Gas Conductance of the Jelly Capsule of Terrestrial Frog Eggs Correlates with Embryonic Stage, Not Metabolic Demand or Ambient Po_2

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Abstract

Previous work indicates that the O_2 conductance of the jelly capsule (Go_2) and O_2 consumption ($\dot{V}o_2$) of Pseudophryne bibronii *eggs increase in parallel during development such that perivitelline Po_2 remains high and constant throughout incubation at 12° C (Seymour and Bradford 1987). To determine whether the pattern of capsular change is adaptively regulated, the eggs were incubated at selected T_a (7°, 12°, 17°, and 22° C), ambient Po_2 (10, 15, 21, 30, and 40 kPa), and substrate water potential (0 and −25 kPa), and we measured $\dot{V}o_2$, Go_2, incubation time, and differentiation rate. The Go_2 is not directly affected by T_a, ambient Po_2, or water potential, but it depends strongly on developmental stage. The embryo is apparently programmed to secrete specific substances that modify capsule morphology at specific stages of development, and it does not respond to environmental conditions by altering the time course of secretion. Consequently, Po_2 in the perivitelline space is not regulated but declines at higher temperatures. At 17° C and 22° C, $\dot{V}o_2$ becomes limited by capsule Go_2 in late stages of development. However, winter breeding at field temperatures averaging about 12° C reduces the possibility of O_2 limitation and retardation of embryonic development.*

Introduction

Amphibian eggs are surrounded by a vitelline membrane and a layered mucopolysaccharide capsule (Salthe 1963; Beattie 1980). This gelatinous

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membrane-capsule complex is a significant barrier to the diffusion of gases between the living embryo and its environment. In single, round eggs, O_2 transfer can be satisfactorily modeled by the Fick equation that involves the rate of oxygen uptake ($\dot{V}O_2$), the partial pressures of oxygen outside the capsule ($P_{O_2\,(out)}$) and within the perivitelline space ($P_{O_2\,(in)}$), and the oxygen conductance of the jelly capsule (G_{O_2}) (Seymour and Bradford 1987):

$$\dot{V}O_2 = G_{O_2}\,(P_{O_2\,(out)} - P_{O_2\,(in)}). \tag{1}$$

The G_{O_2} is related to the morphology of the jelly capsule and Krogh's O_2 diffusion coefficient of the jelly (K_{O_2}). The G_{O_2} is proportional to the ratio of effective surface area (ESA) of the capsule and its thickness (L):

$$G_{O_2} = \frac{\text{ESA}}{L} \times K_{O_2}. \tag{2}$$

If G_{O_2} were constant during development, $P_{O_2\,(in)}$ would decrease in proportion to the rising $\dot{V}O_2$. However, the capsules of some frog eggs are known to change because of the absorption of water into the perivitelline space during development (Salthe 1965; Taigen, Pough, and Stewart 1984; Seymour and Bradford 1987), and G_{O_2} may not be constant. For example, G_{O_2} of the capsule in the terrestrial eggs of the frog, *Pseudophryne bibronii,* increases in proportion to $\dot{V}O_2$ such that $P_{O_2\,(in)}$ is practically constant throughout incubation at 12°C and remains well above the critical level at which $\dot{V}O_2$ becomes diffusion limited (Seymour and Bradford 1987). Furthermore, eggs incubated on substrates of different water potential have capsules that are markedly different in size but very similar in G_{O_2} (Bradford and Seymour 1988*a*).

The developmental changes in capsule morphology and the independence of G_{O_2} from environmental water potential suggest adaptations of *P. bibronii* to maintain adequate gas exchange throughout development. However, the extents to which these changes are actively controlled by the embryo or are passive responses of the capsule itself have not been ascertained. Would eggs incubated at higher temperatures increase G_{O_2} to compensate for higher embryonic $\dot{V}O_2$? Would eggs incubated at lower $P_{O_2\,(out)}$ increase G_{O_2} to keep $P_{O_2\,(in)}$ high? To investigate these questions further, we experimentally altered incubation temperature and $P_{O_2\,(out)}$ and measured $\dot{V}O_2$ and G_{O_2}. We also altered water potential to better represent field conditions.

