of complexes in the ETS. Oxygen plays a role as the final electron receptor, producing H_2O as a waste product. Without oxygen in the mitochondria the ETS is unable to function. Since the rules of thermodynamics mean any gradient must move toward equilibrium, the pmf is a store of potential energy. The final step in aerobic metabolism involves the enzyme complex ATP synthase. ATP synthase forms a channel in the inner membrane for protons to flow from the region of high concentration in the inner membrane space to the region of low concentration in the mitochondrial matrix. As they do so, ATP synthase captures some of the energy released to catalyze the phosphorylation of ADP to ATP. This process is similar to how a hydroelectric dam converts the energy of flowing water into electricity. While oxygen is not directly involved in the reaction that produces ATP the consumption of oxygen and the phosphorylation of ATP are coupled together through the pmf. Therefore, this process is termed oxidative phosphorylation. When oxygen is available, aerobic catabolic pathways are favored, accounting for 95% of ATP production in resting metabolism. Aerobic metabolism is a desirable pathway as the breakdown of molecules into CO_2 and H_2O releases the most energy from their chemical bonds, while producing waste products that are minimally toxic and easily disposed of. As an example of the efficiency of aerobic catabolism this pathway produces 38 mol of ATP for per mole of glucose consumed.

In contrast, anaerobic metabolism, or energy production in the absence of oxygen, is far less efficient. Anaerobic metabolism consists only of the cytosolic breakdown of molecules into the products that would be fed into the mitochondria. While some ATP is produced during this process, termed substrate level phosphorylation, much of the chemical energy remains in an unusable form. Returning to the example of glucose, anaerobic glycolysis breaks down glucose to the level of pyruvate, producing only 2 mol of ATP per mole of glucose. Since this process does not break down pyruvate into smaller units, the energy stored in the unbroken chemical bonds of the carbohydrate molecule remains unavailable. This is clearly far less than aerobic metabolism can provide. In addition, anerobic metabolism also produces NADH that would normally be recycled in the mitochondria when oxygen is available. Instead, most vertebrates use lactate dehydrogenase (LDH) to regenerate the NAD^+ needed to continue glycolysis by converting pyruvate to lactate. This enzyme is found in high levels in tissues that rely heavily on anaerobic glycolysis. For example, the hagfish tongue is particularly well adapted for anaerobic metabolism being an active muscle with poor blood supply. Therefore, hagfish tongue has an exceptionally high activity of lactate dehydrogenase. We will return to the topic of lactate fermentation later in this article when we discuss anoxic acidosis. The difference between the energy production of aerobic and anaerobic metabolism is very important for our discussion of anoxia. The lower efficiency of anaerobic metabolism means that during anoxia the ATP supply cannot meet the ATP demand, creating an energy deficit in the cell. In anoxia intolerant animals this energy deficit quickly results in the depletion of energy stores and the failure of biological processes. Energy depleted cells cannot perform their function, leading to organ failure. Furthermore, when a cell runs out of energy the membrane bound ion pumps fail, allowing ions to flow down their concentration gradients. The breakdown of membrane gradients leads to the failure of many cellular processes causing

the cell to undergo apoptosis or necrosis. Cell death due to anoxia can lead to the death of the whole organism or permanent impairment that impacts future survival.

So how does the Pacific hagfish avoid an anoxic energy deficit?

