Not only students can express alcohol dehydrogenase: goldfish can too!

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Chamberland V, Rioux P. Not only students can express alcohol dehydrogenase: goldfish can too! Adv Physiol Educ 34: 222–227, 2010; doi:10.1152/advan.00088.2009.—This article describes a novel approach to study the metabolic regulation of the respiratory system in vertebrates that suits physiology lessons for undergraduate students. It consists of an experimental demonstration of the goldfish’s (Carassius auratus) adaptations to anoxia. The goldfish is one of the few vertebrates showing strong enzymatic plasticity for the expression of alcohol dehydrogenase (ADH), which allows it to survive long periods of severe anoxia. Therefore, we propose two simple laboratory exercises in which students are first asked to characterize the distribution of ADH isoforms in the goldfish by performing cellulose acetate electrophoresis. The second part of this laboratory lesson is the determination of liver glycogen. To further student comprehension, an interspecies comparative component is integrated, in which the same subjects are studied in an anoxia-sensitive species, the brook charr (Salvelinus fontinalis). ADH in goldfish is restricted to skeletal muscles, where it catalyzes alcoholic fermentation, permitting ethanol excretion through the gills and therefore preventing lactate acidosis caused by sustained glycolysis during anoxia. Electrophoresis also reveals the occurrence of a liver isozyme in the brook charr, which ADH catalyzes in the opposite pathway, allowing the usual ethanol degradation. As for the liver glycogen assay, it shows largely superior content in the goldfish liver compared with the brook charr, providing goldfish with a sustained energy supply during anoxia. The results of this laboratory exercise clearly demonstrate several physiological strategies developed by goldfish to cope with such a crucial environmental challenge as oxygen depletion.

Carassius auratus: anoxia; glycogen; cellulose acetate electrophoresis; physiology

The expertise of the Department of Biology of the Université du Québec à Rimouski’s is mainly in the conservation of marine, freshwater, and terrestrial diversity; undergraduate students often share a common interest in wildlife management and environmental sciences. Therefore, we developed experimental laboratories integrating ecological concerns and comparative physiology. As demonstrated by Dohn et al. (9), focusing on the improvement of contextual interests in physiology classes fosters student motivation for this discipline and thus increases their academic performance.

The exercises proposed in this article are suitable for physiology undergraduate classes and may be achieved within a 6-h period or in two separate 3-h laboratory blocks. They have successfully been performed several times at the university’s laboratories in undergraduate classes and are now part of our physiology lessons. We find them particularly interesting because the exercises include an integrative component: they consider the metabolic aspect of one of the goldfish’s physiological adaptations to a specific environmental challenge. Only a few educational articles have exploited marine or freshwater fishes as models to teach physiology concepts to undergraduate students (2, 3, 6–8, 16, 17, 29–31, 36). In addition, most laboratory textbooks discussing the physiology of respiration use an anthropocentric approach to address the regulation of the respiratory system in vertebrates. Authors often propose exercises involving gas exchange and generally focus on anatomy, histology, and pulmonary functions (5, 10, 12, 22, 26, 37). Thus, this article describes an innovative approach to study respiratory adaptations of fishes in response to ecological pressures, consisting of a metabolic adjustment to oxygen depletion. Here, we suggest two exercises that clearly demonstrate the goldfish’s adaptations to survive anoxia, with the first exercise consisting of cellulose acetate electrophoresis of alcohol dehydrogenase (ADH) and the second exercise consisting of a glycogen assay.

The typical response of fish to hypoxic conditions consists of an intensification of ventilatory activity as well as a slowing of the heart beat, permitting the optimal oxygenation of vital organs (39). However, very low concentrations of dissolved oxygen in water lead to the disruption of oxidative ATP production. This occurs when the fish has completely depleted its oxygen reserves in its myoglobin and gas bladder. Under these conditions, the only pathway remaining for ATP production is glycolysis; however, this alternative metabolism doesn’t supply sufficient energy to maintain standard functions. Usually, in most anoxia-sensitive vertebrates, a lack of oxygen causes a severe drop of cerebral ATP levels, leading to brain failure within minutes (24, 25).

