The plasticity of life histories during larval development and metamorphosis, using amphibians as study organisms.

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Candidate's declaration

I declare that the work presented in this study is entirely my own unless otherwise stated and that it is of my own composition. No part of this thesis has been submitted for any other degree.

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Abstract

The ability of animals to vary growth, development rate and behaviour in response to environmental conditions has been well documented, particularly during the larval phase in animals with complex life cycles. The evolution and maintenance of plasticity in response to environmental conditions is likely to be adaptive in animals that face unpredictable environments. However, there are two aspects of life histories in animals with complex life cycles, which would be expected to favour plasticity, that have received limited attention: traits during metamorphic climax and variation in the life history phase at which temperate species spend the winter. Therefore the aims of this thesis were to consider the environmental factors that are likely to result in plasticity in the timing and duration of metamorphic climax and contribute to variation in the over-wintering life stage, using amphibians as study animals.

To assess the ability of animals to respond to environmental conditions during metamorphic climax conditions were manipulated during metamorphosis independent of larval treatment. Accordingly all larvae entered metamorphic climax having experienced the same conditions. The African clawed toad, *Xenopus laevis*, was used. I examined the influence of environmental temperature, predation risk and starting body size on several traits during the transitional stage (e.g. mass, snout-vent length (SVL), head width, tail morphology, duration and locomotor performance). Morphological measures and the duration of the life stage were shown to vary with temperature and predation risk. As predicted, higher temperatures and the risk of predation resulted in faster development through metamorphosis and smaller sizes on completion. The acceleration of metamorphosis was demonstrated to have potential costs, not in the form of reduced locomotor performance as predicted, but in a reduction in juvenile size as a result of faster metamorphic development. This suggests that, during this potentially

vulnerable stage, it would be advantageous to take more time to complete in the absence of predators. Greater body size at the onset of metamorphosis requires a longer time to complete metamorphic climax suggesting that having a greater quantity of tissue to reconfigure during metamorphosis takes more time. Therefore, the conditions experienced during metamorphosis may have important implications for juvenile fitness and should be considered in studies of life history plasticity.

In many temperate species with complex life cycles, the life history stage at which a species can survive the winter is generally fixed, imposing time limits on the timing of development. Most of these species must therefore often modify developmental rate to reach the appropriate stage or size at the onset of winter, usually at a cost to other traits. However, variation in the stage or developmental group that some amphibian, fish and insect species spend the winter has been observed, such as in the common frog *Rana temporaria* in the UK, which can spend the first winter as either a tadpole or as a juvenile frog. To investigate the factors that contribute to this variation in life history, I examined the influence of environmental temperature, food availability and water depth on the rate of larval development and growth. Data on development, growth and environmental temperature of a field population of *R. temporaria*, which have been observed to over-winter as larvae, were collected to determine how and when the two divergent early life history patterns of development were established. Development rate was slowed by reduced temperatures and food availability and greater water depth during rearing. Temperature and food availability also had a significant impact on the proportion of larvae that over-wintered, but in the field other factors are likely to contribute to the within-population variation in wintering strategy. While a greater water depth did prolong larval development, as predicted, this does not appear to be due to the cost of surfacing to respire acting as a constraint on development, since a

similar slowing in development was observed in the lung-less *Bufo bufo* tadpoles. The results of these studies did not allow a definitive assessment of whether over-wintering as larvae represents an adaptive strategy or occurs as the result of developmental constraints. There is some evidence that over-wintering as larvae might be adaptive, since on completion of metamorphosis individuals that wintered as larvae were larger than those that completed metamorphosis late in the summer. Further work is necessary to identify other factors contributing to the over-wintering of larvae in *Rana temporaria* and to determine the adaptive significance, if any, of the alternative life history patterns.

Chapter 1: General Introduction

In many organisms it is well established that a genotype can give rise to different phenotypes depending on environmental conditions (West-Eberhard 2003). This plasticity in morphology and/or life history, is expected to occur in species that are likely to experience a range of different environmental conditions either through a single life-time or across generations (Pigliucci 2001). Plasticity will be adaptive if it allows an organism to maintain relatively high fitness across a range of environments (Bradshaw 1965; Sultan 1987; West-Eberhard 1989). However, the plasticity of a trait may have maintenance or developmental costs that make it disadvantageous in some conditions, such as stable environments (Via & Lande 1985; Stearns 1989; Van Tienderen 1991; Moran 1992; Relyea 2002). Alternatively, plasticity might simply be a physiological response to environmentally stressful conditions, rather than an adaptive trait (Sultan 1995; Pigliucci et al. 2006).

Life history strategies are known to vary with environmental conditions. For example, in the Atlantic salmon *Salmo salar* and Chinook salmon *Oncorhynchus tshawytscha*, the age at which an individual initiates smolting varies with temperature and photoperiod. Some individuals smolt and migrate towards the sea in their first year, while others may spend a year or more as parr in their freshwater habitat (Metcalfe et al. 1988; Metcalfe & Thorpe 1990; Beckman & Dickhoff 1998). Similarly, morphology has been shown to vary with factors such as predation risk, environmental temperature, inter- and intra-specific competition, landscape structure and food availability (Hjelm & Johansson 2003; Andrade et al. 2005; Relyea & Auld 2005; Merckx & Van Dyck 2006; Georgakopoulou et al. 2007; Relyea 2007). For example, several fish species (e.g. perch *Perca fluriatilis* and crucian carp *Carassius carassius*) develop deeper bodies in

the presence of a predator than when predators are absent (Bronmark & Miner 1992; Vollestad et al. 2004; Eklov & Jonsson 2007). This confers a survival advantage in predator-containing environments (Nilsson et al. 1995).

The costs of plasticity are much less understood. The capacity to be plastic may be costly to maintain and there may be trade-offs with other traits across various time scales. There may also be costs associated with a particular environmentally-induced phenotype. The possession of a deeper body in crucian carp, in response to predator presence, has been demonstrated to have a cost in the context of density-dependent intra-specific competition. At high densities shallow bodied individuals are able to gain twice as much mass as deep bodied individuals (Pettersson & Bronmark 1997). Similarly, in daphnia (*Daphnia* spp.) the induction of morphological predator defences results in changes in life history, where the period between birth and first reproduction is extended (Black & Dodson 1990; Caramujo & Boavida 2000).

Environmental conditions can also result in behavioural plasticity, which can further modulate life history and morphology variation (Lima & Dill 1990; Skelly & Werner 1990; Abrams & Rowe 1996; Ball & Baker 1996; Walsh & Downie 2005; De Kerckhove et al. 2006). Generally, in the presence of a predator, individuals modify their behaviour by foraging less, which reduces the risk of mortality (Sih 1987; Lima & Dill 1990). This behaviour carries the cost of reducing foraging, which can influence growth or development.

The work presented in this thesis investigates morphological and life history variability in both the onset and the duration of metamorphic climax, in response to several environmental factors, using anuran amphibians (*Xenopus laevis*, *Rana temporaria* and *Bufo bufo*) as experimental species. Specifically, my aims were twofold: 1) to determine whether amphibians are capable of displaying plasticity in the

duration of metamorphic climax; and 2) to investigate variation in the seasonal timing of metamorphosis in temperate anurans, relating to the life history phase (either larval or juvenile) at which individuals spend their first winter.

Animals with complex life-cycles

The term 'complex life cycle' has generally come to mean a life history that involves the progression through more than one discrete ecological phase. This usually, but not always, involves an abrupt ontogenetic niche shift, including a change in an individual's morphology, physiology and behaviour. The abrupt niche shift is allowed by a set of developmental processes that irreversibly changes an animal from one form to another, commonly referred to as metamorphosis (Istock 1967; Wilbur 1980; Moran 1994). The evolution and persistence of complex life cycles has been attributed to two explanations. Firstly, developmental biologists have long established that certain developmental processes are highly conservative, and that certain stages of a life cycle (e.g. larval stage) persist because they are an essential part of inflexible developmental pathways or vestigial traits reflecting previous evolutionary history (Gould 1977; Moran 1994). Alternatively, an ecological viewpoint is that complex life cycles are established as adaptive mechanisms for alternating between methods of utilizing resources or for producing phenotypes suited for different functions, such as growth versus dispersal (Istock 1967; Wilbur 1980; Ebenman 1992; Moran 1994). Given the prevalence of animals with complex life cycles (approximately 80% of organisms are considered to have complex life cycles: Werner 1988), it is important to understand the ecological processes determining life history and morphological plasticity in these animals, which may also help to explain more subtle ontogenetic changes in animals that do not have complex life cycles.

Amphibians as study animals

Amphibians have commonly been used for empirical and theoretical work on developmental and morphological plasticity, with a great deal of research using anuran amphibians particularly (e.g. Wilbur & Collins 1973; Petranka et al. 1987; Warkentin 1995; McCollum & Van Buskirk 1996; Denver 1997; Laurila & Kujasalo 1999; Merila et al. 2000a; Van Buskirk & Saxer 2001; Relyea 2001a; Relyea 2001b; Relyea & Hoverman 2003; Relyea 2004; Rose 2005). Anurans, the frogs and toads, are commonly used in investigations of this nature because of the ease of acquiring and rearing large numbers of individuals. Anuran ontogenesis does not have a period of developmental quiescence as occurs in most insect life cycles during pupation (Phillips 1998). The developmental biology of anurans has been extensively studied, and clear stages are distinguished during ontogeny, with reorganisation of structures and tissues involving programmed cell death and tissue re-modelling occurring during metamorphosis (Ishizuya-Oka 1996).

Typical anuran development is categorised by embryonic development within the egg, with hatching into the external environment as a larva occurring over a range of developmental stages. The hatching stage is usually specific to a species, but can exhibit plasticity, as in the red-eyed treefrog in response to predation threat (Warkentin 1995). This is followed by rapid growth during the feeding larval phase (Gosner stage 24 – 41; Nieuwkoop & Faber (NF) stage 41 – 58). This phase can be further sub-divided into 1) early larval period, or pre-metamorphosis, characterised by growth, but little external morphological change; and 2) pro-metamorphosis, when internal and external development progresses (Gosner stage 35 – 41; NF stage 55 – 57), most visibly

with the development of hind limb buds as endogenous thyroid hormone levels rise (Nakajima et al. 2005).

Following the larval stages is the onset of metamorphosis, characterised by peak levels of thyroid hormone, during which major internal and external changes take place. The most notable changes that occur are the re-absorption of the tail and the emergence of the fore-limbs, but during metamorphosis in anurans almost every organ is transformed (Duellman & Trueb 1986; Nakajima et al. 2005). While the mechanisms by which these transformations occur can allow different degrees of independence between larval and adult traits, many adult structures are simply remodelled larval structures, linking life history stages (Alley & Cameron 1983; Duellman & Trueb 1986; Ishizuya-Oka & Shi 2005; Schreiber et al. 2005). After metamorphosis, juvenile frogs begin another phase of growth, which is followed by sexual maturation as adults (Duellman & Trueb 1986).

General theory on morphology at and timing of ontogenetic transitions

It has been proposed that in anurans, and other animals with complex life cycles, the timing and size at ontogenetic transitions (such as metamorphosis or maturation) should be adaptively modified to the conditions experienced (Wilbur & Collins 1973) and potentially traded-off against future conditions or requirements (Werner 1986). Therefore, for example, under good larval conditions metamorphosis should be delayed to take advantage of the opportunity for growth; conversely, in poor larval conditions, metamorphosis should be accelerated (Wilbur & Collins 1973). Originally, this model largely related ‘good’ and ‘poor’ conditions to the potential for recent or current growth (Wilbur & Collins 1973; Hentschel 1999). However, it has become clear through further models and empirical work that many other factors determine ‘good’ and ‘poor’

conditions (such as environmental temperature, predation risk, competition and time constraints, among other factors), which can modulate the timing and/or size and morphology at transitions (Smith-Gill & Berven 1979; Ludwig & Rowe 1990; Rowe & Ludwig 1991; Relyea 2007). Furthermore, adaptations to benefit the current stage's conditions may not always benefit the subsequent stage or stages (Benard & Fordyce 2003; Relyea & Hoverman 2003; Vonesh 2005b; Ficetola & De Bernardi 2006; Richter-Boix et al. 2006).

Competition and resource availability are closely linked with the traditional concepts relating to 'good' or 'bad' growth conditions, with high levels of competition and/or low food availability resulting in poor conditions. This is predicted to result in an accelerated development rate (Wilbur & Collins 1973). However, food levels and the timing of life history transitions are not often so easily reconciled. Extremely high levels of competition or low food availability may not allow sufficient resources for individuals to accelerate development and still reach minimum size requirements for progressing through development, causing longer developmental times (Murray 1990; Leips & Travis 1994).

In poikilothermic animals, environmental temperature has been demonstrated to have a strong effect on the timing of ontogenetic stages, but its effect on size and other morphological traits has been less consistent. Reduced temperature results in slower development and therefore increases the duration of a given stage (embryonic stage: Douglas 1948; Bachmann 1969; Bradford 1990; Mitchell & Seymour 2000; Laugen et al. 2003a; larval stage: Loman 2002; Alvarez & Nicieza 2002a). In amphibians, this has commonly been linked to thermal effects on enzymatic action (Viparina & Just 1975; van der Have & de Jong 1996) rather than adaptive plasticity. However, adaptive responses to environmental temperature have been shown in insects (Partridge & Coyne

1997) and the evolution of higher intrinsic development rates in populations, within a species, that occur at higher latitudes or altitudes where temperatures are lower (counter-gradient variation: Loman 2003; Laugen et al. 2003b). The effects on morphology have been more varied. Generally, lower temperatures result in larger body sizes (Atkinson 1994; Ashton 2002; Alvarez & Nicieza 2002a; Measey & Van Dongen 2006), but this is not always the case (Ashton 2002; Joly et al. 2005; Laugen et al. 2005). Other morphological features, such as leg, gut and tail length, have also been found to be affected by temperature (Blouin & Brown 2000; Merila et al. 2004; Castaneda et al. 2006).

The risk of predation, particularly risk that is stage or size specific, is another factor that can influence the duration and morphology of ontogenetic stages (see reviews: Benard 2004; Relyea 2007). Life history theory predicts that if predation risk during a stage is high, individuals should accelerate development, even at the cost of growth, and leave the high predation risk stage as soon as possible. Additionally, it has been shown that adaptive morphology, such as a deeper body or tail (McCollum & Leimberger 1997; Teplitsky et al. 2003), or behaviour (e.g. reducing activity: Petranka et al. 1987; Sih 1987; Lima & Dill 1990; Skelly & Werner 1990; Anholt et al. 1996; Laurila et al. 1997; Griffiths et al. 1998) may be adopted to mitigate predation risk, but can reduce growth and/or development (McCollum & Van Buskirk 1996). However, empirical evidence for accelerated development and reduced size in the presence of a predator has been mixed (Relyea 2007). Commonly, predation risk during embryonic development results in accelerated hatching (Warkentin 1995; Kusch & Chivers 2004; Vonesh 2005a). During the larval phase, only two studies using caged predators have demonstrated acceleration of larval development (Laurila et al. 1998; Kiesecker et al. 2002); the remainder have demonstrated no change or longer larval durations (see

Relyea 2007). Actual predation during the larval phase can have further effects on growth and development beyond the predicted adaptive plasticity described above. For example, predators remove individuals from the population, which can reduce competition, increasing per capita resource availability and improving conditions for growth (Relyea 2007), which does not occur during non-feeding embryonic development.

Finally, there are other factors that have thus far received less attention, but which could provide interesting influences on plasticity. Photoperiod has been shown to have an important influence on development and life history decisions in some taxa (Clark & Platt 1969; Mcwatters & Saunders 1998; Nylin & Gotthard 1998; Lambrechts & Perret 2000; Tachibana & Numata 2004; Cabanita & Atkinson 2006), but our understanding of its influence on anurans is limited and somewhat contradictory. Laurila et al. (2001) did not find an effect of photoperiod on development in *Rana temporaria*, but development was affected in *Rana pipiens* (Wright et al. 1988) and both development and growth were affected in *Rana catesbeiana* (Bambozzi et al. 2004). Water depth, habitat size and habitat permanence have also been indicated as factors influencing phenotypic plasticity in anurans (Feder & Moran 1985; Pandian & Marian 1985a). Empirical evidence has also indicated that there may be a limited period during which plasticity may be expressed (Hensley 1993; Leips & Travis 1994). In amphibian larvae, food availability only influences larval duration when experienced during early larval stages. After a critical point, which may be flexible (Hentschel 1999), reducing or increasing food availability no longer influences larval period, but may still have an impact on size at metamorphosis. Additionally, environmental conditions will not be experienced individually and some may work in opposition, therefore the response to a

particular environmental factor will depend on its relative selective strength (Pigliucci 2001).

While there has been considerable work investigating plasticity in amphibian ontogeny, there are still aspects that are poorly understood. The duration of metamorphic climax has received some theoretical attention, yet there has been almost no experimental work on the ecology of this life stage (Wassersug & Sperry 1977; Downie et al. 2004). Similarly, there have been correlation studies (Collins & Lewis 1979) and an increasing number of anecdotal reports on over-wintering as larvae in some anurans in recent years (Archibald & Downie 1996; Pintar 2000; Fellers et al. 2001; Lai et al. 2002), but no experimental work.

Metamorphic climax

Metamorphosis offers a unique opportunity to investigate life history and morphological plasticity for a number of reasons. Firstly, it is likely to meet the theoretical conditions that would predict adaptive selection for responsive variation (Schlichting & Pigliucci 1998; Pigliucci 2001). Metamorphosis takes place in a variable environment, experienced by a single individual, and it is probable that individuals are capable of receiving environmental cues. Also, it is expected that completing metamorphosis 'as fast as possible' would carry costs, since it has been shown in a variety of species and life stages that accelerated development is less efficient (Present & Conover 1992; French et al. 1998; Robinson & Partridge 2001) or reduces subsequent fitness (Metcalfe & Monaghan 2001; Fischer et al. 2005). Secondly, metamorphosis represents a distinct, obligate stage of an anuran life cycle, with easily identifiable beginning and termination points, making it quantifiable. Finally, it is free of many of the confounding factors present in studies of phenotypic plasticity in anuran larvae. During metamorphosis,

individuals are non-feeding, relying on stored resources, therefore competition for food resources is not a factor. This simplifies analysis of predator-mediated plasticity, since thinning of population density through predator mortality, which can result in greater per capita resources, will not influence development or morphology during this stage. In studies with lethal predators, this may allow a greater understanding of predator selection effects in addition to induction effects (Relyea 2007). Additionally, behavioural plasticity will have less of an impact during metamorphosis, since foraging is not required. However, this will also provide a good contrast with insect studies of this nature, since mobility is still possible and factors such as microhabitat selection may be responsive to environmental conditions.

Until recently, our understanding of metamorphic duration has been shaped by theories relating to evolutionary biology. These were derived from Williams' (1966) supposition that selection would drive developmental phases of elevated predation-risk to be as short as is possible to comply with developmental requirements. Metamorphosis was perceived as such a stage (Rose 2005), due to potentially reduced locomotor performance relative to other life stages (Wassersug & Sperry 1977; Arnold & Wassersug 1978). While in certain anuran species metamorphosis, as well as the larval stage, has been truncated, such as in the direct developing and parental-caring species, metamorphosis still persists and has been demonstrated to display both intra- and inter-specific variation (Downie et al. 2004). Therefore, it is likely that metamorphic duration is able to respond to environmental conditions, since there may costs to accelerating development similar to those observed in the larval and embryonic stage.

Over-wintering as larvae

Many current models of anuran life history plasticity have been developed to include time constraints, where individuals are expected to accelerate development as time progresses closer to an end point of high or complete mortality risk (Wilbur & Collins 1973; Lardner 2000). Factors such as pond drying and the onset of winter are often expected to be time constraints on the development and survival of larval anurans. However, a number of urodelan (salamanders and newts) species are known to over-winter as larvae in ponds (e.g. Whitford & Vinegar 1966; Bruce 1982; Beebee & Griffiths 2000; Bernardo & Agosta 2003) and an increasing number of anuran species have been observed over-wintering as larvae (e.g. Collins & Lewis 1979; Archibald & Downie 1996; Pintar 2000; Fellers et al. 2001; Gollmann et al. 2001; Lai et al. 2002). This may indicate one of two situations: 1) that over-wintering has always been prevalent, but has only recently been observed in these species; or 2) that over-wintering is becoming more common, as a strategy, amongst anuran larvae. The second situation may indicate an adaptation in anurans to milder winters associated with climate change, which may be an early sign of developmental plasticity in response to climate change. Therefore, it is very important to understand the factors involved in this understudied aspect of anuran life histories.

Study system

The African clawed toad (*Xenopus laevis*) belongs to the family Pipidae and originally comes from Southern Africa, but is found throughout the world due to its use during the 1940's as a pregnancy test in hospitals and its later use as the amphibian of choice for developmental biologists. It is a medium-sized (up to 12 cm in length) anuran that predominately remains in stagnant waters as an adult, leaving only when forced to by

pond drying. It is commonly used in experiments, and is a model species in genetic and embryology research, for a number of reasons. It is an extremely hardy species that is very resistant to disease and infection, which makes it easy to maintain in the laboratory. They are widely available as a result of their use during the 1940's. Finally, they are easy to breed in captivity. Females can be induced to breed by injecting them with human gonadotropic hormone (Polack 1949) and can typically lay several hundred eggs. *X. laevis* was of particular interest in this study since it allows a directly comparable method for assessing locomotor performance throughout development, because the adults are permanently aquatic.

While this species is fully aquatic and does not move between habitats as is common of many other anurans (moving from aquatic larvae to terrestrial adults) it still undergoes a complex metamorphosis, during which almost all organ systems are transformed. Larvae *Xenopus* are algal filter feeders that respire mainly through the use of gills and cutaneous gas exchange, while juvenile and adult *Xenopus* are voracious carnivores that breathe atmospheric oxygen through lungs. Additionally, on completion of metamorphosis *Xenopus* is much less vulnerable to predators.

The European common frog (*Rana temporaria*) belongs to the family Ranidae, which is one of the largest amphibian families (Che et al. 2007), and is found throughout Europe, with the exception of some Mediterranean and Arctic areas. It is a medium sized frog (6 – 9 cm) that is predominately terrestrial, but occupies damp or wetland areas. This species has a relatively short but explosive breeding season, which occurs in early spring. Metamorphosis is normally completed within 12 – 17 weeks of spawning, at the end of the summer. *Rana temporaria* is commonly used in studies investigating phenotypic plasticity and is easily available in the UK. However, this species is of particular interest, since it is one of only a few temperate region species

that has been recorded to over-winter during the larval stage. While life history plasticity in this species, and other larval over-wintering species, has been extensively examined, no empirical research has been conducted on which factors induce this extreme form of plasticity in anurans.

The European common toad (*Bufo bufo*) belongs to the family Bufonidae and is found throughout Europe, parts of Western Asia and North-Western Africa. It is a medium (6 – 9 cm), entirely terrestrial species, that only returns to water bodies to breed. Breeding in this species begins in spring, generally several weeks after *Rana temporaria* in the UK. Development to metamorphosis generally takes 9 – 12 weeks and this species has not been reported to over-winter as larvae. It is of interest, in this context, as a comparison with *R. temporaria* on the effect of water depth, due to differences in the progression of larval development. *B. bufo* does not develop functional lungs until just prior to the on-set of metamorphosis and metamorphosis is at a small size, whereas *R. temporaria* begins to develop lungs much earlier as larvae and generally metamorphose at a larger size (see **chapter 7**).

Thesis content

In this thesis I attempt to determine environmental impacts on the duration and progression of metamorphosis in *Xenopus laevis* and the variation in over-wintering life history stage in *Rana temporaria*. In **chapter 2**, using the natural variation in sizes at the onset of metamorphosis that occur under larval conditions, I investigate the effect of temperature and body size on the duration of metamorphosis in *X. laevis*, decoupled from the effects of larval conditions. I also assess the swimming performance at several stages (larval, metamorphosis, and juvenile) during development to determine the vulnerability of different life stages to predation risk. In **chapter 3**, following similar

procedures to **chapter 2**, I attempt to determine whether the duration and progression of metamorphosis in *X. laevis* is responsive to the presence of a predator. In an attempt to determine costs associated with a plastic response in metamorphic duration, post-metamorphic size and swimming performance are assessed. In the final chapter on metamorphic duration, **chapter 4**, I focus on morphological changes that occur during metamorphosis in *X. laevis* and how these are affected by environmental temperature in relation to the temperature-size rule.

The second half of the thesis centres on over-wintering as larvae in *Rana temporaria*. **Chapter 5** reports on a set of experiments aimed at understanding the factors that influence this delay in metamorphosis. By manipulation of environmental conditions (environmental temperature, food availability), I assess the conditions that contribute to the proportion of over-wintering tadpoles and the differences resulting in froglets that have over-wintered as larvae and those that complete metamorphosis in the summer. In **chapter 6** I investigate the occurrence of over-wintering in the field. Comparisons are also drawn between field and laboratory incidences of over-wintering. In **chapter 7** the effect of water depth on larval development, growth and surfacing behaviour is assessed, in *Rana temporaria* and *Bufo bufo*, as a possible factor in over-wintering.

In **chapter 8**, I conclude with a discussion of the results of these experiments within the general context of developmental plasticity.

Chapter 2: Plasticity of the duration of metamorphosis in the African clawed toad

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Abstract

In organisms with complex life cycles, such as amphibians, selection is thought to have minimized the duration of metamorphosis, because this is the stage at which predation risk is presumed to be highest. Consequently, metamorphic duration is often assumed to show little if any environmentally induced plasticity, because the elevation in the extrinsic mortality risk associated with prolonging metamorphosis is presumed to have selected for a duration as short as is compatible with normal development. I examined the extent to which metamorphic duration in the anuran amphibian *Xenopus laevis* was sensitive to environmental temperature. Metamorphic duration was influenced by body size, but independent of this effect, it was strongly influenced by environmental temperature: the duration at 18°C was more than double that at 24 and 30°C. I also compared the vulnerability of larval, metamorphosing and post metamorphic *Xenopus* to predators by measuring their burst swimming speeds. Burst swim speed increased through development and while I found no evidence that it was reduced during metamorphosis, it did increase sharply on completion of metamorphosis. I therefore found no evidence of a substantial increase in vulnerability to predators during metamorphosis compared with larval stages, and hence the slowing of metamorphosis in response to temperature may not be as costly as has been assumed.

Introduction

Organisms with complex life cycles often have a number of distinct life-history stages, each with substantially different requirements and risks (Wilbur 1980). The durations of different stages have been shown to vary with environmental circumstances. Variation in the duration of the larval period, for example, has been demonstrated in several studies covering a wide range of taxa (e.g. marine invertebrates: Twombly 1996; insects: Blakley 1981; fish: Policansky 1983; amphibians: Werner 1986; Harris 1999). The duration of the transition between stages, often involving a metamorphosis, has received considerable attention in terms of developmental mechanisms, but much less in terms of the effects of environmental circumstances. The duration of metamorphosis is presumed to be minimized by selection because of the high vulnerability to predators during this period (Williams 1966), due to reduced locomotor ability and hence to be insensitive to environmental circumstances (Rose 2005). Contrary to Rose's (2005) statement that metamorphosis is a developmental phase of fixed duration, with no intraspecific variation, a recent analysis of metamorphic duration has shown that it varies considerably among and within anuran species. This analysis also suggested that minimizing predation risk is not the sole factor in determining the duration of metamorphosis, but that it is related to local growth conditions, as indicated by body condition and size, and to environmental temperature (Downie et al. 2004).

Anuran amphibians are widely used in studies of the effect of environmental factors on life cycles because they go through clear and distinct stages. Following hatching from the egg, there is a larval period characterized by rapid growth. Metamorphosis then occurs, during which time individuals do not feed and change from a tail-driven tadpole to a four-legged froglet; this is then normally followed by further growth and then by sexual maturation. It has been suggested that metamorphosis is a

particularly vulnerable period in this group. For example, Arnold & Wassersug (1978) found that chorus frogs *Pseudacris triseriata* were more frequently captured by garter snakes during metamorphosis than as pre-metamorphic larvae or post-metamorphic juveniles. Additionally, it was established that the impaired locomotor performance (measured as swimming endurance) of metamorphosing individuals compared with tadpoles observed in this species was responsible for the increased vulnerability (Wassersug & Sperry 1977).

The locomotor impairment thought to be typical of metamorphosis is of critical importance to the hypothesis that the duration of anuran metamorphosis has been minimized by selection. While there have been several studies evaluating the effects of conditions during the larval period on the speed of larval and juvenile movement (Van Buskirk & Saxer 2001; Alvarez & Nicieza 2002b; Altwegg & Reyer 2003), and differences in locomotion between pre- and post-metamorphic urodeles (Shaffer et al. 1991; Azizi & Landberg 2002; Wilson 2005), the locomotor performance of metamorphosing individuals has only been examined in two other anuran species since Wassersug & Sperry's (1977) original study. Watkins (1997) did not find a significant difference in maximum burst swim speed between premetamorphic individuals (Gosner stage 37; Gosner 1960) and individuals at the start of metamorphic climax (Gosner stage 42) in *Hyla regilla*. However, Huey (1980) demonstrated that burst speed in *Bufo boreas* decreased during metamorphosis, from a peak just before the onset, in tandem with a decrease in tail length.

In this study, I examined the plasticity of metamorphic duration in the fully aquatic *Xenopus laevis* in relation to conditions during metamorphosis. My aims were twofold. Firstly, I examined the effect of experimentally imposed temperatures during metamorphosis, taking into account variation in body size due to differential growth

during the larval period. Secondly, I examined the predator avoidance capability of individuals at different stages in their development to determine whether this is impaired during metamorphosis compared with the pre-metamorphic larval and post-metamorphic juvenile stages.

There are several methods for assessing the predator avoidance capability in anurans, such as turning speed, endurance swimming, maximum attainable swim speed or burst swim speed (Wassersug & Sperry 1977; Huey 1980; Wassersug 1989). Burst swim speed, the starting velocity from a stationary position, has been shown to be important for avoiding predation across all developmental stages (Miller 1982; Azizi & Landberg 2002; Wilson et al. 2005). Therefore, I measured predator avoidance capability using burst swim speed.

Methods and Materials

Animals and rearing conditions

Approximately 250 wild-type eggs of *X. laevis* Daudin were obtained from St Andrews University (St Andrews, Fife, Scotland, UK) in 2005. Tadpoles were kept in a single 40 L holding tank for 20 days before being transferred to 10 smaller holding tanks, by which time they had reached approximately Nieuwkoop and Faber (NF) stage 42 (Nieuwkoop & Faber 1994).