Now that we have discussed the anoxic energy deficit, we can discuss how the Pacific hagfish is able to survive for over a day in conditions that kill most vertebrates in minutes. We will focus first on how the Pacific hagfish maintains the function of its systemic heart during anoxia. The heart is a valuable organ to study for anoxia tolerance as it must continue functioning throughout anoxia to mobilize metabolic fuels and remove wastes (Stecyk et al., 2008). The heart is often considered a particularly vulnerable organ to anoxia as it requires a constant supply of energy and oxygen to keep functioning and its function cannot be easily decreased without consequences for the rest of the body. The function of Pacific hagfish hearts during anoxia has been studied by two methods. Measuring devices can be surgically implanted in the hagfish to measure the function of their hearts, or the heart itself can be removed from the body and perfused with a saline solution where it will continue to beat. When Pacific hagfish were exposed to 36 h of anoxia it was found that their heart dropped by 50% from approximately 10 beats per minute to five beats per minute within the first 2 h. Meanwhile the stroke volume doubled from 1.3 ± 0.1 mL per kg to 2.7 mL per kg. As a result of this decrease in heart rate and increase in stroke volume the cardiac output, the amount of blood pumped out by their systemic heart, dropped by 33% from a routine value of 12.3 ± 0.9 mL per min per kg. This shift of the heart function into a new steady state means that the cardiac power output (a measure of energy consumption) dropped by 25% (**Fig. 2**) (Cox et al., 2010). This shows how the Pacific hagfish heart shifts its function into a new steady state that can be maintained anaerobically (Cox et al., 2010). This shift in heart function is likely controlled by catecholamine levels in the heart since the hagfish systemic heart lacks any neuronal inputs (Farrell, 2007). Experimental manipulation of catecholamine levels does influence the heart rate of Pacific hagfish (Axelsson et al., 1990). Catecholamines that stimulate heart rate require oxygen to be synthesized, so their depletion during anoxia may be the trigger to reduce the heart rate (Cox et al., 2010). The reduction in cardiac output reduces the energy demand of the heart during anoxia allowing those energy needs to be met through anaerobic metabolism. In the next paragraph we will discuss how the hagfish are able to supply those energy needs.

As a group, hagfish represent the lower extreme of vertebrate cardiac energy use. Their systemic heart has the lowest routine pressure and heart rate of any vertebrate (Forster, 1991). The aortic blood pressure of New Zealand hagfish and Pacific hagfish has been measured at 1.4 ± 0.09 and 0.89 ± 0.9 kPa, respectively (Forster et al., 1988; Cox et al., 2010). This is less than one-third that of rainbow trout measured at $\sim$12 °C (Randall et al., 1965). Meanwhile the heart rate of Pacific hagfish has been measured at 10.4 ± 1.3 beats per minute during routine conditions (Cox et al., 2010). The result of this is that the hagfish heart does not need much energy or oxygen to function. The systemic heart of a Pacific hagfish utilizes ATP at a rate of approximately 10 μmol per gram per hour. An average sized heart of around 150 mg could run for 12 h with an exceptionally small 18 μmol of ATP (Gillis et al., 2015). The minimal oxygen requirements of the hagfish hearts are reflected in their morphology. While the hearts of more derived fish often possess a blood supply to the heart muscle, termed coronary circulation, this is not seen in hagfish. Meaning that the heart receives all its oxygen from venous blood that has already passed through the entire circulatory system. Hagfish are not the only fish that lack coronary circulation. This adaptation is used to deliver additional oxygen to the heart and is therefore seen in more active fish whose hearts work harder. While fish with low energy lifestyles and less active hearts do not require this additional circulation. Furthermore, due to the large blood volume and low pressure, the time it takes for blood to complete a circuit may exceed 5 min. So even in conditions of environmental normoxia the hagfish heart may frequently become hypoxic during periods of increased activity when other tissues consume most of the oxygen in the blood before it can reach the heart. The exceptionally low ATP and oxygen requirements of the hagfish heart mean that it is already a practically anaerobic organ. Research examining the anaerobic capacity of the hagfish heart indicated that the hagfish should be able to maintain routine heart function through anaerobic metabolism (Forster, 1991). This hypothesis has been supported by experiments that demonstrated that the hearts of Atlantic hagfish (*Myxine glutinosa*) continue beating after their mitochondria have been poisoned with cyanide but stop working when poisoned with idoacetate, a toxin that stops glycolysis (Hansen and Sidell, 1983). When the hearts of Pacific hagfish were isolated and a calorimeter was used to determine metabolic rates by the measurement of heat production, it was found that energy production dropped during the first hour of anoxia before recovering to routine levels over the next 15 h. This is likely due to the shift from aerobic to anaerobic ATP production with the rate dropping initially as oxidative phosphorylation ceases and recovering as anaerobic pathways are upregulated (Gillis et al., 2015). When Pacific hagfish hearts were removed and perfused with a metabolite free saline solution to measure the depletion of their internal glycogen stores it was found that glycogen was 85% depleted following 8 h of anoxia. However, the hearts continued to function for a further 8 h without any decline in their performance (Gillis et al., 2015), indicating that an alternative fuel is utilized in addition to glycogen to fuel the anaerobic metabolism of the hagfish heart. A follow up experiment perfused the hearts with glycerol, which did stimulate increased function during anoxia. However, it was not clear if the glycerol was being utilized as a fuel source or if it was stimulating the heart through other means (Gatrell et al., 2019). In summary, the systemic heart of the Pacific hagfish has exceptionally low requirements for energy and oxygen compared to that from more derived vertebrates. So low that it can function normally even with the far less efficient ATP synthesis afforded by anaerobic metabolism. During anoxia the energy needs of the heart can be reduced even more by reducing the cardiac output. This enables the heart to avoid a deficit of ATP that would result in a failure of cardiac function.

