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Lungfish

SYMPOSIUM PROCEEDINGS

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PREFACE

Three genera of lungfish remain today as relicts of a once large and important Devonian group. Speakers who have worked on various aspects of Australian, African or South American lungfish presented and discussed the physiology, anatomy and life cycle of these animals and the factors that allowed their survival since the Paleozoic in spite of the emergence of tetrapods and teleosts. We hope to form the core of an international lungfish study group.

Symposium Organizers:

Steven Perry, University of Bonn, Germany
Marisa Fernandes, Federal University of San Carlos, Brazil
Don MacKinlay, Fisheries and Oceans Canada

CONGRESS ACKNOWLEDGEMENTS

This volume is part of the Proceedings of the 6th International Congress on the Biology of Fish, held in Manaus, Brazil in August, 2004. Ten years have passed since the first meeting in this series was held in Vancouver, BC, Canada. Subsequent meetings were in San Francisco, California; Baltimore, Maryland; Aberdeen, Scotland; and again in Vancouver, Canada. From those meetings, colleagues from over 30 countries have contributed more than 2,500 papers to the Proceedings of over 80 Congress Symposia, all available for free viewing on the internet.

We would like to extend our sincere thanks to the many people who helped us organize the facilities and program for this 6th Congress.

The local arrangements team worked very hard to make this Congress a success. The leaders of those efforts were Vera Almeida Val, Adriana Chippari-Gomes, Nivia Pires Lopes and Maria de Nazare Paula Silva (Local Arrangements); Marcelo Perlingeiro (Executive Secretary) and Maria Angelica Laredo (Fund Raising). The enormous contribution of time and effort that was required has led to an unforgettable experience for the participants, thanks to the imagination, determination and dedication of this team.

Many sponsors helped ensure the success of the meeting through both monetary and in-kind contributions, including: Fundação Djalma Batista, Honda, Merse, Cometais, Turkys Aquarium, Banco da Amazônia, Banco do Brasil, FUCAPI, SEBRAE/AM, IDAM/SEPROR, FAPEAM, SECT-AM, SUFRAMA, PETROBRÁS, CAPES, FINEP, CNPq, the Physiology Section of the American Fisheries Society, UFAM - Federal University of Amazonas, Fisheries and Oceans Canada and INPA - National Institute for Research in the Amazon.

Travel arrangements were ably handled by Atlantic Corporate Travel (special thanks to Maria Espinosa) and Orcal Planet, and the venue for the meeting was the spectacular Tropical Hotel Conference Center in Manaus.

The Student Travel Award Committee of the Physiology Section of the American Fisheries Society, led by Michael Redding, evaluated 65 applications from 15 countries and awarded 40 Travel Grants, after an ambitious and trying fund-raising effort. Special thanks must go the US Department of Agriculture, the US Geological Survey, US National Science Foundation and the World

Fisheries Congress for providing funds. In addition, the American Fisheries Society contributed books to be used as prizes for the best student papers.

The editorial team compiled the short abstracts into an abstract book and formatted and compiled the papers for the Symposium Proceedings. Thanks to Karin Howard, Christie MacKinlay, Anne Martin, Callan MacKinlay and Marcelo Perlingeiro.

In particular, we would like to extend a sincere 'thank you' to the organizers of the individual scientific Symposia and their many contributors who took the time to prepare a written submission for these proceedings. Their efforts are very much appreciated. We hope that their participation will result in new insights, new collaborations and new lines of research, leading to new papers to be presented at the 2006 Congress in St. John's, Newfoundland.

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THE ORIGIN AND EVOLUTION OF LUNGFISH

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The dipnoans, or lungfish, are an ancient group of fish of uncertain relationships but highly distinctive characteristics. They first appeared in the early Devonian and have several living representatives. They are not numbered among the earliest of fishes that appeared in the Ordovician and the Silurian, but they are a part of the enormous radiation of fish groups that took place during the Devonian.

Links between the dipnoans and tetrapods have been discussed almost continuously since living lungfish were first described over a hundred years ago, but this argument sheds little light on the origins of the lungfish, or on their relationships with other groups of fish (Miles, 1977). Lungfish are osteichthyans, and usually classified within this large group with the living coelacanth and other related fish as sarcopterygians, or lobe finned fishes. Apart from their obvious links with other lobe finned fishes, fossil and living, some structural characters of living lungfish, such as the cartilaginous skull of the Australian species, suggest links with elasmobranchs (Jarvik, 1980), and evidence derived from the development of lungfish, such as the formation and structure of the scales, indicate close relationships with actinopterygians or other early osteichthyans (Zylberberg, 1988). Other characters, like the unusual forms of dentine in the tooth plates, suggest few close relationships with other fish groups or with tetrapods (Kemp, 2003). It is most probable that this exceptionally bradytelic group diverged early in the Devonian from basal types of bony fish.

The dipnoans reached a peak of diversity and distribution late in the Devonian and during the Carboniferous. Early Devonian lungfish are already distinctive in their dentition (Denison, 1974) and in the unusual pattern of the skull roofing bones, though similar in body form to some crossopterygians like Osteolepis (Miles 1977). Initially, lungfish were marine, and it is unlikely that they could, or did, breathe air, because they lived in deep water.

During the Carboniferous, several unusual forms appeared, and most species adopted a freshwater lifestyle. Lungfish declined in diversity during the Mesozoic, but not in geographic distribution, at least until the Cretaceous when they disappeared from the Northern Hemisphere. Lungfish continued to be a prominent component of fossil faunas in Gondwana, and are still found in Africa, South America and Australia, in specific freshwater environments.

Although evidence regarding the origin of lungfish in relation to other groups of fishes is tenuous and the arguments are often speculative (Miles, 1977), an evolutionary progression within the group can be traced without difficulty. Changes in the morphology and fine structure of the dentition (Kemp, 2001), reduction in number of the skull bones and specific rearrangements of the skull structure (Kemp, 1998), and alterations in the structure of the scales can be followed throughout the history of the group. Major genera of Devonian lungfish had permanent tooth plates arranged in radiating ridges that developed from initially isolated cusps, and this design has been retained in almost all of the descendents of Devonian lungfish. So also have the ultrastructural characteristics of a specialised dentine found only in lungfish. This biomaterial, known as interdenteonal dentine, contains prisms of hydroxyapatite crystals, arranged to prevent propagation of cracks though the tooth plate, a function performed in other vertebrate teeth by enamel or enameloid. Lungfish tooth plates have enamel, but this is a thin and fragile layer, soon removed from the tooth plate by wear. The skulls of Devonian lungfish have a tessera of small superficial bones, fused to an ossified chondrocranium. In their descendents, this has been gradually refined to a simpler pattern, consisting of a few large thick external bones covering a cartilaginous chondrocranium. In the living Australian lungfish, the skull bones can be removed from the underlying cartilage, and the more posterior bones are separated from the underlying chondrocranium by muscles. The chondrocranium of the living African lungfish is partially ossified. In all of the living lungfish, and in some of the fossil species, the upper jaw bone is linked in an unusual way with the skull roof, an arrangement that confers considerable strength and versatility on the jaws.

The divergence of the two major divisions of living dipnoans, represented by Neoceratodus in Australia, Lepidosiren in South America and the related

Protopterus in Africa, can be followed from their common origin in the Mesozoic. The evolutionary progression within the lungfish is not one of degeneration or regression to a basic state. It is a process of refinement, resulting in species that are well adapted for their natural environments.

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ASPECTS OF THE MORPHOLOGY AND PHYSIOLOGY OF THE AUSTRALIAN LUNGFISH, *NEOCERATODUS FORSTERI* (OSTEICHTHYES:DIPNOI)

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The Australian lungfish, *Neoceratodus forsteri*, has numerous morphological and physiological adaptations that are suitable for its natural environment of large river systems in southeast Queensland. These are subjected to periodic flooding as well as frequent partial drought, when the fish may be confined to deep water holes. Temperature and water quality vary widely over the year, and food is sometimes difficult to find. The water is often muddy, and may be stained with tannin from the leaves of the trees that grow on the banks. The adults have few natural enemies, but young lungfish are vulnerable to many predators.

Specialised adaptations of the heart and respiratory system of lungfish are well known. These are of minimal importance to the Australian lungfish, which most often uses gill breathing, not lungs, despite the exigencies of its environment. Less notable adaptations of the Australian lungfish, like the sensory system, the unusual structure of the jaws and teeth, the Mauthner neuron system, the ultrastructure of the dentition, and specific arrangements for spawning and recruitment of young lungfish to the adult population are also of interest, though few are unique to lungfish.