Each of the smaller holding tanks (30 × 20 × 20 cm) contained 25 tadpoles and was filled with *c*. 11 L of aerated, de-chlorinated, copper-free water. The temperature in the tanks was controlled by heaters, which were switched off during the night, and maintained the temperature at 30°C during daytime and 18°C at night. This range is representative of the temperatures that *X. laevis* would experience under natural conditions (Tinsley & Kobel 1996). Thus, all tadpoles were exposed to the same range

of experimental temperatures before metamorphosis; that this range encompassed the full range of temperatures to be used in the experiments during metamorphosis avoided any acclimatization problems. Perspex sheets were placed over the tanks to reduce water loss from evaporation, and a constant water level was maintained in all tanks. Tadpoles were exposed to a 12 h:12 h light/dark cycle throughout the larval period. They were fed daily on *c*. 0.2 g of dried algal pellets per tank, ground and hydrated before being suspended in water.

Experimental protocol

Checks were made three times a day for individuals showing emergence of one or both forelimbs, indicative of the commencement of metamorphosis (stage 60). In total, 139 tadpoles were transferred to separate, individual experimental tanks (15 × 20 × 20 cm) at the onset of metamorphosis. The remainder were not included due to natural mortality before the onset of metamorphosis or because they did not commence metamorphosis within the time span of the study. Individuals were checked three times a day for completion of metamorphosis (stage 66), identified by complete tail absorption (tail length <0.5 mm). Experimental tanks were maintained at constant water temperatures of 18, 24 and 30°C, and tadpoles were allocated sequentially to the three temperature treatments at the onset of metamorphosis. The 18 and 24°C treatments each had 46 individuals, while the 30°C treatment had 47 individuals.

Wet mass, after removal of surface water (±0.001 g), snout–vent length (SVL), head width, tail length and maximum tail depth measurements (±0.1 mm, using callipers) were taken at the commencement of metamorphosis. There were no significant differences in mass, SVL, head width, tail length, tail depth (Table 2.1) or body condition (mean: 0.106±0.001 SE; $F_{2,135}$=1.573, P=0.211) at the start of metamorphosis between the three temperature treatments. Tail length and depth

(±0.1 mm) measurements were taken every 3 days during metamorphosis, to determine whether metamorphosis progressed with the same pattern in all three temperature treatments. At the completion of metamorphosis, mass, SVL and head width were measured. Body condition at the start and end of metamorphosis was calculated from body measurements using the formula provided in Veith (1987), defined as condition=(mass/SVL^3) × 1000.

Newly metamorphosed juvenile individuals to be tested for locomotor performance were maintained at their metamorphic temperature until testing; after testing, they were transferred to a stock tank maintained at 24°C.

Swimming speed data collection

Measurements of swimming speed were taken from NF stages 48–66, comprising the larval stages (stages 48–59), the middle of metamorphosis (stage 63, when 50% of the tail was reabsorbed) and soon after metamorphosis was complete (stage 66+). Pre-metamorphic tadpoles were grouped as follows: stages 48–51 (small hind-limb buds); stages 52–55 (differentiation and flattening of hind foot); and stages 56–59 (separation of toes and development of hind legs). Mid-metamorphosis (stage 63) was selected because it is likely to represent the most critical period during which metamorphosis would be impaired, because the tail is being rapidly absorbed and the hind limbs are still developing.

Owing to the sensitivity of the equipment being used, filming of swimming had to be performed in one room (air temperature 20°C) for all temperature treatments. Individuals to be filmed were brought into the room in a shallow dish 1 h before being filmed to acclimatize to the temperature (the water temperature ranged between 19 and 27°C, due to heat from the lighting equipment required for filming). The 1 h time period

was selected as an acceptable period for acclimatization, as a longer period might have affected the developmental processes being investigated. Water temperature at the time of trials was recorded and included in the analysis as a covariate. This did not significantly affect locomotor performance at any of the three developmental stages (tadpoles: $F_{1,28}$=1.784, P=0.194; metamorphs: $F_{1,52}$=1.330, P=0.255; and juveniles: $F_{1,78}$=1.911, P=0.171).

A Photron FASTCAM-PCI high-speed camera (Photron USA Inc., San Diego, CA, USA) was used to capture video footage. The camera was placed facing directly down into a tank 20 cm long, with laminated grid-paper along the bottom; there was a 4 cm wide corridor through the middle of the grid, filled with *c*. 1 cm depth of water. The camera was mounted sufficiently far above the water surface (0.8 m) relative to water depth (1 cm) to minimize parallax errors in determining position. Each individual was placed at one end of the tank and allowed to settle, and then gently prodded at the base of the tail with a glass stirring rod wrapped in red electrical tape to elicit an escape response. These procedures limited the tadpoles to swimming forward in response to the stimulus.

Filming was carried out at 250 frames per second (fps) and the camera recorded up to 5 s of swimming; the swim speed of each individual was measured five times with *c*. 1 min break between each. There was no evidence of habituation through the series of trials in any of the three developmental groupings (pre-metamorphic: r^2=0.029, P=0.06; metamorphic: r^2=0.006, P=0.23; juvenile: r^2=0.0, P=0.67). As in other studies (e.g. Wilson et al. 2005), the first ≈300 ms (37 frames) following initial movement were used representing burst speed, a critical variable in fleeing from a predator (Watkins 1996; Dayton et al. 2005).

Data analysis

Data are presented as mean ± SE. *F*-tests were used to determine whether variation in metamorphic duration differed between temperature treatments. Principal components analysis was carried out on the four body measurements (SVL, head width, tail length and depth) for use in analysis on metamorphic duration. A single factor was extracted (PC1), which explained 62.4% of the variation, representing predominately body rather than tail measurements. χ^2 was used to determine differences in metamorphic mortality between temperature treatments. Tail loss during metamorphosis was assessed using 3-parameter, sigmoid regression (SigmaPlot 10, Systat Software Inc.) defined as $y = \frac{a}{1 + e^{(-(x - x_0)/b)}}$, where x_0 is the inflection point, b the steepness of curve and a the range of y values. The remaining analyses were performed using analysis of variance with *post hoc* Tukey tests (SPSS 14, SPSS Inc.).

For film analysis, Photron Motion Tools software was used, which allowed digital analysis, giving the distance per frame (velocity) and the distance travelled. The maximum measurement achieved from the five trials was used for data analysis.

Before stage 60, individual tadpoles were taken at random from the small holding tanks for swim testing; they were then returned to the tank, and so it was not possible to track them as individuals. Therefore, the data on locomotor performance between larval and metamorphic stages are treated as independent samples. Comparisons were made between four developmental groups: three pre-metamorphic stages (all experiencing the fluctuating temperature environment) and one at mid-metamorphosis (stage 63). Three such analyses were performed to allow comparisons between larval and metamorph swim speeds at the three different temperature treatments experienced only after metamorphosis commenced. Comparative analysis of locomotor performance between metamorphic and juvenile stages (stages 63 and 66)

was performed on known individuals using a linear mixed model with repeated measures.

Results

Metamorphic duration

Metamorphic duration differed significantly between the three experimental treatments. It was the slowest at the lowest temperature, taking twice as long at 18°C than at 30°C (Fig. 2.1). The difference between the two higher temperatures, while significant, was relatively small (c. 1 day). The variability in metamorphic duration differed significantly among the temperature treatments, with the 24°C treatment being the least and the 30°C treatment the most variable ($F=15.113$, $P<0.001$; 18°C: range 14.04–20.02 days, CV: 9.32%; 24°C: 7.19–10.00 days, CV: 6.96%; 30°C: 6.00–10.19 days, CV: 13.76%).

As the body size (PC1) increased, the duration of metamorphosis increased ($F_{1,117}=7.646$, $P=0.007$). Metamorphic duration was not influenced by starting body condition ($F_{1,117}=2.803$, $P=0.097$; Fig. 2.2). The effects of temperature were significant ($F_{2,117}=3.812$, $P=0.024$) and there was no interaction between temperature and body size ($F_{2,117}=0.721$, $P=0.488$) or temperature and condition ($F_{2,117}=0.089$, $P=0.915$).

Mortality levels were significantly different among treatments. No mortality occurred in the 24°C treatment. The 30°C treatment had the highest mortality rate (11 mortalities, 23.4%), while the 18°C treatment had less (six mortalities, 13%) ($\chi^2=11.905$, d.f.=2, $P=0.003$).

Tail loss during metamorphosis

Tail re-absorption followed a sigmoid pattern, with a rapid decrease in tail length occurring between days 3 and 6 in the two higher temperatures (24 and 30°C) and between day 6 and 12 in the 18°C treatment (Fig. 2.3). Inflection points (x_0) and steepness of curves (b) were significantly different between the three treatments (x_0: $F_{2,124}$=210.986, P<0.001; b: $F_{2,124}$=60.279, P<0.001); post hoc analysis showed that this difference was between 18°C and the two higher temperature treatments.

Locomotory performance

SVL had a significant positive effect on burst swim speed during the larval ($F_{1,28}$=9.223, P=0.005) and juvenile stages ($F_{1,78}$=17.154, P<0.001), but only marginally during metamorphosis ($F_{1,52}$=4.000, P=0.051). The temperature treatment experienced during metamorphosis had a significant effect on metamorph burst swim speed ($F_{2,52}$=5.586, P=0.006), but this was not found in juveniles ($F_{2,78}$=0.245, P=0.783). During metamorphosis, individuals from the 24°C treatment had a significantly faster burst swim speed than the higher and lower temperature treatment groups, which were not significantly different from one another (Fig. 2.4).

To assess whether locomotion is impaired during metamorphosis, comparisons were made between larvae and metamorphs and between metamorphs and juveniles.

The comparisons between the three larval stage groups and metamorphic individuals at the three temperature treatments all showed a significant relationship between maximum swimming speed and stage (18°C: $F_{3,44}$=6.807, P=0.0007; 24°C: $F_{3,45}$=11.989, P=0.0001; 30°C: $F_{3,47}$=6.378, P=0.001; Fig. 2.4). I found no evidence of locomotor impairment during metamorphosis: at 18 and 30°C, metamorphic burst swim speeds did not differ from burst speeds attained by pre-metamorphic larvae. In fact,

metamorphs at 24°C actually swam significantly faster than the late-stage larvae. The maximum swimming speed increased as individuals progressed through larval development and continued to increase up to mid-metamorphosis (NF stage 63). Therefore, swimming speed at metamorphosis was not reduced compared with late-stage, pre-metamorphic larvae of similar sizes and was significantly faster than smaller, early-stage larvae.

Correcting for metamorphic temperature treatment ($F_{2,80.506}$=0.299, P=0.742) and SVL ($F_{1,80.782}$=6.807, P=0.011), at the end of metamorphosis individuals swam significantly faster than they did during metamorphosis ($F_{1,80.125}$=57.741, P<0.001).

Discussion

Metamorphic duration

The results show that metamorphic duration is sensitive to environmental temperature. At temperatures commonly experienced by *X. laevis* (*c*. 21°C), metamorphosis takes *c*. 8 days (Huang et al. 2001). Metamorphosing in cold temperatures resulted in more than double the standard length of time to complete metamorphosis. Metamorphosis at the two higher temperature treatments (24 and 30°C) was much closer in duration, with the duration at 30°C taking about a day less than at 24°C. This result indicates that above a certain temperature threshold, the speed of metamorphosis is optimized, as is suggested by Downie et al. (2004) for other species.

There was also substantial variability in metamorphic duration within the temperature treatment groups. Body size was the main component explaining this variation. Contrary to the prediction of Downie et al. (2004), metamorphic duration was not related to the index of body condition. However, Downie et al. (2004) did not use the term body condition in the precise manner defined by Veith (1987). The lack of

relation with body condition might be a result of the rearing conditions. All individuals were reared at relatively low densities, with an abundance of food on a daily basis. Therefore, the condition at the start of metamorphosis was relatively high and less variable than would be expected in the wild. Rearing individuals at different densities or lower food availabilities may allow the effects of body condition on metamorphic duration to be studied in more detail.

Within-group variability in metamorphic duration differed between the three temperatures. At 30°C, metamorphosis was completed in the fastest time, but the degree of variation was the highest. Additionally, the mortality rate was the highest in this group. The higher mortality indicates that there may be costs associated with such a rapid rate of metamorphosis, as has been found in rapid growth rates (Arendt 1997). At 24°C, the temperature treatment closest to what would be experienced by *X. laevis* in the field (Tinsley & Kobel 1996), the variation was the smallest. This indicates that at the temperature a species is adapted for, a thermal developmental optimum is established (Stahlberg et al. 2001).

Locomotor performance

The temperature experienced during metamorphosis had different effects on swimming speed during and after metamorphosis. At stage 63, the 24°C temperature treatment resulted in individuals having a faster maximum swim speed, with either extreme having slower speeds. Alvarez & Nicieza (2002b) demonstrated a similar result in juvenile jumping performance in the Iberian painted frog *Discoglossus galganoi*, with performance peaking at an optimal temperature and decreasing as the temperature increased or decreased. Miller (1982) found a similar trend in adult *Xenopus*, with the highest locomotor performance occurring at 27°C and performance decreasing with

elevated or lowered temperatures. Additionally, in our study, size was found to have only a marginally significant effect on swimming speed during metamorphosis, possibly due to other factors, such as the progression of hind limb development, being more critical. This result suggests that the temperature experienced during metamorphosis may have some effect on musculoskeletal development, such as the type or capacity of hind limb musculature being developed during metamorphosis, which influences swimming performance.

On completion of metamorphosis, temperature no longer had an effect on swim speed, but size, specifically SVL, did have an effect. This result contrasts with the report of a temperature effect on juvenile locomotion reported by Alvarez & Nicieza (2002b). However, in their study, the temperature treatments were experienced throughout the larval period, while in this study individuals were only subjected to different temperatures from the start of metamorphosis. Thus, the temperature effects found by Alvarez & Nicieza (2002b) might be a consequence of effects operating during larval development.

Comparisons between larval and juvenile locomotor performance may be complicated by differences in thermal capacity between the musculature that the two stages use for locomotion. Sherman (1980) showed that, in *Bufo woodhousii fowleri*, the developing hind limbs were less able to cope with thermal stress than the tail, suggesting that extremely high temperatures would favour tadpole speed over juvenile speed. However, this is not likely to be the case in this study, because the thermal range used was well within the species tolerance and well below the thermal maxima of *Xenopus* (Sherman & Levitis 2003). Additionally, differences in thermal preference between larval and adult amphibians have not been shown in all species (e.g. *Triturus cristatus*: Wilson 2005), and it has even been suggested that the thermal environment of

adult and larval *Xenopus* is likely to be the same, as they occur in the same ponds (Sherman & Levitis 2003), but this will be dependent on pond depth.

Comparison of metamorph predator avoidance

In this study, even when corrected for size, individuals did not experience the decrease in locomotor performance predicted and observed by Wassersug & Sperry (1977), using chorus frogs. In *X. laevis*, metamorphic swimming speed was slightly, but not significantly, faster than speeds displayed by larval individuals of a similar size. There was a dramatic increase in swimming performance on completion of metamorphosis. There could be several explanations for this: (1) this species is fully aquatic, and so the transition costs between terrestrial and aquatic locomotion are not present. (2) Research on *Xenopus* development has shown that during metamorphic climax, individuals are able to use both tail and hind limb-based locomotion (Combes et al. 2004), which may be different from other species (e.g. species with rapid tail loss; see Downie et al. 2004), and could improve metamorphs' locomotion performance. (3) Hind limb development, which has been shown during the larval stage to aid swimming (Park et al. 2003), may confer a greater advantage to metamorphosing *Xenopus* because of the large, well-developed webbed feet found in this species. It has been shown that drag forces are considerably increased during metamorphic climax due to forelimb emergence (Dudley et al. 1991), but the advantage of the thrust generated by the developing hind limbs could offset this cost. However, Dudley et al. (1991) used *Rana catesbeiana* tadpoles, which have a narrower, more streamlined anterior end compared with *X. laevis* tadpoles. (4) Finally, this study examined locomotion performance as burst speed, whereas Wassersug & Sperry (1977) examined swimming endurance. However, burst speed is

more accurate than endurance as an indicator of predator evasion (see Dayton et al. 2005).

In two studies on locomotor performance, where burst speed was examined, conflicting results were observed. Huey (1980) did show a decrease in burst speed in *B. boreas* during metamorphosis, with individuals with longer tails at stage 43 (*c*. NF stage 63) having faster swim speeds. Watkins (1997), working on the Pacific tree frog, found that the maximum burst speed was not significantly different between pre-metamorphic and stage 42 individuals, but argued that using the mean of some of the speed trials, there was a significant decrease in locomotor performance in metamorphic individuals. Watkins (1997) did not investigate post-metamorphic locomotor performance in the Pacific tree frog. In the present study, using either mean or maximum, burst speed did not demonstrate a decrease in performance during metamorphosis in any of the three temperature treatments.

In conclusion, our data show that metamorphic duration varies with both environmental temperature and body size, and that locomotion is not impaired during metamorphosis compared with the pre-metamorphic stage. These findings present some fundamental complications for the idea that selection fuelled by high predation risk has minimized the duration of metamorphosis. It is possible that variation in natural predation risk results in different strengths of selective pressure for minimizing metamorphic duration, and it would be interesting to compare populations from high and low predation risk habitats. It would also be interesting to know more about the costs associated with rapid metamorphosis.

Tables and figures

Table 2.1: Starting measurements of different temperature treatment groups (ANOVA results displayed; NS indicates non-significant difference)

	18°C	24°C	30°C
Mass ^{NS}	0.65±0.03 g	0.69±0.02 g	0.67±0.02 g
SVL ^{NS}	18.1±0.2 mm	18.5±0.2 mm	18.4±0.2 mm
Head width ^{NS}	8.6±0.1 mm	8.8±0.1 mm	8.8±0.1 mm
Tail length ^{NS}	32.1±0.6 mm	31.7±0.6 mm	31.9±0.7 mm
Tail depth ^{NS}	5.8±0.1 mm	5.8±0.1 mm	5.6±0.2 mm

Figure 2.1: Duration of metamorphosis in days (± SE) for the three temperature treatments.

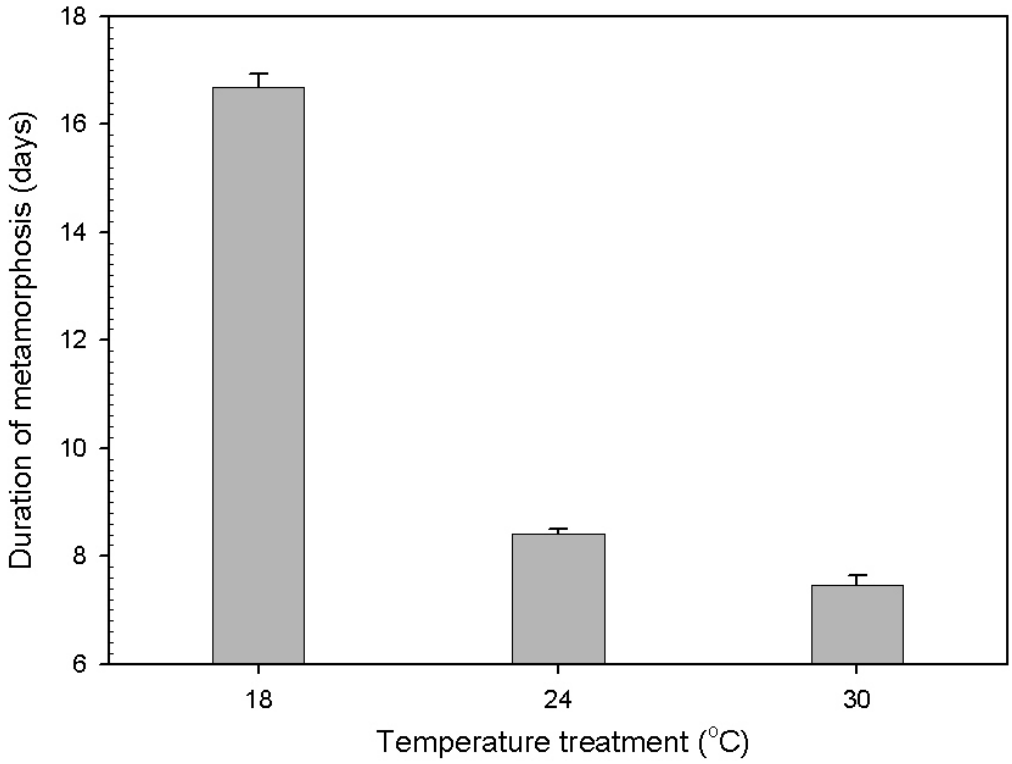


Figure 2.2: Duration of metamorphosis in days in relation to body condition at the onset of metamorphosis (18ºC: ●, solid line; 24ºC: ○, dashed line, 30ºC: ▼, dotted line)

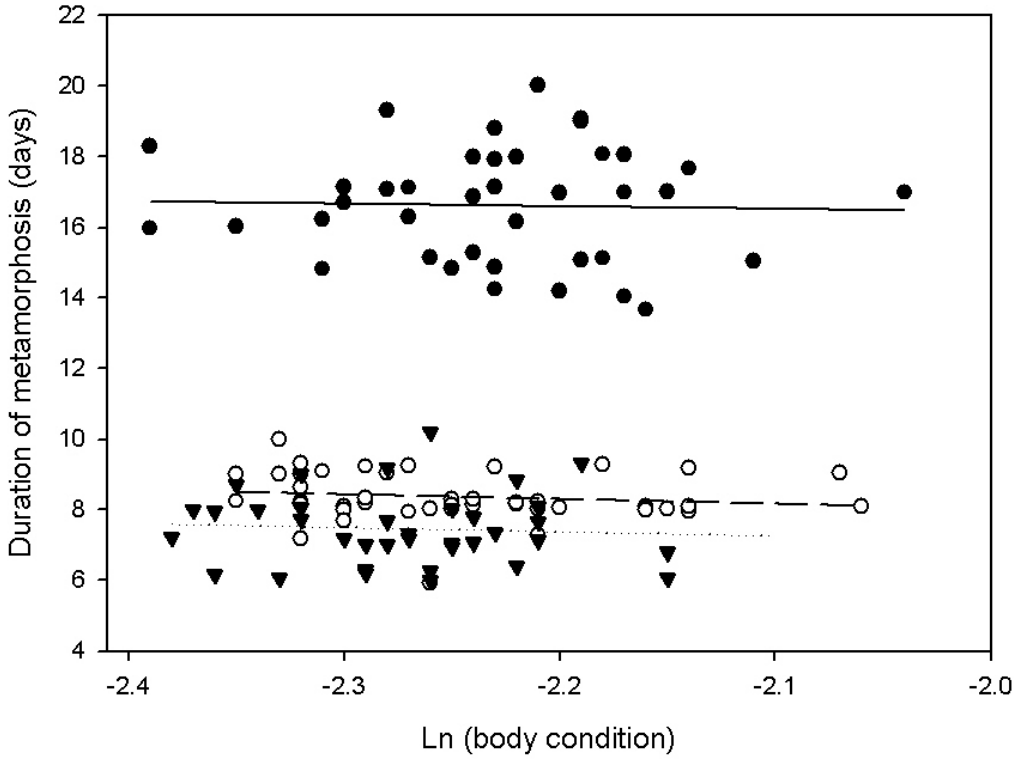


Figure 2.3: Decrease in mean tail length (± SE) through metamorphosis (18°C: $y = \frac{31.437}{1 + e^{((x - 4.188)/0.653)}}$ **, r^2 = 76.8%, p < 0.001; 24°C:** $y = \frac{31.692}{1 + e^{((x - 2.533)/0.289))}}$ **, r^2 = 85.7%, p < 0.001; 30°C:** $y = \frac{32.627}{1 + e^{((x - 2.335)/0.319)}}$ **, r^2 = 80.1%, p < 0.001)**

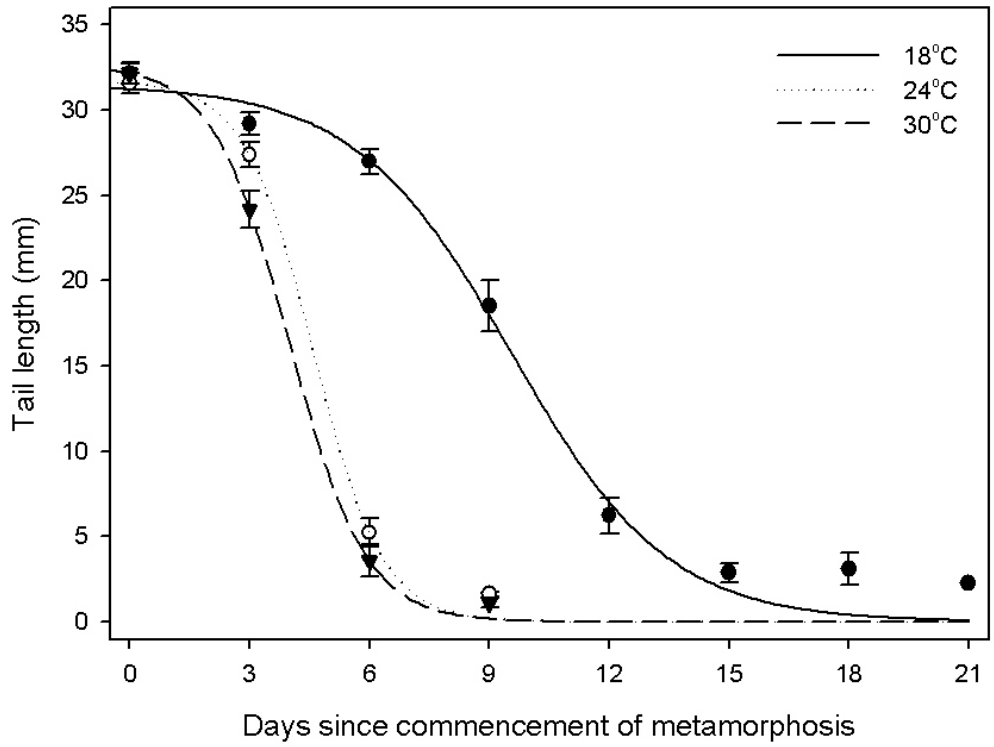
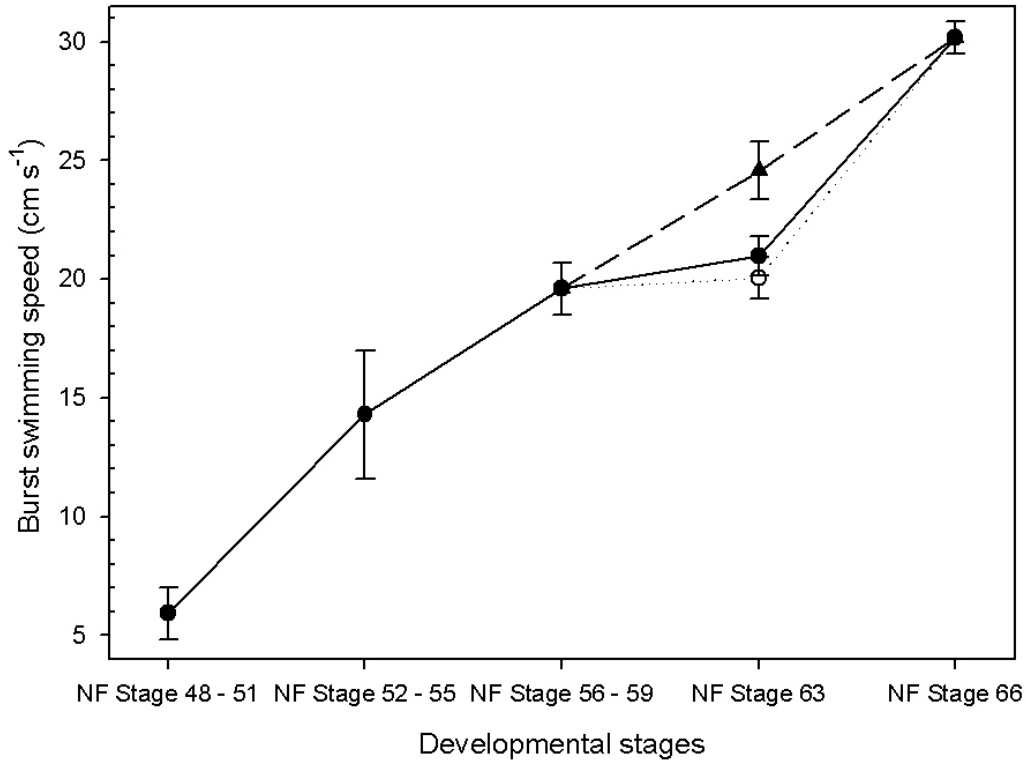


Figure 2.4: Burst swimming speed (± SE) at different developmental stages. At mid-metamorphosis the three temperature treatments are represented individually (●: 18ºC, ▲: 24ºC, ○: 30ºC)



Chapter 3: Predation-induced plasticity in metamorphic duration in *Xenopus laevis*

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Abstract

Many organisms are able to vary the duration of life history stages in response to environmental conditions such as predation risk. However, in those undergoing a metamorphosis, the extent to which the duration and progression of the metamorphic phase itself shows plasticity has received little attention. Using the amphibian *Xenopus laevis*, I examined the extent to which the duration of the metamorphic climax was affected by the presence or absence of a predator. Metamorphosis was accelerated in the presence of a predator and this occurred consistently across the natural range of temperatures experienced by *Xenopus*. Although metamorphic climax was reduced in duration, a functional tail was maintained for longer in the presence of a predator. Furthermore, burst swimming speed was significantly faster for animals metamorphosing in the presence than in the absence of a predator. This suggests that the more rapid development induced by predator presence does not carry costs in terms of ability to escape predators during metamorphosis. There was no evidence of post-metamorphic costs of faster metamorphic climax in terms of escape response since juveniles from the two predator treatments did not differ in burst swimming speed. However, individuals metamorphosing in the presence of predators lost proportionally more mass during metamorphosis, resulting in smaller juveniles than those without predators. This reduced juvenile size represents a potential cost of accelerating metamorphic development.

