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The metabolic rate of Xenopus laevis: Interactional influences of development and short term hypoxia

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The Metabolic Rate of *Xenopus laevis*: Interactional Influences of Development and Short term Hypoxia.

by

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ABSTRACT

Amphibians undergo pronounced physiological and morphological adjustments during metamorphosis, due in large part to the change from water breathing to predominantly air breathing. A developmental change in the ability of the respiratory and cardiovascular systems to transport oxygen or a change in tissue demand for oxygen may be reflected in the aerobic metabolic rate (MO_2). This study focuses on how MO_2 of the South African clawed frog *(Xenopus laevis)* is affected by short term hypoxia during development. MO_2 of animals from Nieuwkoop and Faber stages 1-66, 1 month post-metamorphic froglets, and adult frogs were measured under conditions of normoxia and various degrees of hypoxia down to PO_2>10 mmHg. Whole-body lactate contents were also assayed to assess anaerobic metabolic supplementation of aerobic metabolism. Larvae up to stage 57 were oxygen conformers. After stage 58, larvae were oxygen regulators. Anaerobic metabolism was used to supplement aerobic metabolism only by animals older than stage 52.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

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Introduction

Many different changes in physiology occur during the development of an organism from embryo to adulthood. One of the most pronounced changes that amphibians undergo is the transition from water to air-breathing. Any change in the ability of the respiratory physiology to transport oxygen may be directly reflected in the metabolic rate of the organism. *Xenopus laevis* (the South African clawed frog*), has become a standard laboratory animal for many developmental studies, and in the last few decades has been extensively studied with respect to circulation and respiration. The metabolic properties of adult *Xenopus laevis* (Hutchison, Whitford, and Kohl, 1968; Emilio and Shelton,1974; Deuchar, 1975; Shannon and Kramer, 1988; Gatten et al., 1992), along with numerous studies on

*The genus of African Clawed frogs which we know as *Xenopus* has been given many names since its discovery. *Xenopus* was originally named the "South African Clawed Toad", but after subsequent studies of its anatomy, *Xenopus* has been placed in the family Pipidae. However, *Xenopus* is still often refered to as a toad and it is really problematical whether it should be called a toad or a frog.

the resting and active MO_2 of post-hatch larvae of *Xenopus laevis* (Gradwell, 1975; Feder, 1981; Wassersug and Feder, 1983; Feder and Wassersug, 1984) and other larval amphibians have been measured (see Burggren, 1984; Feder, 1981; Burggren and Just, 1992; Gatten et al., 1992 for reviews of the extensive literature). However, the effects of development on MO_2 and the ability of post-hatch larvae to exploit anaerobic metabolic pathways are less well known. *Xenopus laevis* encounter hypoxic conditions routinely, but it is unknown whether these animals continue to remove oxygen from the environment or resort to anaerobic metabolism to supplement the resulting decrease in aerobic pathways (See Burggren and Just, 1992 for review).

The need for oxygen is commonly the primary factor underlying air-breathing in adult aquatic vertebrates that can breathe both water and air (Randall et al, 1980; Hicks and Woods, 1989; Bennett, 1978), but there are many other factors associated with air breathing.

Both intense activity and exposure to hypoxic environments result in anaerobic metabolism. Under both of these circumstances, demand for high-energy phosphate groups exceeds the supply that can be sustained through aerobic metabolism. The fact that both of these circumstances in vertebrates result in

the formation of large quantities of the same compound, lactic acid (Hughes, 1965; Herreid, 1980; Hutchison and Miller, 1979; Bennett and Licht, 1974), has tended to emphasize their similarities. However, the selective demands of each situation are quite different. During hypoxia or anoxia, it is important to sustain anaerobic metabolism for as long as possible since the time of return to normoxic conditions is unknown. During activity, the purpose of anaerobic metabolism is to generate as many high energy phosphate compounds as possible in the shortest amount of time. This results in a high concentration of anaerobic end-products in an extremely short time. Therefore, to estimate the least amount of lactic acid production required to supplement aerobic metabolism, hypoxic stress should be investigated as opposed to activity.

An increased metabolic rate in response to hypoxia can be elicited in response to the many different ways that a lack of oxygen stresses an animal. Hypoxic conditions stress the whole animal and affect not only the respiratory system, but also the cardiovascular, endocrine, digestive, musculoskeletal, and various other systems of an organism. *Xenopus* larvae are found in habitats ranging from permanently flowing water with an ambient PO_2 of approximately 150 mmHg to stagnant

water buffalo wallows and temporary ponds which have a very low PO_2 (Feder and Wassersug, 1984; Nieuwkoop and Faber, 1967). Thus, while the larvae must routinely encounter aquatic hypoxia, the ability of larvae of different stages to cope with hypoxia is unknown, as is the result of such hypoxia on the animal's metabolism.

In tadpoles of *Xenopus laevis, Rana pipiens, Rana berlandieri,* and *Rana catesbeiana*, air-breathing is essential in compensating for aquatic hypoxia (West and Burggren, 1982; Feder 1983; Feder and Wassersug, 1984; Feder et al., 1984). Larval *Xenopus laevis* have long been known to breathe air at an early age (Bles, 1905) which may significantly benefit the larvae, especially since *Xenopus* larvae lack true gill filaments (Feder, 1984). But, it has also been proven that larval *Xenopus* can survive periods of two to three weeks in normoxic water without access to air (Feder and Wassersug, 1984), so breathing is not obligatory. The large gill filters and buccopharynx of larval *Xenopus laevis* are well vascularized for gas exchange, but these surfaces must also function for food entrapment in feeding (Wassersug, 1972; Gradwell, 1975). Because of the functional conflict between feeding and gas exchange, air breathing, therefore, would allow for simultaneous feeding (in the gills and buccopharynx)

and respiration (in the lungs) (Feder et al., 1984; Wassersug, 1972).

There are also no structures to protect the gill filters against damage and clogging, so buoyancy from the lungs may also be beneficial. Larvae of *Xenopus laevis* almost always hover midwater with the head tipped down and the tail elevated and paddle with the tail to maintain their position in the water column, except when surfacing for air (van Bergeijk, 1959; Gradwell, 1971). Air breathing is essential to the maintenance of this hovering posture and the normal buoyancy of *Xenopus* (Feder and Wassersug, 1984), but there is a direct energetic cost associated with having to swim to the surface (Vivekanandan and Pandian, 1977; Kramer and McClure, 1981; Feder and Moran, 1985; Pandian and Marian, 1985) so the increase in aerial gas exchange may be offset by the increased metabolic rate resulting from the increase in activity.

Larvae may air breathe for two distinct yet inter-related reasons. First, air can be used to deliver a much larger amount of oxygen to the tissues since it contains a much larger concentration of oxygen than water (Dejoures, 1981), but this may not be the primary reason for breathing air. The skin of amphibian larvae accounts for the majority of respiratory

capillarization (Strawinski, 1956; Saint-Aubain, 1982), is thin, and is the predominant route of oxygen uptake in air-breathing larval *Rana berlandieri* and *Rana Catesbeiana* in normoxic water (Burggren and West, 1982; West and Burggren, 1982; Burggren et al., 1983). The lungs of many amphibians show structural and physiological adaptations related to buoyancy regulation. Aquatic larvae of the salamander, *Ambystoma tigrinum*, often float at neutral buoyancy in the water column to feed (Burggren and Roberts, 1991). Therefore, in some cases, the function of air breathing may be primarily for maintaining buoyancy. Buoyancy resulting from filling the lungs would function much like a swim bladder keeping the tadpole from resting on the bottom and clogging its gill filters. The lungs may only serve a major role in acquiring oxygen in times of hypoxia or when repaying an oxygen debt from anaerobic metabolism.

During hypoxia, anaerobic end products (primarily lactic acid) may accumulate in response to anaerobic metabolism (Armentrout and Rose, 1971; Bennett and Licht, 1974; D'Eon et al., 1978; Gatz and Piiper, 1979; Feder, 1981; Feder, 1983). In hypoxic times when the MO_2 is not sufficient to provide the energetic requirements, an animal often resorts to anaerobic

metabolism. Larval *Xenopus laevis* between Nieuwkoop and Faber (1967) stages 45-62, which do not have access to air for long periods of time, and larvae exposed to short term hypoxic water, are known to accumulate copious amounts of lactate (Feder and Wassersug, 1984) indicating anaerobic energy production. As a result, the anaerobic end products (primarily lactic acid) build up and are stored in the tissues. Upon return to normoxic conditions, amphibians may do two things with this material. They can oxidize it for energy through aerobic metabolic pathways or convert it back into storage products such as glycogen (Gleeson, 1991). Both of these alternatives require increased oxygen consumption to power these anabolic pathways, creating what is defined as an "oxygen debt".

Most bimodally respiring adult amphibians repay large oxygen debts almost entirely via the lungs (Feder and Burggren, 1985), but at least in early stage larval *Xenopus laevis* this must not be the case. Oxygen diffusion through the gills and body wall must satisfy metabolic needs and repay the oxygen debts of the young larvae, since the animals do not surface for air and do not have functional lungs.

The metabolic responses of animals to oxygen limitations in the environment are complex and varied,

but metabolic responses can generally be placed in one of two categories. Animals that are able to maintain their MO_2 at ambient PO_2 values below air saturation are termed "oxygen regulators". Metabolism of oxygen regulators is maintained down to a critical PO_2 (termed the P_{crit}), below which oxygen uptake begins to fall with decreasing PO_2. Hence, an efficient oxygen regulator would have a low P_{crit}. In some animals the P_{crit} effectively is at air saturation. The MO_2 decreases in proportion to decreasing PO_2 below normoxic levels. Classically, such animals are termed "oxygen conformers" (Herreid, 1980).

The distinction between oxygen conformity and oxygen regulation is not a sharp one, particularly when the P_{crit} is relatively high (Burggren and Roberts, 1991). This occurs for many reasons, but primarily because no species is a perfect regulator over the entire range of oxygen tensions (Burggren and Roberts, 1973; Herreid, 1980). Several methods have been developed to indicate the degree of regulation for a species (Mangum and Van Winkle, 1973; Bayne, 1973; Portner et al., 1991; Yeager and Ultsch, 1989). However, most authors simply try to identify the P_{crit} of the animal.

The P_{crit} is dependent upon the ability of the animal to supply oxygen to the tissues. If this ability is impaired, the P_{crit} may shift upward to a higher PO_2. This may occur if circulation, ventilation, or surface area for gas exchange is diminished or impaired in anyway, such as during development. Studies on oxygen regulators would suggest that a critical PO_2 exists that is not only characterized by the transition from a regulatory pattern to oxygen conformity but also by a transition to anaerobic energy production (Pelster et al, 1988; Portner et al. 1991).

Measurement of the resting metabolic rate throughout development will reveal the impact of development and differentiation of the respiratory structures on the metabolism of the animal along with the importance of the respiratory structures in satisfying the metabolic needs of the animal under conditions of hypoxia. The role of anaerobic lactic acid production in supplementing the normal metabolic rate of the larvae during periods when the aerobic respiratory structures are insufficient can also be determined from the amount of whole body lactic acid present at the different stages of development.

The ability of the respiratory structures to extract available oxygen from the environment and the ability of anaerobic metabolic pathways to make up for a decrease in aerobic metabolism can be estimated by finding how the animal copes with short term hypoxic conditions. By measuring the decrease in oxygen consumption and the increase in whole body lactate levels in the larvae, I can delineate the role of anaerobic metabolism in supporting the larvae during times when the aerobic respiratory structures cannot satisfy the needs of the animal.

The goals of this thesis are to investigate the influences of developmental changes on the MO_2, the ability of different stage larvae to cope with short term hypoxic conditions, changes in the P_{crit} in response to development, the ability of larvae to exploit anaerobic metabolic pathways, and changes in the surfacing behavior in response to development and hypoxia.

METHODS AND MATERIALS

Animals

Adult *Xenopus laevis* and their larvae and eggs were primarily derived from the breeding stock at the University of Nevada, Las Vegas. Additional animals were bought from commercial breeders (NASCO Scientific (Fort Atkinson, Wi.) and XENOPUS I (Ann Arbor, Mi.) as necessary. Animals were cared for and utilized according to conditions accepted by the University of Nevada, Las Vegas Animal Care and Use Committee (Protocol #R701-1092-080 Approved October 17, 1992). All animals were kept under a constant 12 hour light/dark cycle at 20± 1.0°C until needed for experimentation. Animals were fed NASCO Frog Brittle frog food three times per week on Monday, Wednesday, and Friday mornings.

To minimize the influence of external disturbances and diurnal fluctuations in metabolic rate and activity, animals were isolated in darkened chambers for these experiments. All measurements were performed on animals that had not been fed for a minimum of three

days in order to avoid measurement during a period of increased metabolic rated due to specific dynamic action. Adult *Xenopus* were kept in groups of 2-3 in 90 liter glass aquariums. Forty stage 66 larvae were isolated in one 340 liter aquarium and allowed to complete metamorphosis for the measurement of one month post-metamorphic froglets. Stage 1-66 tadpoles were kept at a concentration of about 3 larvae per liter of water in 273 and 340 liter aquariums prior to measurement. All animals were allowed to acclimate to the measuring chambers for a minimum of 12 hours before measurement was started.

Grouping of Animals

Larvae were individually staged according to Nieuwkoop and Faber (1967) and then isolated in the experimental chambers in developmental groups of similar respiratory development, ranging from early embryos to adulthood, as indicated in Table 1. One month post-metamorphic froglets and adult frogs were also isolated into two definitive groups for purposes of comparison to larval stages measured in these experiments (see Table 1).

Table 1. Definitive Groupings of Developmental Stages of Xenopus laevis. Respiratory/Circulatory Characteristics are indicated in bold face type.

Group Number:	Nieuwkoop & Faber Stages:	Age (DAYS):	Important Physiological and Morphological Characteristics:
I	1-39	0-2.25	Eggs and Embryonic Larvae.
II	40-44	2.5-3.5	Mouth Broken Through. **Blood Circulating in Gills& Filter Apparatus.** **Embryonic Lungs form a Closed System.**
III	45-48	4-11	Beginning of Feeding. **Initiation of Breathing.** **Lung Walls Flat Squamous Epithelium.**
IV	49-51	12-19	Hindlimbs Clearly Growing. **1st Out-Pocketing of Lungs into Dorsal Coelom.** Appearance of 1st Post. Pr. Dorsal Lymph Hearts. **Folds Appearing in Caudal Portions of Lungs.**
V	52-53	21-24	Flattening of Foot on Hindlimb. Fore and Hindlimbs form Paddles. **Blood Vessels Developed in the Lungs.**

Table 1 Continued. Definitive Groupings of Developmental Stages of Xenopus laevis.

VI	54-57	26-41	All Four Fingers Indicated on Hindlimbs. Elbow and Wrist Clearly Formed on Fore Limb. Hindlimb Chondrified.
VII	58-60	44-46	Forelimbs Broken Through. Hindlimbs Fully Formed. **Gill Chambers Still Wide.**
VIII	61-62	48-49	**Caudal 2/3 Lung in Honey-Combed Compartments.** **Openings of Gill Chambers Quite Narrowed.** **Beginning of Degeneration of Filter Apparatus & Branchial Chambers.** Degenerative Hypertrophy of Skin at End of Tail. Atrophy of Fins and Post. Tail Notochord.
IX	63-66	51-58	**Operculum of Gills Closed.** **Branchial Chambers Disappeared.** **Gill Slit Closed.** Degeneration of Tail Muscle. Shortening/Loss of Tail.
X	Froglets	90-100	1 Month Post-metamorphic Froglets.
XI	Adults	180-365	Adult Breeder Frogs.

Determination of Aerobic Metabolism

Aerobic metabolism was determined by using closed respirometry. All animals except adults were placed in 50 ml Erlenmyer flasks with rubber stopper lids. Adult animals were placed in 1 liter glass canning jars. Multiple animals were placed in the respirometers for several reasons. First, multiple animals allow for a 10-15 mmHg decrease in the PO_2 in the respirometer within a period of two hours. Secondly, *Xenopus laevis* is a very social animal (R. Wassersug, personal communication) and is found to surface for air in clusters (Baird, 1983; Shannon and Kramer, 1988), so measurement of isolated animals would not reflect normal MO_2. Lastly, multiple animals were used in order for a comparable amount of animal mass to be in each respirometer chamber. The number of animals isolated in each chamber varied depending on size and developmental stage (see Table 2).

Each respirometer contained 50 ml (minus the volume of the animals) of water and 2 ml of air. Water used to fill respirometers was tap-water that had been boiled to decrease microbial contamination and then cooled to 20^{o}C.

Table 2. Number of Animals in Each Respirometer and Total Number of Animals Measured for Each Group for Determination of MO_2.

Group:	Stage:	Total Number of Animals Measured:	Number of Animals in Each Respirometer:
I	1-39	7000	55-405
II	40-44	932	50-162
III	45-48	977	10-50
IV	49-51	735	10-50
V	52-53	110	1-10
VI	54-57	55	1-5
VII	58-60	43	1-5
VIII	61-62	32	1-5
IX	63-66	43	1-5
X	Froglets	41	1
XI	Adults	14	1

Respirometer Apparatus

A glass tee was epoxied onto a 30 ml glass syringe (Figure 1). A 19 gauge metal needle 7.75 cm long was then epoxied into the end of the glass tee so that it extended into the syringe through the base of the syringe. A three way stopcock was epoxied onto the third side of the three way stopcock forming a closed system. This allowed for gas to pass through the 19 gauge needle directly into the 30 ml glass syringe. The gas then flowed back past the needle into the glass tee and out one of the two open ports of the stopcock. This effectively provided mixing of the air within the 30 ml syringe.

