THE IMPACT OF INTRODUCED PREDATORS ON THE MALLORCAN MIDWIFE TOAD *ALYTES MULETENSIS*

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Submitted for the degree of
Doctor of Philosophy
University of Kent at Canterbury

October 2002

ABSTRACT

The endemic midwife toad of Mallorca, *Alytes muletensis*, was once widespread across the island, but a dramatic decline has resulted in the persistence of only a small number of populations in the mountainous north-west. Predation and competition from introduced species such as the viperine snake (*Natrix maura*) and green frog (*Rana perezi*) are widely believed to have been important agents of decline. This thesis set out to test that theory by assessing the impact of introduced predators on the distribution of the toad, and on the population structure, morphology and behaviour of tadpoles.

GIS analysis revealed the preferred habitat of the toad to be high elevation torrents surrounded by steep sides and containing many pools. This habitat is thought to favour the persistence of cool plunge pools throughout the year, providing optimal conditions for larval growth and development. Although steep sides surrounding a torrent did not prohibit the access of introduced snakes and frogs, the presence of these predators was negatively associated with elevation, resulting in reduced predation pressure at higher altitudes. These findings may be used to optimize the location and design of future reintroductions and to enhance existing sites.

The presence of introduced predators was found to influence the growth, development and population structure of larval *A. muletensis*. Bimodal size-structures of larval populations in sites with snakes and frogs reflected size-selective predation or changes in recruitment, growth and development under the threat of predation. Differences in tadpole morphology between populations reflected rapid and reversible plastic responses to snakes and frogs, but no such responses were found towards a native invertebrate predator. It is likely that intense selection pressure has driven the evolution of inducible morphological and behavioural defences since the introduction of vertebrate predators onto Mallorca some 2000 years ago. Although captive-bred tadpoles showed similar responses to wild tadpoles, experiments showed that behavioural responses to predators were slightly weaker in the former. This may be a result of a captive-breeding bottleneck and supports the decision of the recovery group to stop releasing individuals from the original founder stock and to establish three new bloodlines.

ACKNOWLEDGEMENTS

I would firstly like to thank Richard Griffiths for his invaluable supervision and advice throughout the course of my project and for always being so approachable. Without his statistical expertise I believe I would still be pulling my hair out over incomprehensible equations and reams of SPSS outputs.

I am grateful to the Natural Environment Research Council (NERC) for funding my PhD and to the Durrell Wildlife Trust for providing captive toads for my research.

I am extremely grateful to Alvaro Román of Fons Ferreret for all of his assistance and support in Mallorca. Thanks also Javier Manzano for leading us to toad sites when Alvaro was unavailable. Thanks to Carlos Zayas and Rafael Barber Llorente for kind hospitality and comfortable accommodation with hot showers during our stay in Mallorca, and to all of the land owners that allowed us access to their land to conduct our fieldwork.

I would like to extend my gratitude to the Conselleria de Medi Ambient (Govern Balrear) in Mallorca for providing their permission for the project as well as supplying scanned maps for my GIS analysis.

Thanks to Cliona O'Brien, Violetta Muñoz Perez and David Jay for making my field work so enjoyable and for assisting me with my data collection. I am also grateful to Annette Huggins and Bob Smith for kick-starting me with GIS, and to Leida Dos Santos, Rosemary Godfrey, Patrick Walsh and Rob Muir for helping with the digitizing and saving me from serious repetitive strain injury. I am grateful to Femmie Kraaijeveld Smit for her valuable comments on earlier drafts of my thesis and for knowing considerably more about genetics than me. I would also like to thank Amy Warren for making me regular cups of tea that helped me to stay awake throughout the writing-up period.

Lastly, but by no means least, I would like to thank my family and all of my friends who have been so supportive over the last three years.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 GLOBAL AMPHIBIAN DECLINES

Amphibians are globally widespread, being found in every continent except Antarctica (Beebee, 1996). In the 1970's, however, herpetologists began reporting declines in amphibian populations throughout the world, especially in the Americas and Australia. The scale of the phenomenon led to growing concern about a global decline - and in some areas extinction - of amphibians, and in particular anurans (Blaustein & Wake, 1995; Halliday, 2000; Alford *et al.*, 2001). Well documented examples of declines include the disappearance of the golden toad (*Bufo periglenes*) from Costa Rica's Monteverde Cloud Forest Reserve (Pounds & Crump, 1994), the dramatic decline of the mountain yellow frog (*Rana muscosa*) (Bradford, 1991) and cascade frog (*Rana cascadae*) in California (Fellers & Drost, 1993) and, in Europe, mass mortality of the midwife toad (*Alytes obstetricans*) in the Pyrenese (Márquez *et al.*, 1995).

According to Diamond's (1984) 'Evil Quartet', the four principal causes of species' extinction are habitat fragmentation, overexploitation, introduced predators and chains of extinctions. While habitat loss and fragmentation are probably the most frequently cited causes of species declines and extinction (Diamond, 1989; Sjogren, 1991; Hedges, 1993), widespread amphibian declines are alarming because many are occurring in seemingly 'pristine' habitats (Sjogren, 1991; Griffiths & Beebee, 1992; Blaustein, Hoffman *et al.*, 1994; Blaustein, Kokit *et al.*, 1994; Halliday, 2000). Factors implicated in these cases include increases in UV-B radiation (Blaustein, Hoffman *et al.*, 1994), eutrophication (Hecnar, 1995) and disease (Blaustein, Hokit *et al.*, 1994; Cullen *et al.*, 1995; Laurance *et al.*, 1996). Mass mortality of the midwife toad *Alytes obstetricans* in the Pyrenean mountains, for instance, was attributed to the bacterium *Aeromonas hydrophila* (Márquez *et al.*, 1995). Predation and competition from introduced species has also been cited as a major contributing factor to amphibian declines and extinctions around the world (Hayes & Jennings, 1986; Bradford, 1991; Griffiths & Beebee, 1992; Fellers & Drost, 1993). The decline of the formerly abundant Californian red-legged frog *Rana aurora draytonni* is one of the most dramatic examples of amphibian decline (Fisher & Shaffer, 1996) and some workers have attributed this to predation from introduced bullfrogs and fish (Lawler *et* *al.,* 1999). The collapse of a regional frog fauna in the Yosemite area has also been attributed to predation from introduced fish (Drost & Fellers, 1996).

1.2 INTRODUCED PREDATORS AND ISLANDS

According to Atkinson (1989) alien-induced extinction is primarily an island phenomenon. Thirty-one percent of vertebrate species excluding fishes currently thought to be endangered are insular (WCMC 1986, cited by MacDonald *et al.*, 1999) and more than 90% of documented reptile and amphibian extinctions since 1600 have been of island forms (Atkinson, 1989). Certain demographic characteristics of island species make them more prone to extinction than mainland species; these include more limited powers of dispersal, less total suitable habitat, fewer populations and/or smaller total population size (Atkinson, 1989). Wilson (1961) theorized that populations of island species become increasingly specialized and fragmented over time, and as this occurs their vulnerability to extinction increases. Additionally, island species that have evolved in the absence of any major predators or competitors are likely to be particularly vulnerable to the introduction of exotic species. Periods of coevolution between predator and prey play an important role in reducing dynamical responses of one species on the other (Case & Bolger, 1991) and a lack of coevolution can result in predators severely impacting populations of prey species (Savidge, 1987; Rodda & Fritts, 1992; Keisecker & Blaustein, 1997; Fritts & Rodda, 1998). There are many cited cases of introduced species devastating native island fauna, although most studies to date have focused on introduced mammals and there are few documented cases of reptile introductions. Exceptions include introduced *Boa constrictor* believed to threaten endemic fauna on Cozumel Island in Mexico (Martinez-Morales & Cuaron, 1999) and the brown tree snake (*Boiga irregularis*), introduced accidentally onto Guam in the 1950's and responsible for the extinction of many native fauna (Savidge, 1987; Rodda & Fritts, 1992; Fritts & Rodda, 1998).

1.3 THE MALLORCAN MIDWIFE TOAD

The Mallorcan midwife toad (*Alytes muletensis*, Sanchíz & Alcover, 1977), or ferreret was first described as *Baleaphryne muletensis* from fossil and sub-fossil remains in Mallorca's Middle and Upper Pleistocene (Sanchíz & Andover, 1977, published in 1979). Following its description, the species was presumed to be extinct until live tadpoles were discovered in a remote gorge within the Serra de Tramuntana mountain range (see Figure 1.1) in 1980 (Mayol & Alcover, 1981). Subsequent analysis of the anatomy (Mayol *et al.*, 1984), behaviour (Martens, 1984), osteology (Clarke, 1984), and cytology (Hemmer, 1984) of the toad suggested that it should be placed within the genus *Alytes,* family Discoglossidae. The discovery prompted research to determine the distribution, status, biology and ecology of the species.

1.3.1 Historical Range of the Toad

The historical range of *A. muletensis* can be deduced by piecing together fossil and subfossil evidence along with considerations of the ecology and life history of the species. A subfossil humerus from the lowland Mallorcan Holocene site Cova de a Mort (the most recent fossil record of the species) was identified as *A. muletensis* (Alcover, Sanders & Sanchiz, 1984). This finding seems to provide good evidence that *A. muletensis* at one time inhabited areas of lowland Mallorca. Although Hemmer (1984) suggests that the subfossil may actually be *A. talaioticus* (an extinct form recorded on Menorca) because the differences in locomotory adaptation between the two species is much smaller than was previously thought and *A. talaioticus* is more likely to have been inhabiting lowland pools than *A. muletensis*, it is now generally accepted that the fossils belong *to A. muletensis* (A. Alcover, pers com). *A. muletensis* remains have now been recovered from a number of sites in lowland Mallorca (A. Alcover, pers com) providing more evidence that this species once had a more widespread distribution across Mallorca than at present.

Figure 1.1. Map of Mallorca. The Mallorcan midwife toad is restricted to the Serra de Tramuntana mountain range in the north-west. Source: www.users.rcn.com/felicia/memories-mallorca.htm

1.3.2 Current Distribution and Hypotheses for Decline

Decline and fragmentation has resulted in the persistence of only a small number of isolated breeding populations of *A. muletensis*. Extensive survey work conducted by the Conselleria d'Agricultura Pesca (CDAP) has revealed 13 natural breeding populations of the toad (Buley & Garcia, 1997), with an estimated total of 1000-3000 individuals (Bush, 1994). Populations occur only in remote gorges in the Serra de Tramuntana mountain range, predominantly in the vicinity of semi-permanent plunge pools (Alcover *et al.*, 1984). The largest populations occur in seven separate gorges and are thought to be reproductively isolated (Buley & Garcia, 1997). The severe fragmentation of existing populations has resulted in *A. muletensis* being classified as 'Critically Endangered' in the 1996 IUCN Red List (IUCN, 1996).

The precise causes of the decline of the toad are unclear. One hypothesis is that a lack of available water brought about by changes in climate combined with water demands and population pressure has limited the availability of breeding sites for the toad (Tonge, 1986). Pools in which the toads do breed may dry up completely or, if they do persist, become stagnant in the mid-summer when the water is at its lowest level (Tonge, 1986). Since Mallorcan midwife toads are essentially stream dwellers, the stagnation of the water may affect the growth and survival of tadpoles within the pools and therefore the fitness of individuals within populations (Tonge, 1986). Larval growth and development within the different environments has not been investigated, however. An alternative hypothesis proposed by numerous workers attributes the decline of the toad to predation and competition from introduced species such as the viperine snake, *Natrix maura,* and green frog, *Rana perezi* (Hemmer & Alcover, 1984; Tonge, 1986; Bush, 1994; Griffiths *et al.*, 1998; Schley & Griffiths, 1998).

1.3.3 Introduced Species on Mallorca

The arrival of man onto Mallorca approximately 4000 years ago had catastrophic consequences for much of the native fauna, including the Mallorcan midwife toad (Tonge, 1986). Together with habitat change came the introduction of a number of alien predators and competitors, such as the green frog, *Rana perezi*; green toad, *Bufo viridis*; weasel, *Mustela nivalis*; pine marten, *Martes martes*; genet, *Ginetta ginetta*; false smooth snake, *Macroprotodon cucullatus*; and, perhaps most significantly for the toad, the viperine snake, *Natrix maura*, a semi-aquatic species that preys upon tadpole and post-metamorphic toads. According to Tonge (1986), the Quaternary fauna of the Balearic islands is well known and no fossil snakes have ever been found on Mallorca, suggesting that current populations of viperine snakes on the island are descendants of those introduced by the Romans for religious purposes approximately 2000 years ago (Alcover & Mayol, 1981). Preliminary genetic analysis has also indicated that the snakes were introduced relatively recently (D. Guicking, pers com). A close relative of the ferreret, *Alytes talaioticus*, is believed to have gone extinct on the neighbouring island of Menorca shortly after 250 AD, coinciding with the introduction of alien predators including snakes (Sanders, 1984). The viperine snake, which is present throughout lowland Mallorca and much of the mountainous regions (Tonge, 1986), may have eliminated the midwife toad from much of the island (Bush, 1994), and it could still pose a threat in a number of areas (Schley & Griffiths, 1998). The hypothesis remains to be tested that toad populations have persisted in remote mountain gorges because they are relatively inaccessible to snakes (Tonge, 1986; Schley & Griffiths, 1998).

1.3.4. Species Recovery Programme

A species recovery programme for the Mallorcan midwife toad was established in 1985, incorporating captive breeding, reintroduction, management and monitoring of wild populations with biological, ecological and genetic research. The captive breeding programme was initiated by the Jersey Wildlife Preservation Trust (JWPT) using individuals collected from one site only in order to maintain the genetic integrity of that population (Garcia, 1998). Four adult toads and four tadpoles were collected initially in April 1985 and transferred to Jersey; these were supplemented in June 1987 with a further 12 individuals from the same site. Of the 16 tadpoles collected, 11 survived to adulthood and the species was bred in captivity for the first time in 1988 (Tonge & Bloxam, 1989, 1991). Captive populations stemming from this founder colony are now held at a number of institutes including Jersey Zoo, Marineland in Palma de Mallorca, Chester Zoo, Barcelona Zoo and the Durrell Institute of Conservation and Ecology (DICE).

One of the main aims of the captive-breeding programme is to provide toads for reintroduction back into natural habitat (Tonge & Bloxham, 1991). A number of reintroductions have been made from captive stock into suitable, unoccupied habitat in the Serra de Tramuntana mountains. The first reintroduction of 76 captive-bred toads from JWPT occurred in 1989 at the request of the Mallorcan government (Garcia, 1998) into two sites chosen by the Conselleria d'Agricultura Pesca (CDAP). Since then releases of young toads and larvae have been made almost annually into sites that are within the known historical range of the species but which do not already contain toads (Garcia, 1998). Several reintroductions have been made into man-made watering-troughs, or cisterns, which are used by at least two natural populations of toads (Garcia, 1998). Reintroductions from the original stock have ceased due to fitness considerations associated with successive generations of captive breeding, although individuals collected more recently from three additional sites have provided fresh breeding stock for the programme; all three bloodlines are currently held between JWPT and DICE. The success of future reintroductions may be enhanced by further knowledge about factors that affect the distribution of the toad and influence individual fitness, as well as the implications of inbreeding depression that may result from successive generations of captive breeding.

1.4 AMPHIBIAN POPULATION DYNAMICS

Determining what regulates populations of animals remains a central theme in ecology. Understanding the evolution of growth and size is critical to an understanding of the life history and population biology of most organisms (Ebenman & Persson, 1989). Amphibian populations represent a special challenge because they typically possess a larval stage adapted for rapid growth and a terrestrial stage adapted for dispersal; populations may therefore be regulated in any combination of life history stages (Wilbur, 1980; Berven, 1990). As Werner (1989) states, "an organism is not the static representation of the adult we associate with taxonomic characterizations and most ecological theory, but the dynamic unfolding of the genome over ontogeny, and the consequent succession of life history stages or forms." Because factors affecting individual fitness during the larval stage have implications for the adult phase of the lifecycle that may determine the dynamics of populations from one generation to the next (Wilbur, 1989), larval populations are important in the regulation of amphibian populations as a whole (Berven, 1990).

1.4.1 Larval Population Structure

The breeding biology of adult amphibians dictates the spatial and temporal patterns and initial size distribution of hatchling tadpoles in breeding pools (Wilbur, 1989). Anurans are typically either explosive breeders, producing large clutches of eggs over a short time period, or prolonged breeders, producing smaller clutches over a larger portion of the year. Whereas explosive breeding typically produces a skewed size distribution of larvae (Wilbur, 1989), prolonged breeding such as that exhibited by *A. muletensis* (Bush, 1993) can provide the potential for considerable variation in tadpole size at any time during the breeding season. Size-structure within larval populations is important because the size of a tadpole determines it's competitive success (Rose, 1960) and risk of predation by size-limited (Paine, 1976; Zaret, 1980) and size-selective (Brooks & Dodson, 1965) predators. Spatial and temporal distributions of anuran larvae may also be influenced by the distribution and abundance of predators since predator presence can influence decisions regarding the timing and siting of oviposition in adults (Magnusson & Hero, 1991; Holomuzki, 1986; Resetarits, 1996; Downie *et al.*, 2001).

1.4.2 Tadpole Growth and Development

Factors influencing tadpole growth and development are central to the regulation of amphibian population structure and dynamics. Because higher growth rates reduce time to reproductive size, and often increase survivorship and size at reproduction, it has been argued that there should be strong selection to maximise larval growth rate (Wilbur, 1980; Werner & Gilliam, 1984; Berven, 1990). However, growth rate is also influenced by a suite of biotic and abiotic factors including food availability (Hota & Dash, 1981), water temperature (Harkey & Semlitsch, 1998; Alford, 1999), tadpole density (Dash & Hota, 1980; Hota & Dash, 1981; Travis & Trexler, 1986; Brönmark & Edenham, 1993), intra- and inter-specific competition (Brockelman, 1969; De Benedictis, 1974) and predation risk (Skelly, 1992). The influence of predators on tadpole growth and development can be complex, however. Tadpoles often reduce activity in the presence of predators (Lawler, 1989; Skelly & Werner, 1990; Griffiths *et al.*, 1998) or spend more time hiding in refuges (Petranka *et al.*, 1987; Kats *et al.*, 1988; Lefcort, 1998). As a result individuals may spend less time feeding and grow more slowly when predators are present (Skelly, 1992). Conversely, predation may enhance growth by ameliorating the negative effects of competition through the removal of individuals from the population, resulting in an increase in the growth rate of surviving individuals (Wilbur, 1987; Peacor, 2002). Similarly, when predation risk decreases with increasing tadpole size, tadpoles may benefit by growing rapidly during early development in order to reach a size refuge. Increased growth in the presence of predators has been observed during early development in some tadpole species (Babbitt & Tanner, 1998). Tadpole development rates may respond to environmental influences such as temperature (Lea et al., 2002), photoperiod (Alford, 1999) and predation risk (Laurila & Kujasalo, 1999) as well as to intrinsic cues such as growth rate or body size thresholds (Alford, 1999).

1.4.3 Mortality

Mortality in amphibians is generally highest during the larval stage of development (Turner, 1962; Duellman & Trueb, 1986; Stangel, 1988) and predation typically accounts for most of this mortality (Calef, 1973). The risk of predation on anuran larvae is often size-specific, either decreasing monotonically with increasing tadpole size (Calef, 1973; Heyer *et al.*, 1975; Caldwell *et al.*, 1980; Crump, 1984; Cronin & Travis, 1986, Formanowicz, 1986; Semlitsch & Gibbons, 1988; Semlitsch, 1990; Richards & Bull, 1990; Tejedo, 1993; Alford, 1999) or increasing to a maximum and then decreasing (Brodie & Formanowicz, 1983; Wilbur, 1988). The significance of size-selective predation has long been a dominant theme in research on zooplankton ecology (Brooks & Dodson, 1965; Kerfoot, 1980; Lynch, 1980) and it is likely to be important in shaping prey population structure and influencing population dynamics. However, the literature on size-preferences of vertebrates predating tadpoles and predator-mediated population dynamics is sparse. Crump & Vaira (1991) found that avian predators preferred larger tadpoles over small ones, although Kupferberg (1997) found garter snakes to be unselective with respect to tadpole size. Size-preferences of *N. maura* predating *A. muletensis* tadpoles are not known.

1.5 MINIMIZING PREDATION RISK

Predation is a strong force that can exert considerable selective pressure on prey populations. In order to effectively minimize their susceptibility to predation, prey must be able to accurately assess the imminent threat from predators and respond accordingly (Lefcort, 1998). Responding to neutral stimuli may be costly whereas not responding to predators may result in death; there is therefore strong selection on prey to be able to distinguish between real predators and those that pose little or no threat (Griffiths *et al.*, 1998). The presence of a predator may be determined through any or all of visual, physical or chemical cues. In aquatic systems chemical cues may provide the most accurate information about the presence and, potentially, the identity of a predator (Magurran, 1989; Dodson *et al.*, 1994). A large number of studies have demonstrated that amphibian larvae detect the presence of predators by means of chemical cues in the water (eg. Petranka *et al.*, 1987; Griffiths *et al.*, 1998) and experiments on anuran larvae have shown that visual and tactile cues are less effective than chemical cues at signalling the proximity of a predator (Stauffer & Semlitsch, 1993).

1.5.1 Defenses

One mechanism by which prey species reduce predation pressure is through defences. Defences may be constitutive, and always expressed, or they may be inducible and only expressed in the presence of a predator. Constitutive defences are generally favoured when predation pressure is constant and when the defence is not too costly to build or maintain or when prey cannot accurately assess predation risk. Inducible defences are generally adaptive when the risk of predation is spatially or temporally variable, when prey can accurately detect the presence of predators by some sort of cue and when the antipredator phenotype is beneficial in the presence of predators but disadvantageous in the absence of predators (Dodson, 1989; Clark & Harvell, 1992; Moran, 1992; Brönmark & Petterson, 1994; Tollrian & Harvell, 1999). Inducible defences are widespread throughout invertebrates, vertebrates and plants (Harvell & Tollrian, 1999). As a result, phenotypic plasticity, which is considered to be a heritable trait in its own right, represents an essential component of evolutionary change (Bradshaw, 1965; Scheiner, 1993; Thompson, 1991; Gotthard & Nylin, 1995; de Jong, 1995; Relyea, 2001*a*).

1.5.2 Defenses in Larval Amphibians

Amphibian larvae are vulnerable to a wide range of invertebrate and vertebrate predators, and may display predator-specific defences (e.g. Griffiths *et al.* 1998; Relyea, 2001*a*). Constitutive defences in tadpoles include unpalatability (Griffiths & Denton, 1992; Kats *et al.*, 1988; Alford, 1999; Hero *et al.*, 2001); inducible defences include behavioural and morphological responses to the presence of predators. Predator-induced behaviours include reducing levels of activity (Lawler, 1989; Skelly & Werner, 1990; Griffiths *et al.*, 1998), shifting use of microhabitat (Morin, 1986; Lawler, 1989; Semlitsch & Gavasso, 1992) and increasing time spent in refuges (Petranka *et al.*, 1987; Kats *et al.*, 1988; Lefcort, 1998). Some anuran larvae also change morphology in the presence of predators (Smith & Van Buskirk, 1995; McCollum & Van Buskirk, 1996; McCollum & Leimberger, 1997) and, although it is often difficult to identify the functional significance of changes in specific traits, tadpoles with induced morphologies have been found to enjoy higher survival rates in the presence of predators (McCollum & Van Buskirk, 1996, Van Buskirk *et al.*, 1997; Van Buskirk & Relyea, 1998; Van Buskirk & McCollum, 2000*a*). The visibility and tractability of inducible defences such as these make anuran larvae powerful tools for investigating the conditions necessary for the evolution and maintenance of phenotypic plasticity.

1.6 CAPTIVE BREEDING PROGRAMME: FITNESS CONSIDERATIONS

An integral component of the recovery programme for the Mallorcan midwife toad is the reintroduction of individuals into unoccupied sites within the historical range of the toad from captive-bred stock. While amphibians have been identified by some workers as being particularly good subjects for captive breeding programmes (Bloxam & Tonge, 1995), others have suggested that such programmes are not an effective conservation strategy for reptiles and amphibians (Dodd & Seigel, 1991). Captive breeding can generate problems through inbreeding depression (Ralls *et al.*, 1979; Reinert, 1991), which can lead to a decline in the fitness of individuals within populations (Caughley, 1994; Hedrick & Kalinowski, 2000; Marshall & Spalton, 2000). This was demonstrated in the natterjack toad *Bufo calamita*, when larval growth rate was found to be strongly correlated with expected heterozygosity across microsatellite loci (Rowe *et al.*, 1999). Data from other taxa have also shown that populations with reduced genetic diversity brought about by inbreeding may experience reduced growth and increased extinction risk (Shaffer, 1987; Hedrick & Miller, 1992; Jimenez *et al.*, 1994; Keller *et al.*, 1994; Loftin, 1995; Keller & Waller, 2002). Additionally, some workers have suggested that captive breeding disrupts the process of natural selection and may result in adaptation to the artificial environment and lowered fitness in natural situations (Reinert, 1991; Loftin, 1995; Lewis & Thomas, 2001). Understanding the susceptibility of individual species to inbreeding depression is important when populations are small or are being intensively managed (Kalinowski & Hedrick, 1999) and it is generally accepted that population bottlenecks should be avoided when managing species of conservation concern (Franham *et al.*, 1999). The influence of a genetic bottleneck on the fitness of reintroduced individuals of the Mallorcan midwife toad is clearly of crucial importance to the success of the recovery programme and is an issue that needs to be addressed.

1.7 AIMS AND OBJECTIVES OF THESIS

The overall aim of this thesis is to investigate factors influencing the distribution and population dynamics of the Mallorcan midwife toad, with specific emphasis on the impact of introduced predators. It is hoped that the research will aid in the design of suitable reintroduction sites for the toads by highlighting the conditions that are likely to enhance breeding success and overall viability of toad populations. The principal aims of the study are:

(1) To identify the relationship between the distribution of toads and associated predators with landscape features of the Serra de Tramuntana mountains (Chapter 2).

The current distribution of the Mallorcan midwife toad is well known due to annual surveys conducted by the Conselleria de Medi Ambient, Fons Ferreret and the Durrell Wildlife Conservation Trust. During these surveys counts of tadpoles are made at all sites and data are collected on site characteristics and the presence/absence of predators. One untested hypothesis is that toads have survived in sites that are inaccessible to predators due to steep cliffs and gorges. In order to test this hypothesis, GIS will be used to map the terrain of the Serra de Tramuntana mountain range representing the current distribution of the toad, and multivariate analyses will be conducted to determine which topographical features are important in influencing the distribution of toads and associated predators. In addition, larval population size and number of larvae per pool will be related to a number of site characteristics in order to assess the optimal conditions for the success of toad populations. The information will be used to provide recommendations on the location and design of future re-introduction sites.

(2) **To investigate factors important in influencing growth, development and population structure of *A. muletensis* larvae (Chapter 3).**

Mallorcan midwife toad populations inhabit three main environments; natural torrent pools subject to incursions from predatory snakes and frogs; predator-free natural torrent pools and predator-free artificial cisterns. By comparing tadpole population size-structure across the three environments, factors influencing the structure and dynamics of these populations will be assessed. Additionally, temporal changes in the size- and stage-structure of newly colonised pools will be measured to provide an indication of natural rates of growth and development across the three environments. The results will allow an assessment of the optimal conditions for larval populations of *A. muletensis.*

(3) To determine the nature of morphological and behavioural responses of *A. muletensis* tadpoles to introduced and native predators (Chapters 4, 5, 6 and 7).

Predation from introduced species may have placed strong selection on *A. muletensis* to evolve mechanisms of reducing predation pressure. Previous research has shown that tadpoles of this species detect chemical cues from the introduced snake *N. maura* and reduce activity. However, it is not known whether such a short period of predator-prey coevolution is sufficient for the evolution of morphological responses. In order to test this, morphological differences between larval populations under different predation pressure will be measured and related to plastic responses towards snakes. To test for species-specific predator recognition, behavioural and morphological responses of tadpoles to another introduced species, the green frog, will be assessed and compared with responses to snakes. To test the hypothesis that responses to introduced predators reflect adaptations of defences already exhibited towards native predators, behavioural and morphological responses of tadpoles to a native invertebrate predator will be investigated. Finally, behavioural responses to snakes will be explored; rates of response and recovery will be tested together with the influence of predator history on the strength of the response.

(4) To assess fitness costs associated with antipredator responses (Chapter 8).

An implicit assumption of inducible defences is that they are beneficial in the presence of a predator but costly in the absence of predation. Therefore, the presence of predators at a site may influence the fitness of larvae in more subtle ways than direct predation. The impact of predator-induced behavioural and morphological responses on feeding rate, growth and development of tadpoles will be tested under controlled conditions to assess the fitness costs associated with these antipredatory defences.

(5) To determine the influence of captive breeding on fitness (Chapters 5 and 8).

Whether the performance of individuals is affected by a genetic bottlenecking is of central importance in any captive breeding and reintroduction programme. Captive stock of the Mallorcan midwife toad are derived from small numbers of individuals and are generally bred for several generations before being released into natural sites. An important consideration for the success of the reintroduced populations is whether a captive-breeding bottleneck results in inbreeding depression and therefore lowered fitness of reintroduced individuals. The influence of captive-breeding on tadpole performance will be assessed by firstly comparing morphological and behavioural antipredatory responses of tadpoles from a natural and a reintroduced population. Secondly, antipredatory responses of wild-caught tadpoles will be compared with those of tadpoles from a population that has been subject to approximately six generations of captive breeding. It is hoped that the results will be valuable in assessing the limitations of the captive breeding and reintroduction programme as a means of expanding the range of the species.

CHAPTER 2: INFLUENCE OF TOPOGRAPHY ON THE DISTRIBUTION OF THE MALLORCAN MIDWIFE TOAD AND INTRODUCED PREDATORS

2.1 SUMMARY

The influence of selected spatial and environmental attributes on the distribution of the Mallorcan midwife toad and associated introduced predators was investigated. Elevation, aspect and maximum slope within a 100 m radius was calculated for 17 torrent sites containing toads and 50 randomly generated torrent sites using GIS. The presence or absence of toads was tested against the three topographical variables using stepwise logistic regression. Maximum slope was positively related to toad presence and was the only significant variable to be included in the final model. The distribution of introduced predators between toad sites was tested against the same three topographical variables using the same procedure. Predator presence was negatively related with elevation and was the only significant variable included in the final model. In an attempt to assess factors that may influence larval population size, stepwise linear regressions were used to test mean larval counts over a 10-year period and the number of tadpoles per pool against elevation, number of pools at the site and degree of isolation (mean distance to nearest five populations). Larval population size was strongly positively associated only with number of pools at the site; mean number of tadpoles per pool was significantly positively associated only with elevation. These finding suggest that the optimal habitat for the Mallorcan midwife toad is high altitude torrent gorges containing many pools and surrounded by steep sides. Consideration of these factors may optimise the location and design of future reintroduction sites for the toad and the viability of existing populations may be greatly enhanced by the creation of more pools within a site.

2.2 INTRODUCTION

The conservation of ecologically viable species necessitates an understanding of physical environmental parameters affecting population demographies (Scribner *et al.*, 2001). The paucity of data on which to base conservation decisions is of immediate concern (Scribner *et al.*, 2001) and for many threatened species further data are required on these aspects before informed management decisions can be made. Mapping of species distributions can improve our understanding of the appropriateness of habitat areas for individual species and recent advances in geographical information systems (GIS) and remote sensing technologies have facilitated the analysis of animal and plant distributions in relation to spatial environmental attributes (e.g. Farina, 1997; Corsi *et al.*, 1999; Knutson *et al.*, 1999; Lenton *et al.*, 2000). Habitat relationships have been modeled for a variety of taxa to predict the occurrence of species within a habitat or to rank appropriate habitat for a species (e.g. Howell *et al.*, 2000). Relatively simple environmental descriptors such as altitude, slope and aspect have been used to explain distribution and abundance patterns of certain taxa (e.g. Farina, 1997), and it is likely that these variables strongly influence the microclimate of a site (Stocks & Hetwood, 1994). While quantification of landscape characteristics may be important for assessing habitat suitability for wide-ranging species that use resources over large areas (Naugle *et al.*, 1999), for species with a highly restricted distribution such as the Mallorcan midwife toad, characteristics of the immediate surrounding habitat are probably more important.