Introduction

In organisms with complex life cycles, that is with distinct life history stages and marked between-stage transitions during which the phenotype is re-shaped, environmentally induced plasticity in stage duration and morphology have been well studied (West-Eberhard 2003). Predation risk has been identified as an important environmental factor, being linked to the development of morphological and behavioural anti-predator defences and changes in the duration of life history stages (reviewed in Benard 2004; Relyea 2007). The main focus of these studies has been on the effects of predation risk during embryonic and larval life history stages (Nylin & Gotthard 1998; Benard 2004). However, it is also likely that predation risk will influence the duration of the transition stages, especially where such stages might render individuals more vulnerable to predators, for example if they are less active or mobile.

In most amphibians and insects, metamorphic climax, the beginning of which marks the end of the larval phase and its conclusion the formation of the juvenile in most species, represents a clearly defined transition stage (Bishop et al. 2006). During metamorphic climax individuals are generally more vulnerable, due to reduced locomotor performance, and are captured more frequently by predators (Wassersug & Sperry 1977; Arnold & Wassersug 1978). Predation risk experienced throughout larval and metamorphic stages has recently been shown to affect the duration of metamorphosis in insects (McKie & Pearson 2006), while Van Buskirk & Saxer (2001) did not find an effect of predator-induced larval phenotypes on the duration of metamorphic climax in amphibians. However, the effects of predation-risk experienced during metamorphic climax on its duration and progression have not been assessed independently of predation-induced effects on the larval phenotype, nor have potential costs of accelerated metamorphosis been identified.

During metamorphic climax, the developmental response to predation risk might be similar to that observed during embryonic development i.e. acceleration under heightened predation risk (Martin 1995; Warkentin 1995; Chivers et al. 2001; Li 2002; Kusch & Chivers 2004; Vonesh 2005a), since both are non-feeding stages with reduced locomotor ability compared to the subsequent life stage. However, more than one environmental factor is likely to be important, and there may be interactions among different factors acting simultaneously. Temperature is known to be an important variable affecting the rate of development (Gillooly et al. 2002). The duration of metamorphic climax has been found to change with environmental temperature in amphibians and insects (Downie et al. 2004; Stevens 2004; Walsh et al. 2007a). The observed acceleration of development with increasing temperature is however likely to level off at higher temperatures since the capacity to accelerate development at higher temperatures is limited either due to prohibitive costs or a biological constraint (Bottrell 1975; Wagner et al. 1984; Li & Jackson 1996; Roy et al. 2002; Downie et al. 2004; Walsh et al. 2007a). Hence, we would expect that the acceleration of metamorphic climax in response to predation risk will be most marked at lower temperatures.

In amphibians, it has also been shown that, in the presence of predators, tadpoles alter their tail morphology to aid predator evasion (e.g. Wilson et al. 2005). However, it is not known whether morphology expressed during metamorphic climax responds to predation-risk. Greater tail length and depth have both been shown to improve locomotor performance during larval development (Dayton et al. 2005; Teplitsky et al. 2005; Kaplan & Phillips 2006). Therefore in the presence of predators, metamorphosing individuals might delay the beginning of rapid tail re-absorption, thus retaining a functional tail for a longer period during metamorphosis to aid in locomotion until the hind limbs are better developed (Wassersug & Sperry 1977; Huey 1980).

In this study, I examined the plasticity of metamorphic duration and tail re-absorption during metamorphosis in the fully aquatic amphibian *Xenopus laevis* in relation to predator presence and variation in environmental temperature only during metamorphosis. While *Xenopus* does not leave the aquatic environment, it does undergo an extensive metamorphosis that occurs in every major organ system (Furlow & Neff 2006), including a transition from a tail driven tadpole to a limb propelled juvenile (Combes et al. 2004); there is a rapid increase in burst swimming performance on completion of metamorphosis (Walsh et al. 2007a). *Xenopus* transforms from an herbivorous forager in the water column to a camouflaged, largely bottom-dwelling ambush predator on completion of metamorphosis (Tinsley & Kobel 1996). Therefore, due to developmental changes in speed and niche, the relative predation risk of *Xenopus* during ontogeny within the aquatic environment is likely to differ. *Xenopus* also has the advantage that the continuity of the aquatic environment allows the locomotor performance at different stages to be more easily compared. Finally, a fully aquatic life cycle enables the potential inability to perceive altered mortality-risk in a different habitat to be removed as confounding factors in comparisons between different life stages (Benard 2004). Locomotor performance and body size during metamorphosis and following its completion were assessed in order to identify possible costs associated with predator-induced changes in metamorphosis.

Methods and Materials

Animals and rearing conditions

Approximately 250 *Xenopus laevis* eggs were obtained from St. Andrews University, Scotland in 2006. Tadpoles were kept in a single 40 L holding tank at 24°C until

approximately Nieuwkoop & Faber (NF) stage 42 (Nieuwkoop & Faber 1994) before being transferred to 10 smaller holding tanks.

Each smaller holding tank (30 x 20 x 20 cm) contained 25 tadpoles in approximately 11 L of aerated, de-chlorinated, copper-free water. Water temperature in the tanks ranged from 30°C (daytime) to 18°C (night), which represents the natural range of temperatures experienced by *Xenopus laevis* (Tinsley & Kobel 1996). Tadpoles were exposed to a 12L : 12D photoperiod and fed *ad libitum* on algal pellets, ground and hydrated before being suspended in water. Checks were made three times a day for individuals commencing metamorphic climax, indicated by the emergence of one or both of the fore limbs and coinciding with a surge in thyroid hormone (Shi 2000) (NF stage 60; equating to Gosner (1960) stage 42); this stage is recognised as the end of the larval phase (e.g. Hensley 1993). The conclusion of metamorphic climax was identified by the complete absorption of the tail (tail length < 0.5 mm, NF stage 66; equating to Gosner stage 46).

Experimental Design

A 2 x 3 factorial experimental design was used to examine the effects of predator risk and temperature on the duration of metamorphic climax, morphology and locomotory performance during and after the completion of metamorphosis. To evaluate the effects of predator presence, with and without predator treatments were used. Adult *Xenopus laevis* (SVL: 4 – 6 cm) were used as predators since adults eat larvae in this species, both in the laboratory and in the field (Hey 1946; Tinsley & Kobel 1996). All experimental tanks had clear, perforated Perspex dividing them into two equal segments (15 x 20 x 20 cm). An adult was placed on one side of the Perspex divide in half the experimental tanks and fed on a diet of bloodworms (Family: Chironomidae) and

Xenopus laevis tadpoles (frozen prior to first feeding NF stage 38) to ensure that any predation-associated chemical cues were present in the water (Petranka et al. 1987). Three temperature treatments were established; 18°C, 24°C and 30°C.

In total, 163 tadpoles were transferred from the small holding tanks to individual experimental tanks at the onset of metamorphosis. The 18°C and 24°C treatments had 54 individuals, while the 30°C had 55. The remainder were not included due to natural larval mortality or failure to commence metamorphosis during the experimental period.

Wet mass after removal of surface water (± 1 mg), snout-vent length (SVL), head width, tail length and maximum tail depth measurements (± 0.1 mm, using callipers) were taken at the commencement of metamorphic climax, without anaesthetising as it was deemed unnecessary. There were no significant differences in mass (942 ± 26 mg), SVL (19.5 ± 0.2 mm), head width (9.6 ± 0.1 mm), tail length (36.8 ± 0.4 mm) or tail depth (7.1 ± 0.1 mm) at the start of metamorphosis among the six treatments.

Tail length and depth measurements were taken every three days during metamorphosis. The number of days that a functional tail length and depth was retained was taken as the time until 50% of the tail was absorbed (Van Buskirk & McCollum 2000). This was calculated from the sigmoid regression equation, solving for x ($x = b(\ln(\frac{a-y}{y})) - x_0$, where y = 50%, b is steepness of the curve, a is the maximum asymptote and x_0 is the inflection point).

Individuals were checked three times a day for completion of metamorphic climax, at which point mass, SVL and head width were measured. The number of days between the onset of metamorphic climax and its completion was also recorded. Post-metamorphic individuals were maintained at their experimental temperature until testing then transferred to a stock tank maintained at 24°C. Only six mortalities occurred

during metamorphosis; in the presence of a predator all three temperature treatments experienced a single mortality; at 30°C the 'no predator' treatment had three mortalities.

Swimming Speed Data collection

Locomotor performance was measured halfway through metamorphosis (NF stage 63) and after metamorphosis was complete (NF stage 66+) on a randomly selected sample of individuals, using a Photron FASTCAM-PCI high-speed camera (Photron USA Inc., San Diego, CA, USA). Due to the sensitivity of the equipment, all filming was performed in one room. Prior to filming, individuals were acclimatized to the room temperature (19 - 22°C) for one hour. Slight variation in room temperature at the time of filming, which was included as a covariate, did not affect locomotion in metamorphs or juveniles (Table 3.2). The one-hour time period was selected as an acceptable period for acclimatization, as a longer period may have affected the developmental processes being investigated. Each individual was filmed six times. There was no evidence of habituation in either the metamorphic ($F_{1,434} = 0.972$, $p = 0.325$) or juvenile individuals ($F_{1,427} = 1.241$, $p = 0.266$).

The camera was placed directly above a 20 x 4 cm tank, lined with laminated grid-paper filled with approximately 1 cm depth of water. Each individual was placed at one end of the tank, and then gently prodded at the base of the tail to elicit an escape response. Filming was done at 250 frames per second (fps). Only the first 37 frames (~300 milliseconds) following initial movement were analysed, using Photron Motion Tools software, which generally represents the burst speed critical in fleeing from a predator (Wilson et al. 2005; Royle et al. 2006). The results using the maximum burst speed from the six trials are reported, since it represents the fastest burst speed of which an individual was capable.

Statistical analysis

All analysis was performed using SPSS v15 (SPSS Inc., Chicago, IL) unless otherwise stated. Metamorphic duration and swim speed analyses were corrected for body size. SVL (accounting for 84.4% of the variation using Principal components analysis of mass, SVL, head width, tail length and tail depth) was used as an indicator of body size since all structural body and tail measurements taken were highly correlated. Metamorphic duration was examined using a General Linear Mixed Model (GLMM), with the holding tank that the tadpole originated from as a random factor. The holding tank ($p = 0.45$) and interaction term ($p = 0.22$) were not significant and thus removed to give the minimum adequate model. The time to lose 50% of the tail depth was determined using 3-parameter, sigmoid regression (SigmaPlot 10, Systat Software Inc., Chicago, IL) and analysed using a General Linear model (GLM). Since the re-absorption of tail length followed the same pattern as tail depth re-absorption in relation to predator presence and temperature, tail length results are not presented here. Remaining analyses were performed using GLM; non-significant interaction terms were removed. In analysis of locomotor performance, SVL and tail depth at the time the trials were performed were also included as covariates.

Results

Metamorphic Duration

After correcting for body size (SVL) at the start of metamorphic climax ($F_{1,156} = 64.48$, $p < 0.0001$), the presence of a predator resulted in a reduction in metamorphic duration of between 4 and 7% across the three temperature groups ($F_{1,156} = 10.13$, $p = 0.002$; Fig. 3.1). Duration was longest in the 18°C temperature treatment, followed by the 24°C and 30°C treatments ($F_{2,156} = 1819.43$, $p < 0.0001$).

Despite the shorter duration of metamorphosis, individuals that completed metamorphosis in the presence of a predator lost more mass and structural body size in relation to their size at the start of metamorphic climax than those in the no predator treatment (Table 3.1, Fig. 3.2). This was again consistent across the temperature treatments. In line with this, the shorter metamorphic duration at higher temperatures was also associated with smaller post-metamorphic body mass and size (Table 3.1).

Functional Tail Retention

Tail re-absorption, shown here as tail depth (length followed the same pattern), followed a sigmoid pattern (Fig. 3.3). The length of time for which a functional tail was retained, measured as the time taken to absorb 50% of the tail depth, was greater in the presence of a predator than when there was no predator ($F_{1,148} = 5.86$, $p = 0.017$). Low temperature also extended the period for which a functional tail depth was retained ($F_{2,148} = 655.56$, $p < 0.0001$).

Locomotor Performance

During metamorphosis (NF stage 63), independent of temperature treatment and after correcting for body size (see methods), those tadpoles metamorphosing in the presence of a predator showed maximum swimming speeds approximately 5% faster compared with the no-predator treatment (Table 3.2, Fig. 3.4). Independent of the significant effect of body size, tail depth at the time of the trials was not found to significantly affect swimming speed during metamorphosis (Table 3.2). There was also a significant interaction between predator treatment and SVL (Table 3.2). In the predator treatment burst swim speed increased faster with increasing SVL than in the no predator treatment.

On completion of metamorphosis (NF stage 66), mean maximum swim speed was 34.71 cm s^{-1} ± 0.89 SE (2.26 body length s^{-1} ± 0.04 SE). There was no significant difference between predator and no predator treatments, independent of experimental temperature treatment, when corrected for the significant effect of SVL (Table 3.2).

Discussion

In *Xenopus laevis* both duration of metamorphic climax and tail morphology during metamorphosis were found to vary, as predicted, in response to predator presence. This demonstrates for the first time that increased predation risk experienced only during metamorphic climax induces variation in the duration of this transition stage. The presence of a predator during metamorphosis resulted in shorter duration of metamorphosis at all three temperature treatments, with no difference in the degree of acceleration observed among the temperature treatments. Thus there was no interaction between the predator presence and temperature across the temperature range used in this study, possibly because the range used did not extend to the temperature threshold where acceleration of development is constrained (McKie & Pearson 2006).

It has been proposed that selection will favour shortening the duration of life history stages, such as amphibian metamorphosis, with heightened vulnerability to predation, since this is likely to confer significant fitness benefits (Williams 1966; Istock 1967; Wassersug & Sperry 1977; Arnold & Wassersug 1978; Rose 2005). That metamorphosis is longer in the absence of predators suggests that shortening is sufficiently costly to favour retention of the capacity to adjust metamorphic duration in response to local predation risk. In the absence of predators, it pays to take longer over metamorphosis. One potential cost associated with rapid growth or development is an impairment of locomotor performance in subsequent stages (Kolok & Oris 1995; Arendt

2003; Buckley et al. 2005; Ficetola & De Bernardi 2006; Ji et al. 2006). However, we did not find any difference in post-metamorphic locomotion amongst our treatment groups. Similarly, Capellan & Nicieza (2007b) and Van Buskirk & Saxer (2001) did not find a cost in terms of locomotor performance due to predator presence during the hatching or larval stages, respectively, in the juvenile stage of ranid species. Therefore, it appears that the acceleration of metamorphosis in *Xenopus laevis* does not carry associated locomotor costs, measured in burst swim speed, as in *Rana temporaria* (Capellan & Nicieza 2007b) and *Rana ridibunda* (Van Buskirk & Saxer 2001). However, costs might become apparent later in life (Arendt 1997; Metcalfe & Monaghan 2001; Morgan & Metcalfe 2001) or be evident in other fitness components or measures (e.g. sustained locomotor performance).

Metamorphosing individuals that experienced the presence of a predator showed a greater decrease in mass, SVL and head width than those that metamorphosed in the absence of a predator. Although this did not directly result in reduced absolute or size-corrected burst swim speed, a reduction in size on the completion of metamorphosis has been shown to reduce survival, size at first reproductive bout and reproductive output, and increase the age at first reproduction (Collins 1979; Berven & Gill 1983; Smith 1987; Semlitsch et al. 1988; Berven 1990; Scott 1994; Beck & Congdon 2000; Altwegg & Reyer 2003; Chelgren et al. 2006).

Even though metamorphosis was accelerated in the presence of a predator, a functional tail was retained for longer, but more rapidly re-absorbed towards the end of metamorphosis. Given the shorter metamorphic durations experienced in the presence of a predator, the increased time period of functional tail retention would mean that in the presence of a predator individuals retain over 50% of their tail depth for a substantially greater proportion of metamorphosis. Retention of tail depth would be

expected to benefit locomotion at this stage; however this was not found to be the case, at least with respect to burst swim speed. While locomotor performance increased under predation risk during metamorphosis, this was not related to variation in tail depth (or length). This could be due to several reasons. Tail retention might only have an effect on locomotor performance at earlier stages, prior to when burst speed was assessed in this study, or in more sustained swimming. The faster swimming speeds observed in the predator treatment could be the result of other factors, such as accelerating the development of functional hind limbs to aid in locomotion. Conversely, the predator treatment individuals may have a heightened sensitivity or alertness to a potential predator stimulus, due to exposure to predation-associated chemical cues (Pfeiffer & Riegelbauer 1978; Hews & Blaustein 1985; Hews 1988).

In conclusion, our data demonstrate that during metamorphosis *Xenopus laevis* shows plasticity in both morphology and duration in response to predator presence. This provides further evidence that metamorphosis itself, which has been largely overlooked in studies of phenotypic plasticity, is not fixed in duration and that the variability in responses to environmental conditions observed during larval development may be expected during metamorphosis. Thus, the habitat experienced during metamorphosis may have important implications for the pattern and tempo of phenotypic development.

Tables and figures

Table 3.1: Results of GLM analysis for the juvenile mass and structural size measurements of individuals that completed metamorphosis in the presence or absence of a predator and among the three temperature treatments. The interaction term was not significant (p > 0.27) and is not included. The holding tank that individuals originated from was not found to be significant in any of the analyses.

Dependent variable	Predator treatment			Temperature treatment		
	df	F	p	df	F	p
Mass	1,156	3.95	0.049	2,156	14.98	<0.0001
Snout-vent length	1,156	11.95	<0.0001	2,156	10.18	<0.0001
Head width	1,156	7.70	0.006	2,156	5.32	0.006

Table 3.2: Results of GLM analysis of locomotor performance of individuals at mid-metamorphosis and at the completion of metamorphosis in relation to predator and temperature treatments. The interaction between temperature and predator treatment were non-significant (p > 0.37) and removed.

	Metamorphosing individuals			Post-metamorphic individuals		
	df	F	p	df	F	p
Temperature	2,91	1.41	0.25	2,90	1.88	0.16
Predator	1,91	6.23	<0.02	1,90	0.44	0.51
SVL	1,91	30.41	<0.0001	1,90	87.39	<0.0001
Tail depth	1,91	1.46	0.23	-	-	-
Predator * SVL	2,91	7.64	<0.01	2,90	0.71	0.40
Room temperature	1,91	0.33	0.57	1,90	1.95	0.17

Figure 3.1: Duration of metamorphic climax in days (± SE) for the three temperature treatments and the presence or absence of a predator. Metamorphic climax begins with the emergence of the fore limbs, which coincides with a peak in thyroid hormones, and is concluded with complete tail absorption. Open shapes represent the no predator treatment and solid shapes represent the predation treatment.

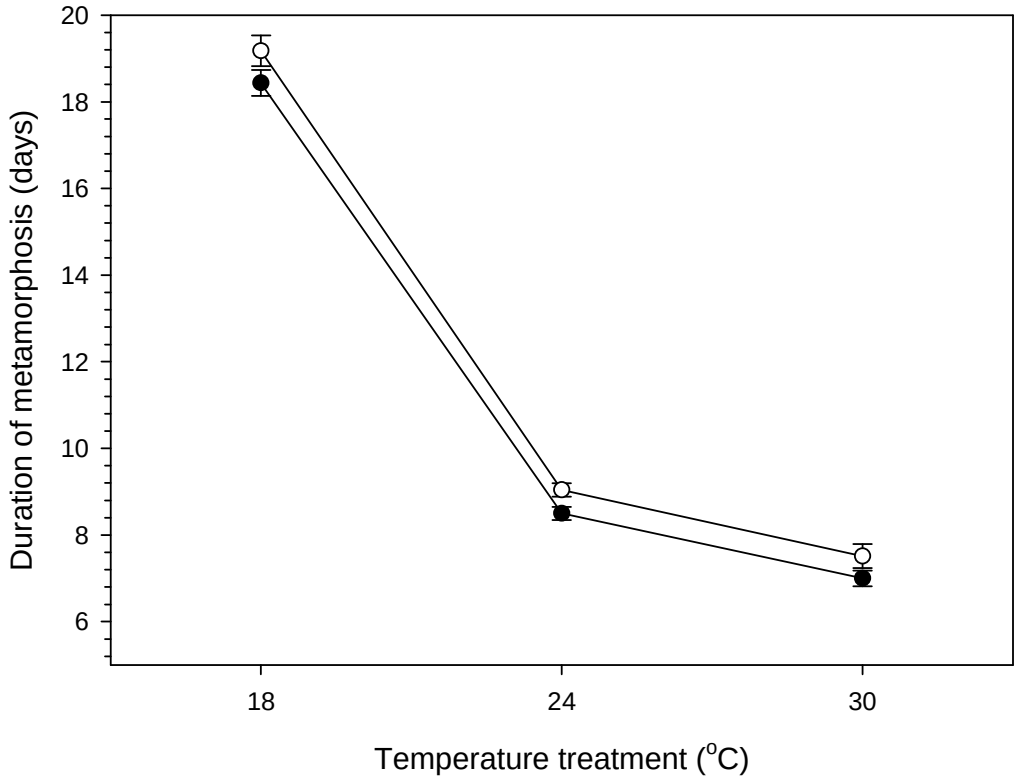


Figure 3.2: Mean percentage loss of mass (●), SVL (▲) and head width (■) during metamorphosis for each temperature and predator treatment (± SE). Open shapes represent the no predator treatment and solid shapes represent the predation treatment.

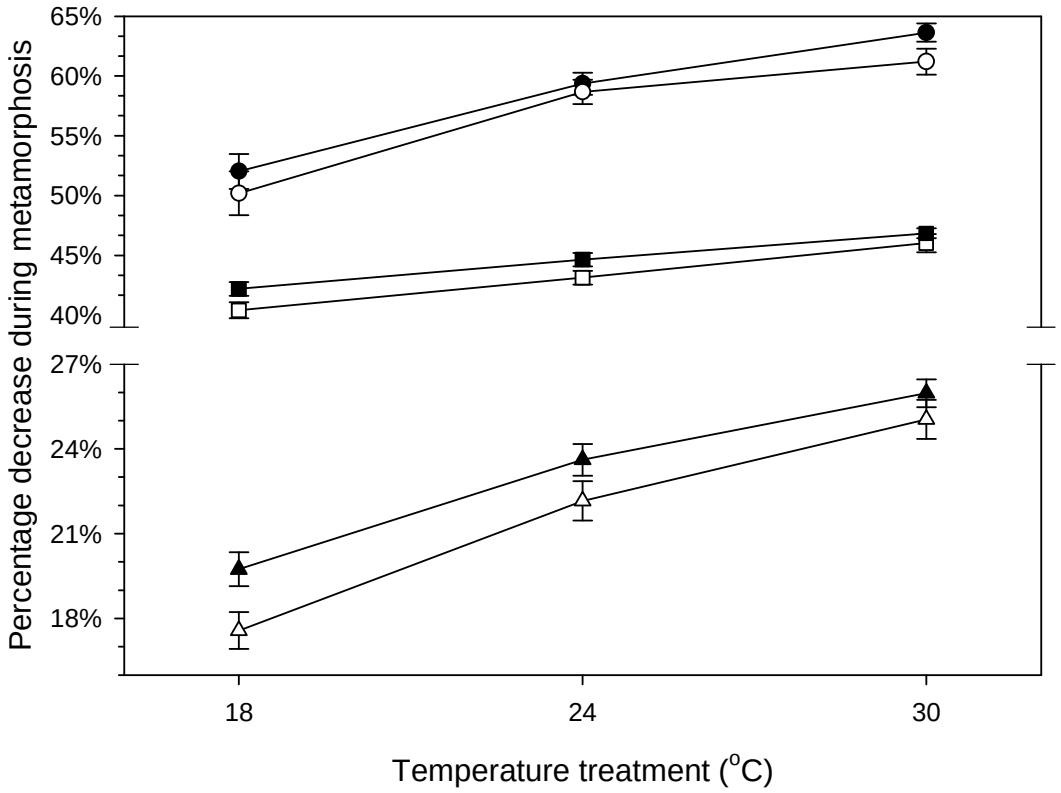


Figure 3.3: Percentage decrease in mean tail depth (± SE) through metamorphosis (■: 18°C, ▲: 24°C, ●: 30°C. For all three temperature treatments, the predator absent treatment is indicated by filled shapes and solid regression lines, while the predator present treatment is indicated by open shapes and broken regression lines).

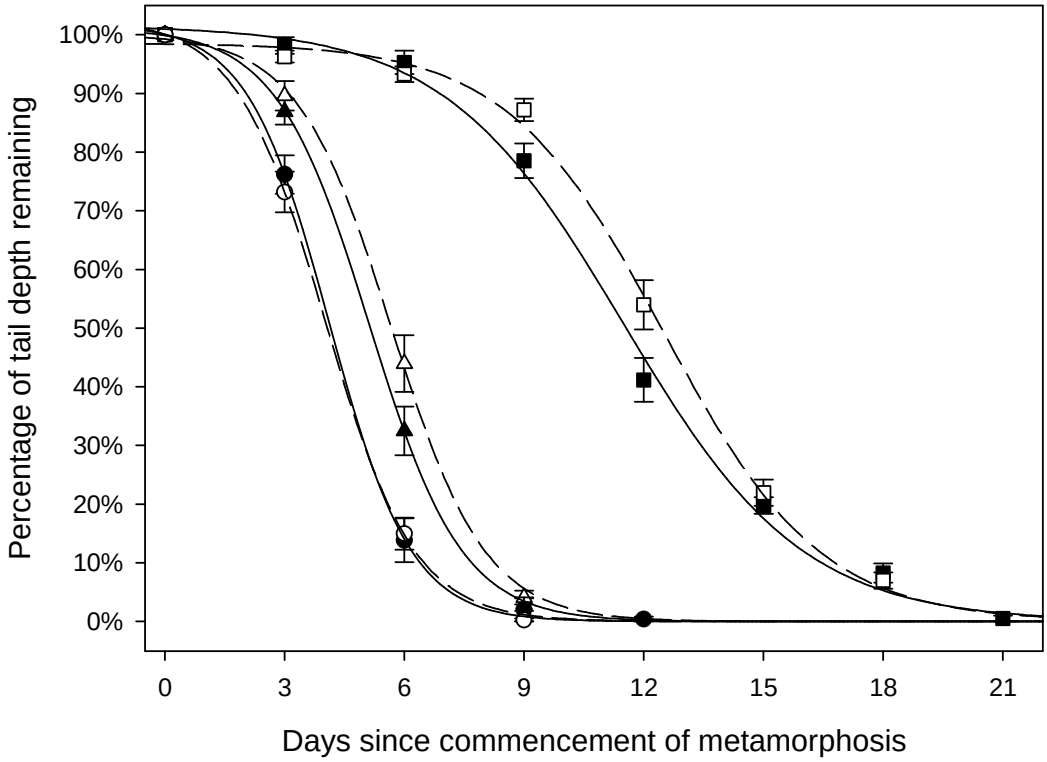
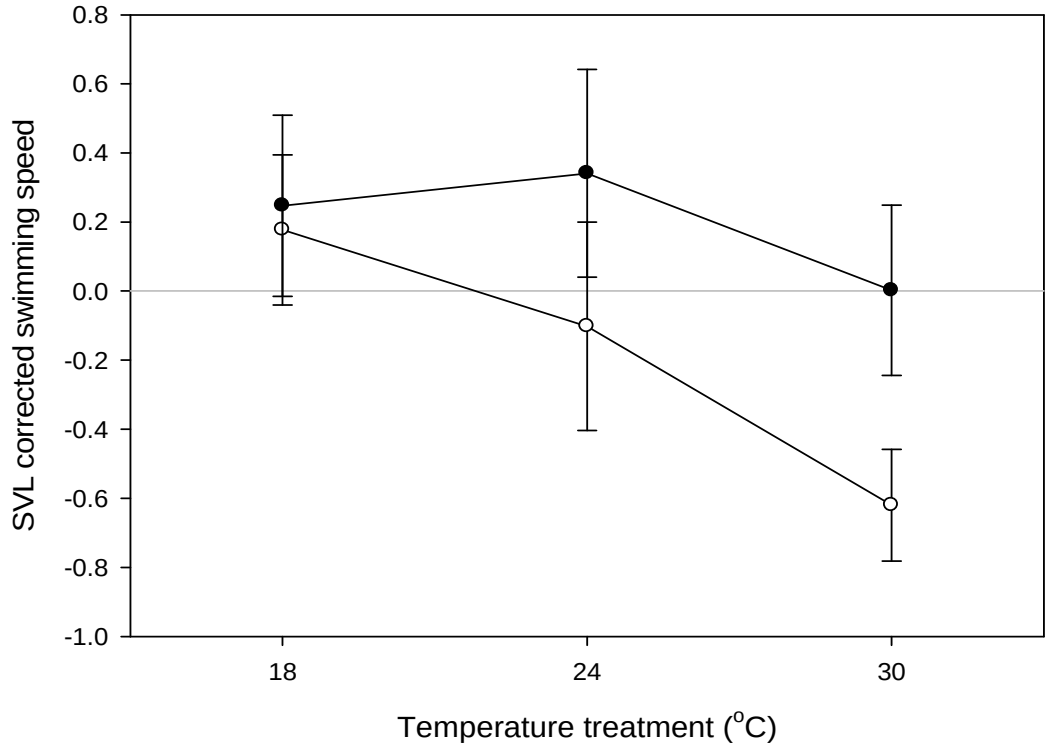


Figure 3.4: Maximum burst speed (± SE) of metamorphosing (NF stage 63) individuals (represented as the residuals corrected for SVL) that are metamorphosing in either the presence or absence of a predator at each of the three temperature treatments. Open circles represent the no predator treatment and solid circles represent the predation treatment.



Chapter 4: Temperature mediated morphology changes during metamorphic climax in the African clawed frog, *Xenopus laevis*

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Abstract

Investigations of the effect of temperature on body-size are largely limited to the larval phase of organisms with complex life cycles, with our understanding of the effect of temperature during metamorphic climax entirely restricted to the insects. Environmental temperature was manipulated only during metamorphosis in the aquatic amphibian *Xenopus laevis*. Lower temperatures during metamorphosis resulted in individuals with greater mass, head width and snout-vent length on the completion of metamorphosis. This demonstrates that temperatures during metamorphosis can be important in modulating the temperature-size relationship established during the larval stage in amphibians.