Two 16 gauge needles were placed in the top of the respirometer so that they pierced the rubber stopper. A piece of Tygon tubing was then used to connect one of the 16 gauge needles in the rubber stopper to the 19 gauge needle epoxied in the glass tee of the 30 ml syringe. A second piece of tygon tubing connected the other 16 gauge needle in the rubber stopper to one of the two ports on the three way stopcock epoxied to the glass tee on the 30 ml syringe. This formed a closed system from the 50 ml respirometer flask to the 30 ml glass syringe with the 30 ml syringe acting as an air reservoir. A peristaltic pump was then placed in the

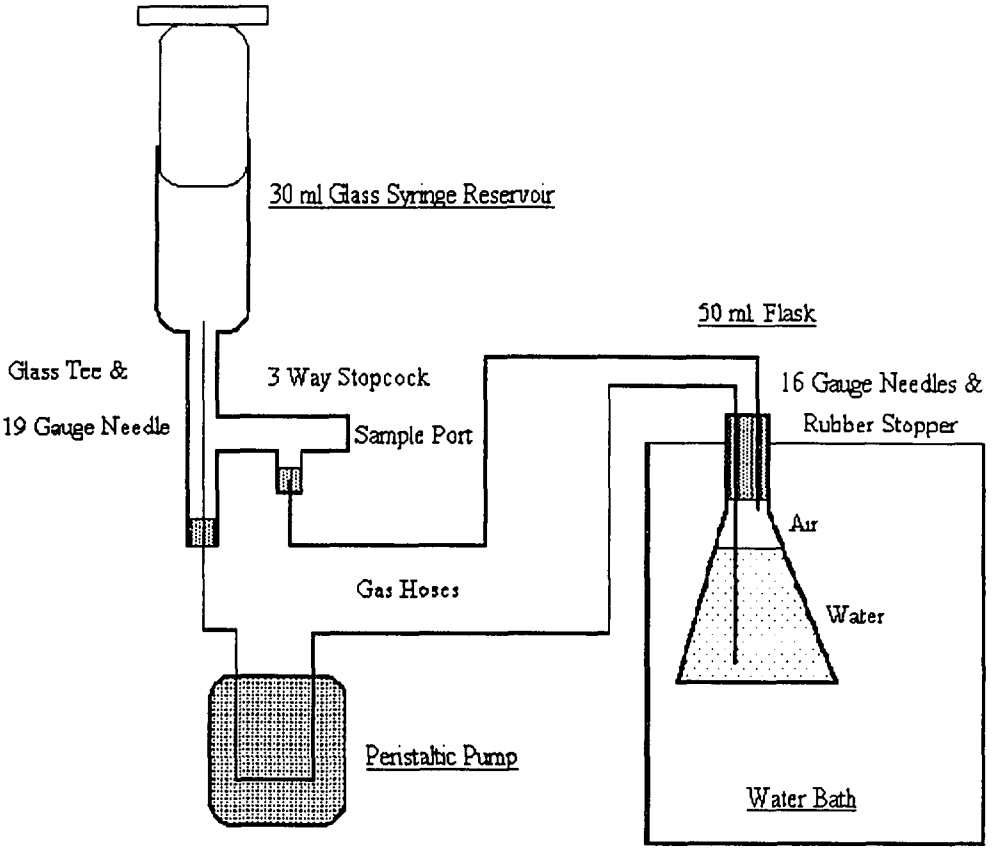

Figure 1. Respirometer Apparatus Used for Measurement of Total Amount of Aerobic Metabolism.

middle of the piece of tygon tubing connecting the respirometer to the 19 gauge needle of the 30 ml syringe apparatus. This provided a closed system that mixed the air between the respirometer flask and the 30 ml syringe. One of the 16 gauge needles piercing the rubber stopper was then extended to reached to the bottom of the flask in order to aerate and mix the water within the respirometer and the other 16 gauge needle was cut off so that it pulled air from the top of the flask. This resulted in complete aeration of the water phase and mixing of the air phase within the respirometer with the air in the 30 ml syringe. The bubbling of the air through the water served to mix the water so that boundary layers did not occur and to keep PO_2's constant within the respirometer and 30 ml syringe. The last open port in the three way stopcock epoxied onto the glass tee of the 30 ml glass syringe was used to remove gas samples from the closed respirometer system. The glass syringe, therefore, served as an air reservoir and also allowed for a decrease in volume when air samples were removed for analysis of PO_2.

Measurement of adult frogs were performed by replacing the 50 ml flasks with 1 liter canning jars that were similarly designed. Each respirometer flask

was placed in a constant temperature water bath that kept the temperature at 20±1.0°C. A dark black towel was then placed over the top of the water bath and respirometers to effectively keep the animals in a dark area isolated from the external environment. This allowed for gas sample to the removed from the respirometer system without effecting the behavior of the animals.

Measurement of MO_2

Measurements of MO_2 in respirometers containing only water and air were run as controls. In order to account for microbial respiration, the control MO_2 was subtracted when the final MO_2 was calculated.

One milliliter gas samples were removed from the respirometer system approximately every two hours resulting in a decrease in the volume of the reservoir. The partial pressure of the air sample was measured with a Beckman model OM-11 oxygen analyzer. This analyzer allows for static sampling of humidified gas samples as small as 0.1 ml, so the 1.0 ml humidified gas samples from the respirometers were directly sampled. The accuracy of the analyzer was checked and calibrated between every measurement with a one point calibration of humidified room air. The decrease in

PO_2 of the air sample was used to calculate the total MO_2 of the animal through both air and aquatic respiration. Pulmonary carbon dioxide elimination is minimal in amphibians, especially aquatic ones like *Xenopus laevis*. The majority of carbon dioxide is released through the skin and dissolves into the water (Burggren and Moalli, 1984; Burggren and West, 1982; Gottlieb and Jackson, 1976; Jackson and Braun, 1979; MacKenzie and Jackson, 1978). Therefore, CO_2 elimination was not measured in these experiments since the quantity of CO_2 that was dissolved in the water could not be determined.

The PO_2 of each respirometer started at a known PO_2 and was consecutively measured every two hours until the MO_2 was so low that oxygen extraction from the respirometer could not be determined or the reservoir no longer contained a gas sample. Although consecutive measurements were performed on the same animals within the same respirometers, animals were not used again for measurements at other developmental stages.

MO_2 Calculation

Oxygen consumption was measured as the percent decrease in available oxygen within the respirometer

and standardized into millimoles of oxygen consumed per kilogram of animal body weight per hour. During all experiments and in all calculations of these experiments the density of one gram of animal tissue was assumed to be equal to one.

The following equations were used:

Total $MO_2 = (MO_{2air} + MO_{2water})$.

$MO_{2air} = V_{air} \cdot (\%O_{2S} - \%O_{2E})/100 \cdot (P_b/760) \cdot (1/T) \cdot (1/M)$.

$MO_{2water} = V_{water} \cdot (PO_{2Ws} - PO_{2We}) \cdot 0.001823 \cdot (1/T) \cdot (1/M)$.

List of Abbreviations:

MO_{2air}= Aerial MO_2.

MO_{2water}= Aquatic MO_2.

$\%O_{2S}$= Percent Oxygen at Start of Measurement.

$\%O_{2E}$= Percent Oxygen at End of Measurement.

PO_{2Ws} (Starting PO_2 of water)= $(P_b - P_w) \cdot \%O_{2S}$

PO_{2We}(Ending PO_2 of water)= $(P_b - P_w) \cdot \%O_{2E}$

V_{air}= Volume of air in Respirometer System

V_{water}= Volume of Water in Respirometer.

P_b= Barometric Pressure.

P_w= Water Vapor Pressure.

T= Time in Hours.

M= Mass of Animals in Kilograms.

Each respirometer measurement contributed only one data point to the body of data regardless of the number of animals which the respirometer contained. Multiple groups of animals were measured until a minimum of ten data points were acquired at intervals of 15 mmHg starting at normoxia.

P_{crit} Determination

A method of least squares regression similar to Yeager and Ultsch (1989) was used to determine the P_{crit} for each of the groups studied using the Sigmastat statistical program from Jandel Corporation (San Rafael, Ca.). The P_{crit} for each of the groups of animals was defined by creating two linear least squares regressions through the average oxygen consumptions of each of the definitive groups. Regressions were calculated for linear and log transformed data. When one regression was found to fit the data better than two regressions, the group of animals was deemed to be oxygen conformers and therefore to have a P_{crit} at or above 150 mmHg. If it was found that two linear regressions fit the data better than one regression, the point at which the two regressions met was defined as the P_{crit}. Optimal linear regressions were defined by R^2 values that

approached 0 or 1 and with p values below 0.05. these criteria were used to find if one linear regression fit the data better than two regressions and was also used to find the optimal linear regression through the data points. When two regression were found to fit the data better than one, the regressions were required to cover all of the points and were not allowed to overlap one another. (For example, one regression may go from 21-10% so the other must start at 10% and go through the rest of the points). Resulting P_{crit} values from this method and the method of Yeager and Ultsch (1989) were compared to assure for accuracy.

Determination of Whole Body Lactate

During exercise of long duration where anaerobic metabolism is occurring, the only significant anaerobic pathway in amphibians is glycolysis. Muscle glycogen is degraded and lactate accumulates, but concentrations of other metabolites such as pyruvate, succinate, and alanine change very little (Bennett, 1978). Therefore, measurement of lactate closely estimates the amount of anaerobic metabolism. Measurement of lactate in the muscle or blood cannot accurately reflect the extent of anaerobic metabolism occurring in the entire body (Hutchison, Miller, and Gratz, 1981; Hutchison and

Turney, 1975; Hutchison, Turney, and Gratz, 1977; Miller and Hutchison, 1979; Preslar and Hutchison, 1978; Putnam, 1979; Hutchison and Turney, 1975; Quinn and Burggren, 1983). Therefore, for the analysis of lactate concentration for these experiments, only whole body lactate concentrations were performed.

Whole body lactate was assessed by exposing animals at the same stages of development and under the same conditions as the animals in the aerobic metabolism portion of this study (Table 1). A large number of animals of the same developmental group (see Table 3) were isolated in a closed aquarium consisting of a 45.5 liter glass aquarium covered with black paper with a 0.35 cm dark Plexiglass lid. Three ports were present in the lid. Two ports allowed for hoses from a water bath to connect to a set of glass cooling coils inside the aquarium resulting in a constant water temperature within the aquarium of 20±1.0°C. The third port allowed for a gas hose from a Cameron Instrument Company gas flow meter model GF-3 to pass into the experimental tank and attach to a bubble bar in the bottom of the aquarium. This allowed for aeration and mixing of the water, equilibration of the air within the aquarium to the desired PO_2, and formed a positive pressure system. The gas mixture was allowed to escape

Table 3. Number of Animals Measured at Each Level of Hypoxia and Total Number of Animals used for Determination of Whole Body Lactic Acid Content.

Group	Stage:	Number of samples analyzed at each level of hypoxia:	Number of animals euthanized at each level of hypoxia:	Total number of animals measured:
I	1-39	6	90-120	1215
II	40-44	10	150-200	1351
III	45-48	10	150-200	1805
IV	49-51	10	40	381
V	52-53	10	10	90
VI	54-57	10	10	90
VII	58-60	10	10	90
VIII	61-62	10	10	90
IX	63-66	10	10	90
X	Froglets	6	6	54
XI	Adults	6	6	60

from the aquarium around the seal of the Plexiglass to the top of the aquarium. Thirty-six liters of water were placed in the aquarium leaving approximately a 3 liter space of air above the water. Water used to fill the aquarium was tap-water that had been boiled to decrease microbial contamination and then cooled to 20°C. Animals were placed in screen enclosures in groups of the number to be euthanized at each interval (see Table 3) and let to acclimate to this normoxic environment overnight. Measurement started at a known PO_2 and was decreased by 15 mmHg every two hours. Oxygen partial pressure within the aquaria were reduced by bubbling a known gas mixture from a Cameron Instruments gas mixing pump through the water in the aquarium, this equilibrated the water and also filled the air above the water level to the desired oxygen partial pressure. At the end of the two hour period (approximately 1-1.5 hours after the PO_2 of the water and air had reached the desired experimental level), one screen enclosure of animals were euthanized by placing them in liquid nitrogen. The group of animals was then divided into approximately ten samples for repetitive measurements for lactic acid concentrations (Table 3). The tissue was then homogenized in six

times its mass of cold 10% trichloroacetic acid using a Tissue Terror tissue homogenizer. The tissue homogenates were then frozen at 0°C and analyzed for lactic acid content within two weeks. Immediately after euthanization of the animals from the experimental aquarium, the oxygen concentration in the aquarium was again decreased 15 mmHg and the remaining animals in the aquarium were exposed to a slightly more hypoxic environment for an additional two hours. After another two hours at the decreased PO_2, another group of animals were removed from the aquarium in the same amounts as stated before for each developmental grouping (Table 3) and euthanized and the PO_2 was decreased another 15 mmHg. This pattern continued until the minimal oxygen level for each group of animals was reached and the final ten animals were euthanized. Adult animals were measured in exactly the same way except a 273 liter aquarium was used for the experimental chamber.

Method of Assay for Lactic Acid

Assays for whole body lactate concentrations were performed using a Sigma Chemicals Corporation kit #826UV (St. Louis, Mo.) and a Sequoia-Turner model 340

spectrophotometer. A standard curve of the absorbance of known concentrations of lactate was made for each day samples were assayed and a quality check assay was performed after every twenty assays during the measurement of unknown samples. Whole body lactate concentrations are reported in millimoles of lactate per kilogram of body tissue. One gram of tissue was assumed to have a density of one. Calculations were performed as described below:

The absorbances for six different known standard concentrations ranging from 0.278-4.44 mM/kg were measured. Each of the standards were diluted six times in 10% trichloroacetic acid and treated exactly like the unknown samples. A least squares linear regression was then calculated through the absorbances of the known standards. The following equation was then used to calculate lactate concentrations of the unknown samples in mM/Kg:

Whole Body Lactate Concentration= $A \cdot X + C$.

A= absorbance of the sample.

X= X coefficient of linear regression.

C= linear regression constant.

Measurement of Surfacing Occurrences

A measurement of the number of surfacing occurrences of groups IX, X, and XI (Table 1) in response to varying degrees of hypoxia was also performed. The number of surfacing occurrences in adults was measured by placing single animals in a section of black plastic pipe 76.25 cm long and 7.75 cm in diameter. Surfacing occurrences in groups IX and X were measured in chambers made out of white plastic pipe that was 5.0 cm in diameter and 76.25 cm long. Both types of chambers had a cap sealed on one end so that the pipe could stand upright. The other end was fitted with a removable cap that had a hole drilled in it to allow for a gas tube from a Cameron gas mixer to pass into the chamber. A large piece of hose was glued down the inside of each chamber starting approximately 7.75 cm from the top of the chamber and extending to approximately 7.75 cm from the bottom through which the tubing from the Cameron gas mixer could pass. The end of the tubing from the Cameron was placed approximately two thirds of the way down the inside of the larger hose glued inside the surfacing chamber. This effectively created a lift tube to mix and aerate the water within the surfacing chamber. The chamber was

filled with boiled water to within 7.75 cm of the top of the chamber.

Fifteen centimeters from the top a hole was drilled completely through the center of the surfacing chamber perpendicular to the side of the pipe and also perpendicular to the lift tube. A photoelectric sensor was then glued in the hole on one side of the pipe and a LED light was glued into the other hole so that anything passing through the chamber would interrupt the light beam to the sensor. The light sensor was then connected to a NARCO Physiograph model CPM chart recorder through a hi-gain coupler. This resulted in a pen movement on the chart recorder each time that an animal surfaced for air and interrupted the light beam. The chart recorder was then used to record the number of times the light was broken (i.e. the number of surfacing occurrences) for a given amount of time.

Normoxic and hypoxic conditions were generated in the chambers in the same concentrations and at the same two hour intervals as in the anaerobic experiments so that animals would be exposed to similar conditions as were present in the previous experiments. Temperature was kept at 20±1.0 ^{o}C by isolating the surfacing chambers in an environmental chamber.

Statistical Methods

Effects of development and hypoxia upon the MO_2 and whole body lactic acid concentrations were tested for significance by a method of least squares linear regression with Sigmastat statistical software from Jandel Corporation (San Rafael, Ca.) on an Gateway 486DX computer. The effect of hypoxia on the number of surfacing occurrences was tested for significance using an analysis of variance (ANOVA) with Sigmastat statistical software.

RESULTS

Aerobic Metabolic Rate in Normoxia

Aerobic metabolic rate increased with development. The MO_2 of groups I, II, and III in normoxia was approximately 0.20 mM/kg/hr, rising to about 2.0 mM/kg/hr for groups IV, V, VI, VII, VIII, and IX. The MO_2 then increased again for group X to about 5.0 mM/kg/hr in normoxia, but returned to the range of 1.0 mM/kg/hr for group XI (Figure 2).

Aerobic Metabolic Rate in Hypoxia

The MO_2 of the different definitive groupings of animals (see Table 1) ranged from the standard resting metabolic rate to aerobic metabolic rates so low that removal of ambient oxygen could not be detected at severe hypoxia. The ranges of oxygen consumption given below will refer to the MO_2 in normoxia and the MO_2 of the animals at the lowest ambient PO_2 in which oxygen removal from the environment within the respirometer could be measured. The aerobic metabolic rate tended to decrease as ambient PO_2 decreased (Figures 2-12).

Figure 14 summarizes the interaction between ambient PO_2, MO_2, and development.

Group I (stage 1-39) MO_2 ranged from 0.0113 to 0.0064 mM/kg/hr. These animals were oxygen conformers (Figure 3). The MO_2 of group II (stage 40-44) ranged from 0.2559 to 0.0127 mM/kg/hr (Figure 4). This group proved to be oxygen regulators with a P_{crit} of about 70.5 mmHg. The MO_2 of groups III (stage 45-48), IV (stage 49-51), V (stage 52-53), and VI (stage 54-57) was 0.1974-0.0101, 1.9777-0.0284, 1.8792-0.0493, and 2.4571-0.3791 mM/kg/hr (Figures 4,5, 6, and 7). Groups III through VI were all oxygen conformers.

The major developmental transition from oxygen conformation to oxygen regulation occurred around stages 57 to 58. Group VII (stage 58-60), VIII (stage 61-62), and IX (stage 63-66) had a MO_2 range of 2.4168-0.0793, 2.5152-0.3193, and 1.8914-0.6206 mM/kg/hr (Figures 8, 9, and 10). Groups VII, VIII, and IX were all oxygen regulators with a P_{crit} of 77.5 mmHg, 76 mmHg, 85.5 mmHg, respectively. Groups X (1 month post-metamorphic froglets) and XI (adult frogs) had a MO_2 of 3.8503-0.2301 and 1.0849-0.3442 mM/kg/hr (Figures 11 and 12). Both groups were oxygen regulators with a P_{crit} of 64.6 mmHg and 30 mmHg, respectively.

Changes in P_{crit} in Response to Development

P_{crit} remained fairly constant at 75 mmHg as development proceeded for groups II, VII, and VIII (Figure 15). The P_{crit} for group IX increased to 85 mmHg. The Pcrit then decreased to 30 mmHg for group XI (Figure 15).

Ability to Survive Hypoxic Exposure

The inability to survive extreme hypoxia remained constant throughout development until adult stature was achieved. All groups except for IV and XI incurred approximately 60% mortality at exposure to 30 mmHg (Table 4). Group IV (stage 49-51) was unable to withstand an ambient PO_2 below 45 mmHg, and incurred 70% mortality at this level(Table 4). Group XI (adults) could survive short term exposure to ambient PO_2's to about 8 mmHg without mortality (Table 4).

Whole Body Lactate Concentrations in Normoxia

Whole body lactate concentrations in normoxia were not effected by increased development until after metamorphosis, after which concentrations started to increase. Whole body lactate concentrations for groups I through X was approximately 1.0 mM/kg. Group XI had

Table 4. Mortality Resulting from Exposure to Ambient PO_2 of 30 mmHg for Two Hours.

Group #:	Percent Mortality:
I (stage 1-39)	60%
II (stage 40-44)	66%
III (stage 45-48)	57%
IV (stage 49-51)	100*
V (stage 52-53)	67%
VI (stage 54-57)	73%
VII (stage 58-60)	68%
VIII (stage 61-62)	74%
IX (stage 63-66)	77%
X (1 mo. froglets)	75%
XI (adult frogs)	0%

an increased level of lactate measured at about 12.5 mM/kg in normoxia (Figure 16).

Whole Body Lactate Concentrations in Hypoxia

Whole body lactate concentrations in response to severe hypoxic conditions increased about midway through larval development. Up through stage 51, whole body lactic acid concentrations did not increase in response to any level of hypoxia (Figures 16-19). After stage 52, whole body lactate concentrations rose in response to severe hypoxic conditions (Figures 20-26). The following whole body lactate concentrations correspond to levels measured in normoxia and the most hypoxic conditions to which the group of animal was exposed (Figures 16-26). Figure 28 summarizes the interactions of PO_2, whole body lactic acid concentrations, and development.

Whole body lactate levels in groups I-III had ranges of 1.817-1.1464, 1.1476-1.7148, and 2.26283-3.70720 mM/kg (Figures 16-18). Whole body lactate concentrations for groups IV-VI were 0.8677-1.7498, 1.4196-1.8854, and 1.0612-1.7022 mM/kg (Figures 19-21). Groups VII, VIII, and IX had whole body lactate levels of 1.1282-4.0293, 1.5295-1.8998, and 1.5601-5.1724 mM/kg (Figure 23-24). Groups X and XI had whole body

lactate levels of 2.7260-8.3203 and 12.3939 to 24.9430 mM/kg, respectively (Figures 25 and 26).

