The evolution of life histories in garter snakes: 
Reproduction, aging, and the physiology of trade-offs

by

Amanda Marie Sparkman

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Program of Study Committee:
Anne M. Bronikowski, Major Professor
W. Sue Fairbanks
Fredric J. Janzen
Jeanne M. Serb
Carol M. Vleck

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ABSTRACT

Life-history theory predicts that optimal life histories are shaped by trade-offs among traits expressed in different evolutionary and ecological contexts. Fast growth and high reproduction are predicted to trade off with lifespan, with the result that “fast-living” organisms will have shorter lives, while “slow-living” organisms will have longer lives. However, the generality of this theory for both determinately and indeterminately growing species, and the physiological mechanisms that underlie trade-offs, are poorly understood.

We tested life-history theories of aging in natural populations of fast- and slow-living ecotypes of the indeterminately growing garter snake *Thamnophis elegans*. Long-term data on age-specific reproduction in both ecotypes revealed that neither showed signs of reproductive senescence even at the latest ages. Instead, both ecotypes continued to increase reproduction with age/size throughout life, with the fast-living ecotype increasing reproduction at a greater rate. These findings suggest that fast-living does not come at a cost to reproductive performance later in life. However, as the fast-living ecotype is known to exhibit shorter adult median lifespan than the slow-living ecotype in the field—a phenomenon proceeding from either extrinsic or intrinsic sources of mortality—we also tested for potential physiological mechanisms for trade-offs. Specifically, we tested for differences in immune defense between the two ecotypes, according to the ecoimmunological hypothesis that suggests fast-living populations should invest more in innate immunity than slow-living populations. As predicted, the fast-living ecotype showed higher levels of constitutive innate immunity than the slow-living ecotype. We also tested for differences in endocrine function between the two ecotypes. We predicted that the fast-living ecotype would exhibit higher levels of plasma insulin-like growth factor-1 (IGF-1), as high IGF-1
signaling is known to stimulate growth and reproduction at a cost to lifespan in model organisms. We found ecotype differences with respect to gravidity, body size, and annual climate, indicating that fast-living snakes may experience cumulatively higher levels of IGF-1 than slow-living snakes over their lifetimes. Thus IGF-1 may be an important mediator of life-history trade-offs in natural populations. Finally, through sequencing IGF-1 mRNA in a variety of reptile species, we found evidence that IGF-1 has been duplicated several times in the reptile lineage, which may have important for the physiology of life-history traits. All of these findings are discussed with reference to their contribution to the study of life-history evolution in general, with emphasis on the unique insights to be gained from the study of indeterminately growing species.
INTRODUCTION

A brief history of life-history theory

Early origins

In his book *Generation of Animals*, Aristotle (384 - 322 BC) describes what would become one of the underlying principles of life-history theory in the 20th century: the existence of biological trade-offs. Observing differences in fecundity among different-sized birds, he writes “…what Nature takes away from one place, she puts in another….” In small birds, for example, the “material” which might increase their bulk is instead turned into “seminal residue” (or, in other words, reproduction). He rephrases this more formally in another section, stating that larger animals have fewer offspring as they use their nourishment for growth instead. In this way, he conceives a world in which resources are limited, and choices must be made regarding investment priorities.

Aristotle applies the principle of trade-offs not only to single reproductive events, but to lifetime fecundity, noting that what we now call semelparous species become exhausted by excessive “evacuation of residue,” which leads to sterility and, ultimately, death. Furthermore, he suggests that the phenomenon of reproductive senescence in lions is due to a decline in residue as it is used up and the “prime of life abates—” a brilliant foreshadowing of what is now considered a trade-off between current reproduction, and future reproduction and survival.

Other fundamental life-history patterns, such as a trade-off between offspring size and number, a negative correlation between developmental time and lifespan, and resource-based sex allocation also occurred to Aristotle. He observes that pipe-fish (seahorses) have large rather than numerous eggs—“here Nature has taken away from their number and added to their
size”—and that those animals that live longer take longer to form (though he distances himself from any causal connection between the two). As an early (and perhaps somewhat misogynistic), prelude to the study of sex allocation, he argues that males are what we would call more costly to produce, so that young and old parents—in whom “heat” is not yet perfected, or is beginning to fail—tend to produce females.

In addition to general observations regarding trade-offs among life-history traits, Aristotle demonstrates a keen awareness of the ecological context in which these traits are expressed. He observes that development occurs faster in sunny conditions, and that certain fish are prolific in numbers of eggs laid because of high juvenile mortality: “Nature makes good the destruction by sheer weight of numbers.” Thus, at a very early date, two of the three major principles of life-history theory had already been expounded: first, that trade-offs appear to exist between life-history traits; second, that these traits are malleable to ecological forces. The third major principle, the notion that life histories evolve, emerged more than two thousand years later, as theoreticians began to build Darwin’s (1859) theory of natural selection into the study of life histories.

*Life history & evolution*

Life-history traits are now defined as those organismal traits most closely linked to evolutionary fitness. Fitness may be defined in a number of ways, usually as a measure of reproductive success, survival, or a combination of both. In general, the most fit organism is the one that produces the greatest number of offspring that are themselves fully capable of producing viable offspring. Thus, the magnitude of reproductive output at any given reproductive event is a central life-history trait; but as growth rate, body size, age/size at maturation, number and size of offspring, sex ratios, and lifespan all interact to affect lifetime
reproductive success (and thus overall fitness), these also are considered fundamental life-history traits.

All else being equal, the most fit organism is one that grows the fastest to the most fecund size and continues to be fecund indefinitely at a rapid rate. Dubbed the “Darwinian demon” (Law 1979), this organism presumably has no intrinsic physiological trade-offs, lives in a world of unlimited resources, optimal temperatures and water availability, and no extrinsic sources of mortality. In other words, a world not our own. As differences in life-history strategies within and among taxa are everywhere evident in the natural world (from scallops to baboons to bighorn sheep to swallows to turtles; plants, fungi, and microorganisms not excepted), the major goal of life-history theory has been to describe why and how these differences occur.

**Optimality theory**

In the 1940s and 50s, David Lack formally introduced the idea of “optimality” to life-history theory to explain variation in life histories using principles of natural selection and the existence of trade-offs among traits. He hypothesized that birds lay exactly the number of eggs that optimizes fitness—or, in other words, the number that will lead to the highest number of successfully-fledged young. Natural selection will act to optimize the number of eggs laid in the context of a trade-off with offspring survival: if too many eggs are laid, all offspring may be of poorer quality, and fewer will live to fledge (Lack 1954). This optimization hypothesis for reproductive output has since garnered considerable (though not universal) empirical support, though with some modifications. For instance, experimental manipulation of kestrel clutch sizes did not negatively affect number of offspring fledged, or number of grand-offspring; however, catering to the demands of an increased clutch size did
negatively impact parent survival, with ramifications for lifetime reproductive success (Dijkstra et al. 1990).

Optimality theory has been the most influential interpretive framework for the field of life-history evolution in this century (Roff 1992; Stearns 1992). In general, it proposes that natural selection (given underlying additive genetic variation for life-history traits), will select for the optimal combination of life-history traits, in the context of intrinsic (physiological) trade-offs and extrinsic (ecological) impacts on survival and reproduction. Optimality theory was taken one step further by Lamont Cole (1954), who discussed life-history strategies as optimal schedules of age-specific reproduction and survival that maximize \( r \), the intrinsic rate of increase in populations. Thus, the study of life histories has come to rely heavily on life tables and demographic equations, studies of both genetic and phenotypic correlations among traits, and estimates of the costs of reproduction, to understand the shape of life histories, and how they evolve (Kirkwood & Rose 1991; Roff 1992; Stearns 1992; Charlesworth 1994).

Theories of aging

Following Lack and Cole, Peter Medawar and George Williams became two of the most influential life-history theorists of the 20th century. Unlike Lack, they did not begin with the costs of reproduction; instead, they began with the end of life, devising two general life-history theories centered on the evolution of aging, or senescence. Medawar (1952) brought the concept of changes in the mutation/selection balance with age to the table with the mutation accumulation (MA) theory of aging. He noted that survivorship will decline at later ages due to sources of extrinsic mortality. Since survivorship declines with age, selection will peak at maturity, when reproductive value—defined by current and expected
reproductive output over a lifetime—is greatest, and decline thereafter. As a consequence, late-acting deleterious mutations will accumulate, generating a senescent phenotype, characterized by degeneration in physiological function at later ages. Expanding on Medawar’s ideas, Williams’ (1957) antagonistic pleiotropy (AP) theory is based on the same basic premise as MA, but suggests that decreased survivorship with age due to extrinsic mortality may favor alleles that have fitness benefits early in life, but are deleterious later in life. In this case, aging evolves as a non-adaptive by-product of an adaptive (optimality-driven) process.

While both MA and AP theories may highlight important aspects of the evolution of aging (reviewed in Bronikowski & Promislow 2006; Hughes & Reynolds 2005), AP is most suited to understanding how life histories evolve. In particular, it provides a powerful interpretive framework for understanding mechanisms behind the expression of two of the most commonly observed combinations in the life-history continuum, across taxa: (1) fast growth, early age at maturity, high reproductive output, and short lifespan, versus (2) slow growth, late age at maturity, low reproductive output, and long lifespan. Williams predicts that the optimal life history will vary according to strength of extrinsic mortality in a given environment. Thus life-history strategy (1) is predicted to evolve in the context of high extrinsic mortality, while strategy (2) is predicted to evolve in low extrinsic mortality.

A major contribution of selection-based theories of life-history, made particularly clear by the AP theory of aging, is the implication that trade-offs need not always be mediated by availability of resources. In other words, the currency of life-history trade-offs has changed from energy, or nutrients, or time, to a currency of fitness (Stearns 1992). This is a reframing of ancient ideas that is truly new, and opens up important avenues of research, including the
study of how trade-offs themselves might evolve (Roff & Fairbairn 2007), and how pleiotropic components of physiology may direct the evolution of life histories into certain trajectories (Finch & Rose 1995; Ketterson & Nolan 1992; Zera & Bottsford 2001; Ricklefs & Wikelski 2002).

“Growth” of the AP theory: Indeterminate growth and delayed/negligible senescence

There has been empirical support for the AP theory in diverse systems—experimental evolution in flies and long-term studies of long-lived birds have clearly demonstrated trade-offs between early investment in reproductive traits and faster senescence (Stearns et al. 2000; Charmantier et al. 2006; Reed et al. 2008), and studies in opossums, grasshoppers, flies, social insects and herpetofauna have demonstrated the predicted relationship between lifespan and extrinsic mortality (Austad 1993; Tatar et al. 1997; Stearns et al. 2000; Keller & Gonoud 1997; Blanco & Sherman 2005). However, the limits of focusing solely on the strength of extrinsic mortality, while ignoring the effects of population dynamics, individual condition or intergenerational transfers on the evolution of senescence, have been expounded in recent theoretical papers (e.g., Williams & Day 2003; Lee 2003; Williams et al. 2006).

The most influential empirical study to find Williams’ AP theory lacking involved a well-known model of experimental evolution, the Trinidadian guppy (Poecilia reticulata) (Reznick et al. 1990). In this study, guppies from high-predation environments actually evolved longer lifespans than those from low-predation environments, in spite of having faster growth rates, early age at maturity and higher fecundity (Reznick et al. 2004). Reznick et al. provide three possible explanations for this unexpected outcome, based on condition-dependence, density-dependence and, significantly, indeterminate growth. The third interpretation has important theoretical implications for understanding how life histories
evolve in a wide range of fish, reptile, amphibian and invertebrate species exhibiting indeterminate growth—defined as the propensity to continue growth past sexual maturity and throughout life. Indeterminate growth is generally accompanied by indeterminate reproduction (a phenomenon fostered by continued oocyte generation—a process which ceases early in the development of mammals and birds), and may powerfully affect the age-specific mutation/selection balance described by Medawar and Williams. Reznick et al. (2004) suggest that the higher rate of increase in fecundity with age in high-predation guppies may have offset differences in mortality rate, and resulted in smaller differences in senescence than predicted.

In his original paper, Williams’ (1957) central argument is based on the assumption that reproductive output does not greatly increase with age after maturity. However, he clearly acknowledged that the increasing fecundity that accompanies increasing size/age in indeterminately growing organisms could change the mutation/selection balance, causing the strength of selection to decline less rapidly after maturity than in determinately growing organisms. Expanding on this idea, Hamilton (1966) also discussed the effects of increases in age-specific fecundity, concluding that while it is impossible to escape senescence completely, it may be substantially delayed in indeterminate growers.

Reznick et al. (2002) provide a history of ideas regarding lifespan and indeterminate growth in fish, beginning with Bidder (1932), who noted the exceptional longevity of many fish species (some of which live to over a hundred years), and concluded that indeterminate growth must come with the benefit of continued repair and replacement of old cells throughout, thus off-setting senescence. In contrast, he theorized, the biomechanical constraints on body size in birds and mammals has resulted in determinate growth, and thus a
reduced ability to repair and replace cells and avoid senescence after maturity. These ideas were overturned in the 50s and 60s when Gerking (1957) showed that natural populations of fish often have increased mortality at older ages, and Comfort (1960, 1962) demonstrated that indeterminately-growing fish can show signs of physiological senescence. It was around this time, that Williams and Hamilton published their thoughts on the matter, which ought to have been sufficient to renew interest in the evolution of lifespan in indeterminately growing species. However, while indeterminate growers were given extensive attention in Finch’s expansive review of comparative senescence (1990), very little has been made of it until recently.

Currently, renewed interest has arisen not only in the phenomenon of delayed senescence, but also in what has been termed “negligible”, or even “negative” senescence—the apparent lack/undetectability of senescence at late ages, which may in some cases be associated with a decrease in mortality with age in indeterminately growing species (Finch 1998; Congdon et al. 2001; Miller 2001; Reznick et al. 2002; Vaupel et al. 2004; Baudisch 2005). Clearly, Reznick et al.’s intriguing findings contradicting extrinsic-mortality based predictions with guppies exhibiting delayed senescence must be replicated in other systems before we can come to generalizable conclusions. Furthermore, we have no knowledge, at present, of how extrinsic mortality affects the evolution of life histories in organisms that show negligible/negative senescence.

Unfortunately, we meet many of the same challenges here that beset studies of senescence in determinately growing species. It is often difficult, particularly with highly secretive species (such as snakes) to capture (and re-capture) a representative sample of all age classes, estimate ages for each individual, and determine whether lifespan in the field is a
function of intrinsic (senescence) or extrinsic (ecological) mortality. Nevertheless, there are ways in which these challenges can be met. First, we require long-term demographic studies that estimate rates of age-specific reproduction and mortality up to the oldest surviving age classes. Second, (particularly useful if senescence is not evident from demographic data), we must test for declines in performance and attenuation of physiological function (such as immune function, or hormone activity) with age (Bronikowski & Promislow 2005).

Employment of both of these strategies will lead to a fuller understanding of the nature of senescence, evaluating both its ubiquity (or lack thereof), and the ecological mechanisms shaping its evolution.

**The physiology of life-history trade-offs**

*Trade-offs with immunity*

While much work has been done to establish the existence/appearance of phenotypic life-history trade-offs under optimality hypotheses (Roff 1992; Stearns 1992), very little is yet known regarding the underlying genetic and physiological processes that shape them (Finch & Rose 1995; Ketterson & Nolan 1992; Zera & Bottsford 2001; Ricklefs & Wikelski 2002). One emerging area of research explores how the immune system is involved in trade-offs with life-history traits (Sheldon & Verhulst 1996; Norris & Evans 2000). Motivated by the principle that investment in immune function is costly (Lochmiller & Deerenberg 2000), many of these studies have successfully demonstrated trade-offs between immune function and current reproduction. A negative correlation between brood size and different components of immunity has been shown, for instance, in collared flycatchers, zebra finches and barn swallows (e.g., Apanius *et al.* 1994; Saino 1997; Gustafsson 1998; Nordling 1998). Furthermore, increasing brood size has also been correlated with increase in parasite load.
(e.g., Norris 1994, Richner 1995; Nordling 1998), and more recent studies in long-lived birds have also demonstrated that immune defense can come at a cost to future reproduction and survival (Hanssen et al. 2003, 2005; Hanssen 2006).

While the immune system can be highly plastic, and some trade-offs among traits may occur primarily on a phenotypic level, negative genetic correlations have also been shown to exist between reproductive traits and immune defense in selected lines of insects (Simmons & Roberts 2005; McKean et al. 2008). A genetic basic for variation in immune function is further supported in domestic fowl, which can be selected for increased antibody production (Siegel & Gross 1980). Norris and Evans (2000) outline three additional principles necessary to demonstrate an evolutionary trade-off between life-history traits and immune defense: (1) they must compete for limited resources, (2) increased investment in one life-history trait should result in a decreased immunocompetence, and (3) decreased immunocompetence must lead to a decrease in fitness. They also recognized that immunocompetence, defined as the ability to effectively combat disease, is not a monolithic trait, and that resource limitation may have different effects on different components of the immune system. According to the immune-defense-component model (IDCM), immune defense may be defined by two main binaries: induced (in response to an immunological challenge) or constitutive (appears at all times), non-specific (innate) or specific (acquired) (Schmid-Hempel & Ebert 2003).

Recently there have been attempts to link immune defense to fitness from another angle, seeking evidence of optimization of immune function in species/populations that have evolved different paces of life. Building on the premises that different immune components may respond differently to selection, and some may be more costly than others, Lee (2006) has proposed that there will be differential investment in constitutive innate versus induced
acquired immunity in fast-living (fast growth, early maturation, high reproductive output, short lifespan) versus slow-living (slow growth, late maturation, low reproductive output, long lifespan) organisms. Fast-living organisms, which are less likely to be repeatedly targeted by the same pathogens, are predicted to invest more in constitutive, innate defenses. These defenses are thought to be developmentally inexpensive, thus freeing resources for growth. Slow-living organisms, on the other hand, which may be repeatedly targeted by the same pathogens, are predicted to invest more in induced, acquired defenses, which are thought to be more expensive to develop, but may provide more efficient protection over a long life. Only a few studies have yet explicitly tested these predictions, with mixed results (reviewed in Chapter 2). However, the disparity in methodology (e.g., different measures of immune defense) and comparative context (e.g., intraspecific vs. interspecific) represented by these studies suggest that we need more, comparable studies of a range of taxa before we can come to any concrete conclusions.

According to the ecoimmunological pace-of-life hypothesis, while extrinsic mortality may be the selective force for fast-growth/high fecundity, high investment in these traits is not the only potential contributor to a trade-off with lifespan. Instead, the trade-off with lifespan is a three-point triangle, with fast-growth/high fecundity trading off with immune function, and decreased immune function resulting in a decrease in survivorship. As this (at least as far as it concerns immune function) is a resource allocation-based (rather than pleiotropic) optimality hypothesis, without assumptions regarding age-specific strength of selection, it may apply equally to both determinately and indeterminately growing species. However, as the ecoimmunology of ectotherms is only beginning to be explored, and we know very little regarding the development and function of their immune systems, the pace-
of-life hypothesis needs to be tested not only in birds and mammals, as has been the case thus far, but also in fish, amphibians, and reptiles.

Endocrine-mediated trade-offs

In the search for physiological mechanisms underlying life-history evolution, the endocrine system has also become a focal point of new research. The endocrine system constitutes a rich source of potential mechanisms for antagonistic pleiotropy in the evolution of life-history trade-offs including, but not restricted to, trade-offs involving lifespan. Its potential as a chief player in the evolution of life-history trade-offs is supported by three general characteristics: (1) Individual hormones may exert widespread pleiotropic effects. Each hormone may bind to multiple target tissues, and initiate multiple signal transduction pathways and changes in gene transcription within each tissue. Thus, adjustments in the regulation of a single hormone and or/its receptors and binding proteins have the potential to either constrain or facilitate the evolution of correlated traits (Ketterson & Nolan 1992, 1999; Finch & Rose 1995; Zera 2001). (2) Selection experiments indicate that hormone levels can have a quantitative genetic basis. For example, chickens selected for low juvenile body weight exhibit lower titers of insulin-like growth factors-1 and -2 at early ages than those selected for high juvenile body weight (Scanes et al. 1989). Thus selection on hormonally-mediated traits can create sustained cross-generational change in hormonal expression. (3) The endocrine system can be plastic in response to external stimuli, such as temperature or food availability, and is thus continually mediating adaptive responses to the environment.