Of course, the heart is not the only organ in the body and there are many other energy consuming processes that must be accounted for to avoid a deadly energy deficit. Hagfish are well suited to tolerate anoxia as they possess the lowest routine metabolic

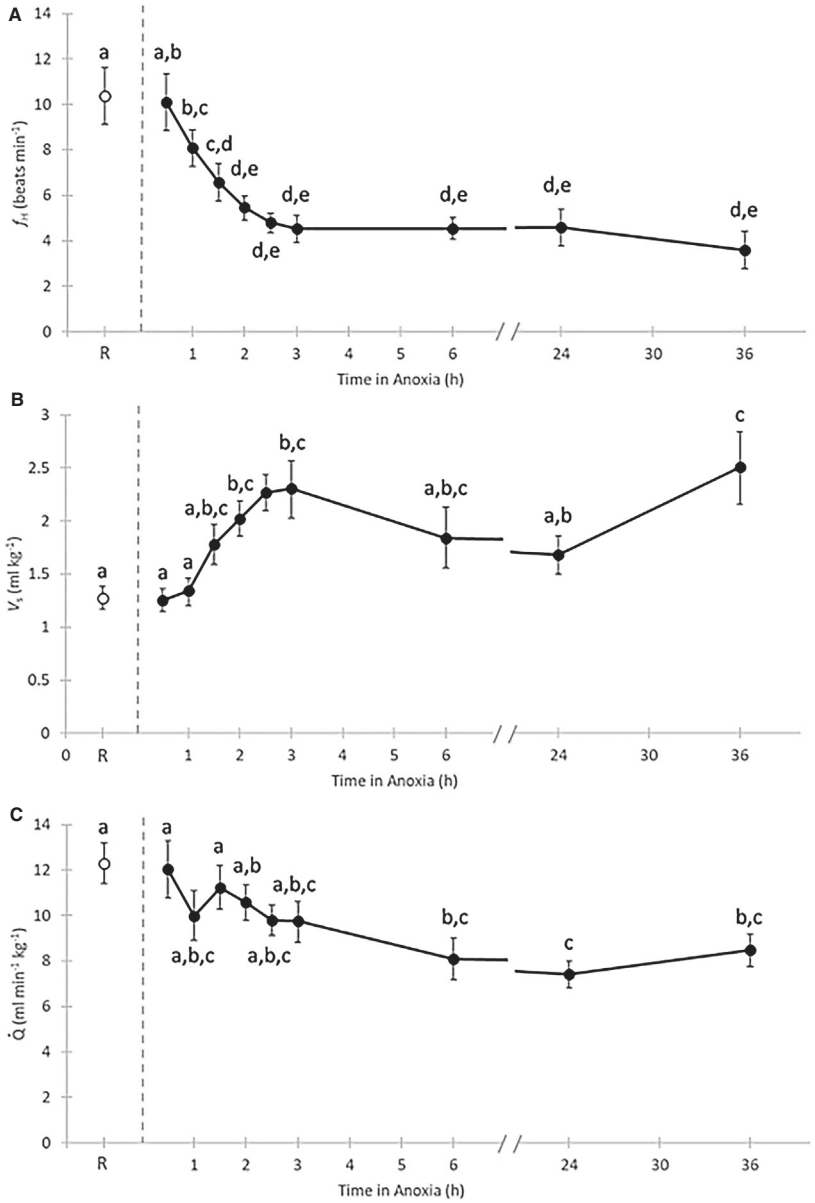


Fig. 2 Cardiovascular variables from hagfish during a 36 h anoxic exposure. Time 0 indicates the start of anoxia. R indicates routine values. Statistical differences ($P < 0.05$) are indicated by dissimilar letters. (A) f_H, heartrate, declines by 50% during anoxia. (B) V_S, stroke volume, increases by 100% during anoxia. (C) $\dot{Q}$, cardiac output, decreases by 33% during anoxia. Modified with permission from Cox et al. (2010).

rate among fishes (Forster, 1990). In addition to having exceptionally low energy requirements, hagfish also possess large glycogen stores that can support anaerobic metabolism for several hours (Forster, 1991). These features give the hagfish a significant advantage in surviving anoxia compared to more active species with higher routine metabolisms. Their low metabolism and large glycogen stores mean that for the Pacific hagfish, losing access to aerobic metabolism is not an immediate crisis. Having a slow metabolism while being able to stock large energy reserves can be seen as an adaptation to living in the deep ocean where they may go months between feeding opportunities. These