The Journal of Experimental Biology 209, 1651-1661
Published by The Company of Biologists 2006
doi:10.1242/jeb.02195

Metabolic scaling associated with unusual size changes during larval development of the frog, *Pseudis paradoxus*

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Accepted 2 March 2006

Summary

The early larvae of *P. paradoxus* grow large but metamorphose into relatively small frogs, the diminished post-metamorphic growth producing a marked contrast between maximum larval size and adult. Thus, O_2 uptake does not appear to limit the energy expenditure on growth processes, and unlike in other anuran larvae, may not be a surface area-related function in *P. paradoxus* larvae. The resting rates of metabolism ($\dot{M}_{O_2}$) and partitioning between aquatic ($\dot{M}w_{O_2}$) and aerial O_2 uptake ($\dot{M}a_{O_2}$) were measured on tadpoles and froglets by closed system respirometry, using water of P_{O_2} ranging from 145 to 40 mmHg. Correlative changes in body glycogen and lactate were examined by standard enzyme assays. Scaling patterns in the growth and degrowth stages were analysed on whole-body, log-transformed data using linear regressions. In normoxia, $\dot{M}_{O_2}$ was 2.1–2.5 μmol g^{-1} h^{-1} in the early larvae, increasing more than twofold on forelimb emergence and decreasing sharply in the froglets; $\dot{M}_{O_2}$ varies in strict proportion to body mass (M_b), both in the growth (b=1.02) and degrowth (b=0.97) phases, according to the equation $\dot{M}_{O_2}=aM_b^b$, where b is the scaling coefficient. $\dot{M}w_{O_2}$ constitutes >90% of total uptake in the growth stages, increasing with b=1.02 while $\dot{M}a_{O_2}$ increases with b=1.13; during degrowth there is a change in the pattern related to intensification of metamorphosis. Hypoxic water did not affect $\dot{M}_{O_2}$; however, in all larval stages $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$ changed with a decrease in P_{O_2}. At 60 mmHg, rates are more severely affected in the largest tadpoles, causing the b values for $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$ to change to 0.11 and 1.44, respectively, in the growth phase. Glycogen and lactate levels increase out of proportion with body mass increase (b=2.05 and 1.47, respectively) in the growth stages, and increase anaerobic capacity in late metamorphosis. In hypoxic water, glycogen levels decrease in the growth stages and the largest tadpoles accumulate surplus lactate, possibly related to surfacing activity. Our results may reveal the consequences of size on energy demand at the tissue level in *P. paradoxus* larvae, indicating that air breathing must subsidise energy expenditure during larval development.

Key words: metabolic rate, oxygen uptake, metamorphosis, scaling, hypoxia, glycogen, lactate, Amphibia, anuran, *Pseudis paradoxus*.

Introduction

Early studies on the diffusion and perfusion limitations of cutaneous breathing indicate that routine rates of aerobic metabolism in amphibians might be limited by the surface area available for O_2 transfer (Piiper and Scheid, 1977; Ultsch, 1976). Amphibians spend a large part of their daily cycle at rest, and many of their routine behaviours seem to use only a fraction of their aerobic capacity (Gatten, Jr et al., 1992). The scaling of resting metabolic rates ($\dot{M}_{O_2}$) with body mass M_b in intra-specific comparisons with adult individuals, described by the allometric equation $\dot{M}_{O_2}=aM_b^b$, has not disclosed any particular tendency towards a 2/3 exponent, and scaling coefficient values (b) on average express an intermediate proportion in the surface area:body mass relationship (Withers, 1992). However, surface limitation may be significant and may restrain metabolic capacity to grow and sustain activity, particularly in those species that rely heavily on cutaneous gas exchange and during larval life stages.

The issue is of interest owing to the recent elucidation of processes contributing to metabolic rate in organisms, and of how body size exerts its influence on energy expenditure at the whole-body level (Hochachka et al., 2003; Darveau et al., 2002; Hulbert and Else, 2000; Rolfe and Brown, 1997). It has become clear that, as organisms increase in size, they adjust their structure and function at all levels of organization, from the external surface for O_2 transfer to the composition of cell membranes and protein function, each with their own characteristic scaling coefficient and control contribution, which in turn, depend on metabolic state. These ideas have been largely developed from inter-specific comparisons of adult organisms,

under low and high activity levels; in the ontogenetic context, energy utilization for growth and organogenesis may have a great influence on overall metabolic scaling according to the developmental stage. In the few mammals and fishes examined, metabolic rates change in direct proportion to mass during the early stages (the period of greatest growth), bringing the mass exponent to close to 1.0; thereafter, rates tend to follow the surface rule with the two regressions intersecting after a given proportion of adult body mass is reached (reviewed by Hulbert and Else, 2000; Wieser, 1995; Wieser, 1984).

Interestingly, unlike the situation in mammal and fish species, there appears to be no clear shift in the scaling pattern during the transition from larval to adult life stages in anuran amphibians, the exponent varying from 0.65 to 0.88 (Gatten, Jr et al., 1992; Feder, 1982), which suggests possible surface limitations to the increase in cutaneous O_2 transfer in direct proportion to body mass during early development. In addition, experimental data with anuran larvae suggest that the larger the larvae, the greater the contribution of air breathing to the responses to severe aquatic hypoxia. The requirement of air breathing to meet metabolic demands in developing tadpoles has long been controversial; while cutaneous respiration provides 60% or more of overall oxygen uptake in anuran larvae, air breathing does not seem to be essential for growth and activity in their natural settings (Burggren and Just, 1992; Feder, 1984). However, since post-metamorphic growth is usually responsible for 80% or more of body size in anuran amphibians (Werner, 1986), it is plausible that growth processes may not influence energy cost at the whole-body level during the larval phase in the species examined, which might explain the apparent discrepancy in the scaling patterns. In the present study, this hypothesis was examined in the frog *Pseudis paradoxus*. The development of *P. paradoxus* is unusual in several respects, such as the large larval size and small gain in body mass after metamorphosis, leading to a large contrast in the maximum size of the tadpole compared to the adult frog (Emerson, 1988). Giantism in *P. paradoxus* larvae may thus represent the anticipation of post-metamorphic growth during the early stages of the life cycle, the adaptive value of which is unknown.

P. paradoxus frogs are exclusively neotropical; in southern Brazil, newly hatched larvae are most abundant in late spring and become scarce by mid-summer, most tadpoles having metamorphosed by the early fall (S.C.R.d.S., unpublished observations). The large size at metamorphosis is attained during a relatively short period, implying use of a large fraction of the energy derived from aerobic sources to support growth processes in larval *P. paradoxus*. Further, O_2 uptake might be severely affected in the largest tadpoles under hypoxic conditions, as is usual in the natural habitat. The present study is concerned with the question of how O_2 uptake may be reconciled to growth and developmental changes in *P. paradoxus* tadpoles. We examined the relationship between body mass and aquatic and aerial O_2 uptake in the growth and degrowth phases of the larval cycle, and the effects of aquatic hypoxia on the scaling patterns. In addition, we analysed correlative shifts in the levels of body glycogen and lactate as an indication of supplementary energy production from anaerobic sources. Our findings generally indicate that the scaling of metabolism in *P. paradoxus* may express the consequences of size on energy demand at the tissue level, suggesting that air breathing must subsidise energy expenditure in the largest individuals, and sustain the increase in metabolism during late metamorphosis.