Despite the inclusion of coloured oil droplets in the retina, possibly conferring colour vision on the fish, sight is not a major sensory modality for lungfish, which are most active at dusk or during the night. Instead, they have a range of sense organs, concentrated on the head around the jaws and within the oral cavity, including the olfactory organs, branches of the lateral line system, pit lines and electroreceptors. Another adaptation, shared with fish and some amphibia, is the Mauthner system, with two large neurons located in the hind brain and nerves running ventrally down the spinal cord and distributed to the body musculature. The neurons receive input from the vestibular organ and from the lateral line system via cranial nerve VIII. This enables the fish to react quickly if threatened, by twisting the body into a C-shape and moving away rapidly in an unpredictable direction.

Aspects of the skull structure, notably the strong jaws and permanent tooth plates, and the complex articulation between the upper jaw bones and the skull roof, means that the jaws are unusually versatile (Kemp, 1992). This enables the Australian lungfish to masticate soft food materials such as filamentous algae and worms, or to crush gastropod shells and grind rough water plants. A similar but stronger articulation allows the African and South American lungfishes to crush and slice their food. The dentine of the lungfish tooth plate has a specialised ultrastructure that prevents crack propagation and resists wear, a function undertaken in other vertebrates by enamel, present but almost vestigial in lungfish.

Lungfish spawn in spring in response to increasing photoperiod (Kemp, 1985). Fertilisation is external, and parent fish lay the eggs close to water plants like *Vallisneria spiralis*, *Hydrilla verticillata* and the submerged roots of *Callistemon saligna*, a tree that grows on the banks of the rivers. Availability of water plants suitable for the deposition and adherence of the eggs is important, but fish will spawn even if water plants are absent. When this occurs, eggs are shed into the water column and carried away by the current. If they are not swept into contact with water plants while the jelly coat is still sticky, the eggs are unlikely to survive. The water plants serve as shelters for the young lungfish when they hatch, and are essential to their survival at a vulnerable stage in the life cycle, when the fish are too young to feed and are poorly equipped to avoid predators (Kemp, 1996). In addition to cryptic colouring, the small lungfish, like the young of some other fish and of amphibia, have ciliated cells in the epidermis (Whiting and Bone, 1981). These function to keep the young lungfish clean in an environment that is laden with silt and potentially harmful settling organisms (Kemp, 1996).

These specific adaptations of structure and function are essential for the life of the Australian lungfish in its natural habitat. Unfortunately, they do not equip the lungfish well for the changes made by human interference with rivers and lakes in southeast Queensland. None of the river systems where the lungfish live have escaped alterations such as the building of weirs and dams, and all are heavily polluted by agricultural waste or effluent from sugar refineries. Weirs and dams, intended to store water for agricultural use, have fluctuating water levels in spring when lungfish are spawning, and this destroys water plants close to the shore. Despite their ability to breath air when necessary, lungfish are as vulnerable to polluted water as any other aquatic species. The Australian lungfish is now under threat of extinction (Kemp, 1995), and efforts to protect the species or its environment have been ineffective.

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MORPHOLOGY OF THE RESPIRATORY ORGANS OF THE AFRICAN LUNGFISH, *PROTOPTERUS AETHIOPICUS*

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Introduction

Only three genera of lungfishes (Dipnoi) occur today. They are restricted to separate continental landmasses: *Lepidosiren* in South America, *Protopterus* in Africa, and *Neoceratodus* in Australia. In the genus *Protopterus*, largely confined to tropical Africa, four species, *P. annectens*, *P. amphibius*, *P. aethiopicus* and *P. Dolloi* exist. While for some species habitats overlap, for others, they are completely isolated. Lungfishes are of particular biological interest. The main reasons are that they are arguably a direct lineage to the evolution of the tetrapods (e.g. Brinkman et al., 2004) and they present an existing example of the adaptive stratagems of transition from water- to air breathing (e.g. Little, 1990). We have studied the structure of the lung of *Protopterus aethiopicus* in an attempt to better understand the design of the early air-breathing organs.

Materials and Methods

Fifteen specimens of *P. aethiopicus* were acquired from creeks leading into Lake Victoria, the largest fresh water mass in continental Africa. The Lake forms a common boundary between Kenya, Uganda, and Tanzania. The fish were transported by road to the laboratory where they were killed by injection with Euthatal^R (200mg/L pentobarbitone sodium) into the heart. They were weighed and their body lengths measured from the tip of the nose to that of the tail. The lungs were exposed through a ventral incision and cannulated. With the fish in a supine position, the lungs were fixed by instillation with 2.3 % glutaraldehyde buffered in sodium cacodylate (pH 7.6; osmolarity 350 mOsm) from a height of 20 cm. When the fixative stopped flowing, the cannula was blocked and the lungs left *in situ* for about six hours. Subsequently, the lungs were dissected from their attachments to the vertebral column and their lengths measured. The volume of the lungs was determined by weight displacement method (Scherle, 1970). The left lung was used for light microscopy and scanning electron microscopy and the right one for transmission electron microscopy (TEM).

The left lung was sampled into five equal parts along its length and the pieces processed for light microscopy and SEM. For analysis, eight micron thick sections were stained with haematoxylin and eosin. The volume density of the air duct and the exchange tissue were determined by point-counting using a 100-point Zeiss integrating graticule at a magnification of x100. At a higher magnification of x400, the volume densities of the components of the exchange tissue, namely air spaces, blood capillaries and interfaveolar septa were determined by point-counting. For TEM, the lung was sampled and tissues processed by standard laboratory techniques. Fifty electron micrographs per specimen were analyzed at a final magnification of x17,500 and structural parameters such as the respiratory surface area and thickness of the blood-gas barrier determined by standard stereological techniques (Weibel, 1979). The diffusion capacities of blood-gas barrier, plasma layer, and erythrocytes were calculated and the membrane and total morphometric diffusing capacity estimated (Weibel, 1970/71).

Results

In *P. aethiopicus*, running over about two-thirds of the body length on the dorsal (vertebral) aspect, the lungs are paired, of equal size, and loosely attach to each other (Maina and Maloiy, 1985). An eccentrically located air duct runs along the length of the lung. Internally, prominent septa subdivide the gas exchange tissue of the lung into stratified air spaces. The luminal ones are 1.5 mm in diameter as they decrease in size peripherally (Maina, 1987). Blood capillaries bulge from the surface of the septa. The septa contain smooth muscle, elastic tissue, and collagen fibers. Depressions on the surface of the lung contain perikarya of epithelial cells that possess composite features of type II and I of the mammalian and avian lungs: they contain osmiophilic lamellated bodies, microvilli, and expansive cytoplasmic extensions. The blood-gas barrier consists of an epithelium, a basal lamina, and an endothelium.

The volume of the lung correlates strongly with body mass: VL = $107.15W^{0.64}$, r = 0.78. The air duct constitutes 49.5% and the exchange tissue 50.5% of the lung. The volume density of the lung decreases cranial-caudally. In the exchange tissue, respiratory air spaces constitute 51%, the septa 43%, and the blood capillaries 6%. The harmonic mean thickness of the blood-gas barrier (tht) was 0.370 µm and the mean total morphometric diffusing capacity (DLo_2) was 0.0005 $mlO_2.s^{-1}.mbar^{-1}$.

Compared with *Lepidosiren paradoxa* on which pulmonary morphometric data are available (Hughes and Weibel, 1976), regarding the thickness of the blood-gas barrier, *P. aethiopicus* has a relatively thinner one (0.37 µm vs 0.86 µm) and a more extensive respiratory surface area (14 $cm^2.g^{-1}$ vs 0.85 $cm^2.gm^{-1}$). The thickness of the blood-gas barrier in *P. aethiopicus* compares with that of *P. annectens* (0.5 µm) (Klika and Lelek, 1967). The weight specific DLo_2, respectively 0.003 and 0.002 $mlO_2.s^{-1}.mbar^{-1}$ $.kg^{-1}$ in *Lepidosiren* and *Protopterus*, are however, comparable.

In conclusion, the lung of *P. aethiopicus* manifests profuse internal subdivision that may produce a particularly large respiratory surface area. This may be adaptive to a fish that lives in hypoxic habitat. The large disparity between values of *Lepidosiren* reported by Hughes and Weibel (1976) and *Protopterus* by Maina and Maloiy (1985), however, call for application of modern stereological methods that are less susceptible to technical bias.

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MORPHOMETRY OF THE RESPIRATORY ORGANS OF THE SOUTH AMERICAN LUNGFISH, *LEPIDOSIREN PARADOXA* (DIPNOI)

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Morphometric data of respiratory structures provide a reference point for the interpretation of physiological parameters and also a starting point for further studies on adaptation (Perry et al., 1994). The Australian lungfish, *Neoceratodus* *forsteri*, a facultative air breather, is usually considered more primitive than sister group Lepidosirenidae, which contains two genera: the African (*Protopterus*) and South American (*Lepidosiren*) lungfish.

