The ecophysiology of terrestrial nesting in Australian ground frogs (Anura: Myobatrachinae)

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Abstract

Variability in the microclimate of oviposition sites is a component of biological fitness that has only recently received serious attention. The fittest phenotypes will be produced if nest characters are consistently optimal; therefore selection should favour the ability of parents to select quality nests. In this study of three species of Australian Myobatrachine frogs I examine how the microclimate of terrestrial oviposition sites influences a), phenotypic traits of embryos, and b), the reproductive strategies and success of adults.

1. *Geocrinia vitellina* and *Bryobatrachus nimbus* develop without larval feeding and fuel their metamorphosis from yolk (endotrophism). Their production efficiencies are respectively, 59.2% and 61.5%, the first such measures for endotrophic amphibians, and their egg energy densities (26.4 and 26.0 J mg^{-1}) are greater than those of exotrophic amphibians (with feeding larvae).

2. Eggs of *B. nimbus* require 13 months to complete metamorphosis in sub-alpine moss nests, at an effective temperature of 8.5° C, and overwintering exhausts most of 249 J of energy contained in the ovum. Eggs in warmer nests (about 14.9° C) can reach metamorphosis in 5 months, and save 123 J, however, this saving is inconsequential if winter temperatures are too cold for froglets to feed.

3. The globular egg masses of *B. nimbus* are vulnerable to hypoxia because of substantial diffusion distances created by extremely large jelly capsules. Models of a spherical, hatching-stage egg mass show that the central embryo in larger clutches (13-21 eggs) experiences profound hypoxia at temperatures above 5° C. However, because broader nests are used as oviposition sites, real masses are typically hemispherical, and embryos are confined to 1-2 layers, which enhances their oxygen supply. Moreover, the photosynthetic nest material enriches jelly oxygen in daylight, when metabolic demands of embryos and larvae are greatest. Laboratory experiments demonstrate that larvae in warm nests (15°-20° C) seek out oxygen-rich jelly at the surface and walls of nests.

4. Incubation of *B. nimbus* embryos at water potentials between 0 and -25 kPa produces normal hatchlings at 0 and -5 kPa, but markedly stunted and asymmetric hatchlings at -10 and -25 kPa, with reduced rates of oxygen consumption. However, similar effects are not observed in natural nests, where desiccation contributes only a small portion (<7%) of embryonic mortalities. This suggests that embryos are able to uptake sufficient water from the surrounding substrate of bryophyte and lichens, and the capacious jelly capsule may be a valuable moisture reservoir.

5. Previous studies have shown broader tolerance to low water potentials in *Pseudophryne bibronii* embryos (0 to -200 kPa), but wetter incubation produced larger hatchlings. Patterns of male *P. bibronii* nest site selection and call advertisement are examined in a field experiment. Males prefer to nest on substrates of high water potential (≥ -15 kPa) and call from these nests at greater rates, and on three times as many nights as males occupying drier areas, presumably because they are not constrained by a risk of dehydration. Male mating success is coupled with calling effort; consequently, females choose between hydrated males, and in the majority of cases, oviposit in a wet nest that produces viable embryos.

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Numerous others assisted with particular aspects of this study, and are acknowledged in relevant chapters.

1 Introduction

Processes that influence the fitness of an individual are of considerable interest to life-history theorists. Strictly defined, biological fitness is the relative contribution of an individual to a gene pool, and is best examined with genetic techniques in a long-term population study, or with numerical models (e.g. Bernado 1996; Resetarits 1996). Although different phenotypes may have different fitness - for example, larger females may be more fecund than smaller females - phenotypic traits do not in themselves measure fitness. However, it is reasonable to assume that traits including body size, locomotor performance, age at sexual maturity, and sexually selected characters, will be relevant to an individual's reproductive success. Moreover, any trait that influences survival has an unambiguous effect on reproductive success. Because the phenotype arising from a given genotype depends in part on the environment in which an organism develops (phenotypic plasticity; McNamara and Houston 1996), knowledge of the developmental physiology of a species is an important adjunct when interpreting differentials in fitness.

Oviparous (egg-laying) females have two main mechanisms by which they may influence the phenotype of their progeny: 1) the allocation of nutrients and energy to eggs, and 2) behaviours such as the selection of the nest-site, and parental care. In some species males select nest sites and offer parental care, and hence these phenotypic effects are realised through the female's choice of her mate. Environmental factors such as food availability and temperature largely determine female expression of the vitellogenic parameters of ovum size, clutch size, and yolk quality, and are known to account for developmental plasticity of larvae both between and within populations (e.g. Kaplan 1985; 1987; James and Whitford 1994; Kaplan and King 1997). However, natural selection cannot have produced the optimal compromise between egg size and egg number without a stable environmental context; likewise, the incubation environment must remain relatively constant if fitter phenotypes are to be consistently produced (Resetarits 1996).

There are four means by which stable environments may occur: 1) the habitat may be uniform (e.g. many marine species), 2) tolerance limits of adults and larvae may be so

similar that females can only survive to reproduction in environments which suit their offspring, 3) females may be philopatric in oviposition, or 4) females may actively assess and select oviposition sites based on criteria that evolved in parallel with other components of her phenotype (Resetarits 1996). It is this latter mechanism that is the focus of this study. The post-vitellogenic environment (the oviposition site) is at least as important as the environment experienced by the female during vitellogenesis, because the oviposition environment can determine embryo survivorship (Howard 1978), size (Kaplan 1992), growth rates (Seymour et al. 1991b), behaviour (Elphick and Shine 1996), time of emergence (Shine and Harlow 1996), and sex (Vogt and Bull 1982). Moreover, the effects of sub-optimal development can carry over into post-metamorphic and adult life (Kaplan 1985; Howard 1988; Halliday and Verell 1988). Thus the choice of oviposition site may affect fitness through a variety of different phenotypic traits.

Anuran amphibians are a focus group in two main fields of research: embryonic development, and sexual selection. The reasons for this are several; amphibians are often readily available, their development is almost exclusively external, and hence is easily observed and manipulated, and the diversity of reproductive modes is remarkable. However, partly due to their reduced accessibility, terrestrial species play the understudy to aquatic species in current research, yet their developmental environment is quite different from that of species that oviposit aquatically. For example, temperature fluctuations are more marked, the egg mass is affixed to a substrate or contained in a nest, and consequently oxygen is sourced from a single direction, and eggs may be exposed and thus prone to dehydration. Further, within a single breeding population there may be marked differences in the microclimates of terrestrial oviposition sites (e.g. Townsend 1989a; Martins 1993; Kam et al. 1996; 1998).

The effects of temperature on embryonic and larval development of aquatic-breeding species are well established, and include the determination of the rate of differentiation and growth, and timing of emergence from an ephemeral water body. Berven and Gill (1983), for example, found that temperature differences between two populations of wood frogs, *Rana sylvatica,* were the most important variable in accounting for 73-88% of the variation in larval phenotype. Terrestrial species are less studied, but experiments on the embryos of *Pseudophryne bibronii* showed that incubation at low temperatures (12° C) produced hatchlings with a significantly higher gut-free dry mass than embryos

reared at high temperatures (17° C and 22° C). The greater amounts of oxygen and yolk consumed over the longer development period at 12° C ultimately produced hatchlings of a larger size, but with less energy in reserve (Seymour et al. 1991b).

Oxygen is fundamental to the normal development of amphibian embryos, and low partial pressures of oxygen (PO_2) are known to cause mortality in nesting species [(e.g. *Hyla rosenbergi* (Kluge 1981); *Kyarranus (= Philoria) loveridgei* (Seymour et al. 1995); and *Crinia georgiana* (Seymour et al. 2000)]. Moreover, the requirement for oxygen has played a major role in determining the structure and location of amphibian egg masses, and has constrained the size of terrestrial masses (Seymour 1994; 1999; Seymour and Bradford 1995).

The integument of the amphibian egg offers no appreciable resistance to evaporation, yet dehydration has adverse physiological effects and causes death at higher extremes. Thus terrestrial embryos must develop in moist conditions, and parental care is important in maintaining embryonic water balance in many species that oviposit their eggs in exposed situations (e.g. Taigen et al. 1984; Burrowes 2000). Otherwise, selection of a reliably moist oviposition site is critical, because incubation in drier nests can reduce rates of oxygen consumption and retard growth (Bradford and Seymour 1988). Thus the thermal, gaseous, and hydrological characteristics of an oviposition site may play an important role in determining the phenotypes of hatchlings.

The consequences of oviposition site selection have been examined in both invertebrates (e.g. Rausher 1979; Singer *et al.* 1988) and vertebrates (e.g. Schwarzkopf and Brooks 1987; Shine and Harlow 1996; Östlund-Nilsson 2000); eminent examples include temperature-mediated sex determination in many reptiles (e.g. Vogt and Bull 1982) and the effects of nest water potential on neonate size and energy reserves in iguanas (e.g. Tracy and Snell 1985; Werner 1988). The majority of studies of oviposition site in amphibians have focused on the biotic factors influencing oviposition site choice in aquatic species: the size and type of predator and competitor communities (e.g. Howard 1988; Waldman 1982; Resetarits and Wilbur 1989; Laurila and Aho 1997; Van Burskirk et al. 1997). However, in the more variable terrestrial environment, discrimination between abiotic factors may have greater relevance. This claim can only be evaluated if the developmental physiology of the study species is understood, but of the great

diversity of terrestrial breeding anurans (Altig and McDiarmid 1999), only the developmental physiology of two species have been studied in depth: *Eleutherodactylus coqui* (Van-Berkum 1982; Taigen et al. 1984; Townsend and Stewart 1985; Burggren 1990; Packard et al. 1996; Elinson and Fang 1998) and *Pseudophryne bibronii* (Woodruff 1976; Bradford and Seymour 1985; 1988a; 1998b; Seymour and Bradford 1987; Seymour et al. 1991a; 199b).

In this thesis I examine three species of large-egged, terrestrial breeding Myobatrachid frogs: *Geocrinia vitellina* from temperate forest in southwest Western Australia, *Bryobatrachus nimbus* from sub-alpine heath in southern Tasmania, and *Pseudophryne bibronii* from dry sclerophyll forest in South Australia. I use the natural variability in nest microclimates to define the boundaries of physiological investigations into the environmental effects on phenotypic traits in embryos and larvae, and nest-site selection in adults. In particular, this research advances three main areas: a) comparative respiration and energetics of anuran amphibians, particularly within myobatrachids, and evaluation of the energy cost of development in natural nests (Chapters 2 and 3), b), effects of temperature, oxygen and water potential on phenotypic traits of embryos and larvae arsing from amongst the largest known anuran eggs (Chapters 3, 4, and 5), and c) examination of the role of the oviposition site in determining reproductive strategies of male frogs (Chapters 6 and 7).

2 The energetics of endotrophic development in the frog *Geocrinia vitellina* (Anura: Myobatrachinae)

Abstract

The energetics of endotrophic development, where the nutrition required to complete metamorphosis is provided solely by yolk, has seldom been quantified. The energy cost of development to metamorphosis of the endotrophic Australian frog *Geocrinia vitellina* was measured using calorimetry and closed-system respirometry. Dry yolk had an energy density of 26.4 J mg^{-1}, and an average 2.8 mm diameter ovum contained 144 J. Incubation at 15° C produced a froglet of 5.8 mm snout-vent length containing 88 J in 87 d, with 11% of residual yolk in the gut - markedly less than the 50% recorded in another endotroph, *Eleutherodactylus coqui*. *G. vitellina* lost 56 J of metabolic energy during development to metamorphosis at 15° C, and the total production efficiency was 61.0%, the first measure for an amphibian. A review of published egg energy densities found a mean for amphibians of 25.1 kJ g^{-1}, significantly lower than the mean of 27.1 kJ g^{-1} for reptiles. Moreover, available amphibian data suggest that endotrophic species have high yolk energy densities and low mass-specific rates of oxygen consumption relative to exotrophic species (with feeding larvae); consequently, large ovum size may not necessarily be prerequisite for endotrophic development.

INTRODUCTION

The naked egg of amphibians comprises an ovum, an egg membrane, and one or more gelatinous envelopes. Such eggs are termed anamniotic, whereas the amniotic eggs of reptiles and birds derive extraembryonic membranes from the embryo during development, are encased in a fibrous shell (Salthe 1963; Carroll 1982). Functional comparisons of anamniotic and amniotic eggs have proved fruitful for evaluating homologies in egg structures, and for explaining the greater size of amniote eggs (Packard and Seymour 1997). However, a dearth of data for energy content of amphibian eggs and larvae has thus far prevented phylogenetic comparisons of energy budgets and production (Thompson and Russell 1998). Moreover, there is little understanding of how yolk reserves influence the timing and maturity at hatching, which is particularly surprising given considerable interest in the diversity and evolution of developmental modes in amphibians (e.g. Altig and Johnston 1989; Hanken et al. 1997; Thibaudeau and Altig 1999). A feature common to many terrestrial modes is endotrophism (direct development of many authors), where nutrition to metamorphosis is provided solely by yolk. Endotrophic amphibian eggs are therefore a closed energy system, equivalent to the eggs of reptiles and birds. Moreover, because endotrophic eggs may be analogous to a transitional stage between anamniotic and amniotic eggs (Elinson 1987; Packard and Seymour 1997; Packard et al. 1996), comparisons amongst these groups are of particular interest.

The energy cost of development is defined as the amount of energy consumed during tissue synthesis and maintenance. It can be measured directly using calorimetry (measurement of heat production of embryos and larvae during development), or indirectly by one of two more practical methods: bomb calorimetry and respirometry (Withers 1992). Bomb calorimetry entails computing the difference in energy contained in embryos or larvae with that in eggs, while respirometry requires measurement of ontogenetic changes in rates of oxygen consumption ($\dot{V}O_2$), and integration of the volume of oxygen consumed and assumption of energy equivalence in J mL O_2^{-1}. These indirect approaches produce similar results in studies of bird and reptile eggs (e.g. Vleck et al. 1984; Thompson and Russell 1998), but the respiratory method is often preferred

because of its relative simplicity, and because suitable amounts of tissue for calorimetry can be difficult to obtain for small organisms.

Endotrophs that hatch as a tadpole morphotype (most nidicolous species) are thought to have less energy remaining at metamorphosis than direct developing endotrophs that hatch as froglets (Thibaudeau and Altig 1999). This study provides the first empirical data for a nidicolous endotrophic anuran, the myobatrachid frog *Geocrinia vitellina* (Roberts et al. 1990) from southwestern Australia. Eggs were examined in natural nests, and incubated in the laboratory, where differentiation rates and $\dot{V}O_2$ were measured until metamorphosis was complete, and energy densities and production efficiencies were determined from calorimetry. Data are interpreted alongside available data for other amphibians, and reptile and avian eggs. An emerging data set of respiratory and energetic variables for Australian myobatrachids highlight potential mechanisms of energy conservation in endotrophic species.

METHODS

Study species and site

Geocrinia vitellina is one of four endotrophic species in the monophyletic *Geocrinia rosea* complex (Anura: Myobatrachidae) from temperate southwest Western Australia. All species deposit between 4-30 globular eggs in shallow moist burrows under matted vegetation, and have nidicolous larvae. Males call from nests from August to December, and shift to a new nest after mating. Adult *G. vitellina* are small (19-29 mm snout-vent length), exhibit extremely low levels of dispersal (Driscoll 1997, 1998, 1999), and are restricted to 20 ha of riparian seepage within a single catchment. The 1 ha study site was Geo Creek (115°18′10′′E, 34°04′50′′ S), about 30 km southeast of Margaret River.

Egg collection and measurement of nests

Nests of *G. vitellina* were located by marking the calling sites of males, and later searching beneath a marked location when a male no longer called. The surface dimensions and depth of egg masses were measured with dial callipers accurate to 0.1 mm, and fresh eggs were carefully excavated from the nest with a plastic spoon. Individual Hobo data loggers (Onset Computer Corp, Pocasset, USA) with modified external thermocouples were positioned to record the temperatures of developing clutches on 19 October 1997. However, on 22 October 1997 a wild fire (ignited from a nearby fuel reduction burn) burnt the entire Geo Creek site (Figure 1, and see Roberts et al. 1998). Damp matted grass protected most clutches from the fire, but all data loggers were destroyed. Nest temperature data collected by Simon Conroy after the fire has been made available for this study.

Egg maintenance

Fresh egg masses (stages 7-12, Gosner 1960) were removed from Geo Creek for laboratory study; three were collected before the 22 October 1997 fire, while four were collected 1-3 weeks after the fire. Eggs were cleaned of debris by rolling gently on absorbent tissue, and each clutch was housed individually in a small, nest-sized, glass dish or ice-cube cell lined with absorbent cloth and sealed with clingwrap. Clutches collected before the fire were stored for 10 days in a 15° C constant temperature room at

Figure 1. Geo Creek after the October 1997 fire. The *Geocrinia vitellina* breeding site is in the background, indicated by the coloured streamers.

the University of Western Australia, and development rates were monitored. They were then transported by air inside an insulated foam container to the University of Adelaide, South Australia, and housed inside a dark 15° C constant temperature cabinet within 6 hrs. Other clutches were transported to Adelaide within 2 days of collection, and were housed in identical conditions. Embryonic mortality in the laboratory was high; one clutch did not survive beyond stage 14 (neural plate formation), and other embryos died when handling caused premature hatching and puncturing of the yolk sac. Two clutches of larvae succumbed to an apparent bacterial attack just after hatching.

Egg staging and morphology

Several staging schemes are available for large-egged terrestrial Myobatrachids: for endotrophic *Philoria (=Kyarranus) sphagnicolus* (de Bavay 1993) and *Bryobatrachus nimbus* (Mitchell and Swain 1996), and for eastern Australian *Geocrinia* (*G. laevis* and *G. victoriana* that have feeding (exotrophic) larvae (Gollman and Gollman 1991; 1994). Like western Australian *Geocrinia*, these species lack external gills, and hence their staging schemes were more applicable than the standard scheme of Gosner (1960) that

appearance of hindlimb buds (stage 26). The detailed scheme of de Bavay (1993), which is referenced to Gosner's scheme, was used to assign *G. vitellina* developmental stages every 1-4 d. Slight modifications were necessary between heartbeat and hatching stages because the curled tail enveloped the embryo and diagnostic features aside from hindlimb buds were difficult to observe. Perivitelline diameters of fully submerged embryos, and the snout-vent length (SVL) and tail length of larvae were measured using a stereomicroscope fitted with an ocular micrometer.

Rates of oxygen consumption

Rates of oxygen consumption ($\dot{V}O_2$) of individuals of each clutch were measured at 15° C throughout incubation; individuals were afterward replaced in their glass dishes and maintained at 15° C. In addition, $\dot{V}O_2$ of eight de Bavay (1993) stage 32 larvae (from two clutches) were measured individually at 5° and 25° C to allow calculation of Q_{10}.

$\dot{V}O_2$ was determined from the rate of decline of P_{O_2} in sealed, water-filled respiratory chambers (0.67 mL, No. 1231, Diamond General). Chambers were fitted with Clark-type oxygen electrodes (Diamond General model 733) and thermostated at 15 ± 0.1° C with a Braun 850 constant temperature bath. The procedure for measurement of $\dot{V}O_2$ was as described by Seymour and Loveridge (1994) and is detailed in Appendix 1. However, four amendments were made. Firstly, fully-hydrated eggs were too large to comfortably fit inside a chamber, so outer jelly layers were removed by gently rolling the eggs on moist cloth. Sensitivity analyses have shown that the inner capsule diameter (equal to that of the perivitelline membrane) has a large influence on the conductance of the jelly capsule; conversely, outer capsule diameter has little effect (Seymour 1994). Hence partially decapsulated embryos with an intact perivitelline membrane are not released from any diffusive constraint, and should have similar $\dot{V}O_2$ to embryos in intact capsules. Secondly, the $\dot{V}O_2$ of water-filled chambers was measured to control for any bacterial respiration in the water. These values averaged about 0.18 $\mu L\ O_2\ h^{-1}$, and were subtracted from the mean embryonic or larval $\dot{V}O_2$ of relevant experiments. Thirdly, chamber volume was corrected to account for the displacement volume (≡mass) of embryos and larvae. Because embryos were contained in the jelly capsule, their masses were estimated from mean masses of similar stage embryos that had been dissected from the egg and preserved for calorimetry (see below). Fourthly, mean values of $\dot{V}O_2$

included only those measures where chamber $P_{O_2} > 5$ kPa. Three embryos were excluded from $\dot{V}O_2$ measurement to reliably determine hatching stage without the confounding influence of handling.

Energy cost of development to metamorphosis was estimated by integrating daily O_2 consumption as determined from measurements of $\dot{V}O_2$. As CO_2 production was not measured, the volume ratio of CO_2 produced to O_2 consumed (the respiratory quotient, *RQ*) was unknown. Instead, an *RQ* of 0.8 and energy equivalence of 20.5 kJ L^{-1} of O_2 was assumed, in common with other amphibian studies (e.g. Seymour et al. 1991b; Chapter 3).

Bomb calorimetry

Ova were dissected from fresh eggs, weighed, dried at room temperature (~ 22° C) over silica gel, weighed again, and homogenised using a mortar and pestle to make a pellet of at least 25 mg. Similarly, random samples of embryos and larvae were removed from incubation and killed by freezing and preserved in fixative (Tyler 1962), before dissection into body and gut. Samples were oven dried at 60° C, stored over silica gel, and later weighed to 0.01 mg on a Mettler AE163 analytical balance. Where possible, samples were pooled by clutch before homogenising into pellets of either gut or body. The energy density of pellets was measured with a 1107 semimicro bomb of a Parr 1261 bomb calorimeter after calibration with dry benzoic acid.

Statistical analysis

Because conservation priorities prevented the collection of large numbers of eggs, data were extended by measuring the $\dot{V}O_2$ of an individual between two and 15 times during its development, thereby sacrificing data independence. Furthermore, some individuals were killed during development for calorimetry, and others died unexpectedly, so to maintain numbers four clutches of larvae were collected from the field in 1999. The thermal history of these larvae was unknown, but their effective ages were estimated from their stages and they were thereafter incubated at 15° C. Although the measurements are not independent, it was practical to describe the data with means, 95% confidence intervals and regression equations. Two-sample t-tests or Mann-

Whitney U-tests (non-normal data) were used to compare species or phylogenetic groups, and statistical significance was assumed when $P < 0.05$.

RESULTS

Geocrinia vitellina nests were hemispherical basins about 2.3 cm in diameter in moist soil beneath thick mats of dead grass (Table 1). Eggs in fresh clutches (10.2 ± 1.2 eggs) were usually positioned in two layers with 5-11 eggs at the surface and 0-6 eggs below. After hatching larvae were interleaved in the jelly mass. The average volume of a fresh egg mass was 4.2 cm^3, but varied widely (Table 1), and diminished after hatching (Simon Conroy, unpublished data). Egg masses located after the fire were coated in fine charred debris, and the grass layered over nests was considerably reduced. Post-fire temperatures of four *G. vitellina* egg masses averaged 17.1 ± 0.6° C from 7 November to 5 December 1997, and increased later in the season (16 December 1997 to 5 January 1998) to 20.1 ± 1.2° C (*n*=3).

Early development through neurulation, tailbud (stage 17; Figure 2a) and heartbeat (stage 19; Figure 2b) agreed with the stage definitions of Gosner (1960) and de Bavay (1993). Late embryonic stages 20-24 were redefined from de Bavay (1993) as outlined in Table 2, and compared to a staging of eastern Australian *Geocrinia* (Gollman and Gollman 1991). Embryos hatched after 19.9-25.7 d between stages 23-25 (Figure 3) and hatching coincided with the appearance of paired white endolymphatic calcium deposits

Table 1. Characteristics of fresh clutches of *Geocrinia vitellina* from Geo Creek

Parameter	Unit	$\bar{x}$ (Range)	95% CI	*N*
Diameter *a*	cm	2.38 (1.82-2.91)	0.24	12
Diameter *b*	cm	2.26 (1.89-2.68)	0.18	12
Depth at centre (h)	cm	1.38 (0.75-2.04)	2.28	12
*Egg mass volume	cm^3	4.2 (1.2-8.1)	1.2	12
Number of eggs		10.2 (5-15)	1.2	23
Egg density	$eggs/cm^3$	3.5 (1.4-9.8)	1.4	12

Diameters *a* and *b* are perpendicular axes at the surface of the egg mass. * Calculated assuming the egg mass is a segment of a sphere ($V = \frac{1}{3}\pi h^2(3r-h)$, r = (diameters $a+b$)/4).

Table 2. Definitions of developmental stages 19-26, redefined from de Bavay (1993) for *Geocrinia vitellina*, and compared to Eastern Australian (EA) *Geocrinia*

Stage	Diagnostic feature/s	Other characters	EA *Geocrinia*
19	Heartbeat	Rudimentary hind limb buds. Blunt tail tip, opaque fin about ¼ height of muscle. Unpigmented vitelline circulation visible under high power.	
20	Transparent tail fin Tail 2x body length	Circulation visible in fin. Optic bulge. Nostrils short pits. Mouth open, diamond-shaped. Pink vitelline circulation.	
21	*Hind limb bud stage I	Eye faintly pigmented	
22	Hind limb bud stage II (circular)	Eye pale grey. Pale blue iridescent flecks appear, mainly on dorsal surfaces	
23	Hind limb bud stage III (triangular)	Eye black. Iridescent flecks extend laterally, brown-grey melanophores covering lateral surfaces of yolk sac. Labial ridges visible. Prominent ventral blood vessel in tail.	Rudimentary hindlimb buds
24	Hind limb bud stage IV-V	Melanophores cover entire yolk-sac	1st row of labial teeth.
25	Hind limb bud stage VI	Prominent white endolymphatic calcium deposits appear as small patches behind eyes. Gold flecking on head.	2nd row of labial teeth
26	Hind limb bud stage VII	Eye protrudes above skin surface, iris clear, sclera specked with gold. Hind limbs pigmented.	3rd row of labial teeth Embryos hatch

*Hindlimb stages are defined in Gosner (1960)

behind the eyes (Figures 2 f-g). Pale blue iridescent flecking that first appeared at stage 22 became very prominent between de Bavay stages 27-31, but diminished toward metamorphosis. At stage 33 fine white spotting appeared on the dorsal surface. Metamorphosis was complete at stage 37 after 86.6 days (from equation in Figure 3); the SVL of froglets was 5.76 ± 0.40 mm, and their wet mass was 20.7 mg, of which 9.5 % was residual yolk contained in the gut (Table 3). Dry mass of larvae increased steadily during development as yolk mass declined (Figure 4) and at metamorphosis froglets contained 31% less (1.69 mg) dry mass than was contained in fresh eggs (Table 3). About 39% (56 J) of the energy contained in fresh eggs (143.6 ± 45.9 J) was lost during development.

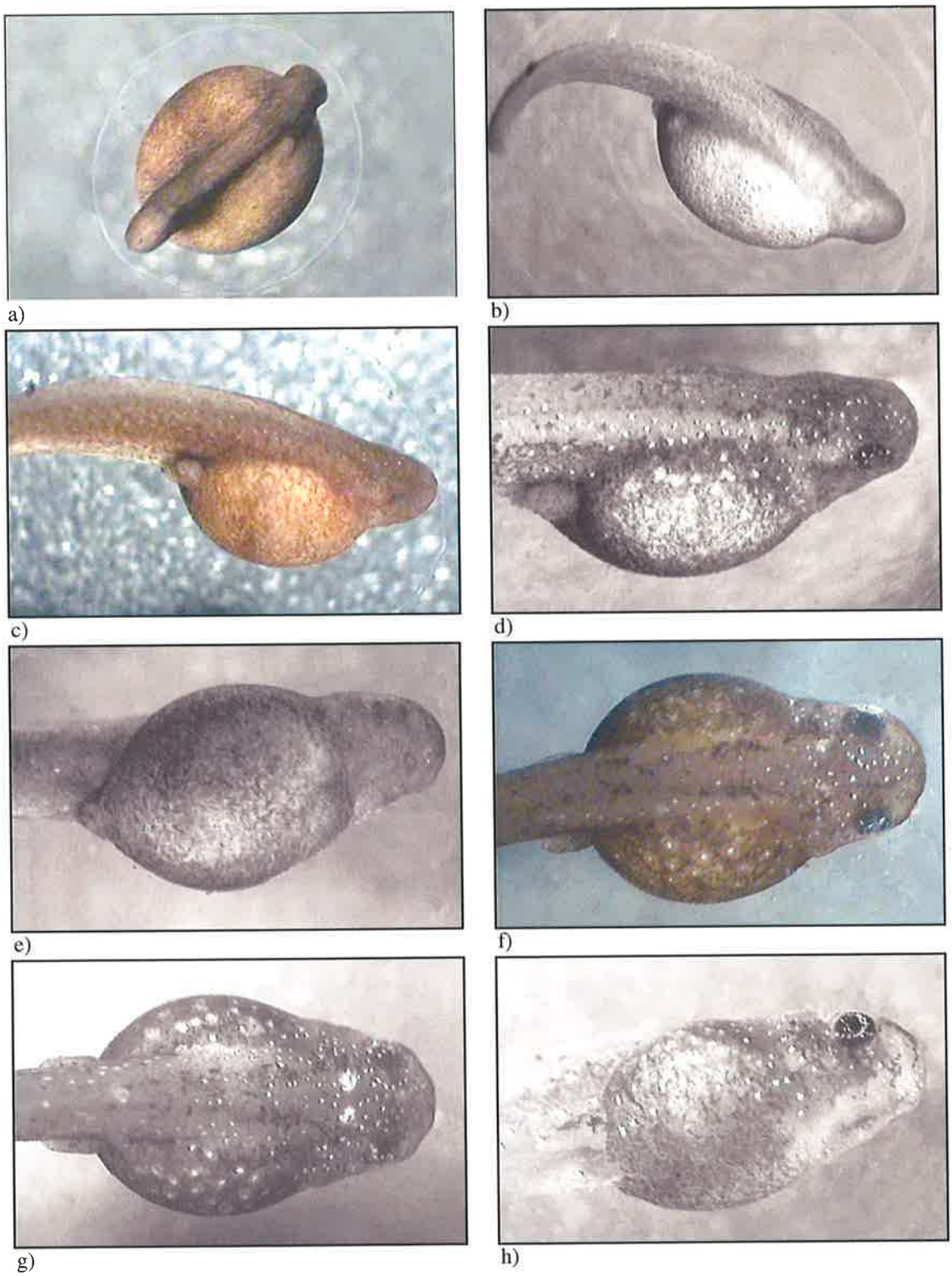

Figure 2. *Geocrinia vitellina* at selected developmental stages: a) stage 17, b) stage 19, c) stage 20, d) stage 23, e) ventro-lateral view of stage 23, showing mouthparts, f) stage 24, g) stage 25, h) stage 26.

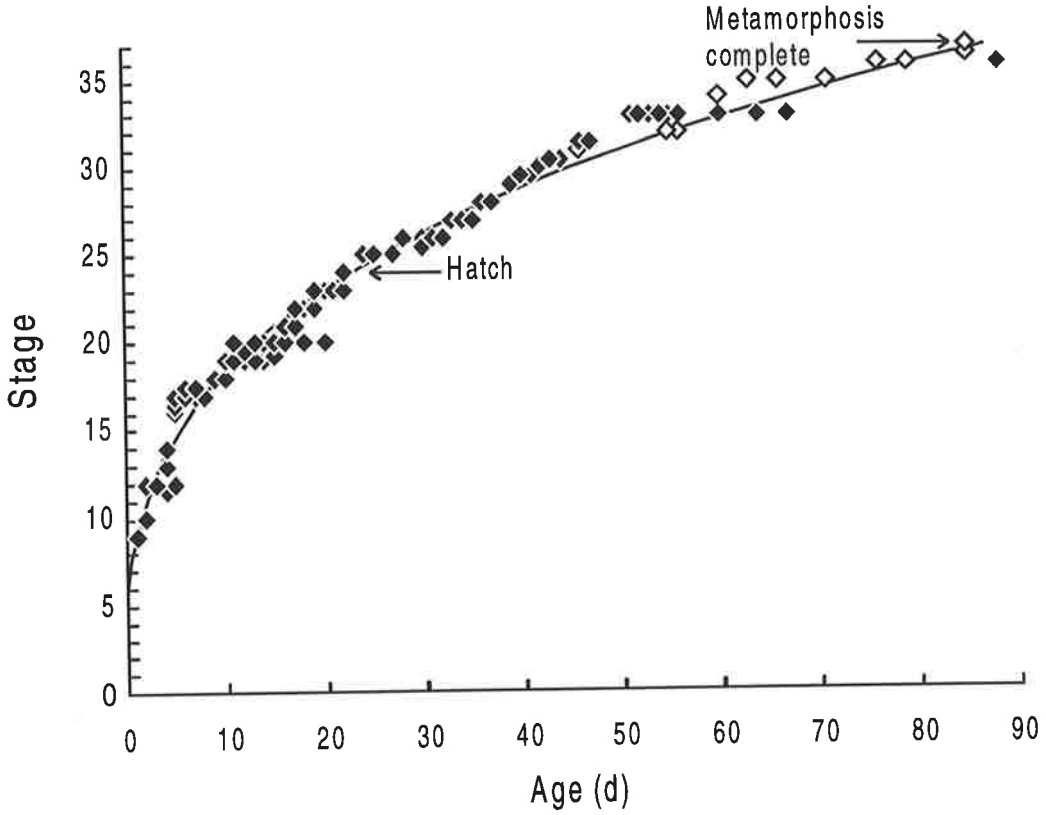

Figure 3. Stage of development and age of *Geocrinia vitellina* during laboratory incubation at 15° C. Each point represents 2-4 embryos or larvae from one of ten clutches used in the experiment. Filled symbols (♦) are clutches collected in 1997 and incubated at constant temperatures, and open symbols (◊) indicate 1999 larvae (see Methods). The equation relating age and stage is Age = $(\text{Stage}/8.7573)^{3.09644}$ (r^2=0.97).

Rates of embryonic oxygen consumption peaked at stage 25 (~ 25.7 d) at 1.61μL h^{-1} (Figure 5). $\dot{V}O_2$ increased steadily during larval development, and maximum $\dot{V}O_2$ of 3.81 μL h^{-1} occurred at stage 33, between days 61 and 67 d (Figure 5). Thereafter $\dot{V}O_2$ declined and averaged 3.13 μL h^{-1} at metamorphic climax. Mean stage 32 $\dot{V}O_2$ of eight larvae were 1.64 μL h^{-1} at 5° C, 2.85 μL h^{-1} at 15° C, and 5.30 μL h^{-1} at 25° C. The Q_{10} for these intervals were 1.74 (5 °-15° C) and 1.86 (15 °-25° C). Integration of the metabolic curve (Figure 5: polynomial a) gave an estimate of 4.98 mL of O_2 consumed by metamorphosis, equivalent to 102.1 J of energy (Table 4). Thus the dry mass-specific energy consumption during development was 1.56 mLO_2 mg^{-1}, or 31.6 J mg^{-1}.

Table 3. Summary of morphometric and energy information for eggs of *Geocrinia vitellina* incubated at 15° C.

Variable		$\bar{x}$	95% CI	Range	*n* samples	*n* clutches
*Egg	Capsule diameter (mm)	9.1	0.4	8.2 - 10.2	15	3
	Ovum diameter (mm)	2.8	0.04	2.6 - 3.1	42	8
	Ovum volume (µL)	12.0	0.5	9.2 - 14.9	42	8
	Wet mass of ovum (mg)	18.2	1.6	10.0 - 31.2	31	8
	Dry mass of ovum (mg)	5.5	0.3	4.0 - 7.5	38	7
	Water content of ovum (%)	69.9	3.2	51.8 - 82.1	25	7
	†Energy density of ova (J mg^{-1})	26.4	1.1	26.0 - 27.5	4	8
	Energy in ovum (J)	144	7.7	104 - 195	38	7
Hatchling	Snout-vent length (mm)	4.3	0.1	3.9 - 4.6	15	3
	Tail length (mm)	6.7	0.6	5.6 - 8.4	15	3
	Dry mass of gut-free body (mg)	1.0	0.2	0.4 - 1.5	15	3
	Wet mass of gut-free body (mg)	4.8	1.0	2.5 - 8.6	15	3
	Water content of gut-free body (%)	78.6	3.1	71.4 - 87.2	15	3
	Energy in gut-free body (J)	22	4	9 - 34	15	3
	Dry mass of residual yolk (mg)	4.3	0.5	2.4 - 5.0	15	3
	Wet mass of residual yolk (mg)	7.5	0.9	3.2 - 9.9	15	3
	Water content of residual yolk (%)	40.7	6.2	20.7 - 63.0	15	3
	Energy in residual yolk (J)	105	11	56 - 125	15	3
Froglet	Snout-vent length (mm)	5.8	0.1	5.3 - 6.0	10	2
	Dry mass of gut-free body (mg)	3.2	0.2	2.7 - 3.7	10	2
	Wet mass of gut-free body (mg)	18.8	1.2	15.6 - 21.9	10	2
	Water content of gut-free body (%)	82.8	1.1	80.1 - 85.1	10	2
	†Energy density of gut-free body (J mg^{-1})	21.8	1.3	20.7 - 22.7	4	2
	Energy in gut-free body (J)	72	5	60 - 82	10	2
	Dry mass of residual yolk (mg)	0.6	0.1	0.4 - 0.7	10	2
	Wet mass of residual yolk (mg)	2.0	0.4	1.1 - 2.8	10	2
	Water content of residual yolk (%)	69.0	4.90	58.3 - 78.3	10	2
	†Energy density of residual yolk (J mg^{-1})	27.4			1	2
	Energy in residual yolk (J)	16	2	10 - 19	10	2
	‡Gross production efficiency (%)	61.0				
	‡Total production efficiency (%)	56.2				

Confidence intervals have been calculated from sample standard deviations

Energy densities are expressed in J mg dry $mass^{-1}$

*Eggs between Gosner (1960) stages 7-12 (~ 1-3 d)

†sample *n* refers to the number of bombed pellets

‡Gross production efficiency = (gut-free body + residual yolk energy of metamorph)/ovum energy; Total production efficiency = gut-free body energy of metamorph /(ovum energy – residual yolk energy of metamorph) (refer Ar et al. 1987)

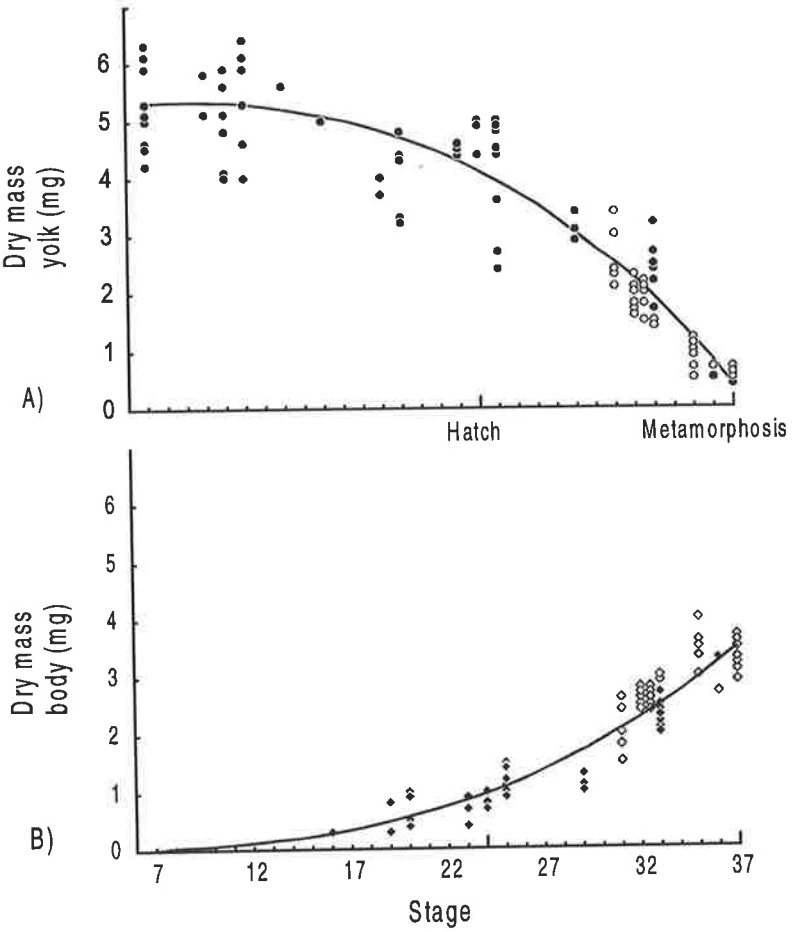

Figure 4. Dry mass of A) yolk (circles), and B) gut-free body (diamonds) from stages 7 to 37. Filled symbols are for individual embryos and larvae collected in 1997 and incubated at constant temperatures, and open symbols indicate individual 1999 larvae (see Methods). Equations for fitted curves are: mass of gut-free body = $7.56 \times 10^{-5} \times \text{stage}^{2.975}$ (r^2=0.86); mass of dry yolk = $8.97 \times 10^{-5} \times \text{stage}^3 - 1.32 \times 10^{-3} \times \text{stage}^2 + 0.0428 \times \text{stage} + 5.13$ (r^2=0.79)

Table 4. Volume (mL) of O_2 consumed during development at 15° C, and cost of development to stage 24 (hatching) and 37 (metamorphosis).