Materials and methods

Experimental animals

Tadpoles and froglets of *Pseudis paradoxus* (Linné 1758) were caught by sieve from the ponds at a fish farm located in Ribeirão Preto, south-eastern Brazil, during the summer. In the laboratory, the animals were maintained in 150 l tanks containing aerated water at 24±1°C and vegetation that served as refuge and support for the late metamorphic stage larvae and froglets. The larvae were fed twice a day with commercial food (Reptomin®) and spinach *ad libitum*. The intensification of metamorphosis caused a cessation of food intake, confirming observations made on the digestive tube of newly captured animals at late metamorphosis. The minimum and maximum masses at capture were 0.1 g and 72 g, respectively, some mass loss following maintenance in the laboratory. The measurements were made on animals kept in the laboratory for less than 20 days, and deprived of food for 24 h prior to the experiments.

Experimental groups

Developmental stage was classified according to Cais after Gosner (Cais, 1982; Gosner, 1960). First stage larvae were very vulnerable to handling and transportation and were not included in the hypoxia experiments; partial data for larval stages 29–32 (body mass=3.7±1.2 g, mean ± s.d.) in normoxic water are given in the Results. The individual data obtained for larvae from the subsequent stages were combined into five developmental groups: the first consisted of stages 33–37 (8.1±4.5 g), all larvae showing inflated lungs; the second group consisted of stages 38–42 (17.0±7.3 g), the larvae showing well vascularized lungs, and stage 42 being marked by the emergence of the forelimbs; the fourth included stages 43–44 (14.8±8.2 g), and the fifth stage 45 alone (10.0±2.6 g); both these groups exhibit the most notable anatomical changes that constitute the shape of the adult frog while maintaining a tail of considerable proportions; the sixth group included stage 46 (7.1±2.4 g), corresponding to froglets up to 20 days of complete metamorphosis. According to our observations, surfacing behaviour begins at low frequencies during the early larval stages, and more regularly in tadpoles at stages 38–42; in the latest stages, the tadpoles remain for long periods at the air–water interface, while froglets alternate periods of partial emersion with prolonged submersion.

Measurement of the aquatic and aerial oxygen uptake

Aquatic and aerial oxygen uptake, $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$, respectively, were measured by closed system respirometry at

25±1°C (room temperature) on single larvae, each animal being used only once. The metabolic chambers consisted of an acrylic tube with a 10 mm thick wall, and of variable length and diameter. Previous measurements showed that *P. paradoxus* tadpoles become restless when held with the body lying flat inside a horizontal chamber. The tube was then vertically oriented with the bottom end closed, and a funnel-like lid that fit over the top end over a layer of compressible rubber. For the experiments, the chamber was screwed shut and filled with water leaving a volume of air close to the funnel stem, and the volumes were adjusted according to the size and stage of the tadpole. During measurements, the water was exchanged via outlet and inlet valves, and the samples were withdrawn through a small aperture located at a medium height in the water column, sealed by a silicone septum (Alltech Associates, Inc., Deerfield, Illinois, USA). The air phase communicated with ambient air *via* a valve attached to the funnel stem, and air samples were withdrawn *via* a silicone septum fitted into the valve aperture.

The experiments were conducted on consecutive runs from 08:00 h to 19:00 h. Preliminary measurements were made in nearly constant, normoxic conditions, to check for any sizeable variability related to experimental procedures. The rates were then measured under acute exposure to incremental levels of aquatic hypoxia while the animals had free access to the air volume. The system was opened at 60–90 min intervals and the water carefully exchanged by the inflow of clean, sterilized water at near-neutral pH from a reservoir. The water in the reservoir was constantly mixed by a flow of air or N_2 such that the resulting water P_{O_2} was 146, 114, 88, 60, 40 mmHg, the levels representing the global mean of the initial P_{O_2} values in each run. Further, the air at the top of the chamber was refreshed and O_2 content set to normoxic levels for each subsequent experimental run. The animals were acclimated to the chamber for at least 12 h prior to measurements with a constant flow of aerated water, and the valve to the air phase open to avoid hypoxia. After closing the system, the water P_{O_2} was allowed to decline with the animal breathing to a limit that nearly matched the initial P_{O_2} value in the next run, and O_2 content in the air phase was allowed to decline to a limit of 60% below normoxic in each run.

$\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$ were measured in triplicate samples of 1 ml and 50 μl, respectively, taken with gas-tight syringes. The air in the gas phase was gently mixed with the syringe before sampling, and the water was mixed for a few seconds with a small, magnetic stirring bar at slow speed, and sampled. O_2 concentration in the air samples were measured using the zirconium sensor of an Oxycon device (Cameron Instrument Co., Port Aransas, Texas, USA), and P_{O_2} in the water samples was measured using a Clark-type electrode held inside a thermo-stable chamber at 25°C, the signal being converted in a gas analyser (Cameron Instrument Co.). The equipment was calibrated in the early morning and checked for stability during the experiments. During measurements, the animals were shielded by an opaque screen to minimize visual disturbance; the stage 33–42 tadpoles remained mostly quiescent, with the snout upwards and the tail hanging downwards into the water column, while the last stage tadpoles and froglets alternated periods near the water surface with variable periods of submersion to the bottom. In all cases, energy expenditure during the measurements was assumed to be an approximation of resting levels, with ΔO_2 values varying according to the tadpole size and degree of aquatic hypoxia. Micromolar O_2 concentrations were calculated from the O_2 tension and the coefficients for distilled water at 25°C, and the total O_2 consumption rates ($\dot{M}_{O_2}$) were calculated from the $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$.

The same protocol was used in control experiments with no animals in the chambers, allowing adjustment for diffusive changes according to chamber size and degree of aquatic hypoxia. Oxygen changes in the air and in the water phase were measured at 60–90 min intervals under incremental levels of aquatic hypoxia as above; the values obtained were then subtracted from, or added to, the experimental O_2 consumption rates accordingly, averaging 0.9–6.1% of the $\dot{M}a_{O_2}$ and 0.1–1.1% of the $\dot{M}w_{O_2}$ values.

Measurement of body glycogen and lactate levels

Total glycogen and lactate contents were measured in larvae at distinct developmental stages in normoxic water (mean P_{O_2}=149 mmHg) or after acute, 3 h exposure to hypoxic water (60 mmHg), during which the animals had free access to normoxic air in the upper phase. Groups of animals were maintained in 60 l tanks containing clean, sterilized water at near-neutral pH and a support made of inert material that allowed access by late metamorphic stage individuals to the surface without increased effort. The animals were killed by pithing and quickly immersed in liquid N_2, then stored at –85°C until analysis.

The frozen larvae were powdered in liquid N_2 and homogenised with ice-cold, 0.6 mol l^{-1} perchloric acid (PCA) using a blender. After complete deproteinization, glycogen and L-lactate concentrations were estimated according to standard enzymatic procedures (Bergmeyer, 1984) by following the oxidation rate of NAD^+ in a spectrophotometer Spectra Max 250 (Molecular Devices, Sunnyvale, CA, USA) at 340 nm and 25°C. The final result was expressed as μmol glycosyl units or L-lactate per unit body mass.