A number of recent studies suggest that looking at diverse hormones such as follicle-stimulating hormone (FSH), corticosterone and testosterone to understand the mechanisms underlying life-history trade-offs is a fertile approach (e.g., Clark et al. 1997; Sinervo &
Licht 1991; Wingfield 1998; Lancaster et al. 2007; Mills et al. 2008). Most notable have been investigations demonstrating the role of juvenile hormone in a trade-off between reproduction (ovary mass) and dispersal ability (wing size) in the field cricket (*Gryllus firmus*) (Zera 1999; Zera et al. 2007), and the role of testosterone in trade-offs among parental care, sexual displays, and condition in juncos (*Junco hyemalis*) (Ketterson et al. 1999; Reed et al. 2006; McGlothlin et al. 2007).

Recent studies in insects have also uncovered a trade-off between growth/reproduction and lifespan mediated by juvenile hormone (Tater & Yin 2001; Flatt & Kawecki 2007). In vertebrates, insulin-like growth-factor-1 (IGF-1), which has been a rising star in aging research over the past decade, is also a prime candidate in this regard. IGF-1, which is a peptide hormone secreted from the liver in response to stimulation by growth hormone, exemplifies each of the three general characteristics of the endocrine system listed above: (1) IGF-1 has well-established pleiotropic effects, and is highly involved in growth, egg formation, and lifespan (reviewed in Tatar et al. 2003; Bartke 2005, 2008). (2) Selection experiments in domestic species show that levels of IGF-1 have a quantitative genetic basis and that lines selected for fast growth exhibit higher levels (e.g., Scanes et al. 1989; Beccavin et al. 2001), (3) IGF-1 responds plastically to environmental factors such as nutrition, temperature, and stress (e.g., Crain et al. 1995b; Davis & Peterson 2006; Shimizu et al. 2006).

Our understanding of IGF-1 function has been fostered by its pedigree as a member of the insulin/insulin-like family of signaling molecules that have been highly conserved across invertebrates and vertebrates. Insulin/IGF signaling (IIS) was first shown to be an integral regulator of lifespan in *Caenorhabditis elegans*, where mutations decreasing the activity of
the DAF-2 (insulin-like) receptor extended lifespan of worms by over 100% (Kenyon et al. 1993; Kimura 1997). Similarly, mutations of the insulin-like receptor in *Drosophila melanogaster* increased female lifespan by as much as 85% (Clancy et al. 2001). In mammals, a landmark study in heterozygous IGF-1R knockout mice demonstrated that a reduction in IGF-1 signaling increased female lifespan by 26% (Holzenberger et al. 2003). The life-extending effects of reduced IGF-1 signaling are thought to be mediated by increased activity of a downstream forkhead transcription factor (DAF-16/FOXO) that is a key regulator of cellular stress resistance (reviewed in Tater et al. 2003). The oxidative damage theory of aging postulates that lifespan is determined by a combination of rates of reaction oxygen species (ROS) production (which inflicts damage on cellular protein, lipids, and nucleic acids), antioxidant capacity, and repair mechanisms (reviewed in Monaghan et al. 2009). Thus, IIS constitutes an endocrine mechanism for understanding rates of aging via modulation of oxidative stress resistance. As IGF-1 is also well known for its role in stimulating growth and reproduction across taxa, in addition to reducing lifespan, it is well posed to provide insight into the physiology of trade-offs among these traits in wild populations.

Unfortunately, while much work on IGF-1 has been done in commercially important species raised in controlled conditions, little is known about the ecology of IGF-1 activity or its relation to the expression of life-history traits in the wild (but see Crain et al. 1995a, 1995b; Guillette et al. 1996; Webster et al. 1996; Schmidt & Kelley 2001). Furthermore, very little information is available regarding IGF-1 in reptiles—and of the three IGF-1 studies that have been conducted in reptiles, none have been on squamates. Thus there is need for baseline information on season, sex, and age-variation in IGF-1 in the wild, and its
relation to lifetime patterns in life-history (particularly indeterminate growth) in fish, reptiles, and amphibians. Furthermore, the basic structure of IGF-1 and its interactions with binding proteins, receptors, and downstream signaling pathways have yet to be examined in any reptile.

In the preceding sections I have outlined the need for three major (but not exclusive) avenues of investigation that will further our understanding of the mechanisms (ecological and physiological) behind the evolution of life history in wild populations. First, to document the presence/absence of senescence (demographic, performance, physiologically-based) in indeterminately growing species and evaluate the role of extrinsic mortality in shaping life-history trajectories. Second, to test for a role of the endocrine and immune systems in generating trade-offs among traits, should they exist. Third, to increase our basic understanding of endocrine and immune function in indeterminately growing species, particularly reptiles. In the following section, I describe the system of garter snakes in which I address aspects of all three of these objectives in the four chapters that constitute this dissertation.

**Model system: Eagle Lake garter snakes**

An ideal system in which to examine outstanding questions in the field of life-history evolution exists in multiple populations of the western terrestrial garter snake, *Thamnophis elegans*, residing in the vicinity of Eagle Lake, California, in the northeastern range of the Sierra Nevada mountains. These populations, which have been the subject of study for over 30 years, constitute two distinct life-history ecotypes, one fast-living and one slow-living (Bronikowski & Arnold 1999). The fast-living ecotype lives along the shore of Eagle Lake,
and exhibits fast growth to large body sizes, high annual reproduction, and low annual adult survivorship. In contrast, populations in montane meadow habitats located within several kilometers of the lake exhibit slow growth to smaller body sizes, low annual reproduction, and high annual adult survivorship. Evidence from neonates raised in a common garden environment suggests a genetic component to growth rate differences between the two ecotypes (Bronikowski 2000). In addition to life-history differences, these ecotypes have significant heritable differences in scalation, vertebral number, and coloration (Manier et al. 2007).

An in-depth population genetic analysis of all major *T. elegans* populations in the vicinity suggests that a single meadow population, Papoose meadow, is the ancestral source population for both meadow and lakeshore populations (Manier & Arnold 2005). A number of general ecological differences between lakeshore and meadow habitats exist that may help explain why lakeshore snakes experienced a change in life history traits upon migration to the lakeshore habitat (Bronikowski & Arnold 1999). One key difference is resource availability. The primary prey of lakeshore populations, the speckled dace (*Rhinichthys osculus*), is consistently available in Eagle Lake itself. For meadow populations, the primary prey are amphibians—the Pacific tree frog, *Hyla regilla*, and the western toad, *Bufo boreas* (though leeches (*Erpobdela punctata*), and, when present, speckled dace are also consumed). Long-term data suggests that populations of amphibian prey in the meadow are subject to dramatic fluctuation, with optimal conditions for breeding being present only in 50% of years (Bronikowski & Arnold 1999). This fluctuation appears to be largely driven by climatic variables: in wet years, when winter snow pack is deep and there is abundant spring precipitation, amphibians in meadow populations have plenty of water available to breed and
thrive during the summer months. However, in dry years, amphibian breeding declines, and meadow snakes experience a dearth of prey; meanwhile, down at the lakeshore where standing water is always available, snakes do not appear to be affected.

Why slow-growing meadow snakes, who are likely to experience the highest resource-based mortality, should exhibit higher adult survivorship is an intriguing question. It is uncertain whether the disparity in adult survivorship between lakeshore and meadow snakes is due to differences in rates of aging, or differences in predation/parasitism between the two habitats. According to Williams’ (1957) AP theory, lakeshore snakes are predicted to have evolved faster senescence, if higher levels of extrinsic mortality in the lakeshore habitat were a major selective force for evolution of fast growth. The heritable differentiation in coloration between the two ecotypes indicate that avian predation has indeed been a powerful selective force in this system (Manier et al. 2007). Furthermore, high rates of juvenile mortality in lakeshore snakes (clearly unrelated to possible aging differences between the two ecotypes), suggest that predation pressure is higher in lakeshore than in meadow habitats (Bronikowski & Arnold 1999, unpublished data). To test Williams’ hypothesis it remains, therefore, to determine whether lakeshore snakes do indeed exhibit faster rates of senescence than meadow snakes and, if not, how indeterminate growth/reproduction may have influenced their evolution.

The Eagle Lake snakes are also amenable to investigations of the role of physiology in determining life-history trade-offs. While the exact nature of the relationship between growth/reproduction and lifespan in this system has yet to be established, it has the appearance of the trade-off between growth/reproduction and lifespan predicted by optimality theory. Evidence for evolution of immune defense and hormone expression in
lakeshore snakes that would facilitate fast growth, but may generate trade-offs with lifespan is thus of great interest. In addition, physiological measures in both ecotypes may also reveal senescent declines at later ages not evident from demographic studies. In the following chapter summaries, I describe four research projects encompassing demographic, physiological, and genetic aspects of life-history evolution in this system.

**Chapter summaries**

**Chapter 1**

**Goal:** To test for patterns of senescence in wild populations of garter snakes, and determine whether faster growth is linked to faster senescence, as predicted by Williams (1957).

*T.elegans* is an indeterminately growing species for which a senescent phenotype has not yet been confirmed. While a significant difference in adult survivorship between fast-growth lakeshore and slow-growth meadow ecotypes at Eagle Lake has been clearly documented (Bronikowski & Arnold 2000), whether this difference is due to the evolution of different rates of aging, or primarily to contemporary differences in rates of extrinsic mortality, is currently unknown. Another approach to documenting senescence is to test for the signature of reproductive senescence, which may be defined as a decline in reproductive success with advancing age. Due to the difficulty of acquiring reliable age estimates of individuals, studies of age-specific reproduction in wild populations are rare, particularly in long-lived reptiles. In the longest-term study of its kind in snakes, we employ 20 years of cross-sectional data on age-specific reproduction, to test for evidence of reproductive senescence in *T.elegans*, with the prediction that (should senescence occur) the fast-growth
lakeshore ecotype will show faster/earlier onset of senescence than the slow-growth meadow ecotype.

Chapter 2
Goal: To test ecoimmunological life-history theories by determining whether investment in innate immunity covaries with pace-of-life in garter snakes.

Ecoimmunological theory has predicted that fast-living populations will invest more in innate, non-specific immune defenses, which are thought to be developmentally inexpensive (leaving resources free for growth), and provide a general, efficient response to a diversity of pathogens (Lee 2006). Slow-living populations, on the other hand, which may experience repeated exposure to the same pathogens over their longer lifespans, should invest more in specific, acquired defenses—more costly to develop, but relatively inexpensive to maintain. By employing three field ecoimmunological techniques (originally designed for the study of wild birds) to measure innate immunity, we tested the first part of the hypothesis in replicate populations of the two T.elegans ecotypes over two years. We also used lymphocyte abundance as a measure of acquired immunity to test the second side of the hypothesis. This constitutes one of only a few studies to explicitly test life-history theories of ecoimmunology, and the first to be conducted in ectotherms. (Note that efforts to test the hypothesis regarding investment in other measures of acquired immunity are described in the Conclusion.)

Chapter 3
Goal: To test the prediction that hormones may mediate life-history trade-offs between growth/reproduction and survival.
We predicted that pleiotropic activity of IGF-1 can provide a mechanism for the evolution of correlated life-history traits in fast-growth and slow-growth ecotypes of *T. elegans*. Since IGF-1 stimulates growth and reproduction, we predicted that fast-growth lakeshore snakes would show higher levels of IGF-1 than slow-growth meadow snakes. Furthermore, since IGF-1 may have a negative impact on lifespan, we suggested that higher levels in lakeshore snakes could be partially responsible for their reduced lifespan relative to meadow snakes. This is the first study to examine IGF-1 in any squamate reptile, and the first to test for a role of IGF-1 in the evolution of life histories in any wild vertebrate. As IGF-1 function has been highly conserved over evolutionary time, and plays pivotal roles in the physiology of both vertebrates and invertebrates, this study has relevance across taxa with regard to the physiology of life histories.

*Chapter 4*

**Goal:** To obtain sequence information for IGF-1 in reptile species, and determine whether reptiles show conservation in structure consistent with that seen in other taxa.

A molecular understanding of IGF-1 is a required for studies of its involvement in life-history traits in reptiles. The IGF-1 system, complete with binding proteins, receptors, and downstream signaling pathways, has been well-characterized in model, domestic, and commercial species, and appears to be largely conserved across taxa. However, information regarding IGF-1 structure and function in reptiles is decidedly lacking. While IGF-1 sequences are available in a variety of mammals, fish, birds, and a single amphibian, no sequences for any reptile species are available. We have evidence that suggests snakes, at least, may differ significantly in IGF-1 coding sequences: though all other mammalian, avian, and fish studies have shown binding of their native IGF-1 to an anti-human antibody
in a radioimmunoassay, only eight species of snake out of thirteen showed such binding. To investigate this further, we sequenced IGF-1 liver mRNA in fifteen species of reptiles, including turtles, snakes, and lizards, and evaluated the ramifications of our findings for reptile physiology.

Appendix

Originally, we intended to test for a role of IGF-1 in life-history evolution in *T. elegans* not only in natural conditions, but also using neonates raised in a common environment. While the first goal was successfully carried out, the second met with crippling experimental difficulties, leading to inconclusive results. This effort is described as a supplement to other IGF-1 chapters, as it may provide useful methodological information for future studies in this area.

Literature Cited


Sciences of the United States of America, 97, 3309-3313.
CHAPTER 1. AN EMPIRICAL TEST OF EVOLUTIONARY THEORIES FOR REPRODUCTIVE SENECE AND REPRODUCTIVE EFFORT IN THE GARTER SNAKE \textit{THAMNOPHIS ELEGANS}

A paper published in \textit{Proceedings of the Royal Society B: Biological Sciences}\textsuperscript{1}

Amanda M. Sparkman\textsuperscript{2}, Stevan J. Arnold\textsuperscript{3} & Anne M. Bronikowski\textsuperscript{4}

SUMMARY

Evolutionary theory predicts that differential reproductive effort and rate of reproductive senescence will evolve under different rates of external mortality. We examine the evolutionary divergence of age-specific reproduction in two life-history ecotypes of the western terrestrial garter snake, \textit{Thamnophis elegans}. We test for the signature of reproductive senescence (decreasing fecundity with age) and increasing reproductive effort with age (increasing reproductive productivity per gram female) in replicate populations of two life-history ecotypes: snakes that grow fast, mature young, and have shorter lifespans and snakes that grow slow, mature late, and have long lives. The difference between life-history ecotypes is due to genetic divergence in growth rate. We find (i) reproductive success (live litter mass) increases with age in both ecotypes, but does so more rapidly in the fast-growth ecotype; (ii) reproductive failure increases with age in both ecotypes, but the proportion of reproductive failure to total reproductive output remains invariant with age; and

\textsuperscript{1} Reprinted with permission from \textit{Proc. R. Soc. B} (2007) 274: 943-950
\textsuperscript{2}Primary author
\textsuperscript{3}Professor, Department of Zoology, Oregon State University
\textsuperscript{4}Corresponding author and Assistant Professor, Department of Ecology, Evolution and Organismal Biology, Iowa State University
(iii) reproductive effort remains constant in fast-growth individuals with age, but declines with age in slow-growth individuals. This illustration of increasing fecundity with age, even at the latest ages, deviates from standard expectations for reproductive senescence, as does the lack of increases in reproductive effort with age. We discuss our findings in light of recent theories regarding the phenomenon of increased reproduction with age in organisms with indeterminate growth, and its potential to offset theoretical expectations for the ubiquity of senescence.

**Keywords:** Reproductive effort, Garter snake, Life history, Senescence

**INTRODUCTION**

Classical evolutionary theories of senescence predict that physiological deterioration with age results from the declining power of natural selection at late ages (Medawar 1952, Williams 1957). The age-specific trajectory of mutation/selection balance for any given group depends on the relative proportions of old and young adults and on the pattern of reproduction (Hamilton 1966). Thus changes in extrinsic forces of mortality that selectively increase the value of older individuals should select for delayed senescence. However, more recent theoretical investigations have suggested that the case of a near absence of senescence, or negligible senescence, can result if older individuals have high reproduction and a high probability of survival that completely offsets their declining numbers (Finch 1998, Gasser *et al.* 2000, Vaupel *et al.* 2004, reviewed in Williams *et al.* 2006).

Although many studies have documented actuarial senescence in wild populations (e.g., Bronikowski *et al.* 2002, McDonald *et al.* 1996, Bonduriansky & Brassil 2002), tests for the persistence and evolution of reproductive senescence in nature are relatively rare (but see Packer *et al.* 1998, Bérubé *et al.* 1999, Holmes *et al.* 2003). Such studies in mammals and
birds have revealed convincing evidence for declining reproduction at late ages and, in some cases, a substantive post-reproductive lifespan. In contrast, ectotherms, and reptiles in particular, remain enigmatic with regard to evolutionary theories of senescence. Specifically, several studies suggest that some ectotherms may not show any signs of reproductive decline, and even where senescence is evident, it may not exhibit the predicted response to extrinsic mortality (e.g., Congdon et al 2001, Miller 2001, Reznick et al 2002, Congdon et al 2003, Reznick et al 2004). Senescence has, however, been documented in some fish, and the lack of clear illustrations of senescence in reptiles may be due in part to the difficulties of studying secretive species, such as snakes, and obtaining long-term data on long-lived organisms. Alternatively, it has been suggested that the apparent absence of a decline in reproduction with age may be a consequence of the indeterminate growth exhibited by many of these species, a trait that may delay (or prevent) the onset of reproductive senescence and even facilitate an increase in fecundity throughout life (Finch 1998, Reznick et al 2002, Vaupel et al 2004).

Here we present intraspecific data on age-specific reproduction for five populations of the garter snake *Thamnophis elegans* that reside in close geographical proximity. These populations are derived from the same ancestral source population, but have differentiated into two distinct, genetically diverged ecotypes that differ in scalation, coloration, and life history (Bronikowski & Arnold 1999, 2001; Manier & Arnold 2005; Manier et al. 2006). These two ecotypes provide an ideal model for the microevolution of life history. One ecotype, which occupies lakeshore habitats, exhibits fast growth, early maturation, large adult body size, high annual reproduction, and low annual survival. In contrast, the second ecotype, which occupies mountain meadow habitats, exhibits slow growth, late maturation,
small adult body size, low annual reproduction, and high annual survival (Bronikowski & Arnold 1999). A common garden experiment has shown that the ecotypic difference in growth has a genetic basis (Bronikowski 2000). Furthermore, the respective lifespans of the two ecotypes reflect these fast/slow life-history strategies, with fast-growth lakeshore snakes maintaining a shorter median lifespan than slow-growth meadow snakes, both in the field (2 years and 5 years, respectively) and in the laboratory (4 years and 8 years, respectively) (Bronikowski and Arnold, unpublished data). This article provides a detailed ecotypic comparison of how reproductive traits covary with age. We explore this comparison using theory for the evolution of life history and senescence.

Classical theory (e.g., Williams 1957) predicts that individuals of the long-lived, slow-growth (meadow) genotype should exhibit concomitantly slow reproductive decline, while short-lived, fast-growth (lakeshore) individuals should exhibit a more rapid decline in reproductive function. We test whether differences in age-specific reproductive trajectories conform to these theoretical expectations using approximately 250 reproductive *T. elegans* individual females collected between the years of 1975 and 1994. We anticipate that lakeshore populations will exhibit an increase in reproduction at early ages at a faster rate than meadow populations, in association with faster growth rates. For the dynamics of age-specific reproduction at later ages we propose two alternate hypotheses to a null model of no senescence: (H1) Reproductive success will decline in populations of both ecotypes at later ages, but will decline more rapidly in lakeshore snakes, indicative of a faster rate of aging. (H2) Reproductive success will not decline in lakeshore snakes, due to a truncated lifespan, but decline will be evident in meadow snakes, which may live long enough to exhibit senescence. Finally, because reproductive effort includes both live and non-live births, we
examine the incidence of reproductive failure, characterized by delivery of yolks and/or stillborns, in addition to testing for declines in reproductive success, because increasing dead litter mass with age could also be indicative of reproductive senescence.

MATERIALS AND METHODS

Study organisms & data collection

Our study populations of *T. elegans* reside in the vicinity of Eagle Lake in Lassen County, CA, at the northern end of the Sierra Nevada range. We collected snakes from two lakeshore habitats, which are characterized by continuous prey availability and warm temperatures, and three mountain meadow habitats, which exhibit highly variable prey availability, and cooler temperatures (Kephart 1982, Kephart & Arnold 1982, Bronikowski & Arnold 1999). Approximately 250 pregnant female snakes were collected from 1975 to 1994 as part of a large mark/recapture study. Within this larger study, all animals were collected by active searching (i.e., not only catching animals out foraging or basking, but also beneath retreat rocks, hidden at the base of vegetation, etc.). Each female contributed a single litter to this study; we excluded repeated measures within females. Thus this is a cross-sectional study of age-specific reproductive parameters. Pregnant females were maintained in captivity for the duration of pregnancy on thermal gradients that permitted thermoregulation (Arnold 1988). All neonates, stillborns, and yolks (undeveloped embryos) were counted, weighed and measured upon parturition. Total, live, and dead litter mass (defined as the combined weight of stillborns and undeveloped yolks) were calculated for each female, along with average individual live offspring mass. Female snout-vent length (SVL) and mass were measured postpartum. In addition to these pregnant females, we sampled 20 non-pregnant females
(randomly with respect to size/age) from each population to determine ages of non-reproducing female to compare to the age-distributions of pregnant females.