Introduction

Body size is an important aspect of individual fitness, with greater size generally resulting in higher fecundity and competitive ability (Roff 1992; Stearns 1992). Environmental conditions during growth play a role in determining final size, and there have been a number of studies investigating the effect of environmental temperature in determining this trait (reviewed in Atkinson 1994). The majority of these studies (> 80%) have shown a developmental response to temperature similar to that observed under Bergmann's rule (Bergmann 1847), with lower rearing temperatures resulting in larger sizes. In ectothermic organisms with complex life cycles, temperature experienced during early development (i.e. embryonic and larval stages) has been shown to influence juvenile and adult body size. For example, in *Drosophila melanogaster*, individuals reared at lower temperatures have larger body and egg sizes (Powell 1974; Azevedo et al. 1996; James et al. 1997; Nunney & Cheung 1997). However, these organisms also undergo a metamorphosis, during which time much of the body is transformed. Organism size is often fixed after metamorphosis (Boggs 1981) but, even if post-metamorphic growth occurs, the effects of immediate post-metamorphic size persist into sexual maturation (Smith 1987; Semlitsch et al. 1988). Additionally, investigations of the temperature-size rule during the larval period are complicated by behaviour, such as feeding rate, so metamorphosis may represent a better opportunity to investigate this phenomenon. However, little attention has been given to the effects of environmental temperature experienced during metamorphosis on adult morphology. Since metamorphosing individuals rely solely on stored resources, it would be expected that lower temperatures would result in smaller losses of body mass. Hence lower temperatures would be associated with larger sizes on completion of metamorphosis, due to differences in thermal efficiency of resource utilisation

(Robinson & Partridge 2001) and/or from slower development being more efficient (Arendt 1997; Fischer et al. 2004).

Those studies that have been carried out on the effects of environmental temperature during metamorphosis are largely restricted to a single class of animals; the insects, specifically dipterans and lepidopterans (French et al. 1998; Ottenheim & Volmer 1999; Chakir et al. 2002; Stevens 2004). In dipterans, lower temperatures experienced during larval development and pupation have been shown to result in the increased size of several morphological traits, such as the wings, abdomen, thorax and head shape and size (French et al. 1998; Ottenheim & Volmer 1999; Stevens 2004). However, not all traits have been shown to be responsive to temperature during metamorphosis. French et al. (1998) demonstrated that thorax size is only affected by temperature experienced during the larval stage not the metamorphic pupal stage, but the abdomen and wing sizes are susceptible throughout the larval and pupal stages. Similarly, in the butterfly *Pararge aegeria*, post-metamorphic head size increases with lower environmental temperature during pupation, while other traits are unaffected (Stevens 2004). Stevens's (2004) study represents the only attempt to decouple the effect of environmental temperature on larval versus metamorphic processes, thus our understanding of temperature effects on metamorphosis is very limited.

Amphibians have commonly been used to investigate both the evolutionary responses and ontogenetic relationship among temperature and body size or growth rate (Ashton 2002; Alvarez & Nicieza 2002a; Laugen et al. 2005; Measey & Van Dongen 2006), particularly during larval growth and development. Additionally, amphibians have been shown to display a high degree of plasticity in the timing of life history transitions and morphology during ontogeny. Our understanding of plasticity during amphibian metamorphic climax itself is extremely limited (Downie et al. 2004; Walsh

et al. 2007a; Walsh et al. 2007b). Given the thermal plasticity in morphology observed during other ontogenetic phases in amphibians (Blouin & Brown 2000; Alvarez & Nicieza 2002a; Alvarez & Nicieza 2002b) and during metamorphosis in a butterfly species (Stevens 2004), it is likely that during amphibian metamorphosis individuals will display morphological plasticity in response to environmental temperature.

Here, by manipulating temperature only during metamorphic climax, I examine whether lower environmental temperature experienced during anuran metamorphosis results in increased body size in the aquatic toad *Xenopus laevis*.

Methods and Materials

In 2005 and 2006, wild type *Xenopus laevis* eggs were obtained from St. Andrews University, Scotland. In both years, tadpoles were kept in a 40 L holding tank at 24°C until they were free-swimming larvae (Nieuwkoop & Faber (1994) (NF) stage 42) before being transferred to smaller holding tanks.

Each smaller holding tank (30 x 20 x 20 cm), filled with approximately 11 L of aerated, de-chlorinated, copper-free water, contained 25 tadpoles to facilitate identification of the onset of metamorphic climax. A constant water level was maintained in all tanks. Daytime water temperature in the tanks was maintained at 30°C using individual tank heaters, and fell to 18°C at night. This represents the natural range of temperatures experienced by *Xenopus laevis* (Tinsley & Kobel 1996). Tadpoles were exposed to this range of temperatures to avoid acclimatization problems when they were transferred to the different experimental thermostable environments (18, 24 and 30°C) during metamorphosis. Tadpoles and metamorphs were exposed to a 12L : 12D photoperiod throughout the experiment and larvae were fed *ad libitum* on dried algal pellets, ground and hydrated before being suspended in water.

Checks were made three times a day for individuals showing emergence of forelimbs, indicative of the commencement of metamorphic climax (NF stage 60), and after the onset of metamorphosis, for the completion of metamorphic climax (tail length < 0.5 mm, NF stage 66). Tadpoles were transferred to individual experimental tanks (15 x 20 x 20 cm) at the onset of metamorphosis. In total, 204 tadpoles were transferred to the experimental tanks (126 in 2005 and 78 in 2006).

Wet mass, after removal of surface water (± 1 mg), snout-vent length (SVL) and head width (± 0.1 mm, using callipers) were taken at the commencement and completion of metamorphosis. Duration of metamorphosis was also recorded. Individuals that died during metamorphosis were not included in the analysis, leaving 186 individuals. Differences in the proportion surviving among temperature treatments were compared using Chi-squared. Data were analysed using General Linear Models, with post-hoc Tukey tests, in SPSS (SPSS v15, SPSS Inc.). Analysis of mass, SVL and head width at the completion of metamorphosis was corrected for the initial measurement, included as a covariate. The proportion of mass, SVL and head width lost during each day of metamorphosis was also examined to investigate whether differences in size were related to the duration of metamorphic climax.

Results

There was no initial difference among individuals allocated across the three temperature treatments, within a year group, in measurements of mass, SVL or head width at the onset of metamorphic climax (Table 4.1). The duration of metamorphosis was significantly shorter as experimental temperature increased in both years. Individuals from 2006 were significantly larger and heavier than individuals in 2005. Correcting for the larger sizes of individuals in 2006, there was no significant difference in

metamorphic duration between years (Table 4.1). Mortality during metamorphic climax was significantly different among temperature treatments ($X^2 = 14.84$, $p = 0.001$), with the 30°C treatment suffering the highest mortality (14 mortalities; 20.0%) followed by the 18°C treatment with only six mortalities (8.8%). The 24°C treatment showed no mortality.

After correcting for the initial measurements at the onset of metamorphic climax, post-metamorphic mass, SVL and head width all showed a significant negative relationship with temperature during metamorphosis (mass: $F_{2,185} = 25.42$, $p < 0.001$; SVL: $F_{2,185} = 77.29$, $p < 0.001$; head width: $F_{2,185} = 74.50$, $p < 0.001$). Figure 4.1 displays the percentage loss in each of the three measurements, showing that individuals at higher temperatures lost a greater percentage of mass, SVL and head width during metamorphosis. Post-hoc analysis revealed that in 2005 the proportional mass loss was not significantly different between the 24°C and 18°C treatments ($p = 0.996$). There was no significant difference between years in the loss of mass, SVL and head width during metamorphosis, after correcting for the larger size of individuals in 2006. However, for both mass and SVL there was a significant interaction between temperature treatment and year (mass: $F_{2,185} = 9.31$, $p < 0.001$; SVL: $F_{2,185} = 4.04$, $p = 0.019$; Fig. 4.1), which showed that at 18°C individuals lost a greater proportion of mass and SVL in 2005 than in 2006.

Analysis of the percentage loss in mass, SVL and head width, correcting for the differences in metamorphic climax duration experienced across the temperature treatments, demonstrated a significant positive relationship with temperature during metamorphosis. Additionally, post-hoc analysis shows that the difference in the proportion of mass loss is significant among all three temperature treatments in both years (Fig. 4.2). Individuals completing metamorphosis at 30°C experienced a daily

decrease in mass that was 62.8% greater than the decrease experienced at 18°C. Similarly, daily decreases in SVL and head width were 68.5 and 64.5% greater, respectively, at the 30°C treatment compared to the 18°C treatment.

Discussion

These results demonstrate that temperature-mediated changes in size and morphology occur during amphibian metamorphic climax. Additionally, even though metamorphic duration was significantly longer at colder temperatures, the proportional decrease in mass, SVL, and head width was greater at higher temperatures. The daily proportional decrease in the three traits was over 60% greater at 30ºC compared to 18ºC. The inverse relationship between temperature during metamorphosis and final mass and snout-vent length could have a number of consequences for fitness. Smaller mass on completion of metamorphosis at higher temperatures may indicate a greater reduction in energy reserves, such as stored lipids. Temperature has been shown to have a similar effect on lipid mass in the Iberian painted frog *Discoglossus galganoi* during the larval stage (Alvarez & Nicieza 2002b). Although measurements of lipid mass were not taken in this study, it would be reasonable to assume a greater reduction of total mass during metamorphosis would reflect a reduction in stored reserves. A reduction of SVL on the completion of metamorphosis will reduce the achievable burst speed of individuals that metamorphosed at higher temperatures. Burst speed has been shown to be largely dependent on SVL in *Xenopus* and many other species (Wilson & Franklin 2000; Ojanguren & Brana 2003; Walsh et al. 2007a) and can impact on an individual's survival (Eidietis 2005; Husak 2006). Both of these factors may reduce the survival probability of juveniles that metamorphosed at higher temperatures (Beck & Congdon 2000; Altwegg & Reyer 2003; Chelgren et al. 2006).

The consequences of a reduction in head width are less clear. Most post-metamorphic amphibians are gape-limited predators, therefore a narrower head as a juvenile would limit the size of prey that could be utilized (Schmidt et al. 2006; Vincent et al. 2006). However, *Xenopus* is not strictly gape-limited, since it is capable of removing bite-size pieces from carrion or forcing larger items into its mouth with its forelimbs. Ultimately, a smaller head and mouth would limit the speed at which an individual was able to feed. Blouin & Brown (2000) did not report an effect of temperature experienced during larval development on head width in *Rana cascadae*. However, temperature effects during the larval period may have masked the effects on head width during metamorphosis. Therefore, it is important to determine whether the effect of temperature on post-metamorphic head width is consistent across species. While metamorphic duration was shorter at higher temperatures, this is likely to provide limited survival and fitness benefits compared to the costs arising from reduced body size (Chelgren et al. 2006).

This study provides further support that ectothermic organisms conform to the temperature-size rule, which is still widely contested (see Ashton 2002), but additionally that temperature during metamorphosis itself is important in determining this relationship. While it has been documented that some ectotherms conform to the temperature-size rule (French et al. 1998; Ashton 2002), investigations of how and when this relationship is formed focus on effects occurring during larval development (Blouin & Brown 2000; Robinson & Partridge 2001; Alvarez & Nicieza 2002a; Fischer et al. 2004). Alvarez & Nicieza (2002a) reported that rearing temperature had no effect on mass loss during metamorphosis in *Discoglossus galganoi*. However, larval effects confound this result, and mass was the only morphological characteristic examined. Stevens (2004) investigated a suite of morphological traits, and found a negative effect

of temperature during metamorphosis on head mass, but not on total mass, wing lengths or leg lengths in the butterfly *Pararge aegeria*. This result accords with my findings, but shows that temperature during metamorphosis may affect traits differently, depending on species. Additionally, temperature during metamorphosis may modify or re-enforce the temperature-size relationship that is established during larval development if the temperatures experienced during the two stages are different.

The de-coupling of temperature effects on size and morphology during larval and metamorphic stages increases our understanding of how the temperature-size rule is established. In organisms with complex life cycles that complete a transition from aquatic to terrestrial forms during metamorphosis, temperatures experienced during the larval period are likely to be different from those encountered by metamorphosing individuals in the shallows or when leaving the water. In *Xenopus laevis* and other species with complex life cycles that remain either terrestrial or aquatic, temperature-mediated variation in size and morphology changes may be influenced by microhabitat selection during metamorphosis.

The establishment of the temperature-size rule requires that the duration of development is extended to allow more resources to be collected to achieve a larger size, or faster growth is achieved through: 1) higher consumption, 2) increased efficiency in resource utilization or 3) resources are diverted to growth at the expense of other traits (e.g. body maintenance and repair, immune function or reproduction). During metamorphosis most organisms do not feed, therefore the observed increase in size lost during metamorphosis at higher temperatures can not be the result of a higher rate of consumption by individuals in the colder treatments. Additionally, although metamorphic duration is longer at colder temperatures, an extension of this non-feeding stage would not in itself allow a larger size on completion, since it does not allow a

longer time to collect resources. In fact, the greater duration of this stage at cold temperatures should represent a greater cost, since the stored resources would have to be spread over a longer time. While a diversion of resources toward maintaining body size during metamorphosis at colder temperatures is still a possibility, it is not clear why an ectothermic organism would adopt this strategy, especially considering the potentially greater metabolic costs associated with a longer fasting period. Therefore, the inverse relationship between temperature and body size is most likely the result of greater efficiency of utilizing resources at lower temperatures, which has been observed in my study and in previous studies (*Drosophila melanogaster*: French et al. 1998; Robinson & Partridge 2001; *Menidia menidia*: Present & Conover 1992; see Benavides et al. 2005 for exception).

In conclusion, I have shown that temperature experienced during metamorphosis influences juvenile size in line with the predictions of the temperature-size rule. This temperature-size relationship during metamorphosis is most likely the result of increased efficiency in utilizing stored resources.

Tables and figures

Table 4.1: Initial measurements of mass, SVL and head width, and the duration of metamorphosis across the three temperature treatments. Data are presented as means (SE). Masses and lengths are provided in mg and mm, respectively. Durations of metamorphosis are presented as days since metamorphosis commenced. Degrees of freedom for GLM results are $F_{2,185}$ for temperature and interaction term and $F_{1,185}$ for year.

		18ºC	24ºC	30ºC		F	*p*
Mass					Temp	0.42	0.659
	2005	644 (27)	677 (26)	642 (22)	Year	66.85	**<0.001**
	2006	917 (77)	958 (63)	983 (57)	Temp * year	0.32	0.726
Snout-vent length					Temp	2.02	0.136
	2005	18.0 (0.2)	18.8 (0.2)	18.3 (0.2)	Year	28.06	**<0.001**
	2006	19.1 (0.5)	19.6 (0.4)	20.0 (0.3)	Temp * year	0.45	0.640
Head width					Temp	0.99	0.374
	2005	8.6 (0.1)	8.8 (0.2)	8.7 (0.1)	Year	62.91	**<0.001**
	2006	9.5 (0.2)	9.6 (0.2)	9.8 (0.2)	Temp * year	0.58	0.564
Metamorphic duration					Temp	7.66	**0.001**
	2005	16.7 (0.3)	8.4 (0.1)	7.5 (0.2)	Year	2.50	0.116
	2006	19.1 (0.4)	9.0 (0.2)	7.5 (0.3)	Temp * year	0.23	0.736

Figure 4.1: Mean percentage loss, with respect to measurement at the onset of metamorphosis, of mass (●), SVL (▲) and head width (■) during metamorphic climax for each temperature and both years (± SE). Open shapes represent the 2005 group and solid shapes represent 2006.

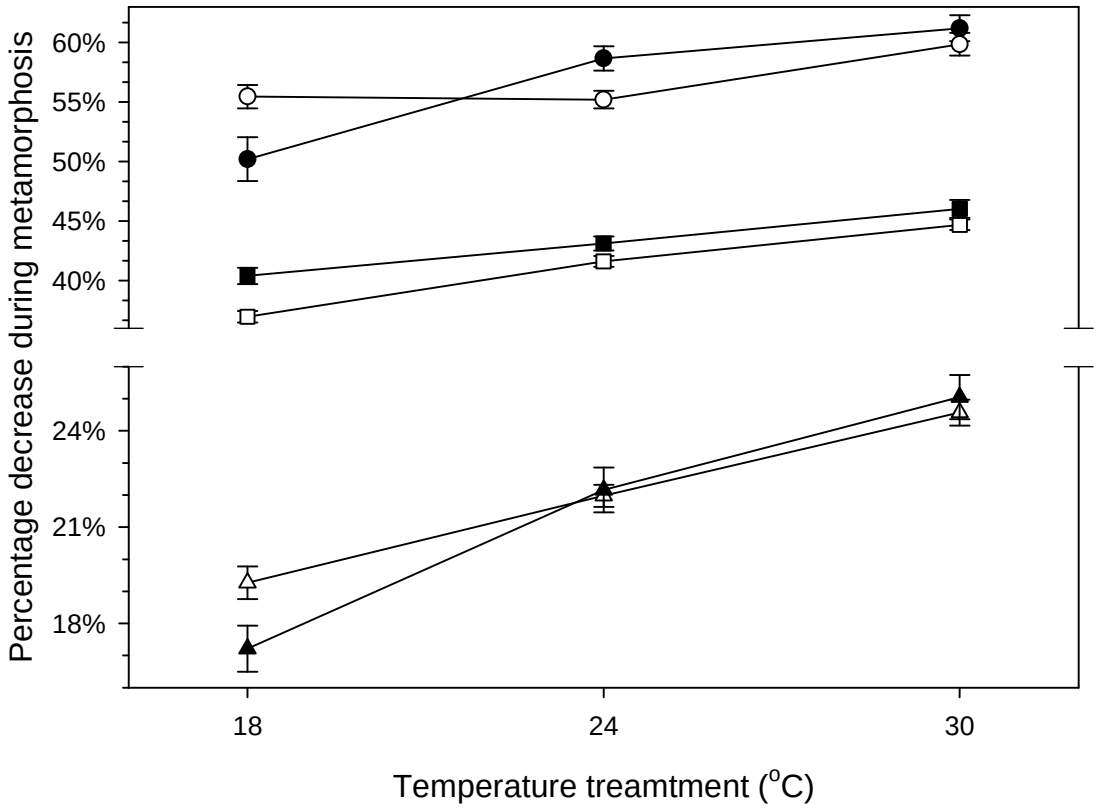
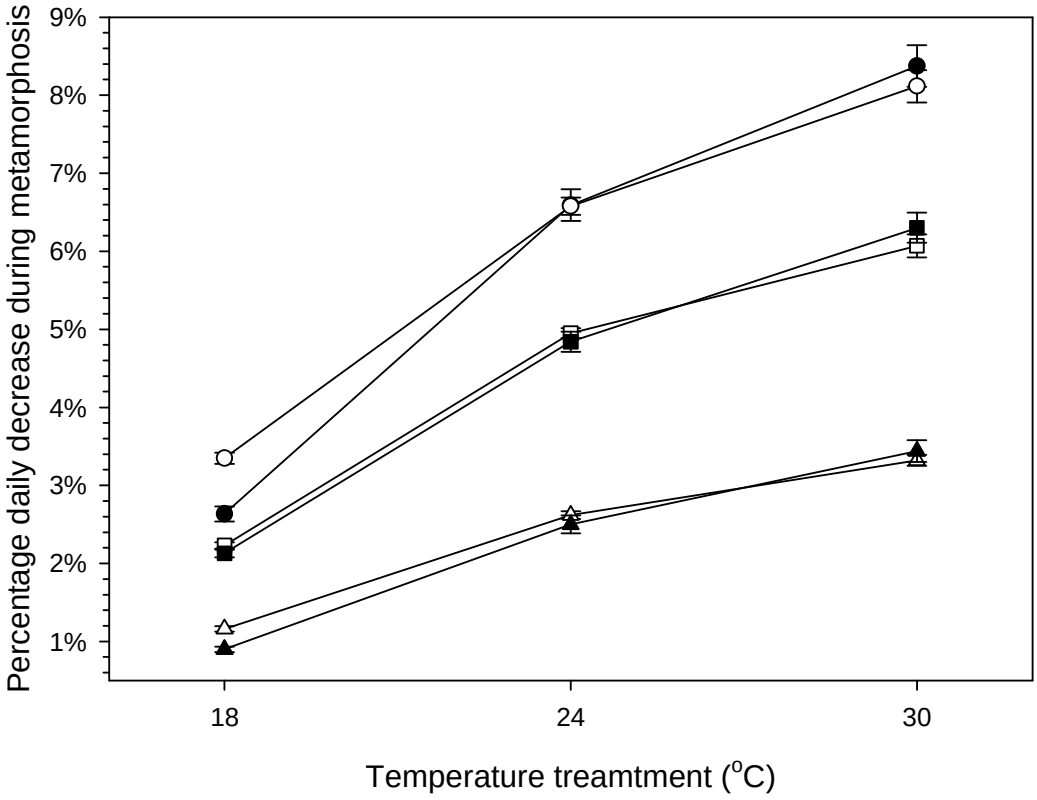


Figure 4.2: Mean percentage loss of mass (●), SVL (▲) and head width (■) per day during metamorphic climax for each temperature and both years (± SE). Open shapes represent the 2005 group and solid shapes represent 2006.



Chapter 5: The effects of temperature and food availability on the over-wintering of *Rana temporaria* tadpoles

Abstract

The duration of amphibian larval development has been shown to vary considerably with environmental conditions, yet most temperate anurans complete metamorphosis prior to the onset of winter. However, an increasing number of temperate anuran species have been reported to vary the duration of larval development so that some individuals within a pond spend the winter as larvae before presumably completing metamorphosis the following spring. I examined the effects of environmental temperature and food availability on larval development and the proportion of individuals that over-wintered as larvae, in *Rana temporaria*. Early development rate was relatively constant within treatment groups, but slower in the lower summer temperature regimes and restricted food treatments. In the laboratory, over-wintering tadpoles did not appear to arrest development early in the season. At simulated summer temperatures that did not exceed 10°C (low temperature regime), larval survival was very low (< 3.0%) and all surviving individuals spent the winter as tadpoles. In the medium (up to 14°C) and high temperature (up to 18°C) treatments survival was higher (46.0 – 68.3%) and the proportion of over-wintering tadpoles was negatively affected by temperature and food availability. While in this study it was not possible to determine whether temperature and food availability operate as a constraint, or trigger strategic adjustments to the life history pattern, there is evidence that over-wintering as a tadpole may be adaptive. Individuals that over-wintered as tadpoles were large enough to commence metamorphosis in the autumn and, on completion of metamorphosis, were significantly larger than individuals that completed metamorphosis during the summer.

Introduction

In ectothermic animals with complex life cycles inhabiting temperate regions, the timing of life history transitions can be influenced by individual performance at a given time of year. The life history stage achieved by an individual at the onset of winter can have an impact on an individual's life history trajectory or other life history characteristics (Gotthard 2001). Environmental conditions earlier in development can influence the life history stage at which organisms spend the winter, such as in the Atlantic salmon *Salmo salar* (Thorpe 1977; Metcalfe et al. 1988). In salmon, spring growth rate of parr is known to influence whether or not fish smolt in their first year; with slower growing individuals delaying smolting and seaward migration until their second year. In amphibians, the larval stage is generally completed before the onset of winter (Stebbins & Cohen 1995). The seasonal reduction in day length and temperature, and the seasonal risk of pond drying, have been considered important factors limiting the duration of the larval phase in most temperate anurans (Wilbur & Collins 1973). However, the increase in reports of over-wintering tadpoles (Berven et al. 1979; Collins 1979; Collins & Lewis 1979; Archibald & Downie 1996; Pintar 2000; Fellers et al. 2001; Gollmann et al. 2001; Lai et al. 2002; Lauck et al. 2005) indicates that, in some species, tadpoles are able to survive the winter period in their ponds and that such larval over-wintering may represent an example of phenotypic plasticity in the timing of life history events.

There has been no published experimental work to determine the factors that favour larval over-wintering. Temperature during larval development is thought to play an important role (Berven et al. 1979) due to observed latitudinal (*Rana catesbeiana*: Collins 1979) and altitudinal (*Rana sauteri*: Lai et al. 2002) clines in the occurrence of over-wintering larvae. A second potentially important factor is food availability or competition level, which is known to affect the duration of the larval phase in a number

of amphibian species. Some studies have shown that larval development is prolonged if resources are low (Leips & Travis 1994; Audo et al. 1995; Beck 1997). Conversely, other studies have demonstrated that when resources are in abundance the larval phase will be extended to take advantage of the good conditions (Wilbur & Collins 1973; Harris 1987; Audo et al. 1995; Shafiei et al. 2001; Doughty & Roberts 2003). As a consequence of these contradictory results, pertaining to resource availability, both high (Freeman & Bruce 2001) and low food availability (Pintar 2000) have been suggested as causes of larval over-wintering; yet the impacts of food availability on tadpole over-wintering have not been investigated.

The developmental stage and size achieved by individuals at the onset of winter may influence survival to the subsequent spring (Gotthard 2001). While larger size may positively influence winter survival (Altwegg & Reyer 2003), a more advanced pre-metamorphic stage may place the individual at greater risk of injuring the hind-limbs through ice damage (Lai et al. 2002). Therefore, the stage at which tadpoles over-winter may provide some insight into whether amphibian larval over-wintering is based on a decision or constraints on development. Over-wintering as larvae may be advantageous, as individuals can then metamorphose in the following spring at a larger size and earlier that year than would otherwise be possible (Collins 1979; Lauck et al. 2005). However, such an extended larval period could also carry the cost of prolonged exposure to aquatic predators (Relyea 2007), anoxic conditions and increased injury or mortality risk from wintering in water rather than on land (Bradford 1983; Pinder et al. 1992; Lai et al. 2002).

In the present study, I investigated the effect of different seasonal temperature regimes and elevated or reduced food availability, under laboratory conditions, on the duration and progression of the larval period in the common frog *Rana temporaria*. I also compared the sizes of post-metamorphic juveniles that either over-wintered as

larvae or metamorphosed in the summer to determine whether over-wintering as larvae conferred a size advantage.

Methods and Materials

Study species and specimen collection

Rana temporaria is a medium sized anuran (adult size: up to 9 cm) found throughout Europe, from northern Spain to Scandinavia. In Britain, breeding can start as early as January and can last until early April, but generally occurs in late February. Females lay between 1000 – 2000 eggs in a single breeding event. Eggs are clumped together, each coated in a gelatinous envelope. Larvae hatch from the eggs approximately 10 – 14 days later, depending on temperature, and become free-swimming tadpoles after a few days. Development and metamorphosis into juveniles commonly takes place between 10 and 15 weeks later (Beebee & Griffiths 2000). Several egg clumps of *Rana temporaria* were collected in March 2005 from Robroyston Marsh in Glasgow, Scotland (55° 50' N, 4° 15' W). Eggs were mixed together and maintained in a 40 L holding tank at 5.5°C until they hatched and the free-swimming larvae were large enough to be handled (approximately Gosner (1960) stage 25).

Experimental design

Seventy-five tadpoles were then transferred to each of 24 experimental tanks, measuring 30 x 20 x 20 cm, and filled with 11 L of de-chlorinated, copper-free, aerated water maintained at 5.5°C. Three temperature regimes with two food availability levels at each temperature were established, giving six treatments with four tanks per treatment. Tadpoles were randomly allocated to the experimental tanks from the different egg clumps to ensure no differences in genetic variability between tanks or treatments. Tanks were cleaned bi-weekly and checked weekly for any mortality; dead

individuals were removed from the tanks. Photoperiod was common to all tanks, set to reflect ambient levels at the study location and ranged from 7L : 17D in December to 17L : 7D in July; photoperiod was changed monthly to reflect the average for each month of the study.

From mid-April, the water temperature was changed on a monthly basis to simulate three temperature regimes. Each regime had a different monthly increase in temperature, so that the peak temperatures in August were as follows: 1) high – peaked at 18°C; 2) medium – peaked at 14°C; and 3) low – peaked at 10°C (Fig. 5.1). Tadpoles were fed on a 3:1 rabbit pellet : fish flake mixture, given three times a week (Relyea 2001a). The amount of food per tank was fixed at either 9% (high food availability) or 3% (low food availability) of the current tadpole biomass in a given tank. Tadpole biomass per tank was calculated weekly as the average individual mass of all tadpoles measured from the six treatments, multiplied by the number of individuals remaining in a tank. This was done to standardise the two food treatments provided between temperature groups, since masses differed among temperature and food treatments.

Ten individuals from each tank, selected at random, were measured once a week. Mass was measured using an electronic balance accurate to 0.1 mg. SVL, body width and total length were measured with dial callipers accurate to 0.1 mm. Development was measured by staging individuals, at x 10 magnification, according to Gosner's (1960) staging table. Tanks were also checked weekly for individuals beginning metamorphosis, indicated by the emergence of the forelimbs (Gosner stage 42). Tadpoles were removed at the onset of metamorphic climax and released on completion at the site where they were collected, after mass and SVL measurements were taken from a random selection from each treatment group. Individuals that did not commence metamorphosis by mid-November were considered to be over-wintering and were then maintained at 5°C. The following March (2006) the temperature was

increased to 14°C over three days to induce metamorphosis. 14°C was selected so that a comparison could be made between post-metamorphic individuals from the medium temperature treatment that over-wintered or completed metamorphosis during the previous summer (2005). On completion of metamorphosis, mass and SVL measurements were taken and individuals were released.

Statistical Analysis

All values are given as means ± SE. Survival and over-wintering were analysed using GLMM on arcsine-transformed percentages. Due to low survival, the low temperature regime was excluded from analysis of the probability of larval over-wintering. Comparisons of the size and stage of over-wintering tadpoles in the temperature treatments were made using t-tests or non-parametric Kruskal-Wallis tests. Comparisons between the size of individuals completing metamorphosis in August and those that metamorphosed after over-wintering were made using t-tests. Developmental rate, taken as the developmental stage reached by 11 weeks after the tadpoles were placed in the experimental treatment, was analysed using general linear models. Week 11 was selected because metamorphosis was first observed in week 12.

Results

Survival and over-wintering

Higher temperature resulted in better survival through to metamorphosis or over-wintering by November ($F_{2,7.07} = 87.14$, $p < 0.001$) but survival was not affected by food availability ($F_{1,15.69} = 0.05$, $p = 0.836$; Table 5.1). The proportion of surviving tadpoles over-wintering was negatively affected by food level ($F_{1,13.0} = 12.42$, $p = 0.004$) and temperature ($F_{2,13.0} = 374.59$, $p < 0.001$): thus, as temperature and available food decreased the proportion of over-wintering individuals increased (Table 5.1). The

high food/high temperature treatment had the highest survival rate, and all individuals metamorphosed before November; no individuals survived in the high food/low temperature treatment. In the medium temperature treatment, the proportion of over-wintering larvae was substantially lower in the high food treatment compared to the low food treatment.