Stage	24	37
VO_2 (mLO_2)	0.34	4.98
Energy cost of development (J)	7.0	102.1
Mass-specific cost of development ($mLO_2\,mg^{-1}$ dry yolk-free body)	0.33	1.56

Energy cost of development assumed that 1 mLO_2 = 20.5 J (Seymour et al. 1991b)

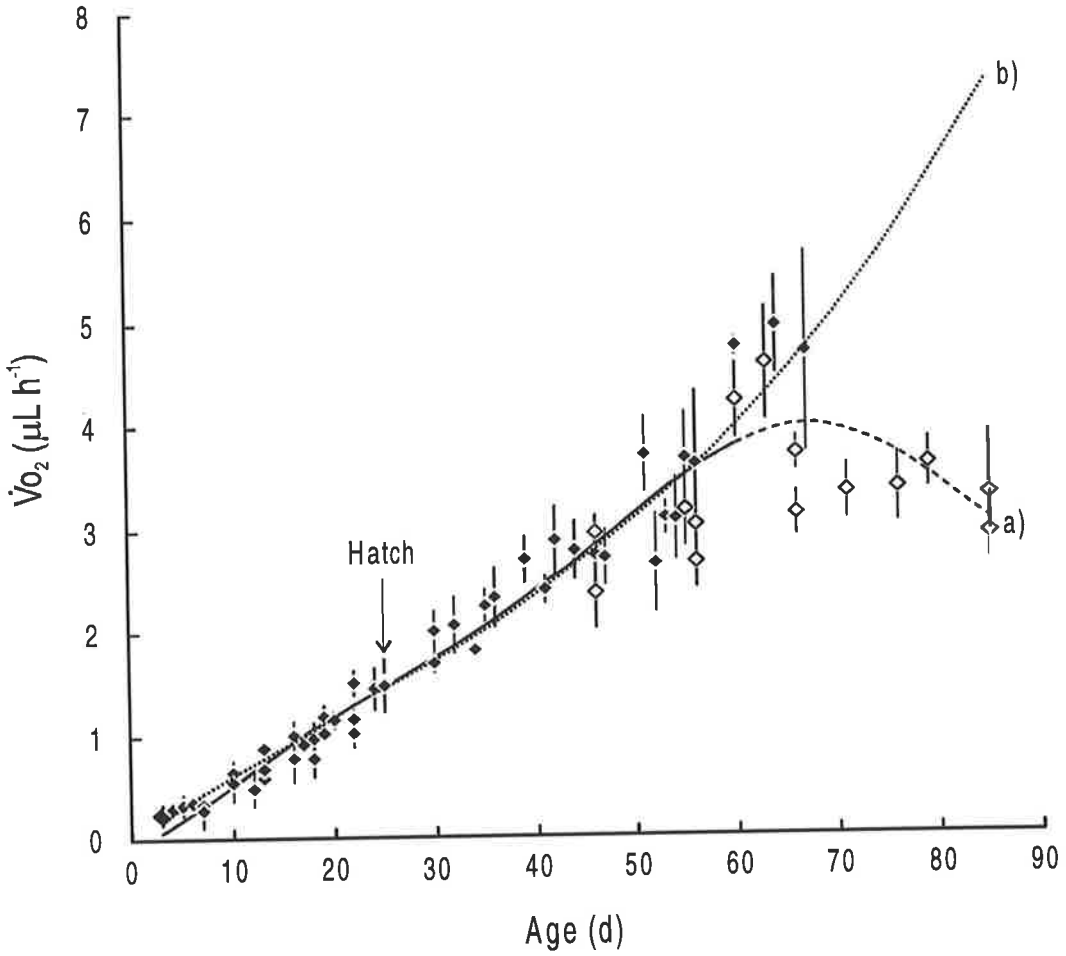

Figure 5. Mean (± 95% CI) rates of oxygen consumption ($\dot{V}O_2$) in water at 15° C of 2-4 *Geocrinia vitellina* embryos or larvae from one of ten clutches. Data symbols follow Figure 3. a) polynomial equation relating age and $\dot{V}O_2$ for all data (solid line; dashed line beyond 60d): $\dot{V}O_2 = 4.169 \times 10^{-10} \times age^6 - 1.043 \times 10^{-7} \times age^5 + 9.461 \times 10^{-6} \times age^4 - 3.886 \times 10^{-4} \times age^3 + 7.313 \times 10^{-3} \times age^2 + 7.571 \times 10^{-3} \times age$ (r^2=0.93). At ~ 67 d (stage 34) forelimbs emerge and larvae are able to air breathe, and their bimodal $\dot{V}O_2$ may be more similar to the extrapolation of polynomial b (dotted line), relating age and $\dot{V}O_2$ for 1997 data only: $\dot{V}O_2 = 8.992 \times 10^{-6} \times age^3 + 5.016 \times 10^{-4} \times age^2 + 6.403 \times 10^{-2} \times age$ (r^2=0.95).

DISCUSSION

Embryonic and larval development

Western Australian *G. vitellina* differ from exotrophic eastern Australian *Geocrinia* by the precocious appearance of hindlimb buds during embryonic development (stage 19, compared to stage 23, Table 2). In this respect they resemble *Philoria sphagnicolus*, except that *P. sphagnicolus* develop an operculum, spiracle and recessed gill filaments before hatching (de Bavay 1993). Consistent with an endotrophic pattern, larval mouthparts of *G. vitellina* are vestigial and never develop beyond fleshy labial ridges (Figure 2e), whereas the labial fringes and horny beak of *P. sphagnicolus* larvae, and the multiple teeth rows of eastern Australian *Geocrinia* larvae (Gollman and Gollman 1994), are conspicuous and useful for staging.

Development of *G. vitellina* most closely parallels that of the endotrophic moss frog, *Bryobatrachus nimbus,* from sub-alpine elevations in southern Tasmania (Mitchell and Swain 1996). Larvae of both species lack gills, and the cuticle and extensively vascularized tail provide the respiratory surface. However, the tail of larval *B. nimbus* is longer than that of *G. vitellina*; for example, at stage 33 the *B. nimbus* tail is 70.4 ± 3.3% of total larval length, compared to 62.7 ± 3.3% for *G. vitellina* ($P < 0.0001$). This is not, however, reflected by a greater $\dot{V}O_2$ of *B. nimbus* larvae. At 15° C, the dry mass-specific $\dot{V}O_2$ at stage 33 was 0.74 μL h^{-1} mg^{-1} for *B. nimbus* (from Chapter 3), about half the 1.54 μL h^{-1} mg^{-1} for *G. vitellina* at 15° C. Therefore factors aside from respiratory surface area must influence interspecific differences in metabolic rate. Foremost amongst these may be the duration of development; lower $\dot{V}O_2$ in *B. nimbus* larvae may avert premature depletion of yolk when development time is extended to over one year by prolonged exposure to cold temperatures (Chapter 3).

Ecological implications

The globular egg masses of *G. vitellina* contain 15 or fewer eggs arranged in 1-2 layers. The larger globular masses of *Philoria (=Kyarannus) loveridgei* eggs (mean size: 31 eggs) become hypoxic near the nest base, resulting in the death of about 30% of the clutch (Seymour et al. 1995). *P. loveridgei* has lost the foam nesting habit of its

congeners and, without air bubbles, PO_2 falls below critical levels at the nest base. Egg densities of *G. vitellina* (3.5 eggs cm^{-3}) are similar to *P. loveridgei* (3.0 eggs cm^{-3}), but as dry mass-specific $\dot{V}O_2$ at hatching stage 24 of 1.38 $\mu L\ h^{-1}\ mg^{-1}$ is lower than that for *P. loveridgei* (about 1.73 $\mu L\ h^{-1}\ mg^{-1}$, adjusted to 15° C from Seymour et al. 1995 using an assumed Q_{10} of 2.5) there is less risk of hypoxia in *G. vitellina* egg masses. However, burning of the breeding swamp results in an increase in nest temperatures because soil moisture and shade cover declines, and solar radiation increases. An effect of nest warming would be to shorten incubation times and increase $\dot{V}O_2$. Coupled with a possibility that debris from the fire slows the rate of O_2 diffusion into the egg mass, hypoxia of embryos at the nest base is feasible. The increase in frequency of *in situ* deaths in *Geocrinia* nests after a fire (Driscoll and Roberts 1997, Roberts et al. 1998), especially embryos in base layers (S. Conroy, unpublished data), supports this suggestion.

The decline in $\dot{V}O_2$ near metamorphic climax of *G. vitellina* (Figure 5: polynomial a) is comparable to that observed near hatching in many reptiles (Thompson 1989) and precocial birds (Vleck et al. 1980, Vleck and Vleck 1987). In the case of *G. vitellina*, the decline is associated with the re-absorption of the tail between stages 35-37 (~ 73-87 d), and hence the gradual reduction of a major surface area for cutaneous gas exchange. However, at forelimb emergence (stage 34; ~ 67 d) larvae often air-breathe by climbing onto the nest wall while cutaneous respiration continues through the tail trailed in the jelly. This bimodal mode of respiration was found to elevate $\dot{V}O_2$ of *B. nimbus* larvae about 20% above aquatic $\dot{V}O_2$, and $\dot{V}O_2$ did not decline near metamorphic climax (Chapter 3). Hence the declining $\dot{V}O_2$ observed for *G. vitellina* may be an artefact of forced aquatic respiration, and bimodal respiration might show no such decline (e.g. extrapolation of polynomial b, Figure 5).

Bomb calorimetric data show that eggs contain 143.6 J at oviposition, and froglets contain 87.6 J (71.9 J in gut-free body + 15.7 J in residual yolk, Table 3). Thus, 56 J was lost in the form of metabolic energy during development at 15° C. In natural nests, intraseasonal variation in nest temperatures was about 2.5° C, which must partly contribute to the range of development times of 46-86 d in 1997 ($\bar{x}$ 61.7 d, n=18; S. Conroy, unpublished data). Because warmer incubation temperatures promote shorter

development times, less total energy is usually expended in warmer nests, despite higher $\dot{V}O_2$ (Kaplan 1980; Vleck and Hoyt 1991; Booth and Thompson 1991). For example, the metabolic energy used by *B. nimbus* to reach metamorphosis decreased by about 12% when incubation temperatures rose from 10 to 15° C (Chapter 3). Correspondingly, *G. vitellina* froglets emerging more quickly from warm nests should have more energy remaining in the gut than those emerging later from comparatively cool nests. The residual 15.7 J of energy at completion of metamorphosis at 15° C (in 86.6 d) is presumably comparable to that of froglets emerging from cooler natural nests (development completed in 86 d).

Notably 15.7 J is about 11% of the energy in fresh ova, yet endotrophic *Eleutherodactylus coqui* use only 50% of the yolk in fresh ova to develop to a froglet (Packard et al. 1996). The large yolk reserve of *Eleutherodactylus* species is postulated as necessary for sustaining juvenile growth, either because mouthparts are too small for efficient feeding following metamorphosis (Thibaudeau and Altig 1999), or because the neurologic system requires further development to permit efficient prey capture (Packard et al. 1996). Conversely, endotrophs such as *G. vitellina* and *B. nimbus* emerge from a nest as miniature adults, and their comparatively small yolk reserves is most likely a buffer against a range of incubation temperatures and times.

Energy cost of development

The energy cost of development to metamorphosis estimated by respirometry (102 J; Table 4) was almost double that estimated by calorimetry (56 J, discussed above). A source of bias in the respirometric estimate may have an inappropriate choice of *RQ* (0.8). Lipid in amphibian yolk is about 20% of the dry mass (Boyd and Goodyear 1971; Komoroski et al. 1998), glycogen 3-8%, and protein 30-77%, and early embryonic amphibian *RQ*'s range between 0.7 and 1.0, with glycogen and lipids being the major metabolites (Løvtrup and Werdinius 1957; Salthe and Mecham 1974). Protein metabolism is more typical of larval development, especially under conditions of starvation (Løvtrup and Werdinius 1957). Therefore, because the dominant metabolite changes ontogenetically, so too does the *RQ*. However, the error associated with assuming a *RQ* is relatively small; for example an *RQ* of 0.7 (19.5 J LO_2^{-1}) leads to an

estimated energy loss of 97 J, whereas an *RQ* of 1.0 (21.4 J LO_2^{-1}) produces an estimate of 106.5 J.

In contrast to the energy budget at metamorphosis, the estimate of metabolic energy used to reach hatching stage 24 (7 J; Table 4), appears reasonable because the total energy budget of 134 J (22 in body + 105 in residual yolk + 7 J) was similar to the 143.6 J contained in fresh ova. Embryos were generally inactive inside respirometry chambers, as they were constrained with the jelly capsule, but larvae were comparatively active, lashing frequently, especially during and following stirring of chamber water. A flow-on effect of tail lashing may have been greater rates of cutaneous oxygen uptake due to disruption of boundary layers. Therefore a plausible reason for the overestimate of metabolic energy used to complete metamorphosis was abnormally high levels of larval activity over the 2 h measurement period. Accordingly, measured $\dot{V}O_2$ were greater than resting $\dot{V}O_2$ (typical of quiescent incubation in temperature cabinets), and hence the metabolic cost of development to metamorphosis was overestimated - the bomb calorimetric estimate of 56 J is a better approximation. Notably, respirometric and bomb calorimetric methods provide similar estimates of the cost of development of amniotic eggs (e.g. Thompson and Russell 1999), presumably because the presence of the shell prevents disturbance of the embryo during respirometry. In future energetic studies of amphibian larvae it would be advisable to use both methods, and to directly measure *RQ*.

When metabolic comparisons are limited to embryonic stages, to alleviate confounding effects of abnormally high larval $\dot{V}O_2$, we find the cost of development to hatching stage 24 of 0.33 $mLO_2\,mg^{-1}$ at 15° C for *G. vitellina* (Table 4) was virtually identical to the 0.34 $mLO_2\,mg^{-1}$ estimated at the same temperature for *B. nimbus* (Chapter 3). However, these values are appreciably lower than the range of 0.47-0.55 $mLO_2\,mg^{-1}$ determined between 12° and 22° C for the exotrophic Myobatrachid *Pseudophryne bibronii* (Seymour et al. 1991b). Such differences may in part reflect the higher embryonic $\dot{V}O_2$ of the exotrophic species; for example, when standardised as dry mass-specific $\dot{V}O_2$, hatching stage *P. bibronii* consume 1.37 $\mu LO_2\,h^{-1}\,mg^{-1}$ at 15° C (calculated using Q_{10} in Seymour et al. 1991b), a higher rate than *G. vitellina* (1.03 $\mu LO_2\,h^{-1}\,mg^{-1}$) and *B. nimbus* (0.83 $\mu LO_2\,h^{-1}\,mg^{-1}$, Chapter 3). The declining dry mass-specific $\dot{V}O_2$ across these species is associated with an increase in capsule thickness from about 1 mm in

fresh eggs of *P. bibronii* (Seymour et al. 1991a), to 3.15 mm in *G. vitellina* (Table 3), and to 5.75 mm in *B. nimbus* (Chapter 3), a pattern that supports the assertion that the rate of O_2 diffusion through jelly limits $\dot{V}O_2$ of larger amphibian eggs (Seymour 1994; Seymour and Bradford 1995; Packard and Seymour 1997). Although these data are by necessity limited to a few related species, there is nonetheless a trend toward metabolic depression within the endotrophs.

Phylogenetic comparisons of energy densities and production efficiencies

Mean yolk energy density for 50 species of birds was 32.4 kJ g^{-1} (Ar et al. 1987) was significantly greater than that of 26.7 kJ g^{-1} for the eggs of 32 reptiles (Booth and Thompson 1991). Continuing this phylogenetic comparison in Table 5, the mean value of 25.1 kJ g^{-1} for 22 amphibian species was significantly lower ($P < 0.001$, Mann-Whitney U-test) than a revised mean of 27.1 kJ g^{-1} for 42 reptile species. Consequently on a dry weight basis, amphibian eggs have lower energy densities than both bird and reptile eggs. Moreover, the mean egg energy densities of 26.4 kJ g^{-1} for four endotrophic amphibians was significantly higher than that of 18 exotrophic species (24.8 kJ g^{-1}, $P = 0.0048$, Mann-Whitney U-test). This trend gives tentative support to the claim that endotrophic eggs are homologous to evolutionary predecessors of amniote eggs. Further, greater energy densities of endotrophic eggs allow higher energy content without associated increases in yolk volume, and may have therefore been adaptive in containing endotrophic eggs to a size where the oxygen requirements of the embryo could be met by diffusion.

On average, avian eggs develop at temperatures about 5-8° C higher than reptile eggs (Vleck and Hoyt 1991), and hence the consensus from metabolic models is that avian development should be more energy efficient than that of oviparous reptiles (e.g. Booth and Thompson 1991). However, available data is inconclusive; the mean total production efficiency for 50 species of birds of 57% compares to a range of 42.0-79.1% for four species of oviparous reptiles (Table 5). The total production efficiency of cool-developing endotrophic froglets, 56.2% for *G. vitellina* and 61.5% for *B. nimbus,* fall within the range of both birds and reptiles, while gross production efficiencies are similar to a published value for hatchling salamanders (Table 5). Notably, *B. nimbus,* which is incubated at about 8.5° C in natural nests (Chapter 3), develops more

efficiently than *G. vitellina* incubated at 15° C, contrary to predictions of the higher-temperature/greater efficiency model. However, in at least one study, large ova developed more efficiently than smaller ova (Kaplan 1980), and the larger size of *B. nimbus* ova (about twice the volume of *G. vitellina* ova) could account for their greater efficiency. Given that the ovum size of endotrophic amphibians ranges between 0.8 and 4.0 mm diameter (Thibaudeau and Altig 1999), and that natural developmental temperatures span between 8.5° C for *B. nimbus* to at least 25° C for *Eleutherodactylus coqui* (Burggren et al. 1990), the energetics of endotrophic development warrants further analysis.

Table 5. Summary of published data on energy density of yolk and hatchings, and production efficiency of birds, reptiles and amphibians. Energy density is expressed on a dry weight basis, and excludes the shell and membranes of amniotic eggs.

Taxon		*EMG	Energy density (J mg^{-1})		Production efficiency (%)		Source
			Yolk	Gut-free neonate or froglet	Gross	Total	
Birds	50 species		33.4	25.1	63.7	57.0	Ar et al. 1987
Reptiles	32 species		26.9				Booth and Thompson 1991
	Trionyx trianguis	OV		23.5		42.0	†Leschem et al. unpublished
	Emydura macquarii	OV	28.3	23.4	84.7	79.1	Thompson et al. 1999a
	Emydura signata	OV	26.6	23.1			‡Booth, unpublished
	Chelodina expansa	OV	27.8	24.1			‡Booth, unpublished
	Chelydra serpentina	OV		21.4		76.0	Wilhoft 1986
	Eumeces fasciatus	OV	28.6	24.4	66.4	63.8	Thompson and Stewart 1997
	Menetia greyii	OV	28.5		60.0		Thompson and Russell 1998
	Morethia boulengeri	OV	28.8	24.8	57.7		Thompson and Russell 1999a
	Morethia adelaidensis	OV	29.0	25.0	58.1		Thompson and Russell 1999b
	Niveoscincus metallicus	PL	25.1		83.4		Thompson et al. 1999b
	Pseudemoia spenceri	PL	25.7	25.1			Thompson et al. 1999c
	Pseudemoia pagenstecheri	PL	27.5	23.1			Thompson et al. 1999d
Amphibians	*Rana temporaria*	EX	22.0	23.0	51.1		⌡Fauré-Fremiet and Dragoui 1923
	Rana pipiens	EX	25.1				Slobodkin 1969
	Ambystoma maculatum	EX	25.1				Slobodkin 1969
	Desmognathus ochrophaues	EN	27.0				Fitzpatrick 1973
	Triturus vulgaris	EX	24.2				Bell 1977
	Hyla parviceps	EX	24.8				Crump and Kaplan 1979
	Hyla rhodopepla	EX	25.2				Crump and Kaplan 1979
	Hyla cruentomma	EX	25.3				Crump and Kaplan 1979
	Hyla punctata	EX	25.4				Crump and Kaplan 1979
	Hyla rubra	EX	24.8				Crump and Kaplan 1979
	Hyla garbei	EX	25.2				Crump and Kaplan 1979
	Hyla bifurca	EX	24.8				Crump and Kaplan 1979
	Hyla sarayacuensis	EX	24.4				Crump and Kaplan 1979
	Hyla triangulum	EX	24.7				Crump and Kaplan 1979
	Ambystoma opacum	EX	26.3		67.6		Kaplan 1980
	Ambystoma maculatum	EX	25.3		59.7		Kaplan 1980
	Ambystoma tigrinium	EX	25.3		65.3		Kaplan 1980
	Physalaemus pustulosus	EX	22.9				Ryan et al. 1983
	Pseudophryne bibronii	EX	26.3				N.J Mitchell, unpublished
	Bryobatrachus nimbus	EN	26.0	20.6	69.1	61.5	Mitchell and Seymour 2000
	Geocrinia lutea	EN	26.3				Appendix 1
	Geocrinia vitellina	EN	26.4	21.8	61.0	56.2	This study

Production efficiency is defined in Table 3.

*EMG = ecomorphological guild; OV = oviparous, PL = placental, EX = exotrophic, EN = endotrophic

†Cited in Booth and Thompson 1991; ‡cited in Thompson et al. 1999a; ⌡cited in Needham (1931)

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3 Effects of temperature on energy cost and timing of embryonic and larval development of the terrestrially breeding moss frog *Bryobatrachus nimbus*

Abstract

The Australian moss frog, *Bryobatrachus nimbus*, oviposits 4-16 large eggs in terrestrial nests constructed in moss or lichen in sub-alpine regions of southern Tasmania. Nidicolous larvae overwinter beneath snow, reaching metamorphosis without feeding after 395 d, the longest development time known for an endotrophic anuran. However, a few clutches develop more quickly and metamorphose before winter. This study examines the effect of temperature on development time and energy expenditure by measuring temperatures and developmental stages in field nests as well as rates of oxygen consumption ($\dot{V}O_2$), developmental stage, body mass and energy content in the laboratory at three relevant temperatures (5°, 10°, 15° C). Eggs and larvae reared at 5° C differentiated very slowly and their development time far exceeded those in natural nests, but development time at 10° and 15° C averaged 277 and 149 d, respectively, and were shorter than field incubation times. Generally, respiration rates of aquatic hatchlings were low in comparison with other species, but increased with larval age and jumped about 25% higher near metamorphosis when larvae were able to air-breathe. The mean energy density was 26.0 J mg^{-1} for the dry ova, and 20.6 J mg^{-1} for a dry gut-free froglet, and total production efficiency was 61.5%. We developed a model based on the relationships between incubation temperature and $\dot{V}O_2$ to estimate the respiratory cost of development to metamorphosis, the first such study for an amphibian. The cost was 177 J at 15° C, 199 J at 10° C, and at least 249 J at 5° C and we predicted that continual development at 5° C would lead to premature yolk depletion, because it equalled the 249 J contained in fresh ova. Continuously logged field nest temperatures and interpolation of laboratory data provided estimates of development rates, $\dot{V}O_2$, and respiratory energy costs in field nests. Development to metamorphosis required between 185-234 J when larvae overwintered, but completion of metamorphosis before winter saved 123 J. However, the advantage of emergence in warmer months, when conditions are suitable for feeding and growth, may offset the greater energy cost of overwintering.

INTRODUCTION

The moss frog, *Bryobatrachus nimbus* (Anura: Myobatrachinae; Rounsevell et al. 1994) occurs in cloud forest in temperate southwest Tasmania, from rainforest at sea level to alpine heath at 1100 m (Ziegeler 1994). At a sub-alpine site, females deposit 4-16 eggs in terrestrial cavities in moss or other bryophytes (Mitchell and Swain 1996). Breeding occurs from October to February, the eggs hatch after 2-3 months and the larvae develop in a homogenous jelly until metamorphosis after a further 9-11 months. The total development period in excess of 1 year is the longest known for any anuran that is endotrophic, i.e., all where all energy is provided by yolk (Altig and Johnston 1989). Development periods of *Alytes obstetricans* and *Gastrotheca riobambae* are similar (~360 d), but their larvae feed (Duellman and Trueb 1994).

Moss frogs are further distinguished by the large size of their eggs; the ovum diameter is 4 mm and the jelly capsule about 15 mm in diameter, the largest of all the Australian anurans (Mitchell and Swain 1996). Eggs of unusual size merit study for two reasons: they enhance our understanding of the allometric relationships of physiological processes (Seymour and Bradford 1995), and they may represent an adaptation to a particular environment. Many authors have noted the association of large amphibian ova with terrestrial habitats (Lutz 1947; Poynton 1964; Salthe and Duellman 1973; Elinson 1987; Seymour and Bradford 1995; Seymour 1999) and with cold, often montane, environments (Goin and Goin 1962; Bradford 1990).

The large literature on amphibian embryology is necessarily biased toward aquatic breeding species, because they are readily available for study. In general, aquatic environments select for rapid development in often ephemeral conditions (Bradford 1990; Seymour 1999). However, because terrestrial microclimates are more variable, and the selective pressures for rapid development are reduced, conclusions from aquatic studies may not hold for terrestrial eggs. Because variability in incubation temperatures of oviparous reptiles has been linked to offspring fitness traits (e.g. Packard and Packard 1988; Shine and Harlow 1996; Elphick and Shine 1998), it seemed desirable to examine how factors pertinent to larval fitness, such as timing of emergence and energy expenditure, varied with natural incubation temperatures in amphibians. Only a single

study of terrestrial embryos of the Australian frog, *Pseudophryne bibronii*, has demonstrated how energy use, hatchling size and residual yolk content are related to temperature. Eggs from the coolest incubation conditions hatched at a larger size, but the cost was higher yolk consumption over an extended incubation time (Seymour et al. 1991). Aquatic studies are consistent with the finding that large hatchlings are associated with cool development (e.g. Smith-Gill and Berven 1979; Kaplan 1980) and further, have shown that hatching size influences locomotor performance, adult size, age at first reproduction and fecundity (Kaplan 1987; Semlitsch et al. 1988).

This study examines how natural incubation temperatures influence larval fitness of a temperate-zone amphibian with the longest known period of terrestrial incubation. To achieve this, we combine data on field development and nest temperatures with laboratory data on rates of oxygen consumption ($\dot{V}O_2$), development time, and energy expenditure at three relevant temperatures: 5°, 10° and 15° C. The study not only documents the developmental ecology and physiology of a species that is unusual in many respects, but also considers the consequences of larval overwintering to the energy budget of development.

METHODS

Field measurements

Field work was carried out at the 5 ha type locality of *B. nimbus* in the Hartz Mountains National Park, southwest Tasmania, Australia, a poorly drained plateau of sub-alpine heath at 870 m (Rounsevell et al. 1994; Figure 1). Here, the heath is interspersed with patches of moss, lichen and other bryophytes that are used as the nesting substrate. About 80 site visits were made from 1996-2000, between November and March.

Egg masses located in the field were flagged and thereafter examined periodically. Developmental stages were assigned using a simple, 17-stage table (Mitchell and Swain 1996) that used features that could be clearly identified under field conditions. The staging was referenced to equivalent stages in a 37-stage scheme for *Philoria* (=*Kyarranus*) *sphagnicolus* (de Bavay 1993) that mirrors *Bryobatrachus* development.

Fresh eggs (Gosner 1960 stage 12 or earlier) were carefully removed from their nest and ovum diameters were measured with a scale lupe 7x (Peak) pressed gently onto the jelly to prevent refractive errors present in the previous study (Mitchell and Swain 1996). Capsule dimensions were measured with dial callipers. Most eggs were immediately replaced back in the nest, but about 20 clutches intended for laboratory experiments were briefly stored at the field site in moistened tissue, before transportation to a temporary laboratory about 50 km away.

The temperatures of selected egg masses were measured with small loggers fitted with external thermistors (Hobo-Temp, Onset Computer Corporation, Pocasset, USA, or Tinytag/Tinytalk, Gemini data loggers, Chichester, UK). Thermistors were pushed laterally through the nest wall into the centre of an egg mass, and were secured in place with wire. On one occasion we examined thermal stratification through egg masses and moss. We positioned thermistors at the surface and base of three egg masses, and at equivalent depths in adjacent moss. For all measures of egg mass temperature, we simultaneously measured ambient temperature, usually inside a dense bush about 0.5 m above ground level. However, during winter the ambient logger was elevated to 1.2 m above ground level to ensure the thermistor was above the snowline. Loggers were

A)

B)

Figure 1. A) Cloud crossing the plateau at the sub-alpine type locality of *Bryobatrachus nimbus* in the Hartz Mountains National Park, south west Tasmania. B) November snowfall at the type locality.

programmed to record temperatures at intervals ranging from 15 to 70 min, and data were periodically downloaded onto a personal computer. Additional temperature data were obtained from the Australian Bureau of Meteorology weather station 094191, located about 3 km from the study site.

Laboratory maintenance of eggs

Eggs were first cleaned of surface debris by gentle rolling on moist tissue, and ovum and capsule diameters were measured under a stereo-microscope when eggs were fully submerged in water. Each clutch was randomly divided into even groups of 2-3 eggs. Groups were placed in labelled ice-cube trays, then sprayed with water and sealed with plastic wrap. Surplus eggs were dissected to remove the ova, which were dried over silica gel and stored for calorimetry.

Ice-cube cells containing eggs were housed inside one of three foam containers that were fitted with fan heaters. A thermocouple attached at egg height inside each container was connected to a custom-built thermoregulator. Containers were stored inside a large refrigerator (temperature about 4° C) and set to temperatures of 5°, 10° and 15° C. These temperatures were chosen because a pilot experiment showed that the development rate at 10° C roughly approximated those in the field (Mitchell 1995). Temperature loggers (Tinytags) verified that containers were at target temperatures ± 1° C.

After about 2 months, eggs were moved to a laboratory at the University of Adelaide, South Australia. Eggs were in transit for 2 d and were kept cool inside a foam container, before being housed inside three constant temperature cabinets at the university. All eggs were maintained in a fully hydrated state throughout development, and when larvae approached metamorphosis, small pieces of disposable absorbent cloth were used to line the ice-cube cell so that larvae could climb onto the cell wall, a position often observed in field nests (Figure 2; Mitchell and Swain 1996).

Rates of oxygen consumption

Oxygen consumption rates ($\dot{V}O_2$) of embryos or hatched larvae were measured periodically throughout development at 5°, 10° and 15°C. Procedures were as described in Seymour and Loveridge (1991). Rates were determined in water through stages 12-37 by calculating the average rate of decline of oxygen partial pressure (P_{O_2}) inside 0.67 mL respirometry chambers fitted with Clark-type oxygen electrodes (Diamond General models 733, 1231, 1271). Readings of P_{O_2} were made approximately every 10 min for about 2 h, and the water surrounding the embryo or larvae was stirred for 15 s before

Figure 2. Froglets sitting on the wall of nest in remnant jelly

each reading. In addition, after forelimb emergence (stage 34), larval $\dot{V}O_2$ was measured in the same chambers filled with air at 100% humidity.

Four necessary amendments were made to the basic procedure. Firstly, *B. nimbus* eggs were too large to fit into a chamber, so capsules were first partially de-jellied by rolling on dry tissue. Secondly, we measured $\dot{V}O_2$ of water-filled chambers to control for algal or bacterial respiration in the water. These values averaged about 0.18 μL O_2 h^{-1}, and were subtracted from the mean embryonic or larval $\dot{V}O_2$ of relevant experiments. Because the outer layer of jelly was removed, we assumed that jelly did not consume oxygen (Bradford and Seymour 1985). Thirdly, because we were measuring large eggs, we corrected the chamber volume to account for the displacement volume (≅mass) of embryos and larvae. We estimated embryo mass from embryos of the same stage that had been preserved for calorimetry. Fourthly, we had determined the value of P_{O_2} at which $\dot{V}O_2$ became limiting for another experiment, so we only included values of $\dot{V}O_2$ calculated from P_{O_2} values that were not limiting.

Larvae were staged after each experiment, snout-vent length (SVL) and tail length were measured with an ocular micrometer, and wet masses were recorded after briefly blotting larvae on tissue. Briefly, stages 1-17 of de Bavay follow the standard scheme of

Gosner (1960), but diverge thereafter because development of the hind-limbs of Philoria occurs precociously. Stages 21-22 that denote development of external gills in Gosner's scheme do not occur. Three de Bavay stages that will be referred to regularly in this study are stage 25 (hatching from the perivitelline membrane), stage 34 (forelimb emergence) and stage 37 (metamorphosis complete).

Bomb calorimetry

Selected embryos and larvae were killed by freezing and then preserved in Tyler's (1962) fixative before dissection into body and gut. Samples were oven-dried for about 1 h at 60° C and then stored over silica gel before weighing. Samples were then combined into a pellet of at least 25 mg and its energy content was measured with a 1107 semimicro bomb of a Parr 1261 bomb calorimeter after calibration with dry benzoic acid.

Statistical analysis

The only accessible study site of *B. nimbus* was the type locality in a national park, so we were permitted to remove only 20 clutches over 2 years for laboratory experiments. Because clutch sizes were small, we extended our data by measuring the $\dot{V}O_2$ of each individual many times during its development, thereby sacrificing data independence. Furthermore, some animals were removed from the temperature treatments for analysis of body mass and calorimetry and others died from temperature cabinet failure. To maintain numbers we added embryos collected in the field, or larvae salvaged from other laboratory experiments. The thermal history of these additional individuals was variable, but we estimated their effective ages from their stages and incubated them thereafter at constant temperature. Values of $\dot{V}O_2$ and mass that were obtained from additional individuals are distinguished throughout this report. Although the measurements violate statistical assumptions, it was practical to describe the data with means, 95% confidence intervals and polynomial regression equations.

RESULTS

Characteristics of eggs

The 4-16 eggs in a fresh clutch had large pale yellow ova contained in clear, slightly sticky, jelly capsules. Capsule diameters were about 8-10 mm diameter at oviposition, but slowly swelled to reach a maximum size of around 15 mm diameter after a few days (Table 1).

Field incubation times and natural nest temperatures

Metamorphosis (stage 37) was complete in about 395 d (~13 months, Figure 3B), however, two clutches laid in November 1998 had reached stage 35 by March 1999, and probably metamorphosed before winter (Figure 3B). There was negligible development from about May to September when the heath was frequently covered with snow. The nest vegetation appeared to insulate the egg mass from daily extremes of ambient temperature (data not presented), but overall, mean clutch temperatures were similar to ambient temperatures in summer and autumn, but several degrees cooler during winter and spring (Table 2b).

Effect of temperature on development time

Embryos developed normally in the laboratory at all experimental temperatures, and incubation time decreased with increasing temperature (Figure 4). The times to hatching (stage 25) estimated from fitted equations were 169 d at 5° C, 75 d at 10° C and 44 d at 15° C. Mean ages at metamorphosis (stage 37) were 277 d at 10° C and 149 d at 15° C. Eggs incubated at 21° C metamorphosed in 104 d in a pilot study (Mitchell 1995). Hence the Q_{10} according to van't Hoff's principle were 3.46 between 10° and 15° C, and 1.82 between 15° and 21° C. Unfortunately, time to metamorphosis could not be measured at 5° C, because larvae died before stage 35 when a temperature cabinet malfunctioned. For comparison, the mean time to stage 34 was 639 d at 5° C, 263 d at 10° C and 125 d at 15° C.

Table 1. Parameters of *Bryobatrachus nimbus* clutches, fresh eggs, hatchlings (stage 25) and froglets (stage 37)

Variable		Mean	95% CI	Range	*n*	
					samples	clutches
Clutch	Size (number of eggs)	9.1	0.3	4-16		144
Egg	*Capsule diameter (mm)	15.0	1.0	8.0-23.0	56	15
	Ovum diameter (mm)	3.49	0.03	3.0 - 4.7	365	48
	*Ovum volume (μL)	22.5		14.1 - 54.4		
	Wet mass of ovum (mg)	38.7	6.5	23.3 - 63.0	20	8
	Dry mass of ovum (mg)	9.6	0.5	5.7 - 12.1	24	10
	†Water content of ovum (%)	75.2				
	‡Energy density of ova (J mg^{-1})	26.0	0.8	24.7 – 26.9	6	10
	Energy in ovum (J)	249	14	168 - 298	24	10
Hatchling	§Snout-vent length (mm)	5.0	0.2	4.4 - 5.4	11	4
	||Dry mass of gut-free body (mg)	1.6	0.4	1.2 - 2.1	6	3
	||Wet mass of gut-free body (mg)	10.0	44.0	5.0 - 13.8	6	3
	†Water content of gut-free body (%)	84.0				
	||Energy in gut-free body (J)	35	9	26 - 46	6	3
	||Dry mass of residual yolk (mg)	7.0	1.0	5.5 - 8.3	6	3
	||Wet mass of residual yolk (mg)	13.1	2.1	11 - 16.5	6	3
	†Water content of residual yolk (%)	46.6				
	||Energy in yolk (J)	167	26	126 - 198	6	3
Froglet	||Snout-vent length (mm)	7.3	0.1	6.3 - 8.3	74	21
	||Dry mass of gut-free body (mg)	6.0	0.8	3.6 - 9.3	20	6
	||Wet mass of gut-free body (mg)	27.5	3.9	12.9 - 45.2	20	6
	†Water content of gut-free body (%)	78.2	78.2			
	‡Energy density of gut-free body (J mg^{-1})	20.6	3.2	20.1 - 21.0	2	6
	||Energy in gut-free body (J)	123	17	75 - 196	20	6
	||Dry mass of residual yolk (mg)	1.9	0.3	1.0 - 3.0	20	6
	||Wet mass of residual yolk (mg)	4.3	0.7	2.0 - 7.6	20	6
	†Water content of residual yolk (%)	55.8				
	‡Energy density of residual yolk (J mg^{-1})	26.2			1	6
	||Energy in residual yolk (J)	49	8	23 - 79	20	6
	†¶Gross production efficiency (%)	69.1				
	†¶Total production efficiency (%)	61.5				

Except for clutch size, confidence intervals have been calculated from sample standard deviations.

