METABOLIC PROFILE OF THE FACULTATIVE AIR-BREATHING

NEOTROPICAL TELEOST FISH

HOPLERYTRINUS UNITAENIATUS (JEJU):

SUBMITTED TO EXERCISE.

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Introduction

Adaptation at the biochemical and physiological levels are established in the capacity for metabolic adjustments during exercise to all organisms. In fish, exercise to exhaustion involves short bouts of high intensity swimming. During this type of activity many physiological and biochemical systems approaches their limits (Milligan, 1996) Exhaustive exercise may produce in respiratory and metabolic acidosis.

Studies on the lactic kinetics after severe exercise in fish try to explain the metabolic fate of such metabolite. As it has been shown, it seems that accumulation of lactic acid and/or glucose, as the depletion of liver glycogen store is very distinct between fishes. The channel catfish presents an increase of blood lactate after 5 minutes under exercise (Cameron, 1990) but the maximum value is reached only 10 minutes after swimming. The fast exercise recovery (4 hours) demonstrated the greater oxidation capability of this fish compared to others. This is indicated by significant glyconeogenesis and glucose rise only during recovery. In contrast, in salmonids after severe swimming, blood lactate levels increase 8 to 10 folds higher than increased values under conditions other than that (Milligan and Girard, 1993). This amount of lactate represents 10-20%

of the total produced which gave to them the term "lactate-releasers" (Wood and Perry, 1985) in contrast to the "non-releasers", like the plaice *Pleuronectes platessa* L. (Wardle, 1978).

Besides lactate mobilization, the rise of glucose concentration is also observed in some species (Haux et al., 1985; Schwalme and Mackay, 1985a, 1985b) and the pre-exercise values are returned to the post-exercise after 24 hours, as it happens in the yellow perch *Perca flavescens* (Schwalme and Mackay, 1991). After the strenuous exercise, glucose concentration still increases until 8 hours. Several mechanisms should explain this event: *1)* glycogenolysis, *2)* slow disappearance of lactate via Cori cycle, *3)* glyconeogenesis from glycerol or aminoacids *4)* preferential oxidation of lactate, inhibiting the glucose catabolism.

The family Erythrinidae is freshwater teleosts and usually living in shallow streams and swamps of South and Central America. *Hoplerythrinus unitaeniatus* (jeju) presents a very intensively vascularized swim bladder and is able to move from pond to pond through across dry lands (Grahm, 1995).

In the present study, considering all these information, the ability of the erythrinid *Hoplerythrinus unitaeniatus* were investigated in respect to the exercise along seven days. It is reported its ability to bypass low environmental oxygen through its behavior. How is the metabolic change of jeju when submitted to exhaustive mechanical chasing? Trying to approach such answer several metabolites, enzymes and other parameters were measured in normal and exercised fish. Among such one, glycogen, glucose, lactate, pyruvate, ammonia, urea, hematocrit, blood pH, glutamine synthetase, ornithine carbamoyl transferase and arginase were determined.

Material and Methods

Animals and Experimental Design

Specimens of *Hoplerytrinus unitaeniatus* (100g ± 50) captured at Mogi Guaçu River, Pirassununga - São Paulo - Brazil. The animals were kept in tanks for 48 hours for complete recovery under pO_2 130mmHg at 25°C and starvation. After recover the animals were transferred to acclimation tanks referred to as exercised and unexercised. This fishes were subjected to either mechanical chasing was done twice a day (15 min.) along seven days. In parallel, control

fishes (unexercised) remain unchanged state. After such period the experiment, the blood was sampled by caudal puncture. After blood withdrawal, the animals were killed by punching the spinal cord. Liver and white muscle samples were kept under liquid nitrogen. The values of blood pH were determined immediately after the blood sampling and a sample blood was centrifuged at 7,000g for 3 minutes (4°C) for separated plasma. hematocrit values (Hct) were determined in microhematocrit capillary tubes (12,000g for 3 min.).

Biochemical DeterminationsCell extract

Hepatic tissue slices put into suitable 10mM phosphate buffer pH 7.0 and submitted to mechanical disruption for 30 sec. in a Potter homogenator. The extract was used for enzyme analysis. All studied enzymes were colorimetrically assayed by end-point procedures.

Glutamine synthetase (GS)

The activities of GS were determined in 50mM HEPES buffer pH 7.0 in a reaction mixture containing 20mM K_2AsO_4, 60mM glutamine, 15mM hydroxilamine, 0,4mM ATP and 3mM $MnCl_2$. The incubation period the reaction was stopped and developed by acid ferric chloride reagent. Such reaction consisted of the development of γ-glutamil hydroxamate determined by Vorhaben et al.., 1973.

Ornithine carbamoyl transferase (OCT)

The activity of OCT was determined in a reaction system containing 50mM of HEPES buffer pH 8.5, 10mM of ornithine and 10mM of carbamoyl phosphate. The reactions were incubated and stopped by 70% TCA addition. The citrulline was determined in the supernatant (Boyde and Rahmatullah ,1980).

Arginase (ARG)

The activities of ARG were determined in 50mM HEPES buffer pH 11 in a reaction mixture containg 278mM of arginine and 10mM $MnCl_2$. The urea was determined in the supernatant (Rahmatullah and Boyde ,1980).

Glycogen

Muscle and liver glycogen were determined after alcoholic precipitation, by the acid hydrolitic procedure described by Duboie et al (1956) and adapted by Bidinotto et al (1997).

Glucose, lactate and pyruvate

The free protein acid extract of 20%trichloroacetic acid was employed to determine glucose by Duboie's method (Duboie et al, 1956) and lactate as described by Harrower and Brown (1972). The same extract, after neutralized by 6N KOH, was used to determined pyruvate by 2,4dinitrophenilhydrazine as described by Lu (1939).

Free Aminoacids

Free aminoacids were measured in water extracts after neutral protein extraction with 0.3N $Ba(OH)_2$ and 5% $ZnSO_4$ as Copley (1941).

Data analysis

All data were compared by Mann-Whitney test resulting different at 0.95 CI.

Results and Discussion

Exercise imposes metabolic adjustments to all organism. Among some kinds of exercise, chasing may simulate several natural situations. Significant decrease of blood pH followed by hematocrit increase was observed after seven days of regular chasing (table I). Such result must be considered in accordance to the metabolite changes observed in plasma (fig. 1) and tissues (table II, fig. 2 a,b). Lactate concentration was slightly increased in plasma and white muscle. Such fact associated to ammonia decrease in plasma should explain its pH decrease. Anaerobic degradation of glycogen results in accumulation of lactate in the muscle and eventually in the blood. As H^+ and lactate are formed in equivalent amounts in glycolysis the lactate levels in the blood may reflected a metabolic acidosis.

TABLE I. Values of pH and hematocrit observed in *H. unitaeniatus* exposed to stress caused by chasing exercise.

Parameters	Control	Chasing
pH	7.610 (± 0.045)	7.045(± 0.052)
Ht	26.25 (± 0.32)	34.00 (± 0.45)

Plasma urea was also significantly decreased. These results, associated to decrease in GS and OCT activities (table III), suggest some changes on nitrogen metabolism. Decrease of plasma ammonia is coherent with GS and OCT reduction. This should be suggesting a possible mechanism to hold the acid-base balance.

TABLE III Enzyme activities of GS, OCT and ARG in the liver of H. unitaeniatus exposed to chasing

Activity (μmols/min/g)	Control	Chasing
GS	0.308 (± 0.023)	0.227 (± 0.015)
OCT	0.049 (± 0.0017)	0.034 (± 0.0012)
ARG	20.149 (± 3.563)	20.949 (± 3.843)

It was possible to establish a particular biochemical profile involving liver, white muscle and plasma.
Exercise imposes metabolic adjustments to all organisms. Among some kinds of exercise, chasing may simulate several natural situations. Strategies to face the stress caused by exercise may change from metabolic to behavioral one.

Decrease of liver glycogen, glucose, pyruvate and lactate suggests such organ as uncharged by glucose supplier as well as lactate consumer. A different profile is observed in white muscle. The increase of lactate, particularly the rate lactate/pyruvate, followed by glycogen and glucose decrease, points to glucose fermentation in such tissue. More over, decrease of free aminoacids hints the employment of such substances as a fuel for such chronic circumstances.

TABLE II Comparison between metabolite contents for different tissue

Metabolite	Plasma (μmols/ml)		While muscle (μmols/g wet tissue)		Liver (μmols/g wet tissue)	
	Control	Chasing	Control	Chasing	Control	Chasing
Ammonia	1.272 (± 0.08)	1.078 (± 0.09)	*	*	*	*
Urea	0.274 (± 0.02)	0.232 (± 0.04)	*	*	*	*
Glycogen	*	*	12.655(± 3.965)	10.525(± 3.079)	189.1(± 2.514)	101.7(± 2.989)
Glucose	6.406 (± 0.35)	4.489(± 0.67)	10.380(± 2.413)	10.030(± 2.781)	166.3(± 6.332)	117.6(± 7.865)
Pyruvate	0.391 (± 0.008)	0.311(± 0.09)	0.406(± 0.091)	0.309(± 0.071)	1.139(± 0.159)	0.978(± 0.123)
Lactate	0.108(± 0.032)	0.132(± 0.012)	8.146(± 1.170)	10.177(± 1.781)	5.311(± 1.032)	3.930(± 0.985)
Free aa.	*	*	1.679 (± 0.198)	1.245(± 0.394)	*	*

* = not assayed

Fishes are continuously spending energy through swimming, sometimes more intensively as in "burst" activity, sometimes less and supportably. Their white muscle is the main tissue accountable for fuel discharge in support the exercise demand. and glycogen is the major fuel of this metabolic process (Driedzic and Hochachka, 1978).

The anaerobic conditions impels glycogen or glucose catabolism to pyruvate which is then converted into lactate in order to maintain the "redox" balance (Stryer, 1992). Some distinct fates might be expected to lactate in white muscle of fish. The turnover of this metabolite into glucose via the Cori cycle in liver is demonstrated in some fishes, like the yellow perch (Schwalme and Mackay, 1991). Although, the rainbow trout experimented by Black et al., (1962) did not recover the glycogen stores even 24 hours after the exercise, suggesting that the lactate was converted into CO_2. The blood lactate concentration, which increased 30 percent after the exercise stress in *H. unitaeniatus* is indicative of anaerobiosis of white muscle. In *Perca flavescens* (Schwalme and Mackay, 1991) the concentration of this metabolic intermediary is also elevated in white muscle and liver after prolonged handling-stress. These results may include *H. unitaeniatus* in the group of the "lactate-releasers", a term proposed to salmonids (Wood and Perry, 1985).
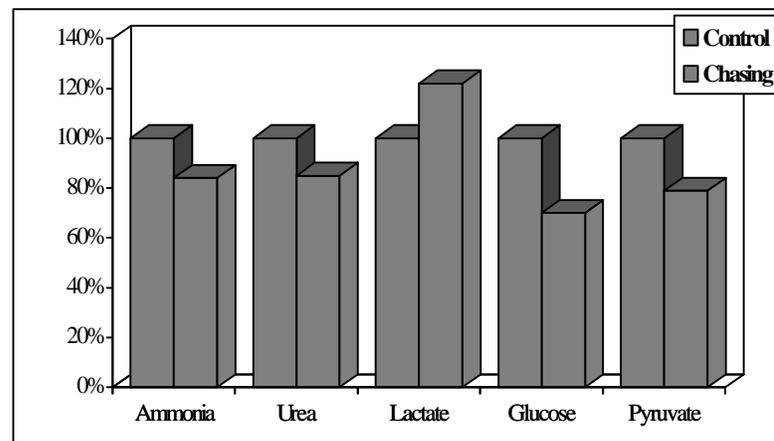

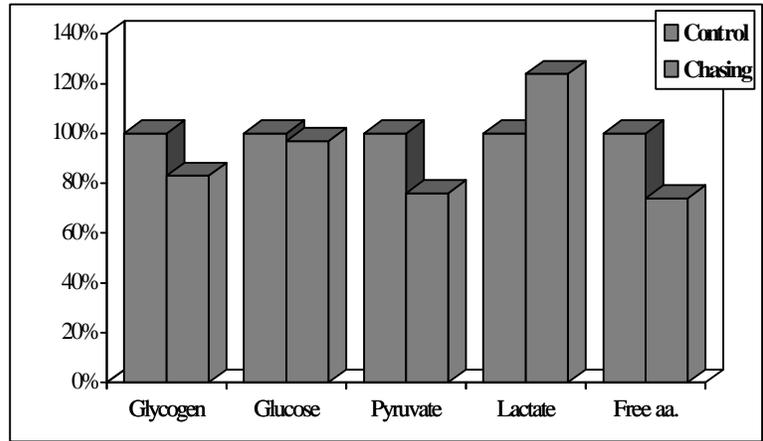Figure 1. Plasma metabolic intermediary in *H. unitaeniatus* submitted to chasing.

Figure 2a. White muscle metabolic intermediary in *H. unitaeniatus* submitted to chasing.
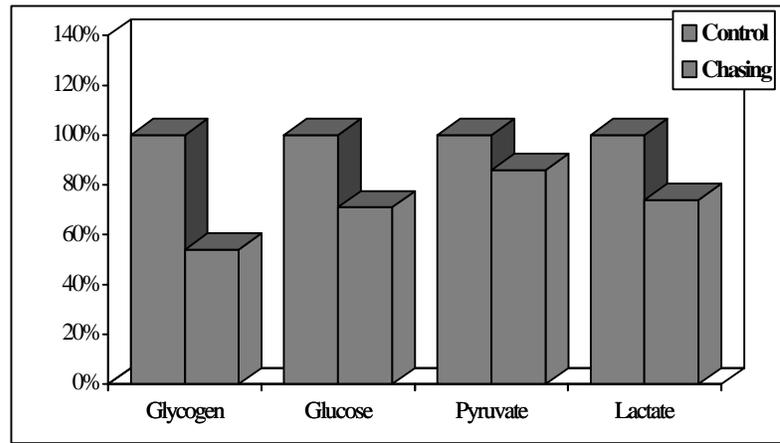

Figure 2b. Liver metabolic intermediary in *H. unitaeniatus* submitted to chasing.

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ORNAMENTAL FISH FROM THE RIO NEGRO BASIN:

OVERCOMING DISEASE-RELATED MORTALITIES.

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Introduction

The aquarium fish industry is an important element in the economy of several exporting countries. In these countries the industry has been contributing not only to the entrance of foreign currency but also in the creation of jobs in very remote areas where people have little opportunity to earn money. In this context, Brazil, one of the major exporting countries in South America, offers good opportunities in this growing market, mainly due to the richness of its fish fauna, which is only partially exploited.

In Brazil the main source of ornamental fish for export is a single region, the middle Rio Negro basin, where the towns of Barcelos, St. Izabel and Sao Gabriel da Cachoeira are considered important trading posts for ornamental fish post-capture. The ornamental fish industry from this region is a long established business. However, attempts to modernise have principally been restricted to the facilities located near the main exporting centre, Manaus. The techniques for fish capture and transportation are, basically, the same as those used at the beginning of exploitation, although better collectors and exporters have implemented some improvements in fish husbandry management post-capture (Ferraz, 1997). However, while the practices utilized by those fishermen who regularly deliver good quality fish to the Manaus exporters' holding facilities have resulted in lower mortalities for such enterprises, neither their practices nor

their improvements can be said to apply to all fishermen/collectors and exporters. Consequently, diseases and mortalities are still present in certain quarters today.

Methods

To assess the major health problems present in the ornamental fish from the Rio Negro basin, and their possible causes, a variety of approaches have been used. These involved: 1) interviews with fishermen, middlemen and exporters; 2) recording the condition of the fish on their arrival at the exporters' holding facilities; and 3) routine disease screening of 7 species of fish. The disease screening techniques were conducted following the procedures recommended by Francis-Floyd (1995) and Ferraz (1995).

Results and Discussion

The results indicate that:

1) the seasonal fluctuations of the water level play an important role in determining fish health; fish captured from areas with low water levels during the long dry season are often found to be in a debilitated condition;

2) in the chain of events linking capture to export the most critical phases influencing the health and survival of the fish are the storage of fish by fishermen and the transport to the local reception areas of the exporters in Barcelos. The transport from Barcelos to the principal fish holding facilities in Manaus was previously considered a critical phase, but the recent provision of new boats for fish transport, and improvements in fish husbandry prior to transport, have contributed to reduced mortalities during this period;

3) the most common diseases diagnosed are related to the series of handling stressors and sub-optimal conditions which fish are exposed to prior to delivery at the exporters' holding facilities (Table I). The negative effects of handling stress on the condition of the fish can be reduced if more attention is paid to the welfare of the fish following the arrival of the consignments at the reception areas in Barcelos.

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Another problem faced by the Brazilian ornamental fish industry concerns the legislation controlling the export of ornamental species. According to the lists published by the Brazilian Environment Agency, IBAMA (1992; 1998), it is permissible to export 184 species of wild ornamental fish. However, these new legislations still do not include all of the species exported as ornamentals. Consequently, problems related to the export of non-permissible species, possessing similar external characteristics to and recorded under the same Latin or common name as a permissible species, are still present.

The species of stingrays recently released for export clearly reflect this situation. Six species are of interest as ornamentals, but only four, *Potamotrygon motoro*, *P. schroederi*, *P. orbignyi* and *Potamotrygon* sp. were included in the list of species that can be legally exported (IBAMA, 1998). The most ornamental of all stingrays, *P. leopold.,* was not included in IBAMA's list, but this species can easily be exported as *P. motoro*, because the two species have similar characteristics (Araujo, 1997).

The misidentification of the species of fish exported, caused by a lack of adequate monitoring, is perhaps one of the most common problems faced by the Brazilian ornamental fish industry. However, it is difficult for the government to provide adequate monitoring because of:

1)   the large number of exotic species exported; and

2)   The lack of specialised people able to accurately identify the species in the authorized airports, prior to export.