**Age determination**

Many dams were marked as neonates; these animals provided data on exact age. When exact age was unknown we used one of two methods to estimate age. Because the snakes in this region hibernate from October – April, we were able to determine age histologically using the technique of skeletochronology by counting annual growth rings (annuli) in a single vertebra from each animal (Waye & Gregory 1998). In cases where the annuli were indistinguishable (e.g., in animals that had been preserved many years ago), we used the close relationship between age and size within each ecotype to estimate age (Pearson correlation: Lakeshore: \( r =0.8482, \) Pr<0.0001, n=19; Meadow: \( r =0.9272, \) Pr=0.0009, n=8).

Von Bertalanffy growth equations were used to estimate age for these latter dams (equations described in Bronikowski & Arnold 1999). In cases in which age was known to be at least 15 years, dams were designated as 15+ years to increase sample sizes in this final age class.

**Statistical analyses**

**Age distributions.** Female age within the two ecotypes exhibited non-normal distributions, so we log\(_e\) transformed and tested for differences between the lakeshore and meadow ecotypes and between gravid and non-gravid females with analysis of variance.

**Reproductive success.** The dependent variables were single observations per female pooled across the 20 years of observation. Live litter mass was analyzed with analysis of covariance (ANCOVA) with the following linear model:
\[ Y = \mu + \text{Age} + \text{Ecotype} + \text{Population(Ecotype)} + \text{Age}\ast\text{Ecotype} + \text{Age}\ast\text{Population(Ecotype)} + \epsilon \]

where \( \mu \) is the population mean, \( \text{Age} \) is a covariate, \( \text{Ecotype} \) is the fixed effect of meadow versus lakeshore habitat, \( \text{Population nested within Ecotype} \) represents the effect of two lakeshore (L1 & L2) and three meadow (M1, M2, & M3) populations, and \( \epsilon \) is an error term (Proc GLM in SAS 9.1). Population nested within ecotype was treated as a fixed, rather than a random, effect to reflect complex microhabitat variation among populations. The interaction between maternal age and population nested within ecotype did not exhibit any significant effects, and was therefore removed from the model. (Note however, that the statistical power of the interaction of age with population was \( \beta < 0.80 \).) Because the results for total and live litter were in agreement, we discuss live litter mass hereafter. In addition to live litter mass, live-litter size, average size of live offspring, and Relative Clutch Mass (RCM, defined as the residuals of the regression of total litter mass on post-parturient maternal mass) were also analyzed using the same model. Parallel analyses were performed with SVL rather than age as the covariate to provide a comparison to other studies, but since the outcomes of both ANCOVAs supported the same conclusions, results are discussed primarily in terms of age (with the exception of RCM, for which both age and SVL are discussed).

**Reproductive failure.** Analyses of total dead litter mass and the number of stillborns were first performed using the model detailed above. Dead litter mass and number of stillborns yielded a significant relationship to maternal age, so in order to determine the relationship between these variables and changes in litter mass/size with age, we expressed
these parameter as a proportion of total litter mass/size for each individual. After arcsine transformation of this ratio, the data were analyzed using ANCOVA. Finally, the presence or absence of dead litter components (i.e., yolks and/or stillborns) was analyzed using logistic analysis.

Because the lakeshore sites had few old females, we ran all analyses (i.e., for both reproductive success and failure) with and without the six lakeshore females that were >10 years of age. The exclusion of these females did not significantly alter the results, or our conclusions regarding age-specific reproduction.

RESULTS

Age distributions
Ages of reproductive females ranged from 2 – 15 years within lakeshore populations (characterized by fast-growth individuals); non-gravid females ranged from 2 – 9 years of age. In meadow (slow-growth) populations, the age of reproductive females ranged from as 3 – 15 years of age; non-gravid females ranged in age from 4 – 13 years. Within ecotype, the mean age of gravid and non-gravid females was indistinguishable in lakeshore habitat (Gravid: 3.9 years; Non-gravid: 4.2 years, Pr = 0.5229). In meadows, the mean age of non-gravid females was less than that of gravid females (Gravid: 7.6 years; Non-gravid: 6.1 years, Pr = 0.0004). Thus for any given population, the pool of non-gravid females in any given year is on average the same ages or younger than the reproducing females, i.e., the non-gravid group of females is not composed of post-reproductive or otherwise reproductively senescent females.
Between ecotypes, the ages for both categories of females (gravid and non-gravid) differed significantly. Reproductive females averaged 3.9 years in lakeshore (fast-growth) sites, and 7.6 years in meadow (slow-growth) sites (Pr < 0.0001) (Figure 1). Most reproductive lakeshore snakes fell in the range 2-6 years, with a few individuals from one lakeshore population (L2) reaching the age of 15+ years. The age distribution of reproductive meadow females was significantly shifted towards older ages, with broad representation in the range 4-15+ years (Kolmogorov-Smirnov D=0.667, Pr<0.0001, n=249), with the maximum difference (D) occurring at age 4 (Figure 1).

Reproductive success

Age and source population significantly affected live litter mass and live litter size (number of live offspring) (Table 1). Live litter mass increased significantly with dam age in both ecotypes with those few fast-growth individuals in the 15+ category having the highest litter masses overall (Fig 2). L1 had significantly greater live litter mass than L2; both lakeshore sites had significantly greater live litter mass than the meadow sites, which were equivalent (ranking of live litter mass: L1>L2>M1=M2=M3). The significant interaction between age and ecotype (Table 1) reflects that populations of fast-growth individuals exhibited a much faster increase in reproductive success with age than populations of slow-growth individuals, in which only a slight increase is evident with age (Fast: slope=3.2 grams of offspring per yr, Pr<0.0001; Slow: slope=0.42 grams of offspring per yr, Pr=0.0137) (Fig 2). Like live litter mass, the dam age by ecotype interaction significantly affected litter size; litter size increased with age in fast-growth snakes but remained constant in slow-growth snakes (L: slope=0.89 offspring per yr, Pr<0.0001; M: slope=0.02 offspring per yr, Pr=0.8125).
Average offspring mass varied with age; older dams gave birth to larger offspring (Table 1, Fig 3). There was no interaction between age and ecotype, which indicates that average offspring mass increases in a similar fashion in fast- and slow-growth snakes (L: slope=0.05 g per yr, Pr=0.047; M: slope=0.07 g per yr, Pr<0.001).

RCM (gram offspring per gram dam) was significantly affected by the interaction between age and ecotype (Table 1). RCM remained constant with age (slope=0.37 g/g per yr, p=0.1465, n=141) in lakeshore females, but increased slightly with SVL (slope=0.03 g/g per mm, p=0.0267, n=141). In contrast, RCM showed a strong decrease with both age and SVL in meadow females (age: slope=-0.67g/g per yr, p=0.0011, n=89; SVL: slope=-0.05 g/g per mm, p<0.0004, n=89) (Fig 4).

Reproductive failure
Maternal age significantly affected dead litter mass and number of stillborns (Table 2). However, when these dependent variables were expressed as arcsine-transformed proportions of total reproductive output (total litter mass and total litter size, respectively), no effects were found to be significant (Table 2). Thus, although dead litter mass and number of stillborns increased with age for both ecotypes (Fig 5a), they remained constant proportions of total litter mass/litter size (Fig 5b). Similarly, there was no significant increase in the incidence of litters containing yolks or stillborns with age (Pr> 0.20).

DISCUSSION
In many organisms, reproductive success increases with age early in adulthood (Clutton-Brock 1984, Cameron et al. 2000 and references therein). This increase can be attributed both to morphological development and learning, which results in improvements in such skills as resource and mate acquisition, and parental care. Increasing body size
throughout life in organisms with indeterminate growth has been shown to be a particularly important factor in increasing reproductive success, and may be attributed to an increase in internal space for offspring development, as well as general improvement in body condition (reviewed in Roff 1992). In general, most studies to date have examined female reproductive success in squamates primarily as a function of body size. Only recently has reproductive success been examined in individuals of known age in wild populations. In these studies up to eight years of data on natural snake and lizard populations have been analyzed (e.g., Madsen & Shine 1992; Olsson & Shine 1996; Diller & Wallace 2002; Stanford & King 2004). However, because of lack of data on older animals, senescence has rarely been examined in squamates. Here, with 20 years of age-specific data, we test evolutionary hypotheses of reproductive senescence and reproductive effort.

Age-specific trends in fecundity and offspring size showed no evidence of senescence. We found that lakeshore (fast-growth) snakes began reproducing at earlier ages, and exhibited a greater increase in reproduction with age than meadow snakes. This lakeshore trajectory of increasing reproduction with advancing age neither plateaued, nor decreased in late age. In slow-growth meadow snakes the rate-of-change in reproduction differed from that of lakeshore females, but again did not decrease within the longest-lived age-classes. Furthermore, it is evident that older dams in both ecotypes produced larger offspring than their younger counterparts, while older lakeshore females also produced increasingly large litters. It is likely that these age-specific trends continue throughout the lifespan of these female snakes, as the oldest individuals are close to the maximum lifespan that we have observed in the field (18 years of age in both ecotypes).
Although dead litter mass and number of stillborns increased with age, they did not change in proportion to total litter mass/size. This result begs the as yet unanswerable question: why not invest more energy into converting this fixed proportion of reproductive effort from failure to success, rather than into producing more total eggs, which means more reproductive dead ends (yolks and stillborns). Nevertheless, our findings are consistent with previous reports in snakes and turtles (e.g., Luiselli et al 1996, Congdon et al 2003). Although an interesting phenomenon, these results provide no evidence for reproductive senescence via a disproportionate increase in reproductive failure with age. Thus, for these ectothermic vertebrates, we have found that at least one endothermic hallmark of aging, declining age-specific reproduction, is not only absent, but is opposite in predicted sign. We focus below on theories for the evolution of reproductive effort and senescence both with respect to local habitat differences and the larger question of the effects of indeterminate growth.

Life-history models predict that reproductive effort will increase with age as a function of expected remaining lifespan (reviewed in Roff 1992, Charlesworth 1994). One measure of reproductive effort, Relative Clutch Mass (RCM), did not change with age in fast-growth snakes, although it did exhibit slight increases with body size. This is contrary to what has been reported for a wide variety of other snakes. Intraspecific RCM tends not to increase with size in snakes (e.g., Pianka & Parker 1975, Seigel et al 1986, Madsen & Shine 1992), and is thought to be limited largely by abdominal volume (e.g., Vitt and Congdon 1978). In some cases, however, size-dependent increases in RCM have been reported, and in the viviparous smooth snake Coronella austriaca the slope is more steep than in lakeshore T. elegans (Luiselli et al 1996). Because larger RCMs are thought to be associated with a
variety of costly factors (e.g., decreased mobility, increased basking, high energetic costs) that may increase vulnerability to predators and other hazards, Luiselli et al speculate that larger *C. austriaca* females may be able to afford larger RCMs because 1) they may be large enough to deter predators by size alone or 2) they may have undergone an ontogenetic switch to larger prey and so may experience an enhanced energy intake. The first possibility may apply to our lakeshore populations. In these populations American Robins (*Turdus migratorius*) and Brewer’s blackbirds (*Euphagus cyanocephalus*) attack small snakes but cannot prey on adult *T. elegans*.

Interestingly, RCM in meadow snakes showed a strong trend in the opposite direction: they exhibited a marked decrease in RCM with age (and body size). In other words, older and larger snakes invested less in reproduction per gram body mass than younger, smaller snakes. Meadow habitats are characterized by larger avian predators such as sandhill cranes (*Grus canadensis*), red-tailed hawks (*Buteo jamaicensis*), and golden eagles (*Aquila chrysaetos*) on the one hand, and a lack of retreat rocks on the other. Thus, large gravid females may be at a greater risk than smaller females, which may be less conspicuous and more mobile. We are currently testing the hypothesis that predator suites have been a major evolutionary pressure for slow growth and small adult body size in meadow populations (as well as fast growth and large body size in lakeshore populations). In meadows, slower age-specific growth rates may represent a compromise between escape from predation (where small body size is beneficial) and increased reproduction (where large body size is beneficial).

Remarkably, lakeshore females increased reproductive success even at the latest ages. This suggests that the paucity of older reproductive females captured in lakeshore sites may
be a direct consequence of higher levels of extrinsic mortality rather than faster senescence, the latter being a predicted cost of fast growth and high reproduction. From the mark/recapture data, we have reported elsewhere that mortality for lakeshore juveniles and adults is twice that of meadow animals (Bronikowski & Arnold 1999 and unpublished data). That fast growth and greater reproductive success in lakeshore snakes relative to meadow snakes does not result in either shortened maximum lifespan (although median lifespan is smaller) or decreased late-age reproduction appears to contradict classical theory (e.g., disposable soma theory: Kirkwood 1977, antagonistic pleiotropy theory: Williams 1957). This is particularly striking because there is a genetic component to the ecotypic difference in growth (Bronikowski 2000).

The apparent contradiction may be a consequence of indeterminate growth. Reznick et al. (2002) suggest that many fish are long-lived because their capacity for indeterminate growth has allowed them to increase fecundity with age, long past maturity. This continuing rise in fecundity may result in increased selection against deleterious alleles that are expressed at older ages, resulting in the evolution of delayed, or negligible senescence (see Finch 1998). Theoretically, increasing age-specific fecundity could completely offset the decline in the strength of selection that naturally occurs because there are fewer older than younger individuals (Vaupel et al. 2004). Thus, lakeshore snakes may be an example of what might be termed “perfect indeterminate fecundity”, which completely offsets the trend for strength of selection to decrease with age. Other trends, however, indicate that the question may not be so easily resolved. Growth in meadow snakes after maturity slows at a more rapid rate than in lakeshore snakes (Bronikowski & Arnold 1999). Nevertheless, meadow snakes show no indications of reproductive senescence. Likewise, Congden et al. (2003)
have suggested that indeterminate growth cannot be the only factor involved in delaying senescence in turtles. They examined aging in painted turtles (*Chrysemys picta*), which exhibit indeterminate growth, and Blanding’s turtles (*Emydoidea blandingii*), which do not, and found no decline in reproductive success or survivorship with age in either species.

Evolutionary theories of senescence are based on the assumption that in a situation in which there are fewer old than young individuals, the conditions for the evolution of senescence are created. These theories are increasingly being challenged and refined. Ongoing generalizations of the theory include factors as diverse as inclusive fitness, immune function, and interactions between sources of mortality (Lee 2003, Williams & Day 2003, Bronikowski & Promislow 2005), as well as alternative theoretical perspectives on the effects of age-specific mutations (see Baudisch 2005). Despite the apparent absence of a trade-off between fast growth and age-specific reproductive success in our system, there may be costs associated with other components of fitness. For instance, laboratory guppies (*Poecilia reticulata*) derived from sites with high extrinsic mortality show earlier maturation and higher reproductive investment than their low extrinsic mortality counterparts. Nevertheless, strains from high mortality environments do not suffer the double cost of reduced survival and increased reproductive senescence. However, they do exhibit a more substantial decline in swimming performance than strains from low-mortality environments (Reznick *et al.* 2004). With regard to our system, it is possible that *T. elegans* in lakeshore environments generate a cost that we have not assessed. For example, we have not determined whether there is an ecotypic difference in investment in somatic maintenance. We are currently assessing oxidative damage and repair mechanisms in the two ecotypes. However, even if there were no differences in cellular damage and repair, ecological trade-
offs might make the fast-growth life-history strategy suboptimal in other settings. Thus, in meadow environments a slow-growth life history may be a bet-hedging response to factors such as unpredictable food resources (Bronikowski & Arnold 1999). Whatever the case, it is clear that there is still much to be learned regarding the relationship between age and reproduction in organisms exhibiting diverse patterns of growth.

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REFERENCES


TABLES & FIGURES

Table 1. Analysis of covariance of parameters of reproductive success. Asterisks denote significant effects.

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<tr>
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<td>(RCM)</td>
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Table 2. Analysis of covariance of two parameters of reproductive failure, dead litter mass and number of stillborns. And analysis of the same expressed as arcsine transformed proportion of total litter mass and proportion of total litter size, respectively. Significant effects ($P<0.05$) are denoted by.*

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FIGURE LEGENDS

**Figure 1.** Histogram of maternal ages. The distributions of pregnant females differed between the two ecotypes (see Results for details).

**Figure 2.** Total live litter mass as a function of maternal age. Total litter mass increased with age in both ecotypes, but at a faster rate in lakeshore populations.

**Figure 3.** Average live offspring mass as a function of maternal age. Offspring size increased with age at a similar rate in both lakeshore and meadow snakes.

**Figure 4.** Relative clutch mass (RCM) as a function of maternal age. RCM remained constant with age for lakeshore snakes, but declined in meadow snakes.

**Figure 5.** Dead litter mass as a function of maternal age. Dead litter mass increased with age in both ecotypes (A), but remained constant when expressed as an arcsine-transformed proportion of total litter mass (B).
Figure 1.
Figure 2.

- Lakeshore
- Meadow

Live Litter Mass (g)

Age (years)
Figure 3.

![Graph showing offspring mass (g) vs. age (years) for Lakeshore and Meadow habitats.](image)
Figure 4.

![Graph showing Relative Clutch Mass (RCM) vs Age (years) for Lakeshore and Meadow populations.](image-url)
Figure 5. A

- Lakeshore
- Meadow

Dead Litter Mass (g)

Dead Litter Mass/Tot. Litter Mass

B
CHAPTER 2. A TEST OF LIFE-HISTORY THEORIES OF IMMUNE DEFENSE IN TWO ECOTYPES OF THE GARTER SNAKE, *THAMNOPHIS ELEGANS*

A modification of a paper accepted for publication in *Journal of Animal Ecology*

Amanda Marie Sparkman & Maria Gabriela Palacios

SUMMARY

1. Life-history theorists have long observed that fast growth and high reproduction tend to be associated with short lifespan, suggesting that greater investment in such traits may trade off with self-maintenance. The immune system plays an integral role in self-maintenance by defending organisms from pathogens which may reduce survivorship.

2. Ecoimmunologists have predicted that fast-living organisms should rely more heavily on innate immunity than adaptive immunity as compared to slow-living organisms, because innate defenses are thought to be relatively inexpensive to develop and can provide a rapid, general response to pathogens.

3. We present the first study to examine this hypothesis in an ectothermic vertebrate, by testing for differences in three measures of constitutive innate immunity (bactericidal competence, natural antibodies, complement-mediated lysis) and one measure of acquired immunity (lymphocyte abundance) in replicate populations of two life-

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3 Collaborating researcher and graduate student, Department of Ecology, Evolution and Organismal Biology, Iowa State University
history ecotypes of the garter snake *Thamnophis elegans*, one fast-living and one slow-living.

4. As predicted, free-ranging snakes from the fast-living ecotype had higher levels of all three measures of constitutive innate immunity than the slow-living ecotype. These differences in immunity were not explained by parasite loads measured. However, no differences in lymphocyte abundance, a measure of acquired immunity, were present.

5. Both ecotypes exhibited a positive relationship between innate immunity and age/body size in the field prior to maturity, with fast-living snakes showing faster rates of increase in innate immunity with age in association with faster growth rates. Only fast-living snakes showed signs of immunosenescence later in life, suggesting the possibility of a trade-off between fast growth and physiological performance at late ages.

**KEYWORDS:** ecoimmunology; life-history evolution; natural antibodies; complement-mediated lysis; bactericidal competence; lymphocytes

**INTRODUCTION**

Life-history theory rests on presumed trade-offs between key resource-demanding traits such as growth, reproduction, and somatic maintenance (Roff 1992; Stearns 1992). Early interpretations of these trade-offs proposed that high investment in growth, for instance, would produce a trade-off with self-maintenance, resulting (among other things) in reduced investment in immune function i.e., reduced “immunocompetence” (reviewed by Sheldon & Verhulst 1996; Norris & Evans 2000; Schmid-Hempel 2003). However, as the complexity of the immune system and the frequent lack of correlation between the activities of its diverse components have become clear to ecoimmunologists, the need to build on this interpretation has been widely acknowledged. Recent theories in the field of ecoimmunology...
have become more nuanced, focusing on the benefits of investing preferentially in different components of the immune system based on life-history differences across species/populations, levels of individual investment in other costly activities, and parasite environment (e.g., Klasing & Leshchinsky 1999; Schmid-Hempel & Ebert 2003; Lee, 2006; Martin, Weil & Nelson 2006a).