Over-wintering individuals

There was no significant difference in the stage at which individuals over-wintered among the three temperature treatments (X^2 = 4.43, p = 0.109). Over-wintering occurred at a mean Gosner stage of 38.2 ± 0.4, which is relatively late in larval development. Stage 38 is characterized by relatively well-developed hind limbs with all toes being differentiated and only three developmental stages until the onset of metamorphic climax. There was no difference between the medium and low temperature treatment over-wintering individuals in mass (542.8 ± 30.0 mg; t = 1.48, p = 0.156), width (8.0 ± 0.2 mm; t = 1.61, p = 0.126), and SVL (13.8 ± 0.2 mm; t = 1.47, p = 0.158). The two individuals that over-wintered at the high temperature treatment were extremely small, less than 30% of the size of the low and medium temperature treatment individuals, but detailed analysis was not possible due to the low numbers.

Winter survival of larvae was relatively high in the low and medium temperature treatments, with all 9 of the low temperature treatment individuals and 8 of the 11 medium temperature treatment individuals surviving until the following spring. Neither of the two high temperature treatment individuals survived until the spring.

There were significant differences between froglets that over-wintered as tadpoles and completed metamorphosis in the spring (n = 8) compared to those that completed metamorphosis in the summer (n = 24; mass: t = 2.83, p = 0.008; SVL t = 2.22, p = 0.034). Over-wintering metamorphs were heavier (255.8 ± 25.6 mg) and

longer (13.0 ± 0.4 mm) than summer metamorphs (mass: 195.5 ± 9.0 mg; SVL: 11.8 ± 0.3 mm).

Development rate

Development rate, measured as the Gosner stage reached by week 11 after the start of the experiment, was positively affected by temperature ($F_{2,183}$ = 64.49, $p < 0.001$, Fig. 5.2) with development in the high temperature regime progressing 20.0% and 40.4% faster than the medium and low temperature treatments, respectively (Table 5.2). Higher food availability resulted in individuals, in all temperature regimes, reaching a more advanced developmental stage by week 11 ($F_{1,183}$ = 15.53, $p < 0.001$, Fig. 5.2). There was no interaction between temperature and food availability ($F_{1,183}$ = 0.86, $p = 0.427$). Development rate, in all treatment groups, followed a linear trend with no apparent arrest during early development (Table 5.2; Fig. 5.2).

Discussion

In *Rana temporaria*, under laboratory conditions with similar genetic variation across treatments, the proportion of individuals over-wintering as larvae was affected by environmental conditions. The temperature regime was shown to have a strong effect on the probability of over-wintering as a tadpole; lower temperatures experienced during the summer growing season resulted in a higher proportion of the surviving individuals remaining as tadpoles during the winter. There are two possible ways in which temperature could influence the timing of metamorphosis: 1) cold temperature during the growing season could delay or prevent metamorphosis, by interfering with endocrine function (Kollros 1961; Frieden et al. 1965; Viparina & Just 1975; van der Have & de Jong 1996; Murata & Yamauchi 2005); 2) temperature could function as a signal which promotes an adaptive response to environmental conditions. The first

indicates a physiological response to the conditions that the individual is subjected to, while the second indicates a decision process similar to that predicted by Wilbur & Collins' (1973) model. If low temperature, acting as a physiological constraint, were solely responsible for tadpoles over-wintering then it might be expected that all individuals would be similarly affected. This is what was observed in the low temperature treatment, in which no surviving individuals commenced metamorphosis prior to the onset of winter. Survival was also very low suggesting that the conditions of the low temperature treatment were adverse to successful tadpole development, regardless of food level. The low survival may also have resulted in selective mortality, with individuals capable of surviving the harsh conditions experienced in the low temperature treatment predisposed to over-wintering.

In both the medium and high temperature treatments, only some individuals remained as larvae into the winter, which is also observed in the field (Viparina & Just 1975; Lai et al. 2002; Chapter 6). My results, and the observations from the field, could be explained by three hypotheses. Firstly, there could be substantial variations in temperature within a tank or pond resulting in some individuals being subjected to insufficient temperatures to complete development. This is highly unlikely in the laboratory due to the small volume of water in the tanks and the stable temperatures experienced. Secondly, there could be differences in genotype among individuals, relating to the thermal thresholds for endocrine or thyroid activity, causing variation in the minimum temperatures at which endocrine pathways function (van der Have & de Jong 1996). This also seems unlikely in our study, since all individuals across treatments were from the same spawn clumps and there would be similar genetic variation across treatments. Finally, low temperature may not be the only factor involved in over-wintering. It may be that additional factors, in conjunction with low temperature, constrain development or form the basis of a decision to over-winter as

larvae based on an individual's competitive ability. The latter of the three hypotheses appears to be the most likely explanation, since food availability was also found to have a significant influence on the proportion of over-wintering observed.

Studies on the effects of food availability on the duration of the larval period in amphibians appear to be contradictory. Early life history theories predict that when food is abundant, individuals should extend the duration of larval development to take advantage of the conditions for greater growth (Wilbur & Collins 1973; Harris 1987; Audo et al. 1995; Freeman & Bruce 2001; Shafiei et al. 2001; Doughty & Roberts 2003). However, low food quality or quantity have been shown to extend the larval period (Leips & Travis 1994; Audo et al. 1995; Beck 1997). The impacts of food availability on larval development can also be influenced by predation risk (Nicieza 2000). My results demonstrate that reduced food availability increases the proportion of tadpoles over-wintering in the high and medium temperature treatments, which does not support the hypothesis that individuals are extending the larval period under favourable growth conditions (Freeman & Bruce 2001). Therefore, in the laboratory, the proportion of over-wintering larvae is the result of insufficient resources either functioning as a constraint on development rate directly or by means of a decision based on reduced growth rate. A reduction in growth rate could induce over-wintering since individuals may not reach the minimum size required for metamorphosis (Wilbur & Collins 1973) before the onset of winter. However, with the exception of the two individuals from the high temperature treatment, all over-wintering individuals had reached an appropriate size to undergo metamorphosis. Alternatively, over-wintering could be induced by a reduced growth rate during a critical phase during development, as has been observed in Atlantic salmon *Salmo salar*. Salmon with high growth rates early in the freshwater parr phase smolt and migrate to the sea in their first year, while slow growing parr take one year or longer before smolting (Metcalfe et al. 1988; Thorpe

1989). In the current experiment it is difficult to distinguish whether low temperature or food availability act as a constraint or promote a strategic decision to over-winter in the larval stage.

Previous descriptive work on over-wintering in anurans predicted that a decision to over-winter as a larva would be reached early in the free-swimming larval phase, at approximately Gosner stages 32 – 35. After stage 35 the hind limbs in anuran larvae, which are vital to the subsequent juvenile and adult stages, begin to develop rapidly and become more vulnerable to ice damage (Lai et al. 2002). Similarly, salmon life history trajectories are fixed after the formation of a bimodal size distribution several months before the first parr begin to smolt (Thorpe 1977; Metcalfe et al. 1988; Heggenes & Metcalfe 1991). In the current study, over-wintering in the laboratory occurred at a stage late in tadpole development (approximately stage 38) when hind limbs are well developed, with some individuals arresting development just prior to the eruption of the forelimbs. The progression of larval development also followed a continuous linear trend until week 11, when metamorphosis was first observed. Our findings do not seem to support the hypothesis that the decision to over-winter as a tadpole occurs at an early developmental stage, but does not exclude the possibility that a decision is involved in over-wintering of larvae in the laboratory or the field. The decision to over-winter, as a tadpole, may simply be taken later in development. Equally, the laboratory methods used may not have allowed individuals to accurately judge conditions, since the temperature and photoperiod were fairly static, punctuated by monthly changes.

From the current study there is evidence that over-wintering by larvae may represent an adaptive strategy. A predicted advantage of larval over-wintering, or longer development times in general, is that extending larval duration allows individuals to achieve larger sizes than those that develop more quickly (Wilbur & Collins 1973). Larger body size at the completion of metamorphosis has been shown to be adaptive by

conferring advantages in improved locomotor performance, survival and reproductive fitness (Semlitsch et al. 1988; Beck & Connor 1992; Goater 1994; Fischer et al. 2004; Alvarez & Real 2006; Chelgren et al. 2006; Ficetola & De Bernardi 2006; Walsh et al. 2007a). My results show that individuals that completed metamorphosis after over-wintering as tadpoles were over 30% heavier and almost 10% longer than those that completed metamorphosis in the summer. The larger size of individuals that over-wintered as larvae was likely the result of either a longer period of growth or exposure to cold temperatures during growth in late autumn, since many ectotherms attain greater body sizes when reared at lower temperatures (Atkinson 1994). This would contrast with what occurs in the salmon, since growth is halted during the winter, as individuals do not feed, but *Rana* tadpoles were observed feeding throughout the year (personal observation). While temperature during metamorphosis has also been shown to significantly influence juvenile body size (Chapter 4) this cannot explain the difference since both groups completed metamorphosis at 14°C. Conversely, larger individuals may simply have been better able to survive the decreasing temperatures in late autumn, but it was not possible to distinguish whether individuals that died would have over-wintered as tadpoles or commenced metamorphosis. The increase in size may have a disproportionately positive effect, even considering the relatively low proportion of over-wintering individuals, given the high levels of juvenile mortality.

In conclusion, the percentage of surviving tadpoles that over-wintered was influenced by environmental conditions since genetic variation would have been the same across treatments. Reduced temperature during the growing season increased the proportion of over-wintering tadpoles and is likely to explain some of the latitudinal and altitudinal clines in over-wintering observed in other species of *Rana*. However, both in the laboratory and the field, temperature does not often result in the 'all-or-none' form of tadpole over-wintering expected if it occurs purely as the result of a physiological

constraint on development. Reduced food availability, and potentially other factors, contributes to larval over-wintering, but the exact mechanism is unclear. There is evidence that over-wintering as a tadpole might be an adaptive strategy since tadpoles that over-wintered completed metamorphosis at a larger size than those that metamorphosed in the late summer.

Tables and figures

Table 5.1: Tadpole survivial during the experiment for each treatment group. The number of surviving tadpoles is given as the number of individuals successfully completing metamorphosis prior to the onset of winter plus the number of pre-metamorphic tadpoles at the onset of winter. Mean survival rate (± SE) as a percentage of the initial number is also given for each treatment group. The total number of individuals over-wintering and the mean percentage of surviving tadpoles over-wintering in each treatment group are also given.

	Low food treatment				High food treatment			
Temperature	Survival		Over-wintering		Survival		Over-wintering	
treatment	n	%	n	%	n	%	n	%
Low	9	3.0 ± 2.6	9	100 ± 0	0	0 ± 0	-	-
Medium	138	46.0 ± 4.7	8	5.8 ± 1.6	155	51.7 ± 4.9	3	1.9 ± 0.6
High	201	67.0 ± 1.8	2	1.0 ± 0.6	205	68.3 ± 6.2	0	0 ± 0

Table 5.2: Regression analysis of development in *Rana temporaria* tadpoles from the start of the experiment until week 11 (* < 0.0001), where B is the slope of the regression equation (± SE). Differences in df are due to mortality during the course of the experiment.

		df	F	r^2	B
Low temperature	High food	1,282	922.6*	0.767	0.604 ± 0.020
	Low food	1,347	1884.0*	0.845	0.558 ± 0.013
Medium temperature	High food	1,439	3628.1*	0.892	0.910 ± 0.015
	Low food	1,439	1094.4*	0.714	0.650 ± 0.020
High temperature	High food	1,439	4664.0*	0.914	1.149 ± 0.017
	Low food	1,439	1304.9*	0.749	0.802 ± 0.022

Figure 5.1: Water temperature throughout the season for the experimental temperature treatments.

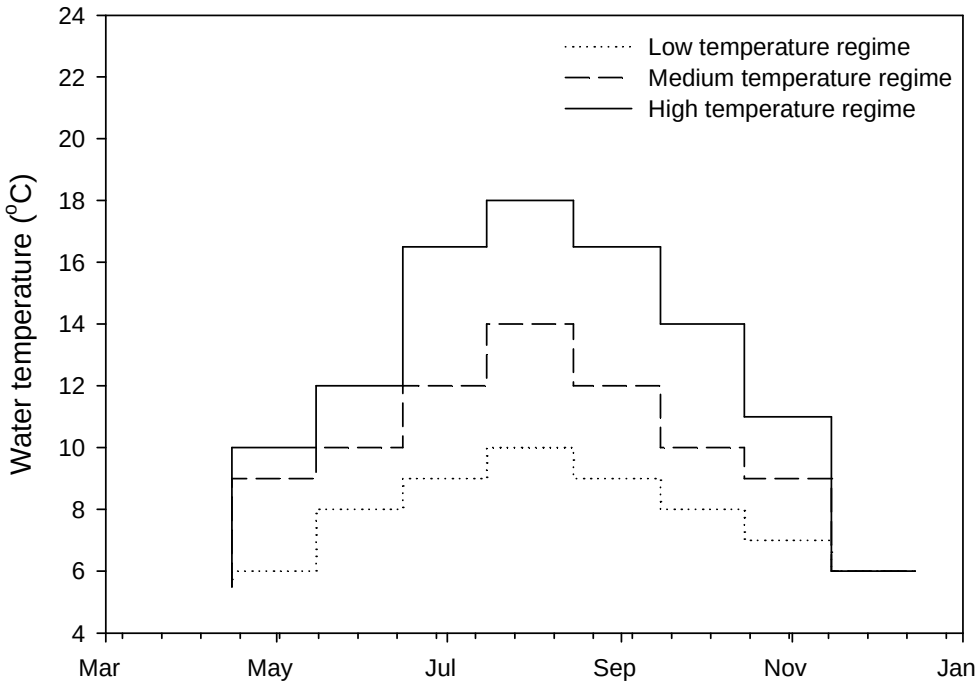
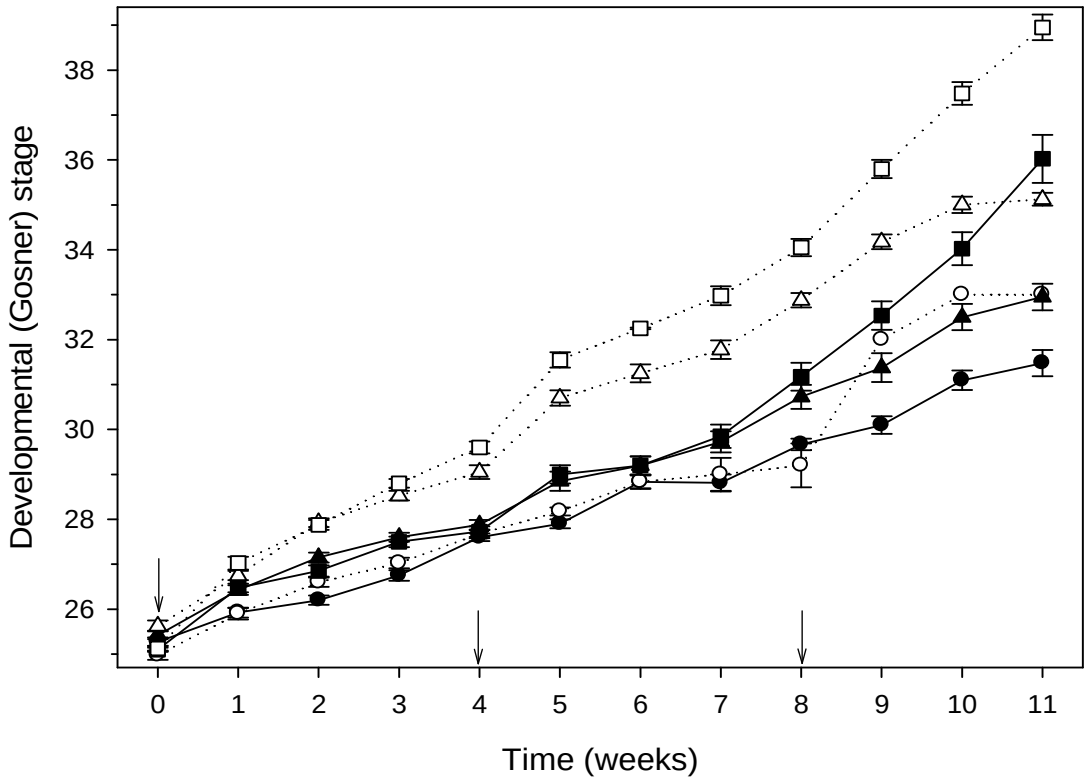


Figure 5.2: Development trajectories (as the weekly mean Gosner stage ± SE) of *Rana temporaria* tadpoles reared at low (●), medium (▲) and high (■) temperature regimes and fed on high (open shapes, broken lines) or low (solid shapes and lines) food rations from the start of the experiment until week 11 after which the first metamorphs were observed. Arrows indicate the weeks when the temperature was increased according to Fig. 5.1.



Chapter 6: The timing and pattern of larval development in over-wintering *Rana temporaria* tadpoles at a field site

Abstract

In ectothermic animals living in temperate regions, winter is a critical time; during early ontogeny the stage or size an individual reaches at the onset of winter can have a significant effect on its survival and other life history characteristics. Generally, temperate amphibian larvae complete metamorphosis in the summer and spend their first winter as juveniles. However, the common frog *Rana temporaria* shows variation in this aspect of its life history, with some individuals remaining as aquatic larvae during the winter. I investigated growth and development during the larval period in a field pond where larval over-wintering of *R. temporaria* had been recorded. Larvae within the field pond showed a bimodal distribution in developmental stage as early as July, when temperatures were still increasing, separating individuals that would metamorphose and over-winter as juveniles from those that would spend the winter as larvae. Individuals that over-wintered as larvae remained at a relatively undeveloped larval stage throughout the summer and autumn. I also examined, in the laboratory, whether variation in temperature equivalent to that in the field pond, at high and low food availabilities explained some of the variation in over-wintering life history stage. Mean temperature and food availability did affect development and growth, but did not appear to be responsible for variation in wintering strategy. This suggests that there are other factors, in addition to temperature and food availability, that contribute to the observed plasticity in over-wintering strategy.

Introduction

In many animals the timing of life history transitions is known to vary in response to environmental conditions experienced during early development. In temperate regions, the life history stage or size that an individual reaches by the onset of winter can have a significant effect on survival and life history trajectory (Gotthard 2001). This has been well studied in the Atlantic salmon *Salmo salar*, where parr migrate to sea either after their first year, or delay migration for one or more years, spending additional time in the larval habitat (Thorpe 1977; Metcalfe et al. 1988). In salmon, the decision on whether to undergo smolting is made long before the onset of winter and a bimodal distribution in size is evident relatively early in the growth season, separating the two modal groups that will either delay or undergo smolting within the year (Thorpe 1977; Thorpe et al. 1992). A similar phenomenon has also been observed in the duration of the larval phase in some amphibians, with some individuals within a pond not completing metamorphosis in the year they were spawned and over-wintering as larvae (e.g. Collins & Lewis 1979). There is no information however on the seasonal or developmental period at which such a decision is made.

Generally, temperate amphibian larvae exploit freshwater habitats during the spring and summer, allowing rapid growth, and complete metamorphosis prior to leaving the aquatic habitat before the onset of winter (Wilbur & Collins 1973; Werner 1986; Stebbins & Cohen 1995). Reports of over-wintering of anuran larvae are becoming more prevalent, in both public media and the scientific literature (Collins & Lewis 1979; Archibald & Downie 1996; Pintar 2000; Fellers et al. 2001; Gollmann et al. 2001; Lai et al. 2002). However, there has been little experimental investigation of this variation in the duration of the tadpole stage, since most reports are largely anecdotal or are descriptive studies. It is not known whether a bimodal distribution,

which could appear in either developmental stage or size, occurs in natural populations where over-wintering of amphibian larvae occurs. Similarly, it is not known in amphibians at what point the over-wintering of an individual is determined, whether (as in salmon) it occurs early in the season or later as a result of ecological factors. However, it has been predicted (Lai et al. 2002) that in anuran amphibians that over-winter as larvae, development should be halted in the pre-metamorphic larval phase (*c.* Gosner (1960) stages 32 – 35) to protect the developing hind limbs from frost damage and that this arrest in development would be accompanied by a reallocation of investment away from development and into growth.

Temperature and food availability have both been shown to influence the proportion of individuals over-wintering as larvae in the common frog *Rana temporaria* under laboratory conditions (Chapter 5). Descriptive studies have also suggested an increase in the occurrence of over-wintering with higher latitude in *Rana catesbeiana* (Collins 1979) and altitude in *Rana sauteri* (Lai et al. 2002), which has been attributed to temperature. However, no attempt has been made to examine variation in the incidence of over-wintering larvae in relation to thermal conditions and resource levels in the field.

In the present study, I investigated growth and development throughout the year in a field population of *Rana temporaria* tadpoles to determine whether a bimodal distribution in size or developmental rate occurs during the growing season, which could be linked to over-wintering as larvae. Observations from the field were also used to determine the developmental stage at which tadpoles over-wintered. I also investigated growth and development in a laboratory population based on temperatures experienced in nature. Resource availability in the field population was not known, so high and low food availabilities were used in the laboratory. This allowed a comparison

of field and laboratory results on the effects of temperature and food availability on the occurrence of larval over-wintering and on growth and development rates.

Methods and Materials

Field Site

The field site was Drumtian Pond, located near the village of Killearn, Scotland [NS 525855], where high numbers of over-wintering *Rana temporaria* tadpoles have been known to occur for several years. It was constructed in 2001, and measures 5 x 8 m. The pond has vertical sides, and water depth is around one metre. Since the pond's construction, *Rana temporaria* spawn (from several locations in western Scotland) has been added in most years. Over-wintering tadpoles were first observed in the pond in 2003. In addition to adult and larval common frogs, there are a number of other species present, including several that are predators of tadpoles (e.g. newts *Triturus spp*, dragonfly larvae and beetles from the family Dytiscidae). There are no fish species present in the pond. The pond bottom is covered in a thin layer of fine organic matter and dead leaves. There is little aquatic vegetation, but high levels of micro-algae.

To quantify the thermal environment, temperature data were collected continuously for one year beginning in March 2005 using waterproof TinyTalk data loggers. Two loggers were used; one to establish temperature just below (1 cm) the surface of the pond, the second to record temperature at the pond bottom. Data loggers were set to record temperature every 11 minutes and the data were downloaded every two weeks.

Tadpole growth and development were monitored on a monthly basis, by collecting up to 80 individuals randomly with a large pond net in the middle of each month until the study concluded in mid January 2006. As the season progressed it

became more difficult to capture sufficient numbers, so a standardized capture effort of 30 minutes was used. Individuals were taken to the laboratory for measurement and returned to the pond within 24 hours. Mass was measured using an electronic balance accurate to 0.1 mg. SVL, body width and tail length were measured with dial callipers accurate to 0.1 mm. Body condition was calculated using the formula from Veith (1987): this is defined as condition = (mass / SVL^3) * 1000. Development was measured by staging individuals, at x 10 magnification, according to Gosner's (1960) staging table.

Laboratory experiments

In order to test whether a thermal regime similar to that recorded in Drumtian Pond would induce over-wintering in tadpoles collected from another location; several clumps of *Rana temporaria* spawn were collected in mid-March 2005 from Robroyston Marsh in Glasgow, Scotland. The egg clumps were maintained in 40 L tanks at 5.5°C until after hatching and the tadpoles were large enough to handle (*c.* Gosner stage 25), at which point the tadpoles were transferred to experimental tanks.

Experimental tanks were 12 L capacity measuring 30 x 20 x 20 cm, and filled with 11 L of de-chlorinated, copper-free, aerated water. From April, water temperatures in the tanks were regulated, using a constant temperature room, to track the temperatures recorded at the bottom of Drumtian pond, where tadpoles in the field were observed to spend the majority of their time. Temperatures were changed on a fortnightly basis, and were set to the mean temperature recorded in Drumtian Pond over the two-week period (Fig. 6.1).

In order to examine whether resource availability influenced over-wintering probability, the tadpoles in the laboratory were fed on a 3:1 rabbit pellet : fish flake

mixture (*c*. 10% protein), given three times a week. Two food availabilities were established: high food availability constituted a 50% increase over the level recommended by Relyea (2001) i.e. 9% of total tadpole biomass per tank; low food availability was set at 3% of the total tadpole biomass per tank. Total tadpole biomass per tank was calculated as the average mass of all tadpoles measured in the laboratory in both food level treatments multiplied by the number of individuals remaining in the tank. This was done to standardise the food provided between the food treatment groups, since masses differed. Photoperiod was set to reflect ambient times and ranged from 7L : 17D in December to 17L : 7D in July and was changed monthly to reflect the monthly average photoperiod.

In total, eight experimental tanks were set up; each containing 75 randomly allocated *R. temporaria* tadpoles, with four tanks for each food availability treatment. Tanks were cleaned bi-weekly and checked weekly for any mortality; dead individuals were removed from the tanks. Tanks were topped-up as required with de-chlorinated, copper-free water to maintain a constant water level.

Ten individuals from each tank, selected at random, were measured once a week. Mass, SVL, body width, tail length and body condition were measured using the same methods as for field tadpoles. Tanks were also checked weekly for individuals entering metamorphosis. Individuals that had commenced metamorphosis were transferred to small individual tubs, with a small amount of water, so that they would not drown in the experimental tanks. On the completion of metamorphosis individuals were released near the site from where the spawn was collected. Individuals that had not commenced metamorphosis by November following their spawning were considered to be over-wintering. Survival was determined in November and comprised

all individuals that had successfully reached metamorphosis and those that were considered to be over-wintering as larvae.

Data analysis

All values are given as mean ± SE, unless otherwise stated. All analysis was performed using SPSS v15 (SPSS Inc., Chicago, IL). Mass, SVL and developmental stage measurements each month were analysed for normality using Shapiro-Wilk's test. Comparisons of the mass and SVL among the field and two laboratory populations in mid July were performed using generalised linear mixed models (GLMM), with tank as a random factor. Differences in developmental stage among the field and laboratory populations was analysed using a non-parametric Kruskal-Wallis test.

Results

Drumtian Pond

The distribution of developmental stage was non-normal throughout the season with the exception of December (Table 6.1). A bimodal distribution of developmental stage became apparent starting in July, and persisted until the onset of winter in November (Fig. 6.2). Both mass and snout-vent length, with the exception of the months shortly after hatching, were normally distributed indicating a single size distribution for all individuals (Table 6.1). Metamorphosing individuals, spawned in the current breeding season (2005), were observed on several occasions during the summer of 2005, but were not taken to the laboratory for measurements.

Over-wintering was observed in the pond in 2005, with a sample of 34 individuals being captured as tadpoles on the 8th of November. At the onset of winter in November remaining tadpoles had reached a mean Gosner stage of 32.7 ± 0.7. Stages

32 – 33 are characterised by small hind-limb buds which are only beginning to differentiate into toes. Tadpoles were present continuously throughout the winter, with samples of tadpoles being captured in December (n = 26) and January (n = 19). Tadpoles spawned in the spring of 2005 were still present at the time of the 2006 spring spawning (Figure 6.3).

Laboratory experiments

Survival through to metamorphosis in the laboratory was relatively low (39.67 ± 8.12%) with no significant difference between the high and low food treatment ($t = 0.46$, $p = 0.664$). Over-wintering was not observed in either the high or low food treatment. Individuals from both food treatments were first observed to commence metamorphosis in the beginning of July. In the high food treatment all surviving individuals had commenced metamorphosis by the end of July. The final individual to begin metamorphosis in the low food treatment did so by the end of August. At that time one tadpole was still present in the low food treatment, but did not survive until November. A single size distribution was maintained throughout development. In the high food treatment mass and SVL were normally distributed at each point measured (Table 6.2). In the low food treatment, SVL and mass data were predominately normally distributed (Table 6.2). A bimodal distribution was not established in developmental stage in either the low (Fig. 6.4) or high food treatment (Fig. 6.5). However, the distribution of developmental stage did develop a 'tail' in the low food availability treatment in June and July, with some individuals trailing behind the majority in development rate.

Comparisons between laboratory and field

Since all surviving individuals from the laboratory treatments completed metamorphosis by the end of August, it was not possible to compare laboratory and field over-wintering individuals. Therefore, comparisons were made among the rates of development and growth at peak season (July) in the laboratory treatments and Drumtian field pond.

Within the laboratory population there were significant tank effects on growth and development variables, with the exception of body width, so tank was included as a random factor. Since there was no food treatment in the field pond, the data were divided into three groups: Drumtian Pond field population (DPF), Laboratory High food treatment (LH) and Laboratory Low food treatment (LL). At peak season in July, DPF had the lowest mass (Tables 6.1 and 6.2) of the three groups ($F_{2,9} = 19.63$, $p = 0.001$). However, the difference between the mass of DPF and LL individuals was only marginally significant ($p = 0.043$). Snout-vent length followed the same pattern with DPF individuals the shortest (Table 6.1), followed by LL and LH ($F_{2,9} = 20.87$, $p < 0.001$, Table 6.2). The stage reached by July was also significantly different in the three groups ($X^2 = 69.62$, $p < 0.001$), with DPF only reaching stage 29.0 ± 0.6, while LL had developed to stage 36.6 ± 0.5 and LH to stage 39.6 ± 0.2. Tadpoles from DPF did not differ significantly in body condition (0.186 ± 0.004) compared to those from LH (0.184 ± 0.003), but both were in better body condition than LL tadpoles (0.168 ± 0.004). Similarly, body widths of individuals from DPF and LH were over 9% greater than those from the LL treatment, but not significantly different from each other ($F_{2,120} = 3.36$, $p = 0.038$). Tank was not shown to have an effect in any analysis.

Although DPF individuals were lower in mass at peak season, for a given stage throughout development DPF individuals were heavier than both laboratory populations ($F_{28,1525} = 9.36$, $p < 0.001$) and had a greater increase in mass as development

progressed than the LL population ($t = 5.43$, $p < 0.001$; Fig 6.6). LH individuals also had a greater increase in mass through development than was found in LL individuals ($t = 11.70$, $p < 0.0005$).