In contrast, a variety of invertebrates show profound anoxia tolerance, frequently associated with novel metabolic pathways (15); only a few vertebrates show strong resistance to sustained oxygen depletion. Two strategies to maintain tolerable ATP levels are possible when vertebrates experience oxygen depletion: the first consists of a reduction of the rate of ATP use via depression of metabolic activity and the second is an intensification of the rate of glycolysis to produce more ATP (20). However, along with this second strategy arises a new challenge: continuous high levels of glycolysis lead to the excessive production of pyruvate, which is then converted into lactate and released from the cells into the bloodstream, where it accumulates. While the increased glycolysis helps compensate for the decrease in ATP, it cannot bind the resulting protons from ATP hydrolysis. Since the buffering capacity of fish blood is very low compared with air breathers, the proton concentration rapidly rises, leading to acidosis of the tissues (25). Surprisingly, few vertebrates show an alternative metabolic pathway to bypass this problem.

The goldfish (Carassius auratus) has developed an ability to deal with oxygen depletion in its environment. At low temperatures, it can survive for several days, weeks, or even months under anoxic conditions by activating a series of metabolic,
physiological, behavioral, and morphological strategies (20, 24). When dissolved oxygen levels are very low, the goldfish reduces its energy expenditure by reducing its swimming activity and survives with only 30% of its normal metabolic rate, saving its glycogen reserves (40). Moreover, Carassius has the largest liver glycogen storage capacity found among all vertebrates, reaching up to 30% of its liver wet weight and providing a continuous energy supply to the whole organism for long periods of anaerobic activity (18). However, the key adaptation of the goldfish to anoxia lies in a strikingly different metabolic pathway. In 1980, Shoubridge and Hochacha (33) discovered that under anoxic conditions, the activity of ADH is crucial to Carassius auratus' survival since it prevents lactic acidosis of tissues generated by continuous glycolytic activity. In the skeletal muscles of Carassius, ADH catalyzes the last reaction of a metabolic pathway leading to the formation of ethanol, using lactate as the initial substrate (24). The series of enzymatic reactions allowing ethanol production in these teleosts is as follows (21, 23, 24):

\[
\text{LDH: Lactate} + \text{NAD} \rightarrow \text{pyruvate} + \text{NADH (enzyme: LDH)}
\]

\[
\text{PDH: Pyruvate} \rightarrow \text{acetaldehyde} + \text{CO}_2 \text{ (enzyme: modified PDH)}
\]

\[
\text{ADH: Acetaldehyde} + \text{NADH} \rightarrow \text{ethanol} + \text{NAD} \text{ (enzyme: ADH)}
\]

where LDH is lactate dehydrogenase and PDH is pyruvate dehydrogenase. The activation of this pathway allows the removal of lactate by LDH, PDH, and ADH. Lactate is first transported through the whole organism from organs in the direction of both white and red skeletal muscles, where it is then converted to pyruvate via LDH. The second step consists of the decarboxylation of pyruvate to CO$_2$ and acetaldehyde via a modified PDH complex (24). Classically, PDH catalyzes the formation of acetyl-CoA from pyruvate, with acetaldehyde as an intermediate product. However, during sustained anoxic conditions, acetaldehyde in Carassius breaks out of the PDH complex and spreads into the cytoplasm, where it is reduced to ethanol by NADH via ADH (20). This metabolic pathway enables these fish to maintain their average body pH by excreting freely diffusible ethanol into the surrounding water through their gills via the blood circulation (24, 33).

Shoubridge and Hochacha (33, 34) stated that ADH appears to be entirely restricted to red and white skeletal muscle in goldfish (33, 34). In contrast, the activity of ADH in anoxia-sensitive vertebrates predominantly occurs in the liver, where it allows the degradation of ethanol to acetaldehyde (24, 33, 34). This harmful product is then immediately oxidized to acetic acid by aldehyde dehydrogenase and is later converted to acetyl-CoA and integrated into the tricarboxylic acid (TCA) cycle (23).