The vertebrate immune system can be broadly subdivided into two main arms: a non-specific innate arm, which constitutes the first line of defense against invading pathogens, and includes phagocytic cells as well as antimicrobial proteins such as natural antibodies and complement; and a specific acquired arm, which is a second line of defense whose main players are T- and B-lymphocytes and the specific antibodies produced by the latter. These various components can be further classified as constitutive (expressed at all times) or induced (expressed upon encounter with pathogen) (Schmid-Hempel & Ebert 2003; Lee 2006). Investment in innate and acquired immunity may vary according to “pace-of-life” differences in life history. At one extreme, “fast-living” organisms are characterized by rapid growth and development, early maturation, and short lifespan, while at the other extreme, “slow-living” organisms show slow growth and development, late maturation, and longer lifespan (Stearns 1992; Roff 1992; Ricklefs & Wikelski 2002). A principal prediction of ecoimmunological theory is that fast-living species/populations should rely more on innate (and constitutive) defenses than slow-living species/populations, given that these defenses tend to be cheaper to develop, become functionally mature earlier during ontogeny, and act more rapidly and generally against pathogens than acquired (and induced) defenses. In contrast, slow-living species/populations should invest more in acquired (and induced) immune defenses, which take longer to develop but are more effective against repeated
infections during a long life, allowing increased probability of survival to subsequent reproductive events (Lee 2006; Martin et al. 2006a). Some findings in past studies with birds and mammals provide support for these predictions, while others show conflicting patterns (reviewed by Lee 2006), highlighting that more empirical studies are critical to determine the generality of the relationship between pace of life and immune defense components.

While it is clear that the field of ecoimmunology is now in a fertile and diversifying phase, the majority of studies on vertebrates have been conducted in endothermic species, and relatively little attention has been paid to ectothermic vertebrates, and reptiles in particular. In addition to being distinct from endotherms in thermoregulatory physiology, many ectotherms exhibit indeterminate growth and reproduction, which may have important ramifications for how trade-offs between life-history traits are manifest (Finch 1998; Vaupel et al. 2004; Sparkman, Arnold & Bronikowski 2007). Early studies on reptile immunology focused primarily on the seasonality of immune function (e.g., Hussein et al. 1979; El Ridi, Badir & El Rouby 1981), while more recent studies have focused mainly on within-population variation in immune function according to social interactions, reproductive investment, temperature, and parasite environment (e.g., Svensson, Sinervo & Comendant 2001; Uller, Isaksson & Olsson 2006; Ujvari & Madsen 2006; Madsen et al. 2007; Freedberg et al. 2008; French & Moore 2008). However, to our knowledge, no studies have tested the pace-of-life hypothesis regarding immune defense in any ectothermic vertebrate to date. Such studies are integral to establishing the generality of empirical findings in endothermic organisms and the relevance of the current theoretical framework.

An ideal system in which to test hypotheses of ecoimmunology exists in two ecotypes of the western terrestrial garter snake, *Thamnophis elegans*, living in the northeastern Sierra
Nevada range of California. These ecotypes are derived from the same ancestral meadow source population (Manier & Arnold 2005), and display natural variation in life-history strategy. The focus of study for over 30 years, these ecotypes differ dramatically with respect to pace of life (Table 1; Bronikowski & Arnold 1999; Sparkman et al. 2007), as well as a variety of physiological parameters (Bronikowski 2008; Robert, Vleck & Bronikowski 2008; Sparkman, Vleck & Bronikowski 2009). Replicate populations of one ecotype resides along the shoreline of Eagle Lake (a large, freshwater lake) and are characterized by a fast pace of life, exhibiting rapid growth to large body sizes, early maturation, high annual reproduction, and short median lifespan. Within several kilometers of the lake, replicate populations of a second ecotype reside in montane meadow habitats, and are characterized by a slow pace of life, exhibiting slow growth to smaller body sizes, late maturity, low annual reproduction, and longer median lifespan. Furthermore, these ecotypes exhibit heritable differences in coloration, scalation, and vertebral number (Manier, Seylor & Arnold 2007), and common garden studies with neonates have revealed a genetic component to differences in growth rate (Bronikowski 2000), suggesting that many of the characteristics that distinguish them are evolved, and not merely a plastic response to differences in habitat.

Recent interest in the immunology of wild birds has resulted in the development of field techniques that require a single capture and relatively small blood samples, and are therefore also ideal for studies in free-ranging reptiles. We used leading field protocols to measure three aspects of constitutive innate immunity in wild populations of T. elegans: (1) natural antibodies (NAbs), (2) complement-mediated lysis (CL), and (3) bactericidal competence of plasma (BC), all of which are part of the first line of defense against invading microorganisms. Natural antibodies are non-specific defenses, recognizing a broad array of
pathogens and promoting their opsonization (marking for destruction) and phagocytosis (Ochsenbein & Zinkernagel 2000). In addition, natural antibodies can activate the complement enzyme cascade, which results in the formation of killing complexes on the surface of invading pathogens, leading to their lysis (Ochsenbein & Zinkernagel 2000). Bactericidal competence of plasma is a measure of the integrated bacterial killing capacity of plasma proteins, including natural antibodies and complement, as well as lysozyme and constitutively produced acute phase proteins (Matson et al. 2006a). In addition to the three measures of constitutive innate immunity, we assessed a measure of acquired immunity: the levels of circulating lymphocytes. Lymphocytes are the major players of the acquired immune system and their abundance may be used as a measure of investment in induced acquired immunity (Lee 2006).

We predicted that all three measures of constitutive innate immunity would be higher in the fast-living lakeshore than the slow-living meadow ecotype, whereas lymphocyte abundance, as a measure of induced acquired immunity, would be lower in the fast-living ecotype. Furthermore, as differences in exposure to parasites could also contribute to variation in immune function between the ecotypes and complicate predictions based on life history alone (Lee 2006), we surveyed blood smears for evidence of blood parasites and inspected snakes for the presence of tail trematodes, which are common in the meadow habitat. Finally, given that T.elegans is an indeterminately growing species and, within each ecotype, body size can be used as a proxy for age (Bronikowski & Arnold 1999), we also explored the relationship between immune defense parameters and size/age in these snakes.
MATERIALS & METHODS

Field methods

This study was conducted in 2007 and 2008 with free-ranging western terrestrial garter snakes, viviparous members of the Colubridae family residing in the vicinity of Eagle Lake, California. Snakes were collected from six discrete populations in 2007, four representing fast-living lakeshore and two representing slow-living meadow ecotypes. Snakes were also collected from an additional meadow population in 2008. Snakes ranging from 200-800 mm snout-vent length (SVL) were hand captured from under rocks, in grasses, or while out basking or actively foraging. In 2007, a total of 99 snakes, evenly distributed between the two ecotypes, were blood-sampled for natural antibody and complement-mediated lysis assays from mid-May through early July. Over a period of two weeks in May 2008, 135 snakes, evenly distributed between the two ecotypes, were blood-sampled for the bactericidal competence assay.

Blood samples of 50-200ul, depending on snake body size, were taken from the caudal vein using heparin-rinsed syringes within a few minutes of capture. After centrifugation, plasma was snap-frozen in liquid nitrogen. All snakes were weighed, measured snout-to-vent length, and sexed via hemipene eversion before release. Females were palpated to establish gravidity (i.e., the presence or absence of embryos), and all snakes were examined for the presence of the trematode Alaria, which induces swelling, inflammation, and necrosis in the tail region (Sparkman, pers. obs.). In addition, blood smears were prepared from freshly drawn blood from 87 individuals, fixed in methanol, and stained with Wright-Giemsa stain (Fisher Scientific, Cat#SDWG80). Each blood smear was surveyed for the presence of extracellular and intracellular blood parasites as described in
Palacios & Martin (2006). Lymphocyte abundance was also estimated using these blood smears (see below).

**Natural antibodies & complement-mediated lysis**

Natural antibodies and complement-mediated lysis were measured using a hemolysis-hemagglutination assay (Matson, Ricklefs & Klasing 2005), with minor modifications. Serial two-fold dilutions of 10μl of plasma were made with phosphate buffered saline (PBS) in a 96-well plate. Each well then received 10μl of a 1% heterologous red blood cell (RBC) suspension. Plates were incubated for 90 minutes at 28°C, the preferred body temperature for *T.elegans* (Peterson, Gibson & Dorcas 1993), and then scored. Titers were estimated as the negative log$_2$ of the highest dilution factor of plasma that showed hemagglutination/lysis. Half scores were given for titers that appeared intermediate. All samples were run in duplicate with positive and negative controls in each plate. We tested for agglutination and lysis of both rat and sheep red blood cells as both of these types of blood have been previously used in snake/reptile studies (e.g., Kawaguchi, Muramatsu & Mitsuhashi 1978; El Ridi et al. 1981).

**Bactericidal competence of plasma**

Bactericidal competence was assessed according to the method described by Matson, Tieleman & Klasing (2006a), with a few modifications. A pellet of lyphophilized *Escherichia coli* (Microbiologies, Cat# 0483E7) was reconstituted using 40ml 1X PBS. A portion of this was further diluted 1:64 with PBS to produce a working solution containing roughly 150 colony-forming bacteria per 10μl. Plasma samples were diluted 1:10 with CO$_2$-independent medium (Gibco-Invitrogen, Cat#18045) containing 4mM L-Glutamine. Sample reactions were prepared by adding 10μl bacterial working solution to 100μl of the diluted plasma samples. All sample reactions were incubated for 20 minutes at 28°C to provide
adequate time for bacterial killing to occur. Two replicate controls were prepared by adding 10μl of the bacterial working solution to 100μl PBS. Duplicate controls and sample reactions were subsequently plated in 50μl aliquots on 4% tryptic soy agar and incubated overnight at 37°C. The number of bacterial colonies on each plate was then counted, and the percentage of colonies on each plate relative to the average number of colonies in the control plates was calculated. This percentage was subtracted from 100 to obtain the percentage of bacteria killed.

*Lymphocyte abundance*

The number of each type of leukocyte in snake blood was quantified by scanning the blood smears under 1000x magnification and classifying the first 100 leukocytes encountered as lymphocytes, heterophils, eosinophils, basophils, and monocytes+azurophils (Strik, Alleman & Harr 2007). Given that we were only interested in the abundance of lymphocytes, expressed as the number of lymphocytes per 10,000 red blood cells (Dein 1984), we present only these data here.

*Statistical models*

All analyses were conducted using JMP 6.0.0 (SAS Institute Inc, 2005) on normally distributed dependent variables. Natural antibodies, lysis, bactericidal competence, and lymphocyte abundance were analyzed for the effects of ecotype, population nested within ecotype, sex, gravidity, body size (SVL), date captured, body condition, and the presence/absence of parasitic infection. Ecotype is the fixed effect of lakeshore versus meadow habitat, and population nested within ecotype represents the effect of the seven different lakeshore and meadow populations. Population is treated as a fixed rather than a random effect to reflect complex microhabitat variation among populations within ecotypes.
Gravidity is the fixed effect of whether or not a given snake was gravid—the gravid group is necessarily composed of females alone, while the non-gravid group is a combination of both males and females. Body condition is defined as the residuals of the regression of body mass on SVL. For each dependent variable, ecotype and population within ecotype were kept in the best fit model even if they were not significant, while other non-significant effects and their interactions were removed in a step-wise fashion to arrive at the final model.

The relationship between natural antibody and lysis activity was assessed using simple correlation. Since these measures proved to be strongly correlated (see Results) we analyzed them jointly using MANCOVA. Lymphocyte abundance and bactericidal competence were both analyzed using ANCOVA (note that we could not correlate bactericidal competence directly to natural antibody and lysis, as it was assayed from a unique sample of individuals in a separate year). Repeatability ($r$) for each assay type was assessed using the intraclass correlation coefficient (Lessells & Boag 1987).

**RESULTS**

*Parasite abundance*

No blood parasites, either intra- or extra-cellular, were detected on any of the garter snake slides of either ecotype. Tail trematode infection occurred only in slow-living meadow snakes, at an incidence of 23% (12/52) in 2007 and 70% (30/43) in 2008. The presence of trematode infection was not significantly related to natural antibodies or complement-mediated lysis ($n = 64; P = 0.4116$), or bactericidal competence ($n = 64; P = 0.8751$).

*Natural Antibodies & Complement-Mediated Lysis*

Natural antibody and lysis titers obtained using sheep RBCs were positively correlated to corresponding titers using rat RBCs (NAbs: $R^2 = 0.18, P < 0.0001, n = 88$; CL:
\( R^2 = 0.29, P < 0.0001, n = 86 \), suggesting that results using either kind of blood reflect the general level of natural antibodies and lysis in *T. elegans*. However, rat RBC plates were more difficult to score than sheep RBC plates because rat RBC pellets were visually more diffuse, which resulted in lower repeatability of titers than in sheep RBC pellets (rat: \( n = 168, r = 0.59, P < 0.0001 \), sheep: \( n = 212, r = 0.77, P < 0.0001 \)). Therefore, only results obtained using sheep RBCs are presented.

There was a strong positive correlation between natural antibody and complement-mediated lysis titers in *T. elegans* (\( r = 0.87, n = 98, P < 0.0001 \); Fig. 1). In the best fitting MANCOVA model for natural antibodies and lysis as dependent variables, only ecotype, body size, and body size squared were significant (Table 2). Sex, gravidity, date captured, and body condition were not significant effects, and were excluded from the model. The MANCOVA showed a significant ecotype difference for both natural antibodies and lysis. Univariate tests revealed that in accordance to the prediction, the fast-living lakeshore ecotype exhibited significantly higher natural antibody and lysis titers than the slow-living meadow ecotype (NAbs: \( n = 99, F = 4.57, P = 0.035 \); CL: \( n = 98, F = 7.20, P = 0.009 \); Fig. 2A). The significant quadratic fit for both natural antibodies and lysis with body size (NAbs: SVL: \( F = 6.18, P = 0.068 \), SVL\(^2\): \( F = 5.46, P = 0.010 \); lysis: SVL: \( F = 7.56, P = 0.007 \), SVL\(^2\): \( F = 6.11, P = 0.015 \)) suggests an increase in these variables with size from 200 to 500 mm, and the beginning of a decline at approximately 500-550mm (Fig 3A, B), which is past the age of reproductive maturity (400-450mm). While there was no significant interaction between body size and ecotype, a MANCOVA analysis considering each ecotype separately shows a significant quadratic fit only for fast-living lakeshore snakes (SVL\(^2\): Lakeshore: \( n = 52, F = 3.16, P=0.0516 \); Meadow: \( n = 46; F = 0.48, P = 0.6242 \).
**Bactericidal competence of plasma**

The bactericidal competence assay showed high repeatability between duplicates (n=312, r = 0.93, *P* < 0.0001). In the best-fitting ANCOVA model for bactericidal competence, only ecotype, population nested within ecotype, and SVL had significant effects on % bacteria killed (Table 3). Sex, gravidity, date captured, and body condition were not significant, and were excluded from the model. Fast-living lakeshore snakes showed higher bactericidal competence than slow-living meadow snakes (Fig. 2B), in accordance with the prediction. The significant population effect denotes heterogeneity among populations within ecotypes—that is, within ecotypes, some populations have significantly higher levels of bactericidal competence than others. Furthermore, bactericidal competence increased linearly with size/age in both ecotypes (Fig. 4).

**Lymphocyte abundance**

The best ANCOVA model for lymphocyte abundance (repeatability: n=24, r = 0.58; *P* = 0.0132) included ecotype, population nested within ecotype, gravidity, and the interaction between SVL and gravidity (Table 4). Sex, date captured, body condition, and presence/absence of trematode infection were not significant effects, and thus were not included in the model. Examination of the interaction between SVL and gravidity revealed that gravid females showed increasing abundance of lymphocytes with size (n = 26; R² = 0.14, *P* = 0.0026), whereas non-gravid individuals showed the opposite pattern (n = 61; R² = 0.13, *P* = 0.0020; Fig. 5).
DISCUSSION

Immune defense & pace of life

We found that fast-living lakeshore snakes had higher levels of all three measures of constitutive innate immunity than slow-living meadow snakes (Fig. 2A, B). Thus this study, the first of its kind to be conducted in ectotherms, lends support to the hypothesis that fast-living animals rely more on constitutive innate immunity (i.e., the components of the immune system that allow them to respond rapidly and generally to pathogenic challenges) than slow-living animals. However, we did not find support for the other side of the hypothesis, which predicts that fast-living animals will simultaneously invest less in induced acquired immunity—in this system, abundance of lymphocytes did not differ between fast and slow-living ecotypes. Since lymphocyte abundance is only a rough index of acquired immunity, other components, such as specific antibody responses to a novel antigen, should be evaluated in order to test this prediction more thoroughly. Nevertheless, differences in constitutive innate immunity between the two ecotypes are clearly present and complement other work showing relationships between pace of life and divergent patterns of cellular and hormonal physiology in these ecotypes of T.elegans (Bronkowski, 2008; Robert et al. 2008; Sparkman et al. 2009).

The few other studies designed to test for variation in immune defense components with respect to pace of life have provided mixed support for the prediction that fast-living animals should invest more in innate (and constitutive) immune components compared to slow-living animals (reviewed in Lee 2006). Contrary to prediction, (and to our findings), bactericidal competence was lower in tropical bird species with faster-paced lives (Tieleman et al. 2005). On the other hand, bactericidal competence was higher, as predicted, in species
of Peromyscus mice with faster-paced lives, as reflected by their body size (though another innate defense, wound healing, showed the opposite pattern) (Martin, Weil & Nelson 2007). In another recent study with tropical bird species, Lee et al. (2008) showed higher levels of complement-mediated lysis in species with larger clutch sizes, a trait associated with a fast pace of life. Interestingly, in the same study, natural antibody titers were higher in bird species with longer incubation periods, a trait associated with a slow pace of life. If natural antibodies are classified as innate, non-specific defenses (as in this study, and others e.g., Matson et al. 2005; Moller & Haussy 2007), this result is contrary to prediction; however, Lee et al. (2008) classify natural antibodies as acquired defenses and suggest that their result supports the hypothesis that slow life histories are associated with well developed antibody-mediated defenses. Finally, in the only other study besides the present one comparing populations within a species, fast- and slow-living populations of house sparrows, Passer domesticus, did not differ in natural antibodies and complement-mediated lysis titers (Martin, Hasselquist & Wikelski 2006b).

Several methodological differences among these studies may have contributed to disparity in results leading to contradictory conclusions, including comparative context (i.e., interspecific versus intraspecific), whether individuals were sampled during the reproductive or non-reproductive seasons, and which components of immunity were measured. Furthermore, differences in evolutionary history, parasite environment, social system, and several physiological and ecological factors could have contributed to the conflicting results. All in all, while our study provides partial support for the pace-of-life hypothesis, it is clear that further study on comparable systems using comparable methodologies is needed in order to establish its generality.
**Immune defense, resources, & parasite abundance**

One of the strengths of the present study is that it was performed with free-ranging snakes and provides vital information regarding immune function in their native environment. However, it also raises the question of whether the documented differences in constitutive innate immunity have a genetic basis or represent a plastic response to ecological differences, such as parasite environment or resource availability, between lakeshore and meadow habitats. We are currently testing these alternatives using neonate snakes from both ecotypes born and raised in a common garden environment. It is important to note that lakeshore snakes do have more predictable prey availability from year to year than meadow snakes (Bronikowski & Arnold 1999) and thus may have more resources to allot to all components of immune function, whether innate or acquired. The fact that the fast-living lakeshore snakes did not show higher lymphocyte abundance in addition to higher levels of constitutive innate immunity, however, suggests that differences between the ecotypes are not solely due to differences in resource abundance.