For *Protopetrus* and *Neoceratodus* there are several functional and morphological studies on the gills and lungs, however, for the South American lungfish, *L. paradoxa* aside from a single morphometric study of the lungs (Hughes and Weibel, 1976), only brief descriptions or comparisons with the respiratory structures of the other two genera exist. The main goal of the present study was to determine the morphometric diffusing capacity of the all respiratory organs (gills, skin and lungs) for O_2 and CO_2 in the South American lungfish using modern stereological techniques.

Adult *L. paradoxa* (Body mass = $M_B = 864 \pm 169$ g) obtained near Cuiabá, MT, Brazil, were kept at 25°C. Following anesthesia (0.5% Benzocaine), the heart was exposed and the fish was perfused through the ventral aorta with 0.1M phosphate buffer (pH 7.4, 300 mOsM) containing Heparin. When the drained venous return was nearly colorless, the perfusion solution was changed to 2.5% glutaraldehyde in phosphate buffer as above (15-20 min) The animal was then decapitated and the entire gill apparatus was removed to the same fixative. The lungs were filled with fixative through the glottis and tied off, and the fish was left in fixative overnight at 4ºC for complete fixation of lungs and skin. The right lung was used for light microscopy (LM) and the left was sampled for transmission electron microscopy (TEM). For skin analysis, the fixed fish was transected by 12 equidistant cuts, the location of the starting section being determined at random within the first increment. The sections were labeled and placed in glutaraldehyde storage solution, consisting of 0.5% glutaraldehyde in phosphate buffer (see above).

Stereological measurements were carried out according to standard techniques (Howard and Reid, 1998). The reference volume (V) of gills , lungs and skin were determined using the Cavalieri principle and the respiratory surface areas (S_R) were calculated using the vertical section method (Costa et al., 2001; Howard and Reed, 1998). The barrier thickness (harmonic mean = τh) of gills and skin (water-blood distance = τh) were determined using LM and the diffusion barrier thickness of lungs (air-blood distance = τh) were determined using TEM. These determinations were employed in calculation of the anatomical diffusion factor (ADF = surface area/harmonic mean barrier thickness) of each respiratory structure and the diffusing capacities for O_2 and $CO_{2,}$ as the product of ADF and the appropriate Krogh's diffusion constant.

The percentage of the total surface present in gills, skin and lungs are shown in Figure 1. The ADF and diffusion capacity for O_2 and CO_2 of the gills, lungs and skin are shown in Table 1.

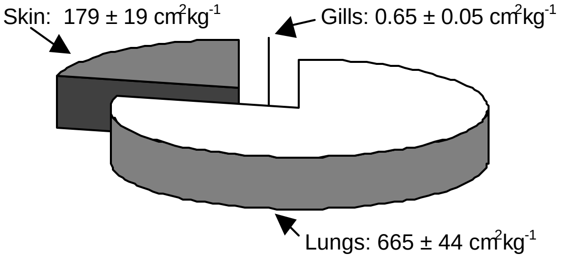

Figure 1. M_B-specific surface area (S/M_B) of the respiratory organs: gills, lungs and skin, of *L. paradoxa*.

Respiratory Organ	**ADF** **(cm^{2}μm^{-1}kg^{-1})**	**D_{O2}** **(cm^{3}min^{-1}mmHg^{-1}kg^{-1})**	**D_{CO2}** **(cm^{3}min^{-1}mmHg^{-1}kg^{-1})**
Gills	8.16± 1.19 (10^{-3})	1.48 ± 0.21 (10^{-6})	2.83 ± 0.4 (10^{-5})
Lungs	443.39 ± 44.97	0.11 ± 0.01	2.10 ± 0.23
Skin	4.84 ± 0.24	8.49 ± 0.82 (10^{-4})	0.016 ± 0.0016

Table 1. Anatomical diffusion factor (ADF), and diffusion capacity for O_2 and CO_2 of gills, lungs and skin of *L. paradoxa*.

In conclusion the lungs (with 99.1% of the diffusing capacity) are the main respiratory structure. Only 0.85% and 0.0013% of the diffusing capacity lies in the gills and the skin, respectively. Thus, the skin may be potentially important for CO_2 excretion, but the diffusing capacity of the gills is negligible, raising the question as to their function.

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EFFECT OF CHRONIC AQUATIC HYPERCARBIA IN THE THE SOUTH AMERICAN LUNGFISH, *LEPIDOSIREN PARADOXA*: PULMONARY VENTILATION AND BLOOD ACID-BASE REGULATION

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Abstract

The South American lungfish (*Lepidosiren paradoxa*, Fitzinger) possess well-developed lungs, whereas its gills are rudimentary. To evaluate its patterns of acid-base regulation, we applied hypercarbia for up to 48 h. After normocarbic control measurements, we applied aquatic hypercarbia (7% ~ 49 mmHg), whereas the gas phase remained normocarbic. Blood was withdrawn from a catheterized caudal branch of the dorsal aorta, and pulmonary ventilation was measured by pneumotachography.

Normocarbic blood gas values were: pHa ~ 7.5; $PaCO_2$ ~ 17 mmHg; $[HCO_3^-]$pl ~ 22 mM (Experimental temperature 25^oC). Aquatic hypercarbia (water PCO_2 ~ 49 mmHg) significantly changed of acid-base status. Thus, $PaCO_2$ increased over the first h to 37.4 mmHg, while pH fell to about 7.21. The ventilatory responses were transient but prevented $PaCO_2$ to reach the level of the water. There was no clear evidence of pHa compensation by active modulation of $[HCO_3^-]$pl. The regulatory patterns of *Lepidosiren* resembles that of aquatic lung breathing salamanders and differs completely from pH compensation in teleost fish.

Introduction

Land vertebrates (Tetrapoda) along with the coelacanths (*Latimeria*) and the lungfish (Dipnoi) evolved from sarcopterygian (lobe finned) ancestors. (Carroll, 1988). Recent studies indicate Dipnoi as the most probable sister group relative to the land vertebrates (Meyer and Dolven, 1992), which has inspired an increasing interest in this group.

Combined lung and gill respiration is common to lungfish, but O_2-uptake in *Protopterus* (Africa) and *Lepidosiren* in (South America) is highly dependent on pulmonary respiration (Johansen and Lenfant, 1967). Recent studies show that respiratory control in lungfish and amphibians share many features. Thus, both amphibians and *L. paradoxa* depend on central chemoreceptors that underlie ventilatory control of acid-base status (Sanchez et al., 2001). On the other hand, virtually no information is available on a possible active modulation of extracellular bicarbonate levels. Therefore, we decided to expose *Lepidosiren* to aquatic hypercarbia at for up to 72 h, while blood samples were obtained from the dorsal aorta and analyzed for acid-base status, PaO_2 and $[O_2]a$. Concurrently, the effects of hypercarbia on pulmonary ventilation were recorded.

Materials and Methods

Specimens of *L. paradoxa* (Fitzinger), weighing 300 to 600 g were collected close to city Cuiabá, Mato Grosso State, and transported to Ribeirão Preto, São Paulo State, where they were kept in 1000-L tanks containing dechlorinated water at 25^oC.

Surgical procedures.
Immersion into a benzocain solution ($1g\cdot L^{-1}$) usually caused anaesthesia within 10 min.. Then, the animal was placed into a support for surgery. A 3-cm incision was cut in the caudal region of the animal. A branch of the dorsal aorta was dissected free and the vessel was catheterized. The catheter was then tied to surrounding tissue and exteriorized. Recovery was obtained placing the animal in benzocain free aerated water.

Blood gas analysis

Blood PO_2 was measured using an O_2 electrode coupled to a FAC 204 O_2 analyzer (FAC Instr., São Carlos, SP, Brazil) while PO_2, PCO_2 and pH were measured using a micro-electrode assembly (Cameron Instr. Co, Texas, USA). A GF3/MP Gas Flow Meter (Cameron Instr., Port Aransas, Texas, USA) was used to provide gas mixtures for calibration of electrodes and also delivered the hypercarbic mixture (7% CO_2) to equilibrate the water.

Total plasma CO_2 was obtained using a Capni-Con 5 Analyzer (Cameron Instr.), calibrated with standard bicarbonate solutions (see Nicol et al. 1983).

Experimental set-up

Lung ventilation was measured using pneumotachography for diving animals (cf. Glass et al., 1983). Kept in a 10-L aquarium, the animal was guided to surface and breathe within an air-filled funnel, connected to a.pneumotachograph. A highly sensitive differential pressure transducer (Mod. DP45-14-2112, Valydine Instr., Northridge, CA, USA) was used to measure inspired and expired volumes.

