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The ontogeny of cardiorespiratory support of metabolism

Paul Richard Territo
University of Nevada, Las Vegas

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THE ONTOGENY OF CARDIO-RESPIRATORY SUPPORT OF METABOLISM

by

Paul R. Territo

A dissertation in partial fulfillment
of the requirements for the degree of

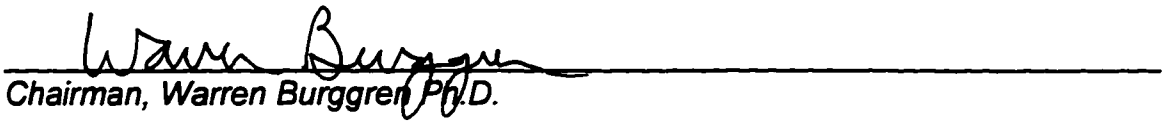
Doctor of Philosophy

in

Biology

Department of Biological Sciences
University of Nevada, Las Vegas
December 1996

The dissertation of Paul Richard Territo for the degree of doctor of philosophy in the biology is approved.



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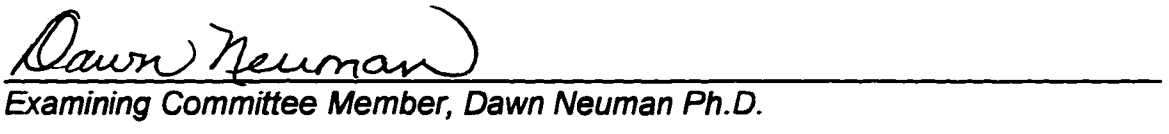

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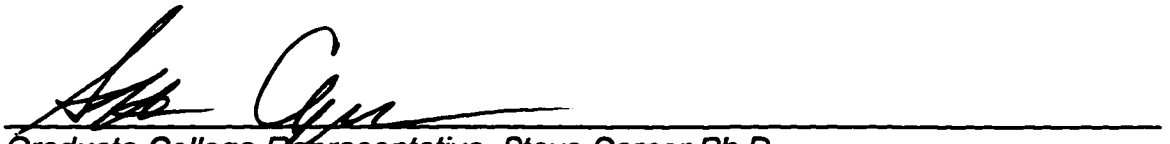

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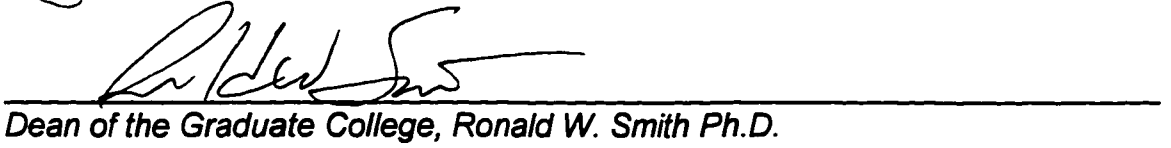

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ABSTRACT

The formation of the cardiovascular system and it role in gas exchange has long been speculated to occur concomitantly. Although this premise has been suggested and quoted for more than a century, there are few studies to date which have attempted to validate these claims. Furthermore, the few which do exist have been primarily concerned with two things: first, how environment may affect the functional morphology; and second, how these changes may affect hemodynamics directly. As a result, our understanding of how gas exchange is coupled with to cardiovascular function is seriously lacking.

The goal of this dissertation was to understand how, or if, the cardiovascular and respiratory systems coordinate function during development. Populations of amphibians were reared in various environments which included carbon monoxide (CO). This allowed for direct assessment of the efficacy of Hb in bulk O_2 transport. The results indicated that CO, and the subsequent elimination of Hb function, had few ill effects on either aerobic or anaerobic metabolism. Further, the data indicated that cardiovascular function was mildly elevated. A separate study, set out to determine what factors limit overall O_2 transport in developing embryos by limiting the available quantities of gas and eliminating Hb function. The results indicated that aerobic metabolism was unaffected in all populations. Cardiovascular function was mildly elevated, but only in populations of animals exposed to CO. Finally, gas exchange was modeled in developing embryos to determine the role of diffusion and plasma

transport in overall O_2 uptake. Calculations of maximal O_2 flux indicate that diffusion would allow for enough gas to be exchanged to support aerobic metabolism in early life. Moreover, the role of plasma transport was considered in addition to diffusion, it be came clear that their combined transport would be adequate to support metabolism for animals in late life.

A separate study evaluated how body composition changes with progressive development. From this it was determined that amphibians are unlike their fish counterparts in composition. When the total energy pool available for growth and development was calculated and compared with aerobic metabolism, it was shown that energy was not a limiting resource during development.

Collectively these data indicated that Hb was not essential of O_2 uptake and that the cardiovascular system as a whole may play a reduced role in total O_2 turnover. Furthermore, the data indicates that neither diffusion nor perfusion limits total gas exchange. In addition, the model indicate that diffusion may be a viable mean to obtain O_2 early in development, and that late in development convection of plasma coupled with diffusion could support metabolism. Finally, the body composition work indicate that resource limitations were not set by available substrate.

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AKNOWLEDGEMENTS

This section of the dissertation is perhaps the most difficult that I have had to write. The reason for this rests in the complexities of how the scientific process occurs. The formation of ideas, thoughts, and hypotheses to be tested are largely an amalgam of the people, place, and experiences one has during the course of completing a degree. With that, it becomes difficult to know which thoughts are truly original, or are simply a mosaic of ideas assembled from external sources. Assuming the latter to be true, I wish to thank the following people whom have advanced my science and my person over the past three years of this degree.

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During the time I have known Tobias, he has been a valuable resource and has taught me not only the importance of physiology, but friendship, and for this I thank you. Similarly, Regina has been a wonderful friend and collaborator, and it is her patience and understanding from which I have learned the most about myself. Lastly, I would like to thank Jordi. Over the past year and a half we

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Paul R. Territo,

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CHAPTER 1

Introduction and Historical Perspective

The process of developing an adult organism from a fertilized egg is almost the rule among all vertebrate classes. This is perhaps one of the most demanding times in any animal's life, and is best illustrated by the organism's need to obtain oxygen and to transport it to the tissues. Although, the time spent as an embryo represents only a small fraction of the total life span, it is clear that all physiological processes must function effectively during this time in order to ensure embryo viability. This was clearly noted more than 2300 years ago in the writings of Aristotle , in which he accurately described the general process of development, and the suspected need for the cardiovascular system in developing chicken embryos. Advancements over the past century have allowed for dramatic increases in our understanding of cardiovascular development, and through this it has been established in the developmental literature that the circulatory system, and the heart, are the first functional unit, and organ, in developing embryo (Burggren and Keller, 1997; Gilbert, 1990). Concomitant with the cardiovascular differentiation is the formation, and subsequent convection, of blood elements and hemoglobin. Clearly, the cardiovascular system is the first vital step which may impose considerable constraints to gas exchange, and ultimately future embryonic development, and this has lead researchers to speculate about its importance in overall metabolism (Adolph and Ferrari, 1968;

Boell *et al.*, 1963; Burggren and Just, 1992; Burggren and Pinder, 1991; Burggren and Territo, 1995).

The formation of this dogmatic theme has arisen from a variety of sources, particularly the early investigations into gas transport. Warburg (1923) empirically derived that the maximal tissue thickness for mammalian hepatic tissue at 38 ^{0}C was not greater than 0.5 mm in overall depth. Based on these observations Warburg (1923) suggested that the overwhelming vascularization in these tissues was for the purpose of O_2 delivery. This was consistent with reported models of the era, which suggested that optimal gas transport in capillaries occurred radially about the cross-section of vessel at distances less than 0.5 mm (Krogh, 1922). Although these observations suggested that convection via the cardiovascular system would afford greater gas transport, the question still remained as to how organisms without convection may achieve overall dimension greater than those reported by Warburg (1923). Harvey (1928) developed a mechanistic empirically derived model which utilized luminescent bacterial plaques as bio-assays. From this work it was determined that a maximal radius could be predicted given the partial pressure difference (ΔP_{O_2}), body mass (M_b), metabolic rate ($\dot{M}_{O_2}$), and tissue solubility coefficient (K_{O_2}), and thus could be expressed as:

$$r = \sqrt{\frac{\Delta P_{O_2} \cdot 6 \cdot K_{O_2} \cdot M_b}{\dot{M}_{O_2}}} \qquad (1)$$

However, his studies also indicated that there was an upper limit to the overall dimensions obtainable for a give organism. Collectively these studies indicated that gas transport was limited by diffusion, and that convection must develop to support high metabolic rates.

Additional support for the premise that convection was essential came from measurements of aerobic metabolism with development. Worked completed on developing fish (Rombough, 1988a), amphibians (Adolph and Ferrari, 1968; Atlas, 1938; Boell *et al.*, 1963; Lovtrup, 1959; Lovtrup and Werdinius, 1957; Wills, 1936.), and birds (Romanoff, 1960) have all noted the sharp increases in metabolic rates which occurs concomitant with the onset of heart rate and convective flow. In all, these studies suggest that the restrictions to oxygen consumption are in fact limited by the need for convection of the O_2 via the blood, and without it, total uptake may be impaired.

A line of evidence which is collateral to the formation of blood convection is the formation of new accessory vascular beds associated with respiratory structures, which has come from the work of functional morphology. The process of development and its embryological underpinnings have been extensively described from fish to birds (Ballard, 1968; Gosner, 1960; Hamburger and Hamilton, 1951; Medvedev, 1937; Nieuwkoop and Faber, 1967; Taylor and Kollros, 1946), and in all cases there has been enormous attention focused to the formation of respiratory structures and there potential role in facilitating greater uptake. Clearly, the emphasis placed on the respiratory and

cardiovascular structures with progressive development implies that gas exchange would be limited otherwise.

Although collectively, the ideas put forth through both physiology and functional morphology have become current dogma, there exists few studies to date which have attempted to test these premises directly in an ontogenic context. This is largely due to the fact that our understanding of developmental cardiovascular regulation in lower vertebrates is only now starting to become clear, and most of what was known prior has been interpolated from adult data. Historically mammalian or avian species have served as models for elucidating the intricacies of cardiovascular regulation. A short coming of these studies is that, they failed to looked at embryonic stages *in vivo* due to the innate difficulties of instrumentation *in utero*. Consequently, *in vivo* cardiovascular measurements in mammals have been made on late fetal stages (Faber *et al.*, 1984; Willis *et al.*, 1985), and as a result extremely little is known about cardiovascular performance during initiation of rate and convective flow. Due to the aforementioned difficulties, chicken models have been employed as analogs for mammalian embryonic circulation (Faber *et al.*, 1974; Howe *et al.*, 1995; Hu and Clark, 1989; Keller, 1994). Although a base of knowledge has begun to accumulate on heart rate, blood pressure, and flow in the central and peripheral vessels, the avian embryo has inherent limitations for these types of studies. Hemodynamic data cannot be collected without physically disrupting the shell. The shell is a major diffusion barrier to water and respiratory gases (Booth and

Rahn, 1990; Carey *et al.*, 1989; Davis and Ackerman, 1987), and disturbing this to gain access to the embryonic heart may have significant hemodynamic consequences both local and central. A compounding problem which plagues both avian and mammalian model systems is the lack of concurrent respiratory measurements.

Against this background, studies of basic cardio-respiratory dynamics in lower vertebrates began. It is assumed, and experimentally verified, that the early development of the cardiovascular system (i.e. prior to cardiac septation) is similar in all vertebrate groups (Burggren and Pinder, 1991; Burggren and Warburton, 1994). The anatomical literature on lower vertebrates is enormous (Ballard, 1968; Nieuwkoop and Faber, 1967; Taylor and Kollros, 1946) and provides detailed background information for physiological studies which are lagging behind. Hemodynamic information and cardiovascular regulation in amphibians, fish and reptiles is almost non-existent except for a few studies on cardiovascular function with development (Hou and Burggren, 1995a; Hou and Burggren, 1995b; Pelster, 1997; Pelster and Bemis, 1991; Pelster and Burggren, 1991).

Similarly, our understanding of respiratory function with development is also deficient. Work completed on fish (Holeton, 1971; Rombough, 1988b), and amphibians (Hastings and Burggren, 1995; Mellish *et al.*, 1995; Pinder and Friet, 1994) are only beginning to elucidate basic physiological function with development. Although our understanding of how oxygen is taken up and

distributed by the cardiovascular system is still lacking, there exists a pervasive dogma in the literature that the cardiovascular system develops to serve the increasing needs set by metabolic demand.

The first studies which attempted to clarify the functional relationship between the cardiovascular and respiratory system came from the work of Baker (1949); Darnell (1949); de Graaf (1957); and Morgan (1919) in which they described the role of the aortic arches and their possible role in gas exchange. Millard (1945), experimentally removed these arches and found that in most cases animals were unaffected. Similarly, Knower (1907) and Mellish *et al.* (1995) have shown that elimination of the heart primordia, exposure to CO, and mutant axolotls of the *Ambystoma mexicanum* are capable of growing and developing with the absences of a functional circulatory system. Flores and Frieden (1969) demonstrated that functional capacitance of the blood can be reduced with phenylhydrazine, resulting in few ill side effects on *Rana catesbieana* tadpoles. Furthermore, Hillman (1980) has demonstrated that animals made functionally anemic with phenylhydrazine, were capable of sustained exercise while maintaining O_2 uptake suggesting that the Hb may play a highly reduced role in gas exchange.

Statement of the Problem and Overall Hypotheses

It is clear from embryological data that the cardiovascular system develops and becomes functional, but what is not well understood is whether the

respiratory system is solely dependent on the formation of convective flow. The objective of this dissertation research is to determine the ontogeny of cardio-respiratory support of metabolism. This work is carried out in two stages, a descriptive approach and a chronic experimental approach, which are as follows:

1. Chapter 2 is a descriptive study which had the goal of delineating the metabolic energy stores and their potential use with development. In addition, it provides valuable information about how body composition changes may affect measures of both cardiac and respiratory function.
2. With the baseline information in Chapter 2 now available, we set out in Chapters 3, 4, and 5 to test hypotheses associated chronic rearing conditions, and how these conditions may affect gas transport. Based on this, the following hypotheses are proposed:
    - first, that the cardiovascular system in general, and hemoglobin more specifically, play a reduced role in bulk O_2 uptake with progressive development;
    - second, that the limitations to gas transport are not solely limited by diffusion or perfusion;
    - and lastly, that diffusion as a mean of O_2 uptake is viable to support aerobic metabolism through development.

By testing these hypotheses, we can then speculate on the absolute requirements of the cardiovascular system with progressive development.

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CHAPTER 2

WHOLE BODY COMPOSITION AND ITS IMPLICATIONS FOR DEVELOPMENT AND MASS IN LARVAE OF *XENOPUS LAEVIS.*

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WHOLE BODY COMPOSITION AND ITS IMPLICATIONS FOR DEVELOPMENT AND MASS IN LARVAE OF *XENOPUS LAEVIS.*

Paul R. Territo[1,2*]
Allan W. Smits[3]

Current Address
[1]National Institute of Health
Laboratory of Cardiac Energetics (OE-108)
NHLBI, Building 10, Room B1D-161
10 Center Dr. MSC 1061
Bethesda, MD 20892-1061

[2]Department of Biological Sciences
University of Nevada, Las Vegas
4505 Maryland Parkway
Las Vegas, NV 89154-4004

[3] Department of Biological Sciences
Quinnipiac College
Hamden, CT 86518
USA

Running head: Body composition and development in amphibians

* Author to whom all correspondences should be sent.

Abstract

Body composition in developing animals has been extensively investigated in fish larvae and bird embryos. However, no studies to date have attempted to determine whole animal body composition, or lean body mass (LBM), in amphibians. The present study investigates how body composition changes with development in *Xenopus laevis* , and the potential implications for substrate turn over, energy stores, and oxygen consumption. Whole animal composition was determined in a developmental range from eggs (NF stage 1) to two weeks post feeding (NF 50-51), which represented two-thirds of development. Wet and dry mass were found to be highly correlated, with water content remaining constant at 93% of wet mass. Whole animal nucleic acids were linearly correlated with both wet and dry mass, and declined relative to dry mass with progressive development. Similarly, total protein was linearly correlated with wet and dry mass; however total protein increased with developmental stage. Individual neutral lipids were stage specific with no linear dependency with either mass or development; although total neutral lipids progressively declined with developmental stage. Total bulk energy paralleled the changes seen in lipids with a sharp decline in available total gross energy by NF stage 44-45. This was matched by a similar mass specific decline in $\dot{M}_{O_2}$ over this same range. Total body composition greatly influenced LBM, and had profound effects on mass specific expression of variables such as $\dot{M}_{O_2}$, $\dot{Q}$, and SV.

Introduction

A common course of development occurs amongst a wide variety of organisms; however, one of the distinguishing characteristics in vertebrates is the amount and distribution of yolk (Browder *et al.*, 1991). This has been shown to vary widely both within, and between species, and it is this attribute which ultimately determines the embryo's ability to survive beyond deposition. In most cases, the animal transforms from a mass of cells into a fully functional organism capable of surviving autonomously, and most, if not all, of this process is fueled via degradation of the yolk. Thus, each embryo must have enough stored fuel to meet its metabolic demands as it undergoes development. Moreover, this fuel must last until the embryo is capable of obtaining extra-embryonic food stuffs to continue to meet its nutritive requirements.

The study of body composition and how it changes with progressive development has been extensively investigated in fish (for review see, Heming and Buddington, 1988) and in chicken embryos (for review see, Romanoff, 1967). However, all studies to date have utilized centrolecithalic animals who's distribution is limited to a yolk sac, or specified region, of the egg/embryo. This has allowed researchers to excise the yolk sac and assay for the contents relative to the whole animal composition. Despite the fact that techniques exist to winnow out true metabolizing tissue mass, few studies exists that have attempted to express physiological characters such as $\dot{V}_{O_2}$ and $\dot{Q}$ as a function of true metabolizing tissue. At best, most developmental studies in fish

(Rombough, 1988b), amphibians (Burggren and Just, 1992), reptiles (Birchard and Reiber, 1995; Birchard *et al.*, 1995; Deeming and Thompson, 1991), and birds (Romanoff, 1960) have ignored the mass component completely (expressing data on a per individual basis), or have chosen to express them on a per egg basis. Unfortunately, this has lead to serious over estimations in metabolizing tissue mass, when expressed on a per gram mass basis, and subsequent reductions in the variable of interest (ex. $\dot{V}_{O_2}$, $\dot{Q}$, and SV). Interestingly, studies on adult cardiovascular and respiratory physiology have expressed these quantities mass specifically for the past half a century.

Although the techniques described for fish and bird have proven useful, they are not feasible in telolecithalic animals such amphibians. Moreover, there exist no method to determine "lean" body mass in such developing organisms as *Xenopus*. This lack of attention to determining yolk-free tissue mass has prompted most developmental physiologist to simply ignore this problem. This paper has determined a method to evaluated yolk-free mass in developing telolecithalic animals, and also addressed some of the common problems encountered when expressing physiological variables in yolk laden embryos.

Materials and Methods

Experimental Animals. Fertilized eggs were obtained from the *in vivo* fertilization of six breeding adult female *Xenopus laevis,* according to Thompson and Franks

(1978). Eggs were transferred into well aerated 25 L holding tanks, where they were maintained in aged tap water at 24 ± 1°C. Larvae were fed Nasco frog brittle™ (Nasco Inc.) *ad lib.* starting at stage 45, and continued through the end of development/sampling period. All animals were maintained on a 14:10 light:dark cycle through development.

Grouping of Developmental Animals. Experiments were conducted on *Xenopus laevis* which ranged in developmental stage from eggs (NF 1) to two weeks post initiation of feeding (NF 51). Stage determination was apportioned according to staging regimes outlined by Nieuwkoop .and Faber, (1967). Animals were grouped into 13 different ontogenic categories according to major morphological and physiological landmarks. Developmental groupings are as follows: NF 1-8, 9-12, 13-18, 19-24, 25-38, 39-41, 42-43, 44-45, 45-46, 46-47, 47-48, 48-49 and 50-51. For a complete discussion regarding characteristics at these developmental stages see Nieuwkoop and Faber (1967) and Burggren and Just (1992).

Experimental Procedure. At each sampling period, individual groups of animals were removed from their holding tanks and were promptly (≤ 1 min.) flash frozen in liquid nitrogen. The frozen sample was then transferred to a storage vial and kept at -70 °C. All samples were assayed for total neutral lipids, whole animal nucleic acids, total protein, and wet mass within one month of storage.

1. Total neutral lipids (NL_{tot}): total neutral lipids were prepared by homogenizing the tissue with a Teflon® pestle in a 1.5 ml microcentrifuge tube. Each sample was diluted with exactly 3x its volume with 0.15N NaCl (for justification, see nucleic acids section below). Upon completion, the pestle was washed with 200 µl of the diluent. All samples were then lyophilized and extracted according to methods outlined by Bligh and Dyer (1959). Extracted lipids were then separated according to their polarity (Mason *et al.*, 1976). Collected neutral lipids were then dried to completeness under a stream of N_2. Samples were resuspended with 100 µl of chloroform for analysis. Identification of individual neutral lipids species were performed in triplicate, and determined via TLC according to Mangold (1969). Visualization was achieved by conjugation with acidic 2M $CuSO_4$, followed by charring for 15-30 minutes at 190 ^{0}C in a convection oven. Images of charred plates were scanned into a personal computer at 600 dpi. utilizing an HP ScanJet 3C (Hewlett Packard Inc). Densitometric analysis was performed with SigmaScan™ image analysis (Jandel Scientific Inc). Outputs were then read and integrated for concentration utilizing Datacan™ v5.1 (Sable Systems Inc). In all cases, TLC plates were run with known standards of neutral lipids (Nu-Check-Prep Inc.) ranging from 2-240mg. Neutral lipid standards included the following species: Monoolein (M), Diolein (D), Triolein (T), Methyl Oleate (MO), Oleic Acid (OA), Cholesterol Oleate (CO), and Cholesterol (C). Species of lipids without standards were identified based on R_f values according to Mangold and Malins (1960).

2. Total nucleic acids (NA_{tot}): Determination of the interaction between diluent strength and yield of nucleic acids revealed that maximum product was achieved by homogenizing tissue in 0.15N NaCl; therefore, all tissue samples were ground with this solution to ensure accuracy. Total nucleic acids were determined in triplicate fluorometrically employing Hoechst 33258, methods according to Paul and Myers (1982). Samples were excited at 350 nm, and the emission was read with a Sequoia Turner® fluorometer at 450 nm (10 nm slit). All samples were compared concurrently against bovine calf thymus standards (Sigma Chemical, St. Louis).

3. Total Protein (P_{tot}): Total protein was determined from dried samples (see mass determination) based on a modified Biuret procedure described by Watters (1978). Protein concentrations were determined against Bovine Serum Albumin (BSA) standards prepared in-house. A 200 µl aliquot, of both sample and standards, were dispensed in duplicate into a Corning® 96-well Microtiter™ plate. Samples were scanned for absorbance at 570 nm on a Dynatech®, MZ580 micro-ELISA reader. Each plate was individually blanked, and read twice. The results of the four readings were averaged and used to determine concentration.

4. Mass Determination (M): Wet mass, used for NL_{tot} and P_{tot}, was determined by wick-drying animals with a Kimwipe®, and weighing them to the nearest milligram on a Sartorius®, Model S120 B, Micro Balance. To ensure sufficient quantity of material for all assays, large numbers of animals (n=30) at each stage were pooled and total tissue mass was determined as per stated.

A separate population of animals which ranged in stage from NF 1 to 60, was used to determine empirically dry mass and water content from wet mass. All samples were dried via capillary action, weighed, and placed into aluminum planchets. Samples were dried at 90 ^{0}C for at least one week where a constant mass was achieved. Both wet and dry masses were measured on a Denver Instrument Analytical Micro Balance, model AB-300. Regressional analysis were performed, and equations describing the relationship between wet and dry mass were used to calculate dry mass for tissues used in NL_{tot} and NA_{tot}.

5. Total gross energy (TGE): Individual gross energy was estimated by multiplying the chemical composition by the standard energy equivalents for protein and lipids, 23.64 $J \cdot mg^{-1}$ and 38.91 $J \cdot mg^{-1}$ respectively (Brouwer, 1965). TGE was calculated as the sum of individual gross energies.

Statistical Analyses. Effects of development on NA_{tot}, P_{tot}, and M were determined by a first order least squares linear regression (Statistica™ v5.0, on a PC). This analysis determines 1) the regression coefficient, 2) equation of the line describing the relationship, and 3) the probability that the slope of the line is not significantly different from zero. A t-test was ran to determine if the slope of the predicted line was significantly different from zero. In all cases fiduciary level significance was takes at $P \leq 0.05$.

Results

Mass and Water Content:

Wet mass was linearly correlated with dry mass over a range from 2.1 to 695.4 mg (Fig. 1). These data are described by the equation of the line $M_d = M_w(0.07)+0.03$ ($r^2=0.99$) where M_w and M_d represent wet and dry mass respectively. Results of regression analysis are provided in (Table 1).

Total wet tissue mass used for NL_{tot} and NA_{tot} ranged from 49.1 to 323.4 mg from NF 1 to 51. Dry mass over this same stage range increased by 6.5 fold, and ranged from 3.5 to 22.7 mg (Table 2). Water content was constant at 92.9% with progressive development up to NF stage 50-51.

Chemical Composition with Development:

Nucleic acids, like dry mass, showed a strong linear correlation ($r^2=0.99$, $p\leq0.001$) with both wet and dry mass (Table 1). NA_{tot} ranged from a low of 1.35 to a high of 7.7 ng over a range of dry mass from 1.2 to 22.8 mg (17.0 to 324.7 wet mass) (Fig. 2), and was described by the allometric equation $NA_{tot}=0.12M_d^{0.58}$ (Table 1). Analysis of percent body composition indicated that relative to dry mass, NA_{tot} decreased over the range from NF 1 to 51. This overall decrease was not simply a linear decline from eggs (NF 1-8) to pre-metamorphic climax (NF 51); instead it showed a 1.2 fold increase between NF 1 and 18. Similarly this trend was observed between NF 19 and 38, and 42 to 45 where it increased

by up to 2 times from the stages which preceded them (Table 2). Between NF 45 and 51 total nucleic acids showed a consistent decline, achieving a post-absorptive value of $3.63x10^{-5}$ %.

Protein, like NA_{tot}, showed a strong correlation (r^2=0.85, $p \leq 0.001$) with both wet and dry mass, and was described by the equation $P_{tot}=-0.08M_d^{0.64}$. Total protein increased 2.47 mg between 0.43 mg and 49.07 mg dry mass (2.03 to 695.4 mg wet mass) (Fig. 3). Analysis of body composition overall reveled P_{tot} increased progressively with development between NF 1 and 51. However, P_{tot} did not show a dramatic change relative to dry mass between NF 1 and 18. By contrast, total protein fell between NF 19 to 38. P_{tot} over the next 8 stage ranges (NF 39 and 51) showed a doubling in relative composition with a final value of 60.60%.

Individual neutral lipids, unlike NA_{tot} and P_{tot}, did not show a consistent linear relationship with either wet or dry mass. Stage-specific analysis indicated that individual lipid classes were highly episodic with progressive development.

Triolein (T) showed a consistent decline with progressive development, ranging from a high of 20.44 $\pm$ 1.2 at NF 9-12, to a low of 0.60 $\pm$ 0.2 $\mu g \cdot mg^{-1}$ at 46-47, where it was maintained through NF 51 (Fig 4A). Diolein (D), like T showed a consistent decreasing trend between NF 1 and 13, where it increased by 1.5 times to a new value of 237.34 $\pm$ 1.44 $\mu g \cdot mg^{-1}$ (Fig. 4C). Between stages NF 19 and 51 D showed an almost linear decrease with each successive stage, where it reached a final value of 28.44 $\pm$ 1.11. Monoolein (M) by contrast, was

constant at 1.2 $\mu g \cdot mg^{-1}$ between stages 1 to 25 where it increased sharply to a maximal value of 10.89 $\pm$ 1.73 at NF 45-46 (Fig. 4A). Over the next four stage ranges M decreased in a linear manor to a final value of 0.16 $\pm$ 0.01 $\mu g \cdot mg^{-1}$. Of all the individual lipid classes, oleic acid (OA) showed the most variability with development. Between NF 19 to 38, and 42 to 45, OA showed no change; conversely it showed a clear reduction between NF stages 1 to 12, 13 to 24, 39 to 43, 45-47, and 48-51 (Fig. 4B).

In stark contrast to the general trends seen in OA, methyl oleate (MO) showed very little change through most of development. Moreover, the only major deviation from the baseline value of 0.40 $\pm$ 0.04 $\mu g \cdot mg^{-1}$ was the sharp increase seen between NF 45 and 48 where it reach a high of 29.16 $\pm$ 0.94 $\mu g \cdot mg^{-1}$ (Fig. 4B). Like M, cholesteryl oleate (CO), showed an initial increase of 1.3 fold from NF 1 to 12, where it began a consistent decline in concentration, and ranged from 286.91 $\pm$ 30.61 to 19.10 $\pm$ 0.1 $\mu g \cdot mg^{-1}$. Cholesterol (C) displayed a fairly constant level through most of development (NF 1 to 45). However, between NF stages 44 and 51, C showed a consistent decline in concentration where it reached a final value of 22.21 $\pm$ 2.27 $\mu g \cdot mg^{-1}$. Diakylmonoacylglycerol (DAG), like MO, showed little activity and was found only at NF stages 13-18, 25-38, and 50-51. In the stages which retained DAG, the concentration never exceed 6.21 $\pm$ 0.14 $\mu g \cdot mg^{-1}$ (Fig. 4D).

Sterols (S) showed a constant level of production around 15 $\mu g \cdot mg^{-1}$ from NF 1 to 38, where they fell by 60% to a value of 7.52 $\pm$ 1.09 $\mu g \cdot mg^{-1}$. S increased

to a maximum of 42.42 ± 2.01 $\mu g \cdot mg^{-1}$ by stage 44-45, and then dropped to an average value of 0.9 $\mu g \cdot mg^{-1}$ through NF 50-51. Similarly, long chain alcohols (LCA), showed a parallel trend to S, with concentrations remaining constant around 30 $\mu g \cdot mg^{-1}$ from NF 1 to 18. Between NF 13 and 43, LCA showed a biphasic increase with the first plateau at NF 25-38 (53.23 ± 1.76) and 42-43 where it reached a maximum of 84.61 ± 5.94 $\mu g \cdot mg^{-1}$ (Fig. 4D). Over the next six stage ranges, LCA fell by 83 $\mu g \cdot mg^{-1}$, where it reached a final value of 0.35 ± 0.03 $\mu g \cdot mg^{-1}$.

Energy content and Metabolism:

Estimations of stage-specific gross energy from lipids and protein are presented in Table 3. Gross energy available from lipids showed a clear decreasing trend with development, and ranged from 23.82 to 2.79 $J \cdot mg^{-1}$. Conversely, the available energy based on proteins increased with progressive development. This inverse relationship between lipid and total protein, however, did not result in a constant level of TGE. In fact, mass specific TGE showed a decreasing trend with progressive development (31.19 to 17.12 $J \cdot mg^{-1}$) illustrating the clear mismatch between energy pools as development progresses.

The relationship between total energy available, TGE, and aerobic metabolism, $\dot{M}_{O_2}$, (from Territo, 1996), as illustrated by Fig. 5, shows the clear differential between stored resources and those required for normal

maintenance and growth. Clearly, TGE is in excess for most of development up to NF 45-46, where it takes a downward deflection indicating resources depletion. This reduction in TGE was concomitant with the onset of feeding, and was largely due to changes in lipid gross energy (LGE). $\dot{M}_{O_2}$ in the other hand showed a clear increase in energy consumed, where it peaks at a maximal rate of 0.0016 $\pm$ 0.0002 $J \cdot mg^{-1} \cdot hr^{-1}$. This peak in $\dot{M}_{O_2}$ was followed by a steady decline over the next seven stage ranges (NF 42 to 51). These data show a clear synchronization of resource depletion (LGE) and aerobic metabolism.

Discussion

Mass and Water Content:

Water content, and therefore the functional relationship between wet and dry mass, has important physiological, computational, and heuristic value. It is clear from work on adult and larval amphibians the water balance affects gas exchange, osmoregulation, and pH regulation (Boutilier *et al.*, 1992; Burggren and Just, 1992). Moreover when this is considered in a purely developmental context, it becomes evident that these processes change radically from one of cellular to whole animal function and that they occurred over a span of time from hours to weeks.

Based on this rational, we determined the relative contribution of water content to dry mass. The relationship between wet and dry mass (Fig. 1) was clearly linear, and similar to data reported in the literature for this species

(Feder, 1981). Water content of *Xenopus* larvae from NF 1 through 51 were constant at approximately 93%, which is in general agreement with the work of Burggren and Just (1992) and Leist (1970) in which they report similar values through NF 57. Clearly, these data suggest that embryos and larvae defend water balance through development in spite of a dramatic changes in body composition. These changes would in fact facilitate a net inward flux due to increases in colloid osmotic pressure associated with increases in muscle mass.

Chemical Composition with Development:

Chemical composition in developing animals has been investigated in a large number of vertebrates, although most work in amphibians have been primarily interested in structural components rather than discrete chemical composition (Deuchar, 1966; Jared *et al.*, 1973; Karasaki, 1963; Wallace, 1963; Wallace, 1965). Thus, we chose to evaluate whole body composition of developing *Xenopus*, thereby allowing for the first time the determination of how energy is partitioned and is utilized with progressive development.

Total nucleic acids showed a clear allometric relationship with mass (development) (Table 1). These data are similar to those seen in larval *Ambystoma tigrinum*, in which DNA was linearly correlated with wet mass (A.W. Smits, unpublished data). Although there exist few studies which have correlated total nucleic acid content with wet or dry mass, our data are in the general range reported in developing fish (Zeitoun *et al.*, 1977). The fact that the percent of

NA_{tot} showed periods of increase (NF 1 to 18, 19 to 38, and 42 to 45) amongst the general declining trend, suggests that specific windows of hyper-activity were occurring concurrent with morphological changes. Inferential support for this premise comes from correlates of morphological changes such as: gastrulation, axis formation, gill formation, alimentary tract formation, and axial musculature formation (Nieuwkoop and Faber, 1967). Clearly, these data suggest that the process of development is not simply adding new cells and structures associated with them, but is a highly coordinated process.

Protein, like NA_{tot}, was positively correlated with dry and wet mass and development. In developing fish larvae reported levels of embryonic proteins ranged from 56 to 67% of dry mass (Dabrowski *et al.*, 1984; Dabrowski and Luczynski, 1984; Smith, 1957; Wang *et al.*, 1987; Zeitoun *et al.*, 1977). Although these are similar to our findings in *Xenopus* between NF 47 and 51, it is unclear from the studies in fish how P_{tot} changes in early development, as most of these measurements in fish were taken post hatch. Additionally, *Xenopus*, unlike meroblastic fish, lack a discrete yolk sac, and therefore may also lack the quantity and distribution of yolk proteins associated with stabilizing neutral lipids. It is clear, however, from the percent body composition data (Table 2) that P_{tot} increases with progressive development. Interestingly, there is a slight decline in total protein between NF 18 and 41, which corresponds well with the dramatic rise in $\dot{M}_{O_2}$ seen over this same range. P_{tot} over the last third of development shows a clear rise which is correlated with the start of free swimming and

ingestion, suggesting that both intrinsic and extrinsic food stuffs were being directed into producing muscle mass.