Discussion

Over-wintering of *Rana temporaria* tadpoles was confirmed at my field site within the UK, with some tadpoles completing metamorphosis in the summer and autumn while others remained in the pond until the following spring. Over the course of the spring after the year of spawning, the numbers of over-wintering larvae present in the pond decreased and it seems likely that most individuals completed metamorphosis. This represents a clear example of plasticity in the timing of life history transitions. At this study site, I recorded the formation of two distinct modal groups that persisted from shortly after the larvae became free-swimming until the onset of winter. Tadpoles in the field pond demonstrated a normal distribution in mass and SVL throughout the year. The mean mass and SVL of field tadpoles increased during the summer until November, when they levelled off, indicating that both modal groups were increasing in body size at a similar rate.

The formation of the two developmental groups suggests that after May, a large proportion of individuals arrested their development but continued to grow, while the second group comprised successive waves of individuals progressing through intermediate developmental stages before commencing metamorphosis. The formation of the first group, which halted development early in the tadpole phase, suggests that the individuals destined to over-winter were determined early in development and at a time when temperatures were still increasing and growth in body size was still progressing. Therefore, the occurrence of over-wintering in anuran larvae in the field may be based

on a decision early in development similar to that observed in the Atlantic salmon (Thorpe 1977; Metcalfe et al. 1988). The mechanisms that determine this difference in life history duration are not clear. At this field site the modal group of an individual may be determined genetically, with some predetermined to over-winter as larvae regardless of conditions. There may also be maternal effects, such as ovum size, that could determine the development rate (Parichy & Kaplan 1992) of the two modal groups. Alternatively, the presence of the over-wintered individuals, early in the spring, and the second developmental group, throughout the growing season, may suppress the development of smaller or earlier stage larvae (Licht 1967; Breden & Kelly 1982; Woodward 1987; Lea et al. 2002). This could explain the successive waves of developing tadpoles; since tadpoles would be freed from developmental suppression as more developed tadpoles began metamorphosis and left the pond. However, suppression of the first group by more developed individuals can only explain the maintenance of arrested development and larval over-wintering, rather than its origin. Finally, the formation of the two distinct groups, which ultimately express different larval durations, may be determined by other environmental conditions, alone or in conjunction with genetic or maternal effects.

Earlier descriptive work has indicated that to maximise survival, individuals should reduce the risk of damage to structures important to the adult phase (e.g. the hind limbs) and over-winter at a stage prior to the development of vulnerable structures (Lai et al. 2002). This was observed in the field, with the mean stage being between 32 and 33 in November and below 36 in January. Prior to stage 36 the hind limbs have a lower surface area to volume ratio and are held close to the body reducing their exposure to low temperatures. This would reduce the risk of the hind limbs suffering from ice damage, and suggests that over-wintering may represent an adaptive strategy. Tadpoles

over-wintering may also be expected to reallocate investment from development into growth to reach larger sizes, but display slower development. Individuals in the field did seem to demonstrate a reallocation of resources away from development towards growth. Field tadpoles were significantly heavier for a given stage than both the high and low food level laboratory treatments, but at peak season individuals from the field were significantly less developed. A reallocation of resources into growth in order to reach larger sizes, at the expense of development, may be an adaptive response to environmental conditions and could provide a survival advantage during the winter (Partridge & Coyne 1997; Sogard 1997; Renault et al. 2003).

Low temperature during the larval growth season has often been predicted (Berven et al. 1979; Collins 1979; Lai et al. 2002) and has been shown, in the laboratory (Chapter 5), to be a factor in the proportion of individuals over-wintering as larvae. In the current study larval over-wintering was not observed in either food level treatment in the laboratory, when tadpoles were reared at the mean fortnightly temperatures experienced in the field. Mean fortnightly temperatures recorded in the field exceeded those used in the highest experimental temperature regime in Chapter 5. While the laboratory results of the current study adhere to the same trend, with higher temperatures resulting in fewer incidences of tadpoles over-wintering, the mean fortnightly temperature in the field alone does not appear to influence larval over-wintering. However, there are several possible reasons for our not having observed over-wintering in the laboratory. Firstly, mean temperature may not be the best indicator of the thermal conditions experienced by individuals in the field. Niehaus et al. (2006) demonstrated that *Limnodynastes peronii* individuals from a diurnally fluctuating thermal environment and those from a stable temperature with the same mean had difference rates of development. They found that the individuals from the

fluctuating thermal environment developed more quickly, as opposed to my results where the field (fluctuating) tadpoles displayed a slower development compared to the laboratory (stable) populations. Furthermore, due to diurnal fluctuations, the mean temperature may not be the most commonly experienced temperature in the field; thus the modal temperature may have been a better indicator. However, over the two-week period that the temperature was recorded continuously the standard deviation in temperatures at the bottom of Drumtian pond were very low (generally < 1.5ºC), but surface temperatures were generally warmer during the summer and displayed a greater degree of fluctuation (mean SD of 2.0ºC).

The fact that temperatures from the field did not result in over-wintering in the laboratory, at either food treatment, may indicate that factors other than temperature contribute to larval over-wintering or that there has been rapid selection at this field site. While it may be counter-intuitive, predation-risk may contribute to over-wintering. Increased predation risk is predicted to result in shorter larval durations, since tadpoles would be expected to try to escape the high-risk aquatic habitat (Wilbur 1980). However, this is rarely shown in empirical studies (Relyea 2007), due to either behavioural adaptations or induced anti-predatory defences reducing resource acquisition rates or carrying developmental costs. The tadpoles collected from the field in the current study displayed the characteristic phenotype (short, wide bodies; short deep tails, personal observation) developed by many aquatic prey species in the presence of predators (Bronmark & Miner 1992; Van Buskirk & Relyea 1998; Vollestad et al. 2004; Moore et al. 2004; Eklov & Jonsson 2007). There were also a number of tadpole predators found in the pond while collecting tadpoles (see methods).

Finally, the food levels used in the laboratory treatments may have been greater in quantity or quality relative to the actual conditions in the field. In collecting tadpoles

from the field, it was common to observe tadpoles feeding on deceased conspecifics. Cannibalism and feeding on dead conspecifics have been demonstrated to result from low food availabilities (Wildy et al. 2001). Additionally, tadpoles in the laboratory were fed on a diet relatively rich in protein, which can increase the rate of growth and development (Steinwascher & Travis 1983; Kupferberg 1997). The influences of predation-risk and lower food availability in the field on larval over-wintering are both supported by the differences among the tadpoles in the field and the two laboratory treatments at peak season. At peak season, individuals in the field were the lightest, shortest and least developed, but with the greatest body width, of the three groups, with the high food laboratory treatment being largest and most developed. This result provides support for the conclusion that resources were limited in the field compared to both laboratory treatments, due to a reduction in feeding opportunities (predation-risk) and/or simply low food availability.

In conclusion, over-wintering of *Rana temporaria* larvae in the field appears to be determined early in development and displays a bimodal distribution, with individuals that will continue to remain in the pond over winter halting development at an early stage. There is some evidence that this may be adaptive, since development is halted at a stage that would be most suited to reduce risk to important developing structures. However, the mechanisms that determine the arrest in development and subsequent over-wintering are not clear, but do not appear to be determined by temperature and food availability alone.

Tables and figures

Table 6.1: Monthly mean mass and snout-vent lengths of individuals from Drumtian pond. Analysis of normality of monthly mass, SVL and developmental stage is displayed as the Shaprio-Wilk statistic, with p values (* < 0.05, ** < 0.01, * < 0.001, NS = non-significant result). Significant p-values denoting deviation from normality are given in bold.**

	Mass (mg)		Snout-vent length (mm)		Gosner stage
	mean (± SE)	S-W statistic	mean (± SE)	S-W statistic	S-W statistic
May	71.2 ± 4.5	**0.57*** **	6.8 ± 0.1	**0.77*** **	**0.29*** **
June	172.7 ± 10.0	**0.88*** **	9.4 ± 0.2	0.97^{NS}	**0.64*** **
July	233.6 ± 12.7	**0.94***	10.6 ± 0.2	0.97^{NS}	**0.78*** **
August	320.0 ± 13.8	0.97^{NS}	11.9 ± 0.2	0.97^{NS}	**0.90*** **
September	371.3 ± 13.8	0.97^{NS}	12.3 ± 0.2	0.98^{NS}	**0.92** **
October	392.6 ± 13.3	0.95^{NS}	12.6 ± 0.1	0.98^{NS}	**0.89** **
November	360.9 ± 23.2	0.95^{NS}	12.4 ± 0.2	0.98^{NS}	**0.90** **
December	374.3 ± 20.9	0.97^{NS}	12.7 ± 0.2	0.97^{NS}	0.93^{NS}
January	371.5 ± 21.7	0.95^{NS}	12.8 ± 0.3	0.96^{NS}	**0.85** **

Table 6.2: Monthly mean (± SE) mass (mg) and snout-vent length (mm) of individuals from the high and low food treatments in the laboratory. Analysis of normality is displayed as the Shaprio-Wilk statistic: NS = non-significant result, * < 0.05.

	Low food treatment				High food treatment			
	mass	S-W statistic	SVL	S-W statistic	mass	S-W statistic	SVL	S-W statistic
Apr.	26.2±1.6	0.93*	4.9±0.1	0.97^{NS}	23.0±1.3	0.94^{NS}	4.7±0.1	0.97^{NS}
May	98.5±47.8	0.97^{NS}	7.5±0.1	0.97^{NS}	101.4±4.2	0.97^{NS}	7.6±0.1	0.97^{NS}
June	304.8±16.5	0.99^{NS}	11.8±0.2	0.95^{NS}	439.3±15.1	0.99^{NS}	13.4±0.2	0.99^{NS}
July	293.9±12.9	0.95^{NS}	12.0±0.2	0.92*	402.0±14.8	0.92^{NS}	12.9±0.2	0.96^{NS}
Aug.	233.5±42.8	0.97^{NS}	11.0±0.6	0.99^{NS}	-	-	-	-

Figure 6.1: Water temperature throughout the season, calculated as the fortnightly mean temperatures (with s.d.) at the surface (○) and the bottom (●) of Drumtian pond. The temperatures recorded at the bottom of Drumtian pond were used for the laboratory experiments as described in the text.

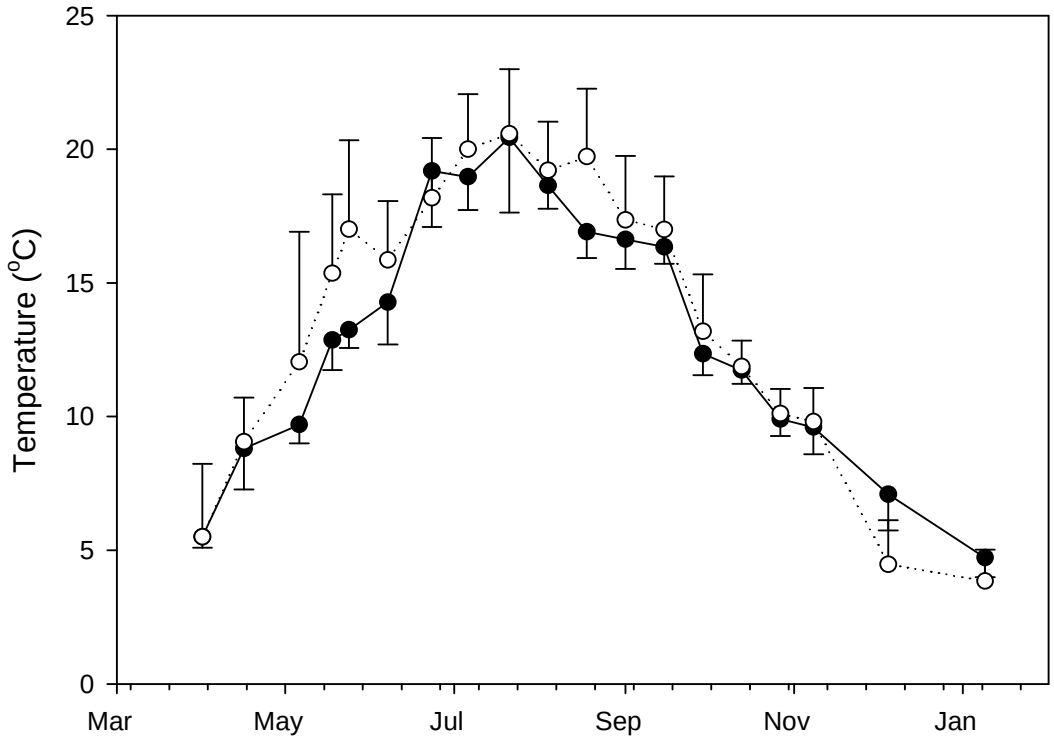


Figure 6.2: Monthly developmental stage frequency distributions of tadpoles found in the field at Drumtian pond.

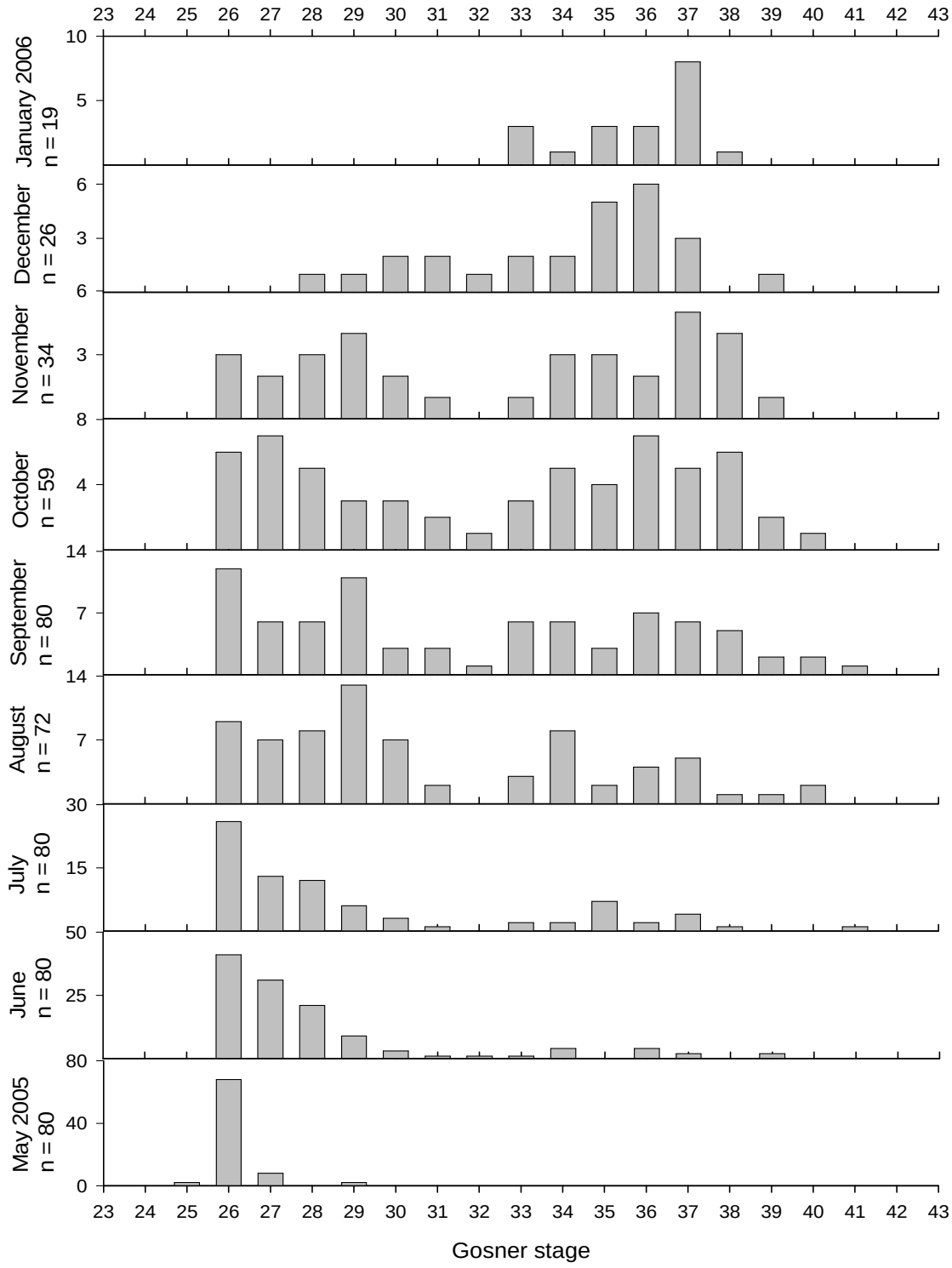


Figure 6.3: Photograph of over-wintered (bottom) and recently spawned (top) *Rana temporaria* larvae in spring 2006.

Figure 6.4: Monthly developmental stage frequency distributions of tadpoles reared in the laboratory under low food availability. In each month n = 40, except August (n = 3).

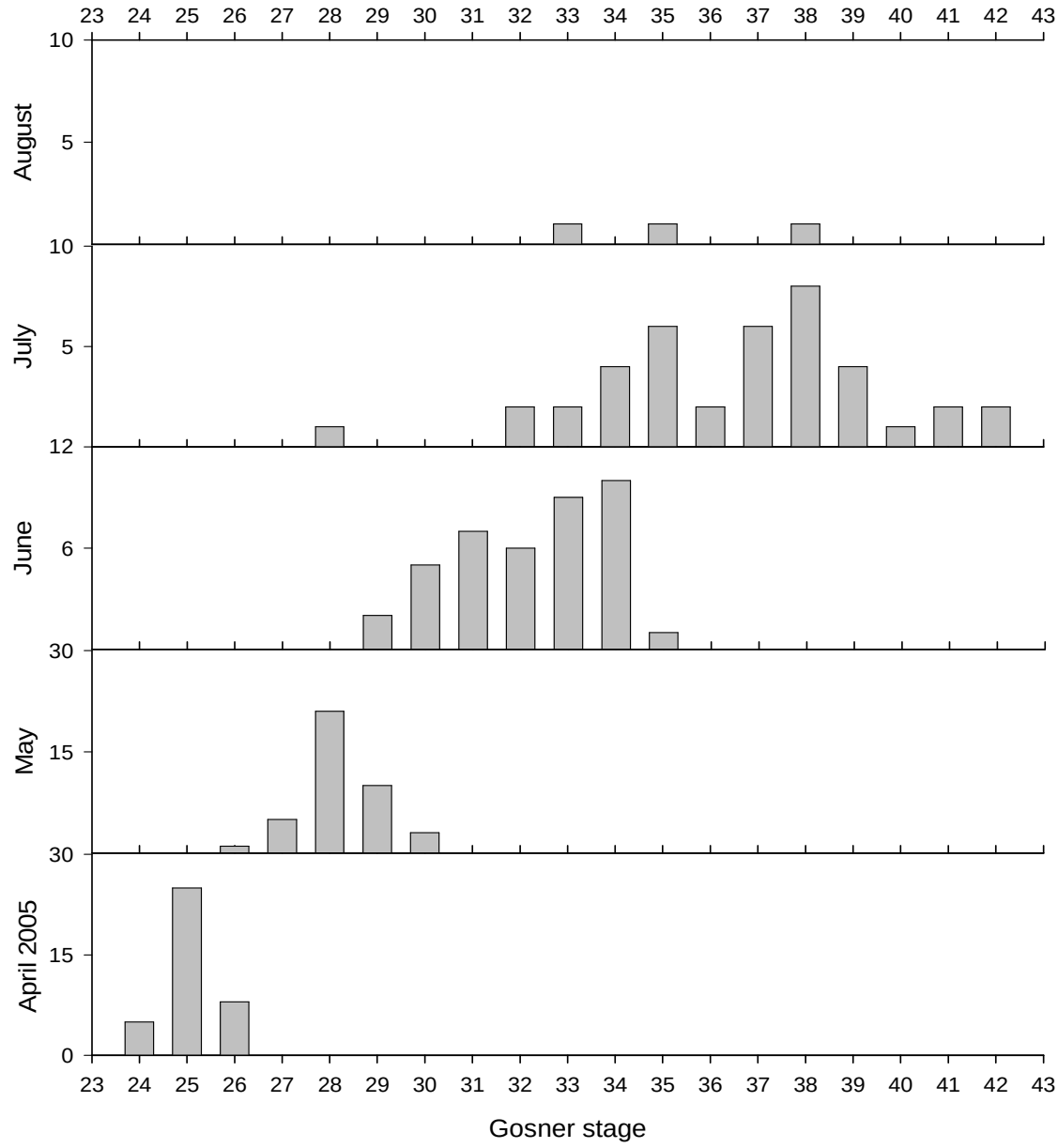


Figure 6.5: Monthly developmental stage frequency distributions of tadpoles reared in the laboratory under high food availability. N= 40 in each month.

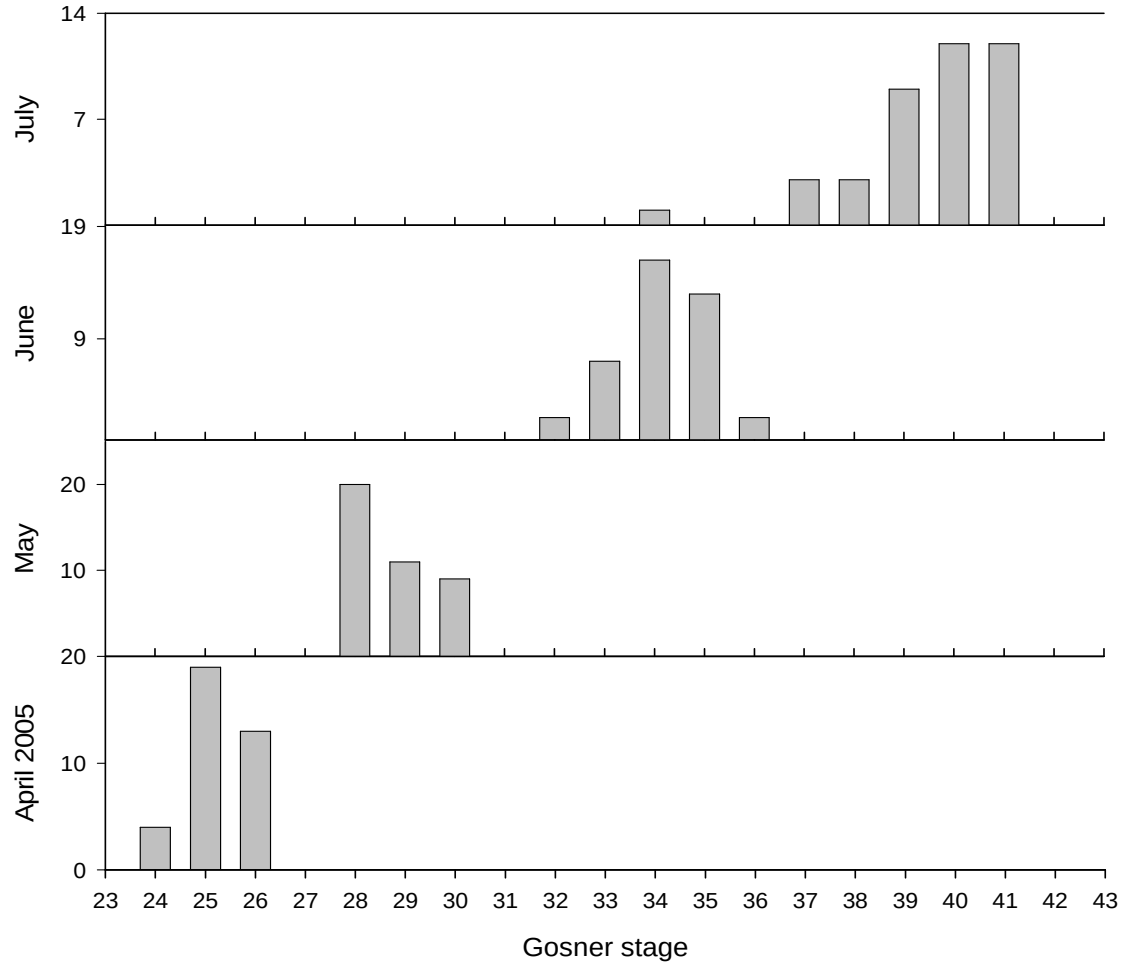
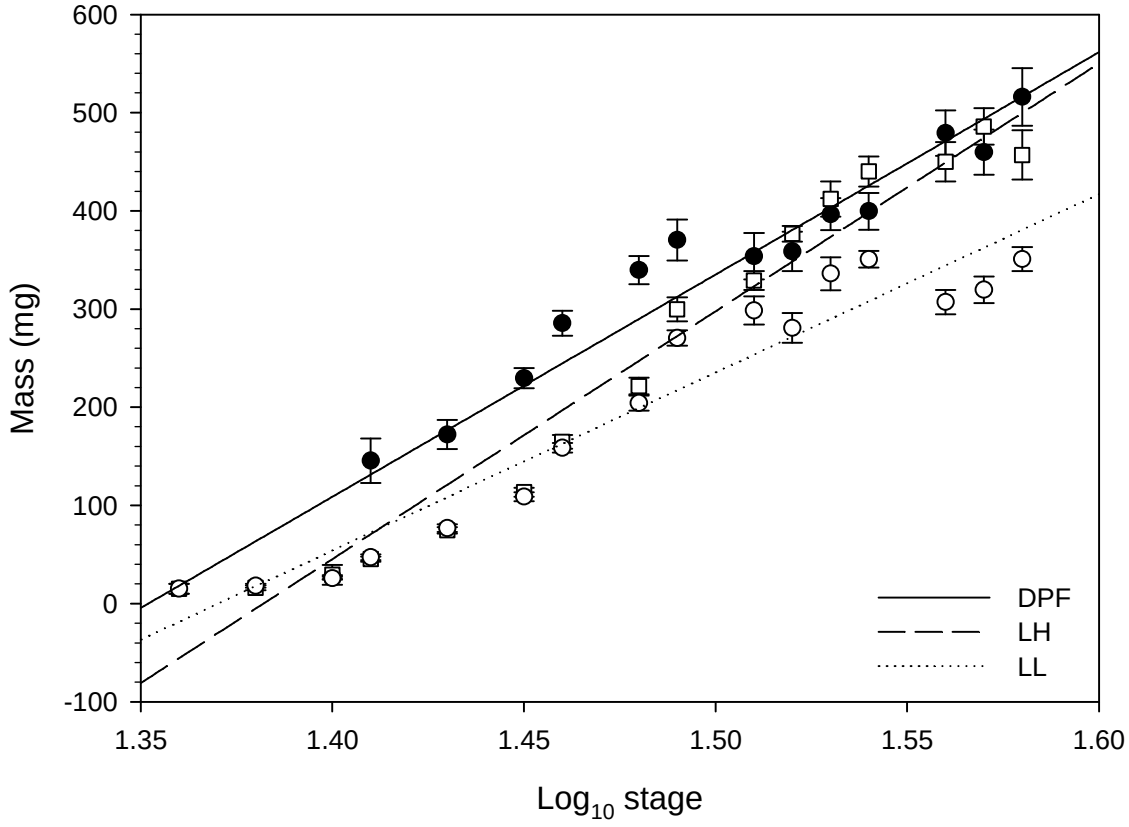


Figure 6.6: Mass at a given stage for DPF, LH and LL, with SE bars. Regression lines show the increase in mass through developmental stages (●: DPF : □: LH ○: LL).



Chapter 7: The effect of water depth on development and metamorphosis in two anuran larvae: *Rana temporaria* and *Bufo bufo*

Abstract

Water depth can impact on the growth and/or development of some aquatic organisms, particularly in animals that are able to utilise atmospheric oxygen. In amphibian larvae that develop functional lungs, greater depth may increase the cost of surfacing to inhale air, thus reducing growth and/or development. However, the effect of water depth on a lung-less tadpole species has not been assessed. I examined the effect of water depth during rearing on growth, development, time to metamorphosis and behaviour in the larvae of two British anuran species, *Rana temporaria* (lunged larvae) and *Bufo bufo* (lung-less larvae). Both species showed an increase in the time taken to commence metamorphosis with increased depth. *B. bufo* also showed a reduction in development rate by week 5, while *R. temporaria* did not. *Rana temporaria* were heavier in the deep treatment by week 5, indicating a higher growth rate at the greater depth. Both species were observed to travel to the surface to gulp air. This behaviour generally increased with developmental stage and occurred more frequently in the shallow tanks. The greater frequency of surfacing events in the shallow tanks was accompanied by a shorter duration of time at the surface. These results demonstrate that when reared in deeper water tadpoles take longer to reach metamorphosis. However, the fact that *B. bufo* delayed the onset of metamorphosis and *R. temporaria* reached a larger size in the deep treatment group suggests that water depth might not solely function as a constraint, due to increased surfacing costs. Greater water depth may indicate a more permanent habitat, instigating a life history decision to remain in the aquatic environment as tadpoles longer in order to reach greater metamorphic sizes. While greater water depth

may contribute to incidences of larval over-wintering in *Rana temporaria*, it does not explain why it occurs in *Rana* and not *Bufo* in the UK when both experience similar environments during the larval growth season.

Introduction

Water depth is an important abiotic factor for a number of aquatic species in determining life history characteristics, including growth and development rate (Arunachalam et al. 1976; Pandian & Vivekanandan 1976; Kramer & McClure 1981; Feder & Moran 1985; Pandian & Marian 1985a), survival (El Sayed et al. 1996), and predation-risk (Peterson et al. 1992; Rypel et al. 2007). The majority of studies on the effects of water depth have focused on growth and/or development of aquatic species or life stages that, in addition to acquiring oxygen from the surrounding water, come to the surface to breathe air. It is believed that, although atmospheric air represents a richer and more easily acquired source of oxygen than water (Schmidt-Nielsen 1979), there are likely to be costs inherent in the action of surfacing to breathe that influence the frequency and advantage of surfacing trips. Energy consumed during trips to and from the surface for respiration has been shown to carry a substantial cost (Kramer & McClure 1981; Kramer 1983). Other costs may involve increased risk of predation during surfacing and loss of time for other activities (Kramer & McClure 1981).

The constraints on development and growth of aquatic animals associated with the costs of surfacing are predicted to increase with depth, and this has been documented in a number of fish (particularly catfish) and amphibian species (Arunachalam et al. 1976; Pandian & Vivekanandan 1976; Kramer & McClure 1981; Feder & Moran 1985; Pandian & Marian 1985a). These costs could have a substantial impact on life history strategies in aquatic species that breathe air. For example, pond depth (Appendix 1) in conjunction with other factors (e.g. temperature and food availability: Chapter 5) may be linked with over-wintering in anuran larvae that are facultative air-breathers (e.g. *Rana temporaria*: Archibald & Downie 1996).