Experimental protocol

Aquatic hypercarbia, 7% CO_2 ~49 mmHg:. Initially normocapnic control measurements were performed, including pulmonary ventilation, blood acid-base status, $[O_2]a$, PaO_2 and hematocrit(time = 0 h). Next, 7% CO_2 was applied to equilibrate the water, while animal continued to breathe atmospheric air. Blood gas measurements were performed during hypercarbia at 1, 3, 6, 24 and 48 h, while ventilation was measured.

Statistics

Krustall-Wallis test was performed followed by Dunn´s test for differences between individual groups. Values are expressed as mean ± SE. Significance level was taken as $P < 0.05$. For N-values see figure legends.

Results

Fig. 1 (A, B, C) shows the effects of aquatic hypercarbia (level 7% ~ 49 mmHg) on acid-base status of the blood. A steep increase of $PaCO_2$ from 18.1±1.8 to 37.4±3.1 mmHg occurred within the first h of exposure. Subsequently $PaCO_2$ remained close to 40 mmHg, which was about 10 mmHg below PCO_2 of the water (Fig. 1A). During the first hour of exposure, pHa decreased from a normocarbic control value of 7.48±0.03 to about 7.21±0.02 and remained at that value until the end of the experiment. Plasma [HCO_3^-] increased slightly during the experiment, but this did not alter pH (Fig. 1 B, C).

Ventilatory responses: V_T was large and constant both during normocapnia and hypercarbia. Meanwhile, the effects of hypercarbia on pulmonary ventilation and f_R were large (Figs.2 A, B, C) and this was reflected in the time course of PaO_2.(Fig. 2 D).

Discussion

Hypercarbia:. About 60% of total CO_2 elimination in *Lepidosiren* is aquatic at 25^oC (Amin-Naves et al. in press), which explains the large effect of hypercarbia on blood acid-base status. Nevertheless, $PaCO_2$ remained well below the level in water throughout the experiment. This can be explained by the continuing pulmonary CO_2-elimination to atmospheric air. Similar data exist for aquatic salamanders, that were exposed to aquatic hypercarbia, while they respired air at the surface (Heisler et al., 1982). In both cases, pulmonary ventilation and CO_2-elimination in air efficiently limits the effects of aquatic hypercarbia.

Compensation of pHa: Lepidosiren compensate pHa during hypercarbia. This is consistent with the data for aquatic salamanders, which stresses that many functional similarities between Amphibians and Dipnoi. When exposed to 6.4% CO_2 during 32 h, the urodele salamanders *Amphiuma means* and *Siren lacertina* failed to show any active compensation of pH by elevated $[HCO_3^-]_{pl}$ (Heisler et al., 1982). The authors suggested, that $[HCO_3^-]_{pl}$ was not adjusted, because these salamanders inhabit a permanently hypercarbic environment, which may also apply to *Lepidosiren*. Moreover, $PaCO_2$ and $[HCO_3^-]_{pl}$ of *Lepidosiren* are high, even under normocarbic conditions. Instead, *Siren*, increased intracellular [HCO_3^-] levels in response to hypercarbia (Heisler et al. 1982).

Some amphibians are able to acid-base status by active modulation of $[HCO_3^-]_{pl}$. Thus, .when exposed to hypercarbia, the urodele salamander *Cryptobranchus alleganiensis* (hellbender) partially compensated pHa by actively increased $[HCO_3^-]_{pl}$, (Boutilier and Toews, 1981). Likewise, Stiffler et al. (1983) reported hypercarbia-induced active modulation of extracellular $[HCO_3^-]_{pl}$ in the neotenic tiger salamanders, *Ambystoma tigrinum.* The authors emphasized that *A. tigrinum* has well-developed mechanisms for cutaneous Na^+ and Cl^- exchanges, which suggests that acid-base relevant ion exchanges could take place (Alvarado et al., 1975).

Anuran amphibians are capable of a limited compensation of pHa by elevation of $[HCO_3^-]_{pl}$. Thus, the toad *Bufo marinus* reached a compensation of about 30% after 24 h of exposure to 5% CO_2 (Boutilier et al., 1979). Based on several studies, it appears that the upper limit for $[HCO_3^-]_{pl}$ in *B. marinus* would center around 30 mM (Heisler, 1986; Boutilier and Heisler, 1988). This conclusion was drawn from studies that applied one level of hypercarbia for 24 h. Alternatively, Toews and Stiffler (1990) used a stepwise increase of CO_2 (2, 4, 6, and 8% CO_2) in *B. marinus* and *Rana catesbeiana*. They hypothesized that a stepwise elevation of CO_2-levels would increase the upper limit for $[HCO_3^-]_{pl}$. Indeed, under these conditions, *B. marinus* attained a $[HCO_3^-]_{pl}$ of about 40 mM, while *R. catesbeiana* reached as much as 46 mM, which suggests that anuran amphibians are better regulators than previously assumed. Moreover, tadpoles of *R. catesbeiana* exhibit an efficient pH-compensation of hypercarbia (Busk et al., 1997).

Ventilatory responses to hypercarbia: Studying *Protopterus* Johansen and Lenfant (1968) reported that aquatic hypercarbia reduced frequency of gill ventilation, while pulmonary respiration became more frequent. Likewise, *Lepidosiren* increased pulmonary ventilation in response to aquatic hypercarbia. (Johansen and Lenfant, 1968). At least partially, the hypercarbia-induced ventilatory responses are due to central chemoreceptors that increase pulmonary ventilation in response to reduced CSF pH (Sanchez et al., 2001). Consistently, a pioneering study on *Protopterus* reported that aquatic hypercarbia (5%CO_2) reduced gill ventilation, whereas pulmonary respiratory frequency increased (Johansen and Lenfant, 1968). Central chemoreceptors are present both in lungfish and in tetrapods classes and probably developed early during the sarcopterygian evolution.

In *Lepidosiren* the hypercarbia-induced ventilatory responses were characterized by an initial slow increase of $\dot{V}_E$ followed by a peak at 6 h. Subsequently, ventilation declined between 6 and 24 h to approximate initial normocarbic control values. Why this decline of ventilation? It might be that a partial compensation of intracellular pH took place and replaced ventilatory responses (Heisler et al., 1982), but the time seems rather short for this to occur.

In teleost fish, the kidneys contribute little to transfers of acid-base status related ions, while the bulk part of the transfers takes place by means of specialized cells in the gill epithelia (Heisler, 1984). When exposed to hypercarbia, teleost fish may increase plasma bicarbonate levels up to four-fold which efficiently approximates extracellular pH towards the original set-point value (Heisler, 1984; Claiborne and Heisler, 1986).

In *Lepidosiren*, it seems that neither gills, nor the kidneys, have any well-defined role in acid-base regulation. Rather, ventilatory responses provided some alleviation of acidosis as long as pulmonary ventilation eliminated was eliminated CO_2 to a normocapnic gas phase. The ventilatory responses were, however, short-term, which could suggest that the intracellular space as the regulated compartment.

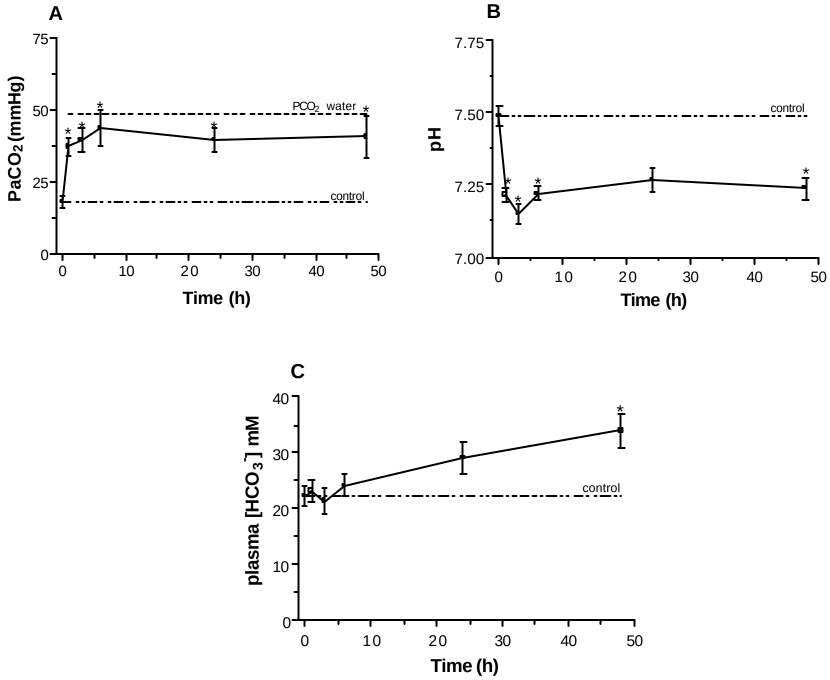

Fig. 1. A). The effects of aquatic hypercarbia (water PCO_2 = 49 mmHg; air at surface) on $PaCO_2$ in *L. paradoxa.* Zero h represents the normocapnic control value.. Notice that $PaCO_2$ was lower than PCO_2 of the water. B). The corresponding effects on pHa. C). Changes of plasma bicarbonate. Statistics as above. $P< 0.05$..Mean values ± SE. N-values: 0 h = 12; 1 h = 12; 3h =11; 6h =10; 24 h = 8; 48 h = 4.