Material and Methods

Source and Handling of Eggs

Clutches of fresh *Pseudophryne bibronii* eggs were collected in the Mount Lofty Ranges, near Adelaide, South Australia, in May. They were returned to the laboratory and cleaned of debris by gently rolling them in a sieve under water. The eggs were at Gosner (1960) stages 8–12 when collected, corresponding to approximately 1.5–5.5 d old at 12°C (Bradford and Seymour 1985). The eggs from each clutch were kept in separate plastic containers with about 20 pieces of Whatman no. 1 filter paper for which the characteristic curve relating water content and water potential had been determined (Seymour and Piiper 1988). Each container was closed with a cover pierced by a few pinholes, and the eggs were incubated in darkened constant temperature cabinets under eight conditions: either 7°, 12°, 17°, or 22°C, and on water potentials of either 0 kPa (resting on filter papers saturated with excess distilled water) or −25 kPa. These water potentials were chosen because they produce marked differences in capsule morphology and are well tolerated in the field and laboratory (Seymour and Bradford 1987; Bradford and Seymour 1988*a*). Containers at −25 kPa were weighed periodically and evaporative losses replaced. Eggs attacked by fungi or failing to develop normally were discarded.

Staging

Whenever measurements were taken, embryos were staged according to Gosner (1960), except for stages 21 (optic vesicle appears) and 23 (iris pigmentation completely around pupil), which come from Woodruff (1972). Stages 19, 20, 24, and 25 were difficult to detect in *P. bibronii* because of developmental peculiarities, so they were temporally interpolated as intermediates between other stages.

Measurement of Capsule Morphology and Conductance

The effects of temperature and water potential on capsule morphology were measured in eggs from three clutches and the results were combined. Capsule morphology was determined according to Seymour and Bradford (1987). In brief, individual photographs of eggs were made in air and water, and measurements of capsule radius and thickness were taken from projected negative images, averaging four values for each egg and calibrating the measurements against photographs of a ruled scale (0.01-mm divisions). About eight eggs were randomly chosen from all clutches in each treatment at

selected times during development, measured, and returned to their containers.

Oxygen conductance was calculated from equation (2). Effective surface area was calculated as $4\pi r_o r_i$, the surface area of a sphere the radius of which is the geometric mean of the outer (r_o) and inner (r_i) radii of the capsule wall. The thickness of the jelly was taken as the mean radial difference, $r_o - r_i$. Krogh's coefficient was derived from Burggren's (1985) data of K_{O_2} in *Rana palustris* egg jelly and corrected for temperature (and solubility) according to Seymour and Bradford (1987). The values of K_{O_2} used at 7°, 12°, 17°, and 22°C were, respectively, 2.46, 2.51, 2.65, and 2.78 × 10^{-7} cm^2 min^{-1} kPa^{-1}.

For the effect of $P_{O_2\,(out)}$ on capsule morphology, one clutch was randomly divided into eight groups of six eggs each, and incubated from stage 8 at constant temperatures of either 12° or 17°C and at constant P_{O_2} values of either 10, 15, 21, or 40 kPa (±1 kPa). All groups were incubated on filter paper at 0 kPa water potential. The containers were placed in eight glass desiccators that were periodically flushed with humidified gas mixtures according to procedures described earlier (Bradford and Seymour 1988*b*). On days 5, 13, and 36 for 12°C eggs and days 5 and 13 for 17°C eggs, the eggs were removed from the desiccators, staged, and photographed.

Measurement of O_2 Consumption

The effect of $P_{O_2\,(out)}$ on respiration was measured on stage-28 eggs from three clutches that had been incubated at atmospheric P_{O_2} on filter paper of 0 kPa water potential. A Gilson model IG-14 bath was used with four entirely glass, submerged, single-valve respirometers. Each 15-mL chamber contained 3–7 eggs, randomly selected from each clutch, and a dish of 1% KOH CO_2 absorbant. Both arms of the respirometers were flushed with humidified gas mixtures as previously described (Bradford and Seymour 1988*a*), and they were closed. After equilibration for at least 1 h, $\dot{V}_{O_2}$ was measured for a further 3–6 h. The average $\dot{V}_{O_2}$ per egg (μL STPD/h) was calculated for each chamber, and this value was used as an independent datum.

Statistics

For measurements of capsule morphology, the mean value from each egg was taken as the experimental unit. For respirometry, the unit was the average of all eggs in each manometer. A one-way ANOVA was performed with the SPSSPc package, and ANCOVA was carried out according to Zar (1984). It

was impossible to avoid pseudoreplication because eggs were collected at only one site, carried to the laboratory in only one vehicle, and incubated in one constant temperature cabinet per experimental temperature and one desiccator per experimental P_{O_2}. In most cases, the results are obvious and statistical procedures unnecessary, but in cases where statistical significance is indicated, the reader may assume that the results apply to the populations of eggs used in each laboratory experiment, not necessarily to field populations.