Table I: Clinical signs commonly observed in the consignments of 7 species of ornamental fish examined at the exporters' holding facilities in Barcelos

Clinical signs	Fish Species							Predisposing Factors
	Paracheirodon axelrodi	Hyphesobrycon erythrostigma	Symphysodon. discus	Ancistrus spp.	Corydoras robinae	C. adolfoi	C. burguessi	
Sudden death of many fish with few preceding clinical signs	X	X	-	X	-	-	-	Exogenous toxins Peracute bacterial diseases, Overstocking
Haemorrhages of scales, fins	-	-	X	-	X	X	X	Overstocking, Bacterial infection.
Skin lesions/ulcers	X	-	X	X	-	-	-	Traumatic damage, Predation,EctoparasitesB acterial infection.
Swellings of the skin	-	-	X	-	-	-	-	Parasitic cysts, Physical damage, Tumours.
Overproduction of gill/ skin mucous	-	-	X	X	X	X	X	Ectoparasites, Environmental irritations.
Corneal opacity	-	-	-	-	X	X	X	Eye fluke (diplostumula of Strigeoidea)

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Table 1. continued.

Clinical signs	Fish Species	Predisposing Factors						
	Paracheirodon axelrodi	Hyphesobrycon erythrostigma	Symphysodon. discus	Ancistrus spp.	Corydoras robinae	C. adolfoi	C. burguessi	
Eroded fins	X	X	X	X	X	X	X	Traumatic damage; Overstocking, Cannibalism, Bacterial infection.
Lethargy	X	X	X	X	X	X	X	Ectoparasites (mainly external monogeneans and protozoans)
Erratic swiming	-	-	-	-	X	X	X	Cysts of Strigeoidea present in the cranial cavity or compressing the nervous system
Growths on the skin	-	-	X	-	-	-	-	Tumours or viral infection (papilloma, lymphocystis).
Starved fish (large head, compared with a thin, cachexic body)	-	-	-	X	-	-	-	Long period of deprivation of food post-capture.

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MECHANISMS OF HAEMOGLOBIN-OXYGEN AFFINITY ADAPTATION IN FISH OF THE AMAZON

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Abstract

Fish of the Amazon face large environmental variations particularly concerning oxygen availability. They have evolved a broad spectrum of respiratory strategies to secure oxygen transfer from the ambient to the tissues. Adaptive adjustments of haemoglobin-oxygen affinity have been associated with molecular heterogeneity of haemoglobin (alo and isoHbs) and thus with the intrinsic oxygen affinity, and sensitivity to allosteric modulators. A difference between species generally correlates with structural differences of haemoglobin molecule and/or with different types of haemoglobin modulators. In addition to ATP (adenosine triphosphate) and GTP (guanosine triphosphate), the major allosteric modulators of $Hb-O_2$ affinity, IPP (inositol pentaphosphate) and 2,3DPG (2,3 diphosphoglycerate) have been detected in the erythrocytes of fish of the Amazon. Individual adaptive adjustments of blood oxygen affinity result mainly from changes in the relative concentrations of isoHbs and in the allosteric modulator levels, which are elicited by specific exogenous and endogenous factors (e.g. oxygen tension, pH, temperature, salinity, etc). Other environmental factors such as water types (e.g. white and black water), river water level and velocity changes, and water pollution may also induce changes in these factors.

Introduction

The amount of dissolved oxygen in waters of the Amazon is determined by complex interactions of physical, chemical, and biological processes. Such processes are all affected by the regular and seasonal river water level oscillation (Junk *et al*., 1989) and so is the amount of dissolved oxygen for a given place

and daytime. These changes in dissolved oxygen are long term changes. Indeed, mixed patterns of dissolved oxygen occur simultaneously. For example, during high river water level periods extremes of oxygen may occur in different places of the same lake because of water plants covering. During low river water season anoxic condition is observed in such places due to the decomposition of the water plants. Such anoxic condition occur together with high levels of dissolved hydrogen sulphide and methane (reviewed by Val and Almeida-Val, 1995). In addition, extreme variations in dissolved oxygen tend to occur in a very short time period. In *várzea* lakes, for example, not rarely oxygen levels drop to zero at night and reach over saturated levels at noon the very next day (Junk *et al*., 1983; Val and Almeida-Val, 1995). These short term changes in dissolved oxygen force rapid respiratory adjustments in aquatic animals, particularly in fishes (Val, 1996).

The ability of the fish of the Amazon to survive and thrive under such extremes of dissolved oxygen depends on a myriad of organismic, biochemical, and physiological adjustments. These adjustments are directed towards enhancing both oxygen uptake from the environment and the oxygen transfer to the tissues. They are initiated as soon as the animal detects an environmental change through its sensory system or through the effects on metabolic processes (Wooton, 1990). According to Slobodkin and Rapoport (1974), the nature of these adaptive adjustments is related to the time scale of the environmental change. Short-term environmental changes result in individual responses while long-term changes result in population responses.

Haemoglobin (Hb) transports oxygen from the respiratory surfaces (lung, gills, accessory air-breathing organ) to the oxygen-consuming structures and carbon dioxide, anions, protons, and heat in the opposite direction (see Jensen, 1991). This dual function is related to the haemoglobin ability to bind reversibly with molecular oxygen through the four haem groups and H^+ and CO_2 to specific amino acid residues. Such ability is further affected by other erythrocytic cofactors, mainly organic phosphates. The main organic phosphates modulating O_2 affinity in the red blood cells of the fish of the Amazon are ATP and GTP, both of them decreasing Hb-O_2 affinity (see Val, 1996).

The solutions adopted by fish to survive low oxygen availability have been recently reviewed (Val, 1996; Val and Almeida-Val, 1995; Randall, 1993; Almeida-Val *et al*., 1993). This paper reviews the available data on haemoglobin heterogeneity in fish of the Amazon. Additionally it addresses the relevance of

the adjustments of erythrocytic organic phosphates as an immediate way to adjust the Hb-O$_2$ affinity in fish exposed to changes in dissolved oxygen.

Haemoglobin heterogeneity

The great majority (95%) of tropical fish has multiple haemoglobins (two or more) in their erythrocytes (Fyhn *et al*., 1979; Galdames-Portus *et al*., 1982). No significant relationship is observed between such haemoglobin heterogeneity and the phylogenetic status of the fish species so far analysed (reviewed by Perez *et al*., 1995; Val and Almeida-Val, 1995). Our recent analysis of haemoglobin multiplicity on species of catfish (Siluriformes) using polyacrylamide gel electrophoresis revealed numbers of haemoglobin fractions ranging from one up to twelve (Galdames-Portus and Val, unpublished data), supporting this observation. Fishes can be divided in two groups regarding such haemoglobin heterogeneity. Group I includes fish species that exhibit a marked relationship between structural and functional heterogeneity while group II includes fish species that exhibit a slight or no relationship between structural and functional heterogeneity. Based on the characteristics of the haemoglobins of group I, many authors have claimed the adaptive characters of such heterogeneity (Brunori *et al*., 1979; Garlick *et al*., 1979; Val *et al*., 1986; Wilhelm Fo and Weber, 1983; Brix *et al*., 1998). However, no clear correlation exists between haemoglobin heterogeneity and environmental and physiological parameters.

Two other aspects should be considered when analysing haemoglobin heterogeneity in fish. First, the haemoglobin exists in the red cell at the limit of its solubility and, second, the deoxyhaemoglobin is less soluble than its counterpart, the oxygenated form (Riggs, 1979). For fish living in a chronic hypoxic environment, any mutation decreasing haemoglobin solubility of the total haemoglobin within the red blood cell would be disadvantageous. This seems to be the reason for the generalised haemoglobin multiplicity occurring together with the preservation of high haemoglobin concentration in fish of the Amazon, and may explain the large number of fish species belonging to group II.

Despite such haemoglobin heterogeneity described for tropical fishes, low levels of allohaemoglobins (polymorphism) have been documented. The proportion of polymorphic species among fish of the Amazon, for example, may be even smaller than that previously reported by Fyhn and co-workers (Fyhn *et al*.,

1979). The difficult taxonomy associated with fish of the Amazon is a complicating factor. Several species previously considered polymorphic are currently known as different species. The best example is the case of *Semaprochilodus* species (Val and Almeida-Val, 1995)

Adjustments of haemoglobin proportions

Several fish species having multiple haemoglobins are able to adjust the relative concentration of each Hb fraction according to environmental and/or physiological conditions. We have described significant changes in haemoglobin proportions for *Colossoma macropomum*, *Prochilodus nigricans*, *Callophysus macropterus*, and *Hypophthalmus* spp (Val, 1996; Ramirez-Gil *et al*., in press; Schwantes and Val, 1996). In *Prochilodus nigricans*, the proportion of the four bands is clearly different between the animals collected during high water (July) and those collected during low water season (November). *Callophysus macropterus* possesses three haemoglobin fractions, which proportions are clearly different between the animals collected from Solimões River (white water) and those collected from Negro River (black water). The mean of the concentration of Fraction I was significantly greater (t test, P<0.001) in the animals collected at Marchantaria island. The mean of the concentration of fraction II did not differ significantly between the individuals from the two sites. The mean of concentration of fraction III was significantly greater in the animals from Anavilhanas Archipelago (t test, P<0.001). These results are summarised in figure 1. This difference in the proportion of the haemoglobin fractions results in different blood oxygen affinities.
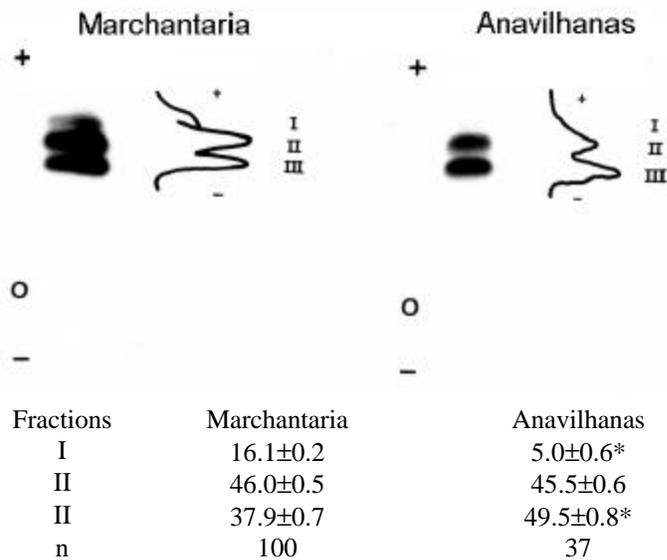
Fractions	Marchantaria	Anavilhanas
I	16.1±0.2	5.0±0.6*
II	46.0±0.5	45.5±0.6
II	37.9±0.7	49.5±0.8*
n	100	37

Figure 1. Haemoglobins of *Callophysus macropterus* collected from Solimões River (white water) and those collected from Negro River (black water). * = Significant difference from animals collected from Solimões river. (Data from Ramirez-Gil *et al*., in press).

Semaprochilodus insignis, on the other hand, has four haemoglobins fractions in the erythrocytes. Fraction I is the second largest component and exhibits a significant variation that correlates to the migration phase of the animals. Haemoglobin fraction II is the largest one and does not exhibit any variation. Fractions III and IV exhibit an intrapopulational variation suggesting that the concentration of these components is regulated on an individual basis (Val and Almeida-Val, 1995).

These two groups of animals clearly indicate that the stimulus for the adjustment of haemoglobin proportion may be exogenous (river water levels changes, temperature, migration phase, oxygen availability, etc.) and/or endogenous (regulated according to individual characteristics). How these adjustments are

improved, particularly in animals living in an ever-changing environment such as the Amazon, remain to be described.

Haemoglobin sensitivity to allosteric modulators

Differences in intrinsic oxygen affinities and sensitivities to allosteric effectors correlates with differences in structural properties, which are genetically coded. These differences are neatly illustrated in fishes of the Amazon. The first example includes two closely related fish species: the water breathing aruanã, *Osteoglossum bicirrhosum* and the air-breathing pirarucu, *Arapaima gigas*. These fish species evolved haemoglobins that are distinct regarding sensitivity to allosteric modulators. The predominant haemoglobin effectors in *O. bicirrhosum* are ATP and GTP whereas IPP is the main effector in *A. gigas* (Isaacks *et al*., 1977; Val *et al*., 1992b). Interestingly, the juveniles of *A. gigas* have only ATP and GTP (Val *et al*., 1992b); the appearance of IPP is not related to any apparent change in electrophoretic Hb pattern.

Hoplosternum littorale and *Hoplosternum thoracatum* are two closely related facultative air-breathing species that also illustrate this aspect. These fishes gulp air into a well-vascularized stomach/intestine where oxygen is taken up. They present a similar haemoglobin electrophoretic pattern but distinct haemoglobin effectors. In *H. thoracatum* the usual levels of ATP and GTP have been observed whereas in *H. littorale* 2,3DPG has been detected in addition to ATP and GTP. Interestingly, changes in intraerythrocytic levels of 2,3DPG correlate to changes in environmental temperature.

In addition to the above mentioned species, several fishes of the Amazon (*Semaprochilodus* spp, *Brycon* spp, *Leporinus friderici*) evolved haemoglobins which sensitivities to ATP appear to be greater or equal to that of GTP. These findings differ from those reported for temperate fish species so far analysed (reviewed by Weber, 1996), and from those observed for Amazonian catfishes (Galdames-Portus and Val, unpublished data).

Adjustment of red cell levels of haemoglobin allosteric modulators

ATP and GTP are the major organic phosphates detected in the erythrocytes of fish of the Amazon, similarly to freshwater and marine fish from other geographic regions. The concentration of both ATP and GTP are reduced in fish exposed to hypoxia what results in an increase in haemoglobin oxygen affinity. This safeguards oxygen transfer in fish exposed to hypoxia (Val, 1996; Weber,

1996). In addition to ATP and GTP, IPP, IP2 (inositol diphosphate) and 2,3DPG have been described in fish of the Amazon (Isaacks *et al*., 1977; Bartlett, 1978; Val, 1996).

The adjustment of red cell levels of these effectors appears to be rapid enough to compensate for rapid environmental (daily variations in dissolved oxygen, for example) and physiological changes (increased tissue oxygen demand) (Val *et al*., 1986; 1992a). However, there is no clear relation between changes in NTP and adrenergic stimulation of red blood cells of fish of the Amazon (Val *et al*., 1998). Changes in 2,3DPG and IPP, on the other hand, appear to be related to temperature and changes in breathing behaviour, respectively. In general, GTP concentration decreases faster than the concentration of ATP in animals exposed to hypoxia, as observed for *O. bicirrhosum* (figure 2).
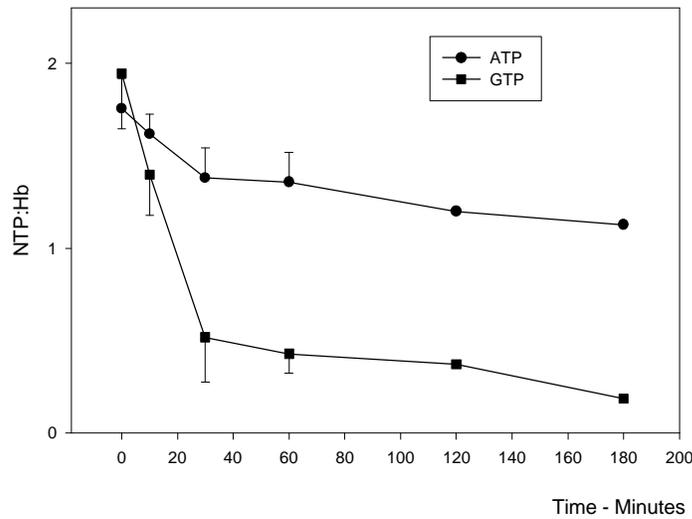

Figure 2. Time variation of ATP and GTP levels in *Osteoglossum bicirrhosum* exposed to deep hypoxia (Po$_2$=30mmHg)

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Tetens and Lykkeboe (1985) reported a significant reduction in NTP within the first hour in rainbow trout exposed to deep hypoxia in contrast to previous studies that suggested that the decrease in ATP and GTP levels would be a relatively slow process taking up to 3 weeks to be completed (reviewed by Nikinmaa, 1990). In *O. bicirrhosum* even shorter time-course is observed (figure 2). In this fish species a significant decrease in GTP levels is observed within the first 30 minutes of exposure to deep hypoxia (PO_2= 35 mmHg). How these adjustments are achieved, however, remains to be described.

Concluding Remarks

The fish of the Amazon have evolved considerable haemoglobin heterogeneity to cope with extremes of environmental conditions. Such heterogeneity that is genetically encoded is the result of an explosive radiation of fish living in an ever-changing environment. In many cases, such molecular heterogeneity correlates to functional heterogeneity. In addition, they have evolved an exceptional ability to adjust the intraerythrocytic levels of haemoglobin modulators to face rapid environmental and physiological changes.

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EFFECTS OF NITRITE ON HEMATOLOGY

AND METABOLIC PARAMETERS

OF AN AMAZONIAN CATFISH,

HOPLOSTERNUM LITTORALE (CALLICHTHYIDAE)

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Abstract

Adults of *Hoplosternum littorale* were exposed to 1 mM nitrite (NO_2^-) during 24 h. Blood parameters, plasma metabolites and tissue enzyme levels were analyzed. Hemoglobin concentration, hematocrit and red blood cell counts increased as a result of an increase in methaemoglobin levels (MetHb content of 57%). Because tissue oxygenation was impaired, a significant increase in opercular rate was also observed. Interestingly, blood lactate accumulation was not observed in fishes exposed to nitrite. Instead, an uncommon increase in pyruvate concentrations was observed. To measure anaerobic metabolism, LDH activities were analyzed in brain and liver, but enzyme levels remained

unchanged. We suggest that *Hoplosternum littorale* adjust blood respiratory properties as a consequence of an impairment of tissue oxygenation.