*Calculated from ovum diameter

†Calculated from table values

‡Samples refer to the number of bombed pellets rather than to the 4-20 individuals constituting pellets.
§Measured for eggs at stages 9 to 14

||Data from all temperature treatments has been pooled. Snout-vent length at metamorphosis includes individuals incubated in field nests.

¶Gross production efficiency = (gut-free body + residual yolk energy of froglet)/ovum energy; total production efficiency = gut-free body energy of froglet /(ovum energy – residual yolk energy of froglet) (refer Ar et al. 1987)

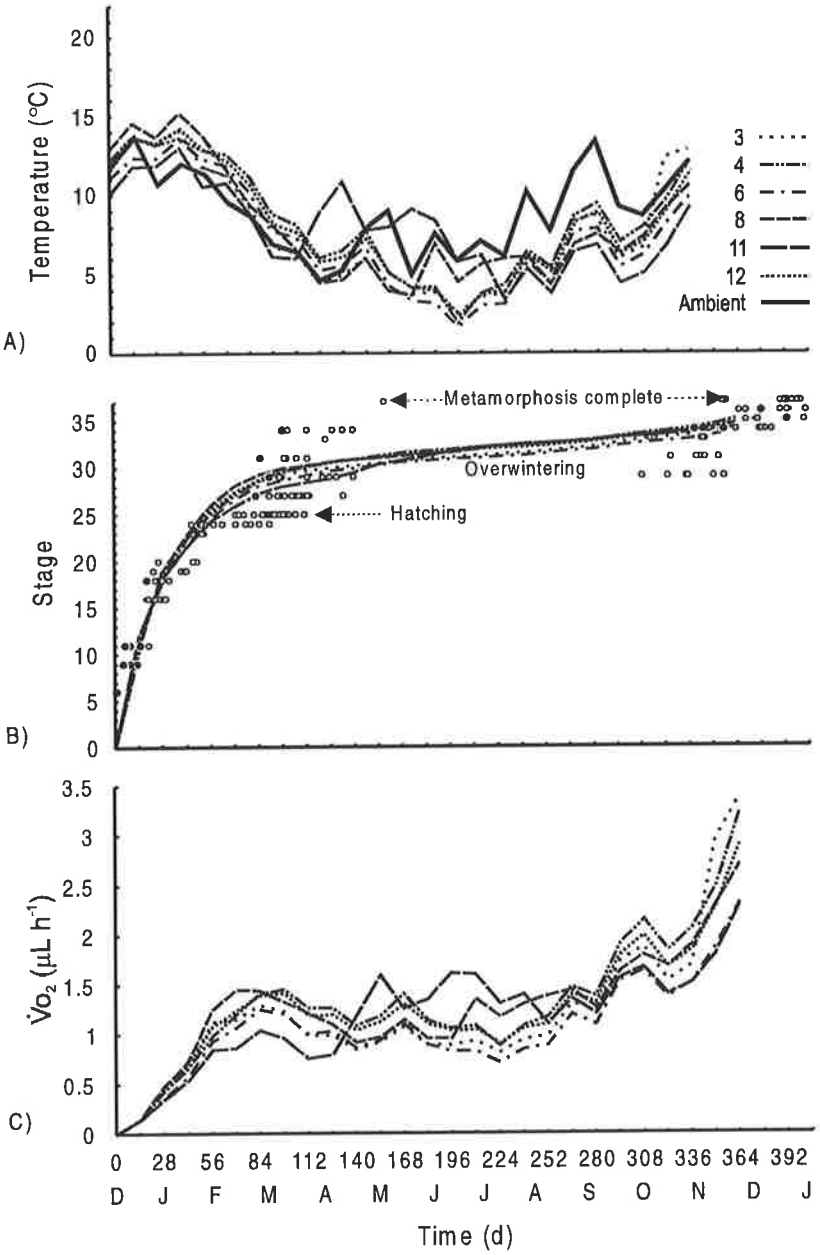

Figure 3. A) Two weekly effective temperatures (T_e) for six nests (broken lines) and ambient temperature (solid line) measured from 15 December 1998 to 15 December 1999. B) Estimated development stage and age of a clutch in each of the six temperature-logged nests (lines), and observed development stage and age of field clutches of *Bryobatrachus nimbus* between 1996-2000 (●○). Clutches found at stage 12 or earlier are denoted by ●; clutches found at later stages and overlaid onto the curve are indicated by ○. C) Estimated $\dot{V}O_2$ of clutches in the six nests each 2-weeks (see text). Zero on the X-axis represents December 15, and was assumed as a common oviposition date for all clutches.

Table 2. Comparison of ambient temperature, egg mass temperature (T) and effective egg mass temperature (T_e)

Logging period	Ambient T (° C)	T (°C)		T_e (°C)		Egg masses (*n*)
		Mean ± 95% CI	Range	Mean ± 95% CI	Range	
Egg masses different groups[a]						
Mid-summer						
(January 2-23 1998)	10.9	12.5±.9	11.0-15.5	13.6 ± 1.0	12.0-16.8	9
Late autumn						
(March 29-May 27 1998)	4.5	4.9 ± 1.3	4.2-6.3	5.0 ± 1.4	4.3-6.4	3
Late spring						
(November 26-30 1998)	5.4	6.9 ±.4	6.0-7.8	7.1 ±.4	6.1-7.9	10
Egg masses same group[b]						
One year						
(December 15 1998-December 15 1999)	9.2	7.6 ±.2	7.0-8.0	8.5 ±.3	7.9-8.9	6
Summer						
(December 15 1998-February 28 1999; December 1-15 1999)	12.1	12.2 ± 1.0	10.5-15.0	13.3 ± 1.1	11.1-16.2	7
Autumn						
(March 1-May 31 1999)	7.1	7.7 ±.6	6.8-9.3	8.7 ±.7	7.2-10.1	8
Winter						
(June 10-August 31 1999)	7.3	4.2 ±.9	2.6-6.6	4.4 ± 1.0	2.9-6.9	7
Spring						
(September 1-November 31 1999)	10.4	6.5 ±.6	5.3-7.6	7.2 ±.7	5.7-8.4	7

Note: Temperature data are divided into seasons. Data were discarded if the thermistor was dislodged from the egg mass, hence *n* differs between time periods.
[a]logged every 15-70 min between 4 and 235 d,
[b]logged every 70 min for one calendar year.

Effect of temperature on $\dot{V}O_2$

Embryonic $\dot{V}O_2$ in water increased with development stage across all temperatures, and the relationships were described by 5th-order polynomial equations (Figure 5). At hatching (stage 25), $\dot{V}O_2$ was 0.55 µL h^{-1} at 5° C, 0.99 µL h^{-1} at 10° C and 1.33 µL h^{-1} at 15° C.

After hatching, $\dot{V}O_2$ flattened slightly between stages 26 - 31 (hindlimb development), before accelerating in stages 32 - 34 (forelimb development) and 35 - 37 (tail reabsorption) (Figure 5). Respiration rates at stage 34 were 1.42 µL h^{-1} at 5° C, 2.24 µL h^{-1} at 10° C and 2.91 µL h^{-1} at 15° C.

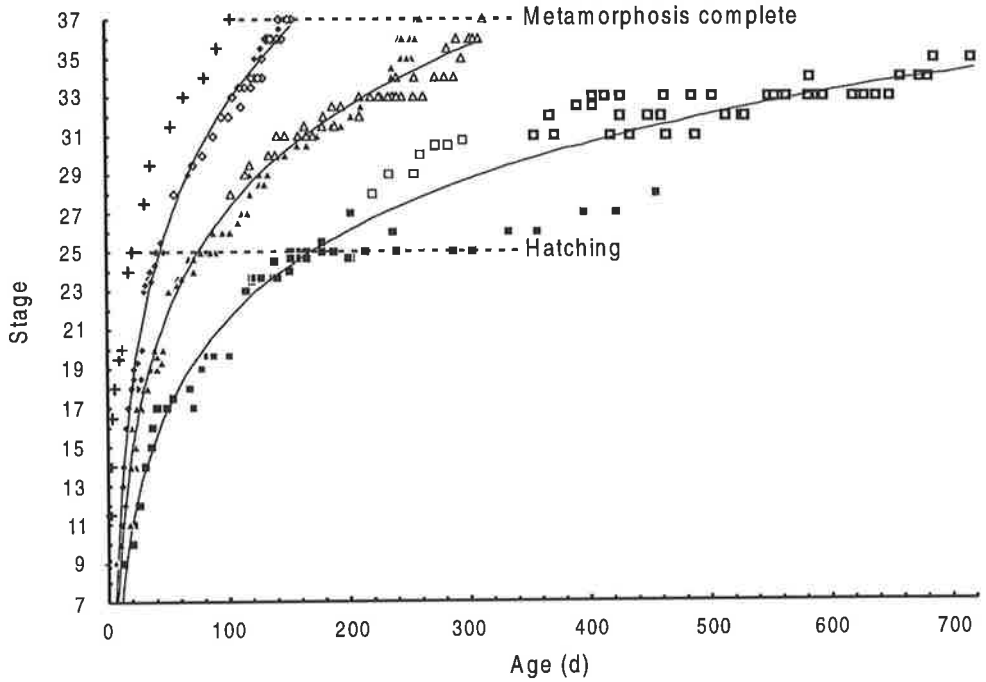

Figure 4. Stage (de Bavay 1993) of development and age of *Bryobatrachus nimbus* during laboratory incubation at 5°C (■□), 10°C (▲Δ) or 15°C (♦◊). Filled symbols (■▲♦) are clutches incubated at constant temperatures; open symbols (□Δ◊) indicate supplementary larvae (see Methods). Plus symbols (+) indicate the development stages of larvae incubated in a pilot experiment at 21° C (Mitchell 1995). Exponential equations were fitted for all data at each temperature. Equations are: 5° C, Age = $e^{(stage+8.4896)/6.5283}$ (r^2=0.95); 10° C, Age = $e^{(stage+8.1454)/7.6654}$ (r^2=0.98); 15° C, Age = $e^{(stage+9.8129)/9.2166}$ (r^2=0.99).

Larvae near metamorphosis (stages 34-36) consumed about 25% more oxygen when measured in air than in water (Figure 5). The means were: 10° C: 2.91 μL h^{-1} in water, 3.63 μL h^{-1} in air, n=26; 15° C: 4.10 μL h^{-1} in water, 5.35 μL h^{-1} in air, n=11.

Masses and energy contents of ova, hatchlings and froglets

The energy density of fresh ova was 26.0 J mg^{-1} and so the total energy in 9.6 mg dry mass was 249 J (Table 1). The dry gut-free carcass at hatching contained about 35 J of energy (14% of fresh ovum). At metamorphosis, the total energy in a dry gut-free froglet was 123 J, which corresponded to 49% of the energy in the fresh ovum.

At hatching (stage 25), larval wet mass was 32.5 ± 3.2 mg (n=12). Overall, larval wet mass, including yolk reserves, was highest at stage 33 (64.4 ± 2.8 mg, n=61), when maximum tail length was reached (14.6 ± 0.4 mm, n=56). Total wet body mass declined

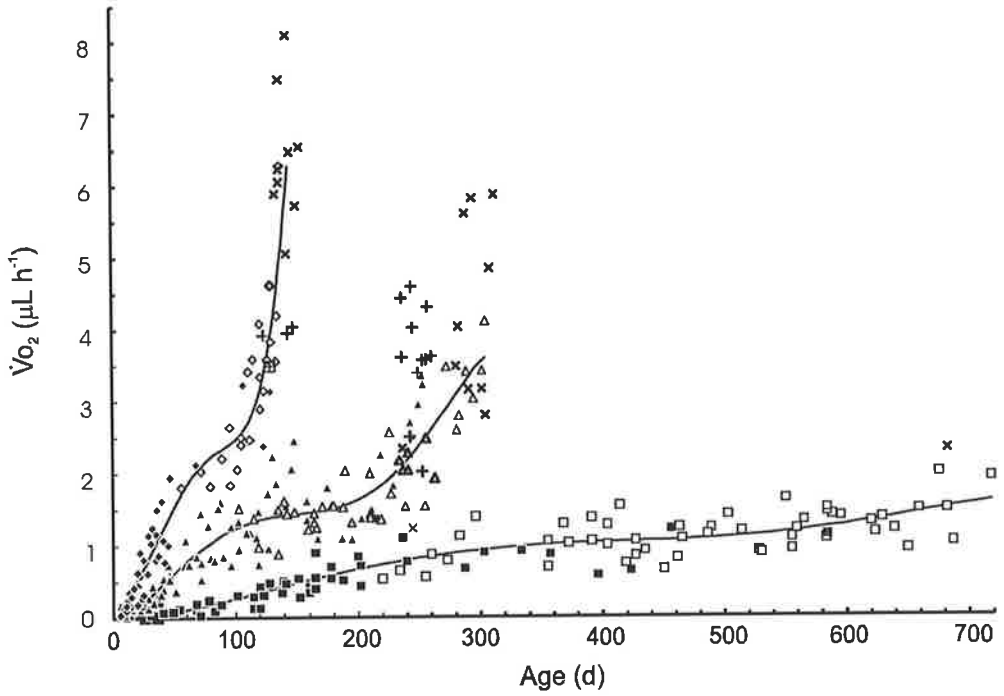

Figure 5. Mean rate of oxygen consumption ($\dot{V}O_2$) for 1-4 *B. nimbus* embryos or larvae from a single clutch, measured in water and air at 5°, 10° and 15°C. The number of clutches measured at each temperature was, respectively, 11, 13 and 11. $\dot{V}O_2$ in water is represented by ■▲♦□Δ◊, as for Figure 4. $\dot{V}O_2$ measured in air is represented by + for larvae incubated at a constant temperature, and x for supplementary larvae. Fifth order polynomials relating age to $\dot{V}O_2$ measured in water were fitted. Equations are: 5° C, $\dot{V}O_2 = -1.16\times10^{-13}a^5 + 2.329\times10^{-10}a^4 - 1.598\times10^{-7}a^3 + 4.063\times10^{-5}a^2 - 7.654\times10^{-5}a$ ($r^2 = 0.80$); 10° C, $\dot{V}O_2 = -2.112\times10^{-11}a^5 + 1.653\times10^{-8}a^4 - 4.239\times10^{-6}a^3 + 3.883\times10^{-4}a^2 + 1.524\times10^{-3}a$ ($r^2 = 0.86$); 15° C, $\dot{V}O_2 = 5.596\times10^{-10}a^5 - 9.851\times10^{-8}a^4 + 1.374\times10^{-6}a^3 + 3.458\times10^{-4}a^2 + 0.019a$ ($r^2 = 0.91$).

to 42.5 ± 4.8 mg (n=21) at metamorphosis, but gut-free dry mass was greatest at this stage (about 6 mg, Figure 6B).

The effects of temperature treatments on mass and size variables at metamorphosis were difficult to determine given that very few individuals reared at constant temperatures survived to this stage. However, the gut-free dry mass of seven froglets constantly incubated at 10° and 15° C was about 4 mg (Figure 6B), and dry yolk mass declined to about 1.5 mg in these specimens (Figure 6A; Table 1).

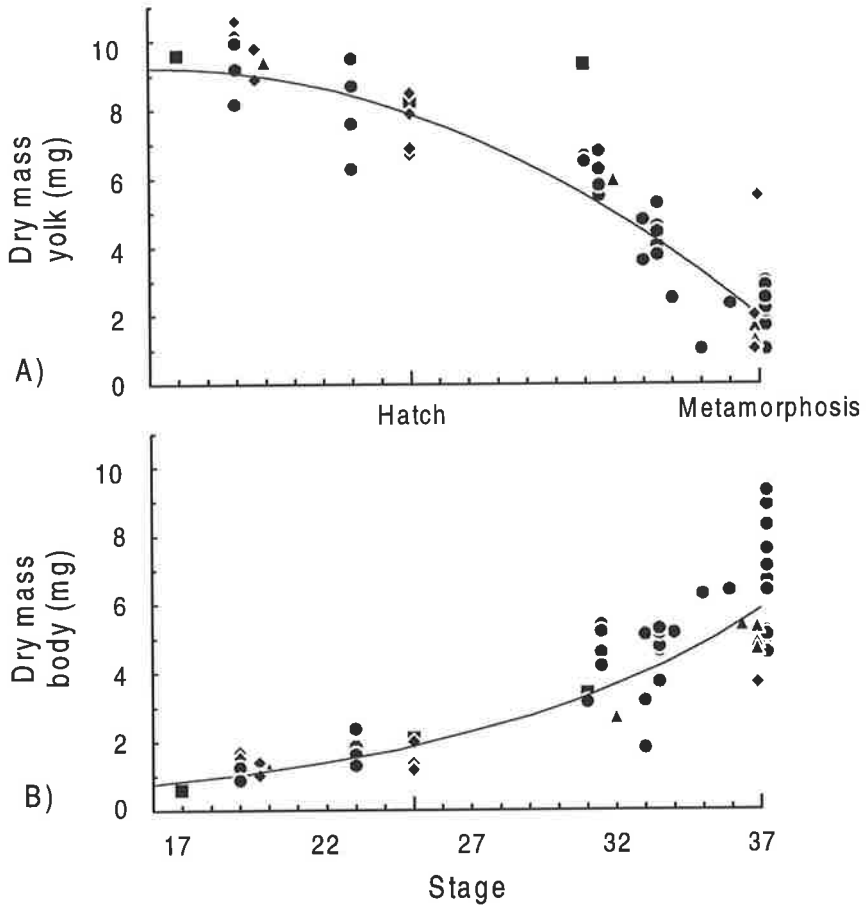

Figure 6. Dry mass of a) yolk, and b) body from stages 17 to 37. Embryos or larvae incubated at constant temperatures are denoted by ■▲♦, for 5°, 10°, and 15°C respectively, and ● denotes supplementary larvae with variable thermal histories (see Methods). Curves have been fitted through all data: mass of gut-free body = $0.1849e^{0.0936 \times stage}$ (r^2=0.84); mass of dry yolk = -0.0165 x $stage^2$ + 0.5309 x stage + 4.982 (r^2=0.88).

DISCUSSION

Effect of temperature on incubation time and differentiation rate

Long incubation and development times are characteristic of larger eggs (Bradford 1990), but the field development time of 395 d for *B. nimbus* eggs is extreme. Overwintering extends development time in this case, because incubation at warm temperatures (15° and 21° C) resulted in far shorter development times (149 and 104 d) than those observed in the field. In contrast, incubation at 5° C, often considered as the lower limit for amphibian eggs (Duellman and Trueb 1994), resulted in very slow differentiation rates and extended the development time beyond the range of field development times to at least 456 days.

Measurements of development rate and $\dot{V}O_2$ under constant conditions in the laboratory permitted calculations of expected rates under variable temperatures in the field. The expected development rate and $\dot{V}O_2$ in the field were based upon measured field temperatures. However, because rates are not proportional to temperature, field temperatures (T) were converted to a constant effective temperature (T_e) with an equation relating $\dot{V}O_2$ to temperature in stage 33 larvae between 5° and 25° C ($\dot{V}O_2 = 0.79883\ e^{0.08149 \times T}$). Thus measured temperatures were converted to $\dot{V}O_2$ values throughout the interval, and then the total oxygen consumed was reconverted into T_e. Our calculated T_e's were generally about 1°C warmer than mean temperatures during summer and autumn, but deviated less from mean temperatures in winter and spring (Table 2). The mean T_e of 6 nests logged for 1 year was 8.5° C. Within a nest, daytime T_e was higher at the surface of the egg mass than at the base, and a similar pattern was evident in the neighbouring moss (Table 3). Conversely, the pattern was reversed overnight, with base moss and jelly usually maintaining a higher T_e. However, stronger daytime temperature stratification may have caused embryos at the base of the egg mass to be at earlier stages than those at the surface (N. J. Mitchell, unpublished data).

Beginning with the nominal oviposition date of December 15, T_e in six nests were calculated at 2-week intervals for a year. For each interval, the change in developmental stage was determined by developmental rate (stage/day), obtained by interpolation between the equations in Figure 4 for development at constant temperature. The

Table 3. Effective temperatures at the surface and base of moss nests and egg masses measured over 57 h in December.

Nest	Substrate moss species	Effective temperature (°C)							
		Moss				Egg mass			
		Day		Night		Day		Night	
		S	B	S	B	S	B	S	B
1	*Dicranoloma robustum*	11.9	11.8	3.9	6.0	9.4	9.1	5.6	6.2
2	*Dicranoloma robustum*	13.2	10.5	4.6	6.0	10.5	9.9	6.9	6.9
3	*Sphagnum australe*	12.8	10.6	5.3	8.0	11.0	10.2	7.5	8.2

S was the temperature just under the moss or egg mass surface; B was the temperature at the base of the moss or egg mass, usually 2-3 cm below.

Day-time was 0530-1929 Australian eastern summer time (AEST); Night-time was 1930-0529 AEST. Mean day-time ambient temperature over the logging period was 8.4°C; mean night-time ambient temperature was 3.1°C.

calculated developmental stages from the six nests were similar to each other and overlapped stage data obtained in the field (Figure 3B). It confirmed the very slow rate of development at cold temperatures during winter.

Effect of temperature on $\dot{V}O_2$

Although $\dot{V}O_2$ measured at 5° C was low, it was substantial and showed that *B. nimbus* embryos and larvae respire at temperatures lower than 5° C (Figure 3). Notably, the 15° C $\dot{V}O_2$ of 1.33 μL h^{-1} at hatching (stage 25) was much lower than the 15° C $\dot{V}O_2$ of 2.8 μL h^{-1} of similarly sized, cold-adapted *Ascaphus truei* hatchlings (Brown 1977). Moreover, the value was below the 95% confidence limits for a regression mean that related $\dot{V}O_2$ at 15° C to ovum volume for 14 amphibian species (Seymour and Bradford 1995), but was within the confidence limits of the sample mean. Therefore, low $\dot{V}O_2$ values for *B. nimbus* do not simply reflect low incubation temperatures, and it is possible that metabolic depression is a feature of protracted development. Yet it is worth noting that the relationship between size and $\dot{V}O_2$ among anurans is not especially tight. For example, the masses of *A. truei* hatchlings were fourfold greater than *Rana tagoi* hatchlings, but $\dot{V}O_2$ of the former was lower (Brown 1977). However, because inert yolk was included in the hatchling mass, a different pattern may have emerged if

gut-free masses were compared. Similarly, *B. nimbus* $\dot{V}O_2$ may be lower than predicted because of the large amount of yolk present at hatching (~7.3 mg dry mass at stage 25, Figure 6a).

The exchange of gases by embryonic and larval *B. nimbus* must be achieved primarily through the skin and well-vascularized tail, at least until stage 32. Stage 32 larvae have been observed in the laboratory with their nostrils emerging from the jelly, and stage 34 larvae in natural nests typically rest vertically on the nest wall with only their tails in jelly. Therefore late stage larvae are apparently capable of gas exchange through both lungs and skin (bimodal breathing). Measurements of $\dot{V}O_2$ at 10° and 15°C in air versus water suggested that bimodal breathing increased $\dot{V}O_2$ by about 25%.

Energy cost of development

The data obtained in this study permit an analysis of the energy cost of development to hatching and metamorphosis (Table 1). The energy density of the dry ovum (26.0 J mg^{-1}) is higher than the mean energy density of 22.3 J mg^{-1} for three fish species (Finn et al. 1991; 1995; 1996), but less than the mean of 26.9 J mg^{-1} for 34 reptile species (Booth and Thompson 1991), and significantly lower than the mean of 33.4 J mg^{-1} from the dry yolks of 35 species of birds (Ar et al. 1987). Similarly the energy density of dry, yolk-free froglets (20.6 J mg^{-1}) is less than 25.1 J mg^{-1} for hatchling birds, but the total production efficiency of *B. nimbus* of 61.5% was slightly higher than the 57% measured for birds (Ar et al. 1987).

Our models of age-specific $\dot{V}O_2$ (Figure 5) allow an estimate of energy cost of development to a specified stage (e.g. Table 4). Larvae consumed more oxygen to reach stages 25, 34 and 37 with decreasing incubation temperature, and so the energy cost of development was greatest at 5°C (Table 4). At hatching (stage 25) the mass-specific cost of development at 15° C was 0.34 $mLO_2\,mg^{-1}$ (0.64 mL O_2 /1.9 mg, Table 4; Figure 6) which was lower than the average cost of 0.52 $mLO_2\,mg^{-1}$ at hatching for *P. bibronii* incubated between 12° and 22° C (Seymour et al. 1991).

Our estimates of total VO_2 appear to be high beyond stage 25, because the total energy budget at metamorphosis (respiratory + somatic + residual yolk) is greater than the

Table 4. VO_2 consumed during development (estimated from curves in Figure 5), and energy cost of development, to three stages: 25 (hatching), 34 (forelimb emergence) and 37 (metamorphosis complete).

	Temperature (° C)	5	10	15
VO_2 (mL O_2)	stage 25	0.90	0.78	0.64
	stage 34	12.14	7.97	4.99
	stage 37	n/a	9.71	8.65
Energy cost of development (J)	stage 25	18.4	16.0	13.1
	stage 34	248.9	163.4	102.3
	stage 37	n/a	199.1	177.4

Energy cost of development assumed that 1 mL O_2 = 20.5 J (Seymour et al. 1991).

energy in fresh ova (Tables 1 and 4). We attribute this discrepancy to the high activity level of larvae during measurement of $\dot{V}O_2$, perhaps induced by regular stirring of the water in respiratory chambers, compared to their development in constant temperature cabinets. In contrast, embryos were minimally disturbed during $\dot{V}O_2$ measurement (because they were still contained in their capsule) and the total energy budget at hatching was similar to ovum energy content. Despite this caveat, the respiratory cost of development to stage 34 of 249 J at 5° C (Table 4) was very high, and equalled the 249 J present in fresh ova (Table 1). Thus it appears likely that continuous incubation at 5° C would deplete yolk reserves before metamorphosis.

Incubation in natural nests

Our calculations of stage at any given T_e (Figure 3B) provided values for constant-temperature 'age', according to Figure 4. These 'ages' were used to estimate field $\dot{V}O_2$ by interpolating between equations from Figure 5. Thus the calculated $\dot{V}O_2$ of field embryos reflected the influences of nest temperature (Figure 3C). The estimates increase quickly for embryos in the warmer months, plateau in overwintering larvae, and increase steeply as larvae approach metamorphosis. The stability of $\dot{V}O_2$ in the face

Table 5. Estimated values of V_{O_2} and energy cost of development to stage 34 for autumn-climax and overwintering clutches.

			Stage 34		
	Time to Stage 37 (d)	T (° C)	Time to stage (d)	VO_2 (mL)	Energy cost of development (J)
autumn climax	160	14.6	124	4.6	94
overwintering	395	8.3	345	10.6	217

Time to metamorphosis (stage 37) was estimated from Figure 3B, and T was estimated using Q_{10} derived from Figure 4 for stage 34 (10°-15° C, 4.6; 5°-10° C, 7.9). Ages from equations in Figure 4 were adjusted to T using appropriate Q_{10}, and then $\dot{V}O_2$ at estimated ages were, similarly, adjusted to T from equations in Table 3. Polynomial equations relating the estimated ages to estimated $\dot{V}O_2$ were fitted and the area under the curve (VO_2) was used to calculate energy cost of development.

of declining T_e from March to July is attributed to slow, but appreciable growth of the larvae.

We used our estimates of $\dot{V}O_2$ to calculate that the mean (and range) of respiratory energy used in a calendar year was 214 J (185 – 234 J), which corresponded to about 86% (74-94%) of the energy in the average egg. These values are obviously too high, for the reasons explained above, but they demonstrate how the differing thermal environments of nests can influence energy expenditure of the egg mass. It was therefore of interest to compare temperature and energy expenditure of froglets that emerged before winter (autumn-climax) with those that overwintered. We estimated that autumn-climax froglets were effectively incubated at 14.6° C, (Table 5). In contrast, froglets that overwintered as larvae were effectively incubated at 8.3° C, which agreed well with the mean T_e of 8.5° C calculated for the six nests logged for one year (Table 2). Because we utilised development rate at 5° C to construct our overwintering models of age and $\dot{V}O_2$, we were only able to estimate energy cost of development to stage 34. However, the values to this stage were strikingly different: 94 J for autumn-climax clutches, compared to 217 J for those that overwintered (Table 5).

Ecological implications of protracted development

Production of large eggs generally lowers fecundity (Salthe and Duellman 1973; Smith and Fretwell 1974), and species with low fecundity, such as *B. nimbus*, are predicted to be the most selective when given a choice between parameters that might influence fitness (Trivers 1972). I have demonstrated that the energy cost of development bears a strong relationship to incubation temperature (Table 4), and as effective incubation temperatures varied by about 5° C in summer (Table 2), it is possible that breeding pairs use thermal criteria to discriminate between potential nest sites, as has been shown for reptiles (e.g. Castilla 1996; Shine and Harlow 1996). Differences in the aspect, elevation and shading of nesting sites contribute to temperature variability, however, a substantial component of annual variability occurs in the coolest months of the year (Figure 3A). Snowfall in Tasmania is heaviest in July and August, (Bureau of Meteorology 1993), but occurs commonly at the study site from March to December (N. J. Mitchell, unpublished). Therefore annual temperature variability must partly depend on what portion of a year a nest is covered by snow. Snowdrift formation at the study site is unpredictable because wind directions are fickle on the exposed plateau (N. J. Mitchell, unpublished), hence chance events might offset any thermal preferences of breeding pairs.

It is worth considering the consequences of completion of metamorphosis before or after winter. Aside from a diminished risk of predation (see Werner 1986), larvae that develop more quickly are less at risk of desiccation. Periods of hot and dry summer weather promote drying of the nest material and dehydration of egg masses, sometimes to the point of larval mortality (N. J. Mitchell, unpublished). Thus froglets emerging before winter may be able to avoid desiccation the following summer by residing in moist environments, whereas larvae that overwintered are trapped in the nest. Conversely, froglets leaving the nest in summer are presumably exposed to better conditions for feeding and growth than those emerging in autumn. While it appears that froglets emerging in autumn would have greater internal energy reserves (Table 5), cooling temperatures may limit food availability and the ability to assimilate it. The result would be that stored energy would be lost during overwintering and they would not benefit from any energetic advantage.

The long development time in *B. nimbus* may be a result of selection in the high latitude, high altitude Tasmanian environment. Such cool conditions would favour developmental processes that result in either rapid metamorphosis in spring and summer, as in many northern hemisphere cold-temperate frogs, or development that delays metamorphosis for over a year. In both cases, the juveniles would be exposed to favourable conditions for feeding and growing. Intermediate rates of development would be selected against, because complete metamorphosis would occur in a less favourable season. Because development rate tends to decrease in larger eggs (Bradford 1990), the large ova characteristic of many Australian Myobatrachines might have forced an adaptive shift into a slow development, low metabolic rate mode, resulting in larger juveniles. It would be interesting to test these ideas by examining *B. nimbus* populations that occur in warmer conditions at lower elevations in their 1100 m distribution.

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4 Constraints on the size of masses of large terrestrial eggs: oxygenation of embryos and larvae of *Bryobatrachus nimbus*

Abstract

The egg capsules of the moss frog, *Bryobatrachus nimbus*, are amongst the largest recorded in a terrestrial breeding amphibian, with a wall thickness of about 4 mm in late-stage embryos. The oxygen requirement of the globular mass of 4-16 eggs is met solely by diffusion, but the large jelly volume creates diffusion distances that may limit respiration of central embryos. I tested this assertion by measurement of the partial pressure of oxygen (PO_2) in field and laboratory egg masses, and inside individual capsules, across a range of natural incubation temperatures. Single embryos incubated in the laboratory between 5° and 20° C maintained perivitelline PO_2 between 10.2 and 17.0 kPa, which were not limiting, but numerical models demonstrated that embryos developing at the sub-alpine study site, where atmospheric PO_2 is lower, would be hypoxic and diffusion limited at 20° C. Moreover, the oxygen supplied to hypothetical spherical masses of 13-21 eggs would be adequate only at temperatures less than 5° C. Mean PO_2 of hatched clutches in moss nests was 18.6 kPa at the surface and 12.6 kPa at the base, at a mean nest temperature of 15.7° C. However, values of PO_2 in an experimental nest submerged in aerated water at 5°, 10°, 15° and 20° C were appreciably lower (5.5-10.6 kPa), but larvae avoided hypoxic conditions by moving to oxygen-rich jelly at the surface and edges of the nest. I suggest several mechanisms by which embryos in the large egg masses avoid hypoxia: 1) the vegetative nest material is porous and photosynthesis supplements PO_2 in daylight, 2), eggs are usually deposited in 1-2 layers and hence embryos either contact air, or the nest wall, 3), embryos have comparatively low rates of oxygen consumption, and 4), *B. nimbus* is restricted to a cool temperate climate.

INTRODUCTION

Oxygen is vital for normal development of amphibian embryos, and extended periods of anoxia can result in retardation or death (Kluge 1981; Seymour et al. 1995; Seymour et al., in press). Uptake of oxygen occurs by diffusion through the jelly capsule along a gradient of partial pressure that is maintained by metabolism of the embryo. The process is described mathematically by the Fick diffusion equation: $\dot{V}O_2 = GO_2 (PO_{2\,out} - PO_{2\,in})$, where $\dot{V}O_2$ is the rate of oxygen uptake by the embryo, GO_2 is the oxygen conductance of the capsule, and PO_{2out} and $PO_{2\,in}$ are the respective oxygen partial pressures of the external environment and the perivitelline space (Seymour 1994). The perivitelline space is defined by a proteinaceous perivitelline membrane that underlies the jelly capsule, and convection within the space is achieved by cilia on the embryo surface, and occasional rotation of the embryo (Salthe 1965; Burggren 1984). The conductance of the capsule (GO_2) depends on the diffusivity of amphibian jelly (KO_2), and the ratio of the effective surface area over which oxygen diffuses (ESA) to the thickness of the capsule (L): $GO_2 = KO_2 (ESA/L)$ (Seymour 1994).

Capsule geometry changes ontogenetically; the perivitelline space enlarges as water moves from the jelly across the semipermeable perivitelline membrane, and increases the inner capsule radius, r_i. Ultimately the capsule becomes larger and thinner, which increases GO_2, and facilitates the increasing $\dot{V}O_2$ of the embryo. This process has been verified experimentally in single eggs of the frog *Pseudophryne bibronii*; PO_2 of the perivitelline fluid ($PO_{2\,in}$) was maintained between 16-19 kPa throughout embryogenesis (Seymour and Bradford 1987). Sensitivity analyses have shown that $PO_{2\,in}$ is critically dependent on r_i, but depends less on the outer capsule radius, r_o. For example, reducing r_i by 35% in a theoretical egg caused anoxia, whereas a 500% increase in r_o was required to achieve the same effect (Seymour 1994). This led to the prediction that provided r_i was sufficiently large, the capsule of an amphibian egg could be many centimetres thick without an embryo becoming hypoxic. However, in practice, increasing r_i would produce an egg with less structural integrity, especially close to hatching when the perivitelline membrane is stretched and weakened by enzymes produced by the embryo. Discounting eggs that develop within the body of the parent (e.g. *Gastrotheca cornuta*), the largest ova of extant amphibians are about 8 mm diameter (Duellman and Trueb 1994), similar in size to the smallest reptile (amniotic)

ova (Thompson and Russell 1988). Hence oxygen and structural constraints appear to have limited the size of embryos that rely solely on diffusion (Seymour and Bradford 1995; Packard and Seymour 1997).

Amphibian embryos rarely develop in isolation, and can occur in masses of up to 20000 individuals in some *Bufo* species (Howard 1978). The diversity of egg mass design is notable, and can be interpreted as different solutions for the prevention of hypoxia. For example, the foamy egg masses of *Limnodynastes tasmaniensis* are perforated with air spaces that space embryos, provide oxygen sources within the mass, and cause the mass to float at the water surface where oxygen concentrations are high (Seymour and Roberts 1994). Other large masses, such as those of *Rana palustris* and *R. sylvatica*, rely instead on water convection through interstitial channels, and must float beneath the water surface for water to exit the top of the mass (Burggren 1985; Seymour 1995). Eggs of some salamander species (e.g. *Ambystoma maculatum*) are bright green due to the presence of symbiotic chlamydomonad algae that provide a positive net oxygen supply due to photosynthesis (Bachmann et al. 1986). However, globular egg masses are restricted to a size where the metabolic requirements of embryos in the centre or bottom of the mass are not limited by diffusion (Burggren 1985; Seymour 1994; Seymour et al. 1995; Strathmann and Strathmann 1995). For example, about one-third of the average 33 eggs in the globular masses of *Philoria (=Kyarranus) loveridgei* die because PO_2 at the base of the soil nest falls below the critical limit. Other *Philoria* species are foam nesting, but *P. loveridgei* has apparently lost the habit, and natural selection should act to produce smaller clutches (Seymour et al.1994).

The suggestion that the requirement for oxygen constrains the size of globular egg masses has been examined in marine invertebrates (Chaffee and Strathmann 1984; Strathmann 1995; Lee and Strathmann 1998; Strathmann and Hess 1999) and amphibians (Seymour and Roberts 1991; Seymour and Loveridge 1994; Seymour et al. 1994). However, conclusions arising from these models were confined to a single temperature, whereas masses typically develop at a range of temperatures. Given the large influence of temperature on embryonic $\dot{V}O_2$ (Seymour and Bradford 1995), it seems pertinent to incorporate temperature as an input into a model. Moreover, published models largely concern egg masses developing in water. However, constraints of terrestrial masses that adhere to a substrate, and hence may receive oxygen only from one direction, require further analysis.

The jelly capsules of the Australian moss frog, *Bryobatrachus nimbus* (Anura: Myobatrachinae) are amongst the largest recorded for any amphibian (Mitchell and Swain 1996), with an outer diameter of 15-20 mm when eggs are fully hydrated (Chapter 5). Because the species is confined to cool climates in southwestern Tasmania, we might speculate that warmer temperatures would limit respiration of the large embryos. This idea was tested using a numerical model that utilised data on the effect of temperature on the $\dot{V}O_2$ of *B. nimbus* embryos (Chapter 3) to identify temperatures likely to cause hypoxia in unhatched clutches of various sizes. After hatching, jelly capsules disintegrate and bathe the nidicolous larvae, and larvae can use behavioural means to avoid hypoxic conditions. Field observations, coupled with a suite of laboratory experiments at four temperatures (5°, 10°, 15° and 20° C) demonstrated several mechanisms by which masses of embryos and larvae avoid hypoxia, and, in particular, argued for the selection of appropriate nest sites.