Dual linear regression analysis (Noland and Ultsch 1981) was applied to the O_2 consumption data to estimate the critical $P_{O_{2\,(out)}}$ where $\dot{V}_{O_2}$ becomes limited. This technique progressively fits two adjacent linear regressions to the entire data set and selects the pair of regressions that minimizes the total residual sum of squares. The critical $P_{O_{2\,(out)}}$ is taken as the intersection of the regression lines.

Results

Effects of Temperature and Water Potential on Capsule Conductance

Calculated O_2 conductance of the jelly capsule increased during incubation at all temperatures and water potentials (fig. 1). The increase occurred more quickly at higher temperatures, and, in all cases, the rate of increase declined as the embryos aged.

The increases in G_{O_2} arose from increases in ESA of the capsule (fig. 2) and from decreases in L (fig. 3). Although ESA and L were strongly affected by water potential, their effects counteracted each other in the calculation of G_{O_2}, which turned out to be largely independent of water potential (fig. 1). The changes in ESA and L were brought about simply by absorption of water into the perivitelline space. The volume of the jelly remained constant at all temperatures, which is consistent with earlier findings at 12°C (Seymour and Bradford 1987).

Although temperature affected the rate of capsular change, maximum G_{O_2} at the end of incubation was independent of temperature (fig. 4). The ANCOVAs of mean capsule G_{O_2} versus stage show no significant differences between the four temperatures or two water potentials, either in slope (F = 0.25, df = 3, 22, for 0 kPa; F = 2.38, df = 3, 17, for −25 kPa; F = 1.71, df = 7, 39, for both) or elevation (F = 3.35, df = 3, 25, for 0 kPa; F = 0.65, df = 3, 20, for −25 kPa; F = 1.75, df = 7, 46, for both). However, G_{O_2} was strongly correlated with embryonic stage throughout development.

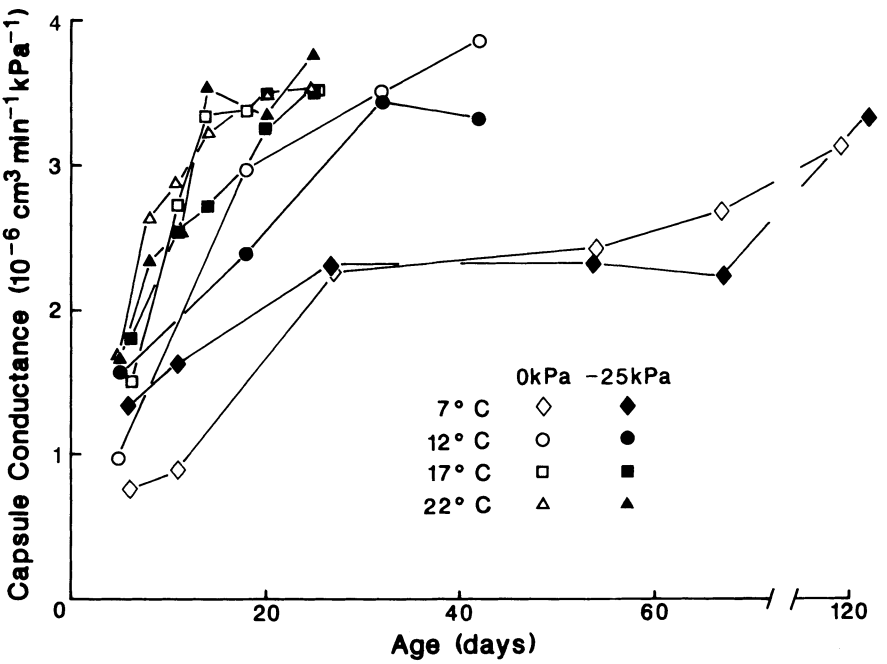

Fig. 1. Mean oxygen conductance (G_{O_2}) of the jelly capsule during development of terrestrial Pseudophryne bibronii *eggs incubated at four constant temperatures and either 0 kPa or −25 kPa water potential. Symbols are means of 3–13 eggs.*

Effect of $P_{O_2\,(out)}$ on Oxygen Consumption

The rate of O_2 uptake by stage-28 embryos became limited at low $P_{O_2\,(out)}$ (fig. 5). At 7°, 12°, and 17°C, critical P_{O_2} values were 15, 19, and 27 kPa (113, 143, 203 Torr), respectively. At these temperatures, the regressions for the data above the critical P_{O_2} all had slopes not significantly different from zero ($-0.12 < r < 0.17$), indicating independence of $\dot{V}_{O_2}$ from $P_{O_2\,(out)}$ in this range. At 22°C, all but one flask of embryos hatched during measurements at $P_{O_2\,(out)}$ below 15 kPa (113 Torr), so the critical $P_{O_2\,(out)}$ is uncertain. However, the upper regression had a significantly positive slope ($r = 0.71$), indicating a relationship between $P_{O_2\,(out)}$ and $\dot{V}_{O_2}$ even at high levels of $P_{O_2\,(out)}$.