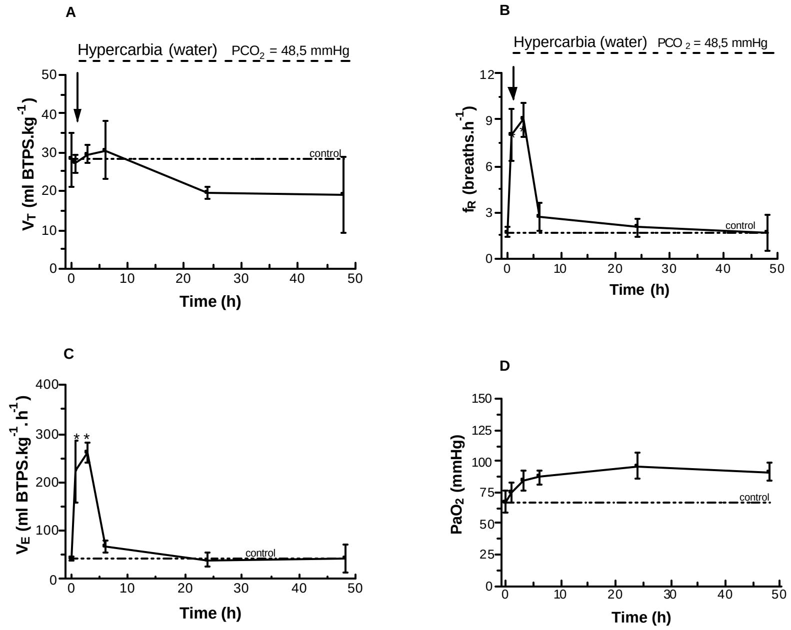

Figure A).The effects of aquatic hypercarbia (water PCO_2 = 49 mmHg; air at surface) on tidal volume (V_T). B). Effects on respiratory frequency (f_R). C). Time course of ventilation (V_E). D). The corresponding time course of PaO_2,. Statistics as above. $P< 0.05$.Mean values ± SE. N-values: Figs. 2, A, B, C: N =4; Fig. 2, D: N- 0 h = 12; 1 h = 12; 3h =11; 6h =10; 24 h = 8; 48 h = 4.

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CARDIAC FUNCTION
IN THE SOUTH AMERICAN LUNGFISH:
PECULIARITIES IN THE E-C COUPLING

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Abstract

This study analyzed the effect of an acute change in temperature and the impact of changes in stimulation frequency on the inotropic responses of the South American lungfish *Lepidosiren paradoxa* ventricle strips. Inotropic responses varied directly with temperature, decreasing from 25 to 15 °C and increasing from 25 to 35 °C. A post-rest potentiation of force was observed only at 15 °C, but ryanodine (sarcoplasmic reticulum function blocker) was able to depress twitch force at low stimulation frequencies, irrespective of temperature. Moreover, when *in vivo* stimulation frequencies were reached, the sarcoplasmic reticulum was relevant to the Ca^{2+} management only at 15 and 25 °C. In contrast, the lack of effect of ryanodine at the *in vivo* frequencies at 35 °C indicates that the excitation-contraction coupling depends exclusively upon transarcolemmal Ca^{2+} influx. Therefore, this organelle seems to have a slower Ca^{2+}-cycling capacity, in spite of being anatomically well developed and potentially functional in all the temperatures tested.

Introduction

The scope of fishes cardiovascular function and the wide-ranging environmental temperatures impose unique demands on the regulation of cardiac contractility and hence to the excitation-contraction (E-C) coupling. Contractile mechanisms (i.e., the actin-myosin interaction and its regulation by Ca^{2+}) appear to be similar in all vertebrate hearts (Driedzic and Gesser, 1994). However, anatomical and ultra-structural distinctions between hearts of different species underlie important physiological differences, particularly to the regulation of Ca^{2+} delivery to contractile apparatus (Tibbits et al., 1992).

On a beat-to-beat basis, Ca^{2+} may originate from the extracellular space or from intracellular stores (Driedzic and Gesser, 1988), specifically the sarcoplasmic reticulum (SR). The putative contribution of SR Ca^{2+} stores to excitation-contraction coupling (E-C coupling) has a profound impact on Ca^{2+} management, since this organelle is able to significantly reduce diffusional distances and accelerate both contraction and relaxation rates.

The relative importance of Ca^{2+} release from the SR in activation of cardiac muscle contraction varies considerably among different species, stages of development, stimulation frequency and temperature (Fabiato, 1982; Bers, 1991). Several studies have demonstrated that in fish this organelle is usually poorly developed and does not seem to directly contribute to the activation of the contractile apparatus at physiological stimulation frequencies and temperatures in ventricle strips of most species (Gesser and Poupa, 1978; Vornanen, 1989; Tibbits et al., 1991).

Since most of the studies on cardiac E-C coupling were carried out on temperate fishes, the goal of this work was to extend the knowledge to the neotropical lungfish *Lepidosiren paradoxa*. Presuming that the tetrapods ancestors were physiologically very similar to the modern lungfish (Burggren and Johansen, 1986), this group is supposed to provide a unique opportunity to study the physiological adaptations correlated with the emergence of air-breathing.

Material and Methods

Active specimens of *Lepidosiren paradoxa* were obtained from water filled clay pits near Cuiabá River, in the Brazilian Pantanal area, and acclimated at 25 °C.

Fish were sacrificed by decapitation, and ventricle strips ($\phi \cong 1$ mm) were excised from the heart and placed into a bathing medium containing (in mM) 100 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.5 NaH_2PO_4, 27 $NaHCO_3$, 2.5 $CaCl_2$ and 10 glucose and bubbled throughout the experiment with a gas mixture of 98% O_2 and 2% CO_2 (pH 7.5). Preparations were connected to an isometric force transducer and to a Grass stimulator delivering electrical square pulses with a voltage 50% above the threshold. Twitch tension was allowed to stabilize for about 30 min at 0.2 Hz before each protocol.

In order to analyze the effect of an acute change in temperature preparations were subjected to a temperature transition from 25 to either 15 or 35 ^{o}C over a 25-30 min time period. Moreover, to detail the capacity for the storage of Ca^{2+} in the SR, ryanodine was added to the bath, and the force developed by upon the first stimulation following a prolonged diastolic pause (10 min) or stepwise increases in stimulation frequency were determined at 15, 25 and 35 ^{o}C with and without pre-treatment with 10 μM of ryanodine. These *in vitro* frequencies were correlated to *in vivo* heart rate, as obtained by electrocardiography.

Results

The twitch force developed by ventricle strips of *L. paradoxa* was 6.29 ± 0.88 mN/mm^2 (mean ± SE; n = 50) after stabilization at acclimation temperature (25 ^{o}C).

The temperature transition from 25 to 15^{o} C resulted in a decrease ($p < 0.05$) in twitch force at the lowest temperature (Fig. 1A), while the increasing temperature resulted in a progressive and significant ($p < 0.05$) positive inotropic response at 32.5 ^{o}C and reached its maximum values at 35 ^{o}C. (Fig. 1B) An acute temperature transition of 10 ^{o}C does not seem to have any deleterious effect on heart tissue, since after the subsequent return to acclimation temperature, the Fc initially observed at 25 ^{o}C was restored for both experimental protocols.

The relative contribution of the Ca^{2+} stored in the SR to force generation after a diastolic pause of 10 min is presented in figure 2 (shaded area) as a function of experimental temperature. Pre-treatment with ryanodine resulted in a post-rest force about 21%, 17% and 38% lower than that observed for control preparations at 15, 25 and 35 ^{o}C, respectively. However, a post-rest potentiation of twitch force, indicative of a higher participation of the SR, was only observed at 15 ^{o}C.