The Mallorcan midwife toad is currently confined to a small number of localities in the Serra de Tramuntana mountain range in NW Mallorca. Populations typically inhabit torrent gorges that flow during winter but dry up in the spring, leaving behind pools of water in scoured basins. While many of these pools dry up over the course of the summer, some persist long enough to provide suitable breeding grounds for the toad. The range of the Mallorcan midwife toad is thought to have once extended throughout the Serra de Tramuntana mountain range and even across lowland Mallorca (Alcover, Sanders & Sanchiz, 1984; Alcover pers com) but a dramatic decline has resulted in the survival of a small number of populations. The decline is believed by many workers to have been driven by predation and competition from introduced species such as the viperine snake, *Natrix maura*, and green frog, *Rana perezi* (Tonge, 1986; Bloxam & Tonge, 1995). One hypothesis suggests that the toad has survived in sites which are inaccessible to snakes (Bloxam & Tonge, 1995; Schley & Griffiths, 1998) although this remains to be tested.

The aim of this chapter is to assess the distribution of the toad and associated introduced predators in relation to topographical features. Further, the success of toad populations, measured as mean larval population counts over a ten year period and mean number of tadpoles per pool, will be related to a number of site characteristics. These analyses will provide an indication of the optimal conditions for the toad and will hopefully aid the species recovery programme by facilitating the location and design of future reintroduction sites as well as making recommendations for enhancing the viability of existing populations.

2.3 METHODS

2.3.1 Mapping Procedure

1:5,000 scale scanned paper maps of the Serra de Tramuntana were obtained from the Conselleria de Medi Ambiente in June 2000. Torrents and 25 m contours were digitised from these scanned maps using Arcview 3.2 (ESRI, 2000) across the entire range of the toad. From these contours a digital terrain model (DTM) was constructed using Triangulated Irregular Networks (TIN) (Figure 2.1A, B). DTMs such as this serve as an important tool for deriving secondary spatial data sets such as altitude, slope and aspect (Stocks & Heywood, 1994). Slope and aspect surfaces with a grid size of 0.01 km were generated from the TIN for analysis. Toad populations were then plotted using GPS coordinates and existing paper maps (Garcia, 1997). To provide a comparison, 50 sites were randomly generated along torrents using a random points generator extension for ArcView 3.2 and by combining all torrents so that the sites were randomly located throughout the range of the toad.

2.3.2 Statistical Analysis

(1) Distribution of Toads and Predators In Relation to Topography

Stepwise multiple logistic regression (Hosmer & Lemeshow, 1989) was used to test a number of topographical factors considered to be potentially important in influencing the distribution of the midwife toad and introduced predators *N. maura* and *R. perezi*. The distribution of the two predators amongst toad sites was perfectly correlated (R=1.0) indicating that wherever snakes are present, frogs are found also and vice versa. Logistic regression was considered the most appropriate analytical procedure for assessing the distribution of toads and predators because it permits the prediction of binary attributes such as presence/absence (McCullagh & Nelder, 1983) and the technique has similarly been used to analyse the distribution of other taxa (e.g. Jokimäki, 1999).

The dependent or response variable in the regression, presence or absence of toads, was scored as 1 (n = 17) or 0 (n = 50) respectively. The independent or explanatory variables were three topographical features associated with each site derived from surfaces constructed using GIS; elevation (m), aspect (in degrees, where 0° = true north and 180° = true south) and maximum slope within a horizontal radius of 100 m (equal to an area of 31,416 m^2). A radius of 100 m was chosen because, in the absence of any data on dispersion capabilities of adult toads, this was considered to provide an appropriate representation of the immediate topography at each site. Scribner *et al.* (2001) also characterized habitat within a 100 m radius around each pond when investigating environmental correlates of toad abundance and genetic diversity. The number of independent variables in the analysis was kept low due to the limited number of sites being compared. This is in accordance with the suggested minimum requirement of at least four to five times more cases than independent variables (Tabachnick & Fidell, 1983). Using the stepwise logistic regression procedure, selection of variables for inclusion in the final model was based on the Wald test (Hosmer & Lemershow, 1989; Jokimäki, 1999). None of the independent variables were highly correlated (all $P>0.05$), avoiding the problems of multicollinearity (see Trexler & Travis, 1993), and all were normally distributed and therefore met the assumptions of the analysis.

Factors influencing the distribution of predators between toad sites was investigated by comparing 10 sites known to contain snakes with 11 snake-free sites; snake presence or absence (scored as 1 and 0, respectively) was regressed against the three independent topographical variables described above. A basic assumption of these analyses is that the diversity of environmental conditions within areas classified as "present" represents the best average conditions for a stable presence of the species being investigated.

(2) Factors Influencing Larval Population Size and Density

Annual larval counts of toad populations conducted by the Conselleria de Medi Ambient were used to provide a measure of relative population size in this analysis. In order to assess the factors important in influencing larval population size, a stepwise linear regression of mean tadpole counts (which were log transformed to ensure normality) at all sites including cisterns from 1991 – 2000 (Román, 2001) against three site characteristics was conducted. Stepwise regression such as this identifies which site characteristics explain the greatest amount of variation in population size; it has been used similarly to investigate the distribution of other taxa, such as birds (Howell *et al.*, 2000). Independent variables included in the regression were: elevation, number of pools (taken from Garcia, 1997) and degree of isolation (calculated as mean horizontal distance to the five nearest toad populations). Snake presence (based on a three point scale 0 = snakes absent; 1 = sporadic snake incursions; 2 = frequent snake presence) was omitted from the analysis because an *a priori* regression found it to be significantly negatively correlated with elevation ($P<0.05$) and positively correlated with number of pools at the site ($P<0.05$). In a separate analysis, factors influencing tadpole density within a site was investigated by regressing the mean number of tadpoles per pool (mean annual counts between 1991 and 2000 divided by number of pools at the site obtained from Garcia, 1997) against the three site characteristics described above.

2.4 RESULTS

2.4.1 Distribution of Toads and Predators in Relation to Topography

A forward stepwise logistic regression was used to analyse the distribution of toads; elevation, maximum slope within a 100 m radius and aspect were compared between 17 toad populations inhabiting torrent gorges and 50 randomly allocated sites within torrent gorges. Only maximum slope, which was significantly positively associated with toad presence, was included in the overall model (Table 2.1). The model was significant ($\chi^2_1=16.2$; $P<0.001$) and resulted in 76.1% correct predictions of toad occurrence. A stepwise logistic regression on the same three topographical variables to predict the presence or absence of predators at toad sites found that only elevation, which was negatively associated with predator presence (Table 2.1), was included in the model. The overall model was significant ($\chi^2_1=8.0$; $P=0.005$) and resulted in 76.2% of correct predictions of predator occurrence.

Table 2.1. Results of logistic regression to predict presence/absence of toads and snakes based on three topographical features. Df = 1 for each analysis. Values in bold represent those included in the model.

	Toads (17 present, 50 absent)			Predators (10 present, 11 absent)		
Variable	Beta	R	P	Beta	R	P
Elevation (m)	-	0.14	0.07	**0.01**	**- 0.31**	**0.03**
Slope	**0.07**	**0.35**	**< 0.001**	-	0.00	0.52
Aspect	-	0.00	0.83	-	0.00	0.51

2.4.2 Factors Influencing Larval Population Size and Density

In order to assess the factors important in influencing larval population size, a stepwise multiple regression was conducted to test the log of mean annual tadpole counts for each population (n = 21) from 1991 – 2000 (Román, 2001) against three site characteristics. Site characteristics chosen were; elevation, number of pools at the site (Garcia, 1997) and degree of isolation (based on mean horizontal distance to the five nearest populations). Only the number of pools was included in the model and this variable showed a strong positive association with larval population size (Table 2.2). The overall model was significant (R^2=0.43; $F_{1,19}$=14.4; P=0.001). When number of tadpoles per pool was regressed against the same three independent variables, only elevation was significantly associated (Table 2.2) and was therefore included in the model. The overall model was significant (R^2 = 0.20; $F_{1,19}$=4.7; P<0.05).

Table 2.2. Results of multiple linear regression of log tadpole population size (means of annual counts 1991 – 2000) and number of larvae per pool against elevation, number of pools and degree of isolation (mean horizontal distance to the five nearest populations). Value in bold represent inclusion in the model.

	Larval population size			Number of larvae per pool		
Variable	Standardized Coefficient (Beta) / Partial Correlation	T	P	Standardized Coefficient (Beta) / Partial Correlation	T	P
No. Pools	**0.66**	**16.67**	**0.001**	- 0.23	-1.02	0.32
Elevation	0.37	1.69	0.11	**0.42**	**2.16**	**0.04**
Isolation	- 0.05	- 0.30	0.76	0.01	0.04	0.97

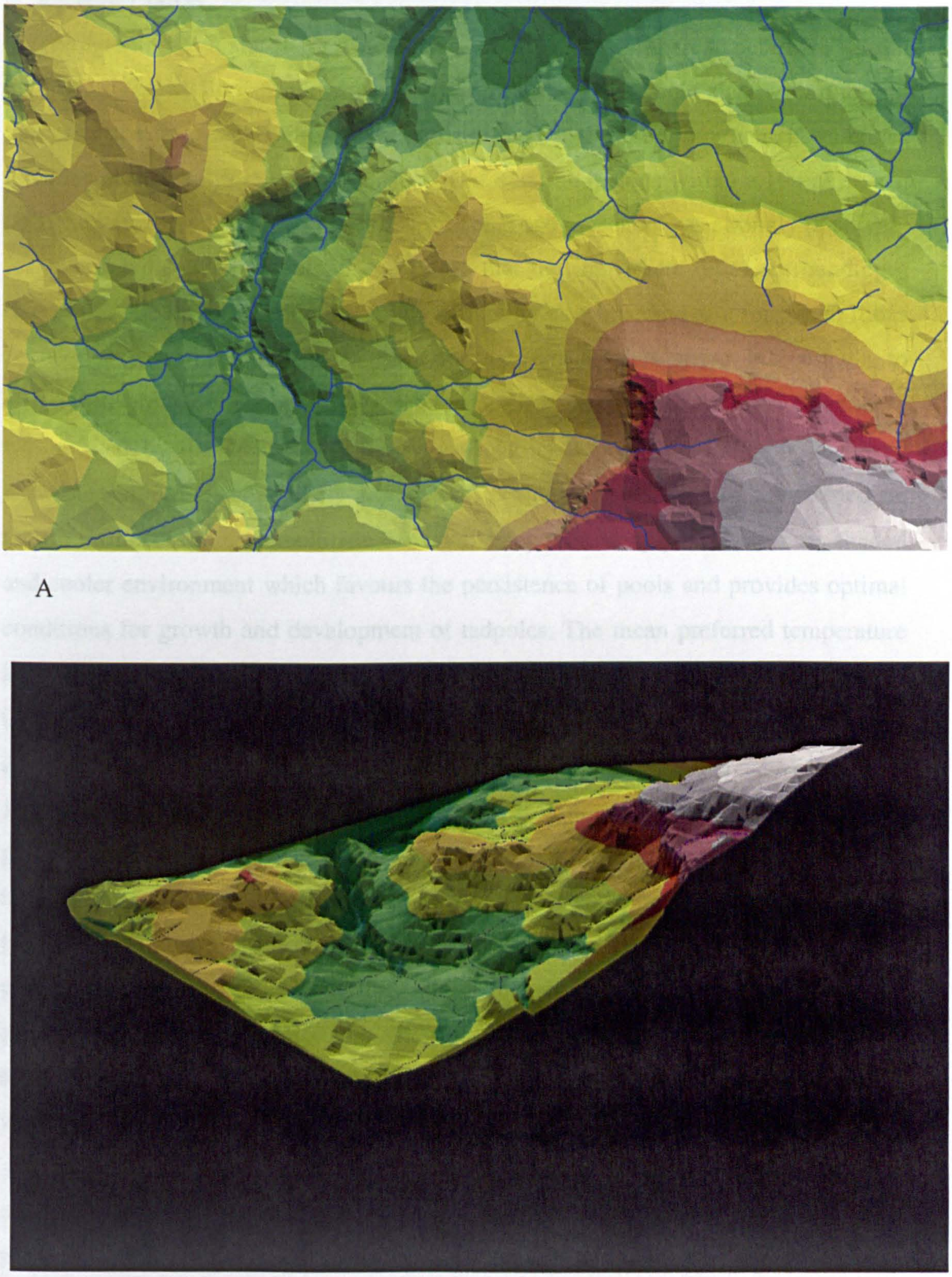

Figure 2.1. Two-dimensional (A) and three-dimensional (B) representations of TIN surface of a selected area of the Serra de Tramuntana, Mallorca.

2.5 DISCUSSION

Topographical features derived using GIS were compared between torrent populations of Mallorcan midwife toads and randomly generated sites within torrent gorges of the Serra de Tramuntana mountain range. Of three topographical variables included in the analysis; elevation, aspect and maximum slope within a 100 m radius, only the latter was included in the final model. Toad presence was strongly positively associated with maximum slope, indicating that the immediate relief surrounding a torrent may be important in dictating its suitability as a breeding site for toads. It has been suggested that steep sides may serve to make sites more inaccessible to introduced predators (Tonge, 1986; Schley & Griffiths, 1998), although this theory was refuted by the lack of a negative association between predator presence and maximum slope. A more likely explanation is that the steepness of the immediate terrain influences the microclimate of a site; steep sides may provide a more shaded and cooler environment which favours the persistence of pools and provides optimal conditions for growth and development of tadpoles. The mean preferred temperature and optimum temperatures for development of *Alytes muletensis* tadpoles are 21.6°C (Martens, 1984) and 21-24°C (Kadel & Hemmer, 1984) respectively, both of which are low relative to other European amphibians including *Alytes obstetricans* (Martens, 1984). Pools in open areas are more likely to be exposed to the sun, making them prone to heating and desiccation during the summer. Because of the low optimum temperature for the growth and development of larvae and since the distribution of the toad is likely to be constrained by water availability, environments favouring the persistence of cool plunge pools throughout the year are most likely to favour the presence of the toad. Additionally, steep sides surrounding a site may provide more suitable habitat for adults, which often take refuge in humid cracks and crevices within the walls of the torrent gorges (pers obs).

Amongst sites occupied by toads, the distribution of two introduced predators *N. maura* and *R. perezi* was found to be identical. In other words, wherever snakes were present, frogs were present and vice versa. This suggests that snake populations may only be sustained by the presence of *R. perezi* and that the presence of *A. muletensis* alone is not sufficient. One study conducted on mainland Spain also found *N. maura* to prey heavily upon adults and tadpoles of *R. perezi* (Santos *et al.*, 2000), and the presence of the frog may be a significant a factor in the successful establishment and continued existence of the snake on Mallorca. This study found a strong negative association between predator presence and elevation amongst toad sites and, although both the frog and the snake were found together at elevations as high as 1000 m (pers obs), it is likely that environmental and climatic conditions associated with lower elevations are preferred by both predators. *N. maura* is restricted to lower elevations in the northern part of its range. In Italy it is constrained to elevations below 700 m and in the Iberian Peninsula, where it reaches its highest elevation, it is found up to 1700 m (Gasc *et al.*, 1997). Although *R. perezi* is known to occur up to elevations of 1500 m in the Pyrenees and over 2000 m in parts of Spain (Gasc, *et al.*, 1997), it may be constrained by a lack of available habitat at higher altitudes on Mallorca. A reduction in predation pressure and competition with increasing elevation may be an important factor in the persistence of toad populations in montane torrent gorges and an important consideration in the design of future reintroduction sites.

The viability of isolated populations of a species typically depends upon factors such as population size and degree of inter-population connectivity (Halley, Oldham & Arntzen, 1996; Hanski, 1999), which in turn is a function of both physical distance and the characteristics of intervening habitat (Forman, 1995). Of three site characteristics considered; elevation, isolation and the number of pools within a site, the size of larval populations of the Mallorcan midwife toad was associated most strongly with the number of pools at the site. This is an important finding because it indicates that the viability of a population may be greatly enhanced by increasing the number of potential breeding pools within a gorge. The capacity of adult toads for dispersal is unknown. However, it is highly likely that torrent gorges provide ideal corridors for the movement of adults, which are more likely to move between pools within the same torrent gorge than to traverse open areas to reach potential breeding sites. Therefore, the number of pools within a torrent gorge is likely to be an important factor determining the viability of a population. When the number of pools at a site was factored in by substituting larval population size with number of larvae per pool in the analysis, the latter showed a significant positive association with elevation. Two possible explanations, which are not mutually exclusive, will be discussed for this finding. Firstly, environmental and climatic conditions associated with high elevations may enhance the viability of toad populations. As has been discussed, the mean preferred and optimal temperatures of *A. muletensis* tadpoles are low relative to lowland European anuran species such as *A. obstetricans* (Martens, 1984). As a result, lower temperatures associated with high altitudes probably favour the growth and development of midwife toad tadpoles. Secondly, because the presence of introduced predators is negatively associated with altitude, populations of tadpoles and adult toads may be depleted by higher rates of predation at lower elevations.

The results of this study have a number of implications for the recovery programme of the Mallorcan midwife toad and may be used to maximise the success of future reintroductions and enhance the viability of existing toad populations. It appears that the optimal habitat for the species is high altitude torrent gorges containing many pools and surrounded by steep sides. These factors should be considered in the design and location of reintroduction sites for the toad. Additionally, the viability of existing toad populations may be significantly enhanced by the creation of more pools within a site, since overall larval population size appears to be strongly associated with the number of pools available.

CHAPTER 3: POPULATION STRUCTURE, GROWTH AND DEVELOPMENT OF *ALYTES MULETENSIS* TADPOLES

3.1 SUMMARY

Factors influencing the dynamics of Mallorcan midwife toad (*Alytes muletensis*) larval populations were investigated. Larval size-structure was measured in 13 populations encompassing three distinct environments types; predator-free torrent pools, predator-free cisterns and torrent pools subject to incursions from introduced snake and frog predators. Unimodal distributions of tadpole size within both predator-free environments contrasted sharply with bimodal size-distributions in pools subject to predation. The absence of medium-sized tadpoles from predator environments is thought to be a result of either size-specific predation from predators (principally snakes), changes in tadpole recruitment, growth and development brought about by the presence of predators, or a combination of the two. Rates of growth and development were measured in three pools, each characterising one of the three environments, by monitoring temporal changes in total length and developmental stage of tadpoles. Growth was fastest in a predator-free torrent pool (peaking at a growth rate of 6.3% per day), intermediate in a predator-free cistern (4.4% per day) and lowest in a torrent pool from which snakes were recently removed (0.3% per day). Development was fastest in a predator-free cistern, where tadpoles were found to metamorphose in little over three weeks, and lowest in the 'predator' torrent pool. Rapid developmental rates in the cistern were probably a result of high water temperatures. The results of this study indicate that *A. muletensis* tadpoles are capable of rapid growth and development under natural conditions and the presence of introduced predators may play an important role in regulating the structure and dynamics of larval populations.

3.2 INTRODUCTION

Factors influencing the structure and dynamics of anuran larval populations are central to regulating amphibian populations as a whole (Berven, 1990). The dynamics of any population is in turn likely to be affected by recruitment, mortality, growth and development. Recruitment is typically dictated primarily by the reproductive strategy of the species and anurans typically fall into one of two categories; explosive or prolonged breeders. Explosive breeders produce large clutches of eggs over a relatively short time period, whereas prolonged breeders produce smaller clutches throughout a longer period in the year. The Mallorcan midwife toad, *A. muletensis* is a prolonged breeder and breeds from February through to September (Bush, 1993). Prolonged breeding such as this typically provides greater potential for large size difference within tadpole populations and provides the potential for greater variation in size-structure between populations. Recruitment may also be influenced by the presence of predators, which can affect decisions regarding the timing and siting of oviposition in anurans (Magnusson & Hero, 1991; Holomuzki, 1995; Resetarits, 1996; Downie *et al.*, 2001).

Mortality in amphibians is highest during the larval stage of development (Turner, 1962; Duellman & Trueb, 1986; Stangel, 1988) and this is primarily due to predation (Calef, 1973). Predation may be size-specific, although the literature on size-preferences of vertebrates predating tadpoles is sparse. Crump & Vaira (1991) found that avian predators preferred to prey upon larger tadpoles, although Kupferberg (1997) found garter snakes to be unselective. *A. muletensis* tadpoles are commonly predated on by the introduced viperine snake *Natrix maura* and green frog *Rana perezi,* at certain localities (pers obs) and, if these predators exhibit size-preferences then these are likely to be important in regulating *A. muletensis* tadpole populations.

Growth and development of anuran larvae affect time to, and size at, metamorphosis, both of which have important consequences for future adult fitness (Berven, 1990). Tadpole growth is influenced by numerous biotic and abiotic factors including food availability (Hota & Dash, 1981), water temperature (Alford, 1999), density (Dash & Hota, 1980; Hota & Dash, 1981; Travis & Trexler, 1986; Brönmark & Edenham, 1993), intra- and inter-specific competition (Brockelman, 1969; De Benedictis, 1974) and predation risk (Skelly, 1992). However, predators can either increase or decrease rates of tadpole growth. Because tadpoles often reduce activity in the presence of predators they inevitably spend less time feeding and often grow more slowly when predators are present (Skelly, 1992). Conversely, tadpoles of some species have been found to grow more rapidly during early development in order to reach a size refuge from predation (Babbitt & Tanner, 1998). Predation may also enhance growth by ameliorating the negative effects of competition through the removal of individuals from the population (Wilbur, 1987; Peacor, 2002). Tadpole development rates may be influenced by, among other factors, temperature, predation risk and growth rate or body size thresholds (Alford, 1999; Laurila & Kujasalo, 1999).

Factors important in the regulation of larval *A. muletensis* populations are poorly understood. While tadpoles raised in captivity generally take from a few months to over a year to reach metamorphosis (Lea *et al*, 2002; pers obs), it is not known if this is a true reflection of the larval period under natural conditions. This chapter will compare the size-structure of *A. muletensis* tadpole populations across different environments to gain an insight into mechanisms underlying the regulation of population structure. Tadpole populations in torrent pools subject to predation from snakes and frogs (hereafter called predator torrent pools) will be compared with populations in predator-free torrent pools and predator-free cisterns. In an attempt to monitor natural rates of growth and development across environments, changes in tadpole population size-structure will be monitored in all three pool-types.

3.3 METHODS

3.3.1 Population Size-and Stage-structure

Three different environments were recognised as containing *A. muletensis* tadpole populations; three predator-free artificial cisterns (pools C1–C3; 120 tadpoles), four predator-free torrent pools (pools PF1–PF4 pools; 150 tadpoles) and six predator torrent pools (pools P1–P6; 206 tadpoles). Between 20 and 50 tadpoles were collected from pools within each environment in May/June 2000 and May/June 2001 using a 1 mm mesh dipnet. Tadpoles were anaesthetised using MS222 and measured for eight morphometric variables (see Figure 3.1) to the nearest 0.1 mm using dial callipers; this was found to be the greatest level of accuracy achievable (O'Brien, 2000). *A priori* tests confirmed that MS222 does not affect short- or long-term survival of tadpoles. Each tadpole was staged according to Cambar & Martin's (1959) staging table for *A. obstetricans*. Patterns of differentiation for metamorphosing *A. muletensis* and *A. obstetricans* larvae are exactly the same apart from possible differences in rate and stage-specific size (Lea *et al.*, 2002).

Size-structure plots were created for each population and for each of the three environments by assigning the total length of each tadpole to a 5 mm size-class and then plotting size-class against percentage frequency within the population. Stage-structure was plotted for each of the environments by calculating percentage frequency of tadpoles at each developmental stage. Rates of development relative to growth were calculated for all tadpoles within each environment by regressing the first principal component (PC1) in a principal components analysis (PCA) of the eight morphometric variables against stage of development. PC1 has similarly been used as an index of overall body size in previous studies (e.g. McCollum & Leimberger, 1997; Van Buskirk & McCollum, 2000*a*). An ANOVA was then conducted to compare unstandardised residuals between the three environments.

Figure 3.1. Diagram showing eight morphometric measurements of each tadpole. 1 = body depth; 2 = body width; 3 = body length; 4 = tail length; 5 = tail muscle depth; 6 = maximum tail fin depth; 7 = upper tail fin depth; 8 = lower tail fin depth.

3.3.2 Monitoring Tadpole Growth and Development

A predator-free torrent pool (PF1) and cistern (C1) within the same gorge were identified as containing only tadpoles under a week old (A. Román pers com). Because all tadpoles were at such an early stage of development these pools provided an ideal opportunity to monitor tadpole growth and development rates in predator-free environments. Another torrent pool (P3) was selected to represent a tadpole population subject to predation from snakes based on the presence of two snakes in the pool on the first visit. Palpation of the snake's stomachs confirmed that they had eaten *A. muletensis* tadpoles.

Pool PF1 measured 6 m x 5 m with a maximum depth of 1.2 m and the artificial cistern C1 measured 4 m x 0.7 m with a depth of 0.25 m; the two pools were approximately 800 m apart and at 750 m altitude. The predator pool P3 measured 1.5

m x 1.2 m and 0.5 m deep and was located in a separate gorge at approx 350 m altitude. On 22 and 23 May 2000, 40 tadpoles were collected using a dipnet from pools PF1 and C1 respectively and on the 25 May 2000 20 tadpoles (thought to comprise most of the population) were collected from pool P3 using the same technique. Tadpoles were anesthetized using MS222, measured for total length to the nearest 0.1 mm using dial callipers and staged using Cambar & Martin's (1959) staging table. Following recovery, tadpoles were released into the pool from which they were collected. Pools PF1 and C1 were revisited after 21 and 35 days and P3 was revisited after 28 days; 40 tadpoles were collected from each of the predator-free pools and 25 tadpoles were collected from the predator pool (again thought to comprise most of the population) at these times. Tadpole measurement procedures were repeated in order to document changes in size- and stage-structure within the population. No snakes entered P3 in the 28-day period between measurements. This could be ascertained with some certainty since any snakes entering the water during this time would have been unable to leave due to extremely steep sides surrounding the pool.

3.3.3 Statistical Analysis

Tadpole size-structures (based on total tadpole length) at each sampling period were plotted to monitor tadpole growth over the period of the study in each of the three environments. Changes in the population structure between sampling periods were used to estimate maximum tadpole growth rate; this was done by taking the difference between the mean length of all tadpoles in the first sample and the mean length of the longest five tadpoles (to avoid including newly deposited tadpoles in the analysis) on the second sample and dividing this by the number of days between measurements. Developmental rates of tadpoles within respective pools were estimated by assessing changes in the stage structure of all tadpoles between sampling periods.

3.4 RESULTS

3.4.1 Population Size- and Stage-structure

Figure 3.2 displays the size structure of the 13 individual populations and Figure 3.3 displays the pooled size- and stage-structures for the three environments. Predator-free torrents and cisterns typically displayed a unimodal size distribution, compared to a distinctly bimodal size distribution in all populations subject to sporadic incursions from snake and frog predators. Tadpoles of between 50 and 55 mm in length were entirely absent from pools subject to predation and only one pool out of the six contained tadpoles between 45 and 50 mm.

An ANOVA revealed that overall tadpole body size (PC1, accounting for 92.6% of variance) differed significantly between the three environments ($F_{2,453}$=8.1; $P<0.001$). A Tukey's post hoc revealed that overall, tadpoles from predator-free torrent pools were significantly larger than those in predator-free cisterns (P=0.002) and those from predator torrent pools ($P<0.001$). Body size did not differ significantly between cisterns and predator torrent pools (P=0.98). Developmental stage-structure differed noticeably between environments; tadpoles in predator torrents were typically more developed than those in predator-free environments. The vast majority of tadpoles in predator-free torrents and predator-free cisterns were at stage IV6 (89.2% and 82.7% respectively) compared to 61.2% in predator torrent pools.

3.4.2 Development vs Growth Rate Across Environments

Body size (PC1) was regressed against developmental stage for all of the tadpoles (R^2=0.09; $F_{1,475}$=47.2; $P<0.001$) and the unstandardised residuals were compared between environments using a one-way ANOVA. Significant differences were found in the magnitude of the residuals between environments ($F_{2,453}$=14.5; $P<0.001$); these differences are illustrated in Figure 3.4. A Tukey's post hoc revealed that residuals for tadpoles from predator-free torrents were significantly higher than those from cisterns ($P<0.001$) and significantly higher also than those from predator torrents ($P<0.001$). Predator pools and predator-free cisterns did not differ significantly in the size of the residuals ($P>0.05$). Thus, tadpoles in predator-free torrents appear to develop more slowly relative to growth than tadpoles in both other environments.

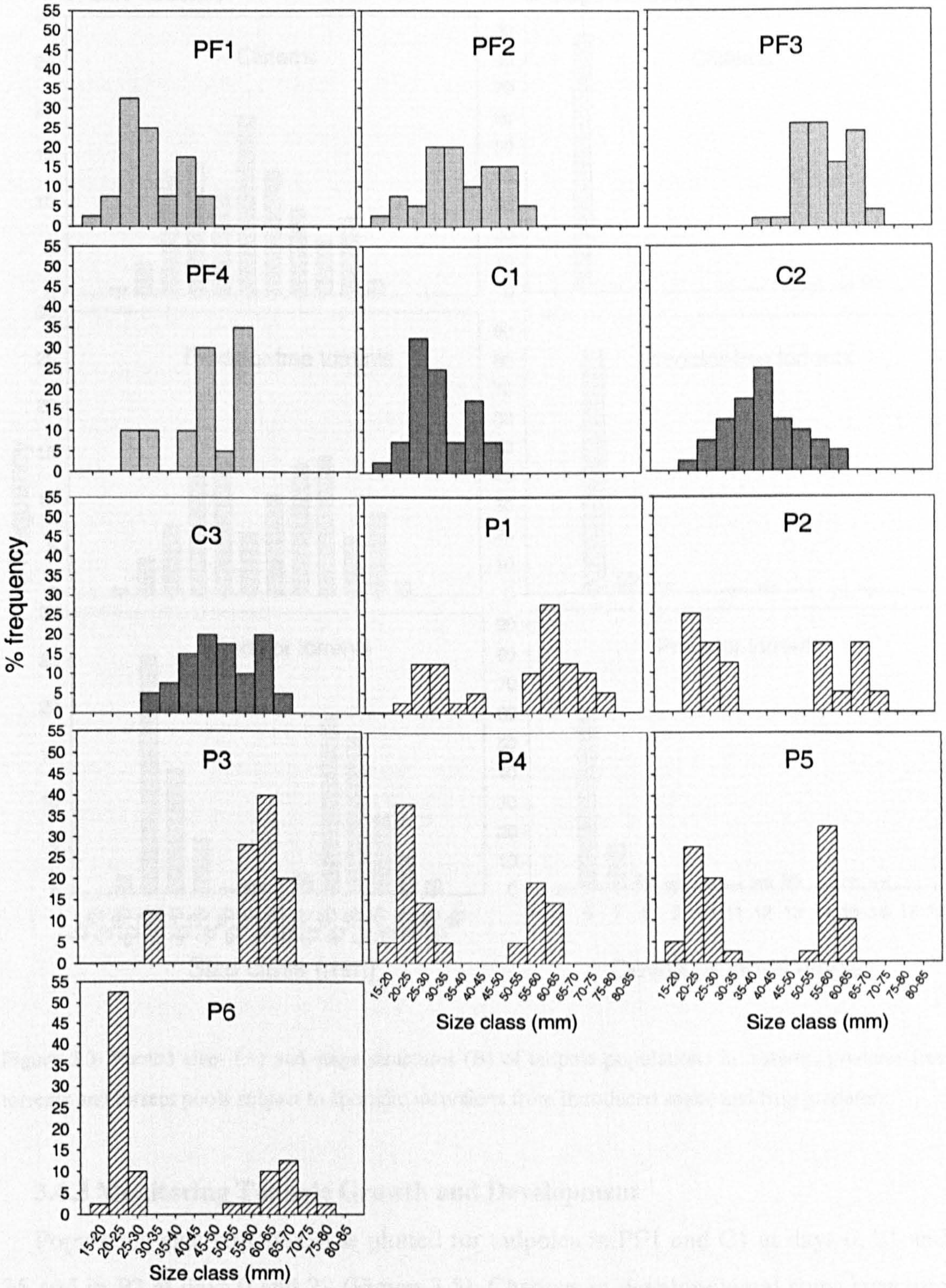

Figure 3.2. Size-structure (based on total length) of tadpole populations within three environments. PF = predator-free torrent pools (light shading); C = predator-free artificial cisterns (dark shading); P = torrent pools with introduced snake and frog predators (diagonal shading).