Lipid composition through development showed little change up to NF 39-41 where it decreased in a fairly linear fashion. By mid-yolk absorption (NF 42-43) body composition reached a value nearing 50% neutral lipids. This was consistent with the work of Eldridge *et al.* (1981) who showed that bulk yolk lipid comprised 52% of dry mass in the common carp *Cyprinus carpio*. Although, our data was similar to that of Eldridge *et al.* (1981), our data was significantly higher than those reported for trout (*Salmo gairderni*), Atlantic salmon (*Salmo salar*), and white sturgeon (*Ancipenser transmontanus*) larvae (Hamor and Garside, 1977; Wang *et al.*, 1987; Zeitoun *et al.*, 1977). The possible reason for this difference could be due to reproductive differences. *Xenopus* routinely lays large numbers of eggs which hatch at 1 day post-fertilization and completely absorb all yolk by 12 days at 25 ^{0}C. In contrast, *Salmo gairderni* hatches at 20 days post-fertilization (Rombough, 1988a) and consumes all yolk by day 45 at 15 ^{0}C. If one considers an average Q_{10} of 2 for trout, it is evident that *Xenopus* is clearly consuming more energy in a much shorter time. Thus, large amount of neutral lipids seen in *Xenopus*, and the dramatic reduction seen by stage NF 51 is highly suggestive that amphibians, unlike teleosts, utilize a different strategy through development, and that lipids are fueling most of metabolism through NF 50-51.

Energy Content and Metabolism:

Total energy content, which is comprised of the bulk energy from lipids and proteins, is an important competent of total energy turnover in any organism. TGE showed a consistent declining trend with progressive development. The majority of the drop was due to a rapid decline in NL_{tot}, while P_{tot} slowly increased with development. Interestingly, both the energy content associated with lipids and $\dot{M}_{O_2}$, both expressed mass specifically, fell precipitously from NF 45 through 51, suggesting that total metabolism was being fueled by lipids over this range (Fig. 5). Conversely, total protein energy increased by more than 50% over this same stage range, further suggesting that resources distribution was being put into muscle mass (Table 2). These data are in general agreement with the observations of Burggren and Just (1992), in which they describe anuran larvae hatching with large quantities of yolk, and abstaining from feeding for several days post-hatch while lipid reserves are depleted.

Overall, these data indicate that resource depletion and energy stores are in delicate balance, and unlike most teleost fish larvae, utilize lipids as their main energy source to fuel aerobic metabolism.

Lean body mass and its implications for expression:

The study of development and the physiological processes that underlie it have been actively investigated for more than a century. Over this period, few studies have attempted to deal with the expression of lean body mass (LBM) in

amphibians. Extensive work in fish larvae (Kamler and Kato, 1983; Lapin and Matsuk, 1979; Lasker, 1962; Moroz and Luzhin, 1976; Rombough, 1988a) have investigated yolk utilization, from which an index of LBM can be gleaned. Although a fairly comprehensive data set exists for fish larvae, we are only aware of one paper by Rombough (1988a) that has directly attempted to compensate for over-estimates associated with yolk mass when considering $\dot{M}_{O_2}$. When this is considered in the context of developing amphibians, there is a clear chasm in our knowledge of how body composition changes with progressive stage, and therefore how this may affect LBM. Consequently, work performed on developing amphibians has largely ignored this problem, yielding to the assumption that wet, or in some cases, dry mass was a true index of metabolizing mass. Clearly, our work suggests that this is not the case. In fact observation that amphibians retain large quantities of yolky material (see Burggren and Just, 1992) coupled with our present findings suggest that LBM may in fact be overestimated by as much as 50% or more over the first half of development. The obvious consequences on the expressing measured variables ($\dot{M}_{O_2}$, $\dot{Q}$, SV, etc.) mass specifically may be the serious underestimate of these variables, as a consequence of over-estimating LBM.

Over the past century measurements of O_2 consumption have revealed that $\dot{M}_{O_2}$ increases sharply over the first third of development , which is counter to standard allometric paradigms. Atlas (1938), Lovtrup (1959), Lovtrup and Werdinius, (1957), and Wills (1936) have all commented on the usual shape of

respiratory curves, attributing them to plotting differences (Lovtrup, 1959), and in some cases have implicated lipids directly (Atlas, 1938; Wills, 1936). Interestingly, in spite of their observations, both of these authors choose not to address this problem directly. However, Wills (1936) did express $\dot{V}_{O_2}$ per mg of nitrogen (N), which was believed to be a better index of LBM. Our work has shown that proteins are in dynamic flux through development, and hence expressions of $\dot{V}_{O_2}$ per mg of N, would in fact misrepresent LMB. Evaluation of Wills (1936) plots clearly reveals that this in fact did not afford any greater accuracy, and in fact may have exacerbated the shapes of the curves.

It is clear from the present study that expression of LBM needs to be considered when evaluating variables through development, and that the majority of the correction must come from lipids. It should be noted that the $\dot{M}_{O_2}$ data presented in this paper was corrected for lean body mass based on percent body composition, and in fact represents trends associated with increasing development.

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Figure 1. Wet mass vs. dry mass in developing *Xenopus laevis* larvae. The linear function of dry mass was plotted against the linear dimension of wet mass. All data were fitted with a first order least squares linear regression and the results are presented in Table 1.

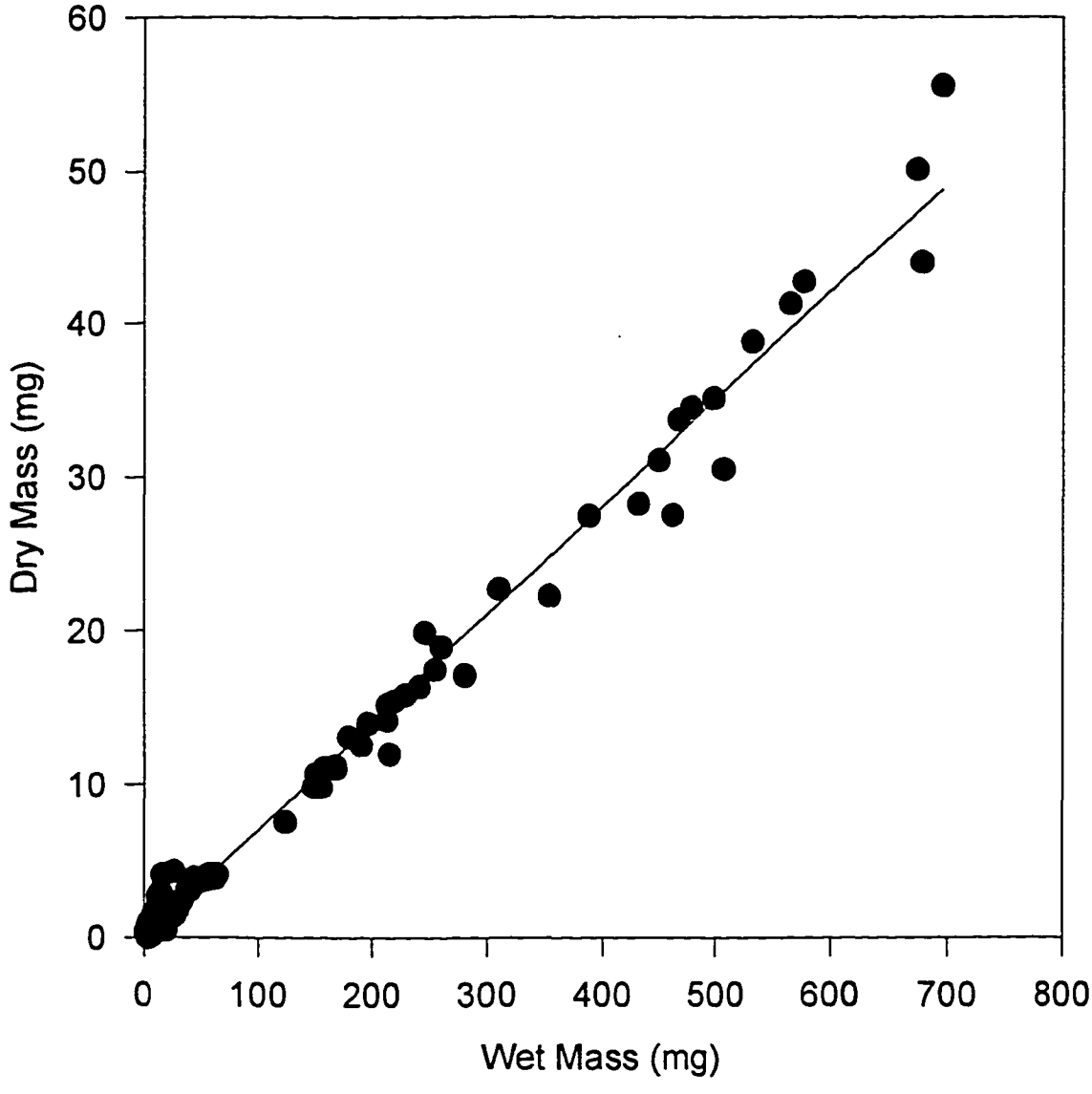
Figure 2. Total nucleic acids vs. wet and dry mass in developing *Xenopus laevis* larvae. Data were plotted as the $\log_{10}$ of total nucleic acids against the $\log_{10}$ of both wet and dry mass. Regressional analysis indicating allometric trends are provided in Table 1.

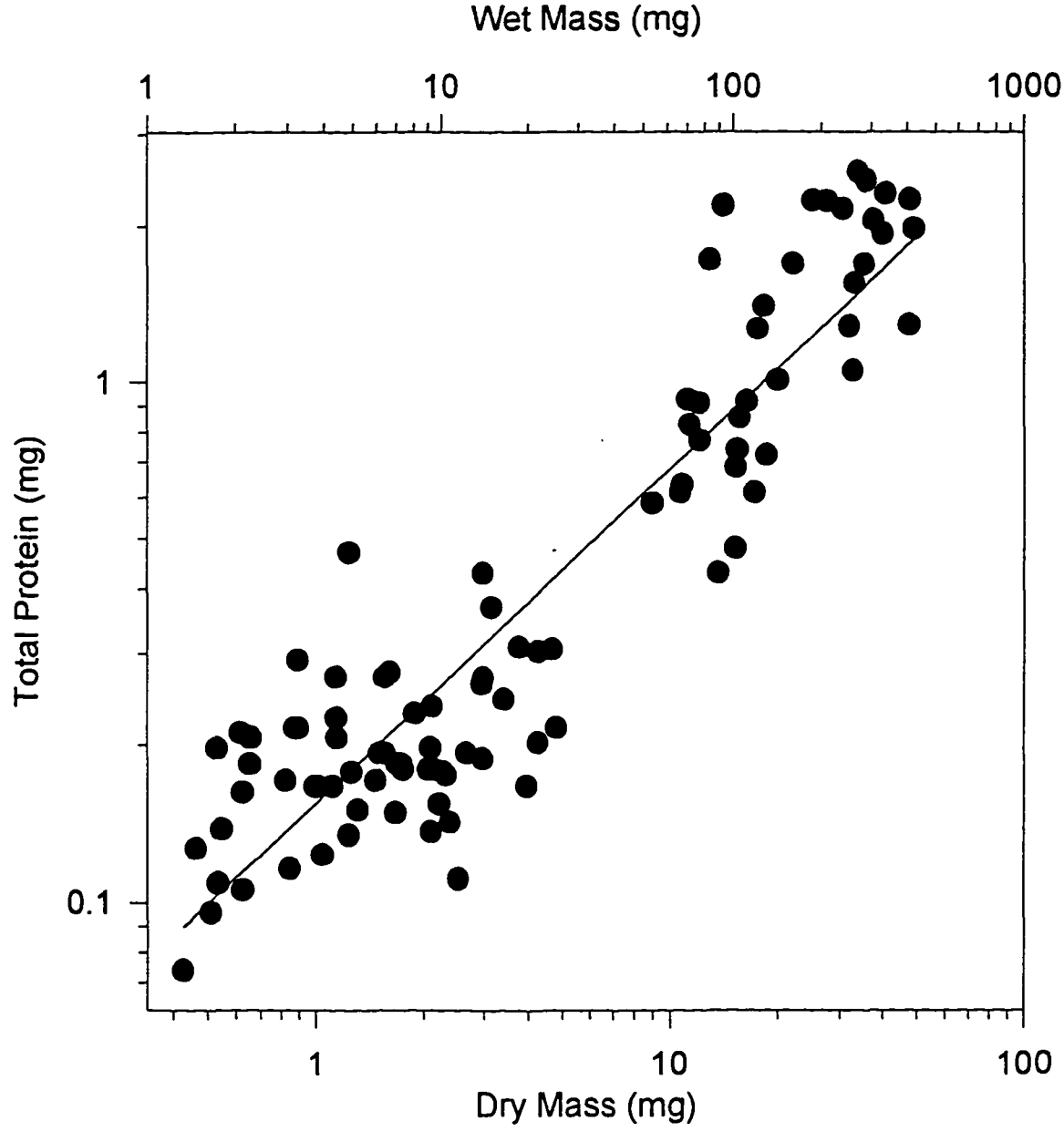
Figure 3. Total protein vs. wet and dry mass in the larvae of *Xenopus laevis* . Data were plotted as the $\log_{10}$ of total protein against the $\log_{10}$ of both wet and dry mass. Results from first order linear regression indicating allometric trends can be found in Table 1.

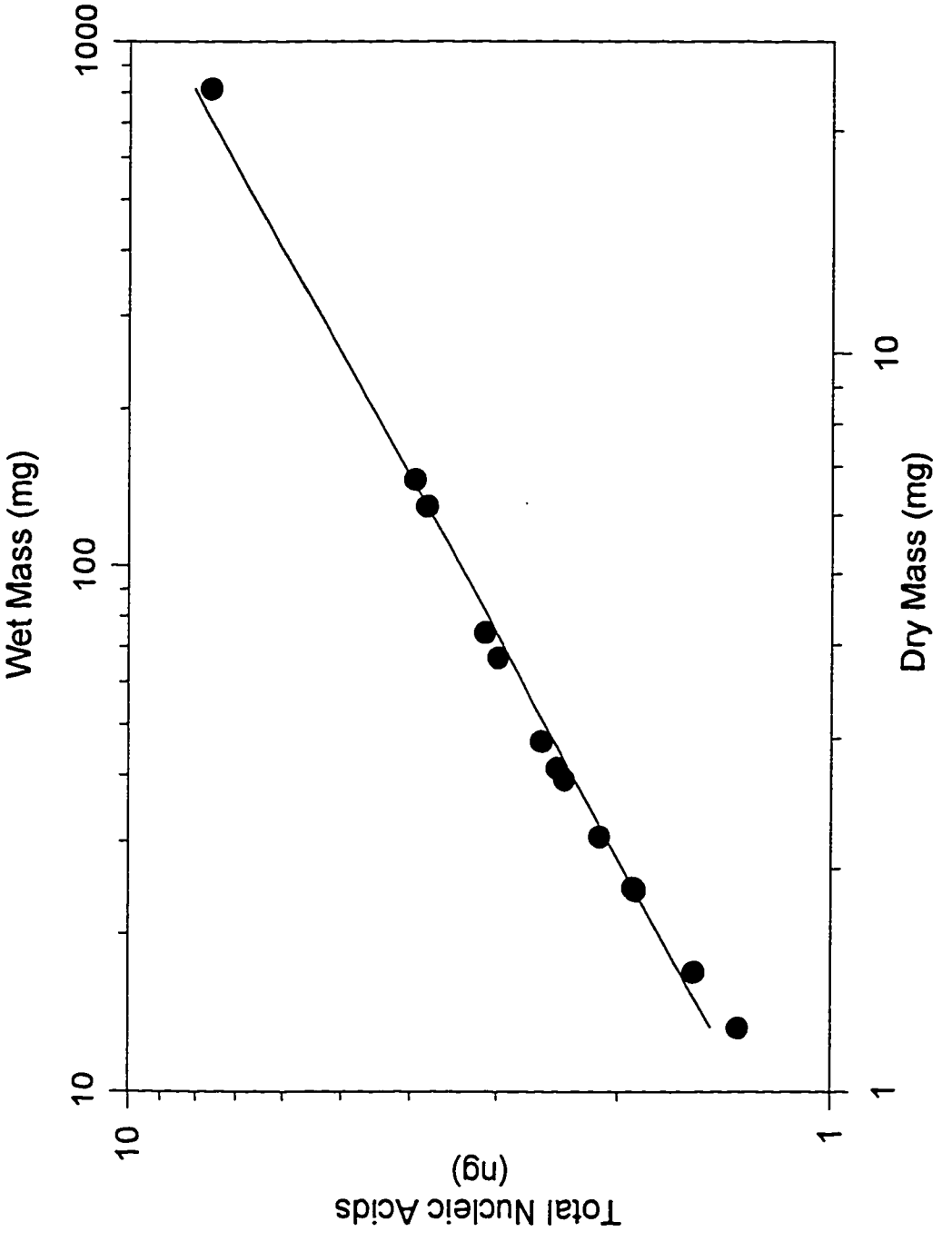
Figure 4. Lipid composition in the developing larvae of *Xenopus laevis* . The linear function of individual lipid classes were plotted against the linear dimension of development (NF stage ranges). Panel "A" shows monoolein (open circles) and triolein (filled squares); while panel "B" indicates methyl oleate (open triangles) and oleic acid (filled diamonds). Panel "C" shows cholesterol (open circles), cholestryl oleate (open squares), and diolein (filled triangles); whereas, panel "D" displays sterols (filled triangles), long chain

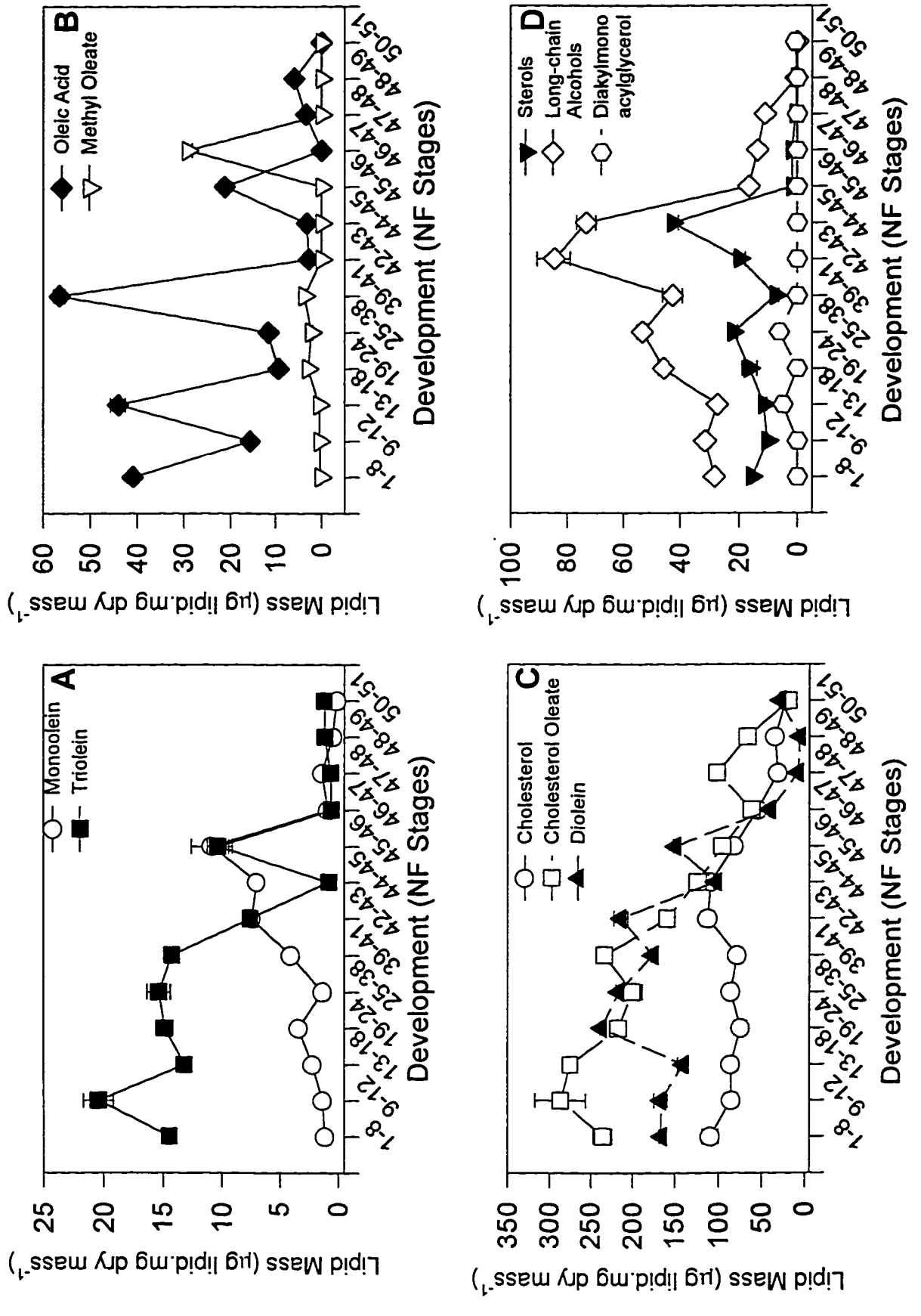
alcohols (open diamonds), and dialkylmonoacylglycerol (open hexagons) all plotted with development.

Figure 5. Oxygen consumption ($\dot{M}_{O_2}$), lipid gross energy (LGE), and total gross energy (TGE) with development in the larvae of *Xenopus laevis* . The linear function of both $\dot{M}_{O_2}$ (filled symbols), LGE (filled bars), and TGE (open bars) was plotted against the linear dimension of development (NF stage ranges). Hatched area indicates the period of feeding relative to developmental stage. All $\dot{M}_{O_2}$ data were corrected for lean body mass based on body compositions presented in Table 2.









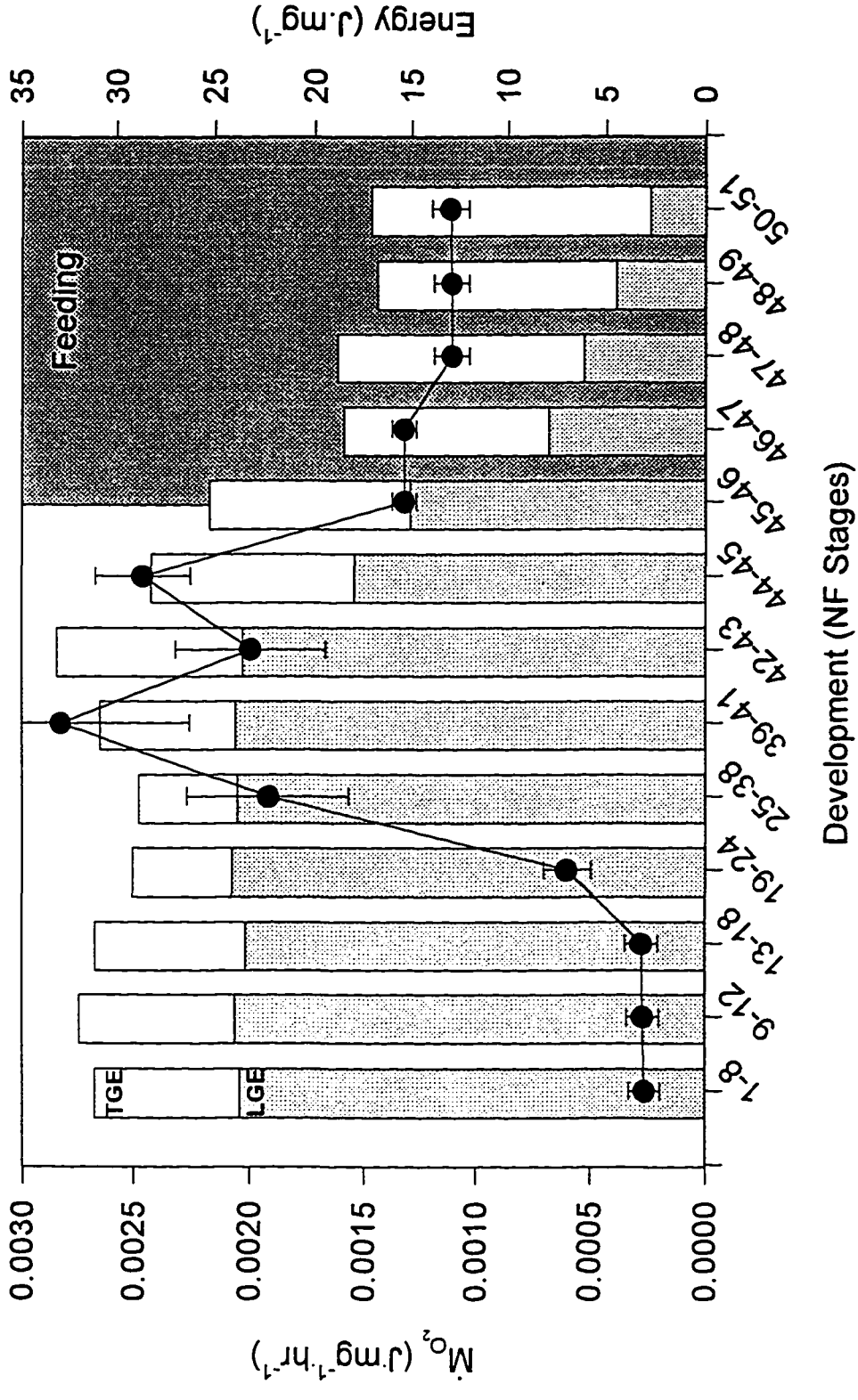


Table 1. Regression analysis of total protein, and TNA with dry mass, and dry mass with wet mass in developing *Xenopus laevis* .

Stage Range	*y*	*n*	*a*	*b*	r^2	*p*
1-60	M_d	98	0.028	0.070	0.99	≤0.001
1-60	Protein	96	-0.081	0.642	0.85	≤0.001
1-50	TNA	13	0.119	0.581	0.99	≤0.001

Relationships for protein and TNA expressed as log y = $\log_{10}$ a +b· $\log_{10}$ M_d, where y is in mg and ng respectively. The relationship between wet and dry mass is describe the by the equation y=a+b·x, such that M_d and M_w are in mg. n, r and p, are sample size, correlation coefficient, and level of significance respectively.

Table 2. Body composition* and water content with development.

	Water Content			***Percentage of Dry Mass***			
Development NF Stage	*Wet Mass (mg)*	*Dry Mass (mg)*	*Total Body Water (%)*	*Neutral Lipids*	*Nucleic Acids ($x10^{-4}$)*	*Protein*	*Other*
1-8	49.10	3.47	92.93	61.22	1.64	31.18	7.60
9-12	41.85	2.96	92.92	61.87	1.74	33.46	4.67
13-18	50.25	3.55	92.93	60.50	1.91	32.30	7.20
19-24	35.40	2.51	92.90	62.20	1.27	21.25	16.55
25-38	37.10	2.63	92.91	61.54	1.36	20.93	17.53
39-41	32.55	2.31	92.90	61.73	0.10	29.13	9.14
42-43	47.70	3.37	92.93	50.49	0.06	40.16	9.35
44-45	55.40	3.91	92.93	46.21	1.26	43.47	10.31
45-46	54.20	3.83	92.93	38.83	0.09	43.02	18.15
46-47	60.00	4.24	92.94	20.25	0.06	45.05	34.70
47-48	110.45	7.78	92.96	15.78	0.05	53.75	30.47
48-49	95.60	6.74	92.95	11.49	0.66	52.14	30.47
50-51	323.40	22.72	92.98	7.18	0.04	60.60	32.23

*Body composition percentages are expressed with respect to dry mass, and were carried through two weeks post initiation of feeding (NF 50-51).

Table 3. Lipid, protein and total gross energy with development in *Xenopus laevis*.

Development NF Stage	*Lipid Energy ($J \cdot mg^{-1}$)*	*Protein Energy ($J \cdot mg^{-1}$)*	*Total Gross Energy ($J \cdot mg^{-1}$)*
1-8	23.82	7.37	31.19
9-12	24.07	7.91	31.98
13-18	23.54	7.64	31.17
19-24	24.20	5.02	29.23
25-38	23.94	4.95	28.89
39-41	24.02	6.89	30.90
42-43	23.68	9.49	33.17
44-45	17.98	10.28	28.26
45-46	15.11	10.17	25.28
46-47	7.88	10.65	18.53
47-48	6.14	12.71	18.85
48-49	4.47	12.33	16.80
50-51	2.79	14.32	17.12

CHAPTER 3

CARDIO-RESPIRATORY ONTONGENY DURING CHRONIC CARBON MONOXIDE INDUCED HYPOXEMIA, IN THE CLAWED TOAD *XENOPUS LAEVIS*.

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CARDIO-RESPIRATORY ONTONGENY DURING CHRONIC CARBON MONOXIDE INDUCED HYPOXEMIA, IN THE CLAWED TOAD *XENOPUS LAEVIS*.

Paul R. Territo[1,2*]
Warren W. Burggren[2]

Current Address
[1]National Institute of Health
Laboratory of Cardiac Energetics (OE-108)
NHLBI, Building 10, Room B1d-161
10 Center Dr. MSC 1061
Bethesda, MD 20892-1061

[2]Department of Biological Sciences
University of Nevada, Las Vegas
4505 Maryland Parkway
Las Vegas, NV 89154-4004

Running head: Cardio-respiratory function with induced hypoxemia

* Author to whom all correspondences should be sent.

Abstract

O_2 transport by hemoglobin (Hb) can be functionally ablated with carbon monoxide (CO), generating hypoxemia. The present study investigates the ontogeny of cardio-respiratory physiology with obstructed O_2 transport in *Xenopus laevis*. Animals were raised from eggs (NF stage 1) to NF stage 63 (metamorphic climax), while maintained in either chronic 2 kPa CO, or air. Whole animal oxygen consumption ($\dot{M}_{O_2}$), whole body lactate, individual mass, heart rate (f_h), and stroke volume (SV) were measured. Additionally, whole animal cardiac output ($\dot{Q}$), and $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratios, were also calculated to determine work loads imparted by limitations to O_2 transport. $\dot{M}_{O_2}$, whole body lactate, mass, and f_h were not significantly different between controls and CO exposed animals. However, CO exposed animals showed a significant ($p<0.05$) increase in SV, $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ and $\dot{Q}$ when compared to controls. These results indicate that limited blood O_2 transport is not deleterious to metabolism and development as a whole, and convective oxygen transport via Hb is not essential for normal cardiovascular or respiratory function through development. Furthermore, our data suggests that the onset of convective blood flow occurs prior to the need for convective vs. diffusive transport.

Introduction

The heart is the first functioning organ in the vertebrate embryo (Burggren and Keller, 1997; Gilbert, 1990). Concomitant with heart development is the formation of blood elements and hemoglobin (Hb). The early convection of these newly formed elements has lead to speculations on their importance to gas exchange (Adolph, 1979.; Boell *et al.*, 1963; Burggren and Just, 1992; Burggren and Pinder, 1991; Burggren and Territo, 1995). The assumption that blood convection is critical to gas exchange has gained circumstantial support from morphological observations that gill differentiation and blood flow both in the gills and the caudal arteries occurs immediately after the heart beats (Ballard, 1968; Medvedev, 1937; Nieuwkoop and Faber, 1967; Taylor and Kollros, 1946). Furthermore, it has been suggested that these changes in circulation may contribute to the sharp initial rise in $\dot{V}_{O_2}$ during early development (Romanoff, 1960).

The synchronous appearance of blood convection and the need for convection to supplant diffusive O_2 delivery between environment and tissues has been termed "synchronotropy" (Burggren and Territo, 1995). Although the link between Hb convection and early embryonic/larval O_2 consumption has become dogma, there exists no data which have explicitly tested these (synchronotropy) ideas. However, we recently have generated an opposing hypothesis that specifically addresses these assumptions (Burggren and Territo, 1995). This alternative hypothesis, termed "prosynchronotropy", argues that the

cardiovascular system begins to generate convective blood flow well before the absolute need for internal O_2 convection of oxygenated blood. Preliminary results have begun to clarify our understanding of when convective O_2 transport becomes necessary in embryos and larvae (Mellish *et al.*, 1995; Pelster and Burggren, 1996), but these studies lack both complete metabolic and cardiovascular profiles through development and the interplay between these two systems.

The purpose of this study was to evaluate the validity of "prosynchronotropy" utilizing embryos of the clawed frog *Xenopus laevis* . This particular species is well suited to test these hypotheses because it has a well known developmental sequence, has recently become the world standard for developmental studies; lays large numbers of eggs in a laboratory setting; has embryos that are free living and transparent; and finally, has a large base of descriptive physiology and morphology that can serve as a basis for formulating more detailed mechanistic questions. Our approach was to elucidate the dependence of O_2 uptake on convective blood flow in larval *Xenopus*. First, we have evaluated the relative contributions of aerobic and anaerobic metabolism. Second, we have measured cardiac performance (heart rate, stroke volume, cardiac output) to determine the role of blood flow in supporting aerobic metabolism. Lastly, we have determined the relative coupling of cardiac function to oxygen uptake. This was achieved in larvae by functionally ablating Hb through chronic exposure to 2 kPa carbon monoxide (CO). Disruption of Hb with

CO has been used by Holeton (1971b), and Pelster and Burggren (1996) in larval fish and in embryonic chickens (Cirotto and Arangi, 1989). However, these studies used acute rather than chronic exposure. By actually rearing larvae in CO, we can determine what role the cardiovascular system plays in oxygen uptake and its distribution. Furthermore, we can access the inevitable and changing role of Hb in bulk O_2 as development progresses. Lastly, we can determine whether the cardiovascular system forms prior to the requirements for convective O_2 transport.

Materials and Methods

Experimental Animals. Fertilized eggs were obtained in our laboratory from the breeding of four adult female *Xenopus laevis,* according to Thompson and Franks (1978). Newly laid eggs were equally divided into two 25 L holding tanks, where they were maintained in dechlorinated water at 24 C $\pm$ 0.2.

Experimental Conditions. Holding tanks for rearing larvae were aerated with one of two gas mixtures: 21 kPa O_2/ 79 kPa N_2 (control) or 2 kPa CO/ 21 kPa O_2/ 77 kPa N_2 . Carbon monoxide (CO) gas mixtures were generated with a Cameron GF-4 gas mixing flowmeter. Larvae in these tanks were fed Nasco frog brittle™ (Nasco Inc.) *ad lib.* during the course of development, and fasted 24 hours prior to measurements. All animals were maintained on a 14:10 light: dark cycle through development.

Grouping of Developmental Stages. Animals were staged according to the NF staging system (Nieuwkoop and Faber, 1967). Experiments were conducted on *X. laevis* ranging from eggs (NF 1) to metamorphic climax (NF 63). Animals were grouped into 11 different developmental categories according to major morphological and physiological landmarks. Developmental groupings were as follows: NF 1-21, 22-30, 31-33/34, 35-36, 37-41, 42-45, 46-47, 48-49, 50-51, 52-54, and 55-63. For a complete discussion regarding characteristics at these developmental stages see Nieuwkoop and Faber (1967) and Burggren and Just (1992).

Experimental Procedures. Animals drawn at random from each condition were sampled for oxygen consumption ($\dot{M}_{O_2}$), whole body lactate, wet mass, heart rate, total ventricular volume at end diastole and end systole, and stroke volume. Cardiac output, and the ratio of O_2 consumption to O_2 transport by the blood ($\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$) were also calculated. An additional group of recently metamorphosed juveniles were sampled for O_2 content in whole blood and plasma during CO exposure.