MATERIALS AND METHODS

Study site

The study site of *Bryobatrachus nimbus* was 5 ha of sub-alpine heath on an exposed plateau 870m above sea level in the Hartz Mountains National Park in southern Tasmania, Australia. The site is described in detail in Chapter 3. Field data were collected between March 1997 and March 2000.

Measurement of clutch parameters

In addition to clutch parameters collated in an earlier study (Chapter 3), I also recorded arrangement of embryos in a nest (number of embryos per layer). All embryos whose jelly capsules were at least partially at the surface of the egg mass constituted the surface layer, and the number of embryos in underlying layers was determined by careful excavation. The surface dimensions and depth of jelly in clutches between de Bavay (1993) stages 7 and 35 were measured with dial callipers. Two clutches of embryos near hatching stage were removed from the nest with a plastic spoon and the diameter of the perivitelline membrane was measured with callipers while embryos were submerged in a petri dish. An ocular micrometer was used for opportunistic measurement of perivitelline membrane diameters of submerged embryos incubated in laboratory experiments.

Field measurement of clutch PO_2

The PO_2 at standard depths at the centre of field clutches was measured twice: in December when eggs were fresh, and in March when most clutches had hatched. On both occasions PO_2 was measured 1, 2.5, 5, and 12 mm from the jelly surface, and at the nest base, using a Diamond General model 733 Clark Style microelectrode (tip diameter 3 mm) connected to a Diamond General Microsensor II Picoammeter (1271). PO_2 was measured at each depth three times, at slightly different positions at the centre of the mass, after gently wiggling the electrode for 30 s to promote equilibration. Jelly temperature was measured after each pass through jelly with a Fluke model 52 thermometer. The electrode was calibrated with a PO_2-zero solution (Tucker 1967) and with air-saturated water before and after each pass through the jelly, to measure electrode drift. Air-saturated water was produced by vigorously shaking creek water in a small vial that was kept cool by partial burial in peat. Because water temperatures were

sometimes dissimilar to jelly temperatures, I used a linear equation relating water temperature to PO_2 at air equilibration to estimate PO_2 of air-equilibrated water at a particular jelly temperature. Jelly PO_2 was calculated assuming linear electrode drift.

Field measurement of larval lashing rate

Tail lashing of hatched larvae was often observed while examining nests. This behaviour was quantified for 15 clutches in February and March 1999 by counting each time any larva moved over a 5-min period. Clutches containing a volume of clear jelly were selected and observed while shaded. The temperature at the centre of each clutch was measured with a Fluke model 52 thermometer before and after each observation period.

Measurement of critical PO_2

Aerobic metabolism of amphibian embryos and larvae is possible under a range of environmental PO_2, but $\dot{V}O_2$ is depressed below a critical PO_2. The critical point is determined by plotting sequential measures of an individual's $\dot{V}O_2$ as PO_2 declines inside a sealed chamber, and identifying where the slope changes abruptly. Pre-hatching *B. nimbus* embryos were unavailable, hence their critical points were represented by those determined for larvae between stages 28-33, at five temperatures (5°, 10°, 15°, 20° C, and 25° C).

The procedure for the measurement of $\dot{V}O_2$ was identical to that described in Chapter 3, except that larvae were kept in the chamber until PO_2 approached 0 kPa. The same seven larvae from two clutches were used at each temperature, and data were combined for early-stage larvae (stages 28-29) and late stage larvae (stages 31-33) so that mean $\dot{V}O_2$ could be calculated for each 1 kPa increment of PO_2. The computer program DRNP53.bas (Duggleby and Ward 1991) was used to estimate the parameters y_T, x_T, m_L, and m_R and their standard errors from the equation:

$$y = y_T + \left[(m_L + m_R)(x - x_T) - (m_L - m_R)\left|x - x_T\right|\right]/2$$

where y is $\dot{V}O_2$, x is PO_2, y_T is the $\dot{V}O_2$ and x_T the PO_2 at the critical point, and m_L and m_R are, respectively, the slopes to the left and right of the critical point. Standard deviations for $\dot{V}O_2$ at each PO_2 increment were included in the model. In several cases m_R was set at zero to improve the fit.

Laboratory measurement of perivitelline PO_2 near hatching

The PO_2 gradient through the jelly capsules of three pre-hatching embryos was measured at each of four T_a (5°, 10°, 15° and 20° C). Each embryo was contained inside a custom-made chamber constructed from Perspex and perforated stainless steel (Figure 1) and submerged in a shallow aquarium of aerated water pumped through a Braun 850 constant temperature water bath. Embryos equilibrated for 1 d at each temperature before measurement of PO_2.

A micro Clark Style oxygen electrode (Diamond General 737GC, tip diameter 75 μm) with a polarizing voltage of -0.75 V (Diamond General 1271) was held in a micromanipulator and moved vertically through the capsule at 1 mm increments until the electrode pushed against the perivitelline membrane (Figure 1). At this point, the PO_2 was considered equivalent to the PO_2 of the perivitelline fluid ($PO_{2\,in}$). Electrode currents were recorded after allowing 30 s for equilibration. The electrode was calibrated at temperatures the same as those in the jelly with PO_2-zero solution, and before and after each transect through a capsule with air-equilibrated water at the surface of the water bath. The procedure was repeated three times for each embryo and PO_2 was averaged at each depth. A single vertical transect was made at similar depths in the aquarium water at each T_a for comparison.

The entire procedure was repeated 1 week later when embryos reached stage 24. One embryo died in the interim, and was replaced by a spare stage 24 embryo.

Effect of temperature on jelly PO_2 and larval behaviour

Because larvae were difficult to observe in field nests, I measured effects of temperature on larval behaviours and jelly PO_2 in the laboratory. Four experimental temperatures, 5°, 10°, 15° and 20° C were established using the same water bath and flow-through aquarium described earlier. A single clutch of 12 larvae, collected from the field at stage 29 in March 1999, was carefully transferred to an experimental nest that was suspended in the water bath. The nest consisted of gauze sewn on to a U-shaped frame of plastic-coated wire that was inserted at right angles into a larger U-shaped piece of Perspex (Figure 8). The Perspex walls provided a clear view of larvae, while the gauze contained the jelly in the nest but allowed oxygen to diffuse into the jelly from the aerated water in the bath. The water level was set 5-10 mm below the jelly surface (see Figure 9) to create a slight thermal gradient through the jelly, mimicking the

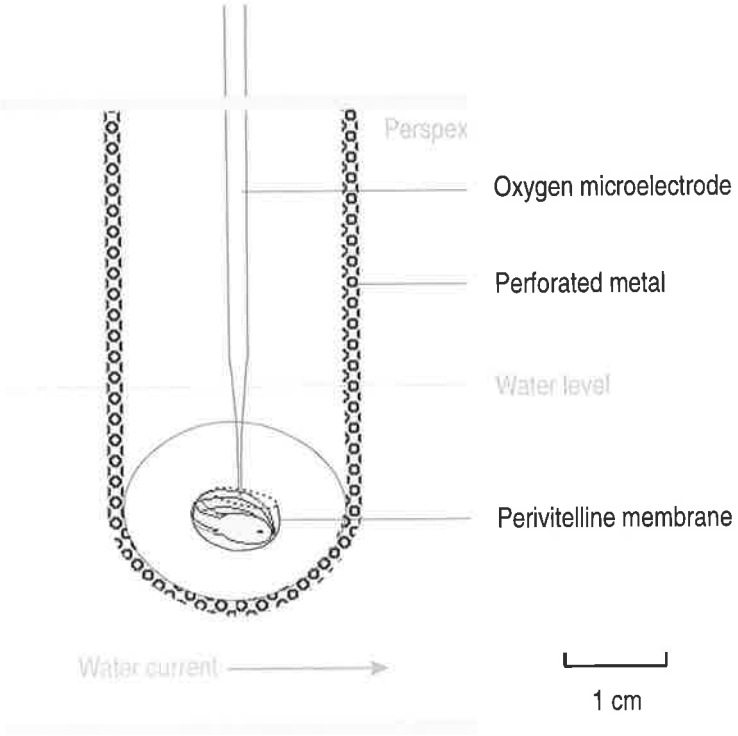


Figure 1. Apparatus for measurement of capsule PO_2. The embryo is contained in an aerated water bath within a chamber of perforated metal and Perspex, and an oxygen microelectrode moves incrementally through the capsule until it approaches the perivitelline membrane.

temperature stratification measured in field nests (e.g. Table 4, Chapter 3). The water bath was set for a minimum of 1 d at each experimental temperature before measurements commenced.

A video camera mounted above the clutch recorded larval activity in the nest for 15 min. Immediately afterward, three PO_2 transects were made through the centre of the clutch, at 2 mm increments, using the microelectrode and micromanipulator described earlier. PO_2 transects were also made at set depths (5, 10 and 15 mm) at four edges of the nest, two adjacent to the Perspex walls, and two adjacent to the gauze walls. PO_2 was averaged at each depth at the centre of the clutch, and at each point by the nest wall. Temperatures at the top, middle, and base of the clutch, and water bath temperatures were recorded, and positions of individual larvae were drawn as viewed from the side and above the nest.

Larvae were at de Bavay (1993) stage 30 for the first set of experiments at each temperature. The entire procedure was repeated one week later when larvae were at stage 31, and again two weeks later when larvae had reached stage 32. Two larvae died at stage 31, which reduced clutch size from 12 to 10 for the final set of experiments. Further, about 20% of the jelly was lost from the cradle before the second experiment at 20° C. Consequently, jelly volumes and surface areas were lower in subsequent experiments.

Video footage was captured using a frame-grabbing board (Imaging Technologies PC-Vision Plus) and analysed with the program MTV (Data Crunch). Relevant dimensions of the jelly surface were measured to calculate its area and circumference, and X and Y co-ordinates of the centre of each larva's body were recorded at the start of filming. The number of tail lashes made by individual larva during the 15 min period was then determined, while the duration of each bout of tail lashing was measured in real time with a stopwatch.

Statistical analysis

Most laboratory measurements and experiments were confined to two clutches collected in 1999, in accordance with permit requirements. Consequently, some laboratory data are not independent. Most data are expressed as means ± 95% confidence intervals about the mean, and statistical significance was assumed at $P \leq 0.05$. Two statistical tests were used to determine whether larval distributions were random or clumped during the laboratory experiment on the effects of temperature on larval behaviours. Both tests [Clark and Evan's (1954) test with the Donnelly (1978) modification, and Thompson's test (1956)], described in Krebs (1999), compared observed nearest neighbour distances (r_i) to expected distances to the nearest neighbour r_e, where $r_e = 1/(2\sqrt{\rho})$, and ρ = number of larvae/surface area of the jelly. Other data were compared with paired t-tests or analysed with linear regression.

RESULTS

Characteristics of clutches in the field

Freshly deposited clutches appeared as clusters of small eggs at the nest base, with most eggs not contacting the nest wall. Over subsequent days, the jelly capsules absorbed water and eggs expanded outwards and upwards toward the lip of the nest. Hence the horizontal dimensions of a nest ultimately determined the number of layers of embryos in a clutch; for example, narrower nests produced a taller stack of embryos. However, two layers of embryos was the most common arrangement (85% of nests, $n = 65$), while a minority of nests housed a single layer (9%), or three or more layers (6%) of embryos. All eggs in sub-surface layers contacted the walls of the nest. Of the average 9.1 ± 0.6 embryos in a clutch (4-16 eggs), 6.5 ± 0.4 (3-11) were at the surface, and 2.7 ± 0.5 (0-9) below. Larger clutches were more likely to be multi-layered (r=0.49, $P < 0.01$).

The volume of jelly in a clutch varied appreciably at each development stage, but gradually declined after hatching, and near metamorphic climax jelly was reduced to a smear that coated the nest base and walls (Figure 2). The surface of fresh clutches was

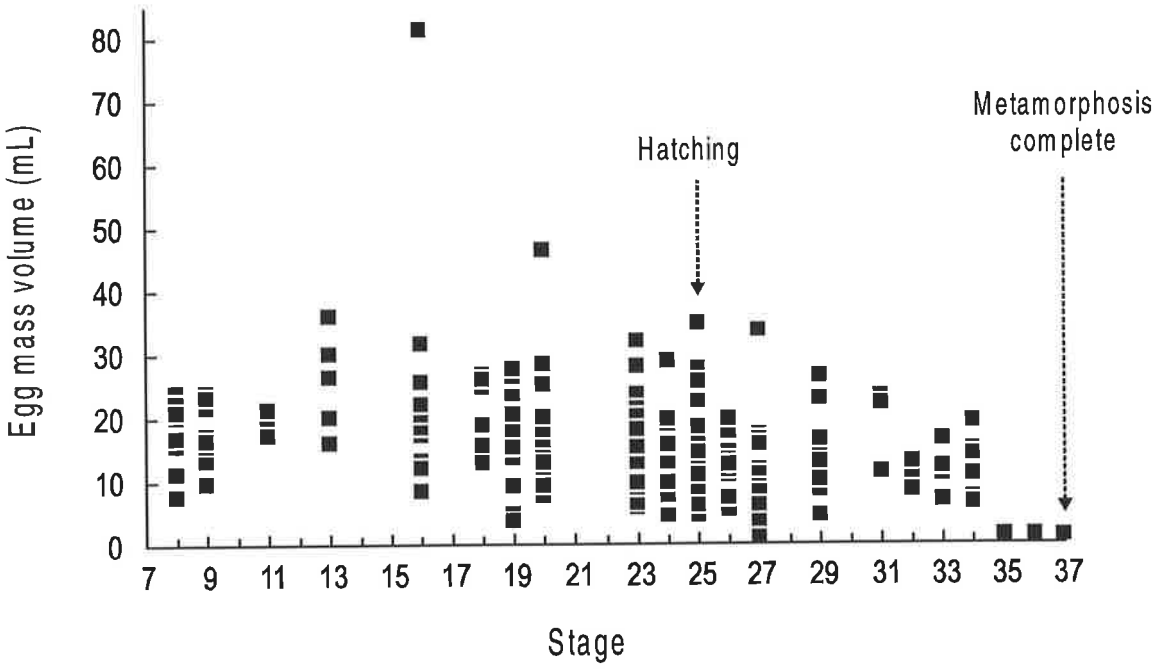


Figure 2. Clutch volumes measured in field nests related to developmental stage. The outlier at stage 16 was a narrow deep nest of 16 embryos in waterlogged moss.

Table 1. Characteristics of fresh clutches (stages 7-13), and pre-hatching clutches (stage 23-24) of *Bryobatrachus nimbus* from the Hartz Mountains National Park.

Parameter	Fresh clutches (n=37)		Pre-hatching clutches (n=25)	
	Mean	95% CI	Mean	95% CI
Diameter a (mm)	36.6	± 1.95	34.9	± 1.23
Diameter b (mm)	34.4	± 1.63	34.3	± 1.30
Depth at centre (mm)	27.3	± 1.90	26.6	± 4.64
*Volume (mL)	19.34	± 1.92	15.49	± 2.79
Number of eggs	9.4	± 0.73	10.0	± 0.95
Density (eggs/embryos mL^{-1})	0.53	± 0.07	0.76	± 0.14

Diameters *a* and *b* are perpendicular axes at the surface of the egg mass

*Volume of eggs and jelly, calculated assuming that the eggs mass is a segment of a sphere ($V = \frac{1}{3}\pi h^2(3r-h)$, r = (diameters a+b)/4), h = depth at centre.

Table 2. PO_2 at the surface, 5mm below the surface, and base of fresh and hatched clutches in natural nests.

		PO_2 (kPa)			
	Jelly temperature	Jelly surface	5 mm below	*Nest base	*n*
Fresh clutches	13.4 ± 2.7	19.1 ± 0.7[a]	18.6 ± 0.8[a]	17.5 ± 0.9[b]	*11*
Hatched clutches	15.7 ± 0.9	18.6 ± 0.7[a,b]	13.1 ± 0.2[c]	12.6 ± 1.6[c]	*17*

Different letters indicate means that are statistically different, as compared with paired t-tests.

Fresh clutches were between de Bavay (1993) stages 7-13; hatched clutches were at stages 25-31.

*Average depth at the base of clutches was 29.0 ± 3.1 mm for fresh clutches, and 17.2 ± 3.4 mm for hatched clutches.

lumpy (e.g. Figure 3), but became smoother as jelly viscosity decreased toward hatching. Jelly volumes were about 19.3 mL in early embryonic stages, and declined to about 15.5 mL before hatching, which increased embryo density (Table 1).

Jelly PO_2 at the surface of egg masses was usually greater than atmospheric PO_2 of 18.1 kPa at 870 m, which suggested that jelly was supersaturated with oxygen produced by the nest vegetation. There was a slight decline in PO_2 from a mean of 19.1 kPa at the top

Figure 3. Two *Bryobatrachus nimbus* clutches in adjacent nests at stages 17 (right) and 11 (left)

of a fresh clutch to 17.5 kPa at the bottom (Figure 4), but after hatching there was significant PO_2 stratification through a jelly mass (Figure 4, Table 2). At 5 mm depth in hatched clutches, jelly PO_2 averaged 5.5 kPa lower than the 18.6 kPa measured at the jelly surface, and only slightly higher (0.5 kPa) than PO_2 at the nest base. PO_2 near the centre of the clutch (5 mm depth) decreased with increasing developmental stage, but the relationship was not significant (r=0.25; P=0.31).

Observations of 15 hatched clutches between stages 25 and 30 revealed that larvae lashed frequently; on average there were 17.5 ± 8.2 movements of larvae in 5 min (range 0 – 43) when jelly temperatures were 15.6 ± 1.4° C (range 11.0-18.6° C). The number of movements was not related to jelly temperature (r=0.10; P=0.72) or to the number of larvae in a mass (r= 0.28; P = 0.31; data not presented).

Table 3. Critical PO_2 of early and late-stage larvae determined from DRNP53.bas (Duggleby and Ward 1991)

Temperature	Critical PO_2 ($\bar{x}$ ± s.e.)	
	Stage 28-29 larvae	Stage 31-33 larvae
5	3.68 ± 0.88*	no fit
10	3.63 ± 0.69*	6.90 ± 1.07*
15	6.81 ± 2.14*	6.06 ± 1.57*
20	8.42 ± 0.98	7.47 ± 1.29
25		8.99 ± 1.23*

*indicates when m_R was fixed at 0

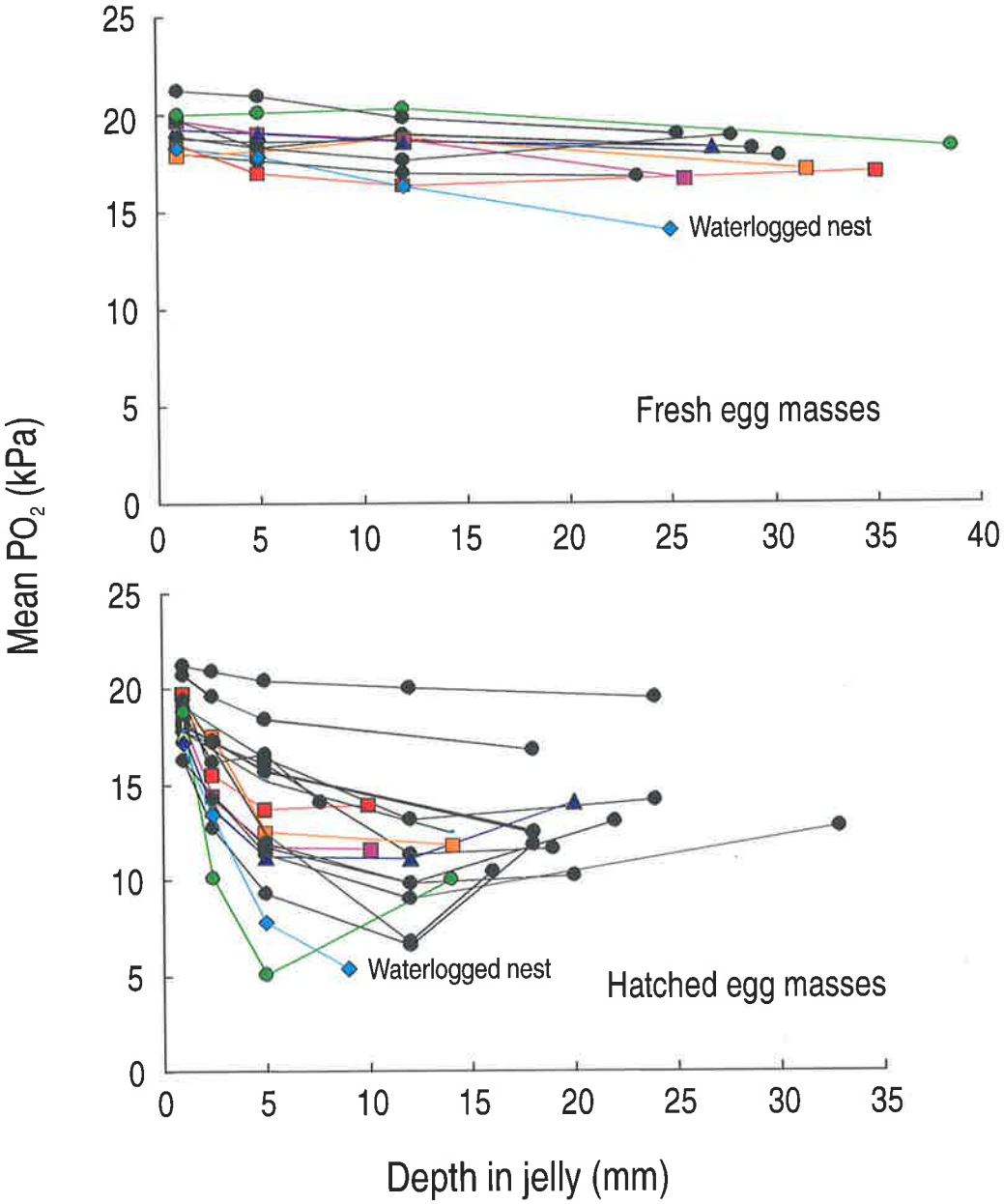


Figure 4. PO_2 profiles through fresh (stages 7-16) and recently hatched (stages 25-26) clutches in the Hartz Mountains National Park. Coloured symbols and lines indicate the PO_2 of clutches measured on both occasions.

Critical PO_2 of larvae

The PO_2 at which larval $\dot{V}O_2$ became limited varied between 3.63 kPa for early-stage larvae at 10° C, to 8.99 kPa for late stage larvae at 25° C (Table 3; examples of larval $\dot{V}O_2$ in response to declining PO_2 are shown in Figure 5.). Generally there was a trend toward higher critical PO_2 at warmer temperatures.

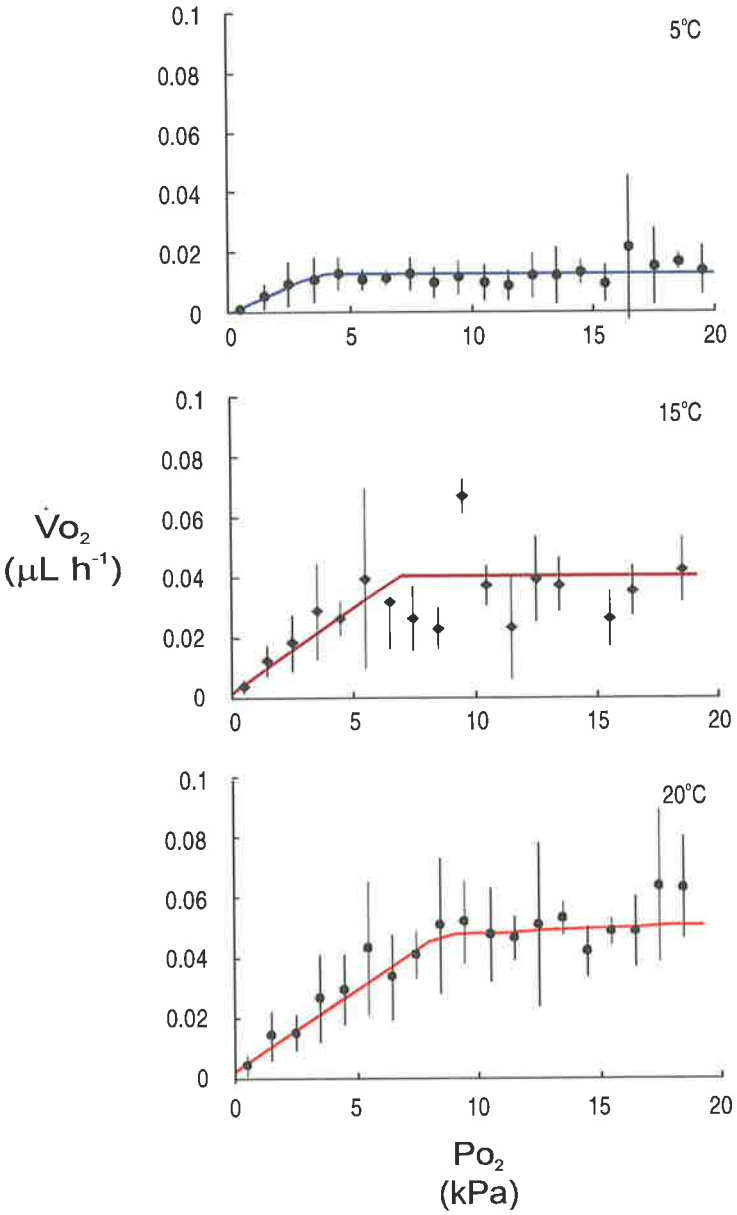


Figure 5. Examples of the relationship between $\dot{V}O_2$ and PO_2 of larvae in sealed respirometry chambers: a) 5° C, larval stages 28-29, b) 15° C, larval stages 31-33, and c) 20° C, larval stages 31-33. Points are mean $\dot{V}O_2$ at each 1 kPa increment of PO_2, and bars are ± 1 standard deviation. Lines and critical points are those estimated using the program DNRP53.bas (Duggleby and Ward 1991).

Perivitelline diameters, PO_2 through capsules of isolated embryos, and $PO_{2\,in}$

The average diameter of the perivitelline membrane of late-stage embryos (stages 23-24) was 6.61 ± 0.29 mm (n=45, field and laboratory data combined). Values of PO_2 measured near to the perivitelline membrane ($PO_{2\,in}$) varied between individual embryos and were lower at stage 24 than at stage 23 (Figure 6). However, in general, $PO_{2\,in}$ declined with increasing temperature (Table 4). The relationship was described by a linear equation: $PO_{2\,in} = 19.705 - 0.474 \times T$ ($r^2 = 0.74$, $P < 0.0001$), where T is the temperature of the water bathing the embryo.

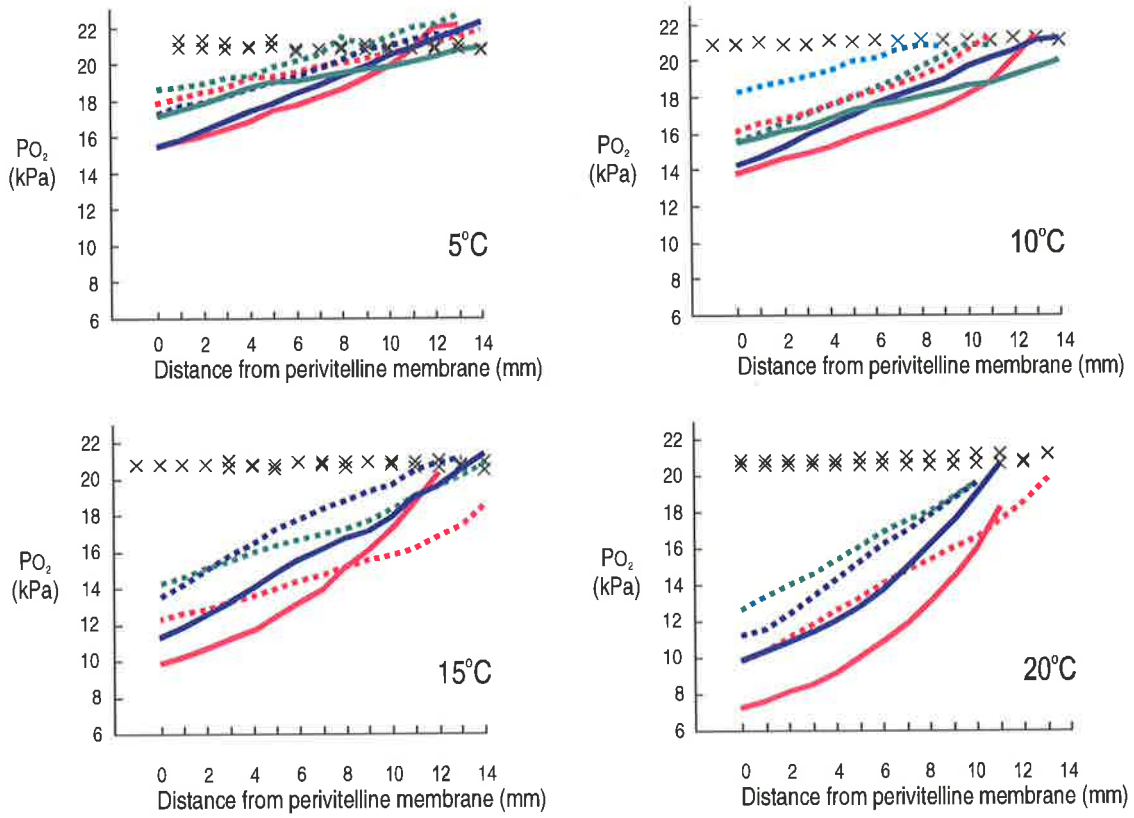


Figure 6. PO_2 transects through the jelly capsules of isolated embryos (lines) and the adjacent water column (x). Different coloured lines represent different embryos; dashed lines are embryos at stage 23, solid lines are embryos at stage 24.

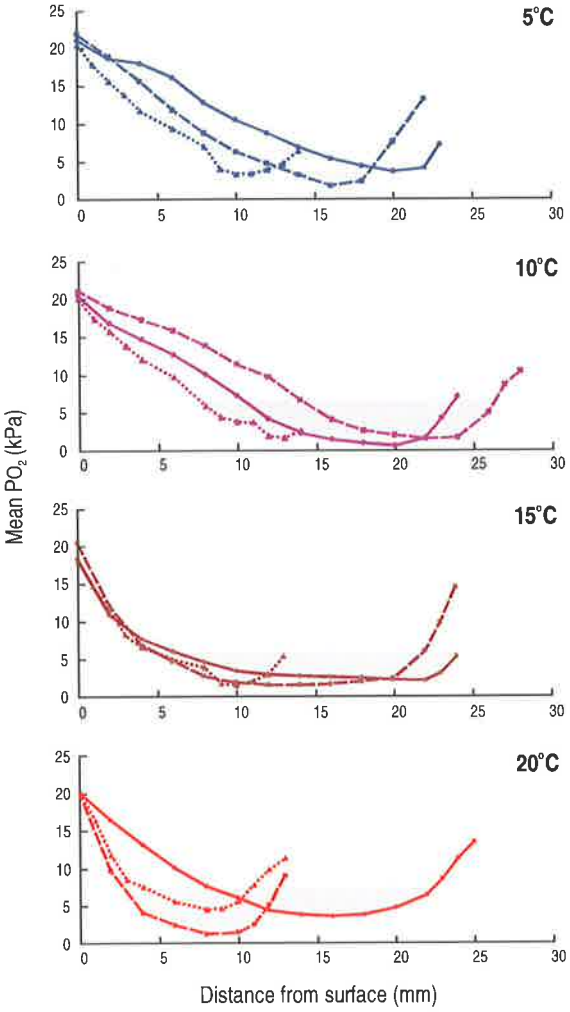


Figure 7. PO_2 profiles through the centre of the clutch in the experimental nest at four experimental T_a. Circles and solid lines indicate stage 30 larvae, squares and dashed lines indicate stage 31 larvae, triangles and dotted lines indicate stage 32 larvae. Areas shaded grey show PO_2 below the critical PO_2 determined for larvae at stages 31-33 (Table 3), and hence indicate depths where larvae would experience hypoxic conditions.

Table 4. Microelectrode measurements of perivitelline PO_2 ($PO_{2\,in}$) at stages 23 and 24 compared to predictions from a stage 24 model of $PO_{2\,in}$ at four T_a.

$PO_{2\,in}$ (kPa)	Temperature (°C)			
	5	10	15	20
Measured (stage 23)	17.9 ± 1.8	16.7 ± 3.5	13.4 ± 2.6	11.3 ± 3.7
Measured (stage 24)	16.0 ± 2.4	14.6 ± 2.2	10.6 ± 9.5	8.5 ± 16.8
*Model estimate (stage 24)	16.3	13.1	11.1	7.4

*Unlike the model in Figure 11, which predicts $PO_{2\,in}$ of embryos at the study site (870 m), this model predicted $PO_{2\,in}$ at the same elevation as the laboratory (45 m above sea level).

The effect of temperature on jelly PO_2 and larval behaviour

Measurement of jelly PO_2 in the experimental nest showed that temperature strongly influenced the PO_2 gradient (Figure 7), and that jelly PO_2 was modified by larval behaviour. Most notably, larvae showed distinctly different distributions at the four experimental temperatures (Table 5, Figures 8, 9 and 10). At 5° C, stage 31 larvae were clustered near the nest base (e.g. Figure 8a and b) and their distribution was significantly clumped (Table 5), as were larvae at 10° C at stage 32. Larvae became more widely dispersed as temperature increased, a trend shown graphically in Figure 10, and expressed numerically as a decreasing $|z|$ value in Table 5. At temperatures between 10° and 15° C, most larvae were oriented with their head at the gauze walls of the nest with their tails inclined slightly downward (e.g. Figures 8c and d). PO_2 at the gauze boundaries of the nest was appreciably higher than at the Perspex walls (Figure 9). At 15° and 20° C some larvae were positioned with their nostrils emerging above the

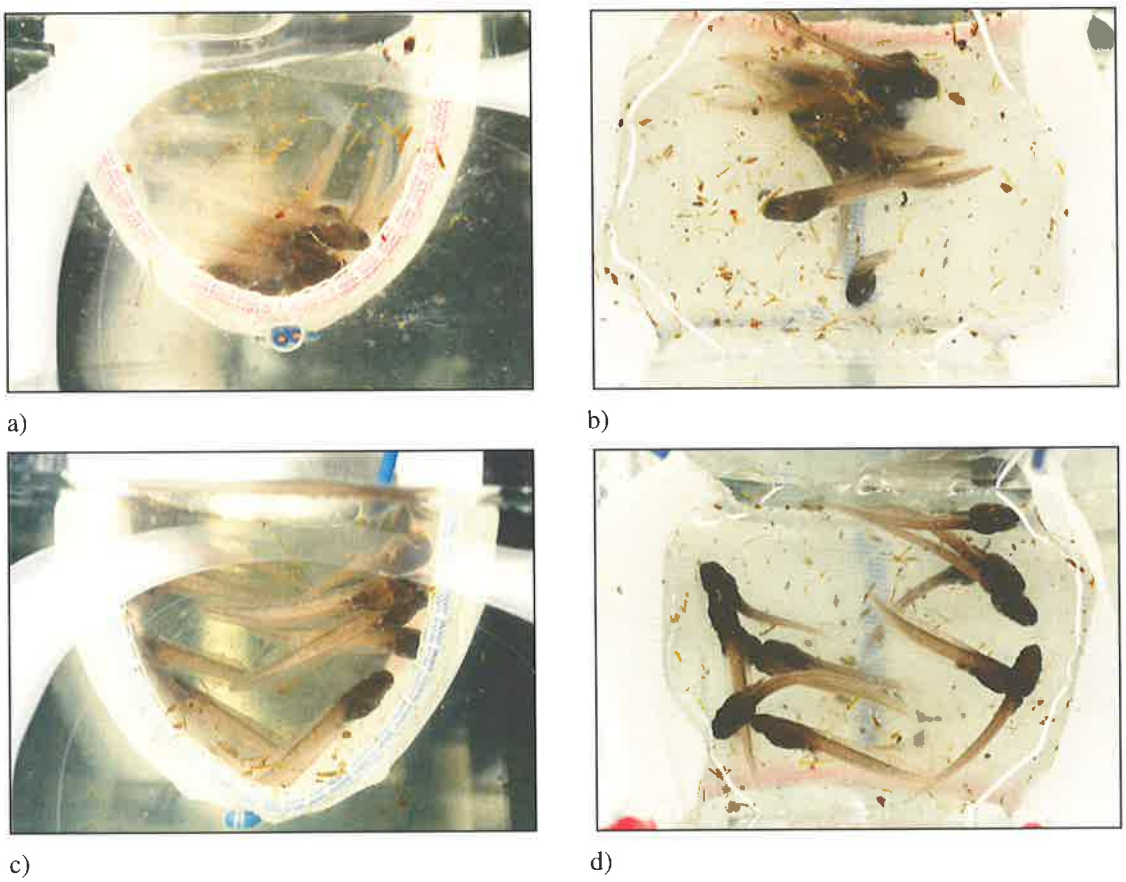


Figure 8. Examples of surface (a and c) and lateral (b and d) views of 12 stage 31 larvae in the experimental nest: a) and b) larvae at 5° C; c) and d) larvae at 15° C.

jelly surface for extended periods. Larvae were sometimes bunched at a surface edge of the nest (e.g. at 20° C in Figure 9), which was similar to arrangements observed in field nests.