Changes in Whole Body lactate 15 mmHg Below P_{crit}

Whole body lactate concentrations 15 mmHg below the P_{crit} of each of the groups remained fairly constant for groups I-IX at 1.0 mM/kg. Whole body lactate concentrations for groups X and XI increased to 9 and 25 mM/kg respectively (Figure 29).

Surfacing Occurrences

Surfacing occurrences for each of the three groups of animals studied (stage 63-66, froglets, and adults) showed no significant change from control values at normoxia to ambient PO_2 levels above that group's P_{crit}. After the ambient PO_2 was reduced below the P_{crit}, surfacing occurrences increased significantly (Figure 30).

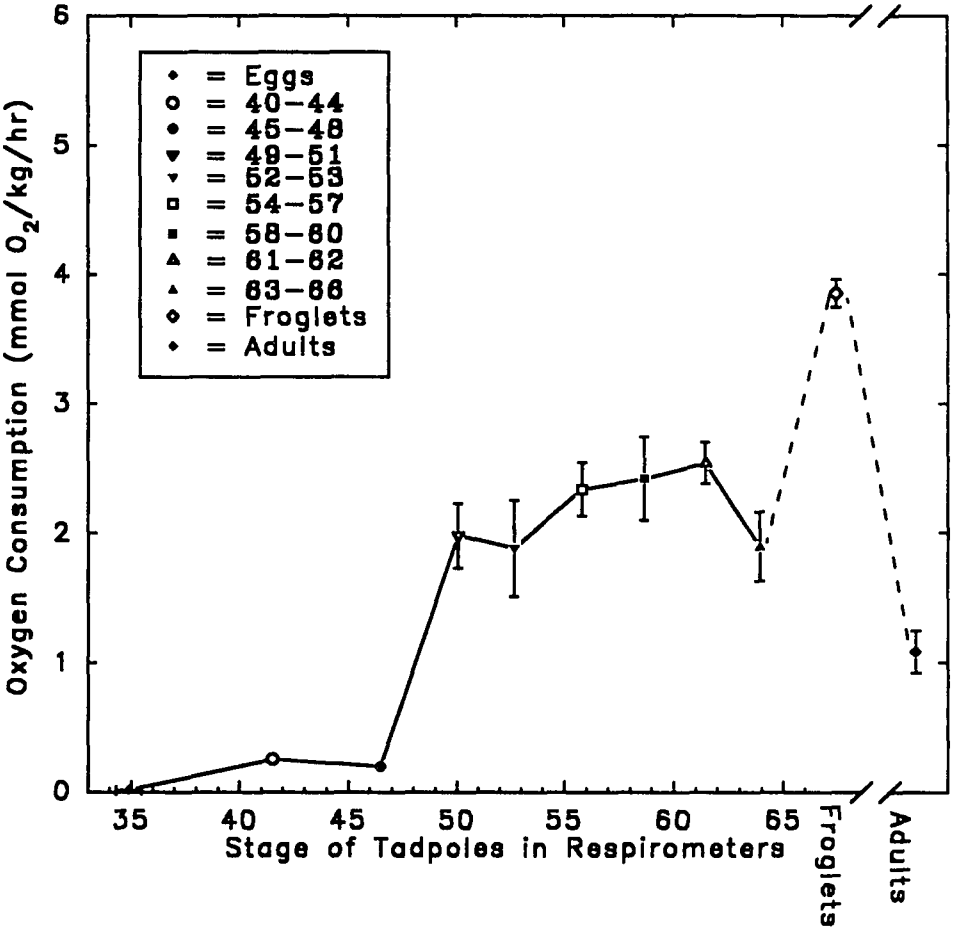

Figure 2. Changes in MO_2 in Response to Development in Normoxia (PO_2 =150 mmHg). Each point represents the average normoxic MO_2 at 20°C for each developmental group. Error bars indicate ±1 standard error of the mean. Error bars are smaller than symbols in some cases.

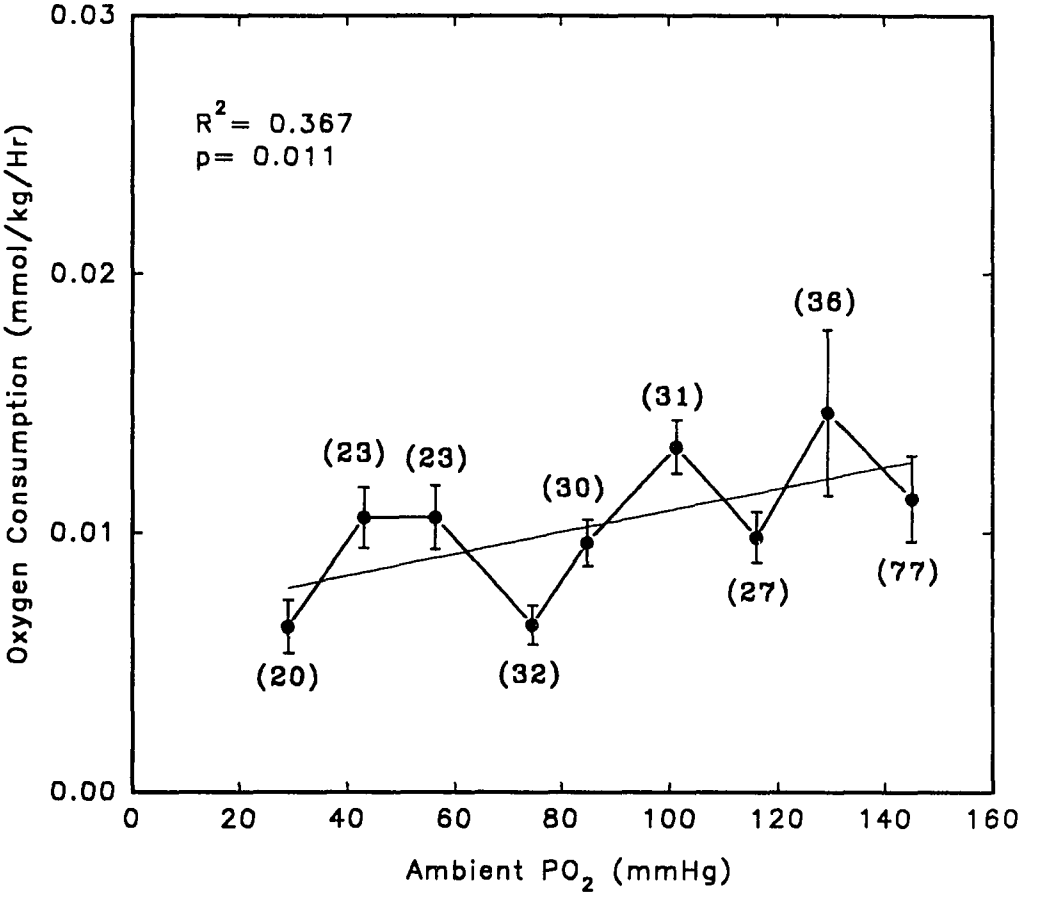

Figure 3. **MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 1-39 Larvae.** Each point represents the averaged MO_2 at each PO_2. The number of observations averaged for each point plotted are indicated in parentheses. Error bars for X and Y axes indicate ±1 standard error of the mean. Error bars are smaller than symbols in some cases. R^2 and p values are indicated for a least squares linear regression through the mean data points.

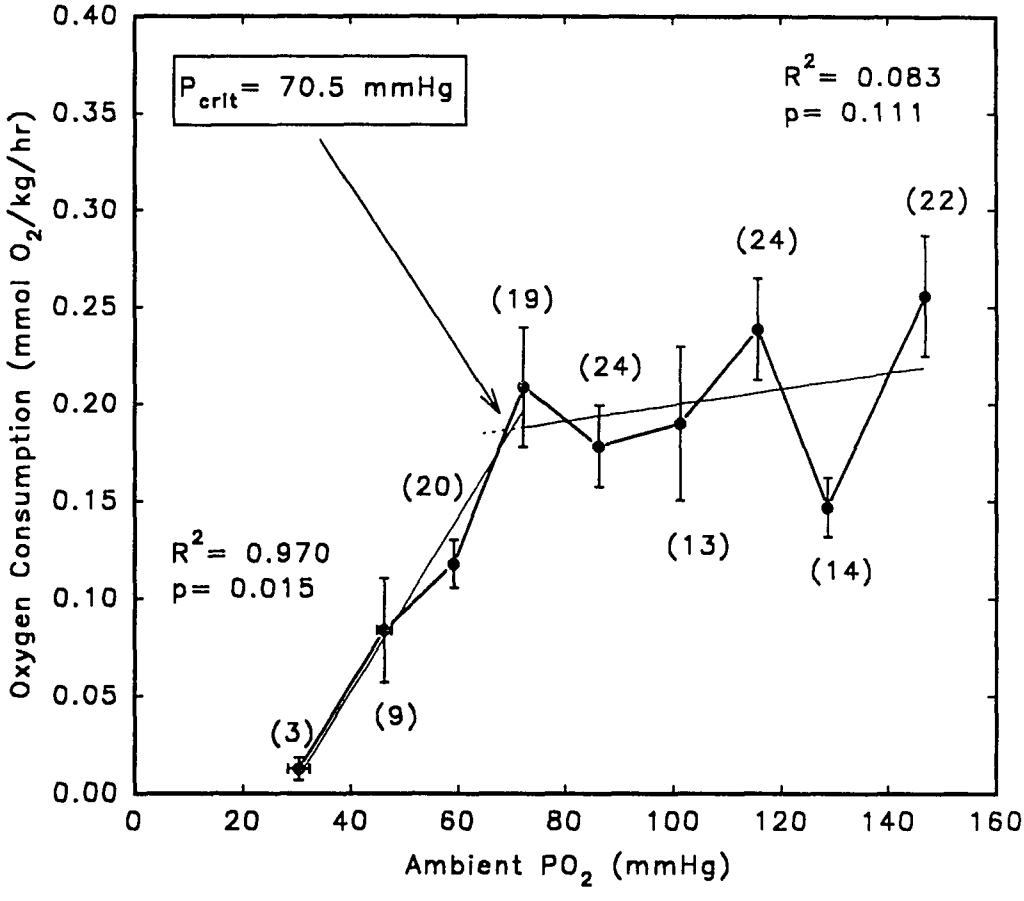

Figure 4. **MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 40-44 Larvae.** P_{crit} defined as the point where two least squares linear regressions meet. R^2 and p values are indicated for each least squares linear regression through data points. See Figure 3 and text for further details.

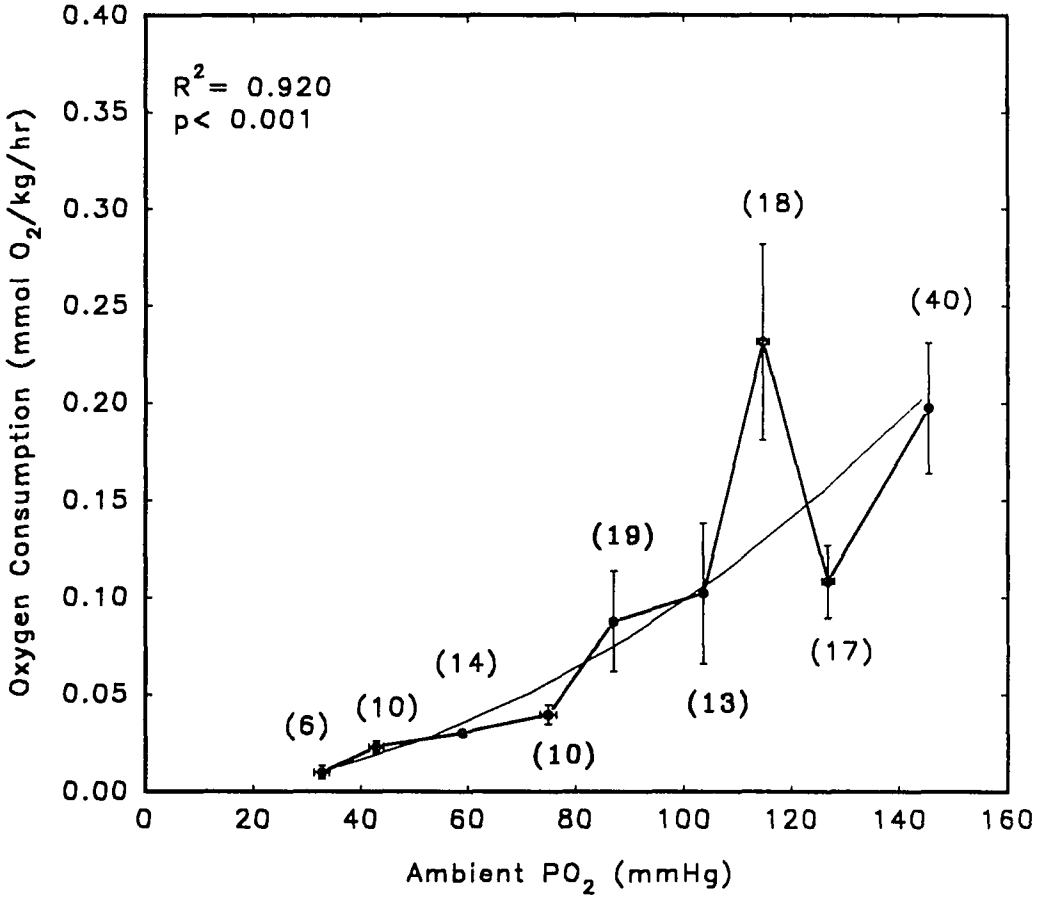

Figure 5. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 45-48 Larvae. R^2 and p values are indicated for a least squares linear regression through log transformed averaged data points. See Figure 3 and text for further details.

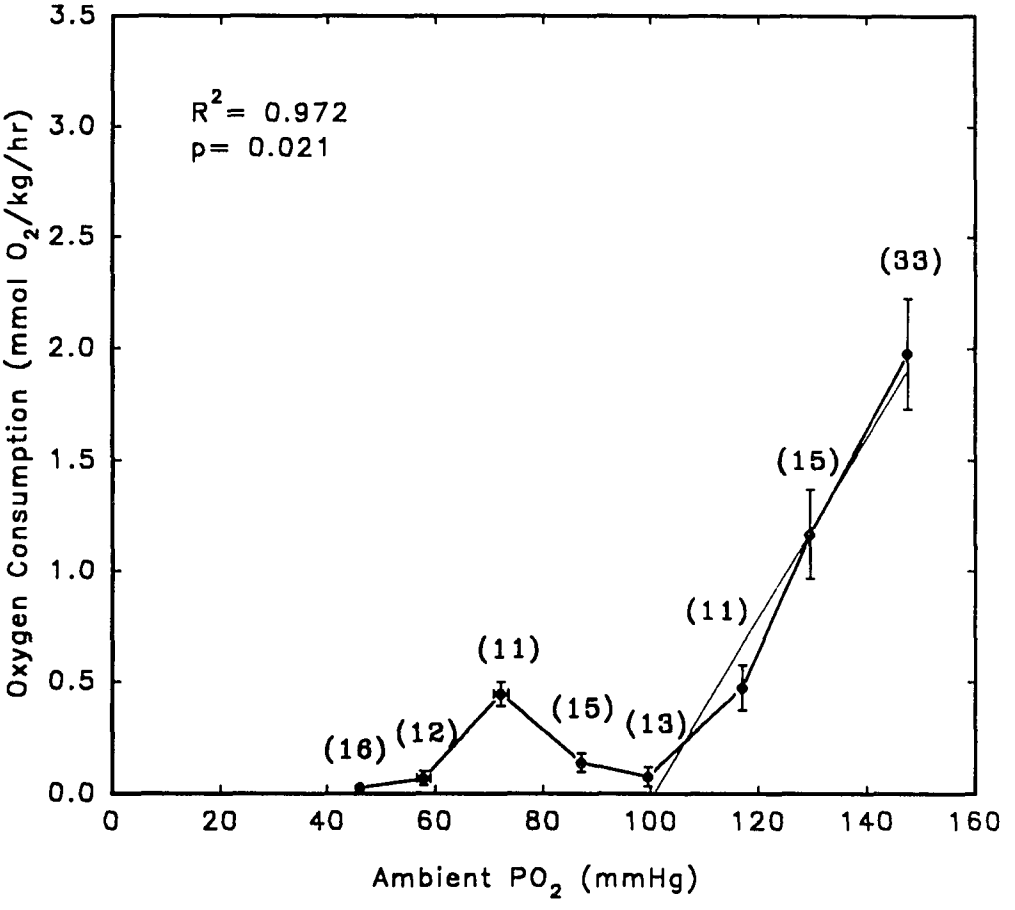

Figure 6. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 49-51 Larvae. See Figure 3 and text for further details.

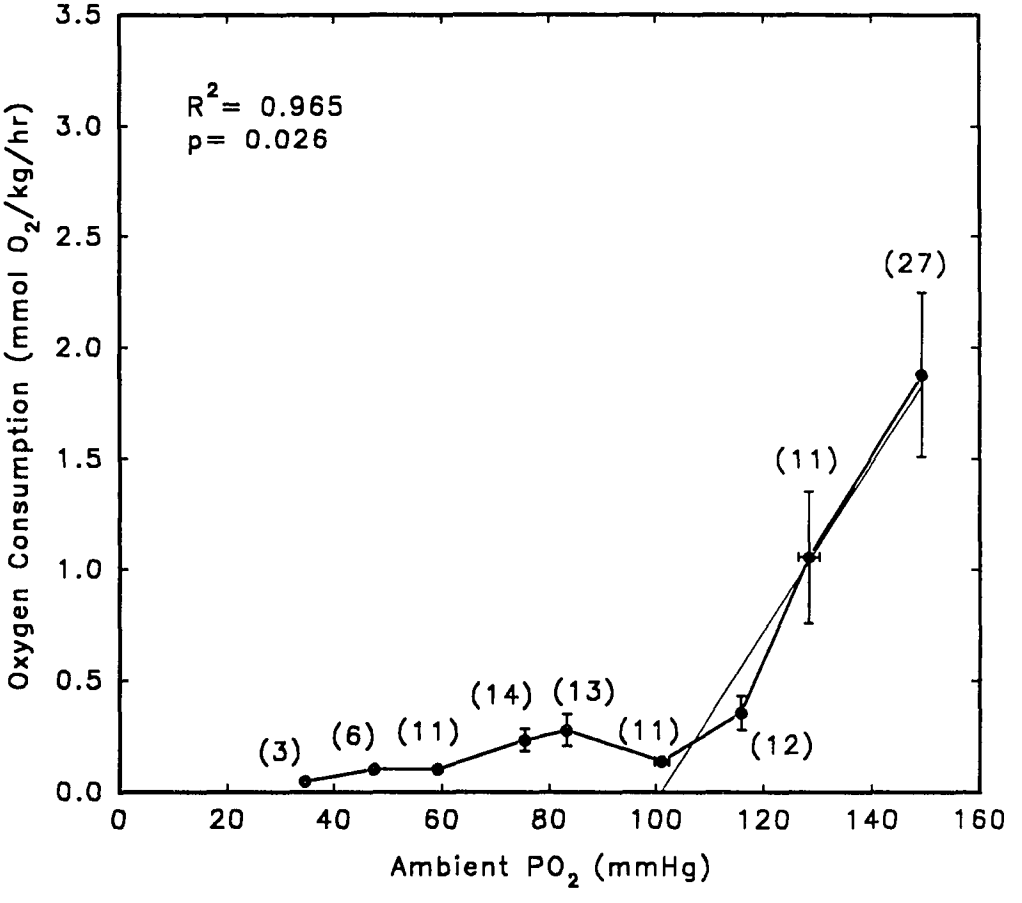

Figure 7. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 52-53 Larvae. See Figure 3 and text for further details.