1. Total Oxygen Consumption ($\dot{M}_{O_2}$): Aerial and aquatic O_2 consumption in larval *Xenopus* were determined by closed system respirometry described in detail in Hastings and Burggren (1995). Briefly, the respirometer consisted of a 20 ml vial filled with dechlorinated tap water, and sealed with an aluminum cap

and double Viton® O-rings. Five ml of air were introduced into the chamber, displacing water. Each respirometer was attached to the reservoir and pumping apparatus and allowed to equilibrate with the gas mixture for 2 hours prior to closure of the system. During this period the chambers were continually bubbled with the gas mixture which corresponded to the rearing conditions. All experiments were carried out with additional blank respirometers (minus animals) to account for any bacterial O_2 consumption in the respirometers. The total gas volume of the system was 7 ml. Each respirometer was thermostatted at 24 ^{0}C. All chambers were placed in a dark enclosure to minimized visual disturbances to the animals. The length of the measurement period varied based on NF stage and number of animals required to achieve approximately 3 kPa drop in O_2. In most cases, animals were placed into respirometers at approximately 10 am (PST) and were sampled for periods which extended through one diel cycle. Upon closure of the system, and at the conclusion of the experimental period, a 1 ml sample of gas was analyzed for O_2 with an Ametek™ S3A/I Oxygen Analyzer. Gas samples were injected into a three-way stopcock fitted on the analyzer inlet, which was also fitted with a one meter length of polyethylene tubing (PE-90, Beckton-Dickson Inc.). The injected gas sample was first diverted into the PE tubing and then drawn at atmospheric pressure back into the Ametek™ pump through a micro Drierite® column at a rate of 20 ml·min^{-1}.

$\dot{M}_{O_2}$ in the respirometer was calculated as follows:

$$\dot{M}_{O_2} = \frac{(\%O_{2_i} - \%O_{2_f}) \cdot (P_b - P_{H_2O}) \cdot \beta_a \cdot V_g}{100 \cdot M_w \cdot T} \qquad (1)$$

where,

$\%O_{2_i}$ and $\%O_{2_f}$ are the initial and final percent O_2 in the respirometer, as read by the Ametek oxygen analyzer.

P_b is the barometric pressure in kPa,

P_{H_2O} is the water vapor at given temperature in ^0C,

β_a is the capacitance of O_2 in air in $nmol \cdot L^{-1} \cdot kPa^{-1}$,

V_g is the volume of gas in the aerial fraction in L,

M_w is the animal wet mass in mg,

and T is the time in hours.

2. Whole Body Lactate Analysis: Whole body lactate was determined on each animal sampled for $\dot{M}_{O_2}$. Upon completion of the experiment larvae were removed from their respirometers and promptly ($\leq$ 5 min.) flash frozen in liquid nitrogen. The frozen larvae were then transferred to a storage vial and kept at -70 ^0C. All samples were assayed for lactate within two months of cold storage. Animals were ground in glass tissue grinders with a 5:1 dilution of 8% perchloric acid : 1g animal wet mass. Assays for whole body lactate concentration were performed using kit number 826-B, Sigma Chemical CO. USA, and were read on a Sequoia-Turner Spectrophotometer at 340 nm.

3. Mass Determination: Individual wet mass for $\dot{M}_{O_2}$ and whole body lactate was determined by first wick-drying animals with a Kimwipe®, and then weighing them to the nearest 10th of a milligram on a Denver Instrument Analytical Micro Balance, model AB-300. In most cases, several animals were

pooled for $\dot{M}_{O_2}$ and lactate analysis. Individual mass' were determined by averaging over the total mass.

4. Heart rate (f_h), stroke volumes (SV), and cardiac output ($\dot{Q}$): Animals were sampled as per $\dot{M}_{O_2}$. Each animal was videotaped for 30 seconds (120 frames·sec^{-1}) using a Zeiss® M3Z microscope fitted with a video camera. Output from the camera was recorded on a Panasonic® sVHS video tape recorder and stored for later analysis (for review see Burggren and Fritsche, 1995). f_h was obtained by the number of beats in a given time and SV were determined based on the difference between total ventricular volumes at end diastole and end systole, and were calculated using Optimas® software (BioScan Inc.). These volumes were estimated by calculating rotational volumes based on a formula for a prolate spheroid ($V = \frac{4}{3} \cdot \pi^{-0.5} \cdot A^{1.5}$) (Burggren and Fritsche, 1995; Hou and Burggren, 1995a). Individual cardiac outputs were determined by the product of f_h and SV.

5. O_2 consumption/transport quotient: An index of the relationship between aerobic metabolism ($\dot{V}_{O_2}$) and blood O_2 transport ($\dot{Q}_{O_2}$), which we term O_2 consumption/transport quotient, and was calculated as follows:

$$\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1} = \frac{\dot{V}_{O_2}}{\dot{Q} \cdot C_{O_2}} \qquad (2)$$

such that,

$\dot{V}_{O_2}$, is oxygen consumption in $\mu l\ O_2 \cdot g^{-1} \cdot h^{-1}$,

C_{O_2}, is the content of blood at complete saturation expressed in

$\mu l\ O_2 \cdot 100 \mu l\ blood^{-1}$,

and $\dot{Q}$, is total cardiac output in $\mu l\ blood \cdot g^{-1} \cdot h^{-1}$.

$\dot{V}_{O_2}$ was calculated from transformed $\dot{M}_{O_2}$ data (Dejours, 1981) to allow for direct comparison between O_2 uptake and $\dot{Q}$ in equivalent units.

6. Hematology: Ventricular blood was obtained from anesthetized juveniles by methods described by Burggren *et al.* (1987). Total sampling time was less than 3 min. Samples were taken from 31 animals ranging from 5 to 30g in size and 3 to 11 months in age. Each animal yielded 400-700 µl of whole blood, which was immediately heparinized (1000 $IU.ml^{-1}$). Plasma oxygen content was determined in 15 of the 31 animals sampled. Plasma was obtained by centrifugation of whole blood at 10,000 rpm for 15 min. Whole blood and plasma oxygen content was determined according to the methods described in Tucker (1967.). Haldane affinity coefficient (Haldane and Smith, 1897) was calculated to determine the affinity of *Xenopus* blood for CO relative to O_2. The coefficient (H) was described by the following formula:

$$H = \frac{[CO]_{Hb} \cdot P_{O_2}}{[O_2]_{Hb} \cdot P_{CO}} \qquad (3)$$

thus,

$[CO]_{Hb}$, is the concentration of CO bound to Hb in $\mu l\ CO \cdot 100 \mu l\ blood^{-1}$,

P_{O_2}, is the partial pressure of O_2 in the blood in kPa,

$[O_2]_{Hb}$, is the concentration of O_2 bound to Hb $\mu l\ O_2 \cdot 100 \mu l\ blood^{-1}$,

and P_{CO}, is the partial pressure of CO in the blood in kPa.

Hemoglobin (Hb) concentration was measured on 15 µl blood samples, using a spectrophotometeric hemoximeter (model OSM 2, Radiometer). Although this hemoximeter used wavelengths appropriate for human Hb, Wood, (1971) has demonstrated that amphibian Hb can be assayed accurately with this instrument. Hematocrit (Hct) was determined on 15 µl blood samples centrifuged at 10,000 rpm for 4 min.

Oxygen and carbon monoxide equilibration was achieved by bubbling gas into a 1.5 ml centrifuge tube containing 100 µl of whole blood and spun gently on low speed in a mixer on alternate 3 min. intervals for 20 minutes at 25 ^{0}C. All whole blood samples were exposed in sequence to 0, 0.5, 1.0, 1.5, and 2 kPa (~2%) CO. Plasma samples were exposed to air only.

Statistical Analyses. Effects of experimental treatment upon $\dot{M}_{O_2}$ and lactate were compared for each variable's covariance with wet mass. To meet the assumptions of the MANCOVA, all variables were $\log_{10}$ transformed. MANCOVA (1993 BMDP-VAX-mainframe) determines 1) the correlation coefficient, 2) equation of the line describing the relationship, and 3) the probability that the slope of the line is not significantly different from zero. f_h, SV, $\dot{Q}$ and $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ were all tested for significant differences with development and between treatments utilizing a Brown-Forsyth MANOVA. If significant differences

occurred, a Tukey's (HSD) multiple range test was used to determine which points were different within, and between, the experimental groups. All values reported as mean $\pm$ 1 standard error of the mean (SEM). The fiduciary level of significance was taken at $p \leq 0.05$.

Oxygen content data was analyzed by a first order least squares linear regression (Statistica™ v5.0 on a PC). This analysis determines 1) the regression coefficient, 2) equation of the line describing the relationship, and 3) the probability that the slope of the line is not significantly different from zero. In addition a one-sample t-test was ran to determine if the slope of the predicted line is significantly different from the experimental line. In all cases the values are means $\pm$ 1 SEM.

Results

Wet Mass:

Wet mass increased significantly ($p \leq 0.05$) in an exponential fashion with progressive development of normoxic animals. All stages increased significantly in wet mass with development except NF stages 1-21 and 35-36. Interestingly, between NF stages 22 and 34 wet mass actually fell prior to the observed increase (Table 1).

Animals exposed to CO, like the normoxic animals, showed a significant ($p \leq 0.05$) rise in wet mass during development, although there was no significant ($p=0.10$) difference between normoxic and hypoxemic animals at any stage. All

stages except 1-21 and 22-30 were significantly different from the preceding stage. Like mass in normoxic animals, hypoxemic stages 22-30 also showed a drop in wet mass prior to the exponential increase through development (Table 1).

Hematology:

In the absence of CO (P_{O_2}=21 kPa), the blood of *Xenopus* showed a significant relationship between Hb concentration and oxygen content (r^2=0.94, $p \leq 0.05$) (Fig. 4). Details of regression analysis are given in Table 2. Addition of 0.5 kPa CO reduced whole blood oxygen content to that of plasma O_2 content with an average value of 0.8±0.14 µl O_2 ·100 µl blood^{-1}. Higher levels of CO (1.0, 1.5 and 2.0 kPa) also reduced O_2 content to that of plasma carrying capacity. There was no significant difference between CO levels with blood O_2 content (p=0.09). Calculation of the Haldane affinity coefficient revealed that whole blood from recently metamorphosed *Xenopus* froglets had a 168 times greater affinity for CO than O_2.

Developmental changes in $\dot{M}_{O_2}$ *:*

Both wet mass (r^2=0.85, $p \leq 0.05$) and developmental stage (r^2=0.67, $p \leq 0.05$) covaried in normoxic animals. However, $\dot{M}_{O_2}$ of eggs (NF 1-13) and very young embryos (14-30) showed a non-linear relationship with wet mass. During these

early stages $\dot{M}_{O_2}$ increased 50 fold, with just a 1.5 fold increase in mass from 4.4 mg to 6.8 mg (Fig. 1, *inset*). Conversely, larvae from NF 31 to NF 63 showed a clear allometric relationship with the equation $\dot{M}_{O_2}=0.41M^{0.71}$. $\dot{M}_{O_2}$ increased approximately 215 fold (from 3 $nmol{\cdot}h^{-1}$ to 536 $nmol{\cdot}h^{-1}$) over a mass range of 4 mg to 1048 mg wet weight (Fig. 1).

Similar to normoxia, $\dot{M}_{O_2}$ significantly covaried ($r^2=0.79$, $p\leq0.05$) with whole animals mass and stage ($r^2=0.61$, p,0.05). However, metabolism in eggs and early larvae showed the same non-allometric growth in $\dot{M}_{O_2}$ evident in normoxic animals. $\dot{M}_{O_2}$ increased from 0.2 $nmol{\cdot}h^{-1}$ at 3.7 mg to 8.7 $nmol{\cdot}h^{-1}$ at 6.8 mg. By contrast, larval (NF 31 to NF 54) metabolism increased in parallel with wet mass, as $\dot{M}_{O_2}$ ranged from 1.9 $nmol{\cdot}h^{-1}$ to 200.1 $nmol{\cdot}h^{-1}$. These data are described by the allometric equation $\dot{M}_{O_2}=0.50M^{0.61}$. The increase in $\dot{M}_{O_2}$ over development in CO exposed animals, was not significantly different ($p=0.92$) from that in normoxia.

Whole body lactate concentration:

Whole body lactate production was highly correlated with wet mass ($r^2=0.81$, $p\leq0.05$), but increased episodically with respect to development. Whole body lactate concentrations ranged from a low of 0.2 ± 0.2 $mmol{\cdot}g^{-1}$ at NF stage 48-49 to a high of 8.2 ± 1.9 $mmol{\cdot}g^{-1}$ at NF stage 55-63 (Table 1). Although lactate production was variable through development, *Xenopus* larvae showed a trend

toward increasing lactate concentrations in animals approaching metamorphosis. Post-hoc analysis indicate a significant stage-specific (significant from the previous stage) response in whole body lactate with all stages except 1-21 and 48-49.

Hypoxemic animals showed a moderate correlation (r^2=0.57, $p \leq 0.05$) between whole body lactate and wet mass. Hypoxemic, like normoxic animals, show a significant relationship with stage ($p \leq 0.05$); but there was no orderly progression with development. This is exemplified by lactate concentrations which ranged from a low of 0.2±0.2 $mmol.g^{-1}$ (NF 46-47) to a peak of 12.0±2.6 $mmol.g^{-1}$ at NF 52-54. Post-test comparison for stage specific responses showed that between NF stages 37-54 all groups were different than the preceding stage range (Table 1). Whole body lactate in hypoxemic animals, over all, was not significantly different (p=0.09) from control (normoxic) animals.

Heart rate:

Heart rate through development followed a curvilinear relationship with an initial rate of 86±2 bpm at NF stage 31-33/34, increasing progressively to a maximum of 183±5 bpm by NF stage 50-51. Rates obtained between NF stage 52-63 followed a steady decline to a new minimum value of 88±12 bpm. Heart rates were significantly different ($p \leq 0.05$) with progressive development, and all stages ranges, except 35/36, and 50-51, were significantly different than the preceding grouping (Table 3).

Animals chronically exposed to CO displayed a similar response in f_h to those seen in normoxia through development. Heart rate began at NF stage 31-33/4 at 85±5 bpm and elevated to a maximum of 171±1 bpm at NF stage 50-51. Comparable to the trend observed in controls, f_h fell with progressive development past stage NF stage 52 to a value of 92±11 bpm. Heart rate changed significantly ($p \leq 0.05$) in all stages with the exception of stage NF 37-41, in which f_h was not found to change from the preceding stage (Table 3). Although development significantly influenced f_h, exposure to 2 kPa CO did not ($p=0.11$).

Stroke volume:

Stroke volume increased progressively with development, and ranged from a low of 1.1±0.2 nl at NF stage 31-33/4 to 554.9±149.7 nl at NF stage 52-54. SV, like f_h, increased significantly ($p \leq 0.05$) with progressive development. However, a full order of magnitude increase occurred between NF stages 36 and 45. Over the next four stages ranges (NF 42-51) SV increased episodically, but over all continued to increase exponentially with development (Table 3). SV increased dramatically over the final two stage ranges (NF 50-54) where it reached a new value of 554.9±149.7 $nl.min^{-1}$, and occurred over a mass increase of 120 mg.

Stroke volumes in hypoxemic animals increased similarly to those observed in normoxic animals. SV increased progressively with development

($p \leq 0.05$) from 1.1±0.1 nl (stages 31-33/4) and reached maximum of 505.7±109.1 nl at NF 52-54. Significant stage specific responses occurred for development and treatment. SV through development did not show a progressive rise, holding constant between stages 42 and 47. Moreover, CO-induced hypoxemia had a significant effect only at stages 35/36, 48-49, and 52-54.

Cardiac output:

Cardiac output displayed a statistically significant ($p<0.05$) exponential rise with progressive development. This highly stage-dependent increase in $\dot{Q}$ showed a near doubling in flow at each successive stage over NF stages 33-41 (Fig. 2). Cardiac outputs between NF stage 37 and 45, however, showed a 6-fold increase from 219.3±0.3 $nl \cdot min^{-1}$ to 1326.9±3.6 $nl \cdot min^{-1}$. Moreover, animals from NF 42 to 47 increased $\dot{Q}$ by 100% over a mass range from 5.1±0.3 to 8.5±0.1 mg. This change was not maintained between NF stages 47 and 49, however, where $\dot{Q}$ decreased 1.5 times. Similar to the pattern seen in earlier development, flow increased 3.5 fold between NF stages 48 and 51. Total cardiac output, however, increased a full order of magnitude to 48547.6±1770.7 $nl \cdot min^{-1}$ over the next staging interval of 50-54. All stages were significantly different from the preceding stage.

$\dot{Q}$ in hypoxemic animals showed a significant ($p<0.05$) increase with development, and was significantly affected by treatment. Cardiac output

showed the same near doubling between subsequent stages that was observed in normoxic animals, and these ranged from a 96.7±0.6 nl·min^{-1} at stage 33/34 to 310.6±1.3 nl·min^{-1} at NF stage 41. Between NF stages 37 and 45 $\dot{Q}$ increased 4.5 fold. Over the next stage range (NF 42-47), $\dot{Q}$ increased a modest 1.1 times to 1541.8±7.7 nl·min^{-1} This was followed by a small reduction in $\dot{Q}$ to 834.8±5.9 nl·min^{-1} at NF stage 48-49. Flow consistently increased between NF stages 48 and 54 where $\dot{Q}$ increased nearly 7 fold between each of these stage ranges. There was a significant effect of development on $\dot{Q}$, and all NF stages were found to be different that their preceding stage (Fig. 2).

O_2 consumption/transport quotient:

The relationship between $\dot{V}_{O_2}$ and convective transport has been extensively investigated in mammals (Taylor *et al.*, 1989) and modeled as conductances (resistances^{-1}) in series (Piiper and Scheid, 1975). According to our definition, a high value of $\dot{V}_{O_2}\cdot\dot{Q}_{O_2}^{-1}$ would suggest that the relative contribution of cardiac output to total oxygen consumption is minimal, and low values suggest just the opposite. Additionally a value of 0.25 in mammals suggests that both the respiratory and cardiovascular systems are coupled, and that O_2 taken up at the respiratory surface is transported via the blood for metabolism with a 25% extraction..

Cardio-respiratory quotient in normoxic animals showed a significant ($p \leq 0.05$) decline with progressive development indicating a progressive increase in the dependence of $\dot{V}_{O_2}$ on blood flow. The greatest fall in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ (0.36 units) occurred between stages 33/34 and 46-47 which ranged from 0.38 ± 0.095 to 0.02 ± 0.001 respectively. Between stages 46 and 49 $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ increased 4.5 fold to 0.09 ± 0.009 (Fig. 3). $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ decreased a full order of magnitude over the next three stage ranges (NF 48-54), where it obtained a final of value of 0.01 ± 0.001. This reduction in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio indicates that $\dot{Q}_{O_2}$ exceeds $\dot{V}_{O_2}$ by 127 times by stage NF 52-54 (Table 4). All stages from NF 33 to 54 had $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ values below the theoretical O_2 consumption/transport coupling threshold of 0.25.

Much like normoxia, $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ in hypoxemia showed a significant ($p \leq 0.05$) declining relationship with progressive development. Although the trend was similar to normoxia, the magnitude of the decline between stages 33/34 and 46-47 was greater, and changed 1.76 units as compared to the 0.36 units seen in normoxic animals. Animals from stages 52 to 54 displayed values of $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ below the coupling threshold of 0.25, indicating a clear excess of blood O_2 capacitance relative to O_2 uptake. By contrast, NF stages 33 to 41 and 48-49 all obtained $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ values in excess of 0.25, indicating that O_2 consumption is occurring irrespective of convective transport (Table 4).

Treatment with CO significantly affected the $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ($p \leq 0.05$). There was also a highly stage specific response in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ with progressive development and treatment (Fig. 3).

Discussion

Critique of method:

A potential critique of this study could be the use of CO to eliminate blood O_2 transport's contribution to O_2 consumption. It is clear from the oxygen content data that the addition of low levels of CO seriously impairs blood capacitance. Circulating CO-Hb provides no O_2 transport, therefore animals exposed to CO are in fact reliant solely upon diffusion alone. Thus, changes in perfusion would afford only modestly increases in gas transport (in the plasma), thereby making them primarily diffusion limited.

Another potential criticism may be that our study used the blood of juvenile *Xenopus* to determine the effect of CO on blood capacitance. It is clear from work in amphibians that P_{50} increases with ontogeny and degree of terrestriality (Boutilier *et al.*, 1992; Pinder and Burggren, 1983). In addition, *Xenopus* blood has a higher degree of affinity relative to its terrestrial counterparts, and this is thought to be link to the reduced solubility of O_2 in water. Therefore, it unlikely that embryonic or larval stages of *Xenopus* will have lower blood affinities than post-metamorphic animals.

Last, is a concern of CO toxicity relative to metabolism through this gas' actions on hemoprotiens other then Hb. A P_{CO}:P_{O_2} ratio of 5:1 is required to reduce oxidative metabolism by 50% in mammals (Ewer, 1942). The present study used a P_{CO}:P_{O_2} ratio of 0.1:1, which is far below the level affecting cytochrome oxydase reducing potentials. Another benefit of using CO to lower oxygen content is that it is specific to Hb, and does not interfere with carbonic anhydrase activity, which is important in CO_2 transport and buffering (Ewer, 1942). These facts indicate the suitability of CO to evaluate the role of convective O_2 transport in general, and the role of Hb more specifically, in total oxygen uptake.

Hematology:

There is a high degree of correlation between Hb concentration and carrying capacity of *Xenopus* blood, and the majority of O_2 transported in the blood occurs via Hb-dependent processes. Carbon monoxide as low as 0.5 kPa reduced the functional capacity of the blood to levels nearing plasma content. This is consistent with the findings of Brody and Coburn (1969) in which they demonstrated that inspiration of small quantities of CO in humans was capable of reducing total O_2 content by 75%. Moreover, Roughton, (1954, 1970) has shown that CO interacts with Hb in a pressure-dependent fashion and is capable of forming equilibrium curves at pressures which are orders of magnitude below that required for O_2 (for review see Root, 1965). An index of the relative ease by

which CO combines with Hb is termed the Haldane affinity coefficient (H) (Haldane and Smith, 1897). *Xenopus*, exhibits a value of H which clearly shows that CO will combine with Hb 168 time faster than O_2. This explains the fact despite a quartering of P_{CO}, blood O_2 capacitance was unaffected, and remained at levels near plasma values.

Metabolism:

Although it has been speculated for some time that the convection of Hb-O_2 by the cardiovascular system plays a significant role in total $\dot{M}_{O_2}$ in pre-metamorphic amphibians (Adolph, 1979.; Boell *et al.*, 1963; Burggren and Just, 1992; Burggren and Pinder, 1991) these claims have yet to be validated for early developmental stages. Elimination of Hb-dependent O_2 transport would disrupt metabolism, if this was solely dependent on the O_2 convection by the cardiovascular system. Therefore $\dot{M}_{O_2}$ and whole body lactate was assessed in *Xenopus* from eggs to metamorphic climax to determine the extent to which elimination of convective O_2 transport would have on total substrate turn over.

Our results demonstrate that aerobic metabolism during early life (NF 1-30) increased in an exponential fashion regardless of the state of Hb-O_2 transport. The fact that $\dot{M}_{O_2}$ increased 100 fold despite the absence of functional Hb shows that the role of Hb in bulk transport is negligible in these early stages.

A clear rise in $\dot{M}_{O_2}$ in spite of CO exposure shows that the role of Hb in bulk O_2 transport, and the role of the circulatory system in general, is highly reduced in maintaining aerobic metabolism. These interpretations gains support from several quarters. Mellish *et al.* (1995) showed that $\dot{M}_{O_2}$ between mutant axolotls, CO exposed animals, cardio-ablated larvae, and controls animals of *Ambystoma mexicanum* were unchanged over a wide range of working P_{O_2}'s. This premise is further supported by the existence of mutant strains of adult *Xenopus* who's blood lacks formed elements (de Graaf, 1957; Ewer, 1959). Moreover, work by Flores and Frieden (1969) demonstrated that functional capacitance of the blood can be impaired with phenylhydrazine (PHZ) with few ill effects on survivorship in tadpoles of *Rana catesbieana*. Hillman (1980) has shown that PHZ-induced anemia in exercising *Rana pipiens* impairs performance, but that animals whom functionally retain little Hb are still capable of sustaining moderate levels of activity. This combined evidence all suggest that amphibians are capable of surviving with minimal contribution of Hb to O_2 transport and uptake.

Although the aerobic component of total metabolism was unaffected in our study, amphibians are capable of dealing with sustained periods of stress by supplementing aerobic pathways with anaerobic metabolism (for review see Gatten *et al.*, 1992) Lactate, the major end-product anaerobic metabolism in amphibians, is an excellent indicator of metabolic stress. Therefore, animals in our study were assayed for whole body lactate to evaluate the extent of

hypoxemic stress on anaerobic metabolism with development. Whole body lactate was not significantly affected by exposure to CO. Our measured values of lactate are consistent with the finding of Hastings and Burggren (1995), but are slightly lower than those reported by Feder and Wassersug (1984) when converted to similar units at 25 ^{0}C. Normoxic animals approaching metamorphic climax (NF 55-63) had lactate concentrations (8.2 mmol·g^{-1}) which were slightly higher than data for tadpoles *Xenopus* at comparable stages (Hastings and Burggren, 1995). The highly episodic nature of the increase in whole animal lactate, and the consistent low levels which persisted until late in development, suggest that anaerobic metabolism was not significantly contributing to total energy turnover through most of larval development. Moreover, these data also suggest that the formation of lactate observed was not due to the supplementation of aerobic metabolism, but rather were baseline levels observed with normal maintenance through development. Additionally, these data also suggest that cytochrome activity was not impaired by CO, as the levels of lactate would presumably be much higher than those observed.

Animals between NF 1-30 did not follow an allometric pattern predicted from interspecific data. In fact, the dramatic rise seen in $\dot{M}_{O_2}$ over this stage range was accompanied by only a modest change in wet mass. However, $\dot{M}_{O_2}$ between stages 31-63 did show a clear allometric rise, with metabolism increasing two orders of magnitude over a 2.5 orders of magnitude increase in mass from 2 mg to 1100 mg. Although, both Hb and the circulatory system were

fully functional over this range, there was no significant effect of hypoxemia. This allometric rise in $\dot{M}_{O_2}$ (b=0.72, normoxia; and b=0.61, hypoxemia) is consistent with the findings of Feder (1981) although the slopes we report are slightly lower than those reported (b=0.85) . A possible reason for the difference between the two studies could be that fact that Feder (1981) restricted the scope of development to only a few stages, whereas our study covers all of development from early embryos to metamorphic tadpoles.

Cardiovascular function:

In spite of the fact that both aerobic and anaerobic metabolism were unchanged with exposure to CO, this does not preclude the possibility that total $\dot{Q}$ has been augmented. Moreover, it is reasonable to assume that a reduction in blood capacitance via CO would result in an increase in total flow to accommodate tissue metabolic demands. Support for this premise came from the work of Holeton (1971a, 1971b) which demonstrated in both larval and adult rainbow trout that exposure to 5% CO elicited a 1.5 fold increase heart rate, and in all probability $\dot{Q}$, thereby facilitating a greater O_2 delivery in the face of falling blood capacitance.

Holeton (1970), and Ruud (1954, 1958, 1965) have shown that icefish, which lack erythrocytes and Hb, are capable at 1.5 ^{0}C of maintaining $\dot{M}_{O_2}$'s at similar to amphibians at 25 ^{0}C, and this was supported in icefish by a

comparatively large $\dot{Q}$. The fact the icefish are capable of maintaining relatively high metabolic rates despite the lack of Hb can be explained by two phenomena. First, capacitance for O_2 in water at 1.5 ^{0}C is 1.2x higher as compared to 25 ^{0}C. Therefore it is reasonable, that plasma capacitance will also increase by this same factor, thereby affording greater delivery irrespective of Hb. Second, all out-flow vessels from the heart in fish empty into the gills, and counter-current exchange mechanisms in the gills have greater extraction efficiency than infinite pool exchangers (skin and buccal cavity in amphibians) (Piiper, 1981). Although these mechanisms work well for icefish at 1.5 ^{0}C, they do not for amphibians, who live at much higher temperatures. Thus, it is unlikely that O_2 transport via plasma alone can meet total oxygen demands in *Xenopus*, as in *C. aceratus.*

$\dot{Q}$ was the only factor changing significantly with exposure to CO. Total flow in both groups of animals, from NF stage 33/34 to the first plateau at NF stage 42-45, increased more than one order of magnitude within 2 days post-initiation of f_h. These finding are consistent with work of Hou and Burggren, (1995b) and Orlando and Pinder (1995). Interestingly, this dramatic increase in $\dot{Q}$ occurred over only a doubling of wet mass. Both treatments and controls showed a lack of increase and, in the cases of hypoxemia, a decrease in $\dot{Q}$ between NF stages 42 and 47 in spite of a near doubling of body mass and advancement of one week of development. One possible explanation may be gleaned from the study of Hou and Burggren (1995b), which showed that total

peripheral resistance (TPR) precipitously decreases from 701 TPR units to values approaching zero (0.9 TPR units) over a range in wet mass from 3 to 20 mg in larval *Xenopus* . Hou and Burggren (1995b) speculate that this distinct fall in TPR was concomitant with an increase in total cross sectional area of the vasculature. Additional inferential support, based on morphology, from Nieuwkoop and Faber (1967), has shown that *Xenopus* form gills, a simple lung, and utilization of the alimentary tract over this stage range (NF 42-47) each adding to the total vasculature. Additionally, Hou and Burggren (1995a) has also demonstrated a minimal rise in mean truncus pressure over the mass range which corresponds to NF 42-47. The formation of these complex structures and their vascularization could explain the fall in TPR. The net effect would be a reduction in filling pressure, and thus in SV. In fact, the over all absence of change seen in $\dot{Q}$ in our study over this range was due to a modest reciprocal changes in SV and f_h (Table 3). Animals between stages 48 and 54 increased $\dot{Q}$ in a nearly consistent fashion as that seen in early development. Flow in hypoxemic animals showed a consistent steady rise to a maximum of about 47000 $nl.min^{-1}$ at NF stage 52-54. Normoxic flow over this same range increased in a similar fashion. Interestingly, total flow was higher for hypoxemic animals up to NF stage 42-45, where they reached an almost identical value to controls. At stages between NF 46 and 49 the trend was reversed, with normoxic flows that were higher than hypoxemic animals. This reversal of trend could be explained by increases in total vascular area. Anecdotally, we have

observed that animals raised in CO show an increase in the extent of vascular beds in the skin and buccal cavity. We argued previously that a fall in TPR via increased vascularity would reduce the venous filling pressure and therefore result in a fall in SV, and consequently $\dot{Q}$. In spite of the fact that $\dot{Q}$ was lower for hypoxemic animals (NF 46 to 49), total flow increased over controls between NF 50 and 52. Fritsche and Burggren (1996) have shown that cardiovascular regulation during hypoxia begins between NF stages 49-53 and that these changes are suggestive of a Frank-Starling mechanism. Moreover, Nieuwkoop and Faber (1967) have shown that anatomical inervation of the ventricle exists at NF 49, suggesting that active regulation of cardiac function may exist over this stage range. Interestingly, changes in $\dot{Q}$ in hypoxemic animals over this range (NF 48-51) are due to an 8 fold increase in SV. Clearly, this evidence is highly suggestive that the changes that were observed were due in fact to cardiac regulation in CO exposed animals.

Heart rate increased in a curvilinear fashion with progressive development with no significant effect of treatment. The general trend of heart rate is similar to that reported by Fritsche and Burggren (1996), Hou and Burggren (1995a), and Orlando and Pinder (1995). Even though the general trend was preserved, in some cases the shape of the curves differed. This differences in shape may be due to two factors; first, Hou and Burggren (1995a) selected an order of regression for their allometric equation which described a line, where perhaps a higher order regression might have fit the data better; and

secondly, Orlando and Pinder (1995) only sampled animals from NF stage 44-57. In doing so they might have missed the initial increase in heart rate seen between NF stages 33/4-43. Nonetheless, it is clear from our data that heart rate shows a generalized response similar to those seen in other studies and is unaffected by exposure to CO.

The clear changes seen in $\dot{Q}$ in response to hypoxemia and the fact that f_h did not show a significant effect, suggests that SV was a major contributor to the resultant changes seen in flow. In adult fish and reptiles, bouts of hypoxia result in increases in total flow which were largely due by changes in cardiac stroke volume (Fritsche and Nilsson, 1990; Holeton, 1971b; Millard and Johansen, 1974; Wood and Shelton, 1980). These changes in stroke volume are due increases in pre-load and/or decreases in down stream resistance, the latter reducing after-load and increasing SV. Pelster and Burggren (1991) have shown that preload increases with development, which in turn would increase SV. Therefore, it is probable that hypoxemia would induce changes in SV not only from the perspective of development, but via induced changes resulting from the hypoxic stress it self.

Based on this rational, we evaluated the effects of chronic hypoxemia on stroke volume through development. Stroke volume increased in a fairly constant exponential fashion with ontogeny, a trend consistent with observations by Fritsche and Burggren (1996), Hou and Burggren (1995b), Orlando and Pinder (1995), and Tang and Rovianen (1996). However unlike f_h, SV was

significantly different in animals exposed to CO through development. A stage-specific analysis indicated that stages NF 35/36, 46-47, and 50-51 showed a clear treatment effect in CO exposed animals.

Based on the changes seen in $\dot{Q}$ with development and experimental treatment, and the fact that the majority of the changes seen in cardiac output are due to associated changes in SV, we suggest that the responses seen are due largely to Frank-Starling mechanisms. Furthermore these changes also suggest that reductions in arteriole blood capacitance result in an augmentation of flow which is based largely on changes in cardiac stroke volume. These results on larvae are in direct opposition to the findings of Farrell (1991), who speculates that adult amphibians regulate cardiac function via rate dependent processes.

Matching of O_2 consumption and O_2 transport:

The regulation and coordination of cardiac and respiratory function has been assessed in several anuran larvae (Burggren and Feder, 1986; Wassersug *et al.*, 1981; West and Burggren, 1982). The importance of coordinating both mechanisms has important consequences for regulating blood gases and pH (Boutilier and Shelton, 1986; Boutillier and Shelton, 1986; Weintraub and MacKay, 1975). This regulation is magnified with development, as changing metabolic needs must be met by tighter regulation between the respiratory exchange areas and the convective mechanisms which supply them.

In the case of a developing *Xenopus*, this regulation must take place over a large number of changing respiratory surfaces (e.g. gills, lungs, and the skin). The quantification of these respective exchange surfaces relative to the flow which supplies them is beyond the scope of this paper (see, Malvin, 1994). However, the coordination of total respiratory gases relative to total perfusive conductance would give an indication of the relative amount of cardiac work that must be put forth in transporting a quantity of gas.

The fact the normoxic animals showed a clear reduction in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio (between NF stages 33 and 54) suggests that either $\dot{V}_{O_2}$ was falling relative to $\dot{Q}$, or that cardiac outputs were highly elevated relative to aerobic metabolism. In fact, our data suggest that the latter was true, and that $\dot{Q}$ increased significantly with little change in $\dot{M}_{O_2}$ relative to $\dot{Q}$. $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio in hypoxemic animals showed a similar pattern to that of normoxic animals, however these changes occurred at a level 6 times higher than that for control animals. Clearly, the fact that $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ was elevated in CO exposed animals suggest that the cardiovascular system was contributing little to total oxygen uptake.