**EXPERIMENTAL PROCEDURES**

**Educational Objectives**

The main objective of this experiment is to show the distribution of ADH isozymes in muscle and liver tissues of the goldfish (C. auratus) using electrophoretic protein migration on cellulose acetate. We also propose an additional analysis to further student comprehension consisting of the determination of glycogen content in the liver. Furthermore, an interspecies comparison is added to the exercise by examining the distribution of ADH and liver glycogen content in a fish species associated with well-oxygenated rivers and lakes, the brook charr (Salvelinus fontinalis). It should be specified that the choice of this anoxia-sensitive species can be made according to the availability of live fish in respective regions. Students are asked to discuss the metabolic mechanisms involved in the goldfish's physiological adaptations to anoxia by writing a laboratory report. At the end of this exercise, students should also have gained new laboratory skills. A series of questions is proposed at the end of this article that can be used as guidelines to further student reflection on the different physiological adaptations used by the goldfish to increase its energy efficiency when coping with anoxia; they can also be included in the laboratory report.

**Teaching New Laboratory Techniques**

The first part of this laboratory exercise is achieved using cellulose acetate electrophoresis. This technique was discovered 50 yr ago by Joachim Kohn, a pathologist at Queen Mary’s Hospital in Roegampton, London, United Kingdom (32). Nowadays, this technique plays an essential role in routine clinical diagnostics. Moreover, it is useful in a wide variety of fields in life science research. Cellulose acetate electrophoresis has been shown to be a simple but accurate method for protein separation and quantification. Protein migration on gel supports such as cellulose acetate, agarose, and acrylamide is widely used in genetics, physiology, and biochemistry to reveal concrete information on polypeptide, genetic variation, phylogeny, and isozymes (13). As for glycogen content, it is also commonly used in many fields: by biologists as a global condition index, by biochemists to study energetic metabolism (43), by workers in the food industry to assess meat quality (42), and by medical health professionals for various diagnostic purposes (38). Thus, teaching electrophoresis and glycogen determination to undergraduate students gives them the opportunity to learn the basic manipulations of two techniques that are useful in many spheres of science.

**Experimental Protocol**

Students, in teams of 2 or 3 students/group, proceeded to tissue sampling, cellulose acetate electrophoresis of ADH, and the liver glycogen assay on one specimen of each fish species. Students were required to wear gloves, security glasses, and a laboratory coat when handling 3-aminobenzoic acid ethyl ester (MS222), dimethylthiazolyl-diphenyltetrazolium bromide (MTT), phenazone methosulfate (PMS), and perchloric acid since these chemicals, according to their material handling 3-aminobenzoic acid ethyl ester (MS222), dimethylthiazolyl-diphenyltetrazolium bromide (MTT), phenazone methosulfate (PMS), and perchloric acid since these chemicals, according to their material safety data sheets, may cause irritation in case of ingestion, inhalation, or skin and eye contact. Perchloric acid is particularly hazardous, and great care should be taken when handling this acid.

**Animals.** Goldfish were purchased at a pet store and kept in an aquarium, whereas brook charrs were obtained from a fish farm (Pisciculture Diane et Adrien Gagnon, Saint-Valérien, QC, Canada). Specimens from both species were at least 10 cm in length to facilitate sampling, cellulose acetate electrophoresis of ADH, and the liver glycogen assay on one specimen of each fish species. Students were required to wear gloves, security glasses, and a laboratory coat when handling 3-aminobenzoic acid ethyl ester (MS222), dimethylthiazolyl-diphenyltetrazolium bromide (MTT), phenazone methosulfate (PMS), and perchloric acid since these chemicals, according to their material safety data sheets, may cause irritation in case of ingestion, inhalation, or skin and eye contact. Perchloric acid is particularly hazardous, and great care should be taken when handling this acid.

**Acetate Cellulose Electrophoresis**

**Equipment.** The equipment necessary for this laboratory experiment includes the following:

- A tissue homogenizer (Heidolf DIAx 900)
- One Helena Laboratories (Beaumont, TX) electrophoresis kit, including a Super Z-12 applicator, 76 × 76-mm Titan III cellulose acetate gel plates (cat. no. 3033), and an electrophoresis tank
- A power supply (no. 15-5340, Ward’s Natural Science)
- A microwave or hot plate
- A centrifuge (MiniSpin Eppendorf centrifuge)
Equipment. The equipment necessary for this laboratory experiment includes the following:

- A tissue homogenizer (Heidolph DIAX 900)
- A centrifuge (Sorvall Biofuge Stratos)
- A spectrophotometer (Genesys 20)
- A vortex mixer (VWR Mini Vortexer)
- Spectrophotometric cuvettes
- Macrocentrifuge tubes
- Digital micropipettors
- Micropipetter tips
- A dissection kit
- Ice buckets

Glycogen Liver Storage Assay

For each fish species, 2 g of liver were finely cut, weighed, and homogenized in 10 ml of cold perchloric acid. The crude homogenate was then centrifuged at 15,000 × g for 10 min at 4°C. The resultant supernatant was decanted and kept on ice. A standard (0.4 ml; 0–600 μg/ml) or tissue extract was added to 2.6 ml of reagent. A distinctive amber-brown compound developed immediately after the mixture was vortexed, revealing the binding of iodine to glycogen. Absorbance was measured with a spectrophotometer only after 10–30 min of rest at a wavelength of 460 nm. A Student’s t-test was performed to compare liver glycogen contents between the goldfish and brook charr. Statistical analyses were achieved using a commercial software package (SPSS version 17.00 for Windows, SPSS).

RESULTS AND DISCUSSION

The electrophoresis assay revealed ADH distributions corresponding to each species’ metabolic strategy. As shown in Fig. 1, ADH was restricted to skeletal muscle in goldfish, whereas it was only found in liver tissue in brook charr. Moreover, both assays displayed two different isozymes for the tissues studied. The brook charr’s liver extract migrated further on the gel plate than did the goldfish’s muscle tissue extract, suggesting different ADH protein conformations in these two samples. These results support Shoubridge and Hochachka’s study (33). Although the transformation of lactate into ethanol imposes a cost due to a loss of carbohydrate, the goldfish’s ADH genotype allows it to avoid intoxication by metabolic end products, increasing its fitness during a sustained lack of oxygen. In contrast, in anoxia-sensitive species like S. fontinalis, ADH is confined to the liver, where it catalyzes the usual pathway that results in ethanol degradation. ADH allows the transformation of ethanol to acetaldehyde, which is then converted to acetic acid and acetyl-CoA and integrated into the TCA cycle (24). Thus, in the brook charr, ADH isn’t involved in anoxia tolerance, as it is in the goldfish.
The results of the liver glycogen determination (Fig. 2) clearly demonstrated that storage of glycogen in the goldfish was higher than in the brook charr ($t = -4.422, P = 0.001$). Members of the *Carassius* genus have the largest liver glycogen reserves found among all vertebrates, providing a continuous energy supply for long periods of anaerobic activity (19). A large glycogen supply is essential for the goldfish, whose glycogen metabolism and cerebral blood flow are greatly upregulated during anoxia, enhancing its rate of glucose delivery and lactate removal (24). Having students perform the glycogen content determination adds a quantitative component to the laboratory exercise and also gives students the opportunity to realize statistical comparisons between species.

The laboratory experiment proposed in this article clearly demonstrates the strongly divergent metabolic evolution of these two teleosts according to their respective environmental pressures. The goldfish originates from Central Asia, Northern China, and Japan, where winter ice formation on ponds limits oxygen availability (41). The goldfish has been introduced worldwide due to its popularity as a pond and aquarium fish and is now mainly found in stagnant waters and slow streams colonized by dense vegetation. Thus, this species is likely to encounter anoxic conditions (27) and is therefore well adapted to low levels of dissolved oxygen in its environment. Alcoholic fermentation and important liver glycogen stores are purported to be of less importance to the brook charr since it colonizes rivers and lakes characterized by fresh, clear, and well-oxygenated water (1).

The aim of these experiments was to compare two fish species with different abilities to support hypoxia and to allow students to investigate the physiological and evolutionary reasons for the different expression of ADH. This was achieved by comparing the goldfish and the brook charr, which clearly demonstrate different abilities to express this enzyme even if maintained under normoxic conditions before sampling. This experimentation could be furthered by comparing the capacity of the same fish species to modulate the expression of this enzyme according to the level of oxygen concentration in the environment. This would be a great opportunity to investigate the concept of phenotypic flexibility at the level of metabolic organization.