There were no clear differences in the number of larvae that tail lashed, total lashing time, or the number of tail lashes at different temperatures; variation in behaviours were at least as variable within as between treatments (Table 6). For example, 10 of 12 larvae tail-lashed a total of 138 times when at stage 30 at 5° C, but there was little movement at stage 31, and no movement at stage 32. Larvae were tightly clumped at stage 30, and their close juxtaposition meant that a writhing individual bumped a neighbour,

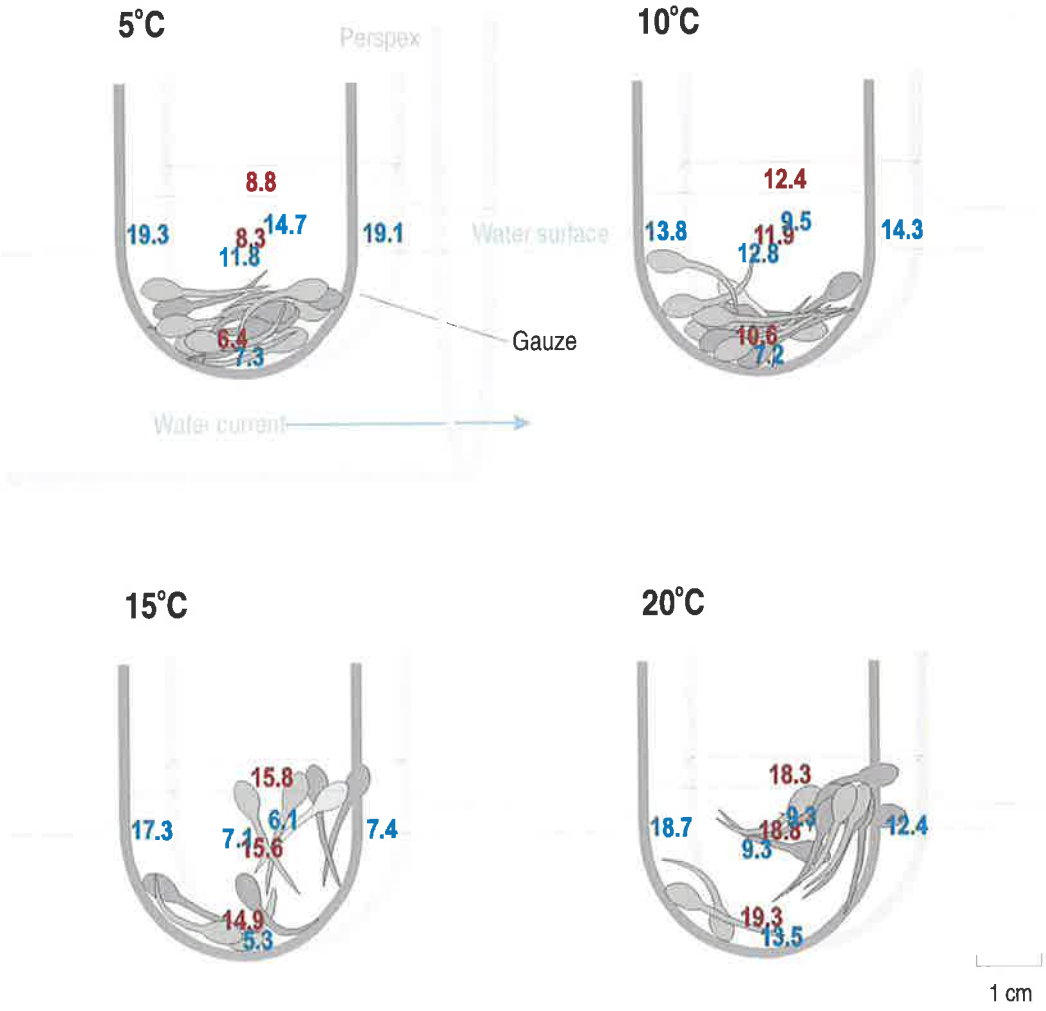


Figure 9. Arrangements of stage 30 larvae in the experimental nest at four T_a. PO_2 (kPa) at four points near the nest walls, and at the nest base, are indicated in blue. Jelly temperatures (° C) at three depths at the centre of the clutch are shown in red

producing a cascade of writhing larvae. There was a trend of an increased rate of tail lashing with temperature, and experiments where vigorous tail-lashing were recorded had slightly higher mean jelly PO_2 than experiments where larvae were comparatively inactive (Table 6). However, the lowest mean PO_2 was recorded at 15° C when larval activity was greatest; all bar the surface and base of the jelly was below the critical PO_2 of 6.1 kPa (Figure 7). Most larvae oriented their head to abut gauze at either the nest wall or base where PO_2 was higher (Figures 8 and 9).

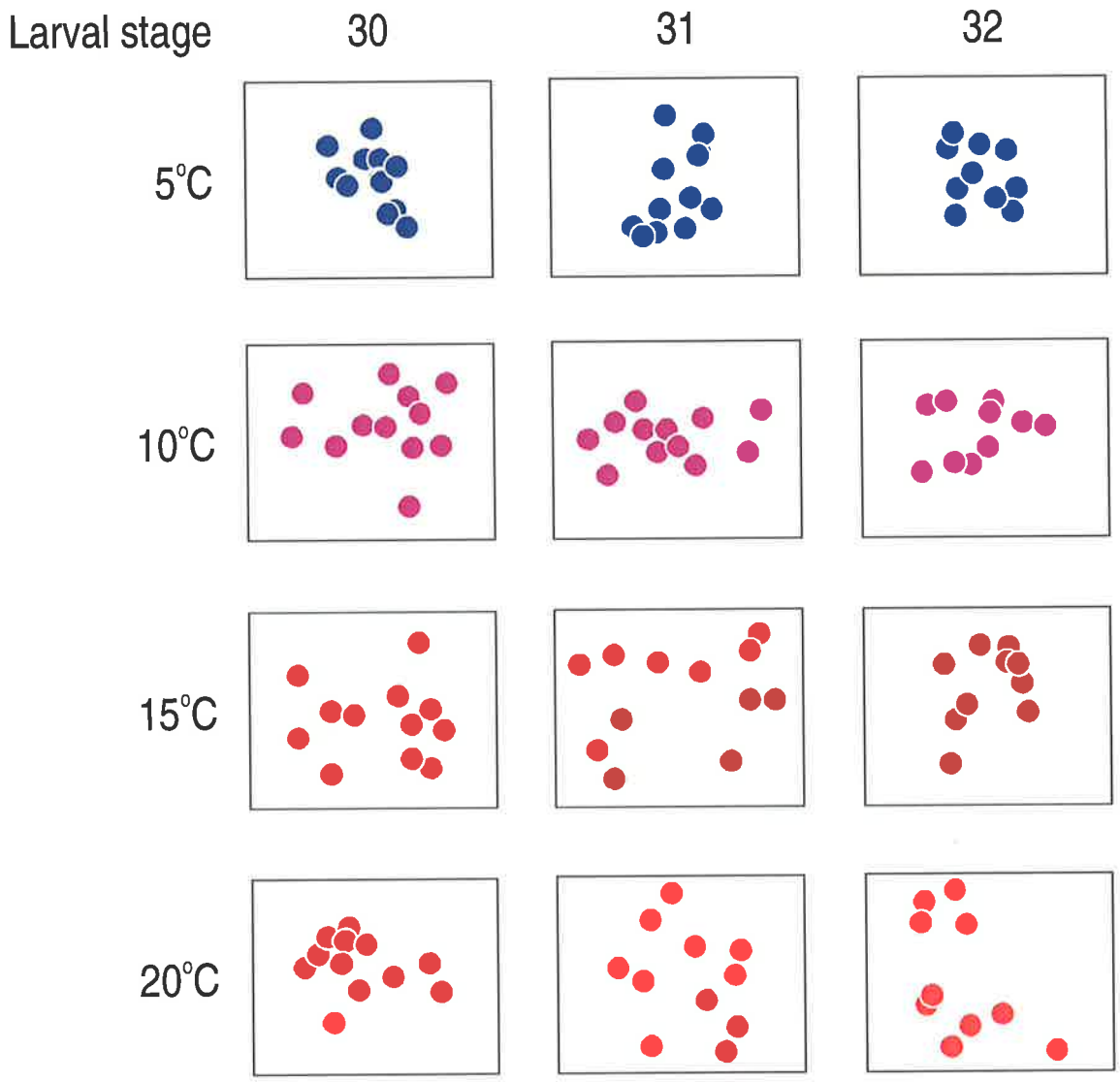


Figure 10. Spacing of larvae in a horizontal plane in each of three experiments at the four experimental temperatures. Nearest neighbour distances were calculated from the centre of the dot, which represents the centre of each larval body.

Table 5. Tests of the spatial distributions of larvae in an experimental nest at four experimental temperatures.

Temperature	Stage	*n* tadpoles	Jelly surface area (cm^2)	Jelly circumference (cm)	$\sum r_i$	$\sum(r_i)^2$	Clark and Evan's test		Thompson's test		Spatial distribution
							|z|	*P*	Observed χ^2	$\chi^2_{.975} - \chi^2_{.025}$	
5	30	12	11.35	14.42	2.97	1.02	3.29	**0.001**	**6.81**	12.4-39.4 (d.f.= 24)	**Clumped**
	31	12	11.77	14.67	3.96	1.71	2.49	**0.006**	**10.97**	12.4-39.4 (d.f.= 24)	**Clumped**
	32	10	10.34	13.73	3.57	1.31	2.14	**0.016**	**7.94**	9.6-34.2 (d.f. =20)	**Clumped**
10	30	12	12.22	14.94	6.45	3.89	0.46	0.323	23.97	12.4-39.4 (d.f.= 24)	Random
	31	12	12.23	15.02	5.94	3.18	0.89	0.187	19.61	12.4-39.4 (d.f.= 24)	Random
	32	10	9.95	13.50	3.24	1.14	2.39	**0.008**	**7.19**	9.6-34.2 (d.f. =20)	**Clumped**
15	30	12	12.16	14.91	6.40	3.88	0.48	0.313	24.05	12.4-39.4 (d.f.= 24)	Random
	31	12	11.94	14.77	6.72	4.17	0.16	0.435	26.33	12.4-39.4 (d.f.= 24)	Random
	32	10	10.11	13.60	3.82	1.78	1.89	0.030	11.07	9.6-34.2 (d.f. =20)	Random
20	30	12	12.67	15.25	4.88	2.20	1.86	0.032	13.09	12.4-39.4 (d.f.= 24)	Random
	31	11	11.79	14.67	6.08	3.79	0.46	0.321	22.21	11.0-36.8 (d.f = 22)	Random
	32	10	9.92	13.48	4.65	2.72	1.09	0.138	17.24	9.6-34.2 (d.f. =20)	Random

Nearest neighbour distances (r_i) were calculated from the distributions shown in Figure 10.

P of |z| <= 0.025 indicate a distribution that is significantly clumped (distribution is one-tailed)

If observed χ^2 is between $\chi^2_{.975} - \chi^2_{.025}$, the spatial distribution is random, if it is less than $\chi^2_{.975}$ the distribution is clumped, and if it is greater than $\chi^2_{.025}$ the distribution is uniform (Thompson 1956).

Table 6. Larval behaviours in the experimental nest over 15 min at four experimental temperatures, and jelly PO_2

T (°C)	Stage	Measured T (°C)	Number of active larvae	Number of larvae breaking surface	Total number of lashes	Total time lashing (min)	Lash rate (lash sec^{-1})	*PO_2 (kPa)
5	30	6.4	10	0	138	5.90	0.393 ± 0.044	10.6 ± 2.0
	31	6.8	2	0	18	0.73	0.374 ± 0.069	9.7 ± 2.2
	32	6.1	0	0	0	0.00	n/a	9.5 ± 2.0
10	30	10.6	0	0	0	0.00	n/a	7.5 ± 2.1
	31	11.9	2	0	44	0.52	0.516 ± 0.071	9.4 ± 2.0
	32	11.0	3	0	16	0.74	0.938 ± 0.218	8.7 ± 2.2
15	30	14.9	11	5	192	4.41	0.678 ± 0.066	5.5 ± 1.4
	31	15.9	7	4	172	2.35	1.146 ± 0.093	5.9 ± 1.8
	32	15.3	6	3	46	1.10	0.730 ± 0.067	6.8 ± 2.0
20	30	19.3	2	7	19	0.29	0.988 ± 0.460	8.9 ± 1.6
	31	19.8	2	5	17	0.32	0.872 ± 0.184	6.1 ± 2.5
	32	19.5	6	9	185	2.43	1.142 ± 0.110	9.5 ± 1.8

*Average of three PO_2 transects through the centre of the egg mass (data from Figure 7).

DISCUSSION

The oxygen environment of natural nests

The PO_2 measured in fresh *B. nimbus* clutches in natural nests were consistently high, about 19.1 kPa at the jelly surface and declining only a further 1.5 kPa at the nest base. After hatching, when the oxygen consumption of the mass was higher, mean PO_2 ranged between 18.6 kPa at the jelly surface and 12.6 kPa at the nest base (Figure 4; Table 2). In comparison, the globular clutches of late *Philoria loveridgei* embryos developing in soil depressions averaged 7.6 kPa at the nest base (Seymour et al. 1995). The PO_2 profiles measured in *B. nimbus* natural nests were unlike those in laboratory experiments, where PO_2 fell to below 5 kPa at all temperatures between 5 and 20° C (Figure 7). Although jelly PO_2 is influenced by multiple factors, including larval developmental stage, density, and temperature, it is apparent oxygen concentrations in natural moss nests were greater than those in the experimental nest.

Two mechanisms may account for this difference. Firstly, moss and other vegetation used for nesting by *B. nimbus* is perforated with air spaces. The ease of oxygen diffusion through gas is about 300 000 times that through jelly (Seymour 1994), therefore sub-surface jelly is replenished with oxygen from air pockets bordering the egg mass. Secondly, photosynthetic activity of moss provides the clutch with oxygen during daylight, as evidenced by jelly PO_2 exceeding atmospheric PO_2. Presumably jelly PO_2 is lower overnight when photosynthesis ceases. For example, a nest of the frog *Crinia georgiana* constructed in moss had a high PO_2 of 26.2 kPa in the early afternoon, but a PO_2 of 13.5 kPa before dawn; notably, these values were higher than the mean PO_2 of 12.9 kPa for 29 nests, most of which were irrigated depressions in silt (Seymour et al. 2000). Algal cells in the jelly envelopes of eggs the salamander *Ambystoma maculatum* supersaturated an egg mass with oxygen in light (32.8 kPa), but PO_2 dropped to 0.62 kPa after 1 hr of darkness (Bachmann et al. 1986). Hence the combined respiration of embryos and surrounding plant material can deplete jelly oxygen reserves in darkness, but because night-time temperatures are low (e.g. 5.6-7.5° C for *B. nimbus* clutches in December, Table 3, Chapter 3), respiratory demands would decrease proportionally, and hypoxia may be avoided.

Oxygen supplies in the experimental clutch could be replenished from the aerated water bathing the gauze perimeters of the nest, and hence it was broadly analogous to a natural nest situated in a seepage line. In natural nests, PO_2 at the nest base was lowest in a partially-flooded *B. nimbus* nest occurring in a poorly drained region of the plateau, about 14 kPa when eggs were fresh and about 5 kPa after hatching (Figure 4). Inundation of stagnant water into a *B. nimbus* nest would therefore potentially result in very low jelly PO_2, particularly in conditions of low light.

PO_2 in the perivitelline space of single embryos

Unlike larvae, embryos are contained within the perivitelline membrane and cannot move within the mass to seek a richer supply of oxygen. Substantial diffusion distances resulting from the large jelly capsules may inhibit respiration of *B. nimbus* embryos at higher temperatures. However, laboratory data in unsupportive; for example, $PO_{2\,in}$ of stage 23 and 24 embryos ranged between 17.0 kPa at 5° C and 10.2 kPa at 20° C, exceeding the critical PO_2 for stage 28-29 larvae at the corresponding temperature (Tables 3 and 4). However, because the critical PO_2 of larvae may be greater than that of embryos (see Bradford and Seymour 1988b), it is possible that embryonic $\dot{V}O_2$ is limited at warmer temperatures, or in conditions of low external PO_2. For example, embryos incubated in nests at 870 m elevation at the sub-alpine study site are subject to an external PO_2 ($PO_{2\,out}$) of 18.1 kPa, compared to 20.4 kPa at an altitude of 45 m in the laboratory. If $PO_{2\,out}$ is reduced, then $PO_{2\,in}$ is also lower because, reworking the Fick diffusion equation:

$$PO_{2\,in} = PO_{2\,out} - \frac{\dot{V}O_2}{GO_2} \qquad (1)$$

We can predict the $PO_{2\,in}$ of a stage 24 embryo developing at 870 m at a specified temperature by assuming $PO_{2\,out}$ is 18.1 kPa, using equations in Chapter 3 to estimate temperature-specific $\dot{V}O_2$, and calculating capsule conductance as $GO_2 = KO_2 \times ESA/L$, where KO_2 is related to temperature using an equation in Seymour (1994), and the ESA of a spherical capsule is $4\pi r_o r_i$, and $L = r_o - r_i$, where r_o and r_i are outer and inner capsule radii. Hence,

$$PO_{2\,in} = PO_{2\,out} - \frac{\dot{V}O_2(r_o - r_i)}{KO_2 \pi 4 r_i r_o} \qquad (2)$$

The average capsule r_i of embryos near hatching was 0.33 cm. Because r_o is indistinct near hatching, it was estimated by assuming that each egg occupied $1/n^{th}$ of the jelly mass, where n was the number of eggs. The mean volume of pre-hatched clutches measured in field nests was 15.5 ± 2.8 cm^3, where clutch size averaged 10 eggs (Table 1), thus the average volume of an embryo was 1.55 cm^3, which equated to a spherical capsule with a r_o of 0.72 cm. It should be noted that a r_o of 0.72 cm is that of a partially hydrated capsule, for clutches were measured at a range of hydration states. Field observations suggested that the r_o of a fully hydrated capsule of a stage 24 embryo was about 1 cm.

The estimated $\dot{V}O_2$ and $PO_{2\,in}$ of a stage 24 embryo are plotted along with critical PO_2 in Figure 11. At subalpine elevations, estimated $PO_{2\,in}$ is 5.3 kPa at 20° C, substantially *less* than the critical larval PO_2 at 20° C of 8.4 kPa (Table 3). At 25° C, estimated $PO_{2\,in}$ falls to about 2 kPa, and, extrapolating beyond this temperature, $PO_{2\,in}$ is reduced to about 0 kPa at 26.9° C (Figure 12). This same model applied at the four laboratory T_a, and altitude of 45 m, predicted $PO_{2\,in}$ at stage 24 to within 0.3-1.6 kPa (3-13%) of the mean measured $PO_{2\,in}$ (Table 4), which demonstrates the model's applicability across a range of temperatures. Low $PO_{2\,in}$ is known to stimulate hatching of amphibian larvae (Petranka et al. 1981; Bradford and Seymour 1988; Seymour et al. 2000), hence nest temperatures in excess of 20° C might promote early hatching of *B. nimbus* embryos.

Rates of oxygen consumption of *B. nimbus* embryos are low compared to other species with similarly sized ova. For example, a predicted hatching stage $\dot{V}O_2$ of 2.70 μL h^{-1} at 15° C based on ovum size (using the equation $\dot{V}O_2 = 0.393V^{0.62}$ in Seymour and Bradford 1995, where V=ovum volume) was more than double the measured value (1.33 μL h^{-1} ; Chapter 3). Notably, doubling $\dot{V}O_2$ would produce anoxic conditions (i.e. $PO_{2\,in}$ = 0 kPa) between 14° C and 15° C if capsule geometry remains constant. Therefore, unless large embryos are confined to low temperatures, metabolic depression is critical to their viability when bounded by a thick layer of jelly.

3.3 mm, and the width of each shell was $1/50^{th}$ of global r_o- global r_i. The total $\dot{V}O_2$ of the 12 outer embryos was allocated to the 20^{th} shell from the perimeter of the mass (the 'zone of consumption'; Figure 12), and initial $PO_{2\,out}$ was set at saturated atmospheric conditions of 18.1 kPa. This model is simplifies the real situation on two counts: 1) PO_2 is lower (about12.6 kPa) at the nest base of hatched clutches (Table 3), and 2) the perivitelline fluid of outer capsules is treated as jelly, whereas convection of oxygen occurs within the perivitelline space.

Working inward, a PO_2 profile through the hypothetical mass can be calculated at any temperature using a computer spreadsheet. At 6.3° C, for example, the $PO_{2\,in}$ of the central embryo falls to 0 kPa, but is adequate (about 7 kPa) for the 12 outer embryos. Therefore at least one embryo in a spherical mass of 13 embryos incubated at 6.3° C is anoxic; but an isolated embryo at the same temperature experiences a $PO_{2\,in}$ of about 13.3 kPa (Figure 12). Predictions of the model of $PO_{2\,in}$ of a central embryo in variously sized spherical masses of stage 24 *B. nimbus* embryos show that larger clutches are only viable at temperatures less than 5° C; that is, the $PO_{2\,in}$ of the central embryo is positive (Figure 13). If we use critical PO_2 of early stage larvae to represent that of embryos, it suggests that an embryo surrounded by only 8 embryos (the mean *B. nimbus* clutch size of 9 eggs) is hypoxic at about 6° C, and anoxic at about 8° C. Pre-hatching embryos occur in the field between December 22 and March 29, when effective nest temperatures over two-week periods averaged 11.9 ± 0.6° C (range 6.2-15.2° C; n=6, data from Chapter 3). These temperatures would lead to hypoxia of a central egg, even within a mass as small as 7 eggs. Thus, although amphibian embryos can withstand periods of hypoxia, and even anoxia (Burggren 1984, but see also Seymour et al. 2000), a spherical egg mass is undesirable because the central embryo would only sporadically be reprieved from hypoxia (when temperatures are low), and its development may be delayed or retarded.

A spherical model does not accurately reflect conditions in real nests because egg masses are approximately hemispherical, and most eggs are situated in a surface layer. No egg has ever been found entirely surrounded by other eggs; normally, each egg capsule contacts either air or a moss wall of the nest. Because the shape of the nest ultimately determines the arrangement of embryos, wider nests would cause embryos to distribute in 1-2 layers, allowing good oxygenation, but narrower nests will create a deeper egg mass more similar to the spherical model. Other nest characters may also

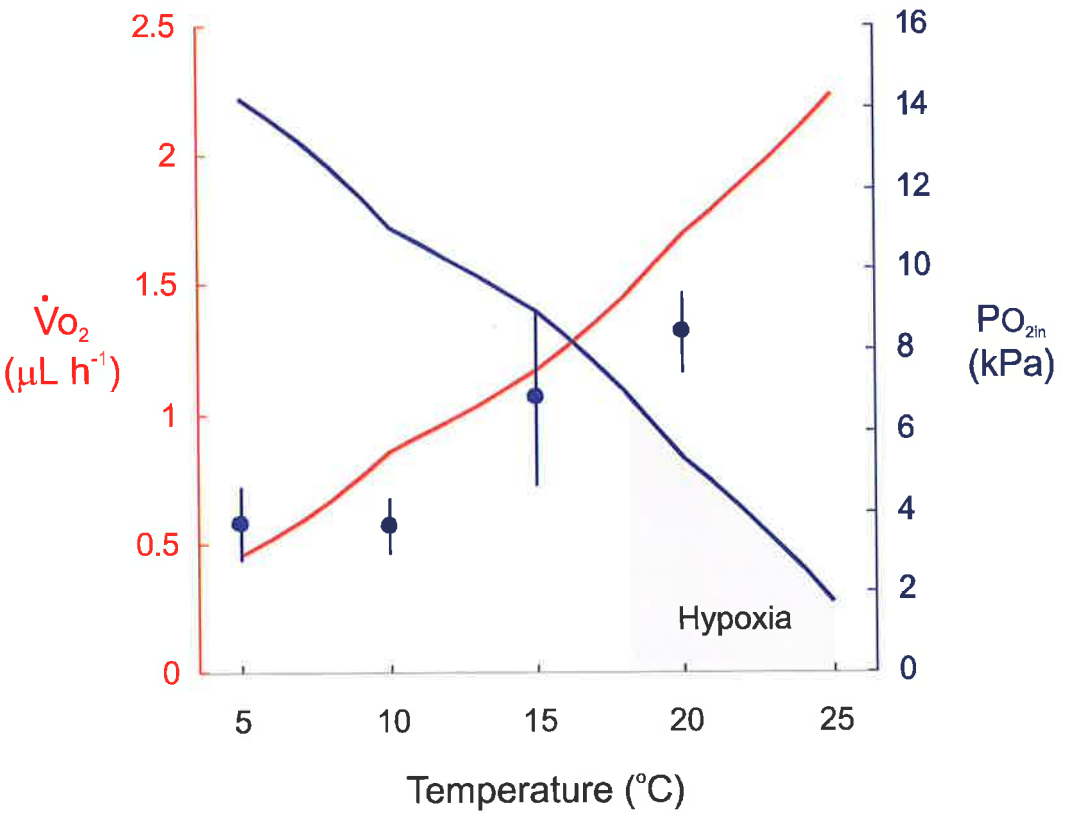
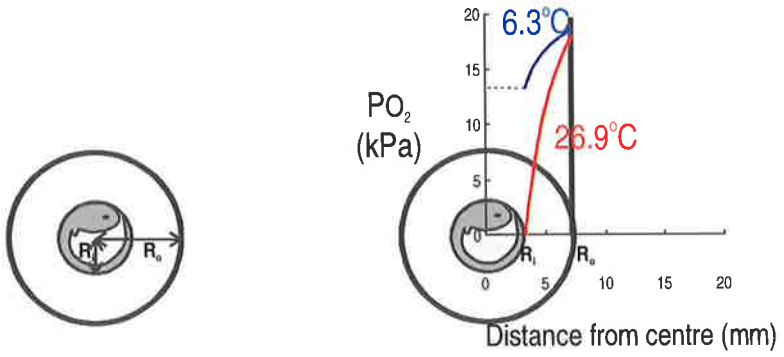


Figure 11. Model of the effect of incubation temperature on $\dot{V}O_2$ and $PO_{2\,in}$ of an isolated stage 24 *Bryobatrachus nimbus* in subalpine conditions. $\dot{V}O_2$ at 5°, 10° and 15° C were estimated from equations in Chapter 3, and adjusted to other temperatures using Q_{10}. $PO_{2\,in}$ was calculated assuming a saturated atmospheric PO_2 of 18.1 kPa (870 m altitude). The capsule had a r_i of 0.33 mm and r_o of 0.72 mm. KO_2 was adjusted to the appropriate temperature using an equation in Seymour (1994). Filled circles and error bars are measured critical PO_2 of early (stage 28-29) larvae (Table 3); grey shading indicates where $PO_{2\,in}$ falls below critical levels.

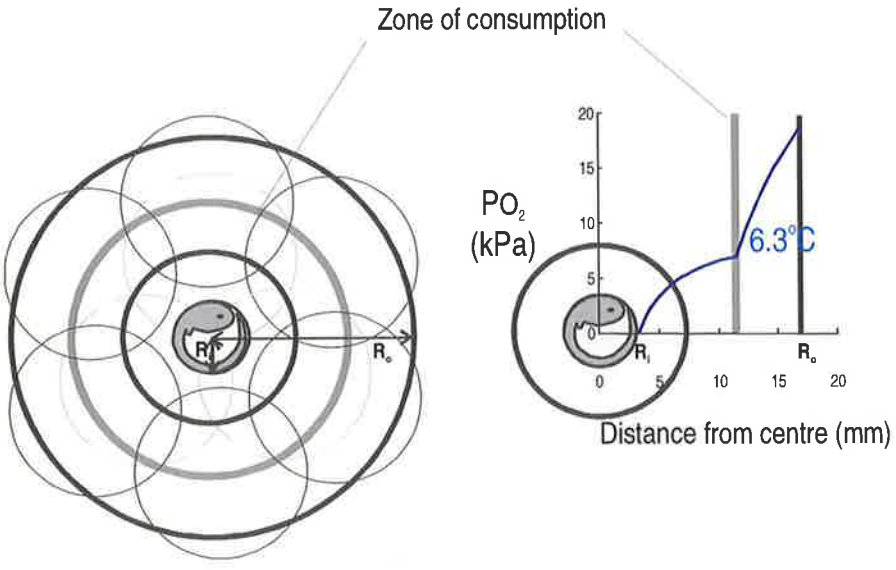
Oxygenation of a globular mass of embryos

The above model considered an embryo developing in isolation, and assumed that the entire outer boundary of the capsule experienced atmospheric PO_2. In reality, embryos develop within a mass, and the outer PO_2 of a central embryo is lower than atmospheric levels because of oxygen uptake by neighbouring embryos. Embryos at the centre of globular masses can avoid hypoxia only if the egg mass is suitably small. A spherical egg mass containing late *Limnodynastes tasmaniensis* embryos, for example, would be viable at a size of less than 29 eggs and radius of < 1.3 cm (Seymour and Roberts 1991). Their model is based on equation (2), and assumes that embryonic $\dot{V}O_2$ is not affected by low PO_2. It is readily applied to variously sized *B. nimbus* clutches developing at a range of temperatures. Briefly, by regarding the egg mass as a series of concentric shells of jelly bordering the perivitelline membrane of a central embryo, it is possible to

predict the $PO_{2\,in}$ of each shell, and ultimately, the central embryo within a mass of any size and temperature. For example, I assumed that a spherical clutch of thirteen eggs was arranged as 12 embryos enclosing the central embryo (Figure 12). Under these conditions, the volume of the mass was 20.21 cm^3 (13 x 1.55 cm^3) and the mass had a global r_o of 16.9 mm. The global r_i was the perivitelline radius of the central embryo,



Single embryo model



13 embryo model

Figure 12. Diagrams of an isolated stage 24 embryo and a spherical clutch of 13 stage 34 embryos. Models predicted a $PO_{2\,in}$ of 0 kPa at 26.9° C for the isolated embryo, and $PO_{2\,in}$ of 0 kPa at 6.9° C for an embryo at the centre of a mass of 13 embryos.

influence embryonic oxygen supply. Ten species of vegetation are used for nesting in the Hartz Mountains population of *B. nimbus* (Chapter 6), and consequently, there may be variability in rates of photosynthetic oxygen production. Further, nests are dehydrated to varying degrees, as evidenced by the wide range in clutch volumes at any development stage. Dehydrated clutches have smaller jelly volumes, and the resultant high embryo densities may lead to hypoxia at warmer temperatures, even though diffusion paths are reduced (*c.f.* Bradford and Seymour 1988a).

Larval responses to low PO_2

Anuran larvae developing at low PO_2 can modify their behaviours to enhance their supply of oxygen. For example, the water-filled depressions in which *Hyla rosenbergi* larvae develop averaged about 3.3 kPa (Kluge 1981), and early-stage larvae were suspended at the surface with their large gills extended for maximum contact with air. Older larvae swam to the surface to gulp air, and released bubbles into the water as they descended. The latter behaviour is similar to that observed in *Philoria loveridgei* larvae after a period resting at the base of the nest, but larvae at the surface lashed their tails every 1-2 min, which mixed the oxygen-rich surface jelly deeper in the mass (Seymour et al. 1995).

Air gulping and bubble formation was never observed in *B. nimbus* larvae. Tail lashing was vigorous in experiments where mean PO_2 approached critical levels, but the most obvious response of larvae to low mean PO_2 was to move higher in the jelly and position their nostrils at the surface (Table 6). It was not possible to determine whether larvae were air-breathing, or simply utilising oxygen-rich areas of the jelly. Surface larvae were often clustered (e.g. Figure 9), and may have been drawn together by menisci around them. However, overall, the ability of larvae to disperse widely within the jelly pool appeared to be an important response to increasing jelly temperatures (Figures 8 and 10). Tightly clumped larvae experienced hypoxic conditions in some 5° C experiments, and would almost certainly have become hypoxic at warmer temperatures had they not dispersed. The lower limit of larval density will be defined by the amount of jelly in a nest; hence the large capsules of *B. nimbus* eggs may be critical to the maintenance of a suitably sized jelly pool during the 8-11 months of larval development (Chapter 3).

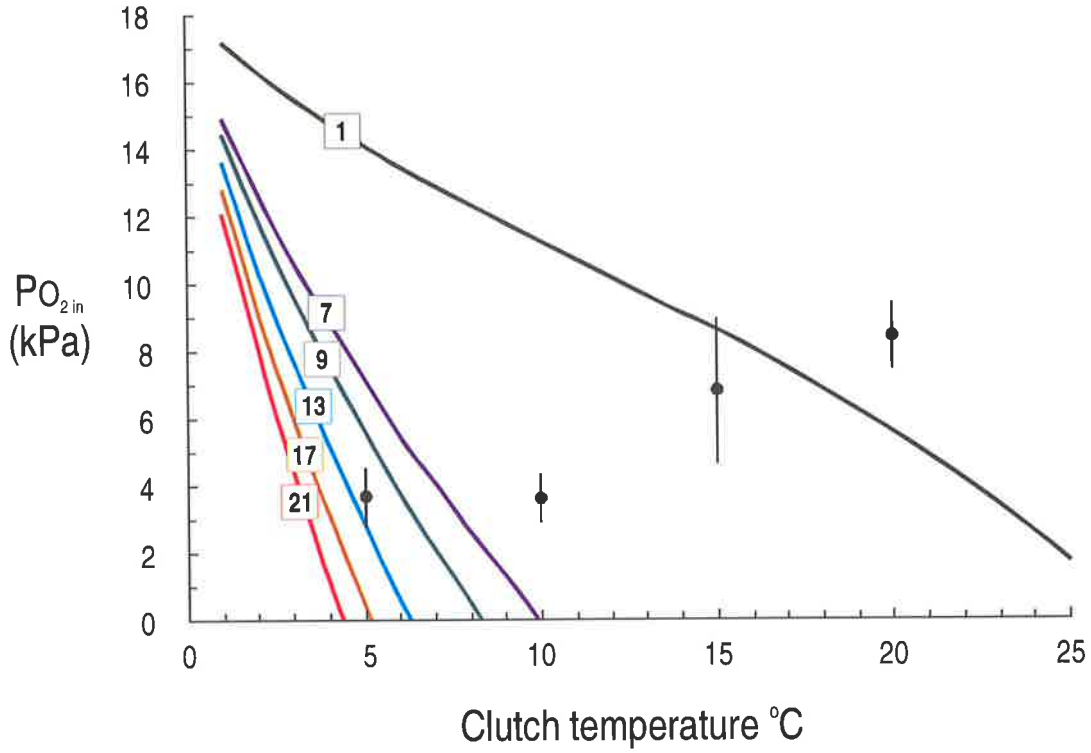


Figure 13. Results of a numerical model of the effect of incubation temperature on the $PO_{2\,in}$ of a central embryo in a mass of 1-21 embryos near hatching (stage 24). Boxed numbers on each curve indicate the size of the egg mass. Critical (limiting) PO_2 of early-stage larvae (Table 3) provide approximations of the conditions when the embryo experiences hypoxia. The curves show that larger spherical egg masses (13, 17 and 21 embryos) are viable only at temperatures below 5° C, while the central embryo in smaller egg masses (7 and 9) eggs will become hypoxic between 5° and 10° C. Filled circles and error bars are critical PO_2 of early larvae (stage 28-29).

Limits to clutch size

Clutch sizes of anurans are significantly larger when bubbles are introduced into egg mass; for example, models have demonstrated that foam egg masses can be several orders of magnitude larger than masses without bubbles (Seymour and Roberts 1991: Seymour and Loveridge 1994). The Australian Myobatrachidae is divided into two subfamilies, the Limnodynastinae, where female frogs possess flanges on their forelimbs that are used to whip foam into an egg mass, and the Myobatrachinae, which lack finger flanges and deposit globular clutches. Clutch sizes of terrestrial breeding Limnodynastinae are considerably larger (80-500 eggs) than those of myobatrachids with globular, terrestrial clutches (5-31 eggs; Tyler 1994). Analytical models of spherical *B. nimbus* egg masses have shown that larger masses (13-21 eggs) are not viable at late embryonic stages, but this constraint is presumably lost if embryos can be

restricted to one or two layers. Hydrated *B. nimbus* eggs are about half the size of male (19-27 mm snout-vent length; Rounsevell et al. 1994), hence males would need to construct enormous nests relative to their body size to contain a large clutch of, for example, 20 eggs, to one or two layers. An alternative solution is for females to deposit their egg component into more than one nest. Clutches of similar age are often found in neighbouring nests (e.g Figure 3), but whether they represent the contributions of a single female requires genetic appraisal. However, multiple clutching would likely enhance survival of embryos within thick jelly capsules, because small egg masses should be adequately oxygenated.

Acknowledgments

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5 Incubation water potential affects survival, growth, metabolic rate and morphology of terrestrial embryos of the Australian frog, *Bryobatrachus nimbus*

Abstract

The embryos and larvae of the Australian moss frog *Bryobatrachus nimbus* develop in concealed cavities within bryophytes or lichens for over one year. Oviposition occurs in drier summer months and consequently embryos experience a variety of hydrous conditions in concert with the drying and rehydration of the nest material. In the laboratory, *B. nimbus* embryos were reared at four moist water potentials ranging between 0 and -25 kPa. Survival to hatching was significantly greater at 0 kPa than at drier water potentials (-5, -10 and -25 kPa). Embryos incubated in drier conditions developed with pronounced trunk and hindlimb-bud asymmetry, and had significantly shorter and shallower tails, and lower metabolic rates, relative to embryos incubated at 0 kPa. The volume of perivitelline fluid in -10 and -25 kPa embryos was only 9% of that bathing a 0 kPa embryo. This reduced developmental space coupled with adhesions between the yolk sac wall and perivitelline membrane limited rotation of drier embryos, and hence promoted asymmetric development. The severity of abnormalities at -25 kPa indicated that *B. nimbus* was less tolerant of low water potentials than other anuran species studied. Nests must therefore provide appreciable resistance to egg desiccation during development in the field. Moreover, the unusually large capsule of the *B. nimbus* egg provides embryos with a short-term reservoir of moisture when conditions dry.

INTRODUCTION

It is well established that water balance during incubation of amniote eggs is a critical component of their hatching success (birds, Simkiss 1980; Synder and Birchard 1982; reptiles, Muth 1980; Packard and Packard 1984; Packard and Packard 1988). But beyond the absolute boundary of live-or-die, it is the phenotypic differences between hatchlings arising from different nest sites that have captured recent attention. A synthesis by Packard (1999) flagged the 'wetter is better' principle of chelonian egg incubation and provided evidence from laboratory studies to demonstrate that embryos developing in the wettest conditions consume the most yolk, hatch at larger sizes and perform better in locomotor trials when compared to their drier-reared siblings (e.g., Packard and Packard 1984; Miller et al. 1987; Miller and Packard 1992). Similar evidence exists for the anamniotic eggs of amphibians; in the terrestrial breeding frog *Pseudophryne bibronii,* incubation of embryos at -25 kPa reduced oxygen consumption by 72-81%, and retarded growth by 32%, relative to fully hydrated embryos incubated at 0 kPa (Bradford and Seymour 1988). Because the gelatinous outer capsule of amphibian eggs is almost completely permeable to water (Martin and Cooper 1972; Taigen et al 1984; Kam et al. 1998), moist conditions are essential to the successful development of amphibian embryos, particularly for terrestrial species.

Terrestrial eggs characteristically have large capsules relative to ovum size, which slows their rate of dehydration (Salthe 1965). During normal development the volume of the perivitelline space increases in proportion to the growth of the embryo. The process is driven by the steepening osmotic potential of the perivitelline fluid (caused by secretions from the embryo), and water moves from the jelly across the semipermeable perivitelline membrane into the perivitelline space, creating pressure that gradually stretches the membrane (Salthe 1965). However, under conditions of water restriction, swelling of the perivitelline space is reduced. For example, *P. bibronii* embryos reared below -50 kPa contained very little perivitelline fluid, and may have resulted in adhesions between the embryo and the perivitelline membrane (Bradford and Seymour 1988).

Anuran species using terrestrial oviposition sites show great diversity in behaviours that reduce egg dehydration. Females of some arboreal breeders deposit empty capsules amidst normal eggs, which provide an external water source for developing embryos, while others fold leaves to create relatively impermeable nests (Pyburn 1970; 1980). Parental brooding is a widespread mechanism, and functions in three ways: 1) evaporation is reduced from the surface of a clutch (e.g. Forester 1979), 2) adults may actively secrete water collected from their surroundings onto eggs (e.g. Wells 1981), or 3) adults use gradients in water potential to directly transfer water to eggs through highly permeable ventral skin (Taigen et al. 1984). However, most commonly, egg water balance is maintained via uptake of external water through contact with a terrestrial substrate, and hence parental selection of a moist oviposition site is critical.

Eggs of the Australian moss frog, *Bryobatrachus nimbus,* develop to metamorphosis within a cavity constructed in bryophytes and lichens. During the 13-month developmental period (Mitchell and Swain 1996) the vegetation dries and rehydrates in compliance with changing weather, and thus eggs experience a variety of hydrous conditions. Some embryonic mortality occurs in about 32% of nests, and entire clutches have succumbed to dehydration in the field (Mitchell 1995; Chapter 6). It was therefore relevant to quantify the tolerance of *B. nimbus* embryos to a range of substrate water potentials experienced in natural nests. Embryonic development was studied in deference to larval development because the former occurs in the warmest months of the year (Chapter 3) when drying of the nest vegetation is common. Eggs were incubated in the laboratory at four water potentials: 0, -5, -10 and -25 kPa, and effects on embryonic survival, developmental rate, metabolic rate, and morphology were measured. Additionally, adult breeding behaviours are described, and I discuss several mechanisms by which *B. nimbus* embryos avoid dehydration over the protracted development period.