Introduction

Hoplosternun littorale is an armoured catfish. It presents facultative air-breathing and is distinguished from other species because it is adapted to extremely poor quality water. It can be easily captured in shallow waters, poorly aerated, in Amazon lakes and in polluted igarapés surrounding Manaus. In 1995, Brauner and his co-workers measured the respiratory responses (air-breathing frequency and oxygen uptake) of these animals after exposure to hypoxic, acidic, and hydrogen sulphide rich waters, and concluded that not only air-breathing but also regulation of metabolic rate are the explanation for the hardiness of *H. littorale* to environmental constraints (Brauner *et al*., 1995).

Natural water bodies present nitrite (NO_2^-), an intermediary compound, at concentrations lower than 0.005mg/L. Such low concentrations may rise by contamination with agricultural pesticides, industrial waste, and even the waste of aquaculture facilities. Ammonia can also reach high levels in natural or artificial ponds where fish are raised in high densities. Nitrite is toxic to aquatic organisms and is known as a potent oxidising compound (Lewis & Morris, 1986). Most fish species present an increase in methaemoglobin (oxidised haemoglobin) after exposure to high nitrite concentrations (Brauner et al., 1993; Paula-Silva et al., 1996). Methaemoglobin can not bind reversibly to oxygen (Jensen, 1990) and causes an impairment of oxygen transfer from blood to tissues. We have investigated the influence of nitrite on haematological parameters, metabolite concentrations, and enzyme activities of *Hoplosternun littorale*.

Material and Methods

Adult animals (80-130g) were obtained from a fish farm in Itacoatiara, Amazonas state. Fish were held in outdoor tanks (1,000L) supplied with ground water and fed daily on commercial food pellets. After acclimation period, 20 fishes were evenly distributed in 4 glass tanks (60L each). Feeding was suspended 72 hours prior to the experiments. After collecting two animals from each tank (n=8) we have added 1.0 mmol.l^{-1} NaNO$_2$. After 24 hours of nitrite exposure, the animals (3 from each tank, n=12) were stunned and blood and

tissue samples were collected. Haematological parameters were estimated by classical method. Methaemoglobin levels were estimated as described by Benesch et al. (1973). Plasma and tissue metabolites were determined using commercial kits (Sigma and Doles). Enzyme levels were estimated using a Genesys 2 spectrophotometer; assay conditions were based on established protocols for fish tissues (Sidell et al., 1987). LDH (lactate dehydrogenase) and Cyt Ox (Cytochrome oxidase) are expressed in μmoles substrate.min^{-1}.gwt^{-1}. Lipid peroxidation was estimated measuring the reaction between the products of lipid oxidation with TBA (tiobarbituric acid) as described in Ohkawa (1979) and Bird & Draper (1984); the results are presented as nmoles TBARS/ L plasma (nM).
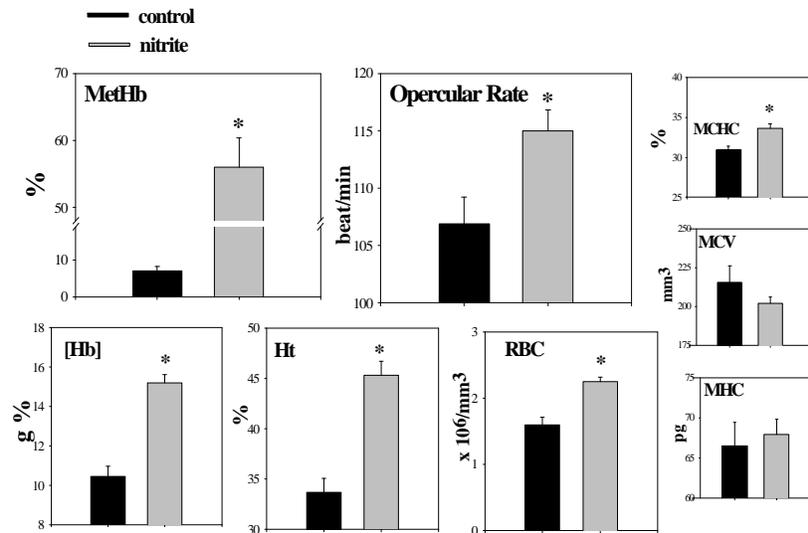

Figure. Blood and behavioral responses of *Hoplosternum littorale* exposed to nitrite during 24 h. Significantly different from the control group ($p < 0.05$, unpaired *t* test).

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Results and Discussion

As described in many fish species, *Hoplosternun littorale* presented an increase in methaemoglobin concentration after 24 hour nitrite exposure (Jensen et al., 1987; Bartlett et al., 1987; Braunser et al., 1993; Paula-Silva et al., 1996). The increase in methaemoglobin was paralleled by an increase in opercular rate, haemoglobin concentration, hematocrit, and red blood cell counts, indicating that the animal tried to increase oxygen transport in the blood (figure). The trend to decrease in MCV and the increase in MCHC, suggest the release of young red cells in blood stream. These results are different from other species whose haematological parameters did not change (*Astronotus ocellatus*, Paula-Silva et al., 1996) or presented decrease in such values (*Symphysodon aequifasciata*, unpublished data).

Methaemoglobin formation in *Cyprinus carpio* and *Astronotus ocellatus* resulted in high plasma levels of lactate suggesting the activation of anaerobic metabolism in those animals (Jensen *et al*., 1987; Paula-Silva *et al*., 1996). Such increase in plasma lactate levels was not observed in *Hoplosternun littorale,* which did no present, either, any increase in liver or brain LDH (lactate dehydrogenase) levels (table). These results suggest a complete absence of anaerobic metabolism in these animals. In fact, glucose, total lipids, total proteins, lactate, and LDH levels did not change after nitrite exposure (table).

The species responded to nitrite exposure decreasing lipid peroxidation processes and oxidative metabolism at liver, increasing pyruvate plasma levels and Cyt Ox activities in brain. All these results suggest a depression in liver metabolism, channeling most oxidative process to the brain. Brauner *et al*. (1995) have suggested that the reduction in metabolic rate observed during hypoxia exposure may be an important strategy of this species to survive oxygen shortage periods.

During oxygen shortage the classical response of most vertebrates is the activation of anaerobic metabolism coupled with a metabolic depression. The anaerobic metabolism consists in the production of ATP trough the breakdown of glucose to pyruvate. This compound is then reduced to lactate, matching the redox balance. The accumulation of pyruvate in the plasma can be explained as the inability of this species to conclude the anaerobic pathway in the tissues. The consequences of such accumulation could not be evaluated in the present study. Further analysis are needed to verify the destination of such compound and to analyze the redox balance and completion of anaerobic metabolism.

Table - Plasma metabolites, lipid peroxidation (TBARS) and enzyme levels in *Hoplosternum littorale* exposed to nitrite (1 mM) during 24 h.

	Control	Nitrite
Glucose (mM)	4.51±0.45	3.82±0.16
Total lipids (g/L)	7.72±0.65	6.27±0.34
Total proteins (g/L)	0.33± 0.01	0.35±0.01
Lactate (mM)	1.46±0.19	0.84±0.21
Pyruvate (mM)	0.02±0.001	0.12±0.02*
Pyruvate/lactate	0.02	0.28
Ammonia (mM)	448.53±38.15	162.13±24.86*
LDH (liver)	2.74±0.29	3.78±0.75
LDH (brain)	27.21±1.54	24.10±1.05
Cyt Ox (liver)	9.50±1.82	5.63±1.08*
Cyt Ox (brain)	0.93±0.10	2.32±0.31*
TBARS (nM)	117.68±7.68	60.88±5.61*

* Significantly different from the control ($p < 0.05$, unpaired *t* test).

Acknowledgments

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CHANGES IN LACTATE DEHYDROGENASE AND MALATE DEHYDROGENASE DURING HYPOXIA AND AFTER TEMPERATURE ACCLIMATION IN THE ARMORED FISH, *RHINELEPIS STRIGOSA* (SILURIFORMES, LORICARIIDAE)

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Introduction

Many studies have been conducted submitting organisms to hypoxia in order to study intermediary metabolites and enzymes (Shoubridge and Hochachka, 1983; Claireaux and Dutil, 1992; Sébert, *et al*., 1993; Almeida-Val *et al*.. 1995) but none of them focused on the effects of acute hypoxia on enzymes of fish acclimated to different temperatures. Hochachka and Somero (1973, 1984) proposed that ectothermic organisms, particularly fish, use adaptive biochemical strategies to obtain metabolic homeostasis during oscillations in dissolved oxygen, of temperature and in some other water physicochemical parameters. Studies on exposure of fish acclimated to different dissolved oxygen concentrations did not give a single answer for enzyme responses (Shaklee et al., 1977; Almeida-Val and Hochachka, 1993; Almeida-Val *et al*., 1995). There is an extensive background of work in general and specific properties of lactate dehydrogenase (LDH) (Wilson 1977; Graves and Somero, 1982; Panepucci et al. 1984; Panepucci et al. 1987; Coppes and Somero, 1990) and in the soluble

form of malate dehydrogenase (cMDH) (Shaklee et al., 1977; Schwantes and Schwantes, 1982a;b; Farias and Almeida-Val 1992; Lin and Somero,1995a;b). Lactate dehydrogenase (LDH, lactate; NAD-oxidoredutase, EC 1.1.1.27) is among the most extensively studied glycolitic enzyme. In fish it is usually coded by three loci, one expressed principally in skeletal muscle (LDH-A), another in heart muscle (LDH-B) and a third one in the eye (LDH-C). Malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase, EC 1.1.1.37) catalyzes the reversible oxidation of malate to oxalacetate requiring NAD$^+$ as a cofactor. It is involved in gluconeogenesis and lipogenesis, and in the malate-aspartate shuttle during aerobic glycolisis. The mitochondrial form (mMDH) acts in the Krebs cycle (Zink and Shaw, 1968). The present work aimed at understanding how fish enzymes respond to acute hypoxia at different acclimation temperatures.

Material and Methods

The armored fish, *Rhinelepis strigosa,* a facultative air-breather*,* found in the Mogi Guaçú river basin, Brazil, is a stenothermal, detritivore-herbivore sedentary fish with moderate economic importance. The habitat temperature in the Mogi Guaçú river varies from 20 to 30 ^{o}C during the year. Low temperatures occur only within a short period (June and July) and high temperatures in the middle of summer (January and February).. Adult fish, "Cascudos pretos", *Rhinelepis strigosa,* (wt $\cong$ 200 g) were net fished in the Mogi Guaçú river, São Paulo state, Brazil. Fish were kept for at least 30 days at an acclimation temperatures of 20, 25 and 30 ^{o}C $\pm$ 1 ^{o}C in 250 L tanks with water circulation and continuous aeration ($P_{WO2}.$>130mm Hg). The tanks were illuminated with natural light and fish fed on lettuce and aquatic plants "ad libitum". Feeding was stopped 24 h before experiments. After acclimation to the experimental temperatures, six fish were placed in a special aquarium for 24 h with proper aeration ($P_{WO2}.$>130mm Hg). Oxygen tensions of inlet and outlet water were measured continuously by O_2 electrodes connected to a O_2 Analyzer. The water oxygen tensions (Po_2) inside the experimental chamber were gradually decreased until critical oxygen tensions were reached as already determined by Sanches et al. (1994) and Takasusuki et al. (1998), and kept at stable levels by bubbling N_2. Fish were kept in hypoxia during 6 h, then killed with a blow to the head. Tissues were excised and saved frozen at -20 ^{o}C until needed for use.

Enzyme preparation and assay of LDH and MDH activity.

White muscle, heart and brain tissues from fish acclimated to 20, 25, and 30 ^{o}C, were weighed and homogenized at ice-temperature with a 9-fold volume of Imidazol 5 mM, KCN 1 mM, pH 7.4 (at 25 ^{o}C) buffer. The homogenate was centrifuged at 17000 g at 5 ^{o}C for 30 min. The supernatant was used directly as an LDH and MDH source in the kinetic study. LDH and MDH activity was determined by following the oxidation of NADH at 340 nm with a circulating thermobath at 25 ^{o}C. The reaction mixture was contained in a total volume of 1 ml, 50 mM Imidazol, 1 mM KCN buffer pH 7.4 at 25 ^{o}C, 0.13 mM of NADH and different concentrations of pyruvate. Substrate saturation plots for oxalacetate were determined for MDH by following the oxidation of NADH at 340 nm. The reaction mixture was contained in a total volume of 1 ml, 50 mM Imidazol, 1 mM KCN, 100 mM KCl buffer pH 7.2 at 25 ^{o}C, 0.12 mM of NADH and different concentrations of oxalacetate. NADH saturation plots were determined for MDH activity with 0.3 mM oxalacetate and different concentrations of NADH. For obtaining K_M values, mathematical analyses using the Michelis-Menten model were used with the aid of a computer program, Origin version . Activity of enzymes were expressed as U/gwt (Unit per gram of wet tissue). One unit of enzyme activity is defined as the amount of enzyme utilizing 1 mole of substrate per minute at 25 ^{o}C. Non-parametric Mann-Whitney test was used to estimate differences between experiments with fish submitted to both hypoxic and normoxic conditions. Rates of MDH/LDH activity were calculated in concentrations of 0.3 mM oxalacetate for MDH and in 1 mM pyruvate for LDH and 0.13 mM NADH for white muscle, heart muscle and brain tissue. Low and high ratios of LDH activity (L/H) were calculated in 1 mM and 10 mM pyruvate respectively for white muscle tissues.

Results and Discussion

Experiments on fish submitted to hypoxia showed significant differences in enzyme activity from fish in normoxia at 20 ^{o}C. LDH pyruvate saturation plots of white muscle showed significant differences (P<0.05) between hypoxia and normoxia (Fig.1a).
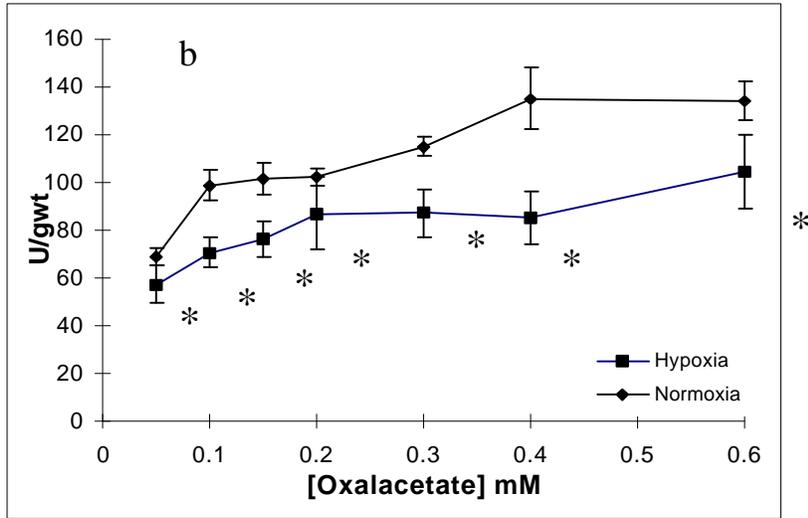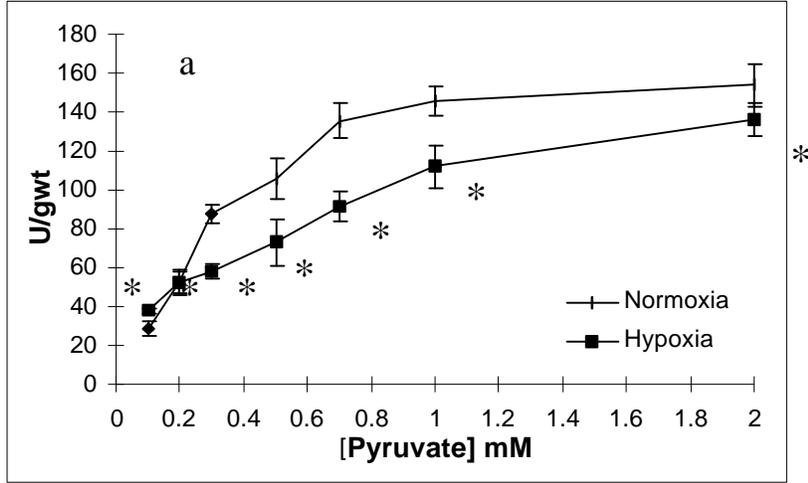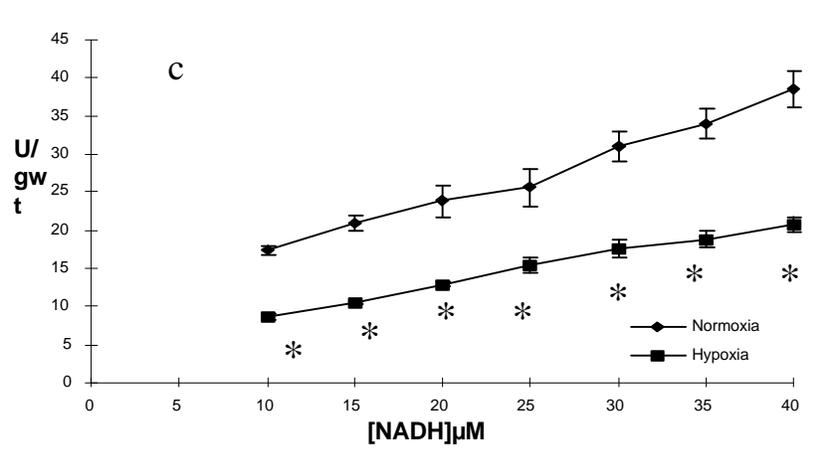
Figure 1 : Effects of hypoxia on *P. scrofa* acclimated to 20 ^{o}C on: a)LDH activity of white muscle in different pyruvate concentrations. b) MDH activity of white muscle in different oxalacetate concentrations and c) MDH activity of white muscle in different NADH concentrations. * P< 0.05, n=5. Error bars are within limits of symbols when not visible. U, mole substrate/min. GWT, grams of .wet tissue.