In adult humans $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratios approach values of 0.25 (Taylor *et al.*, 1989), which suggests that the cardiovascular and respiratory systems are coupled and regulated between $\dot{M}_{O_2}$ and its distribution via the blood. The decreases in the O_2 consumption/transport quotient in hypoxemic animals to a

value approaching 0.25 suggests that the role of the cardiovascular system through development was functionally being coupled with O_2 uptake. Although, this in fact may be the case for hypoxemic animals between stages NF 33 to 51, this is not the case for stages NF 52 to 54 where the $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio falls below a value of 0.25 (i.e. $\dot{Q} \cdot C_{O_2}$ is in excess of $\dot{V}_{O_2}$). Normoxic animals on the other hand never achieve values of 0.25, and this clearly suggests that $\dot{M}_{O_2}$ was either highly dependent on $\dot{Q}$, or that oxygen extraction was sufficiently low, and therefore aerobic metabolism occurred irrespective of $\dot{Q}$. Clearly, these data suggest the cardiovascular system is functionally decoupled from $\dot{V}_{O_2}$ in CO exposed animals and that $\dot{V}_{O_2}$ must be supported by means other than by a tight coupling between the respiratory and cardiovascular systems. Mellish *et al.* (1995) has shown that $\dot{M}_{O_2}$ is unaffected in *Ambystoma* mutant axolotls, which do not retain a functioning heart, and in animals with hearts removed. They speculate that the major contribution of total O_2 uptake must be through simple O_2 diffusion across the body surface.

The role of transcutaneous diffusion in embryonic gas exchange:

Although cardiac function appears to be regulated with exposure to CO, it is apparent from blood O_2 capacitance data the magnitude of $\dot{Q}$ increases in CO exposed larvae seen are incapable of meeting total aerobic metabolic demands.

Calculations of maximal delivery with blood capacitance near plasma content levels suggests that this level of flow would be insufficient to meet metabolic demands (Table 4).

Elimination of whole blood capacitance through CO treatment did not impede aerobic metabolism, indicating that early $\dot{M}_{O_2}$ occurs via diffusion alone. Although these data are not surprising for eggs and early (small) embryos, it is however astonishing that large (>3 mg) larvae deprived of significant convective O_2 transport are capable of surviving through metamorphic climax. Since ablation of O_2 carrying capacity of the blood resulted in only modest changes in $\dot{Q}$, it appears the cardiovascular system may in fact not play a significant role in total $\dot{M}_{O_2}$ in early developmental stages, as has also been suggested in contemporary studies on other lower vertebrates (Mellish *et al.*, 1995; Pelster and Burggren, 1996).

The available data suggest that the initiation of cardiac function is not for the sole purpose of supplying convective transport of O_2 (prosynchronotropy). Instead, these data suggest that the heart begins to initiate function as part of a genetic program, and its role in supplying oxygen to the tissues may in fact act as a redundant system during periods of low environmental P_{O_2} (Burggren and Territo, 1995). Moreover, it has been suggested that the role of the cardiovascular system in early development may serve several purposes other than for O_2 distribution. Burggren and Warburton (1994) has suggested that the formation of early f_h and pressure generation may in fact serve to distend newly

developing vascular beds. Alternatively, the role of early convective transport may be for nutrient distribution (Burggren and Territo, 1995). What ever the purpose of early convection, it is clear from these data that the bulk O_2 transport is not essential via Hb dependent processes in early developmental stages.

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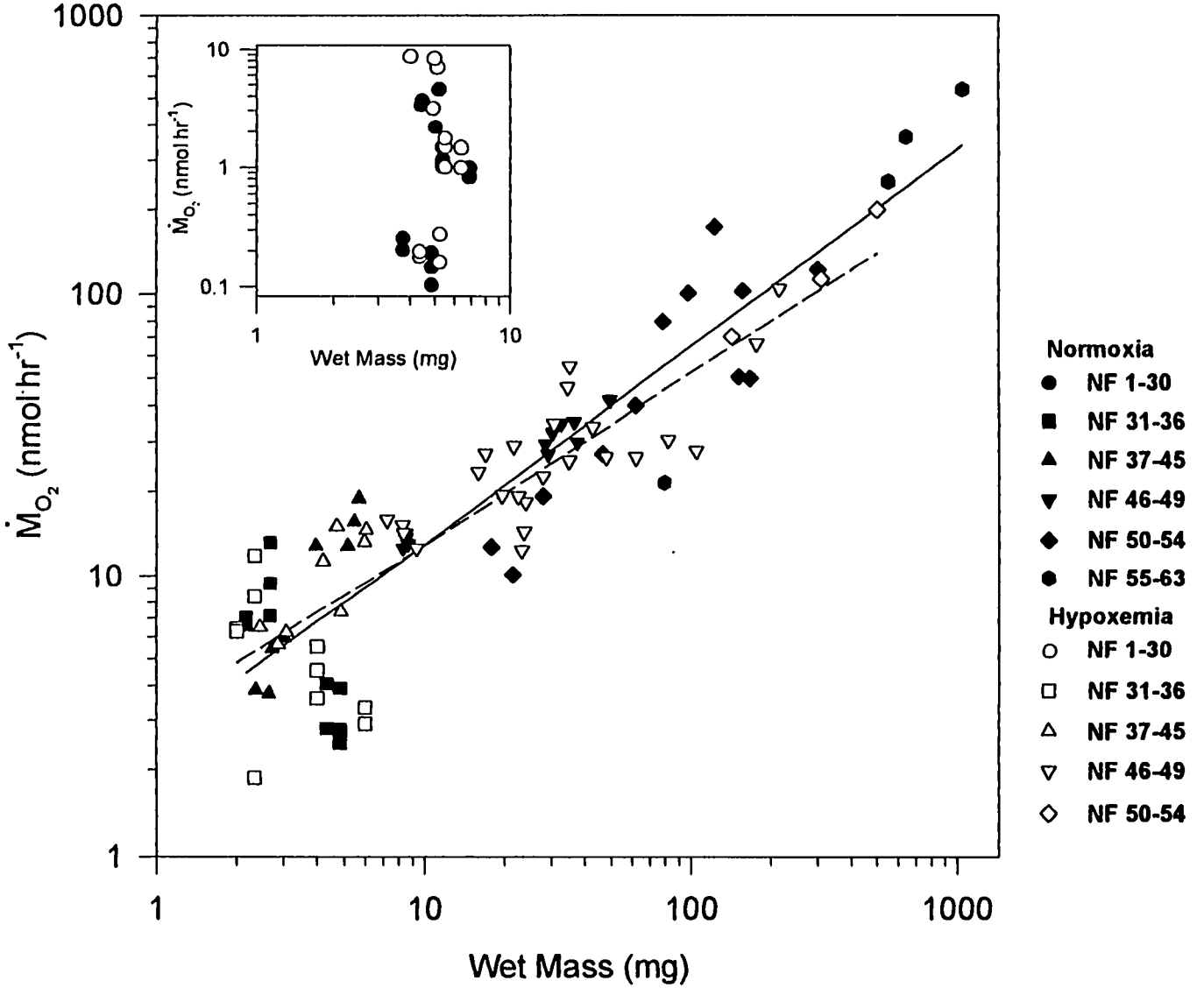
Figure 1. The relationship between $\dot{M}_{O_2}$ and wet mass in tadpoles of *Xenopus laevis* exposed to chronic CO-induced hypoxemia (open symbols) and control animals exposed to normoxia (closed symbols). Both oxygen consumption and wet mass were plotted on $\log_{10}$ scales. Regression lines indicate allometric trends for normoxia (solid) and hypoxemia (dashed); analysis results are given in Table 1. The inset shows the data set for the smallest population of eggs and larvae.

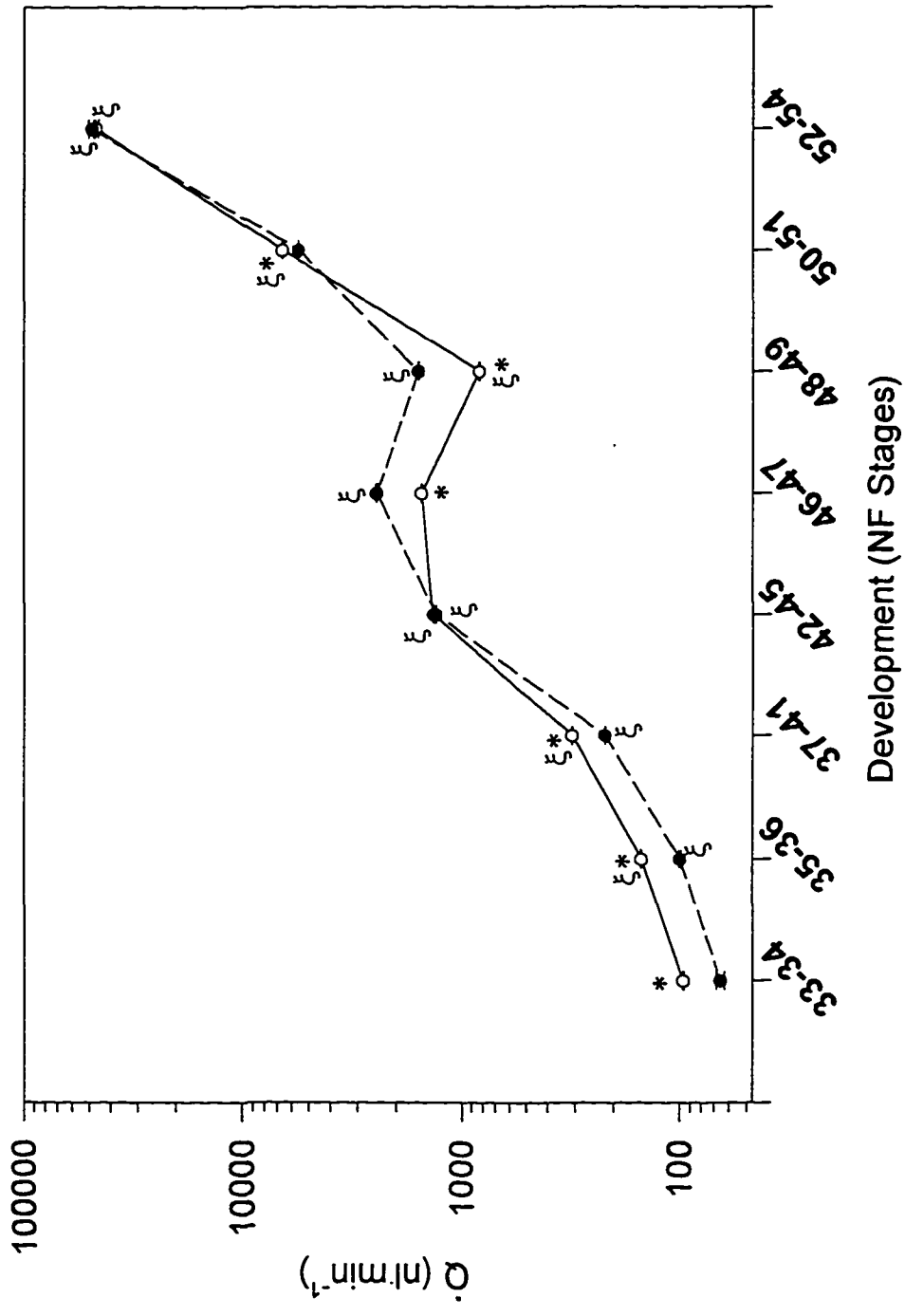
Figure 2. The relationship between blood flow and developmental stage for normoxic animals (filled symbols) and chronically CO-exposed animals (open symbols). $\dot{Q}$ was plotted on a $\log_{10}$ scale against the linear dimension of development. Values are shown as means and SEM. In some cases the error bars are inside the symbols. ξ indicates significant ($p \leq 0.05$) difference than the previous stage, and * indicates significant difference from controls.

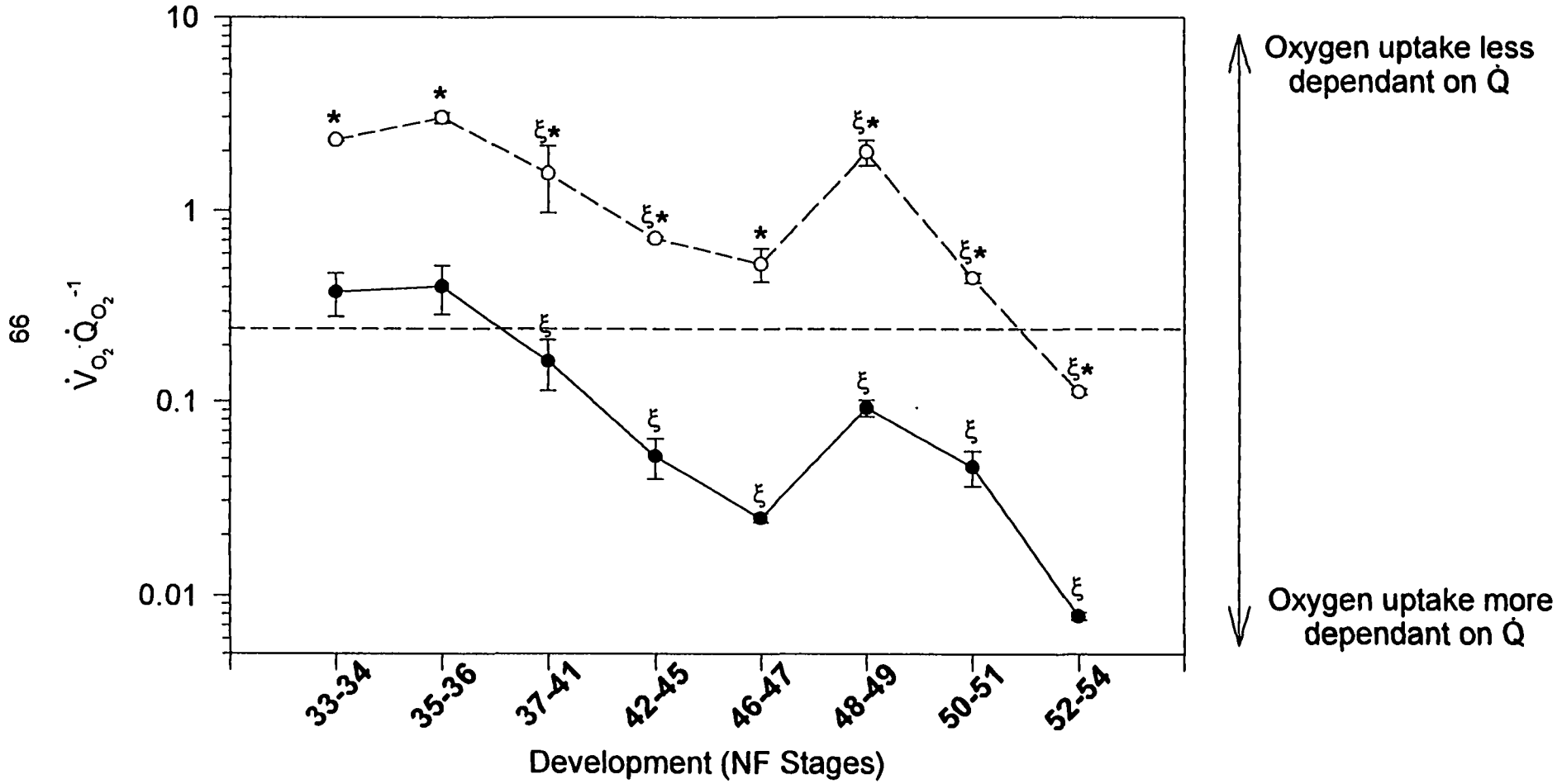
Figure 3. The relationship between cardio-metabolic quotient ($\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$) and development for control animals (filled symbols) and chronically CO exposed (open symbols). Cardio-metabolic quotient was plotted on a $\log_{10}$ scale against the linear dimension of development. The dashed line at a value of 1 indicates the point at which the ability of the blood to carry O_2 exactly meets O_2 demands (100% extraction). Values are shown as means and SEM. In some cases the

error bars are inside the symbols. ξ indicates significant ($p \leq 0.05$) difference than the previous stage, and where * indicate significant difference from controls.

Figure 4. Blood oxygen content during exposure to carbon monoxide in *Xenopus laevis* blood. The linear function of oxygen content in whole blood, and plasma, was plotted against the linear dimension of Hb content. Exposure to normoxic air (filled symbols) was fitted with a first order linear regression and the results are found in Table 3. Exposure to increasing P_{CO} (open symbols) were plotted against their respective Hb concentrations.







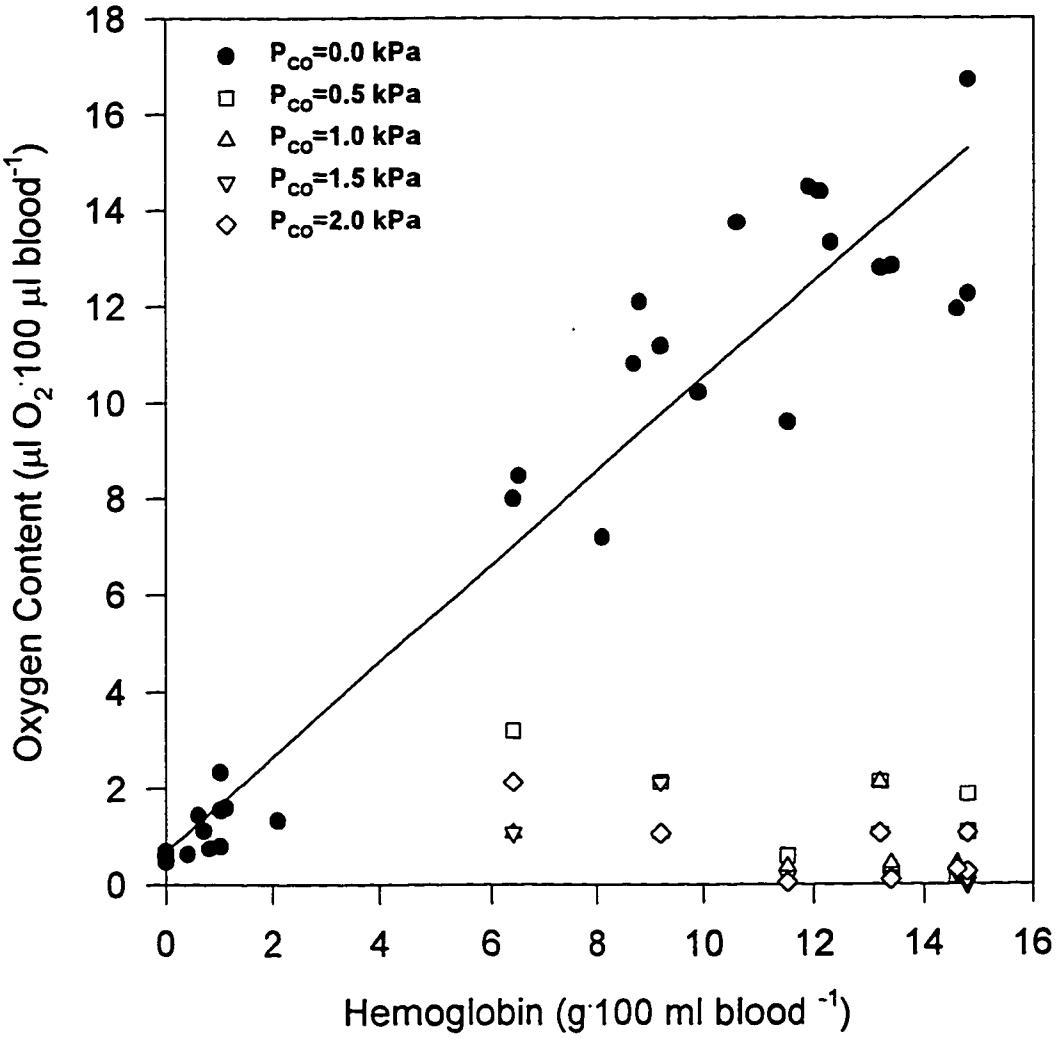


Table 1. The ontogeny of anaerobic metabolism and wet mass with normoxia and hypoxemia.

	Normoxia		***Hypoxemia***	
Developmental Stage Range	*Lactate ($\mu mol\, g^{-1}$)*	*Wet Mass (mg)*	*Lactate ($\mu mol\, g^{-1}$)*	*Wet Mass (mg)*
1-21	1.2 ± 0.2	5.0 ± 0.3	1.2 ± 0.4	5.1 ± 0.2
22-30	0.2 ± 0.1[§]	4.6 ± 0.2[§]	1.1 ± 0.3	5.0 ± 0.7
31-34	1.0 ± 0.1[§]	2.4 ± 0.2[§]	1.2 ± 0.5	2.2 ± 0.1[§]
35/36	0.8 ± 0.2[§]	2.6 ± 0.3	0.7 ± 0.1	2.8 ± 0.2[§]
37-41	2.2 ± 0.7[§]	3.3 ± 0.3[§]	1.1 ± 0.2[§]	3.4 ± 0.3[§]
42-45	4.5 ± 0.4[§]	5.1 ± 0.3[§]	0.7 ± 0.3[§]	6.7 ± 0.9[§]
46-47	0.4 ± 0.2[§]	8.5 ± 0.1[§]	0.2 ± 0.2[§]	8.3 ± 0.4[§]
48-49	0.2 ± 0.2[§]	34.0 ± 2.4[§]	0.8 ± 0.3[§]	32.8 ± 4.4[§]
50-51	3.6 ± 1.0	71.2 ± 10.6[§]	6.2 ± 1.7[§]	123.8 ± 13.4[§]
52-54	5.5 ± 1.2[§]	190.5 ± 25.1[§]	12.1 ± 2.6[§]	224.7 ± 38.3[§]
55-63	8.2 ± 1.9[§]	685.7 ± 107.8[§]	--	--

Values are means ± SEM, * Significantly different from control ($p \leq 0.05$)

[§] Significantly different from preceding stage ($p \leq 0.05$)

Table 2. Regression analysis of hematology, oxygen consumption, and lactate with [Hb], and wet mass in developing *Xenopus laevis* .

y	*Condition*	*n*	*a*	*b*	r^2	*p*
$[O_2]_b$	-	31	0.6897	0.9861	0.94	$\leq$0.05
$\dot{M}_{O_2}$	Normoxia	61	0.4125	0.7064	0.85	$\leq$0.05
	Hypoxemia	59	0.5015	0.6091	0.79	$\leq$0.05
Lactate	Normoxia	55	-2.469	1.0590	0.81	$\leq$0.05
	Hypoxemia	55	-2.497	0.9902	0.57	$\leq$0.05

Relationships for $\dot{M}_{O_2}$ and whole body lactate are expressed as $\log y = \log_{10} a + b \cdot \log_{10} M$, where y is $\dot{M}_{O_2}$ ($nmol \cdot hr^{-1}$) or whole body lactate (μmol). Whole blood is expressed as a linear combination and is described by the formula $y = a + b \cdot x$. *n*, *r* and *P* , are sample size, correlation coefficient, and fiduciary level of significance respectively.

Table 3. The ontogeny of cardiac function with normoxia and hypoxemia.

	Normoxia		***Hypoxemia***	
Stage Range	*Stroke Volume (nl)*	*Heart Rate (beats·min^{-1})*	*Stroke Volume (nl)*	*Heart Rate (beats·min^{-1})*
1-21	--	--	--	--
22-30	--	--	--	--
31-34	1.1 ± 0.2	59 ± 2	1.1 ± 0.1	85 ± 5
35/36	1.1 ± 0.1	90 ± 13	1.4 ± 0.1§*	106 ± 4§
37-41	2.2 ± 0.3§	99 ± 2§	2.9 ± 0.4§	108 ± 4
42-45	11.0 ± 1.1§	121 ± 3§	9.7 ± 1.1§	141 ± 5§
46-47	18.2 ± 2.5§	136 ± 5§	12.0 ± 1.4§*	128 ± 6§
48-49	8.7 ± 1.5§	183 ± 5§	5.0 ± 1.4§	167 ± 4§
50-51	30.8 ± 5.5§	182 ± 5	41.7 ± 4.7§*	159 ± 3§
52-54	554.9 ± 149.7§	88 ± 12§	505.7 ± 109.1§	92 ± 11§

Values are means ± SEM, * Significantly different from control ($p \leq 0.05$)
§ Significantly different from preceding stage ($p \leq 0.05$).

Table 4. The ontogeny of cardio-respiratory coordination with normoxia and hypoxemia.

	Normoxia		***Hypoxemia***	
Stage Range	$\dot{V}_{O_2} \cdot \dot{G}_{perf}^{-1}$	$\dot{G}_{perf} \cdot \dot{V}_{O_2}^{-1}$	$\dot{V}_{O_2} \cdot \dot{G}_{perf}^{-1}$	$\dot{G}_{perf} \cdot \dot{V}_{O_2}^{-1}$
1-21	--	--	--	--
22-30	--	--	--	--
33/34	0.38 ± 0.01	2.64 ± 0.11	2.29 ± 0.04*	0.44 ± 0.02*
35/36	0.41 ± 0.12	2.46 ± 0.09	2.98 ± 0.19*	0.34 ± 0.01*
39-40	0.16 ± 0.05[§]	6.09 ± 0.10[§]	1.55 ± 0.58[§]*	0.64 ± 0.02[§]*
43-45	0.05 ± 0.01[§]	19.38 ± 0.81[§]	0.72 ± 0.02[§]*	1.39 ± 0.06[§]*
46-47	0.02 ± 0.01[§]	40.64 ± 7.55[§]	0.53 ± 0.10*	1.88 ± 0.01*
48-49	0.09 ± 0.01[§]	10.79 ± 1.09[§]	1.99 ± 0.29[§]*	0.50 ± 0.03[§]*
50-51	0.05 ± 0.01[§]	22.05 ± 0.53[§]	0.45 ± 0.03[§]*	2.22 ± 0.04[§]*
52-54	0.01 ± 0.01[§]	127.25 ± 29.31[§]	0.11 ± 0.01[§]*	8.88 ± 2.39[§]*

Values are means ± SEM, * Significantly different from control ($p \leq 0.05$)
[§] Significantly

CHAPTER 4

THE ONTOGENY OF CARDIO-RESPIRATORY FUNCTION UNDER CHRONICALLY ALTERED GAS COMPOSITIONS IN *XENOPUS LAEVIS.*

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THE ONTOGENY OF CARDIO-RESPIRATORY FUNCTION UNDER CHRONICALLY ALTERED GAS COMPOSITIONS IN *XENOPUS LAEVIS*.

Paul R. Territo[1,2*]
Jordi Altimiras[2]

Current Address
[1]National Institute of Health
Laboratory of Cardiac Energetics (OE-108)
NHLBI, Building 10, Room B1D-161
10 Center Dr. MSC 1061
Bethesda, MD 20892-1061

[2]Department of Biological Sciences
University of Nevada, Las Vegas
4505 Maryland Parkway
Las Vegas, NV 89154-4004

Running head: Ontogeny of cardio-respiratory function

* Author to whom all correspondences should be sent.

Abstract

The role of diffusive and perfusive conductance can be evaluated independently by chronically altering either inspired O_2 content (C_{O_2}), or pressure (P_{O_2}). When these are combined with carbon monoxide (CO), it allows for determination of which components are limiting to overall gas exchange. The present study investigates the ontogeny of cardio-respiratory physiology with functional limitations to gas exchange in *Xenopus laevis* . Animals were raised from eggs (NF stage 1) to pre-metamorphic climax (NF stage 54), while maintained in 0.5, 1, and 1.5X O_2 saturation, both with and without 2 kPa carbon monoxide (CO) . Whole animals oxygen consumption ($\dot{M}_{O_2}$), individual mass, heart rate (f_h), and stroke volume (SV) were measured. Additionally, cardiac output ($\dot{Q}$), and O_2 consumption/transport quotients ($\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$) were also calculated to determine work loads imparted by limitations to gas exchange. $\dot{M}_{O_2}$, wet mass, and f_h were not significantly different between controls and all experimental treatments. However, hyperoxic+CO and normoxic+CO exposed animals showed a statistically significant ($p<0.05$) increase in SV, $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ and $\dot{Q}$ when compared to controls. These results indicate that altering C_{O_2} and/or P_{O_2} was neither deleterious, nor advantageous, to metabolism and development as a whole, and that Hb was not essential for normal cardiovascular and respiratory function. Moreover, our data suggests that diffusion plays a significant role in

total O_2 uptake, and is not limiting to total substrate turnover through development.

Introduction

The development of the cardiovascular and respiratory systems in vertebrates is suggested to coincide with the concomitant rise in metabolism associated with development (Adolph and Ferrari, 1968; Boell *et al.*, 1963; Burggren, 1992; Burggren and Pinder, 1991; Burggren and Territo, 1995). This premise has gained inferential support from morphological observation that circulation begins in gills of amphibians (Nieuwkoop and Faber, 1967, Taylor, 1946 #3167) post initiation of heat beat. Additionally, it has been implicated in amphibians (Atlas, 1938; Wills, 1936.) and, in birds (Romanoff, 1960), that the sharp increase in O_2 consumption occurs just after the start of heart beat, suggesting that the limitation to total O_2 turnover may in fact be limited by diffusion prior to this event.

Burggren and Mwalukoma, (1983) report that exposure to chronic hypoxia, and hyperoxia, results in an accentuation and reduction, respectively, in morphological structures associated with respiratory gas exchange. Pinder and Burggren (1983a) have demonstrate that the functional capacitance of blood changes with the rearing conditions. Collectively, they suggest that environmental perturbations may act as an important stimuli to both morphology and physiology alike (Burggren and Pinder, 1991). This hypothesis is supported by work on vascular growth in developing chickens showing that intermittent and chronic bouts of hypoxia has profound affects on vascular growth (Adair *et al.*, 1987; Adair *et al.*, 1988). Similarly, work on the hormone erythropoetin show that

hypoxia can act as an important stimulus of this gene, thereby affording a functional increase in O_2 transport by increasing the number of circulating erythrocytes (Fandrey, 1995).

Although these studies independently suggest that gas exchange would benefit from the concomitant changes in both morphology and physiology, few studies have evaluated the cardio-metabolic response chronic alteration of O_2 content and pressure of the inspired medium, and therefore presumably arterial blood C_{O_2}, and its physiological effects on the cardiovascular and respiratory system.

The purpose of this paper was to evaluate the role of modulating both O_2 content (C_{O_2}) and pressure in uptake and transport via the cardiovascular system through development utilizing the embryos of the African clawed frog, *Xenopus laevis*. Our goal was to elucidate the dependence of O_2 uptake and convective blood flow on either pressure or content, or both. First, we evaluated aerobic metabolism which was used to indicate the respiratory state. Second, we measured cardiac performance (heart rate, stroke volume, cardiac output) to determine the extent of limitations on cardiac function. Lastly, we determined cardio-metabolic coordination which indicates the relative contribution of cardiac function to oxygen uptake. This was achieved by rearing animals from eggs to metamorphic climax in environments containing normal air, 0.5 and 1.5 times O_2 saturated air. Additionally, we functionally ablated larval Hb through chronic

exposure to 2 kPa carbon monoxide (CO) both at normoxic and hyperoxic pressures.

Disruption of Hb with CO has been utilized by Holeton, (1971b); and Pelster and Burggren, (1996) in fish, and Cirotto and Arangi (1989) in embryonic chickens. However, these studies used acute rather than chronic exposure. By rearing larvae in CO , we can determine the role of the cardiovascular system in oxygen uptake, and test the following hypotheses: 1) first, that the limitations to gas transport are not solely limited by diffusion; 2) second, that perfusion is not a significant impediment to gas exchange in developing embryos; and 3) that bulk oxygen transport via Hb is not essential to support resting metabolism.

Materials and Methods

Experimental Animals. Fertilized eggs were obtained from the breeding in our laboratory of four adult female *Xenopus laevis,* according to Thompson and Franks (1978). Newly laid eggs were collected and evenly separated into five 25 Liter holding tanks, where they were maintained in dechlorinated tap water at 24 $\pm$ 0.2 ^{o}C.

Experimental Conditions. Experimental tanks were equilibrated for the whole developmental period with one of five gas mixtures: 1) normoxia: 21kPa O_2/ 79kPa N_2; 2) hypoxia: 11kPa O_2/ 89kPa N_2 3) hyperoxia: 35kPa O_2/ 65kPa N_2; 4) normoxia+CO: 21kPa O_2/ 2kPa CO/ 77kPa N_2; and 5) hyperoxia+CO: 35kPa O_2/

2kPa CO/ 79kPa N_2. The carbon monoxide gas mixtures were achieved with a Cameron GF-4 gas mixing flowmeter. All other gas combinations were obtained by volumetric mixing using flow meters (Cole Palmer Inc.). Animals were fed Nasco frog brittle™ (Nasco Inc.) *ad lib.* during the course of development, and fasted 24 hours prior to measurements.

Grouping of Developmental Stages. Animals were staged according to the NF staging system (Nieuwkoop and Faber, 1967). Experiments were conducted on *X. laevis* ranging from NF 1 to pre-metamorphic climax (NF 54). Animals were grouped into 9 different ontogenic categories which are as follows: 1-24, 25-34, 35-41, 42-43, 44-45, 46-47, 48-49, 50-51, and 52-54. Animals were selected on major morphological and physiological landmarks which delineated key windows of development to be observed. For a complete discussion regarding group of developmental stages see Nieuwkoop and Faber (1967) and Burggren and Just (1992).

Experimental Procedures. Animals randomly chosen from each condition were sampled for oxygen consumption ($\dot{M}_{O_2}$), wet mass, heart rate, total ventricular volume at end diastole and end systole, and stroke volume. O_2 consumption/transport quotient, and cardiac output were also calculated.

Aerobic metabolism was determine according to Territo (1996; see Chapter 3). Briefly, aerial and aquatic O_2 consumption ($\dot{M}_{O_2}$) were determined by

closed system respirometry, which consisted of a 20 ml vial filled with dechlorinated tap water, and sealed with an aluminum cap and double Viton® O-rings. The total gas volume of the system equaled 7 ml, and chambers were placed in a water bath thermostatted at 24 ± 0.2 °C. The length of the measurement period varied based on NF stage and number of animals required to achieve an 3 kPa drop in O_2; although, in most cases, animals were sampled for periods which extended through one diel cycle. Upon closure of the system, and at the conclusion of the experimental period, a 1 ml sample of gas was analyzed for O_2, with an Ametek™ S3A/I Oxygen Analyzer.

$\dot{M}_{O_2}$ was calculated as follows:

$$\dot{M}_{O_2} = \frac{(\%O_{2_i} - \%O_{2_f}) \cdot (P_b - P_{H_2O}) \cdot \beta_a \cdot V_g}{100 \cdot M_w \cdot T} \qquad (1)$$

where,

$\%O_{2_i}$ and $\%O_{2_f}$ are the initial and final percent O_2 in the respirometer, as read by the Ametek oxygen analyzer.

P_b is the barometric pressure in kPa,

P_{H_2O} is the water vapor at given temperature in °C,

β_a is the capacitance of O_2 in air in $nmol \cdot L^{-1} \cdot kPa^{-1}$,

V_g is the volume of gas in the aerial fraction in L,

M_w is the animal wet mass in mg,

and T is the time in hours.

2. Mass Determination: Individual wet mass was determined according to the procedures outlined in Chapter 3. In most cases, several animals were pooled for $\dot{M}_{O_2}$, and therefore individual mass' were determined by averaging over the total mass.

3. Heart rate (f_h), stroke volumes (SV), and cardiac output ($\dot{Q}$): Animals were sampled as per Chapter 3. Briefly, animals were videotaped for 30 seconds (120 frames·sec^{-1}) and were recorded on a Panasonic® sVHS video tape recorder and stored for later analysis. f_h was obtained by the number of beats in a given time and SV were determined based on the difference between total ventricular volumes at end diastole and end systole, and were calculated using Optimas® software (BioScan Inc.). Individual cardiac outputs were determined by the product of f_h and SV.