In previous studies on the effect of depth on growth and development in amphibian larvae, it has been assumed that surfacing behaviour was solely related to air breathing. However, surfacing behaviour in tadpoles is not ubiquitous and the role of surfacing in respiration has been questioned. Surfacing may instead function as a method of maintaining neutral buoyancy (Guimond & Hutchison 1972; Guimond & Hutchison 1973; Gee & Waldick 1995; Rondeau & Gee 2005). Additionally, the impacts of water depth on amphibian larval growth and development may not function solely as a constraint associated with the cost of surfacing. Larval amphibians may use water depth as an indicator of habitat permanence or desiccation risk, which has been demonstrated to affect the duration of the larval period (Laurila & Kujasalo 1999; Loman 1999; Merila et al. 2000b; Loman 2002; Doughty & Roberts 2003; Loman & Claesson 2003). Deeper water may indicate a more permanent habitat, which would allow more time for tadpoles to develop and attain greater size (Wilbur & Collins 1973).

Previous work has also shown that the strategies used to cope with increased depth in closely related species can differ substantially. Work on two *Rana* species (*R. tigrina* and *R. pipiens*) has shown an opposite behavioural response in surfacing behaviour with depth. *R. tigrina* surfaced more frequently at greater depths, resulting in a daily distance travelled of 1.458 km (Pandian & Marian 1985a), while *R. pipiens* surfaced to breathe more frequently in shallow water (Feder & Moran 1985). The increase in surfacing frequency with greater aquaria depth observed in Pandian & Marian (1985a) might be due to the decrease in surface area in deeper tanks, reducing the exchange of oxygen between the air and water. A similar difference, in the surfacing frequency relationship with depth, was found between two fish species *Heteropneustes fossilis* (Arunachalam et al. 1976) and *Ophiocephalus striatus* (Pandian & Vivekanandan 1976). This difference may be explained by the fact that the species

that increased surfacing frequency with depth was an obligate aerial respiring species while the other was facultative. Therefore it is important to establish the behavioural response to depth, in conjunction with growth and development rate, of species that differ in their source of oxygen.

The effect of water depth on growth, development and behaviour has not been assessed in the larvae of an amphibian species that does not incur the costs of surfacing to respire. *Rana temporaria* develop lungs as tadpoles, and, like other ranids, are believed to be facultative air-breathers (Noland & Ultsch 1981). Alternatively, *Bufo bufo*, like other bufonids, does not develop lungs until just prior to the onset of metamorphosis (Savage 1952) and is believed to rely solely on aquatic respiration throughout larval development. Therefore, *B. bufo* would be expected not to carry the respiratory costs of depth, and growth and development should not be constrained when reared at greater depths. However, in *R. temporaria*, growth and development would be predicted to be affected by greater depths. It is also of interest to compare the effect of depth on growth and development between these two species, since in the UK over-wintering of larvae occurs in *Rana temporaria* (Archibald & Downie 1996; Chapter 6) but has not been reported in *Bufo bufo*.

In this study, I investigated the effect of depth on growth rate and development pattern in the larvae of the two aforementioned British anuran species; one that has a facultative aerial respiring larval stage (*Rana temporaria*), and one that has obligate aquatic respiring larvae (*Bufo bufo*). The time taken to commence metamorphosis was also assessed to examine the effects of potentially increased costs of greater water depth on life history strategies. I assessed the behavioural response of surfacing in both species throughout larval development and into metamorphosis at two different depths.

Methods and Materials

Eggs were collected from Glasgow, Scotland, UK in February 2007 (*Rana temporaria*) and in April 2007 (*Bufo bufo*). After collection eggs were placed in 40 L tanks, at 16ºC, until approximately stage 25 (Gosner 1960) when samples of larvae were divided to be housed separately for two experiments to: 1) examine growth and development; and 2) observe surfacing behaviour.

Experiment 1: Growth & development

To investigate the effect of depth on the growth and development rate of *Rana temporaria* and *Bufo bufo*, two depth treatments were used, shallow and deep, both with 5 tanks per treatment. In the shallow treatment, 10 individuals were maintained in rectangular tanks filled with 12 L of water 20 cm deep (20 cm length x 30 cm width; tadpole density: 0.83 tadpoles L^{-1}). In the deep treatment, 10 individuals were maintained in rectangular 60 L tanks filled with water 100 cm deep (20 cm length x 30 cm width; tadpole density: 0.17 tadpoles L^{-1}). Tanks from both treatments were kept at 25ºC in a 12 L : 12 D photoperiod, fed 9% of total body mass of 3:1 rabbit pellet : fish flake mixture three times a week. All tanks were lined with a shallow layer of gravel and filled with de-chlorinated, copper-free water. In all tanks, the water was aerated continuously with a small air-stone to avoid severe hypoxic conditions. This would be expected to reduce the need for surfacing to acquire sufficient oxygen.

Growth rate was determined by the wet mass and snout-vent length (SVL) reached approximately 5 weeks after the start of the experiment in both *Rana temporaria* (day 31) and *Bufo bufo* (day 36). Wet mass was measured using an electronic balance accurate to 0.1 mg. SVL was measured using callipers accurate to 0.1 mm. Development rate was taken as the mean Gosner (1960) stage reach after

approximately 5 weeks in each species. The number of individuals commencing metamorphosis was recorded weekly.

Experiment 2: Behavioural observations

Investigation of the surfacing behaviour of both species was performed separately from the growth and development experiments, so that a known individual's surfacing behaviour could be recorded. In order to ensure that tadpoles at different developmental stages were available throughout the experiment, individuals were distributed randomly into several 20 cm tall tanks (2.08 tadpoles L^{-1}). In both species, observations were made across all developmental stages from 26 – 46. However, due to limitations in the number that could be observed, similar developmental stages were grouped in analysis: stages 26 – 30 (small hind-limb buds); stages 31 – 35 (differentiation and flattening of hind-foot); stages 36 – 40 (separation of toes and development of hind-legs); and stages 41 – 46 (metamorphic climax).

Observation tanks were constructed with the same dimensions as the shallow and deep tanks in the growth experiments, but divided into six equal vertical cells (10 cm x 10 cm). Each cell was aerated, with a small air-stone, for 24 hours after tadpoles were introduced to the tank (one tadpole per cell) and the air stones were removed 4 hours prior to observations being made. This was done to minimize variation in the oxygen concentration in the observation tanks between trials. Feeding was performed 24 hours prior to observations being made, to ensure that hunger level did not influence surfacing behaviour between trials. Observations were conducted for 1 hour, during which time the number of surfacing events, and the duration at the surface were recorded. Not all trips towards the surface resulted in individuals breaking the surface with their mouths or nostrils, but only those occasions, where the surface was broken,

were recorded. A tadpole was considered to be at the surface if it remained within 5% of the total depth from the top after reaching the surface, and at the bottom when any part of the tadpole was in contact with the bottom of the tank.

Tadpoles were tested at the two depths consecutively, so on completion of the first observation period the six individuals from the 100 cm tank were transferred to the 20 cm tank and *vice versa*. Wet mass, SVL and stage were recorded before observations. Oxygen concentration at the surface and at the bottom of the observation tanks was measured after observations were made, using a Whatman DO400 oxygen meter. Oxygen concentrations did not vary significantly between trials ($Z = 1.16$, $p = 0.144$). The oxygen concentration was not significantly different between the two observation tanks ($F_{1,32} = 1.34$, $p = 0.256$), or between the oxygen concentrations at the top compared to the bottom of the tanks ($F_{1,32} = 3.32$, $p = 0.079$). The concentration of dissolved oxygen in the observation tanks was 6.62 ± 0.23 parts per million (ppm).

Statistical Analysis

All analysis was performed using SPSS v15 (SPSS Inc., Chicago, IL) and all data are presented as means ± SE. Comparisons of size and developmental stage between the two depth treatments were performed using t-tests or unequal variance t-tests. Survival analysis was used to analyse differences in the time to metamorphosis between the two depth treatments, using the time until the onset of metamorphic climax (i.e. fore limb emergence: Gosner stage 42) as the survival variable. Analysis of surfacing frequency between depth treatments was performed using Mann-Whitney tests and among the developmental stage groupings using the Kruskal-Wallis test. Due to the number of tests performed on the surfacing frequency between the depth treatments for the four

stage groups, the results of the analyses were Bonferroni corrected to avoid type I errors.

Results

Experiment 1: Growth & Development

The starting mass, SVL and developmental stage were not significantly different between the two treatments for either *Rana temporaria* or *Bufo bufo* (Table 7.1). Growth to week 5 in *Rana temporaria* was affected by water depth. The deep treatment had on average 14% greater mass and 3% longer SVL. However, differences between the two treatments were only significant in mass, not SVL (Table 7.2). In *R. temporaria* developmental rate was not significantly different between treatments (Table 7.2). *Bufo bufo* did not show a significant difference in growth to week 5 (Table 7.2) but the average developmental stage was 7% lower in the deep treatment compared to the shallow treatment (Table 7.2).

In both species the median time to metamorphosis was significantly different between the two treatments (*R. temporaria*: Wilcoxon Gehan statistic = 10.33, $p = 0.001$; *B. bufo*: Wilcoxon Gehan statistic = 20.46, $p < 0.001$, Fig. 7.1). The median time to metamorphosis from the date of hatching was approximately 34% shorter in the shallow tanks than the deep treatment in *R. temporaria*, and 21% shorter in *B. bufo*.

Experiment 2: Behaviour observations

Surfacing behaviour in *Rana temporaria* was largely consistent, with individuals leaving the bottom and swimming rapidly directly towards the surface. At the surface individuals would noticeably gulp air, before swimming directly to the bottom. *Rana* spent the majority of the observation time resting on the bottom of the tank. *Bufo*

surfacing behaviour was more variable, but generally individuals would swim a meandering path towards the surface. After breaking the surface, individuals generally drifted to the bottom. *Bufo* also spent considerably more time hanging from the sides of the tanks than did *Rana*.

The frequency of trips to the surface differed with developmental stage in both species, with the exception of *B. bufo* at 1 metre depth (Table 7.3). Both species surfaced significantly more frequently at 0.2 m depth than at 1 m deep, for all stage groups except during metamorphosis (stages 41 – 46) in *Rana* and during the early larval stages in *Bufo* (stages 26 – 30; Table 7.3). In both species the surfacing frequency increased through larval development, during which time mass and stage were significantly correlated ($r^2 = 0.85$, $p < 0.0001$), until the onset of metamorphosis. *Rana* surfaced more frequently than *Bufo* at 1 metre depth, but the difference was only significant between stages 31 – 40 (stages 31-35: $Z = -2.40$, $p = 0.017$; stages 36-40: $Z = -2.56$, $p = 0.012$).

The time spent at the surface at each surfacing event generally did not differ among stage groups (*R. temporaria* – 1 m: $F_{3,52} = 0.20$, $p = 0.898$; *B. bufo* – 1 m: $F_{2,21} = 1.29$, $p = 0.297$; 0.2 m: $F_{3,62} = 2.07$, $p = 0.114$), with the exception of at 0.2 metre depth in *Rana* ($F_{3,68} = 3.78$, $p = 0.014$). At 0.2 m, *Rana* individuals increased the time spent at the surface with developmental stage until stage group 36 – 40. *B. bufo* individuals spent five times longer at the surface in the 1 metre tank (43.33 ± 17.46 sec) than when in the 0.2 metre tank (8.31 ± 1.83 sec; $t = 4.41$, $p < 0.0001$). The same was also true of *Rana* (1 m: 355.15 ± 70.38 sec; 0.2 m: 143.73 ± 76.36 sec), with the exception of stages 41 – 46 where there was no significant difference in time spent at the surface ($t = 1.52$, $p = 0.267$). *R. temporaria* spent significantly longer at the surface than *B. bufo* at both depths (1 m: $F_{1,71} = 33.42$, $p < 0.0001$; 0.2 m: $F_{1,71} = 11.23$, $p = 0.001$).

Discussion

The results of the current study demonstrate that water depth influences tadpole growth and development independently, with greater depth resulting in slower development in *Bufo bufo* and faster growth in *Rana temporaria* by week 5, and a prolonging of the median time to commence metamorphosis in both species. While the effect of depth in prolonging the onset of metamorphosis has previously been demonstrated in a ranid species (*R. pipiens*: Feder & Moran 1985), this study, for the first time, demonstrated an effect of increased depth on development in bufonid (*B. bufo*) larvae. Surprisingly, in the current study, developmental rate in *B. bufo* responded more strongly to increased depth than in *Rana temporaria*. This study has also confirmed that *B. bufo*, as well as *R. temporaria*, tadpoles travel to the water's surface. In both species, when observed at greater depth, tadpoles reduced the frequency of trips to the surface. This was accompanied by an increase in the duration of time spent at the surface when the trips were made. The frequency of trips to the surface changed during larval development, with both species surfacing more frequently as development progressed, until just prior to metamorphosis.

By week 5, *B. bufo* tadpoles reared in shallow tanks demonstrated a faster rate of development than those reared in the deep treatment. In *R. temporaria*, the rate of development at week 5 was not significantly different between the shallow and the deep treatment individuals. The median time to metamorphosis, in both species, was longer in the deep treatment. Tadpoles of both species, during the observation experiments, varied their surfacing behaviour with depth in a way that would be expected to mitigate many of the energetic costs of surfacing behaviour. This was achieved by reducing the number of trips to the surface, but spending more time at the surface, when at a greater depth (Arunachalam et al. 1976; Feder & Moran 1985; Shannon & Kramer 1988,

exceptions Pandian & Vivekanandan 1976; Pandian & Marian 1985a). The reduction in development rate and the adoption of this mitigation behaviour does, therefore, suggest a cost to surfacing behaviour; but that, even with costs, surfacing is essential, even in normoxic water, by anuran larvae of species that are lung-less or facultative air-breathers. However, the effect of depth in reducing development rate was not predicted to occur in *B. bufo*, as larvae in this species are normally capable of obtaining all their required oxygen from the water, even under mildly hypoxic conditions (Feder 1983a). This indicates that, in anuran larvae, respiration might not be the sole reason for travelling to the surface (Ultsch et al. 2004).

While facultative air breathing in bimodally respiring species is still likely to be an important function in hypoxic conditions, there may be other explanations for the commonly observed relationship between water depth and developmental rate. Several studies have shown that access to the surface is important for normal development and successful metamorphosis in species that develop lungs during the larval phase (Pronych & Wassersug 1994; Gdovin et al. 2006) and that surfacing is performed to maintain lung inflation (Feder & Wassersug 1984; Crowder et al. 1998; Ultsch et al. 2004). Yet, even without functional lungs, *Bufo* larvae were commonly observed travelling to the surface and apparently gulping air in this study. Willem (1920) (translation cited in Jorgensen 2000), who also reported surfacing behaviour in *B. bufo*, found that in dissections air was present in the intestinal tract. In tadpoles the intestinal wall is very thin and consequently not much of a barrier to oxygen diffusion; therefore the presence of air in the gut of *Bufo* could serve a respiratory function, as some teleost fish have also been found to use the intestine for respiration (Graham 1997). Alternatively, an important function of travelling to the surface may be to obtain air to achieve neutral or positive buoyancy, thereby reducing the energetic costs of

maintaining position in the water column. This may allow *Bufo* to remain nearer the surface where oxygen concentration may be greater or the water is warmer to speed development (Noland & Ultsch 1981). However, retaining air internally has also been demonstrated to carry cost, such as increased drag and susceptibility to predation (Feder 1983b). The ability of individual tadpoles to balance the costs of travelling to the surface and the advantages that are gained may therefore be state-dependent. It has been shown that hunger state influences the time spent at the surface in the bimodally respiring fish *Heteropneustes fossilis* (Arunachalam et al. 1976) and the adult anuran *Xenopus laevis* (Boutilier 1984; Shannon & Kramer 1988). It is also likely that the presence of predators would alter the trade-offs in surfacing behaviour with different depths, since extending the time at the surface will make tadpoles vulnerable to surface predators as well as aquatic predators (Kramer et al. 1983; McIntyre & McCollum 2000) and the retention of air for buoyancy might become less advantageous (Feder 1983b).

Water depth might not only function as a constraint, but also influence a decision on development rate since tadpoles might be able to directly assess water depth (Denver 1997). A water body of greater depth is likely to have a longer hydroperiod (Brooks & Hayashi 2002; Skidds & Golet 2005) and has also been shown to reduce the risk of predation in some species (Peterson et al. 1992). Both hydroperiod and predation risk have been shown to influence the duration of the larval period. The reduced risk of predation and desiccation would allow individuals to exploit the aquatic habitat for longer, lengthening the larval period, so that they could obtain a larger size (Wilbur & Collins 1973). This hypothesis is partially supported by the data, since by week 5 *Rana* reached a significantly larger size at the expense of a small, but not significant, decrease in development rate. *Bufo* tadpoles did not differ in size, at week

5, between the two treatments, so they did not appear to benefit from the slower development that occurred when reared at 1 metre depth.

The surfacing frequency in both species was affected by the developmental stage of the tadpoles. Generally, surfacing frequency increased as the tadpoles progressed through larval stages until just prior to the onset of metamorphosis, when the rate of surfacing dropped considerably. This was expected in *Rana*, and several other studies have shown an increase in surfacing with increases in size, age or developmental stage in a range of facultative air breathing aquatic species (Babiker 1979; Burggren & West 1982; Hillman & Lea 1983; Burggren & Doyle 1986; Wong & Booth 1994; Wilson & Franklin 2000). The greater frequency of surfacing events has been linked to the greater demand for oxygen with larger size and/or coinciding with the timing and progression of developing aerial respiring apparatus. Again this cannot explain why the same trend was observed in *Bufo*, since there is no lung development. It has been shown that even without lungs bufonids can acquire more oxygen through the skin, in hypoxic conditions, by travelling to the more oxygen-rich surface water (Wassersug & Seibert 1975). This seems unlikely to be the case in the current study since in both the deep and shallow observation tanks the dissolved oxygen concentration at the bottom was not significantly lower than that at the water surface and was more than double the concentration that forced *Bufo woodhousii* to surface in Wassersug & Seibert's (1975) experiments. However, the increase in surfacing with development also seems to accord with other results in the current study suggesting that surfacing might be linked with maintaining neutral buoyancy. As development progresses, mass increases until just prior to the onset of metamorphosis, therefore it might take a greater frequency of trips to the surface for air to offset the larger sizes (Power & Walsh 1992) of more developed tadpoles.

The surfacing frequency and time spent at the surface differed between the two species, with *Rana* surfacing more frequently and spending more time at the surface than *Bufo*. This would be expected, since in addition to the reason shared by both species for surfacing (i.e. maintaining buoyancy), *Rana* might need to maintain lung inflation and can take advantage of air breathing (Feder & Wassersug 1984; Crowder et al. 1998; Ultsch et al. 2004).

Greater depth may also be linked to the occurrence of over-wintering as larvae in *Rana temporaria* in the UK (see Appendix 1). While a greater depth does result in delayed metamorphosis, it does not appear to explain why over-wintering occurs in *R. temporaria* tadpoles and not *B. bufo* tadpoles. However, a greater depth, in addition to indicating a more permanent habitat or acting as a constraint on development leading to over-wintering of larvae, might allow higher survival of larvae during the winter (Bradford 1983). The higher survival of larvae into the winter may explain why over-wintering tadpoles are more commonly observed in deeper ponds.

In conclusion, the costs of surfacing to breathe air do not seem to fully explain the differences in growth and development rate observed in amphibian larvae when reared in greater water depth, since the same trend is observed in a lung-less species. Additionally, under controlled conditions, tadpoles seem to mitigate the costs of travelling to the surface from a greater depth. In light of these results, reduction in development rate with greater water depth might be a variable response of larvae to a more permanent, low risk habitat. There appears to be a number of reasons why amphibian larvae travel to the water's surface, but the occurrence of *Bufo bufo* travelling to the surface observed in our study, indicates that surfacing may play a greater role in maintaining neutral buoyancy than previously thought.

Tables and figures

Table 7.1: Starting mass, SVL and developmental stage of different depth treatments for both *R. temporaria* and *B. bufo*, including results for t-tests for comparison between the depth treatments (df = 98).

		Mass (mg)	SVL (mm)	Gosner stage
Rana	Shallow	47.2±2.0	5.93±0.09	26.0±0.9
temporaria	Deep	46.5±1.0	5.86±0.10	26.2±0.9
	t	0.25	0.58	-1.65
	p	0.81	0.57	0.10
Bufo bufo	Shallow	12.9±0.2	4.22±0.04	24.6±0.1
	Deep	12.9±0.3	4.24±0.05	24.6±0.1
	t	-0.05	-0.33	0.40
	p	0.96	0.75	0.69

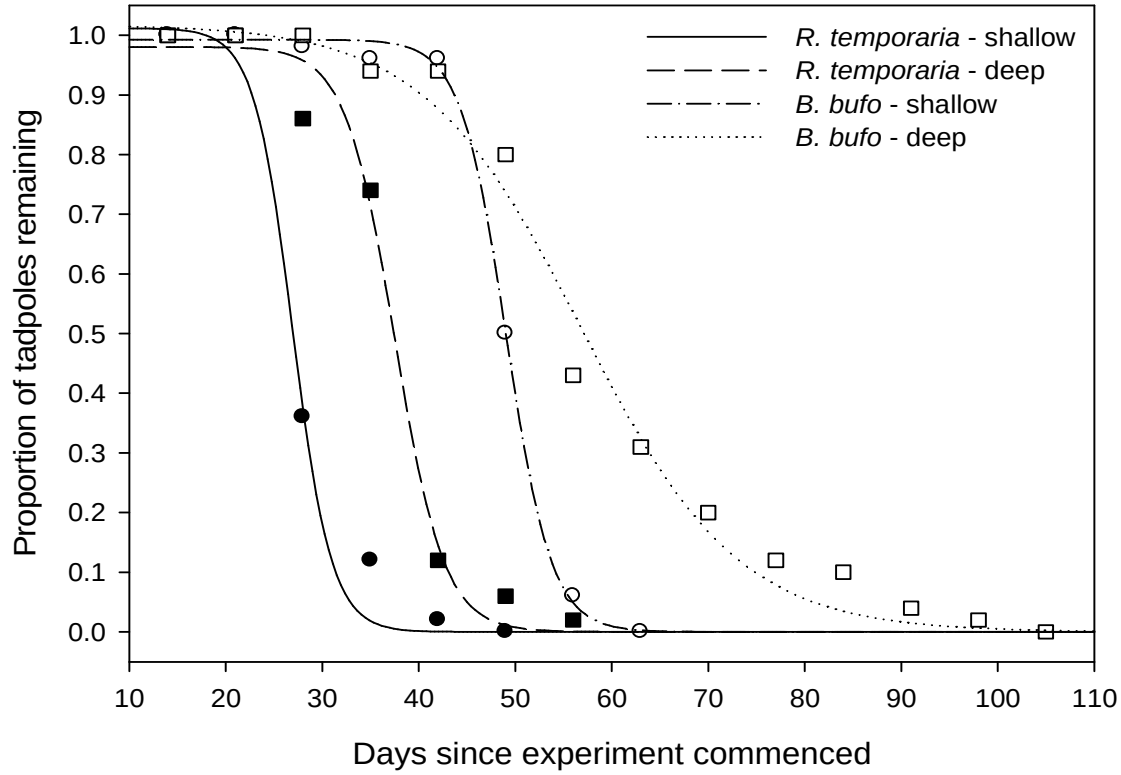
Table 7.2: Mean mass (mg), SVL (mm) and Gosner stage of *Rana temporaria* (day 31) and *Bufo bufo* (day 36) for the shallow and deep treatments, including results for t-tests for comparison between depth treatments. Differences in the df between analyses is due the use of unequal variance t-tests (NS: not significant, * < 0.05, ** < 0.001).

	Rana temporaria				*Bufo bufo*			
	Shallow	Deep	df	t	Shallow	Deep	df	t
Mass	243.6±9.0	276.5±10.0	48.82	-2.46*	86.3±1.0	89.1±4.0	58.82	-0.71^{NS}
SVL	11.3±0.2	11.6±0.1	53.00	-1.39^{NS}	8.4±0.1	8.3±0.1	63.30	0.86^{NS}
Stage	37.6±0.5	37.3±0.3	53.00	0.46^{NS}	35.3±0.1	33.0±0.3	59.05	6.85**

Table 7.3: Mean frequency of trips to the surface (trips h^{-1}) in *Rana temporaria* and *Bufo bufo*, for each stage grouping, in 0.2 m and 1 m deep tanks. Values are given as mean ± SE. Analyses from Mann-Whitney test of differences between the depth treatments at each stage group are given (Z) (NS: not significant, * < 0.05, ** < 0.005). P value thresholds are based on the Bonferroni corrected α–values. Analyses from Kruskal-Wallis H tests comparing the different stage groups within a depth treatment are presented as the X^2 value.

		Developmental stage group				
	treatment	26 - 30	31 - 35	36 - 40	41 - 46	X^2
Bufo bufo	0.2 m	4.5 ± 1.0	8.5 ± 1.4	11.8 ± 3.0	3.0 ± 0.7	11.74***
	1.0 m	1.8 ± 1.0	1.3 ± 0.4	1.4 ± 0.6	0.0 ± 0.0	7.05^{NS}
	Z	-2.46^{NS}	-3.48*	-3.25*	-3.31*	-
Rana temporaria	0.2 m	15.8 ± 4.9	19.9 ± 5.4	16.6 ± 3.3	1.5 ± 0.8	20.73***
	1.0 m	1.8 ± 0.6	3.0 ± 0.6	2.8 ± 0.6	0.2 ± 0.1	18.69***
	Z	-2.83*	-3.94**	-4.05**	-1.49^{NS}	-

Figure 7.1: Proportion of tadpoles that have not begun metamorphosis for both *R. temporaria* (solid shapes) and *B. bufo* (open shapes) for each treatment (●: shallow treatment; ■: deep treatment).



Chapter 8: General discussion

The aim of this thesis was to address several aspects of the seasonal timing and the duration of metamorphosis in relation to environmental conditions. Metamorphosis, in addition to occurring in amphibians, occurs in the majority of marine invertebrates, many insects and even some fish species. It has also recently been questioned whether metamorphosis occurs in plants (Bishop et al. 2006); however, this will not be considered in this discussion. The timing and duration of metamorphosis can have important implications in many of these species. I have demonstrated that the duration and progression of metamorphic climax in anurans varies with abiotic (temperature) and biotic (predation risk and body size) factors (Chapters 2 and 3) and that there may be costs, expressed in juvenile morphology, associated with accelerating development in this life phase (Chapter 3 and 4). I explored some of the factors that may contribute to the postponement of metamorphosis until after winter as opposed to the summer or autumn following the spring spawning (Chapters 5, 6 and 7). I also investigated the possible adaptive nature of spending winter as an aquatic larva, but this could not be definitively determined over the course of this study (Chapter 5 and 6). In this chapter I will briefly discuss these results, including some limitations to their interpretation, and place the results within the broader literature on variation in life histories. I will also discuss some avenues for further work, relating to questions raised by the results presented and by studies piloted during this research.

Phenotypic plasticity is a widely occurring phenomenon in organisms (e.g. variation in age at maturation: Cole 1954; variation in life history pattern of development in salmon: Metcalfe et al. 1988), but some traits show little variation with environmental conditions. Plasticity is not expected to occur in traits where precise

timing of developmental events is essential for successful development or survival. Therefore certain developmental processes (e.g. early embryogenesis: Smith-Gill 1983; and nervous system development: Nishikawa 1997) are canalized and progress with little variation in response to environmental conditions. For example, in early embryogenesis, to ensure the required timing of events, the embryo is buffered from the environment (e.g. jelly envelop in anuran eggs) and the genes that regulate early embryogenesis are highly redundant (Flickinger 1970). Similarly it has been shown by Sultan (1995) that, even under severe environmental conditions (low light levels), the plant *Polygonum persicaria* did not vary the resources allocated to producing the fruit, but did reduce the resources allocated to the fruit casing. Additionally, the timing of variation in environmental conditions will affect the degree of plasticity produced in traits. Only tissues that are metabolically active at the time of an environmental perturbation will be sensitive and capable of responding to those environmental conditions (Zwilling 1955). Also in order for an organism to display plasticity in the developmental response to environmental conditions, the organism must have a mechanism for detecting the prevailing conditions. While early embryogenesis is highly canalized, it has been demonstrated that embryonic development can respond to changing environmental conditions. Warkentin (1995), using the red-eyed tree frog, demonstrated that individuals hatch earlier in response to the presence of the snake *Leptodeira septentrionalis* that preys on eggs.

The potential for the metamorphic climax of anurans to change in timing or duration with environmental conditions might have been ignored due to a belief that metamorphic climax was canalized. It has been proposed that, as a highly vulnerable life stage, metamorphosis would be selected to progress as fast as possible. Also, it was believed that the timing and order of developmental events during metamorphosis were

fixed (see Nodzenski & Inger 1990), and possibly important for successful completion. However, there are several reasons why plasticity might be favoured during metamorphosis. During the transition from a larva to a juvenile (metamorphosis), tissues within an individual are very metabolically active. Therefore, the new tissues being constructed during metamorphosis are likely to respond to environmental variation. Furthermore, in anurans, individuals are not buffered from the environment during metamorphosis. Similarly, the developmental phase that many temperate animals reach by the onset of winter will have an impact on an individual's survival. However in species that are capable of surviving winter at more than one life stage, plasticity in the pattern of life history development may be expected.

Metamorphic climax

The results presented in the first half of this thesis (Chapters 2, 3 and 4) clearly demonstrate that metamorphic climax (which, in amphibians and other organisms with complex life cycles, usually allows the transition from the larval to the juvenile phase) is itself a developmental phase that can vary in response to environmental conditions. Much like other early ontogenetic life stages, the duration of metamorphosis appears to be the result of trade-offs between the costs and benefits relative to the environmental circumstances experienced during metamorphosis (Wilbur & Collins 1973). The results in this thesis demonstrate that metamorphic climax is accelerated in response to perceived predation risk, and takes longer to complete at lower temperatures and when individuals are of greater size at the onset of metamorphosis. I have also demonstrated that there are potential costs associated with rapid metamorphic development, in the form of reduced size on completion. Environmental responsiveness in the duration of the larval phase across a range of taxa has commonly been reported. However, given

the prodigious attention focused on plasticity in the larval phase in amphibians and little appreciation or recognition of the subsequent metamorphic stage as a phase of the life history that merits consideration, it is surprising that there is not a consensus on the inclusion or exclusion of metamorphosis from the 'larval period'. Yet treatment during metamorphic climax is consistently different from treatment conditions during pre-metamorphic larval development.