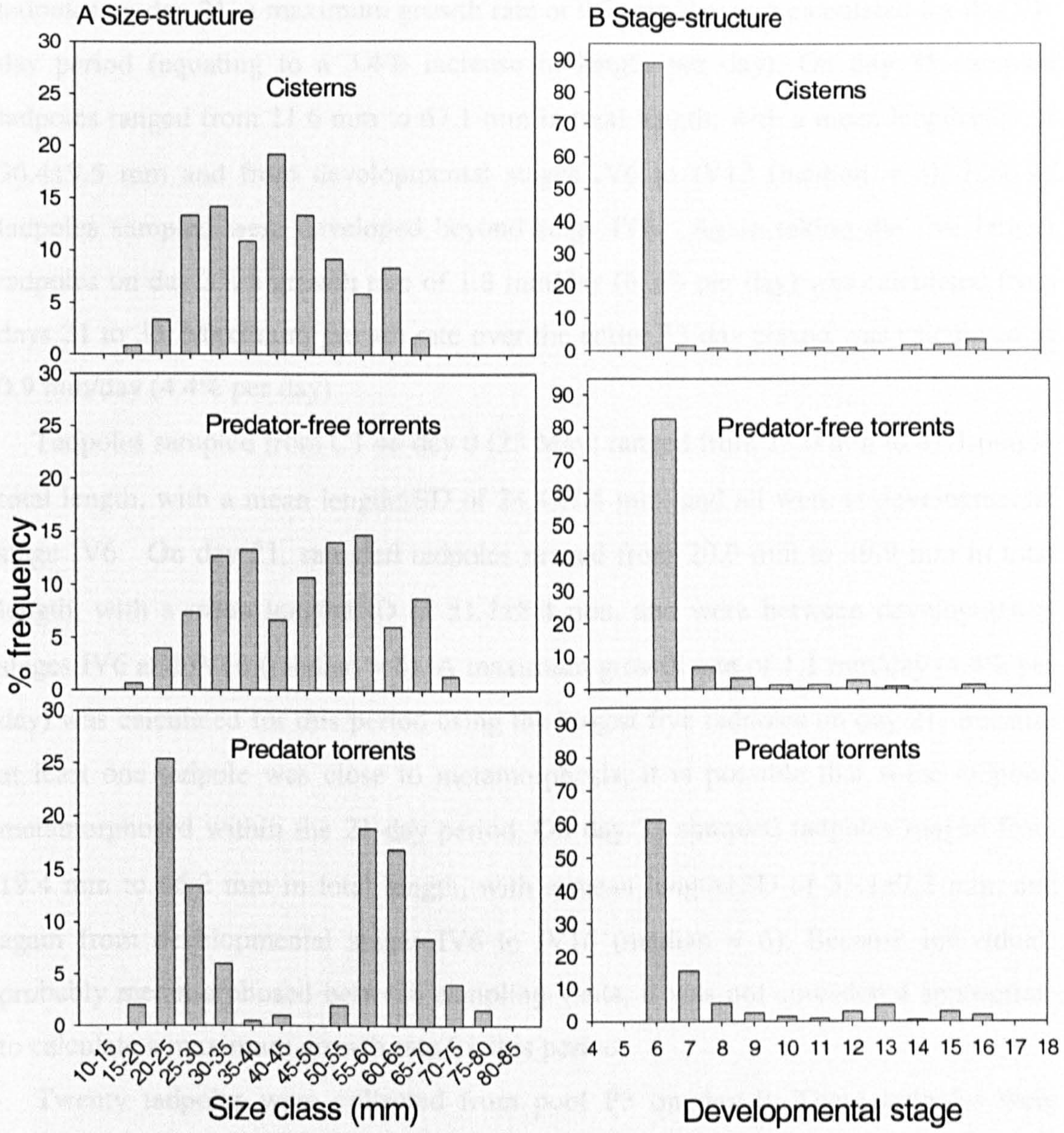

Figure 3.3. Pooled size- (A) and stage-structures (B) of tadpole populations in cisterns, predator-free torrents and torrent pools subject to sporadic incursions from introduced snake and frog predators.

3.4.3 Monitoring Tadpole Growth and Development

Population size-structure was plotted for tadpoles in PF1 and C1 at days 0, 21 and 35 and in P3 at days 0 and 28 (Figure 3.5). Changes in developmental stage structure of the populations over the course of the study are shown in Table 3.1. Tadpoles sampled from PF1 on day 0 ranged in total length from 16.7 mm to 26.1 mm with a mean length±SD of 21.6±2.5 mm and were all at developmental stage IV6. On day 21 sampled tadpoles ranged from 19.9 mm to 39.0 mm in total length with a mean length of 29.1±4.9 mm; again all were at developmental stage IV6. Taking the largest five tadpoles on day 21, a maximum growth rate of 0.7 mm/day was calculated for this 21-day period (equating to a 3.4% increase in length per day). On day 35 sampled tadpoles ranged from 21.6 mm to 67.1 mm in total length, with a mean length±SD of 36.4±9.5 mm and from developmental stages IV6 to IV12 (median = 6); 10% of tadpoles sampled were developed beyond stage IV6. Again taking the five largest tadpoles on day 35, a growth rate of 1.8 mm/day (6.3% per day) was calculated from days 21 to 35. Maximum growth rate over the entire 35-day period was calculated as 0.9 mm/day (4.4% per day).

Tadpoles sampled from C1 on day 0 (23 May) ranged from 13.0 mm to 41.1 mm in total length, with a mean length±SD of 24.4±7.4 mm, and all were at developmental stage IV6. On day 21, sampled tadpoles ranged from 20.9 mm to 49.9 mm in total length, with a mean length±SD of 31.7±8.4 mm, and were between developmental stages IV6 and IV16 (median = 6). A maximum growth rate of 1.1 mm/day (4.4% per day) was calculated for this period using the largest five tadpoles on day 21. Because at least one tadpole was close to metamorphosis, it is possible that some tadpoles metamorphosed within the 21-day period. On day 35 sampled tadpoles ranged from 19.4 mm to 46.2 mm in total length, with a mean length±SD of 33.1±7.2 mm, and again from developmental stages IV6 to IV16 (median = 6). Because individuals probably metamorphosed between sampling visits, it was not considered appropriate to calculate a maximum growth rate for this period.

Twenty tadpoles were collected from pool P3 on day 0. These tadpoles were thought to comprise the majority of the population since considerable netting effort did not result in any more captures. Sampled tadpoles ranged in total length from 53.5 mm to 64.7 mm (mean length±SD = 60.3±2.8 mm) and from developmental stages IV7 to IV11 (median = 8.5). On day 28, 25 captured tadpoles (including three thought to be newly deposited due to their small size) ranged in length from 31.2 mm to 66.6 mm (mean length±SD = 58.2±10.3 mm) and from developmental stages IV6 to IV16 (median = 13). Based on the five largest individuals on day 28, a mean growth rate of 0.2 mm/day (0.3% per day) was calculated for the 28-day period.

Table 3.1. Developmental stage-structure (according to Cambar & Martin, 1959) of tadpole populations in pools from three different environments. Values represent the percentage of tadpoles at each stage of development on each sampling day.

Pool	PF1			C1			P2	
Stage	Day 0	Day 21	Day 35	Day 0	Day 21	Day 35	Day 0	Day 28
IV 6	100	100	85	100	60	67.5	20	12
IV 7			10		7.5	5	30	
IV 8					2.5	2.5	30	8
IV 9					2.5			4
IV 10							5	4
IV 11			2.5		12.5	2.5	15	4
IV 12			2.5		5	2.5		16
IV 13					5			20
IV 14					2.5	5		4
IV 15						5		20
IV 16					2.5	10		8
IV 17								

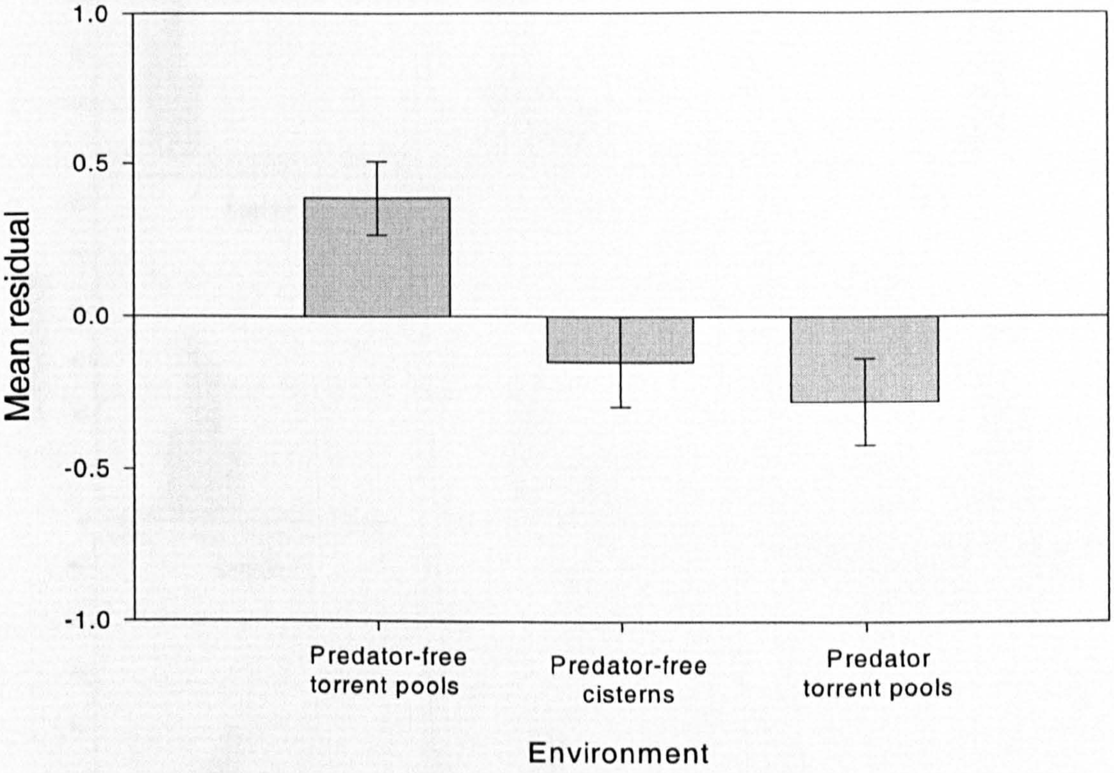

Figure 3.4. Residuals of regression (±95% confidence intervals) of body size (PC1) against developmental stage (Cambar & Martin, 1959) of all populations contained within three environments; predator-free torrent pools, predator-free cisterns and torrent pools subject to incursions from snake and frog predators. The magnitude of the residuals denotes the rate of growth relative to development. Tadpoles in predator-free torrent pools possessed the largest body size relative to developmental stage and those in torrent pools subject to predation from snakes and frogs possessed the smallest body size relative to developmental stage.

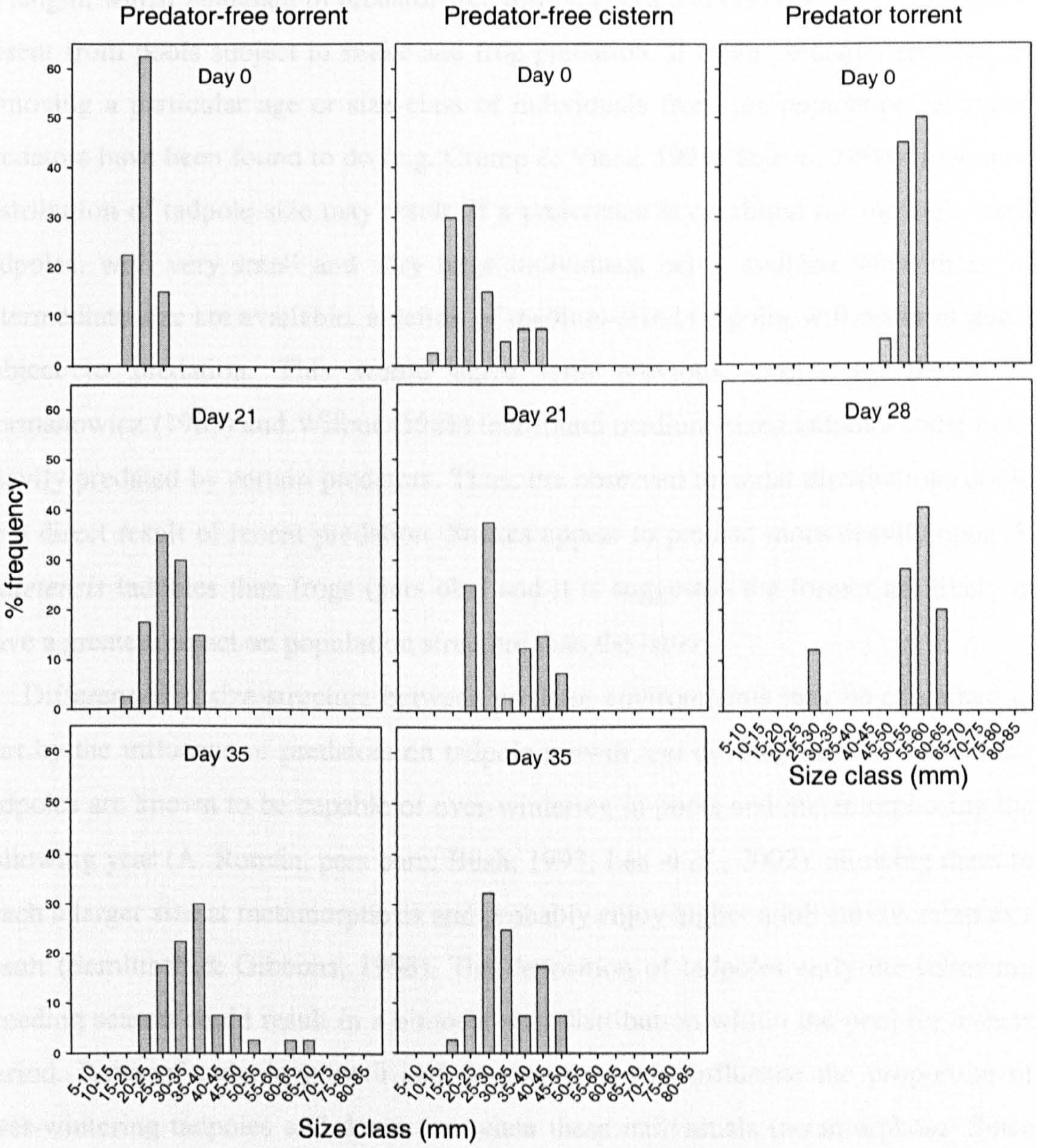

Figure 3.5. Temporal changes in population size-structure in a snake-free torrent pool, snake-free cistern and torrent pool subject to incursions from snake and frog predators.

3.5 DISCUSSION

Size- and stage-structure of *A. muletensis* tadpole populations was compared across three environments (predator-free torrent pools, predator-free cisterns and predator torrent pools) to investigate factors important in regulating larval population structure and dynamics. Populations from the two predator-free environments were typically unimodal in their size distribution, whereas pools subject to predation displayed distinctly bimodal distributions of tadpole size. Tadpoles between 50 mm and 55 mm in length, whilst abundant in predator-free torrent pools and cisterns, were completely absent from pools subject to snake and frog predation. If these predators are directly removing a particular age or size-class of individuals from the population, as many predators have been found to do (e.g. Crump & Vaira, 1991; Tejedo, 1993), a skewed distribution of tadpole size may result. If a preference is exhibited for medium-sized tadpoles, with very small and very large individuals being avoided when those of intermediate size are available, a deficit of medium-sized tadpoles will occur in pools subject to predation. This would agree with previous studies by Brodie & Formanowicz (1983) and Wilbur (1988) that found medium-sized tadpoles to be most heavily predated by certain predators. Thus, the observed bimodal distributions could be a direct result of recent predation. Snakes appear to predate more heavily upon *A. muletensis* tadpoles than frogs (pers obs) and it is suggested the former are likely to have a greater impact on population structure than the latter.

Differences in size-structure between predator environments may be explained in part by the influence of predators on tadpole growth and development. *A. muletensis* tadpoles are known to be capable of over-wintering in pools and metamorphosing the following year (A. Román, pers com; Bush, 1993; Lea *et al.*, 2002), allowing them to reach a larger size at metamorphosis and probably enjoy higher adult survivorship as a result (Semlitsch & Gibbons, 1988). The deposition of tadpoles early the following breeding season could result in a bimodal size-distribution within the pool for a short period. Rates of tadpole growth and development will influence the proportion of over-wintering tadpoles and determine when these individuals metamorphose. Since large over-wintered tadpoles can inhibit the growth of smaller individuals (Griffiths, 1997; Lea *et al.,* 2002) this may further reinforce size differences between tadpole cohorts for as long as over-wintered tadpoles remain in the pool. Monitoring of pools over a number of weeks showed that tadpoles in a pool from which snakes were recently removed grew at less than a fifth the rate of individuals in both a snake-free torrent pool and cistern. Although this cannot conclusively be attributed to the influence of predators, the presence of predators can inhibit tadpole growth through trade-offs associated with anti-predatory behaviours and feeding (e.g. Lima & Dill, 1990; Skelly and Werner, 1990; Skelly, 1992) and a number of studies have demonstrated that the perceived presence of a predator can have a large impact on tadpole growth, particularly at low competitive densities (Werner & Anholt, 1996;

Van Buskirk & Yurewicz, 1998; Peacor & Werner, 2000). While some studies have found predators to enhance growth rates by ameliorating competition (e.g. Wilbur, 1987), this may be counteracted by the negative impact of predator presence on tadpole behaviour since *A. muletensis* tadpoles are known to reduce activity in the presence of snakes (Griffiths *et al.*, 1998). Inter-population comparisons of tadpole body size at each developmental stage showed that tadpoles from predator torrent pools developed faster relative to growth than those from predator-free torrent pools. If development rate is similar in all pools, this will result in tadpoles metamorphosing at smaller sizes in predator environments. This did not appear to be the case, however, as tadpoles attained relatively large body sizes in predator pools (individuals reaching 85 mm in length were found in P6). Thus, the presence of snakes may influence time to metamorphosis by slowing development as well as growth. Indeed, some studies have found that the presence of predators can lengthen the larval period in this way and result in later development at a larger size (Smith & Van Buskirk, 1995; Laurila & Kujasalo, 1999). If this does occur in *A. muletensis*, a higher proportion of tadpoles may over-winter and persist in pools well into the following year. Changes in tadpole growth and development in the presence of predators could therefore influence population structure by delaying metamorphosis and inhibiting the growth of newly deposited individuals, resulting in a bimodal distribution of tadpole size well into the breeding season.

Populations inhabiting both predator-free torrent pools and cisterns displayed unimodal distributions of size, although comparisons of stage-specific body size found differences in relative rates of growth and development between the two environments. Tadpoles inhabiting cisterns were, on average, smaller at each developmental stage than those in predator-free torrents, suggesting a faster rate of development relative to growth in the former. Additionally, temporal changes in population size-structures suggested that growth was faster in a predator-free torrent pool (peaking at 1.8 mm or 6.3% of total length per day) than in a nearby cistern (peaking at 1.1 mm or 4.4% body length per day) whereas development rate was faster in the cistern. These differences resulted in tadpoles metamorphosing in a shorter time and at a smaller size in the cistern, with some individuals estimated to have reached metamorphosis within just over 21 days after deposition. Such differences in tadpole growth and development between predator-free torrent pools and cisterns may be due to variation in a number of biotic and abiotic factors. Smith-Gill & Berven (1979) found that the relationship between size and developmental stage in anuran larvae depends to a large extent upon environmental temperature. They suggested that development rate typically decreases faster than growth rate as temperature decreases so that at lower temperatures tadpoles grow more in each stage and metamorphose at larger body sizes than at high temperatures. *A. muletensis* tadpoles have also been found to develop faster at higher temperatures (Lea *et al.*, 2002). Because the relatively small size and exposed nature of cistern C1 may mean that it reaches higher daily temperatures than the nearby torrent pool PF1, this could speed up the development of tadpoles in the former. In environments where predation rates are higher on tadpoles than adult toads, it may be beneficial for tadpoles to develop rapidly and high water temperatures may actually increase the chances of survival by reducing the amount of time spent in the risky aquatic environment. In environments where tadpole predation rates are low, individuals may benefit from cooler temperatures that allow them to metamorphose at a larger size, since size at metamorphosis may be positively correlated with adult fitness (Berven, 1990).

Differences in relative rates of growth and development rate between environments may additionally be influenced by intra-specific competition. While the torrent pool was estimated to contain around 500 tadpoles at the start of this study, the smaller cistern contained in excess of 2000 individuals (pers obs); tadpole density was therefore considerably higher in the latter. While crowding can impede growth and speed up development in some species (Morin, 1986), Lea *et al.* (2002) found that increasing density actually slowed both growth and development in larval *A. muletensis*. Under both scenarios, individuals are likely to metamorphose at smaller sizes as tadpole density increases and this may impair adult fitness (Berven, 1990).

In addition to the effects of relative rates of growth and development, recent recruitment into pools may also explain differences in size- and developmental stage-structure between environments. Particularly high proportions of tadpoles at stage IV6 in predator-free torrent pools and, to a lesser extent, cisterns, may reflect high levels of recent recruitment into these pools relative to predator pools. Temporal changes in population size-structure indicated lower levels of recruitment in pool P3 than in PF1 and C1. While tadpole populations in both predator-free pools increased substantially in size between sampling periods, suggesting high levels of recruitment, only three newly deposited tadpoles were confirmed in pool P3 over the 28 day study period. Local conditions are influential in determining the timing of breeding and oviposition (Beebee, 1996) and may explain the different recruitment rates. The presence of predators has been found to influence the timing and choice of oviposition sites in some anurans (Magnusson & Hero, 1991; Holomuzki, 1995; Resetarits, 1996; Downie e*t al.*, 2001; Binkley & Resetarits, 2002) and the presence of snake and frog predators may therefore influence oviposition choice in adult *A. muletensis*. Upon the first visit to pool P3 two snakes were found in the water and were removed; the persistence of snake cues may have influenced decisions regarding the deposition of tadpoles over the following weeks. Due to the transient nature of the predators, it may be difficult to completely avoid tadpole predation in a torrent frequented by snakes and frogs. However, adults may still benefit from avoiding the deposition of tadpoles in pools containing strong cues from either predator. *A. muletensis* appears to avoid depositing tadpoles in pools occupied by *R. perezi* (pers obs), a potential competitor as well as predator of toad tadpoles (Tonge, 1986; pers obs). It is likely to be beneficial for the toad to preferentially choose pools that are not breeding sites of the frog, although this hypothesis remains to be tested.

Thus, while many factors may influence the population structure and dynamics of *A. muletensis* larvae, this study identified certain factors that appear to play a major role. The presence of introduced snake and frog predators heavily influenced population structure, indicating intense predation pressure in certain localities. The impact of these predators on tadpole behaviour, morphology and overall fitness is investigated further in later Chapters (Chapters 4, 5, 7 and 8). This chapter also found that *A. muletensis* tadpoles are capable of rapid growth and development under natural conditions, although there was considerable variation in growth and development rates between environments. Whereas high water temperatures appear to result in rapid development, shorter larval periods and metamorphosis at a smaller size, the presence of predators appeared to slow growth and development, resulting in longer larval periods and metamorphosis at a large size. The influence of abiotic and biotic factors on tadpole growth and development should be taken into consideration in the design of future reintroduction sites. In environments where the risk of tadpole predation is high, faster development may increase the chances of survival (Babbitt & Tanner, 1998; Peckarsky *et al.*, 2001) and pools designed to reach higher water temperatures may maximise population fitness. In predator-free environments, adult fitness may be maximised by allowing tadpoles to develop slowly and metamorphose at larger sizes; pools designed to maintain colder water temperatures are probably optimal in such environments. These findings highlight the need for further research into the influence of tadpole growth and development rates on the overall fitness of toad populations across environments in order to optimise the design of future reintroduction sites.

CHAPTER 4: MORPHOLOGICAL RESPONSES OF *ALYTES MULETENSIS* TADPOLES TO THE INTRODUCED SNAKE *NATRIX MAURA*

4.1 SUMMARY

In the face of variable predation pressure, it may be beneficial to evolve inducible defensive traits that only develop when a predator is present. These defences can comprise changes in life history, behaviour or morphology that enhance survival. While tadpoles of the Mallorcan midwife toad (*Alytes muletensis*) have been found to reduce activity in response to cues from the introduced viperine snake (*Natrix maura*), it is not known whether morphological responses have evolved in this relatively short period of predator-prey coevolution. Site comparisons revealed that the morphology of tadpoles varies between populations according to the presence or absence of snakes. In populations threatened by snakes, tadpoles possessed longer tails, thicker tail muscles and narrower tail fins. Experiments showed that such characteristics developed within 17 days of snake cues being introduced into a natural pool of tadpoles and within 15 days of exposure to snake cues under controlled conditions. Equally, when snakes were removed from a pool that they had naturally entered, tadpoles reverted to the non-predator phenotype within 30 days. Inducible and reversible defences are likely to evolve in prey if predators are wide-ranging and visit patches containing prey irregularly. The observed defences probably evolved relatively recently under intense and variable selection pressure from introduced predators. It is likely that the plastic responses contribute significantly to the overall morphological variation observed between natural populations.

4.2 INTRODUCTION

Anuran larvae can respond behaviourally (Morin, 1986; Petranka *et al.*, 1987; Kats *et al.*, 1988; Lawler, 1989; Skelly & Werner, 1990; Semlitsch & Gavasso, 1992; Griffiths *et al.*, 1998; Lefcort, 1998) and morphologically (Smith & Van Buskirk, 1995; McCollum & Van Buskirk, 1996; McCollum & Leimberger, 1997; Van Buskirk, 2000) to the presence of predators in order to reduce their risk of being eaten. Whereas behavioural responses are typically easily reversible, morphological responses are thought to be long-term and typically irreversible, because of the time constraints and physiological costs involved (Gabriel, 1999; Relyea, 2001*a*). Van Buskirk (2002) found little relationship between behavioural and morphological plasticity and suggested that the two may evolve under different scales of environmental evolution. Behavioural responses, being more plastic, undoubtedly require less time to develop than morphological ones (West-Eberhard, 1989). Brönmark & Pettersson (1994) found that the reversibility of morphological defences of crucian carp was slow and incomplete after chemical cues from predatory pike were removed. Therefore, while some anuran larvae respond behaviourally to introduced predators (Kiesecker & Blaustein, 1997; Chivers *et al.,* 2001), we do not know whether inducible morphological defences can develop within a similarly short time period. Previous work on *A. muletensis* has revealed that the tadpoles and adult toads respond behaviourally to introduced viperine snakes (Griffiths *et al*., 1998; Schley & Griffiths, 1998), but morphological changes have not been described. The survival of the toad and its continued coexistence with the snake raises questions about antipredator defences that may have developed within this relatively short period of predator-prey coevolution.

The aim of this chapter is to investigate whether *A. muletensis* tadpoles under different predator regimes (see Chapter 2) differ in their morphology and, if they do, whether this reflects a plastic and reversible response to the presence of predatory snakes, which are believed to be the primary predator of the toad (Tonge, 1986; Griffiths *et al*., 1998). As Relyea (2002*b*) states "it is important that we identify the mechanisms underlying the outcomes of predation, rather than simply observing the outcomes and then inferring which processes are responsible". Firstly, morphological variability between different populations of *A. muletensis* tadpoles will be investigated in relation to the presence or absence of snakes. Secondly, the inducibility and reversibility of any predator-related morphological patterns will be tested using addition and removal experiments with snakes in two natural pools. Finally, whether or not the predator-related morphological patterns observed in natural populations can be induced experimentally using only chemical cues from snakes will be tested.

4.3 METHODS

4.3.1 Comparison of Morphology Between Natural Pools With and Without Snakes

The morphology of tadpoles from ten pools in the Serra de Tramuntana, in NW Mallorca was compared. The populations were the same torrent pools used in the population size-structure comparison (Chapter 3). The ten pools were located in different gorges, and represented different populations of toads. According to long-term surveys by the Mallorcan conservation agencies, six of these pools (P1 - P6) were known to suffer incursions by *N. maura*, while the remaining four populations were in areas known to be snake-free (PF1 – PF4). All of the pools were located along torrent beds that were otherwise dry at the time of sampling. Tadpoles were caught using a net (mesh size 1 mm), anaesthetised using 0.01% MS222; measured for eight morphometric variables; allowed to recover and released. Selection of variables for measurement was based upon those traits that were considered likely to be under selection from predators. These were body length, body width, body depth, tail length, tail muscle depth, maximum tail fin depth and upper and lower tail fin depth at the widest point (see Figure 3.1). Measurements were made to the nearest 0.1 mm - found to be the greatest level of precision achievable (O'Brien 2000) - using dial callipers, and tadpoles were placed on moist tissue paper while being measured to prevent desiccation. Tadpoles were never out of the water for more than two minutes and there was no mortality during the measuring procedure.

Statistical Analysis

In order to standardise the morphological measurements against a single measure of body size, the first component of a principal components analysis (PC1) of the eight log-transformed morphological measurements was used as 'body size' (see Chapter 3). The residuals obtained from regressions of the morphological variables on PC1 were then used to obtain measures of relative size for each variable (e.g. Bookstein 1991; Van Buskirk & Relyea 1998; Van Buskirk & McCollum 1999). A multivariate analysis (MANOVA) was performed on the residuals of the eight morphometric variables to test for variation in tadpole shape between the ten pools. An *a priori* comparison of variation in tadpole shape between pools with and without snakes was then performed using a further MANOVA. Univariate analyses (ANOVA) were then conducted on each variable separately to compare differences in the relative size of each morphological trait.

4.3.2 Field Tests for Induction and Reversibility of Traits

Two whole-pool manipulation experiments were conducted to test for the plasticity of morphological defences in tadpoles. Firstly, an attempt was made to induce a predator phenotype in tadpoles that had never previously been exposed to snakes (induction experiment). This experiment was conducted in a natural torrent pool in NW Mallorca in an area where snakes do not occur naturally (site PF4). The toad population at this site is derived from introductions of captive-bred stock between 1992 and 1994. The founders of the captive stock came from a site where snakes occurred, so the ancestors of the tadpoles tested had probably been exposed to snakes. Although the torrent was dry at the time of the study, water had scoured a number of circular pools along the torrent bed, some of which retained water and provided breeding sites for toads. Two adjacent and similar sized pools (c. 1 m in diameter and 0.2 m deep) were chosen for this experiment. Twenty tadpoles were collected from each of the two pools and measured for eight morphometric variables as described previously. Three snakes held in nylon bags were then introduced into one of the pools (test pool) for 3-4 hrs per day. This was repeated every day for 17 days. The other pool (control pool) was left undisturbed over this period. After 17 days, 20 tadpoles were collected from each pool and measured as above.