Statistical analysis

Visual inspection suggested the effects of P_{O_2} on $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$ per unit mass to be non-linear in some of the experimental groups. Without a theoretical model, however, the outcome of the analysis using a different function would be equally arbitrary, and hence, for the purpose of this study, the correlation coefficient '*r*' was used as a reliable measure of the degree of association between the variables. The data were log-transformed for calculation of the coefficient given the indication of deviation from bivariate normality. The percentage of variance of the whole-body $\dot{M}_{O_2}$, $\dot{M}w_{O_2}$, $\dot{M}a_{O_2}$, and metabolites attributable to body mass and to developmental stage were estimated by analysis of covariance

(ANCOVA), using the data obtained in normoxic and hypoxic water. The relationships between whole-body $\dot{M}_{O_2}$, $\dot{M}w_{O_2}$ or $\dot{M}a_{O_2}$ and body mass were analysed at two selected water P_{O_2} levels (146 and 60 mmHg) on log-transformed data, using the least-squares, linear regression method. The growth and degrowth phases of the larval period were considered as criteria of biological significance in the calculation of two regression lines and equations, one consisting of the developmental stages 33–42, and the other of stages 43–46, and the regression coefficient r^2 was used as a measure of functional dependence of O_2 uptake rates with respect to body mass. The effect of hypoxic water on glycogen and lactate per unit mass was tested against normoxic water and as a function of developmental stage by two-way analysis of variance (ANOVA). The scaling relationships of the metabolite concentrations were analysed using the least-squares, linear regression method on log-transformed, whole-body data as above. The analyses were performed according to Zar (Zar, 1999), using SigmaStat (Jandel Scientific Co., San Rafael, CA, USA) and MiniTab (MiniTab Inc., State College, PA, USA) statistical software. The test results and probability of error are given in the text or in the tabulated results, differences being considered significant at $P \leqslant 0.05$.

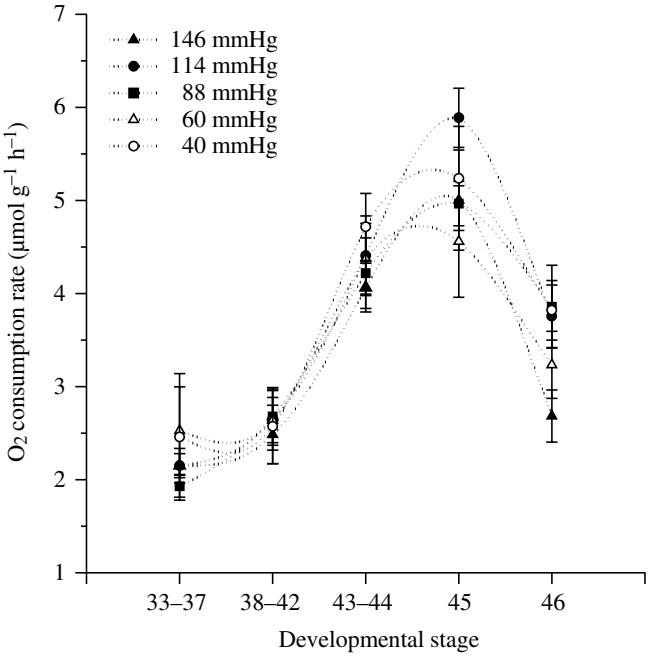

Fig. 1. Mass-specific O_2 consumption rates in *Pseudis paradoxus* tadpoles and froglets measured at 25°C on exposure to water of variable O_2 tension (mean of initial P_{O_2} values) with free access to air. Each point represents the mean ± 1 s.e.m. of $N>7$ animals staged according to Cais after Gosner (Cais, 1982; Gosner, 1960) and measured individually. The data are pooled into groups of stages representing distinct developmental steps during the larval period. There is no significant effect of O_2 tension on the rates; most variability is attributable to developmental stage (F=48.2, P<0.0001).

Results

Mass-specific, O_2 consumption rates and partitioning between aquatic and aerial uptake

There were no significant changes in mass-specific O_2 consumption rates ($\dot{M}_{O_2}/M_b$) of tadpoles and froglets measured in consecutive experimental runs under normoxic conditions. It was thus assumed that the experimental procedure and its total duration did not contribute significant variation to the data. In normoxic water, $\dot{M}_{O_2}/M_b$ measured 2.1–2.5 μmol g^{-1} h^{-1} in stage 29–42 larvae, increasing to 3.9 μmol g^{-1} h^{-1} in stages 43–44 larvae, and peaking at 5.7 μmol g^{-1} h^{-1} in stage 45 larvae (Fig. 1). $\dot{M}_{O_2}/M_b$ values decreased sharply on metamorphosis, and those of the froglets were similar to the early larval stages. Acute exposure to water of reduced P_{O_2} caused no significant change in $\dot{M}_{O_2}/M_b$ within the P_{O_2} range analysed, most variability being attributable to developmental stage (F=48.2, P<0.0001).

Under normoxic conditions, the stage 33–37 tadpoles acquire 96% of O_2 consumed from the water, with a minor contribution from aerial exchange (Table 1; Fig. 2). This value is unchanged in the subsequent stages until the emergence of the forelimbs in stage 42, when gill respiration becomes impaired. Thereafter, the contribution by aerial uptake increases sharply, reaching 73% in stage 45 larvae and in froglets. Partitioning between aquatic and aerial O_2 uptake was altered on exposure to aquatic hypoxia in all groups of stages examined (Table 1; Fig. 2). In stage 33–37 larvae $\dot{M}w_{O_2}/M_b$ decreased with P_{O_2} decrease (r=0.73; P<0.0001), this effect being accompanied by an increase in $\dot{M}a_{O_2}/M_b$ (r=0.80; P<0.0001) that accounts for 74% of O_2 consumed under the most severe hypoxia. Similarly, in larval stage 37–42, there was a positive correlation between $\dot{M}w_{O_2}/M_b$ and the degree of aquatic hypoxia (r=0.68; P<0.0001), $\dot{M}a_{O_2}/M_b$ showing an

Table 1. *Partitioning of O_2 uptake in Pseudis paradoxus tadpoles and froglets as a function of water O_2 tension*

Water P_{O_2} (mmHg)	Respiratory medium	Developmental stage				
		33–37	38–42	43–44	45	46
146	Air	3.7	4.9	65.6	73.2	72.4
	Water	96.3	95.1	34.4	26.8	27.6
114	Air	5.4	16.8	79.3	79.0	80.5
	Water	94.6	83.2	20.7	21.0	19.5
88	Air	24.5	23.1	84.4	90.7	75.7
	Water	75.5	76.9	15.6	9.3	24.3
60	Air	55.3	69.5	92.0	93.7	87.0
	Water	44.7	30.5	8.0	6.3	13.0
40	Air	73.8	77.0	90.7	95.0	90.2
	Water	26.2	23.0	9.3	5.0	9.8

Water P_{O_2} is the mean initial water O_2 tension.