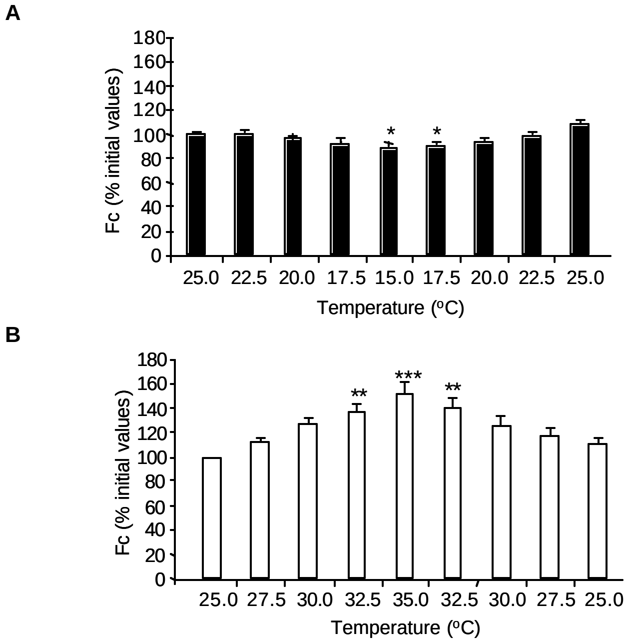

Figure 1 - The effect of temperature transition from 25 to 15 °C (A) or 25 to 35 °C and subsequent return to 25 °C on twitch force (Fc - % of the initial values) of ventricle strips of *L. paradoxa*. Mean values + SE (n = 10). Asterisks denote significant (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) differences in relation to the initial force.

In the force-frequency experiments, the positive relationship observed for control preparations at 15 °C (Fig. 3A) was shifted to the left in response to ryanodine and the strips started to contract irregularly bellow the *in vivo* heart frequency (0.29 ± 0.01 Hz; shaded area). At 25 °C (Fig. 3B), the force-frequency curve was shifted downwards by addition of ryanodine, resulting in a decrease in Fc of about 10% when frequencies within the *in vivo* frequency range (0.54 ± 0.03 Hz; shaded area) were reached. In spite of the shift in the force-frequency curve to the left after treatment with ryanodine at 35 °C (Fig. 3C), this drug had no effect on the force at *in vivo* frequencies (1.17 ± 0.03 Hz; shaded area).

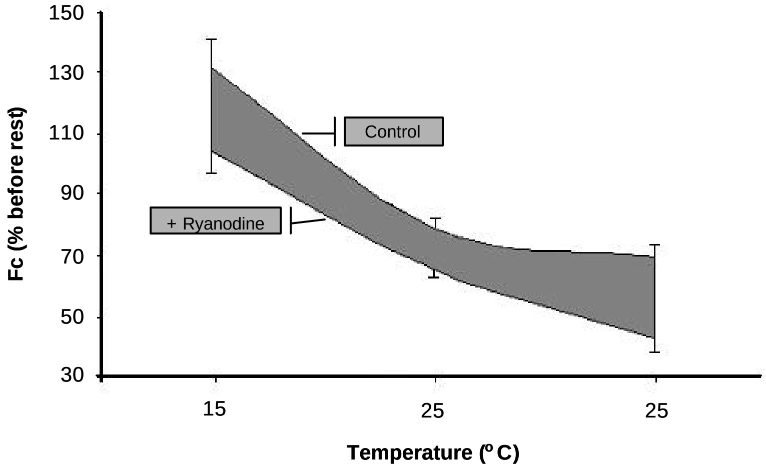

Figure 2 - Effect of temperature on force development (Fc - %; mean values ± S.E.) of ventricle strips of *L. paradoxa*, after 10 min of rest in the absence (top; n = 10) and presence (bottom; n = 10) of 10 μM ryanodine. Shading indicates ryanodine-sensitive force (SR contribution to force generation).

Discussion

The positive inotropism presented by *L. paradoxa* ventricle after temperature increases (Fig. 1A) differs from the responses reported for most of the ectothermic vertebrates and mammals, since these animals show a decrease in myocardial force (e.g. flounder, frogs, turtle, rats, rabbits) or a lack of change in twitch force (e.g. trout) at higher temperatures (Tibbits et al., 1992; Shi & Jackson, 1997; Bers, 2001). The maintenance of a positive inotropism at higher temperatures, even after the acceleration of cardiac dynamic, indicates that the species possesses very efficient mechanisms of Ca^{2+} transportation from and into the cytosol in the cardiac E-C coupling.

A potential explanation would be the presence of a very active and temperature-dependent NCX (Na^{+}/Ca^{2+} exchanger), resulting in a fast Ca^{2+} efflux from the cell during cardiac relaxation. This hypothesis is of special relevance due to the post-rest decay observed at higher temperatures (Fig. 2). Accordingly to Bers (2001), a decreased amplitude of the first post-rest contraction with increased resting periods occurs in ventricular myocardium of several mammalian species, and is linked to transsarcolemmal Ca^{2+} movements via NCX that reduce cytosolic Ca^{2+} during relaxation. This result contrasts with those described for most fish species, since in these animals the NCX presents relative temperature insensitivity

Indeed, at physiological stimulation frequencies, the temperature sensitivity of the NCX would allow the species to increase its cardiac performance as temperature is augmented even without a significant increase in SR activity (figure 3C). This finding suggests that *L. paradoxa* possesses an efficient transsarcolemmal Ca^{2+}-transporting system, which allows an adequate delivery of Ca^{2+} to the myofilaments at *in vivo* heart rates at high temperatures. Such mechanism could partially explain the positive inotropic response observed when temperature was acutely increased from 25 to 35 ^{o}C. (Xue et al., 1999)

Conversely, the unexpected negative inotropism observed after the decrease in temperature (Fig. 1B) indicates that an acute drop in temperature does not allow the expression of Ca^{2+} transporting proteins and/or myosin isoforms more adapted to low temperatures. In spite of this, the strong post-rest potentiation of twitch force (Fig. 2) at 15 ^{o}C, which has been related to a greater accumulation of Ca^{2+} in the SR (Edman and Johannsson, 1976; Rumberger and Reichel, 1972), associated to the shift to the left of the force-frequency curve (Fig. 3A), are indicative of a greater contribution of the SR at this temperature. The greater reliance on intracellular Ca^{2+} stores may be responsible for the lower Q_{10} values for both Fc and f_H observed after the acute decrease in temperature when compared to the values obtained when temperature was increased. This would result in a less pronounced effect of low temperatures than the higher ones on both ino- and chronotropic responses of the species heart and may prevent the occurrence of an acute drop in cardiac output. Therefore, within physiological limits, this sedentary and aestivating fish will be able to face acute reductions in environmental temperatures without great impairments in the cardiac performance.

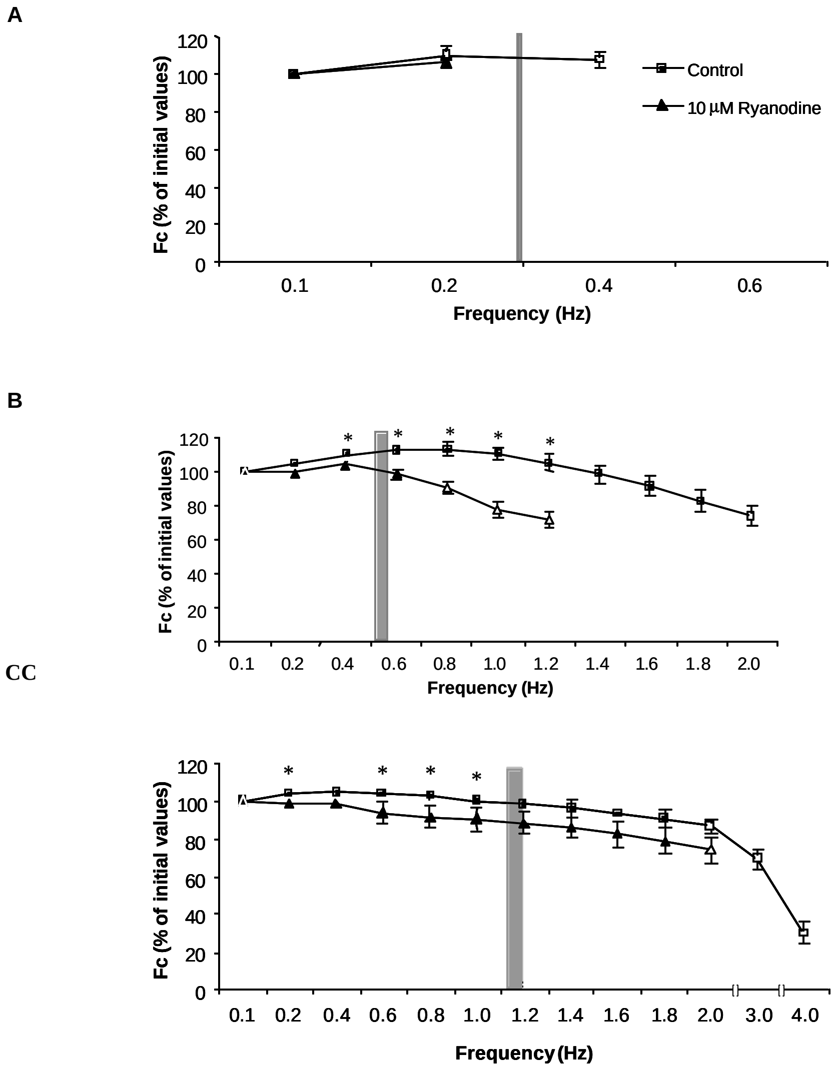

Figure 3 - Effect of increases in frequency on the force (Fc - % of initial values ± S.E., n = 10) developed by control preparations (squares) and after treatment with ryanodine (triangles) at 15 (A), 25 (B) and 35 ^{o}C (C). Open

symbols: difference ($p < 0.05$) in relation to the initial Fc; *: control ≠ ryanodine ($p < 0,05$). Shaded area: *in vivo* heart rate measured by ECG.