4. Cardio-metabolic quotient: An index of the relationship between aerobic metabolism ($\dot{V}_{O_2}$) and blood O_2 transport ($\dot{Q}_{O_2}$), which we term cardio-metabolic quotient, and was calculated as follows:

$$\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1} = \frac{\dot{V}_{O_2}}{\dot{Q} \cdot C_{O_2}} \qquad (2)$$

such that,

$\dot{V}_{O_2}$, is oxygen consumption in ml $O_2 \cdot g^{-1} \cdot h^{-1}$,

C_{O_2}, is the content of blood at complete saturation expressed in

ml $O_2 \cdot 100$ml blood^{-1},

and $\dot{Q}$, is total cardiac output in ml blood$\cdot g^{-1} \cdot h^{-1}$.

$\dot{V}_{O_2}$ was calculated from transformed $\dot{M}_{O_2}$ data (Dejours, 1981) to allow for direct comparison between O_2 uptake and $\dot{Q}$ in equivalent units. Normoxia, hyperoxia, hyperoxia+CO, and normoxia+CO C_{O_2} were obtained from hematology data (Territo, 1996, Chapter 2), were hypoxic values were estimated from Hill transformed equations of adult blood (Boutilier *et al.*, 1987).

Statistical Analyses: Effects of experimental treatment upon $\dot{M}_{O_2}$ were compared for covariance with wet mass. To meet the assumptions of the MANCOVA, all variables were $\log_{10}$ transformed. MANCOVA (1993 BMDP-VAX-mainframe) determines 1) the correlation coefficient, 2) equation of the line describing the relationship, and 3) the probability that the slope of the line is not significantly different from zero. f_h, SV, $\dot{Q}$ and $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ were all tested for significant differences with development and between treatments utilizing a Brown-Forsyth MANOVA. If significant differences were seen, a Tukey's (HSD) multiple range test was used to determine which points were different within, and between, the experimental groups. All values reported as mean $\pm$ 1 standard error of the mean (SEM). The fiduciary level of significance was taken at $P \leq 0.05$.

Results

Wet Mass:

Developmental stage had a significant effect ($p \leq 0.05$) on wet mass in all groups tested, and showed an exponential growth pattern between NF stages 1 and 51; however exposure all experimental conditions did not significantly ($p=0.08$) influence wet mass. All normoxic stages were significantly ($p \leq 0.05$) different than their previous stage range except NF 1-24, 25-34, and 35-41. Hyperoxic animals showed a clear stage specific response with all stage, except NF 1 to 41, displaying a significant ($p<0.05$) increase in wet mass from their previous stage (Table 1). All hyperoxic animals showed a significant ($p<0.05$) increase in wet mass between progressive stages. All hyperoxia+CO animals, except NF stages 42-43 and 44-45, showed a clear stage specific response with wet mass, and were significantly different than their previous stage range. normoxic+CO, like hyperoxic animals showed a significant stage by stage increase in all stage ranges tested.

Developmental changes in $\dot{M}_{O_2}$ *:*

Wet mass and development in normoxic animals significantly covaried with $\dot{M}_{O_2}$ embryos (NF 1-24; $r^2=0.99$, $p \leq 0.05$), and larvae (NF 25-51; $r^2=0.83$, $p \leq 0.05$) of *Xenopus*. The changes seen in O_2 consumption with embryos increased almost isometrically ($\dot{M}_{O_2}=-1.52M^{0.98}$) with $\dot{M}_{O_2}$ increasing a full order of magnitude over a range in wet mass from 0.3 to 3 mg (Fig. 1, *inset*). Similarly,

$\dot{M}_{O_2}$ in larvae, NF stages 25 to 51, showed a 150 fold increase in O_2 consumption over an increase in wet mass from 0.8 mg to 100 mg, and was described by the equation, $\dot{M}_{O_2}=-0.92M^{1.14}$ (Fig. 1). Post hoc analysis indicated that stages 25-34, 35-41 and 48-49 were significantly different ($p\leq0.05$) than their preceding stages (Table 2).

Similar to normoxia, chronically hypoxic embryos showed a near 10 fold increase in $\dot{M}_{O_2}$ over an increase in wet mass from 0.7 to 2.2 mg, and showed a allometric trend which was described by the equation $\dot{M}_{O_2}=-1.6M^{0.83}$. Larvae increased O_2 consumption from a low of 1.29 nmol.h^{-1} at stage NF 25-34 to a high of 55.83 nmol.h^{-1} (NF 50-51). This progressive increase in $\dot{M}_{O_2}$ with wet mass was depicted by the equation $\dot{M}_{O_2}=-0.89M^{1.17}$ (Table 3). Neither, embryos or larvae were significantly different from control (normoxic) animals ($p=0.10$). However, both embryos ($r^2=0.98$, $p\leq0.05$) and larvae ($r^2=0.86$, $p\leq0.05$) did significantly covary with wet mass and stage. Stage-specific analysis indicated that all stages were significantly ($p\leq0.05$) different than the stage which preceded them, with the exception NF 50-51.

Hyperoxic, like hypoxic, animals showed a strong covariance with wet mass and development in embryos ($r^2=0.99$, $p\leq0.05$) and larvae ($r^2=0.84$, $p\leq0.05$) and were described by the formulas, $\dot{M}_{O_2}=-1.58M^{1.12}$ and $\dot{M}_{O_2}=-0.86M^{1.19}$ respectively. $\dot{M}_{O_2}$ in embryos increased 4 fold from 0.02 nmol.h^{-1} to

0.08 nmol.h^{-1} over an increase in mass of 1.8 mg (Fig. 1, *inset*). Larvae similarly increased $\dot{M}_{O_2}$ from 0.13 nmol.h^{-1} (NF 25-34) to 44.67 nmol.h^{-1} (NF 50-51) which spanned a mass range from 0.7 to 80.2 mg. All hyperoxic animals, from NF 1 to 51, were not significantly different ($p \leq 0.05$) from control animals, although they did show a developmental stage response (Table 2).

Hyperoxia+CO embryos and larvae displayed a significant ($p \leq 0.05$) allometric trend with wet mass seen in normoxic animals, and were described the equations $\dot{M}_{O_2} = -1.60M^{1.14}$ ($r^2=0.97$) and $\dot{M}_{O_2} = -0.80M^{1.11}$ ($r^2=0.89$) (Table 3). Embryonic $\dot{M}_{O_2}$ spanned a range from 0.05 nmol.h^{-1} to 0.11 nmol.h^{-1} while mass increased only 4 fold. By contrast, larval O_2 uptake increased a full order of magnitude over a range in mass from 1.49 mg (NF 25-34) to 88.2 mg at NF 50-51. Animals exposed to hyperoxia+CO, like hypoxia, showed no treatment effects; however there was a significant effect of development on $\dot{M}_{O_2}$ (Table 2). Stage-specific analysis indicated that all stages from 1 to 47 were significantly ($p \leq 0.05$) different than the preceding stage (Table 2). Stages NF 48-49, and 50-51 did not however, show this trend.

Both embryos and larval animals exposed to Normoxia+CO displayed a strong covariance with wet mass and development. Embryos and larvae both showed strong allometric trends and were described the formulas $\dot{M}_{O_2} = -1.54M^{0.87}$ ($r^2=0.98$) and $\dot{M}_{O_2} = -0.88M^{1.20}$ ($r^2=0.84$) (Fig 1). $\dot{M}_{O_2}$ in embryos increased 4 fold over a wet mass increase of 1.1 mg. Larvae, on the other hand, increased O_2

consumption by an order of magnitude with a change in wet mass from 1.56 mg to 98.9 mg. Stage-specific analysis revealed that all stage, except NF 46-47 were significantly different than their preceding stage (Table 2).

Cardiac output:

Normoxic animals showed a statistically significant ($p \leq 0.05$) exponential increase in $\dot{Q}$ with progressive development. Total flow increased by a near doubling with each successive stage from NF 33-34 to 39-42. Animals between NF 39 and 45, however increased $\dot{Q}$ from 227.5±0.5 to 1326.9±3.6 nl.min^{-1} (Fig 2A). Between stages NF 43-45 to 46-47 $\dot{Q}$ increased by more than 2 times over a change in wet mass of 3 mg. Cardiac output between NF 46 to 49, unlike the preceding stages, showed a clear reduction from 2961.3±10.3 to 1768.7±4.5 nl.min^{-1}. This reduction in flow was followed by a tripling of $\dot{Q}$ at each stage from NF stage 48 to 51 where it reached final value of 48547.6 ± 1770.7 nl.min^{-1}. All stages were found to be significantly different than the stage which preceded them (Fig. 2A).

Hypoxic animals, like normoxic, showed a significant ($p \leq 0.05$) progressive rise in $\dot{Q}$ with development. However, animals between NF stages 33 to 38 did not show an increase in $\dot{Q}$, where flow ranged from 69.1 ± 0.2 to 82.9 ± 0.2 nl.min^{-1} . Flow did however, show a 2.3 fold increase in animals between NF 35 to 42. Similar to normoxic animals, $\dot{Q}$ increased nearly a full order of magnitude

in hypoxic larvae, from 187.5 ± 1.4 nl.min to 1371.9 ± 2.8 nl.min^{-1} (Fig. 2A). This was followed by a much smaller increase (867 nl.min^{-1}) in total $\dot{Q}$ over a change in wet mass from 5.7 mg. Similar to normoxic animals, hypoxic larvae showed a slight fall in flow from 2328.2 ± 9.2 to 1909.8 ± 16.4 nl.min^{-1} between stages 46 to 49. Over the next three stage ranges, $\dot{Q}$ increased from 1873 ± 19.1 nl.min^{-1} to a final value 31853.0 ± 324.6 nl.min^{-1}. All stage ranges, except 35-38 and 48-49, showed a significant ($p \leq 0.05$) stage-dependent increase in $\dot{Q}$. However these data were not significantly different than controls ($p=0.09$).

Hyperoxic larvae, unlike hypoxic animals, showed a dramatic rise (100.7 nl.min^{-1}) in $\dot{Q}$ over the stage range NF 33 to 38. This was followed by an increase in $\dot{Q}$ which ranged from 166.4 ± 0.9 nl.min^{-1} to 259.3 ± 1.0 nl.min^{-1} between NF stages 35 and 42. Between stages 46 and 49 $\dot{Q}$ showed a significant ($p \leq 0.05$) drop where it reached a new value of 1159.1 ± 2.3 nl.min^{-1}. This fall in $\dot{Q}$ was followed by a 33 fold increase in flow over the next two stage ranges (NF 48 to 54). Like normoxic animals, hyperoxic larvae showed a consistent stage-specific response in total flow (Fig. 2A). Hyperoxic larvae were not significantly ($p=0.09$) different than normoxia.

Animals exposed to hyperoxia+CO showed the same significant ($p \leq 0.05$) exponential rise in $\dot{Q}$ with progressive development seen in normoxic animals. Larvae between stages NF 33 to 38 showed a modest rise in $\dot{Q}$ with flow

ranging from 89.2 ± 0.8 to 101.3 ± 0.4 nl.min^{-1} . Flow more than doubled over the next stage range (NF 39 to 45, Fig. 2B), where it was followed by a 6 fold increase which occurred without a change in wet mass (Table 1). Interestingly, $\dot{Q}$ between NF stages 43 and 47 increased by 2.4 fold while mass nearly tripled. Larvae showed a consistent decline in flow over the next stage range (NF 46 to 49) where $\dot{Q}$ achieved a new value of 745.8 ± 5.6 nl.min^{-1}. Unlike normoxic animals, hyperoxia+CO larvae showed a 66 fold increase in flow over the next three stage ranges (NF 48 to 54), by comparison to the 30+ fold increase seen in controls. All stage ranges except 46-47 showed a strong stage specific response in $\dot{Q}$ relative to the previous stage grouping. Hyperoxia+CO animals were significantly different ($p \leq 0.05$) than controls.

Normoxic+CO larvae showed a significant ($p \leq 0.05$) increasing trend in $\dot{Q}$ with progressive development. $\dot{Q}$ showed a near doubling at each stage over the range NF 33 to 42. Between stages NF 39 and 45, flow sharply increased from 310.6 ± 1.3 to 1354.7 ± 3.9 nl.min^{-1}. This was followed by a moderate increase (270.8 nl.min^{-1}) in $\dot{Q}$ between stages NF 43 to 47 . Flow decreased over the next stage range (NF 46 to 49) to a new value of 622.7 ± 3.5 nl.min^{-1}. Over the next three stage ranges (NF 48 to 54) flow increased by 71 fold (Fig. 2B). Exposure to normoxic+CO was significantly different than controls. Post-hoc analysis indicated that all groupings, except NF 46-47, were significantly ($p \leq 0.05$) different than their preceding stage.

Heart rate:

Heart rate in all groups tested displayed a significant ($p \leq 0.05$) curvilinear relationship with progressive development. Although there was a relationship between development and f_h, treatment did not significantly influence f_h ($p=0.99$).

Normoxic animals showed a progressive rise in f_h from a low of 58 $\pm$ 2 bpm to a high of 184 $\pm$ 4 bpm. This initial rise was followed by a plateau between NF stages 48 and 51, where f_h was 181 $\pm$ 5 bpm. Heart rate fell 1.5 fold over the next stage range (NF 50 to 54). A post-hoc analysis of development revealed that only NF 43-45, 48-49, and 52-54 were significantly different than the stage which preceded them (Fig. 3).

Animals exposed to hypoxia showed a 2.1 fold rise in rate between NF 33 and 49. Unlike normoxic animals, hypoxic larvae showed an increase of 1.1 fold in f_h over the next two stage ranges (NF 48 to 51), where it was followed by a 1.3 fold decrease in f_h between NF 50 to 54 (Fig. 3). Hypoxic animals showed a significant ($p \leq 0.05$) stage-dependent increase in f_h between most NF stage groupings, although NF stages 39-42, 46-47, and 50-51 were not found to be significantly different than their preceding stage.

Hyperoxic reared larvae ranged in f_h from 82 $\pm$ 2 bpm at NF 33/34 to a high of 192 $\pm$ 9 bpm at NF stage 48-49. This was followed by a slight drop in f_h

of 13 bpm to a new value of 179 ± 3 bpm over the next stage range. Similar to normoxic animals, f_h fell 1.5 fold to a new low of 118 ± 7 bpm. f_h at 43-45, 48-49, and 52-54 was significantly different ($p \leq 0.05$) than the stage which preceded each grouping.

Hyperoxic+CO larvae, like all other treatments, showed a 2 fold increase in f_h from NF 33 to 49. Heart rate in hyperoxic+CO animals plateaued between NF 48 and 51 where f_h reached a new value of 160 ± 9 bpm (Fig 3). This was followed by a decrease in f_h of 42 bpm at NF 52-54, where it achieved a value of 119 ± 6 bpm. As with hypoxic animals, post-hoc comparisons of f_h with development revealed that hyperoxic+CO larvae showed significant ($p \leq 0.05$) increase in rate at NF stages 43-45, 48-49, and 52-54.

Normoxic+CO animals increased f_h by 2 fold over the stage range from NF 33 to 49, and ranged from a low of 85 ± 6 bpm to a maximum of 176 ± 6 bpm. Animals between NF stages 48 and 51 showed a slight fall in f_h, which followed by a further reduction in f_h of 37 bpm to a final value of 122 ± 7 bpm. Normoxic+CO larvae, like hyperoxic+CO animals, showed a significant ($p \leq 0.05$) response between stages at NF 43-45, 48-49, and 52-54.

Stroke volume:

Stroke volume in all groups tested increased in an exponential manor with development, and was found to be significantly ($p \leq 0.05$) affected by ontogenic stage. Like $\dot{Q}$, SV was found to change with experimental treatment, however

these changes were only seen in animals exposed to hyperoxia+CO and normoxia+CO.

Normoxic larvae showed a consistent increase in SV between NF stages 34 to 47, as was evident from the 16 fold increase over a change in wet from 2.2 $\pm$ 0.2 mg to 12.6 $\pm$ 0.5 mg. Over the next three stage ranges (NF 40 to 54) stroke volume showed a 64 fold increase in SV (Table 1). All stage ranges, except NF 35-41, showed a significant ($p \leq 0.05$) stepwise increase in SV at each successive NF grouping.

Hypoxic animals, like normoxic ones, showed a significant increase between NF stages 34 and 47, where individual SV increased by more than one order of magnitude (Table 1). Stroke volume decreased in hypoxic larvae by 1.5 times between NF 46 and 49. This decrease in SV was followed by a 40 fold increase in SV over the NF stage range 48 to 54. Like normoxic animals, hypoxic larvae showed a significant change from the preceding stage in all NF grouping, except NF 35-41.

Animals exposed to hyperoxia showed a consistent significant increase in SV from 0.9 $\pm$ 0.2 nl (NF 33-34) to 25.1 $\pm$ 3.4 nl (NF 46-47). This was followed by an 0.8 fold reduction in volume between NF 46 and 49, while wet mass nearly doubled over this same range. Stroke volume however, increase by more than 65 times over the next three stage ranges (NF 48 to 54). Post-hoc tests reveled that all stages were significantly ($p \leq 0.05$) different than the stage which preceded them.

Similar to hyperoxic animals, hyperoxic+CO larvae, showed a 25 fold increase in stroke volume over the first four stage ranges (NF 33 to 47). This increase which initially peaked at 26.5 ± 2.9 nl and fell to 5.7 ± 0.9 nl by NF 48-49. This reduction in volume was followed by a 133 fold increase, where SV reached a new value of 795.3 ± 140.3 nl. All stages groupings from NF 33 to 54 were significantly different than the previous stage. Additionally, hyperoxic+CO larvae from NF 44 to 54 were significantly ($p \leq 0.05$) different from controls.

Normoxic+CO animals ranged in SV from a low of 1.1 ± 0.1 nl (NF 33/34) to a high 862.8 ± 109.1 nl at NF 52-54. Stoke volume increased by more than one order of magnitude over the first 5 stage ranges (NF 33 to 47), where it reached a new value of 12.0 ± 1.4 nl. Like all other experimental conditions, SV fell by 2.4 times between NF 46 and 49, prior to increasing 172 fold by stage NF 52-54. All NF stage groupings were significantly different ($p \leq 0.05$) than the stage which preceded them. Moreover, SV was significantly different than normoxic larvae at NF stages 35-41, and 46 to 54.

O_2 Consumption/Transport quotient:

$\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratios indicated the relative contribution of perfusive conductance to total O_2 uptake. Both $\dot{V}_{O_2}$ and $\dot{Q}_{O_2}$ are expressed $\mu l\ O_2 \cdot g^{-1} \cdot h^{-1}$, thus making this ratio unitless. A value of 0.25 indicates a relative coupling between the respiratory and cardiovascular systems with 25% extraction, where values approaching zero indicate a relative over perfusion relative to total O_2

consumption for a complete discussion see Chapter 3. Values above 0.25 indicates that O_2 uptake occur with little, or no, contribution by total flow. It should be noted that $\dot{Q}_{O_2}$ indicates the maximal perfusive conductance, and therefore maximal O_2 available for uptake via perfusion.

Cardio-metabolic quotient in all animals was significantly ($p \leq 0.05$) influenced by developmental stage; while hyperoxic+CO and normoxic+CO larvae were the only conditions which were significantly ($p \leq 0.05$) affected by treatment. All normoxic, hypoxic, and hyperoxic animals were below the theoretical cardio-metabolic coupling threshold of 0.25. Hyperoxic+CO and normoxic+CO larvae both started well above 0.25 and fell to the theoretical isoline by stage NF 42-43, where they remained for most of development.

$\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio in normoxic animals showed the greatest decline (0.22 to 0.02 units) between NF 25 and 43 (Fig. 4A). Animals between NF 42 and 51, however showed no change in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ which ranged from 0.02 $\pm$ 0.003 to 0.02 $\pm$ 0.005 units. Larvae, at NF 35-41, and 42-43 were the only stages which were significantly ($p \leq 0.05$) different than the stage which preceded each grouping.

Hypoxic animals, like normoxic larvae, showed a precipitous fall in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ from 0.35 $\pm$ 0.07 to 0.02 $\pm$ 0.005 units over the stage range from 25 to 43. This was followed by a plateau between stages NF 42 and 45 where $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ only changed 0.01 units. Between NF 45 and 47 however, $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ values decreased by 50% to a new value of 0.017 $\pm$ 0.002 (Fig 4A). Larvae over

the next stage range (NF 46 to 49) increases by 1.7 fold, prior to falling to a final value of 0.02 ± 0.01 at NF 50-51.

Animals raised under hyperoxia, like normoxic larvae, showed a similar decrease (28.1 fold) between NF 25 and 43 (Fig. 4A). This dramatic reduction in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ was followed by a constant ratio between NF 42 and 47. By contrast, animals over the stage range NF 46 to 49 showed almost a full order of magnitude increase to a new value of 0.09 ± 0.001 units. $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio in larvae over the next stage range (NF 48 to 51) decreased to 0.01 ± 0.008 units. Stages NF 42-43 and 48-49 were the only groupings which were found to significantly different than the stage which preceded them.

Hyperoxic+CO larvae, like hyperoxia, showed a dramatic fall in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ from NF 25 to 43, although the absolute change was greater. This ranged from 4.19 ± 0.6 to 0.13 ± 0.01 units (hyperoxic+CO) compared with the 0.29 ± 0.08 to 0.01 ± 0.006 seen in hyperoxia. Animals over the next stage range (NF 42 to 45) decreased only slightly to 0.10 ± 0.007 units. $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ increased by more than one order of magnitude between NF 46 and 49, prior to falling to 0.43 ± 0.17 at NF 50-51. All stages, except 44-45, were significantly different than the preceding stage range. Treatment has a significant ($p \leq 0.05$) effect on $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio at all stages tested (Fig 4B).

Larvae raised under normoxic+CO conditions showed the same abrupt decrease in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio seen in hyperoxic+CO animals between NF 25 and

43, although the magnitude of the change was only 17 fold as compared to the 39 fold seen in normoxic larvae. Normoxic+CO larvae over the next stage range (NF 42 to 45) showed a decrease of 1.3 fold in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio to 0.22 ± 0.03 units. Over the next two stage range cardio-metabolic quotient increased 4.4 fold, before falling to 0.22 ± 0.05. All stage ranges were significantly influenced by treatment, however only NF 35-41, 42-43, 48-49, and 50-51 were found to be significantly different than the stage which preceded them.

Discussion

Critique of method:

One possible criticism of this study could be the use of "mild" perturbations (i.e. hypoxia and hyperoxia at 0.5 and 1.5 times normal pressure of oxygen) to elicit changes in either cardiovascular or respiratory parameters. It is clear from the work of Burggren and Mwalukoma, (1983) and Pinder and Burggren (1983a) that mild hypoxia results in significant changes in respiratory structures of *Rana catesbieana* tadpoles. Complete discussion of how environmental factors such as temperature, O_2 tensions and acid/base status may affect physiology has been reviewed by Pelster (1997).

Another potential critique of this method may be that our study used blood O_2 dissociation curves from adult *Xenopus* (Boutilier *et al.*, 1987) to determine the effect of hypoxia on blood C_{O_2}. It is clear from work in amphibians that P_{50} increases with ontogeny and degree of terrestriality (Boutilier *et al.*,

1992; Pinder and Burggren, 1983a). Moreover, these studies also show that rearing larvae in chronic hypoxia results in a left shift of the equilibrium curve, thereby increasing the overall affinity of larval blood. Based on this we argue that utilizing adult curves would over-estimate blood C_{O_2}, and therefore $\dot{Q}_{O_2}$, making the $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ calculations more conservative overall.

Metabolism:

Although it has been speculated for some time that chronic environmental factors are key modulators of physiological function in lower vertebrates (Feder and Block, 1991; Shoemaker, 1988), these claims have yet to be demonstrated for the cardiovascular and respiratory systems in an ontogenic context. Thus, modulating both P_{O_2} and C_{O_2} simultaneously (hypoxia and hyperoxia) would result in different levels of metabolism, if metabolism was dependent on a coupled response of both perfusion and diffusion. Similarly, elimination of Hb-O_2 transport should disrupt metabolism if this was entirely dependent on O_2 convection via the cardiovascular system (normoxic+CO). Elimination of C_{O_2} coupled with an increase in P_{O_2} (hyperoxic+CO) should restore the levels of aerobic metabolism if this process was reliant on diffusion alone.

Analysis of the allometric data indicated that there was no clear difference in $\dot{M}_{O_2}$ in early embryos (NF 1-25) between treatments, and this was evident from the slopes of the equations (Table 3) which were all about a value of 1. For

later stages, $\dot{M}_{O_2}$ increased in a linear fashion with wet mass, but was not significantly affected by experimental condition. The allometric rise in $\dot{M}_{O_2}$ (b=1.14, normoxia; b=1.17, hypoxia; b=1.19, hyperoxia; b=1.14, hyperoxia+CO; and b=1.20, normoxia+CO) is higher than the (b=0.85) value reported by Feder, (1981). A possible reason for the difference between the two studies could be that fact that Feder (1981) restricted the scope of the development stages observed, where our study covers development from very early stages to metamorphic climax animals. The slopes seen in this study are slightly different than those reported in our previous work (Territo, 1996; Chapter 3). These differences arise from the method of stage grouping we chose in this study, which emphasizes both early and mid-developmental stages, as compared to our previous work which spanned a much larger range of mass' and developmental stages, and tended to look at all stage ranges from eggs to metamorphic climax. This larger span of developmental stages and mass, especially late in development, tends to have a weighting effect on the slope of the allometric curve, and is a function of the much lower mass specific metabolic rate seen in larger animals

Aerobic metabolism increased in an exponential fashion during early life (NF 1-24) regardless of the experimental condition, and that $\dot{M}_{O_2}$ increased by a full order of magnitude in both hypoxic and hyperoxic embryos, shows that neither C_{O_2} or P_{O_2} are significant barriers to uptake. Similarly, embryos raised

under hyperoxic+CO and normoxic+CO showed the same degree of increase in metabolism as both hypoxic and hyperoxic animals. This indicates that newly forming blood elements play a negligible role in bulk O_2 transport over these stages, and that diffusion in fact must be the major mode of conductance.

The fact that the $\dot{M}_{O_2}$ increased normally, in spite of the modulation of C_{O_2} and P_{O_2}, suggest that the cardiovascular and respiratory systems may not be coupled in terms of O_2 uptake. Moreover, our results indicate the elimination of blood C_{O_2}, with normal and 1.5x P_{O_2}, did not significantly alter $\dot{M}_{O_2}$, suggesting that neither perfusion, or diffusion alone, were the limiting factors in total O_2 consumption. These findings gain support from observation and descriptions of mutant strains of *Xenopus* which lack formed blood elements (de Graaf, 1957; Ewer, 1959) have described. The existence of such biological anomalies is highly suggestive that the cardiovascular system can operate under highly impaired conditions. Work by Mellish *et al.* (1995) have shown in mutant axolotls that $\dot{M}_{O_2}$ was not significantly affected. Additionally, they also demonstrated that *Ambystoma mexicanum* larvae exposed to CO and cardiac ablated animals when compared to controls maintained normal $\dot{M}_{O_2}$ over a large range of P_{O_2}s. Similarly, work completed by Flores and Frieden (1969) further supports the contention that the cardiovascular system plays a highly reduced role in gas exchange, in which they demonstrated that functionally ablated O_2 transport with phenylhydrazine (PHZ) in *Rana catesbieana* tadpoles had no

significant effects on survivorship. Moreover our previous work (Territo, 1996; Chapter 3), has shown that elimination of Hb-O_2 transport via CO does not significantly affect $\dot{M}_{O_2}$ or whole body lactate.

By contrast, Burggren and Mwalukoma (1983) have shown that exposure to chronic hypoxic and hyperoxic alters the morphology of the respiratory structures (skin, lungs), and they argue that these alterations would afford greater O_2 uptake during periods of reduced P_{O_2}. Furthermore, it has also been demonstrated that chronic hypoxia an hyperoxia alters the affinity of larval blood (Pinder and Burggren, 1983a; Pinder and Burggren, 1983b), suggesting that O_2 transport via convection could be maintained in spite of falling P_{O_2}. The need for augmented structures in *R. catesbieana* tadpoles may be due to the more than one and a half orders of magnitude difference in size between this species and *Xenopus*, and thus the increased diffusion distances associated with them. It is clear from our work in *Xenopus*, and the combined evidence in *Rana* that neither C_{O_2} nor P_{O_2} is a limiting factor in O_2 uptake. Moreover, these data suggest that amphibians are capable of surviving with minimal contribution of Hb to O_2 uptake, and that P_{O_2} is not limiting to diffusive conductance in the absence of Hb.

Cardiovascular function:

Although metabolism was unaffected by experimental treatment, this does not preclude the possibility that $\dot{Q}$ has been altered. Moreover, it is reasonable to assume that altering inspired C_{O_2} and P_{O_2} and elimination of C_{O_2} via CO would result in changes in $\dot{Q}$ to accommodate tissue metabolic demands (Pinder *et al.*, 1992). Additional support for this premise has come from the work of Holeton (1971a, 1971b) which demonstrated that exposure to moderate levels of CO increased f_h, and most likely $\dot{Q}$, thus increasing perfusive conductance in spite of the reduction in blood C_{O_2}.

$\dot{Q}$, unlike $\dot{M}_{O_2}$, was significantly influenced by treatment. Total cardiac output in all groups of animals increased by 1.5 orders of magnitude between NF 33/34 and the plateau at stage 46-47, which occurred over a change in wet mass of more than 10 mg. These findings are consistent with the work of Hou and Burggren (1995b), Orlando and Pinder (1995), and Territo (1996; Chapter 3). Between stages NF 46 and 49 $\dot{Q}$ was highly variable, no change in normoxic and hyperoxic animals, while all other groups showed a significant reduction in flow. The work of Hou and Burggren (1995b) has demonstrated that total peripheral resistance (TPR) decreases with progressive development, and that the greatest fall (700+ units) occurs between 3 and 20 mg in larvae of *Xenopus*, which they speculate was concomitant with an increase in total cross-sectional area. Additionally, Hou and Burggren (1995a) has demonstrated that mean

truncus pressure (P_t) increases marginally over a mass range which corresponds to NF 43 to 47. This was consistent with the work of Nieuwkoop and Faber (1967) which has shown that *Xenopus* larvae develop complex respiratory structures (gills , simple lungs), and initiate the use of the alimentary tract by stage 45.

Collectively, the coupled response of developing new parallel vascular beds, along with the lack of a significant rise in P_t could explain the fall in TPR observed by Hou and Burggren (1995b). Thus, the net effect would be a reduction in filling pressure and would therefore reduce SV through Starling effects. The plateau seen in our study over this range was in fact due to a modest reciprocal change in SV and f_h. These changes were consistent with our previous work (Territo, 1996) in which $\dot{Q}$ fell by more than 50% over this same stage range. $\dot{Q}$ between stages NF 33 to 42 in all CO exposed groups was significantly elevated when compared to controls. By contrast, flow over the next two stage ranges (NF 46 to 49) were lower in hyperoxic+CO and normoxic+CO animals by comparison to normoxia. Interestingly, both groups show a marked increase in vascular area in the skin and buccal cavity which was visually discernible by NF stage 43-45. Based on these observations, we argue TPR would fall thereby reducing venous filling pressure and consequently reducing $\dot{Q}$ by changes in SV. Interestingly, flow returned to near control levels for both hyperoxic+CO and normoxic+CO larvae between NF 50 and 54. Fritsche and Burggren (1996) have shown that regulation of cardiac function begins by NF

48-49 when exposed to hypoxia, and that these changes suggest of a Frank-Starling mechanism. Anatomical innervation of the ventricle in larval *Xenopus* occurs by NF 49-50. Combined, these data indicate that active cardiovascular regulation is probable from this stage (NF 49) forward. SV between NF 48 and 51 increased by at least one order of magnitude in both CO exposed groups. These data are highly suggestive that the changes observed were in fact due to active regulation of cardiac function. The absence of change in $\dot{Q}$ in both hypoxic and hyperoxic larvae indicates that the level of stress/augmentation (hypoxia/hyperoxia) was insufficient to meet the threshold stimulus required to elicit changes or that the gas exchange may be acting independent of convective transport.

Heart rate progressed in a curvilinear manor with development, and was not significantly affected by experimental condition (Fig. 3). The trends observed are in general agreement with previous work (Fritsche and Burggren, 1996; Hou and Burggren, 1995a; Orlando and Pinder, 1995; Territo, 1996). However the initial rise in f_h seen in our study was not observed by Hou and Burggren, (1995a) or Orlando and Pinder (1995). A possible explanation for these differences seen may be due to two observations. First, Orlando and Pinder, (1995) examined animals from NF 44 through 57, and in doing so may have missed the initial rise in f_h; secondly, Hou and Burggren (1995a) described their allometric data with a first order linear regression, and in doing so they may have missed the initial rise as well. Regardless, it is evident from our data that f_h is in

general agreement with the published literature, and was unaffected by chronic gas treatment.

The fact the $\dot{Q}$ showed a clear treatment effect in hyperoxic+CO and normoxic+CO, and the observation that f_h was unaffected by any experimental conditions, suggest that SV contributed to the subsequent changes seen in $\dot{Q}$. Although few studies exist for cardiovascular variables in response to chronic gases, it is clear from acute studies that bouts of hypoxia results in concomitant changes in $\dot{Q}$ which were largely due to changes in SV (Fritsche and Nilsson, 1990; Holeton, 1971b; Millard and Johansen, 1974; Wood and Shelton, 1980). (Pelster and Burggren, 1991) have shown that pre-load increases with development, and therefore would in result in augmented SV. Therefore, we suggest that exposure to CO both with normoxia and hyperoxia would result in an increase in cardiac SV both from development and arterial hypoxemia itself.

Stroke volume increased in a exponential fashion with progressive development, which was consistent with previously published findings (Fritsche and Burggren, 1996; Hou and Burggren, 1995b; Orlando and Pinder, 1995; Tang and Rovianen, 1996; Territo, 1996). Although, f_h was unaffected by treatment, SV in hyperoxic+CO and normoxic+CO exposed animals was significantly augmented. Both hyperoxic+CO (NF 44 to 54) and normoxic+CO (35-41, 46 to 54) stroke volumes showed a clear treatment effect.

Based on the changes seen in $\dot{Q}$ with development and experimental treatment, and the fact that the majority of the change seen in cardiac output are due to associated changes in SV, we suggest that the responses seen are due largely to Frank-Starling mechanisms. Furthermore these changes also suggest that reductions in arterial blood capacitance result in an augmentation of flow which is based largely on changes in cardiac stroke volume.

O_2 Consumption/Transport Coordination:

The regulation of gas uptake and transport during development has been studied extensively in fish (Perry and Wood, 1989; Rombough, 1988) and amphibians (Burggren and West, 1982; Feder and Burggren, 1985; Wassersug *et al.*, 1981; West and Burggren, 1983.). The importance of regulating both respiratory and cardiovascular function has profound influences on both blood gases and pH (Boutilier and Shelton, 1986a; Boutilier and Shelton, 1986b; Weintraub and MacKay, 1975). When extended to the context of development, the importance of regulating these mechanisms is enhanced, as greater coordination between uptake and distribution must meet the changing metabolic needs of the organism.

In the case of developing *Xenopus*, the regulation of O_2 uptake and transport must take place over an ever changing number of respiratory surfaces (e.g. skin, gills, and lungs). The analysis of blood flow to each of the respective exchange surfaces is beyond the scope this paper (see, Malvin, 1994).

However, the coordination of total respiratory gases relative to total perfusive conductance would give an indication of the relative amount of cardiac work that must be put forth in transporting a quantity of gas. Therefore, we examined at a unitless ratio of total O_2 uptake and maximal blood transport ($\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$) to evaluate the relative contribution of each to total substrate turnover. For a complete discussion on this topic see Territo (1996).