Effect of $P_{O_2\,(out)}$ on Capsule Conductance and Rate of Development

During development at both 12° and 17°C, L decreased, r_o increased, ESA increased, and G_{O_2} increased (table 1). The ANOVAs showed no significant effect of $P_{O_2\,(out)}$ on L under any conditions. The $P_{O_2\,(out)}$ also did not influ-

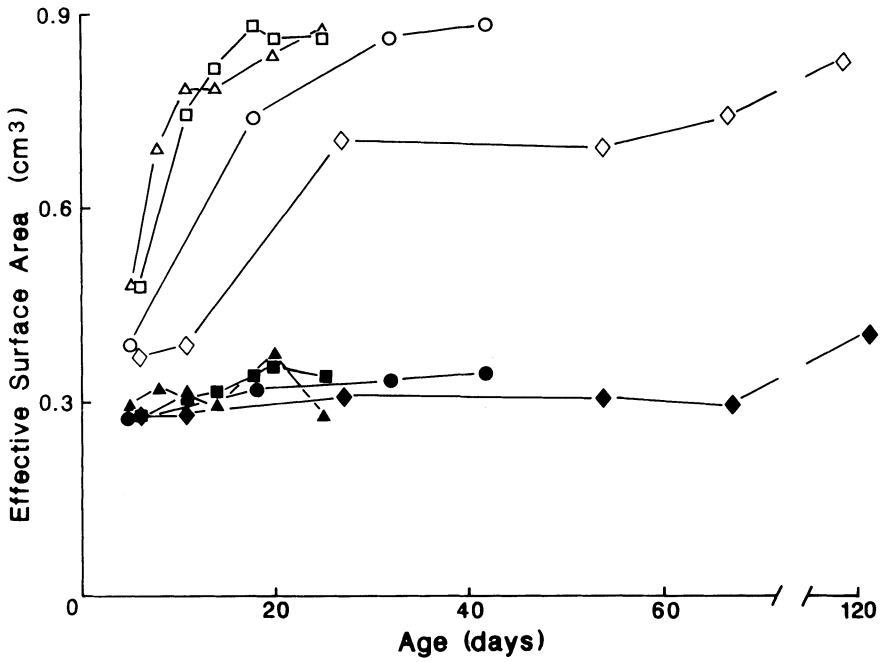

Fig. 2. Effective surface area of the jelly capsule of Pseudophryne bibronii *with age. Symbols as in fig. 1.*

ence G_{O_2} at any time in development at 12°C, although there were significant, but inconsistent, differences in ESA in some treatments (table 1). However, on day 13 in eggs incubated at 17°C, ESA and G_{O_2} were highly and consistently dependent on $P_{O_{2\,(out)}}$, with G_{O_2} increasing at higher P_{O_2} (table 1).

The $P_{O_{2\,(out)}}$ also affected the rate of development (table 2). At 12°C, development was slightly accelerated at P_{O_2} = 40 kPa (300 Torr), but not at lower levels. At 17°C, however, eggs at P_{O_2} = 10 kPa (75 Torr) were retarded and those at 40 kPa (298 Torr) were accelerated.

Discussion

Effects of Temperature, Ambient P_{O_2}, and Water Potential on Capsule Conductance

Increases in gas conductance of the jelly capsule that occur during development in *Pseudophryne bibronii* are caused entirely by absorption of water into the perivitelline space; there are no detectable changes in the volume of the capsule itself (Seymour and Bradford 1987; present study). According