characteristics of the hagfish may also be exaptations, features that evolved for one purpose that have incidental benefits in another, for surviving anoxia exposure. As one strategy employed by other anoxia tolerant species is hypometabolism, the reduction of their metabolic rate during anoxia to conserve energy. Freshwater turtles, which can survive months of anoxia, reduce their metabolism by over 90% (Milton and Prentice, 2007; Bundgaard et al., 2020). By slowing their metabolism to a crawl, the turtles can survive exceptional periods of anoxia but the cost of this is that they enter a catatonic state and cannot respond to stimulus. Hagfish on the other hand can remain active during anoxia. Hagfish are often a very sedentary species, preferring to wait in burrows or in a coiled position unless actively swimming. This makes it hard to judge their activity levels during anoxia. However, since they feed inside the anoxic body cavities of deceased whales, they must be capable of active movement. To test whether hagfish utilize hypometabolism to conserve energy during anoxia, experiments were performed

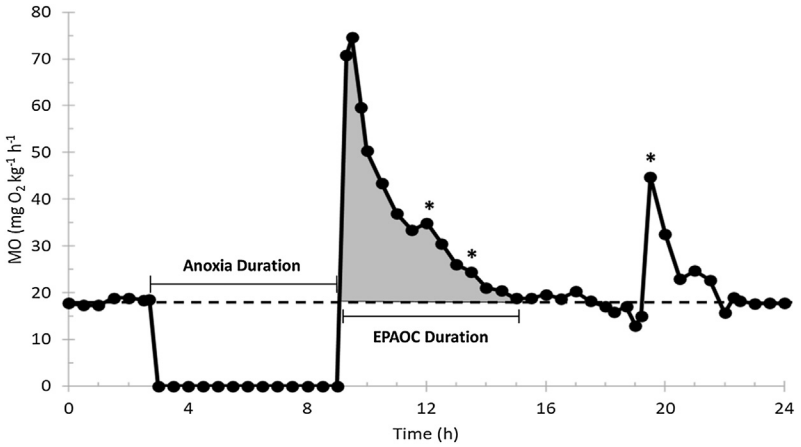


Fig. 3 Representative oxygen consumption trace from a hagfish exposed to 6 h of anoxia. Dashed line indicates the resting metabolic rate. Shaded area indicates the EPAOC. Asterisks indicate periods of spontaneous activity. Modified with permission from Cox et al. (2011).