MDH oxalacetate saturation plots of white muscle submitted to hypoxia also showed significant differences P<0.01) in all substrate concentrations from fish in normoxia (Fig 1b). MDH saturation plots of white muscle submitted to hypoxia using NADH as a substrate showed significant differences (P<0.01) in all substrate concentrations (Fig.1c). The fact that MDH using oxalacetate as a substrate and MDH using NADH as a substrate differed in normoxia and hypoxia may reflect its dual role in both aerobic and anaerobic energy metabolism at low temperature in this case, as pointed out by Hochachka and Somero (1984).

Table 1 shows K_M values for hypoxia and normoxia from the above experiments for all temperatures. Except for LDH of fish acclimated to 20 and 30 ^{o}C, K_Ms did not reveal significant differences between fish submitted to hypoxia. Table 2 shows enzyme activities for muscle, heart and brain tissues in normoxia and hypoxia. Significant differences between normoxia and hypoxia were found for white muscle LDH and MDH$_{[OAA]}$, for fish acclimated to 20 ^{o}C and, also, for heart muscle MDH$_{[OAA]}$ of fish acclimated to 25 ^{o}C. Brain tissue did not show

significant differences for enzymes tested.. In fish acclimated to 25 ^{o}C significantly higher values during hypoxia suggest that MDH has a role in redox regulation during hypoxic stress.

Table l: K_M values for LDH and MDH for white muscles of *P. scrofa* acclimated at different temperatures. The values are means ± SD, n = 5 fish. * P< 0.05, significantly different from values in normoxia.

20 ^{o}C

Enzyme	K_M in Normoxia	K_M in Hypoxia
LDH$_{[PYR]}$ (mM)	0.824 ± 0.14	1.70 ± 0.27 *
MDH$_{[OAA]}$ (mM)	0.020 ± 0.005	0.018 ± 0.003 NS
MDH$_{[NADH]}$( )	36.81 ± 11.83	45.36 ± 7.11 NS

25 ^{o}C

Enzyme	K_M in Normoxia	K_M in Hypoxia
LDH$_{[PYR]}$ (mM)	1.14 ± 0.22	1.27 ± 0.20 NS
MDH$_{[OAA]}$ (mM)	0.057 ± 0.009	0.046 ± 0.008 NS
MDH$_{[NADH]}$( )	19.04 ± 5.06	22.98 ± 5.13 NS

30 ^{o}C

Enzyme	K_M in Normoxia	K_M in Hypoxia
LDH$_{[PYR]}$ (mM)	0.14 ± 0.02	0.83 ± 0.19 *
MDH$_{[OAA]}$ (mM)	0.042 ± 0.014	0.059 ± 0.019 NS
MDH$_{[NADH]}$( )	19.07 ± 5.06	23.44 ± 5.49 NS

* P< 0.05; NS, not significant.

Table 3   shows the ratios of MDH/LDH activity which demonstrate the oxidative capacity of the tissues at all temperatures of acclimation (high rates denote high oxidative capacity). These ratios are extremely high (up to 280 times higher than white muscle) for heart muscle of fish acclimated to all temperatures, showing the importance of this organ for the survival of fish in critical hypoxia situations and at the extreme temperatures found in their habitat. Brain tissue also presented a high ratio (11 times higher than white muscle) at all temperatures of acclimation. Short term hypoxia seems to be more stressful for heart muscle and brain because they need oxygen for their metabolism in order to avoid excessive metabolite accumulation. A high MDH/LDH ratio may cause an attenuated pyruvate to lactate flux and as a consequence carbohydrate metabolism will be largely channeled toward complete oxidation (Almeida-Val

and Hochachka, 1995). This will benefit hypoxia situations like in heart muscle acclimated to 20 and 30 ^{o}C.

Table 2. Enzyme activities[#] in tissues of *R. strigosa* in normoxia (N) and hypoxia (H) acclimated at different temperatures. The values are mean ± SD, n = 5 fish.

LDH [#]						
	20°C		25°C		30°C	
	N	H	N	H	N	H
Muscle	187.62	112.06***	104.74	95.66	239.55	330.23
	±6.59	±17.43	±35.76	±16.64	±57.62	±105.56
Heart	5.59	4.30	5.47	8.52	13.85	4.22
	±2.06	±2.05	±2.20	±3.45	±9.63	±6.24
Brain	41.57	41.92	61.73	54.27	57.81	42.25
	4.05	±5.96	±2.01	±8.57	±15.55	±8.12
MDH[#]						
	20°C		25°C		30°C	
	N	H	N	H	N	H
Muscle	98.87	70.90**	69.45	66.44	80.71	74.12
	±13.37	±14.55	±16.57	±8.89	±26.70	±22.38
Heart	750.48	842.12	764.95	945.34**	794.86	643.41
	±157.71	±148.05	±93.12	107.84	±139.86	±144.31
Brain	281.67	333.76	345.34	286.5	278.78	269.13
	±57.33	±106.73	±67.05	±82.74	±18.17	±19.11

** $P<0.01$; *** $P<0.001$
[#] (U/GWT) U, mole substrate/min.   GWT, g.wet wt. of tissue.

Table 3. Comparative MDH/LDH ratios from *P. scrofa* tissues acclimated at different temperatures (n = 5 fish).

MDH /LDH[#]						
	20°C		25°C		30°C	
	N	H	N	H	N	H
Muscle	0.68	0.63 NS	0.66	0.69 NS	0.29	0.22 NS
Heart	142.50	223.35 *	150.92	126.16 NS	57.39	152.37 *
Brain	6.77	8.03 NS	5.61	5.21 NS	5.23	6.55 NS

U/gwt. U, mole substrate/min. GWT, g.wet wt.   of tissue.
* $P< 0.05$; NS, not significant

The ratio of LDH activity at low to that at high pyruvate concentrations (L/H) is often used as an index of the kinetic poise of LDH (Kaplan and Goodfriend, 1964). L/H LDH ratios for white muscle at different temperatures in normoxia and hypoxia suggest anaerobic organization (Table 4). These values are higher in normoxic than in hypoxic conditions, indicating that an increase exists in the reduction of pyruvate to sustain glycolisis under anaerobic conditions. The results of K_M values obtained for LDH and MDH of fish acclimated to 20 ^{o}C and submitted to hypoxia suggest that naturally intense fluctuations in dissolved environmental oxygen may result in significant changes in enzyme activity, such as the ability of enzymes to respond to acute hypoxia. Lushchak et al., (1997) found differences in enzyme activity throughout anaerobiosis and recovery of a sea mussel. Shaklee et al. (1977) found significant differences in enzymatic activity for liver LDH and white muscle aldolases in fish acclimated to different oxygen concentrations.

Table 4. Rates of white muscle LDH activity in low and high substrate concentrations (L/H) and at different acclimation temperatures (n = 5 fish).

Temperature of acclimation	L/H
Normoxia 20 ^{o}C	0.72
25 ^{o}C	0.47
30 ^{o}C	0.73
Hypoxia 20 ^{o}C	0.48
25 ^{o}C	0.43
30 ^{o}C	0.63

This ability to respond to hypoxia may have been acquired in times of oxygen deficiency. Experiments at 25 and 30 ^{o}C did not result in significant changes in enzyme activities and K_M for acute hypoxia. It is interesting to notice that these temperatures are those encountered in the environment of the fish almost all year round. A number of studies with many species have shown that fish frequently respond to change in environmental oxygen levels with changes within hematological parameters and alteration in physiological responses (Randall, 1993; Fernandes et al. 1995). Furthermore, fish are known to avoid low oxygen concentrations (Reynolds and Thomson, 1974). According to M.N.Fernandes and J.R.Sanches (personal communication) no differences were found in *Rhinelepis strigosa* acclimated at different temperatures and submitted to

hypoxia for the oxygen carrying capacity of the blood measured by changes in hematocrit, hemoglobin concentration and red cell count, although changes were found in cardiac frequency, metabolic rate, oxygen uptake, ventilation rate and volume. Probably, hematological changes are subtle while physiological and biochemical adjustments provide the strategy used to deal with changes in oxygen concentrations in natural environments.

Changes in K_M are difficult to explain in short term periods of acute hypoxia. Changes in K_M of pyruvate and NADH for M_4-LDH from shallow and deep sea living species were related with changes in pressure in fish (Siebenaller and Somero, 1979). According to Greaney and Somero (1980) studies of NADH binding suggest that for M_4-LDHs and other dehydrogenases NADH (NAD) binding sites should remain cofactor-saturated, so that the direction of dehydrogenases function is established by the redox state, i.e. the NADH/NAD ratio of the cell. Differences in K_M of enzymes have also been attributed to a modulation resulting from changes in the pH milieu (Wilson, 1977; Yancey and Somero, 1978; Walsh and Somero, 1982; Somero, 1983; Coppes, et al., 1992). This would explain the high affinity (lower K_M) for substrate of muscle LDH in hypoxia and the lower affinity (higher K_M) of muscle MDH for NADH as a substrate in cascudo preto. These hidden strategies such as the ability of enzymes to respond to acute hypoxia may explain differential responses to hypoxia situations which fishes encounter in different environments.

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ENERGETICS OF SWIMMING IN

PARROTFISHES (LABRIFORM LOCOMOTION) AND

TRIGGERFISHES (BALISTIFORM LOCOMOTION)

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Introduction

Many fish swim using movements of median or paired fins to generate thrust, while holding the body rigid. This mode of swimming is considered advantageous for low speed stability and a high degree of maneuverability, and is presumably associated with the type of habitat (i.e., coral reefs). In addition, it has been suggested that swimming with a rigid body should result in less drag than in an undulating body (Lighthill and Blake, 1990) and therefore may be a more efficient means of locomotion.

We examined the energetic costs of swimming in two species with different modes of rigid-body swimming. The parrotfish *Scarus schlegeli* swims by flapping the pectoral fins (labriform locomotion) and the triggerfish *Rhinecanthus aculeatus* swims by undulations of the dorsal and anal fins (balistiform locomotion). Both species switch to body-caudal undulations at higher swimming speeds.

Materials and Methods

Seven parrotfish (243 ±58 g, 23.2 ±1.8 cm) and five triggerfish (136 ±31 g, 17.5 ±1.3 cm) were caught by gill net and hook-and-line, respectively, and held at ambient temperatures (27°C) at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. Oxygen consumption

(MO_2) was measured during swimming in a recirculating water flume (31 liters) using intermittent flow respirometry, automated by computer. Following overnight acclimation, water velocity was increased step-wise by ~0.5 body lengths (L)/s every 30 min until the fish fatigued.

Results

The parrotfish swam exclusively with the pectoral fins up to 3 to 3.5 body lengths (L)/s. Oxygen consumption increased significantly with velocity during pectoral fin locomotion (Figure 1, $p<0.05$, $r^2= 0.91$). Body-caudal undulations were recruited at maximal velocities between 3 and 4 L/s resulting in a disproportionate increase in MO_2 and then rapid fatigue.

The triggerfish swam with undulations of the dorsal and anal fin up to 1.5 L/s. Between 1.5 and 3 L/s, body-caudal undulations were recruited for increasing amounts of time, and were used continuously above 3.5 L/s. During body-caudal swimming, the dorsal and anal fins beat at the same frequency as the caudal fin. Although typically considered adapted for low speed maneuverability, this species of triggerfish was also capable of high prolonged speeds (>4.5 L/s) using body-caudal swimming. The oxygen consumption data was analyzed in two groups: measurements during balistiform locomotion only, and measurements when body-caudal swimming was also used. Oxygen consumption increased in both groups (Figure 2, $p<0.05$, $r^2 = 0.41$ and 0.93, respectively). The slope was significantly greater with body-caudal swimming (ANCOVA, $p<0.05$), however, the intercepts of the two regressions were not different.

Discussion

Both parrotfish and triggerfish had higher oxygen consumptions upon switching to body-caudal undulations from rigid-body swimming (Figures 1 and 2). In addition, comparison of the semi-log regressions with other species that use only body-caudal swimming (subcarangiform locomotion) reveals that the rigid-body swimmers have lower slopes (0.15 - 0.18 compared to 0.32 - 0.35). These slopes, however, were much higher than those reported by Gordon et al. (1989) for labriform swimmers (0.02 - 0.08) and are closer to those reported by Webb (1974) (0.24). These data suggest that the parrotfish and triggerfish may be able

to take advantage of the reduced drag of rigid-body swimming to lower swimming costs.
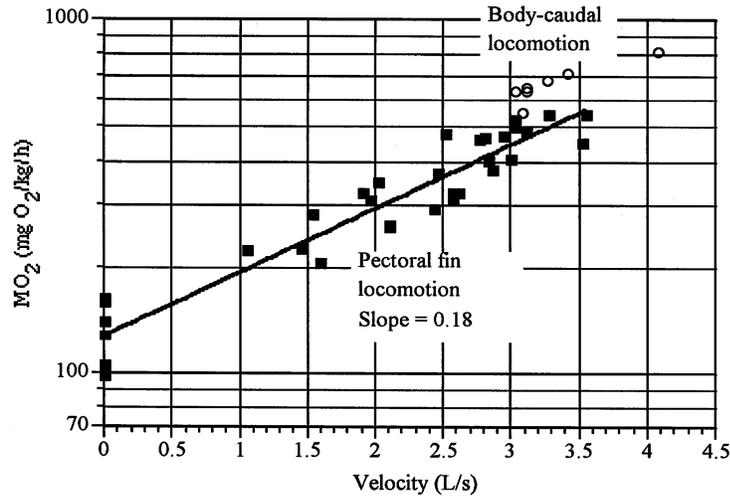

Figure 1. Oxygen consumption of parrotfish (*Scarus schlegeli*) as a function of swimming velocity.
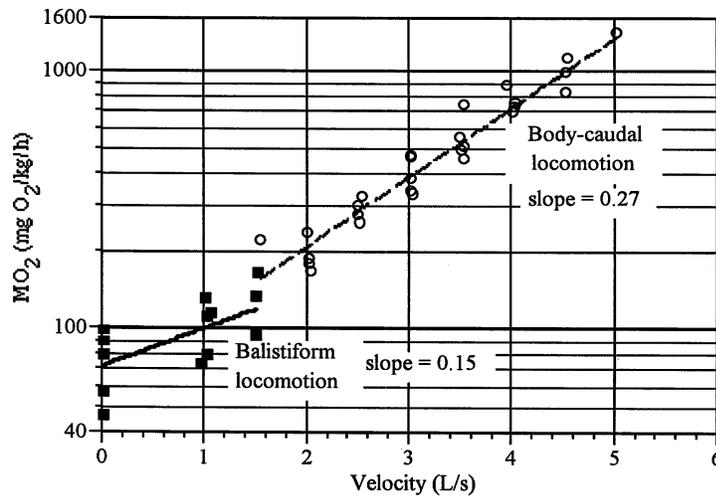

Figure 2. Oxygen consumption of triggerfish (*Rhinecanthus aculeatus*) as a function of swimming velocity.

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REEF-SPECIFIC LIFE HISTORY TRAITS

OF THE STRIPEY BASS

LUTJANUS CARPONOTATUS (LUTJANIDAE)

AT THE PALM ISLAND GROUP,

CENTRAL GREAT BARRIER REEF

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Introduction

Spatially segregated populations are likely to experience different natural and anthropogenic influences, thus potentially exhibiting distinct site-specific life history traits. Given that schedules of growth, mortality and reproduction form the basis of many stock assessment models, the degree of variability in these traits is of interest to fisheries biologists and managers. In this study, I examine local variability in the life history traits of the stripey bass *Lutjanus carponotatus*, an abundant lutjanid that is an important component of the catch of line fishers on the Great Barrier Reef.

Methods

Four sites spanning 15 km along a north-south axis at three islands in the Palm Island group, central Great Barrier Reef (S 18° 36', E 146° 30') were sampled monthly over a one-year period by spear fishing. Ages were determined by counting opaque bands in sagittal otoliths, validated as annuli in a recent study (Davies and Kritzer, in prep). Von Bertalanffy growth functions fitted to length at age data were used to compare growth trajectories. Age based catch curves were used to compare total instantaneous mortality rates, Z. Gonads were

staged macroscopically, and a gonosomatic index (GSI = 100 x gonad mass/whole body mass) and liposomatic index (LSI = 100 x gonadal fat body mass/whole body mass) were calculated for each mature female.

Results and Discussion

Life history traits were generally consistent between sites (Table 1). Total instantaneous mortality rates did not differ among sites (ANCOVA: d.f. = 3,46; F = 1.674; p = 0.186). However, the four sites differ in marine park zoning with different levels of fishing pressure permitted within each. Apparent similarities in total mortality rates could be masking underlying additive effects of natural and fishing mortality. Energy storage and gonad development also showed little spatial variation. The gonads of *L. carponotatus* are characterized by a fat body associated with each gonadal lobe, akin to that found in surgeonfishes (Acanthuridae; Fishelson *et al.*, 1985). Like surgeonfishes, the relative mass of this structure peaks immediately prior to the spawning season (LSI peak in Aug-Sept; GSI peak in Oct-Dec). The relative masses of both the fat body and gonad during peak months of development were similar among the four sites (Table 1), though uneven sampling effort and small sample sizes within some months precluded a formal statistical comparison.

Unlike mortality and reproductive parameters, patterns of growth did vary in space. Although lengths at age showed considerable variation within sites, site-specific growth curves (Figure 1) were highly significantly different (Analysis of residual sums of squares: d.f. = 9, 515; F = 5.074; p < 0.0001). In particular, the Pelorus Island population attained much larger sizes, while the Juno Bay population showed an early cessation of growth. Interestingly, these extremes represent sites that are open to both line and spear fishing. The other two sites have prohibitions on the use of either spear (Harrier Bay) or both types of gear (Cattle Bay). This data suggests that there is no readily predictable effect of fishing on growth at these islands.