Data are percentages of mass-specific, O_2 consumption rates for groups of developmental stages, stage 46 corresponding to newly metamorphosed froglets.

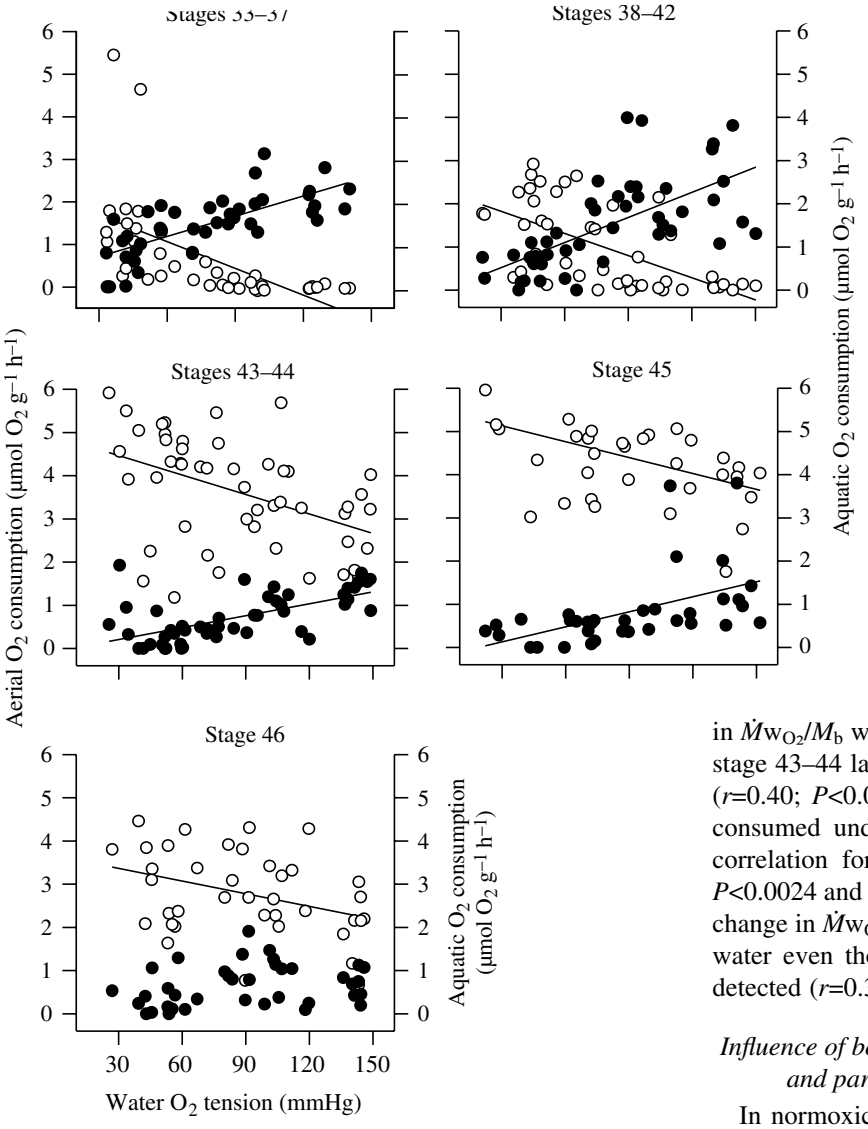

Fig. 2. Mass-specific, aquatic (filled circles) and aerial (empty circles) O_2 consumption rates of *Pseudis paradoxus* tadpoles and froglets as a function of water O_2 tension (mean of initial P_{O_2} values), at 25°C. Each point represents a single animal, staged according to Cais after Gosner (Cais, 1982; Gosner, 1960) and individually measured. The data obtained from the animals in each group of stages are given separately. Lines through the data points represent significant correlations between the variables (broken line, aquatic; solid line, aerial consumption rates); detailed descriptions of correlation coefficients and statistics are given in the text.

Table 2. *The mass exponent b for the scaling relationship of whole-body O_2 consumption rates, and partitioning between aquatic and aerial O_2 consumption in Pseudis paradoxus tadpoles and froglets exposed to normoxic and hypoxic water*

Water P_{O_2} (mmHg)	Rate of O_2 consumption	*b*	
		Stage 33–42	Stage 43–46
146	$\dot{M}_{O_2}$	1.02±0.15	0.97±0.16
	$\dot{M}w_{O_2}$	1.02±0.15	1.18±0.14
	$\dot{M}a_{O_2}$	1.13±0.21	0.77±0.18
60	$\dot{M}_{O_2}$	0.83±0.14	1.01±0.16
	$\dot{M}w_{O_2}$	0.11±0.30	0.75±0.42
	$\dot{M}a_{O_2}$	1.44±0.21	0.99±0.15

$\dot{M}_{O_2}$, whole-body O_2 consumption rate; $\dot{M}w_{O_2}$, aquatic O_2 consumption rate; $\dot{M}a_{O_2}$ aerial O_2 consumption rate.

Water P_{O_2} values are mean initial O_2 tensions.

Values for *b* are means ± s.e.m.

Developmental stages 33–42, larval growth; stages 43–46, degrowth until complete metamorphosis.

inverse correlation (r=0.61; P<0.0001) and accounting for 77% of O_2 consumed at the lowest P_{O_2} level. The $\dot{M}w_{O_2}/M_b$ and $\dot{M}a_{O_2}/M_b$ correlation lines cross over at approximately 60 mmHg, when the contributions of aquatic and aerial uptakes are nearly equivalent. Significant decreases in $\dot{M}w_{O_2}/M_b$ with decrease in water P_{O_2} were also observed in stage 43–44 larvae (r=0.64; P<0.0001) and in stage 45 larvae (r=0.40; P<0.0172), the rates reaching less than 10% of O_2 consumed under the most severe hypoxia, with an inverse correlation for $\dot{M}a_{O_2}/M_b$ in both groups (r=0.43 and 0.44; P<0.0024 and 0.0082). In the froglets, there was no significant change in $\dot{M}w_{O_2}/M_b$ as a function of decreasing O_2 level in the water even though a small increase in lung ventilation was detected (r=0.37; P=0.0234).

Influence of body mass on whole-body, O_2 consumption rates and partitioning, in normoxic and hypoxic water

In normoxic water (P_{O_2}=146 mmHg), body mass accounts for 87% of the overall variability in $\dot{M}_{O_2}$ of *P. paradoxus* larvae and froglets (ANCOVA; F=35.8; P=0.0001); significantly, developmental changes in the gas exchange organs account for 14% (F=4.4; P=0.001). This reflects the strong dependence of $\dot{M}w_{O_2}$ on body mass M_b (F=38.0; P=0.0001), with a lesser effect of developmental changes (F=3.4; P=0.004). The $\dot{M}a_{O_2}$ is predominantly influenced by developmental stage (F=90.8; P=0.0001), and varies significantly as a function of larval size (F=14.6; P=0.0001) (Table 2; Fig. 3).