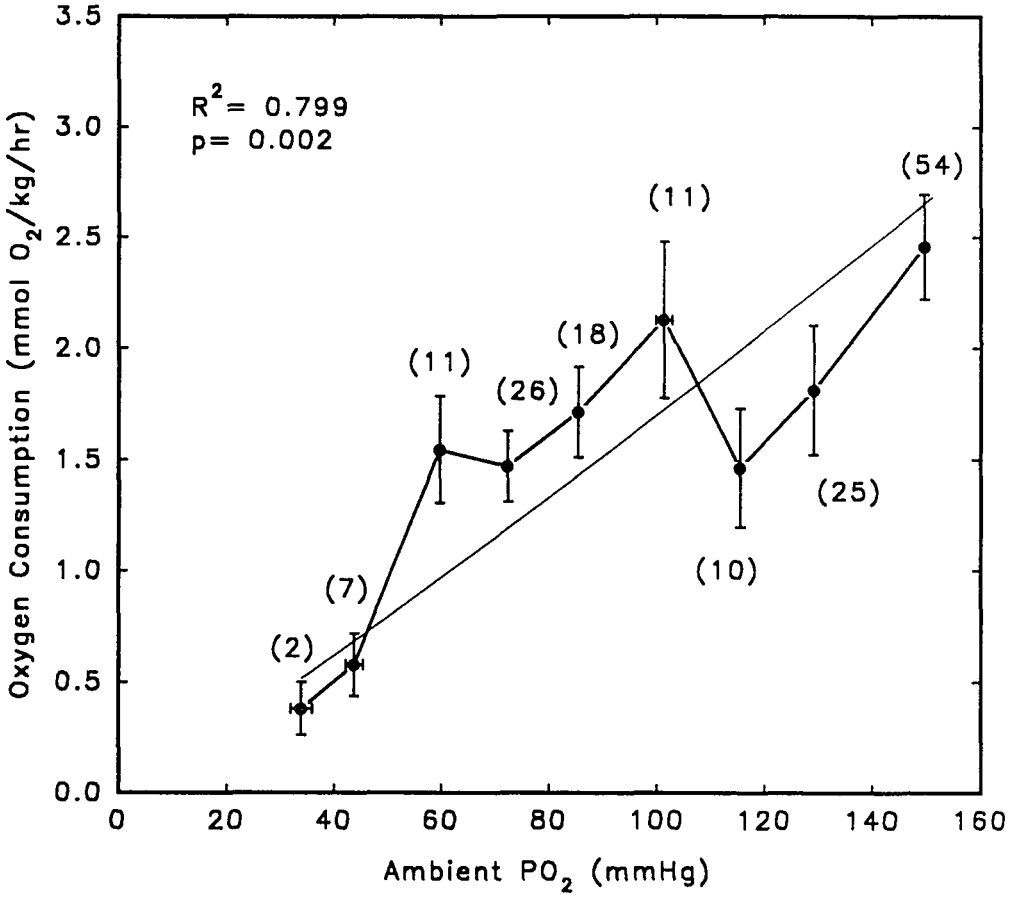

Figure 8. **MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 54-57 Larvae.** See Figure 3 and text for further details.

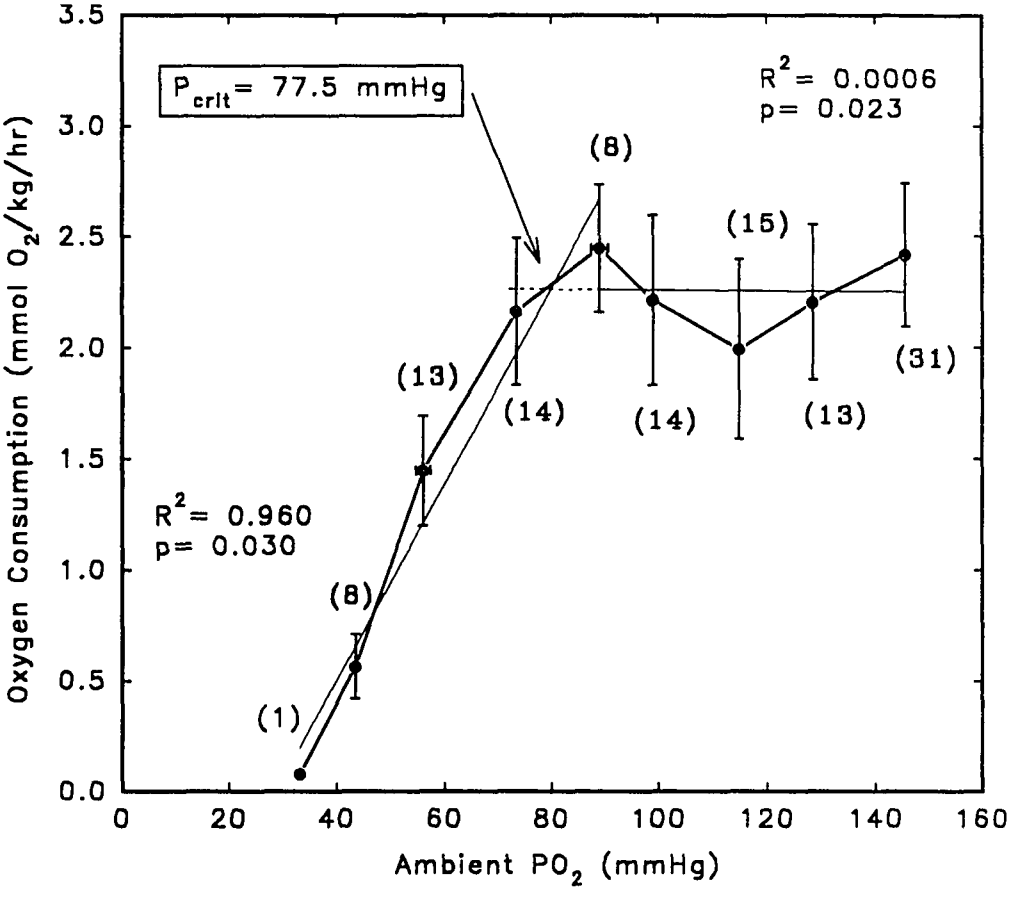

Figure 9. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 58-60 Larvae. See Figure 4 and text for further details.

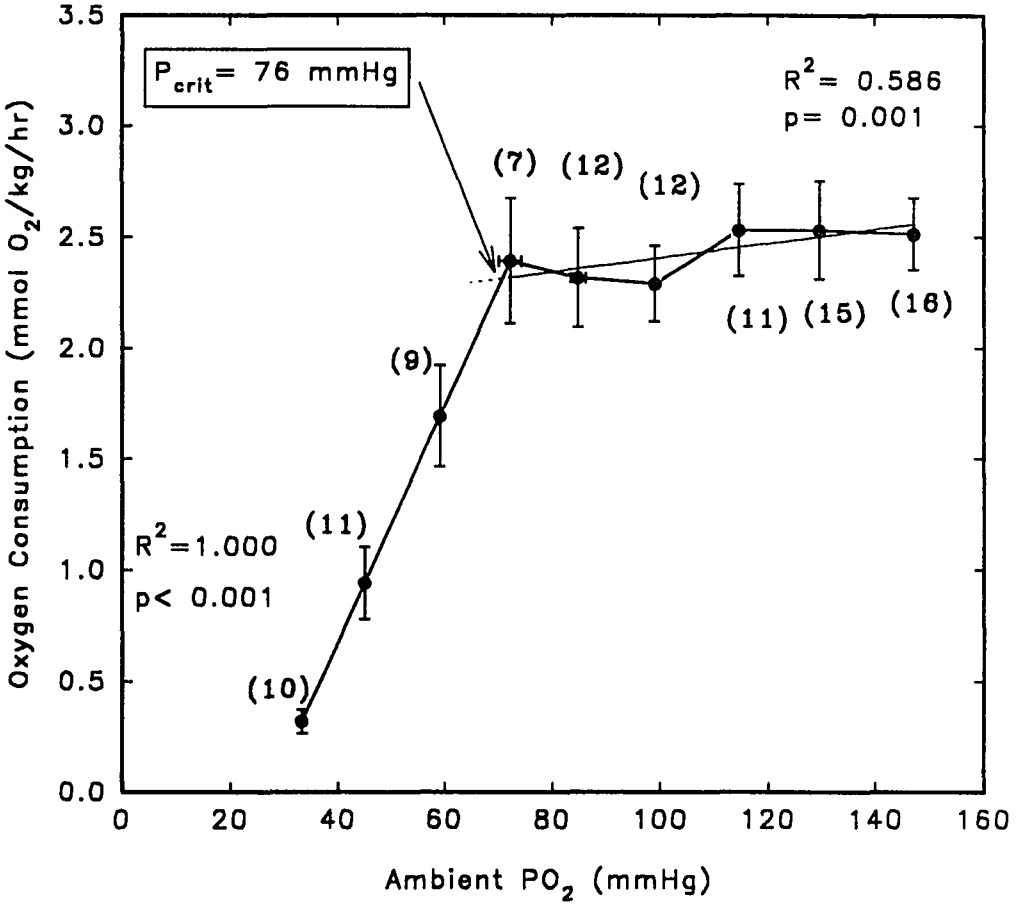

Figure 10. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 61-62 Larvae. See Figure 4 and text for further details.

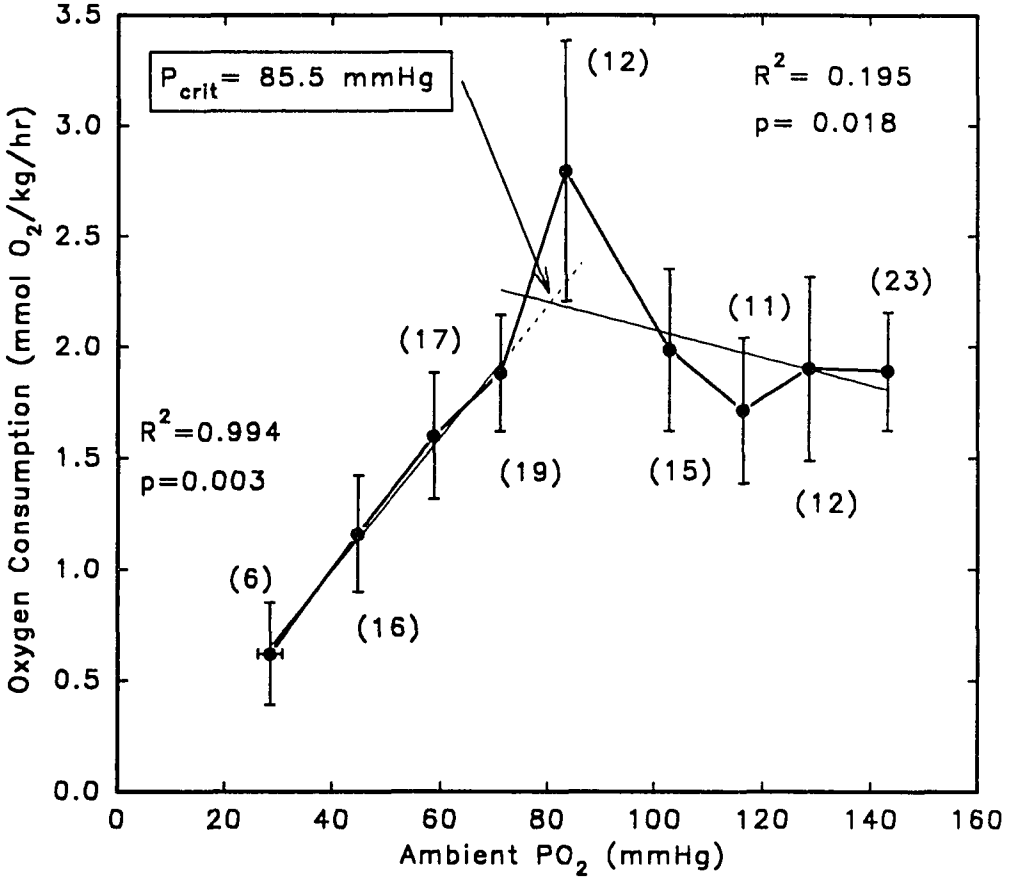

Figure 11. MO_2 as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 63-66 Larvae. See Figure 4 and text for further details.

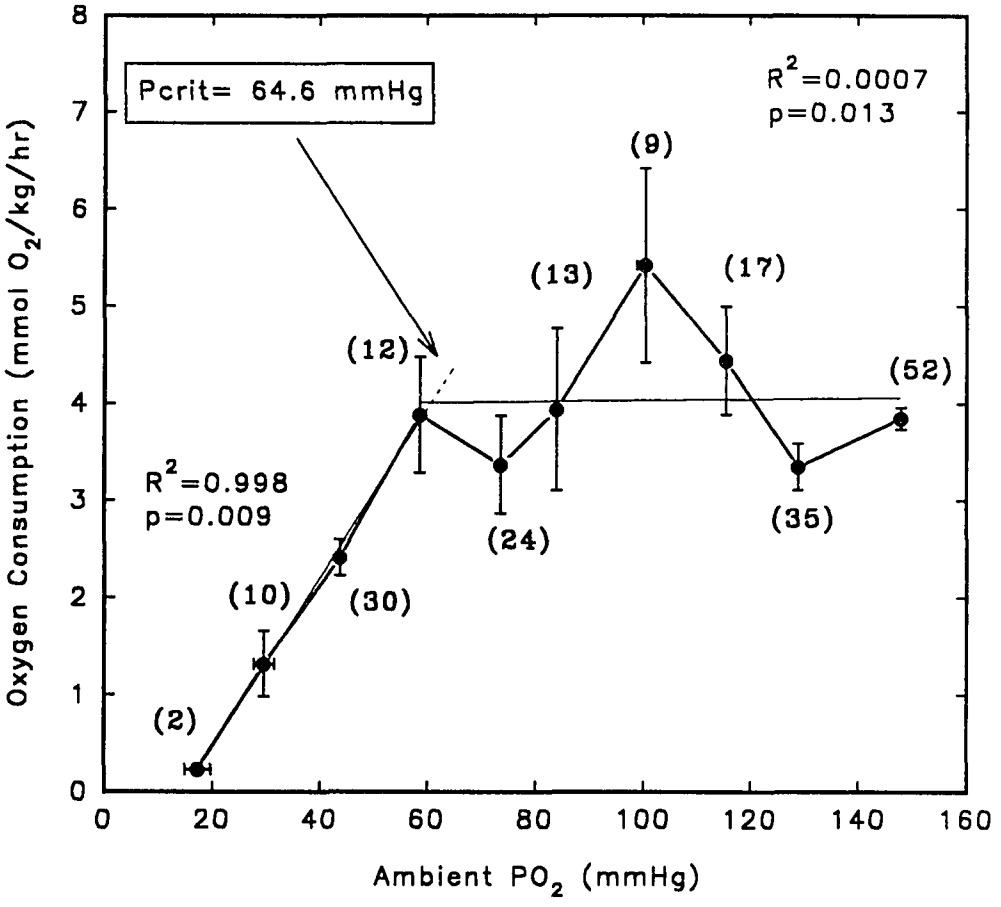

Figure 12. MO_2 as a Function of Ambient PO_2 in 1 Month Post-metamorphic froglets. See Figure 4 and text for further details.

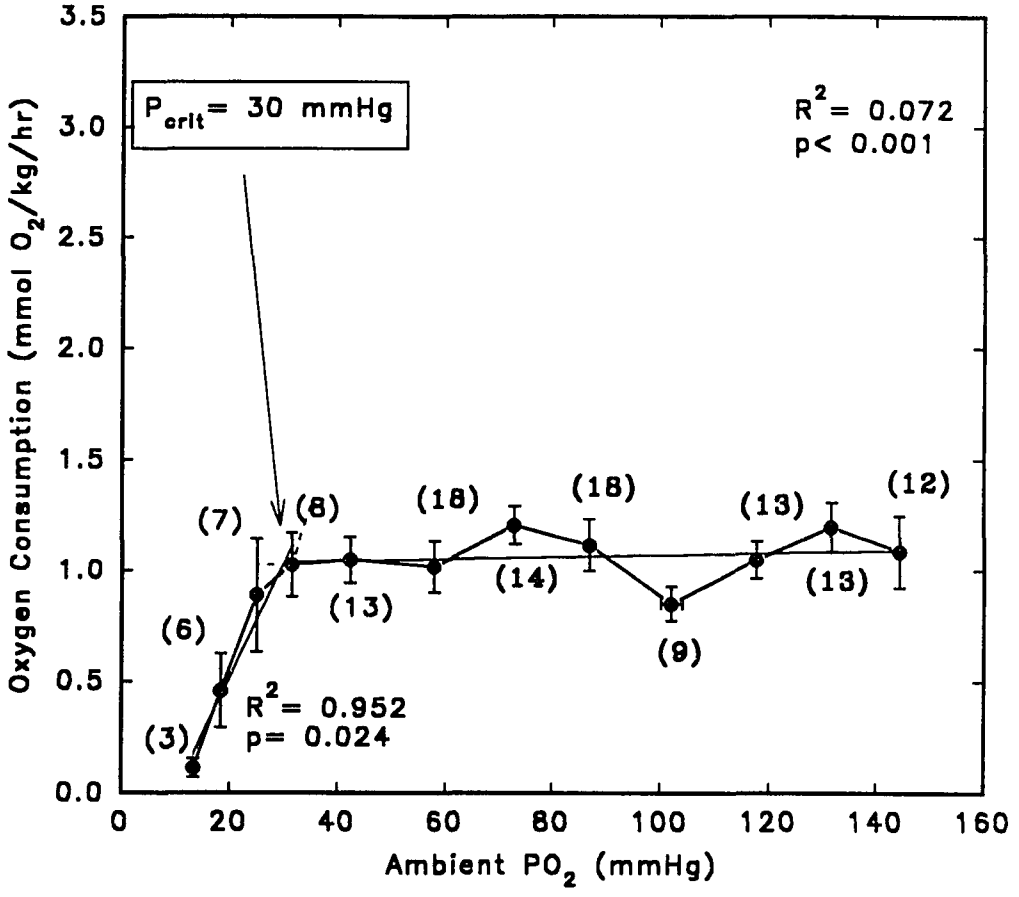

Figure 13. MO_2 as a Function of Ambient PO_2 in Adult Breeder Frogs (mass 20-70g). See Figure 4 and text for further details.

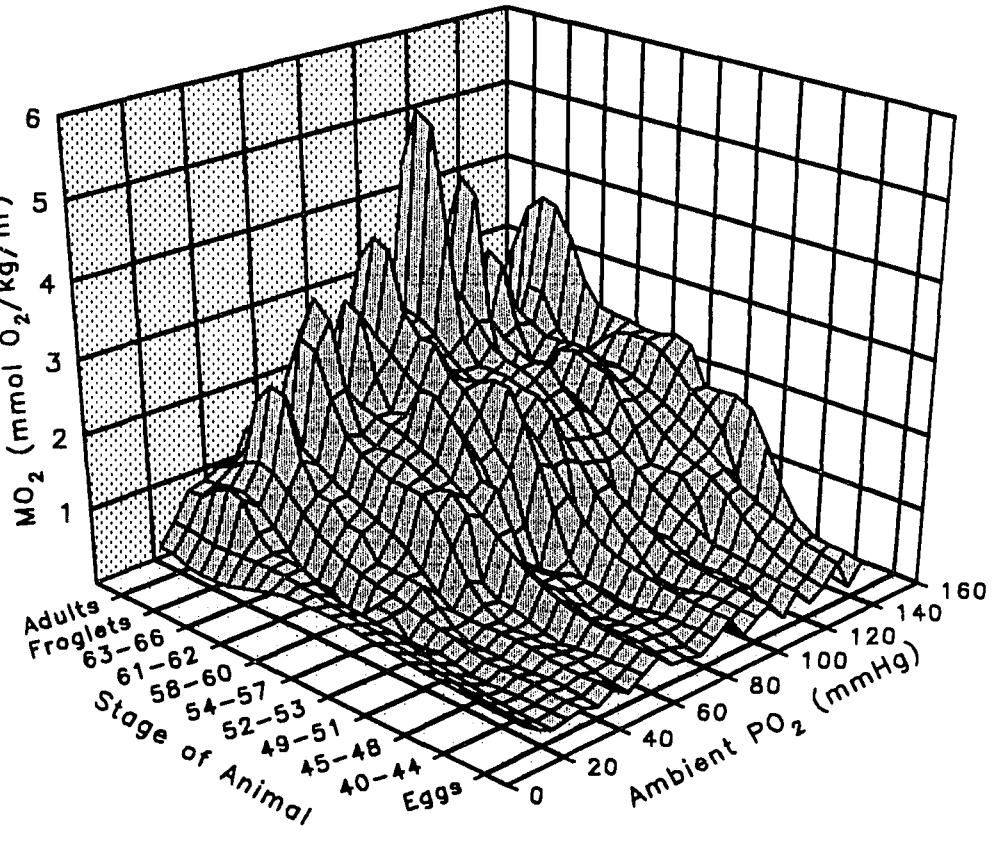

Figure 14. **Summary of the Changes in MO_2 as a Function of Ambient PO_2 and Development in *Xenopus laevis*.** Ambient PO_2 ranges from 150-20 mmHg STDP. Developmental stages according to Nieuwkoop and Faber (1967) are indicated.