The dispersive larval stage and consequent broad connectivity of coral reef fish populations is likely to result in little genetic variation between reefs, particularly at small spatial scales (Shulman and Bermingham, 1995).

Assuming genetic traits are similar, these results suggest that variable ecological processes influencing the amount of surplus energy available to fish on different reefs are driving the observed differences in growth. Populations of herbivorous surgeonfish *Acanthurus nigrofuscus* on neighboring reefs exhibited variation in growth as a response to different levels of algal cover following crown-of-thorns starfish *Acanthaster planci* outbreaks (Hart and Russ, 1996). If surplus energy was similar among sites, *L. carponotatus* at Pelorus Island would be expected to show a compensatory response in reproductive investment as a result of its greater somatic investment (Reznick, 1983).
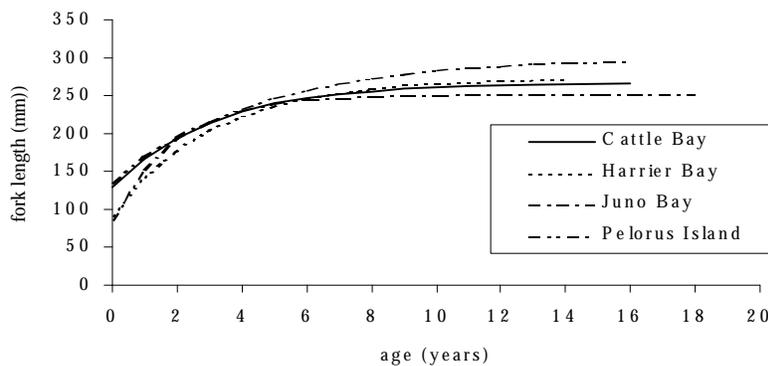
Figure 1. Von Bertalanffy growth curves fitted to length at age data for the stripey bass *Lutjanus carponotatus* from the Palm Island group, central Great Barrier Reef. Parameter estimates for each curve are given in Table 1.

These patterns can have important implications for population dynamics and harvest. Stocks of New Zealand abalone *Haliotis iris* that grow to larger sizes produce higher yield-per-recruit but fewer eggs-per-recruit than nearby smaller-growing stocks (McShane and Naylor, 1995). On individual coral reefs, the effects of variable egg production arising from growth variability will depend upon the nature of larval dispersal. Scenarios ranging from complete retention to complete larval export need to be investigated in conjunction with reef-

specific population biology to confidently conduct stock assessments for coral reef fish.

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Table 1. Summary of site-specific and pooled life history traits of the stripey bass, *Lutjanus carponotatus*, from the Palm Island group, central Great Barrier Reef. Standard errors are given in parentheses. Marine park zoning is indicated below site name (L = line fishing permitted; S = spear fishing permitted; N = all gears prohibited).

PARAMETER		SITE				
		Pelorus Is. (L,S)	Cattle Bay (N)	Harrier Bay (L)	Juno Bay (L,S)	Pooled
Z		0.291 (0.030)	0.279 (0.046)	0.225 (0.033)	0.150 (0.023)	0.226 (0.017)
VBGF Parameters	k	0.22 (0.04)	0.32 (0.05)	0.32 (0.04)	0.51 (0.08)	0.33 (0.03)
	L_∞	301 (12)	267 (6)	274 (7)	252 (5)	270 (3)
	t_0	-2.72 (0.65)	-2.07 (0.50)	-1.26 (0.33)	-0.81 (0.34)	-1.74 (0.24)
Mean Female LSI (Aug-Sept)		0.57 (0.20)	0.85 (0.18)	0.53 (0.14) (Sept only)	0.46 (0.14) (Aug only)	0.66 (0.095)
Mean Female GSI (Oct-Dec)		1.6 (0.15)	1.9 (0.21)	1.5 (0.25) (Dec only)	2.1 (0.15) (Nov only)	1.5 (0.10)

ECOPHYSIOLOGICAL BEHAVIOR

OF *PETENIA KRAUSSI*

EXPOSED TO DIFFERENT

TEMPERATURES AND SALINITIES

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Abstract

Tropical river sardine *Petenia kraussii* captured from La Aguá lagoon (Sucre State, Venezuela) were acclimatized for four weeks at 22, 24, 30 and 32ºC and at 0, 5, 10, 15 and 17‰ salinity. To evaluate effects of thermal response to acclimatization level, the fish were transferred suddenly from the lower temperatures (22 and 24ºC) to higher ones (30 and 32ºC). Then thermal resistance time was measured at the lethal temperature of 40.9ºC for 30 days. We considered that the acclimatization process was complete when resistance time was stabilized at the new temperature regime. For the saline effect, the concentration of sodium and potassium was measured in the tissues at each treatment: gills, white muscle, gut and heart. The results showed that thermal tolerance increased rapidly in 3 hours with a 6ºC rise in temperature (from 24 to 30ºC) and in 24 hours with a 10ºC rise (22 to 32ºC). With decreasing temperatures, the acclimatization level reached its lowest in 11 days with a 6ºC decrease (from 30 to 24ºC) and in 14 days with a 10ºC decrease (32 to 22ºC). *Petenia kraussii* regulates as much sodium as potassium in gills and white muscle tissues at all salinity levels tested; however, gut and heart tissues showed significantly different regulations among salinities examined.

Introduction

Temperature and salinity are the abiotic factors that have the strongest influence on organisms because they involve functional and structural alterations through changes in the osmotic concentration, promoting modification of behavior of a species inside an ecosystem. Also, salinity may establish differences in physiological effects in the embryonic development depending on other environmental factors, particularly temperature (Bennett & Judd, 1992; Battaglane & Talbot, 1993; Chung & Strawn, 1994)

Knowledge of these two factors is very useful in studying tropical organisms found in coastal lagoons, temporary shallow water lagoons, lakes and estuaries. They are exposed to temperature fluctuations during the day and night and to salinity changes due to evaporation during dry periods or dilution during rainy seasons (Segnini *et al.*, 1993; Segnini & Chung, 1997).

Fish living in a freshwater environment have higher rates of glomerular filtration and renal excretion of water and salts than those living in a seawater environment, because there is a hypoosmotic relation to their environment. Therefore, they have less electrolytic concentration than the seawater variety, absorbing Na^+, K^+ and Cl^- ions, mainly in the digestive tract and desalinizing the water at gill levels. In euryhaline fish, the glomerular filtration rate and urine flow changes in the direction of ion concentrations, which means that it passes from a lower saline ion concentration to a higher one and vice versa. Although the nature of this mechanism is not known, it may be assumed that these changes are promoted in renal functions; furthermore they may participate in circulatory and hormonal functions (Canahan *et al.*, 1986; Salman & Eddy, 1988)

In spite of the physiological importance and ecological consequences of temperature and salinity on the stenohaline and euryhaline organisms, the available information is scarce. The aim of this work is to study the influence of acclimatization temperature and salinity in river sardine *Petenia kraussii*, which is a great food source for its excellent flavour, not very bony axial skeleton, low fat and high protein contents.

Materials and Methods

The organisms were captured in "La Aguá" lagoon, near Chiguana, Estado Sucre, Venezuela (10°30´N and 63°41´W) and transferred to the laboratory in insulated containers to avoid abrupt changes of temperature.

Later on, groups of 200 fish of 13,0±1,5 mm in standard length (S.L.) were acclimatized to temperatures of 22, 24, 30 and 33°C, respectively (the temperature range in La Aguá lagoon during the year). For their acclimatization, aquaria with a 300 liters capacity, a thermal control, a heater and a contact thermometer to regulate water temperature with a variation of ±0,01°C, were used.

Acclimatization rates of two temperature changes were studied: a difference of 6°C (24 to 30°C) and of 10°C (22 to 32°C). Before the experiment and after transfer up to 30 days, 10 fish, taken at random with replica, were exposed to the lethal temperature of 40.9°C to measure their thermal resistance time. We considered that acclimatization level had been completed when the survival time of the organisms progressively stabilized at a higher or lower temperature.

To study the incipient lethal salinity, three groups of 20 fish (10,0±3.3 mm S.L.), acclimatized at 23°C with their respective replica and control were placed in freshwater aquaria. Then salinity was increased daily by 2‰ up to 15‰. After this, the increment was 1‰ per day, because in Colombia this species can be found in 14‰ salinity water (Hurtado, 1975).

To obtain the Na^+ and K^+ ion concentrations, groups of 10 fish and their replica (80±10 mm S.L.) were acclimatized to five salinities (0, 5, 10, 15 and 17‰) at a constant temperature of 23°C. Each experimental salinity was reached in the way described previously.

After a 4 week saline acclimatization, the fish were sacrificed; heart, muscular, gill and gastric tissue samples were taken, using a scalpel, taking care of always cutting the same place and approximately the same amount (complete heart, complete gut, 4 thin sheets of gills and segments of white muscle). They were weighed immediately after the dissection, placed in plastic bags and then frozen at -17°C until the ion concentration was determined.

The tissues were placed separately (muscle, heart, gill and gut) in a Kjeldahl flask. Nitric acid (3mL to 50%) was added and organic matter destroyed by

being boiled for approximately 5-8 minutes. After this, a 5 mL liquid sample with the intratissular ions was obtained and placed in assay tubes to determinate ionic concentrations later on.

The determination of ionic concentration of sodium and potassium was carried out in a direct reading by flame photometer. The samples were diluted in a 1:10 ratio. Before carrying out photometric readings, the apparatus was calibrated using NaCl and KCl solutions of known concentrations

The results obtained during the experimentation were subjected to a statistical treatment of a confidence level of 95% (Sokal & Rohlf, 1981), using a variance analysis and an *a posteriori* test using Duncan´s simultaneous comparison method (Steel & Torrie, 1980) in order to determine significant differences between the temperatures and salinities tested.

Results and Discussion

The results show a straight and positive relationship between acclimatization temperature and thermal tolerance of *Petenia kraussii* (Figures 1-4, Table 1). The variance analysis applied to *P. kraussii* thermal resistance time during reacclimatization periods at the lethal temperature of 40,9ºC showed a highly significant effect on the increasing temperatures from 22 to 32ºC (gl: 13/70; F: 33.63) and from 24 to 30ºC (gl: 13/126, F: 5.53). Similar results were obtained for the decreasing conditions from 32 to 22ºC (gl: 14/75; F: 107.83) and from 24 to 20ºC (gl: 11/108; F: 53.96) for each thermal exchange rate.

Duncan´s *a posteriori* analysis indicates that thermal resistance times measured before increasing temperatures from 24 to 30ºC and from 22 to 32ºC were significantly lower than those measured after temperature increased. On the other hand, for temperatures decreasing from 30 to 24ºC and 32 to 22ºC, thermal resistance times during 8-9 days after transfer were significantly higher than the rest. (Table 1).

Figures 1 and 2 show that *Petenia kraussii* have a wide adaptation capacity to an abrupt temperature increase: 3 hours after being transferred, their thermal resistance time begins to increase and they reach full acclimatization at a new temperature regime from 48 to 72 hours, depending on whether the increment is of 6ºc or 10C. These results imply that rapid acclimatization behavior of *Petenia kraussii* is due to adaptation mechanisms to temperature.
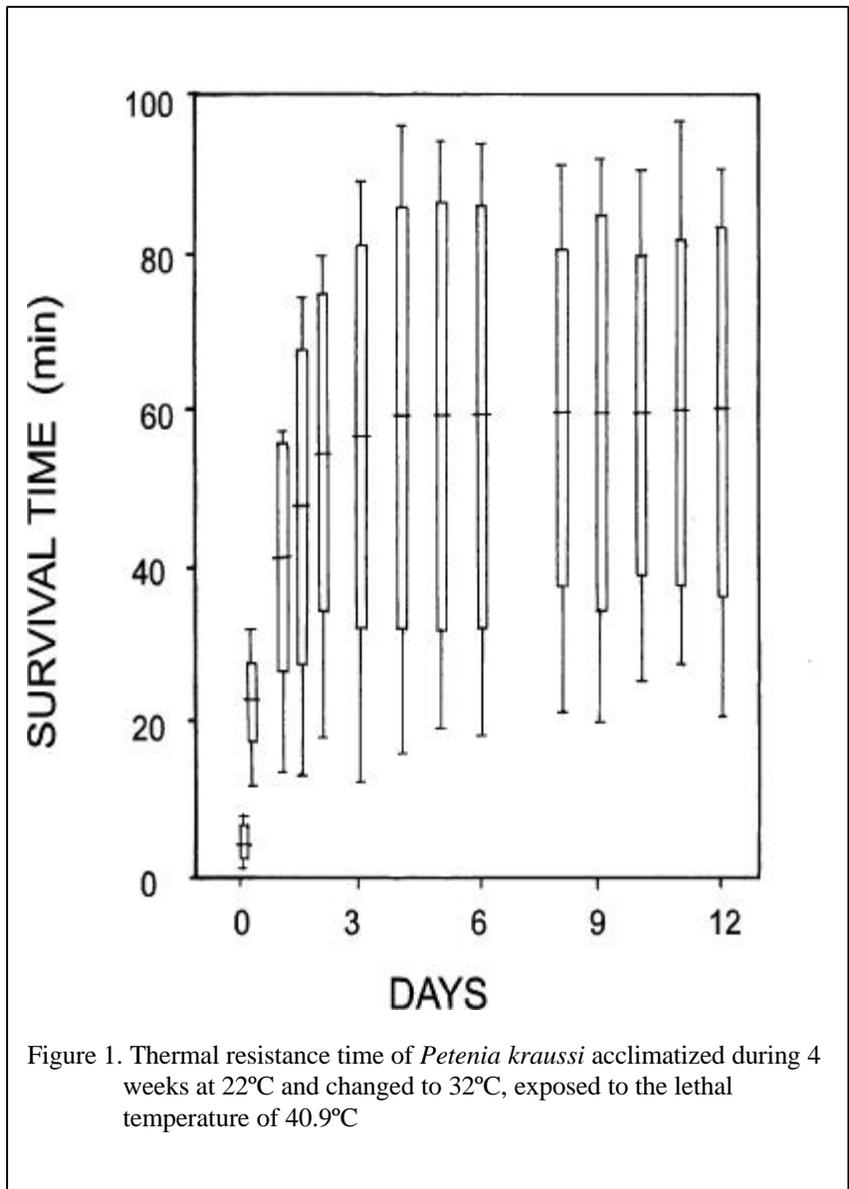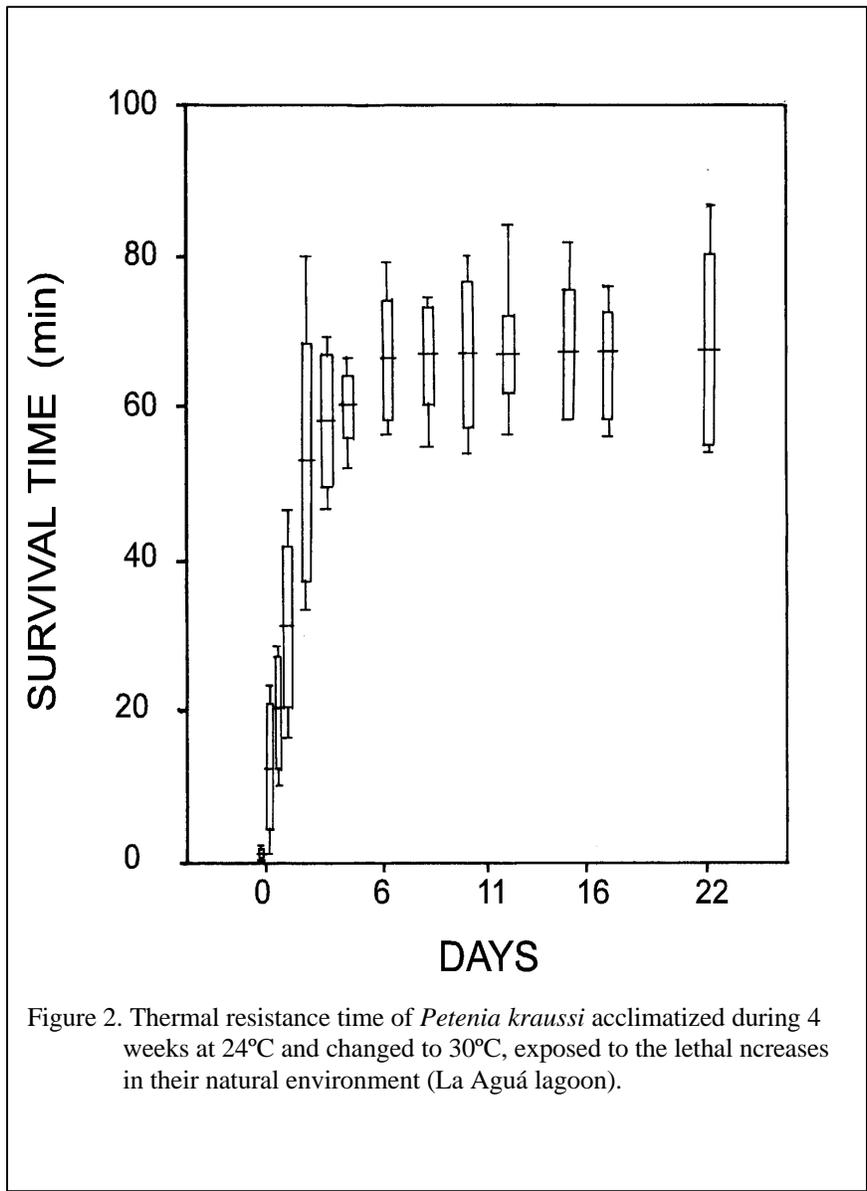
Figure 1. Thermal resistance time of *Petenia kraussi* acclimatized during 4
weeks at 22ºC and changed to 32ºC, exposed to the lethal
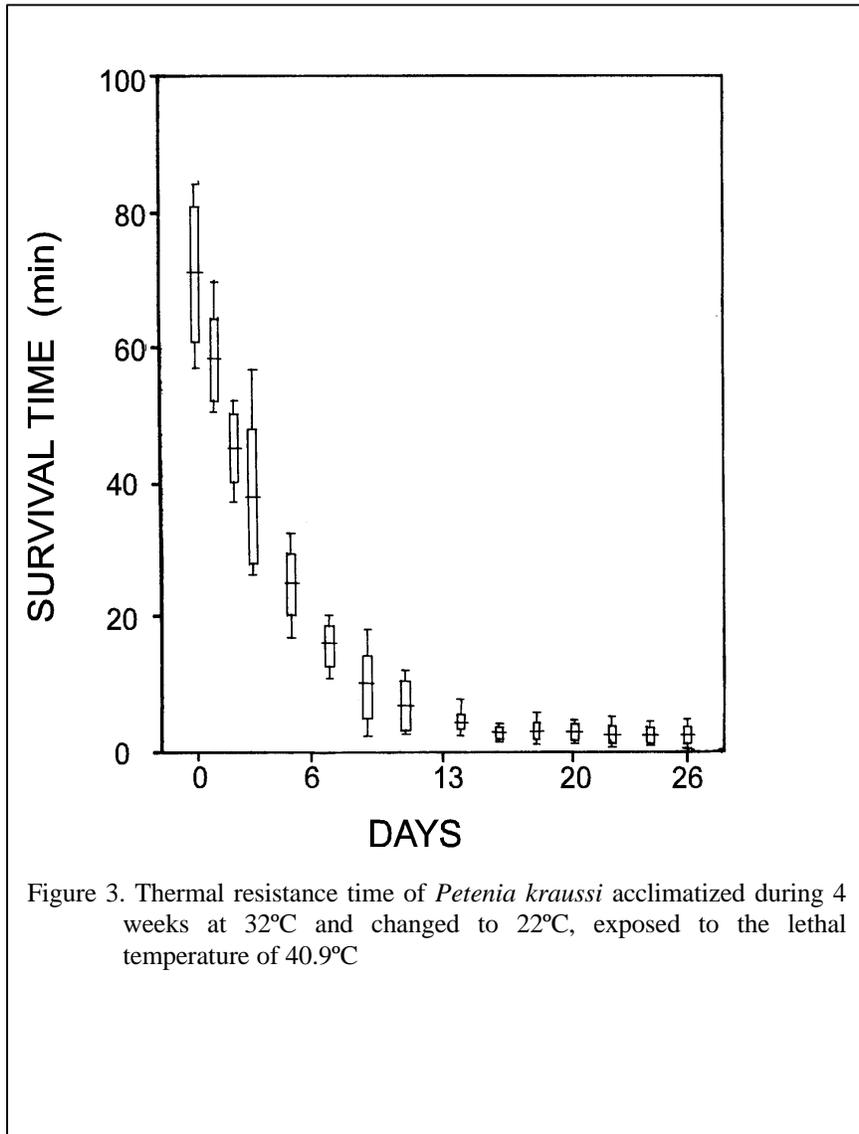temperature of 40.9ºC