All larvae showed clear reduction in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio between NF 25 and 43, where it plateaued through most of development. A declining ratio suggests that their maximal $\dot{Q}_{O_2}$ far exceeds the total O_2 consumed, or that tissue O_2 extraction from the blood was very low, and therefore aerobic metabolism was occurring irrespective of convection. This pattern in $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ was paralleled in animals exposed to hyperoxic+CO or normoxic+CO, however these ratios were an order of magnitude above those seen in controls. The reason for this significant difference was due largely to the depressed blood C_{O_2}. These findings are similar our previous work (Territo, 1996), in which we argued that a high $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ value indicates that O_2 uptake was occurring via means other than convection alone.

Work in humans has shown that $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratios approach values of 0.25 (Taylor *et al.*, 1989), which implies that the cardiovascular and respiratory systems are coupled between O_2 uptake and distribution via the blood,

providing a 25% extraction. The fact that amphibians have $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratios well below a value of 0.25 would indicate a functional decoupling of perfusion and consumption according to mammalian paradigms. Amphibians, unlike mammals and fish, retain a pulmonary and systemic circuits which are in parallel. Moreover, significant gas exchange occurs by simple diffusion across the skin, in addition to convective transport via the blood. As a result, it is unlikely that animals would show a $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ ratio above 0.25 under normal circumstances, since uptake can, and does occur irrespective of $\dot{Q}$. It has been suggested though mechanistic models, and empirically verified, that amphibians would attain a $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ value approaching 0.25 at rest (Hillman, 1978.; Piiper and Scheid, 1975). These data indicate that gas exchange and subsequent extraction (arterial-venous difference) would play a reduced role in resting animals, and therefore diffusion would supply the remaining component of total O_2 uptake.

Clearly these data suggest that the cardiovascular system may play a reduced role in total O_2 uptake, and that $\dot{M}_{O_2}$ must be occurring by means other than by a tight coupling between the respiratory and systems. Mellish *et al.*, (1995) has shown that removal of heart primordia, and mutant *Ambystoma* axolotls which lack a functional heart, show no difference in $\dot{M}_{O_2}$ when compared to controls. They speculate that in the absence of convection, diffusion must be the major contributor to total uptake.

The role of diffusion in embryonic gas exchange:

Total $\dot{M}_{O_2}$ is set by two process of diffusion and perfusion both in parallel and in series. Our $\dot{M}_{O_2}$ data clearly shows that modulating either pressure alone, (CO exposed animals) or pressure and content simultaneously (hypoxic and hyperoxic larvae), did not significantly alter aerobic metabolism.

These data suggest that O_2 uptake is not limited by a coupled response of diffusion and convection, nor by diffusion alone. Moreover, these data also suggest that convection is not essential for normal metabolism, and that diffusion may be the sole means of O_2 uptake. Although these data are not surprising for embryos (NF 1-24), it is however, counter-intuitive that larvae (NF 25 to 51) showed no effects of chronic exposure to any gas combination.

It is clear from the absence of a treatment effect in $\dot{M}_{O_2}$ and the modest increases seen in $\dot{Q}$, that the cardiovascular system may not play a significant role in O_2 uptake and distribution, as has been also suggested in contemporary studies (Mellish *et al.*, 1995; Pelster and Burggren, 1996).

Our data indicates that the role of the cardiovascular system in early development may serve several purposes other than for O_2 distribution. Burggren and Warburton (1994) indicated that the formation of early f_h and pressure generation may in fact serve to dilate newly developing vascular beds. Furthermore, it has been argued that the role of early convective transport may be for nutrient distribution (Burggren and Territo, 1995). We contend that

whatever the role of embryonic circulation may be, it is clear that acquisition of O_2 via convection is not the limiting factor to growth or metabolism, and that the formation of accessory structures may be a secondary response to adverse environments.

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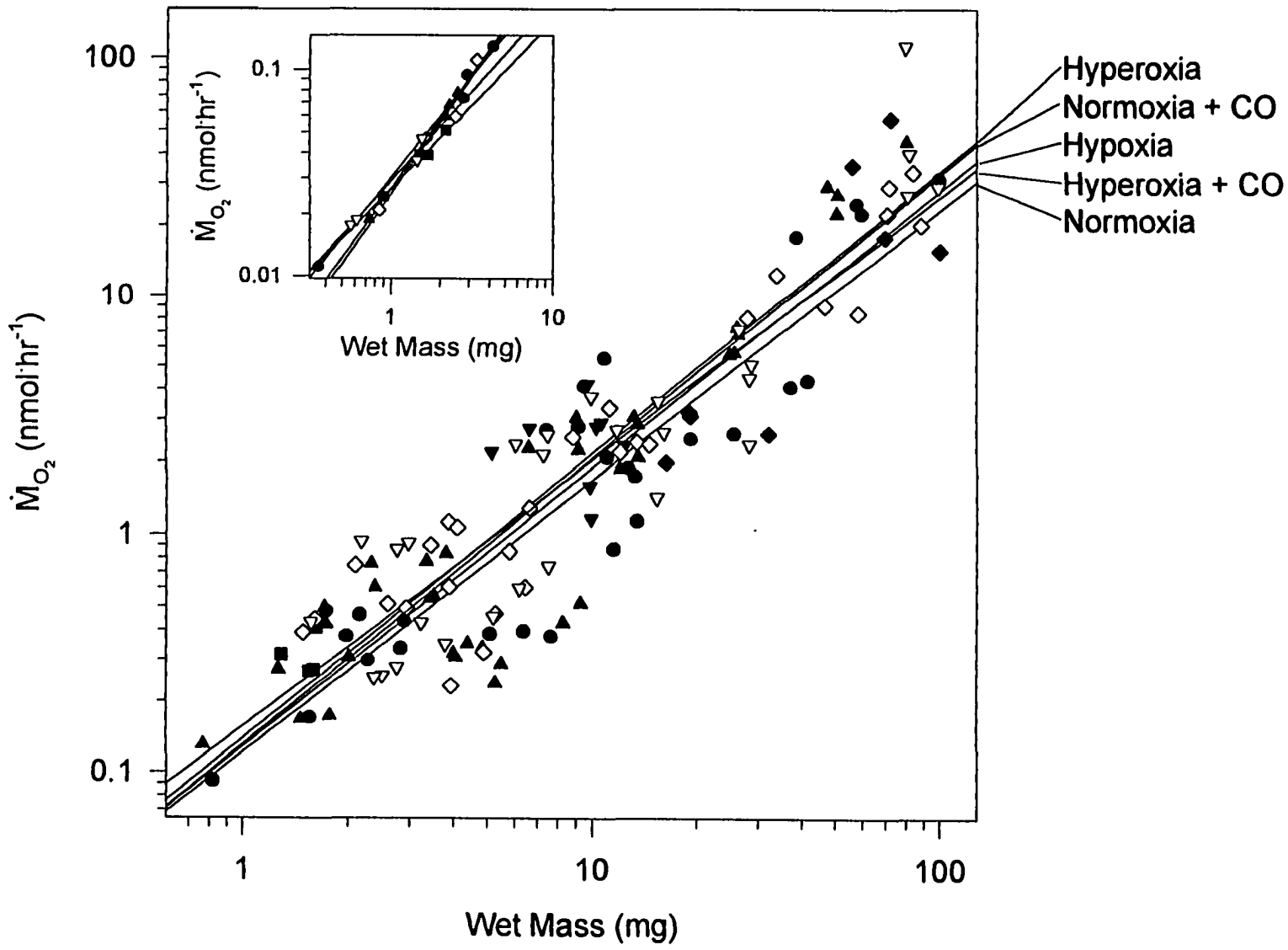
Figure 1. The relationship between $\dot{M}_{O_2}$ and wet mass in tadpoles of *Xenopus laevis* exposed to chronic normoxia with CO (open diamonds); chronic hyperoxia with CO (open triangles); chronic hypoxia (filled squares); chronic hyperoxia (filled triangles); and control animals exposed to normoxia (filled circles). Both oxygen consumption and wet mass were plotted on $\log_{10}$ scales. Regression lines indicate allometric trends for normoxia, hypoxia, hyperoxia, hyperoxia+CO, and normoxia+CO; results are presented in Table 3. The inset shows the data set for the smallest population of eggs and larvae.

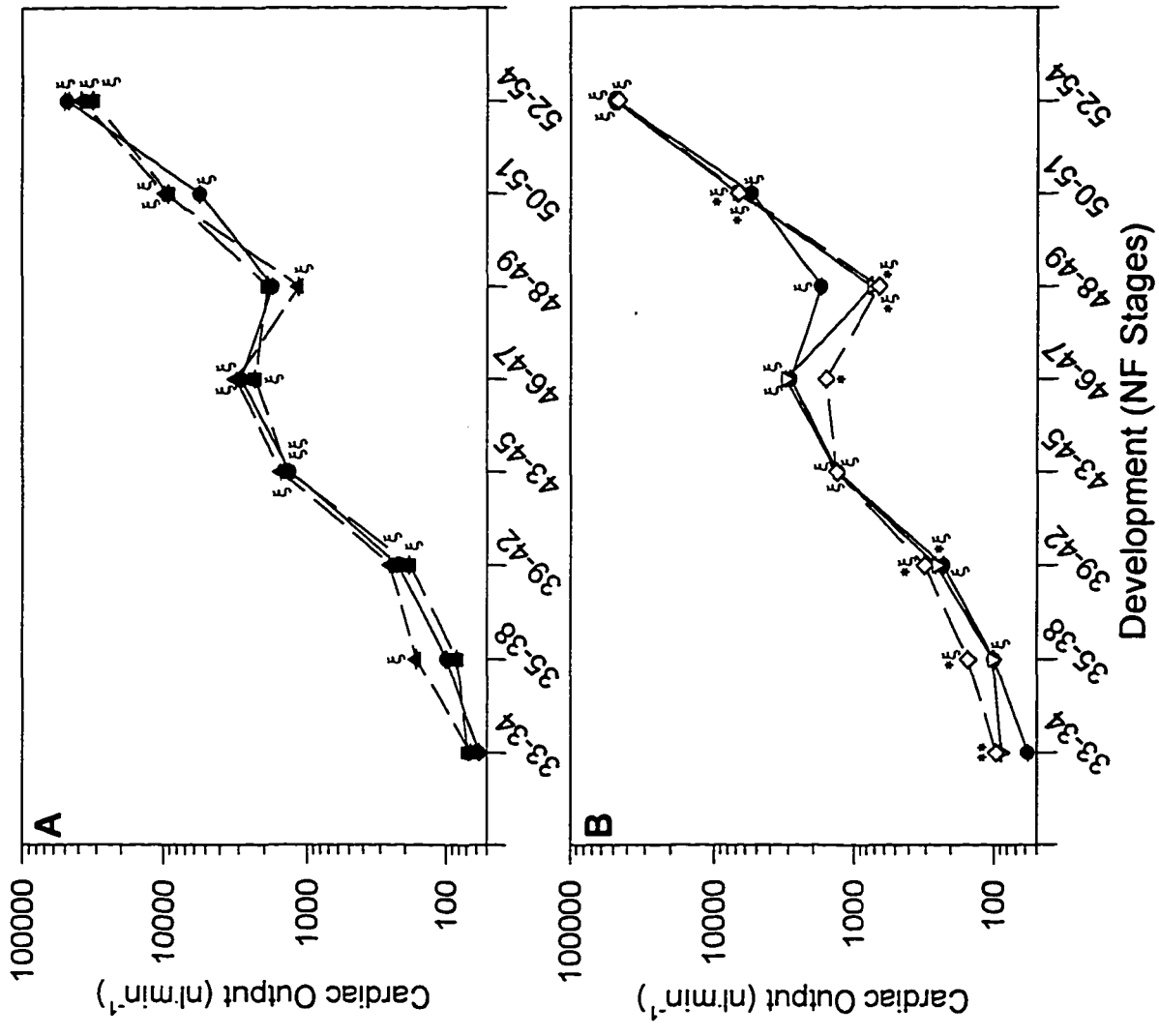
Figure 2. The relationship between blood flow and developmental stage for chronic normoxia with CO (open diamonds); chronic hyperoxia with CO (open triangles); chronic hypoxia (filled squares); chronic hyperoxia (filled triangles); and control animals exposed to normoxia (filled circles). $\dot{Q}$ was plotted on a $\log_{10}$ scale against the linear dimension of development. Values are shown as means and SEM. In some cases the error bars are inside the symbols. ξ indicates significant ($P \leq 0.05$) difference than the previous stage, where * indicate significant difference from controls.

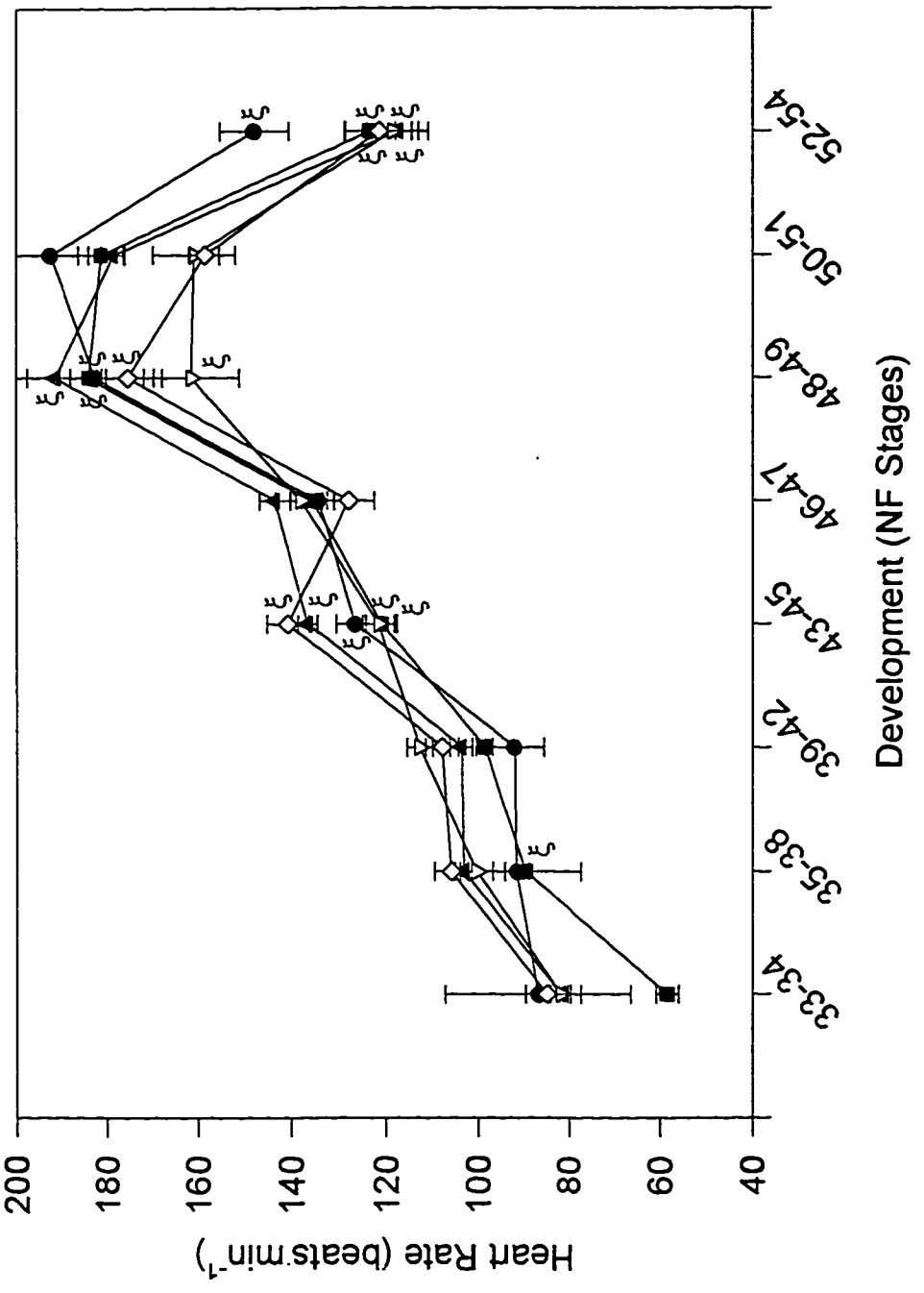
Figure 3. The relationship between f_h and developmental stage for chronic normoxia+CO (open diamonds); chronic hyperoxia+CO (open triangles); chronic hypoxia (filled squares); chronic hyperoxia (filled triangles); and control animals exposed to normoxia (filled circles). f_h was plotted on a linear scale against the

linear dimension of development. Values are shown as means and SEM. ξ indicates significant ($P \leq 0.05$) difference than the previous stage, where * indicate significant difference from controls.

Figure 4. Indicates the relationship between $\dot{V}_{O_2} \cdot \dot{Q}_{O_2}^{-1}$ and development for chronic normoxia+CO (open diamonds); chronic hyperoxia+CO (open triangles); chronic hypoxia (filled squares); chronic hyperoxia (filled triangles); and control animals exposed to normoxia (filled circles). Cardio-metabolic quotient was plotted on a $\log_{10}$ scale against the linear dimension of development. Values are shown as means and SEM. In some cases the error bars are inside the symbols. ξ indicates significant ($P \leq 0.05$) difference than the previous stage, where * indicate significant difference from controls.







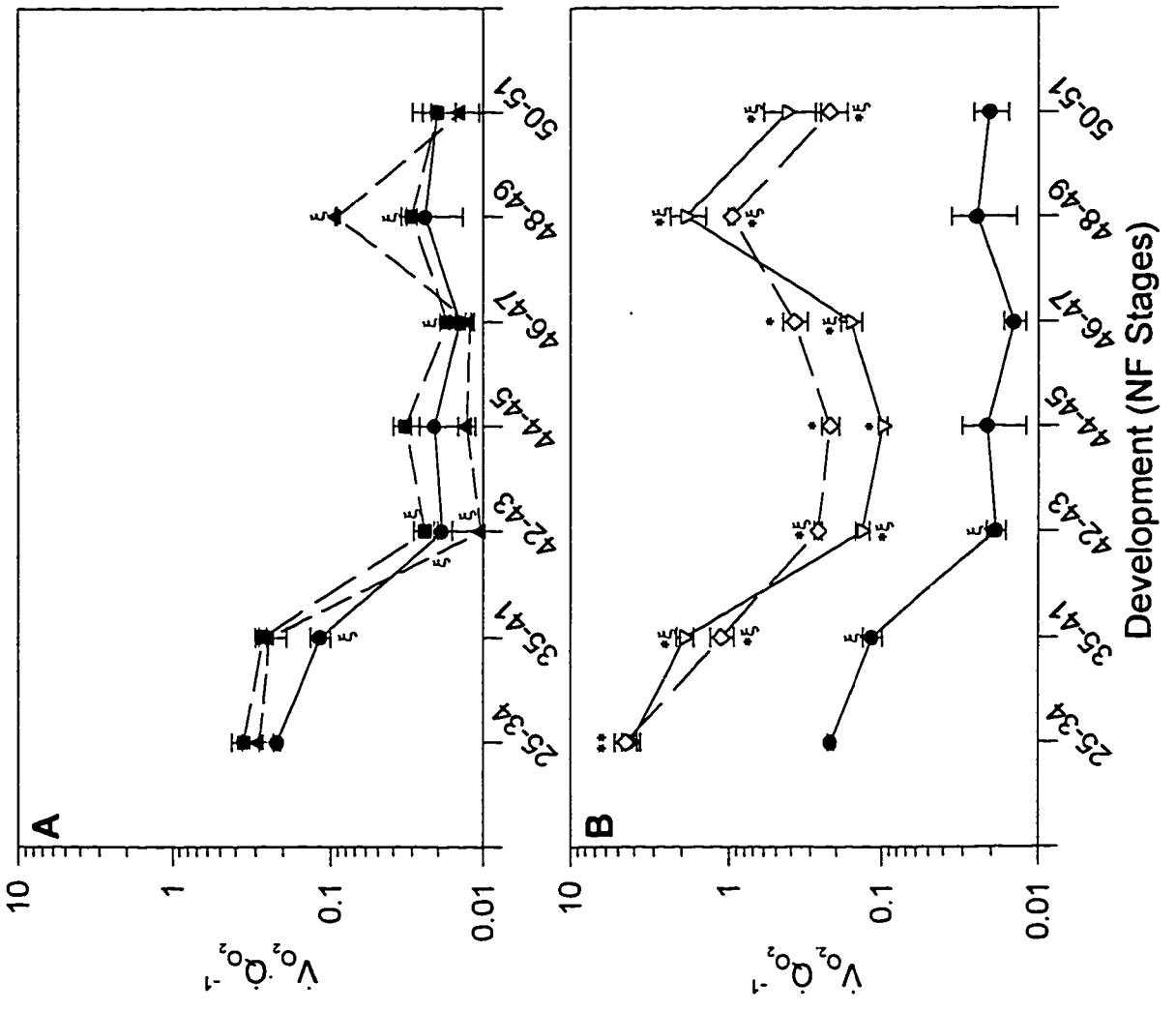


Table 1. The ontogeny of wet mass and stroke volume with normoxia, hypoxia, hyperoxia, hyperoxia + CO, and normoxia + CO.

	Normoxia		***Hypoxia***		***Hyperoxia***		***Hyperoxia+CO***		***Normoxia+CO***	
Stage Range	*Mass (mg)*	*SV (nl)*	*Mass (mg)*	*SV (nl)*	*Mass (mg)*	*SV (nl)*	*Mass (mg)*	*SV (nl)*	*Mass (mg)*	*SV (nl)*
1-24	2.5±0.7	--	1.5±0.2	--	1.9±0.4	--	2.1±0.5	--	1.1±0.2	--
25-34	2.2±0.2	1.1±0.2	1.5±0.1	0.8±0.0	1.9±0.3	0.9±0.2	2.3±0.5	1.1±0.2	2.4±0.3§	1.1±0.1
35-41	1.9±0.4	1.1±0.1	2.2±0.4	0.9±0.1	2.6±0.6§	1.6±0.4§	4.8±0.7§	1.0±0.1§	2.7±0.2§	1.4±0.1§*
42-43	7.7±1.2§	2.2±0.3§	4.3±0.2§	2.2±0.6§	7.1±0.9§	2.6±0.5§	5.1±0.4	2.2±0.3§	5.7±0.7§	2.9±0.4§
44-45	9.2±0.6§	10.8±1.1§	8.0±1.1§	9.9±0.9§	7.0±1.2	10.3±0.8§	4.8±1.21	11.1±0.9§*	7.7±0.7§	9.7±1.1§
46-47	12.6±0.5§	18.2±2.5§	10.6±0.5§	16.9±2.9§	13.1±0.3§	25.1±3.4§	12.9±0.7§	26.5±2.9§*	14.7±0.9§	12.0±1.4§*
48-49	30.9±4.5§	8.7±1.5§	21.7±3.1§	10.3±1.5§	25.7±0.3§	5.1±0.7§	41.7±5.8§	5.7±0.9§*	28.0±0.5§	5.0±1.4§*
50-51	74.4±8.0§	30.8±5.5§	63.7±11.1§	49.2±7.2§	57.2±6.7§	54.3±4.4§	85.2±4.0§	43.5±3.2§*	78.5±3.9§	41.7±41.7§*
52-54		554.9±149.7§		408.4±133.8§		325.3±40.0§		795.3±140.3§*		862.8±109.1§*

Values are means ± SEM, * indicates significantly different from normoxia, and § indicates significantly different from preceding stage ($p \leq 0.05$).

Table 2. The ontogeny of aerobic metabolism with normoxia, hypoxia, hyperoxia, hyperoxia + CO, and normoxia + CO.

	Normoxia	***Hypoxia***	***Hyperoxia***	***Hyperoxia+CO***	***Normoxia+CO***
Stage Range	$\dot{M}_{O_2}$ $(nmol\,g^{-1}\cdot hr^{-1})$	$\dot{M}_{O_2}$ $(nmol\,g^{-1}\cdot hr^{-1})$	$\dot{M}_{O_2}$ $(nmol\,g^{-1}\cdot hr^{-1})$	$\dot{M}_{O_2}$ $(nmol\,g^{-1}\cdot hr^{-1})$	$\dot{M}_{O_2}$ $(nmol\,g^{-1}\cdot hr^{-1})$
1-24	371.2±13.1	444.3±16.4	423.4±13.6	412.7±27.3	437.2±18.1
25-34	2094.7±191.6[5]	1994.5±280.6[5]	2590.5±287.3[5]	2961.9±177.8[5]	3321.5±293.7[5]
35-41	1302.0±126.1[5]	1256.3±82.1[5]	2087.6±155.4	1666.3±90.6[5]	1106.4±65.9[5]
42-43	685.1±18.8[5]	591.5±49.6[5]	461.6±17.1[5]	691.1±66.2[5]	843.7±19.1[5]
44-45	773.4±65.4	800.6±72.9[5]	578.4±52.0[5]	504.0±32.6[5]	702.0±36.9[5]
46-47	738.6±111.7	553.4±74.4[5]	755.1±69.2[5]	821.4±108.8[5]	716.8±113.0
48-49	400.0±53.1[5]	334.0±16.0[5]	740.6±36.3	745.4±128.0	517.9±101.3[5]
50-51	395.6±28.4	456.4±128.2	535.6±30.6[5]	630.3±226.1	337.9±36.2[5]

Values are means ± SEM, * indicates significantly different from normoxia, and [5] indicates significantly different from preceding stage ($p \leq 0.05$).

Table 3. Regression analysis of $\dot{M}_{O_2}$ with wet mass in developing *Xenopus laevis*.

Condition	*Stage Range*	*n*	$log_{10}\ a$	$log_{10}\ b$	r^2	*p*
Normoxia	1-24	4	-1.522	0.987	0.99	≤0.05
	25-51	32	-0.915	1.141	0.83	≤0.05
Hypoxia	1-24	4	-1.575	0.831	0.98	≤0.05
	25-51	32	-0.891	1.169	0.86	≤0.05
Hyperoxia	1-24	4	-1.578	1.117	0.99	≤0.05
	25-51	32	-0.855	1.191	0.84	≤0.05
Hyperoxia + CO	1-24	4	-1.601	1.139	0.97	≤0.05
	25-51	32	-0.804	1.113	0.89	≤0.05
Normoxia + CO	1-24	4	-1.540	0.877	0.98	≤0.05
	25-51	32	-0.881	1.197	0.84	≤0.05

Relationships for $\dot{M}_{O_2}$ expressed as $\log y = \log_{10} a + b \cdot \log_{10} M$, where y is $\dot{M}_{O_2}$ ($nmol \cdot hr^{-1}$). n, r and

CHAPTER 5

A MORPHOMETRICS BASED MODEL OF DIFFUSION IN THE DEVELOPING LARVAE OF *XENOPUS LAEVIS*.

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A MORPHOMETRICS BASED MODEL OF DIFFUSION IN THE DEVELOPING LARVAE OF *XENOPUS LAEVIS*.

Paul R. Territo[1,2*]
Jordi Altimiras[2]

Current Address:
[1]National Institute of Health
Laboratory of Cardiac Energetics (OE-108)
NHLBI, Building 10, Room B1D-161
10 Center Dr. MSC 1061
Bethesda, MD 20892-1061

[2]Department of Biological Sciences
University of Nevada, Las Vegas
4505 Maryland Parkway
Las Vegas, NV 89154-4004

Running head: Morphometrics and modeling diffusion.

* Author to whom all correspondences should be sent.

Abstract

O_2 transport by hemoglobin (Hb) can be functionally ablated with carbon monoxide (CO), generating hypoxemia. The present study investigates the models the ontogeny of respiratory physiology *Xenopus laevis*. Animals were raised from eggs (NF stage 1) to NF stage 63 (metamorphic climax), while maintained in either chronic 2 kPa CO, or air. Whole animal surface area (A_s) and volume (V) were calculated. From these variables whole animal diffusive O_2 conductance could be determined according to 2 different variables and considering the effects of boundary layers. The first model considers the diffusion of oxygen through the entire cross section of the animals depending solely on the P_{O_2} gradient between the environment and the core of the slice, a second model determined the effects of diffusion based on a fix distance into the animals. In addition, we calculated the contribution of plasma to total gas transfer ($\dot{T}_{O_2}$) in animals exposed to CO. Calculations of diffusive conductance ($\dot{D}_{O_2}$) and $\dot{T}_{O_2}$ between NF 33 and 47 indicated an adequate O_2 supply for the developing embryo provided the ΔP_{O_2} was maintained at 8 kPa between core and surface P_{O_2}s. Calculation of $\dot{D}_{O_2}$ or $\dot{T}_{O_2}$ considering the maximum diffusion distance into the animal (DD_{max}) indicated that O_2 conductance was significantly influenced by boundary layers with progressive development; however, in most cases animals between NF 33 and 47, there existed conditions which sufficient quantities of gas would be available for exchange. Interestingly, estimations of

conductance in late stage larvae (NF 50-51) reveled that they were unable to exchange enough gas by simple diffusion alone but the addition of convected plasma resulted in conditions which would provide enough gas for exchange. These results indicate that metabolism could be met via simple diffusion early in development, while late development would require additional transport via convection. Furthermore these data also indicate that plasma convection provides a substantial increase in the total amount of O_2 available for exchange in CO exposed animals. Lastly our model indicates that gas exchange in developing amphibians may be significantly influenced by boundary layers, core P_{O_2}s and $DD_{max.}$ and it is these factors which limited the first step in a multi-compartment system.

Introduction

The heart and circulatory system are the first functioning organ and system in the developing embryo (Burggren and Keller, 1997; Gilbert, 1990). Concomitant with these changes is the formation of respiratory structures (skin and gills) (Nieuwkoop and Faber, 1967; Taylor and Kollros, 1946) and the initiation of circulation to them, leading researchers to speculate about their role in gas exchange (Medvedev, 1937; Vogel, 1982). Adolph, 1979.; Boell *et al.*, 1963; Burggren *et al.*, 1991; and Burggren and Pinder, 1991 have suggested that requirements for oxygen are the driving force behind the development of the cardiovascular system Recently, our work in developing larval *Xenopus laevis* larvae have demonstrated that the absolute requirements for O_2 uptake occurs irrespective of Hb-dependent processes (Territo, 1996). This hypothesis has been termed "prosynchronotropy" (Burggren and Territo, 1995), and states that the circulatory system forms well before the need for convective transport of O_2. Additional support for this theory comes from many contemporary studies (Holeton, 1971; Mellish *et al.*, 1995; Pelster and Burggren, 1996) which demonstrate that neither Hb, nor a functioning circulatory system, is required to obtain O_2 through embryonic and early larval development. Moreover, these data have been further supported by the work of Hillman, (1980; 1981) in which anemic frogs were shown capable of maintaining sustained periods of exercise in spite of an experimental reduction in hematotrict by 60%. These collective data suggest that the circulatory system may in fact develop for purposes other

than bulk O_2 transport, and therefore suggesting that diffusion alone plays a significant role in oxygen uptake in early development.

The diffusion of any gas through tissue is governed by physical properties (surface area, solubility, pressure etc.) which have been outlined over the past century (see Fick, 1870; Krogh, 1904; Piiper *et al.*, 1971). Based on this work, many gas transport models have been formulated that describe O_2 movement via diffusion and how changes in flow to associated structures may affect oxygen uptake (Burggren and Moalli, 1984; Malte and Weber, 1985; Piiper, 1988; Pinder and Friet, 1994; Withers, 1992; Withers and Hillman, 1988). Although there are numerous models which describe gas transport, most are mechanistic models and have the assumption that the respiratory surfaces can be represented by simple geometries. These models are limited, because they rarely resemble an animals true shape, and thus either over or under estimate transcutaneous gas flux.

The purpose of this study was to formulate a mechanistic model based on analytical geometries that would incorporate surface area (A_s) and volume (V) directly from empirically derived data. This would allow for further calculations of diffusion based on true animal shapes. Our approach was to elucidate whether O_2 uptake was feasible via simple diffusion in larval *Xenopus*. First, we calculated A_s and V based on morphometric data from larval *Xenopus* raised both in normoxic and normoxic+CO water. Second, we calculated maximal diffusion based on two models: Model 1, which considers the effects of changing

P_{O_2} in the core of the animal; while Model 2 considers the effects of diffusion at a fixed maximal distance into the animal. Both models were calculated over a range of surface P_{O_2}s corresponding to different predicted boundary layers (for the relationship between boundary layer, water speed, and reductions in surface P_{O_2} see Pinder and Feder, 1990). Since convection of O_2 via the plasma may play a role in total transport, we have determined its relative contribution to oxygen uptake, by calculating maximal plasma O_2 transport in larvae chronically exposure to 2 kPa carbon monoxide (CO).

Collectively our data would represents the first assessment of gas transport in a developing amphibian, in addition to the evaluation of relative components in the gas transport processes. Furthermore, by using animals exposed to CO, we can thereby determine the relative contribution of diffusion, and plasma transport to total gas exchange.

Materials and Methods

Experimental Animals. Fertilized eggs were obtained from the breeding in our laboratory of four adult female *Xenopus laevis,* according to Thompson and Franks (1978). Newly laid eggs were equally divided into two 25 L holding tanks, where they were maintained in dechlorinated water at $24\pm0.2\ ^{0}C$.

Grouping of Developmental Stages. Experiments were conducted on *X. laevis* larvae ranging from NF 33-34 to pre-metamorphic climax (NF 51) according to

the staging regime of Nieuwkoop and Faber (1967). Animals were grouped into 4 different ontogenic categories according to major morphological and physiological landmarks. Developmental groupings are as follows: 33-34, 44-45, 46-47, and 50-51.

Experimental Conditions. Experimental conditions were as prepared as per Territo (1996). Briefly, larvae were placed in a holding tanks and aerated with one of two gas mixtures: 21 kPa O_2/ 79 kPa N_2 (control) or 2 kPa CO/ 21 kPa O_2/ 77 kPa N_2 . Carbon monoxide (CO) gas mixtures were achieved with a Cameron GF-4 gas mixing flowmeter. Larvae in these tanks were fed Nasco frog brittle™ (Nasco Inc.) *ad lib.* during the course of development. All animals were maintained on a 14:10 light: dark cycle through development.

Respiratory and Cardiovascular measurements. Data obtained for comparison with calculated diffusional transport were from Territo (1996). Larvae, were raised in either normoxic water, or under chronic 2 kPa CO through development. In all cases, oxygen consumption ($\dot{M}_{O_2}$) was found to be unaffected by treatment, thus data used for comparison with our model was averaged and one set of representative values were reported. Heart rate (f_h), stroke volumes (SV), and cardiac output ($\dot{Q}$) were obtained from Territo (1996). Stroke volume, and therefore $\dot{Q}$, in hypoxemic larvae were mildly elevated over controls. In order to determine the contribution of convective transport to total

oxygen turnover, $\dot{Q}$ and blood oxygen content data were calculated for CO exposed animals and used for all subsequent analysis.

Videotaping and calibration for surface area and volume. A group of six animals from the two different experimental treatments, and at four different developmental stages were sampled from the experimental tanks and were anesthetized with buffered MS-222 (0.02%, w/v). Animals were then placed on an agar plate and videotaped with an sVHS video (see Territo, 1996), and stored for further analysis. The earlier developmental stages (NF 33/34 and 44-45) were videotaped under a Leica M3Z compound microscope fitted with a color video camera (Javelin Electronics Inc.) Later stages (NF 46 to 51) were videotaped using a compact VHS video-camera, model GR-AX9000U (JVC Inc.) attached to a tripod. Each animal was taped in a dorsal and a lateral view, one perpendicular to the other. Care was taken to insure that both planes taped were perpendicular to each other and the to the mid-line of the animal. In order to obtain real dimensions from the videotaped animals, a piece of polyethylene tubing (PE-10, Becton-Dickinson Inc.) was also taped between each treatment group in order to calibrate the image analysis program (see image analysis section below).