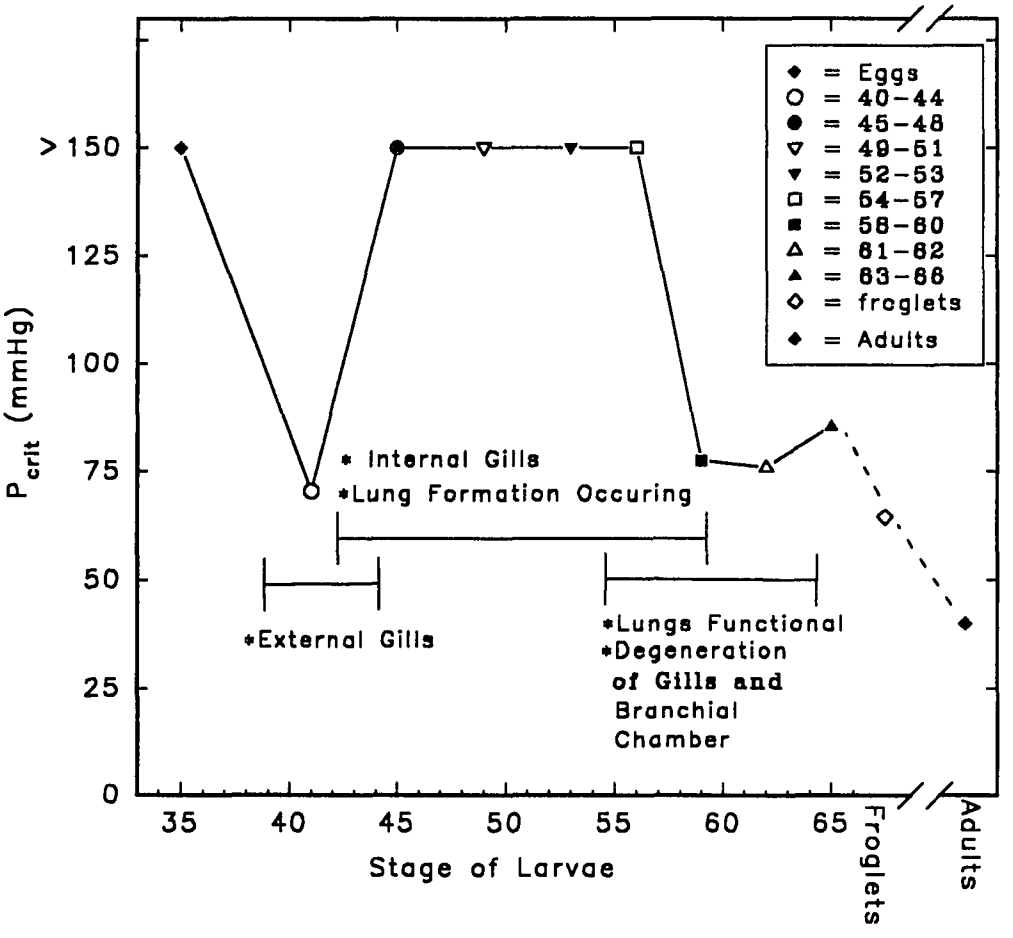

Figure 15. Changes in P_{crit} as a Function of Development. Stages according to Nieuwkoop and Faber (1967) are indicated. Lines indicate the developmental stages through which each defined respiratory structure is developing. Oxygen conformers have a P_{crit} at or above 150 mmHg indicated by >150.

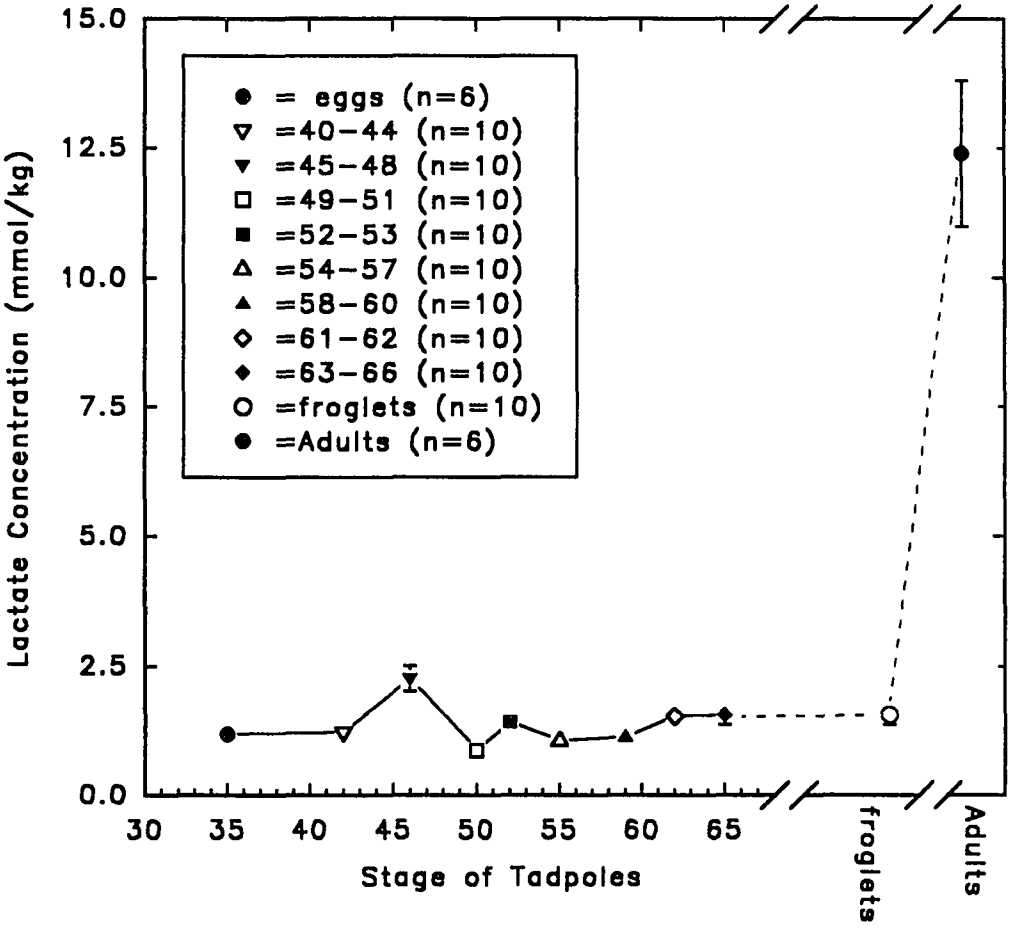

Figure 16. Changes in Whole Body Lactate Concentrations in Normoxia as a Function of Development. Ambient PO_2=150 mmHg. Staging according to Nieuwkoop and Faber (1967). Each point represents the average normoxic whole body lactate concentration of each developmental group. Number of observations averaged for each point plotted are indicated in parentheses. Error bars indicate ±1 standard error of the mean.

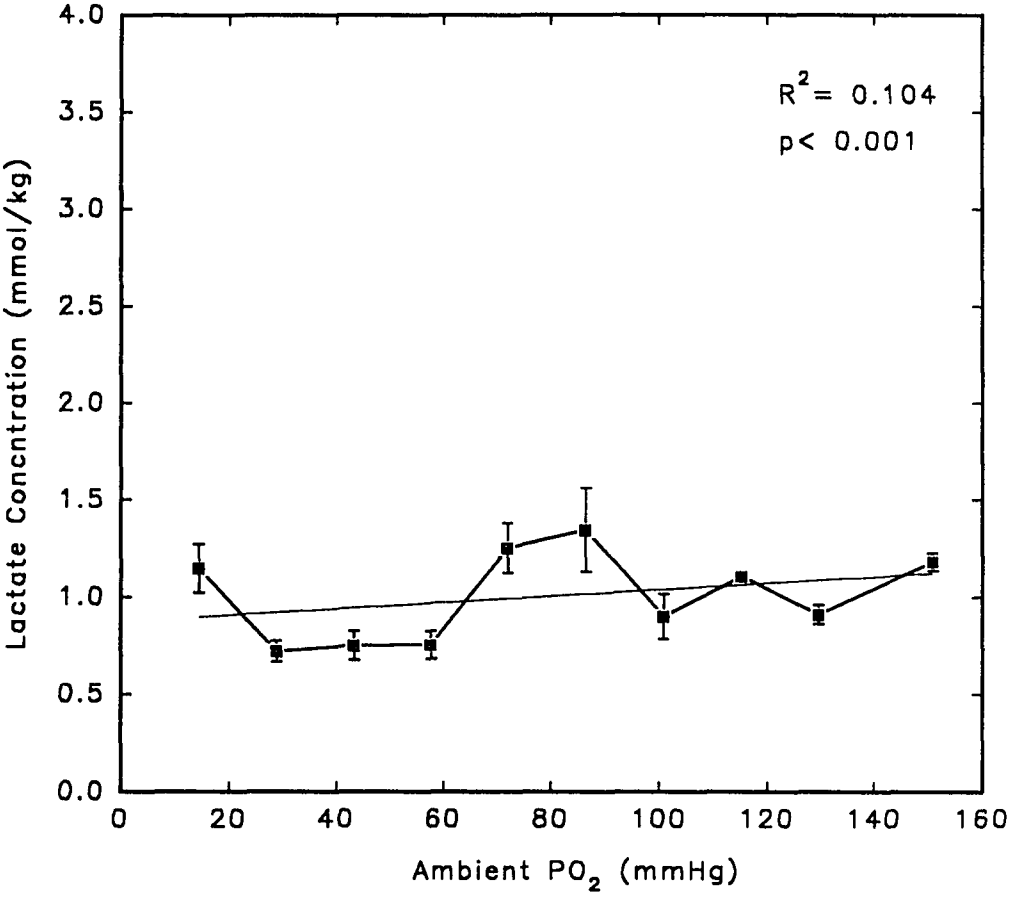

Figure 17. Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 1-39 Larvae. Each point represents the average whole body lactate concentration at each PO_2 exposure. Number of observations averaged for each point plotted are equal to six. Error bars indicate ±1 standard error of the mean. R^2 and p values are indicated for the least squares linear regression through the average data points.

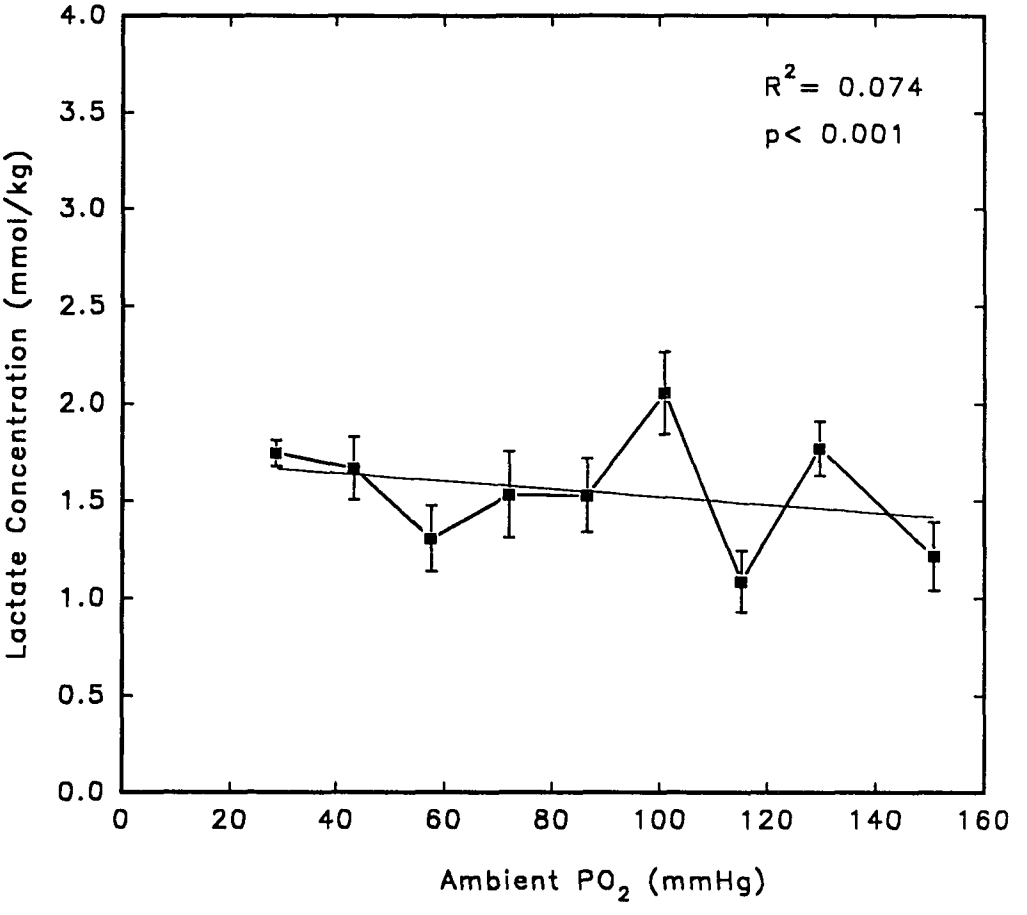

Figure 18. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 40-44 Larvae.** Number of observations averaged for each point plotted are equal to ten. See Figure17 and text for further details.

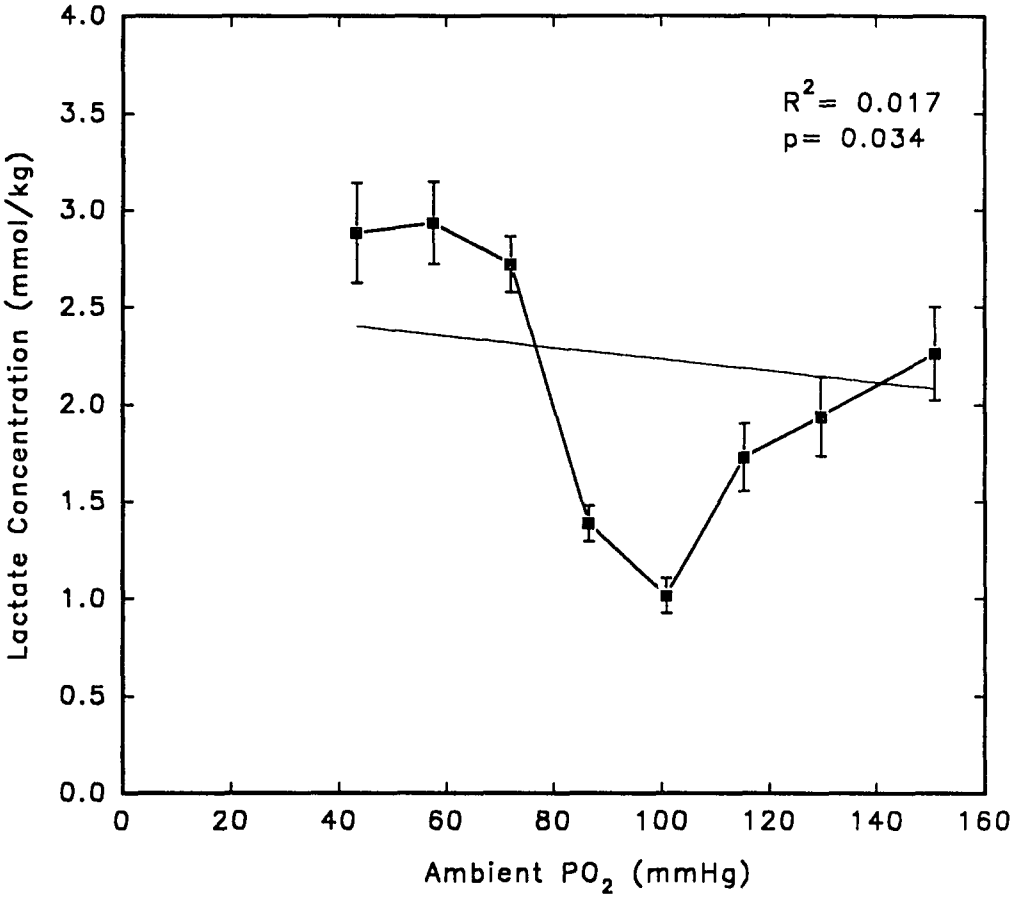

Figure 19. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 45-48 Larvae.** See Figure17 and text for further details.

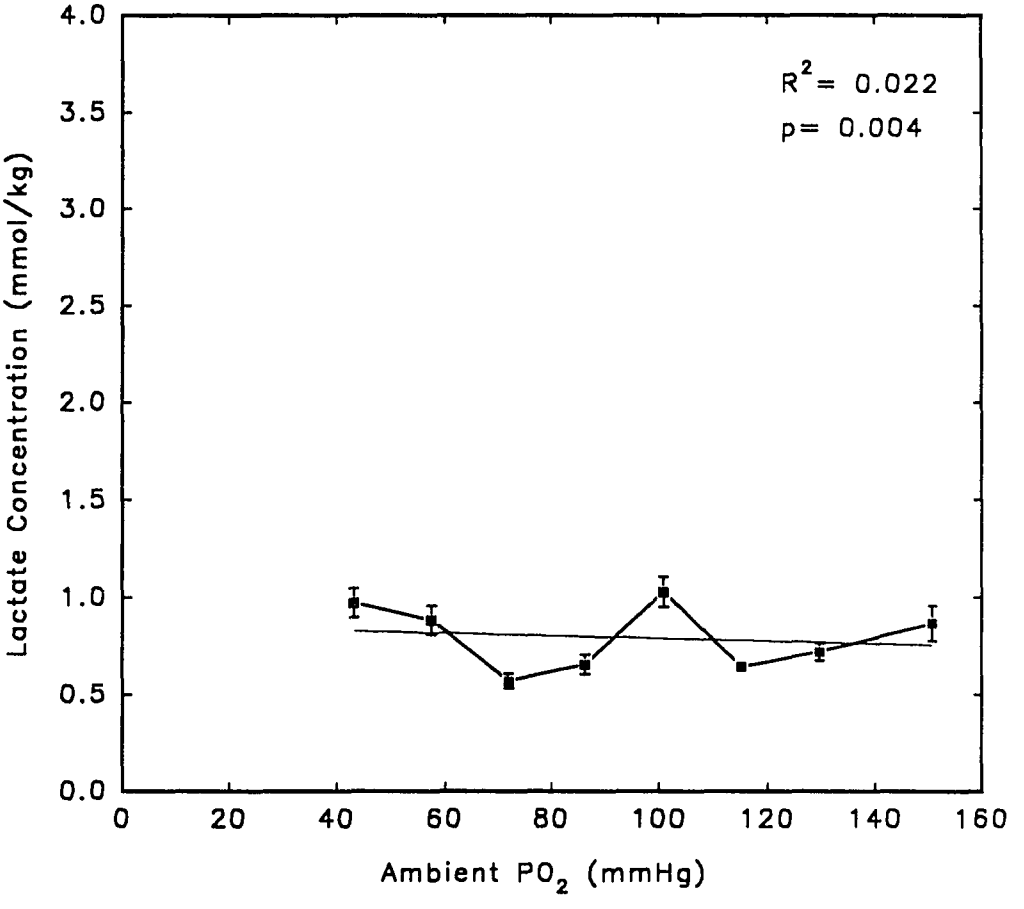

Figure 20. Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 49-51 Larvae. Error bars for some data points are smaller than symbols. See Figure17 and text for further details.