Figure 2. Thermal resistance time of *Petenia kraussi* acclimatized during 4 weeks at 24ºC and changed to 30ºC, exposed to the lethal ncreases in their natural environment (La Aguá lagoon).
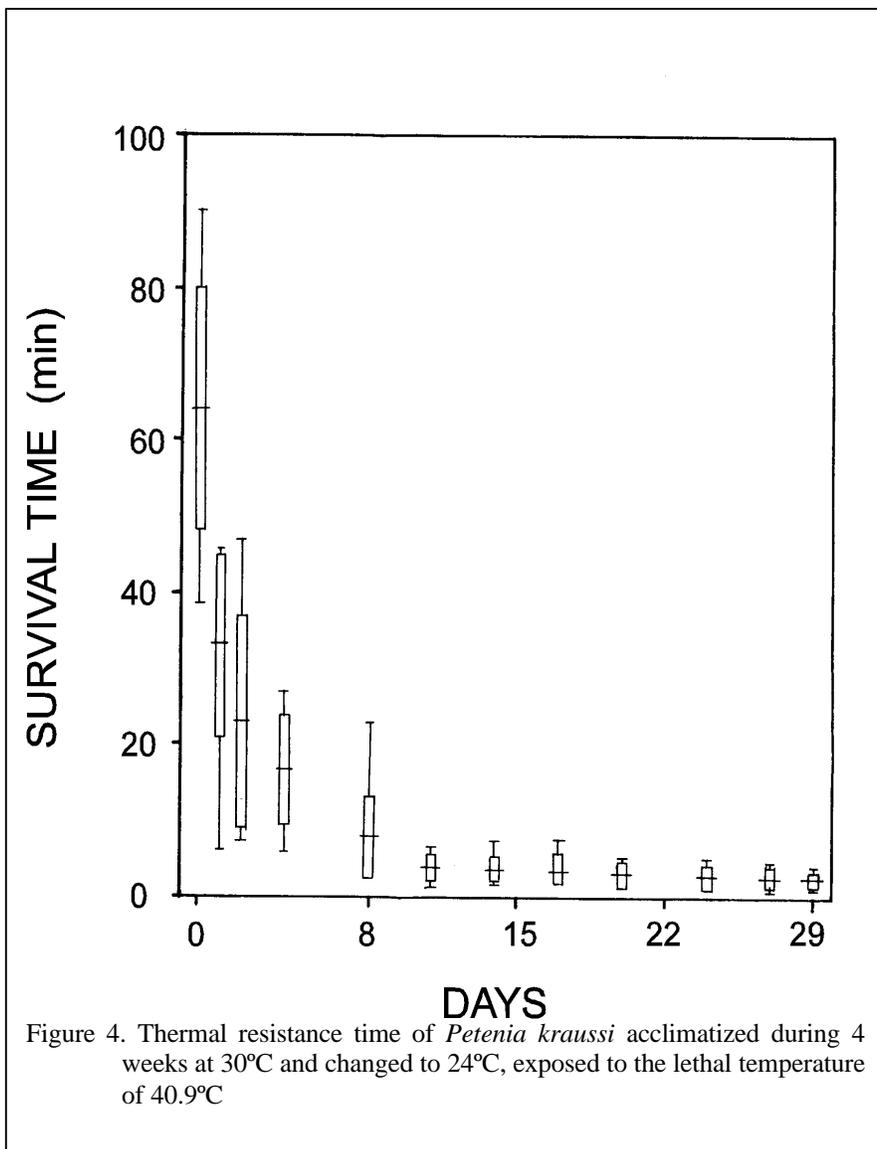
Figure 3. Thermal resistance time of *Petenia kraussi* acclimatized during 4 weeks at 32ºC and changed to 22ºC, exposed to the lethal temperature of 40.9ºC

Figure 4. Thermal resistance time of *Petenia kraussi* acclimatized during 4 weeks at 30°C and changed to 24°C, exposed to the lethal temperature of 40.9°C

case of acclimatization to lowering temperatures, a fast decrease of thermal resistance is observed in those organisms exposed to a 6ºC decrease (Fig. 3,4). When the acclimatization temperature difference is 10ºC, thermal resistance of this species diminishes slowly. This thermal decrease occurs abruptly at first (at 24 hours), followed by two smaller decreases at a close interval (48 and 72 hours), continuing with another remarkable descent (120 hours) and later on, three small decreases (144, 216 and 288 hours) until reaching full acclimatization at 336 hours (14 days), where the variances are so small that they are not significant. On the other hand, in the 6ºC difference decrease, the thermal resistance time occurs in three stages: an abrupt loss in the first 24 hours, then a marked descent from 2 to 4 days to conclude in the stability phase from that time onwards. Possibly, *Petenia kraussii* have a lesser capacity to diminish their acclimatization temperature the higher it is.

The results obtained from 6ºC *Petenia kraussii* acclimatization temperature were similar to those reported by Chung (1985) for another species belonging to the same family, *Oreochromis mossambicus:* they required two days for temperature increase, but acclimatization rate for temperature decrease was not similar. While *Oreochromis mossambicus* needed 14 days, *temperature of 40.9ºC*

In the *Petenia kraussii* only required 11 days. Segnini *et al.* (1993) reported one day to allow *Mugil curema* to reach their full acclimatization in a temperature increasefrom 24 to 30ºC and from 28 to 34ºC and 6 to 8 days in a temperature decrease at the same rates.

The mechanism of different acclimatization types to temperature increase and decrease can be correlated with the fish´s metabolism, since temperature acts as a controlling factor of the state of molecular activation of the metabolic chain components (Wootton, 1990), increasing in this way, the rate at which the chemical transformations take place. Therefore, decrease in water temperature diminishes the fish´s metabolism. On the other hand, a temperature increase speeds up its metabolism and, consequently, it is possible to assume that the acclimatization rate can be diminished at lower temperatures. The organisms that live in lagoons and shallow water lakes can be acclimatized to temperature changes during the day and they do not lose their acclimatization level during the night. These organisms have the advantage of tolerating higher temperatures, and they displace the thermal tolerance zone at high temperature levels (Bettinger & Fitzpatrick, 1979; Chung & Strawn, 1994).

In Table 2, average values of ionic concentration of sodium and potassium are shown (in µEq/g) for *Petenia kraussii*, acclimatized at 23ºC and subjected to different environmental salinities during 4 weeks. The variance analysis show that a significant difference does not exist (P>0.05) for the values related to sodium and potassium ions in muscular and gill tissues, but a significant difference does exist for gut and heart tissues. For the heart tissue it was found that for Na^+, Fs = 7,771 and for K^+, Fs = 6,715. In the gut tissues, values for Fs were determined: 52,247 for Na^+ and 15,770 for K^+, which indicate that significant variations exist.

On the other hand, it is necessary to notice that *Petenia kraussii* acclimatized at 5, 10, 15 and 17‰ showed a 100% survival rate during the experimentation (40 days). When the salinity was changed at 18‰, the survival rate diminished to 3%, and at 19‰ salinity the survival rate was 95%. When the salinity was 20‰ the survival rate observed was 50% at 24 hours and a 100% death toll at 36 hours. The opercular movement stayed in a range between 74 and 76 bit/minute, until 15‰ salinity and increased to 83-90 bit/minute at higher salinities (19-20‰). The previous results show that the lethal incipient salinity for this species is 17‰. This is the limit at which they can be acclimatized, because after this concentration, the fish ingest little food. When the salinity was increased to 19‰, the fish stopped eating completely. When the salinity reached 20‰ the fish remained most of the time at the bottom of the aquarium and they were aggressive in defending their territory. If a fish lost a scale through the aggression of another fish, it ascended immediately to the surface of the water and it was almost immobile until it died. They presented a dark color which is characteristic of a stress condition, as well as a slimy consistency. Their weight diminished 50%.

As we can observe, Na^+ and K^+ ions can regulate muscular and gill tissues, but they did not do so with gut and heart tissues. *Petenia kraussii* regulate the sodium and potassium ions separately but do not do both at the same time, mainly at 10‰ salinity. This seems to be the salinity limit in which this species begins to stimulate all its osmoregulatory system or stress strategy to compensate the salinity. Also, we can observe that *Petenia kraussii* abruptly modify their preferential temperature at this salinity. Segnini & Chung (1989) reported these changes (26,7 at 0‰, 25,6 at 5‰, 31,3 at 10‰ and 27,8ºC at 15‰) according to environmental salinity. These species modify their final preferendum (28,3 at 30,0ºC) when living in a hyperosmotic environment. These results confirm previous findings presented by Finstad *et al*. (1989) who pointed

out that the ionic content in fish depends on temperature when the salinity is altered.

The processes of physiological adaptation in the gills to salinity changes are gradual and involve synthesis and destruction of molecular components of the epithelial transport system while monovalent ions (Na^+ and K^+) are excreted by the gills(Towle, 1981; Langdon & Thorpe, 1984).

At 5 and 10, 15 and 17‰ salinities, the physiological mechanisms of regulation to excrete the excess absorbed by the gills are stimulated, indicating that gills are highly permeable to sodium ions in both directions, and that saline balance is achieved by the adjustment of gain or loss of salts. All these mechanisms are very closed to the circulatory and hormonal system. They indicate that to maintain the sodium balance in teleost in a seawater environment, external potassium is necessary. It is thought that in seawater, the potassium ion is exchanged for the sodium ion which is eliminated through the blood into the seawater, and that this effect might be responsible for the maintenance of the sodium ion gradient concentration through the gills. The exchange suggests that activated adenosintrifosfatase by sodium and potassium (ATPase carrier) may have a function in the ionic exchange of the gills (Meador & Kelso, 1990)

P. kraussii in salinities higher than 17‰ diminished the potassium levels in all the tissues studied, suffering a hypotacemia that brings the organism to death. These results confirm those reported by Robertson (1984), who studied the content of sodium and potassium in *Anguilla anguilla* muscle and found that sodium ion increased in the animals adapted to freshwater, even though the potassium ion diminished. For *Petenia kraussi*, it was found that potassium ion lowering was so significant that muscular paralysis occurred because the loss of potassium in the cell interior produces hyperpolarization of nervous and muscular tissues that prevent their transmission from the electric potentials to the nervous signals through these tissues.
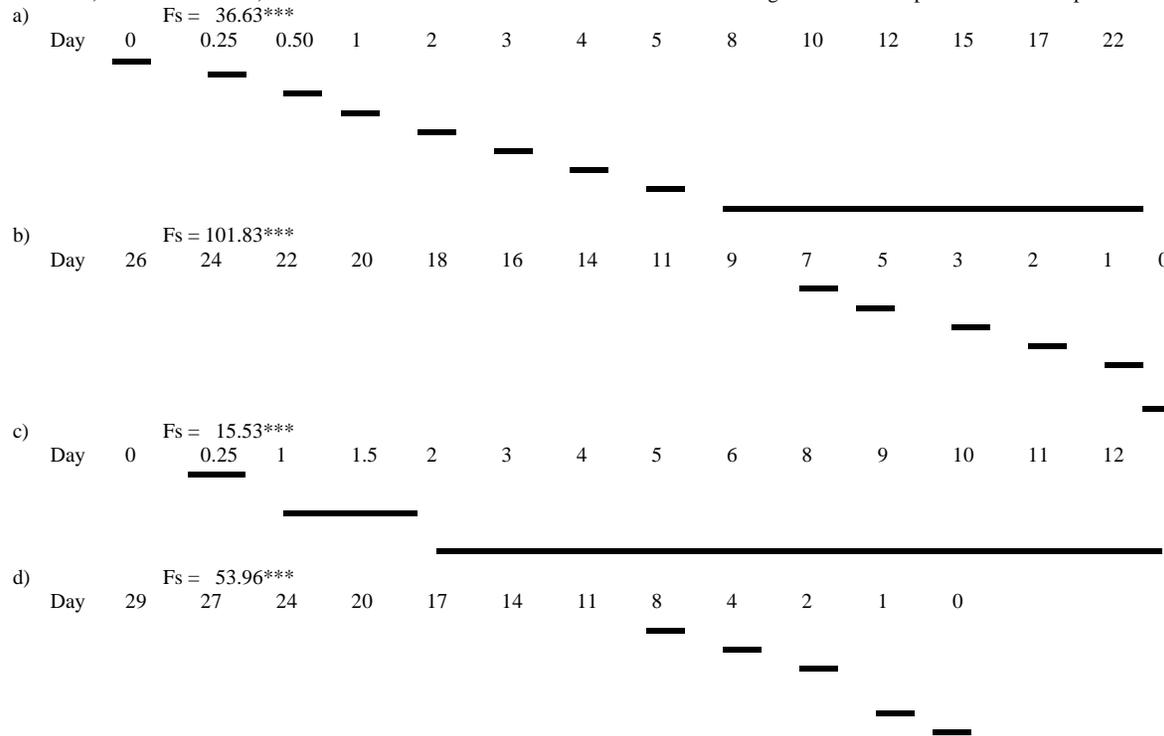
We conclude that Petenia kraussii is an oligohaline fish that regulates the sodium and potassium ions at gills and muscle until the salinity of 17‰ is reached, which is considered to be the incipient lethal level.

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Table 1. Duncan´s statistics results and F factor from variance analysis for acclimatization rate values of a) 22 to 32ºC and b) 32 to 22ºC and from c) 24 to 30ºC and d) 30 to 24ºC for *Petenia kraussii* acclimatized at 23ºC during 4 weeks and exposed at lethal temperature of 40.9ºC

a) $F_s = 36.63***$

Day	0	0.25	0.50	1	2	3	4	5	8	10	12	15	17	22

b) $F_s = 101.83***$

Day	26	24	22	20	18	16	14	11	9	7	5	3	2	1	0

c) $F_s = 15.53***$

Day	0	0.25	1	1.5	2	3	4	5	6	8	9	10	11	12

d) $F_s = 53.96***$

Day	29	27	24	20	17	14	11	8	4	2	1	0

***: $p < 0.001$

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Table 2. Sodium and potassium ion average values (µeq/g) obtained for *Petenia kraussii* tissues acclimatized at 23ºc during four weeks at different salinities

Tissue	Ion	SALINITY						
		0%0	5%0	10%0	15%0	17%0	18%0	20%0
Muscle	Na^+	33.95	35.91	37.01	37.15	36.57	91.44	109.51
	K^+	110.59	108.68	102.57	100.00	91.03	64.10	60.05
Gills	Na^+	115.27	120.31	120.17	128.86	112.19	188.12	201.79
	K^+	68.21	46.62	49.64	54.05	46.66	38.64	10.32
Gut	Na^+	97.64	139.14	347.46	303.87	340.30	350.43	392.40
	K^+	83.49	112.54	162.94	118.16	100.59	60.94	70.72
Heart	Na^+	117.67	244.44	197.58	241.38	243.20	270.15	305.33
	K^+	106.87	106.87	164.76	103.82	110.56	70.53	47.82

PHYSIOLOGICAL RESPONSES

OF TROPICAL FISHES

TO SALINITY CHANGES

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Abstract

Physiological response of the tropical organisms to salinity changes was carried out for the fishes acclimated for four weeks at various salinities. The fishes were placed at high lethal salinities and/or increased acclimation salinity to determine median lethal salinity. Studies indicated that acclimation level was the most significant factor. This phenomenon is very important for tropical aquatic organisms in shallow waters, where they could be acclimated at a high salinity during the dry period and will not lose its acclimation level at low salinity during abrupt rain. This behavior will contribute to salinity adaptation of tropical organisms, their proliferation and distribution in saline fluctuating environments.