The second experiment was conducted to investigate whether tadpoles revert back to the original 'non-predator' phenotype after the removal of snakes (reversal experiment). This experiment was conducted in a natural pot-hole located in a second torrent bed (site P3). The pot-hole was about 1.5 m in diameter, and had been scoured to a depth of about 2 m by torrent water. At the time of the study, the pot-hole contained a pool about 0.3 m deep. This was found to contain toad tadpoles and two *N. maura* when first visited. The steep sides surrounding the pool prevented the snakes from escaping and they may have been trapped there for some time. Palpation of the snakes' stomach contents resulted in the regurgitation of both tadpoles and an adult *A. muletensis*. The snakes were removed from the pool. Twenty tadpoles were captured (thought to comprise most of the population), measured for all eight variables as described above, allowed to recover and then released back into the pool.

The tadpoles were then left undisturbed for 28 days. During this period no snakes entered the pool. This could be ascertained with some certainty because any snakes that may have entered during this time would have been unable to leave on account of the vertical sides of the pot-hole. After 28 days, 25 tadpoles were collected and measured. Because of the relatively long larval period of this species and the small number of tadpoles in the pool, the tadpoles caught on the second visit were assumed to contain mostly the same individuals that were caught and measured 28 days previously. However, three tadpoles were probably newly deposited judging by their small size.

Statistical Analysis

Morphometric measurements were log-tranformed and analysed as described above for each of the two experiments. MANOVAs were conducted on the residuals of the eight morphometric variables after regression against body size (PC1) to test for significant changes in morphology upon the addition and removal of snakes. Univariate ANOVAs were used to test for significant changes in the relative size of each morphometric variable.

4.3.3 Induction and Reversibility in Relation to Morphological Variation Between Populations

Using the eight morphometric variables obtained from the ten natural populations, discriminant function analysis was used to determine which combinations of variables provided the best separation of the populations. The discriminant functions obtained were then used to predict population membership for each tadpole. In order to visualise the positions of tadpoles from pools P3 and PF4 in relation to the other populations, tadpoles from these two sites were classified both before and after addition and removal of snakes respectively.

4.3.4 Induction of Morphological Changes by Chemical Cues from Snakes

In order to test whether the morphological patterns observed in natural populations could be induced just using chemical cues from snakes, the development of tadpoles was compared in snake-conditioned water and unconditioned water. Thirty-two small tadpoles (mean length±SD 28.9±1.84 mm) were collected from torrent pool PF4 on 18

May 2001. These were measured for the eight morphometric variables described above, and divided into eight groups of four. Each group of four was raised in a plastic box containing 2.5 l of either snake-conditioned water or unconditioned water. Four boxes were allocated to the snake-conditioned water treatment while the remaining four boxes were kept as controls. The water used in both treatments originated from a well containing local natural ground water. Snake-conditioned water was obtained by suspending three *N. maura* held in nylon bags into a 20 l tank of water for three hours (the nylon bags were positioned so that the snakes could move freely within the water and surface for air when needed). Unconditioned water used for the controls was left to stand for a similar period of time. Conditioned and unconditioned water was renewed every two days and tadpoles were fed fish flake food *ad libitum* after each water change. All tadpoles were re-measured after 15 days.

The plastic boxes were randomly positioned within a regular array on the floor of a room in a farmhouse at 1000 m altitude in the Serra de Tramuntana, Mallorca. After each water change (i.e. every two days), the position of each box was re-randomised. The room was exposed to natural light-dark cycles via a south-facing window, and the temperature varied from 12.5°C at night to 24°C during the day. This is similar to temperature cycles in natural pools (Schley, Griffiths & Román, 1998).

Statistical Analysis

Morphometric measurements were log-tranformed and analysed as described above. Using boxes-within-treatments and tadpoles-within-boxes as nested factors, a MANOVA was conducted on the residuals of the eight morphometric variables after regression against body size (PC1), to test for significant differences in morphology between the two treatments. Univariate nested ANOVAs were used to test for significant changes in the relative size of each morphometric variable

4.4 RESULTS

4.4.1 Comparison of Morphology Between Natural Pools With and Without Snakes

There were a number of highly significant correlations between morphometric traits (Table 4.1) confirming the appropriateness of multivariate analyses. A number of different phenotypic correlations were found between tadpoles in the different predator environments indicating that traits may be independent and under different selection pressure within the two environments. For instance, in snake-free environments, as PC1 increased tadpoles developed relatively wider, deeper and shorter bodies, shorter tails with deeper tail fins and shallower tail muscles; exactly the converse was found in environments with snakes (Table 4.1). Additionally, whilst in snake-free environments tail length and tail muscle depth were highly significantly negatively correlated, in environments with snakes the two traits were highly significantly positively correlated.

An initial MANOVA revealed that tadpole morphology varied significantly between the ten pools sampled (Wilks' Lambda=0.058; $F_{63,1780}$=18.6; P<0.001). A further *a priori* comparison also revealed significant differences between sites with and without snakes (Wilks' Lambda=0.48; $F_{7,323}$=49.6; P<0.001). Univariate ANOVAs showed that tadpoles from pools with snakes possessed shallower and narrower bodies, longer tails, deeper tail muscles and shallower maximum and lower tail fins relative to overall body size than tadpoles from snake-free environments (Table 4.2, Figure 4.1). Relative body length and upper tail fin depth did not differ significantly between the two predator environments.

4.4.2 Field Tests for Induction and Reversibility of Predator-Related Traits

In the pool where snakes were added, there were significant differences in tadpole shape before and after exposure to snakes (Wilks' Lambda=0.35; $F_{7,32}$=8.5; P<0.001). Following the addition of snakes, tadpoles developed significantly deeper tail muscles, shallower overall, upper and lower tail fins and wider bodies relative to body size (Table 4.3, Figure 4.2). The body and tail became relatively longer, but not significantly so. In the control pool, there was no significant change in shape over the experimental period (Wilks' Lambda=0.67; $F_{7,32}$=2.7; P>0.05).

In the second experiment, there were significant changes in tadpole shape in the 28 days following the removal of snakes from the pool (Wilks' Lambda=0.22; $F_{7,37}$=18.4; P<0.001). After the snakes were removed tadpoles developed significantly shorter bodies and tails, shallower tail muscles and deeper overall, upper and lower tail fin depths relative to body size (Table 4.3, Figure 4.3). The body also became relatively deeper and wider, although not significantly so.

Table 4.1. Phenotypic correlations among morphological traits of *A.muletensis* tadpoles. Coefficients are based on 150 tadpoles from snake-free environments (above diagonal) and 181 tadpoles from environments with snakes (below diagonal). All traits are residuals after regression on body size, which is the first component (PC1) from a principal components analysis on all eight traits. $^{**}P<0.01$; $^{*}P<0.05$.

Relative trait size	Body size (PC1)	Body depth	Body width	Body length	Tail length	Tail muscle Depth	Tail fin depth	Upper fin depth	Lower fin depth
Body size (PC1)	--	.251**	.067	-.293**	-.312**	-.121	.097	.012	.235**
Body depth	-.198**	--	.374**	.185*	.048	-.182*	-.252**	-.357**	-.370**
Body width	-.145	.759**	--	.275**	.133	-.331**	-.357**	-.482**	-.399**
Body length	.149*	-.053	.007	--	.146	-.143	-.376**	-.445**	-.375**
Tail length	.411**	-.274**	-.211**	.333**	--	-.260**	-.473**	-.323**	-.375**
Tail muscle depth	.382**	-.166*	-.088	.094	.451**	--	.363**	.265**	-.245**
Tail fin depth	-.137	-.501**	-.515**	-.429**	-.262**	-.255**	--	.552**	-.070
Upper fin depth	-.021	-.428**	-.490**	-.370**	-.353**	-.357**	.342**	--	-.023
Lower fin depth	-.367**	-.300**	-.375**	-.453**	-.527**	-.421**	.574**	.337**	--

Table 4.2. Univariate analysis comparing each of the eight morphometric variables between environments with and without snakes.

Trait	Model SS (1df)	Error SS (329df)	F	P
Body depth	0.043	0.458	30.8	< 0.001
Body width	0.048	0.549	28.6	< 0.001
Body length	0.000	0.507	0.2	0.64
Tail length	0.254	0.912	91.5	< 0.001
Tail muscle depth	0.380	0.471	265.5	< 0.001
Maximum tail depth	0.024	0.366	21.3	< 0.001
Upper tail fin depth	2.025	0.825	0.8	0.37
Lower tail fin depth	0.171	1.505	37.4	< 0.001

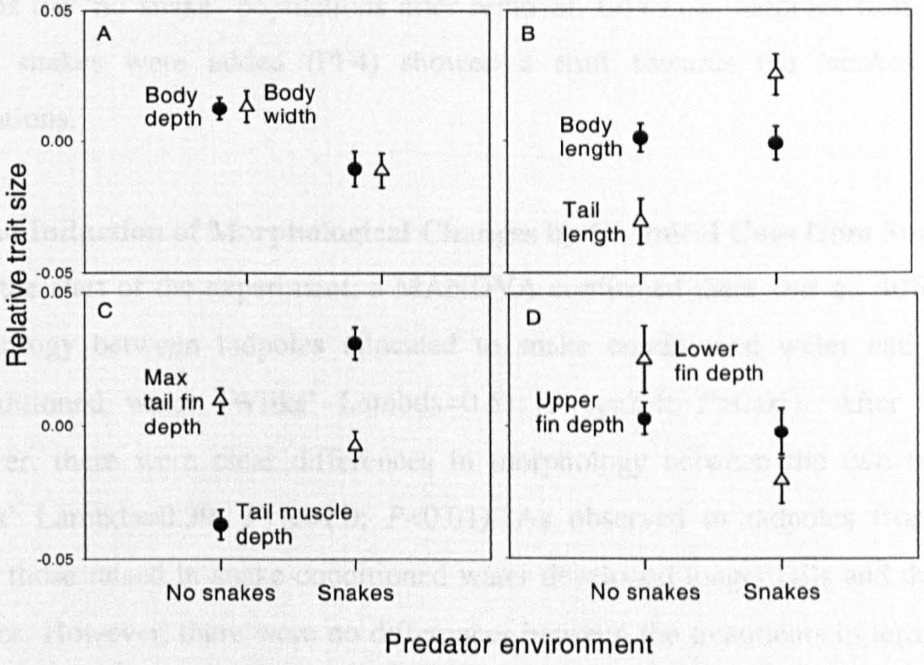

Figure 4.1. Comparison of morphology of tadpoles between populations with and without snakes. Plots show mean relative trait sizes (±95% confidence intervals) of eight morphometric variables. Relative trait sizes were calculated as the residuals from regressions of the log-transformed measurements against a compound measure of body size (i.e. the 1st principal component obtained from a PCA of all eight variables).

4.4.3 Field Induction and Reversibility in Relation to Morphological Variation Between Populations

Using ‘population’ as a grouping variable, in a discriminant function analysis, DF1 accounted for 56.4% of variance (Wilks’ Lambda=0.05 χ^2_{77}=1080; P<0.001), DF2 accounted for 26.5% of variance (Wilks’ Lambda=0.20 χ^2_{60}=581; P<0.001) and DF3 accounted for 12.6% of variance (Wilks’ Lambda = 0.48 χ^2_{60} = 266; P < 0.001). DF1 was plotted against DF2 and DF3 for each tadpole to illustrate the differentiation in the two scores between sites, and to show how the positions of tadpoles from pools PF4 and P3 changed before and after experimental manipulation (Figure 4.4). Examination of the correlations between the predictor variables and the discriminant functions showed that body depth and body width explained a large amount of the variation on DF1, while tail length and maximum tail fin depth explained much of the variation on DF2 and tail muscle depth explained most of the variation in DF3. In tadpoles from the pool where snakes were removed (P3) there was a clear shift towards the 'no snake' populations after removal. Likewise, tadpoles from the pool where snakes were added (PF4) showed a shift towards the 'snakes present' populations.

4.4.4 Induction of Morphological Changes by Chemical Cues from Snakes

At the start of the experiment, a MANOVA confirmed there was no difference in morphology between tadpoles allocated to snake conditioned water and those in unconditioned water (Wilks' Lambda=0.51; $F_{7,18}$=2.4; P>0.05). After 15 days, however, there were clear differences in morphology between the two treatments (Wilks' Lambda=0.39; $F_{7,18}$=4.0; P<0.01). As observed in tadpoles from natural pools, those raised in snake-conditioned water developed longer tails and thicker tail muscles. However, there were no differences between the treatments in terms of tail-fin depth (Table 4.3; Figure 4.5).

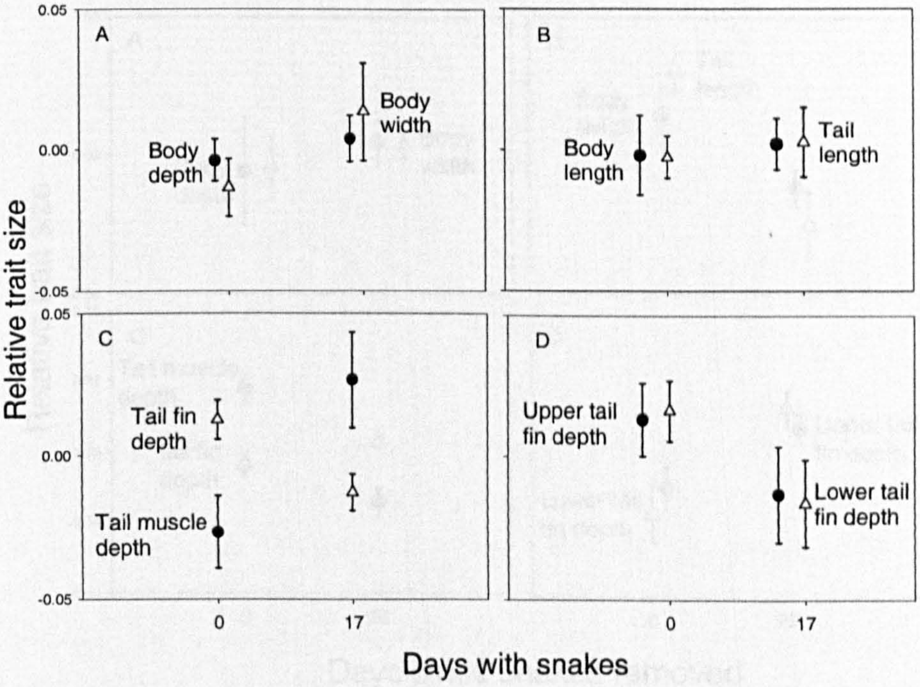

Figure 4.2. Comparison of morphology of tadpoles in a pool before and after snakes were added in nylon bags. Plots show mean relative trait sizes (±95% confidence intervals).

Table 4.3. Univariate ANOVAs showing differences in the relative size of each trait before and after the addition of snakes (field induction), before and after the removal of snakes (field reversal) and nested ANOVAs showing differences in the size of each trait after 15 days of exposure to snake cues (lab induction).

Experiment	Field Induction		Field reversal		Lab induction	
Trait	F	P	F	P	F	P
Body depth	2.1	0.16	1.3	0.26	0.1	0.71
Body width	7.8	<0.01	2.0	0.16	0.1	0.73
Body length	0.2	0.63	20.0	<0.001	3.6	0.07
Tail length	0.6	0.44	59.2	<0.001	4.7	0.04
Tail Muscle depth	28.1	< 0.001	42.0	<0.001	10.4	0.004
Max tail fin depth	32.8	< 0.001	123.6	<0.001	2.0	0.17
Upper tail-fin depth	6.8	0.01	11.7	0.001	0.004	0.95
Lower tail fin depth	13.4	< 0.001	23.5	<0.001	0.69	0.41

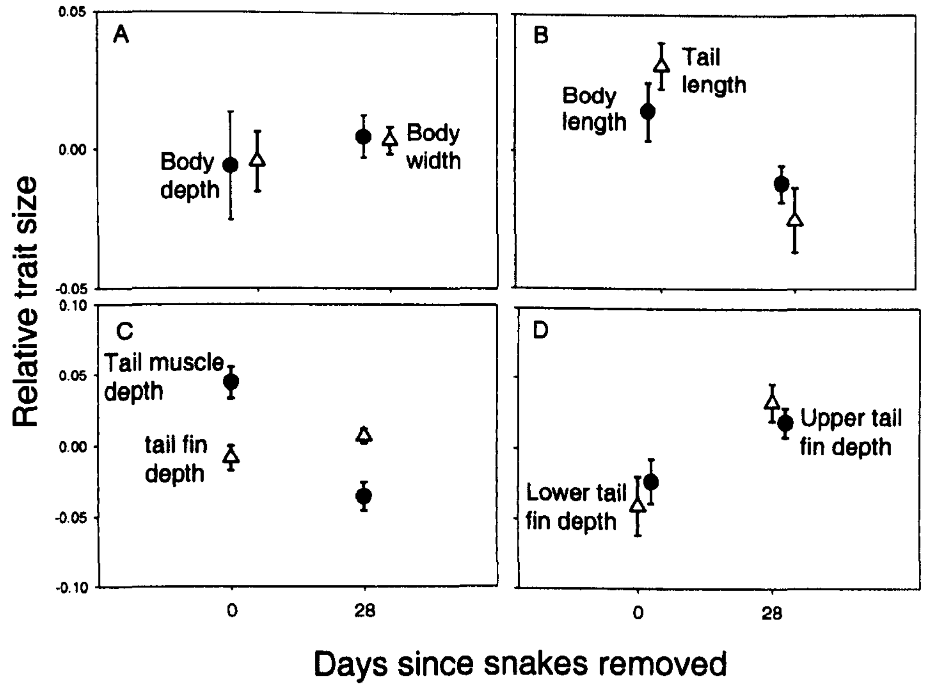

Figure 4.3. Comparison of morphology of tadpoles in a pool before and after two snakes were removed. Plots show mean relative trait sizes (±95% confidence intervals).

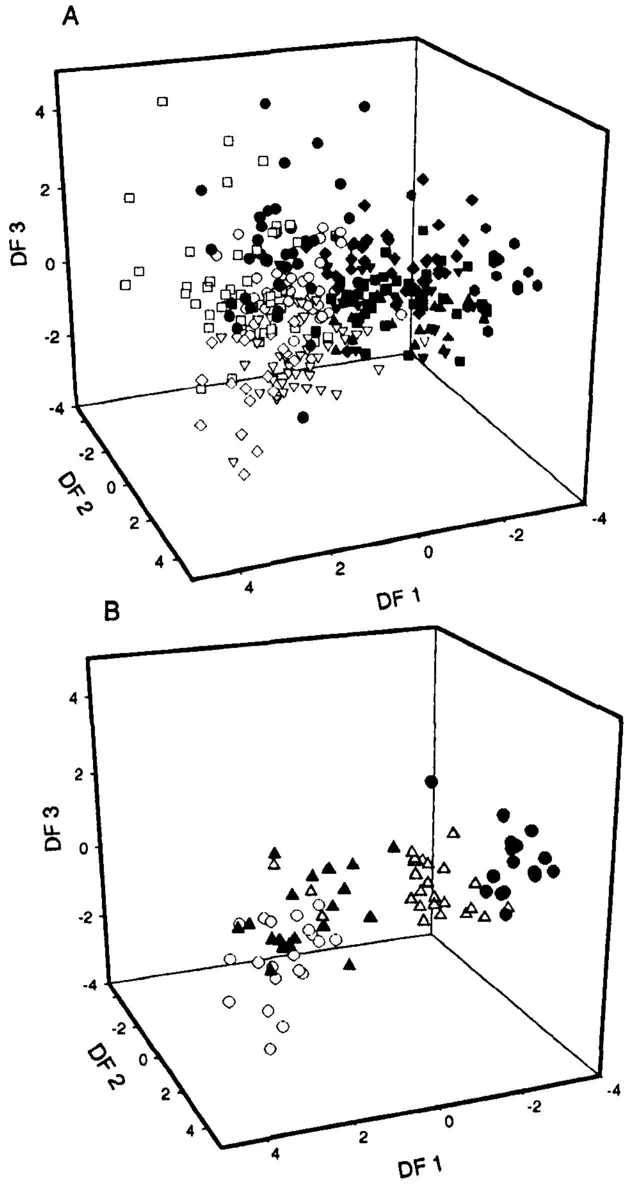

Figure 4.4. Discriminant function analysis showing morphological variation between different populations and between environments with and without snakes. Individual tadpole scores have been plotted on the first three discriminant functions. Different symbols indicate different populations; unfilled symbols indicate populations without snakes; filled symbols indicate populations with snakes. A, unmanipulated populations (including PF4 and P3 before experimental manipulations); B, pool PF4 before (○) and after (Δ) snakes were added, and pool P3 before (●) and after (▲) snakes were removed. DF1 accounted for 56.4% of variance: Wilks' Lambda=0.05; $\chi^2_{77}=1080$; $P<0.001$; DF2 accounted for 26.5% of variance: Wilks' Lambda=0.20; $\chi^2_{60}=581$; $P<0.001$; DF3 accounted for 12.6% of variance: Wilks' Lambda=0.48; $\chi^2_{45}=265$; $P<0.001$. 59% of tadpoles were correctly classified into the appropriate population.

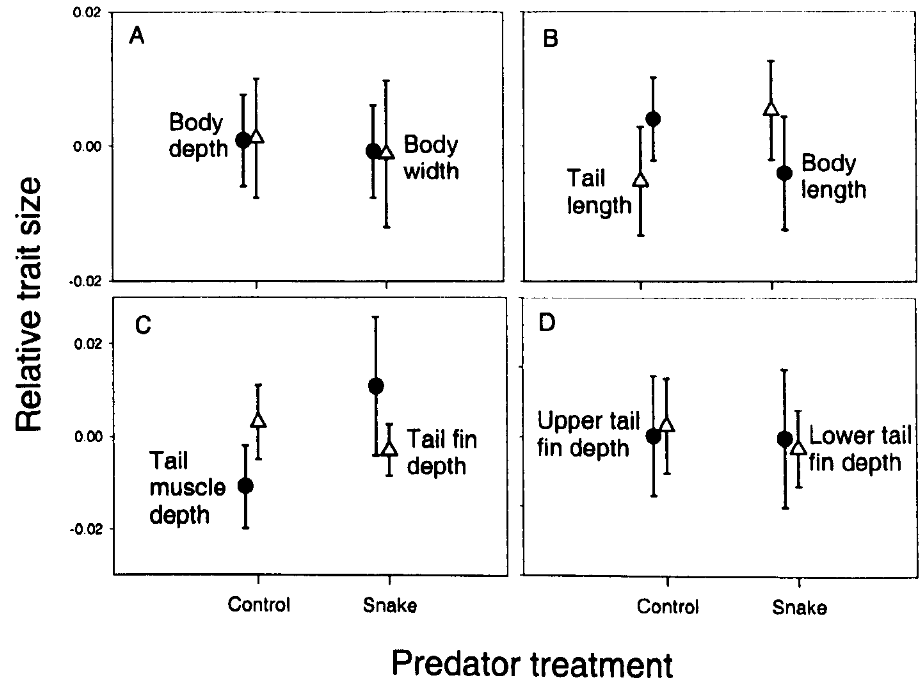

Figure 4.5. Comparison of morphology between tadpoles exposed to snake cues for 15 days and those housed in unconditioned water over the same period. Plots show mean relative trait sizes (±95% confidence intervals).

4.5 DISCUSSION

Phenotypic variation between populations may be due to genetic differences, selection resulting in non-random survival, or plasticity (Van Buskirk & McCollum, 1999). While these processes all act on prey phenotypes simultaneously, there is little information regarding the relative importance of each in influencing the actual phenotypes of prey (Relyea, 2002*b*). The results of this study suggest that differences in tail shape that exist between natural populations of *A. muletensis* tadpoles (Figure 4.1 B, C and D) are induced responses caused by the presence of snakes rather than the result of selective predation, similar to the findings of a previous study on *Hyla versicolor* tadpoles (Relyea, 2002*b*). On the other hand, body depth and width, which were relatively narrower in populations with snakes, but tended to be marginally wider when snake cues were experimentally added, may be regulated by other factors. As activity is reduced when snakes are present (Griffiths *et al.*, 1998), when there is direct predation of tadpoles perhaps feeding activity in survivors is reduced to the extent that they lose body condition (see Chapter 8). When snakes are added in nylon bags, however, it is possible that tadpoles become acclimated to the non-lethal predator cues, and in the absence of predation, continue to feed and maintain body condition (see Chapters 5 and 8).

The predator-induced morphological changes found in *A. muletensis* tadpoles (Figures 4.1, 4.2, 4.3 and 4.5) displayed some similarities to those obtained from laboratory or artificial pond studies of tadpoles exposed to invertebrate predators. In the presence of dragonfly nymphs, for example, anuran larvae typically develop relatively smaller bodies (Van Buskirk & Relyea, 1998; Van Buskirk, 2000), deeper tail muscles (Smith & Van Buskirk, 1995; Van Buskirk, 2000; Van Buskirk & McCollum, 2000*a*;) and longer tails (Van Buskirk & McCollum, 1999) in relation to overall body size. In contrast to the tails of *A. muletensis* which were shallower in the presence of snakes, the same studies showed that anuran larvae often develop wider tail fins in the presence of dragonfly nymphs. However, a study of a wider range of anuran species exposed to different predators showed that plasticity can be species and predator-specific (Relyea, 2001*a*). Such changes in morphology have been found to increase the chances of survival in the presence of predators by facilitating predator escape (Van Buskirk *et al.*, 1997). As in other species, changes in the tail morphology of *A. muletensis* tadpoles may therefore serve to increase acceleration and facilitate predator escape once the tadpole is detected (Van Buskirk *et al.*, 1997; Trembath & Anholt, 2001; Van Buskirk & McCollum, 2000*b*).

Behavioural anti-predator traits are generally more plastic than morphological anti-predator traits (West-Eberhard, 1989; Relyea, 2000*a*). Behavioural responses to an unnatural predator (introduced bullfrogs) have been observed in two North American amphibians (Kiesecker & Blaustein, 1997; Chivers *et al.*, 2001) and these responses may have evolved over the 60-70 year period since bullfrogs were introduced. Behavioural changes have been induced in *A. muletensis* tadpoles by chemical cues from snakes (Griffiths *et al.*, 1998) and this study shows that morphological responses can develop within a similarly short period of predator-prey coevolution. The behavioural response was found to be species and location-specific, with tadpoles responding to *N. maura* from Mallorca but not to the same species from the Iberian peninsula (Griffiths *et al.*, 1998). As other amphibian species also display different responses to different predators (Relyea, 2001*a*), it is unlikely that the evolution of a general response to novel stimuli would be beneficial. Behavioural and morphological responses of *A. muletensis* to an introduced predatory frog and to a native dragonfly nymph are investigated in Chapters 5 and 6 respectively in order to assess the ability of *A. muletensis* tadpoles to differentiate predatory cues and respond accordingly.

Gabriel (1999) points out that irreversible inducible defences are likely to evolve if the relative probability of a predator appearing in a prey population is low, but the probability of it staying is high. On the other hand, reversible inducible defences would be expected if the probabilities of a predator appearing and staying are both relatively low. As a wide-ranging and semi-aquatic predator, the behaviour of *N. maura* would seem to be consistent with the latter model. Snakes can move easily between pools, and may do so when tadpole numbers are reduced to a level whereby foraging would be more profitable elsewhere. Such variability in predation pressure among and within habitats may have contributed to the evolution of inducible defences to snakes and their reversibility when snakes leave. Further comparisons of the specificity of inducible and reversible responses to different predators may therefore shed further light on those selection pressures that are driving phenotypic plasticity; these issues are explored further in Chapters 5 and 6.

Thus, tadpoles of the Mallorcan midwife toad exhibit rapid and reversible morphological responses to introduced predatory snakes, suggesting intense predation pressure since the introduction of the snakes onto Mallorca some 2000 years ago. Mechanisms underlying the evolution of such responses are poorly understood, although it may be that the responses represent an adaptation of defences towards native invertebrate predators; this hypothesis is explored in Chapter 6.

CHAPTER 5: RESPONSES OF *ALYTES MULETENSIS* TADPOLES TO PREDATORY FROGS AND SNAKES

5.1 SUMMARY

Tadpoles of the Mallorcan midwife toad, *Alytes muletensis*, have been found to respond behaviourally and morphologically to cues from the introduced predatory viperine snake, *Natrix maura.* While the snake is widely regarded as the main predator of the toad, the green frog, *Rana perezi*, is another introduced species that predates on adult toads and tadpoles. To determine whether tadpoles of the midwife toad are capable of differentiating predatory cues, behavioural and morphological responses of *A. muletensis* tadpoles to the green frog were investigated and compared with responses to the snake using a replicated experiment under controlled conditions. Two populations were compared – one natural and the other reintroduced following a short period of captive breeding – to assess whether a captive-breeding bottleneck had impaired antipredatory responses of reintroduced individuals. Tadpoles were raised in either snake-conditioned, frog-conditioned or unconditioned water for 30 days and behaviour and morphology were monitored. Tadpoles reduced activity in response to cues from both snakes and frogs and descendent tadpoles responded more strongly than individuals from the founder population. Tadpoles from both populations changed shape in response to cues from both predators within 15 days of exposure. The results indicate that a captive-breeding bottleneck has not diminished either behavioural or morphological antipredator responses. It is possible that two to three generations in captivity is not sufficient to produce inbreeding depression, or else the population may have recovered genetically since reintroduction to a natural site. Subtle differences in behavioural and morphological responses to snakes and frogs suggest that, while similar mechanisms of escape may be effective against both predators, tadpoles appear to be capable of species-specific predator recognition. It is likely that intensive selective pressure has driven the evolution of rapid morphological and behavioural responses since the introduction of predatory snakes and frogs onto Mallorca some 2000 years ago.

5.2 INTRODUCTION

There may be strong selection on prey organisms to be able to accurately assess current predation risk and respond to reduce the immediate threat. The effectiveness of the response may depend upon the identity of the predator, and it may be advantageous for prey individuals to adopt different responses towards different predators (Relyea, 2001*a*). In aquatic systems, chemical signals can confer information regarding the presence of a predator (Dodson *et al.*, 1994; Griffiths *et al.*, 1998) and species-specific recognition may be possible if different predators emit different chemical cues or different levels of the same cue (Relyea, 2001*a*). Different responses to multiple predators have been demonstrated in anuran larvae with respect to behaviour (Feminella & Hawkins, 1994; Richardson, 2001; Schmidt & Amézquita, 2001) and morphology (Relyea, 2001*a*, *b*).

The viperine snake, *N. maura*, is widely believed to be the most significant introduced predator of the Mallorcan midwife toad (Tonge, 1986). However, the introduced green frog, *R. perezi*, also predates upon both adults and tadpoles (Tonge, 1986; pers obs) and is similarly widespread throughout the range of the toad (Chapter 2). While tadpoles of *A. muletensis* have been shown to reduce activity (Griffiths *et al.*, 1998) and change shape (Chapter 4) in response to cues from the introduced predatory snake *N. maura*, it is not known if the tadpoles additionally respond to *R. perezi*. It is likely that behavioural and morphological responses to the frog, if they exist, will reflect the intensity of historical selective pressure from this predator. The comparison of a tadpole's responses to snakes and frogs may therefore provide an insight into the relative selection pressure exerted by the two introduced predators, as well as providing an indication of the ability of *A. muletensis* tadpoles to differentiate between cues from different predators and respond accordingly.

The recovery programme for the Mallorcan midwife toad involves the captive breeding and reintroduction of toads into suitable unoccupied habitat. This has resulted in the establishment of a number of new populations within the historical range of the toad and, as such, appears to be a successful strategy. However, captive breeding can generate problems through inbreeding depression (Reinert, 1991), leading to a decline in fitness of reintroduced individuals (Caughley, 1994; Jimenez *et al.*, 1994; Hedrick & Kalinowski, 2000; Marshall & Spalton, 2000). Additionally, adaptation to the artificial environment may impair fitness upon reintroduction of individuals to the wild (Reinert, 1991; Lewis & Thomas, 2001). Tadpole growth and development have been suggested as useful measures of fitness (Rowe & Beebee, 2001). It is highly likely that ability to detect and respond to predators is also strongly correlated with fitness under natural conditions. A loss or reduction in anti-predatory responses resulting from a captive-breeding bottleneck would greatly reduce a tadpole's chances of survival upon reintroduction into a natural pool.