that estimated the metabolic rate of Pacific hagfish in anoxia by measuring the excess post-anoxic oxygen consumption (EPAOC). Following reoxygenation from anoxia exposure the oxygen consumption of the hagfish increases above the routine metabolic rate. This is necessary to replenish ATP stores that were depleted during anoxia and can be thought of as repaying the energy debt incurred by the anoxic energy deficit. This is similar to the spike in oxygen consumption observed following exercise where energy stores utilized by muscles must be replenished. By comparing the measured EPAOC to a predicted value calculated from the routine metabolic rate it can be determined if the hagfish's metabolic rate was suppressed during anoxia (**Fig. 3**). If the EPAOC is lower than predicted that will indicate that the hagfish did suppress their metabolism since their energy debt was smaller than it would be if energy consumption was unchanged. When this experiment was performed it was found that the Pacific hagfish reduced their metabolism by an estimated 50% during a 36-h anoxia exposure (Cox et al., 2011). This metabolic suppression is less than what is seen in other species with longer anoxia survival times but still serves to extend the hagfish's energy stores while also allowing it to remain active. While other species must enter a catatonic state to survive anoxia the Pacific hagfish is still able to swim and feed. This strategy to delay the anoxic energy deficit is effective enough that in metabolically active tissues (heart, liver, tongue, and skeletal muscle) glycogen stores were reduced by only 60% after 36 h of anoxia (Cox et al., 2011). This suggests that energy exhaustion is not the limiting factor in the Pacific hagfish's anoxic survival and supports the suggestion that the ability of hagfish to store significant amounts of metabolic fuel is an exaptation to anoxia exposure. Therefore, we will continue our discussion by examining another consequence of anoxia that the hagfish must overcome which is the increasing acidity of their tissues.

Anoxic acidosis

In addition to the energy deficit another challenge during anoxia is acidosis. Fish, like all organisms, regulate their internal pH within a very tight range. The enzyme systems that comprise metabolic processes have a narrow pH optimum for their ideal function. Therefore, changes in pH in either direction are expected to result in reduced performance. Extreme shifts in pH can cause the denaturing of proteins, leading to injury or death. Under normal conditions excess H^+ and OH^- are continuously produced by metabolic processes and removed through excretion. A key aspect of acid base regulation is the bicarbonate buffer system where excess H^+ ions can react with bicarbonate (HCO_{3^-}) to form water and CO_2 which can be easily excreted into the external environment. However, when that homeostasis is disrupted, animals must have means of returning their internal pH to its normal range. Disruptions in pH can occur because of a variety of factors including temperature, environmental pH, exercise, and hypercarbia (elevated environmental CO_2). Anoxia is also an inducer of increased internal pH for much of the same reasons as exercise. In both cases, an increase in the utilization of anaerobic metabolism is associated with a decrease in pH. It is often stated that the production of lactic acid as the end product of anaerobic metabolism is the cause of acidosis. However, this is a misconception. Lactate, not lactic acid, is formed during lactate fermentation as no H^+ ions are liberated during the reaction. Instead, the acidosis is a product of ATP hydrolysis which liberates a proton during the transition to ADP. When ATP hydrolysis is low and can be met entirely by aerobic metabolism, the protons can contribute to the generation of the proton motive force within the mitochondria and be recycled in ADP phosphorylation. During periods of intense ATP hydrolysis, such as exercise, or low ADP phosphorylation, such as anoxia, the rate of proton production can exceed the buffering capacity of the cell and result in acidosis (Wang and Richards, 2011).

While in the previous section we discussed how the Pacific hagfish were able to meet their ATP demands anaerobically, anaerobic metabolism resulted in acidosis during anoxia. Over 36 h of anoxia the blood pH of Pacific hagfish declined from 7.9 to 7.03 (Cox et al., 2011). Similarly the pH of isolated hearts from Pacific hagfish decreased from 7.03 to 6.96 after 8 h of anoxia exposure (Gillis et al., 2015). The unique biology of the hagfish plays a role in their ability to respond to challenges to their acid-base homeostasis.