Image Analysis. The videotaped animals were played back on a Panasonic sVHS editing VCR (Model AG-7350) connected to a computer image analysis

system based on OPTIMAS® software. Calibration was performed using PE-10 tubing (Becton-Dickinson Inc.) of known dimensions (O.D.=610 μm). Calibration of the virtual-instrument was checked between the analysis of each treatment group. By playing back a picture of each animal in the two taped planes, the perimeter of the animal was delineated by hand and stored in the form of Cartesian coordinates in an Excel™ (Microsoft® Inc.) spreadsheet linked with the image analysis software. The perimeter was sampled at a resolution of 100 μm. The spreadsheet files were saved for further determination of volume and surface area.

Algorithm for calculation of total surface area and total volume:

The computation was performed in a specifically designed program using LabView™ graphical software (National Instruments©). The spreadsheet files were used to obtain three profiles (series) from each animal: dorsal, ventrolateral, and dorsolateral views. The dorsal plane was considered to be symmetric and only the right half side was used. The lateral plane is conspicuously asymmetric and both halves were obtained and used in the analysis.

Correction and alignment of images. Three correction routines were employed in order to get a perfect alignment of the three profiles of the animal:

1) Normalization of the X-axis in order to get the three profiles adjusted to the same length.

2) Correction for the pitch of the animal in the taped image by subtracting the linear regression line set between the beginning and the end of the animal

3) Interpolation in each profile in order to get a sequence of Cartesian coordinates evenly spaced in the X-axis.

The computation of total volume and total surface area was estimated as described in Figure 1. The animal was considered to be composed of 10 μm wide cross-sectional slices and the sum of surface area and volume of all the slices made up total volume and total surface area.

The volume of each slice was calculated by modeling it as an elliptical cylinder using the width of the slice and the height of the cylinder. The base was modeled as two semi-ellipses with a common semi-axis (r_1, the radius in the dorsal view) and two different semi-axis (r_3, radius in the dorsolateral or r_2, ventrolateral semi-profiles, respectively).

From that, the area of the base of the cylinder was:

$$A_n = A_{n_1} + A_{n_2} \qquad (1)$$

such that, A_n =Area of base at slice 'n' and

A_{n_1} =Area of semi-ellipse 1 at slice 'n',

$$\therefore\ A_{n_1} = \tfrac{1}{2} \cdot \pi \cdot r_1 \cdot r_2, \qquad (1.1)$$

where r_1 and r_2 are the semi-radii in the dorsal and dorsoventral planes.

A_{n_2} =Area of semi-ellipse 2 at slice 'n',

$$\therefore A_{n_2} = \tfrac{1}{2} \cdot \pi \cdot r_1 \cdot r_3, \quad (1.2)$$

where r_1 and r_3 are the semi-radii in the dorsal and ventrolateral planes.

Therefore, the volume for each slice could be calculated as follows:

$$V_n = A_n \cdot T_n, \quad (2)$$

$\therefore$ T_n=the thickness at slice 'n', and was fixed at 10 μm.

Total animal volume was calculated as follows:

$$V = \sum^{n} V_n \quad (3)$$

The surface area of each slice could not be modeled the same way because an elliptical cylinder largely underestimates surface area, although not volume. Thus, the slices were modeled as elliptical truncated cones and the surface area calculated between two slices (P and P'). For a graphical explanation, see Figure 2.

Cross sectional slice area was calculated as follows:

$$A_{cs} = \left(\frac{P + P'}{2}\right) \cdot \bar{T}_n \quad (4)$$

which P and P' indicate the perimeters of two consecutive slices

$$\therefore P = P_{(1\text{-}2)} + P_{(1\text{-}3)} \quad (4.1)$$

$$P' = P'_{(1\text{-}2)} + P'_{(1\text{-}3)} \quad (4.2)$$

such that $P_{(1-x)}$ and $P'_{(1-x)}$ are the semi-perimeter between radii r_1, r_2 and r_3.

$P_{(1-x)}$ and $P'_{(1-x)}$ are described by the equation:

$$P_{(1-x)} = 4 \cdot r_1 \cdot E_{(1-x)} \cdot \psi \tag{4.3}$$

$$\therefore \ \psi = \sin^{-1}\left(\frac{\sqrt{r_1^2 - r_x^2}}{r_1}\right) \tag{4.4}$$

where, r_1 is the dorsal semi-axis and r_x is the dorsolateral or dorsoventral semi-axis.

$E_{(1-x)}$ was elliptic integrals of the second kind between segment 1 and x, and was obtained from Handbook of Tables for Mathematics. 4th. ed. CRC Press 1975 Ohio.

Thickness of the slice was based on the following formula:

$$\overline{T} = \frac{T_1}{2} + \frac{T_2}{4} + \frac{T_3}{4} \tag{5}$$

where T_1, T_2, and T_3 are the thickness of the slice at the dorsal, dorsolateral and ventrolateral margins respectively.

Total animal surface area was calculated as the sum of the individual slice areas and was described by the equation:

$$A_s = \sum^{n} A_{cs} \tag{6}$$

Accuracy of the volume and surface area estimation:

Two sets of calibrations were run to evaluate of our methodology. The first calibration indicated the strict accuracy of the computational algorithm. We

generated geometrical models in the computer for which formulas to calculate volume and surface area are available. These were run through the algorithm at different slice widths (5, 10, 20, 50 and 100 μm) to evaluate the efficacy of changing thickness on the algorithm. Spheres, spheroids (prolate and oblate), ellipsoids and cones of different dimensions (fitting in the object range size of our experimental animals) were used and the deviation of the estimation from the purely geometrical model was analyzed. In all cases our slicing method underestimated the real values, depending on the slice width chosen, ranging from 0.05% to 0.98% for volume and 0.18 to 3.31% for surface area as shown in Figure 3A. Given this, we decided to standardize our calculations using 10 μm slices, thereby underestimating volume by 0.08% and surface area by 0.35%.

The second calibration evaluated the accuracy of the whole protocol including image analysis and computations with the algorithm. We determined v and A_s of three different spherical ball bearings of 2.32, 3.15 and 6.12 mm diameter. Each was videotaped and stored for later analysis. All samples were hand delineated and ran though the algorithm to determine the error associated with operator estimation. In most cases, the deviation ranged from 4.7 to 5.2% for A_s and -0.1 to -5.4% for volume (Figure 3B) and were considered adequate for our model.

Description of the diffusion model:

Oxygen diffusion in each slice was calculated based on its surface area and averaged radius:

$$D_n = K_{O_2} \cdot P_o \cdot \frac{P_i}{d} \cdot T_n \cdot A_s \qquad (7)$$

where D_n is the oxygen diffusion in slice 'n' in $nmol \cdot h^{-1}$,

K_{O_2} is Krogh's diffusion constant for O_2, in $nmol \cdot h^{-1} \cdot \mu m^{-1} \cdot kPa^{-1}$,

P_o is the partial pressure of oxygen in the water in kPa,

P_i is the partial pressure of O_2 in the core of the animals in, kPa,

d is the maximum diffusion distance in μm,

T_n is the average thickness of slice 'n' in μm,

and A_n is the surface area of slice 'n'.

Whole animal diffusion was calculated as the sum of the respective slices with the following formula:

$$\dot{D}_{O_2} = \sum^{n} D_n \qquad (8)$$

where $\dot{D}_{O_2}$ is the O_2 transport via diffusion across the whole animal in $nmol \cdot h^{-1}$ and D_n is the diffusion across each segment along the length of the animal at 10 μm intervals.

Convective transport of oxygen in the whole animal exposed to CO was calculated to determine the contribution of plasma transport. Data on cardiac output was from Territo (1996) and was defined as:

$$\dot{Q}_{O_2} = \dot{Q} \cdot C_{O_2} \qquad (9)$$

where, $\dot{Q}_{O_2}$ is the total transport of O_2 in the plasma in nmol·h^{-1},

$\dot{Q}$ is the total cardiac output of the animal in nl blood.h^{-1},

and C_{O_2} is the O_2 content of blood in nl O_2·100 nl blood^{-1}.

To consider the effects of both diffusional and convective transport on total oxygen uptake, we calculated the total gas transport as follows:

$$\dot{T}_{O_2} = \dot{Q}_{O_2} \cdot E_f + \dot{D}_{O_2} \qquad (10)$$

where $\dot{T}_{O_2}$ is the total transport of O_2 in nmol·h^{-1},

$\dot{Q}_{O_2}$ is total O_2 carried via the blood in nmol·h^{-1},

$\dot{D}_{O_2}$ is whole animal diffusional oxygen transport in nmol·h^{-1},

and E_f is the O_2 extraction efficiency of the tissues set at 25%, based on theoretical estimations (Piiper *et al.*, 1976), and empirical observations in *Xenopus* (Hillman, 1978.).

Boundary layer accommodation:

Boundary layers were calculated based on mean free diffusion distances (MFDD) and water velocities from the work of Pinder and Feder (1990). Values were expressed as reductions in surface P_{O_2} at water velocities of 0 (1.4 kPa), 0.5 (9.8 kPa), and 5.2 (14 kPa) $cm.sec^{-1}$.

Results

Surface area and volume estimation:

Surface area and volume significantly increased with development for both normoxic and CO exposed animals (8.46 $\pm$ 0.31 mm^2 to 70.18 $\pm$ 1.75, and 1.81 $\pm$ 0.10 mm^3 to 35.73 $\pm$ 1.82 mm^3 respectively) (Fig. 4A), although there was no significant effect of treatment (Table 1). The ratio of surface area and volume showed a consistent decline with progressive development, and was not significantly different between normoxic and CO exposed animals (Fig. 4B).

Gas transport with variable core oxygen tensions (Model 1):

Model 1 is a single compartment model. That considers the effects of diffusion irrespective of convective transport. Thus, it relies solely on the differential between ambient and core P_{O_2}s (ΔP_{O_2}) to evaluate if diffusion alone would be adequate for O_2 uptake through development.

Predicted $\dot{D}_{O_2}$ was plotted with respect to both core and animal surface P_{O_2} with development, and are presented in Figures 5A-8A. $\dot{D}_{O_2}$ showed a consistent linear increase as core P_{O_2} decreased. Similarly, as the MFDD was reduced by increasing ambient water velocity from 0 to 5.2 cm.sec^{-1}, $\dot{D}_{O_2}$ showed a similar increase to a maximum of 20.39 $\pm$ 0.75 nmol·h^{-1} in stage 33/34 animals. Measurements of $\dot{M}_{O_2}$ for *Xenopus* larvae at NF 33/34 (initiation of heart beat) was 3.98 $\pm$ 0.42 nmol·h^{-1}. When we plot $\dot{M}_{O_2}$ for reference over the range of core and surface P_{O_2}s, it is clear that the maximal diffusion would be adequate for O_2 uptake under most circumstances (Fig. 5A). Moreover, values below the plane of reference illustrate that diffusion is inadequate to meet metabolic demands, when the MFDD increases, thereby reducing surface P_{O_2} to at or near core P_{O_2}s, thus reducing or in some cased negating total O_2 flux.

Calculations of total diffusional transport for NF 44-45 are illustrated in Figure 6A. Like the previous stage, $\dot{D}_{O_2}$ increased as core P_{O_2} fell and surface P_{O_2} increased. Maximal transport (38.50 $\pm$ 2.99 nmol·h^{-1}) was achieved when the greatest ΔP_{O_2} between the core and surface was 18 kPa. Measured $\dot{M}_{O_2}$ (15.07 $\pm$ 1.26 nmol·h^{-1}) indicates that a smaller proportion of the diffusive flux would be capable of meeting total aerobic demands. Larvae at NF 44-45 would require a surface P_{O_2} greater than 6 kPa at a core P_{O_2} of 2 kPa to obtained enough O_2 by

diffusion alone. This equated to a ΔP_{O_2} between the core and surface of 4 kPa, which was constant over both ranges. The fact that ΔP_{O_2} was larger with the advancement of one stage range indicates that the proportion of increase seen in $\dot{M}_{O_2}$ did not increase isometrically with A_s.

Stage range NF 46-47 represents an advancement of two week of development, a near doubling of wet mass, and a 1.5 fold increase in surface area, all the while $\dot{M}_{O_2}$ remained constant. Like the previous stage ranges (NF 33 to 45), $\dot{D}_{O_2}$ was dependent on a linear decrease in core P_{O_2}, while surface P_{O_2} increased. Diffusional gas flux, which set the boundaries for adequate transport at or above $\dot{M}_{O_2}$, was based on a ΔP_{O_2} of 2.5 kPa and were constant over all ranges (Fig. 7A). The fact that the $\dot{D}_{O_2}$ was set at a lower ΔP_{O_2} indicates that A_s was increasing faster relative to $\dot{M}_{O_2}$. Maximal transport (45.35 $\pm$ 1.25 nmol·h^{-1}) was achieved at a core P_{O_2} of 0 kPa and the equivalent of no boundary layer at 18 kPa.

Calculations of $\dot{D}_{O_2}$ indicate that at all core and surface P_{O_2}s, diffusion would be inadequate for O_2 transport in order to meet aerobic demands at NF 50-51. Interestingly, the point of maximal flux, which corresponds to a boundary layer of 0, obtained a value of 69.98 $\pm$ 2.33 nmol·h^{-1} which was within one standard deviation of the actual $\dot{M}_{O_2}$ of 72.93 $\pm$ 21.12 nmol·h^{-1}, indicating that

diffusional gas transport could meet aerobic demands if augmented slightly by convective transport of O_2 (Fig. 8A).

Gas transport with a fixed diffusion distance (Model 2):

Model 2 is a two compartment model, that considers the effects of a fixed distance on diffusion into the first compartment. It assumes that the second compartment is well stirred, and that gas transport limitations are set by the first step.

Estimates of $\dot{D}_{O_2}$ demonstrated an almost linear dependency of surface P_{O_2} and $\dot{D}_{O_2}$, while maximum diffusion distance (DD_{max}) showed a curvilinear relationship with $\dot{D}_{O_2}$ at NF stage 33/34. Comparison of estimates of $\dot{D}_{O_2}$ and $\dot{M}_{O_2}$ indicate that animals at this stage were capable of surviving with a surface P_{O_2} of 7 kPa and a DD_{max} of 1 mm. Similarly, animals with a DD_{max} of 5 mm could supply enough O_2 via diffusion with a surface P_{O_2} down to 4 kPa (Fig. 5B). It is clear, that diffusion was more than adequate for gas exchange over a wide range of surface P_{O_2}s and DD_{max} provided that the partial pressure never fell below 4 kPa.

Calculations of oxygen transport for animals at NF stage 44-45 showed a consistent increasing trend of $\dot{D}_{O_2}$ with surface P_{O_2} and DD_{max}. Although there was a consistent trend, the changes seen in $\dot{D}_{O_2}$ associated with DD_{max} were not

linear over the range of surface P_{O_2}s. Like NF stage 33/34, estimations of $\dot{D}_{O_2}$ indicated that diffusion was adequate to meet aerobic metabolism, provided that surface P_{O_2} was above 8 kPa at 5 mm and above 10 kPa at 1 mm DD_{max} (Fig. 6B). The fact that $\dot{D}_{O_2}$ increases with surface P_{O_2} (increasing water velocity) indicates that the MFDD was a crucial step in setting gas exchange over the range of DD_{max}s.

Calculations of $\dot{D}_{O_2}$ at NF 46-47 indicated that the major limitations to gas exchange were set by surface P_{O_2}s below 8 kPa for all DD_{max}s. The maximum gas flux obtainable was achieved with a DD_{max} of 5 mm and a surface P_{O_2} which corresponded to a water velocity of 5.2 $cm.sec^{-1}$ (Fig. 7B). Calculations of DD_{max} at 2 mm showed a reduction in $\dot{D}_{O_2}$ at 18 kPa relative to DD_{max} of 1 and 5. This reduction in $\dot{D}_{O_2}$ is opposite of that seen in the previous stage (NF 44-45). This reduction in $\dot{D}_{O_2}$ at a DD_{max} of 2 indicates a physical limitation to gas exchange at high water velocities.

All combinations of surface P_{O_2}s and DD_{max}s in larvae at NF 50-51 indicated that $\dot{D}_{O_2}$ was far below the observed $\dot{M}_{O_2}$. Calculations over this stage range showed the same shape and pattern as those seen in animals at NF 46-47. Interestingly, the magnitude of the reductions seen at the DD_{max} of 2 mm and a surface P_{O_2} of 18 kPa were more pronounced indicating that gas exchange at

high water velocities (high surface P_{O_2}s) was limited by morphology and the ΔP_{O_2} achievable across the tissues.

The effects of plasma transport on Model 1 and 2:

Our models thus far have only considered the effects of modulating O_2 tensions with respect to the core and distance penetrated into the animal. Another important consideration is the transport of oxygen by the blood. Animals exposed to CO have a maximum blood O_2 content at the level of plasma (0.2 vol%). Our previous work has shown that cardiac output is mildly elevated in hypoxemic animals (Territo, 1996). When these data are incorporated into our current model it is clear that late stages of *Xenopus* may subsist with a combination of $\dot{D}_{O_2}$ and plasma O_2 transport. Thus, buy considering model 1 and 2 in the context of $\dot{Q}_{O_2}$, we therefore have added a functional compartment to each model, with plasma acting as a storage and transport component.

The boundary conditions which limited $\dot{D}_{O_2}$ and the overall animal shape in each model remained constant for $\dot{T}_{O_2}$ at all stage. Instead, the addition of $\dot{Q}_{O_2}$ to each model resulted in a 1.3 and 1.5 fold increase in $\dot{T}_{O_2}$ at NF stage 33/34 for both model 1 and 2 respectively (Fig 5C,D). This increase resulted in a overall greater variety of conditions under which gas transport was sustainable. This was true for larvae over the next stage range (NF 44-45), which showed a 3.5 and 4.8 fold increase in $\dot{T}_{O_2}$ with the addition of $\dot{Q}_{O_2}$ in model 1 and 2,

respectively (Fig. 6C,D). The fact that $\dot{T}_{O_2}$ increased so dramatically from the previous stage indicates that $\dot{Q}_{O_2}$ contributed a larger fraction to the total change. Addition of $\dot{Q}_{O_2}$, and the resultant increase in $\dot{T}_{O_2}$, at NF stage 46-47 was maintained with a 3.4 (model 1) and 4.2 fold (model 2) increase, although this increase occurred with a doubling of wet mass and a 1.5 fold increase in A_s. Interestingly, calculations of $\dot{D}_{O_2}$ for larvae at NF 50-51 showed an inability to obtain sufficient O_2 via diffusion alone. However, addition of $\dot{Q}_{O_2}$ resulted in a $\dot{T}_{O_2}$ which clearly shows the conditions are adequate for aerobic metabolism. Unlike all preceding stages, NF 50-51 showed an adjustment to the boundary condition which limit gas exchange (Fig. 8C,D). Addition of $\dot{Q}_{O_2}$ to model 1 resulted in boundary conditions which was described the limitations to gas exchange at a surface P_{O_2} of 11 kPa and a core value of 9 kPa, while model 2 was limited in $\dot{T}_{O_2}$ by surface P_{O_2}s above 14 kPa at a DD_{max} of 5 mm. This however, was not the case at DD_{max} of 1 and 2, where gas tensions were required above 15 and 15 kPa respectively.

Discussion

Critique of the method:

A potential criticism of the study could be the use an extraction efficiency for plasma of 25% in CO exposed animals. The work of Burggren and Feder,

(1986); Feder and Burggren, (1985); and Feder and Pinder, (1988) have shown that the respiratory exchangers (skin and lungs) in amphibians occurs both in parallel with the systemic vascular beds, therefore resulting in a venous add-mixture and an elevation of venous C_{O_2}. Empirical measures of arteriole venous (A-V) difference in adult *Xenopus* indicate a tissue extraction at rest of 25% (Hillman, 1978.). These observations were within the theoretical estimations for *Desmognathus fuscus* (Piiper, 1988), although the A-V difference estimated were slightly higher than those seen in *Xenopus*, signifying the complete reliance of these animals on cutaneous exchange. In any case, it is clear that tissue extraction would be at least 25%, as indicated in adult anuran amphibians, and therefore would at best underestimate the contribution of $\dot{Q}_{O_2}$ in total exchange

Surface area and volume:

The importance in determining surface area and volume and their relevance to diffusional gas exchange are evident. Interestingly, we know of only two studies to date which have attempted to determine this perimeter in adult amphibians, (Hutchinson *et al.*, 1968.; Talbot and Feder, 1992), and none which have looked at this in larvae. Diffusion, according to the Fick principle, is set by A_s, thickness of the exchange area, and the ΔP_{O_2} between exterior and interior of the animal. Therefore it is reasonable to suppose that animals exposed to chronic CO might increase A_s in order to increase the functional area for

exchange. However, our data clearly shows that surface area in animals exposed to CO was not significantly different from controls (Fig 4A; Table 1). Based on this, we put forth two models which attempt to determine the affects of altering both ΔP_{O_2} (model 1) and DD_{max} (model 2).

Gas transport models:

Although numerous models of gas transport exist for amphibians (Piiper, 1988; Piiper *et al.*, 1971; Piiper *et al.*, 1976; Withers, 1992; Withers and Hillman, 1988), few which have broad applicability in a developmental context, and even fewer models have directly attempted to determine the constraints under which diffusional gas exchange would be set through development. Thus, our approach of modeling gas transport based on mechanistic determinants of surface area, and subsequent calculations of diffusion based on established paradigms, has allowed for the first time the evaluation of conditions limiting to gas transport in larvae. Furthermore, by evaluating these perimeters in animals whom have been chronically raised under CO, we can comment on the relative importance of diffusion and convection with ontogeny.

Model 1 (diffusion with variable core oxygen tensions):

Model 1, a single compartment model that evaluates the effects of ΔP_{O_2} on diffusion, assumes diffusion alone would be adequate for O_2 uptake through development. Model 1 predicts that $\dot{D}_{O_2}$ would be more than adequate for gas

exchange over a wide range of core P_{O_2}s and water velocities through NF stage 46-47. Interestingly, the ΔP_{O_2} required to achieve aerobic metabolism increased from 2 to 7 kPa over the first three stage ranges (NF 33 to 47) (Fig. 5A). This progressive increase illustrates the greater requirements needed to meet tissue metabolism with advancing development.

Although diffusion was adequate early in development, it is clear from our calculations that late stage larvae (NF 50-51) under most conditions would require some augmentation to meet aerobic demands. Calculations of maximal flux occurred under conditions of zero boundary layer, and a core P_{O_2} of 0 (Fig 8A). These data were within one standard deviation of $\dot{M}_{O_2}$ measured at NF 50-51 larvae at 24 ^{0}C (Territo, 1996). Although the model predicts that diffusion may be adequate, It is clear from the work of Pinder and Feder, (1990) that, even with a water velocity greater than 5 cm.sec^{-1}, there exists a functional boundary layer which reduced surface P_{O_2} to 14 kPa. Based on this, it is unlikely that animals later in development would be capable of exchanging gases via simple diffusion alone according to our model.

The addition of convective O_2 transport to model 1 allows for assessment of how animals exposed chronically to CO augment gas exchange. Additionally, by considering the convection of plasma, this model effectively adds an additional compartment thereby allowing for determination of the effects of storage and dispersal on diffusion.

Our previous work has shown that exposure to CO did not significantly alter $\dot{M}_{O_2}$. However, $\dot{Q}$ was augmented by as much as 1.2 fold. Coupling this with the C_{O_2} in CO exposed animals, it is reasonable to assume that the addition of $\dot{Q}_{O_2}$ with diffusional transport would result in an overall greater quantity of O_2 available for flux.

Calculations of $\dot{T}_{O_2}$ indicated that gas exchange would in fact be enhanced by the addition of a plasma compartment. Although, the boundary conditions which limited gas exchange for animals with $\dot{D}_{O_2}$ did not change for all stages, $\dot{T}_{O_2}$ represented a much larger fraction of the overall available gas. This increase was clear in animals at NF 50-51, in which surface P_{O_2}s above 11 kPa with a minimum ΔP_{O_2} of 8 kPa allows for sufficient quantities of gas to be transported for exchange (Fig. 8C). A surface P_{O_2} of 11 kPa equates to a water velocity of ~1.5 cm.sec^{-1}, or a swimming speed of at least 0.75 body legnths.sec^{-1}. Clearly, animals actively engaged in feeding or normal behavior routinely exceed this value by many fold (von Seckendorff-Hoff and Wassersug, 1986; Wassersug and von Seckendorf-Hoff, 1985) indicating that the combination of convection, diffusion, and swimming would allow for conditions favoring transport.

Model 2 (diffusion given a fixed distance):

Model 2 considers the effects of a fixed diffusion distance into compartment one, reveling how transport limitations are set by the first step of a multiple compartment model.

In all stages up to NF 46-47, gas transport was calculated to be adequate to meet aerobic metabolism over a wide range DD_{max}, surface P_{O_2}s (Figures 5B-8B). Calculations for larvae at NF 50-51, clearly indicates that despite the greatly elevated A_s, $\dot{M}_{O_2}$ was increasing at a rate faster than could be meet by diffusion alone. This data suggests that with the addition of convective transport would result in a level of O_2 transport which may be enough to meet metabolic demands.

When we considered the effects of $\dot{Q}_{O_2}$ on total transport, it becomes clear, that like in model 1, the amount of available gas usable for consumption increases, although the boundary conditions which setup the limitations remain constant. The increase of $\dot{T}_{O_2}$ with the addition of $\dot{Q}_{O_2}$ indicates that plasma transport may contribute to the total turnover of respiratory gases in developing larvae. This was more evident in the changes seen in NF 50-51, in which prior to addition of $\dot{Q}_{O_2}$, animals were unable to transport enough gas by diffusion alone (Fig. 8B). However, when plasma O_2 transport was added, it become apparent that under some conditions gas exchange would in fact be possible (Fig. 8D). The minimum water velocity needed to achieve this transport is ~3 $cm.sec^{-1}$,

which translates into ~1.5 body legnths.sec^{-1}, and is well within the normal range of swimming speeds seen for *Xenopus* larvae (von Seckendorff-Hoff and Wassersug, 1986). The results seen for $\dot{T}_{O_2}$ at NF 50-51 are in fact double those observed for our previous model, indicating that DD_{max} imposes a greater constraint to total exchange than does considering core P_{O_2}.

Lastly, this model allows for prediction of maximum capillary depth for a given water velocity. Based on our calculation, a maximum depth of 5 mm would not impede gas exchange until late in development. Further, when convection of plasma is considered, we have shown that $\dot{T}_{O_2}$ would provide enough gas over a range of DD_{max}s.

Conclusions

The results of model 1 suggest that diffusion would provide ample gas exchange through two-thirds of development, and the addition of convection would afford greater over all ability to obtain gas as would be required by animals at NF 50-51. Both models suggest that the limitation to gas exchange occurring in the first step, and were constrained by progressively larger ΔP_{O_2}s required to achieve the same overall uptake. Moreover, the fact that a greater water velocity (increasing surface P_{O_2}, and reduction in boundary layer) was required with each progressive stage also indicates that gas exchange was limited by the first step in the process of diffusion (model 2). Addition of

convective transport clearly indicated that gas exchange would be possible through all stages of development, provided that capillaries were not greater than 1 mm below the surface and water velocities were above 3 $cm.sec^{-1}$. So based on the results of our models it is clear that animals whom retain a impaired O_2 transport system would be capable of sustaining life provided the conditions which favor large gradients and minimal diffusion distances are met.

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Figure 1. A schematic representation of a *Xenopus* larvae illustrating calculations for slice and whole animal volume.

Figure 2. A schematic representation of two consecutive slices through a *Xenopus* larvae illustrating calculations for slice and whole animal area.

Figure 3. (**A**) Illustrates the robustness of the algorithm, and shows the relationship between percent deviation for estimating volume (open bars) and surface area (filled bars) as a function of slice width. Values are means ± 1 standard deviation. (**B**) The measurement errors associated with sampling, and shows the relationship between percent deviation for estimating volume (open bars) and surface area (filled bars) as a function of slice width. Values are means ± 1 standard deviation.

Figure 4. (**A**) Shows the relationship between surface area (circles) and volume (squares) with developmental stage for normoxic animals (filled symbols) and chronically CO-exposed animals (open symbols). Both A_s and V were plotted on a linear scale against the linear dimension of development. (**B**) Shows the relationship between the ratio of surface area and volume with development for normoxic (filled bars) and chronically CO exposed larvae (open bars) plotted on a linear scale against the linear dimension of development. All values are shown as means ± SEM. In some cases the error bars are inside the symbols. ξ

indicates significant ($p \leq 0.05$) difference than the previous stage, and where * indicate significant difference from controls.

Figure 5. The relationship between oxygen transport, and surface P_{O_2} or DD_{max} for models 1 and 2 at NF 33-34. In all cases calculated data is presented as dark gray fill, while the light filled plane indicated the measured $\dot{M}_{O_2}$ for animals at this stage range and were plotted for reference. (**A**) The relationship of diffusive conductance with surface and core P_{O_2} for *Xenopus* larvae (Model 1). (**B**) Indicates the results of Model 2, based on the relationship of $\dot{D}_{O_2}$ with surface P_{O_2} and maximal diffusion distance for *Xenopus* larvae. (**C**) Indicated the results of total conductance with respect to surface and core P_{O_2}. (**D**) The relationship of total conductance with surface P_{O_2} and DD_{max}.

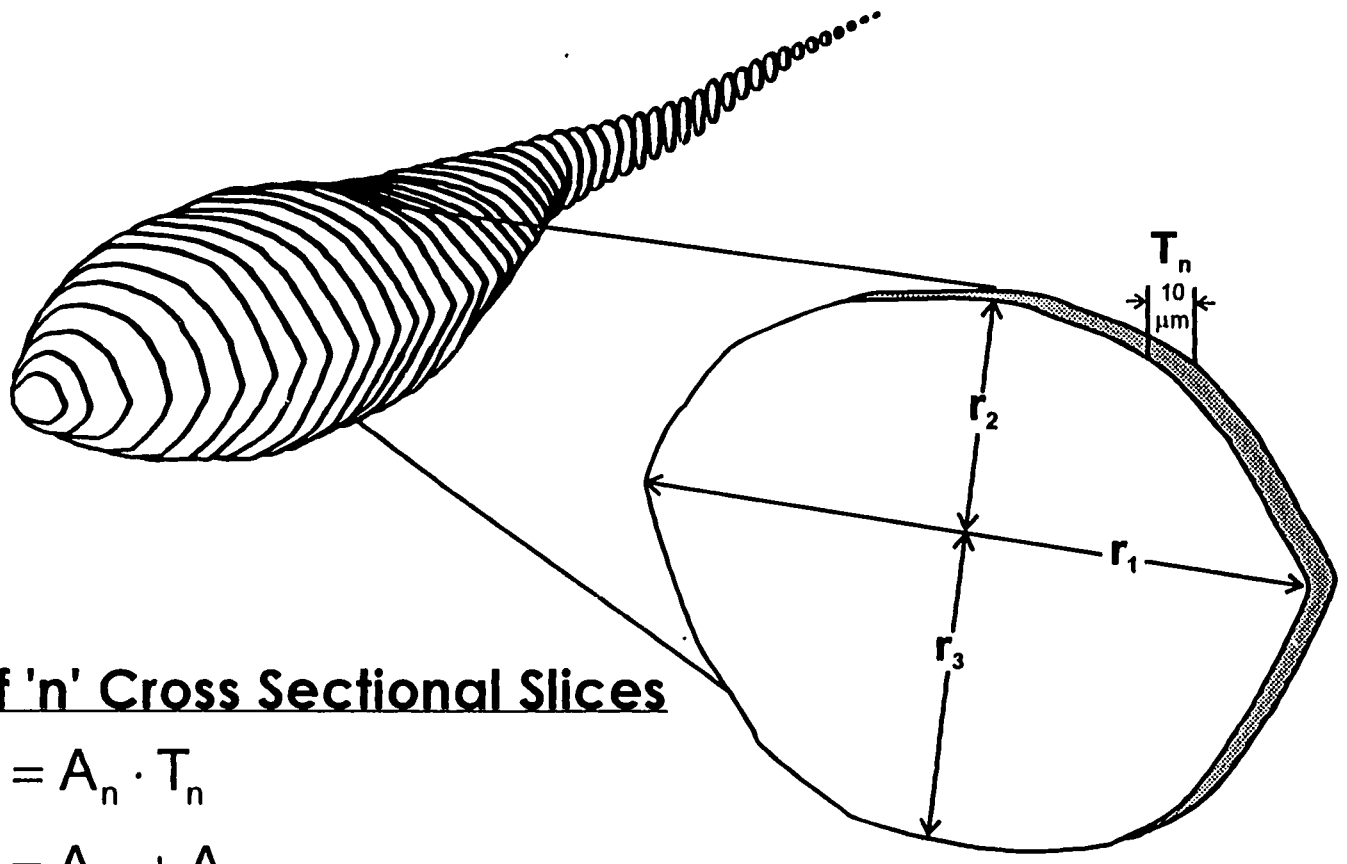
Figure 6. The relationship between oxygen transport, and surface P_{O_2} or DD_{max} for models 1 and 2 at NF 44-45. In all cases calculated data is presented as dark gray fill, while the light filled plane indicated the measured $\dot{M}_{O_2}$ for animals at this stage range and were plotted for reference. (**A**) The relationship of diffusive conductance with surface and core P_{O_2} for *Xenopus* larvae (Model 1). (**B**) Indicates the results of Model 2, based on the relationship of $\dot{D}_{O_2}$ with surface P_{O_2} and maximal diffusion distance for *Xenopus* larvae. (**C**) Indicated the results

of total conductance with respect to surface and core P_{O_2}. (**D**) The relationship of total conductance with surface P_{O_2} and DD_{max}.

Figure 7. The relationship between oxygen transport, and surface P_{O_2} or DD_{max} for models 1 and 2 at NF 46-47. In all cases calculated data is presented as dark gray fill, while the light filled plane indicated the measured $\dot{M}_{O_2}$ for animals at this stage range and were plotted for reference. (**A**) The relationship of diffusive conductance with surface and core P_{O_2} for *Xenopus* larvae (Model 1). (**B**) Indicates the results of Model 2, based on the relationship of $\dot{D}_{O_2}$ with surface P_{O_2} and maximal diffusion distance for *Xenopus* larvae. (**C**) Indicated the results of total conductance with respect to surface and core P_{O_2}. (**D**) The relationship of total conductance with surface P_{O_2} and DD_{max}.

Figure 8. The relationship between oxygen transport, and surface P_{O_2} or DD_{max} for models 1 and 2 at NF 50-51. In all cases calculated data is presented as dark gray fill, while the light filled plane indicated the measured $\dot{M}_{O_2}$ for animals at this stage range and were plotted for reference. (**A**) The relationship of diffusive conductance with surface and core P_{O_2} for *Xenopus* larvae (Model 1). (**B**) Indicates the results of Model 2, based on the relationship of $\dot{D}_{O_2}$ with surface P_{O_2} and maximal diffusion distance for *Xenopus* larvae. (**C**) Indicated the results

of total conductance with respect to surface and core P_{O_2}. (D) The relationship of total conductance with surface P_{O_2} and DD_{max}.