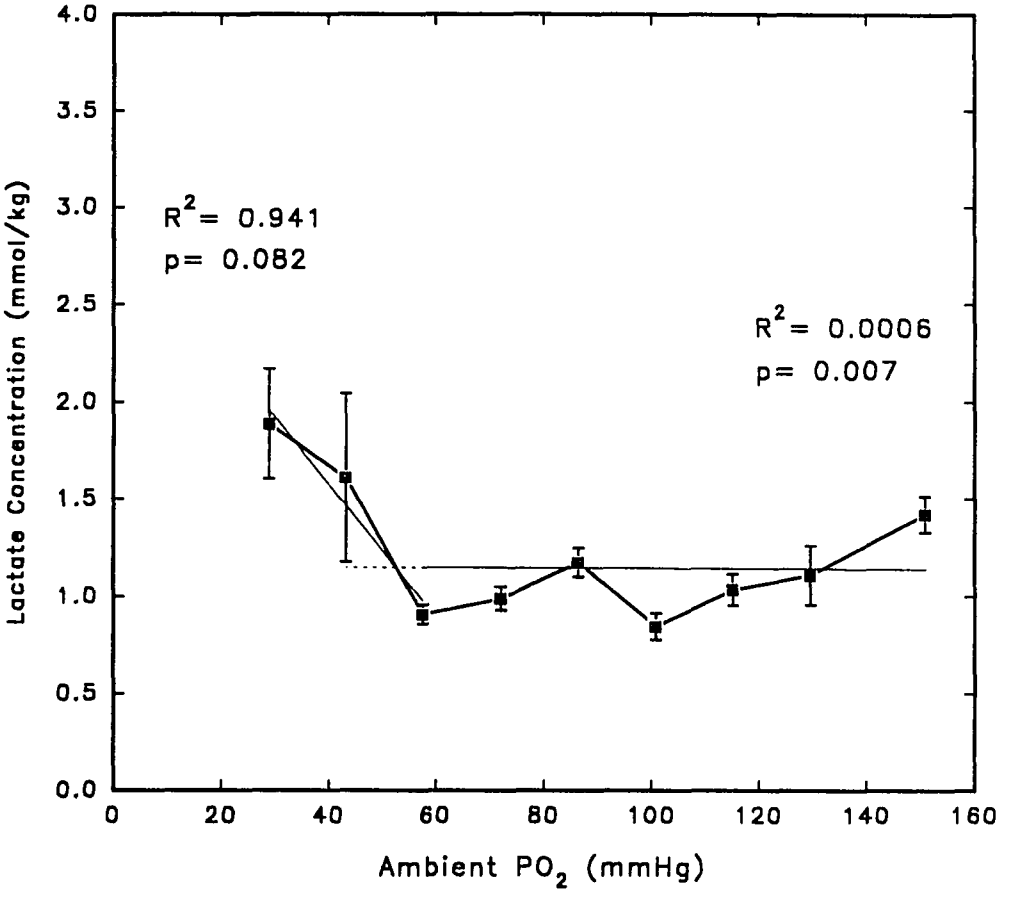

Figure 21. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 52-53 Larvae.** See Figure17 and text for further details.

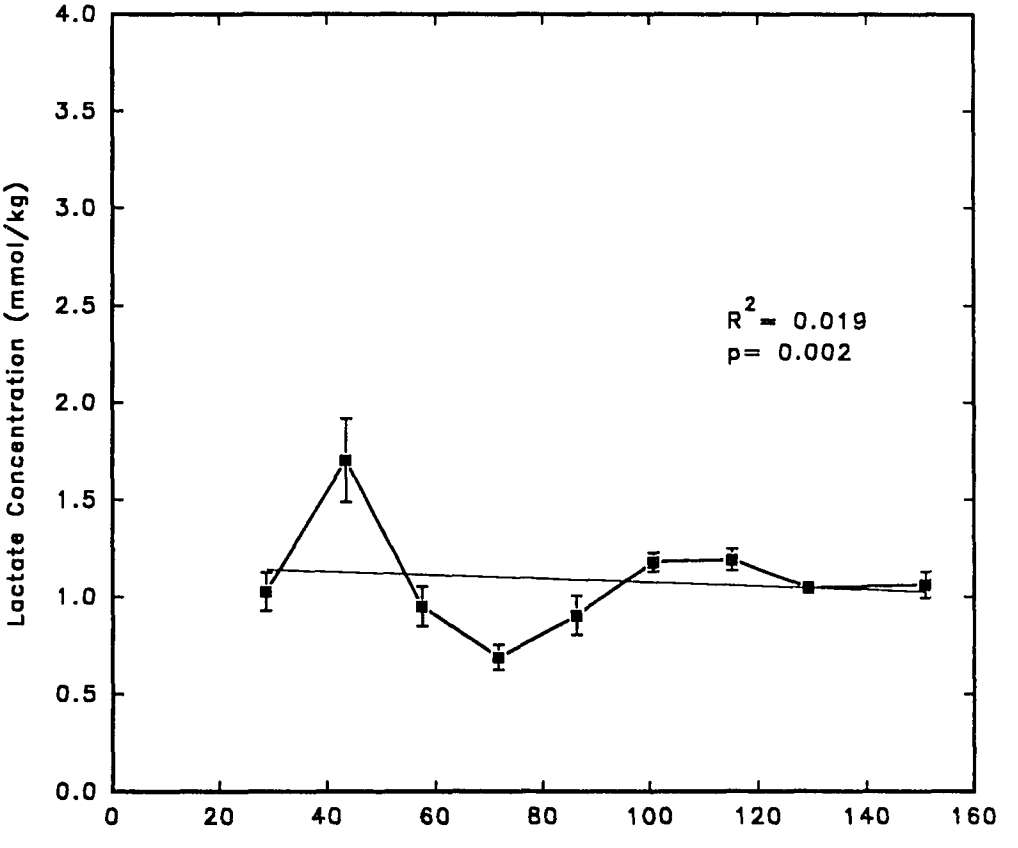

Figure 22. Whole Body Lactate Concentrations as a Function of Ambient PO_2 (mmHg) in Nieuwkoop and Faber (1967) **Stage 54-57 Larvae.** Error bars are smaller than symbols in some cases. See Figure17 and text for further details.

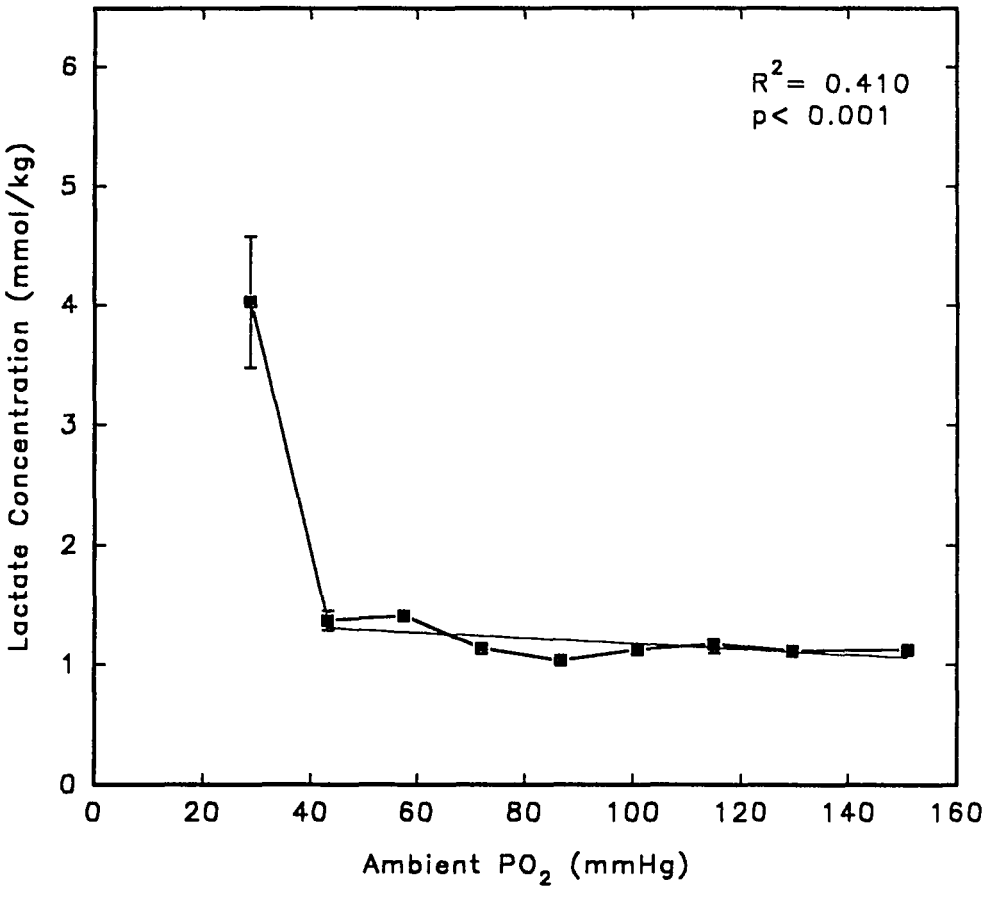

Figure 23. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 58-60 Larvae.** Error bars are smaller than symbols in some cases. See Figure17 and text for further details.

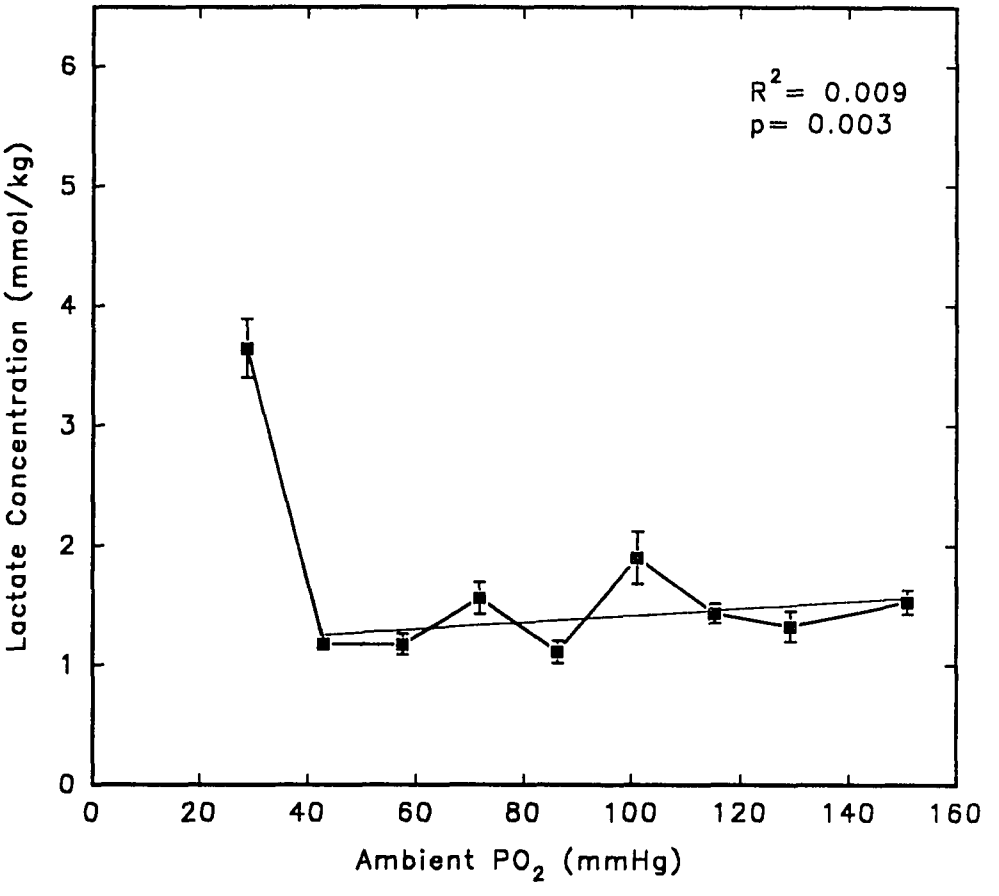

Figure 24. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 61-62 Larvae.** Error bars are smaller than symbols in some cases. See Figure17 and text for further details.

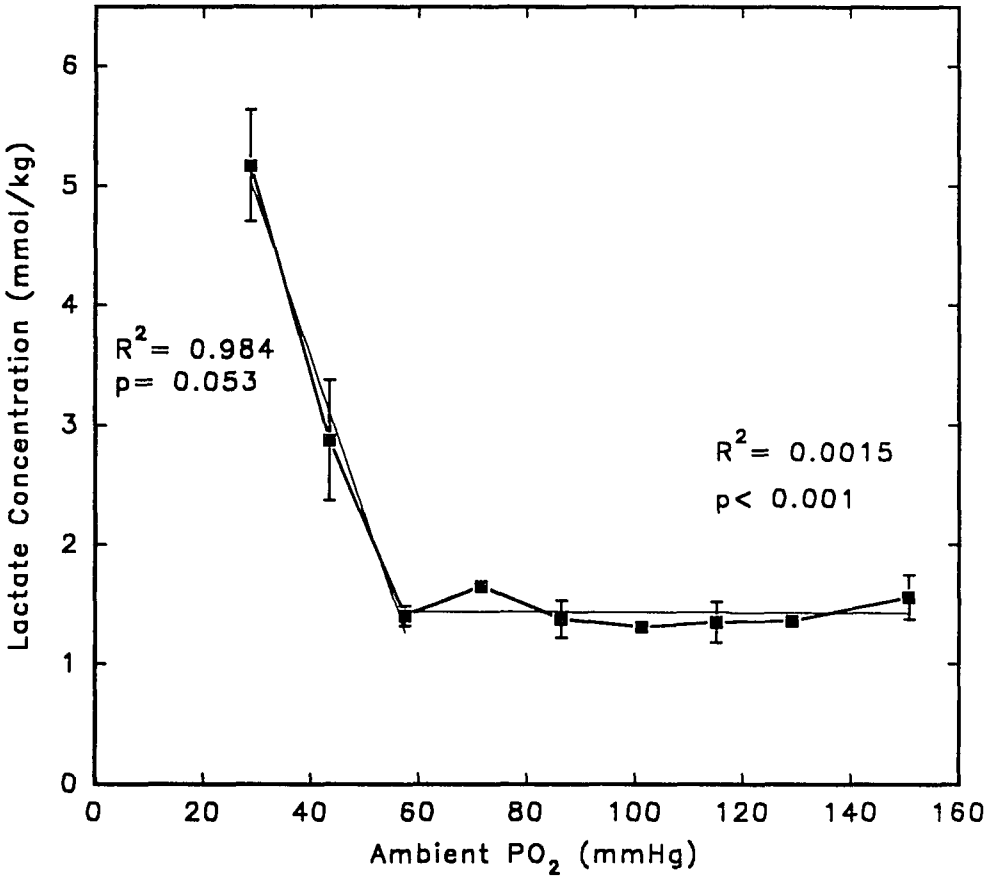

Figure 25. **Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Nieuwkoop and Faber (1967) Stage 63-66 Larvae.** Error bars are smaller than symbols in some cases. See Figure17 and text for further details.

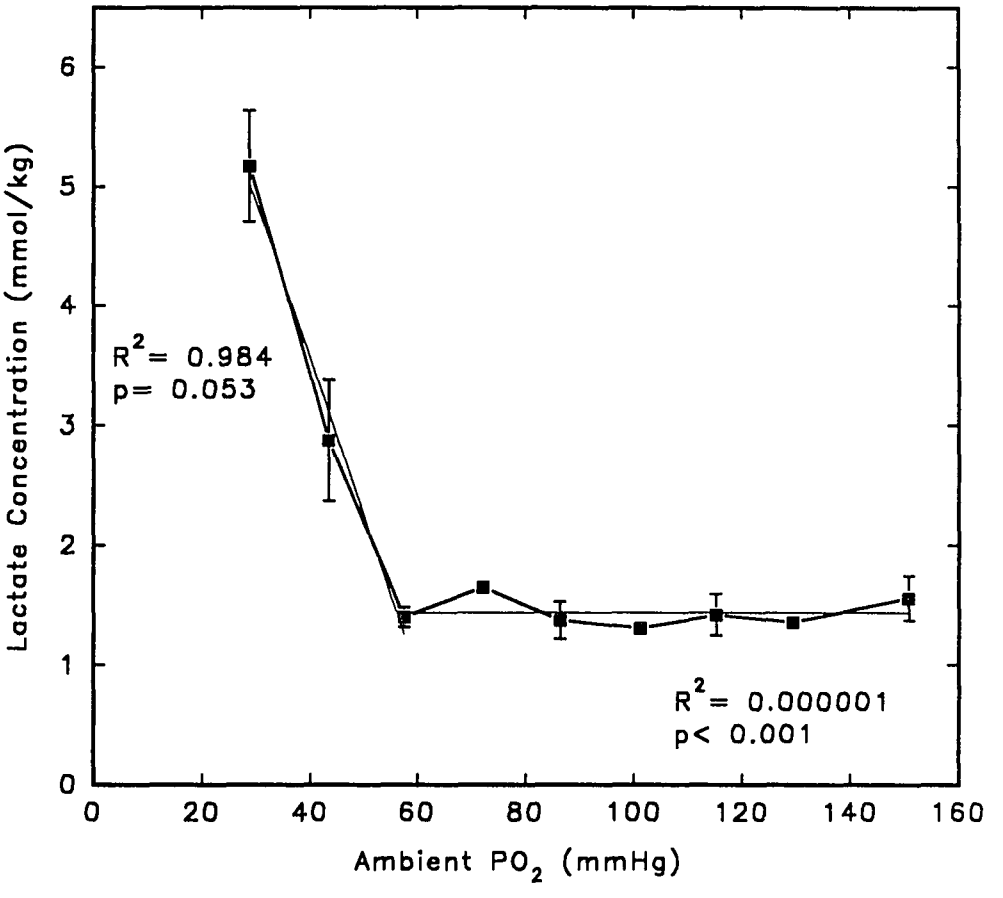

Figure 26. Whole Body Lactate Concentrations as a Function of Ambient PO_2 in 1 Month Post-metamorphic froglets. Number of observations averaged for each point plotted are equal to six. R^2 and p values are indicated for the two least squares linear regressions through the average data points. Error bars are smaller than symbols in some cases. See Figure17 and text for further details.