The ecotype differences in constitutive innate immunity observed in *T.elegans* could also reflect differences in parasite load rather than differences in pace of life. We attempted to investigate this by quantifying parasite loads in the two *T. elegans* ecotypes. Although blood parasites can be found in many snake species, including some in the *Thamnophis* genus (Roudabush & Coatney 1937; Fudge 2000), we did not detect any blood parasite infection in the blood smears from *T. elegans* from either ecotype. While the extent to which the overall pathogen environments differ for meadow and lakeshore ecotypes is unknown at present (as it is for most free-ranging animal populations), a distinctive difference is the presence of the tail trematode *Alaria* only in the meadow habitat. A large percentage of adult
meadow snakes are infected with this trematode, which results in severe tail necrosis. We found no relationship, however, between trematode infection and natural antibodies, complement-mediated lysis or bactericidal competence in *T.elegans*. Thus it is unlikely that the ecotype differences in constitutive innate immunity merely represent a plastic response to habitat-based differences in parasite load. A more thorough study of the parasite faunas is warranted to fully test this hypothesis. In addition, the on-going common garden experiment will also shed light into the influence of parasite load on the observed immune defense patterns.

**Immune defense & size/age**

All three measures of constitutive innate immunity increased with size/age up to maturity in *T.elegans*—however, they were subsequently decoupled, with only bactericidal competence continuing to increase after maturity (Fig. 4). The increase in levels of innate immunity with size occurred continuously from an early age (Fig. 4; 200 mm, the lowest sizes in our study, is within a year of birth). While natural antibodies and lysis appeared to exhibit a plateau after reproductive maturity, as documented in free-living birds (Palacios *et al.* 2007), bactericidal competence in *T.elegans* continued to increase with age far past reproductive maturity and into the latest ages (Fig 4). A positive relationship between natural antibodies and size has also been found in water pythons, *Liasis fuscus*, where larger snakes are also considered older snakes (Madsen *et al.* 2007), and similar relationships between immune parameters and age/size have been reported in fish (e.g., Johnson, Flynn & Amend 1982; Scapigiliati *et al.* 1999). This is a pattern of immune development not previously described in determinately growing species and may be seen as an intriguing consequence of
indeterminate growth, emphasizing that the unique characteristics of ectotherms have much to contribute to understanding development and allocation of immune function.

Recent studies in free-living birds show patterns of immunosenescence i.e., deterioration of immune function towards the end of life (e.g., Haussmann et al. 2005; Palacios et al. 2007). In our study, we found evidence that natural antibodies and lysis activity decline with size after maturity and that this pattern appears to be present only in fast-living lakeshore snakes. Ujvari & Madsen (2006), testing antibody responses to a novel antigen (induced acquired immunity) in water pythons, were the first to document a decline in immune function with age/size in snakes. However, this decline began from six months of age and (as these animals may live to more than 15 years of age) is unlikely to be solely due to immunosenescence. Our study is thus the first to document a decline in immune function after maturity in any snake species, in a manner indicative of immunosenescence. Furthermore, we found evidence to suggest that this decline may only be present in fast-living lakeshore and not in slow-living meadow snakes. If this is indeed the case, our findings are consonant with the antagonistic pleiotropy theory of aging, which predicts that high investment in early life traits will come at a cost later in life (Williams 1957). In this system, therefore, the cost of fast growth in lakeshore snakes may be manifest as a faster rate of immunosenescence. Two caveats, however, must be considered here. First, it is possible that the immunosuppressive effects of reproduction, documented in a number of other species (Schmid-Hempel, 2003), may be partially responsible for a decline in immune function at later ages/sizes. The largest snakes in our sample were gravid, fast-living lakeshore snakes. However, we consider immunosenescence to be the best interpretation of our results, as gravidity was not a significant effect in any of our analyses of innate immune
components, even in comparisons among similarly-sized gravid and non-gravid adults (data not shown). Moreover, a (non-significant) quadratic trend is evident in lakeshore snakes even when gravid females (the largest, oldest snakes among them) are excluded from the sample. Nevertheless, more extensive sampling of larger, older individuals, both gravid and non-gravid, must be undertaken to substantiate these findings. The second caveat is that the relationship between size and age is much less predictable after maturity in slow-living meadow snakes than in fast-living lakeshore snakes, due to a faster tapering of growth rate at later ages in the former group (Bronikowski & Arnold 1999). Thus, while it may appear that only lakeshore snakes exhibit a decline in immune function with size/age, this same trend may be concealed in meadow snakes when using size as a proxy for age at older ages. Future research should employ skeletochronological techniques to age females more precisely (Waye 1998; Sparkman et al. 2009), so that we can more confidently test for differences in immunosenescence patterns between the two ecotypes.

Whether or not immunosenescence is present in these snakes, the lack of a significant interaction between ecotype and body size in any of the three measures of innate immunity has important implications regarding the rate of development of immune function early in life. Since lakeshore snakes grow faster than meadow snakes, a lakeshore snake is younger than a meadow snake of the same size (Bronikowski & Arnold 1999). The equivalency of slopes relating innate immunity to size in the two ecotypes has, therefore, two possible implications: (1) it is size, and not age, that is the rate-limiting factor in the development of immune function, or (2) immune function, like growth, is on a developmental fast-track in lakeshore snakes, whether or not the two are mechanistically linked. Variation in the ontogenetic development of immune defense components has been recognized as an
important area for future research in ecoimmunology (Martin et al. 2006a, Palacios et al. 2008), and these garter snake ecotypes constitute an ideal system in which to test for differential rates of development of various immune defense components (e.g., innate versus acquired) in a life-history context.

**Immune defense and reproduction**

With regard to intrapopulation variation in immune function, theoretical predictions suggest that individuals will rely less on costly innate defenses during reproduction itself—a very costly activity, and more on acquired defenses—which, though costly to develop, may operate at low cost thereafter (Lee, 2006). Gravidity itself (independent of body size) did not have a strong effect on any of the three measures of innate immunity in our study. However, since we measured components of innate immunity that are not considered as costly as other components, such as induced inflammation (Lochmiller & Deerenberg 2000; Klasing 2004), further tests of this half of the hypothesis should be conducted. We found, however, a significant difference in patterns of lymphocyte abundance between gravid and non-gravid individuals: in both meadow and lakeshore snakes, gravid females had increasing numbers of lymphocytes with size/age, while both non-gravid females and males showed decreasing lymphocytes with size/age (Fig. 5; note that there is little overlap among size groups as gravid females tend to be the largest individuals). With the caveat that conclusions based on lymphocyte numbers must be interpreted cautiously, as they can also be higher in individuals battling infections, we propose that this trend may reflect the increase in reproductive output with size seen in these snakes (particularly in lakeshore populations) (Bronikowski & Arnold 1999; Sparkman et al. 2007). As such, these findings are consistent with the prediction that reproducing individuals will rely more on acquired than on innate immunity when compared
to non-reproducing individuals. Even more so, we suggest that this prediction can be further refined for species exhibiting indeterminate reproduction: the greater the reproductive output, the greater the reliance on acquired immunity.

**CONCLUSION**

This study demonstrates that variation in constitutive innate immunity correlates to pace of life in *T. elegans* in a manner consonant with theoretical predictions: fast-living lakeshore snakes showed higher levels of natural antibodies, complement-mediated lysis, and bactericidal competence than their slow-living meadow counterparts. Furthermore, larger, older snakes—of both sexes and ecotypes—showed higher levels of innate immunity as they aged/grew larger up to maturity. This pattern appears to be driven by size rather than age, as older meadow snakes showed similar levels of innate immunity to younger lakeshore snakes of the same size, suggesting that the two ecotypes have evolved difference in rates of immune defense development that correspond to differences in growth rate. After maturity, lakeshore snakes showed a decline in natural antibodies and complement-mediated lysis, suggesting the possibility that fast living results in a faster rate of immunosenescence.

With regard to acquired immunity, lymphocyte abundance did not show the predicted difference between the two ecotypes. However, gravid females showed increasing lymphocyte abundance as they aged/grew larger, paralleling their increased reproductive output, which may reflect an increased reliance in operationally less expensive defenses during reproduction.

The lack of a difference in lymphocyte abundance between ecotypes, and the disparate relationships between size/reproductive status and measures of immunity confirm other reports indicating broad diversity in regulation of immune function with respect to life-
history traits even within a given arm of the immune system (e.g., Lee et al. 2006; Martin et al. 2007). Thus, they accentuate the importance of using multiple measures of immunity to obtain a thorough and accurate portrait of immune investment in relation to other variables. Finally, this study provides a promising foundation for future work, which will elucidate the relationship between measures of acquired immunity (e.g., *in vivo* specific antibody response to a novel antigen, *in vitro* lymphocyte proliferation) and pace-of-life variation, as well as test for a genetic basis for ecotype differences in immune function.

**ACKNOWLEDGEMENTS**

Warm thanks to A. Bronikowski for field and lab support, D. Miller for statistical advice, and the Vleck lab and A. Bronikowski for comments on previous versions of the manuscript. Many thanks also to J. Chamberlain, S. Zylstra, A. Lehman, S. Arnold and the Oregon State University crew for help with snake collection, K. Martin for hospitality at Eagle Lake, and USFS Tom Rickman for field support. The State of California Department of Fish and Game granted collecting permits. This research was supported by a grant from the National Science Foundation to A. Bronikowski (DEB-0323379) and a National Science Foundation Doctoral Dissertation Improvement Grant to A. Sparkman (DEB-0710158).

**REFERENCES**


Evolutionary Ecology, 21, 271-279.
corticosterone levels on offspring behavior in fast- and slow-growth garter snakes (Thamnophis elegans). Hormones and Behavior, 55, 24-32.


Table 1. Life-history differences between fast-living lakeshore and slow-living meadow garter snake ecotypes (Bronikowski & Arnold 1999).

<table>
<thead>
<tr>
<th>Life-history trait</th>
<th>Fast-living (lakeshore)</th>
<th>Slow-living (meadow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>maximum body size</td>
<td>~700 mm</td>
<td>~550 mm</td>
</tr>
<tr>
<td>size at maturity</td>
<td>~400 mm</td>
<td>~450 mm</td>
</tr>
<tr>
<td>age at maturity</td>
<td>3 years</td>
<td>5-7 years</td>
</tr>
<tr>
<td>mean litter size</td>
<td>8 neonates</td>
<td>5 neonates</td>
</tr>
<tr>
<td>adult median lifespan</td>
<td>4 years</td>
<td>8 years</td>
</tr>
</tbody>
</table>
Table 2. Best fit MANCOVA model with natural antibody and complement-mediated lysis titers as the response variables (n = 98). Asterisks denote significant effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>Wilks' lambda</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotype</td>
<td>0.081</td>
<td>2,89</td>
<td>3.583</td>
<td>0.032*</td>
</tr>
<tr>
<td>Population(Ecotype)</td>
<td>0.858</td>
<td>8,178</td>
<td>1.77</td>
<td>0.086</td>
</tr>
<tr>
<td>SVL</td>
<td>0.092</td>
<td>2,89</td>
<td>4.08</td>
<td>0.020*</td>
</tr>
<tr>
<td>SVL$^2$</td>
<td>0.079</td>
<td>2,89</td>
<td>3.533</td>
<td>0.033*</td>
</tr>
</tbody>
</table>
Table 3. Best fit ANCOVA model with bactericidal competence (% bacteria killed) as the response variable (n = 135). Asterisks denote significant effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecotype</td>
<td>1</td>
<td>3.9534</td>
<td>0.0489*</td>
</tr>
<tr>
<td>Population(Ecotype)</td>
<td>5</td>
<td>5.586</td>
<td>0.0001*</td>
</tr>
<tr>
<td>SVL</td>
<td>1</td>
<td>60.9715</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Error</td>
<td>127</td>
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<td></td>
</tr>
</tbody>
</table>
Table 4. Best fit ANCOVA model with abundance of lymphocytes per 10,000 red blood cells (RBCs) as the response variable (n = 87). Asterisks denote significant effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F</th>
<th>P</th>
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<tbody>
<tr>
<td>Ecotype</td>
<td>1</td>
<td>2.111</td>
<td>0.1503</td>
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<tr>
<td>Population(Ecotype)</td>
<td>5</td>
<td>1.865</td>
<td>0.1102</td>
</tr>
<tr>
<td>SVL</td>
<td>1</td>
<td>1.782</td>
<td>0.1859</td>
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<tr>
<td>Gravid(y/n)</td>
<td>1</td>
<td>2.548</td>
<td>0.1145</td>
</tr>
<tr>
<td>Gravid * SVL</td>
<td>1</td>
<td>13.290</td>
<td>0.0005*</td>
</tr>
<tr>
<td>Error</td>
<td>77</td>
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</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Simple correlation between natural antibody and complement-mediated lysis titers in garter snakes (n = 98). (The correlation did not differ significantly between ecotypes.)

Figure 2. Comparison of constitutive innate immune defense components between life-history ecotypes of the garter snake. (A) Natural antibody (NAbs) and complement-mediated lysis (CL). (B) Bactericidal competence (BC, % bacteria killed). Least square means and standard error bars from statistical models. Statistics provided in the text.

Figure 3. Relationship between body size (SVL) and (A) natural antibody titer and (B) complement-mediated lysis titer in two life-history ecotypes of garter snakes.

Figure 4. Relationship between body size (SVL) and bactericidal competence (% bacteria killed) in two life-history ecotypes of garter snakes. Data points are residuals adjusted for other effects in the ANCOVA model.

Figure 5. Number of lymphocytes per 10,000 red blood cells (RBCs) in relation to body size in gravid (female) and non-gravid (male and female) snakes.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
ABSTRACT

The endocrine system plays an integral role in the regulation of key life-history traits. Insulin-like growth factor-1 (IGF-1) is a hormone that promotes growth and reproduction, and has been implicated in the reduction of lifespan. IGF-1 is also capable of responding plastically to environmental stimuli such as resource availability and temperature. Thus pleiotropic control of life-history traits by IGF-1 could provide a mechanism for the evolution of correlated life-history traits in a new or changing environment. An ideal system in which to investigate the role of IGF-1 in life-history evolution exists in two ecotypes of the garter snake *Thamnophis elegans*, which derive from a single recent ancestral source, but have evolved genetically divergent life-history characteristics. Snakes from meadow populations near Eagle Lake, California, exhibit slower growth rates, lower annual reproductive output, and longer median adult lifespans relative to populations along the lakeshore. We hypothesized that the IGF-1 system has differentiated between these ecotypes.
and can account for increased growth and reproduction, and reduced survival in lakeshore versus meadow snakes. We tested for a difference in plasma IGF-1 levels in free-ranging snakes from replicate populations of each ecotype over three years. IGF-1 levels were significantly associated with adult body size, reproductive output, and season in a manner that reflects established differences in prey ecology and age/size-specific reproduction between the ecotypes. These findings are discussed in the context of theoretical expectations for a trade-off between reproduction and lifespan that is mediated by pleiotropic endocrine mechanisms.

**KEYWORDS:** insulin-like growth factor-1; life-history evolution; trade-offs; hormonal plasticity; garter snake; *Thamnophis elegans*

**INTRODUCTION**

How complex traits evolve, singly or in tandem with other traits, is a question central to understanding how populations diverge, and ultimately how speciation may occur. While much research examines genomic and/or morphological manifestations of divergence, few studies have sought the physiological mechanisms between the level of genes and the whole organism phenotype that may mediate, and to some extent orchestrate, changes among populations.

The endocrine system is critically involved in the determination of fundamental life-history traits involved in evolutionary fitness, such as growth, reproduction, and even, as recent studies have indicated, aging and lifespan (e.g., Sinervo & Licht 1991, Tatar et al. 2001, 2003, Flatt et al. 2005, 2007, Bellino 2006). Life-history theory has long focused on how constraints involved in allocation of energy and other resources creates trade-offs among life-history traits both within and among taxa (Kirkwood 1977, Stearns 1992, Roff 1992, Charlesworth 1994). However, the endocrinological mechanisms that may underlie many of
these trade-offs, and restrict life histories to certain commonly observed patterns—such as an
association between fast growth, high reproduction, and short lifespan—are only just
beginning to be explored (Stearns 1989, Finch & Rose 1995, Ketterson & Nolan 1992, 1999,

Recent studies addressing the role of hormones in life-history trade-offs have given
promising results. Experimental manipulation of follicle-stimulating hormone (FSH) in the
side-blotched lizard, for instance, has been shown to generate the classic life-history trade-off
between offspring size and number (Sinervo & Licht 1991). In birds, testosterone has been
linked to the evolution of trade-offs in such traits as mate choice and reproductive success,
and survival (reviewed in Ketterson and Nolan 1999, Reed et al. 2006). And in insects,
juvenile hormone has proved a key actor in trade-offs between reproduction and lifespan

Currently much attention in model organisms has been focused on the role of insulin-
like growth factor/insulin molecules in the regulation of life-history traits (reviewed in Tatar
et al. 2003, Bartke 2005, 2008). While invertebrate species may exhibit several to dozens of
ligands that bind to a single receptor, vertebrates have evolved three homologous but distinct
ligands—insulin, IGF-1, and IGF-2—that bind to three separate receptors (though some
degree of cross-reactivity among them still exists) (reviewed in Nakae et al. 2001). While
insulin chiefly coordinates metabolic activities, IGF-1 is heavily involved in stimulating cell
survival, proliferation, migration and differentiation. It is secreted primarily by the liver in
response to pituitary growth hormone (GH), and stimulates growth, development, and
reproduction in all vertebrates yet examined—a list that includes mammals, fish, reptiles, and
birds (e.g., Perez-Sanchez et al. 1995, Guillette et al 1996, Hiney et al. 1996, Kagawa et al.
1994, Beccavin et al. 2001, Uchida et al. 2003, Beckman et al. 2003). However, it appears that these constructive actions of IGF-1 do not come without a cost. IGF-1 (along with insulin) triggers a signaling pathway that is homologous to the IGF/insulin (IIS) pathway in invertebrates that has negative impacts on longevity (reviewed in Giannakou & Partridge 2007). Evidence for a role of IGF-1 in vertebrate aging is growing (reviewed in Bartke 2008), and one landmark study has shown that reduced expression of IGF-1 receptors leads to extended lifespan and reduced oxidative stress in mice (Holzenberger 2003).

This study tests for a role of IGF-1 in the evolutionary divergence of life-history traits in replicate wild populations of the garter snake, *Thamnophis elegans*. These populations are derived from the same ancestral source lineage, but have differentiated into two distinct ecotypes that differ in scoliation, colouration, and life history (Bronikowski & Arnold 1999, Bronikowski 2000, Manier & Arnold 2005, Manier et al. 2007). Individuals in one ecotype, which occupies lakeshore habitats, exhibit fast growth, early maturation, large adult body size, high annual reproduction, and low annual survival. In contrast, individuals in the second ecotype, which occupies mountain meadow habitats, exhibit slow growth, late maturation, small adult body size, low annual reproduction, and high annual survival (Bronikowski & Arnold 1999). Long-term mark-recapture and reproduction data have shown distinct differences in age-specific reproduction between ecotypes (Sparkman et al. 2007). The respective lifespans of the two ecotypes reflect these fast/slow life-history strategies, with fast-growth lakeshore snakes maintaining a shorter median adult lifespan (4 years) than slow-growth meadow snakes (8 years) (Bronikowski & Arnold, unpublished data). Furthermore, a genetic basis for differences in growth rates between the two ecotypes has been established with neonates raised in a common environment (Bronikowski 2000).
All populations of fast-growth snakes along the lakeshore were derived from an ancestral meadow lineage (Manier & Arnold 2005). There are a number of ecological factors that are likely to have influenced the evolution of life-history traits upon dispersal to lakeshore habitats, including higher mean temperatures, year-to-year stability and abundance of prey, and increased susceptibility to predation (Bronikowski & Arnold 1999, Arnold unpublished data). Such conditions are conducive to faster growth, and the attainment of larger body sizes, as well as a correlated increase in reproductive output. IGF-1, which strongly influences the expression of all of these traits, responds plastically to experimental manipulations of nutrition and temperature (Duan & Plisetskaya 1993, Gabillard et al. 2003). Thus the life-history evolution which has occurred in lakeshore snakes may have involved evolution of the GH-IGF-1 axis in response to novel environmental cues encountered in the lakeshore habitat. If this was the case, it is also possible that increased IGF-1 receptor signaling produced a trade-off in lifespan.

The goals of the study were two-fold. First, it was necessary to establish basic biological patterns of IGF-1 in free-ranging snakes, as this constitutes the first study of IGF-1 in squamate reptiles, and one of only a handful of studies conducted in the wild (e.g., Crain et al. 1995a, 1995b; Guillette et al. 1996, Webster et al. 1996, Schmidt & Kelley 2001). In particular, the activity of IGF-1 in life histories characterized by indeterminate growth and reproduction is poorly understood, and while IGF-1 secretion is positively associated with food intake and varies seasonally in some fish and mammals (Duan et al. 1995, Schmidt & Kelley 2001, Webster et al. 1996, Uchida et al. 2003), how levels might vary from year to year according to food availability is unknown. To understand the influence of these factors, both reproductive and non-reproductive snakes of a wide range of sizes were sampled during
three years that differed dramatically with respect to climate-driven food availability. The second, and most pertinent, goal was to test for differences in plasma IGF-1 between ecotypes that would account for differences in growth and reproductive rates, and support the possibility of its involvement in a trade-off with lifespan.