Separate analysis of the growth and degrowth phases revealed that the slope for the relationship between $\dot{M}_{O_2}$ and body mass is b=1.02 and 0.97, respectively (Table 2; Fig. 3), implying that O_2 uptake first increases proportionally to body mass increase (r^2=0.77; P<0.0001), then decreases in proportion to mass reduction during larval development (r^2=0.61; P<0.0001). $\dot{M}w_{O_2}$ similarly increases in strict proportion to the increase in body mass during growth stages, with b=1.02 (r^2=0.76; P<0.0001), and although representing only a small fraction of the total O_2 uptake, $\dot{M}a_{O_2}$ increases out of proportion to body mass increase, with b=1.13 (r^2=0.67; P<0.0001). During degrowth stages, while the decrease in the

$\dot{M}w_{O_2}$ with body mass decrease is disproportionate, with b=1.18 (r^2=0.75; P=0.0001), $\dot{M}a_{O_2}$ increases with b=0.77 (r^2=0.45; P=0.0002), implying an increasingly higher capacity for O_2 uptake by the lungs during the late metamorphic stages.

In hypoxic water (P_{O_2}=60 mmHg), body mass similarly explains most of the variability (87%) in the $\dot{M}_{O_2}$ of tadpoles and froglets (ANCOVA; F=37.3; P<0.0001). However, the influence of body mass on the $\dot{M}w_{O_2}$ is less pronounced (F=4.38; P=0.045), while the mass dependence of $\dot{M}a_{O_2}$ is greatly increased (F=17.7; P=0.0001), with 65% of the variability in $\dot{M}a_{O_2}$ attributable to body mass compared to 14% in normoxic water. According to the regression analysis, the slope for $\dot{M}_{O_2}$ in hypoxic water declined to b=0.83 over the growth stages (r^2=0.69; P<0.0001), implying a variable effect of hypoxia on O_2 uptake as a function of larval size, larger larvae being more affected than smaller ones (Table 2; Fig. 3). Accordingly, the depression in aquatic O_2 uptake is notably large in the larger tadpoles, causing the slope for $\dot{M}w_{O_2}$ with body mass to approach zero (b=0.11), and associated with an increase in $\dot{M}a_{O_2}$ out of proportion to body mass, with b=1.44 (r^2=0.57; P=0.0023). During the degrowth stages, there is a change in the slope for the relationship between $\dot{M}w_{O_2}$ and $\dot{M}a_{O_2}$ with body mass to b=0.75 (r^2=0.127; P=0.087) and 0.99 (r^2=0.61; P<0.0001), respectively, suggesting that the effects of aquatic hypoxia are more severe in the large tadpoles than in froglets.

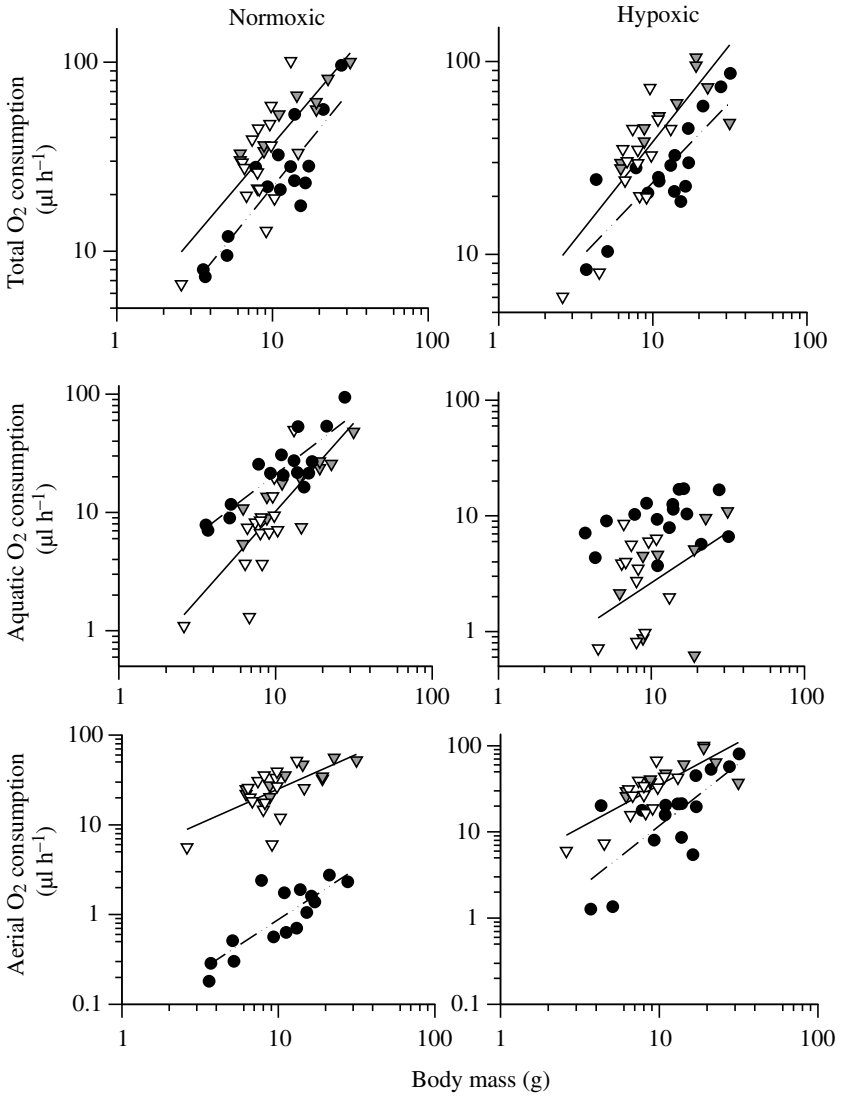

Fig. 3. Scaling relationship between whole-body O_2 consumption rates and body mass in *Pseudis paradoxus* tadpoles and froglets exposed to normoxic (146 mmHg; mean of initial values) and hypoxic water (60 mmHg), at 25°C. Each point represents a single animal, staged according to Cais after Gosner (Cais, 1982; Gosner, 1960) and measured individually. Filled circles represent larval stages 33–42, grey triangles larval stages 43–45, and empty triangles froglets (stage 46). The data were log-transformed and used to calculate two separate linear regressions that correspond to growth (broken line) and degrowth phases (solid line) of the larval period; the line has been omitted where the correlation is not significant. Detailed descriptions of determination coefficients and statistics are given in the text.

Glycogen and lactate contents

In normoxia (P_{O_2}=149 mmHg), glycogen and lactate concentrations per unit mass change remarkably with developmental stage (Fig. 4). Glycogen represents 2.33± 0.51 glycosyl units g^{-1} body mass (mean ± s.e.m.) in stage 33–37 larvae, increasing sixfold in stages 38–42 (15.32±4.41), and almost tenfold in stages 43–45 (22.32±7.53), a sharp decrease following in the newly metamorphosed froglets (4.48±0.75). Lactate measures 2.28± 0.26 μmol lactate g^{-1} body mass (mean ± s.e.m.) in stage 33–37 larvae and is similar during stages 38–42, then increases sharply twofold (4.48±1.7) and threefold (7.63±0.64) in the late metamorphic stages, remaining similarly elevated in the young froglets (Fig. 4).