METHODS

Fieldwork and egg collection

Bryobatrachus nimbus clutches were examined periodically over about 150 site visits to the type locality in the Hartz Mountains National Park, Tasmania, between 1994 and 2000. Frequent site visits during two breeding seasons (1998-2000) allowed observation of breeding pairs in nests. Their nests were marked with flagging tape and re-examined on the next visit to the site.

Eight fresh clutches were collected on December 17 1998, and transported by air to Adelaide, South Australia on the following day. Eggs were cleaned of debris and held inside sealed ice-cube cells in a constant temperature cabinet at 10° C. Developmental stage at collection ranged between stages 8 and 14 according to Gosner (1960). The ages of embryos were estimated from their stages with an equation relating stage to age at 10° C (Chapter 3).

Response of B. nimbus *embryos to dehydration*

Fresh eggs were incubated at four water potentials (0, -5, -10 and -25 kPa) on stacks of 1 mm thick chromatography paper (dimensions 8 x 14 x 0.4 cm) sealed inside a 900 mL plastic food storage container. Papers were wetted to known water potential using the relationship between the wet weight and matric tension of the chromatography paper, determined previously with a pressure plate apparatus (Appendix 3; Seymour and Piiper 1988). Briefly, 0 kPa papers were saturated with reverse osmosis water, and -5, -10 and -25 kPa papers were wetted with 2.89, 1.42, and 0.83 times the dry paper mass. Dry papers were first autoclaved, and water was boiled and dosed with a malachite green aquarium fungicide to prevent fungal infection of eggs (1 drop per 250 mL of water). Containers were stored at 10° C for 2 d before eggs were introduced, to allow equilibration of the water potentials of paper stacks.

Pilot trials showed that when a *B. nimbus* egg was placed on damp chromatography paper, the soft jelly adhered rapidly to the paper and flattened the egg. To reduce this effect, small plastic rings about 2 cm in diameter (cut from clear plastic 35 mm film canisters) were placed onto a square of plastic wrap, and four eggs were positioned

snugly inside. Rings were then lowered onto a paper stack, and the plastic gently pulled out from underneath to allow eggs to contact the filter. Horizontal expansion of the capsule was initially constrained by the ring (Figures 4a and b). Healthy eggs from the eight clutches were divided equally between the four treatments, and randomly assigned to one of four rings. Individual embryos could be identified during the experiment by their position within the ring.

Eggs rapidly lost water to the papers of the drier treatments (-5 to -25 kPa) and so increased the water potential of the papers. To monitor this effect, four replicate 3 x 3 cm squares of dry, pre-weighed chromatography paper were placed between the rings on the paper stacks of the -5, -10 and -25 kPa treatments, and removed periodically and weighed to 0.01 mg on a Mettler AE163 analytical balance to determine the treatment water potential. Test squares indicated that the time to reach water potential equilibrium of eggs and paper was about 3 d in the -5 and -10 kPa treatments, and about 7 d for the –25 kPa treatment. The water content of the papers was adjusted where necessary by drawing out water with absorbent tissue. When test papers stabilised at the target water potential, the mass of the container and contents was recorded to the nearest 1 mg on a Sartorius 1265MP balance - this mass was thereafter maintained by periodically adding drops of treated water. Mean water potentials (± 95% CI) as measured by test squares during the experiment were: '-5 kPa', -5.38 ± 0.37 kPa (n=68); '-10 kPa', -10.49 ± 0.61 kPa (n=68); '-25 kPa', -24.46 ± 4.75 kPa (n=50). These substrate water potentials resulted in embryos with capsule sizes comparable to those observed in field nests.

Embryos were examined and staged according to de Bavay (1993) every 5-6 d, and mortalities were recorded. High mortality of -5 kPa embryos prompted the early removal of four of the original 16 embryos (hereafter referred to as early embryos) from each treatment when aged approximately 48 d. They were scraped off their paper substrate and 'hatched' by carefully stretching the perivitelline membrane until it broke. Their rates of oxygen consumption ($\dot{V}O_2$) were measured using closed system respirometry (Appendix 1), before they were weighed, killed by brief freezing, and preserved in fixative (Tyler 1962). Snout-vent length (SVL), tail length, yolk sac width and length and hindlimb bud dimensions were measured from digitised images of preserved embryos with Optimas 5.2 image analysis software (Optimas Corp., Bothell, Washington). Embryos from each treatment were later dissected into body and yolk,

dried to a constant mass over silica gel, weighed to 0.01 mg and combined into a 25 mg pellet of body or yolk for bomb calorimetry (refer Chapter 3). The procedure was repeated at day 61 for all surviving embryos from each treatment; hereafter termed 'late embryos'.

Permit conditions limited the scope of the experiment, and sample sizes were low in some treatments, especially because it was necessary to analyse data at early and late embryonic stages separately. Descriptive statistics are means ± 95% confidence intervals for sample means. When results were normally distributed, ANOVA was applied to treatment data; otherwise non-parametric Wilcoxon Rank Sum tests were used. Fisher's exact tests were used to compare frequencies of morphological abnormalities between treatments. Significance was assumed at $P < 0.05$.

RESULTS

Field observations of breeding pairs

Fifteen breeding pairs of *B. nimbus* were located during the day between November and January, over two breeding seasons. Pairs were concealed in moss and lichen nests, but were never observed in amplexus; instead, the gravid female filled the base of the nest and the male was usually oriented perpendicular to and above the female, with his head resting on her flank (Figure 1). Four pairs remained in a similar position for 2-4 d before producing clutches. The delay of at least 4 d between pairing and oviposition occurred during dry weather in November 1998. Males sometimes attended fresh egg masses on the days following oviposition (n=5 of 77 observations of fresh clutches), while one male was found atop stage 19 embryos, and another sat on a clutch of tailed froglets.

The egg capsules within two fresh egg masses produced by marked pairs were 8 mm in diameter (Figure 2a) and the eggs were clustered at the nest base and did not contact the nest wall. Capsules slowly swelled to fill the nest, and reached their maximum size in about 4 d. After rains, capsules swelled rapidly, to the extent that the egg masses would brim outside the upper lip of the nest. Conversely, during dry weather, eggs shrank toward the base of the nest, leaving a thin coat of jelly on the nest wall. A crusted jelly

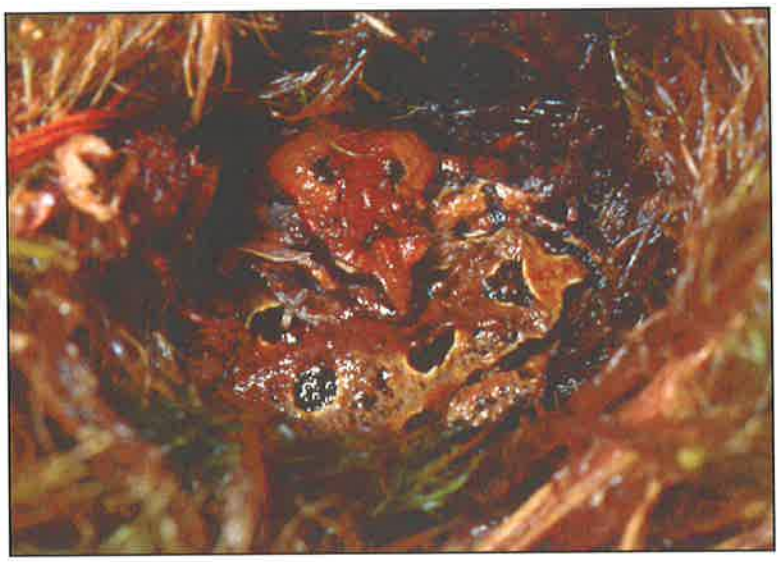

Figure 1. Male (top) and female (below) *Bryobatrachus nimbus* paired in a moss nest

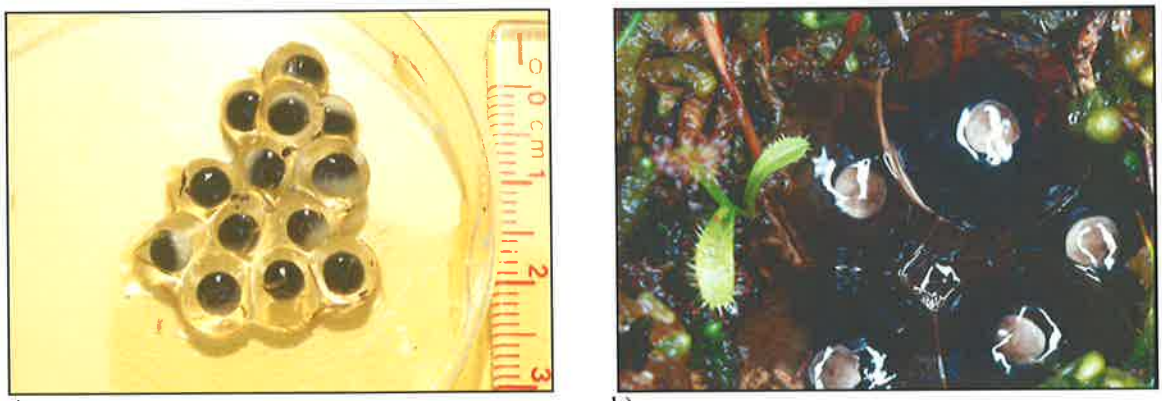

Figure 2. *Bryobatrachus nimbus* clutches photographed within 2 days of oviposition. a) clutch deposited in a comparatively dry moss nest by a marked breeding pair, and transferred to petri dish for photographing; b) clutch deposited in a wet nest within a leafy liverwort.

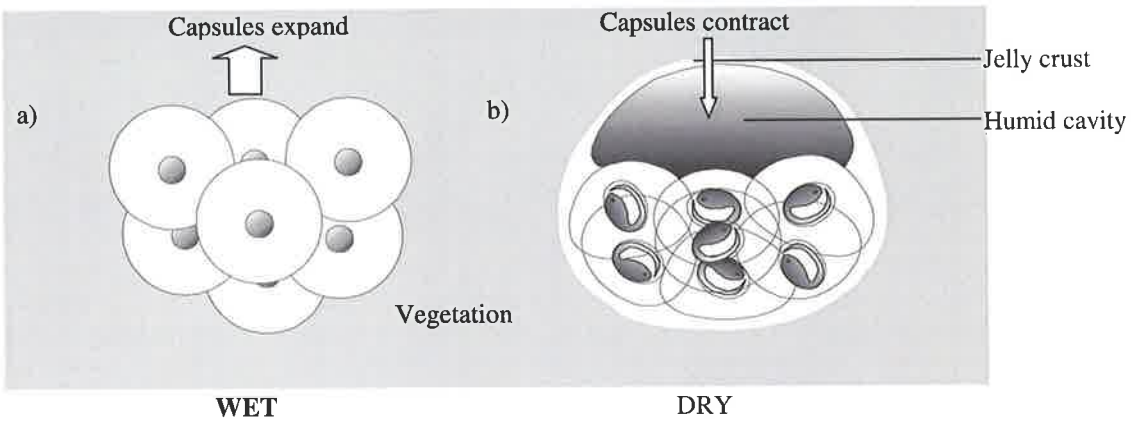

Figure 3. A possible mechanism for the formation of a jelly cap over a hatched clutch. At oviposition, eggs swell to fill the nest cavity (a), and following precipitation, the clutch expands further to contact the overlying vegetation. When conditions dry, and capsule viscosity has decreased near hatching (b), embryos sink to the nest base leaving jelly residue lining the cavity. The residue dries to a crust, and in some nests forms a complete seal over the clutch.

cap was sometimes broken while a moss patch was searched (n=20), and the clutch below the cap usually contained more jelly than uncapped clutches examined on the same day. It was never possible to examine an intact cap, but a diagram of a capped nest is shown in Figure 3.

Effects of water potential on laboratory eggs

Eggs placed onto filter papers at -5, -10 and -25 kPa gradually flattened onto the paper, whereas eggs at 0 kPa remained approximately spherical, and were similar to structure to eggs in moist field nests (e.g. Figure 2b). The vertical dimensions of capsules could not be measured without risking damage to embryos; hence the exact effect of water potential on capsule volume was not known. However, the volume of jelly in the -5 kPa eggs appeared at most to be about half that of 0 kPa eggs, while -10 and -25 kPa eggs appeared to be about one-quarter of the fully hydrated size (Figure 4).

It was impractical to measure perivitelline diameters during development because inconsistencies in capsule structure meant that refractive errors would occur. However, at the termination of the experiment, unhatched eggs were quickly submerged and the perivitelline diameters were measured with an ocular micrometer and stereomicroscope. The mean perivitelline diameter of wet eggs (0 kPa) of 6.55 ± 0.46 (n=5) was significantly greater ($P < 0.0001$) than 3.95 ± 0.15 mm measured for -10 and -25 kPa embryos (n=6, treatments pooled). Embryos of 0 kPa eggs were loosely arched inside perivitelline membrane, whereas drier embryos were curled tightly around the yolk.

Of the sixteen embryos introduced to each treatment, one mortality occurred at 0 kPa, and there were eight, five and three mortalities at -5 kPa, -10 kPa and -25 kPa, respectively. The difference in mortality between paired comparisons was significant between the 0 and -5 kPa treatments (Fisher's exact test, P=0.007), and between 0 kPa and the combined mortalities in the drier treatments (P=0.026)

After stage 19 (heartbeat), developmental stage was assigned using characters of the embryonic eye, because hindlimb buds developed abnormally in the two driest treatments, and other diagnostic characters, such as fin circulation, were difficult to observe. Developmental stages at termination of the experiment were 19.9 ± 0.4 for early embryos, and 23.6 ± 0.2 for late embryos; in both cases the developmental stage reached was independent of treatment water potential (Wilcoxon Rank Sums tests, P=0.39; P=0.97). Embryos reared at 0 kPa had the highest $\dot{V}O_2$ on both days 48 and 61, while $\dot{V}O_2$ was suppressed at the two lowest water potentials on day 48 (Tables 1 and 2).

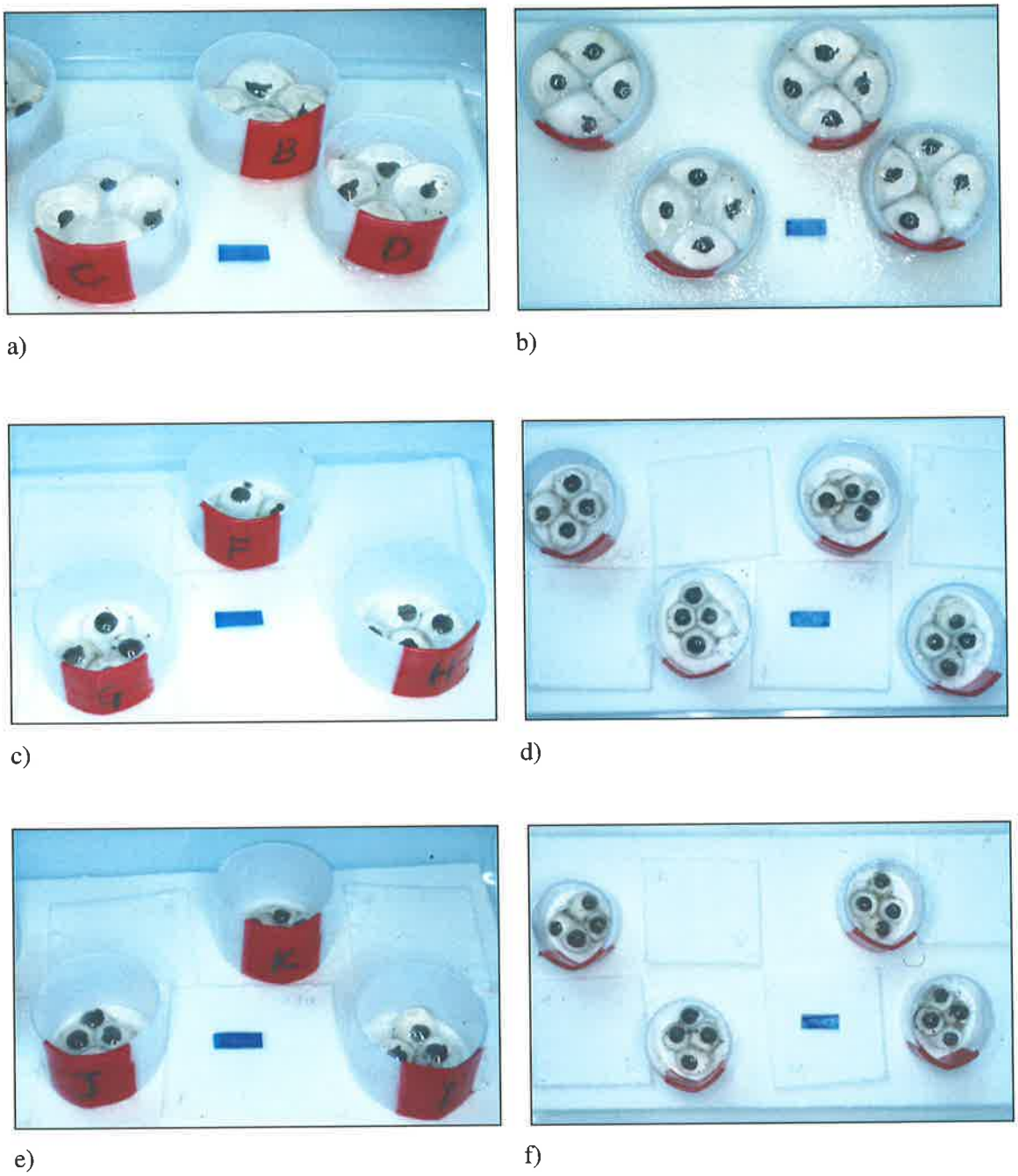

Figure 4. Filter paper stacks, egg rings and test squares: a) 0 kPa treatment viewed from the side, and b) above; c) -5 kPa treatment viewed from the side; d) -10 kPa treatment viewed from above, and e) the side; f) -25 kPa treatment viewed from above. Blue scale bars are 1 cm

The rate of embryonic growth was influenced by water potential. After 48 d, embryos incubated at 0 kPa had grown to a greater length than embryos from -10 and -25 kPa treatments (Table 1). After 61 d, 0 kPa embryos were at least 15% longer than embryos from all drier treatments (Table 2; Figure 5); at both ages the difference was due to the proportionally longer tails of the wettest incubated embryos. Moreover, fin expansion in these embryos was greater than all other embryo groups by day 61, as indicated by their significantly taller tails (Table 2; Figure 5). However, morphological differences were not matched by differences in wet or dry mass, or in the energy assimilated into body tissue (Tables 1 and 2).

Hindlimb buds of 0 kPa embryos developed normally, but the ratio of bud length to breadth was significantly less in the -5, -10 and -25 kPa treatments (Tables 1 and 2), which would have led to an incorrect assessment of developmental stage had eye characters not been used. Many drier-incubated embryos were severely malformed at the termination of the experiment. Symptoms included asymmetry, depression of the head, and tail lesions (Table 3; Figure 5). Several embryos from dry treatments developed ruptures in the yolk membrane after stage 19, while the perivitelline fluid of three embryos from the -25 kPa treatment contained clouds of yolk particles that were released from the perivitelline membrane when hatching was induced. When embryos were periodically examined under a stereomicroscope it was noticed that rotation of -10 and -25 kPa embryos was jerky and infrequent compared to fluid and frequent movement of 0 kPa embryos.

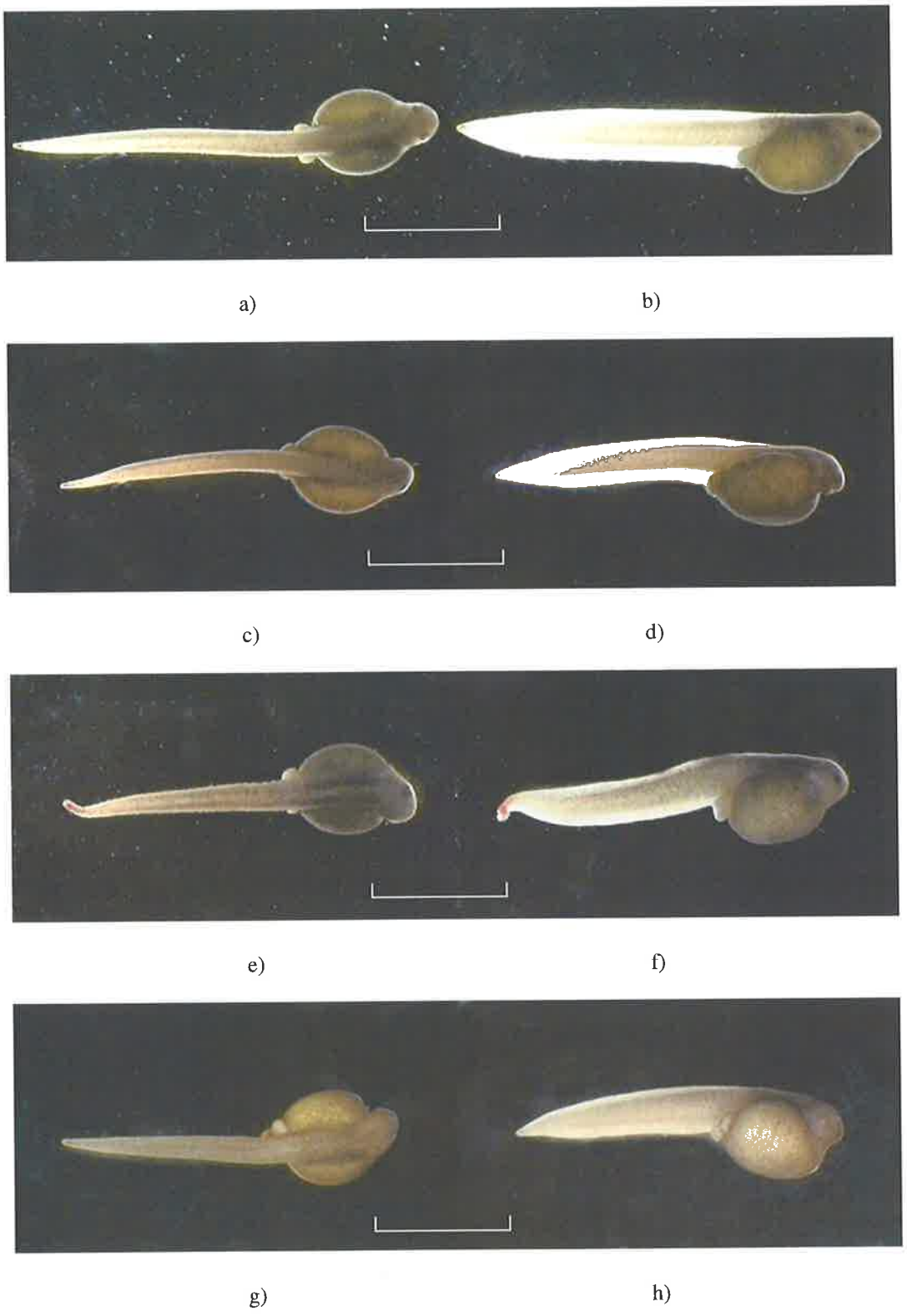

Figure 5. Examples of dorsal and lateral views of late embryos from each water potential treatment: a) and b), 0 kPa; c) and d), -5 kPa; e) and f), -10 kPa; g) and h), -25 kPa. Scale bars are 5 mm

Table 1. The effect of incubation water potential on embryonic growth, morphology and energy of early embryos (48 d)

Variable	Water potential (kPa)				*F*-value	*P*	Test
	0	-5	-10	-25			
Stage	19.75 ± 0.46[a]	19.75 ± 0.46[a]	19.50 ± 0.00[a]	19.75 ± 0.46[a]		0.392	NP
Hindlimb bud length:diameter	0.80 ± 0.07[a]	0.64 ± 0.07[b]	0.52 ± 0.20[c]	0.51 ± 0.10[c]	12.20	**0.001**	P
Embryo length (mm)	12.63 ± 1.12[a]	11.81 ± 1.17[ab]	11.59 ± 0.95[ab]	10.90 ± 1.34[b]		**0.036**	NP
% tail	65.3 ± 1.5[a]	57.9 ± 3.6[b]	61.1 ± 5.6[b]	60.2 ± 4.8[b]	5.49	**0.013**	P
Fin height (mm)	1.48 ± 0.43[a]	1.54 ± 0.14[a]	1.61 ± 0.09[a]	1.57 ± 0.25[a]	0.48	0.699	P
$\dot{V}O_2$ (μL h^{-1})	0.764 ± 0.136[a]	0.745 ± 0.051[a]	0.595 ± 0.071[b]	0.580 ± 0.213[b]	5.33	**0.015**	P
Total wet mass (mg)	19.40 ± 3.43[a]	21.70 ± 1.73[a]	21.1 ± 3.62[a]	22.28 ± 0.08[a]		0.124	NP
Total dry mass (mg)	11.19 ± 2.33[a]	11.28 ± 0.52[a]	12.00 ± 1.52[a]	11.77 ± 1.20[a]	1.09	0.396	P
Water content (%)	43.8 ± 6.5[a]	47.9 ± 3.6[a]	45.3 ± 8.7[a]	47.2 ± 5.5[a]	1.31	0.324	P
Dry gut-free body mass (mg)	1.75 ± 0.67[a]	1.78 ± 0.34[a]	1.97 ± 0.14[a]	1.86 ± 0.58[a]		0.682	NP
Dry yolk mass (mg)	9.31 ± 1.32[a]	9.51 ± 0.37[a]	10.01 ± 1.34[a]	9.91 ± 1.76[a]	0.71	0.565	P
[*]Energy in gut-free body (J)	46.4 ± 13.7[a]	47.1 ± 7.0[a]	52.3 ± 2.8[a]	49.3 ± 11.9[a]		0.682	NP
[†]Energy in residual yolk (J)	250.1 ± 20.2[a]	255.5 ± 7.7[a]	269.0 ± 20.5[a]	266.3 ± 36.4[a]	0.71	0.565	P
n	*4*	*4*	*4*	*4*			

Means (± 95% CI) with the same letter are not statistically different based on pairwise comparisons. Test indicates when parametric (P =ANOVA) and non-parametric (NP=Wilcoxon) tests were applied, in accordance with normality of data. **Bold** indicates variables where significant treatment effects were detected

[*]Energy density of body: 26.5 J mg^{-1}; [†]Energy density of residual yolk: 26.9 ± 0.2 J mg^{-1}

Table 2. The effect of incubation water potential on embryonic growth, morphology and energy of late embryos (61 d)

Variable	Water potential (kPa)				*F*-value	*P*	Test
	0	-5	-10	-25			
Stage	23.59 ± 0.28 [a]	23.67 ± 1.43[a]	23.71 ± 0.41[a]	23.71 ± 0.33[a]		0.968	NP
Hindlimb bud length:diameter	0.83 ± 0.06[a]	0.52 ± 0.24[b]	0.52 ± 0.07[b]	0.43 ± 0.10[b]	30.99	**<0.001**	P
Embryo length (mm)	14.16 ± 0.52[a]	12.24 ± 1.29[b]	11.90 ± 0.69[b]	10.47 ± 0.99[c]		**<0.001**	NP
% tail	67.4 ± 1.1 [a]	63.0 ± 5.8[ab]	62.0 ± 4.4[b]	60.3 ± 4.0[b]		**0.008**	NP
Fin height (mm)	1.92 ± 0.09 [a]	1.59 ± 0.20 [b]	1.67 ± 0.12 [b]	1.61 ± 0.08[b]	11.69	**<0.001**	P
$\dot{V}O_2$ ($\mu L\ h^{-1}$)	0.799 ± 0.122[a]	0.627 ± 0.441[a]	0.742 ± 0.160[a]	0.641 ± 0.118[a]		0.183	NP
Total wet mass (mg)	23.56 ± 2.43[a]	21.34 ± 2.97[a]	23.06 ± 2.04[a]	23.21 ± 2.08[a]	0.58	0.633	P
Total dry mass (mg)	10.85 ± 1.00[a]	10.37 ± 0.41[a]	11.10 ± 0.80[a]	10.70 ± 0.76[a]	0.42	0.739	P
Water content (%)	53.7 ± 3.7[a]	51.4 ± 5.0[a]	51.6 ± 3.6[a]	53.8 ± 1.5[a]		0.418	NP
Dry gut-free body mass (mg)	1.92 ± 0.26[a]	1.66 ± 0.25[a]	1.85 ± 0.22[a]	1.74 ± 0.07[a]	1.25	0.317	P
Dry yolk mass (mg)	8.92 ± 0.88[a]	8.70 ± 0.58[a]	9.25 ± 0.74[a]	8.96 ± 0.77[a]		0.349	NP
*Energy in gut-free body (J)	52.2 ± 7.0[a]	45.1 ± 3.8[a]	50.2 ± 6.1[a]	47.2 ± 2.0[a]	1.25	0.317	P
†Energy in residual yolk (J)	237.9 ± 25.0[a]	231.1 ± 8.0[a]	245.6 ± 19.7[a]	242.9 ± 20.8[a]		0.450	NP
n	*9*	*3*	*7*	*7*			

Means (± 95% CI) with the same letter are not statistically different based on pairwise comparisons. Test indicates when parametric (P =ANOVA) and non-parametric (NP=Wilcoxon) tests were applied, in accordance with normality of data. **Bold** indicates variables where significant treatment effects were detected

*Energy density of body: 26.6 J mg^{-1}; †Energy density of residual yolk: 27.0 ± 0.3 J mg^{-1}

Table 3. Effect of water potential on the frequency (%) of malformations in late embryos.

Character	% with malformation				*P* (0kPa vs. drier treatments)
	0 kPa	-5 kPa	-10 kPa	-25 kPa	
Yolk sac damage	0	33	14	43	0.094
Tail tip curled	11	33	14	43	0.241
Tail lesions	0	0	57	14	0.094
Trunk twisted	0	100	100	86	**<0.001**
Hind bubs asymmetric	0	0	71	86	**0.002**
Head depressed	0	0	86	86	**<0.001**
Head abnormalities	0	0	14	29	0.262
Body abnormalities	0	33	0	57	0.094
n	*9*	*3*	*7*	*7*	

Fisher's exact tests (Sokal and Rohlf 1995) were used to test for differences in the frequency of malformations between 0 kPa embryos and all drier embryos (-5 to –25 kPa). **Bold** indicates those abnormalities significantly affected by incubation water potential.

DISCUSSION

Susceptibility of B. nimbus *embryos to dehydration*

In the relatively few published studies of embryonic water balance of terrestrial anurans, conditions of water stress have been established by different means. Eggs have been incubated on filter papers of certain water contents (Kam et al. 1998), at different relative humidities (Martin and Cooper 1972), or at known water potentials (Taigen et al. 1984; Seymour and Bradford 1987; Bradford and Seymour 1988; Seymour et al. 1991a; 1991b). Direct comparisons can therefore only be made between these latter studies, and the present study of *B. nimbus*.

A feature of this study was that embryonic morphology appeared abnormal at water potentials that allow normal development in other anuran embryos. Embryonic tolerance limits are -200 kPa in *P. bibronii* (Bradford and Seymour 1988) and at least -550 kPa in *Eleutherodactylus coqui* (Taigen et al. 1984). In marked contrast, embryonic survival of *B. nimbus* was significantly reduced at relatively moist water potentials (-5 to –25 kPa), compared to the treatment where water uptake was unrestricted (0 kPa). Moreover, it is probable that some embryonic malformations resulting from drier incubation, such as lesions of the yolk sac, would have led to mortality later in development. Surprisingly, the greatest difference in mortality occurred between 0 and -5 kPa treatments, but because the experiment was pseudo-replicated, it is conservative to conclude that the high mortality in the -5 kPa treatment was a container effect.

Development of *B. nimbus* at drier water potentials produced a significant reduction in embryo total length, tail length and fin height, and greater asymmetry relative to 0 kPa embryos; in many cases asymmetries appeared sufficiently severe to persist into larval stages. Differences in the amount of perivitelline fluid (PVF) may partly account for these divergent morphologies. The large volume of PVF in 0 kPa eggs permitted frequent rotation and flexion of the embryo. We can compute this volume by subtracting the mass (≈volume) of the embryo from the volume enclosed by the perivitelline membrane; at 0 kPa it was about 128 mm^3 (147 mm^3 -19.4 mg). In contrast, typical PVF volumes for -10 and -25 kPa late embryos were about 11 mm^3 (32

mm^3 –21.7 mg). Thus drier embryos developed in 9% of the PVF present in a 0 kPa embryo. Comparable analyses of published data for smaller *P. bibronii* embryos show that 0 kPa incubation resulted in a PVF volume of 55 mm^3 prior to hatching, while -25 kPa embryos contained 31% of this amount (17 mm^3), but neither malformations nor asymmetries were reported (Seymour and Bradford 1987). In this study, the common observation of damage to the yolk sac, and yolky particles in the PVF, suggested that adhesions had formed between the body wall of the embryo and the vitelline membrane. Adhesions coupled with low PVF volume would undoubtedly restrict embryo movement, and perhaps limit conditioning of tail muscle. Further, if embryos became affixed on a particular side, malformations such as head depression and trunk asymmetry (prominent in the -10 and -25 kPa treatments; Table 3) are likely consequences.

The highest rates of oxygen consumption ($\dot{V}O_2$) were those of embryos incubated at 0 kPa, which agreed with a pattern established in *P. bibronii* (Seymour et al. 1991b). However, as the dry mass of metabolic tissue was no greater in 0 kPa incubated eggs (Tables 1 and 2), it does not explain their higher $\dot{V}O_2$. Instead, the area of the tail fin (calculated as an equilateral triangle, with tail length and fin height as the two axes), which declined with decreasing water potential, explains some of the variation in $\dot{V}O_2$ of late embryos ($r=0.40$, $p<0.05$). Hence, the larger surface of hydrated embryos provides a larger area for cutaneous gas exchange, and is likely to facilitate their higher $\dot{V}O_2$.

Incubation of *B. nimbus* eggs at drier water potentials produced embryos of a similar mass to fully hydrated embryos, and there were no differences in the amount of yolk remaining in the gut (Tables 1 and 2). Similarly, *Eleutherodactylus coqui* incubated between -50 and -250 kPa hatched at comparable masses, but masses were reduced by 60% when eggs were incubated at -550 kPa (Taigen et al. 1984). The authors concluded that there is a minimum egg water content that allows maximum utilisation of yolk, analogous to a pattern established in studies of reptile eggs (Packard 1999). Emergence at larger sizes as a result of a favourable embryonic water balance is often asserted to have important fitness consequences for oviparous species (e.g. Janzen 1993; Kam et al. 1998; Packard 1999). In this study, fitness traits mediated by incubation water potential include a larger, more symmetric phenotype, and higher $\dot{V}O_2$ of embryos incubated at 0 kPa.

Factors ensuring adequate hydration

B. nimbus embryos apparently largely avoid dehydrating in natural nests, because the severely altered embryonic morphologies resulting from laboratory incubation on drier substrates were not evident in the field. The unusually large egg capsules of *B. nimbus* eggs may have an important role as reservoirs of water. The time taken for eggs to equilibrate with their paper substrate gives some indication as to how long capsules retain water. At -5 and -10 kPa, eggs equilibrated in about 3d, whereas the eggs on -25 kPa papers dehydrated over about 7 d. In field nests the interval between precipitation events rarely exceeds 1 week (Bureau of Meteorology, Hobart), and consequently, in the short term, capsules can buffer embryos against water loss at water potentials up to -25 kPa. Further, the dried jelly cap that sometimes formed over hatched *B. nimbus* clutches in dry weather (Figure 3) might be an additional mechanism by which embryonic desiccation is reduced. Presumably the crust seals water vapour inside the nest, and so prevents dehydration of the jelly. Although intact caps were found rarely, my frequent inspection of nests at early developmental stages usually widened the nest aperture, and perhaps prevented cap formation.

Capsules of *B. nimbus* are small immediately following oviposition (Figure 2a), and the rate and extent of swelling depends on the differential in water potential between the eggs and the nest. For example, *B. nimbus* clutches deposited in drier nests swelled comparatively slowly. Conversely, oviposition in wet conditions, or in a wet nest (e.g. Figure 2b), promoted rapid swelling of egg capsules up to a maximum size of about 23 mm diameter (Chapter 3). Although capsule jelly is elastic and can rapidly rehydrate when supplied with external water (e.g. Martin and Cooper 1972), there is also evidence from a study of aquatic *Rana temporaria temporaria* eggs that the capsule size attained 24 h after oviposition influences subsequent capsule size (Beattie 1980). Hence the habit of arboreal nesting phyllomedusid females of filling their bladders in a nearby pool between each oviposition event, can be viewed as a mechanism for promoting the maximum swelling of egg capsules (Pyburn 1970). Similarly, *B. nimbus* females should use moisture criteria to discriminate between oviposition sites, because the failure to do so might result in capsular jelly with a reduced capacity for water storage. It is tempting to conclude that females resident in dry nests for several days were delaying mating until wetter condition prevailed, but it is also possible that my observations disrupted breeding behaviour.

Generally, associations between adult *B. nimbus* and eggs were rare and thus parents did not appear to assist with maintenance of egg water balance. The extremely protracted development time of *B. nimbus* (11-13 mo.) would likely prohibit parental care; moreover, parental transfer of water may be largely redundant because *B. nimbus* conceal their eggs in moist vegetation. Mosses and leafy liverworts lack roots, leaf cuticle and stomata, and water is absorbed from water falling on or flowing over the leaves (Proctor 1981; Richardson 1981). In moist conditions, water is 382% of the dry weight of *Dicranoloma* moss (Chapter 6). In contrast, the waxy cuticle of the leaves and fronds of *E. coqui* nest sites do not supply the eggs with water (Taigen at al. 1984; Townsend 1986), and circulation of air around the exposed egg mass is much greater than in the concealed nests of *B. nimbus*. Male *E. coqui* regularly attend egg masses, and transfer water to the eggs through their ventral surface, causing a threefold increase in egg mass during the course of incubation. Eggs that do not at least double in mass either die or hatch at about 60% of the wet mass of hydrated eggs (Taigen et al. 1984).

Given the severity of embryonic abnormalities that resulted from incubation at relatively high water potentials, it appears that successful metamorphosis of *B. nimbus* will only occur if the oviposition site is reliably moist. Entire clutches occasionally dehydrate and die in field nests, but most mortality is not caused by dehydration (Chapter 6). An understanding of the mechanisms and criteria used by adult frogs to select nest sites would be a valuable adjunct to future studies of water balance of terrestrial breeding species.