METHODS

Study populations & sample collection
Free-ranging western terrestrial garter snakes, *Thamnophis elegans*, living in the vicinity of Eagle Lake in Lassen County, California were collected during 2006, 2007 and 2008. They were sampled from four distinct lakeshore populations scattered intermittently along the Eagle Lake shoreline (L1-L4), and three montane meadow populations located at increasing elevations just southeast of the lake (M1-M3) (population descriptors in accordance with those described in Bronikowski & Arnold 1999). Only two southern lakeshore populations, L1 & L2, were sampled in 2006, but sampling was extended to two northern populations, L3 and L4, in 2007. All three meadow populations were sampled in 2006, but only M1 and M3 meadows were sampled in 2007 as M2 was dry and virtually devoid of snakes in that year. All seven populations were sampled in 2008. A total of 229 snakes were sampled in 2006, 342 in 2007, and 194 in 2008.

In 2006 and 2007, sampling occurred from mid-May, within a few weeks of emergence from hibernation, through peak foraging season in June and July. In 2008, sampling occurred just as emergence and initial dispersal from hibernacula were beginning during two weeks from early to late May during 2008. Snakes were captured by hand from under rocks and vegetation or while basking or actively foraging. Previous work shows negligible diel variation in plasma IGF-1, so sampling was performed throughout the day.
(Lee & Rosenfeld 1987). Each snake was bled caudally within 10 minutes of capture. Blood samples were spun in a field centrifuge and plasma was frozen in liquid nitrogen for later analysis. All snakes were weighed, measured (snout-to-vent-length [SVL]), sexed, and checked for ecdysis on site, and females were palpated for embryos to determine reproductive status. A subset of the gravid females was returned to the lab in 2006 and 2007, and reproductive output was estimated by total litter mass upon parturition, using the combined masses of undeveloped embryos and both live and stillborn young (Robert et al. 2008).

**Radioimmunoassay**

Field plasma samples were assayed for IGF-1 using a standard radioimmunoassay (RIA) protocol (GroPep Ltd. Protocol #3002). The assay was validated for *T. elegans* via serial plasma dilution. Briefly, a human standard curve was generated with recombinant human IGF-1 standards ranging from 0.078-10 ng/ml. A pool of acid-ethanol extracted plasma from *T.elegans* was aliquoted in 50-, 25-, 12.5-, 6.25-, and 3.125-μl volumes, and assayed in duplicate along with the human standards using human IGF-1 antibody, and I-125 labeled human IGF-1. Percent I-125 IGF-1 binding (B/B₀) for all samples is shown (Figure 1). The serial dilutions of snake plasma yielded a curve parallel to the human standard curve.

All field samples were extracted and assayed in duplicate following each field season. Randomly assigned groups of samples were assayed back-to-back within a 48-hour time period using the same reagents to reduce inter-assay variability. A pooled positive control was run with each successive assay. For 2006 assays the intra-assay coefficient of variation (CV) averaged 2.2% and the inter-assay CV was 9.3%; for 2007 the intra-assay CV
averaged 4.2% and the inter-assay CV was 6.7%; for 2008 the intra-assay CV averaged 2.9% and the inter-assay CV was 4.3%.

Climate
To assess the climatic variation among years, temperature and snow depth data were obtained from the National Climate Data Center (www.ncdc.noaa.gov) and the California Data Exchange Center (www.cdec.water.ca.gov) respectively, for all three years of study. Snow depth measures were taken from a nearby weather station at Feather River Meadow, and monthly temperature averages were taken from Susanville, a nearby city. It was clear from these data, and from that of multiple nearby locations, that snow depth was much reduced during the spring of 2007 versus 2006 (April—63.7” vs. 20.5”, May—66.5” vs 6.3”). Furthermore, spring temperatures reached an early high of 46.5°F in March of 2007, while in 2006 the March average temperature was 36.0°F, and did not reach into the 40s until April. 2008 was an intermediate year with respect to these variables, with snow depth high in April (56.3”), but low in May (19”), with a March average temperature of 40.5°F. Negligible spring rainfall suggests that 2008, like 2007, will be a drought year. However, standing water from recent snow melt was still present in the meadows during the two-week sampling period.

Statistical analyses

*Full model: Ecotype, population, time, group & ecdysis* All statistical analyses were conducted using SAS software (SAS 9.1.3, SAS Institute Inc., Cary, NC) using log-transformed values for IGF-1. IGF-1 was analyzed separately for each year as the dependent variable in an analysis of covariance (ANCOVA) using the following linear model, $Y = \mu + day + ecotype + population(ecotype) + gravidity + ecdysis + day*ecotype + day*gravidity +$
gravidity*ecotype + ε, where μ is the population mean, day (representing Julian day, the number of days elapsed between January 1 and the date of capture) is the covariate, and ε is the error term. Ecotype is the fixed effect of lakeshore versus meadow habitat and population nested within ecotype represents the effect of the seven different lakeshore and meadow populations. Population was treated as a fixed rather than a random effect to reflect complex microhabitat variation among populations even within ecotypes. Gravidity is the effect of reproductive status, and is divided in two categories: (1) male & non-gravid females, and (2) gravid females. Ecdysis is the effect of the presence or absence of ecdytic characteristics (i.e., blue eyes and/or shedding skin). Originally, all two and three-way interactions between effects were included in the model, but all non-significant interactions were removed to produce the model presented above.

**Adult body size model** SVL was excluded from the full model as it exhibited no significant relationship to IGF-1 when all body sizes were included in the analysis. However, when the data set was restricted only to non-reproductive adult snakes, a relationship between IGF-1 and SVL become evident. This relationship was assessed by year using a separate model, \( Y = \mu + \text{ecotype} + \text{population(ecotype)} + \text{SVL} + \text{ecotype*SVL} + \epsilon \), where SVL is the covariate consisting of a range of body sizes in mature snakes (maturity occurs around 400 mm in meadow, and 425 mm in lakeshore—see Sparkman et al 2007, Bronikowski & Arnold 1999). Sex and day showed no significant effects and thus were not included in the model.

**Reproductive output model** Gravid females were excluded from the above body size analysis since reproductive output is highly correlated to body size, making it difficult to dissociate the effect of reproductive activity from any independent effect of body size (Sparkman et al 2007). The relationship between field levels of IGF-1 and final reproductive output was
originally assessed using a full model analysis including ecotype, population nested within
ectype, and day, but as none of these effects showed any significance, the analysis was
reduced to a simple linear regression between field plasma IGF-1 and total litter mass by
year.

RESULTS

Full model: Ecotype, population, day, gravidity & ecdysis

In 2006, ecotype differences in IGF-1 emerged as a significant gravidity by ecotype
interaction (Table 1). Comparison of least square means revealed that fast-growth lakeshore
gravid females had significantly higher IGF-1 levels than slow-growth meadow gravid
females (p=0.009), while meadow males and non-reproductive females exhibited higher IGF-
1 levels than their lakeshore counterparts (p=0.033). There was also a nearly significant
interaction (p=0.06) between gravidity and days into the growing season, where IGF-1
remained constant in males and non-gravid females, but was low in May and rose to fairly
constant levels in June and July in gravid females (Figure 2A). Finally, ecdytic (shedding)
snakes had significantly higher values of IGF-1 than non-shedding individuals (Table 1).

In 2007, a strong ecotypic effect was revealed in the ecotype by days interaction,
where fast-growth lakeshore snakes retained constant levels of IGF-1 throughout the season
(Figure 2B), but slow-growth meadow snakes showed a gradual but highly significant
decline as the season progressed (Figure 2C). This proved a strongly assorting ecotypic
effect, as all meadow populations showed significant decline throughout the season, while all
of the lakeshore populations remained constant.

Gravidity also emerged as a significant effect in 2007, with males and non-gravid
females having higher levels of IGF-1 than gravid females. There was no significant
gravidity by ecotype interaction, as fast-growth lakeshore snakes from each group had higher levels of IGF-1 than slow-growth meadow snakes from corresponding groups. As in 2006, ecdysis also emerged as a significant effect in 2007, with non-shedding snakes having lower values than shedding snakes (Table 1).

In 2008, a gravidity by ecotype interaction was again evident, with lakeshore gravid females having significantly higher levels of IGF-1 than all other groups (Lakeshore gravid vs. lakeshore non-gravid, p=0.0197; vs. meadow non-gravid, p=0.0234; vs. meadow gravid, p=0.0103).

Adult body size model
In both 2006 and 2007 there was a significant interaction between ecotype and SVL, where plasma IGF-1 levels increased with adult body size in fast-growth lakeshore snakes (2006, p=0.013, n=40, Figure 3A; 2007: p=0.011, n=62, Figure 3C), but either remained constant (2006: p=0.823, n=85; Figure 3B) or actually declined (2007: p=0.003, n=69, Figure 3D) with body size in slow-growth meadow snakes (Table 2). In 2008, the year with a reduced sampling period, IGF-1 was positively correlated to SVL in both ecotypes (p=0.0182, n=82).

Reproductive output model
Regression of IGF-1 on total litter mass revealed the same pattern in both 2006 and 2007: increasing IGF-1 levels were associated with increasing reproductive output (2006: p=0.003, R²=0.25, n=34; 2007: p=0.046, R²=.12, n=34; Figure 5).

DISCUSSION
IGF-1 is well-known for its close involvement in growth, reproduction, and survivorship in a variety of species throughout the vertebrate lineage (e.g., Kagawa et al.
1984, Buonomo et al. 1987, Daughaday & Rotwein 1989, Scanes et al. 1989, Holzenberger et al. 2003). The plasticity of IGF-1 in response to a variety of environmental stimuli, including nutrition, temperature, and stress, has been particularly well documented in laboratory fish, birds and mammals (e.g., Duan & Plisetskaya 1993, Schmidt & Kelley 2001, Gabillard et al. 2003), suggesting that studies situated in a natural ecological context are of great interest. However, IGF-1 activity in wild populations has been largely unexplored (but see Crain et al. 1995a, Guillette et al. 1996, Webster et al. 1996). This study presents the first data set on plasma IGF-1 in squamate reptiles, and explores its relationship to both environmental and phenotypic variation. In so doing, it becomes the first to elucidate a relationship between IGF-1 and ecotypic differences in life history in any wild species.

Here we report a complex and novel picture of how plasma IGF-1 differs within the two ecotypes of *Thamnophis elegans* in question. A basic understanding of the growth-stimulating effects of IGF-1 suggests that if lakeshore snakes are growing faster than meadow snakes, they should exhibit higher circulating IGF-1 levels on the whole. Surprisingly, a simple differentiation in plasma IGF-1 levels between slow-growth meadow and fast-growth lakeshore ecotypes snakes was not evident. Instead, the way in which ecotype influenced IGF-1 levels in the complete sample of individuals was through interactions with three key variables: Julian day, body size, and reproductive status (“gravidity”).

**IGF-1 & Julian day**

Interactions between Julian day and both gravidity and ecotype illuminate various aspects of how IGF-1 changes in relation to seasonal and inter-annual climate variability. In 2006, gravid females from both ecotypes showed an increase in IGF-1 levels from May to
June (Figure 2A). This timeline coincides with the onset of embryogenesis in females that have recently emerged from hibernation and mated, and matches seasonal elevations of IGF-1 seen in reproductive sea turtles (Crain et al. 1995a). IGF-1 levels in gravid females did not show a similar change from May to June in 2007, but this can easily be explained by an earlier emergence from hibernacula, owing to dramatically reduced snow depth and earlier highs in spring temperatures in 2007 than in 2006 (see Climate in Methods section).

Presumably adult females mated and commenced embryogenesis a few weeks before blood sampling was initiated in that year.

From June through July in 2006 for both ecotypes, and throughout 2007 in lakeshore snakes, IGF-1 did not change with Julian day (Figure 2B). In 2007, however, meadow snakes showed declining IGF-1 levels from May to July (Figure 2C). According to predictions from long-term records, amphibians, the primary prey source for *T. elegans* in meadow habitats, were scarce in 2007 due to the low levels of snow and rainfall (Bronikowski & Arnold 1999). As IGF-1 is known to decline with low nutrition (e.g., Webster et al. 1996, Uchida et al. 2003), the seasonal decline in IGF-1 levels in meadow snakes during 2007 is thus readily interpretable as a response to declining availability of amphibians prey as the meadows continued to dry over the summer months. Given that scarcity of prey frequently occurs in meadow habitats due to highly variable annual precipitation from year to year (Bronikowski & Arnold 1999), this has important ramifications for differences in circulating IGF-1 between the two ecotypes. If meadow snakes regularly experience years of diminished IGF-1 levels, it follows that lakeshore snakes—which feed primarily on fish that are consistently available in the lake—may experience cumulatively higher levels of IGF-1 across years than meadow snakes.
**IGF-1 & reproduction**

In both 2006 and 2007 there was a strong correlation between circulating IGF-1 in gravid females and reproductive output: females with larger litters had higher IGF-1 levels (Figure 4). As fast-growth lakeshore females have higher age/size-specific reproductive output than slow-growth meadow females (Bronikowski & Arnold 1999, Sparkman et al. 2007), this relationship between IGF-1 and reproductive output presumably resulted in lakeshore females having significantly higher IGF-1 levels in both years than meadow females. While it is clear that other species have elevated IGF-1 during gravidity (Daughaday & Rotwein 1989, Crain et al. 1995a, Guillette et al. 1996), this is the first time plasma IGF-1 levels have been shown to rise with increasing reproductive output in an indeterminately growing species. Furthermore, it supports a role for IGF-1 in the divergence of reproductive life-history traits between the two ecotypes, with higher levels of IGF-1 in the lakeshore facilitating higher reproductive output.

**IGF-1 & body size**

This study presents three disparate relationships between IGF-1 and body size in *T.elegans* that have intriguing ecological and evolutionary implications (Figure 3). In the lakeshore ecotype, where prey is continuously available, IGF-1 consistently increased with adult body size in all three years. In slow-growth meadow snakes, however, the relationship between IGF-1 levels and body size was plastic, and varied annually with resource availability. During the two week period sampled immediately post-hibernation in 2008, when tadpoles were still available (despite the promise of a dry year to come), meadow snakes, like lakeshore snakes, showed increasing IGF-1 with size. However, during the two years of extended sampling over a two month period, meadow snakes showed markedly
different patterns from lakeshore snakes. In 2006, when conditions for amphibian breeding were favorable, IGF-1 levels in non-reproductive adult meadow snakes showed no relationship to size. In 2007, when conditions for amphibian breeding were unfavorable, larger mature snakes had lower plasma IGF-1 than smaller snakes.

This negative relationship between IGF-1 and size may reflect differential ability to obtain prey, where smaller snakes have the advantage in times of scarcity, or else may reflect ontogenetic changes that involve a juggling of energy investment priorities. There is some support for the former hypothesis in other snake species. Feeding rate in water pythons, for instance, has been shown to be lower in larger species during drought, as they have more difficulty obtaining prey from small openings (Shine & Madsen 1997). However, the latter hypothesis also merits investigation, as we do know that meadow snakes experience age/size-specific changes in energy investment: while reproductive effort (defined as grams reproductive output per gram female) remains constant in lakeshore snakes, it actually declines with age/size in meadow snakes (Sparkman et al. 2007). This may have important ramifications for IGF-1 activity during scarcity.

Plasticity between years aside, the fact that even in a wet (“good”) year, fast-growth lakeshore and slow-growth meadow snakes sampled over a two-month period exhibit fundamentally different relationships between body size and IGF-1 levels, suggests the possibility of evolution in the GH-IGF-1 axis. Growth begins to slow dramatically at maturity in *T. elegans*, so the increase in IGF-1 in mature lakeshore snakes is unlikely to be tied to increasing growth rate (Bronikowski & Arnold 1999). However we do know that reproductive output increases throughout life in lakeshore females, and shows minimal to no increase in meadow females (Sparkman et al. 2007). Thus we can hypothesize that mature
lakeshore snakes are exhibiting increases in IGF-1 levels that are associated with size-related increases in reproductive output, while IGF-1 levels in mature meadow snakes reflect the relative lack of change in output with age/size.

As previously discussed, higher reproductive output is associated with higher IGF-1 levels in reproductive females, which is consistent with reproductive lakeshore females having higher IGF-1 levels than meadow females. Increasing IGF-1 as age/body size increases in non-reproductive adult lakeshore females may reflect increasing devotion of resources towards reproduction, in both “on” and “off” years. Whether most males reproduce every year, and how their reproductive effort varies with age/body size is unknown. We do know, however, that IGF-1 stimulates spermatogenesis, and that testis size tends to increase with body size in many ectotherms (Baker et al 1996, Shine et al 1998, Nader et al 1999, Skinner & Watt 2007). Such an increase in IGF-1 with size suggests that males may also exhibit size-specific changes in reproductive investment.

**CONCLUSION**

This study presents three lines of evidence in support of a role for IGF-1 in mediating the evolution of life-history traits in *T.elegans*. First, IGF-1 was higher in high-reproduction lakeshore snakes than in low-reproduction meadow snakes in all three years of study. Second, in two years of two-month sampling, IGF-1 increased with body size in male and non-reproductive female adult lakeshore snakes, but remained constant or declined in adult meadow snakes. Finally, IGF-1 remained high and constant across the season with constant prey availability in the lakeshore habitats, whereas it declined in meadow snakes in a year with declining prey availability (a common occurrence in meadow habitats—see Bronikowski & Arnold 1999). Whether or not these ecotype differences in IGF-1 levels owe
more to plasticity in different environments than to evolutionary divergence has yet to be established, but at the very least they suggest that fast-growth snakes living in the lakeshore habitat may experience cumulatively higher IGF-1 levels over their lifespan due to its association with larger body sizes, higher reproductive output, and more consistent prey availability. Clearly, any assessment of whether free-ranging lakeshore snakes experience cumulatively higher IGF-1 levels over their lifespan would require many more years of close monitoring, preferably involving repeated measures of individuals. However, if lakeshore snakes do indeed experience a higher IGF-1 “load”, this may directly contribute to differences in survivorship between the two ecotypes by increasing the phenotypic rate of aging via increased IGF-1 receptor signaling.

Since this study focuses on levels of IGF-1 in free-ranging snakes, its findings are necessarily correlational, and cannot definitively establish causal links between IGF-1 and the evolution of growth, reproduction, and aging. To approach this system from a more experimental angle, neonates are currently being raised in a common environment to test for a genetic divergence in IGF-1 between the two ecotypes. Further investigation of key components of the IGF-1 system, such as binding proteins, receptor, and aging-related signaling pathways downstream, is also in order. Thus, the complex involvement of IGF-1 in *T. elegans* ecology, body size, and reproduction presented here provides a solid and promising foundation for further work testing for a role of the endocrine system in generating trade-offs between reproduction and survival in an ecological context.

**ACKNOWLEDGEMENTS**

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salmon: relation of size and growth rate to autumnal smolting. Aquaculture 222:149-
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insulin-like growth factor-I (IGF1) and IGF binding-protein levels in swine. Domestic
dependent, sex-dependent, and seasonal changes in insulin-like growth factor-II in
the Loggerhead Sea Turtle (Caretta caretta). General and Comparative


**TABLES & FIGURES**

**Table 1.** *Full Model.* Analysis of covariance (ANCOVA) with log IGF-1 (ng/ml) as the response variable. Asterisks denote significant effects.

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Table 2. SVL Model. Analysis of covariance (ANCOVA) with log IGF-1 (ng/ml) as the response variable. Analysis includes mature, non-gravid snakes. Asterisks denote significant effects.

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FIGURE LEGENDS

Figure 1. Validation of a human radioimmunoassay (RIA) for IGF-1 in *Thamnophis elegans*. Percent I-125 IGF-1 binding (B/B₀) against concentration of IGF-1 (ng/ml) for human standards of known concentration, and against volume (μl) of pooled snake plasma.

Figure 2. Full Model. Curve of log IGF-1 (ng/ml) against Julian days in 2006 (A) for gravid and non-gravid snakes in both ecotypes, and 2007 (B,C) for both fast-growth lakeshore and slow-growth meadow individually. Months are denoted on the x-axis for the sake of clarity. Asterisks denote significant slopes; NS=non-significant. Note: non-significant effects of Julian days and ecdysis are excluded from the 2008 model, due to the short length of the sampling period, and the relative lack of ecdytic snakes captured during that time.