Taken together, the results suggest a low Ca^{2+}-cycling capacity of the ventricular SR of *L. paradoxa*, implying that this organelle does not play a significant role at the relatively high heart rates seen at elevated temperatures, in spite of its high anatomical development (Hochachka and Hulbert, 1978) and apparent functionality at lower temperatures and/or lower stimulation frequencies. Therefore, as temperature decreases, with a concomitant temperature-dependent decrease in chronotropism, the SR plays a central role in Ca^{2+} regulation, partially compensating the apparent temperature-dependent decrease in NCX activity. These results contrast with those described for the ventricle strips of most fish already studied (Keen et al., 1994; Thomas et al., 1996; Shiels and Farrell, 1997; Shiels et al., 1998; Costa et al., 2000) in which ryanodine does not change the force developed when physiological frequencies and/or temperatures are considered.

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EFFECTS OF ULTRAVIOLET RADIATION ON THE INCIDENCE OF ECTOPARASITES IN PIRARUCU, Arapaima gigas (CUVIER, 1829), (OSTEICHTHYES: OSTEOGLOSSIFORMES)

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Studies on the harmful effects brought about by the higher ultraviolet (UV) radiation incidence on living organisms have been increasing in the past few years. UV radiation makes up a small portion of the total radiation received from the sun. It is subdivided into ultraviolet A (UVA) 320-400nm, ultraviolet B (UVB) 280-320nm and ultraviolet C (UVC) 100-280nm, being UVB radiation the most dangerous due to its adding effect (Seeling, 2003). UV radiation effects on aquatic ecosystems are in directly related to the suspended particle number and depth. In some water bodies the UV intensity diminishes with depth. However, organisms that use the water surface, such as phytoplankton and obligatory air breathing fish, suffer direct influence from this radiation (Kirchhoff, 2003). Pirarucu, *Arapaima gigas* (Cuvier, 1829), is one of the most valuable fish found in the Amazon region. It can reach a total length of up to 3m, is widely distributed throughout the Amazon and is an obligatory air breathing fish, which needs coming up to the surface at regular intervals of few minutes to breathe oxygen from the atmosphere (Queiroz & Crampton, 1999). Thus, pirarucu ectoparasitas must suffer influence from UV radiation. Pirarucu ectoparasitas are: Monogenoidea, *Dawestrema cycloancistrium* Price & Nowlin, 1967, *D. cycloancistrioides* Kritsky, Boeger & Thatcher, 1985, and *D. punctatum* Kritsky, Boeger & Thatcher, 1985; Copepoda, *Ergasilus* sp.; Branchiura, *Argulus* sp. and *Dolops discoidalis* (Bouvier, 1899). Few studies have been performed to evaluate the UV radiation effect on ectoparasites organisms. The purpose of the present study is to evaluate the UVR (UVA + UVB) radiation effects on the pirarucu ectoparasite fauna. Fish were acquired from a fish-culture station and acclimated for eight days at the Molecular Evolution and Ecofisiology Laboratory (LEEM) in the National Research Institute of Amazon (INPA). Then, they were transferred to a room that had been adapted for the experiments. Fish on treatment 1 were exposed to UVR radiation for 1h/d and on treatment 2 to 2h/d. Control treatment fish were exposed for 4h/d to fluorescent light of the same voltage as that of ultraviolet. At the end of the experiments, the fish were slaughtered and underwent necropsy at the Fish Parasitology and Pathology Laboratory (LPP) in INPA. Parasite indexes were determined according to Bush *et al.* (1997) and analysed according to the different UV radiation exposure times. Only *D. cycloancistrium* species was found. Mean intensity and standard deviation in the control treatment were 583 monogenoids and 278.56 respectively. In treatment 1 they were 564 and 227.56. And in treatment 2 they were 265 and 86.76. There is little difference between control and treatment 1. However, there is a much bigger difference between control and treatment 2, which points out that the longer the exposure time the lower the parasite number. Studies have yet to be carried out on the effects of UV radiation on fish monogenoids. Nevertheless, studies conducted on shrimp, crab, and other invertebrates larvae found that the longer the UV radiation exposure time the higher the mortality rate of those organisms (Seeling, 2003), thus corroborating the findings in the present study.

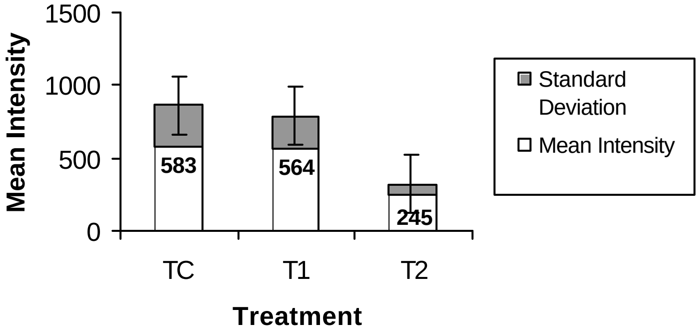

Figure 1 – Mean intensity variation of pirarucu parasite *Dawestrema cycloancistrium*, when exposed to UVR (UVA+UVB) (n = 3).
TC=control treatment; T1=1h/day exposure; T2=2h/day

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THE STRUCTURE AND COMPOSITION OF THE COCOON OF THE TERRESTRIALIZED AFRICAN LUNGFISH (*PROTOPTERUS DOLLOI*)

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EXTENDED ABSTRACT ONLY – DO NOT CITE

African lungfish are almost unique amongst fish species in that they are able to aestivate for long periods of time when faced with drought conditions. An essential aspect of this ability is the production of a protective cocoon. The aim of this study was to investigate if specific aspects of the structure and composition of the cocoon could be instrumental in lungfish survival under aerial conditions.

African lungfish were maintained, in individual aquaria, at McMaster University for approximately 6 months before terrestrialization. During this period the lungfish were fed on alternate days a diet of bloodworms. The water was then removed and the fishes terrestrialized in a darkened environment. On every second day 2 – 5 ml of water were sprayed onto the fish. Cocoons were collected, from representative fish, after 2 weeks, 8 weeks and 4 months terrestrialization. During collection the addition of water was used to assist cocoon removal. Once collected the cocoon was immediately frozen in liquid nitrogen and then stored at -70^{o}C.

For structural analysis samples of the cocoon were embedded in resin. Ten µm transverse sections were then cut, mounted glass slides and stained with toluene blue. For biochemical analysis cocoon samples were initially prepared by homogenizing a known amount (approximately 75 – 100mg) in 2.0ml, 0.2M perchloric acid (PCA). The homogenate was collected by centrifugation and washed twice more in 2.0 ml 0.2M PCA. The first and second supernatants were combined and retained for ammonia and urea measurements. Ammonia was measured using a commercially available ammonia detection kit (Raichem, San Diego, USA) and urea was measured according to the methods described by Rahmatullah and Boyed (1980). The final homogenate was dissolved, for 1 h at 37^{o}C, in 5.0ml, 0.5M NaOH. Total protein, RNA and DNA were measured according to the methods described by Mathers et al (1993).

Light microscopy revealed the majority of the cocoon biomass is composed of a series of layers of approximately equal thickness (Fig 1). In addition structures which appear to be cells appear at intervals on the inner surface (Fig 1).