Acknowledgments

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6 Nest characters, site selection, and egg mortality in a frog with protracted development

Abstract

The embryos and larvae from the Tasmanian moss frog, *Bryobatrachus nimbus*, develop in a terrestrial nest for up to 13 months, the longest nidicolous phase known for any anuran amphibian. Parents do not attend the eggs, so selection of a nest site that provides an appropriate incubation climate and protection from predators is critical to offspring fitness. At a sub-alpine site, males formed 30 cm^3 concealed nest cavities within *Dicranoloma robustum* and *D. billardieri* moss. *Dicranoloma* mosses were used in 82% of oviposition sites, but eight other species of bryophytes, lycopods and lichens were also used. Most nest cavities persisted for at least 5 years, and occasionally contained clutches twice in this period. Males constructed a small proportion (<10%) of new nests each year, and these were more likely to be used as oviposition sites than older nests. Discriminant function analysis showed that *Dicranoloma* nests that contained eggs could be distinguished from vacant nests by their larger dimensions – their mean horizontal area was 2 cm^3 greater than vacant nests. No embryonic mortality was attributed to hypoxia, possibly because clutches (4-16 eggs) deposited in wide nests are positioned in fewer layers, facilitating oxygenation of the mass. However, partial or complete embryonic mortality was recorded in 32% of 290 clutches, of which 13% were due to clutch desiccation or invertebrate predation.

INTRODUCTION

In many amphibians, embryonic mortality constitutes a significant portion of premetamorphic mortality (e.g. Kluge 1981; Malone 1985). Two factors that may enhance the survival of embryos are selection of a quality oviposition site (Howard 1978), and attendance by a parent, which decreases the risk of desiccation and predation (Taigen et al. 1984; Burrowes 2000). Both behaviours should be favoured by natural selection if they do not incur costs to the adults (Shine 1978; Petranka and Petranka 1981). Moreover, the oviposition environment can profoundly influence hatching phenotype in ways that are liable to affect offspring fitness (e.g. Shine and Harlow 1996; Kaplan 1987; 1992). For example, nests of anuran amphibians that have low substrate water potentials tend to produce smaller hatchlings with higher frequencies of developmental abnormalities (Seymour et al. 1991; Chapter 5). Hence the choice of oviposition site should be subject to at least as strong selection as that on egg size and number, because the production of the fittest phenotypes requires a consistent environmental context (Resetarits 1996).

The eggs of most anuran amphibians are fertilised externally, and consequently female selection of her oviposition site is at least partially dependent on behaviours of the male. Pairs may select a site together (e.g. *Litoria lesueuri*; Richards and Alford 1992), but more commonly in terrestrial species, the male chooses the nest, defends it from rival males, and vocalises to advertise to females. Selection should favour males that choose quality nests, and females that use nest quality to discriminate between potential mates (Townsend 1989; Resetarits and Wilbur 1991). This is especially true when the development time in the nest is protracted, and when parental care is absent. The Australian moss frog, *Bryobatrachus nimbus*, has the longest nidicolous lifespan known for an anuran amphibian; on average clutches of 4-16 endotrophic eggs develop for 11-13 months within a terrestrial cavity in moss or lichen (Chapter 3). Hence the oviposition site must provide a suitable microclimate over the spectrum of seasonal extremes. Although males are sometimes associated with fresh clutches (Mitchell 1995), there is no parental care, and it is likely that the cost of brooding is prohibitive over such a long period.

Physiological studies have identified several microclimatic variables that influence the viability of *B. nimbus* eggs: 1) a 6°C difference in the effective temperature of the nest controls whether froglets emerge in autumn, or the following summer, and consequently influences the size of yolk reserves at metamorphosis (Chapter 3), 2) nest shape determines how deeply eggs are stacked, which in turn influences the oxygenation of the egg mass (Chapter 4), and 3), embryonic growth is stunted and asymmetric at water potentials drier than -10 kPa (Chapter 5). Given such stringent requirements, oviposition in appropriate nests will be paramount to successful metamorphosis.

An earlier study over a single *B. nimbus* breeding season found that moss patches containing nests differed significantly from those that did not; specifically, used patches occurred in regions of low heath density. It was suggested that male frogs preferred these patches because call propagation was more effective in sparser heath (Mitchell 1995). Moreover, there were large numbers of vacant nests relative to those that contained a clutch. Nests that were used as oviposition sites could be distinguished from vacant nests using a suite of predictive nest characters. The horizontal area of a nest was the strongest predictor of vacant and occupied nests; occupied nests were generally wider than those without eggs. In this study, conducted over 4 breeding seasons, I determined whether males constructed nests, or whether nest cavities were instead created by an oviposition event. Characters of used and vacant nests were compared in a refinement of the earlier survey, while egg mortalities were recorded and their causes evaluated. These data allowed assessment of the opportunity for female mate choice based on the quality of a male's oviposition site.

METHODS

Study site and data collection

The study site was the type locality of *B. nimbus* - a poorly drained plateau of sub-alpine heath in the Hartz Mountains National Park (refer Chapter 3). All nest cavities present in a clump of moss, liverwort, lycopod or lichen were located after carefully searching the entire clump. Nest occupancy (male, breeding pair or clutch) was noted, and the snout to vent length (SVL) of frogs was measured to the nearest 0.1 mm with dial callipers. Clutch sizes were counted on the first inspection. Nests were periodically re-examined (2-10 times per clutch) over approximately 80 daytime visits between October 1995 and January 2000. The number and potential cause of egg mortalities were recorded.

Frequency of nest use

Sixty-seven patches of *Dicranoloma* moss were surveyed each year, for 4 years, to provide information on the frequency of nest building, the proportion of nests containing clutches, and to clarify whether pre-existing nests were used as oviposition sites. Patches were tagged in October 1995, prior to the onset of the summer breeding season, and drawn to detail their shape, dimensions, and the locations of any nests. Nests were frog-sized cavities with compacted walls and base, and sometimes contained jelly residue. Nest status (*i.e.* vacant, or containing larvae from the 1994/95 breeding season) was recorded. Nests containing jelly residue were assumed to have contained clutches deposited in the 1993/94 breeding season. Patches were not searched again until March 1996 (when breeding activity had ceased), and the occurrence of clutches and locations of new nests was recorded. Thereafter, the census was repeated in the late summer of 1997, 1998 and 1999, and patches were deliberately not examined in the interim. Photographs of patches were taken in 1998 to replace the 1995 drawings. Some patches could not be relocated, causing the survey to reduce to 62 moss patches by the final year.

Measurement of nest characters

The presence of large numbers of vacant nests stimulated a preliminary survey of nest characters, with the view to identify features of preferred oviposition sites (Mitchell 1995). The survey was repeated in February and March 1999, but with three important

refinements. Firstly, the 1995 survey included nests that occurred in various vegetation types, whereas the current survey focused solely on nests in the two very similar moss species, *Dicranoloma robustum* and *D. billardieri.* Secondly, the 1995 survey ranked nests in terms of whether their base was close to the ground, or elevated above it, whereas in the present survey, elevation was measured exactly. Finally, because females will not oviposit in a nest that does not contain a male, I chose vacant nests according to the following conventions: either a male was known to have occupied the nest within the previous year, or the nest was the closest vacant nest to a nest containing a clutch. Hence these nests were potentially rejected as oviposition sites, and therefore provided a relevant comparison to used nests.

Six characters thought to influence nest microclimate were monitored; they related to the size, elevation, exposure, and water potential of nests. They were:

1. nest depth (cm);
2. width and breath of a nest (cm);
3. elevation of nest base above soil (cm);
4. distance to nearest edge of moss patch (cm);
5. patch exposure (1-5);
6. water content of moss (% dry mass)

Dial callipers accurate to 0.1 mm were used to measure nest depth (1), which was defined as the distance from the base of the nest to the tip of the moss stem. Similarly, nest diameters (2) were the widest horizontal span (a), and the span perpendicular to it (b). The horizontal area of a nest was then calculated as the area of an ellipse ($\pi \times a/2 \times b/2$). The elevation of the nest base above the soil (3) was measured by pushing a piece of 3 mm diameter dowel through the nest until the dowel tip contacted the soil, and then sliding a plastic sleeve down the dowel to meet the nest base. The dowel was removed and the distance between the dowel end and the sleeve was measured with callipers. Moss patch exposure (5) was scored as 5 if all sides were exposed (i.e. the patch experienced full sunlight), and 4,3,2 and 1 if shaded by heath on one, two, three or four side(s) respectively. A representative sample of moss adjacent to each nest was collected during a 2.5 h period on the afternoon of 8 March 1999. Samples were immediately sealed inside a labelled snap-lock bag, and in the laboratory, sealed bags were weighed to the nearest mg on a

Mettler AE 163 analytical balance. Bags were then opened and oven dried for two days at 70 °C before being reweighed. The water content, (6) of each moss sample could be calculated after subtracting the mean mass of empty bags (n=5) from total masses.

Statistical analysis

Descriptive data are means ± 95% CI, or expressed as percentages. Stepwise discriminant function analysis (Tabachnick and Fidell 1996) determined whether nests used as oviposition sites could be distinguished from vacant nests in terms of the nest variables measured, using the computer programme Statistica (Statsoft Inc. 1995).

RESULTS

Characters of sub-alpine B. nimbus *nests*

At the study site in sub-alpine heath nests were located in ten species of vegetation (Figure 1). Most nests were concealed beneath surface vegetation, but occasionally a fully hydrated clutch bulged over the lip of the nest and was partially visible before vegetation was searched. The mosses *Dicranoloma robustum* and *D. billardieri* were most commonly used as oviposition sites (81.7% of 290 clutches), followed by the edaphic lichens *Cladia retipora* and *C. sullivanii* (6.6%), a leafy liverwort, *Heteroscyphus billardieri* (6.2%), sphagnum moss, *Sphagnum australe* (2.8%) and a rhizoid moss *Breutalia pendula* (1.6%). Species used infrequently (<1% of clutches) were the lycopods *Lycopodiella diffusa* and *Lycopodium scariosum*, and the branching moss *Lepidozia ulothrix*. Vegetation species appeared to be used in proportion to their abundance within the heath. Vegetation was compacted in the lower region of the nest and provided a solid wall, but was progressively less rigid toward the nest top, usually forming a goblet-shaped cavity of about 30 cm^3. The average diameter of *Dicranoloma* nests was 3.24 ± 0.80 cm (n=89). Nests in lichen were shallow depressions (1-2 cm deep; mean diameter 3.47 ± 0.27 cm, n=5) in a sponge-like stratum of soil and lichen, underlying 3-10 cm of surface lichen.

Frequency of nest use

The annual surveys of *Dicranoloma* moss patches showed that nests used as oviposition sites existed prior to an oviposition event. Patches contained between 0 and 9 nests (mean 2.8 ± 0.5 nests). Patch size ranged between 113 and 1178 cm^3 (mean 459 ± 65 cm^3), and the number of nests in a patch was positively correlated with its size (r=0.46, n=64, P = 0.0002). Approximately 7.3% of surveyed nests contained a clutch, but the ratios differed from year to year (Table 1). Four patches contained two clutches in a single season, while a *Heteroscyphus* patch that was not part of the survey contained nine clutches over three successive breeding seasons. Small numbers of new nests appeared in *Dicranoloma* patches each year (Figure 2), and these were more likely to be used as oviposition sites than pre-existing nests ($\chi^2_{0.05,1}$ =27.8, p<0.001, based on mean frequencies in Table 1). Four moss patches contained nests that were used twice in five breeding seasons; in one of these nests, froglets were observed on the nest wall on 17 November 1999, and a fresh clutch had appeared in the nest by December 22 1999.

Dicranoloma robustum

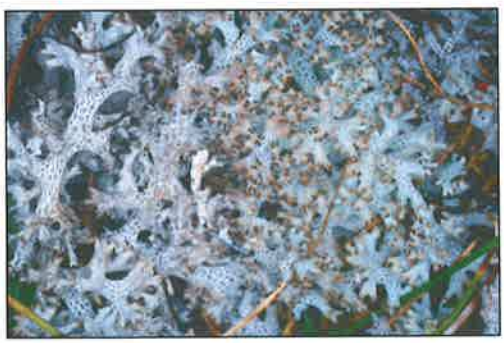
Sphagnum australe

Breutalia pendula

Lepidozia ulothrix

Heteroscyphus billardieri

Lycopodium scariosum



Cladia retipora

Cladia sullivanii

Figure 1. Eight of ten vegetation species used as nest material by *Bryobatrachus nimbus* in sub-alpine heath in the Hartz Mountains National Park, southern Tasmania.

Table 1. Frequency of nest use in old nests, new nests and all nests over six breeding seasons.

Season	*n* patches	% Nests containing clutches			
		Old	New	Total	(*n* nests)
1993/94	67			7.8	(140)
1994/95	67			11.4	(140)
1995/96	64	3.6	10.7	4.8	(168)
1996/97	65	6.5	50.0	8.0	(176)
1997/98	65	5.3	22.7	7.3	(193)
1998/99	62	4.5	6.3	4.6	(195)
Mean		**4.9**	**22.4**	**7.3**	

The proportion of surveyed nests containing clutches could be used to estimate annual nest search effort (i.e. searches beyond the surveys described above) based on the total number of clutches found each season (Table 2). For example, in order to locate 84 clutches at the 1998/1999 rate of 4.6 clutches per 100 nests examined, approximately 1818 nests would have been searched that season. While these figures are simplifications, it allows us to estimate the proportion of nests that contain males. On average 2.3 males were located in moss nests for every 100 nests examined (Table 2). This estimate reflects daytime occupancy rates, for nests were not searched at night. In total 109 males were resident in nests, including one case where three males were found in separate nests within a small patch of *Dicranoloma* moss. A further 10 males were located outside nests, usually on the edge of a moss patch, or else crawling on its surface. Four males were found under a rock slab. Of the 109 males in moss nests, 12 were paired with a female, four sat on the surface of a fresh clutch, and 17 males were in the same vegetation patch as a fresh clutch (e.g. Figure 3). The mean dimension of a nest containing a breeding pair was 3.43 ± 0.24 cm (n=9), and nest dimensions did not change noticeably once a clutch had been deposited.

Do nests containing clutches differ from vacant nests?

A stepwise discriminant function analysis was performed using six nest variables as predictors of membership of two nest groups, those containing clutches, and vacant nests. Predictors were nest depth and horizontal area, elevation of the nest above the

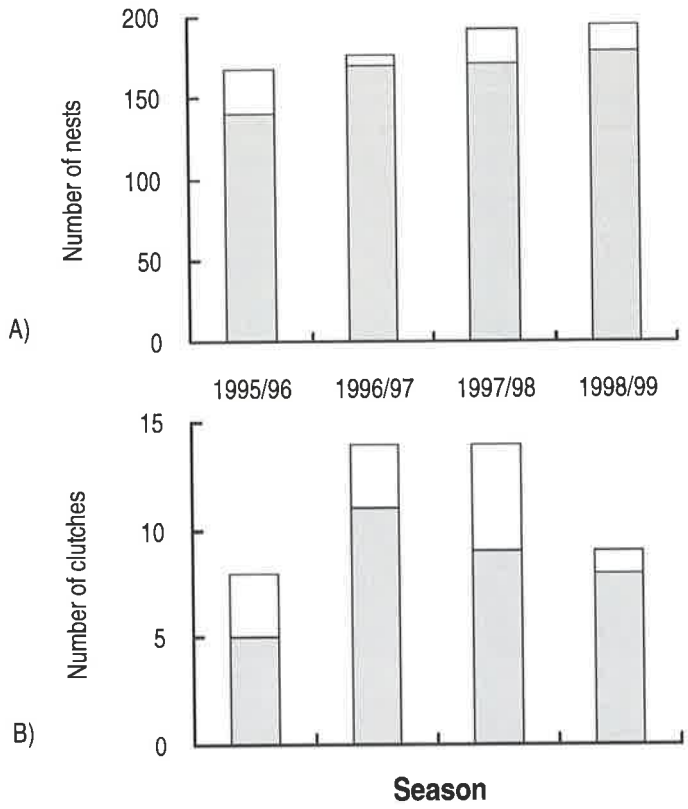


Figure 2. Data from annual surveys of 65 *Dicranoloma* moss patches: A) numbers of old nests (grey) and new nests (white) recorded; B) number of clutches deposited in old nests (grey) and new nests (white). Some nests recorded in earlier surveys were indistinct in later years and were excluded from the data set.

Figure 3. A male *Bryobatrachus nimbus* in a nest immediately adjacent to another nest containing a clutch of late embryos.

Table 2. Summary of field data

Season	% Surveyed nests with clutches*	Total number of clutches found	% Clutches with embryonic mortality	†Estimated number of nests examined	‡Number of males found in moss nests	% Nests containing males
1995/1996	4.8	20	20.0	420	9	2.14
1996/1997	8.0	57	22.8	717	0	
1997/1998	7.3	101	49.5	1393	34	2.44
1998/1999	4.6	84	41.7	1818	44	2.42
1999/2000		28	25.0		22	
Total		**290**			**109**	
Mean	**6.2**		**31.8**			**2.33**

* From Table 1

†Based on the % of surveyed nests with clutches, and total number of clutches found. Fieldwork ended in January 2000, before breeding activity ceased; hence data are incomplete for the final season.

‡Excludes males found in lichen, and males found near a moss nest. No males were located in 1996/1997 when fieldwork was limited to the end of the breeding season in March 1997.

soil, distance to the nearest edge of a moss patch, moss patch exposure, and moss water content. Of the 89 surveyed nests, four were identified as multivariate outliers and were deleted. The outliers were evenly distributed between the two nest groups (clutch and vacant), and were either very elevated, deep, or wet nests. For the remaining cases, nest elevation, distance of the nest to the patch edge, and moss water content were log or square root transformed to satisfy normality. All variables satisfied homogeneity of variance criteria.

A significant difference [F-statistic (3,81) 6.0033, p = 0.0010] was detected between vacant and occupied nests, based on only three predictor variables: the horizontal area of the nest, nest elevation, and the water content of the moss. The canonical loading of predictors (their correlation with the discriminant function) suggested that the best predictor for distinguishing between the nest groups was the horizontal area of the nest (loading of 0.93; Table 3), while nest elevation was the second most discriminatory variable (loading of 0.38). Nests used as oviposition sites were wider (9.2 cm^2) than vacant nests (7.4 cm^2; Table 3), and were on average elevated 5.5 cm above the soil,

Table 3. Group means of predictor variables and their canonical loadings (correlation with the discriminant function)

Variable	Nest group mean (± 95% CI)		*Standardised correlation of variable with the discriminant function
	Containing clutch (*n*=43)	Vacant (*n*=42)	
Horizontal area (cm^2)	9.2 ± 0.7	7.4 ± 0.7	0.928
Elevation above soil (cm)	5.53 ± 6.00	5.03 ± 7.32	0.380
Moss moisture content (% dry weight)	160 ± 25	164 ± 26	-0.342
Moss patch exposure (0-5)	2.2 ± 0.3	2.1 ± 0.3	
Distance of nest to edge of patch (cm)	5.2 ± 0.6	5.1 ± 0.6	
Nest depth (cm)	4.8 ± 0.3	4.9 ± 0.4	
Nest volume (cm^3)	32.7 ± 3.4	28.3 ± 3.2	

* Three predictor variables were sufficient to distinguish between vacant and occupied nests.

compared to 5.0 cm for vacant nests. Mean moss water content was 160% of dry mass in used nests, slightly lower than vacant nests, but moss water content ranged widely, from 20-382% in used nests, to 44-382% in vacant nests.

Frequency of clutch mortality

Embryonic mortality was recorded in 31.8% of clutches (Table 2), but was not usually severe; on average, 33.8 ± 2.3 % (2-3) eggs in affected clutches did not survive (Figure 4). Dead eggs and embryos occurred most often at the nest margins. Egg desiccated in seven of 104 cases, while predation by planarian or dipteran larvae was recorded in a further 10 clutches. I characterised predated eggs by the appearance of opaque yolk-coloured clouds in centre of each egg that were very similar to the aftermath of planarian predation of Western Australian *Geocrinia* embryos (N.J. Mitchell, personal observation). However, planarians were observed only once. Dipteran larvae were noticed burrowing through the jelly capsule in 3 clutches. Several fresh clutches had pale cream eggs about 1 mm in diameter scattered on their surface. The cause of embryonic mortality was unknown in the remaining 88 clutches - some clutches where 100% mortality was recorded may have been unfertilised.

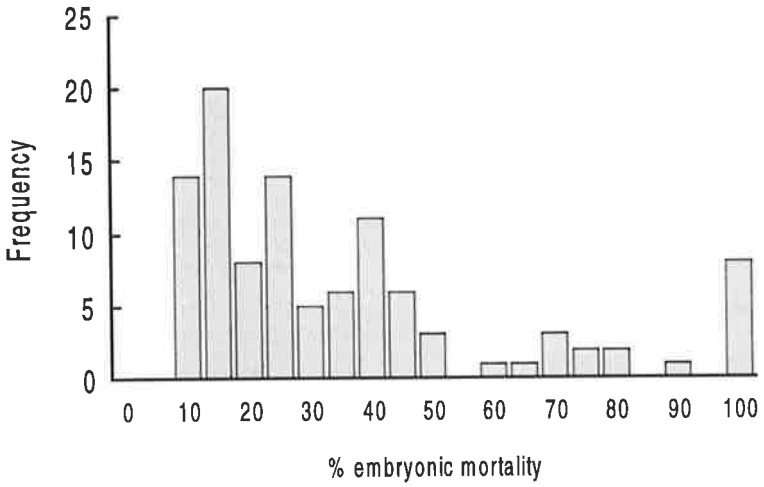


Figure 4. Frequency of embryonic mortality of *Bryobatrachus nimbus* in natural nests (n=104). Clutches where no embryonic mortality was recorded (n=185) are excluded. When clutch size could not be counted accurately, as sometimes occurred when clutches were discovered at later embryonic stages, the mean clutch size of 9 eggs (Chapter 3) was used to estimate % mortality.

Mortality of 1-2 larvae was observed twice, while six clutches of larvae disappeared entirely between inspections in March and November. While it is possible that metamorphosis was complete and froglets had left the nest (*c.f.* Chapter 3), eggs may have been consumed by adult harvestmen (Opiliones: *Triaenonychidae*) or numerous juvenile harvestmen, both of which were conspicuously associated with *B. nimbus* clutches. Other potential predators included several species of leech and spider, and the skink *Niveoscincus orocryptus,* but predation by these species was not observed.

DISCUSSION

Moss frogs nested in an array of bryophytes and lichens, and vacant nests were abundant; on average only 7.3% of *Dicranoloma* nests contained clutches each breeding season (Table 1). Hence oviposition sites were not limiting, and the diverse substrates and sizes of nests provided a range of potential incubation climates. Annual surveys of a subset of nests revealed that most nests persisted for at least 5 years, relatively few nests were constructed each year, and that both old and new nest cavities were used as oviposition sites. Nests were not formed by an oviposition event (*c.f* Richards and Alford 1992), and although nest construction was never observed, male *B. nimbus* were almost certainly responsible.

Bryophytes and lichens appear to be eminently suitable oviposition sites for the large eggs of *B. nimbus* - they not only conceal eggs from potential vertebrate predators, but provide a moist, oxygen rich environment and may afford some protection from freezing in winter. Two other Australian Myobatrachines, *Kyarranus* (= *Philoria*) sphagnicolus, and *Philoria frosti*, construct burrows in moss, but the nest base is a water filled depression (de Bavay 1993; Malone 1985). Because *Philoria* are foam-nesters, air pockets within the egg mass may free it of an association with a photosynthetic substrate.

Like an earlier study (Mitchell 1995), discriminant function analysis showed that nests in *Dicranoloma* moss that were used as oviposition sites could be reliably distinguished from a subset of vacant nests by their greater horizontal area; occupied nests having an area almost 2 cm^2 greater than vacant nests (Table 3). This result can be interpreted in two ways. Firstly, the larger size of nests used as oviposition sites may have been a consequence of their occupation by a male-female pair. The average SVL of a gravid female (3.04 ± 0.07 cm, n=12; N. J. Mitchell, unpublished data) was similar to the average dimensions of nests in *Dicranoloma* (3.24 ± 0.80 cm) and lichen (3.47 ± 0.27 cm), hence pairs manoeuvring within the nest might further compact the vegetation, and so widen the nest prior to the oviposition event. An alternative interpretation is that the male shapes the nest; females assess nest breath before oviposition, and prefer wider nests. A similar preference was found in female *Chirixalus eiffingeri* in Taiwan - females preferred to

oviposit on the walls of wider bamboo stems, and their eggs were more successful at such sites (Kam et al. 1988).

The incubation climate of B. nimbus *nests*

Wide nests promote a hemispherical arrangement of eggs in a mass, and so reduce the diffusion distance from the jelly surface to basal embryos. *B. nimbus* egg capsules are unusually large (at least 4 mm thick near hatching), hence embryos are particularly susceptible to hypoxia because oxygen diffuses through jelly at a rate $1/300000^{th}$ that in air (Chapter 4; Seymour 1999). A model of a spherical mass of *B. nimbus* embryos near hatching stage demonstrated that an embryo surrounded by as few as six others would be in anoxic conditions at temperatures above 10°C (Chapter 4). Embryos developing in a narrow nest are more likely contained in a spherical cluster, and therefore inner embryos may not develop aerobically at warmer temperatures.

Egg viability is also likely to be influenced by nest water potential. Because amphibian eggs freely exchange water with their surroundings, the water potential of embryos and larvae depends upon the water vapour permeability of the nest vegetation, and local variables such as soil drainage and exposure to solar radiation. Wetter moss should provide a superior incubation climate relative to drier moss for several reasons: a) its water potential will favour normal embryonic development (0 to -5 kPa; Chapter 5), b) photosynthesises occurs at greater rates (Proctor 1981), and hence more oxygen is produced during daylight when metabolic demands of embryos are greatest, and c) developmental temperatures will be cooler due to evaporation. However, according to a schematic relationship between the water content and water potential of drought-tolerant mosses (Dilks and Proctor 1979) the average water content of *Dicranoloma* moss samples of 160% (Table 3) equates to approximately -1150 kPa. While mosses were sampled in dry weather, -1150 kPa is far drier than -25 kPa, where severe effects on embryonic growth and morphology were recorded during laboratory incubation (Chapter 5). However, the curve of Dilks and Proctor (1979) may not apply to *Dicranoloma* mosses because the shape of the curve is influenced by the architecture of the moss stem at high water potentials (Proctor 1981). An avenue of future research might be to examine the dynamics of oxygen and water exchange between *B. nimbus* eggs and the surrounding moss.

Nests used as oviposition sites were slightly more elevated than vacant nests. While the canonical loading (0.38; Table 3) was lower than the 0.5 recommended for interpretation (Tabachnick and Fidell 1996), nest elevation should be relevant to the success of a clutch. For example, *B. nimbus* nests in sub-alpine heath rapidly desiccated in hot sun if they were especially elevated, while nests close to the soil were likely to become waterlogged. Notably, *B. nimbus* nests in implicate rainforest habitat were elevated on logs and were not inundated when the drainage flooded (Appendix 4). Several studies have reported embryonic mortality of terrestrial embryos after flooding. For example, Kam et al. (1998) attributed 4.4% of *C. eiffingeri* mortality to flooding, Townsend (1989) found *Eleutherodactylus coqui* eggs in exposed sites often burst due to overhydration, and there was substantial mortality of *Pseudophryne corroboree* embryos when their terrestrial nests flooded (Gerry Marantelli, personal communication). There are corroborative data for *B. nimbus* - bursting of yolk-sacs occurred in partially flooded *B. nimbus* embryos incubated in the laboratory (Mitchell 1995), while PO_2 at the base of a waterlogged nest of *B. nimbus* hatchings was much lower (about 5 kPa) than comparable elevated nests (about 12 kPa), and larval mortality was high (Chapter 4). However, most *B. nimbus* nests occurred in well-drained regions of the plateau, and hence nest flooding was unlikely to account for unexplained embryonic mortality.

Causes of egg mortality

Partial or complete embryonic mortality occurred in one third of *B. nimbus* clutches. Available data for other myobatrachids are 0-10.7% mortality in *Pseudophryne* spp. (Woodruff 1976), 74% mortality in *Philoria frosti* (Malone 1985), and 26% mortality in *Kyarranus* (= *Philoria*) *loveridgei* (Seymour et al. 1995). In the first two studies, clutch desiccation was heavily implicated; similarly, desiccation accounted for some mortality of *B. nimbus* clutches. *Kyarranus loveridgei* embryos were delayed and ultimately died at the nest base where PO_2 fell below a critical limit, but in this study mortalities of *B. nimbus* embryos usually occurred at the nest surface and hence were not caused by hypoxia. Some mortality may have been caused by developmental irregularities associated with cleavage of large eggs (Elinson 1987) or predation by invertebrates such as planarians, dipteran larvae or arachnids. Harvestmen (Opilionids) were especially abundant in moss, and constitute a major portion of the diet of *B. nimbus* adults (Mitchell 1995). In his study of moss-nesting salamanders, *Nototriton picadoi*, Bruce

(1998) cited Opilionids as potential egg predators. The formation of a jelly crust over the nest entrance in dry weather (Chapter 5) may restrict the entry of invertebrate predators.

Potential for intersexual selection in B. nimbus, *and male nesting strategies*

There is little opportunity for sexual selection based on the size of *B. nimbus* males, for the SVL of adult males is relatively invariant (2.30 ± 0.04 cm; n=117; N.J. Mitchell, unpublished). The limit of male growth may be constrained by the high energetic cost of calling over a prolonged breeding season (e.g. MacNally 1981, Woolbright 1989). Yet despite a large calling investment, males probably have a low parental investment relative to female *B. nimbus*, given the negligible cost of sperm production and absence of paternal care; in contrast, females furnish each of 4-16 eggs with sufficient energy (about 249 J; Chapter 3) to develop endotrophically. Females should therefore be discerning in mate choice (*sensu* Trivers 1982), and a preference for a male occupying a broader nest might facilitate clutch oxygenation and enhance fitness. However, some studies are suggesting that males select nests using different criteria to females (Resetarits and Wilbur 1991). For example, if the calling site is also the oviposition site, males may prefer sites that better amplify their call. Many male *Eupsophus emiliopugini* matched the resonant frequency of their burrow to that of their call (Penna and Solís 1996; 1999), and because frequency co-varied with burrow depth, males may have favoured nests of certain dimensions for reasons unrelated to the viability of their eggs.

The possibility that larger males were associated with broader nests was not examined because there was little variation in male size, and no certainty that the resident male had constructed the nest. Moreover, relationships between nest size and male size have not been found in other studies (Kluge 1981; Kam et al. 1998). However, nest size can be influenced by the nature of the nest substrate. For example, nests of *Hyla rosenbergi* were 121.3 mm in diameter in Costa Rica, and 269.9 mm in Panama, yet male size was similar (70.3 and 79.2 mm SVL) in both populations. Costa Rican nests were built in dense grass, which compacted to form an immovable barrier, while nests in Panama were constructed in soft mud (Kluge 1981). In the present study, nests in lichens were wider than those in denser *Dicranoloma* mosses, suggesting that broader nests can be constructed in looser vegetation.

The frequency of re-use of an oviposition site was low within the five-year survey period, but is relatively high in other anurans whose nest sites are less abundant (*c.f.* Kluge 1981; Kam et al. 1988). Male *H. rosenbergi* engaged in nest construction do not usually call (Kluge 1981), so re-occupation of an old nest both saves energy, and allows earlier entry into the chorus. Energy savings will also accrue if males exploit pre-existing depressions when constructing new nests. Male *H. rosenbergi* were observed constructing nests in depressions left by boot prints or old nests, and similarly, *B. nimbus* males might enlarge natural cavities in the moss such as those vacated by wasp pupae. Survey data showed that newly constructed nests were more likely to be used as oviposition sites, presumably because more males resided in, and advertised, newly constructed nests. Kluge (1981) found that the number of clutches deposited in nest basins of *Hyla rosenbergi* was positively correlated with the number of days it was occupied by a calling male. Hence although a female may be influenced by nest quality when she selects an oviposition site, her selection of a particular nest will also depend on the effort expended by a male in advertising it.

Acknowledgments

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7 Males call more from wetter nests: effects of substrate water potential on reproductive behaviours of the terrestrial toadlet, *Pseudophryne bibronii*

Abstract

Laboratory studies of terrestrial-breeding frogs have demonstrated that wetter substrates produce fitter offspring but the relevance of substrate wetness to adult reproductive strategies is unknown. I hypothesised that male toadlets (*Pseudophryne bibronii*) would select wetter areas for nesting and would advertise wet nests strongly, and tested these predictions by manipulating water potentials at a breeding site. Males preferred to nest in the wettest areas, and called at greater rates on almost twice as many nights as males occupying drier nests. Overall, males that mated called on significantly more nights than unmated males. Hence, because males occupying wet nests called more, they also mated more, and in 19 of 20 cases oviposition occurred in wet nests that were suitable for embryonic development. Males occupying drier nests may have risked dehydration by calling, and so were less able to signal to females. The hydration state of a terrestrial breeding male frog therefore has the potential to influence its reproductive success.

INTRODUCTION

Choice of males by females is widely acknowledged as a mechanism that drives selection in the Anura (Sullivan et al. 1995), but a vital caveat for its adaptive value is evidence of fitness benefits to the female (Halliday 1983). A model of female choice that has received little consideration in the literature is one where females select mates based on a resource contained within the male's territory, such as the oviposition site. Discrimination between oviposition sites can provide a consistent environment for the fittest phenotypes to be expressed (Resetarits 1996), so when the breeding environment is variable, males should attempt to control quality nests. Howard (1978) found that larger male bullfrogs (*Rana catesbeiana*) occupied warmer aquatic territories containing fewer leeches than those of smaller males, and that females preferred larger males and egg survival was high. An experimental study revealed that female *Eleutherodactylus coqui* preferred males that called from elevated terrestrial nests, and egg-hatching success was also greater at elevated sites (Townsend 1989). Therefore characters of male nests can be viewed as phenotypic variables of the male that may be subject to female scrutiny.

The Australian toadlet *Pseudophryne bibronii* provides good evidence of the fitness consequences of oviposition site selection in anurans because several studies have determined how the incubation environment influences embryonic and larval viability (Bradford and Seymour 1988a; Geiser and Seymour 1989; Seymour et al. 1991a; 1991b). *Pseudophryne* toadlets nest in depressions under rocks, logs or leaf litter, embryos hatch when the nest floods after rains, and thereafter larvae are aquatic and feeding (Woodruff 1976). The water potentials of natural nests are variable (Bradford and Seymour 1985), and when water potentials were controlled in the laboratory, embryos incubated on wet substrates (0 kPa) increased in mass at a rate 71% greater than embryos reared on drier substrates (-25 kPa, Bradford and Seymour 1988a). Therefore, because offspring size potentially relates to adult traits such as size and age at first reproduction, and fecundity (e.g. Semlitsch et al. 1988), toadlets breeding in wetter nests should have greater fitness.

Male *Pseudophryne* toadlets mate 0-3 times each season, but females may also mate with multiple partners by depositing their egg component in discrete batches over several days (Woodruff 1976). Given this mating flexibility, *Pseudophryne* are excellent models for examining the relevance of nest quality to mating strategies. The present study focuses on the mating strategies of male *P. bibronii* when offered variable water potentials in a manipulated field experiment. Males were monitored over a 10-week breeding season to determine nest locations and to relate variables such as calling effort and mating success to the water potential of the nest site. Three fundamental questions are addressed. Firstly, do males prefer to establish nests on wetter substrates; secondly, do males advertise a wetter nest more, and thirdly, is the mating success of males using wetter nests greater than that of males occupying drier nests?

METHODS

Study species and site

Pseudophryne bibronii (Anura: Myobatrachidae) is a small (22-36 mm snout-vent-length) cryptozoic toadlet found across temperate south-eastern Australia. Males establish nests after the first autumn rains and call for 1-8 weeks with discrete mate attractant and territorial calls. At the study site in remnant eucalyptus woodland in Watt's Gully Reserve, about 50 km north-east of Adelaide, South Australia, nests were localised along the banks of a meandering winter creek. Several nest types were identified in a pilot study in 1998; most were shallow depressions under litter or amongst grass roots, or were burrows angled into the creekbank (Figure 1).

Experimental design and watering procedure

Fifteen experimental plots of 3 x 3 m were positioned across sections of creek before the onset of the 1999 breeding season, and were allocated to one of three treatments using a stratified random design (Figure 2). Plots were either watered to maintain high soil water potentials, disturbed in a similar manner but not watered (procedural control, PC) or not disturbed (disturbance control, DC).

Figure 1. Male *Psuedophryne bibronii* occupying a burrow built in the creekline.

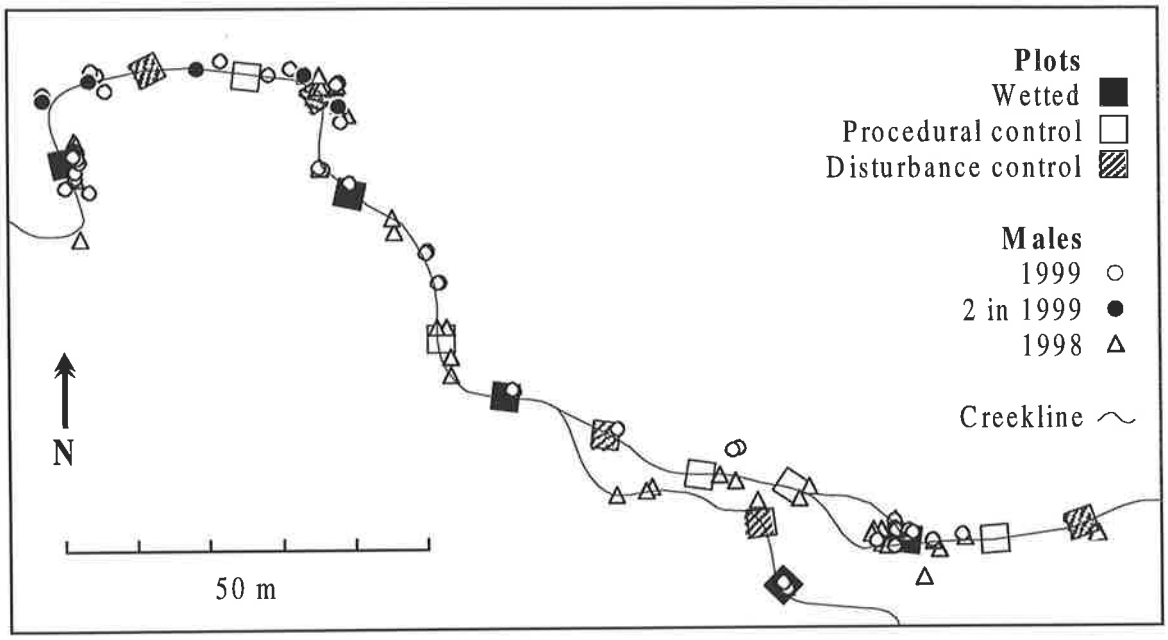

Figure 2. Map of study area showing the creekline, the location of experimental plots and distributions of calling males in 1998 and 1999.

Watering began in March, immediately before the onset of the breeding season, so that variability in water potential might influence where males chose to nest. Watering continued every 2-3 d until natural rains saturated the study area. Plots were watered sequentially in the late afternoon using a portable polypropylene frame with 90° irrigation sprayers fixed into each corner. About 100 L of rainwater was pumped from a nearby water trailer onto each plot over 15-20 min, corresponding approximately to an 11 mm rainfall event (Figure 3). A sham polypropylene watering frame was positioned on a PC plot at the same time an adjacent wetted plot was watered, and DC plots were only entered in the eighth week of the experiment to confirm the identity of resident males.