Developmental change accounts for 35% of the variability in glycogen at the whole-body level (F=3.56; P<0.019), while 55% of the variability derives from the effect of size (ANCOVA; F=5.58; P<0.026). In Table 3, the mass exponents for the relationship between glycogen and body mass suggest changes out of proportion both in the larval growth stages, during which glycogen content increases with body mass according to b=2.05 (r^2=0.53; P=0.002), and in the stages of size reduction, during which glycogen content decreases sharply with body mass decrease, according to b=2.04 (r^2=0.84; P=0.0001). Lactate content also increases out of proportion to the increase in body mass in the larval growth stages according to b=1.47 (r^2=0.77; P=0.000), then decreases in strict proportion to body mass decrease during late metamorphosis, with b=1.03 (r^2=0.73; P=0.0001).

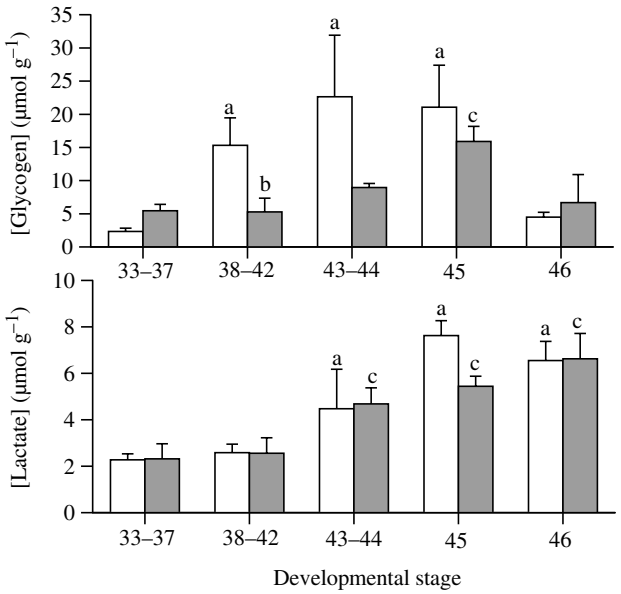

Fig. 4. Body lactate and glycogen concentrations in *Pseudis paradoxus* tadpoles and froglets after 3 h exposure to normoxic (open columns; mean P_{O_2}=149 mmHg) and hypoxic water (filled columns; 60 mmHg) with free access to air at 25°C. Values represent the mean ± s.e.m. from $N \geqslant 6$ animals, staged according to Cais after Gosner (Cais, 1982; Gosner, 1960) and measured individually. The data are pooled into groups of stages representing distinct developmental steps of the larval period. 'a', significantly different (P 0.05) compared to stages 33–37 in the normoxia series; 'b', compared to values for normoxia in the same group of stages; and 'c', from stages 33–37 in the hypoxia series.

Table 3. *The mass exponent b for the scaling relationship of whole-body glycogen and lactate concentrations in Pseudis paradoxus tadpoles and froglets exposed to normoxic and hypoxic water*

Metabolite concentration	Water P_{O_2} (mmHg)	*b*	
		Stage 33–42	Stage 43–46
Glycogen	146	2.05±0.51	2.04±0.23
	60	NS	1.55±0.38
Lactate	146	1.47±0.18	1.03±0.16
	60	2.04±0.53	0.79±0.15

Water P_{O_2} values are mean initial O_2 tensions.
Values for *b* are means ± s.e.m.
Developmental stages 33–42, larval growth; stages 43–46, degrowth until complete metamorphosis. NS, relationship not significant.

The 3 h exposure to aquatic hypoxia (P_{O_2}=60mmHg) with access to normoxic air showed an interaction effect with developmental stage on glycogen concentration per unit mass (ANOVA; F=2.99; P=0.028); however, a large, interindividual variability is associated with this response (Fig. 4). Multiple comparisons among mean values revealed a significant decrease of 66% during larval stages 38–42 compared to values in normoxic water, a similar tendency being seen in stages 43–44. Hypoxia exposure may differentially affect the whole-body glycogen content of growing larvae according to body size, the slope for the regression being less steep than in normoxic conditions, suggesting that large tadpoles are more severely affected than smaller ones. However, the coefficient r^2=0.147 does not predict functional dependence between the variables, and the small sample size and mass range in this group prevent a solid conclusion. The regression for the degrowth phase is likewise less steep after hypoxia exposure (r^2=0.52; P=0.002), and in this case, the calculated equation predicts a 50% reduction in body glycogen in 20 g larvae, and no change in 5 g animals, typically froglets.

Hypoxia exposure caused no significant change in lactate content per unit mass compared to normoxic values (Fig. 4). However, the large change in the mass exponent for the whole-body content, to b=2.04 (r^2=0.46; P=0.021), suggests that larger tadpoles accumulate surplus lactate in hypoxic water (Table 3). According to the calculated equations, body lactate content would increase by 84% in 20 g larvae relative to normoxic levels. In the degrowth stages, hypoxia exposure caused a decline in slope to b=0.79 (r^2=0.64; P=0.0001), and the equations indicate that under these conditions 20 g larvae contain 36% less lactate compared to normoxic levels, while the contents would be similar in 5 g froglets.

Discussion

The present study shows that multiple adjustments of metabolism during the early development of *P. paradoxus* correlate with body mass changes resulting from the growth and degrowth processes. The scaling patterns reveal a general tendency for O_2 uptake to increase in direct or more than direct proportion to mass, and may express the consequences of size on energy demand at the tissue level. One consequence of these patterns may be that air breathing must provide potential for any further expansion of energy expenditure in the largest individuals, and for the large increase in metabolism during late metamorphosis. This may be particularly meaningful in the context of fluctuating O_2 availability in the aquatic environment, as suggested by remarkable changes in the scaling patterns, and by evidence of a size-dependent increase in lactate content, related to hypoxia exposure. These findings are examined below, and the means by which energy derived from aerobic and anaerobic sources may be reconciled with growth and developmental changes in *P. paradoxus* tadpoles, ensuring the large size at transformation defined by natural selection, is discussed.

Metabolic rate and the costs of growth and metamorphosis

The mass-specific O_2 consumption rates of larval *P. paradoxus* are fairly constant and low in the early larvae until the emergence of the forelimbs, values lying within the ranges reported for other frogs, despite large inter-specific differences

in the degree of size variation during the larval period and in the adult mode of life (Gatten Jr et al., 1992). This phase constitutes more than three quarters of the larval period in *P. paradoxus*, lasting for 120 days under laboratory conditions (Cais, 1982) and possibly less in the natural environment, during which the larvae undergo minor developmental changes and body size increases; in the present study, the minimum and maximum weights at capture were 0.1 g (stage 26) and 72 g (stage 40), respectively. By contrast, *Rana catesbeiana* tadpoles can grow at very slow rates to a large size by increasing the developmental time during one or two overwintering cycles, as seen under temperature conditions that lead to interruption of metamorphosis (Emerson, 1988). Overall, then, the great size quickly attained by *P. paradoxus* tadpoles may result from higher rates of whole body protein accretion.