Figure 3. Adult Body Size Model. Curves of log IGF-1 (ng/ml) against snout-vent length for two years. (A) 2006 fast-growth lakeshore, (B) 2006 slow-growth meadow, (C) 2007 lakeshore, and (D) 2007 meadow snakes. Asterisks denote significant effects. Note: significant effect remains even if large snakes are excluded from (C).

Figure 4. Reproductive Output Model. Simple linear regression of log field IGF-1 (ng/ml) against reproductive output (defined as total litter mass in g) of fast-growth lakeshore and slow-growth meadow snakes combined for 2006 and 2007.
Figure 1.
Figure 2.

(A) 2006 Gravid and Non-Gravid

(B) 2007 L-fast

(C) 2007 M-Slow
Figure 3.
Figure 4.

A

2006

L-Fast

M-Slow

B

2007

L-Fast

M-Slow

Reproductive Output (g)
CHAPTER 4. EVIDENCE FOR MULTIPLE UNPRECEDENTED PARALOGOUS DUPLICATIONS OF INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) IN THE REPTILE LINEAGE

Amanda M. Sparkman\textsuperscript{8}, Jeanne M. Serb\textsuperscript{2}, Scott E. Boyken\textsuperscript{3},

Neil B. Ford\textsuperscript{4}, & Anne M. Bronikowski\textsuperscript{2}

ABSTRACT

Insulin-like growth factor-1 (IGF-1) is a member of the vertebrate insulin family engaged in vital growth, reproductive, and survival-related activities. Its structure and function have been well characterized in mammals, birds, and fish, and IGF-1 genomic and/or mRNA sequences are available for multiple species within each of these groups, as well as for one amphibian species. In general, IGF-1 has been shown to be a single copy gene with an amino acid sequence that is highly conserved across the vertebrate phylogeny. However, a notable gap in our current knowledge of the IGF-1 gene has been evident in the complete absence of reptile sequences. We sequenced IGF-1 mRNA in fifteen reptile species—two turtles, three lizards, and eleven snakes. As might be expected, reptile IGF-1 showed high sequence identity with other taxa. Unexpectedly, however, all IGF-1 coding sequences were not identical either within species, or within individuals. Rather, each species, and many individuals within each species, showed unprecedented numbers of IGF-1

\textsuperscript{8}Primary researcher and author
\textsuperscript{2}Assistant Professor, Department of Ecology, Evolution and Organismal Biology, Iowa State University
\textsuperscript{3}Graduate student, Bioinformatics and Computational Biology Laboratory, Iowa State University
\textsuperscript{4}Professor, Department of Biology, University of Texas at Tyler
haplotypes that differed by one to several base pairs, many of which constituted an amino acid change. Moreover, many of these mutations were confirmed by replicate sequences across species, suggesting that they represent real variation. The evidence points towards multiple gene duplication events in the reptile lineage as the most plausible explanation for this explosion of IGF-1 haplotypes. Furthermore, we evaluate the biochemical ramifications of amino acid changes for structure and function, and explore key characteristics in the evolution of reptile physiology that might favor the maintenance of multiple forms of IGF-1.

INTRODUCTION
The insulin/insulin-like growth factor/relaxin (In/IGF/RLN) family is composed of a single insulin, two IGFs (IGF-1 and IGF-2), and varying numbers of insulin-like factors and relaxin genes across the vertebrate phylogeny. Invertebrates also carry a startling array of insulin homologs, designated insulin-like peptides (Wu et al. 2006), situating the root of the insulin family tree deep in the animal kingdom. Comparative studies of the vertebrate In/IGF/RLN family have suggested that it originated from a single ancestral preproinsulin-like gene, still retained in the extant cephalochordate amphioxus (*Branchiostoma californiensis*) (Chan et al. 1990), and subsequently diverged during several gene duplication events prior to and during the vertebrate radiation (Olinski et al. 2006). During this time, the family also split into two more or less distinct groups, where insulin and the IGF proteins retained more similarity to each other than to the relaxin and insulin-like factors (Olinski et al. 2006). Among vertebrate groups, insulin became predominately involved in the regulation of intermediary metabolic processes, while the IGF proteins specialized in the regulation of growth, development, and reproduction.
IGF-1, discovered in the 1950s, has recently entered the limelight, not only as a hormone largely responsible for proper growth, development, and reproduction (null mutations for IGF-1 being lethal or near-lethal in mice (e.g., Lui et al. 1993)), but also as a key player in the regulation of lifespan. Studies of insulin-like peptides in insects showing an association between decreased insulin-like receptor signaling and increased lifespan were the first to suggest the possibility that their vertebrate counterparts might also be involved in lifespan (Tater et al. 2003). In several landmark studies in mammals, this inference has been substantiated: for instance, both mice and humans with reduced IGF-1 signaling due to induced (mice) or natural (human) mutations in the IGF-1 receptor gene reportedly live longer lives (Holzenberger et al. 2003; Suh et al. 2008).

Its prominence in the regulation of key organismal life-history traits, and important ramifications for medicine and animal husbandry, has led to a finely detailed description of the IGF-1 system in mammals, fish, and poultry. Some studies have identified vagaries in IGF-1 function among different taxonomic groups and experimental contexts, but it remains the case that every major vertebrate group exhibits a highly conserved role for IGF-1 in growth, development, and reproduction (e.g., Perez-Sanchez et al. 1995, Guillette et al. 1996, Hiney et al. 1996, Kagawa et al. 1994, Beccavin et al. 2001, Uchida et al. 2003, Beckman et al. 2003).

IGF-1 is a peptide hormone primarily synthesized in the liver, and acts in an endocrine manner, though it may also exert paracrine/autocrine effects when produced locally in other tissues. It is released from the liver into the blood upon stimulation by growth hormone, and affects target cells of nearly every tissue type bearing IGF-1 receptors, where it promotes cell growth, differentiation, migration and survival. In mammals, birds
and fish (with the exception of tetraploids and the catfish, which has a brain-specific IGF-1 (McRory & Sherwood 1994)), IGF-1 has been confirmed as a single copy gene that encodes a mature 70-amino acid peptide made up of four domains, B-C-A-D. These domains are found on two exons, which are flanked by additional exons comprising the signal peptide and the E-domain of prepro-IGF, the precursor to the mature IGF-1 protein.

Allelic variation in non-coding regions of the IGF-1 gene appears to be widespread, and may have regulatory significance. Several studies have found associations between non-coding SNPs and various cancers, as well as growth rate in chickens (Cheng et al. 2006, Tamimi et al. 2007, Zhou et al. 2005), and one intriguing study has found a single SNP that is linked to body size differentiation among dog breeds (Sutter et al. 2007). Variation in IGF-1 expression is also manifest in mammals and fish through alternatively spliced mRNA, which involves transcript alteration of either the signal peptide region or the E-domain (e.g., Rotwein et al. 1986, Lowe et al. 1987, Duguay et al. 1992; Tanaka et al. 1998). These transcripts can have differing expression profiles, and may represent a fine-tuning of IGF-1 function during different developmental periods (Tiago et al. 2008).

In spite of these types of alternative splicing and allelic variation, the relative stability of the IGF-1 gene across widely divergent lineages is unmistakable. IGF-1 genomic and mRNA sequences obtained from a number of mammals, fish, birds, and a single frog species (Xenopus laevis) show remarkable conservation in the IGF-1 coding region, particularly in the B and A domains, where critical residues defining IGF-1 structure are located (Brzozowsky et al. 2002, Table 4).

The near-absence of knowledge about IGF-1 in reptiles stands out amidst the wealth of knowledge regarding IGF-1 in mammals, birds, and fish. While a handful of studies have
confirmed its accustomed role in reptile growth and reproduction (Crain et al. 1995a,b; Guillette et al. 1996; Sparkman et al. 2009) we have no information regarding the coding sequence and structure of reptilian IGF-1, and very little understanding of how the details of its function compare with those of other taxa. This information is fundamental for asking ecological and evolutionary questions, such as how IGF-1 influences the dynamics of ectothermy and indeterminate growth and reproduction in a terrestrial environment.

Currently, IGF-1 function has been examined in five reptile species: the loggerhead sea turtle (Caretta caretta), painted turtle (Trachemys picta), American alligator (Alligator mississippiensis), and western terrestrial garter snake (Thamnophis elegans) (Crain et al. 1995a,b; Guillette et al. 1996; Sparkman et al. 2009). A heterologous (i.e., cross-species) radioimmunoassay using an anti-human IGF-1 antibody that is commonly used in a variety of taxa was successfully validated for each of these species—in each case, native IGF-1 bound the anti-human antibody in a consistent manner. We were interested in stimulating further study of IGF-1 in reptiles, and thus attempted to validate the assay for a range of snake species. We began by validating the assay in the western terrestrial garter snake (Sparkman et al. 2009), and went on to test twelve additional snake species, as well as re-validate (for purposes of comparison) the painted turtle (originally validated in Crain et al. 1995a). Interestingly, we were only able to validate the assay for the painted turtle and eight out of the thirteen snake species examined (Sparkman, unpublished data). Of those eight snake species, the assay was validated for five Natricine species and a single Xenodontinae species; however we were unsuccessful with four out of six Colubrine species examined and a Pseudoxyrophiinae species validation was also unsuccessful. In these latter species, native IGF-1 did not consistently bind to the anti-human antibody in a predictable manner.
As the anti-human IGF-1 antibody has consistently bound IGF-1 in diverse vertebrate groups—mammals, birds, crocodilians and turtles alike (e.g., Wilson & Hintz 1982, Webster et al. 1996, Crain et al. 1995a, Schmidt & Kelley 2001)—we hypothesized that the IGF-1 coding sequence may have diverged in some snake species so as to alter protein shape in ways that prevent heterologous antibody binding. This phenomenon was of interest not only on a practical level, with regard to future assays of IGF-1 in reptiles, but also on an evolutionary level, considering the diverse history of structure and function in the insulin family. To test for potential variation in the IGF-1 coding region, we sequenced IGF-1 liver mRNA in squamates (snakes and lizards) and turtles across the reptile phylogeny (Fig. 1).

Here we present evidence gained from both squamate and turtle mRNA sequences that reptiles have experienced novel duplications and modifications of the IGF-1 gene. We confirm not simply that IGF-1 coding differs among snakes species (as suggested by our inability to universally validate the heterologous radioimmunoassay), but that it can vary within single individuals in both squamates and turtles. Combining our IGF-1 coding sequences with those from a wide range of other taxa in a phylogenetic analysis, we propose that the reptile/bird lineage experienced one or more paralogous duplications of the IGF-1 gene, and that an additional series of duplications subsequently arose in the squamate lineage. Finally, we assess amino acid changes found scattered throughout the coding region for potential to alter protein shape in ways that may have functional ramifications.

METHODS
Reptile species
IGF-1 liver mRNA was sequenced from fifteen reptile species, including two turtles from divergent families, *Chelydra serpentina* (snapping turtle) and *Trachemys scripta* (slider
turtle), and representatives of three lizard clades: an iguanid, *Anolis carolinensis* (green anole), an anguid, *Elgaria coerulea* (alligator lizard), and a skink, *Scincella lateralis* (ground skink). Sequences were also obtained from eleven species of snake, including the basal *Charina bottae* (rubber boa), *Agkistrodon contortrix* (copperhead), *Causus defilippi* (night adder), *Madagascarophis colubrina* (cat-eyed snake), *Pantherophis guttata* (corn snake), *Elaphe helena* (trinket snake), *Lamprophis fuliginosus* (house snake), *Spalerosophis diadema* (diadem snake), *Thamnophis marcianus* (checkered garter snake), and *Thamnophis elegans* (terrestrial garter snake) (Table S1). Phylogenetic relationships among these species are shown in Figure 1. All specimens were maintained at -80°C prior to RNA extraction. For all turtle and lizard species, a single individual was sampled. Numbers of individuals sampled per snake species varied from one to four (Table 3).

**RNA extraction and DNA sequencing**

Total RNA was extracted from the liver of each individual using the Trizol method following the manufacturer’s protocol (Invitrogen Cat. No. 15596-026). cDNA was reverse transcribed from total RNA using the SuperScript First-Strand Synthesis kit (Invitrogen Cat. No. 11904-018) with Oligo(dT) primers, also according to the manufacturer’s protocol. IGF-1 cDNA was amplified using a nested polymerase chain reaction (PCR). A nested design was employed to achieve higher specificity for the IGF-1 sequence alone than was achieved with a non-nested design. The PCR involved 5 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min. The reaction was terminated after 10 min of final extension at 72°C. As a nested
PCR, this program was run twice, once with a first set of primers using the product from the first run as a template, and a second time with a second set of primers.

Primers were designed from highly conserved regions in human, rat, fish, and chicken IGF-1 mRNA sequences obtained from GenBank (Table 1). Where base pair differences existed, preference was given to the chicken sequence for primer design. The primers for the first run of the nested PCR, 109F and 403R, flanked the 210 base pair IGF-1 coding region (Table 2). The primers for the second run, 151F and 353R, overlapped 27 base pairs at the beginning of the IGF-1 coding region, and three base pairs at the end.

A trial nested PCR was also performed using primers 109F and 403R for the first run, and 109F again, this time in combination with 353R, in the second run. We successfully obtained the full snake IGF-1 coding sequence for the snake *T. marcianus* (minus the last three base pairs) using this combination, but as it did not appear to work consistently among species we abandoned it early on for the combination of primers described above. However, we did design an alternate 151F primer based on the *T. marcianus* sequence rather than the chicken sequence, as they differed by two base pairs (one of which resulted in an amino acid change, P2Q) (Table 2). Given the high number of IGF-1 haplotypes per individual, both chicken and snake 151F primers were employed in separate reactions in order to eliminate the possibility that we were favoring the amplification of either more “snake-like” or more “chicken-like” IGF-1 (see Shuldiner et al. 1990, which describes how a certain combination of oligonucleotides in the cloning procedure favored the amplification of only one of the two copies of IGF-1 in *Xenopus*). IGF-1 cDNA from one individual from each reptile species was amplified in two separate reactions, once using the chicken 151F primer, and a second
time using the snake 151F. Snake 151F was used for all remaining individuals (all of which were snakes).

Nested PCR products were purified using a gel extraction kit (Qiagen Cat. No. 28704), and cloning was performed with the purified product using the pCR2.1 Topo Vector with Chemically Competent Cells (Invitrogen Cat. No. K4500-01). Transformed bacteria were spread onto LB-Ampicillin plates spread with 40 μl of X-gal (40 mg/ml) and grown at 37°C overnight. Colonies with inserts were picked at random from each plate, placed in 20μl sterile water, and lysed at 95°C for 10 minutes. Each resulting sample was screened using gel electrophoresis for the presence of an appropriately-sized insert after PCR amplification with vector-specific M13 forward and reverse primers (initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, followed by 10 min of final extension at 72°C). Samples containing inserts were sequenced using BigDye Terminator v. 3.1 (Applied Biosystems Cat. No. 4337455) with M13 plasmid primers (initial denaturation at 96°C for 1 min, followed by 35 cycles of 96°C for 10 sec, 54°C for 5 sec, and 60°C for 4 min). Sequencing clean-up was performed via EDTA-ethanol precipitation, and samples were sent to the Iowa State University DNA facility for sequencing. Alignment of the resulting DNA sequences was performed with BioEdit Sequence Alignment Editor 7.0.5.3 (Hall 1999). Electropherograms accompanying each sequence were evaluated closely for “clean” peaks, especially in positions where alignment indicated a base pair substitution. Any sequence encoding an apparently unique haplotype that was not robustly supported by the presence of clean peaks in the position(s) in question was excluded from further analysis.
**Phylogeny & duplication history**

Maximum parsimony and Bayesian gene trees were constructed from 128 IGF-1 nucleotide coding sequences, including 41 GenBank sequences from non-reptile species (Table 1). No duplicate reptilian haplotypes were used—if more than one species shared a haplotype, it was considered only once. The dogfish *Squalus acanthias* was designated as the outgroup species for both analyses, as cartilaginous fish are considered an outgroup to both bony fish and tetrapod clades, which comprise all other species used for these analyses (Mallatt & Winchell 2007). PAUP* 4.0b10 (Swofford 2002) was used to generate 1000 equally parsimonious trees using a heuristic search with 100 random addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Support was assessed with 2000 bootstrapping pseudoreplicates. The Bayesian analysis was conducted with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) using a general time reversible + gamma model, the best model according to AIC values generated by MrModeltest2.3 (Nylander 2004). The analysis was run with four Markov chains, saving the current tree every 100 generations for a million generations. The first 25% of trees (which included all trees prior to convergence) were discarded and the remaining trees were used to generate a 50% majority-rule consensus tree with posterior probabilities representing the fraction of samples recovering a given clade.

In addition to the phylogenetic analyses, a haplotype network was constructed using the median-joining method in Network 4.5.1.0 (Bandelt *et al.* 1999, Fluxus Technology Ltd, [www.fluxus-engineering.com](http://www.fluxus-engineering.com)) to show frequencies of different haplotypes in different species, as well as indicate relationships among haplotypes. Furthermore, a hypothetical reconstruction of duplication events was created by tabulating novel base pair substitutions in
birds, turtles, and squamates, in positions that are largely conserved across fish, mammal, and amphibian species. Highly variable positions, in which it was difficult to determine the ancestral states, were not included in the tabulation.

*Structural analysis*

As many of the reptilian IGF-1 sequences differed from one another by one or more amino acids, we determined the ramifications of these substitutions for protein structure. The structure of the IGF-1 protein has been resolved for humans (Brzozowsky et al. 2002), and contains three α helices (residues 7-18, 43-47, and 53-57), a 3.10 helix (58-60), and three disulfide bonds constituted by six cysteine residues ((6,48),(47,52),(18,61)), all located in the A and B domains and thought to be important for IGF-1 receptor binding affinity. We used homology modeling (SWISS-MODEL, Schede et al. 2003) to thread reptile IGF-1 sequences onto the human structure (Protein Data Bank ID ‘1GZR’, www.rcsb.org/pdb/home), and produced models that were virtually identical to the human structure (Fig. 2). However, as comparisons among reptile sequences essentially involved comparisons among multiple hypothetical structures, introducing a high level of computational error, we did not continue to pursue this approach. As an alternative, we analyzed the biochemical implications of each mutation for secondary protein structure. Specifically, we assigned each mutation to one or more of five basic categories: (1) change to glycine, (2) change to proline, (3) change in charge, (4) change in hydrophobicity, and (5) disulfide bond breakage. Glycine is the most flexible amino acid and can act like a hinge, affecting the orientation of the residues on either side. In contrast, proline is the most constrained amino acid and often results in twisting. Significant changes in hydrophobicity (from highly hydrophilic to highly hydrophobic amino acids, and vice versa), and changes in charge may also introduce changes in structural
conformation. Finally, the cysteine residues constituting the three disulfide bonds are essential for the structural integrity of IGF-1 (Brzozowsky et al. 2002). All squamate sequences were compared to amino acid variant C (aaC), which shows the highest conservation of base pairs with other vertebrate groups, while all turtle (and turtle-like—see Results) sequences were compared to aaA, which is identical to the bird sequence (see Table 4).

As mutations within the C and D domains are widespread among vertebrates, and many of these fall into the basic categories listed above (e.g., P33S and T66P in many non-fish vertebrates involve a change to/from proline, S38L in Xenopus involves a change in hydrophobicity), we focused our analysis on mutations with these effects that are found within the helical and disulfide bond regions in the A and B domain. We also evaluated mutations located within the IGF-1 anti-human antibody binding site (residues 12-22, Fig. 2), which might pose difficulties for antibody binding during a radioimmunoassay. Finally, we examined whether there are any changes in regions of aaC that were unique to reptile sequences that suggested any significant change in IGF-1 structure from other vertebrates.

RESULTS

Haplotype diversity

The final DNA alignment included 180 base pairs corresponding to amino acids 10-69 on the mature IGF-1 peptide. An average of twenty (range=12-33) IGF-1 clones were sequenced for each species, resulting in an unexpectedly high total of eighty-eight IGF-1 haplotypes in the fifteen reptile species combined (Fig. 3). Of these, sixty-three coded for unique amino acid variants. Fourteen of these haplotypes (HapA-HapM) were shared by two or more species (Fig. 3, Table 3A). Two sets of these shared haplotypes, HapI and HapJ, and
Hap L and Hap M contained synonymous base pair substitutions, and therefore coded for the same amino acid variants, aaI/J and aa L/M respectively (Tables 3A, B). However, several unique haplotypes found in only a single species also contained synonymous base pair substitutions, and coded for two additional amino acid variants, aaO and aaP. In total, fourteen amino acid variants (aaA-aaP) were shared by two or more species (Table 3B).