Experimental measurements

Four variables described the responses of toadlets to the experimental plots. These were: (1) the number of colonising males, (2) male calling effort, (3) male mating success, and (4) egg hatching success. Additionally, (2), (3) and (4) were measured for males outside plots, but within the study site. Monitoring began on the night before the first watering (Figure 4) and continued approximately every second night for the following 10 weeks. Because wetted plots were always watered beyond their boundaries, a male was counted

Figure 3. Portable watering frame positioned on a wetted plot

as a resident of an experimental plot if he was located inside the plot or within 0.5 m of the perimeter.

Nesting males were usually found by triangulating on the call, and females were either found near to a calling male or were captured in pitfall traps set around wetted and PC plots. The uniquely patterned ventral surface of each toadlet was photographed to allow re-identification. Snout-vent length was measured with dial callipers and mass was recorded to the nearest 0.1g with an electronic balance, after first blotting the toadlet with absorbent tissue.

Once a male entered the chorus a concerted effort was made to locate it on subsequent nights. Males would indicate their presence in a nest by answering a crude mimic of an attractant call, but when a male called without this stimulus it was scored as a calling night. When a male called consistently his calls were recorded with a Sony digital audio tape-corder and a Sennheiser ME66 microphone or Sony Professional Walkman and Sony ECM-MS907 microphone. Recordings were digitised and analysed with Avisoft SASLab Pro software (Specht, Berlin, Germany). Temperature at the calling site was measured with a Fluke model 52 thermometer.

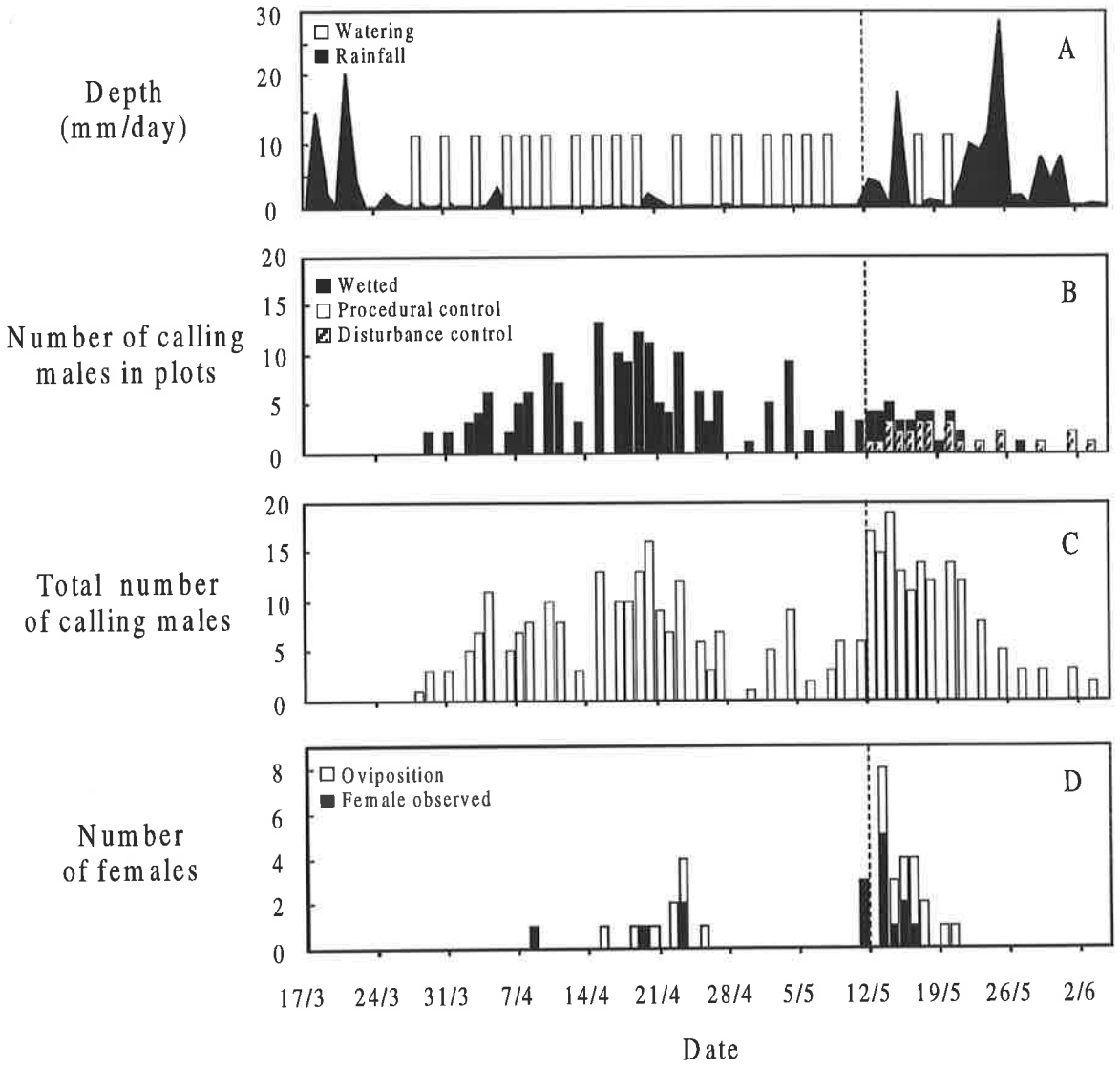

Figure 4. A) Watering events and natural rainfalls in 1999 (rainfall data sourced from the South Australian Bureau of Meteorology Weather Station 23878, 5 km from the study site); B) number of males calling in experimental plots, and C), in the study area each monitoring night; D) number of female observations and oviposition dates of egg batches, including some outside the study area. The dashed line divides the nominal before and after rain periods.

Call rates of males in wetted and PC plots were measured synchronously after dusk on one occasion each week. Four observers positioned themselves in two pairs at either a wetted plot or at the closest PC plot, and a timer was set for 15 min. Observers sat quietly and determined the number of calling males for the first 5 min, and then counted calls for 10 min. The procedure was repeated for the remaining four pairs of wetted and unwetted plots, and the time and the near-ground air temperature were noted on each occasion.

Calling sites were examined for eggs approximately every second night. Matings were attributed to the male attending the eggs (White [1993] found that when male *Pseudophryne* were placed on unattended eggs of another male, the introduced male would always desert the eggs). Fresh eggs were counted, and when they reached hatching stage 28 (Gosner 1960) they were carefully excavated from the nest and were flooded the next day in the laboratory. Hatchlings were counted, staged and measured using Optimas image analysis software (Optimas Corp., Bothell, Washington).

Measurement of the plot environment

Soil water potentials of wetted and PC plots were measured weekly (1-3 d after watering) using a chromatography paper technique (reviewed by Savage et al. 1992). Squares of saturated paper (20 x 20 x 1 mm) were inserted inside dialysis tubing to keep them clean, before being buried in duplicate in each plot under about 1 cm of soil. Papers were removed after 5 d, their water contents measured in the laboratory, and soil water potential was directly inferred from an equilibration curve of paper water content and matric tension (range 0 to -600 kPa).

The physical habitat of plots was measured at the end of the monitoring period. The percent cover of five substrate categories (litter, grass, gravel, soil and bank) was measured using a 1m square frame divided into 0.25 m^2 cells. Litter depth was measured to the nearest 2.5 cm in any cell that contained it, and mean litter depth was calculated for each plot. Substrate cover and litter depth values were 4^{th} root transformed and the treatment groups were tested for similarity in a one-way ANOSIM procedure using the Bray-Curtis coefficient of similarity and the PRIMER software package (Clarke and Warwick 1994).

RESULTS

Experimental conditions

The first six weeks of the experiment were unusually dry, but after May 12 rainfalls continued intermittently until the last monitoring day (Figure 4A). During the dry weather water potentials of wetted plots averaged about –16 kPa, whereas water potentials of unwetted plots were drier than -600 kPa (Figure 5). After the rains the water potentials of both wetted and unwetted plots were >-2 kPa. Breeding activity waned after May 25 (week 9) when large pools formed in the creekbed, and had ceased by June 18 when the creek was in full flood (Figure 6).

Distribution of nesting males

Fifteen males established nests and commenced calling in wetted plots during the six dry weeks of the experiment, and most left wetted plots during natural rainfalls in week seven (Figure 4). No male occupied a PC plot, and five males occupied a DC plot following the rainfalls. However, during the dry weather 13 males nested in litter outside of experimental plots (Figure 2). Their peak density was about 1 male/76 m^2, compared to 1 male/7 m^2 inside wetted plots.

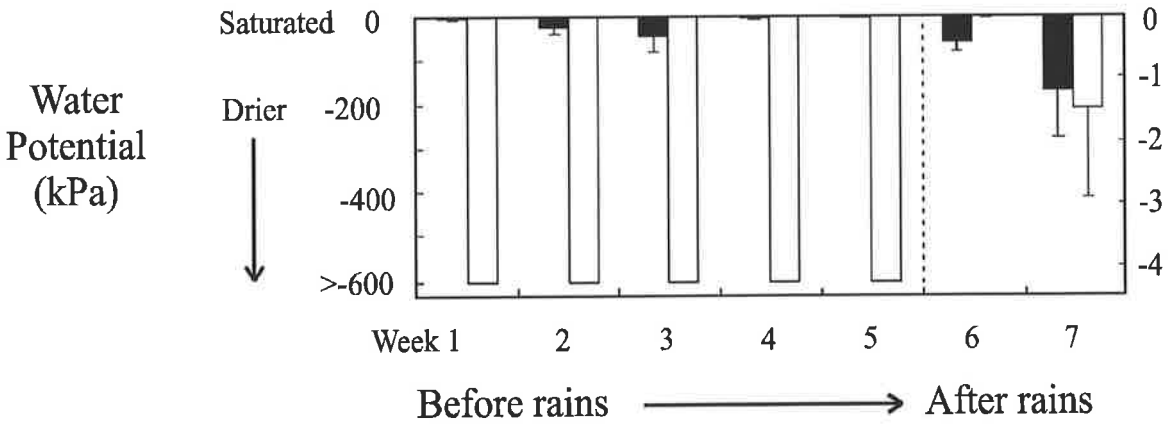

Figure 5. Mean water potentials (± s.e.) of wetted (filled bars) and PC (open bars) plots during the first 7 weeks of the experiment. The water potential scale changes after the rains (indicated by dashed line).

Figure 6. Flooded plots on June 18 1999

Table 1. Male distribution data

Treatment (n = 5)	Number of males per plot ($\bar{x}$ ± s.e.) before rain	after rain
Wetted	3.0 ± 0.9	0.6 ± 0.4
Procedural control	0.0 ± 0.0	0.0 ± 0.0
Disturbance control	0.0 ± 0.0	1.0 ± 0.5

Table 2. Results of two-way ANOVA for male distribution data.

Variable	d.f.	SS	F ratio	p
Time (before or after rain)	1	0.1180	0.84	0.3672
Treatment	2	1.7568	12.58	0.0002
Time x Treatment	2	1.4083	10.08	**0.0007**

Variances of *ln* (x+1) transformed data were homogenous (Cochran's test), but data was not normally distributed. However, ANOVA is generally tolerant to deviations from normality when treatments are well replicated (Underwood 1981).

A two-way analysis of variance was used to test male distribution data, because the effect of watering plots was predicted to be greater in the absence of rain than in the presence of rain (Table 1). It was apparent that males preferred to nest in wetted plots before the rains, but not after the rains, because the interaction between treatment and time was significant (Table 2).

All males re-captured in 1999 nested between 5-130 m from their 1998 nest (n = 8), so males were not returning to previous breeding sites. Further, in 1998 there was no difference in the distribution of males relative to the locations of the wetted, unwetted and control plots established in 1999 (ANOVA $F_{2,12} = 0.414$, $p = 0.67$). The physical habitat of plots in each treatment was similar (ANOSIM, $r = 0.055$, $p = 0.28$).

Calling effort of males in wet and dry nests

Because control plots were not colonised before the rains, I instead compared characters of wetted males to all other males in the study site (hence forth called unwetted males). A wetted male was defined as one that spent 75-100% of its calling nights before the rains within a wetted plot; all other males were classed as unwetted males. Wetted males (n = 11) called on 46 % of monitoring nights before the rains (n = 30 nights), compared to 15% for unwetted males (n = 12). After rains, the same, previously wetted males, called on 36% of nights (n = 15 nights), while calling by previously unwetted males increased to 50% of monitoring nights (Figure 7). The difference in calling effort before and after rains was significantly different between the two groups of males (t_{20}, p = 0.0002).

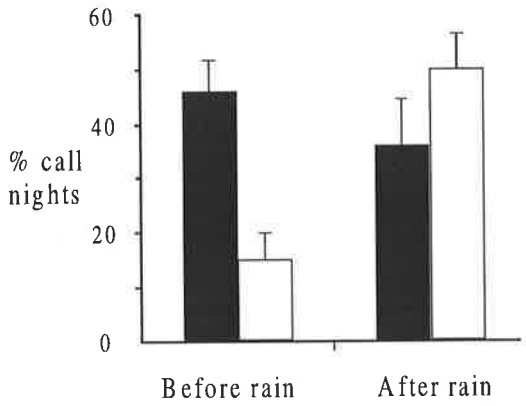

Figure 7. Mean % call nights (± s.e.) of wetted males (n =11; filled bars) and unwetted males (n = 12; open bars) before and after rains.

Table 3. Physical and behavioural characteristics of wetted and unwetted males in the study area.

Time	Variable	Wetted males		Unwetted males	
		$\bar{x}$ ± s.e.	*n*	$\bar{x}$ ± s.e.	*n*
a) General	Snout-vent length (mm)	26.3 ± 0.53	11	26.5 ± 0.68	12
	Night that male joined chorus	7 ± 1.3	11	13 ± 3.1	12
b) Before rain	Number of calling nights in wetted plot	14 ± 1.8	11	1 ± 0.4	12
	*Attraction call rate (calls min^{-1})	6.1 ± 0.9	32	3.5 ± 0.4	8
	*Territorial: Attraction calls	0.68 ± 0.11	37	0.28 ± 0.12	8
	†Mass (g)	1.8 ± 0.10	7	1.9 + 0.05	9
	Number of calling locations	1.7 ± 0.38	11	1.2 ± 0.24	12
c) After rain	†Mass (g)	1.5 ± 0.07	7	1.8 ± 0.07	9
	Number of calling locations	1.1 ± 0.16	11	1.3 ± 0.18	12

*from call recordings, *n* refers to number of recordings rather than number of males

†only males weighed both before and after rains have been included

Statistical tests were not appropriate for call data obtained from recordings, because both spatial (e.g. a calling neighbour) and temporal (e.g. time of night) variables influenced call parameters. However, call recordings showed that wetted males produced attraction calls at about twice the rate of unwetted males, and that wetted males produced more territorial calls relative to attraction calls (Table 3). Deep litter piles in particular attracted multiple males and territorial calling could be frenetic, as occurred when five of six males in a wetted plot occupied a single large litter pile (1 x 0.5 x 0.2 m). If territorial calls are included in the measurement of call rate, then wetted males produced 10.3 calls min^{-1} before rains, compared to 4.5 call min^{-1} for unwetted males before rains. After rains the call rate of all recorded males was 7.5 calls min^{-1}.

The weekly synchronous measures of call rates inside wetted and PC plots were confounded by the absence of males in the latter. However, attraction call rates in wetted plots before the rains ($\bar{x}$ 2.88 calls min^{-1}; range 0.2-6.1 calls min^{-1}; n =19) were lower than those measured from recordings (Table 3), and call rates of males in each wetted

plot (data from all weeks pooled) were not related to plot water potential, the number of males in plots, or the time or temperature that measurements were made (linear regression, all $p > 0.3$, $n = 19$).

Male mating success

All females located in dry weather ($n = 8$; Figure 4D) were near calling males in wetted plots. Six of 11 wetted males mated during dry weather, compared to one of 12 unwetted males mated (Fisher's exact test, $p = 0.023$). After the rains, two of the 11 previously wetted males mated, and four of the 12 previously unwetted males mated (Fisher's exact test, $p = 0.270$), so the difference in mating success between wetted and unwetted males was significant only in the absence of rain. One wetted male mated both before and after rains.

Egg hatching success in wet and dry nests

The egg hatching success ($\bar{x} \pm$ s.e.) of five clutches oviposited in wetted plots before rain was high (95 ± 2.3 %, range 87-99 %). The clutch oviposited in unwetted litter before the rain experienced almost complete mortality. Only three larvae hatched, and they were 45% of the wet mass and 70% of the length of hatchlings from wet nests. The precise hatching success of this clutch was unknown, because the fresh eggs were shrunken and difficult to discern from debris.

DISCUSSION

The negligible rainfall in the first six weeks of the experiment (only 9 mm) meant that wetted plots offered markedly different substrate water potentials to control plots (Figure 5). As male distributions could not be explained by nest-site fidelity or a preference for a nesting material, then differences in water potential were strongly implicated; males preferred to nest in wetter over drier areas. Further, high substrate water potentials induced behaviours such as male calling and female oviposition, independent of ambient cues such as declining temperature. The large proportion of territorial calls produced by wetted males before the rains, and the tendency of these males to call from more locations than unwetted males (Table 3) suggested strong competition between males for territories in wet litter.

Almost all (19 of 20) females mated in wet nests, either by mating with a male in a wetted plot before the rains, or by mating after the rains. About 50% of males that entered the chorus secured a mate (Table 5), and three mated more than once (estimated from the age of egg batches). There was no suggestion that mating success was related to male size or to the dominant frequency of the attraction call (Table 4), although this result should be treated cautiously because the size of correlation coefficients depends on the number of males that females sample (Benton and Evans 1998). Instead, mated males tended to call earlier in the breeding season, and more often (Table 4), a finding consistent with other studies (e.g. Ryan 1983, Wagner and Sullivan 1995).

Because wetted males had both high calling efforts and high mating success before the rains, I examined whether mating frequencies predicted from calling effort matched actual mating frequencies (Table 5). I made two assumptions: that my 30 observation nights were representative of all nights, and that females should mate with males in proportion to the number of nights each male called (e.g. Greer and Wells 1980). It appeared that wetted males were not *directly* advantaged by occupancy of a wet nest before the rains, because their observed mating frequencies were not significantly different from expected frequencies (Table 5; $\chi^2_{0.05,1} = 1.78$, $p > 0.1$). Instead, females

probably *indirectly* selected males in wet nests, because wetted males called more than unwetted males (Table 3, Figures 4B and 7).

There are many potential explanations of the high calling effort of males occupying wet nests. Firstly, wetted males may have equated the wet conditions with the possibility that females would attend the chorus, and responded by increasing their calling effort. Secondly, the high density of wetted males may have promoted calling competition, but as males were noticed to call antiphonally with neighbours up to 15 m distant, the calling of wetted males should also have prompted nearby unwetted males to call. Thirdly, plot watering may have increased invertebrate abundance (*c.f.* James and Whitford 1994) and so given wetted males an energetic advantage over unwetted males. However, the effect of feeding on male calling behaviour is inconclusive; some workers have found that feeding increases calling activity (Murphy 1994; Marler and Ryan 1995) while others found no effects (Green 1990; Murphy 1999). Finally, wetted males may have called more because they were not in danger of dehydration.

Unfortunately, although the water relations of frogs have been under investigation for two centuries (Jørgensen 1997), the precise effects of dehydration on calling behaviour are unknown. Certainly dehydration causes elevated resting metabolic rates (Pough et al. 1983)

Table 4. Physical and behavioural traits of mated and unmated males compared with *t*-tests

Trait	Mated males		Unmated males		*t*-test
	$\bar{x} \pm$ s.e.	*n*	$\bar{x} \pm$ s.e.	*n*	
*Snout-vent length (mm)	26.7 ± 0.35	20	26.4 ± 0.46	20	$p = 0.586$, d.f.=38
*Mass (g)	1.7 ± 0.06	20	1.6 ± 0.08	20	$p = 0.321$, d.f.=38
*†Dominant frequency of attraction call (Hz)	2436 ± 34.8	14	2496 ± 52.9	10	p =0.338, d.f.=22
Number of calling locations	2.6 ± 0.4	11	2.2 ± 0.4	11	$p = 0.396$, d.f.=20
Night of arrival in chorus (1-33)	6 ± 1.1	12	15 ± 3.1	11	$p = 0.013$, d.f.=21
Calling nights (%)	43 ± 4.6	11	25 ± 5.4	11	$p = 0.019$, d.f.=20

* includes 17 males located outside the study area after the rains, eight of which mated.

† the only call parameter independent of nest temperature ($r^2 = 0.001$, $p = 0.73$) and where coefficients of variation were stable between recordings (see Gerhardt 1991).

Table 5. Expected and observed mating frequencies for wetted and unwetted males before the rains.

Male		Number of	Proportion of	Number of matings		O - E
Type	ID	calling nights	mating opportunities*	expected*	observed	
Wetted	98-27	11	0.054	0.376	0	-0.376
	98-40	9	0.044	0.307	0	-0.307
	C1	14	0.068	0.478	1	0.522
	C3	21	0.102	0.717	1	0.283
	C4	9.5	0.046	0.324	1	0.676
	C6	3	0.015	0.102	0	-0.102
	E1	16	0.078	0.546	0	-0.546
	E2	10	0.049	0.341	1	0.659
	O1	19	0.093	0.649	1	0.351
	O2	20.5	0.100	0.700	0	-0.700
	O3	19	0.093	0.649	1	0.351
	98-23	5	0.024	0.171	0	-0.171
Wetted total		**157**	**0.766**	**5.361**	**6**	**0.639**
Unwetted	I1	6	0.029	0.205	0	-0.209
	98-20	12	0.059	0.410	0	-0.209
	98-23	5	0.024	0.171	0	-0.209
	98-33	3	0.015	0.102	0	-0.209
	98-44	1	0.005	0.034	0	-0.209
	98-8	7	0.034	0.239	0	-0.209
	K1	13	0.063	0.444	1	-0.209
	M1	1	0.005	0.034	0	-0.209
Unwetted total		**48**	**0.234**	**1.639**	**1**	**-1.672**
	Total	**205**	**1**	**7**	**7**	**-1.033**

* Calculations follow Greer and Wells (1980)

and a 'water seeking response' where activity levels increase (Hillyard 1999), both of which might hamper calling activity. Some evidence that dry conditions restrict calling behaviour is that significantly more male *E. coqui* adopted water-conserving postures on dry summer nights compared to wet nights, and that the number of vocalising males increased respectively from 20% to 35% (Pough et al. 1983). In the present study light rainfalls that damped the litter could prompt an unwetted male to move to and begin calling from a new nest, which implied that substrate wetting decreased the dehydration risk associated with movement and calling. In contrast, males in wetted plots (at about -16 kPa) must always have been fully hydrated, given that *E. coqui* could absorb water through their ventral surface from substrates as dry as –540 kPa (Van Berkum et al. 1982).

If we accept that male hydration at least partly affects calling behaviour, then this tests an assumption of honest signalling, namely, that for a signal to be reliable it must incur a cost (Zahavi 1975). Undoubtedly, calling effort is an honest signal of male quality, for frog vocalisations are energetically expensive (e.g. MacNally 1981; Bucher et al. 1982; Wells and Taigen 1989), and in this study, wetted males lost more weight than unwetted males during the experiment (Table 3) and did not call to the same extent after the rains (Figure 7). However, if the ability to call (=signal) at any particular time depends on favourable nest water potential, then a male occupying a dry nest is less able to signal to females. In contrast, a male that chances upon a moist site early in the season (in this experiment a wetted plot, but more usually a deep pile of litter) is advantaged because he can begin signalling earlier. Therefore only if the best males occupied wet nests would call effort truly reflect male quality.

This study highlights the mechanism by which female *P. bibronii* select wet oviposition sites. Males prefer wetter nests because they benefit by increased opportunities to advertise acoustically. Female sampling is therefore biased toward males occupying wetter nests, and a perhaps fortuitous consequence is that oviposition occurs in hydrated sites that enhance embryonic survival. High embryonic mortality was the result of the single oviposition event in a dry nest, because the water potential of the unwetted litter exceeded the viable embryonic limit of -200 kPa determined by Bradford and Seymour (1988a). This mating occurred after light rainfall that promoted male calling, but did not penetrate the leaf litter. Consistent calling from a nest during a breeding season should therefore be an honest signal of the persistence of the moisture and the suitability of the nest for embryonic development.

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APPENDIX 1 Measurement of oxygen consumption ($\dot{V}O_2$) of amphibian eggs

Oxygen consumption rates ($\dot{V}O_2$) of eggs, embryos or hatched larvae were determined by measuring the average rate of decline of oxygen partial pressure (PO_2) inside sealed respiratory chambers (Diamond General no. 1271, Figure 1). Chambers were filled with about 0.67 mL of reverse osmosis water, and miniature Clark oxygen electrodes (Diamond General no. 733) were inserted into each chamber and set to a polarizing voltage of –750 mV with a picovoltmeter (Diamond General no. 1231). Chambers were thermostated at an experimental temperature (± 0.1° C) with a Braun 850 constant temperature water bath for at least 1.5 h before electrode calibration.

Electrodes were calibrated with a sodium sulphate-borax PO_2-zero solution, and air-equilibrated water sourced from an aerated beaker suspended in the water bath. Electrode currents were read with a picoammeter (Diamond General no. 1231) after first stirring the fluid in the chamber for 15 s with a small, plastic coated magnetic bar. A motor inserted behind the respiratory chamber rotated the bar.

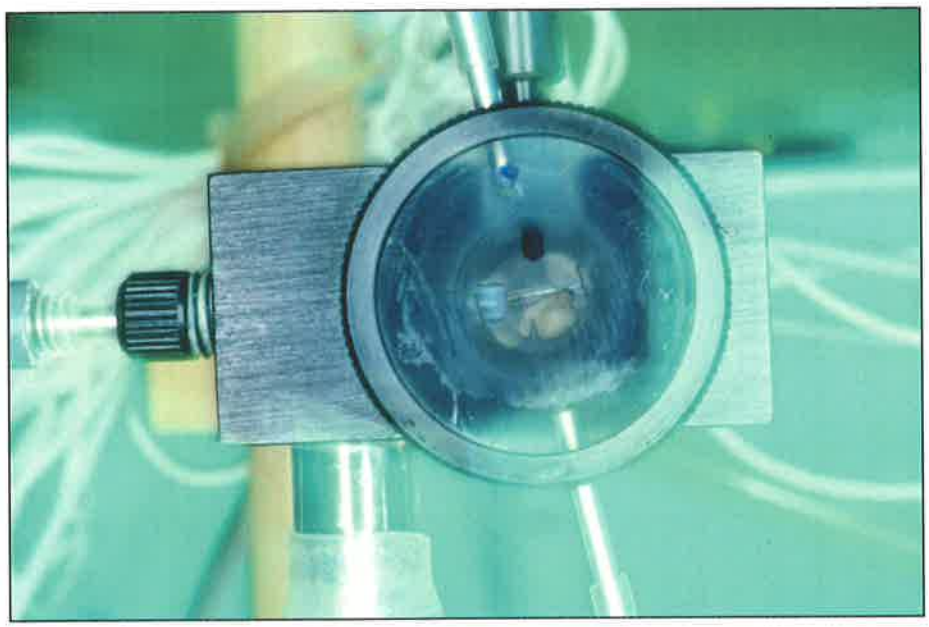

Figure 1. Diamond General no. 1271 respiratory chamber containing a stage 17 *Bryobatrachus nimbus* embryo.

Partially de-jellied eggs and embryos, or hatched larvae were sealed individually inside a chamber. The first measurements were made after 10-15 min, to allow for handling disturbance and for temperature stabilisation, and thereafter measurements were made every 5-30 min, depending on developmental stage and temperature. Eggs were usually removed after 2 h, or when chamber P_{O_2} dropped below about 5 kPa, but were sometimes left until P_{O_2} approached 0 kPa so that critical P_{O_2} could be determined (e.g. Chapter 4). The latter procedure did not kill larvae. Eggs were staged after each experiment, snout-vent length and tail lengths of larvae were measured with an ocular micrometer, and larvae were weighed to 0.01 mg on a Mettler AE163 analytical balance after brief blotting on tissue.

The P_{O_2} at each measurement time was calculated from the oxygen capacitance of pure water corrected to standard temperature and pressure (Riley and Chester 1971), the volume of water in the chamber minus the mass (≈volume) of the embryo or larvae, and the electrode current (corrected for electrode drift, which was assumed to be linear). P_{O_2} increments below the critical P_{O_2}, increments were not included in the calculation of mean $\dot{V}O_2$. $\dot{V}O_2$ of reverse-osmosis water-filled chambers was measured often because the chamber sometimes had appreciable microorganism respiration. Chamber $\dot{V}O_2$ was subtracted from the mean embryonic or larval $\dot{V}O_2$ of relevant experiments.

APPENDIX 2 Nest and egg mass characteristics of *Geocrinia lutea*

Nests and egg masses of *Geocrinia lutea* were studied from 15-19 October 1997 at two sites near Quinn Rd, about 6 km north of Walpole, southwest Western Australia. The intention was to collect fresh eggs for respiratory studies, but the plan was abandoned when it became apparent that most field clutches had hatched. However, two fresh clutches were collected and transported to Adelaide. The ova from a sub-sample of fresh eggs were dissected from the capsule, weighed, dried over silica gel, and reweighed. Ovum energy content was later determined by bomb calorimetry (Table 1). Other eggs were incubated at 0 kPa water potential in a constant temperature cabinet at 15°C, and metamorphosis was complete after 105 d. Metamorphs were killed by freezing and preserved in fixative (Tyler 1962), and given to Dr. Margaret Davies for osteological studies.

Nest characteristics

Nests of *Geocrinia lutea* were small, hemispherical, soil-based burrows concealed in damp moss in swamps dominated by *Lepdiosperma* sedge (Figure 1). Moss patches were often concealed by grasses, and always abutted small runnels. Very wet and very dry moss was never found to contain nests, nor were nests located in *Sphagnum* moss.

Figure 1. Habitat of *Geocrinia lutea* near Walpole, Western Australia

Empty nests were found only rarely; most nests contained either a clutch or a male frog. Two nests were found containing skeletons of adult *Geocrinia*; deaths may have occurred during a high intensity burn at the site in 1993. Nest characters are detailed in Table 2.

Table 1. Summary of egg and energy data for *Geocrinia lutea*

Characteristic	$\bar{x}$	95% CI	*n*	Range
Ovum diameter (mm)	2.71	0.11	10	2.50-3.10
*Ovum volume (µL)	10.42			8.18-15.60
Wet mass of ovum (mg)	16.46	2.32	13	13.0-24.0
Dry mass of ovum (mg)	4.70	0.25	11	4.1-5.1
†Water content of ovum (%)	71.4			
‡Energy density of ova (J mg^{-1})	26.32		1	
†Energy in ovum (J)	123.7			

*Calculated from ovum diameter

†Calculated from table values

‡*n* refers to the number of bombed pellets rather than the number of individuals constituting a pellet.

Table 2. Characteristics of fourteen field nests of *Geocrinia lutea* near Walpole, Western Australia

Parameter	Unit	$\bar{x}$	95% CI
Diameter a	cm	2.59	0.13
Diameter b	cm	2.62	0.10
Depth at centre (h)	cm	1.38	0.09
Volume*	cm^3	5.08	0.69
Number of eggs		16.6	1.3
Egg density	$eggs/cm^3$	3.40	0.43

*Volume of eggs and jelly, calculated assuming that the eggs mass is a segment of a sphere ($V = \frac{1}{3}\pi h^2(3r-h)$, r = (diameters a+b)/4).

APPENDIX 3 Estimating substrate water potential with filter papers

Water potential is widely used in biological and agricultural research to describe the wetness of a substrate. It is especially useful because it is continuous across different media, whereas water content on a mass basis is not. Matric tension is the component of water potential arising from binding of water to the surface of soil particles by adhesive, cohesive and ionic forces (Packard et al. 1992). Total water potential is matric tension plus osmotic potential resulting from solutes, and consequently is only relevant in saline conditions.

Filter papers provide a versatile and simple method of measuring the water potential of a substrate, and are less expensive than other techniques such as ceramic plate extraction, tensiometry, and thermocouple psychrometry. A further advantage is that filters can measure potentials from 0 kPa through to at least -4500 kPa (Savage et al 1992), whereas techniques such as tensiometry are confined to potentials >-100 kPa.

The filter paper technique is based on the premise that a paper kept in thermal and water vapour pressure equilibrium with a porous medium is at the same water potential as the medium (Hamblin 1981). A standard curve relating water potential of the filter to its water content can be used to indirectly measure the matric tension of a medium, simply by determining the water content of an equilibrated paper. Several published curves are available for Whatman® filter papers (reviewed by Savage et al. 1992). However, I calibrated a 1 mm thick chromatography paper, to allow more accurate water content measurement.

Calibration

Two duplicate 2 x 2 cm squares of chromatography paper pre-soaked in reverse osmosis water were placed on a wetted cellulose nitrate filter (Sartorius: diameter 4.7 cm, pore diameter 0.05 μm) and sealed inside a custom-built pressure plate (Seymour and Piiper 1988). The plate was pressurised using a water column for potentials >-1 kPa, and a gas bottle and regulators between -1 and -600 kPa. Pilot experiments showed that equilibration was reached after 9 h at -50 kPa, but samples were generally left to equilibrate for a minimum of 24 h, and up to 7 d at high pressures (e.g. -600 kPa).

Following equilibration, the chamber was depressurised with a vent valve and opened, and the papers quickly removed with forceps and weighed to 1 mg on a Mettler AE163 analytical balance in a tared aluminium pan. Papers were oven dried in their pans for 1 d at 60° C, restored to room temperature, and re-weighed. Water content was expressed as % dry weight, and mean values were plotted against the applied pressure (= matric tension) to produce a standard curve (Figure 1). Estimates were most accurate between –5.4 and –16.7 kPa (100 and 200% water) where the slope of the curve is less steep.

Applications in the laboratory and the field

The standard curve was used to produce experimental substrates of known water potential by wetting a piece of air-dry chromatography paper to a target mass, determined from the inverse of the equation in Figure 1. Conversely, in the field, saturated papers were inserted beneath eggs or buried in soil, and after at least 5d equilibration (see recommendations of Greacen et al. 1988; Deka et al. 1995), were removed and quickly sealed inside a labeled vial for transportation to the laboratory. There, water content was determined after oven drying papers, and the water potential of a nest was estimated. Field papers were kept clean by encasing them inside a length of dialysis tubing, which was stapled at both ends to prevent the paper slipping out of the case.

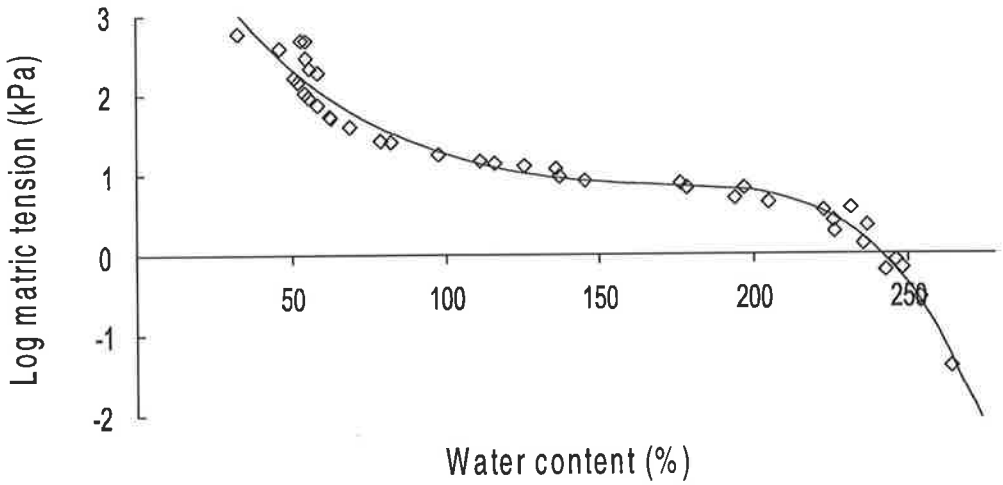

Figure 1. Relationship between matric tension and water content of the chromatography paper. Log matric tension = $-3.7787 \times 10^{-11}a^5 + 2.284 \times 10^{-8}a^4 - 5.9040 \times 10^{-6}a^3 + 9.1014 \times 10^{-4}a^2 - 9.0015 \times 10^{-2}a + 5.1612$ ($r^2 = 0.97$), where a = water content of the filter paper (% dry weight)

APPENDIX 4 Characters of *Bryobatrachus nimbus* nests at a lowland site in implicate rainforest.

A lowland population of *B. nimbus* in a drainage line of Louisa River (146°25′E, 43°29′S; altitude *ca.* 40 m), near the south coast of Tasmania, was visited between 16-23 1995. The habitat was implicate rainforest dominated by canopy species *Nothofagus cunninghamii*, *Melaleuca squarrosa* and *Phyllocladus aspleniifolius,* and an understorey of the fern *Blechnum wattsii* (Rounsevell et al. 1994; Ziegeler 1994). The visit coincided with a major flooding of Louisa River, and consequently fieldwork was limited. However some nests were identified as shallow depressions in *Dicranoloma* moss, elevated 0.5-1.0 m above the forest floor on fallen logs and tree stumps (Figure 2). Nests were similar in shape to sub-alpine lichen nests, but their dimensions were not measured. Two nests contained 8-10 larvae approaching metamorphosis (stage 35; de Bavay 1993).

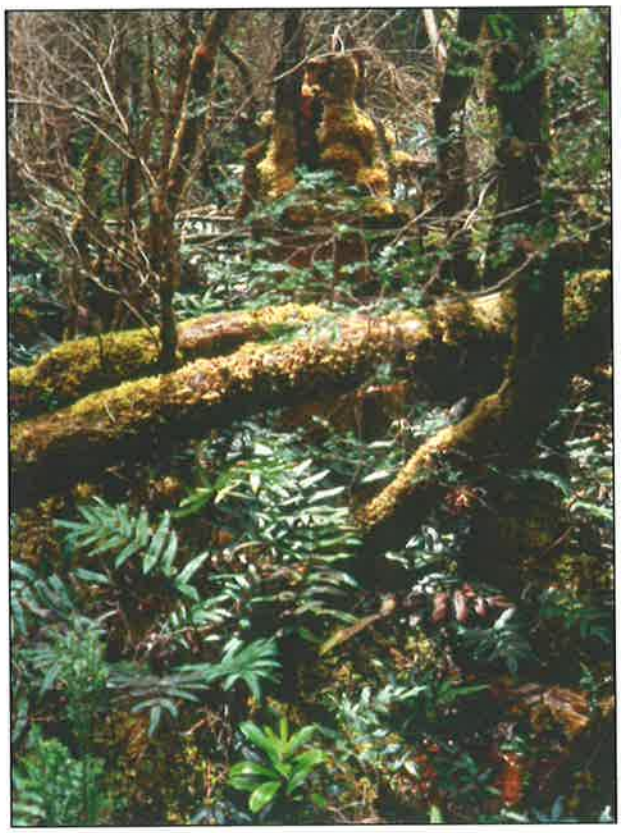

Figure 2. Breeding site of *Bryobatrachus nimbus* in implicate rainforest at Louisa River, 37 m above sea level near the south coast of Tasmania. Nests occurred in mosses blanketing logs and tree stumps.

'...it is, perhaps, dangerous
to speak of the emotions of so
passive a creature as a frog'

Noble 1931