The tail represents 78% of body length in the largest tadpoles of *P. paradoxus* (stages 38–42), and its wet muscle mass increases exponentially (1.19) with body mass in the absence of significant changes in dry:wet tissue mass ratio (C.M.K. and S.C.R.d.S., unpublished data). Using available data on developmental time (Cais, 1982) and a tail muscle protein content of 10 mg% obtained from our samples, a rough calculation gives 1.4 mg day^{-1} for the rate of tail muscle protein synthesis in tadpoles growing at 1% per day. The fraction of $\dot{M}_{O_2}$ expended on this process would be between 19 and 42%, employing a minimum cost of peptide bond formation of between 7 and 16 mmol O_2 g^{-1} of protein (Houlihan, 1991). While representing a smaller fraction of the body mass, other sites of protein synthesis to be considered in the growing larvae are the liver and the gastrointestinal tract, as protein turnover by these tissues reaches very high rates compared to white muscle (Houlihan, 1991). Further, tadpoles are typically herbivores, exhibiting elevated rates of intestinal nutrient transport to extract the amino acids required for growth from large quantities of plant matter (Toloza and Diamond, 1990). The costs of growth would then include the energy expended on eating, digesting and absorbing food in excess of that needed for maintenance, which amounts to 60–80% of metabolic rate in young toads, *Bufo bufo* (Jørgensen, 1988) and may represent a large fraction of the routine rates of metabolism in *P. paradoxus* tadpoles.

Oxygen consumption increases sharply in *P. paradoxus* tadpoles on emergence of the forelimbs concomitant with the abolishment of gill respiration and food intake, and may remain elevated until completion of metamorphosis over a period of approximately 10 days (Cais, 1982). Compositional changes during the advanced stages of metamorphosis, such as those in water content and in the relative size of internal organs, may partially explain this increase in metabolic rate. The dry:wet muscle tissue ratio increases slightly, both in the degenerating tail and in the growing leg of *P. paradoxus* tadpoles. In addition, there is a 19-fold increase in liver tissue mass from the early to the latest larval stages (C.M.K. and S.C.R.d.S., unpublished data), associated to an increase in the liver somatic tissue index from 0.5 to 2% of body mass, implying that the biosynthetic capacity of this organ relative to body mass would increase in late metamorphosis. The heart size decreases with decreasing body size according to b=0.72, the net effect being a 1.7-fold increase in relative organ mass in later stage larvae (C.M.K. and S.C.R.d.S., unpublished data), and together these changes may increase O_2 consumption by an increase in the relative mass of metabolically active tissue. Accordingly, studies focusing on phenotypic plasticity and physiological adaptation have shown that the relative size of the heart, liver and kidney may explain much of the inter-individual and inter-specific variability seen in O_2 demand at the whole-body level (Selman et al., 2001; Williams and Tieleman, 2000).

Despite the extensive tissue reorganization, however, O_2 consumption decreases on completion of metamorphosis in *P. paradoxus*, and the mechanisms promoting the sharp increase in energy expenditure in the tadpoles, prior to metamorphosis, remain unknown. The late metamorphic events in anurans are marked by tail reabsorption, when more than half of the body weight is lost; the energy requirements of caudal proteolysis may be substantial in *P. paradoxus* tadpoles, given the large relative mass of muscle tissue that is degraded in a matter of a few days. Increases in the rates of anabolic processes underlying the extensive tissue reorganization, such as in the gastrointestinal tract, may also contribute significantly to the increase in metabolic rates at the whole body level (Hourdry et al., 1996). Besides, the amino acids resulting from tail resorption provide the animal with a continuous source of carbohydrate *via* the gluconeogenic pathway; recently we have found increased enzyme activity related to gluconeogenesis in the liver of tadpoles during late metamorphosis (F. M. Oshiro, S. C. R. de Souza, J. E. P. W. Bicudo and M. S. C. Bianconcini, unpublished observations). In mammals, nearly one-quarter of body O_2 use takes place in the liver and gastrointestinal tract under standard conditions (Rolfe and Brown, 1997); assuming a similar proportion in the organs of *P. paradoxus* tadpoles, a fourfold increase in the rates of liver and gastrointestinal tissue respiration would be necessary to fully account for the twofold increase in O_2 consumption rates at the whole-body level during late metamorphosis.

Developmental changes in O_2 consumption of frogs do not share a common pattern, and the available data are far from exhaustive (Burggren and Just, 1992). A study with *Xenopus laevis* illustrates the contrasts with *P. paradoxus* tadpoles, showing a peak in $\dot{M}_{O_2}$ per unit mass on hatching, and another in newly metamorphosed frogs, while in the more advanced larval stages, rates decline slightly due to a presumed decrease in energy expenditure linked with cessation of gill respiration (Hastings and Burggren, 1995). The significance of the distinct metabolic patterns seen during the metamorphic transition of anurans is difficult to interpret based on the information available, and species-specific characteristics may also be involved, together with plasticity of morphological and physiological characters in response to the environment.

Partitioning of O_2 uptake and the effects of the larval size

In normoxic water, the early larvae of *P. paradoxus* rely almost exclusively on aquatic O_2 exchange to maintain routine metabolism, as do other anuran larvae studied (Feder, 1984). On emergence of the forelimbs, the contribution of aerial breathing increases sharply together with an equivalent decrease in aquatic O_2 uptake. Under aquatic hypoxia, gill ventilation is apparently inhibited and lung breathing stimulated at P_{O_2} values around 90 mmHg and lower, $\dot{M}_{O_2}$ per unit mass in the developmental groups remaining nearly constant over the P_{O_2} range analysed. Previous data from *Rana catesbeiana* reveal a remarkable capability of the larvae to counteract the effects of aquatic hypoxia by increasing gill ventilation during forced submersion in water of decreasing P_{O_2}, with effective O_2 regulation occurring down to a critical P_{O_2} of around 30 mmHg (Crowder et al., 1998). This pattern may differ among ranid frogs, as suggested by the study with *R. berlandieri*, showing a marked decrease in total O_2 uptake during submersion in low P_{O_2} water and insufficient capacity to offset the effects of aquatic hypoxia by air breathing (Feder, 1983). Despite the influences of phylogeny *per se*, and the more obvious differences in methodological approach, there is evidence of dissimilarities in the ability of anuran larvae to compensate for environmental O_2 fluctuations by gill ventilation. In *P. paradoxus* tadpoles, adjustments in gill and lung ventilation are apparent in water P_{O_2} levels at which gas exchange across the integument is presumably effective, and may indicate early reliance on aerial O_2 uptake associated with the large larval size on transformation. Hypoxia exposure affects growth rates in many ways, as shown by reductions in feeding rate, respiration rate, faecal production and protein synthesis (Zhou et al., 2001). In the habitat where *P. paradoxus* tadpoles are found, O_2 supply is neither homogeneous nor stable, circumstances in which air breathing may be favoured.