This chapter will investigate behavioural and morphological responses of *A. muletensis* tadpoles to chemical cues from the introduced green frog *R. perezi*, and, using a replicated experimental design, compare these with antipredator responses towards the viperine snake *N. maura*. To assess if several generations of captive breeding have had any impact on tadpole performance, tadpoles from a reintroduced population (descendent), which had been subject to approximately three generations of captive breeding, will be compared directly with individuals from the population that originally provided the founders for the captive stock in 1985-1987.

5.3 METHODS

5.3.1 Study Sites

Two populations of the Mallorcan midwife toad within separate gorges of the Serra de Tramuntana mountain range in Mallorca were selected for comparison: one representing a descendent and the other a founder population. The descendent population was established in the early 1990's (A. Roman pers com) using captive-bred stock derived from four adults and 16 tadpoles collected between 1985 and 1987 from the founder population (Tonge, 1996; Tonge & Bloxam, 1991). All tadpoles used in the experiment therefore originated from the same population, although the descendent population had been subject to approximately three generations of captive breeding (based on a generation time of two to three years) followed by four generations in the reintroduction site.

5.3.2 Experimental Design

The experiment was designed to compare the behavioural and morphological responses of tadpoles collected from descendent and founder populations to cues from snakes and frogs. The experiment was conducted in a designated room of a farmhouse situated at around 1000 m altitude in the Serra de Tramuntana, Mallorca. The room was lit by natural light from a south-facing window and the temperature in the room ranged from a minimum of 12.5°C at night to a maximum of 24°C during the day over the course of the experiment. This is similar to natural temperature cycles in pools (Schley *et al.*, 1998). Tadpoles were allocated to one of three predator treatments: snake-conditioned water ('snake' treatment), frog-conditioned water ('frog' treatment) or unconditioned water ('control'). Water, which originated from a well containing local natural ground water, was snake-conditioned by placing three snakes held separately in 1 mm nylon mesh bags into 20 l of water for three hours, frog-conditioned by placing three frogs into 20 l of water for three hours, or left to stand for three hours (control).

5.3.3 Experimental Design

Forty-eight tadpoles were collected using a dip net from each of the founder and the descendent populations on 18 and 19 May 2001 respectively. Small tadpoles of approximately 30 mm in length were selected in an attempt to ensure consistency in size between populations and between treatments; the use of small tadpoles also reduced the chances of tadpoles metamorphosing before completion of the experiment. Tadpoles were divided equally between the three predator treatments (snake, frog and control) so that four tadpoles were housed in each of 24 boxes containing 2.5 l of appropriately conditioned water. Tadpoles were maintained in these boxes in a 6x4 array on the floor of the experimental room for 30 days; water changes were performed every two days at 11 am to ensure regular exposure to predator cues and tadpoles were fed fish flake food *ad libitum* after each water change. The position of the boxes was re-randomised within the room after each water change to eliminate any bias due to positioning.

5.3.4 Monitoring Tadpole Behaviour and Morphology

Tadpole activity was measured on the same days as the water changes. As activity is likely to be affected by food and disturbance, it was measured twice on such days, immediately before, and one hour after, the water change. At these times each tadpole was observed for one minute and activity timed; tadpoles were considered active when they were feeding or swimming. This technique has proved to be a reliable and useful measure of tadpole activity (Griffiths *et al.*, 1998).

In order to monitor changes in morphology, tadpoles were anaesthetized using MS222 and measured for eight morphometric variables (see Figure 3.1) prior to the start of the experiment on day 0 on then again on day 15 and following the completion of the experiment on day 30. Developmental stage was also recorded for each tadpole at these times using Cambar & Martin's (1959) staging table for *Alytes obstetricans*.

5.3.5 Statistical Analysis

Tadpoles in the same box are likely to influence each other's behaviour and cannot be considered independent. Therefore, mean tadpole activities (measured in seconds per minute) in each box were used in the analysis. Activity was compared between treatments and between populations both before and after the water changes using two-way ANOVAs. This also tested for population by treatment interactions.

Morphology was compared between treatments and between populations on day 0, day 15 and day 30 of the experiment. Eight morphometric measurements of the tadpoles were log transformed to meet the assumptions of the analysis. For each set of measurements, a principal components analysis was conducted on all eight morphometric variables to obtain the first principal component (PC1) as a measure of body size. In order to remove the effect of body size, log transformed morphological measurements were regressed against PC1 to obtain the unstandardised residuals (see Chapter 4). Mean residuals for each box were used for comparisons since they represent independent variates. A two-way multivariate analysis (MANOVA) was conducted on mean residuals to compare morphology between populations and between treatments on day 0. Two-way univariate analyses (ANOVAs) were used to compare the relative sizes of individual traits as well as body size (PC1) and developmental stage. On days 15 and 30, differences in shape between predator treatments and between populations were compared using two-way MANCOVAs, with relative trait sizes at day 0 as the covariate. Univariate two-way ANCOVAs were used to compare the relative sizes of specific traits as well as body size. Two-way ANOVA's were used to compare developmental stage between treatments and populations. Although stage data are discontinuous, according to the 'central limit theorem', means should be normally distributed even if the original data are not (Sokal & Rohlf, 1980) and this permitted the use of parametric tests on the means of this trait. Tukey's post hoc tests were used to identify where significant differences lay following univariate ANOVA's. However, due to constraints imposed by the statistical software, Bonferronis post hoc tests were used to identify where significant differences lay between treatments when using ANCOVA's.

5.4 RESULTS

5.4.1 Behaviour

A two-way ANOVA found significant variation in activity between predator treatments before the water change ($F_{2,78}$=3.4; P<0.05), although Tukey's post hoc tests revealed no significant differences between any two treatments. Control tadpoles were non-significantly more active than tadpoles in both snake-conditioned (P=0.063) and frog-conditioned (P=0.07) water at this time, but there was no difference in tadpole activity between the two predator treatments (P=1.0). After the water change a significant difference between treatments ($F_{2,78}$=3.9; P=0.02) reflected higher levels of tadpole activity in unconditioned water than in both snake-conditioned (P<0.05) and frog-conditioned (P<0.05) water. Again tadpole activity levels were similar in the two predator treatments at this time (P=1.0). Founder tadpoles were highly significantly more active than descendent tadpoles before ($F_{1,78}$=9.7; P<0.01) and after ($F_{1,78}$=4.2; P<0.05) the water change. Tadpoles from both populations reduced activity in response to cues from both predators before the water change and, although these differences were significant only in the descendent population (Table 5.1), there was no significant population by treatment interaction at this time ($F_{2,78}$=1.1; P=0.33). After the water change descendent tadpoles reduced activity non-significantly in response to snake cues and significantly in the presence of frog cues; founder tadpoles reduced activity non-significantly in response to snake cues and increased activity non-significantly in response to frog cues (Table 5.1). The different responses of the two populations to predatory cues after the water change is reflected in a significant population by treatment interaction ($F_{2,78}$=10.2; P<0.001).

Table 5.1 Mean tadpole activity in the different treatments. Letters in subscript indicate significantly different levels of activity between treatments within a group as determined by Tukey's post hoc tests of a two-way ANOVA .

			Activity (secs/min)	
Population	Water change	Control	Snake	Frog
Founder	Before	18.09	16.40	16.66
	After	29.02	26.79_a	30.94_a
Descendent	Before	$16.77_{a,b}$	11.21_a	11.08_b
	After	30.61_a	27.31_b	23.08_{ab}

5.4.2 Morphology

Day 0

On day 0, the first principal component (PC1) of a PCA on the eight log-transformed morphometric traits accounted for 43.8% of variance. Phenotypic correlations revealed that, in founder tadpoles, as PC1 increased the body became relatively shallower, narrower and longer and the tail became relatively shorter with a deeper tail muscle and deeper overall and upper tail fins; exactly the converse was found in descendent tadpoles (Table 5.2). These differences indicate that traits are independent and individuals from the two populations may have been subject to divergent selection pressures prior to collection.

A two-way MANOVA on the residuals of the regression of all eight morphometric traits against PC1 revealed a significant difference in tadpole shape between descendent and founder populations ($F_{11,8}$=4.5; P=0.01), between predator treatments ($F_{22,16}$=3.7; P<0.01), but no significant population by treatment interaction ($F_{22,16}$=1.6; P=0.14). Univariate analysis found that descendent tadpoles possessed significantly deeper tail muscles, shallower overall tail fins and wider bodies relative to overall body size than those from the founder population. Between treatments, univariate analyses revealed that tadpoles differed significantly in body width only. There was no significant difference in body size (PC1) between populations or between predator treatments (P>0.05).

Table 5.2. Phenotypic correlations among morphometric traits on day 0 in tadpoles from founder (above horizontal) and descendent (below horizontal) populations. *P<0.05; **P<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	-.91	-.180	.086	-.044	.082	.115	.050	-.031
Body depth	.064	-	-.242	-.587**	.295*	-.076	.062	-.275	-.019
Body width	.151	.091	-	.113	-.172	-.484**	-.048	.049	-.192
Body length	-.184	-.140	-.168	-	-.525**	.000	.015	-.057	-.176
Tail length	.061	.350*	.030	.007	-	.023	-.278	-.348*	-.159
Tail muscle depth	-.053	-.403**	-.151	-.020	-.110	-	-.025	-.267	-.288*
Tail fin depth	-.061	-.060	-.158	-.195	-.489**	-.424**	-	-.259	-.268
Upper fin depth	-.039	-.604**	-.063	-.091	-.212	.482**	-.309*	-	.276
Lower fin depth	.037	-.332*	-.508**	-.052	-.180	-.162	.112	-.035	-

Day15

On day 15 there were a number of noticeable differences in phenotypic correlations between populations and between treatments (Tables 5.3, 5.4 and 5.5). Most notable in control tadpoles were the significant negative correlations between tail length and maximum, upper and lower tail fin depths in tadpoles from the founder population, and between tail length and lower fin depth in descendent individuals (Table 5.3). Body width was consistently positively correlated with body depth in both populations, except in founder tadpoles treated with frog-conditioned water (Tables 5.3, 5.4 and 5.5). In snake-conditioned water, PC1 correlated positively with all body traits and negatively with most tail traits in descendent tadpoles, while the relationship was reversed in founder tadpoles (Table 5.4). Body width was negatively correlated with upper and lower tail fin depths in all cases except descendent tadpoles treated with frog-conditioned water, which displayed a non-significant positive correlation between body width and upper fin depth (Table 5.5). These differences are indicative of divergent selection acting on certain traits according to population of origin and exposure to predatory cues.

A two-way MANCOVA on the residuals of eight morphometric traits on day 15 revealed a marginal difference in tadpole shape between predator treatments ($F_{16,6}=1.34$; $P=0.05$), no difference between populations ($P>0.05$) and no population by treatment interaction ($P>0.05$). Univariate two-way ANCOVA's on each of the eight variables and body size (PC1) and a two-way ANOVA on stage revealed significant differences between predator treatments in relative tail muscle depth, tail length, developmental stage and body size (Table 5.6). Boferroni's post hoc tests found that tadpoles in snake-conditioned water possessed significantly deeper tail muscles relative to body size than individuals in unconditioned water, with tadpoles in frog-conditioned water intermediate in the size of this trait (Table 5.6). Relative tail length did not differ significantly between tadpoles exposed to snake and frog cues, but both predator treatments possessed significantly longer tails relative to body size than controls (Figure 5.1). Tadpoles treated with snake cues were significantly more developed than controls; individuals treated with frog cues were of intermediate developmental stage and not significantly different from either of the other groups. Tadpoles in frog-conditioned water were significantly larger than individuals in snake-conditioned water at this time, with control tadpoles of intermediate size. There was a significant population by predator interaction in body size only and tadpoles from both populations exhibited similar morphological responses to cues from each predator (Figure 5.1).

Table 5.3. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in unconditioned water on day 15. **P*<0.05;***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	-.115	-.520*	.170	-.023	.015	.189	.123	.092
Body depth	.221	-	.507*	-.286	.570*	-.424	-.315	-.474	-.622*
Body width	.324	.330	-	-.220	.321	-.230	-.356	-.360	-.555*
Body length	.082	-.395	.377	-	.194	.303	-.433	-.264	-.340
Tail length	.125	-.188	.045	.172	-	-.155	-.634**	-.691**	-.808**
Tail muscle depth	.141	-.036	-.019	.286	.286	-	-.185	-.118	-.049
Tail fin depth	-.190	.069	-.498*	-.689**	.058	.058	-	.227	.726**
Upper fin depth	-.337	-.599*	-.619*	.043	-.386	-.386	.084	-	.674**
Lower fin depth	-.242	-.091	-.385	-.096	-.607*	-.607*	-.002	.492	-

Table 5.4. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in snake-conditioned water on day 15. **P*<0.05;***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	-0.36	-.121	-.231	.273	.032	.288	-.147	-.039
Body depth	.252	-	.574*	-.144	-.413	-.213	-.304	-.496	-.572*
Body width	.089	.337	-	.083	-.107	-.126	-.374	-.656**	-.723**
Body length	.303	-.245	-.194	-	.060	-.165	-.305	-.044	-.102
Tail length	-.299	-.254	-.014	-.473	-	.221	-.330	-.203	.158
Tail muscle depth	-.142	-.016	.173	-.334	.225	-	-.079	-.272	-.247
Tail fin depth	.008	-.580*	-.657**	.010	-.021	.032	-	.272	.127
Upper fin depth	-.004	-.164	-.463	.125	-.134	-.814**	.151	-	.611*
Lower fin depth	-.283	-.167	-.507	-.018	-.139	-.667**	.373	.657**	-

Table 5.5. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in frog-conditioned water on day 15. **P*<0.05;***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	-.080	-.078	-.154	.389	-.485	-.193	.144	.166
Body depth	-.014	-	-.073	.137	-.022	-.056	.074	-.570*	-.288
Body width	.063	.388	-	.063	.154	.049	-.245	-.197	-.492
Body length	.005	-.295	-.418	-	.294	-.060	-.386	-.387	-.763**
Tail length	.176	-.177	-.440	.057	-	-.160	-.646**	-.251	-.214
Tail muscle depth	.004	-.389	-.197	-.382	-.177	-	.264	-.468	.029
Tail fin depth	-.346	-.280	-.242	-.287	-.131	.051	-	-.120	.237
Upper fin depth	.181	.220	.144	.355	-.489	-.098	-.560*	-	.405
Lower fin depth	-.081	-.547*	-.040	-.307	-.121	.213	.506*	-.551*	-

Table 5.6. Two-way ANCOVA results with Bonferroni post hocs comparing eight morphometric traits of tadpoles after 15 days of exposure to predatory cues, using starting sizes as covariates. Significant differences between predator treatments are underlined; single underline = significantly different to one other treatment; double underline = significantly different to both other treatments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Trait/Factor	Population	Predator	Popn x Predator	Covariate	Predator ranking
Body depth	0.51	1.69	3.09	0.00	Control > Frog > Snake
Body width	0.11	0.87	3.32	0.02	Control > Snake > Frog
Body length	2.23	2.17	1.34	0.02	Snake > Control > Frog
Tail length	0.48	9.95**	2.86	0.28	Snake > Frog > Control
Tail muscle depth	1.41	5.50*	1.52	0.56	Snake > Frog > Control
Max tail fin depth	0.72	2.71	0.89	0.28	Frog > Control > Snake
Upper fin depth	0.01	0.05	0.29	1.82	Control > Snake = Frog
Lower fin depth	0.12	2.10	0.37	1.61	Snake > Control > Frog
Stage	0.71	5.16*	1.37	-	Snake > Frog > Control
Body size (PC1)	18.73***	3.74*	7.16**	14.65**	Frog > Control > Snake

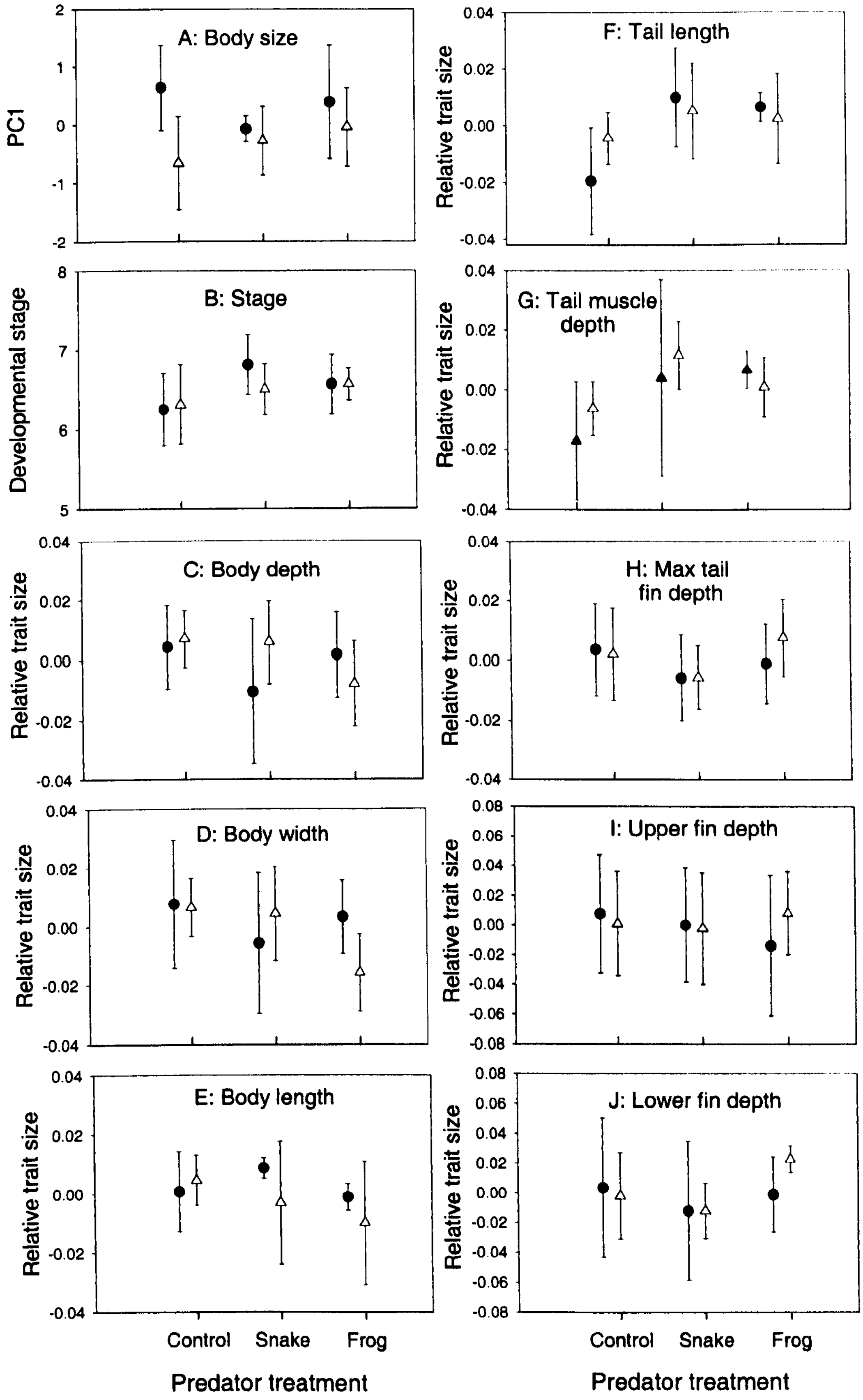

Figure 5.1. Differences in tadpole size, stage and morphology on day 15 of experiment in descendent (filled circles) and founder (unfilled triangles) populations. Plots show mean relative trait sizes (±95% confidence intervals).

Day 30

At the end of the 30-day experiment, tadpoles in all three treatments and from both populations typically displayed positive phenotypic correlations within the three body measurements and within the five tail measurement, with an inverse relationship being displayed between the two groups (Tables 5.7, 5.8 & 5.9). Body depth and width were invariably negatively correlated with maximum, upper and lower tail fin depths in both populations in all treatments. Control tadpoles from both populations developed relatively wider, deeper and shorter bodies and shorter tails with deeper tail muscles and deeper maximum tail fin depths with increasing body size (PC1) (Table 5.7). Consistent with correlations on day 15, body depth and width were consistently positively correlated with one another and only frog-conditioned descendent tadpoles exhibited a non-significant correlation.

A two-way MANCOVA on the eight morphometric traits on day 30 revealed no significant difference in tadpole shape between treatments ($P>0.05$) or populations ($P>0.05$), and no significant treatment by population interaction ($P>0.05$). Univariate two-way ANCOVA's on the eight traits and body size, and a two-way ANOVA on stage revealed that, of the ten traits, only relative tail muscle depth differed significantly between predator treatments (Table 5.4). Consistent with the differences observed after 15 days, tadpoles conditioned with snake cues possessed significantly deeper tail muscles than controls, with tadpoles in frog-conditioned water exhibiting intermediate tail muscle depth. Although tadpoles in frog- and snake-conditioned water possessed relatively longer tails than controls at the end of the experiment, the differences were not significant. There were a number of significant differences in shape between tadpoles from the two populations, however; tadpoles from the descendent population possessed relatively deeper, wider bodies, deeper tail muscles and shallower upper and lower tail fins than founder tadpoles in all treatments (Figure 5.2). Tadpoles from the founder population were larger and more developed than descendent tadpoles in all treatments at the end of the experiment. However, the lack of any significant population by predator interactions at this time suggests that the two populations responded in a similar manner to the presence of predatory cues.

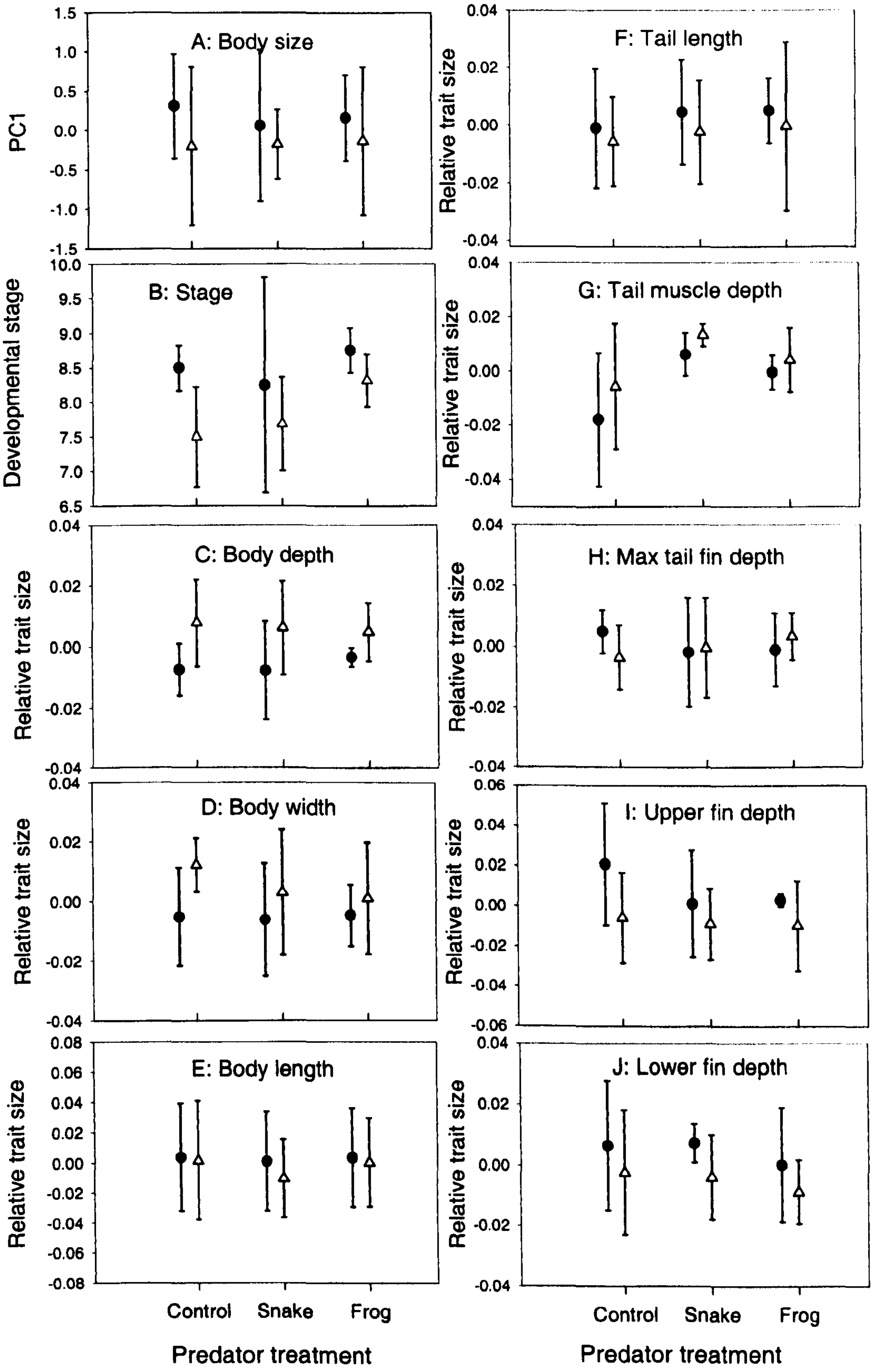

Figure 5.2. Differences in tadpole size, stage and morphology on day 30 of experiment in descendent (broken line) and founder (solid line) populations. Plots show mean relative trait sizes (±95% confidence intervals).

Table 5.7. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in unconditioned water on day 30. **P*<0.05; ***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	.156	.166	-.372	-.039	.064	.438	-.067	-.200
Body depth	.310	-	.693**	.414	-.157	.180	-.503*	-.533*	-.742
Body width	.157	.601*	-	.379	.233	.174	-.655**	-.780**	-.680**
Body length	-.288	.215	.198	-	-.166	-.183	-.637**	-.399	-.284
Tail length	-.228	-.403	-.340	-.253	-	-.059	-.028	-.400	-.311
Tail muscle depth	.031	-.295	.000	-.591	.493	-	-.335	-.345	-.343
Tail fin depth	.163	-.337	-.544*	-.667**	.070	.070	-	.576*	.417
Upper fin depth	-.364	-.610*	-.221	.275	-232	-.232	-.087	-	.565*
Lower fin depth	.144	-.357	-.684**	-.262	-.310	-.310	.651**	.090	-

Table 5.8. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in snake-conditioned water on day 30. **P*<0.05;***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	.049	-.131	-.213	-.055	-.117	.269	.140	-.020
Body depth	.006	-	.719**	.098	-.311	-.521*	-.230	-.546*	-.065
Body width	-.139	.537*	-	.126	-.054	-.349	-.431	-.572*	-.312
Body length	.163	-.044	.004	-	-.319	.225	-7.7**	.079	-.207
Tail length	.039	-.466	-.593*	-.151	-	.447	-.017	-.329	-.517**
Tail muscle depth	-.146	-.512*	-.321	.196	.405	-	-.425	.102	-.335
Tail fin depth	-.319	-.229	-.409	-.770**	.332	-.049	-	.114	.344
Upper fin depth	.059	-.667**	-.296	-.098	.050	-.017	.162	-	.044
Lower fin depth	.421	-.029	-.263	.040	-.287	-.414	-.058	.061	-

Table 5.9. Phenotypic correlations among morphometric traits in tadpoles from founder (above horizontal) and descendent (below horizontal) populations in frog-conditioned water on day 30. **P*<0.05;***P*<0.01.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	.042	.370	.278	-.098	.280	-.140	-.283	-.457
Body depth	-.121	-	.712**	.051	-.011	-.246	-.440	-.642**	-.501*
Body width	.111	.468	-	.112	-.014	-.226	-.436	-.731**	-.564*
Body length	.296	.304	.245	-	-.267	.362	-.604*	-.040	-.595*
Tail length	.095	-.479	-.551*	-.437	-	-.375	.079	-.365	-.121
Tail muscle depth	.305	.031	.093	-.356	.086	-	-.447	.234	-.075
Tail fin depth	-.510*	-.137	-.403	-.386	-.008	-.389	-	.344	.457
Upper fin depth	-.151	-.530*	-.335	-.252	.037	-.014	.055	-	.395
Lower fin depth	-.135	-.437	-.344	-.008	-.132	-.451	.385	.077	-

Table 5.10. Two-way ANCOVA with Bonferroni post hocs of eight morphometric variables after 30 days of exposure to predatory cues, using starting sizes as covariates. Underlined predator treatments represent significant differences. *P<0.05; **P<0.01; ***P<0.001.

Trait/Factor	Population	Predator	Popn x Predator	Covariate	Predator ranking
Body depth	11.92**	0.03	0.57	0.21	Frog > Control > Snake
Body width	8.07*	0.24	0.90	2.52	Frog > Snake > Control
Body length	0.32	0.30	0.13	0.02	Control > Frog > Snake
Tail length	0.64	0.43	0.05	0.26	Frog > Snake > Control
Tail muscle depth	7.42*	12.63**	1.47	2.93	Snake > Frog > Control
Max tail fin depth	0.85	0.25	1.34	1.25	Control > Frog > Snake
Upper fin depth	6.87*	1.47	0.74	0.00	Control > Frog > Snake
Lower fin depth	5.38*	1.05	0.02	.26	Control > Snake > Frog
Stage	10.74**	3.22	0.70	-	Frog > Control > Snake
Body size (PC1)	2.69	0.11	0.17	0.03	Control > Frog > Snake

5.5 DISCUSSION

Previous studies have shown that tadpoles of the Mallorcan midwife toad respond behaviourally (Griffiths *et al.*, 1998) and morphologically (Chapter 4) to the perceived presence of the viperine snake. The findings of this chapter demonstrate that tadpoles additionally respond both behaviourally and to cues from the introduced green frog, *R. perezi*. Activity levels were generally suppressed in response to cues from either predator (Table 5.1) indicating that crypsis may be an effective defense against both species. After fifteen days of regular exposure to snake cues, tadpoles had developed significantly longer tails and deeper tail muscles and non-significantly shallower tail fins relative to body size than controls morphologically (Figure 5.1); these changes are consistent with previous findings (Chapter 4). Tadpoles exposed to frog cues over the same period developed significantly longer tails and non-significantly deeper tail muscles relative to body size than controls. At the end of the 30-day experiment differences in morphology between treatments were less pronounced than they were after 15 days, and only relative tail muscle depth differed significantly between treatments (Figure 5.2). Thus, in the absence of direct predation, tadpoles may cease to respond to the presence of predatory cues and may even begin to lose their predator morphology as suggested in Chapter 4. A study by Stauffer & Semlitsch (1993) indicated that tadpoles of some species respond more strongly to chemical and tactile cues together than to chemical cues alone and it is possible that, in the absence of additional visual or tactile cues, tadpoles habituate to the presence of chemical cues in the water. Alternatively, some studies have shown that as tadpoles increase in size they may become less susceptible to predation (Calef, 1973; Caldwell *et al.*, 1980; Crump, 1984; Cronin & Travis, 1986, Formanowicz, 1986; Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Semlitsch, 1990; Tejedo, 1993; Alford, 1999) and if this is the case they may benefit less from displaying a predator morphology as they grow. Because snakes and frogs appear to predate most heavily upon medium-sized *A. muletensis* tadpoles (Chapter 3), however, the former explanation for the loss of the predator morphology is favoured.

Subtle differences were found in morphological responses to snake and frog predators, suggesting that tadpoles are able to differentiate between species-specific predatory cues and respond accordingly. Previous studies on anuran larvae (Relyea, 2001*a*), zooplankton (Havel, 1987) and plants (Karban & Baldwin, 1997) have found that predators producing different chemicals, or different levels of the same chemical, may be differentiated by prey species in this manner. This will be an adaptive strategy if different mechanisms of escape are effective against different predators.