**Study Questions**

**Question 1:** describe the different physiological consequences of lack of oxygen leading to death by anoxia in anoxia-sensitive species. The major issue for cells under anoxic conditions is to maintain ATP production, especially in brain cells, which have high energy consumption due to their continuous electrical activity. The switch from oxidative metabolism (36 mol ATP/mol glucose) to glycolysis (2 mol ATP/mol glucose) significantly reduces ATP production, leading to severe brain perturbations in anoxia-sensitive vertebrates. Within minutes of anoxia, cerebral Na"^+"-K"^+" pumps slow down or stop, causing an important outflow of K"^+" into the extracellular environment. As a result, extracellular K"^+" concentrations rapidly reach high levels, causing depolarization of the brain followed by the intrusion of Na"^+" and Ca"^{2+}" into cells. Water then floods through the cellular membrane, causing brain swelling and high intracranial pressures, leading to global brain ischemia. Moreover, Ca"^{2+}" appears to be largely responsible for numerous degenerative and lytic processes. The combination of these perturbations occurring during a sustained lack of oxygen leads to neuronal death in anoxia-sensitive vertebrates (19, 24).

**Question 2:** illustrate other behavioral, physiological, and morphological adaptations of goldfish to survive low levels of dissolved oxygen in water. **Behavioral adaptations.** When experiencing anoxia, the goldfish considerably reduces its locomotory activity and moves to cooler water if possible, lowering its metabolic energy requirements (24, 28). **Physiological adaptations.** GABA plays a major role in neuronal survival in anoxia-tolerant vertebrates. As a major inhibitory neurotransmitter in the brain, it significantly downregulates neuronal activity under anoxic conditions, reducing ATP consumption (24). **Morphological adaptations.** Under hypoxic conditions, the goldfish has the capacity to remodel its respiratory surface by

![Fig. 1. Photograph of the electrophoresis gel displaying alcohol dehydrogenase (ADH) isozymes in goldfish and brook charr muscle and liver tissues.](image1)

![Fig. 2. Liver glycogen content in the brook charr and goldfish. Values are presented as means ± SD.](image2)
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increasing the size of its interlamellar cell mass in its gills. This alteration is due to the apoptosis of cells between adjacent lamellae, enlarging its respiratory surface and thus facilitating gas exchanges (35).

**Question 3:** describe the thermoregulatory mechanisms resulting from exposure to a hypoxic environment. In the goldfish, there is an important connection between hypoxia and metabolic depression. During the first 30 min of anoxia, an increase in the ventilatory rate of the goldfish is observed, leading to higher metabolic activity and internal temperature. After 30 min, severe hypoxia induces a remarkable decrease in body heat production, to <30% of the normoxic rate. Postanoxic recovery is rapid and complete and is characterized by a transitory period of excessive body heat production, associated with the additional metabolism necessary to pay back the “oxygen dept” built up during anoxia. Thus, the length of time of excess metabolic activity required for returning to a normoxic states depends on the duration of the anoxic interval experienced by the goldfish (28).

**Question 4:** does the goldfish retain complete cerebral functions under anoxia? compare this organism’s cerebral response with that of American freshwater turtles of the genera Trachemys and Chrysemys under anoxic conditions. C. auratus and American freshwater turtles of the genera Trachemys and Chrysemys all exhibit anoxia tolerance during sustained periods of oxygen depletion. However, their strategies to avoid neuronal death differ. C. auratus survives low levels of oxygen in an active state, although at a reduced level of 30% of its normal metabolic rate, whereas anoxic turtles switch to a comatose-like state, during which their usual metabolism is reduced from 90% to 95%. This metabolic depression is due to a downregulation of key glycolytic enzymes, resulting in a suppression of neurotransmission and in an important reduction of brain electrical activity. This strategy allows the anoxic turtle to considerably decrease its energy expenditure. In contrast, in C. auratus, glycolytic enzymes are upregulated to increase anaerobic glycolysis, which enables the goldfish to maintain sufficient ATP production, ensuring the regulation and control of locomotory and sensory activities. This second strategy is possible via ethanol production as an end product of anaerobic glycolysis, avoiding lactic acidosis of its tissues (20, 24).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


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