Introduction

Saline adaptability of aquatic organisms is influenced by various biotic and abiotic parameters (Chung, 1994a); among them, acclimation salinity and temperature are significant factors on physiological response of tropical aquatic organisms (Segnini & Chung, 1989, 1991; Azuaje & Chung, 1993; Chung & Méndez 1993; Chung, 1990,1994b, 1995, 1996). It appeared that process of saline adaptability of tropical organisms is more convenient in decreasing salinity than in increasing salinity (Chung, 1990); however, thermal acclimation process is opposite (Segnini & Chung, 1991; Segnini et al., 1993; Chung, 1981, 1994a, 1995, 1996). Thus, the objective of this work is to study and summarize

the effects of salinity, comparing saline tolerance and adaptability of certain fishes in the nororiental region of Venezuela, and to investigate some physiological responses of aquatic organisms to salinity changes.

Materials and Methods

Various species of tropical fishes, oligohaline (*Astyanax bimaculatus* and *Petenia kraussii*), polyhaline (*Cyprinodon dearborni)* and euryhaline (*Oreochromis mossambicus*) were captured from el Golfo de Cariaco, La Laguna de Los Patos, el Río Los Bordones and el Río Manzanares (Sucre State, Venezuela) (Chung, 1980, 1985, 1990, 1994). The fishes were acclimated for four weeks at various temperatures between 22 and 35°C, whose values represent the minimum (22-24°C) and maximum water temperatures (30-35°C) during all year around in natural ambient. The organisms acclimated were placed to various lethal salinities to determine upper and lower limits of saline resistance. Other groups acclimated to lower salinities were exposed to increasing salinity of 2 ppt per day to determine the limit of high lethal salinity, the critical saline maximum, CSM (Segnini & Chung, 1989; Azuaje & Chung, 1993; Chung 1994b).

Results and Discussion

Tables 1 and 2 indicated that acclimation level was principal influence in salinity tolerance of the fishes (Chung, 1982; Chung, 1990); thus, it appeared that times of salinity resistance increased depending upon increment of saline acclimation. Also, the study revealed that exchange pattern, gradual o abrupt, was important factor for salinity acclimation process and mean saline resistance (Tables 1and 2), confirming that salinity tolerance of the aquatic organisms is significantly affected by acclimation salinity (Chung, 1982).

The order of general sensibility of the fishes exposed to temperature was: the Oligohalines (fishes of freshwater), the Polyhalines (marine fish) and the Euryhalines (organisms of estuaries), Chung (1993, 1994b). It is interesting to note that tropical freshwater fish, river sardine *Astyanax bimaculatus*, acclimated in saline water of 4 ppt resisted much more longer than those acclimated in freshwater when these were exposed to lethal temperatures, possibly due to less osmotic pressure in saline water (Fig. 1).

Table 1. Numbers of accumulated dead *Cyprinodon dearborni* by abrupt salinity
changes (from 35 to 95 ‰) during 16 days. The twenty fishes were
used for bioassays (modified from Chung 1982).

Salinity (‰)	Time							
	1 h	2 h	4 h	8 h	14 h	24 h	36 h	16 day
2	0	0	0	0	0	0	0	0
35-55	0	0	0	0	0	0	0	0
65	0	0	0	2	2	8	10	10
75	0	6	12	20				
85	2	18	20					
95	20							

Table 2. Numbers of accumulated dead *Cyprinodon dearborni* by gradual saline
changes (5 ‰ increment per day; from 2 to 117 ‰). Sixty fishes were
assayed (modified from Chung 1982).

Day	0	...	17	18	19	20	21	22	23
Salinity (‰)	2	...	87	92	97	102	107	112	117
Accumulated dead numbers	0	...	1	2	2	2	10	45	58
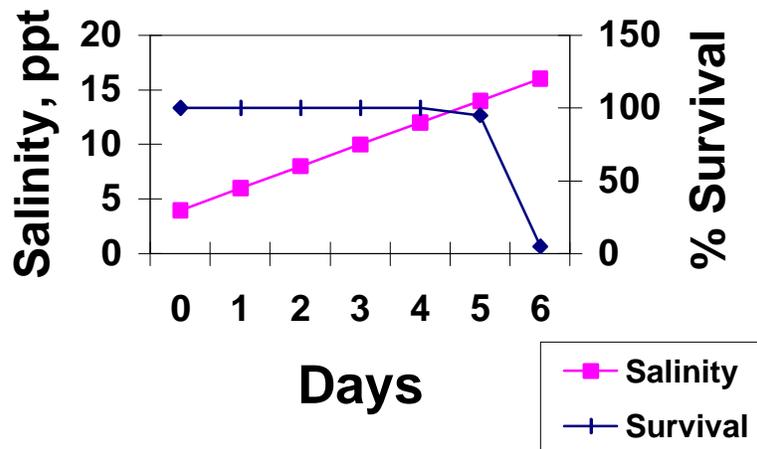
Fig. 1. Percentage survival of tropical fish, *Astyanax bimaculatus*, acclimated for four weeks at 23ºC y 4 ‰ salinity, and then increased the salinity 2 ‰ per day (modified from Chung, 1994).

Final preference temperature of *Petenia kraussii* acclimated in 5-15 % of the salinity was slightly higher than the acclimated in freshwater (Segnini & Chung, 1989). The mollusks adapted in stenohaline condition, "la pepitona" *Arca zebra*, were less resistant than those acclimated in euryhaline state, "el mejillón" *Perna perna*, and mangrove oyster *Crassostrea rhizophorae* (Fig. 2). Therefore, the critical saline minimum and maximum (CSM) and higher and lower lethal saline concentrations during 96 hours (96-h LC_{50}) which caused 50% mortality of the organisms were 26 and 50 ppt for "la pepitona" and 12 and 68 ppt for "el mejillón". The mangrove oyster survived in freshwater and 84 ppt of salinity during 96 hours, demonstrating that this specie is truly euryhaline (Chung, 1983). The process of saline acclimation was affected severely in increasing salinity (from lower to higher salinity), but was not affected much in decreasing salinity (from higher to lower salinity) (Tables 1and 2).

This phenomenon is very important for the tropical aquatic organisms which reside in shallow water of coastal lagoons, temporal tide pools, lagoons, rivers y estuaries, etc., permitting them to acclimate to high salinities during dry seasons and keeping this level of acclimation during rainy seasons.

Table 3. Thermal resistance times of *Astyanax bimaculatus* acclimated for four weeks at 23ºC in freshwater and 4 ‰ salinity, and exposed to experimental lethal temperatures of 36,6 and 37,5ºC (modified from Chung, 1994).

		Resistance (min)						
Sal	Temp	Range	Med	Average	O_2	pH	Length (mm)	Weight (g)
0	36,5	24,4 - 88,5	45,6	$50,6 \pm 22,2$	7,8	7,1	$77,8 \pm 19,6$	$12,3 \pm 9,4$
0	37,5	16,6 - 32,1	27,2	$26,2 \pm 05,0$	7,2	7,2	$79,3 \pm 11,1$	$11,3 \pm 6,3$
4	36,5	56,9 - 460,1	126,6	$185,4 \pm 15,2$	6,4	7,3	$74,8 \pm 17,1$	$11,3 \pm 8,2$
4	37,5	16,3 - 45,1	34,8	$31,9 \pm 10,2$	5,9	7,3	$80,0 \pm 16,8$	$11.8 \pm 7,0$

Sal: Salinity (‰)
Temp: Experimental lethal temperature (ºC)
Med: Medium resistance time
O_2: Dissolved oxygen, ppm

This kind of behavior helps them to resist abrupt salinity changes in tropical natural ambient in river and estuarine flow and/or during severe raining, and contributes to distribution of the organisms of saline waters through salinity adaptation of tropical organisms (Chung, 1989, 1994b). The larva of white and brown shrimp (*Penaeus schmitti* and *Penaeus brasiliensis*) acclimated in diverse salinities, selected lower salinities (from 5 to 25 ppt), when they were exposed in salinity gradient tank without barrier (Chung, 1980; Azuaje y Chung, 1993). This physiological response implies that the larva of *Peaneus* prefer in shallow waters and estuaries, where the salinities fluctuating during larval development, and stay there until juvenile and pre-adult stages before returning to open sea. The larva of *Macrobrachium* placed in saline gradient without barrier, selected their lower saline preference (5-15 ppt) during larval development, however, the juveniles changed their preference to lower salinities (0-5 ppt), confirming that this species requires and need to adjust different salinities during ontogenic development (Graziani *et al*, 1993, 1995).

Conclusion

Salinity acclimation level influences saline tolerance limit of the tropical fishes. The process of saline acclimation in tropical organisms is affected in increasing salinity (upward acclimation) more than in decreasing salinity (downward acclimation). This phenomenon contributes to salinity
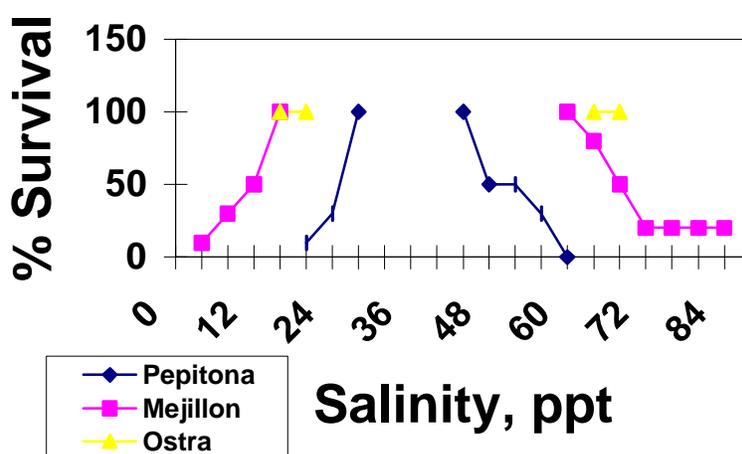adaptation of tropical fishes, their proliferation and distribution in fluctuating saline ambient.

Fig. 2. Percentage survival of mollusks ("el mejillón" *Perna perna*, "la pepitona" *Arca zebra* and "la ostra" mangrove oyster *Crassostrea rhizophorae*) acclimated for 3-4 weeks at 23ºC and 36 ‰ salinity, and then exposed to various salinities, from 4 a 84 ‰ during 96 hours (modified from Chung, 1983).

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DIRECT EVIDENCE OF NA,1(-ATPASE

AND NA,K,2CL-COTRANSRPOTER

IN GILLS OF SEAWATER-ADAPTED

TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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Introduction

The osmolarity of theleosts living in marine environments is about one-third as high as that of seawater. In order to maintain an ionic balance, the fish must extrude those extra ions difflising into their bodies. The current model for Cl secretion by gills of seawater fish was ascribed largely to Silva et al. (1977) with some modification. In this model, cr is actively transported through the epithelial mitochondria-rich (MR) cells. Na,K-ATPase (sodium pump) localized in the MR cells is the ultimate driving force to establish the Na+ electrochemical gradient and chloride ions following the gradient enters the cell basolaterally by means of a Na+-dependent cotransrpoter ~a,K,2Cl-cotransporter; NKCC) which is Inhibited by the "loop" diuretics flirosemide and bumetanide ~arshall, 1995). Cf exit occurs down its electrochemical gradient through anion channels in the apical membrane of MR cells. However, the proposed model was described according to the data arose from studies on the operucular epithelium (Marshall, 1995), a flat sheet epithelium simllar to the branqhial epithelium. Hence, in the present study, biochemical and morphological results were combined to provide direct evidence of the Cl.- transport mechanism in the gill epithelium

Materials and methods

Tilapia *(Oreochromis mossambicus)* was raised in seawater for at least one month before the expreiments (14wang, 1987). Monoclonal antibodies to Na,K-ATPase (aS) and NKCC (T4) were used in the present study. Fish gill was homogenized and Western blot was done first to confirm the cross reaction of antibodies with the tissue. Then the gill filaments were dissected and the whole-mount fluorescent staining were performed. Con-A, a fluorophore which identified the apical crypts of the MR cells (Li et al., 1995), was applied in the filaments following by the immunofluorescent staining of the antibodies and observed by a confocal laser scanning microscopy.

Results and Discussion

Although the antibodies were not directed against the fish, western blot analyses of seawateradapted tilapia revealed immunoreactive bands of Na,K-ATPase and NKCC with the molecular weight of 100 ~ig. 1) and 195 kr)a, respectively. Fluorescent staining showed that Con-A was restricted to the apical crypts of the MR cells. Immunofluorescent staining revealed that either Na,K-ATPase or NKCC (Fig.2) were found in the cytoplasm of gill epithelial cells. Further

colocalization of Con-A and Na,K-ATPase or NKCC observed by the confocal microscopy demonstrated that both the pump and the transporter were distributed in MR cells. Being members of membrane proteins, the sodium pumps and the NKCC were obviously located in the basolateral membrane constituting the tubular system in the cytoplasm of MR cells.

Using the vibrating probe technique and tilapia operucular epithelium, Foskeff and Scheffey (1982) proved that epithelial MR cells were the site of Cl-secretion. A recent study on killifish opercular epithelium described the effects of Inhibitors on membrane pumps or transporters depicted a more convincing model about Cl- secreting mechanism in seawater teleosts (Zadunaisky et al., *1995).*

In the present study, for the first time we provided direct evidence of the current model for Cr secretion in seawater fish gills.
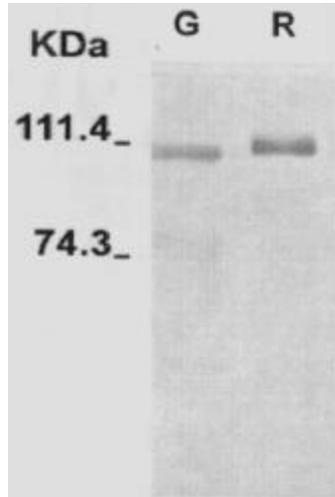
Fig.1 Immunoblot of Na,K-ATPase a-subunit in seawaer-adapted tilapia gills. U, gill; R, rat brain (positive control). The molecular weight is about 100 Ina.
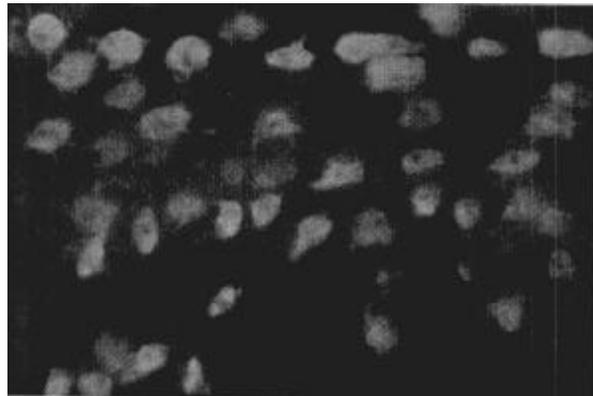

Fig. 2 Immunofluorescent staining of NKCC in the epithelium of seawater tilapia gill filaments.

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PECULIARITIES OF OOGENESIS

IN SOME COASTAL TROPICAL FISHES

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Introduction

The reproduction of fishes has an essential importance for the supporting of the abundance of the species. The peculiarities of gametogenesis, as well as the types of spawning show a significant diversity caused by the specific relationship of the organism with its environment. The aim of this study was a preliminary assessment of the features of sexual cycles in some most abundant fish species of the South China Sea.

Material and methods

Fish samples were collected in 1993 and 1995 in Nha Trang Bay, South China Sea (Vietnam). The features of oogenesis were studied in seven coastal fish species from four families: Synodontidae (*Trachinocephalus myops*), Engraulidae (*Stolephorus buccaneeri*, *S. heterolobus*, *S. indicus*), Atherinidae (*Atherinomorus lacunosus*, *Hypoatherina temminkii*), Clupeidae (*Spratelloides gracilis*). A total of 378 specimens were analyzed. Their length, body and gonad weight, and dynamics of GSI were determined. The number of vitellogenous oocytes and their proportion in a group of oocytes were counted, and size frequency distributions of the oocytes were plotted, based on the histological analysis of the ovaries. In some species, the ultrastructure of oocyte membranes was described.

Results and discussion

All fishes had a prolonged spawning period, most likely reaching entire year in some species. The duration of the spawning season was connected with the

reproduction of fishes of different age and size groups at different time, as well as with the peculiarities of their oogenesis. A permanent type of oogenesis was found in the majority of the studied fish species (six) from the families Synodontidae, Engraulidae, and Atherinidae. This type of oogenesis was characterised by a continuous recruitment of the mature oocytes from the reserve generation of the small previtellogenous oocytes. The proportion of small immature oocytes was much higher than that of mature oocytes of the larger size, and germ cells, as well as the cells of the intermediate size were always registered in the ovaries (Fig. 1a). Such a type of oogenesis leads to the highest reproductive potential and is common for marine fish species of the tropical zone (Gotting,1961; Oven, 1976; Emel`yanova, 1997).