Tadpoles from descendent and founder populations differed in their behavioural, but not in their morphological responses to predators. Whereas descendent tadpoles consistently reduced activity in response to snake and frog cues, and continued to display suppressed activity two days following each water change, founder tadpoles did not reduce activity to a significant degree in response to cues from either predator. While numerous studies have demonstrated reduced fitness as a result of inbreeding depression caused by captive breeding (Ralls *et al.*, 1979; Reinert, 1991; Jiminez *et al.*, 1994), the results of this study suggest that around three generations of captive breeding has not diminished the ability of *A. muletensis* tadpoles to detect and respond to predators. Alternatively, genetic recovery may have occurred since the reintroduction of the population into a natural site, as data from other taxa suggest rapid recovery may be possible following a genetic bottleneck (Hansson *et al.*, 2000). It is unclear, however, why descendent tadpoles should respond more strongly to the presence of predators than founder individuals. It may be that different histories of exposure to predators are influencing observed responses. Founder tadpoles were collected from a site known to be subject to sporadic incursions by snakes and frogs, whereas descendent tadpoles were collected from a site known to be free from both predators (A. Román, pers com). Previous exposure to predators has been shown to influence behavioural antipredator responses in other species (Kraus & Petranka, 1989; Semlitsch & Reyer, 1992) and may explain differences observed between tadpoles in this study. Previous work on A. *muletensis* found lower baseline activity levels and subdued responses in tadpoles with a history of exposure to predators (Griffiths *et al.*, 1998). The current study also found stronger responses in naïve tadpoles despite similar starting levels of activity in tadpoles from the two populations. Although it is unclear why previous exposure to predator cues would desensitise the tadpoles in this way, it may be that tadpoles require further stimuli in the form of visual and/or tactile cues to evoke a pronounced behavioural antipredatory response as was discussed with regard to morphological responses. The influence of experience on behavioural antipredatory responses in A. *muletensis* tadpoles is investigated further in Chapter 7.

Morphological responses to predatory cues were similar in tadpoles from both populations, suggesting that, consistent with behavioural responses, a period of captive-breeding has not diminished morphological antipredatory responses in the descendent population. Significant differences in tadpole morphology were found between populations at the start of the experiment, however. Descendent tadpoles, despite being collected from a predator-free site, possessed significantly deeper tail muscles relative to body size than founder tadpoles collected from a site subject to sporadic incursions by snakes and frogs (Román, pers com; pers obs). This contradicts previous findings that tadpoles from sites with predators possess deeper tail muscles than those from predator-free sites (this chapter; Chapter 4). Morphological differences may have been due to responses to invertebrate predators within the pools. Smaller tadpoles are generally more susceptible to predation from invertebrate predators than larger individuals (Calef, 1973; Heyer *et al.*, 1975; Caldwell *et al.*, 1980; Crump, 1984; Cronin & Travis, 1986, Formanowicz, 1986; Semlitsch & Gibbons, 1988; Richards & Bull, 1990; Semlitsch, 1990; Tejedo, 1993; Alford, 1999); thus, because tadpoles were all in the early stages of development upon collection, responses to invertebrate predators may have been predominant in influencing morphology. Because descendent tadpoles were collected from a smaller pool than founder tadpoles, the concentration of invertebrate cues may have been stronger in the former resulting in a more pronounced response. Responses of A. *muletensis* tadpoles to an invertebrate predator are investigated and discussed in the following chapter.

Rates of tadpole growth and development were influenced by the presence of predatory cues over the first 15 days, with the smallest body size and most advanced state of development in snake-conditioned tadpoles. Previous studies have found that tadpoles and some invertebrates speed up development in the presence of predators to escape the risky aquatic environment (Babbitt & Tanner, 1998; Peckarsky *et al.*, 2001) and this may be the case here. By end of the 30-day experiment, however, there were no significant differences in size or developmental stage between treatments. Previous studies have similarly shown that predator-induced reductions in growth, while common in early development, may disappear and even reverse later in development (Van Buskirk & Yurewicz, 1998; Relyea & Werner, 2000; Relyea, 2002*b*). Thus, snakes may actually suppress growth and development and prolong the larval stage, consistent with the findings of Chapter 3. It is also interesting that tadpoles in frog-conditioned water were significantly larger than those in snake-conditioned water on day 15. It is important to note that *R. perezi*, in addition to predating *A. muletensis* tadpoles, probably also competes with this species. The tadpoles may therefore be responding to frog cues as a competitor as well as a predator and large body size may enable individuals to use greater proportions of the available resources in the presence of frog tadpoles (Alford, 1999).

Thus, while previous studies indicated that intensive selection pressure imposed by introduced snakes has driven the evolution of inducible morphological and behavioural defences in *A. muletensis* tadpoles, the findings of this chapter indicate similar predation pressure may have been imposed by introduced frogs. It is likely, therefore, that the recovery of the toad would be enhanced by the implementation of measures to control the frog as well as the snake. This chapter additionally found that approximately three generations in captivity followed by four generations at a natural site has not diminished the ability of midwife toad tadpoles from a reintroduced population to respond behaviourally or morphologically to predators. Such a short period of captive breeding may not be sufficient to significantly impair fitness through inbreeding depression or adaptation to the natural environment, or alternatively the descendent population may have recovered since its reintroduction into a natural site. The effects of a captive-breeding bottleneck on tadpole performance is investigated further in Chapter 8.

CHAPTER 6: BEHAVIOURAL AND MORPHOLOGICAL RESPONSES OF *ALYTES MULETENSIS* TADPOLES TO A NATIVE INVERTEBRATE PREDATOR

6.1 SUMMARY

Tadpoles of *Alytes muletensis* are known to respond behaviourally and morphologically to the perceived presence of predatory snakes and frogs that were introduced onto Mallorca some 2000 years ago. The development of inducible defences in such a short period of predator-prey coevolution raises questions about mechanisms underlying phenotypic plasticity in this species. If inducible defences have always been exhibited towards native invertebrate predators such as dragonfly nymphs, this may have facilitated the evolution of similar defences towards novel vertebrate predators. To provide an insight into natural defences possessed by tadpoles, behavioural and morphological responses of individuals towards a native odonate predator were investigated. Tadpoles were raised for 30 days in the presence or absence of a caged odonate predator (*Anax imperator*) and activity, morphology, growth and development were monitored. The non-lethal presence of dragonfly nymphs did not elicit any behavioural or morphological responses in tadpoles, nor did it influence growth or development. These results suggest that tadpoles of *A. muletensis* may not be capable of inducing defences towards native invertebrate predators and antipredator responses could have evolved since the introduction of vertebrate predators onto Mallorca some 2000 years ago.

6.2 INTRODUCTION

Anuran larvae display a variety of behavioural and morphological responses to cues from predators to minimize the risk of predation. *A. muletensis* tadpoles have been shown to reduce activity and change shape in response to cues from the introduced predatory snake *Natrix maura* (Chapters 4 and 5) and green frog *Rana perezi* (Chapter 5). These inducible defences are thought to have evolved since the introduction of vertebrate predators onto Mallorca around 2000 years ago and may be indicative of intense selection pressure over this period. However, many studies have documented behavioural and morphological responses of tadpoles to invertebrate predators (e.g. Petranka *et al.*, 1987; Kats *et al.*, 1988; Lawler, 1989; Skelly & Werner, 1990; Horat & Semlitsch, 1994; Kiesecker *et al.*, 1996; Lefcort, 1998) and, if

A. muletensis tadpoles also respond to native invertebrate predators, this may have facilitated the evolution of inducible defences towards introduced predators. Responses to native aquatic invertebrates may therefore provide vital insights into the mechanisms underlying the evolution of behavioural and morphological responses to introduced vertebrate predators in this, and other species.

This chapter will examine behavioural and morphological responses of *A. muletensis* to *A. imperator* nymphs using a replicated experimental design. The findings will be related to theories proposed for mechanisms underlying the evolution of defences towards introduced predators.

6.3 METHODS

6.3.1 Experimental Set Up

Seventy Mallorcan midwife toad tadpoles were collected from a predator-free cistern (cistern C1 in Chapter 3) at approximately 750 m altitude in the Serra de Tramuntana mountain range in NW Mallorca on 25 May 2000. On the same day, five *A. imperator* nymphs were collected from a separate cistern some 2 km away that did not contain *A. muletensis* and so where nymphs had never fed on tadpoles of this species. Tadpoles were anaesthetized using MS222 and measured for eight morphometric traits as illustrated in Figure 3.1. The tadpoles were then divided equally between ten 2.5 l boxes, ensuring a similar size structure of the seven tadpoles within each box. Two perpendicular lines were drawn on the base of each box to act as transects. One piece of broken tile (8 cm^2) was placed in the centre of each box to provide a refuge. A clear, perforated plastic cup was then placed in a randomly allocated corner of each box. Into five randomly chosen cups an *A. imperator* nymph was placed (predator treatment) and the cup was covered with a 1 mm nylon mesh lid; the tadpoles could therefore detect the presence of the nymph both visually and chemically without any risk of being eaten. Into the remaining five cups were placed washed stones to act as controls and the cups were similarly covered with a similar mesh lid. The boxes were randomly positioned on a table shaded by some trees, in a farmhouse garden at 1000 m in the Serra de Tramuntana. Temperatures ranged from 11.5°C at night to 25°C during the day and were therefore similar to temperature cycles in natural pools (Schley, Griffiths & Román, 1998). The tadpoles were fed *ad libitum* every two days on fish flake food and once a week the *A. imperator* nymphs were replaced with newly captured individuals. Tadpoles were maintained in this way for 30 days.

6.3.2 Measuring Behavioural and Morphological Responses

Tadpole activity was monitored by counting the number of transect crossings during six 10-minute trial periods spread over the 30-day experiment. Morphological responses to the presence of the *A. imperator* nymphs were investigated by anesthetizing the tadpoles and measuring all eight morphometric variables at the start and at the end of the 30-day experiment.

6.3.3 Statistical Analysis

Transect crossings were compared between treatments and between trial periods using a two-way ANOVA. To test for differences in tadpole shape between treatments on day 0, morphometric measurements were log-transformed to ensure normality and a principal components analysis was conducted on all eight log-transformed measurements to obtain the first principal component (PC1) as a composite measure of body size (see Chapter 3). Each trait was then regressed against PC1 and the mean residuals from each box were calculated (see Chapter 4). A MANOVA on eight traits was used to compare overall shape (based on mean residuals in each box) between treatments. An ANOVA was used to compare body size. After 30 days, tadpole shape was compared between treatments using a MANCOVA, with the relative size of the traits on day 0 used as the covariates. An ANCOVA was used to compare body size and, because all tadpoles commenced the experiment at stage IV6 (Cambar & Martin, 1959), an ANOVA was used to compare developmental stage on day 30. Although stage data are discontinuous, according to the 'central limit theorem', means should be normally distributed even if the original data are not (Sokal & Rohlf, 1980) and this permitted the use of parametric test on this trait.

6.4 RESULTS

6.4.1 Behaviour

Tadpoles were marginally and non-significantly ($F_{1,48}$=0.01; P=0.92) more active in the non-lethal presence of *A. imperator* nymphs than in boxes without nymphs (Figure 6.1). Although there was a significant difference in activity between trial periods ($F_{5,48}$=3.0; P=0.02) there was no treatment by period interaction ($F_{5,48}$=1.4; P=0.23).

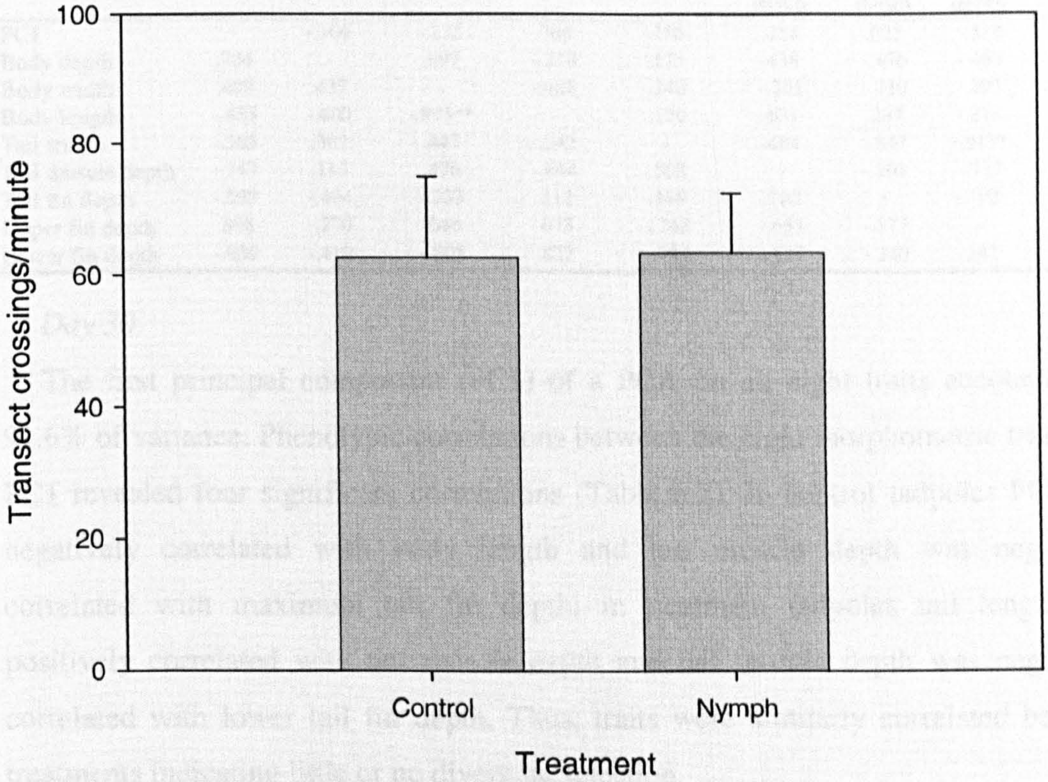

Figure 6.1. Transect crossings per minute (±95% confidence intervals) of tadpoles in the presence and absence of an *Anax* nymph predator, illustrating a lack of a significant behavioural response.

6.4.2 Morphology

Day 0

The first Principal Component (PC1) of a PCA on eight morphometric variables of all tadpoles at the start of the experiment accounted for 88.9% of variance. Phenotypic correlations between the eight morphometric traits and PC1 revealed just two significant correlations; these were negative correlations between tail length and upper tail fin depth in control tadpoles and between body length and body width in treatments tadpoles (Table 6.1).

A MANOVA on the residuals of a regression of each trait against PC1 revealed no significant difference in shape between treatments ($F_{7,2}$=5.3; P=0.17). Body size also did not differ significantly between treatments (P>0.05). All tadpoles were at stage IV6 (Cambar & Martin, 1959).

Table 6.1. Phenotypic correlations among morphometric traits on day 0 in tadpoles in control (above horizontal) and predator (below horizontal) treatments. *$P<0.05$;**$P<0.01$.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	-.106	-.135	.768	.186	.258	.033	-.318	-.163
Body depth	.704	-	.697	-.218	.173	.438	-.476	-.466	-.370
Body width	.409	.427	-	-.628	.240	-.201	-.710	-.303	.123
Body length	-.453	-.600	-.973**	-	.170	.631	.287	-.315	-.251
Tail length	-.365	.302	.447	-.542	-	.484	-.847	-.917*	.694
Tail muscle depth	-.147	.113	.476	-.444	.568	-	-.201	-.717	-.204
Tail fin depth	-.595	-.464	-.233	.312	.189	.702	-	.792	-.617
Upper fin depth	.398	-.270	.046	.073	-.743	-.653	-.577	-	-.352
Lower fin depth	-.039	-.410	-.805	.827	-.794	-.837	-.240	.541	-

Day 30

The first principal component (PC1) of a PCA on all eight traits accounted for 91.6% of variance. Phenotypic correlations between the eight morphometric traits and PC1 revealed four significant correlations (Table 6.2). In control tadpoles PC1 was negatively correlated with body length and tail muscle depth was negatively correlated with maximum tail fin depth; in treatment tadpoles tail length was positively correlated with tail muscle depth and tail muscle depth was negatively correlated with lower tail fin depth. Thus, traits were similarly correlated between treatments indicating little or no divergent selection.

A MANCOVA on the residuals of all eight morphometric traits revealed no significant difference in overall shape between the two treatments ($F_{1,8}$=0.7; P=0.70). Univariate ANCOVAs revealed that only relative tail muscle depth and lower tail fin depth differed significantly between treatments ($P<0.05$); the former was shallower and the latter was deeper in tadpoles exposed to predators. There was no significant difference in mean tadpole size or developmental stage between treatments ($P>0.05$).

Table 6.2. Phenotypic correlations among morphometric traits on day 30 in tadpoles in control (above horizontal) and predator (below horizontal) treatments. *$P<0.05$.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	.067	-.012	-.910*	-.709	.201	.242	-.268	.747
Body depth	.806	-	.447	-.161	-.555	.744	-.650	-.283	-.534
Body width	.241	.701	-	-.278	-.652	-.223	.286	.222	-.310
Body length	-.847	-.616	.094	-	.848	-.152	-.260	-.082	-.517
Tail length	-.509	-.288	.129	.752	-	-.232	-.134	.086	-.190
Tail muscle depth	-.727	-.350	.148	.797	.904*	-	-.898*	-.299	-.320
Tail fin depth	.108	-.276	-.764	-.561	-.707	-.594	-	.151	.621
Upper fin depth	.134	-.361	-.624	-.346	-.711	-.746	.785	-	-.326
Lower fin depth	.814	.367	-.249	-.856	-.778	-.956*	.584	.667	-

6.5 DISCUSSION

Many studies on tadpoles have found reducing activity (Petranka *et al.* 1987; Kats *et al.*, 1988; Lawler, 1989; Skelly and Werner, 1990; Lefcort, 1996, 1998), increasing time spent in refuges (Petranka *et al.* 1987) and changing morphology (Smith & Van Buskirk, 1995; Van Buskirk & Relyea, 1998; Van Buskirk, 2000; Van Buskirk & McCollum, 2000*a*) to be effective defences against invertebrate predators. Despite this, *A. muletensis* tadpoles did not respond behaviourally or morphologically to the non-lethal presence of *A. imperator* nymphs. The possible reasons for this are discussed below.

According to Tollrian & Harvell (1999), inducible defences are beneficial when selective pressure from predation is variable and unpredictable, but sometimes strong. Because *Anax* nymphs are obliged to remain in the same pool until metamorphosis the predation threat to tadpoles may not be sufficiently variable or unpredictable to select for inducible defences. Behavioural and morphological responses to introduced snake and frog predators (Chapters 4 and 5), on the other hand, may have evolved under more variable, unpredictable and potentially stronger predation pressure brought about by the ability of these predators to move freely between pools.

Another potential explanation for the lack of any response is the diet of the predators, since the *A. imperator* nymphs used in the experiment had never fed upon *A. muletensis* tadpoles. Predator-diet has been found to be important in dictating the antipredatory responses exhibited by some amphibian larvae (e.g. Laurila *et al.*, 1997; Lefcort, 1998; Murray & Jenkins, 1999). However, in all of these studies at least a mild behavioural response was elicited regardless of predator-diet. Additionally, snakes and frogs collected from toad-free habitats evoke an antipredatory response in *A. muletensis* tadpoles (pers obs) and it is therefore suggested that the lack of response cannot be completely attributed to the diet of the predators.

The findings of this chapter have important implications for the mechanisms underlying the evolution of inducible defences towards introduced predators in *A. muletensis* tadpoles. Further research is required to test responses of tadpoles to other aquatic invertebrate predators before any firm conclusions can be reached. However, if tadpoles fail to respond to all native invertebrate predators, the theory that defences towards introduced predators reflect adaptations of those already exhibited towards native predators (Chapters 4 and 5) is refuted and an alternative explanation must be sought. It is possible that the evolution of phenotypic plasticity in this species was driven by highly variable environmental conditions associated with the tadpoles' habitat. Torrents inhabited by the toad typically flow during the winter and dry up during the spring leaving a number of standing pools of water. Because tadpoles often overwinter, they are undoubtedly exposed to extreme variations in water flow rate throughout the year. Individuals may benefit by displaying different morphologies in fast flowing and stationary water, since some species exhibit different morphologies according to whether they are stream- or pond-dwelling (Jennings & Scott, 1993), and morphological plasticity may therefore be an adaptive strategy. Thus, the genetic machinery to be plastic may have evolved in midwife toad tadpoles long before the introduction of alien species onto Mallorca, facilitating the evolution of plastic responses upon exposure to novel predators. Further research into plastic responses to changing water conditions may provide vital insight into the conditions necessary for the evolution and maintenance of phenotypic plasticity in this and other species.

CHAPTER 7: ANTIPREDATORY BEHAVIOUR: RATES OF RESPONSE AND RECOVERY IN NAÏVE AND CONDITIONED *ALYTES MULETENSIS* TADPOLES

7.1 SUMMARY

In a highly variable environment there is likely to be intense selection for rapid and reversible responses to the presence of predators. Tadpoles of many anuran species have been found to respond behaviourally to the perceived presence of predators, typically reducing activity and/or increasing refuge use. Factors influencing the nature and magnitude of the behavioural response may be important in determining the adaptiveness of the response as an antipredatory strategy. One factor which may influence the nature of the response is history of previous exposure to predatory cues. Tadpoles of the Mallorcan midwife toad, *Alytes muletensis*, are known to reduce activity in response to cues from the predatory viperine snake, *Natrix maura*. In an experiment designed to test the influence of predator history on subsequent behavioural responses, predator-naïve individuals exhibited a greater reduction in activity upon exposure to snakes than individuals with previous experience of the predator. This confirms that behavioural responses to snakes are innate rather than learned in this species. Tadpoles conditioned with snake cues were less active than unconditioned tadpoles in control groups, indicating that a history of exposure to predatory cues can reduce baseline activity levels even after the threat of predation has been removed. It is likely that, in the face of prolonged and unpredictably variable predation pressure, baseline levels of activity are more important than short-term responses to snake presence in reducing predation risk. In an experiment designed to investigate recovery following exposure to snakes, tadpoles in semi-natural plunge pools reduced activity over three days of exposure to snakes and then recovered to former levels of activity within a day of removing the snakes from the water. Thus, short-term fluctuations in predator presence can invoke rapid behavioural responses in midwife toad tadpoles, although a prolonged behavioural response is likely to be induced by sustained exposure to predatory cues.

7.2 INTRODUCTION

Amphibian larvae of many species can detect the presence of predators and respond behaviourally by reducing levels of activity (e.g. Lawler, 1989; Skelly &

Werner, 1990; Griffiths *et al.*, 1998), shifting use of microhabitat (Morin, 1986; Lawler, 1989; Semlitsch & Gavasso, 1992) and/or increasing time spent in refuges (e.g. Petranka *et al.*, 1987; Kats *et al.*, 1988; Lefcort, 1998). While reducing activity may increase a tadpole's chances of survival in the presence of a predator, in the absence of a predator such behaviour may impede feeding, growth and even survival. In a highly variable environment, therefore, there is likely to be strong selection on tadpoles to accurately assess changes in predation risk and respond rapidly. Rapid behavioural adjustments that reduce time spent inactive or in refuges can provide tadpoles with greater opportunities for feeding and growth, and increase fitness by reducing time to, and increasing size at, metamorphosis (Wilbur, 1980; Skelly & Werner, 1990). Tadpoles typically detect predators using chemical cues, which are thought to convey the most accurate information regarding the presence and, potentially, identity of a predator (Magurran, 1989; Dodson *et al.*, 1994). However, chemical cues may occasionally provide erroneous information about predator presence because of information time lags (Petranka *et al.*, 1987) and a number of studies have reported prolonged behavioural responses to predators following the removal of the threat (Petranka *et al.*, 1987). Tadpoles of the Mallorcan midwife toad, *A. muletensis*, have been found to detect chemical cues from the viperine snake, *N. maura* and respond by reducing activity levels (Griffiths *et al.*, 1998; Chapter 5). Schley (1996) suggested that chemical cues from the snakes may persist in pools and be detected by the Mallorcan midwife toad, *A. muletensis* tadpoles for several days after a snake has left the water, resulting in a prolonged behavioural response. While response and recovery times may be crucial to individual fitness in a highly variable environment such as that inhabited by *A. muletensis*, there are no data on how rapidly tadpoles of this species respond upon the detection of snakes, nor on how rapidly they recover following the removal of the threat.

A number of factors can influence the nature of behavioural antipredatory responses displayed by tadpoles. Previous exposure to a predator may influence subsequent behavioural responses. This has implications for dictating the strength of antipredatory responses exhibited by individuals under different predator regimes. While many anuran tadpoles typically respond only to predator species that they would encounter in the wild (Kiesecker *et al.*, 1996), previous exposure to predatory cues within an individual's lifetime has been found in some species to influence subsequent responses (Magurran, 1990; Semlitsch & Reyer, 1992). Previous studies have confirmed that predator-naïve Mallorcan midwife toad tadpoles respond to cues from predatory snakes (Griffiths *et al.*, 1998; Chapter 5), although the extent to which previous experience of predatory cues influences baseline activity levels and the nature of subsequent behavioural responses to predators in this species remains unclear.

This chapter will investigate several aspects of the behavioural response of *A. muletensis* tadpoles to the predatory snake *N. maura*. Firstly, the influence of previous exposure to snake predators on the strength of subsequent behavioural responses will be tested by directly comparing the responses of tadpoles of similar age and from the same population but with different predator histories under controlled conditions. Secondly, rates of response and recovery following the addition and removal of snakes respectively will be investigated by conducting experiments under controlled and semi-natural conditions.

7.3 METHODS

7.3.1 Influence of Predator History on Behavioural Responses

Pre-conditioning Tadpoles

Thirty-two tadpoles were netted from a snake-free pool (Pool PF3 in Chapter 3) in May 2001 and transported to a farmhouse at 1000 m in Mallorca. The tadpoles were divided into eight groups of four and each group was raised for 30 days in a plastic box containing 2.5 l of either snake-conditioned water or control water (four boxes of each). Water, which was taken from a well containing local natural ground water, was snake-conditioned by suspending three *N. maura* held in nylon bags into a 20 l tank of water for three hours (the nylon bags were positioned so that the snakes could move freely within the water and surface for air when needed). Water for control treatments was taken from the same well and left to stand for three hours prior to use. The plastic boxes were randomly positioned within a regular array on the floor of a room in a farmhouse in the Serra de Tramuntana, Mallorca. The water in each box was renewed every two days to ensure regular exposure to cues and tadpoles were fed fish flake food *ad libitum* after each water change; and the position of each box was re-randomised at this time. The room was exposed to natural light-dark cycles via a south-facing window, and the temperature varied from 12.5°C at night to 24°C during the day. This is similar to temperature cycles in natural pools (Schley, Griffiths & Román, 1998).

Experimental Protocol

Following the 30-day conditioning period outlined above, a 2x2 factorial experiment was established to compare the behavioural responses to snake cues of tadpoles raised in snake-conditioned and unconditioned water. Tadpoles raised in snake-conditioned water were divided equally between eight 20 l boxes each containing 2.5 l of unconditioned well water so that there were two tadpoles in each box. Tadpoles raised in unconditioned water were similarly divided between a further eight boxes. Four boxes from each of the conditioned and the naïve group were randomly allocated as 'snake' treatment; the remaining four boxes were designated controls.

Tadpoles were allowed a two-day 'recovery' period between the 30-day conditioning period and the start of behavioural observations. Behavioural observations were then conducted over two days between 13:00 and 19:00 with half the tadpoles being observed on the first day and the other half being observed on the second day. Tadpole activity was monitored every thirty minutes throughout this period by observing each tadpole for one minute and recording the amount of time spent active (see Chapter 5). At 12:00 on each day, one hour prior to the start of the first behavioural observation, a bag of washed stones was placed into one corner of each box. Immediately following the fourth behavioural observation at 14:30, the bagged stones were removed from each box and replaced with either a bag containing a snake ('snake' treatment) or another bag containing washed stones (control). A further 7 observations were made at 30 minute intervals to monitor the activity of tadpoles within each box.

Statistical Analysis

Tadpole activity was compared between the four treatments described above. Because the activity of the two tadpoles in each box was not considered independent, mean tadpole activity within each box at each observation was used in the analysis. Behavioural responses of naïve and conditioned tadpoles to snakes were compared by regressing activity against time since the addition of snakes (or bagged stones in controls) for each of the four treatments (naïve tadpoles-snake; naïve tadpoles-control; conditioned tadpoles-snake; and conditioned tadpoles-control). Activity was normally distributed and therefore met the assumptions of the analysis. Activity at time 0 (before the addition of snakes) was taken as mean tadpole activity within the group over the four behavioural observations prior to the addition of snakes. The slopes of the regression lines were compared between conditioned and unconditioned tadpoles in each of the two treatments using the t-test and, if no significant difference were found, the elevations of the lines were compared using a two-way ANOVA, with tadpole condition and time as the independent variables.

7.3.2 Persistence of Response

Experimental pools

An experiment was established under semi-natural conditions to investigate the behavioural recovery of tadpoles following the addition and subsequent removal of snakes. Six similarly sized plunge pools (circa 1.5 m diameter) at a predator-free site were selected for the study. The pools, which did not contain any tadpoles prior to the start of the experiment, were topped-up using water from a neighbouring plunge pool (pool PF3 in Chapter 3) to a depth of approximately 10 cm, but to within no less than 2cm from the brim to prevent tadpoles jumping out. Twenty-four tadpoles of similar size (mean length±SD = 57.7±4.0 mm) were collected from a nearby pool (also pool PF3) and randomly divided between the experimental pools so that each pool contained four individuals. Three of the pools were then randomly designated as 'snake' treatment and the remaining three as controls. The tadpoles were fed flaked fish food and then left undisturbed for 24 hours prior to the start of the experiment.

Experimental protocol

The experiment was run over a nine-day period in June 2001. Tadpole activity was monitored on each day in each of the six pools at 17:30 and 18:30. These times were chosen because they have been found to fall within the peak period of activity for *A. muletensis* tadpoles (Schley, Griffiths & Román, 1998). At these times, each tadpole was watched by one of two observers for one minute and the amount of time spent active recorded; mean tadpole activity within each pool was then averaged for each observation period.

The experimental period was divided into three consecutive three-day phases; pre-treatment, treatment and post-treatment. During the pre-treatment period (days 1 - 3), a bag constructed using 1 mm netting and containing washed stones was dipped into each pool three hours prior to the start of the behavioural observations and removed following the completion of the observations. On each day of the treatment period (days 4 - 6) a snake contained within a bag of the same design was placed into each of the designated 'snake' treatment pools three hours prior to the first behavioural observation and removed following the completion of the observations; into control pools bagged stones were dipped as before. On each day of the post-treatment period (days 7 – 9), bags containing washed stones were placed in all pools as in the pre-treatment period. Thus, tadpoles were exposed to three days of pre-treatment (a bag containing stones in all pools), three days of treatment (three pools with a bagged snake, three with a bag containing stones) and three days of post-treatment (a bag containing stones in all pools).

Statistical Analysis

Mean tadpole activity was calculated for each pool on each day. Activity was plotted against day for each of the two treatments (snake and control) to investigate behavioural responses of tadpoles following the addition and removal of snakes. Separate two-way ANOVAs were conducted for each of the three phases to compare mean activity between treatments and between days.

7.4 RESULTS.

7.4.1 Influence of Predator History on Behavioural Responses

Predator-naïve tadpoles reduced activity to a greater extent in response to snake cues than individuals with previous exposure to such cues (Table 7.1 and Figure 7.1). Unconditioned tadpoles reduced activity within 30 minutes of the addition of snakes to the water and were least active 120 minutes following the addition of snakes. All other test groups reduced activity non-significantly over the course of the experiment (Table 7.1). A t-test confirmed that the regression slope of unconditioned tadpoles was steeper than that of conditioned tadpoles in the snake treatment (t=2.1; df=56; $P<0.05$). In control groups, naïve tadpoles maintained higher levels of activity than conditioned tadpoles throughout the course of the experiment (Figure 7.1B). A t-test confirmed that the regression slopes were not significantly different (t=0.1; df=56; $P>0.05$). A two-way ANOVA showed that the difference in activity between naïve and conditioned tadpoles was significant ($F_{1,48}=4.9$; $P<0.05$). However, activity did not differ significantly between observation times ($F_{7,48}=0.4$; $P>0.05$) and there was no condition by time interaction ($F_{7,48}$=0.1; P>0.05). Therefore, tadpoles previously exposed to predatory cues for 30 days continued to display suppressed activity even after the removal of the threat.