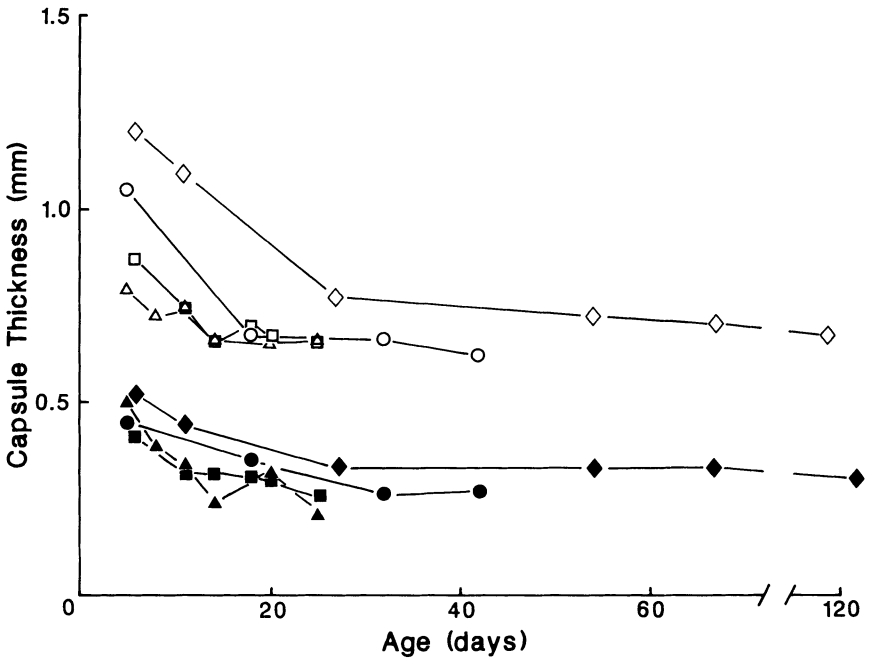

Fig. 3. Thickness of the jelly capsule of Pseudophryne bibronii *with age. Symbols as in fig. 1.*

to Salthe (1965), who investigated aquatic *Rana pipiens* eggs, the amount of water taken up depends on the interaction between (1) the osmotic gradient between the environment and the perivitelline fluid, which is influenced by high-molecular-weight secretions of the embryo, and (2) the hydrostatic pressure caused by elastic tension in the vitelline membrane–jelly capsule complex, which also may be influenced by embryonic secretion of certain enzymes. Capsule conductance, therefore, is ultimately affected by the embryo.

However, the embryo of *P. bibronii* appears incapable of regulating capsule conductance in response to environmental factors. Although conductance increases during development in parallel with rising metabolic rate and perivitelline P_{O_2} remains high and constant throughout development at atmospheric P_{O_2} (Seymour and Bradford 1987), conductance does not adaptively increase during incubation at higher temperatures or lower ambient P_{O_2}.

Conductance increases more quickly during incubation at higher temperatures, but the maximum value just before hatching is independent of temperature (figs. 1, 4). Therefore the high metabolic rates at higher temperatures progressively reduce perivitelline P_{O_2}. In fact, capsule conductance can become a limiting factor to embryonic respiration, at least during

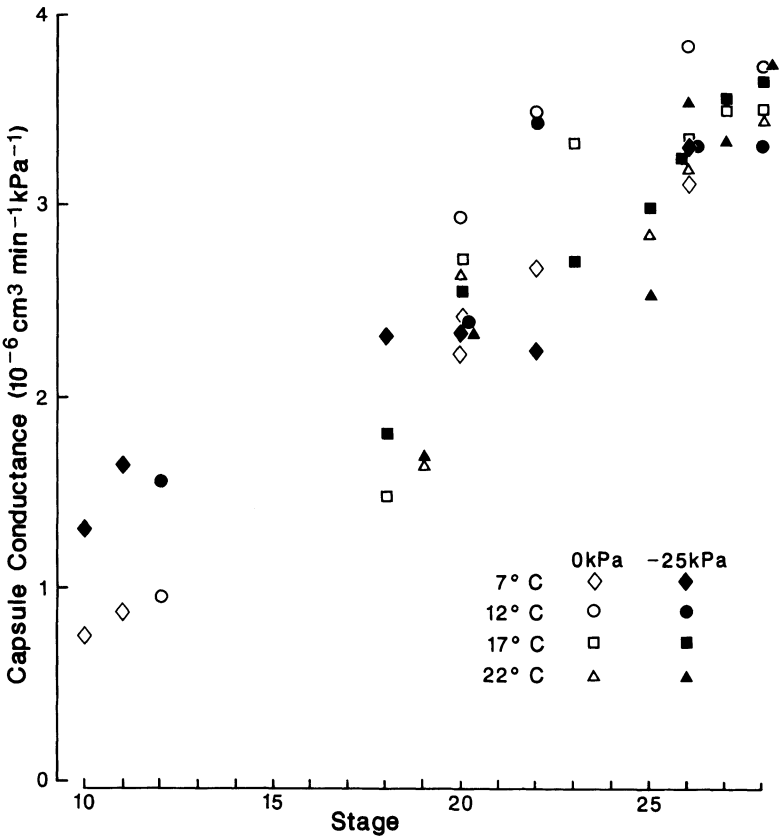

Fig. 4. Oxygen conductance (G_{O_2}) of the capsule of Pseudophryne bibronii *in relation to embryonic stage at four incubation temperatures and two water potentials. Symbols are means of 3–13 eggs.*