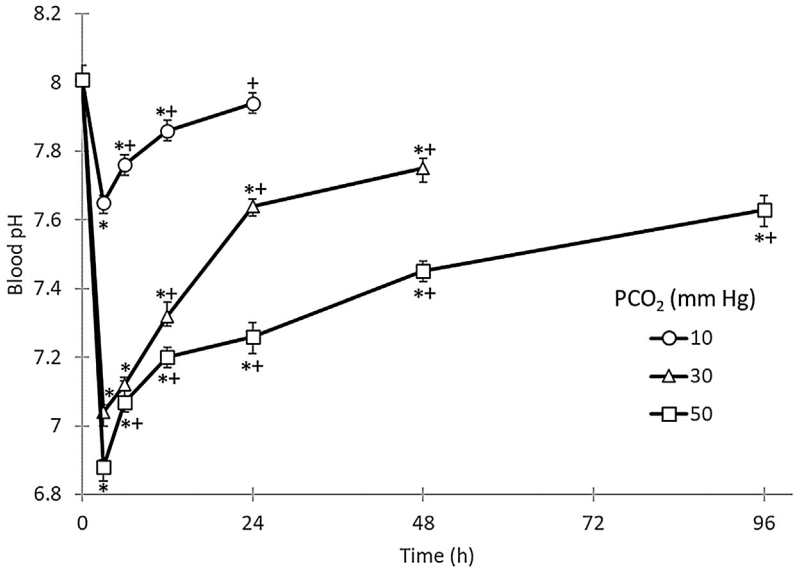


Fig. 4 Effect of elevated water PCO_2 on blood pH in Pacific hagfish over a 96 h period. Values are mean ± SEM. Asterisk indicates a significant difference from the time zero pH for a PCO_2 treatment. Plus signs indicate a significant difference from the lowest pH recorded for a PCO_2 treatment. Modified with permission from Baker et al. (2015).

The hagfish are the only vertebrates that are iono-osmoconformers, likely due to their evolutionary history as the only extant vertebrates to never inhabit freshwater. The hagfish regulate divalent ions in their bodily fluids while the monovalent ions Na^+ and Cl^- conform the environment. The osmotic strategy of the hagfish informs their acid-base regulation. When Pacific hagfish were exposed to a hypercarbic environment to induce acidosis, their blood pH initially declined to 6.81 ± 0.03 when exposed to 50 mm Hg PCO_2, the greatest level of survivable acidosis reported for a water breathing animal (Baker et al., 2015). The blood pH recovered over 96 h of hypercarbia, demonstrating that the hagfish have an impressive ability to regulate their pH (**Fig. 4**). The recovery of blood pH was associated with an increase in HCO_{3^-} concentration and equimolar decrease in Cl^- concentration (**Fig. 5**). This strategy of exchanging chloride for bicarbonate in the environment to buffer acidosis has been utilized by other water breathing animals. However, the Pacific hagfish take this to a further degree than any other known species, increasing HCO_{3^-} concentrations by as much as 78.2 ± 4.5 mM. Typically, fish can only increase their plasma HCO_{3^-} content by 25−33 mM. It is likely that the iono-osmoconforming strategy of the hagfish is responsible for their extreme ability for acid base regulation through chloride/bicarbonate exchange. Since the hagfish possess high levels of Cl^- they possess a large reserve that can be traded for HCO_{3^-}. In both hagfish and teleosts, the proportion of Cl^- that could be removed from the plasma was the same, with the limit being around 17%. However, in the hagfish 17% of their plasma Cl^- represents a far larger amount giving them a higher ceiling for acid-base regulation (Baker et al., 2015). The difference in osmotic strategies between hagfish and marine teleosts is possibly a result of their differing evolutionary histories, with the ancestors of teleosts migrating to freshwater before returning to marine life while the ancestors of modern hagfish remained in the ocean. Therefore, it is likely that the usefulness of iono-osmoconforming for mitigating anoxic acidosis is an exaptation. This osmotic strategy also helps to save significant cellular energy. By having an osmotic strategy that keeps their cells far closer to equilibrium with the external environment hagfish can spend less energy on ion regulation. This likely contributes to them having the lowest energy consumption among vertebrates and saves energy during anoxia.