Body mass in *P. paradoxus* tadpoles covaries with the effects of developmental stage on whole-body O_2 uptake, and accounts for some 90% of the variability under normoxic conditions, similar to observations on other anuran larvae (Feder, 1982). However, the scaling patterns seen in *P. paradoxus* are distinct, and reveal a general tendency for O_2 uptake to increase in direct or more than direct proportion to mass, as in the early ontogenetic stages of mammal and fish species (Hulbert and Else, 2000; Wieser, 1995). The gas exchange organs of mammals and fishes presumably increase their efficacy with growth and development, while in metamorphosing amphibians, growth coincides at some point with gill degeneration. Despite this, and the inherent limitation of the skin for gas exchange, aquatic O_2 uptake increases in direct proportion to the increase in mass in *P. paradoxus* tadpoles, and the relationship nearly overlaps the corresponding curve for total O_2 uptake under resting conditions. Since this pattern does not prevent the developing animal from increasing metabolism with activity changes and metabolic state, some reserve capacity may be available at the O_2 transfer step. One consequence of this pattern, however, may be that air breathing must subsidise any further increase in energy expenditure in the largest individuals, as the scaling for aerial O_2 uptake in normoxia appears to indicate (b=1.13). Under hypoxia, the depression in cutaneous O_2 uptake is notably large in the larger tadpoles, causing the slope of the aquatic O_2 uptake in the growth stages to change from 1.02 to almost zero (b=0.11), and they may depend disproportionately on air breathing to maintain O_2 consumption rates, as the slope of the aerial uptake indicates (b=1.44).

During the degrowth stages of late metamorphosis, the scaling relationships for aquatic O_2 uptake imply that the mass-specific rates of aquatic O_2 uptake are twice as great in the latest larval stages than in froglets (b=1.18). *P. paradoxus* larvae not only have unusually shaped tails, with the keel extending further onto the head compared to tadpoles of *R. catesbeiana* of similar size, but tail reabsorption takes longer than in other frogs (Emerson, 1988). In the present study, tail length corresponds to 63% of total body length in stages 43–45, the oldest larvae resembling adult frogs with a larval tail hanging in the water column. Since its size and surface area actually increase relative to body mass during these stages of body mass reduction, the tail appears to function as an important site of O_2 transfer until completion of metamorphosis. In water of low O_2 content, however, cutaneous O_2 uptake becomes limiting and late-stage larvae may increase lung ventilation more than newly metamorphosed frogs to sustain their much higher metabolic rate, as suggested by the change in the mass exponent for aerial O_2 uptake (b=0.77) in normoxia to close to unity under hypoxia.

Other functions have been attributed to surfacing behaviour in developing tadpoles, such as prevention of lung collapse during organ development in the early larval stages (Burggren and West, 1982; Bruce et al., 1994; Crowder et al., 1998). The emergence of central chemoreceptive reflexes regulating O_2 and CO_2 by lung ventilation precedes the disappearance of central gill respiratory regulation in *R. catesbeiana*, and as development advances, the central lung pattern generator gradually becomes more functional, dominating in the late metamorphic stages (Torgenson et al., 1997). In *P. paradoxus*, there is a disproportionate increase in the aerial O_2 uptake to size increase during larval growth stages, and a sharp increase in air breathing rates on forelimb emergence, reaching 20-fold in 30 g larvae, according to the calculated equation. Some plasticity in the ontogeny of respiratory control may have developed in *P. paradoxus* tadpoles, associated with their large size at metamorphosis. Other physiological and biochemical adjustments may also contribute to providing adequate O_2 transfer and delivery to the body tissues. Burggren et al. (Burggren et al., 1992) described changes in blood pressure variables in *P. paradoxus* of dramatic speed and magnitude, causing a threefold increase in mean, systemic, arterial blood pressure in late-stage larvae compared to younger larvae. This may be explained in part by the developmental changes in the relative heart mass, described above, and thus scaling effects on adjustments by the cardiovascular system may promote increased blood perfusion and contribute to an increase in the

O_2 uptake and delivery steps to the body tissues in the later stages of *P. paradoxus* metamorphosis.

Metabolic implications of changes in glycogen and lactate levels

Body glycogen content rises in *P. paradoxus* larvae from stages 38 to 42, and remains high over the period of late metamorphosis, dropping in newly metamorphosed froglets. Lactate content increases sharply after emergence of the forelimbs and remains high after metamorphosis. Together, these changes express increased anaerobic potential and power generation for burst activity in the late larval stages. Given that food intake is interrupted during late metamorphosis in *P. paradoxus*, the high, sustained glycogen content suggests that an endogenous substrate is required for carbohydrate synthesis during the fasting period, possibly amino acids resulting from protein catabolism in the tail, in agreement with our recent findings of increased enzyme activity related to gluconeogenesis in the larvae of late stages (F. M. Oshiro, S. C. R. de Souza., J. E. P. W. Bicudo and M. S. C. Bianconcini, unpublished observations).

The lack of significant changes in lactate concentration per unit mass after 3 h of aquatic hypoxia is not unexpected, given the ability of *P. paradoxus* tadpoles to maintain aerobic, resting metabolic rates by increasing lung ventilation. Lactate does not build up in other anuran larvae, except after prolonged exposure to severe hypoxia (P_{O_2}<10 mmHg) (Crowder et al., 1998), or on exertion, at rates dependent on the intensity and duration of exercise (Gatten, Jr, 1985; Quinn and Burggren, 1983). In this context, however, the significant decrease in body glycogen in *P. paradoxus* larvae in stages 37–42 exposed to hypoxic water appears paradoxical. One explanation is that carbohydrate stores may be mobilised and used to fuel oxidative processes, due to the higher energy yield per O_2 molecule from glucose (Hochachka and Somero, 2002); this mechanism is apparently essential to the development of hypoxia tolerance in animals (Hochachka et al., 1996; West and Boutilier, 1998). An alternative interpretation comes from the suggestion that whole-body glycogen and lactate in larval *P. paradoxus* are markedly affected by body size. In the growing larvae, the calculated equations predict a large increase in lactate and glycogen levels of 2.8 and 4.1-fold, respectively, for a twofold increase in body size, similar to the pattern observed in teleost fish (Somero and Childress, 1980). After hypoxia exposure, there is a tendency for body glycogen to decrease more in the larger larvae, and a clear suggestion of a change in the mass exponent for lactate, implying that larger tadpoles produced surplus lactate after exposure to hypoxic water, calculated to be 1.8-fold of lactate in 20 g larvae compared to normoxic conditions. In the stages of larval growth, the tadpoles ascend periodically in the water column to ventilate their lungs, and apparently increase the frequency of ascending during hypoxic exposure. This behaviour tends to disappear during late metamorphosis, and the greater reliance on air breathing of the largest tadpoles might explain the differential production of lactate as a function of size in *P. paradoxus*.

In conclusion, multiple adjustments at the O_2 transfer step during the early development of *P. paradoxus* correlate with body mass changes resulting from the growth processes, similar to observations of metabolic scaling on fishes and mammals. Developmental programs operating through changes in organs and body parts may ultimately effect adjustments in the metabolic capacities of tissues and related systems, so that O_2 transfer and energy expenditure may be reconciled with the large larval size and metamorphosis.

This study was financed by a research grant from the Foundation for Research Support of São Paulo State (FAPESP) to S.C.R.d.S. C.M.K. received a scholarship from FAPESP. The authors are deeply grateful to Wagner Bianchini for access to his fish farm and for valuable contributions during field work. Constructive criticism of two anonymous referees greatly improved the clarity of the article.

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