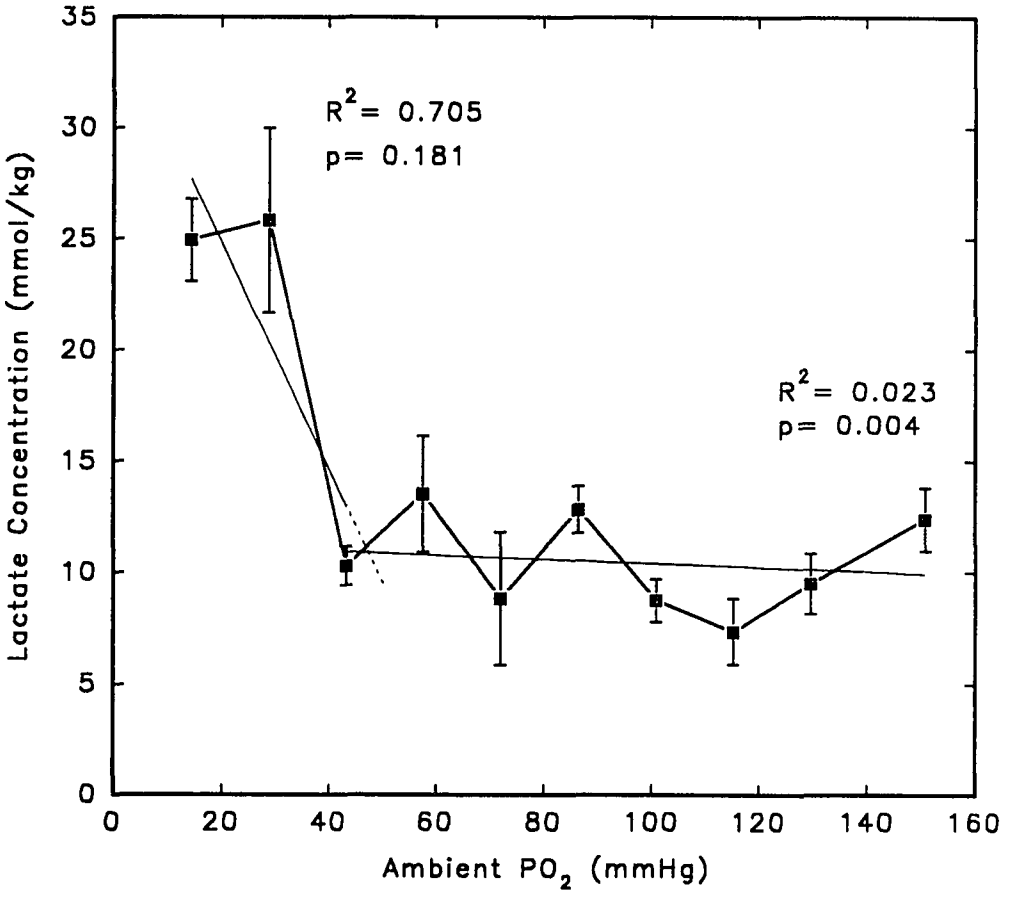

Figure 27. Whole Body Lactate Concentrations as a Function of Ambient PO_2 in Adult Breeder Frogs (20-70 g). See Figure28 and text for further details.

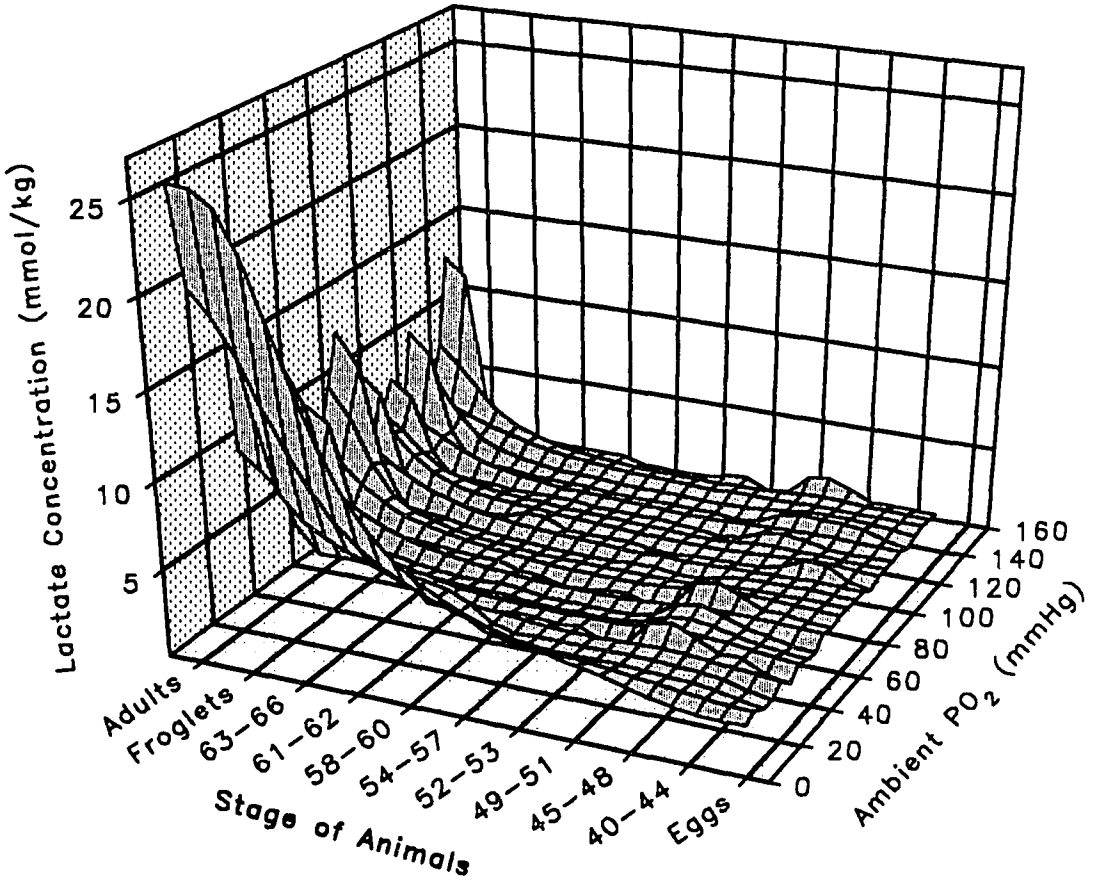

Figure 28. **Summary of the Effect of Development and Short Term Hypoxic Conditions on Average Whole Body Lactate Concentrations in *Xenopus laevis*.** Developmental stages of larvae are indicated according to Nieuwkoop and Faber (1967). Ambient PO_2 ranged from 15-150 mmHg.

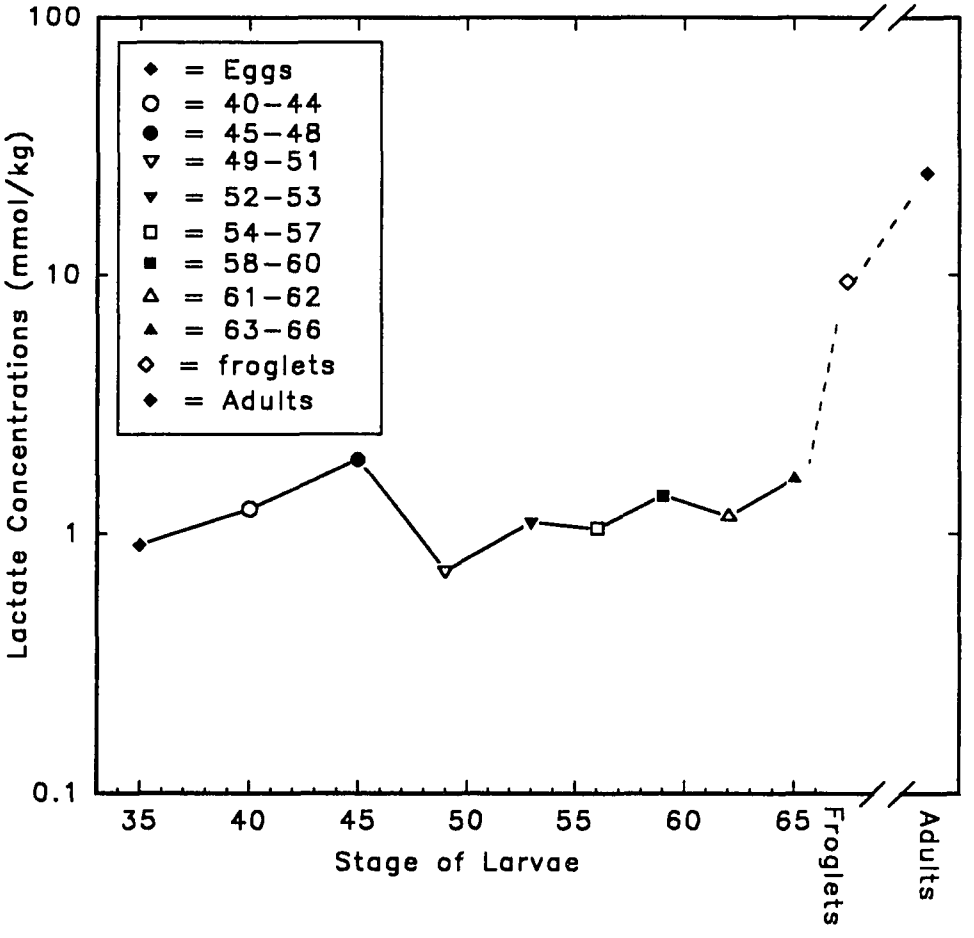

Figure 29. Summary of Whole Body Lactate Concentrations 15 mmHg Below the P_{crit} for Each Developmental Group of *Xenopus laevis*. Stages according to Nieuwkoop and Faber (1967) are indicated. Stage of larvae is indicated in a linear scale on the X-axis and whole body lactate concentrations are indicated on a log scale on the y-axis. Pcrit as determined by method described in text.

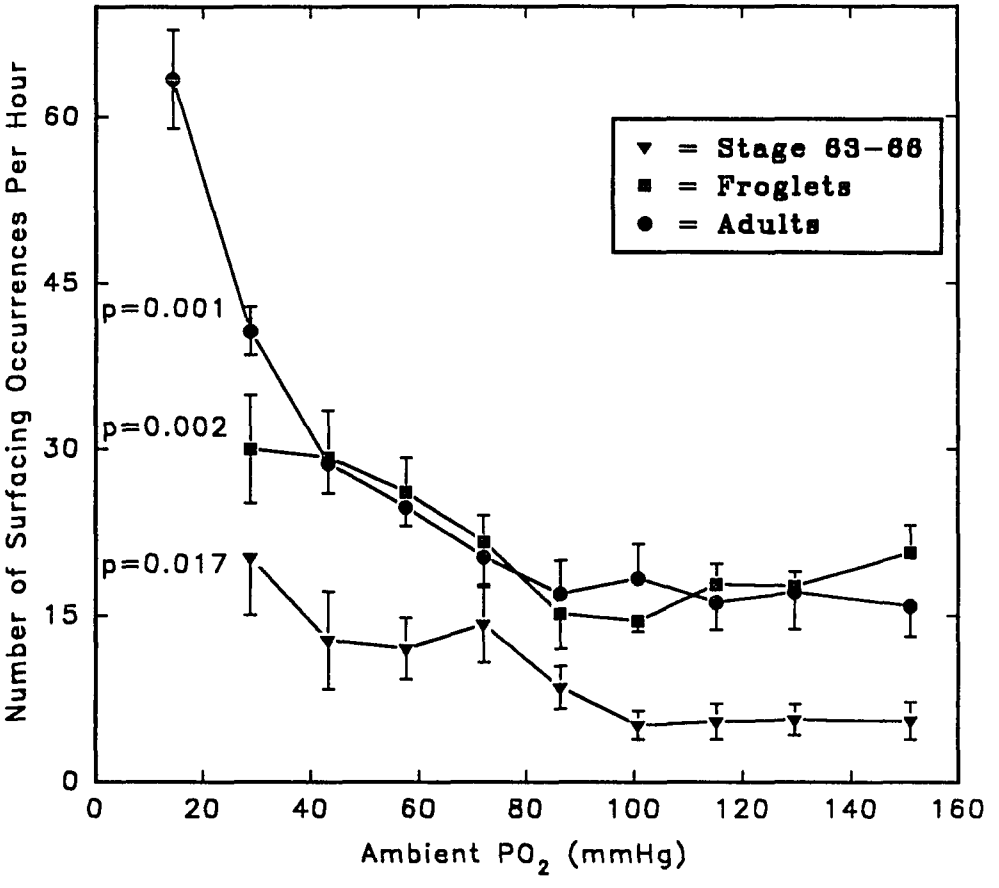

Figure 30. Changes in Number of Surfacing Occurrences in Response to Short Term Hypoxia in *Xenopus laevis*. The average number of surfacing occurrences in Nieuwkoop and Faber (1967) stage 63-66 tadpoles, 1 month post-metamorphic froglets, and adult frogs are indicated. PO_2 ranged from 150-15 mmHg. Time interval equals two hours for all observations. Number of observations equals repeated measures on six individuals for each developmental stage. p values for ANOVA of the effect of hypoxia on average number of surfacing occurrences is indicated for each developmental stage.

Discussion

Developmental Changes in Normoxic MO_2

Although groups I (stage 1-39) and II (stage 40-44) are approximately the same mass and body size, and are only hours apart in development, the MO_2 measured for group I in this experiment is approximately one tenth the MO_2 measured for group II (Figures 1-3). Expressing the total MO_2 on a per egg or per gram body mass basis is reasonable only when comparing individuals of uniform size and composition. The eggs of *Xenopus laevis* are relatively uniform in shape and size and are laid on a string like mucus thread (Deuchar, 1975; Nieuwkoop and Faber, 1967). As a result, structural differences between the early developmental stages themselves are small, so gas diffusion and boundary layers are relatively regular for all eggs. However, eggs/embryos are considerably different in composition than free swimming larvae. In eggs, the vast majority of mass consists of "non-living", metabolically inert lipids stored in yolk cells within the egg. As the embryo matures, the lipids are incorporated into living, oxygen-consuming

tissue. As the young larvae are ready to hatch, excess yolk cells are drawn into the body of the larvae to be metabolized later. A high number of yolk platelets are present in newly hatched larvae, and remain in the larvae up to stage 39 (Nieuwkoop and Faber, 1967). Therefore, accurate measurement of the mass of metabolically active tissue is very difficult in the early developmental stages. Expressing mass as embryo plus yolk considerably underestimates the mass-specific MO_2 and may erroneously suggest the greatly accelerating aerobic metabolism accompanying yolk depletion which is often observed (Bialaszewicz and Bledowski, 1915; Parnas and Kaskinska, 1921; Brachet, 1934; Wills, 1936; Hopkins and Handford, 1943; Gregg, 1960).

The resting MO_2 in normoxia measured in group II (stage 40-44) agrees with values reported by Feder (1982) for stage 45 tadpoles (Figures 1 and 3). The MO_2 of groups II and III (stage 45-48) was significantly greater than the MO_2 of group I as would be expected if yolk depletion occurred (Bialaszewicz and Bledowski, 1915; Parnas and Kaskinska, 1921; Brachet, 1934; Wills, 1936; Hopkins and Handford, 1943; Gregg, 1960).

The resting MO_2 increased once again, beginning with group IV (stage 49-51), but then stayed fairly constant until the completion of metamorphosis (Figure 2). Resting and active VO_2 of stage 49-66 larvae of *Xenopus laevis* in normoxia have been previously reported (Feder, 1981; Feder, 1982; Fletcher and Myant, 1959). Feder (1981 and 1982) reported the VO_2 in ml O_2 /g dry weight/hr at STPD and did not report the wet to dry weight ratio or give the wet weight of the animals. Fletcher and Myant (1959) did, however, measure the wet to dry weight ratios for developmental stages 49-66 and reported that the ratio of dry to wet weight was fairly constant at 0.05 through all of these developmental stages. Therefore, the VO_2 reported by Feder (1981 and 1982) can be converted to "wet weight MO_2" using this conversion factor. After conversion; the MO_2 of groups III-IX in normoxia are approximately equal to the converted values of the above investigators (Figure 2). Both Feder (1981) and Feder (1982) reported a fairly constant VO_2 (converted to MO_2) of 2.25-4.5 mM/kg/hr for stage 49-66 larvae while Fletcher and Myant reported a MO_2 of 3.7-5.5 mM/kg/hr for the same stages of larval Xenopus laevis during activity.

After metamorphosis and the complete development of the lungs, MO_2 increased significantly (Figure 2).

This increase likely occurred because the lungs were fully functional and this stage of animals have again begun to eat. The animals were not fed for at least three days before measurement, but it is possible that the increased MO_2 could be a result of specific dynamic action from eating. With the return to feeding and with fully functional lungs, an increased MO_2 affiliated with active growth would be expected.

MO_2 decreased upon attainment of adulthood (Figure 2). The resting MO_2 determined for group XI (1.1 mM/kg/hr) is also in agreement with measurements of 1.2-3.8 mM/kg/hr that have previously been reported for adult *Xenopus laevis* (Hutchison, Whitford, and Kohl, 1968; Charles, 1931; Hillman and Withers, 1981; Emilio and Shelton, 1974; Merkle and Hanke, 1988; Shield and Bentley, 1973; Bentley and Shield, 1973; Hutchison and Miller, 1979).

Since normoxic MO_2 measurements of each of the different developmental groups of animals in these experiments agree with previously reported values, it can be assumed that the MO_2 values measured during hypoxia are equally accurate since the same experimental protocol, methods, and apparatus was used.

Hypoxia Induced Changes in MO_2

Group I (stage 1-39) larvae are not affected by decreases in the ambient PO_2, since MO_2 and whole body lactate concentrations did not significantly change in response to the hypoxic conditions (Figure 3). This would suggest that these animals are not highly dependent on aerobic metabolism, as has been reported by prior investigations of amphibian embryos (see Burggren, 1984; Feder, 1984 for reviews). This is supported by the fact that embryos reduce or even stop cell division and go into a dormant period in response to unfavorable environmental conditions (Detwiller and Copenhaver, 1940; Gregg, 1960; Rose et al, 1971; Weigmann and Altig, 1975). It is further supported by the fact that lipid metabolism, which requires less oxygen, is the primary source of energy for the developing embryo (Gregg, 1962; Weigmann and Altig, 1975).

External gills are only present during developmental stages 40-44 in larvae of *Xenopus laevis*. The presence of external gills, in addition to the large surface area to volume of the larvae, may account for why these animals can regulate their MO_2 in response to mild decreases in ambient PO_2 (Figure 4). A MO_2 of approximately 0.20 mM/kg/hr was conserved down

to a PO_2 around 70 mmHg in this group of larvae. While a MO_2 of this magnitude was accomplished by the next developmental stages of larvae in group III, it could not be maintained in a PO_2 below 150 mmHg.

With the loss of the external gills at stage 45, the group III (stage 45-48) animals returned to oxygen conformation (Figure 5). Interestingly, these animals frequently surfaced for air. Possibly, the internal gills may not be as effective at removing oxygen from the water as the external gills, and the buccopharynx of larval *Xenopus* is known to have poor gas exchange capabilities (Czopek, 1955). It is also possible that the air may not be primarily for aerobic metabolism. It has been priorly hypothesized that the lungs may primarily function as buoyancy regulators (van Bergiejk, 1954; Burggren and Roberts, 1991). Even though lungs first start to form in this early larval stage, the lungs are still much less complex than the lung of an adult. At this stage of development, the lung walls consist of a flat, squamous epithelium and reach into the caudal part of the abdominal cavity, caudal to the stomach and circulation in the branchial cavity and filter apparatus has been established (Nieuwkoop and Faber, 1967). However, the role of the lungs in buoyancy has never been directly tested.

At stages 49-51, the lung epithelium begins to form folds in the caudal portion of the lungs (Nieuwkoop and Faber, 1967), which theoretically should result in more efficient lungs due to an increased surface area. This may explain the increase in the normoxic MO_2, as has previously been reported. Nonetheless, group IV (stage 49-51) animals continue to be oxygen conformers (Figure 6). MO_2 increases rapidly as PO_2 is decreased to 100 mmHg, at which time it appears to level off.