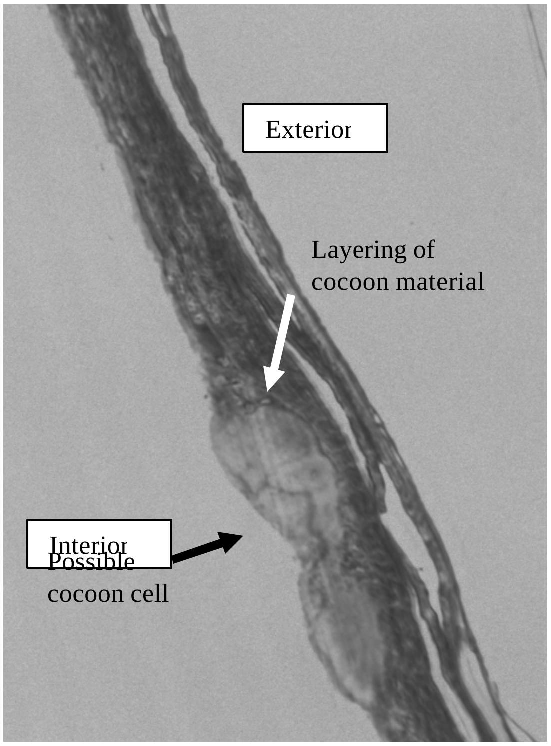

Figure 1. Toluene blue stained transverse section of the cocoon of the terrestrialized

African lungfish (x40).

The possibility of cells existing within the cocoon is supported by the detection of RNA and DNA in the overall composition (table 1). RNA : protein has been shown to be an index of the capacity for protein synthesis (Millward et al, 1973) and, in this sense, the African lungfish cocoon has a greater protein synthetic capacity than many fish tissues (e.g. Mathers et al, 1993; Smith et al, 1996).

Table 1. Nitrogenous waste and nucleic acid composition of the cocoon of the African lungfish at different terrestrialization periods. Superscript lettering indicates similarities and differences of each parameter in cocoons of different ages.

Cocoon age	Nitrogenous waste concentration (μmol g^{-1} wet mass)		Biochemical indices	
	Urea	Ammonia	RNA : protein (μ: mg)	RNA : DNA
2 weeks	41.6 ± 10.8^a	2.1 ± 0.8^a	111.1 ± 8.2^a	21.6 ± 3.8^a
8 weeks	3.8 ± 0.8^b	5.7 ± 2.5^b	119.2 ± 7.1^a	8.9 ± 2.6^b
4 months	9.0 ± 1.8^c	28.2 ± 6.4^c	112.9 ± 15.5^a	10.8 ± 0.6^b

The trend in ammonia and urea concentrations, with cocoon age, are similar to that measured in aestivating lungfish tissues (Chew et al, 2004) although cocoon ammonia concentrations are between 5 – 10 times higher than in the muscle, liver, brain or gut. This suggests a degree of compartmentalization of nitrogenous waste between the body and the cocoon during terrestrialization with the cocoon acting as a sink.

Whether or not these cells are instrumental in cocoon protein synthesis or nitrogen excretion is, as yet, unknown. More experiments are in progress to address these questions. Also a more detailed electron microscopy structure analysis is underway. However these data do suggest the cocoon of the African lungfish may be much more than an inert protective coating. Instead it may be a metabolically active tissue with specific excretory functions.

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DEVELOPMENT OF RESPIRATORY SYSTEMS AND RESPONSES
IN LARVAL AND JUVENILE LUNGFISH
(*PROTOPTERUS AETHIOPICUS:HEKEL)*.

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Despite their important position as the closest living prototetrapod relatives, the Dipnoi have received only sporadic research interest. The physiology of adults of the living genera *Neoceratodus, Lepidosiren* and *Protopterus* have been most frequently studied (Liem,1986; Fritsche et al.,1993; Harder et al.,1999) but larval stages have been poorly studied. Accordingly the following studies were carried out on developmental stages of an African lungfish *Protopterus aethiopicus.(Hekel)*

Larval and juvenile stages of *P. aethiopicus* were collected from L. Victoria near Entebbe, Uganda. Respiratory and cardiac movements were observed and counted using a dissecting microscope. At collection the youngest larvae had 3 pr. external gills (equivalent to Stage 35 for P.annectens, Kerr, 1909) but both buccal pumping and surface visits to breathe air were observed. It was thus assumed that four areas, lungs, external and internal gills and the skin were potentially available for gas exchange.

Results

The responses of branchial and lung ventilatory systems and, in some stages, heart rate were measured on a range of developmental stages during confinement under water and during exposure to normoxic and hypoxic water.

The responses are compared with those of adults (McMahon, 1970) to illustrate the development of respiratory systems and their control.

Effects of emersion

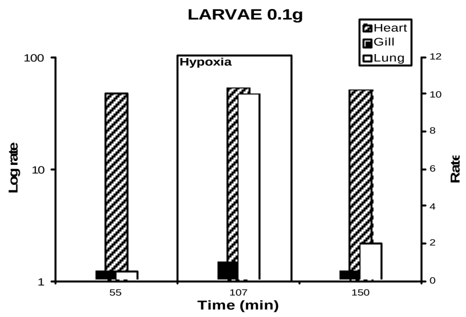
Table 1 Variation in viability of *P.aethiopicus* immersed in normoxic water (PO_2 =120mm.Hg; 16kPa) with no access to aerial ventilation.

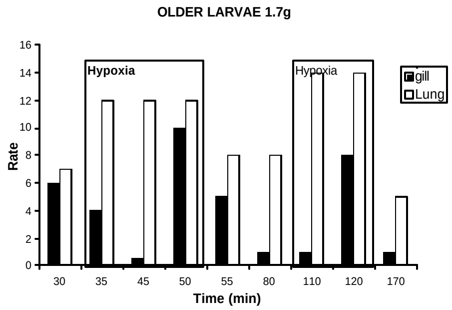
Stage	**External**	**Internal**	**Lungs**	**Viability Immersed**
Young larva (35)	3 pairs	Poorly developed	Functional: poorly perfused	>15days
Older larva	2 pairs reduced	gill slits open	>50% perfused	6days
Juvenile	Reabsorbed	Functional	Functional	1-4days
Small adult	“	“	“	Approx 24h

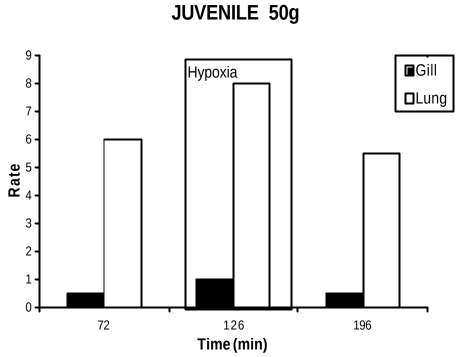
Effects of exposure to severe ambient aquatic hypoxia (20mm.Hg, 2.5kPa) with access to air.

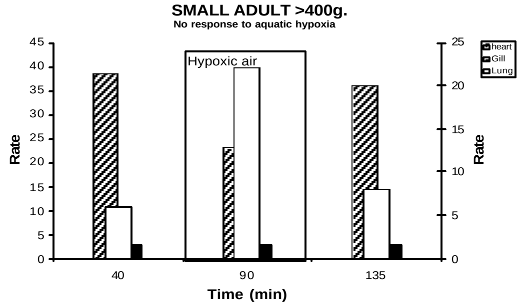
Rate responses of the heart and both branchial and lung ventilation systems of two larval stages, juveniles and small adult *Protopterus aethiopicus* were assessed in normoxic and hypoxic water (Figure 1)

Figure 1. Variation in cardiac and respiratory responses to severe hypoxic exposure during development in *Proptopterus aethiopicus*.









Discussion and Conclusions

During development *Protopterus aethiopicus* must transfer from fully aquatic to bimodal gas exchange. At hatching no accessory gas exchangers are developed and the larvae must rely on exchange across general body surface. In later larval stages external gills, lungs and internal gills become functional but larvae can sustain life confined under normoxic water for extended periods (Table 1).

In severely hypoxic water the available aquatic gas exchangers are insufficient. Prior to the development of accessory exchangers, oxygen levels in the nest are enhanced by ‘thrashing’ movements of the bodies of parental adults and probably by outward diffusion of oxygen across the parental gills and body surface. Specialized pelvic fin exchangers as reported for *Lepidosiren* are not seen in *Protopterus.*

External gills develop and become functional very early in development and these provide an additional exchange surface while the lungs and internal gills develop. Importantly the external gills do not require the complex musculo-skeletal and neurological developments associated with ventilation of internal gas exchangers and thus can be functional in the early larval stages.

Perhaps sensibly in an often hypoxic environment, lung ventilation appears to be functional earlier than branchial ventilation and is used predominantly in response to hypoxic exposure even when both external and internal gills are present. This situation persists into the adult where >90% of oxygen uptake occurs across the pulmonary surface even in normoxic water. Aquatic ventilation alone is not sufficient to sustain oxygen uptake in adults but is maintained at a low level and is important in the elimination of CO_2.

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