later stages of development at temperatures above about 12°C (fig. 5). We can calculate critical values of perivitelline $P_{O_2\,(in)}$ in prehatching (stages 27 and 28) embryos by substituting into equation (1) the mean values of $\dot{V}_{O_2}$ from Seymour, Geiser, and Bradford (1991) and G_{O_2} and critical $P_{O_2\,(out)}$ values from this study. Actual $P_{O_2\,(in)}$ is calculated with the assumption of saturated standard atmospheric $P_{O_2\,(out)}$. These estimates show actual $P_{O_2\,(in)}$ above the critical level at 7° and 12°C but considerably below it at 17°C (fig. 6). Presumably the trend continues at 22°C, but we have no definite value of critical $P_{O_2\,(out)}$ at that temperature. We conclude that O_2 uptake becomes diffusion limited in prehatching embryos at temperatures above about 12°C and that limitation appears at earlier stages of development at higher temperatures.

Although the capsule conductance of prehatching *P. bibronii* is independent of temperature, the situation may be different in other species. Beattie

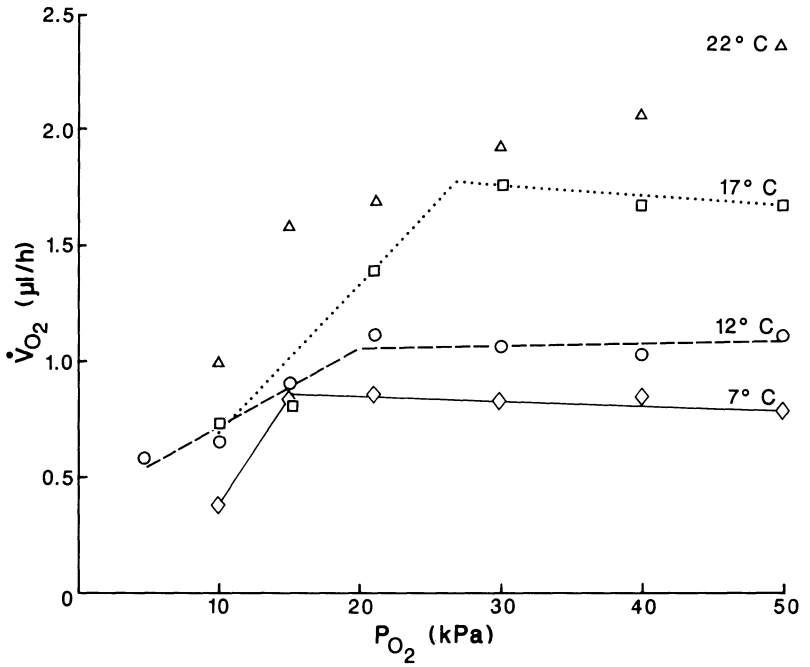

Fig. 5. Rate of oxygen consumption per egg ($\dot{V}_{O_2}$) in Pseudophryne bibronii *at stage 28 in relation to ambient P_{O_2} and temperature. Each point is a mean of 4–16 determinations. Dual linear regressions are fitted to the individual data (see text), and their intersections indicate critical P_{O_2} at temperatures below 22° C.*

(1980) found that the eggs of *Rana temporaria,* stripped from the female and incubated in water for 24 h, swelled more at higher temperatures. There was a linear relationship between egg volume and temperature. However, it must be noted that, in Beattie's experiments, the mechanism of swelling was obviously different. There was no significant change in perivitelline volume with egg swelling, which indicates that swelling was due to uptake of water into the jelly alone, decreasing conductance. Furthermore, there was no difference between fertile and infertile eggs, and there was no indication of embryonic stages at different temperatures. Without information on r_i and r_o of the capsule, we are unable to assess the respiratory effects of changes in conductance for this species. We have found no other published data on changes in capsule dimensions in other amphibians.