At stage 52, the inner surface of the lung consists of a very thin squamous epithelia with an epithelial sac of connective tissue and blood vessels surrounding them (Nieuwkoop and Faber, 1967), but this still does not complete the formation of the lungs! The lungs are now completely perfused and the lung walls have completely formed, but the partitioning of the lungs has not completed, so the lung still does not have the large surface area that is found in late stage larvae or an adult *Xenopus laevis*. This may help to explain the reason that group V (stage 52-53) continue as oxygen conformers (Figure 7). An explanation for this may be due to changes occurring in the skin along with the fact that the lung still has a somewhat open sac-like appearance. The thin compact layer of

connective tissue below the epidermis has begun to thicken at stage 52 and continues to thicken all the way through stage 57 (Nieuwkoop and Faber, 1967). This may account for group V animals continuing to be oxygen conformers, since any increase in the thickness of the skin drastically alters the diffusion of respiratory gases through the skin and also dramatically increases the boundary layer (Hughes, 1965; Feder and Burggren, 1985). As a result of the decreased oxygen diffusivity of the skin, the lungs may be required to contribute more oxygen for aerobic metabolism. This increased gas exchange across the lung may occur at normoxic conditions where the PO_2 gradients between the environment and the body tissues is very high. But a decrease below the normal concentrations, which would only slightly decrease the gradient, may result in a decrease in the gas exchange across the lung. This decrease may be large enough so that the energy requirements can't be met by aerobic metabolism, resulting in oxygen conformity.

By stage 57, the connective tissue layer in the skin has thickened markedly and acquires a structure almost identical to the stratum compactum of the fully differentiated adult skin (Nieuwkoop and Faber, 1967) and does not thicken significantly with increased

development. There are also no significant changes in the sac-like, largely non-partitioned lungs. Therefore it is not surprising that group VI (stage 54-57) animals continue to be oxygen conformers with the aerobic metabolic rate decreasing with increasing hypoxia (Figure 8). The decrease in aerobic metabolism in mild hypoxia is not as drastic as in earlier stage animals and the data tend to convey a trend toward oxygen regulation that nevertheless cannot be proven with statistical significance. There are wide variations in the oxygen consumptions even within animals of the same developmental stage, which implies that some may be oxygen regulating while others of the same specific developmental stage are still oxygen conforming.

Beginning with group VII (stage 58-60), tadpoles consistently oxygen regulate and this continues for all further development (Figures 8-12). After stage 57, the skin begins to metamorphose into adult skin in patches (and the larval skin begins to degenerate) and the branchial clefts begin to close (which proceeds slowly to stage 62) (Nieuwkoop and Faber, 1967). The larvae also stop filter feeding at stage 59-61, before any gross morphological changes occur in the larval feeding apparatus or tail degeneration starts (Naitoh,

Wassersug, and Leslie, 1989). Most importantly, the posterior half of the lungs are now honeycombed in appearance. At stage 61, the cranial one-third of the lungs still consist of a smooth epithelium, but the caudal two-thirds show a honey-comb like compartmentalization by the formation of folds and the filter apparatus begins to degenerate and the operculum takes on a roundish appearance (Nieuwkoop and Faber, 1967). This increased development of the lungs may be the primary factor that allows for the conservation of the metabolic rate during the mild hypoxic exposure in groups VII and VIII even though the gills are nonfunctional and cutaneous respiration is decreased (Figures 8 and 9). The increased surface area may allow a larger portion of gas exchange that was performed by the skin in earlier developmental stages to be performed by the lung.

By the end of stage 66 (which is the last developmental stage in group IX) the skin has almost completely metamorphosed into the adult form (Nieuwkoop and Faber, 1967). Since it has been found that very little oxygen is exchanged across the skin of adult *Xenopus laevis* (Deuchar, 1972; Czopek, 1955), this means that the only effective gas exchange organ available to this developmental stage of tadpole is the

lungs. Fortunately, the lungs by the end of this developmental group are very similar to the adult form. At stage 66, only the most proximal portion is still smooth epithelium. The remainder of the lung has become aveolar as in the adult form. Therefore, it is not surprising that group IX is an effective oxygen regulator (Figure 11).

After the completion of the lung and metamorphosis, MO_2 for group X (1 month post-metamorphic froglets) drastically increased (Figure 12). The MO_2 was consistently higher in these animals than all other stages of animals in these experiments until the PO_2 was reduced down to around 30 mmHg. At 30 mmHg, the MO_2 was in the range of 1.0 mM/kg/hr, but this proved to be an insufficient amount of aerobic metabolism to support the animal since a mortality rate of 75% was incurred. Therefore, these animals either must have a higher standard metabolic rate than any other developmental stage studied in these experiments or a less efficient oxygen uptake mechanism.

The MO_2 of adult animals was not significantly affected by increased hypoxic conditions (Figure 13). The animals were able to compensate for decreases in the ambient PO_2 and maintain their normal MO_2 down to a PO_2 of 30 mmHg. This is in contradiction to a prior

study performed by Hutchison and Miller (1979) on slightly smaller *Xenopus laevis* frogs. Hutchison and Miller reported that the frogs could not oxygen regulate below a PO_2 of 90 mmHg at 25°C or 120 mmHg at 15°C. The frogs in their study weighed between 15-50g while the frogs in the present study experiments ranged from 20-70g. During their study, the aerial and aquatic portions of the ambient environment were isolated by a layer of mineral oil, while aerial and cutaneous MO_2 was not separated during the current experiments. Other than these three factors, both experiments were performed the same way.

Developmental Changes in Whole Body Lactate Concentrations in Normoxia

Whole body lactate concentrations for groups I-X remained at approximately 1.0 mM/kg and did not change with increasing development (Figure 16). These values are somewhat lower than a previous study that examined lactate concentrations in these stages of *Xenopus laevis* in normoxic conditions. Feder and Wassersug (1984) reported resting values of stage 45-62 larval *Xenopus.* Again, the dry weight of the animal was used, so once again the ratio of dry/wet weight 0.05 obtained by Fletcher and Myant (1959) was used to estimate the

concentration in wet mass. This allows calculation of a whole body lactate value of 3.36-4.3 mM/kg, which is significantly higher than the values found for the same stages of animals in this study. Feder and Wassersug had a limited number of animals (5) and performed their experiments at 25°C, but other than these two things the two experiments were essentially the same.

The whole body lactate concentrations for group XI (9.5078 mM/kg) are somewhat higher than most of the reported lactate concentrations for Adult *Xenopus laevis* (1.22-10.11 mM/kg), but are still well within the range of reported values (Miller and Camilliere, 1981; Hutchison and Miller, 1979; Putnam, 1979; Boutilier et al, 1986). The whole body lactate levels measured during this experiment may not represent lactate concentrations when the animal is at complete rest due to the possibility that the animals may not have been completely acclimated to the new environment of the experimental chamber at the end of the twelve hour acclimation period. Nevertheless, the whole body lactate concentrations were found to be very constant in an ambient PO_2 ranging from normoxia to the P_{crit}. Therefore, for the purposes of these experiments, which were to identify increases in the whole body lactic acid concentration, these values are accurate.

Developmental Changes in Whole Body Lactate Concentrations in Hypoxia

Interestingly, whole body lactate concentrations of groups I (stage 1-39), II (stage 40-44), III (stage 45-48) and IV (49-51) did not increase significantly in response to even severe hypoxia (Figures 16-19). The animals significantly reduce the aerobic metabolic rate, but apparently anaerobic metabolism (as measured by lactic acid production) was not utilized to supplement the decrease in aerobic metabolism. The animals may cope with the hypoxic conditions in a way similar to earlier stage embryos, by reducing the amount of living tissue produced and maintaining the tissue which had already been produced. The amount of energy necessary to carry out the minimal metabolic needs of the living tissues produced previously may be satisfied by aerobic metabolism.

The whole body lactate concentrations for group III falls as ambient PO_2 decreases to 100 mmHg, but then starts to rise as the PO_2 is decreased further (Figures 18). This fall and then rise may suggest that the animals decrease the amount of surfacing occurrences as the ambient PO_2 falls, as it has been found that swimming activity is fueled primarily by

anaerobic metabolism (Hillman and Withers, 1981; Miller; 1983). This stage of animal, if watched, is found to frequently surface for air even in normoxic conditions. Therefore, a decrease in the swimming activity may result in a lower concentration of lactate production through anaerobic metabolism. The rise in whole body lactate below 100 mmHg may result again from increased surfacing for air or may be occurring to supplement the further decrease in aerobic metabolism. Interestingly, the whole body lactate concentrations of group IV at severe hypoxia is only slightly higher than normoxic concentrations (Figure 20). This may suggest that the anaerobic metabolism, as measured by whole body lactic acid concentrations, is not involved in supplementing the aerobic metabolism of these animals.

Beginning with group V (stage 52-53), whole body lactate concentrations increase in response to hypoxic conditions (Figure 21). Whole body lactate concentrations for group V remained constant down to approximately 60 mmHg. However, after a PO_2 of about 60 mmHg is reached, the whole body lactate concentration is found to significantly increase, suggesting that anaerobic metabolism, as measured by whole body lactic acid concentrations, may be required for maintenance of the body tissues for the first time.

Beginning with this stage, anaerobic metabolism and lactic acid production may be required to supplement the aerobic metabolic rate in order for the animals to cope with hypoxic conditions.

In group VI (stage 54-57), the lactic acid concentrations do not increase in response to hypoxia until a PO_2 of around 60 mmHg again is reached (Figure 22). At 40 mmHg, the lactic acid concentration with in the tissues is found to drastically increase and then to decrease again as hypoxia is increased. This increase and then decrease may be explained by the high mortality rate found when this stage of animal was exposed to this level of hypoxia in the aerobic portion of these experiments. The animals measured for lactate content may have been dead or dying at which time lactate concentrations have been found to decrease (S. J. Warburton, personal communication).

The whole body lactate concentration of group VII (stage 58-60) is very constant with very little variation from normoxic conditions down to an ambient PO_2 of 40 mmHg (Figure 23). This implies that the lactic acid pathways utilized are constant and unaffected by the changes in the ambient PO_2. At 30 mmHg, the whole body lactate level significantly

increased which would support anaerobic metabolism having a role in supplementing aerobic metabolism.

For group VIII (stage 61-62), the lactate concentrations are relatively unaffected by increasing hypoxia until a PO_2 of 40 mmHg is reached (Figure 24). Whole body lactate concentrations again drastically increase in an ambient PO_2 of 30 mmHg just like the prior developmental group. These findings are not unexpected in that the skin and lungs are still not completely metamorphosed into the adult form and the larvae are at a similar respiratory developmental state.

The whole body lactate levels for groups IX (stage 63-66) and X (1 month post-metamorphic froglets) also remained constant in response to mild hypoxia to a PO_2 of about 60 mmHg (Figures 24 and 25). Below 60 mmHg, whole body lactate concentrations increase with increased hypoxia, implying a role for anaerobic metabolism in supplementing aerobic metabolism.

Whole body lactate concentrations remained fairly constant in group XI (adult frogs) down to a PO_2 of 40 mmHg as in groups VII and VIII (Figure 27). Whole body lactic acid concentrations then increased to a concentration around 25 mM/kg in response to exposure to PO_2 of 30 and 15 mmHg where they again remained

fairly constant with no mortality occurring. This implies that anaerobic metabolism can fully compensate for the decrease in the aerobic metabolic rate through production of lactic acid.

Developmental Changes in P_{crit}

$P_{crit's}$ for all of the different groups that were oxygen regulators were determined by two independent methods, which produced agreement within 15 mmHg (Table 5). Young larvae with a large surface area to body size ratio and larvae in late developmental stages where the lungs are fully functional were all oxygen regulators and determined to have a critical PO_2 value (Figure 15). Whole body lactate levels were also drastically increased only at PO_2's below the defined P_{crit}. Animals studied in the surfacing portion of these experiments were also dramatically increased the number of surfacing events as the P_{crit} was approached providing increased support.

The critical PO_2 decreased as development proceeded. A P_{crit} of about 75 mmHg was established for groups II (stage 40-44), VII (stage 58-60), and VIII (stage 61-62) (Figure 15). At stage 63-66, the

Table 5. Comparison of P_{crits} Resulting from Two Different Methods

Stage of Animals.	Method of Least Squares Regressions. (mmHg)	Method of Yeager and Ultsch (1989). (mmHg)
Stage 40-44	70	67.5
Stage 58-60	77.5	73
Stage 61-62	76	73
Stage 63-66	85.5	69
Froglets	64.5	59
Adults	30.28	28

P_{crit} was higher,but began to decrease with increasing development. The P_{crit} decreased from 85 mmHg for group IX (stage 63-66) to approximately 65 mmHg for group X (1 month post-metamorphic froglets) and finally decreased to 30 mmHg for group XI (adult frogs) (Figure 15).

The whole body lactate concentrations 15 mmHg below the P_{crit} for groups II, VII, VIII, and IX were indistinguishable from groups I and III-VI at 1.0-2.0 mM/kg (Figure 29) indicating a similar rate of anaerobic metabolic lactate production. Groups X and XI had increasing amounts of whole body lactate 15 mmHg below their P_{crit} indicating an increased anaerobic metabolic production of lactate in response to the hypoxia. Whole body lactate concentrations increased to 9.4 mM/kg for group X and then increased again to 24.94 mM/kg for group XI (Figure 29). This implies a shift from aerobic to anaerobic pathways to support metabolic needs.

Early in development, all amphibians have external gills which are usually lost with the formation of internal gills. Stage 40-44 are the only stages of *Xenopus laevis* with fully functional external gills. This along with the fact that all amphibians early in development fulfill all of their metabolic needs by

direct diffusion of the respiratory gases through the body wall (Burggren, 1985; Quinn and Burggren, 1983; Feder and Burggren,1985), may account for the fact that these early embryos were able to regulate their MO_2 down to a PO_2 around 70 mmHg (Figure 4). Diffusion through the body wall may be the primary route of gas exchange in normoxia because of the high surface to body mass ratio of this stage of animal (Nieuwkoop and Faber, 1967; Burggren, 1985). However, it seems that in hypoxic conditions, the external gills may contribute to oxygen regulation. This is supported by the finding that when the external gills are nonfunctional (as in some of the group III animals with approximately the same age and body size) the animals are no longer able to oxygen regulate and become oxygen conformers once again (Figure 15). However, the loss of the ability to oxygen regulate between groups II (stage 40-44) and III (stage 45-48) may also be an immediate response to increased development. The developmental change must be somewhere other than the skin, though. In both groups, the skin is composed of a double layered epidermis which is more or less flattened cells with a thin layer of connective tissue (Nieuwkoop and Faber, 1967), and there is little if any change in the skin structure between these two groups.

In groups VII (stage 58-60) and VIII (stage 61-62) the MO_2 is fairly constant above the P_{crit} and decreases almost linearly as the PO_2 is reduced below the P_{crit} (Figures 8 and 9). The P_{crit} is the same in both groups as would be expected since the lung is in the same developmental stage.

Group IX (stage 63-66) is clearly able to oxygen regulate, but the P_{crit} is found to be significantly higher than prior larval stages (Figure14). The whole body lactate levels also begin to rise at a higher PO_2 than the prior larval stages (Figure 29). This may be explained by developmental changes in the internal gill and filter apparatus. The arteries of the filter apparatus begin to disappear with reduction of the branchial basket and the operculum is completely closed by stage 63 (Nieuwkoop and Faber, 1967). This effectively is the end of the use of gills and buccopharynx by the tadpole for water breathing. However, the lung is only completed at the end of this developmental group. Again, there is great variability in the MO_2 measured, but this time it is only in the measurements above the P_{crit} (Figure 11). Above 85 mmHg, there is wide variation in the oxygen consumption of larvae of the same developmental stage. But below 75 mmHg, the oxygen consumption decreases in a

completely linear fashion. Variability in the oxygen consumption above the P_{crit} may be explained by variation in the development of the lung. In some of the tadpoles of the same exact developmental stage, the lung may be completely partitioned while in other tadpoles of the same stage, the lung is not completely partitioned. This would lead to variability among the lungs of the same developmental stage of larvae to extract more or less oxygen, depending on the partitioning of the lung and would account for an increased P_{crit} and variability in the measurements of the MO_2 above the P_{crit}.

The P_{crit} decreased to 65 mmHg for group X (1 month post-metamorphic froglets) (Figure 15). Group X had a greatly increased MO_2 which was quite variable above 60 mmHg (Figure 12). Below 60 mmHg, MO_2 decreased linearly. Therefore, while the MO_2 may be highly variable in mildly hypoxic conditions, it is quite clear that the P_{crit} is around 60 mmHg where they begin to oxygen conform. The MO_2 first begins to increase as the ambient PO_2 decreases. This may be a result of increased activity due to an escape response elicited by the decrease in ambient PO_2. As ambient PO_2 decreases further, MO_2 decreases indicating that an

escape response may only be elicited by mildly hypoxic conditions.

A low P_{crit} is expected for an animal like *Xenopus* that is known for its ability to live in an environment with low ambient PO_2 (Deuchar, 1972; Deuchar, 1975; Nieuwkoop and Faber, 1967). Therefore, it was not surprising to find that adult animals were able to survive short term exposure to a PO_2 in the range of 10-15 mmHg (Figure 13) and had a P_{crit} of 30 mmHg (Figure 15). However, this is drastically different than prior reported values for adult Xenopus (Hutchison and Miller, 1979) The difference may have occurred due to the method used for determination of the P_{crit}. Two independent methods were used for the determination of the P_{crits} which are reported in the current study. The method used by Hutchison and Miller in 1979 is unclear.

Hypoxic Changes in Surfacing Occurrences:

Significant changes in the surfacing occurrences in response to hypoxic conditions occurred in response to development from groups IX (stage 63-66), X (1 month post-metamorphic froglets), and XI (adult frogs) (Figure 30). All three groups increased the number of surfacing occurrences in response to hypoxia only after

the P_{crit} was reached. Group IX consistently surfaced less often than both groups X and XI. Groups X and XI effectively surfaced the same number of times in response to exposure to the same PO_2. This indicates that there are two distinctly different patterns of surfacing between pre-metamorphic and post-metamorphic animals. The difference in the number of surfacing occurrences is most likely due to the developmental state of the respiratory physiology. In post-metamorphic animals the respiratory structures are fully formed and functional. The correlation between groups X and XI indicate that the increased MO_2 of group X does not significantly hinder the animals ability to remove oxygen from the ambient environment through aerial respiration.

Conclusion:

Development has a significant affect on the metabolic rate of *Xenopus laevis* (Figure 14). Early in development (up to about stage 52), aerobic pathways are the principle source of energy for the developing larvae and anaerobic lactic acid production does not play a major role in supplementing aerobic metabolism even under extremely adverse conditions (Figure 28). Later in development, after stage 52, the aerobic metabolic pathways are supplemented by anaerobic lactic acid production. A shift from sole support by aerobic metabolism to aerobic and anaerobic metabolism occurs. This shift may occur because of an increased amount of living tissue that must be supported or it may occur in response to the ability to utilize energetic pathways that were not available before this point. In either case the shift is a result of increased development of the larvae. A shift from oxygen regulation to oxygen conforming back to oxygen regulation also occurs in response to increased development. Early larvae with small body sizes and large surface areas are able to conserve a normal MO_2 in exposure to mild hypoxia. As

body size and a shift from solely aquatic to bimodal gas exchange begins to occur, larvae are no longer able to regulate their MO_2 and become oxygen conformers even in the mildest hypoxic environments. Close to the completion of the shift to bimodal breathing, when the development of the respiratory structures are almost complete, there is a shift back to oxygen regulation once again that is constant into adulthood. There is also a shift in the surfacing for air during development. Upon the completion of metamorphosis, animals surface almost twice as often as larvae in the same ambient environment. This indicates a shift from primarily aquatic to aerial gas exchange for the developing animals.

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