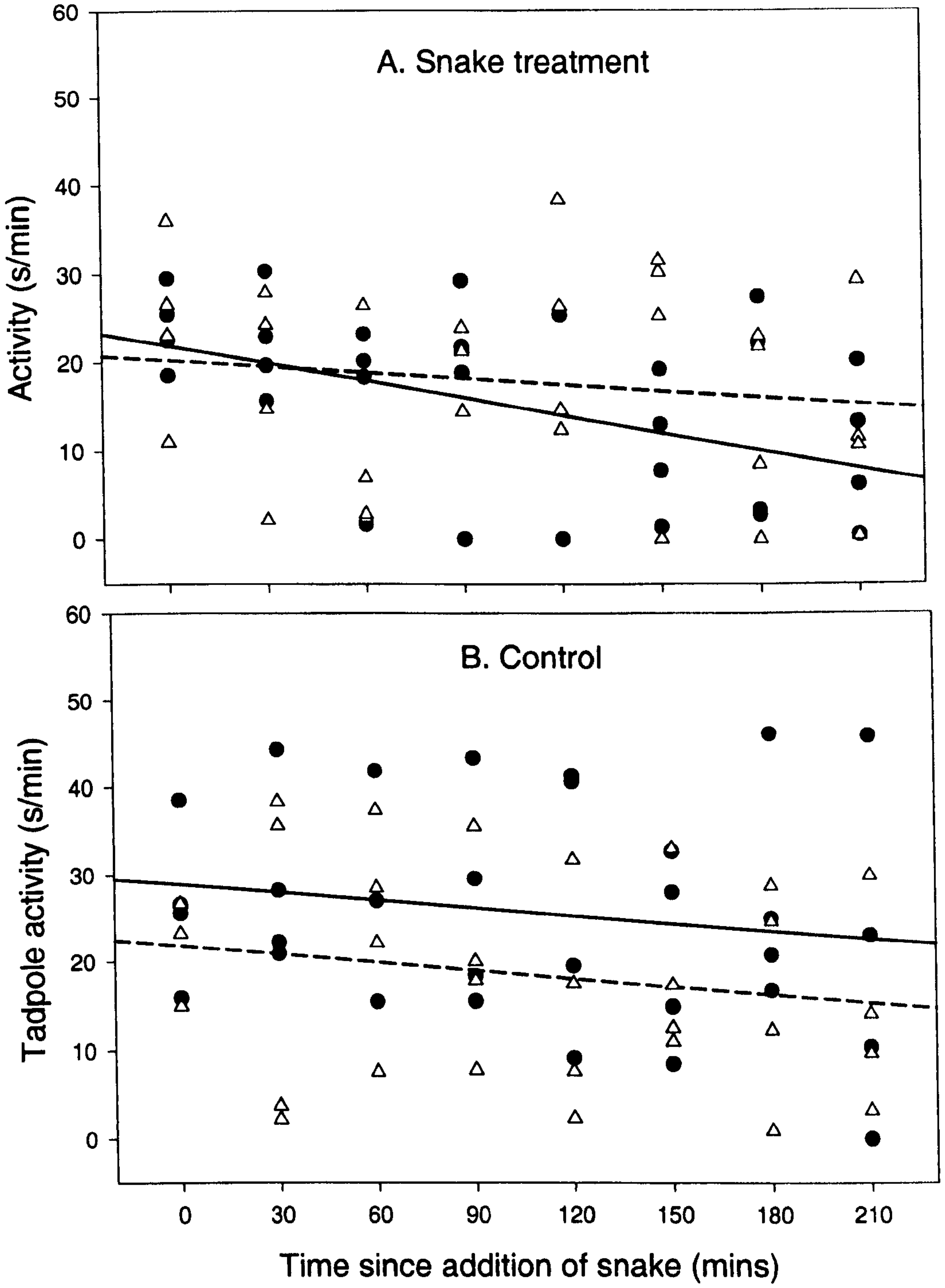

Figure 7.1. Mean activity in naïve and conditioned tadpoles in response to snakes (A) and stones (B). ● = naïve tadpoles Δ = conditioned tadpoles. ——— = regression of time against activity in naive tadpoles; -------- = regression of time against activity in conditioned tadpoles.

Table 7.1. Linear regression of mean tadpole activity against time immediately prior to, and at seven 30-minute intervals following the addition of snakes (snake treatment) or bagged stones (control).

Predator history	Naïve		Conditioned	
Treatment	Snake	Control	Snake	Control
R^2	0.20	0.03	0.02	0.04
B	- 1.98	- 0.90	- 0.71	- 0.94
S. E.	0.73	0.94	0.86	0.89
$F_{1,30}$	7.42	0.92	0.67	1.13
P	0.01	0.35	0.42	0.30

7.4.2 Persistence of Response

In the experiment conducted within semi-natural plunge pools, a two-way ANOVA revealed no significant difference in tadpole activity between treatments (snake and control) or between days (1-3) in the pre-treatment period (Table 7.2). When snakes were introduced into three of the six pools, tadpoles in these pools displayed significantly lower levels of activity than those in control pools and the level of response was consistent over the three days of the treatment phase (Table 7.2; Figure 7.2). During the post-treatment phase, there were no significant differences in activity between treatment and control tadpoles, no significant difference in activity between days and no treatment by day interaction (Table 7.2; Figure 7.2). Thus, tadpoles which had been exposed to snake cues recovered to former levels of activity within a day following the removal of the threat.

Table 7.2. Results of two-way ANOVAs comparing treatment (with snake) and control (without snake) tadpoles contained within semi-natural plunge pools over three treatment phases.

Phase		Pre-test		Test		Post-test	
	Df	F	P	F	P	F	P
Treatment	1, 30	0.1	0.81	7.3	0.01	0.8	0.39
Day	2,30	0.4	0.68	0.0	0.96	0.2	0.86
Treatment * Day	2,30	0.8	0.48	0.1	0.95	0.5	0.61

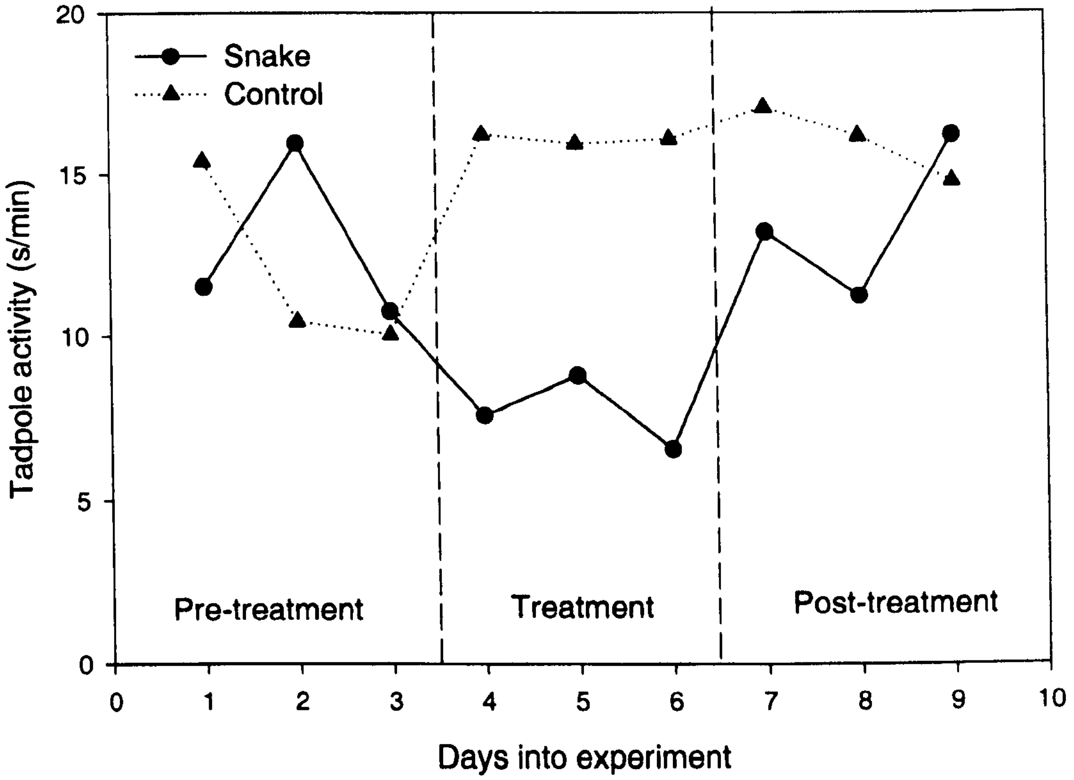

Figure 7.2. Changes in tadpole activity in semi-natural plunge pool upon the addition of snakes (treatment period) and after the removal of snakes from the water (post-treatment period). • = snakes added; ▲ = control (bagged stones added instead of snakes). The vertical dashed lines represent the addition and removal of snakes or bagged stones from treatment and control pools respectively.

7.5 DISCUSSION.

Predator-naïve tadpoles exhibited a stronger behavioural response upon exposure to snake cues than individuals that had been raised in snake-conditioned water (Figure 7.1). This demonstrates quite clearly that behavioural antipredator responses are innate rather than learned and supports the findings of previous studies on this (Griffiths *et al.*, 1998) and other (Gallie, 2001) species. The relatively weak response of conditioned tadpoles may be due to lower starting activity levels in this group. Snake-conditioned tadpoles exhibited suppressed activity throughout the course of the experiment in both treatment and control groups, suggesting that previous exposure to predatory cues has reduced baseline activity levels even after the predation threat has been removed. In agreement, a previous study showed that *A. muletensis* tadpoles inhabiting a pool frequented by snakes displayed lower activity levels and responded less strongly to the addition of snakes than tadpoles inhabiting a snake-free pool (Griffiths *et al.*, 1998). The results of this chapter suggest that such suppressed activity may be a result of a prolonged behavioural response and does not necessarily imply the persistence of snake cues in the water. It is likely that, in the face of prolonged and unpredictably variable predation pressure, baseline levels of activity are more important than short-term responses to snake presence in reducing predation risk. This is supported by a study showing that predation rates on four species of tadpoles were correlated with baseline activity levels (Lawler, 1989). Thus, while some authors have taken prolonged behavioural responses following the removal of predators to be an indication of the persistence of cues in the water (e.g. Petranka *et al.*, 1987), this may not always be the case.

Predator-naïve tadpoles responded rapidly following the introduction of snakes into the water. A drop in activity was observed after just 30 minutes and the lowest activity levels occurred 120 minutes after the addition of snakes. These findings indicate that tadpoles are capable of responding almost immediately to the presence of snakes and the reaction peaks within 2 hours of initial detection. In a similar study, Van Buskirk & Arioli (2002) found continuous dosage response curves for most behavioural and morphological traits that indicated sensitivity to a graded predation risk. The results of this study indicate that *A. muletensis* tadpoles may similarly exhibit a graded response to the strength of snake cues in the water.

The experiment conducted in semi-natural plunge pools found that tadpoles reduced activity significantly over three days of daily exposure to snakes, but recovered to former activity levels within a day of removing the snakes (Figure 7.2). Thus, tadpoles are capable of reacting to short-term fluctuations in snake presence and may recover rapidly once the threat of predation has been removed. However, the experiment discussed above showed that prolonged exposure to snake cues may result in a reduction in baseline activity levels; thus, the nature of antipredatory responses may be dictated by an individual's history of previous exposure to predators. Although there is likely to be strong selection on rapid responses in the presence of a predator, in the face of sustained predation threat, reducing baseline levels of activity may be a more effective antipredator defence strategy despite the costs associated with being less active (Lima & Dill, 1990; Relyea 2002*a,b*).

The results of this chapter indicate that strong predation pressure has resulted in the evolution of rapid and reversible behavioural responses to chemical cues from introduced snakes. While tadpoles recover rapidly following a short period of exposure to snake cues, a longer period of sustained exposure appears to result in reduced baseline levels of activity even after the threat of predation has been removed. In pools where snake incursions are frequent, lower baseline activity levels may influence individual fitness by impacting tadpole growth rate, larval period and size at metamorphosis (Wilbur, 1980; Skelly & Werner, 1990). Indeed, lower rates of growth and longer larval periods have been estimated in pools subject to sporadic incursions from predators (Chapter 3), and fitness costs associated with antipredator responses are explored further in the following chapter. The findings of this study also have a practical application. If recent predation pressure can be estimated by measuring tadpole activity, this could provide a useful for tool for monitoring predator presence at a site.

CHAPTER 8: COSTS OF RESPONDING TO PREDATORS

8.1 SUMMARY

Inducible defences, whilst increasing an individual's chance of surviving in the presence of a predator, typically carry fitness costs in the absence of predation. Tadpoles of the Mallorcan midwife toad *Alytes muletensis* have been shown to respond behaviourally and morphologically to the presence of the viperine snake *Natrix maura* and, while such responses probably enhance the chances of survival in the presence of a predator, the costs to an individual's fitness are harder to quantify. The aim of this study was to assess the costs associated with inducible defences in *A. muletensis* tadpoles. A 12-week experiment was conducted under controlled conditions to test the influence of predator cues on behaviour, morphology, growth and development. Responses of wild-caught individuals were directly compared with those from captive stock to assess whether there had been any impact of a potential genetic bottleneck on tadpole performance. Trade-offs between behaviours associated with resource acquisition and predator avoidance resulted in significant reductions in feeding rate in all tadpoles in the perceived presence of the viperine snake. Wild-caught tadpoles reduced their feeding rate by 31% and captive-bred individuals by 9.8% when exposed to snake cues. Fitness costs associated with lower feeding rates were a 38.8% reduction in growth rate of wild-caught individuals exposed to snake cues over the first six weeks of the experiment and a 6.0% reduction in growth over the second six weeks of the experiment. Snake cues did not significantly influence the growth of captive-bred tadpoles during either period. The different responses of the two populations suggest that caution should be exercised when releasing captive-bred stock into the wild since a potential genetic bottleneck may impair the ability of tadpoles to detect and respond to predators. Although tadpoles failed to exhibit a morphological response in the presence of snake cues, this may be because water changes were performed just once a week. Behaviour is far more plastic than morphology and frequent, sustained exposure to predatory cues is probably required to induce a predator morphology.

8.2 INTRODUCTION

In addition to direct predation, predators may affect the fitness of prey organisms in more subtle ways. Inducible behavioural and morphological defences expressed by tadpoles of many amphibian species (see Chapters 4, 5 and 7), whilst increasing an individual's chances of survival in the presence of a predator, may also incur a cost to the individual's overall fitness (e.g. Relyea, 2002*a*,*b*). For instance, under the direct threat of predation, tadpoles are likely to experience trade-offs between behaviours associated with predator avoidance and those required for resource acquisition (reviewed by Lima & Dill, 1990). *A. muletensis* tadpoles reduce activity in the presence of introduced snake and frog predators to reduce the chances of being eaten (Chapters 5 and 6); such reduced activity may result in less time spent feeding and consequently reduced growth (Skelly & Werner, 1990; Werner & Anholt, 1996; Relyea, 2002*a*), development (Relyea, 2002*a*) and even survival (McNamara & Houston, 1987; Lima & Dill, 1990; Lawler, 1989; Skelly, 1992, 1995) in the perceived presence of predators. Lower growth in the presence of predators often leads to metamorphosis at a smaller size (Skelly & Werner, 1990; Ball and Baker, 1996) and metamorphic size, in turn, has been positively related to survivorship to maturity (Berven & Gill, 1983) and size at maturity (Smith, 1987). Morphological defences may also be costly to build and maintain and Smith and Van Buskirk (1995) found that clear differences in the morphology of two species of anuran larvae were consistent with a trade-off between structures designed for feeding and those for predator avoidance. Although the costs associated with inducible defences are less well understood than the benefits, they are equally important to the adaptiveness of plasticity as a strategy (DeWitt *et al.*, 1998).

A central component of the Mallorcan midwife toad recovery programme is the reintroduction of captive-bred toads and tadpoles into suitable new habitats. Whilst this appears to be a successful strategy to date, the influence of a captive-breeding bottleneck on the fitness of reintroduced toad populations remains an open question. Captive breeding has received criticism from many workers who claim that it lowers fitness through inbreeding depression (Ralls *et al.*, 1979; Reinert, 1991; Jimenez *et al.*, 1994) or by disrupting the process of natural selection (Loftin, 1995). In a study investigating the effects of inbreeding depression on individual fitness, white-footed mice were subject to three to four generations of captive-breeding and released into the wild simultaneously with non-inbred individuals; individuals that had been through a captive-breeding bottleneck had a significantly lower survival rate (Jimenez *et al.*, 1994). A study by Rowe *et al.* (1999) found that larval growth rate strongly correlated with expected heterozygosity across microsatellite loci in *Bufo calamita*. If

A. muletensis are susceptible to inbreeding depression this may reduce their chances of survival upon reintroduction into the wild following a period of captive breeding. Activity, growth and development are all indicators of fitness in larval anurans (Rowe & Beebee, 2001). Additionally, because a number of toad populations are subject to intense predation pressure (Chapters 4, 5 and 6), responses to cues from predators is likely to be an important component of fitness under natural conditions. If a period of captive breeding in a predator-free environment reduces fitness and diminishes the ability of tadpoles to detect and respond to predators, this will have serious implications for their chances of survival upon reintroduction to the wild. While a previous experiment indicated that a period of captive-breeding may not affect antipredatory responses in *A. muletensis* tadpoles (Chapter 5), the individuals tested had only been subject to around two to three generations in captivity followed by four generations under natural conditions. Other taxa have been found to recover genetically following a bottleneck (Hansson *et al.*, 2000) and it is therefore necessary to compare tadpoles hatched in captivity with wild-caught individuals to determine the influence of captive-breeding on fitness.

This chapter aims to quantify the fitness consequences of inducible defences in *A muletensis* larvae by measuring feeding and growth rate in the presence and absence of snake cues. By directly comparing responses of individuals from a population that has been subject to around six generations of captive-breeding with those of wild-caught tadpoles, the influence of several generations of captive-breeding on tadpole growth, development and antipredatory responses will be assessed.

8.3 METHODS

8.3.1 Populations

Two populations of *A. muletensis* were used in the experiment; one naturally-occurring (‘wild’ population) and one held in captivity. A cistern that was free from snake and frog predators (Cistern C1 in Chapter 3) was selected to represent the wild population because high numbers of newly deposited tadpoles permitted the collection of sufficient numbers of equally-sized tadpoles for the experiment. These were compared against tadpoles from a captive toad population held at the Durrell Institute of Conservation and Ecology (DICE). The latter is derived from a founder stock of 15 surviving adults collected from a site in Mallorca between 1985 and 1987 (Tonge, 1986; Tonge & Bloxam, 1989; Garcia, 1998) and bred over 13-15 years, or approximately six generations in captivity. Forty-six hatchling tadpoles were collected from the cistern (hereafter referred to as 'wild' population) on 27 June 2000 and 54 tadpoles were deposited by captive stock held at DICE (hereafter referred to as 'captive' population) between 21 and 28 July 2000. Forty-four tadpoles from the wild population (due to two deaths) and all 54 from the captive population were used in the experiment.

8.3.2 Experimental Protocol

Forty-four 'wild' tadpoles were anaesthetized and measured for eight morphometric traits (Figure 3.1) on 20 July 2000. At the same time, 15 l of water was snake-conditioned by placing a snake into each of three 5 l tanks of aged tap water for three to four hours. This method of conditioning has been found to be sufficient to evoke a behavioural response in *A. muletensis* tadpoles (Griffiths *et al.*, 1998). Tadpoles were then assigned to either the 'snake' treatment (raised in snake-conditioned water) or 'control' (raised in unconditioned water). Twenty-one tadpoles assigned to the snake treatment were placed individually in 1 l boxes of water filled with 0.5 l of snake-conditioned water. Twenty-three tadpoles assigned to the control treatment were placed individually in identical boxes containing 0.5 l of unconditioned water. Lids with ventilation holes were placed on all boxes to prevent tadpoles jumping out and the boxes were positioned randomly on shelves in a temperature-controlled room maintained at 18°C±3°C during the day. Water was changed weekly and replaced with appropriately conditioned water and at this time the position of boxes was randomly rearranged.

The procedure outlined above was repeated with the 54 tadpoles from the captive population – tadpoles were measured and the experiment was started on 28 August 2000. Half the tadpoles were randomly assigned to the snake treatment and the other half used as controls.

Following each weekly water change, tadpoles were fed *ad libitum* with fish flake food, left for one hour and then each tadpole was observed for a minute and time spent feeding recorded. Tadpoles were considered to be feeding when their mouthparts were visibly moving. Feeding was monitored in this way for nine weeks in the wild population and seven weeks in the captive population due to logistical time constraints. Prior to the start of the experiment, tadpoles were anaesthetized using

MS222 and measured for eight morphometric variables (see Chapters 3 and 4). Individuals were than re-measured for all eight variables after six weeks and again after 12 weeks in order to monitor differences in size and shape between treatments.

8.3.3 Statistical Analysis

A three-way ANOVA was conducted to compare feeding rate (sec/min for each tadpole) between treatments, between populations and between weeks and to test for interactions between these factors. A principal components analysis (PCA) was conducted on eight log-transformed morphometric variables of all tadpoles on each day for which measurements were taken to obtain the first principal component (PC1) as a measure of body size. Each trait was then linearly regressed against PC1 to obtain the unstandardised residuals. A two-way MANOVA was conducted to compare these residuals between populations, between treatments and to test for a population by treatment interaction at each time period. Growth rate (changes in total length, expressed as mm/day) was calculated for each treatment within each population. Growth rate was compared between treatments and populations over the first and second six-week periods using separate two-way ANOVAs.

8.4 RESULTS

8.4.1 Feeding Rate

Overall, tadpoles exhibited significantly lower feeding rates in snake-conditioned than in unconditioned water (Table 8.1, 8.2; Figure 8.1). A three-way ANOVA found no significant difference in feeding rate between the two populations and, although tadpoles from the wild population reduced their feeding rate more than captive individuals in the presence of snake cues (Table 8.1; Figure 8.1), this did not result in a significant population by treatment interaction (Table 8.2). Feeding rate varied significantly between weeks (Table 8.2), and a Tukey's post hoc test revealed that significant differences occurred between weeks one and four (P=0.02) and weeks one and five (P=0.01). There was a significant population by week interaction but no significant population by treatment by week interaction (Table 8.2).

Table 8.1. Mean time tadpoles from each population spent feeding (s/min) in snake-conditioned and unconditioned water.

Population/treatment	Unconditioned	Snake-conditioned
Wild	31.3	21.6
Captive	31.6	28.5

Table 8.2. Results of three-way ANOVA comparing feeding rate between treatments, populations and weeks.

Source	DF	MS	F	P
Population	1	224.00	1.0	0.31
Treatment	1	2876.27	13.3	< 0.001
Population * Treatment	1	148.99	0.7	0.41
Week	8	552.71	2.6	0.01
Population * Week	6	605.84	2.8	0.01
Treatment * Week	8	225.85	1.0	0.40
Population * Treatment * Week	6	256.42	1.2	0.31

8.4.2 Growth Rate

Over the course of the 12-week experiment, tadpole growth (based on changes in total length) was significantly higher in captive-bred than wild-caught tadpoles (Tables 8.3 and 8.4) despite a smaller starting size ($P<0.05$ on PC1) in the former. Overall, growth rate was significantly lower in snake-conditioned water than in unconditioned water (Table 8.4). A significant population by treatment interaction in growth rate from weeks 0-6 (Table 8.4) reflected heavily suppressed growth rates of wild tadpoles raised in snake-conditioned water contrasting with higher growth rates in captive-bred individuals raised in snake-conditioned water (Figure 8.2).

Over the first six weeks of the experiment, a two-way ANOVA found that tadpoles in snake-conditioned water grew significantly less than those in unconditioned water, captive tadpoles grew significantly more than wild-caught individuals, and there was a significant population by treatment interaction indicating different growth responses of the two populations to snake-cues (Table 8.4). During the second six weeks of the experiment, a two-way ANOVA on growth rate revealed no significant differences or interactions between populations or treatments.

No captive tadpoles died over the course of the 12-week experiment. In the wild population 23.8% of tadpoles in snake-conditioned water died of fin rot over the first six weeks of the experiment and 6.3% of remaining individuals died of the same cause over the second six-week period. No tadpoles died in unconditioned water.

Table 8.3. Mean growth rates of tadpoles (mm/day) raised in unconditioned and snake-conditioned water.

Population	Wild		Captive	
Weeks	Control	Snake	Control	Snake
0 – 6	0.3938	0.2412	0.4518	0.4789
6 – 12	0.2969	0.2792	0.2426	0.3064

Table 8.4 Results of two-way ANOVAs comparing growth rate between treatments (snake-conditioned and unconditioned water) and populations (captive-bred and wild) over weeks 0-6 and 6-12 of the experiment.

Period (weeks)	0 – 6		6 – 12	
Source	$F_{1,69}$	P	$F_{1,69}$	P
Population	11.3	0.001	0.3	0.57
Treatment	0.4	0.53	0.5	0.43
Population * Treatment	40.3	< 0.001	2.8	0.10

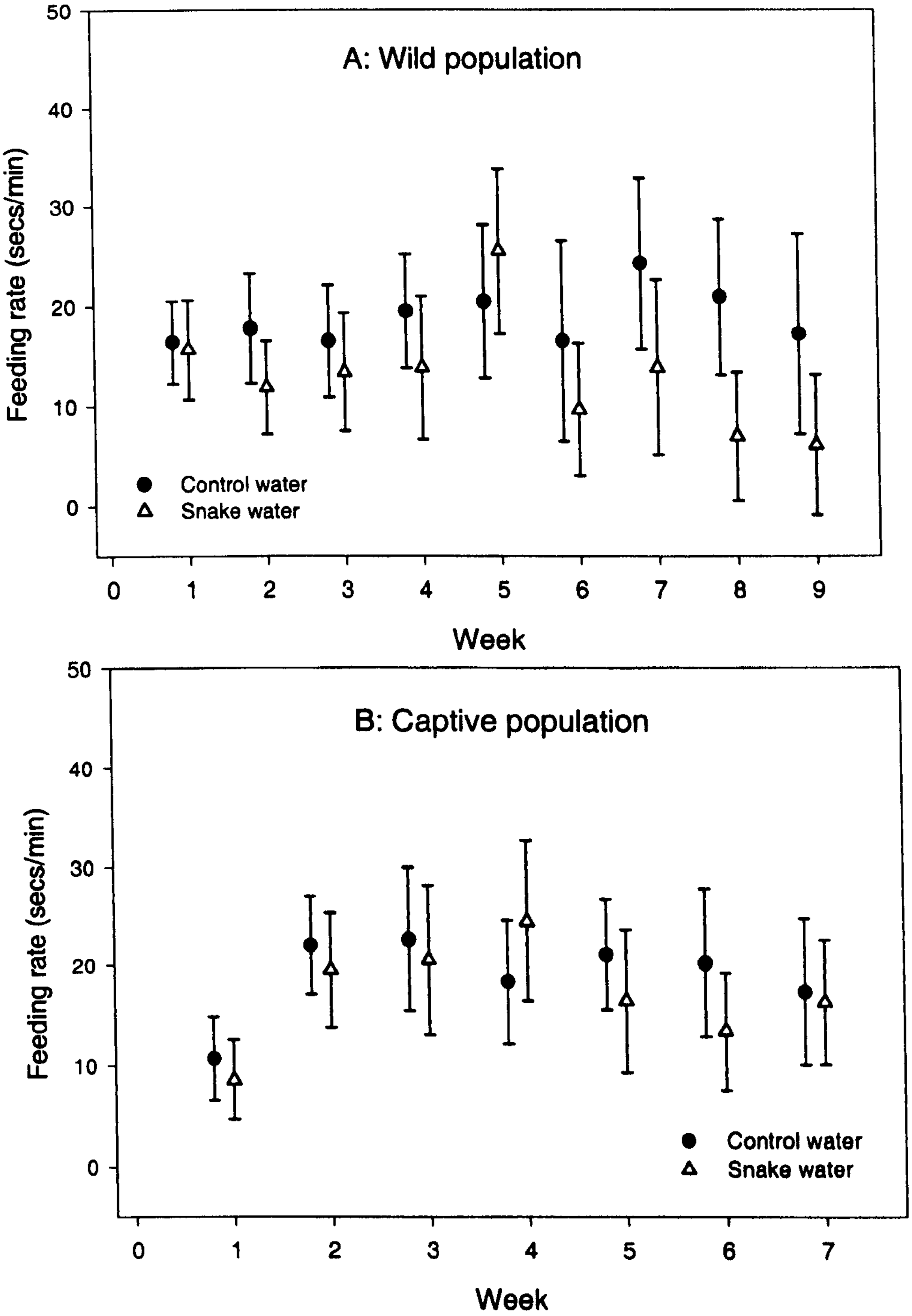

Figure 8.1. Feeding activity (±95% confidence intervals) of *A. muletensis* tadpoles from wild (A) and captive (B) populations in snake-conditioned and unconditioned water. Feeding was monitored one hour following each weekly water change over a nine-week period (wild population) and seven-week period (captive population).

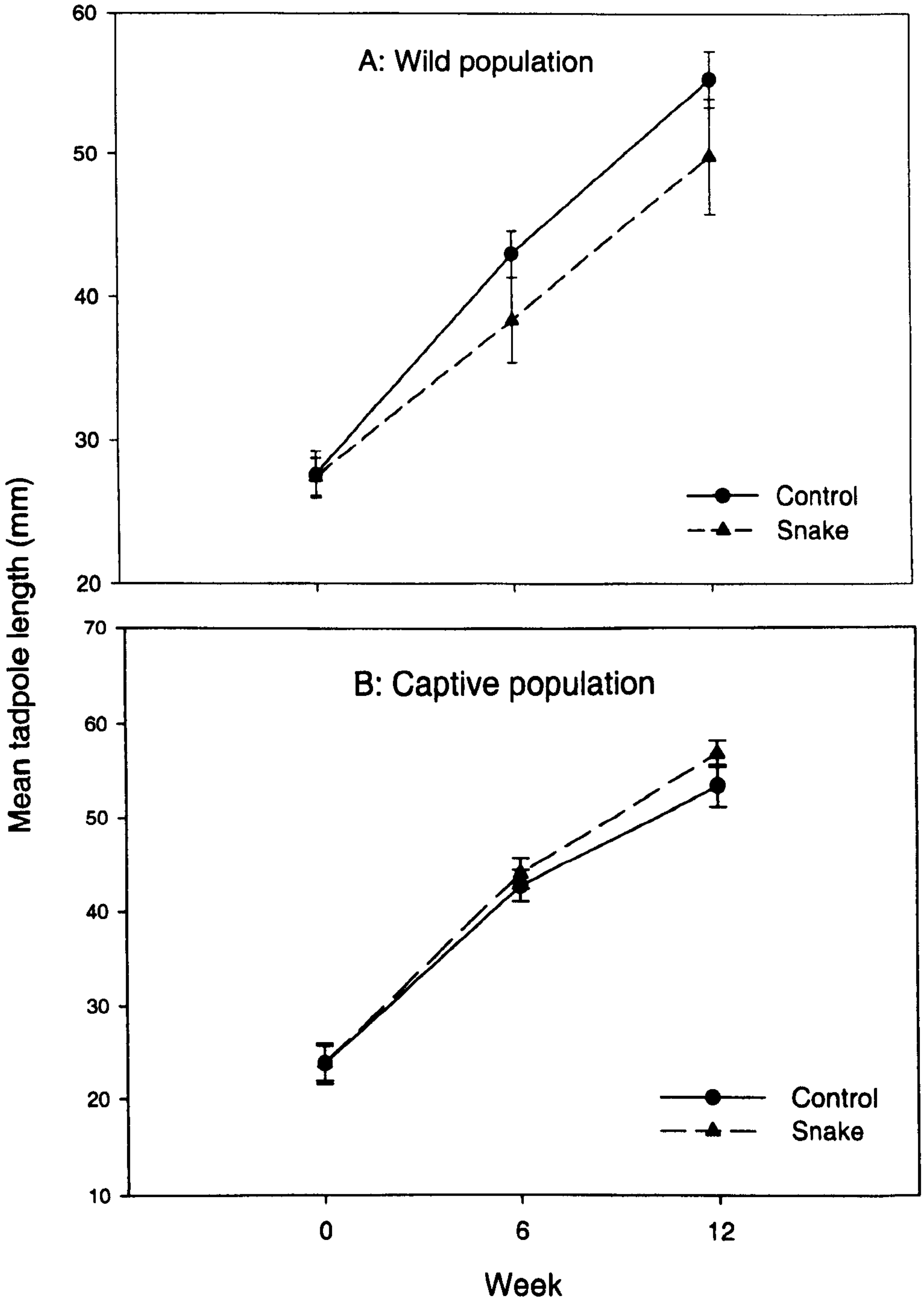

Figure 8.2. Changes in total length (mm) of wild-caught (A) and captive-bred (B) tadpoles (±95% confidence intervals) after 6 and 12 weeks of being reared in unconditioned or snake-conditioned water.