Pacific hagfish and reoxygenation

While we have covered the challenges that occur during anoxia, the return of oxygen is not the end of the ordeal. Earlier we discussed the role that mitochondria play in aerobic metabolism, while in contrast anaerobic metabolism occurs entirely within the cytosol. However, the mitochondria are not simply dormant during anoxia. Instead, the mitochondria become unbalanced when exposed to anoxia in such a way that this causes dangerous disfunction upon reoxygenation. As the ETS cannot function to maintain the pmf in the absence of oxygen, the proton gradient will decline through proton leak across the membrane (St-Pierre et al., 2000). In order to maintain the pmf the ATPase complex will run in reverse, turning the mitochondria from an ATP producer to an ATP consumer (St-Pierre et al., 2000). A second consequence of anoxia is the accumulation of succinate by the Krebs cycle. When oxygen returns, the accumulated succinate is rapidly oxidized causing a hyperpolarization of the proton gradient (Chouchani et al., 2014, 2016). This hyperpolarized gradient creates favorable conditions for reverse electron transfer (RET) a process where protons move in reverse

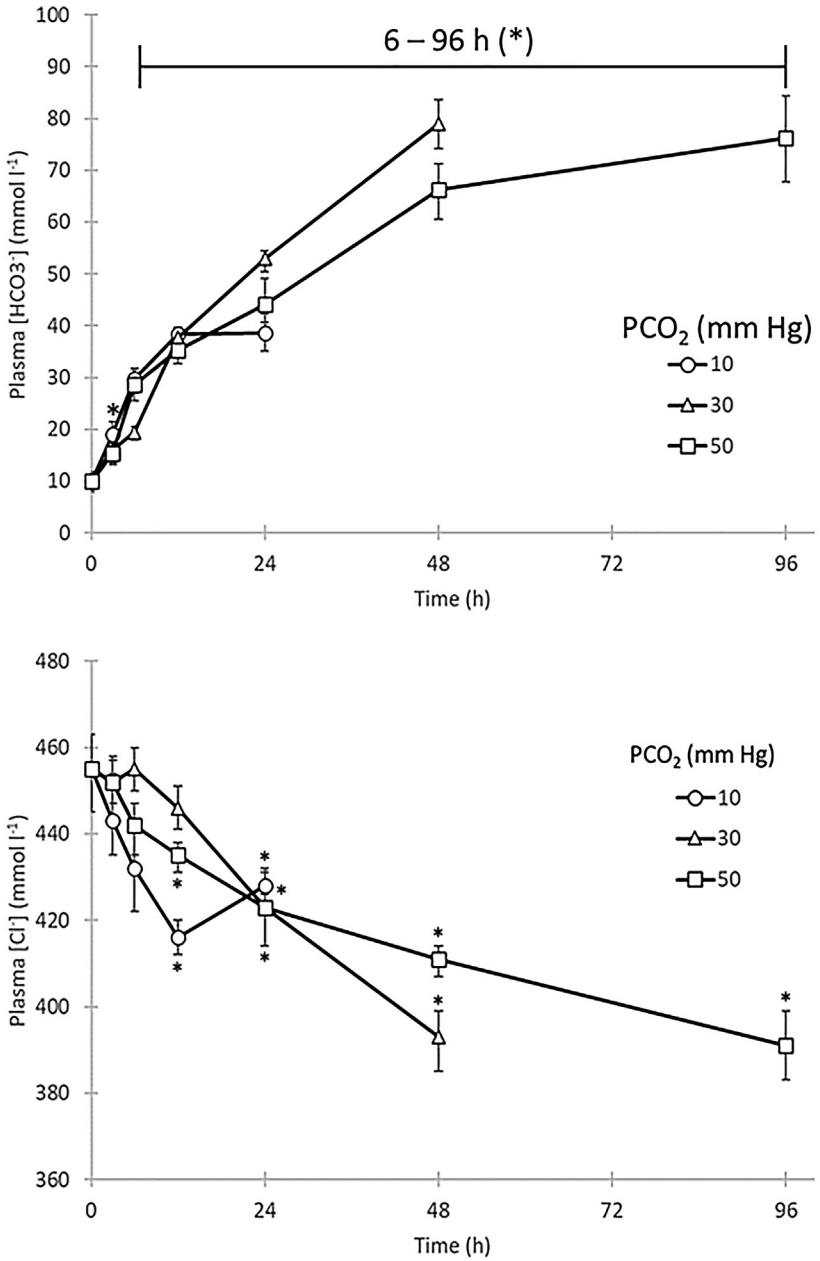


Fig. 5 Effect of elevated water PCO_2 on plasma HCO_3^- and Cl^- concentrations in Pacific hagfish over a 96 h period. Values are mean ± SEM. Asterisk indicates a significant difference from the time zero pH for a PCO_2 treatment. Modified with permission from Baker et al. (2015).

through Complex I of the ETS. This process produces reactive oxygen species (ROS), highly reactive molecules that can cause damage to cellular components by breaking or changing chemical bonds. While the preceding anoxia can cause significant cell death, the surge of oxidative damage during reoxygenation can be even more damaging. An example from human health is the ischemia reperfusion injury that occurs during a heart attack or stroke. Strategies to mitigate this mitochondrial disfunction are known from other anoxia tolerant species. Several species inhibit ATPase during anoxia to reduce ATP consumption (St-Pierre et al., 2000). The surge of ROS can be countered through antioxidant enzymes that detoxify these dangerous compounds. High constitutive antioxidant activity or an upregulation of enzyme activity in response to anoxia (Willmore and Storey, 1997; Cox and Gillis, 2020). However, while we can examine the ways in which other anoxia tolerant species respond to mitochondrial disfunction it is not known how the Pacific hagfish meet this challenge. The effects of reoxygenation on Pacific hagfish and their responses to it represent a gap in our current understanding of this species. Future research should aim to address this knowledge gap and determine how the Pacific hagfish survive the challenges of reoxygenation.