I have surveyed 98 studies relating to the variation in the duration of the larval period in anuran amphibians alone (Table 8.1); 68% used the emergence of the fore limbs (the start of metamorphic climax; Gosner stage 42), while 16% used the absorption of the tail (the completion of metamorphic climax; Gosner stage 46) as the conclusion of the larval period. Of those studies that concluded that the larval period ended at stage 42, a quarter continued and used the resulting juveniles to relate larval conditions to juvenile performance or traits (16 studies). All removed metamorphosing individuals from treatment conditions and only three included data about metamorphic climax. Furthermore, even in the majority of the 16 studies that included metamorphic climax in their 'larval period', individuals were removed from experimental treatments during metamorphic climax. The remaining 13% of the studies used either mid-metamorphic climax (Gosner stage 43-45; ten studies), both points (stages 42 and 46; 3 studies), or did not clearly state a method for how they determined the end point of the larval phase (2 studies). Half of the ten studies that used mid-metamorphosis as the end of the larval period were conducted by Loman and colleagues (Loman 1999; Loman 2001; Loman 2002; Loman 2003; Loman & Claesson 2003) and were estimating the end of the larval period in the field.

This literature review highlights several concerns related to studies conducted into the variability of amphibian life histories. Firstly, while all of the 98 studies

reviewed pertained to the timing of the completion of the larval phase, there was variation in how this was defined. The majority of the studies recognised fore limb emergence as the conclusion of the larval phase, but there were still a significant number that included all or part of metamorphic climax in the 'larval period'. Secondly, the majority of the studies that used fore limb emergence as the conclusion of the larval phase did not consider factors during metamorphic climax as having an influence on juvenile traits. Of the 98 studies, only three included data pertaining directly to metamorphic climax distinct from the larval period. Finally, in studies that used the conclusion of metamorphosis as the end of the larval period and those that tested juvenile traits after experimental treatment during larval development, there was an almost total disregard for what happens during metamorphosis. In these studies, metamorphosing individuals were removed from the larval tanks and placed in smaller isolated tubs out-with the experimental treatment conditions until complete tail absorption. Yet in some studies the time to complete metamorphosis was still included in the treatment effect on larval duration. While there is an obvious necessity in removing the metamorphic individuals of terrestrial anurans (most will drown in aquaria commonly used for tadpoles), the results presented in this thesis indicate that environmental conditions experienced during metamorphosis, which can affect the duration and size on completion of metamorphosis, need to be considered.

In other taxa that undergo a clearly defined metamorphosis (e.g. many insects, crustaceans and other marine invertebrates) there has also been substantial effort focused on the variability in the timing of the initiation of metamorphosis (Harms 1992; Armitage et al. 1995; Twombly 1996; Nylin & Gotthard 1998), but little attention on metamorphosis itself. In insects, metamorphic climax, occurring in the pupal stage, has commonly been shown to be variable in duration in response to environmental

temperature (e.g. Nielsen & Evans 1960) but few other factors have been investigated. Additionally, the duration of insect pupation is often complicated by diapause, a physiological state of dormancy usually occurring during unfavourable conditions, or used to synchronise developmental stages for reproduction. Diapause can occur at all insect life stages, but commonly occurs during pupation (Gullan & Cranston 2005), and can be controlled by a number of factors (e.g. temperature, photoperiod and resource availability: Leather et al. 1993) that would also be expected to influence the duration of larval and metamorphic development. Comparisons between studies of insect pupation duration, determined by diapause, and amphibian metamorphosis may be limited as diapause is usually accompanied by developmental arrest and relies on distinct environmental cues to re-initiate development (Danks 2000), neither of which have been reported to occur in amphibian metamorphic climax. However, diapause has been reported to occur in some vertebrates (e.g. embryonic diapause in annual fish: Wourms 1972; and freshwater turtles: Ewert 1991).

The results reported in this thesis suggest that there might be some degree of flexibility in the progression of metamorphosis. It is believed that, given the apparent consistency in the sequence of events during metamorphosis among species, the order of transformation events is fixed (Etkin 1936). This has allowed the formulation of the numerous staging tables based on external morphological features used in identifying anuran development (e.g. Taylor & Kollros 1946; Limbaugh & Volpe 1957; Gosner 1960). The determination of the sequencing of events is believed to relate to the sensitivity of particular tissues or organ systems to thyroid hormone (Etkin 1935; Kollros 1961). Tissues that are most sensitive respond first at lower concentrations, but as concentrations of thyroid hormone increase less sensitive transformation events occur. It has been demonstrated, however, that Borean torrent-dwelling tadpoles, which

require specialised oral discs to maintain their position on rocks in fast moving water, retain the larval oral disc structure until near the completion of metamorphic climax (Nodzenski & Inger 1990). According to most staging tables, and in most species, the re-shaping of the mouth begins early in metamorphic climax (Gosner 1960; Nieuwkoop & Faber 1994). I have demonstrated that under stressed conditions (predation-risk) the absorption of the tail may be decoupled from other events during metamorphic climax, since a functional tail is retained for longer but more rapidly absorbed nearer the completion of metamorphosis (Chapter 4). Therefore, there appears to be some scope to alter the progression of metamorphic climax both in species that may be required to retain specialised larval traits and in cases where different environmental conditions might make it beneficial to retain a larval feature (Downie et al. 2004). Other morphometric measures (in addition to tail length and depth) that change during metamorphic climax were not measured, so it was not possible to determine whether the order of events in *Xenopus* metamorphosis was further altered when exposed to predation risk. It would be of interest to determine whether the progressions of other metamorphic events are altered when tadpoles or metamorphosing individuals are in stressed conditions.

While there may be some potential for variation in the timing and potentially the sequencing of events during metamorphic climax, it is a well-defined stage in anurans, holometabolous insects and some fish species. This may not be the case in all organisms that are considered to have a complex life cycle. There is still considerable debate about whether the changes that occur during ontogeny in many fish species can be referred to as metamorphosis (Bishop et al. 2006).

The results reported in chapters 2 – 4 provide clear evidence that morphology and the duration of metamorphosis varies, but there may be limitations in the broader

interpretation of the results. *Xenopus laevis* provides the ideal life history for a study of this nature, since it remains in the water, easily allowing non-lethal predation risk to be perceived by the use of chemical signals (Petranka et al. 1987) during metamorphosis. However, given the common transition from aquatic larva to terrestrial juvenile and adult that occurs in many amphibians and some insects there may be differences in the factors influencing metamorphic duration in those species. Even in species that move to a terrestrial environment, much of metamorphosis is completed in the water and chemical signals from predators or injured conspecifics can be perceived by terrestrial metamorphs and adults and possibly fully terrestrial species (Rajchard 2006; Meng et al. 2006). Therefore it would be expected that animals that change habitats with metamorphosis or that are fully aquatic or terrestrial would be capable of responding similarly to predation risk. It would also be expected that in accordance with my results (Chapter 4), those from studies on insect metamorphosis (Stevens 2004) and the importance of temperature on development rates (Gillooly et al. 2002), environmental temperature would influence the duration and size on completion of metamorphic climax in all amphibians.

The studies conducted and presented in this thesis represent some of the first steps in our understanding of the ecology of amphibians during metamorphosis and how the conditions during metamorphic climax can impact on subsequent life history stages. There are several avenues of future work to further our understanding of metamorphic climax. Initially, it would be important to broaden the number of species studied, to ascertain the ability of metamorphs from other species to perceive and respond to predation risk. In some tropical species metamorphic duration is very short, which may limit the scope for variation to occur and/or make it difficult for researchers to perceive any existing variation. Additionally, little is known about the behaviour of

metamorphosing individuals (Downie et al. 2004). In the study presented in this thesis temperature was even and constant in the tanks; predators were always in view of metamorphs; and no shelter was provided for the metamorphs. It would be of interest to investigate whether when presented with a thermal gradient, such as would occur in a natural pond, would individuals choose to complete their metamorphosis at a particular temperature to either maximize development rate or size on completion of metamorphosis? Also, if provided with a shelter would a difference in metamorphic duration under predation risk still be present, since metamorphs would not need to feed and could remain hidden.

Larval over-wintering

The results presented in the second half of this thesis (Chapters 5, 6 and 7) attempt to determine what factors contribute to the observed variation in the seasonal timing of metamorphosis in *Rana temporaria*, a species that can spend their first winter as either a tadpole or a juvenile. Most temperate anurans complete their larval development and undergo metamorphosis in the summer of the year they were spawned, so that there is sufficient time to rebuild energy reserves, spent during the fasting that occurs with metamorphosis, before the winter. Several species have been reported to be unable to survive as larvae in the aquatic environment over winter (e.g. Fauth 1990; Pehek 1995; Altwegg & Reyer 2003), as tadpoles may require adaptations to survive anoxia common in ponds during winter (Bradford 1983). Therefore, regardless of conditions, these species must attempt to attain a size that allows them to metamorphose, even if it is very late in the season, as a limited opportunity of terrestrial survival is advantageous compared to certainty of mortality in the aquatic environment. *Rana temporaria* is a species that is normally capable of completing its larval development within one season,

even in regions with lower summer temperatures (Miaud et al. 1999) than the United Kingdom. However, under certain conditions it may be advantageous to remain in the pond rather than metamorphose late in the season or at a small size, due to reduced survival of small or late season terrestrial metamorphs (Chelgren et al. 2006).

The size at and time to metamorphosis has been demonstrated to vary in response to several environmental factors (e.g. temperature: Niehaus et al. 2006; intraspecific competition: Travis 1984; interspecific competition: Bardsley & Beebee 1998; food quality and availability: Audo et al. 1995; predation risk: Relyea 2007; hydroperiod: Laurila & Kujasalo 1999; and UV-B radiation: Belden & Blaustein 2002). The results in this thesis have shown that reduced food availability and temperature result in slower rates of development and in a greater proportion of tadpoles over-wintering, but that there are other factors contributing to the variation in over-wintering strategy in the field. While increased depth results in reduced development rate, it does not appear, as predicted, to constrain development due to the cost of surfacing for aerial respiration (Feder & Moran 1985). Greater depth, in addition to being a factor inducing larvae to over-winter, might also allow tadpoles a better chance of surviving the winter. Deeper water provides a greater refuge from freezing and extreme fluctuations in temperature and reduces the occurrence of hypoxic conditions (Bradford 1983).

Variation in the life history stage at which individuals over-winter has been more thoroughly studied in other taxa, particularly those that are economically important (e.g. salmon: Metcalfe et al. 1988; Thorpe 1989; and pest insect species: Nylin 2001). In most non-migrating temperate insect species, the life history stage at which individuals can survive the winter is fixed and species specific, but between even closely related species the stage at which individuals must spend the winter may be different (Tauber et al. 1986). Therefore, just as in most amphibians, under poor

conditions most insects may have to accelerate their development in order to reach the appropriate stage at the start of the winter in order to survive. Most temperate insects pass the winter in a state of diapause. The timing or occurrence of diapause during the winter has been shown to vary according to environmental factors, but in many cases this appears to be influenced by maternal and genetic effects. For example, a reproducing female striped ground cricket *Allonembius socius* utilizes a bet-hedging strategy and as the season progresses gradually shifts from producing non-diapausing eggs in favour of diapausing eggs that can survive the winter (Bradford & Roff 1997). This variation is determined by maternal effects in response to environmental factors (e.g. temperature and photoperiod). However, in some insects such as the butterfly *Pararge aegeria* there is variation in the developmental stage at which over-wintering diapause occurs. Individuals can pass the winter either in the third larval instar or the pupal stage (Tauber et al. 1986), with all other larval instars unable to survive the winter (Shreeve 1986). The decision on the stage at which to spend the winter appears to be determined by temperature and photoperiod (Lees & Tilley 1980) as the season progresses, but little is known about what contributes to the differences in development rates. The results presented in this thesis, in addition to the data on salmon life history variation, suggests that conditions during the growth season will affect the decision of what stage to pass the winter for the majority of organisms with complex life cycles, including other amphibians and insects. Therefore, the conditions during the growth season can greatly influence individual survival, reproductive timing and fitness and thus the population dynamics of these temperate species.

Unfortunately, the results presented in this thesis were not able to definitively determine whether conditions during larval development induce larval over-wintering as a result of a physiological constraint on individuals or as the result of an adaptive

strategy. However, the evidence appears to suggest that larval over-wintering occurs even under environmental circumstances where normal larval development can be completed before the onset of winter (Chapter 6). Therefore, a decision to over-winter as either a tadpole or juvenile is taken at some point in the season. As the season progresses, the balance between the advantage of metamorphosing and emerging onto land and the cost associated with not having enough time to rebuild energy reserves following metamorphosis must shift. As a result of this over-wintering as larvae will become more advantageous later in the season or throughout the season if in poor larval conditions.

Currently, it is difficult to compare the adaptive benefit of the two over-wintering stages, since little is known about immediate post-metamorphic growth and survival, or about over-wintering behaviour in amphibians generally (Pinder et al. 1992). Due to the difficulty in maintaining juvenile individuals in the laboratory and a limited ability to directly track post-metamorphic individuals in the field, data on the period after metamorphosis until reproduction are scarce. Post-metamorphic growth rate is believed to be high (Turner 1960; Breckenridge & Tester 1961), but also potentially variable and will depend greatly on the time available between metamorphosis and the onset of winter (Altwegg & Reyer 2003). Therefore the differences in sizes between individuals that over-wintered as larvae and those that completed metamorphosis in the summer may be complicated by post-metamorphic growth rate. However, it is unlikely that the individuals that metamorphosed in the summer, used in the comparison with over-wintered larvae, would have been able to increase substantially in mass or SVL in the short time available between their late metamorphosis and the onset of winter. Additionally, it is believed that juvenile anurans do not feed during the winter, while tadpoles in this study and others (e.g.

Jenssen 1967) were observed feeding throughout the winter. Mortality is also expected to be very high in post-metamorphic individuals (Clarke 1974; Clarke 1977). To fully determine the dynamics of the trade-off between wintering strategies and their adaptive nature, if any, individuals over-wintering both as larvae and juveniles during their first year must be followed for longer than was possible in this study, since time to maturation in *R. temporaria* can be up to three years (Beebee & Griffiths 2000). In addition to the laboratory experiments following individuals utilizing the two alternative wintering life history stages, it would be of importance to collect field data on the behaviour and growth of metamorphs, both with respect to their wintering stage and the duration of the growing season they are able to exploit, for several seasons.

A further limitation in a study of this nature appears to be that although over-wintering larvae survive and metamorphose, only a proportion does so. In the laboratory the number of surviving over-wintering larvae was relatively small given the original numbers. Low numbers makes analysis of the post-metamorphic data limited in its application. It also makes following juveniles that have over-wintered as tadpoles difficult given the problems in maintaining *Rana temporaria* metamorphs in the laboratory (Stamper 2006). A laboratory study of this nature, examining post-metamorphic locomotion after larval over-wintering, was piloted during this thesis, but juvenile mortality did not allow sufficient data to be collected. The use of semi-natural, outdoors 'common garden' experiments may allow a method for achieving greater juvenile survival from larvae that over-winter or complete larval development in their first year. Higher juvenile survival may allow a further study that attempts to address potential costs or benefits associated with over-wintering stage on post-metamorphic traits.

In addition to requiring a greater understanding of the trade-off dynamics in post-metamorphic fitness between the life history stage at which individuals over-winter, there is still considerable work needed to determine what factors contribute to arrested or delayed larval development and thus larval over-wintering. Several of these have already been addressed in the discussions of chapter 5 and 6, and include predation risk, decreases in photoperiod and maternal effects. There may also be latitude or altitude effects on the incidences of over-wintering strategy in the UK. Collins (1979) demonstrated a latitudinal cline in the incidence of larval over-wintering in *Rana catesbeiana*. Larval over-wintering was more common and persisted for longer as latitude increased. *Rana temporaria* in Sweden demonstrate latitudinal variations in development rate in the laboratory that allow them to complete development at higher latitudes in a shorter period of time (countergradient variation: Laugen et al. 2003b; Lindgren & Laurila 2005) to allow them to cope with the shorter season. However, over-wintering is not observed in Sweden and may not be possible due to severe winters. It is not known whether *Rana temporaria* in the UK respond to the variation in season length and temperature that occurs throughout the country. Individuals could either display latitudinal counter-gradient variation, as observed in Sweden, or lack geographical differences in inherent development rate. The ability to over-winter as larvae could possibly lessen the selection for completing larval development in shorter seasons. Therefore, intrinsic acceleration of development may not be observed in northern or high altitude populations in the laboratory. A study of this nature was also piloted during the time that the research reported in this thesis was completed, but the results were too preliminary for inclusion (Appendix 2).

A final limitation of the studies conducted on factors affecting the rate of larval development was the use of two taxonomically unrelated species (*Bufo bufo* and *Rana*

temporaria) in assessing the effect of water depth. While studies of this nature are occasionally employed (e.g. Feder 1983a), it is difficult to be certain whether the difference that one is intending to assess, in this case lunged or lung-less, is actually the difference that contributes to the effect observed. The scope for experimental manipulation to make a similar comparison, within the same species, is extremely limited. An interspecific comparison of this nature can be refined by expanding the number of species, both lunged and lung-less, included in the study. However, a comparison of these two species is of interest since they often share similar habitats, experiencing the same temperature and water depth ranges, in the United Kingdom, yet *B. bufo* have not been reported to over-winter as larvae.

Conclusion

In conclusion this study has demonstrated that, in addition to variability in morphology and the duration of larval and embryonic phases, the duration of metamorphic climax is capable of responding to environmental variation. Additionally, the life history stage at which some species over-winter can be influenced by environmental conditions. Therefore, these two under-studied aspects of life history theory should be considered in future studies.

Tables and figures

Table 8.1: The developmental stage used by studies on plasticity in anuran larval duration to signify the conclusion of larval development and whether the study provided any data on metamorphic duration. Studies that performed trials with post-metamorphic individuals are marked with an asterisk.

Study	Gosner stage concluding larval period				Includes metamorphic data
	Stage 42	Stage 43-45	Stage 46	Did not state	
Alford & Harris 1988	√				No
Altwegg & Reyer 2003			√		No*
Altwegg 2002a	√				No*
Altwegg 2002b	√				No
Alvarez & Nicieza 2002a	√				Yes
Alvarez & Nicieza 2002b	√				No*
Arendt 2003	√				No
Audo et al. 1995			√		No
Babbitt & Tanner 1998			√		No
Babbitt 2001	√				No
Barnett & Richardson 2002			√		No
Beachy et al. 1999	√				No
Beck & Congdon 2000	√				No*
Beck 1997	√				No
Benard & Fordyce 2003	√				No
Berven & Chadra 1988	√				No
Berven & Gill 1983	√				No
Berven et al. 1979	√				No
Blouin & Brown 2000	√				No
Brady & Griffiths 2000	√				No
Brockelman 1969				√	No
Buchholz & Hayes 2002	√		√		No
Capellan & Nicieza 2007a	√				No
Capellan & Nicieza 2007b	√				No*
Chivers et al. 1999	√				No
Collins 1979		√			No
Crump 1984			√		No
Crump 1989b			√		No
Crump 1989a	√				No
de Vito et al. 1999	√		√		No
Denver et al. 1998	√				No
Ficetola & De Bernardi 2006		√			No*
Gascon & Travis 1992			√		No
Gervasi & Foufopoulos 2008	√				No
Hamer et al. 2002	√				No
Harkey & Semlitsch 1988	√				No*
Hensley 1993	√				No
Kiesecker et al. 2002	√				No
Kraft et al. 2005			√		No

Lane & Mahony 2002	√			No
Lardner 2000		√		No
Laugen et al. 2003b	√			No
Laurila & Kujasalo 1999	√			No
Laurila 2000	√			No
Laurila et al. 2001	√			No
Laurila et al. 2002	√			No
Laurila et al. 2004	√			No
Laurila et al. 2006	√			No
Leips & Travis 1994	√			No
Leips et al. 2000		√		No
Loman & Claesson 2003		√		No
Loman 1999		√		No
Loman 2001		√		No
Loman 2002		√		No
Loman 2003		√		No
Merila et al. 2000a	√			No
Morey & Reznick 2000	√			No
Morey & Reznick 2001	√			No*
Morey & Reznick 2004	√			Yes
Morin 1983	√			No
Morin 1986			√	No
Newman 1988	√			No
Newman 1989	√			No
Newman 1994	√			No
Newman 1998	√			No
Nicieza 2000	√			No
Nicieza et al. 2006	√			No*
Niehaus et al. 2006			√	No
Pandian & Marian 1985a	√			No*
Pandian & Marian 1985b	√		√	No
Pearman 1993	√			No
Pearman 1995	√			No
Relyea & Hoverman 2003	√			No*
Relyea 2001a	√			No
Resetarits et al. 2004			√	No
Rudolf & Rodel 2007			√	No
Ryan & Winne 2001			√	No
Semlitsch & Caldwell 1982	√			No
Semlitsch 1993			√	No
Skelly & Werner 1990	√			No
Smith 1983	√			No
Smith 1987	√			No*
Snodgrass et al. 2005	√			No
Sommer & Pearman 2003	√			No
Tejedo & Reques 1992	√			No
Tejedo & Reques 1994	√			No
Tejedo et al. 2000a	√			No*
Tejedo et al. 2000b	√			No*
Travis 1984	√			No

Van Buskirk & Relyea 1998			√		No
Van Buskirk & Saxer 2001	√				Yes*
Van Buskirk 1988	√				No
Vonesh & Bolker 2005	√				No
Vonesh & Warkentin 2006		√			No
Vonesh 2005b				√	No*
Wilbur & Fauth 1990	√				No
Wilbur 1977	√				No
Woodward 1987			√		No

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Appendix 1: National survey of larval over-wintering

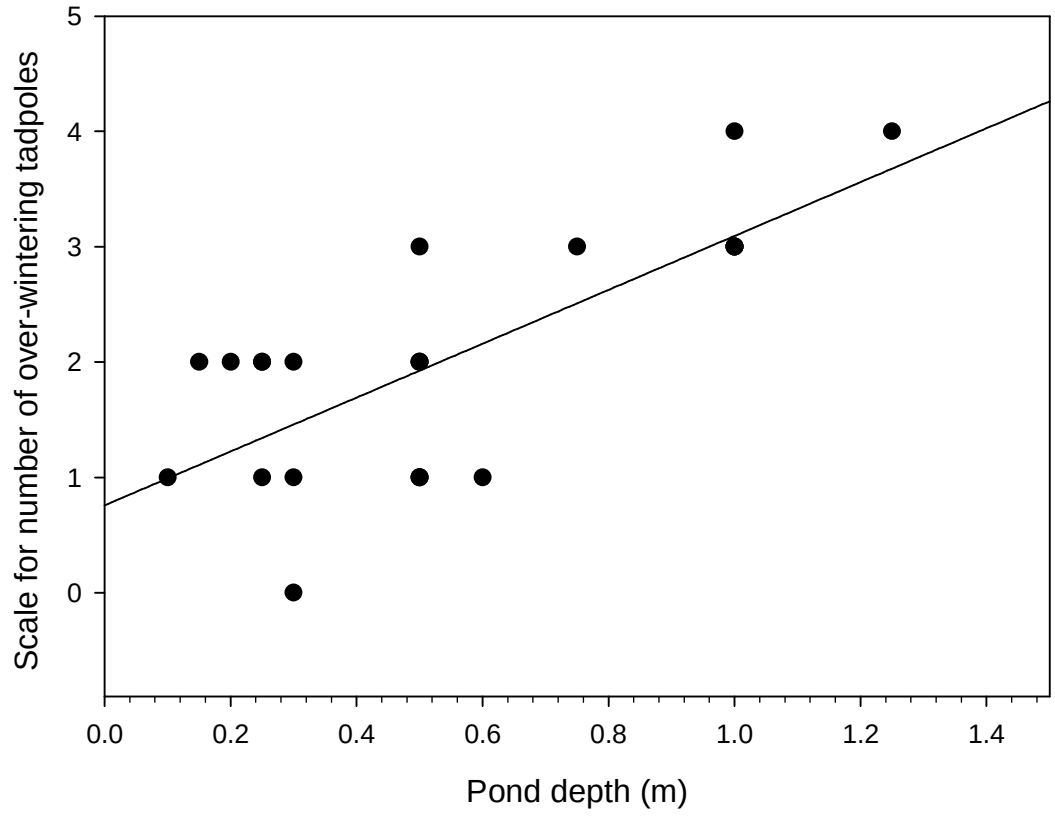
Appendix 1 reports on the results of a national survey on the occurrence of over-wintering *Rana temporaria* tadpoles across the United Kingdom, carried out in response to public interest as a result of a Guardian Editorial. Members of the public were sent a questionnaire and requested to provide data on the depth, surface area, altitude and latitude of ponds (largely garden ponds) where over-wintering larvae were observed. They were also requested to estimate the number of over-wintering larvae observed in November, using a scale from 1 (1-2 tadpoles) to 5 (more than 100 tadpoles). In total 23 completed questionnaires were returned.

Over-wintering of *Rana temporaria* tadpoles was distributed across the whole of the UK, from the south-west in Cornwall (latitude: 51.0ºN) to the North-east in Aberdeen (latitude: 57.2ºN; Fig. A.1). Although these results are not by any means an exhaustive report of incidences of over-wintering, it does demonstrate that the phenomenon is not restricted to a geographical region of the UK. Altitude data was incomplete and biased toward lower elevations, in line with areas of greater urban and suburban human populations.

The number of individuals over-wintering as larvae, reported by respondents, increased with pond depth ($F_{1,23} = 26.61$, $p < 0.001$; Fig. A.2), but not with pond surface area ($F_{1,23} = 0.98$, $p = 0.334$). Latitude was not correlated with pond depth ($p = 0.179$), nor with the incidences of over-wintering larvae ($p = 0.148$). This result suggests that the depth of the pond where tadpoles are spawned might have an impact on the life history pattern of development or on the survival of tadpoles during the late autumn and/or winter.

Figure A.1: National distribution of the occurrence of over-wintering of *Rana temporaria* larvae

Fig A.2: Estimation of the number of over-wintering tadpoles in garden ponds in relation to the depth of the pond. Each point represents at least a response from one questionnaire; some points may represent multiple identical responses. The equation of the regression line is: y = 2.335x + 0.757.



Appendix 2: Latitudinal variation in the proportion of over-wintering larvae

Appendix 2 reports on the results of a pilot experiment to determine whether UK *Rana temporaria* populations demonstrate inherent differences in the tendency to remain as tadpoles during the first winter suggesting adaptation to local temperatures and season length. In Spring 2006 several egg clumps were collected from five areas in the UK: two low altitude sources near Canterbury, England (51°N); Robroyston Marsh in Glasgow (55°N); and two altitude sources (550 m and 50 m) near Aberdeen (57°N). Tadpole care, rearing (medium temperature regime) and measurements were performed using the same methods as in Chapter 5.

There was a significant difference in survival among the three sources, with Canterbury (59.55 ± 3.17%) having the highest survival followed by Aberdeen (39.12 ± 8.67%) then Glasgow (11.17 ± 2.56%) ($F_{3,15}$ = 185.709, $p < 0.0001$). There was no difference in survival between the two locations in Canterbury where the spawn was collected from. However, with spawn from Aberdeen, Upland (55.57 ± 3.17%) tadpoles survived better than those spawned at the lowland source (22.67 ± 10.08%) ($F_{2,15}$ = 7.035, $p = 0.007$). The high mortality rate of the Glasgow and lowland Aberdeen tadpoles was most likely the result of a late frost that occurred after spawning, but before the clumps were collected. The high mortality raises the issue that the results observed are an artefact of differential survival rather than differences in the origin of the spawn.

There was a significant difference among the sources in the proportion of over-wintering tadpoles, with the proportion highest in larvae from Aberdeen (25.2 ± 7.34%), followed by Glasgow (17.25 ± 4.87%) then Canterbury (6.53 ± 1.85%) ($F_{3,15}$ = 15.791, $p < 0.0001$). There was no difference in the proportion of over-wintering between the

two source ponds in Aberdeen or Canterbury ($F_{2,15} = 0.118$, $p = 0.890$). The proportion of individuals that over-winter as tadpoles increases with increasing latitude. This preliminary result suggests that *Rana temporaria* in the UK do not demonstrate a higher intrinsic development rate in populations from higher latitudes that would allow them to complete development within the shorter and colder growth season, as occurs in Scandinavia (countergradient variation: Laugen et al. 2003b; Lindgren & Laurila 2005). In fact, the opposite appears to occur with individuals from more northern populations showing a greater intrinsic tendency to remain as tadpoles during the winter. The milder winters that occur in the UK, compared to Scandinavia, may allow high survival during the winter removing the selective pressure to complete development more rapidly in a shorter season. Additionally, the higher intrinsic tendency to over-winter as a tadpole in northern UK populations may be driven by the advantage of being able to achieve greater size from a longer larval period.

Appendix 3: Other outputs

- Walsh, P.T., Downie, J.R. & Monaghan, P. in press. Predation-induced plasticity in metamorphic duration in *Xenopus laevis*. *Funct. Ecol.*

- Walsh, P.T., Downie, J.R. & Monaghan, P. **2008**. Temperature mediated morphology changes during metamorphosis in the African clawed frog. *J. Therm. Biol.* 33: 244-249

- Walsh, P.T., Downie, J.R. & Monaghan, P. **2007**. Plasticity of the duration of metamorphosis in *Xenopus laevis*. *J. Zool.* 274: 143-149.

- Meddeb, C. Nouira, S., Cheniti, T.L., Walsh, P.T. & Downie, J.R. **2007**. Age structure and growth in two Tunisian populations of green water frogs *Rana saharica* (Boulenger 1913) a skeletochronological approach. *Herpetol. J.* 17: 54-57.

- Walsh, P.T., Evans, D., Hansell, M. & Ruxton, G.D. **2007**. Factors influencing nocturnal egg turning frequency in Meadow Pipits *Anthus pratensis*. *Bird Study* 54: 133-136.

- Jowers, M., Campbell-Palmer, R., Walsh, P.T. & Downie, J.R. **2006**. Intraspecific anti-predator spatial avoidance response variation of *Mannophryne trinitatis* (Anura: Dendrobatidae) tadpoles to *Rivulus hartii* and *Macrobrachium carcinus* by chemical cue detection. *Herpetol. J.* 16: 337-346.