8.4.3 Morphology

Week 0

The first principal component (PC1) of a PCA on the eight morphometric traits of all tadpoles at the start of the experiment accounted for 85.5% of variance. At this time the three measurements of body traits were typically positively correlated with one another, the five measurements of tail traits were positively correlated with one another and body traits were negatively correlated with tail traits in tadpoles from both populations (Table 8.5). Significant correlations between traits confirm that multivariate tests are appropriate.

A two-way MANOVA on all eight traits revealed a significant difference in shape between populations ($F_{7,87}=2.9$; $P<0.01$) but not between treatments ($F_{7,87}=1.1$; $P>0.05$) and there was no significant population by treatment interaction ($F_{7,87}=0.5$; $P>0.05$). Two-way univariate ANOVAs showed that captive tadpoles were significantly smaller and possessed deeper, wider, longer bodies with shallower upper tail fin relative to body size than wild-caught individuals (Table 8.6). There were no significant differences between treatments and no population by treatment interaction for any of the traits. All tadpoles were at developmental stage IV6 according to Cambar & Martin's (1959) staging table for *Alytes obstetricans*.

Table 8.5. Phenotypic correlations at the start of the experiment in wild-caught tadpoles (above the horizontal) and captive-bred tadpoles (Below the horizontal). *$P<0.05$; **$P<0.01$.

	PC1	Body depth	Body width	Body length	Tail length	Tail muscle depth	Tail fin depth	Upper fin depth	Lower fin depth
PC1	-	.324*	.436**	-.174	-.177	.167	-.085	-.359*	-.115
Body depth	.112	-	.605**	.035	-.239	-.063	-.388*	-.439**	-.428**
Body width	.031	.385**	-	.079	-.442**	-.067	-.378*	-.394**	-.431**
Body length	.257	-.013	.035	-	.267	-.222	-.282	-.318*	-.393
Tail length	.159	-.380**	-.218	.241	-	-.029	.082	-.344*	-.068
Tail muscle depth	-.170	-.360**	-.478**	.011	.272*	-	-.363*	-.244	-.165
Tail fin depth	-.048	-.122	-.163	-.403**	-.129	.058	-	.391*	.153
Upper fin depth	-.084	-.111	-.164	-.519**	-.241	-.221	-.128	-	.176
Lower fin depth	-.154	-.182	-.176	-.105	-.417**	-.276*	-.052	-.018	-

Week 6

The first principal component (PC1) of a PCA on the eight morphometric traits of all tadpoles accounted for 68.9% of variance. A two-way MANCOVA on all eight traits using the size of each trait on week 0 as the covariate revealed a significant difference in shape between populations ($F_{8,62}=5.3$; $P<0.001$), no significant difference between treatments ($F_{8,62}$=1.3; P=0.241) and no significant population by treatment interaction ($F_{8,62}$=1.7; P=0.117). Univariate ANCOVAs revealed that captive tadpoles were significantly larger and possessed longer tails with shallower maximum, upper and lower tail fins relative to body size than wild-caught individuals (Table 8.7). All tadpoles were still at developmental stage IV6.

Table 8.6. Two-way ANOVA results showing F ratios between populations and treatments on day 0 of the experiment. *P<0.05; **P<0.01.

Trait/Factor	Population	Treatment	Population x Treatment	Population differences
Body depth	10.3**	0.0	1.0	Captive > Wild
Body width	10.1**	0.1	0.4	Captive > Wild
Body length	4.2*	0.5	2.0	Captive > Wild
Tail length	0.7	0.9	0.2	Captive > Wild
Tail muscle depth	0.9	2.5	0.0	Wild > Captive
Max tail fin depth	1.2	2.8	0.0	Wild > Captive
Upper fin depth	9.1**	0.7	0.0	Wild > Captive
Lower fin depth	0.0	0.4	0.2	Wild > Captive
Body size (PC1)	28.0**	0.0	0.2	Wild > Captive

Table 8.7. Two-way ANCOVA results showing $F_{1,76}$ values after 6 weeks, using starting sizes as covariates. *P<0.05; **P<0.01; ***P<0.001.

Trait/Factor	Population	Treatment	Population x Treatment	Covariate	Population differences
Body depth	0.1	0.5	2.6	0.7	Wild > Captive
Body width	2.8	0.7	0.0	2.7	Captive > Wild
Body length	1.4	2.6	0.0	0.0	Captive > Wild
Tail length	29.8***	3.5	4.1*	2.4	Captive > Wild
Tail muscle depth	0.3	2.1	0.8	0.8	Wild > Captive
Max tail fin depth	7.7**	2.4	3.5	0.5	Wild > Captive
Upper fin depth	4.9*	1.6	0.5	0.8	Wild > Captive
Lower fin depth	8.3**	0.1	2.3	3.1	Wild > Captive
Body size (PC1)	11.3**	0.4	40.3***	30.8***	Captive > Wild

Week 12

The first principal component (PC1) of a PCA on the eight morphometric traits of all tadpoles accounted for 67.9% of variance. A MANCOVA on the eight morphometric traits, using the size of each trait on day 0 as the covariate, revealed a significant difference in shape between populations ($F_{8,54}$=4.4; P<0.001) but not between treatments ($F_{8,54}$=2.1; P=0.1) and no significant population by treatment interaction ($F_{7,56}$=0.4; P=0.92). Univariate two-way ANCOVAs on each trait found that captive tadpoles possessed significantly longer tails, shallower overall and upper tail fins and deeper tail muscles relative to body size than wild-caught individuals (Table 8.8). Tadpoles in snake-conditioned water possessed significantly shallower bodies and shallower lower tail fins relative to overall body size than individuals in unconditioned water. Body size (PC1) was the only trait to exhibit a significant population by treatment interaction. The most developed individual was at stage IV11, although there was no significant difference in developmental stage between populations or between treatments and no significant population by treatment interaction (P>0.05).

Table 8.8. $F_{1,68}$ ratios of two-way ANCOVAs after 12 weeks, using starting sizes as covariates. Values given for stage are $F_{1,69}$ ratios of an ANOVA. *P<0.05; **P<0.01; ***P<0.001.

Trait/Factor	Population	Treatment	Population x Treatment	Covariate	Population differences
Body depth	0.1	6.6*	0.0	0.0	Wild > Captive
Body width	0.1	4.6*	0.2	0.9	Captive > Wild
Body length	2.8	1.3	1.6	0.8	Wild > Captive
Tail length	24.5**	0.2	0.1	4.1*	Captive > Wild
Tail muscle depth	15.4***	0.2	0.1	4.6*	Captive > Wild
Max tail fin depth	4.6*	0.2	0.9	0.1	Wild > Captive
Upper fin depth	6.6*	0.0	1.1	0.7	Wild > Captive
Lower fin depth	1.4	4.1*	0.3	0.8	Wild > Captive
Body size (PC1)	3.2	0.0	16.6***	5.3*	Captive > Wild
Stage	0.2	0.3	1.7	-	Wild > Captive

8.5 DISCUSSION

Selective pressures may exist for larvae to balance conflicts between maximising food intake and minimising exposure to predators (Lefcort, 1996) and tadpoles must therefore choose a strategy that is an adaptive compromise between these two demands (Sih, 1980). In this chapter, trade-offs between behaviours associated with resource acquisition and predator avoidance resulted in reductions in feeding rate of *A. muletensis* tadpoles in the perceived presence of the predatory viperine snake *N. maura* (Figure 8.1). Lowered feeding rates in the presence of snake cues were translated into reduced growth (Figure 8.2). Previous studies have also demonstrated that the addition of predatory cues can have a large impact on growth, particularly at low competitive densities (Werner & Anholt, 1996; Van Buskirk & Yurewicz, 1998; Peacor & Werner, 2000). Wild-caught tadpoles exposed to predatory cues suffered a 31.0% reduction in feeding rate and a 24.6% reduction in growth relative to control individuals. Captive-bred tadpoles, on the other hand, reduced feeding by only 9.8% and exhibited a 13.1% increase in growth in snake-conditioned water over the course of the 12-week experiment. Intuitively, growth rate should be positively correlated with feeding rate; the discrepancy between feeding rate and growth in captive tadpoles exposed to snake cues may indicate that, while individuals in the predator treatment reduce activity immediately after each water change, they spent, on average, more time feeding in the week between water changes than control individuals. The responses of wild-caught tadpoles were consistent with previous studies that have documented reductions in tadpole feeding rate (Lawler, 1989; Feminella & Hawkins, 1994), growth (Lima & Dill, 1990; Skelly & Werner, 1990; Skelly, 1992, 1995) and survival (Lawler, 1989; Skelly, 1992 Werner & Anholt, 1996) in the non-lethal presence of a predator. Although there is little literature available on the responses of tadpoles to snake predators, one study by Kupferberg (1997) found a 56% reduction in activity and a 28% reduction in growth rate of tadpoles exposed to garter snakes.

As discussed in Chapters 1 and 5, captive breeding can generate problems through inbreeding depression (Ralls *et al.*, 1979; Reinert, 1991; Jimenez *et al.*, 1994; Lewis & Thomas, 2001) and adaptation to an artificial environment (Reinert, 1991; Loftin, 1995; Lewis & Thomas, 2001), both of which can result in reduced fitness of individuals in natural situations. The different levels of behavioural response exhibited by tadpoles from captive-bred and wild-caught populations of the Mallorcan midwife toad in this study indicate that a captive-breeding bottleneck may have diminished the ability of tadpoles to detect and respond to predatory cues. Whereas in Chapter 5 no differences were found in the behavioural and morphological antipredator responses of tadpoles from a reintroduced and a wild population, the reintroduced population had only been subject to approximately three generations of captive-breeding and had subsequently been established at a natural site for around four generations, allowing the potential for genetic recovery (see Hansson *et al.*, 2000). The results of this study indicate that around six successive generations of captive breeding may significantly impair tadpole fitness through the effects of inbreeding depression or adaptation to an artificial environment.

Captive-bred tadpoles spent more time feeding, grew faster and suffered less mortality than wild-caught individuals over the course of the experiment. Because growth and survival are two key components of fitness (Jimenez *et al.*, 1994; Rowe & Beebee, 2001), this finding contradicts the theory that captive-bred individuals are less fit than wild-caught individuals. However, the difference may be attributed to the differential treatment of tadpoles from the two populations prior to the start of the experiment. Because wild-caught tadpoles were collected from a cistern in Mallorca and transported back to the UK, they were subject to more potentially stress-inducing treatment than captive individuals. This stress may have weakened individuals and resulted in reduced feeding and growth rates and increased mortality. At the start of the experiment captive tadpoles had significantly deeper, wider and longer bodies relative to overall size and therefore may have been in better condition than wild-caught individuals; these differences had disappeared after six weeks, however. Because the presence of predators can also induce stress (Relyea & Mills, 2001), this may have contributed to mortality of wild-caught tadpoles in snake-conditioned water by making them more susceptible to fungal infection. Wild-caught individuals may also have been carrying pathogens that further lowered their fitness. It is also possible that captive tadpoles have become adapted to an artificial environment and to feeding on flake fish food.

Wild-caught tadpoles suffered greater reductions in growth upon exposure to snake cues over the first six weeks than over the latter six weeks of the experiment. During the second six-weeks of the experiment tadpoles may have become habituated to the presence of cues and, in the absence of further visual or tactile cues, minimized reductions in growth and as a result increased their chances of survival (see also Chapter 5).

Average growth rate of tadpoles ranged from 0.24 mm/day to 0.48 mm/day within each treatment over a six-week period. These rates are considerably lower than the maximum growth rates of 1.8 mm/day and 1.1 mm/day calculated for tadpoles inhabiting a natural torrent pool and artificial cistern respectively, but comparable to the maximum growth rate of 0.2 mm/day calculated for tadpoles inhabiting a torrent pool subject to snake predation (Chapter 3). Tadpoles in the experiment also developed relatively slowly when compared to natural populations. All tadpoles began the experiment at stage IV6 (Cambar & Martin, 1959); after 12 weeks no tadpoles had developed beyond stage IV11, only one tadpoles had developed beyond stage IV9 and 69.8% of tadpoles were still at stage IV6. In contrast, tadpoles inhabiting a cistern in the Serra de Tramuntana developed from stage IV6 to at least stage IV16 (close to metamorphosis) in just 21 days (Chapter 3). Thus, rates of growth and development were slower in the lab than maximum rates calculated for natural populations. Because temperature is important in influencing rates of growth and development in anuran larvae (Harkey & Semlitsch, 1998; Alford, 1999), the relatively slow rates of both these variables may have been due to low temperatures (18°C±3°C) maintained in the lab. Although the mean preferred temperature of *A. muletensis* tadpoles is 21.6°C (Martens, 1984) and the optimum temperatures for development is 21-24°C (Kadel & Hemmer, 1984), both of which are relatively low for a European amphibian (Martens, 1984), tadpoles of this species have been found to develop faster as temperature increases (Lea *et al.*, 2002). Relatively long larval periods have also been observed in natural pools, where tadpoles frequently overwinter (Román, pers com, pers obs) and temperature is probably an important factor influencing rates of growth and development and consequently larval period in this species.

Tadpoles did not change shape in response to snake cues. The only significant difference in morphology between predator treatments occurred on week 12 when tadpoles from both populations were pooled. At this time, tadpoles in snake-conditioned water possessed significantly shallower bodies and shallower lower tail fins relative to body size than individuals in unconditioned water but there was no significant change in tail length or tail muscle depth as expected. The lack of any change in these traits may be due to the long time period between water changes. Water changes were performed once a week; if cues do not persist for any length of time in the water this could mean that tadpoles in the snake treatment were, for most of the experiment, in water which was free from predatory cues. Previous induction experiments that resulted in morphological changes involved exposing tadpoles to snake cues at least every two days (Chapter 4) and this frequency of exposure may be required to evoke a response. Whereas behavioural responses are typically easily reversible, morphological responses are thought to be long-term and typically irreversible, because of the time constraints and physiological costs involved (Gabriel 1999; Relyea 2001*a*). Thus, it is likely that frequent and sustained exposure to predatory cues is required to induce and maintain a predator morphology, although this is an area that requires further research.

In addition to the costs measured in this experiment, it is likely that there are other, less apparent costs associated with inducible defences, since some studies have shown evidence for complex allocation shifts among growth, reproduction, and longevity in response to a changing defence state (Walls & Ketola, 1989; Baldwin *et al.*, 1990). Additionally, costs associated with inducible morphological defences may exist in natural populations (Smith & Van Buskirk, 1995) but these could not be ascertained in this study because no morphological response was found.

Thus, the findings of this chapter demonstrate that antipredatory behaviours carry a cost in the form of reduced feeding and growth in the perceived presence of a predator. This confirms the implicit assumption that inducible defences incur a cost in the absence of predation. The presence of predators is likely to negatively influence the dynamics of natural larval populations and this supports the findings of Chapter 3, which documented differences in larval population structure and relative rates of growth and development between predator environments under natural conditions. This chapter additionally found that tadpoles from a population that has been subject to around six generations in captivity respond less strongly behaviourally to the presence of predators than wild-caught individuals. This may be indicative of impaired fitness as a result of inbreeding depression, and has serious implications for the success of the Mallorcan midwife toad captive breeding and recovery programme. It is suggested that stock which has been subject to many successive generations of captive-breeding should not be reintroduced into natural habitats.

CHAPTER 9: GENERAL DISCUSSION

9.1 DISTRIBUTION OF THE TOAD AND INTRODUCED PREDATORS

The aim of this thesis was to investigate the factors influencing the distribution of the Mallorcan midwife toad, with specific emphasis on the role of introduced predators in driving the historical decline of the species. The toad, having once been widespread over most of Mallorca (A. Alcover pers com), is now restricted to only a small number of gorges in the northwest of the island. The introduction of exotic species such as the viperine snake *Natrix maura* and green frog *Rana perezi* have been implicated by many authors as the primary agents of decline (Tonge, 1986; Garcia, 1998; Griffiths *et al.*, 1998) and it is believed by some workers that toad populations have survived only in areas that are relatively inaccessible to these predators (Tonge, 1986; Schley & Griffiths, 1998). To test this theory, GIS was used to map and analyse the distribution of the toad and associated predators in relation to selected topographical features. When compared against randomly generated sites along torrents, the presence of toads was indeed found to be strongly and positively associated with steep slopes within a 100 m radius. However, while the presence of predators between toad sites was negatively associated with elevation, it was not associated with aspect or maximum slope, indicating that steep sides do not necessarily make a site inaccessible to these predators. It may be that gorges surrounded by steep sides are more shaded, resulting in a cooler microclimate that favours the persistence of pools throughout the year. Larval population size was found to be strongly positively associated with the number of pools at the site and the number of larvae per pool was positively associated with elevation. These results suggest that the optimal habitat for toads is high elevation torrents surrounded by steep sides and containing many pools. At higher elevations predators become less common and climatic and environmental conditions may become more favourable for the toad.

A number of physiological characteristics of the midwife toad suggest that it is well adapted to life in montane torrents. A slender, long-legged morphology together with spatulate terminal phalanges allow adult toads to climb the vertical gorge walls and rest in narrow cracks and crevices (Alcover *et al.*, 1984). The relatively streamlined shape and strong tail muscles of the tadpoles probably facilitate their survival in fast flowing torrents. The larvae also achieve higher filtering rates than most other European anuran tadpoles, and Viertel (1984) related their feeding behaviour in the aquarium to their behaviour in torrents, where they adhere to rocks through the sucking force of a large oral disc. The mean temperatures in torrent pools inhabited by *A.muletensis* larvae fluctuate between 9°C in winter and 22°C in summer (Alcover *et al.*, 1984). Indeed, these temperatures match the mean preferred temperature of 21.6 °C (Martens, 1984) and the optimum temperatures for development of 21-24 °C (Kadel & Hemmer, 1984) for tadpoles of this species. *A. muletensis* is the only discoglossid species which appears to be adapted in this way to a life cycle in montane torrent gorges with steep walls and cold, oligotrophic plunge pools. The present distribution of *A. muletensis* populations may therefore reflect the optimal habitat for the species rather than an ecologically marginal area into which it has been forced by predators, or alternatively the species may have become adapted to torrent habitats since being restricted to these areas by predators.

9.2 LARVAL POPULATION STRUCTURE AND DYNAMICS

Identifying what regulates certain life history traits of a species is central to effective management of wild populations. Factors influencing the structure and dynamics of anuran larval populations are central to regulating populations as a whole (Berven, 1990). Larval size-structure of *A. muletensis* populations was compared across three distinct environments (predator-free torrent pools, predator-free cisterns and torrent pools subject to incursions from introduced snake and frog predators) to identify factors important in regulating population dynamics. Larval populations subject to sporadic incursions from predators displayed distinctly bimodal size-distributions. This contrasted sharply with unimodal distributions found in both predator-free environments. Bimodal size distributions in predator environments may be a direct result of size-specific predation on medium-sized tadpoles, as has been found in other studies (Brodie & Formanowicz, 1983; Wilbur, 1988). Alternatively, differences in size-structure may reflect the influence of predators on ovipositing site choice or rates of tadpole growth and development. Whatever the underlying cause, the findings indicate that predation from introduced predators plays an important role in regulating the structure and dynamics of *A. muletensis* larval populations.

Rates of growth and development appeared to be extremely variable across environments. While some tadpoles reached metamorphosis within little over three weeks (Chapter 3), others can overwinter in pools and metamorphose the following year (Lea *et al.*, 2002; A. Román, pers com). The presence of predators appears to suppress growth and development and may lengthen the larval period and influence individual fitness. Temperature is likely to be important in influencing rates of tadpole growth and development (Harkey & Semlitsch, 1988; Alford, 1999; Lea *et al.*, 2002) as is intra-specific competition (Brockelman, 1969; Dash & Hota, 1980; Semlitsch & Caldwell, 1982; Travis; 1983; Mahapatro & Dash, 1987) and in a small, exposed cistern rapid tadpole growth and development were attributed to high daily water temperatures and a high larval density. Further research is required to determine the influence of larval growth and development on tadpole fitness within different environments in order to optimise the design of pools at future reintroduction sites.

9.3 INDUCIBLE DEFENCES

Tadpoles may reduce predation pressure by exhibiting morphological and/or behavioural defences towards predators. Behavioural responses have previously been described for *A. muletensis* tadpoles towards introduced viperine snakes *N. maura* (Griffiths *et al.,* 1998). Whereas behavioural responses are typically easily reversible, however, morphological responses are thought to be long-term and typically irreversible, because of the time constraints and physiological costs involved (Gabriel 1999; Relyea 2001*a*). As a result, behavioural responses undoubtably require less time to develop than morphological ones (West-Eberhard, 1989). Selection on tadpole morphology from introduced snake predators was investigated to determine whether 2000 years is a sufficiently long period for the evolution of morphological plasticity as an antipredator defence. Morphological differences between larval populations inhabiting different predator environments were found to reflect rapid and reversible plastic responses to the perceived presence of viperine snakes. In the presence of snake cues tadpoles typically developed longer tails with narrower tail fins and deeper tail muscles; these changes are believed to facilitate predator escape by enhancing burst speed. Tadpoles developed the predator morphology within weeks and reverted back to a non-predator morphology within a similar time period. Tadpoles also responded behaviourally and morphologically to the presence of the green frog *R. perezi*, indicating intense selection pressure from both snakes and frogs since their introduction onto Mallorca some 2000 years ago.

One hypothesis proposed in Chapters 4 and 5 was that inducible defences to introduced predators may represent an adaptation of inducible defences already exhibited towards native invertebrate predators. In order to test this theory, behavioural and morphological responses of tadpoles to a native invertebrate predator, the aquatic nymph of *Anax imperator,* were tested (Chapter 7). While numerous studies have documented behavioural (Petranka *et al.*, 1987; Kats *et al.*, 1988; Lawler, 1989; Skelly & Werner, 1990; Lefcort, 1998) and morphological (Smith & Van Buskirk, 1995; McCollum & Van Buskirk, 1996; McCollum & Leimberger, 1997) responses of anuran larvae towards invertebrate predators, a controlled experiment found that *A. muletensis* tadpoles did not respond behaviourally or morphologically towards the predatory odonate. These findings have important implications for the mechanisms underlying evolution of inducible defences towards introduced predators. In the absence of responses to native invertebrate predators, the theory proposed above for the evolution of defences towards introduced predators is refuted and an alternative explanation must be sought. It is possible that phenotypic plasticity evolved in this species to cope with the highly variable environmental conditions experienced by tadpoles. Adult toads live and breed in torrents that typically flow during the winter but dry up during the spring leaving behind standing pools of water. Tadpole overwintering in torrent pools will therefore be exposed to extreme variations in water flow rate throughout the course of the year. Tadpoles of some species vary their morphology according to whether they are stream- or pond-dwelling (Jennings & Scott, 1993) and morphological plasticity such as this may be an adaptive strategy for Mallorcan midwife toad tadpoles. Thus, the genetic machinery to be plastic may have evolved in response to changes in environmental conditions, and such plastic responses could have facilitated the evolution of morphological responses to novel predators. Further research into morphological variation according to changes in water flow rate may provide vital insight into the conditions necessary for the evolution and maintenance of phenotypic plasticity in this species.

9.3.1 Behavioural Responses

The adaptiveness of phentotypic plasticity as an antipredatory strategy depends largely upon response and recovery times relative to temporal variation in predation threat (Thompson, 1991; Padilla & Adolph, 1996). The influence of prior experience on the nature and magnitude of responses is also important in dictating the strength of responses exhibited across different environments (Magurran, 1990; Semlitsch & Reyer, 1992). In Chapter 6, experiments conducted under controlled and semi-natural conditions revealed that behavioural responses of *A. muletensis* tadpoles towards snakes are rapid (within an hour of the addition of snakes to the water) and reversible (activity returned to former levels one day following the removal of snakes from a plunge pool). Prolonged exposure to cues (30 days) resulted in a reduction in baseline activity levels even after the threat of predation had been removed. These results suggest that, while tadpoles are capable of responding rapidly to predator presence, in the face of sustained and frequent predator pressure baseline activity levels may be more important in reducing predation risk than short-term responses. Predator-naïve tadpoles responded at least as strongly to the presence of predatory cues as those with prior exposure, confirming that responses are innate rather than learned.

9.3.2 Costs of Responding to Predators

An implicit assumption of inducible defences is that they are beneficial in the presence of predators but carry a fitness cost in the absence of predators, since if such costs did not exist then the defences would be fixed. While the benefits associated with inducible defences are intuitive, in that they increase an individual's chances of survival in the presence of a predator (Van Buskirk *et al.*, 1997), the costs are often harder to quantify. Fitness costs associated with antipredatory responses in *A. muletensis* larvae were tested by monitoring the feeding rate, growth, development and morphology in wild-caught and captive-bred tadpoles in water that was either conditioned with snake cues or unconditioned (Chapter 8). Overall, tadpoles in snake-conditioned water spent significantly less time feeding than control individuals, resulting in a reduction in the growth and survival of wild-caught individuals in the perceived presence of a predator. Tadpoles did not respond morphologically to the presence of snake cues and it is thought that weekly exposure to cues was not sufficient to evoke a morphological response. Further research is required to determine costs associated with displaying a predator-induced morphology as well as maintaining the genetic machinery associated with such a response.

9.4 CAPTIVE BREEDING AND REINTRODUCTION PROGRAMME: FITNESS IMPLICATIONS

The recovery programme for the Mallorcan midwife toad involves the reintroduction of larval and post-metamorphic toads from captive-bred stock into natural, unoccupied, sites. Because captive breeding can generate fitness problems through inbreeding depression (Reinert, 1991; Caughley, 1994; Hedrick & Kalinowski, 2000; Marshall & Spalton, 2000) and artificial selection (Reinert, 1991; Lewis & Thomas, 2001), some workers have suggested that captive breeding programmes are not an effective conservation strategy for reptiles and amphibians (Dodd & Seigel, 1991). Ralls *et al.* (1979), for instance, showed that inbreeding lowered juvenile viability in populations of captive mammals and Rowe *et al.* (1999) found that larval growth rate in the natterjack toad, *Bufo calamita,* was strongly correlated with expected heterozygosity across microsatellite loci. In a risky aquatic environment such as that inhabited by larval populations of the Mallorcan midwife toad, the ability of individuals to detect and respond to predators is likely to be highly correlated with fitness. In a controlled experiment, tadpoles from a population which had been subject to approximately five generations of captive breeding responded less strongly behaviourally to the presence of snake cues than wild-caught individuals (Chapter 8). This indicates that a captive-breeding bottleneck has impaired fitness by diminishing behavioural antipredatory responses. Whereas in Chapter 5 similar behavioural and morphological antipredator responses were found in tadpoles from a reintroduced and a wild population, the reintroduced population had only been subject to approximately three generations of captive-breeding and had subsequently been established at a natural site for a further four generations. Either such a short period in captivity was not sufficient to produce inbreeding depression, or the population had recovered genetically since reintroduction (see Hansson *et al.*, 2000). The results of the experiment comparing captive-bred with wild-caught individuals suggest that five successive generations of captive breeding may be sufficient to significantly impair tadpole fitness through inbreeding depression or adaptation to an artificial environment. Genetic work is currently underway to relate behaviour and morphology to genotype and it is hoped that this will clarify the impact of captive breeding on individual fitness. A decision was taken by the recovery group not to release any more stock descended from the original founders collected in 1985-1987 due to concerns about potential inbreeding depression and three new bloodlines were established in

1997 for reintroduction purposes. The results of this study confirm that this is a sensible precaution.

9.5 CONCLUSIONS AND RECOMMENDATIONS

The optimal habitat for the Mallorcan midwife toad appears to be high elevation torrents with steep sides and containing many pools. While steep sides surrounding a torrent do not appear to reduce accessibility to predators, this feature may influence the microclimate within a gorge and favour the persistence of pools of water throughout the hot summer months. Increases in population size and number of larvae per pool with increasing elevation may reflect physiological adaptations of the toad to cool montane pools combined with reduced predation pressure at higher elevations. Consideration of these factors may optimise the design and location of future reintroductions.

Differences in larval population structure, morphology, behaviour, growth and development between sites with and without introduced predatory snakes and frogs are all indicative of intense predation pressure since the introduction of these species onto Mallorca some 2000 years ago. The absence of toad populations in seemingly suitable habitat within the Serra de Tramuntana mountain range may be attributed to extinction events caused by predation and the evolution of inducible behavioural and morphological defences may have facilitated the continued survival of the toad in gorges prone to continued incursions from predators. The presence of snakes and frogs still appears to have a significant impact on populations, however, and the recovery of the toad is likely to be facilitated by measures implemented by Fons Ferreret to control introduced snakes.

Fons Ferreret currently control snake numbers by regularly catching and removing individuals from sites occupied by the toad. Logistical constraints associated with trapping snakes make it very difficult to eradicate these predators entirely, however. The effectiveness of the current practice needs to be assessed; one way of doing this would be to intensively remove snakes from a site and monitor the number of new individuals that appear. Steep sides leading into a torrent do not appear to prohibit the entry of introduced predators, but may result in snakes that do enter being unable to leave, as was the case in one of the study pools. By removing snakes from such pools and then monitoring whether more individuals arrive, it may be possible to assess how frequently snake incursions occur and whether removing individuals on a regular basis significantly reduces predation pressure. As a means of optimising future reintroductions, Fons Ferreret have proposed creating 'snake-proof' cisterns; these may prove to be an effective way of reducing predation on tadpoles in areas occupied by snakes. Because snake populations appear to be supported by the presence of frogs, predation pressure on adult and larval toads may be significantly reduced by controlling frog numbers. Logistical constraints associated with trapping and killing frogs make it equally difficult to eradicate this species, however, although it may be beneficial to test the effectiveness of different strategies. While the creation of more pools within torrent gorges currently occupied by the toad is suggested as a way of enhancing the viability of existing populations, care must be taken to ensure that these pools do not simply provide further breeding grounds for the frog. It is suggested that the creation of 'predator-free' cisterns that are inaccessible to both snakes and frogs as proposed by Fons Ferreret would maximise the chances of a successful reintroduction.

A captive breeding and reintroduction programme has successfully established a number of new populations within the historical range of the toad. This study found that a captive-breeding bottleneck can impair individual fitness by reducing the ability of tadpoles to detect and respond to snakes. Problems associated with reintroducing inbred individuals into natural sites have been avoided, however, since the recovery group has already taken the decision to cease the reintroduction of individuals derived from the original founder stock. Populations do not appear to suffer any reduction in fitness following a small number of generations in captivity or, if they do, they recover quickly upon introduction into a natural site. The results of this study confirm that the establishment of three new bloodlines for reintroduction in 1997 was an appropriate measure to avoid problems associated with inbreeding depression and adaptation to an artificial environment.

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