Spratelloides gracilis (Clupeidae) had an interrupted type of oogenesis. The portion of mature oocytes was formed only from a generation of vitellogenous oocytes, and therefore, the number of mature oocytes was limited. The number of the oocytes of the larger size was the highest, two or more peaks of vitellogenous oocytes were registered, and the oocytes of the intermediate size were absent (Fig. 1b).
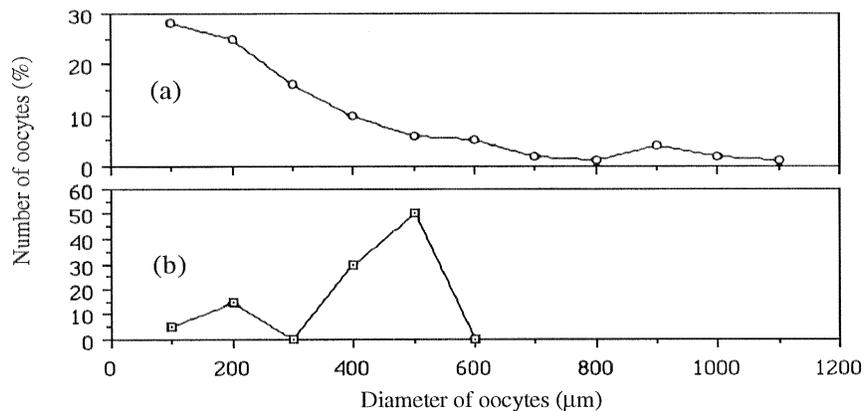

Figure 1. Size distribution of oocytes in (a) *Trachinocephalus myops*, a species with permanent type of oogenesis and in (b) *Spratelloides gracilis*, a species with interrupted type of oogenesis.

A high reproductive capacity in fishes with interrupted type of oogenesis can be reached due to small duration of the sexual cycles caused by intensive feeding.

A prolonged spawning season and multiply spawning in the majority of fish species of the tropical zone is connected with the absence of substantial environmental fluctuations, including fluctuations in plankton abundance.

From the seven species, four (families Synodontidae and Engraulidae) had pelagic eggs. *T. myops* (Synodontidae) had round eggs, covered by the egg shell with the structure similar to that in honey combs, to increase the egg surface. Three other species (Engraulidae) had oval or pear-shaped eggs with smooth membranes.

A high reproductive potential in tropical fishes, especially in species with pelagic eggs, can be regarded not only as an adaptation to comparatively stable environment, but also as a compensation of significant decrease of eggs and larvae under the influence of a high pressure of predators.

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CULTURE OF TRANSGENIC TILAPIA

WITH ACCELERATED GROWTH

UNDER DIFFERENT INTENSIVE

CULTURE CONDITIONS

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Introduction

Fish are appreciated by consumers in many countries and can provide acceptable yield on relative low inputs. Tilapia is economically important fish species in many parts of the world accounting for over 50% of the fresh water fish production in Cuba. Tilapia posses several advantages for aquaculture including short generation times, adaptability to different environments, good disease resistance and is an important supply of animal protein to humans. These qualities have ensured the introduction of tilapia in the aquaculture programs in many countries. Israel, Zambia and Kuwait are examples where tilapia are widely used both in monoculture and polyculture together with common carp, grey mullet and silver carp (Sarig, 1996; Gopalakrishnam, 1988; Hopkins, 1988). The application of intensive culture technology and polyculture systems with fish species utilizing a different ecological niche of natural food in the pond, has permitted to increase the production yields to several tens tons/ha. These results improve the food conversion rates and daily growth rates for male

tilapia at high stocking rates to about 2.0 and 2.0-3.5g, respectively (Sarig, 1996).

Our group have generated a transgenic tilapia line (IG-91/03F70) with accelerated growth after the transfer of a transgene driving the ectopic expression of tiGH cDNA under the control of CMV regulatory sequences (Martinez et al., 1992; 1996; de la Fuente et al., 1995; Guillen et al., 1996). A male containing 1 copy/cell of the transgene was selected to establish the transgenic line. After genetic, biochemical, phenotypic, behavioral and safety studies, this tilapia strain was shown to be suitable for culture and consumption (de la Fuente et al., 1998).

Transgenic tilapia show a mean growth acceleration when compared to non-transgenic siblings ranging from 60% to 80%, depending on the culture conditions (Martinez et al., 1996; Guillen et al., 1996). This growth phenotype is associated with the ectopic expression of tiGH in many tissues of the tilapia with no detrimental effects to the animals (Martinez et al., 1996; Hernandez et al., 1997).

This paper presents the results of the characterization of the growth rate of transgenic tilapia (line IG-91/03F70) under different conditions of intensive culture. The results of our experiments confirmed the improvement in growth performance of these transgenic tilapia under intensive culture conditions.

Experimental Methods

Transgenic tilapia were generated and characterized as described by Martinez et al. (1992; 1996), de la Fuente et al. (1995), Guillen et al. (1996) and Hernandez et al. (1997).

The animals used for intensive culture were always F3 generation, from the crosses between homozygous transgenic tilapia (line IG-91/03F70) and wild type *O.aureus* tilapia to produce heterozygous fry.

The LK strain of *O.aureus* was obtained by selection as reported by Sanchez et al. (1994).

Three experiments were performed:

Exp. 1. For intensive culture, 818 heterozygous transgenic fry (1.4g average weigth) were cultured in a 100m^2 tank together with 12 439 red tilapia fry (3g average weigth) and catfish. The intensive policulture conditions were assured by the artificial aeration of the pond, by feeding with protein-rich pellets and by the culture of various fish species. The pond was sampled periodically to follow the growth rate of tilapia during 257 days of culture, covering from tilapia fry nursing until the attainment of the commercial weight. Statistical comparisons were done employing a Student t-Test.

Exp.2. A second experiment with 10 000 transgenic tilapia (9.9g mean weight) was conducted in an intensive monoculture system for 150 days with similar aeration and feeding conditions.

Exp. 3. Low density intensive tilapia monoculture in a 100 m^2 tank was assayed in the last experiment with 651 transgenic tilapia (73.2g mean weight). Tilapia were cultured for 150 days.

Results and Discussion

A comparison of the three experiments described here appears in the table 1.

The first experiment started from nursing of tilapia fry and proceeded until the commercial weight was attained after 257 days of culture. Transgenic tilapia showed a better growth performance when compared to red tilapia. At the end of the experiment, a difference in the mean±SD weight of 384.4±158.4 g vs. 313.7±100.9 g was obtained for transgenic and red tilapia, respectively (p=0.09) (Cabezas et al., 1997).

The daily growth rate of transgenic tilapia was of 1.5 g throughout the experiment (Table 1). This represented a 1.25 fold increase over red tilapia (Cabezas et al., 1997).

Furthermore, during the face of fry nursing until the 60g of weight approximately, transgenic tilapia maintained a daily growth rate of 1.5g while for red tilapia this value was of 0.7g. The 2.1 fold increase in the daily growth

rate in transgenic versus red tilapia during the early stages of growth may be of interest for tilapia fry producers.

In the second experiment, the culture was subjected to starvation during the first two months. Although the daily growth rate was better than with other tilapia lines, it was lower than that obtained on the first experiment (Table 1), thus sustaining previous reports on the density- and feeding-depending conditions in tilapia culture.

In the third experiment under similar conditions than the first but at low culture density, the daily weight gain was equal to 1.56 g (Table 1).

Table 1. Intensive culture of transgenic (line IG-91/03F70), red and LK tilapia.

Parameter	Transgenic (IG-91/03F70)[a]			LK [b]	Red [c]
	Exp. 1	**Exp. 2**	**Exp. 3**		
Culture area (m^2)	100	100	100	100	100
Culture conditions	High density polyculture	High density monoculture	Low density monoculture	High density monoculture	High density monoculture
Initial mean weight (g)	1.4	9.9	73.2	117	13.8
Final mean weight (g)	384.4	137.4	326.1	179	174.3
Culture days	257	150	150	76	251
Daily growth rate (g/day)	1.5	0.85	1.56	0.81	0.64

[a]Experiments are referred to the Exp. described here.
[b]The LK strain of *O.aureus* was obtained by selection.
[c]Hybrid red tilapia were originally introduced from Mexico.

The results obtained on intensive culture with the transgenic tilapia line IG-91/03F70 are compared with the best results obtained by other groups employing selected tilapia strains (Table 2). This appears as a confirmation that transgenic techniques are a powerful tool to improve growth performance in fish, decreasing the time required selecting a genetically improved strain.

Finally, the coefficient of variation for growth still present in transgenic tilapia (Martinez et al., 1996) predicts a good chance for improvement employing this line in commercial crosses with selected tilapia strains. Alternatively, the transfer of the growth hormone transgene could be done in one of the previously selected strains. The application of chromosome manipulations (e.g. induction of tetraploidity, Tave, 1993) in transgenic tilapia may also results in further improvements.

Table 2. Daily growth rate of different tilapia strains under intensive culture.

Tilapia line	Daily growth rate (g/day)
Transgenic tilapia (line IG-91/03F70)[a]	1.53
Philippine red tilapia[b]	1.5
F1 hybrids of *O.niloticus x O.aureus*[b]	1.2
Singapore red tilapia[b]	1

[a]Mean daily growth rate calculated from Exp. 1 and 3 (Table 1).
[b]Intensive monoculture on 100 m^2 ponds with high density (Pruginin, 1988).

Conclusions

Comparing the results obtained in these experiments employing hybrids of the transgenic tilapia line IG-91/03F70 (F2$^{+/+}$) x *O.aureus*, we concluded that:

(1) Transgenic tilapia show a better average daily growth rate (1.2 g/day) when compared to non-transgenic *O.aureus* (LK strain) (0.81 g/day) or hybrid red (0.64 g/day) tilapia.

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(2) The effect on growth acceleration in transgenics is more evident during larval stages.

(3) The transgenic tilapia line IG-91/03F70 has a growth rate on intensive culture compared with the best results reported for selected tilapia strains.

The results obtained in this study confirmed under intensive culture in high and low density conditions, a growth improvement in the line IG-91/03F70. Studies in progress are evaluating crosses between transgenic tilapia and different hybrid red, *O.aureus* and *O.niloticus* strains under extensive and intensive culture conditions, in mono and polyculture and in freshwater and seawater.

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COMPARATIVE STUDY OF THE

LIVER MONOOXYGENASE SYSTEM

OF BRAZILIAN FISH

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Abstract

The monooxygenase system (MOS) of the liver endoplasmic reticulum
(microsomes) contains enzymes that catalyze xenobiotic biotransformations in
eukaryotes, and play a major role in the detoxification and activation of
pollutants. These enzymes are potentially useful as bioindicators of
environmental contamination, particularly in fish ecology. The MOS of South
American fishes is described herein.

Fish studied

Adult *Prochilodus scrofa* and *Hoplostemum litorale* were obtained from the
ABRAq (Pirassununga, São Paulo State). *Prochilodus nigricus* was provide by
INPA (Manaus, Amazon). Specimens of *Colossoma macropomum* and *Brycon
cephalus* were obtained from both regions. Thus, among the species studied
here, three occur in the rivers of São Paulo State and three - in the Amazon
river. Mouse liver microsomes (MLM) served as a control.

Fish (FLM) and mouse (MLM) liver microsomes were prepared using methodology generally employed for the preparation of FLM (Vrolijk et al., 1994). Some preparations were also obtained based on methodology used for MLM (Degterev et al., 1990). Activity of NADPH-cytochrome c (P-450) reductase was measured according to [3]. The methods of Lowry and Omura & Sato were used for determination of total microsomal protein and cytochrome b_5 and P-450 concentrations, respectively.

Reduction of fish hepatic microsomes with NADPH (NADH) and sodium dithionate resulted in the unexpected appearance of two different spectra. All microsomes studied, except those prepared from the livers of *P. nigricus* and *C. macropomum,* exhibited a typical peak of reduced cytochrome b_5 when exposed to NADPH (NADH). A much greater peak with the same shape and position of a minimum as that of cytochrome b_5, but with a maximum shifted to 428-434 nm, appeared when microsomes of all species were reduced with sodium dithionate. The intensity of this peak usually differed by an order of magnitude from that of cytochrome b_5 and was close to that of a mouse enzyme (Fig. 1B). Washing the microsomes twice with either buffer or distilled water (20:1, v/v) did not significantly decrease the peak, thus confirming the absence of contaminant hemoproteins.

Carbon monoxide difference spectra of sodium dithionate-reduced FLM showed the characteristic peak of cytochrome P-450 overlaid by a spectrum with an intense peak at 418 nm, a deep trough at 432-435 nm, and two small peaks in the visible region (Fig 1A, spectrum 1). When recorded against non-reduced microsomes, this spectrum showed an intense peak at 420 nm and two small peaks at ~ 540 nm and ~ 570 nm, in addition to the peak for cytochrome P-450. These characteristics were similar to those reported for isolated rabbit cytochrome P-420. The peak of cytochrome P-450 appeared in the FLM spectra of all fish species from the Paraná basin and in one from the Amazon river (*P. nigricus).* FLM from other two species from the Amazon river (*C. macropomum* and *B. cephalus)* showed only a P-420-like spectrum, unlike specimens of the same species from the Paraná basin.

Comparative data on the microsomal cytochrome content and cytochrome c (P-450) reductase activity are presented in Table 1. The concentrations of cytochrome b_5 were obtained in experiments with NADPH (NADH) reduced microsomes (∇A = 424 nm - 410 nm, ε=112 mM^{-1} x cm^{-1}). The concentrations of cytochrome P-450 were calculated based on data obtained with sodium

dithionate-reduced microsomes (∇A = 450 nm - 400 nm, ε=91 mM^{-1} x cm^{-1}). This results indicate that, compared to mammals, liver microsomes from all of the fish species studied here had very small peaks (in some cases none), of cytochromes b_5 and P-450. Biotransformation enzymes in tropical and subtropical fish have only been described for a few marine species. To the best of our knowledge, there are no published data to compare with those obtained here. The data available for tropical marine fish are close to those for mammals. For instance, the cytochrome P-450 content in liver microsomes from *Chaetodon capistratus* collected in Florida and Beliz ranged from ~ 500 to ~800 nmol/mg microsomal protein (Vrolijk et al., 1994). Activity of NADPH-cytochrome c (P-450) reductase in the fish we studied was close to that of both mouse (see Table) and *C. capistratus* (0.27 - 0.33 µmol/min/mg microsomal protein (Vrolijk et al., 1994)).

FLM from all fish species studied here containing the pigment(s) were reducible by sodium dithionate, non-reducible by NADPH or NADH, and interacted with CO.

Acknowledgements

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Table 1. Properties of the liver monooxygenase system of Brazilian fish from the Paraná basin and Amazon river.

| Space	Mouse	*H. litorale*	*P. scrofa*	*P. nigricus*	*C. macropomum* (SP)	*C. macropomum* (AM)	*B. cephalus* (SP)	*B.cephalus* (AM)
$[b_5]$	500±180 n=6	150±10 n=3	50±10 n=3	90±40 n=3	160±80 n=3	none n=4	100±50 n=3	34±1 n=3
[P450]	550±200 n=6	140±50 n=4	10±5 n=8	40±20 n=3	140±50 n=3	none n=4	30±10 n=3	none n=7
CCRA	0.4±0.1 n=3	0.25±0.05 n=4	0.3±0.1 n=4	0.26±0.03 n=3	n.d.	0.35±0.1 n=3	n.d.	0.34±0.04 n=3

$[b_5]$ - concentration of cytochrome b_5 measured in microsomes reduced with NADH or NADPH as nmol/mg of total microsomal protein ($\varepsilon = 112$ mM^{-1}. cm $^{-1}$);

[P450] - concentration of cytochrome P-450 measured as ∇A between 450 nm and 490 nm in microsomes reduced with sodium dithionate and exposed to carbon monoxide (nmol/ mg of total microsomal protein, $\varepsilon = 91$ mM^{-1}. cm $^{-1}$);

CCRA - NADPH-cytochrome c (P-450) reductase activity in liver microsomes expressed in μmol per mg protein x min based on the increase in absorbance at 550 nm ($\varepsilon = 21$ mM^{-1}. cm $^{-1}$);

none - no peak was observed

n.d. - not determined
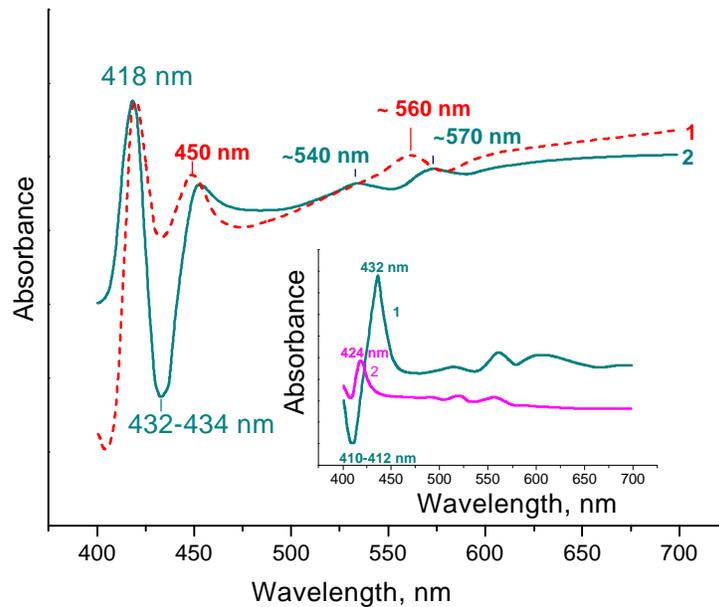
FIGURE 1. Visible spectra of liver microsomes from Brazilian fresh-water fish. **(A)** Carbon monoxide spectra of sodium dithionate-reduced FLM as recorded against reduced (1) or non-reduced (2) microsomes. (B) Reduction of FLM. Difference spectra of dithionite-reduced FLM (1) and NADPH (NADH)-reduced FLM (2) against corresponding non-reduced microsomes. NADPH (NADH) was 1 mM, and microsomal protein was 1 mg/ml in 0.4 M potassium phosphate buffer, pH 7.4. Temperature was 25^0C.