Our study also shows that conductance is not adaptively influenced by $P_{O_{2\,(out)}}$. If there were a tendency to regulate $P_{O_{2\,(in)}}$, conductance should increase at lower $P_{O_{2\,(out)}}$, but it either remains constant at 12°C or decreases at 17°C (table 1). It might be argued that these results are due to our selection

TABLE 1

Effects of ambient oxygen tension ($P_{O_{2(out)}}$) on capsule morphology in Pseudophryne bibronii *eggs*

Variable	Mean(SE)	*F*	$P_{O_{2(out)}}$ (kPa) 10	15	21	40
Day 5, 12°C, n = 14:						
L	1.00 (.04)	.63 NS				
ESA	.40 (.02)	4.30*	.47	.42	.34	.40
G_{O_2}	1.08 (.07)	2.39 NS				
Day 13, 12°C, n = 23:						
L	.78 (.02)	1.16 NS				
ESA	.53 (.02)	1.27 NS				
G_{O_2}	1.80 (.10)	.77 NS				
Day 36, 12°C, n = 18:						
L	.64 (.01)	2.81 NS				
ESA	.97 (.01)	6.04**	.93	.98	.96	1.00
G_{O_2}	3.98 (.09)	.43 NS				
Day 5, 17°C, n = 16:						
L	.95 (.04)	1.14 NS				
ESA	.39 (.01)	.26 NS				
G_{O_2}	1.12 (.07)	.42 NS				
Day 13, 17°C, n = 23:						
L	.64 (.02)	2.44 NS				
ESA	.83 (.04)	9.34***	.59	.75	.90	.98
G_{O_2}	3.45 (.17)	15.2***	2.16	3.34	3.69	4.15

Note. The variables are capsule thickness (*L*, mm), effective surface area (ESA, cm^2), and O_2 conductance (G_{O_2}, $10^{-6} \cdot cm^3 \cdot min^{-1} \cdot kPa^{-1}$). A one-way ANOVA yielded *F*-values under the specified conditions of age and temperature. When the *F*-value is not significant, only the mean of all groups is given; when significant, the differences in populations, according to the Student-Newman-Keuls test, are shown by breaks in the lines below the values. NS = not significant.

* $0.01 < P < 0.05$; Student-Newman-Keuls results are ambiguous.

** $0.001 < P < 0.01$; Student-Newman-Keuls results are ambiguous.

*** $P < 0.001$.

TABLE 2
Stage of development of Pseudophryne bibronii *incubated at 12° and 17° C and at four levels of* $P_{O_{2(out)}}$

Age (d)	Temperature (°C)	$P_{O_{2(out)}}$ (kPa)			
		10	15	21	40
5	12	10	10	11	11
13	12	18	18	18	18
36	12	22	22	22	23
5	17	16	16	16	16
13	17	21	23	23	24

Note. Stages are defined in the text.

of a range of experimental values of $P_{O_{2\,(out)}}$ that was too high to limit embryonic O_2 uptake appreciably. This may be the case at 12°C, at which the critical $P_{O_{2\,(out)}}$ is near the low end of the range. At 17°C, however, embryonic O_2 consumption is clearly limited, relatively early in development. Oxygen consumption of stage-28 embryos becomes limited at about 27 kPa (203 Torr) $P_{O_{2\,(out)}}$ (fig. 5). The embryos at 17°C were at stage 22 on day 13 when G_{O_2} was last measured (table 1). Allowing for a 75% lower O_2 uptake at stage 22 (Bradford and Seymour 1985), the critical $P_{O_{2\,(out)}}$ is about 21 kPa (158 Torr). Therefore a $P_{O_{2\,(out)}}$ of 15 kPa (113 Torr) should be markedly limiting, 10 kPa (75 Torr) even more so. But instead of an increase in conductance at these levels, it is reduced (table 1), and the development of the embryos is retarded (table 2).

The final capsular conductance is also unrelated to incubation water potential (fig. 1). This independence was also observed in *P. bibronii* between 0 and −50 kPa (Bradford and Seymour 1988*a*). It is remarkable that conductance is so similar in eggs that differ so much in ESA (fig. 2) and *L* (fig. 3). We would like to understand more fully the balance of forces that affect the volumes of the perivitelline space and the jelly.