Volume of 'n' Cross Sectional Slices

$$V_n = A_n \cdot T_n$$

$$A_n = A_{n_1} + A_{n_2},$$

$$\therefore A_{n_1} = \tfrac{1}{2} \cdot \pi \cdot r_1 \cdot r_2,$$

$$A_{n_2} = \tfrac{1}{2} \cdot \pi \cdot r_1 \cdot r_3$$

T_n = thickness at slice'n'

Whole Body Volume

$$V = \sum^{n} V_n$$

'n' Cross Sectional Slice Area

$$A_s = \left(\frac{P + P'}{2}\right) \cdot \bar{T}_n$$

$$\therefore P = P_{(1-2)} + P_{(1-3)}$$

$$P' = P'_{(1-2)} + P'_{(1-3)}$$

$$P_{(1-2)} = 4 \cdot r_1 \cdot E_{(1-x)} \cdot \psi$$

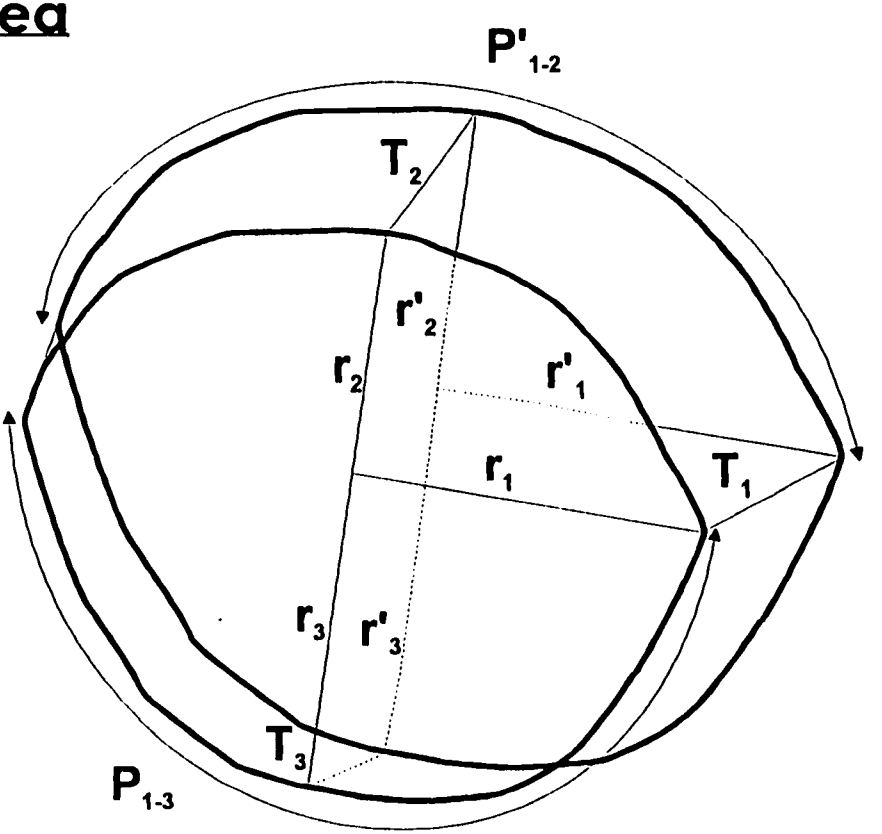
$$\therefore \psi = \sin^{-1}\left(\frac{\sqrt{r_1^2 - r_2^2}}{r_1}\right)$$

r_1 = dorsal semiaxis

r_2 = dorsolateral semiaxis

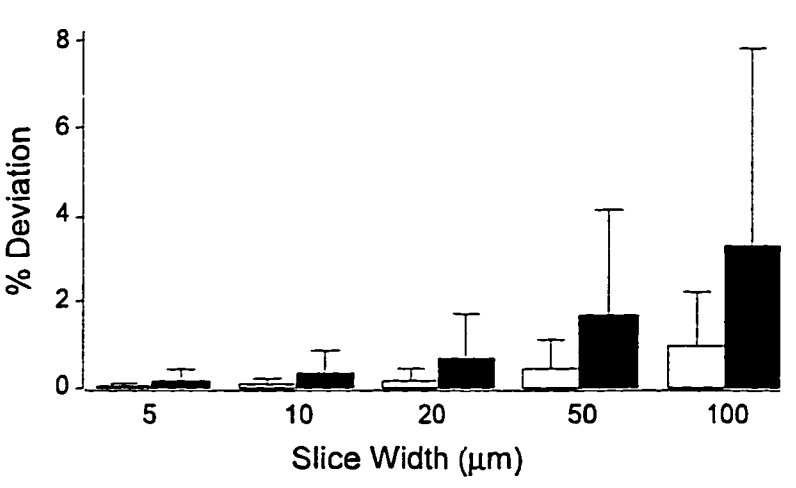
$E_{(1-x)}$ = eliptecal integral segment 1 - x

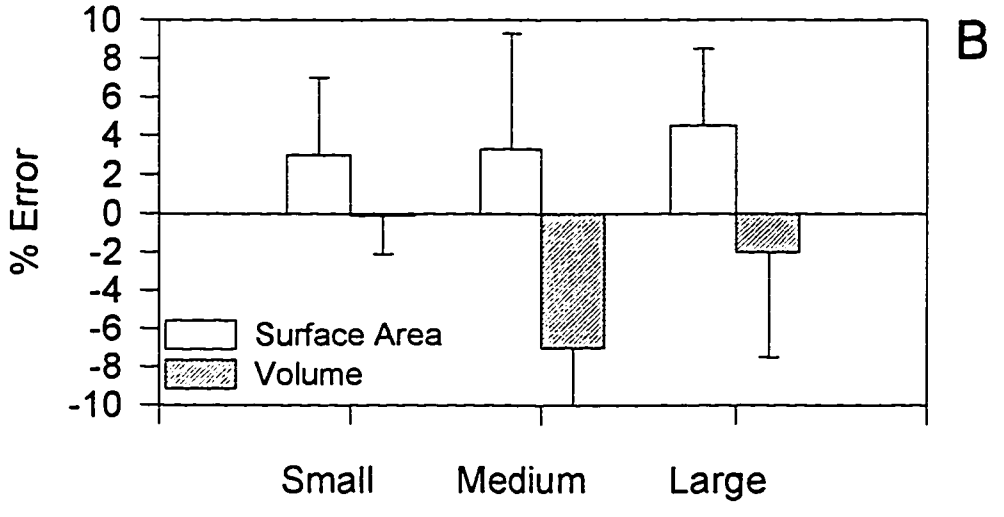
$$\bar{T}_n = \frac{T_1}{2} + \frac{T_2}{4} + \frac{T_3}{4}$$

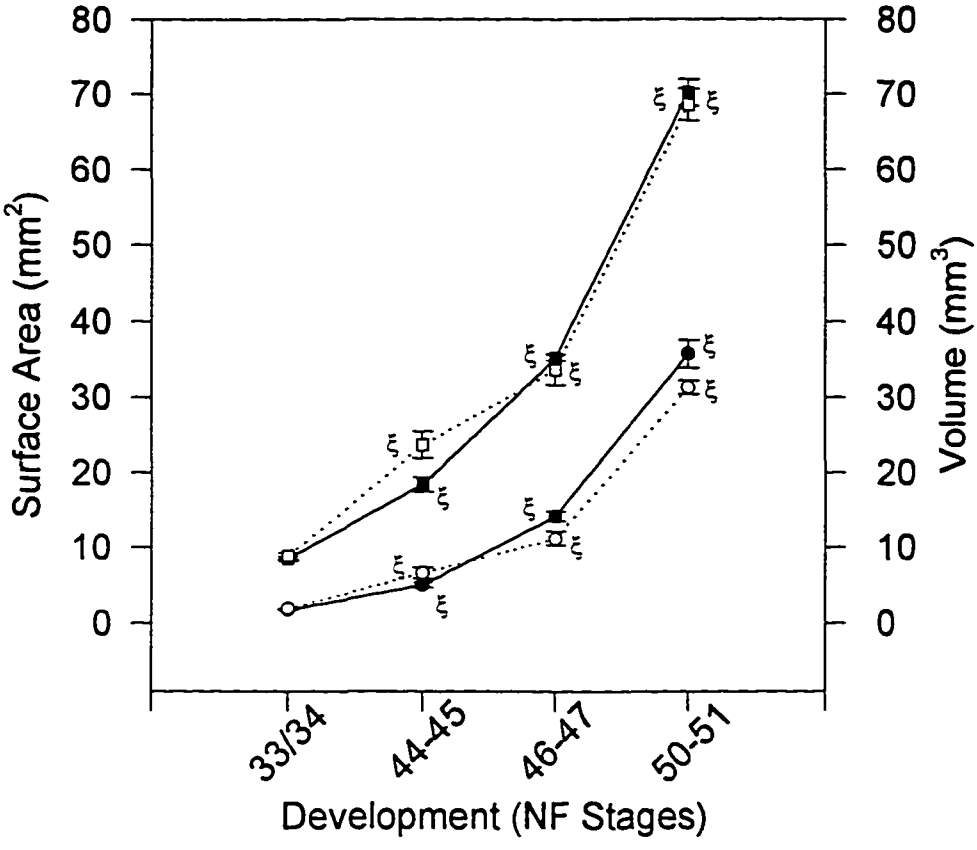


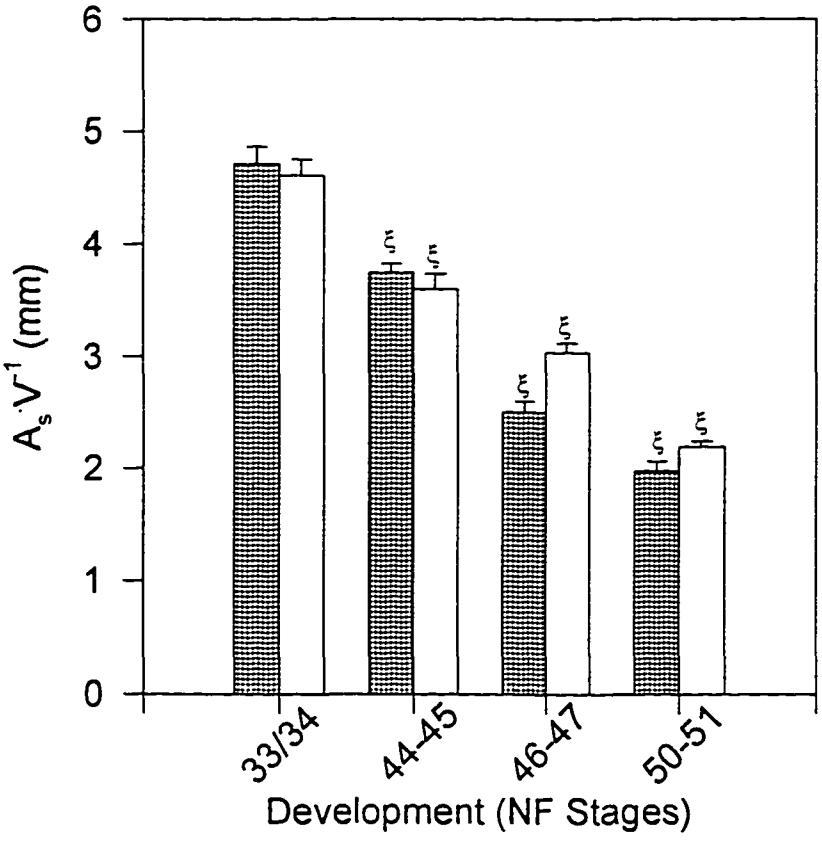
Whole Body Surface Area

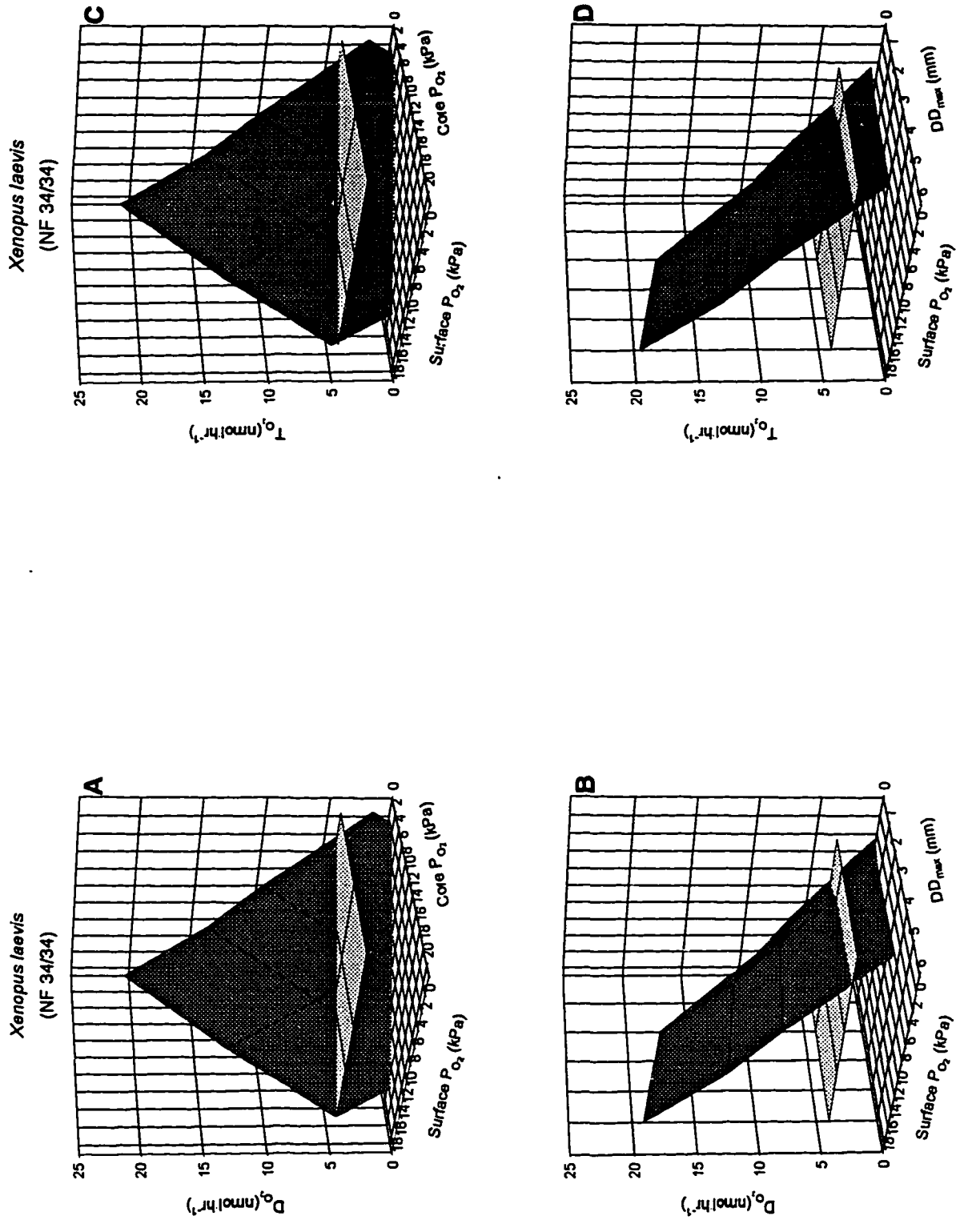
$$A_s = \sum^{n} A_{cs}$$

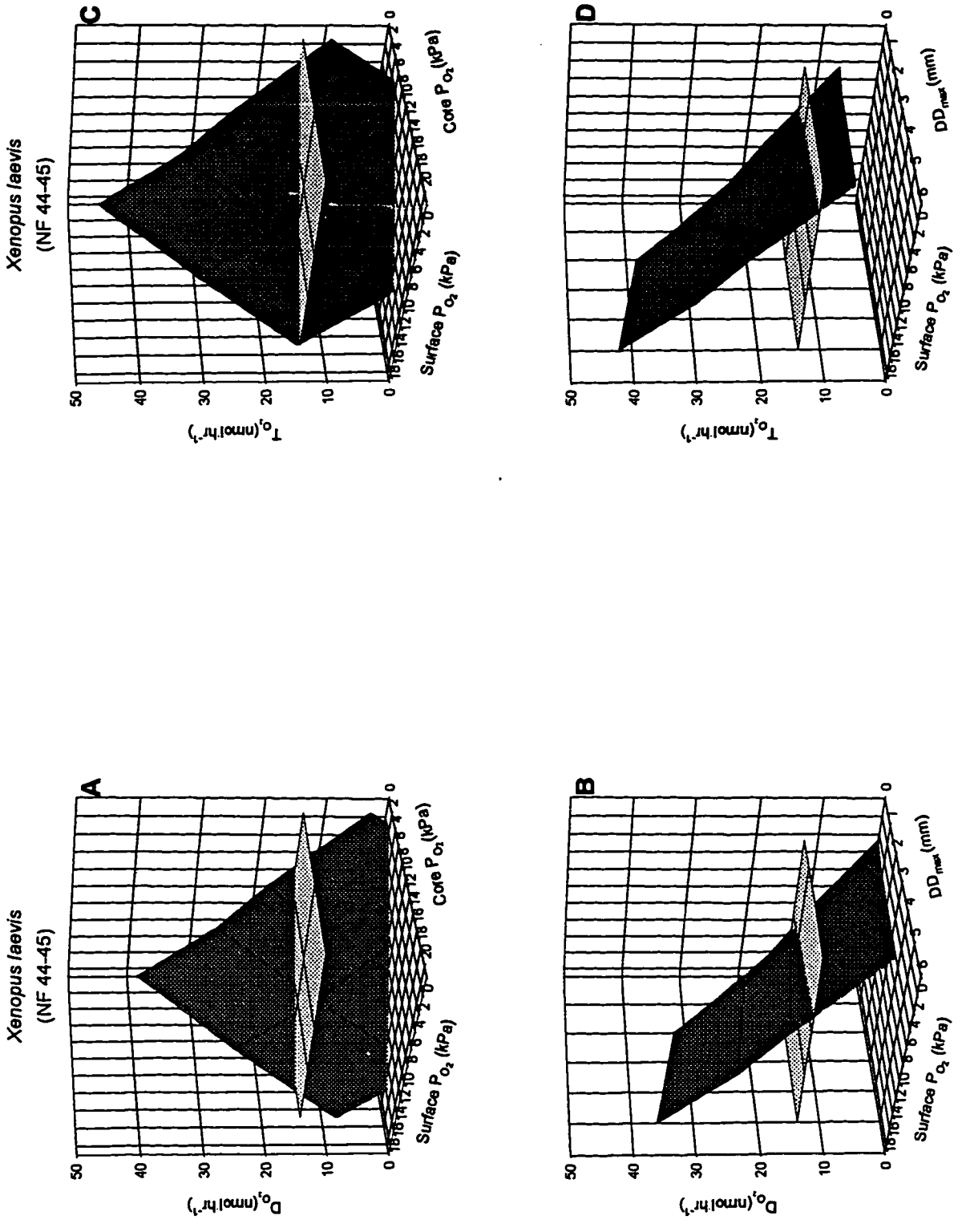


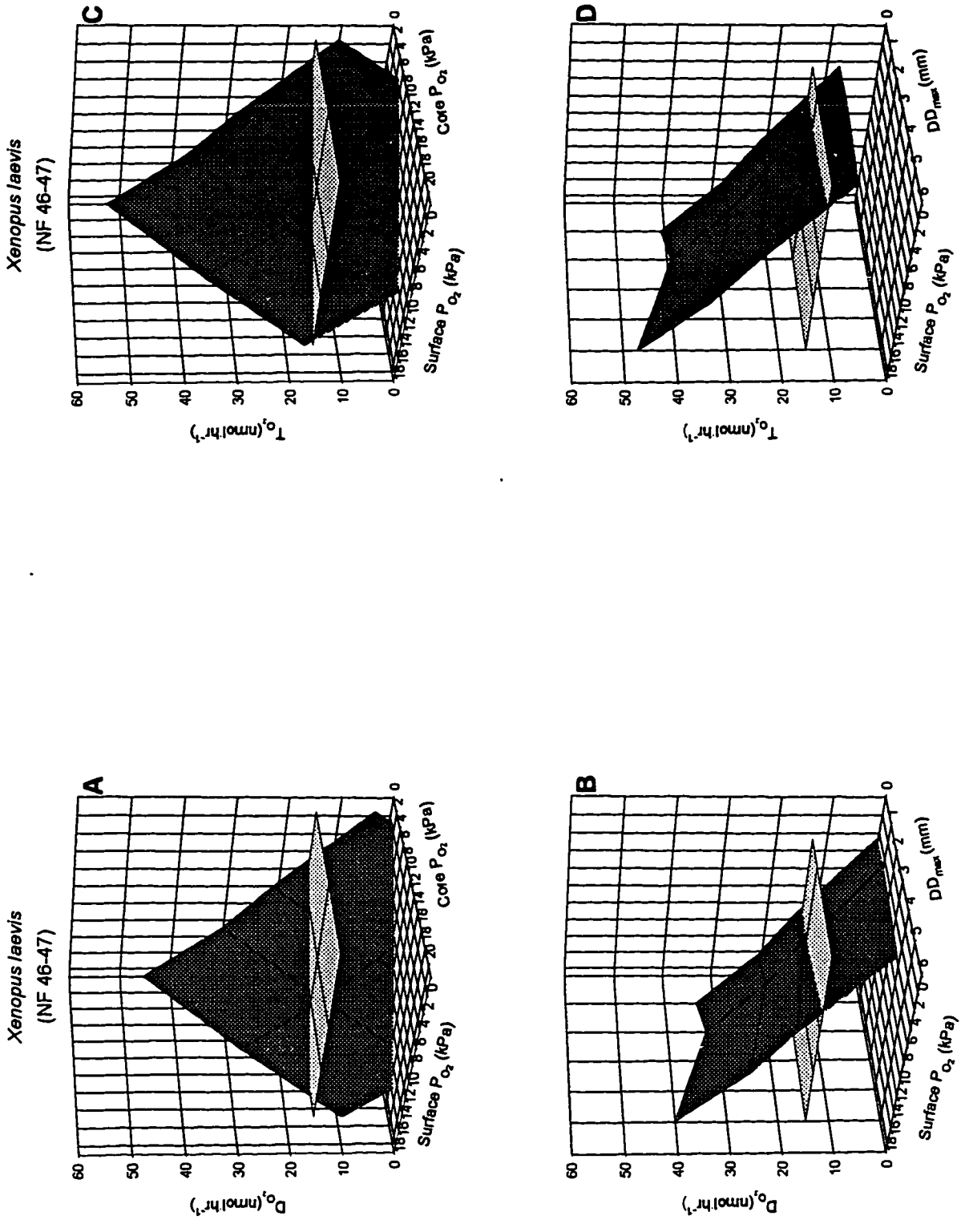












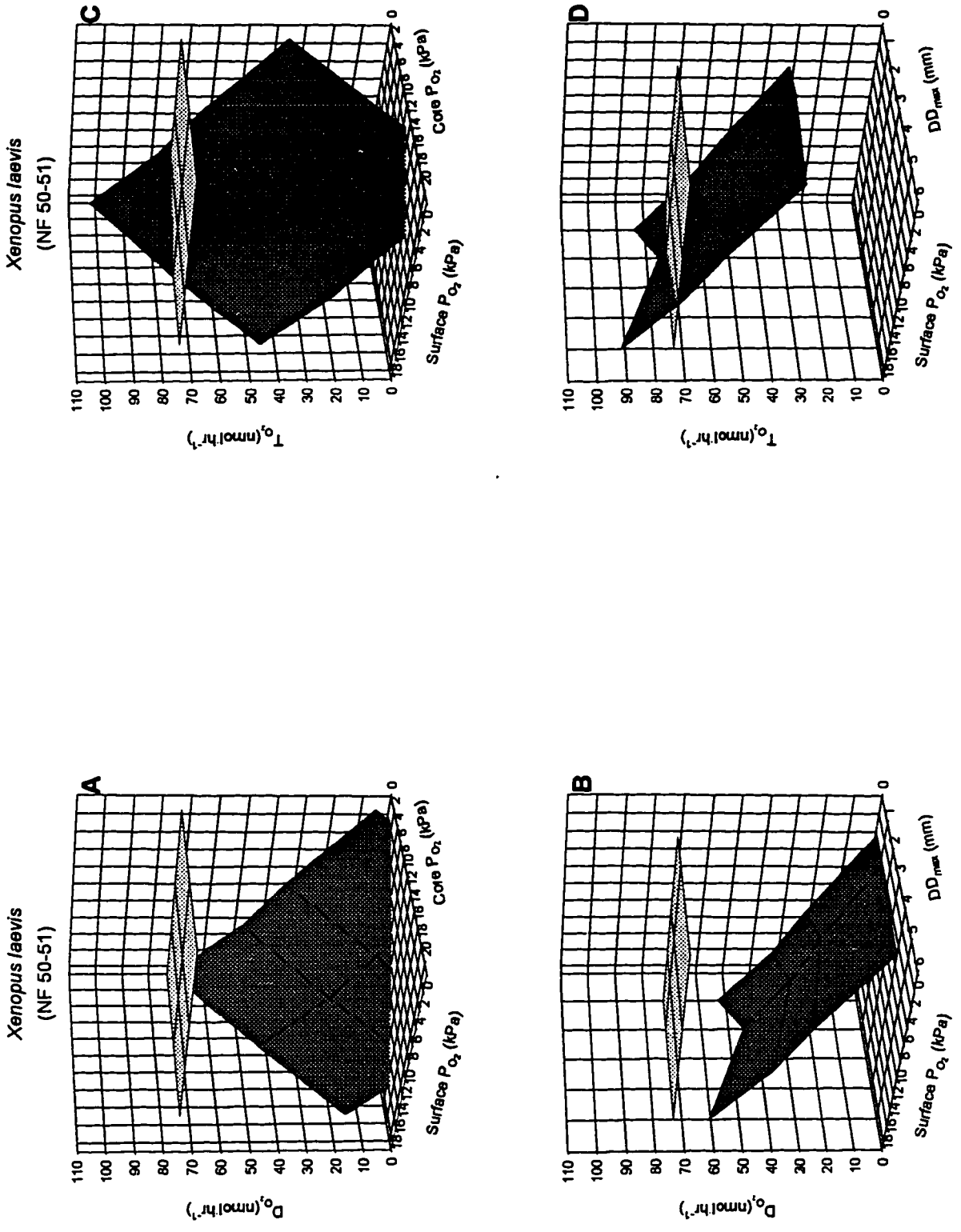


Table 1. Statistical results for surface area, and volume with stage and condition.

Variable	*Factor*	*n*	*df*	*F-value*	*p*
Vol	Condition	160	158	0.6826	0.4958
	Stage	80	78	18.4284	0.0001
A_s	Condition	160	158	0.2101	0.8339
	Stage	80	78	19.7788	0.0001

n, df, F, and p are the sample size, degrees of freedom, F-statistic, and fiduciary level. Significance was taken at $p \leq 0.05$.

CHAPTER 6

General Conclusions and Relevance of the Work

That the cardiovascular system forms for the sole purpose of delivering oxygen to the tissues has become a pervasive dogma in the cardiovascular and respiratory literature. Its foundation arose from observations and speculation of researchers throughout the past century. Interestingly, few studies have tested this assertion directly through controlled experimentation. Needham (1959) stated that embryology, and therefore embryological physiology, relies on a delicate balance of three things: careful observation, speculative thought, and controlled experiments. Thus, it is clear that our intuition of what must be, and what truly is, resets on the on the balance that Needham (1959) spoke about.

It was against this background that the studies in this dissertation began. The objective was to determine the ontogeny of cardio-respiratory support of metabolism which was carried out in two stages, a descriptive, and experimental approach.

Chapter One, the descriptive approach, evaluated how body composition changes with progressive development. Early amphibian body composition was primarily composed of lipids which was unlike the results seen in fish. Moreover, when the total energy pools available for growth and development were calculated from both lipids and protein, it became clear that the total available energy for metabolism was not a limiting resource during development. Another important feature of this study was the contribution of body composition, and its

potential role in overestimating body mass. This work demonstrated that body composition could result in an overestimate of dry body mass by as much as sixty percent. The implications of overestimating body mass can have profound effects on any physiological measure; therefore, the main contribution of this work is to emphasize the need to understand of how body composition changes with development, and how these changes could be utilized by future researchers to minimized error associated therein.

Provided with the information from Chapter Two, we can then go forth to utilized an experimental approach to elucidate our how, or if, the cardiovascular and respiratory systems are coordinated with respect to gas exchange. Chapters Three, Four, and Five set out to test these hypotheses directly.

The work presented in Chapter Three illustrated three important things. First, that the onset of respiratory function is not timed with the development of convective flow. Second, that Hb is not essential for the bulk transport of oxygen. And last, the cardiovascular and respiratory systems show a low level of coordination. Collectively these data indicated that the central dogma of coordination is in fact not accurate for lower vertebrates, and that gas exchange in developing embryos/larvae may be set by processes other than by convection alone. The relevance of this study is far reaching, in that it demonstrates the absolute requirements for O_2 is far below the cardiovascular and respiratory systems ability to deliver gas for metabolism. Recent advancements in micro-surgical techniques have allowed for invasive procedures to be carried out on

human fetuses *in utero*. These corrective techniques, which were thought to be impossible just a few short years ago, are now a reality. In a few decades therapeutic surgery for the repair of atrial and ventricular septal defects, and patent ductus arteriosus in embryos will become common place. Thus, knowing the precise role of the embryonic and early fetal circulation and its role in gas exchange will be crucial during heart corrective surgery in making decisions about whether to interrupt circulation and for how long this would be possible. Clearly, our work indicates that this would in fact be possible, and that disruptions like this may not affect overall embryo viability.

Chapter Four investigated what factors overall limit gas exchange in a developing vertebrate embryo. The suggestion that gas exchange in vertebrate systems may be limited by diffusion, perfusion, or both has yet to be determined. Our results indicated that gas exchange in lower vertebrate embryos was not limited by diffusion, as animals exposed to either high or low levels of O_2 did not result in marked changes either cardiovascular or respiratory function. Moreover, our data also demonstrated that convective flow was also not limiting to overall gas exchange. This was evident in the normal level of aerobic metabolism seen coupled with the moderate changes seen in cardiovascular function for animals raised in chronic CO. Clearly, these data indicate that gas exchange in lower vertebrates may occur by static processes, and that the limitations to gas exchange may be set by environmental factors which are more extreme than those imparted in our studies. Overall the results from Chapters Three and Four

indicate that diffusion may be a viable means by which to obtain O_2 through development.

The relevance of this chapter indicates that the circulatory system is highly plastic and is build with a high degree of "functional reserve". More recently there has been a movement in developmental biology to understand which factors may act as teratogens, thereby stimulating growth and differentiation. One such factor is oxygen, and has been studied in great detail by researchers working on erythropoietin (EPO) and vascular endothelial derived growth factor (VEDGF). The state of oxygenation during early development can have a profound effect on the production and distribution of these growth modulators. Moreover, understanding how, or if, the cardio-respiratory system is limited to gas exchange is of paramount importance in establishing which conditions stimulate the production of these gene products, and which are simply timed with the stage of development.

Chapter Five evaluated two models examining the question of whether gas exchange is feasible by diffusion alone, and if convection of plasma would afford animals exposed to CO any benefit. Our calculations indicated three important things. First, that diffusion could play a substantial role in overall gas exchange in larvae from the on set of heart beat through pre-metamorphic climax. Second, that convection of plasma could afford a larger quantity of gas to be transported for aerobic metabolism. Moreover, that animals at metamorphic climax would be capable of obtaining enough O_2 via a combination of convection

and diffusion to live without Hb-O_2 transport. Lastly, our models indicated that the overall limitations to gas exchange were set by the surface and core P_{O_2}s, and that maximal diffusion distances were much larger than those predicted in the literature.

The development of mechanistic models are of immense importance to elucidate the intricacies of embryonic gas transport. The overall importance of a model such as this, is that it allows for prediction of internal gas tensions, as well as maximal diffusion distances. A potential clinical application could be the determination of potential embryo viability post *in vitro* fertilization. It is common practice to ablate "redundant" embryos post implantation in the cases of multiple fetuses ($\geq$3), so the question becomes, which has the greatest potential for survivability. Our model allows for the determination of diffusive gas flux given the parameters collected routinely collected by obstetricians and therefore determination of sufficient O_2 supply prior to vascular growth.

Overall the results from these studies indicates that the formation of the cardiovascular system may form for purposes other than for the gas exchange alone. Moreover, it is also clear that that the limitations to gas exchange are set more by extrinsic than intrinsic factors. Thus, based on our findings we submit that our understanding of overall gas exchange with development needs to be reevaluated, and that blanket statements which might apply for mammals may not be suitable for exothermic lower vertebrates.

Future Directions

The work completed in the descriptive phase of this dissertation (Chapter 2) will allow for the determination of how body composition changes when metabolic loads are increased. Work completed in fish has indicated that changing metabolic load with temperature can have a marked effect on the rate of substrate used (Rombough, 1988). Since amphibian eggs are primarily composed of and utilize lipids for energy production with development, it would be interesting to see if there is an energetic preference for certain low-energy easy to degrade fuels. Additionally, there exist few studies in lower vertebrates which have looked at metabolic coefficients, and how they change with progressive development and substrate types. Clearly, these types of studies would go a long way to clarifying how lower vertebrate metabolism may regulate substrate preference with development.

The chronic experimental studies (Chapters 3 to 5) have opened a myriad of questions about how gas exchange in lower vertebrates may be regulated. One such question which remains unanswered to date, is how is vascularity in animals affected when exposed to CO? Anecdotally, we have observed a functional increase in total vascular area. Thus, a future project which determined how vascularity changes with progressive development would begin to elucidate how increases in functional surface area may change with exposure to CO. Total vascular area can be determined by using laser confocal microscopy (LCM). Animals could be micro-injected with a fluorescently labeled

inulin, and then sampled for the fluorescence in 10 μm sections. These sections could then be reconstructed with the use of an image analysis program, and from this an index of vascularity could be determined from a ratio to voxel area and fluorescent area.

Another study which would give additional credence to the idea that vascular area increases with exposure to CO would be to measure pressure and flow in animals whom have been raised under normoxia and chronic CO. Based on this information, one can then determine total peripheral resistance, which would give an index of how vascularity changes with additional parallel vascular beds. The addition of new vascular beds would increase the relative area for exchange, and therefore allow for greater quantities of gas to be exchanged via simple diffusion. Thus, our understanding of how these structural changes occur could lend additional support to the contention that diffusion is a viable means for exchange in hypoxemic animals.

Another observation made during the course of this work was the contribution of lymphatic flow to total vascular volume maintenance. It was observed that these hearts began function just as animals began to actively swim. Work on adult vascular volume regulation in *Xenopus* has shown that lymphatics may make up as much as one third to one half of the total blood volume (Hillman, 1978.). The question remains however, as to their role in oxygen uptake and distribution in animals chronically exposed to CO. To answer this, animals could be video taped and analyzed for rate and stroke volume,

which would afford inferential support for the premise that lymph flow increases with exposure to hypoxemia. This line of reasoning would argue that the accessory organs, such as the lymphatics, may play a more substantial role gas transport than had been previously thought.

Lastly, to determine if diffusion truly is sufficient for gas exchange with progressive development, a population of early embryos could be cardio-ablated (After Mellish *et al.*, 1995) and then allowed to develop. Measures of oxygen consumption, critical oxygen tension (P_{crit}), and developmental trajectory (a developmental rate, $T_d = \frac{\text{NF Stage}}{\text{Day of Development}}$) can be determined, thus allowing for the assessment of how important the cardiovascular system in reference to gas transport through development, and how this limitation may affect rate of development.

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