Summary

We have discussed in this article how the Pacific hagfish can tolerate the challenges of energy depletion and acidosis during anoxia. Contributing to their anoxia tolerance are what could be described as passive physiological systems, at least in the context of their role in anoxia tolerance. These systems can be considered passive as their function does not change from routine normoxic conditions to anoxic conditions. An exceptionally low metabolism and substantial fuel reserves are advantageous to a low activity lifestyle where food is a rarity. These physiological systems aid the hagfish in avoiding the anoxic energy deficit by enabling them to subsist off anaerobic metabolism for many hours. Meanwhile the hagfish's ancestral osmotic strategy of being both osmo- and ionoconformers affords them an exceptional resistance to acidosis, while also saving energy. These physiological systems, likely exaptations, that either aid the hagfish in surviving the deep sea or are holdovers of their ancient past almost paint a picture of the Pacific hagfish as a species for whom anoxia is simply not a serious stressor. However, the hagfish do demonstrate some active responses to anoxia through changes in their physiological function that are triggered by the absence of oxygen. Metabolic rate suppression, a strategy seen in other anoxia tolerant species, is employed to conserve energy and prolong their fuel reserves. Shifting their systemic heart into a new steady state, where it beats at a slower rate and reduces its energy consumption, extends its energy reserves and delays acidosis. While the hagfish may not tolerate anoxia for as long as other species, they appear to have achieved their anoxia tolerance through very different means. For example, the freshwater turtles slow their metabolism to the bare minimum required to sustain life and the crucian carp ferment ethanol, but the Pacific hagfish simply carries on. What physiological responses they do show with anoxia exposure are muted compared to their contemporaries. Perhaps the hagfish are a species whose unique physiology has, by chance, given them the ability to survive a challenge that would be lethal to nearly all other forms of vertebrate life.

See Also: Anaerobic metabolism in fish; Behavioral responses to hypoxia; Case study: The anoxia-tolerant crucian carp; Gas transport and exchange: Interaction between O_2 and CO_2 exchange; Integrated response of the cardiovascular system to hypoxia; Metabolic rate depression as a mechanism for surviving hypoxia; Oxygen in the marine environment; Oxygen sensing in fish; The effect of exercise on respiration; The hypoxic ventilatory response and oxygen sensing in fishes.

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