Correlation between Conductance and Embryonic Stage

Of all the factors measured in our experiments, conductance is most highly correlated with embryonic stage (fig. 4). Differentiation is faster at higher temperatures (Seymour et al. 1991) and so are the changes in conductance

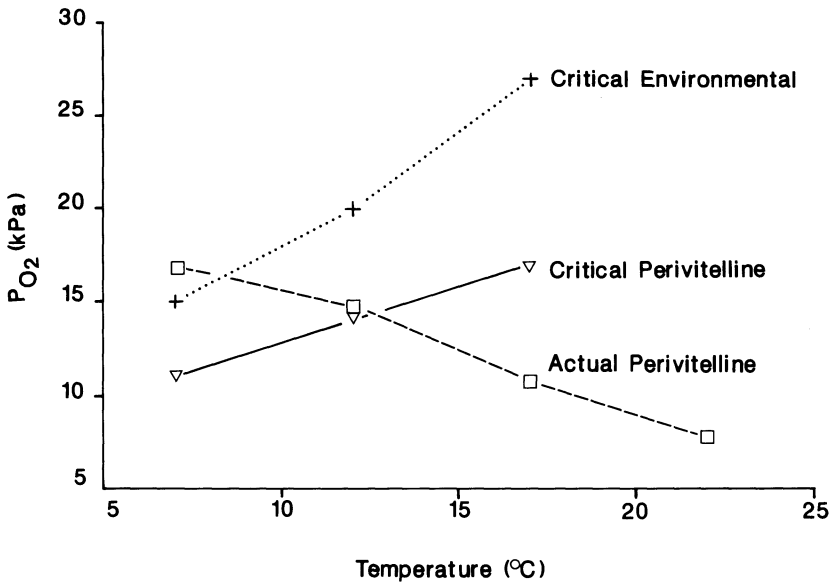

Fig. 6. Calculations of critical levels of P_{O_2} in the environment and in the perivitelline space of Pseudophryne bibronii *at which O_2 uptake becomes limited. Actual perivitelline P_{O_2}, calculated from measured $\dot{V}_{O_2}$ and G_{O_2}, is also given in relation to temperature.*

(fig. 1). The reduction in conductance at low $P_{O_2\,(out)}$ that occurs at 17°C (table 1) can be attributed to a slight dependence of differentiation rate on $P_{O_2\,(out)}$ (Bradford and Seymour 1988*b*). Embryonic metabolism is significantly limited by low $P_{O_2\,(out)}$ at this temperature (fig. 5), and differentiation rate is retarded (table 2).

Salthe (1965) noted that the pattern of change in volume of the perivitelline space in *R. pipiens* was largely stage-specific, from stage 15 through hatching, there being only a slight effect of temperature. This is consistent with the idea that the developmental sequence of the embryo is "programmed" for secretion of specific substances at specific stages. It seems reasonable, therefore, that the embryo is unable to alter the schedule of secretions enough to adjust capsule conductance to suit environmental conditions.

Implications for Natural Incubation

The failure of capsule morphology to respond to T_a and O_2 availability implies that the P_{O_2} in the perivitelline space is subject to fluctuations in incubation environment. Although terrestrial incubation in *P. bibronii* assures

a good O_2 supply, unimpeded by boundary layers and O_2 depletion that occur in aquatic habitats, the eggs are exposed to variable temperatures.

It is significant that *P. bibronii* breeds mainly in winter. The eggs are laid in March–May, and hatching typically occurs during June–August when the average incubation temperature is about 12°C (Bradford and Seymour 1985). At this temperature the eggs are not limited by O_2 availability (fig. 6).

However, temperatures ranging from 7.5° to 23.5°C have been measured under a stone at one breeding site (Geiser and Seymour 1989). This range corresponds to the temperature limits for successful embryonic development, although hatching success is greatest in the region of 12°–17°C (Seymour et al. 1991). On one hand, incubation to hatching stage takes about 140 d at 7°C, and many embryos die during this protracted period. On the other hand, high mortality occurs during late stages of development at 22°C, possibly because of O_2 limitation.

It is possible that O_2 availability can become limiting in late development if the eggs come in contact with other eggs, the male frog, or the substrate, all of which reduce effective egg surface area for gas exchange. If, for example, at 12°C, occlusion of the capsule were to reduce O_2 conductance to a third of its original value, the effective $P_{O_2\,(out)}$ would decrease below 6.9 kPa (52 Torr) and the embryo would stop developing and ultimately die (Bradford and Seymour 1988*b*). As expected, however, dead eggs are not usually found in the field, so the choice of oviposition site, the arrangement of the eggs, and possibly the manipulations of the male keep the eggs sufficiently aerated.

Acknowledgments

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