The two turtle species, *C. serpentina* and *T. scripta*, each possessed five IGF-1 haplotypes (Table 3A). Haplotypes within each turtle species differed from one another by one to two base pairs, and shared approximately 94% base pair identity with avian haplotypes. All five haplotypes coded for five different amino acid variants in *C. serpentina*, while (due to the synonymous nature of some mutations) only three amino acid variants were coded for in *T. scripta*. Amino acid variant aaA, which is identical to the avian IGF-1 amino acid sequence (Table 4), was coded for by 13/17 *C. serpentina* clones, and 14/16 *T. scripta* clones, while the remaining clones differed from aaA by a single amino acid change resulting from a single non-synonymous base pair substitution. Furthermore, 4/16 clones in the viperid snake *A. contortrix* also revealed haplotypes that coded for aaA, and one of these haplotypes was identical to a haplotype also seen in *C. serpentina*, HapA. The three additional turtle-like clones in that species differed from HapA by a single base pair. A similar haplotype coding for aaA (and differing from HapA by two synonymous base pair substitutions), was also present in the colubrine snake, *S. diadema* in 2/12 clones. All snake HapA/HapA-like (i.e., differing from HapA by one to two base pairs) haplotypes were obtained with the 151F primer, designed from the chicken IGF-1 sequence (see Methods, Table 2).

The distinctive squamate IGF-1 amino acid sequences differed from all other non-squamate taxa primarily in the C-domain with a characteristic “G_R_SSSTR signature
(though there are some modifications of this pattern—see aal/J, aaL/M and aaO) along with five other consistent amino acid changes: T29A and R55I, as well as E/D20E (Table 4). This last change converges with 20E seen in many fish species, but is evidently novel in squamates as it is coded by an ‘A’ rather than ‘G’ (as in fish) in the third codon position. Four novel synonymous base pair substitutions characteristic of squamates were also featured (base pair positions 28, 45, 90 and 144). In general, squamate haplotypes (apart from the bird/turtle-like haplotypes in the snake species described above) shared roughly 82% base pair sequence identity with birds, and 67-75% identity with teleost fish.

Within the most highly sampled individuals from any given species, haplotype numbers in squamates ranged from four (E. guttata, E. helena), five (A. carolinensis, C. bottae, C. defilippi, T. marcianus), six (T. elegans, S. diadema) and seven (E. coerulea, L. fuliginosus, M. colubrina), all the way up to twelve in the copperhead snake, A. contortrix, and the skink, S. lateralis (Table 3A). Agkistrodon contortrix shared six of its twelve haplotypes with other species (including HapA, shared with the turtle C. serpentina), while S.lateralis shared five haplotypes with other squamate species. The remaining haplotypes for these and other species were species-specific, or unique to an individual within a species. Amino acid variants, both shared among species and unique, were no less abundant as a large proportion of the base pair substitutions within haplotypes were non-synonymous (Table 3B).

Among squamates, there were two predominant IGF-1 haplotypes, HapB and HapC, found in nearly all snake and lizard species, with the exceptions of the snakes P. guttata and T. marcianus, which did not possess HapB, and C. defilippi, which did not possess HapC (Table 3A). (Note, however, that T.marcianus does have a haplotype that differs from HapB by a single non-synonymous mutation, and therefore codes for aaB (Table 3B)). HapB and
HapC differed from each other by a single base pair substitution, resulting in an amino acid change from 52L (in aaC and all other taxa) to 52P (Fig. 3, Table 4). In addition to these two major haplotypes, seven species, (five snakes, *C. bottae*, *A. contortrix*, *P. guttata*, *T. elegans*, and *M. colubrina*, and the skink *S. lateralis*) shared HapD, which differs from HapC by a single base pair, resulting in a change from the widely conserved 43I (in aaC and all other taxa) to 43T (in aaD) (Fig. 3, Table 4). In addition to HapA, HapB, HapC and HapD, eight other haplotypes shared by only two or three species also emerged, some relatively closely related species, and others more distant (Tables 3A).

For eleven of the thirteen squamates, either HapC and/or HapB were derived from randomly selected clones at the highest frequencies. The majority of clones from all three lizards, *A. carolinensis*, *E. coerulea*, and *S. lateralis*, as well as snakes *C. defilippi*, *A. contortrix*, and *S. diadema* exhibited HapB, while the majority from the snakes *C. bottae* and *T. marcianus* exhibited HapC. The snakes *E. helena*, *T. elegans*, and *M. colubrina* clones exhibited a fairly evenly distributed mix of HapB and HapC. The corn snake *P. guttata* was distinctive in that only 3/32 clones exhibited a shared haplotype, corresponding to HapC, HapD and HapL. In this species, the two most abundant haplotypes were unique and present in 13/32 and 7/32 clones, respectively. The house snake *L. fuliginosus* was also notable in that it had replicate clones for a fairly even mix of both shared and unique haplotypes.

**Phylogeny & Duplication History**

Maximum parsimony and Bayesian analyses produced virtually identical gene trees, with comparable bootstrap support and posterior probabilities (Figs 4 & 5). Both analyses showed strong support at the internal node dividing fish from all other vertebrates, and the node between mammals/amphibian and all birds, turtles, and squamates. There was only
weak support at the node dividing squamate from turtles/turtle-like and bird haplotypes, but strong support for distinct bird, turtle/turtle-like, and squamate clades. Since the majority of (non-turtle-like) squamate haplotypes differed by only 1-3 base pairs, Bayesian and parsimony trees resulted in a relatively uninformative spread of polytomies for the squamate clade. A clearer representation of relationships among haplotypes was given by a haplotype network, which revealed that all fourteen shared haplotypes fall into four main clusters from which all unique haplotypes (found only once within a given species) radiate (Fig. 3). One well-defined cluster is formed around HapC, and includes HapD, HapG, and HapH, and a second cluster is evident around HapB and includes HapE, HapF, HapK and HapM. A third, more loosely defined cluster of similar haplotypes includes HapI, HapJ, HapL and HapN. The fourth cluster consists of all turtle (T) and turtle-like haplotypes, and is closely allied with bird haplotypes. Both HapC and HapB clusters consisted of haplotypes from both lizards (L) and snakes (S); however, the third cluster did not contain any lizard haplotypes.

The most parsimonious duplication tree generated by analysis of lineage-specific base pair substitutions points to the occurrence of up to three independent duplication events (Fig. 6). The first duplication hypothetically occurred before the split of the bird/reptile clade, but after mammals and amphibians have branched off, as the basic squamate IGF-1 (represented by HapC) has five substitutions found in birds and turtles (and not shared by mammals/amphibians), but does not share eight subsequent substitutions present in the IGF-1 of birds, turtles, and two snake species. What became the most widespread squamate IGF-1 was presumably lost in the bird lineage, where only a single copy of IGF-1 has been reported, as well as in the turtle lineage, which only exhibits IGF-1 haplotypes that share high sequence identity with birds. Since birds, turtles, and two species of snake all share eight
substitutions not present in the squamate IGF-1, a second duplication event presumably occurred prior to the bird/turtle radiation, resulting in the multiple turtle/turtle-like IGF-1 haplotypes seen in turtles and the two snake species. There were three base pair substitutions held in common between turtle/turtle-like and squamate IGF-1 that were not found in birds, but this is likely a result of convergence, as the most recent molecular phylogenies (including our own) place turtles in a separate clade with birds and crocodilians (e.g., Hedges & Poling 1999, Iwabe et al. 2005, Krenz et al. 2005). Finally, a third duplication event is posited at the base of the squamate lineage (by which time sIGF-1 has accumulated 15 novel substitutions), resulting in numerous sIGF-1 haplotypes in squamates. A fourth duplication event (not pictured) may also have occurred in snakes, as they expressed a cluster of haplotypes (described above) not found in any lizard species. There is a possibility, however, that increased taxon sampling may recover such haplotypes in lizards.

Structural Analysis

A total of fifty-five squamate mutations distinct from aaC were found, as well as seven turtle/turtle-like mutations distinct from aaA. Of these mutations, scattered throughout all four IGF-1 domains, there were eight changes to proline, nine changes to glycine, twenty-six changes in charge, fifteen significant changes in hydrophobicity, and four breakages of disulfide bonds.

Of the twenty-two mutations occurring within the helical/disulfide bond regions of the A and B domains, nineteen resulted in potentially significant alteration of IGF-1 biochemical properties (Table 5). Only one mutation unique to all squamates (i.e., not found in other vertebrates), R55I, was present, resulting in a change in charge. Four mutations, C18R, C47Y, C48R, and C61Y involved breakage of disulfide bonds (of which the cysteine
(C) residue is a necessary component). Eight mutations lay within the anti-human IGF-1 antibody binding region. Two of these were in unique haplotypes from the snakes *T. elegans* and *L. fuliginosus*, for which the IGF-1 assay was successfully validated, while the remainder were in unique haplotypes found either in species for which the validation was not successful (*S. diadema, E. helena, P. guttata*), or has not been attempted (*E. coerulea, S. lateralis, M. colubrina, C. serpentina*). Several other mutations present in one to three species result in changes in charge and flexibility (i.e., changes to proline or glycine). Most significant of these was the mutation L57P, which is present in aaB, aaE, aaF, aaK and aaN, as well as in a number of unique sequences from individual squamate species. Also significant was the change in hydrophobicity in I43T, present in aaD, a widespread haplotype among squamate species.

**DISCUSSION**

*Haplotype diversity*

This study presents evidence for a proliferation of IGF-1 haplotypes in reptiles that is unprecedented in the vertebrate phylogeny. Both turtle species, *C. serpentina* and *T. scripta*, as well as two of the snake species, *A. contortrix* and *S. diadema*, exhibited an IGF-1 amino acid sequence identical to that of birds (aaA, Table 4). Unexpectedly, however, each turtle expressed five distinct IGF-1 haplotypes differing by a single base pair, while the two snake species carried two or four bird/turtle-like haplotypes, respectively, in addition to several characteristically squamate haplotypes (Fig. 3, Table 3A). All reptiles examined showed high haplotype diversity, with multiple haplotypes being expressed within single individuals, and species exhibiting a range of five to twelve haplotypes in total. Thus our hypothesis that IGF-1 is not the same in all reptiles (driven by our inability to consistently measure the IGF-1
protein in some snake species) has been confirmed in an entirely unexpected manner, with an important modification: it does not, in fact, appear to be the same even within a single individual.

There are a number of potential explanations for the high variability in IGF-1 liver mRNA sequences in reptiles, which stands in stark contrast to reports of a single IGF-1 coding sequence found in the majority of other vertebrates studied (with allelic mutations only rarely present—e.g., Walenkamp et al. 2005). Specifically, it is possible that mutational differences among reptile haplotypes are (1) due to sequencing error, (2) a result of alternative splicing of the same gene, or (3) evidence for multiple gene duplications events.

Errors in the IGF-1 sequencing process may have occurred during the synthesis of cDNA by the reverse transcriptase, or during amplification of IGF-1 by Taq DNA polymerase in three separate PCR cycles. The error rates for these steps are $6 \times 10^{-5}$ (Invitrogen Tech. Support, pers. comm.), and $0.8 \times 10^{-5}$, respectively (Cline et al., 1996). On the biological end, transcription error is thought to be quite low—about $0.3 \times 10^{-5}$ (Ninio, 1991). Cumulatively, the probability of mutational error is roughly $9 \times 10^{-5}$. Out of 271 squamate clones sequenced in our study, 212 differed from HapC by at least a single base pair. If all of these substitutions were to be considered errors (a conservative estimate of error, as many haplotypes differed from HapC by more than one substitution), it would suggest an error rate of $400 \times 10^{-5}$—approximately 44 times higher than predicted. In other words, while less than $5/212$ mutations in squamate sequences may be a result of transcription/sequencing error, this rate presents no significant challenge to the validity of the remaining mutations. (Note that a similar mutation rate was calculated for turtle/turtle-like sequences that differed from HapA.)
While it is possible to question whether specific mutations that appear only once are a result of sequencing error, the existence of multiple haplotypes in reptiles is robustly supported by replicate clones. If we set aside those haplotypes that appear only once, as well as those that are found in three or fewer species, we are still left with clear haplotype variation that is strongly supported across species. With this highly conservative approach, the existence of at least four haplotypes is evident in reptiles: HapA (2/2 turtles, 2/13 squamates; shared with birds), HapB (11/13 squamates), Hap C (12/13 squamates), and Hap D (7/13 squamates) (Table 3A).

As it is clear that real variation in IGF-1 mRNA in reptiles does exist, we should consider next whether these haplotypes provide evidence for alternatively processed mRNA from a single gene, or suggest the existence of duplicate genes. Alternative splicing of IGF-1 mRNA has indeed been reported in mammals and fish, occurring either at the 5' or 3' end of the transcript (e.g., Rotwein et al. 1986, Lowe et al. 1987, Duguay et al., 1992; Tanaka et al., 1998). It results in the alteration of the signal peptide at the 5', and of the E-peptide at the 3' end. While both of these regions are translated from mRNA transcripts, they are cleaved from the pro-IGF-1 protein (during post-translational modification), and so are not part of the mature 70-amino acid IGF-1 protein. There are no reports of alternative splicing in the coding region of the mature protein. All in all, it would be difficult to make a case for a role of alternative splicing in the variability in IGF-1 we see in reptiles, as the nucleotide changes in the different haplotypes are scattered throughout the coding region of the mature protein, and do not show patterns characteristic of consistent alternative splicing at exon/intron junctions.

Given that more haplotypes are present within individuals than can be explained by
allelic variation in a single copy gene alone, all evidence thus far suggests that the IGF-1 gene has experienced paralogous duplications leading to multiple haplotypes in reptiles—up to four haplotypes in a single individual strongly supported, and supplementary evidence (partially relying on sequences that appeared only once) for up to twelve haplotypes per individual (Table 3A). However, one point of counter-evidence to an entirely duplication-based explanation must be considered here. A “BLAT” search of the Anolis carolinensis Genome Browser Gateway (The Broad Institute, http://www.broad.mit.edu/models/anole) results in a single IGF-1 match (scaffold_72:3071743-3120828). This region contains the entire IGF-1 sequence, split into two parts by a large intron. Curiously, while this IGF-1 does share 86% nucleotide identity with the squamate IGF-1 sequences described in this study and confirms the P2Q mutation identified in our full sequence of the snake, *T. marcianus* (see Methods), it differs from our *A. carolinensis* and all other squamate sequences by five amino acids (S34N, S38I, S29A, T40A and I55T). To further complicate matters, a nucleotide blast search (nBlast) on GenBank (NCBI Accession # FG759753.1) reveals an IGF-1 transcript from *A. carolinensis* in a cDNA library of the ovary that differs from the Broad Institute genome sequence by two amino acids (D12Y and R21G). We are uncertain why sequences from both of these *Anolis* projects differ from our squamate sequences, and why the ovary cDNA differs from genomic DNA, if IGF-1 is indeed a single copy gene in this species. This discrepancy may be due to error in the compilation of the *Anolis* genome, where multiple copies may have been conflated to appear as one copy; alternatively, it may indicate there is variation in IGF-1 copy number among individuals, as has been reported with several human genes (Sebat *et al.* 2004, Redon *et al.* 2006). We mention this quandary primarily as a note of caution. If in fact further characterization of
reptilian genomes suggests only a single genomic IGF-1 exists, investigation should turn towards how a single gene could produce the multiple IGF-1s shown here in reptiles—specifically, what type of transcript processing, consistent across species, could result in identical base pair substitutions scattered throughout the coding region. Given that such a mechanism is currently unknown, we conclude that gene duplication is as yet the most parsimonious explanation for our findings.

*Phylogeny & duplication history*

As it now stands, the *Anolis* genome does not challenge at least one gene duplication event resulting in two snake species (*A. contortrix* and *S. diadema*) having two types of highly divergent IGF-1—one characteristically squamate, and one of a kind with turtles, as is clearly shown in maximum parsimony and Bayesian gene trees (Figs. 4 & 5). Other squamate species (such as *Anolis*), may have lost the turtle-like IGF-1, but the fact that both a viper and colubrid, at distant points on the snake phylogeny (Fig. 1), exhibit turtle-like IGF-1 as well as the characteristically squamate IGF-1 stipulates a gene duplication event early in the reptile lineage. A hypothetical gene tree superimposed on a species tree tracking the duplication and loss of IGF-1 copies across the vertebrate radiation suggests that the first duplication event occurred prior to the bird/turtle and squamate split, and that birds and turtles subsequently lost what became characteristic squamate IGF-1 (Fig. 6). Two additional duplication events putatively occurred prior to the bird/turtle and squamate divide, and the squamate radiation: the first resulted in multiple copies of the bird-like IGF-1 in turtles and two snake species, and the second resulted in multiple copies of the characteristically squamate IGF-1. Due to high sequence identity, the relationships among squamate IGF-1 haplotypes remains unclear; however, the fact that there are two main
haplotype clusters with multiple lizard and snake haplotypes suggests that multiple duplication events may have occurred in the squamate ancestor within a relatively short period of time. Furthermore, the existence of a third cluster that does not contain any lizard haplotypes suggests that an additional duplication event may have occurred in the snake lineage (Fig. 3), though increased taxon sampling in lizards should be conducted to confirm this hypothesis.

The existence of multiple IGF-1 haplotypes presents the possibility of innovation in function (Ohno 1970). According to theoretical predictions, one copy of a duplicated gene may be freed from selective constraint and begin to accumulate mutations without immediate consequences for fitness (Kimura & Ohta 1974). In most cases, this copy will degenerate into a non-functional “pseudogene”, as such mutations will rarely be beneficial (Mighell et al. 2000; Lynch & Conery 2000). However, in some cases, this copy may retain a function, either identical to that of the ancestral gene (if the coding and/or regulatory regions continue to be well-maintained for both duplicates) or, through changes in protein shape, timing, or context of expression, one copy may take on a novel function (neofunctionalization) or subdivide the ancestral function (subfunctionalization) (Kimura & Ohta 1974). The deep history of the insulin family itself is a story of subfunctionalization, and may have included components of neofunctionalization, as insulin, the IGFs, insulin-like factors and relaxins divided up roles in metabolism, growth, development and reproduction after they arose from a series of gene duplication events (Steiner et al. 1985).

There have also been a handful of reports of more “recent” duplication events in the insulin family. In mammals, rats and mice have two insulin copies (Smith 1964, Clark & Steiner 1969, Bunzli et al. 1972, Wentworth et al. 1986), one of which is thought to have
originated as a functional retrotransposon (Soares et al. 1985). The channel catfish (*Ictalurus punctatus*) also has two copies of insulin (Mommsen 2002), as well as two forms of IGF-1: a ubiquitous IGF-1 as well as highly divergent brain-specific form (McRory & Sherwood 1994). Whether these duplicate forms are different alleles for the same gene copy, or originate from duplicate genes remains to be determined. Other examples of duplicate genes in the insulin family are also evident in tetraploid organisms, including *Xenopus laevis* and a number of fish species (Shuldiner 1989, Shuldiner 1990, Wallis & Devlin 1993, Papasani et al. 2006). Whether due to a genome-wide duplication event, or a more localized duplication, these examples of multiple copies of key hormones shed light on the possibility of subfunctionalization of the IGF-1 haplotypes in our system. For instance, the two insulins in the tetraploid zebrafish (*Danio rerio*) show differential expression during development (Papasani et al. 2006), and one IGF-1 in *X. laevis* (also tetraploid) appears to be more prevalent during organogenesis, while the other is more prevalent during premetamorphic growth (Perfetti et al. 1994). Thus there is precedent for more recent functional diversification in the insulin family that may also have occurred in reptilian IGF-1 haplotypes.

**Structural analysis**

All squamate variants carry a R55I mutation that results in a change in charge in a position in an alpha helix that remains invariant in all other taxa. However, as certain other species also exhibit single amino acid changes with novel biochemical effects (e.g., R55G is a change to glycine in *Sebastes*, D20P is a change to proline in *Mus musculus*, T29I is a change in hydrophobicity in *Eospalax*) but presumably continue to maintain proper IGF-1 functioning, it is unlikely that this change has significantly altered the ability of squamate
IGF-1 to bind its receptor. As the IGF-1 variants in each reptile species differ by a mere one to three amino acids (turtle-like vs. typical squamate comparisons excepted), they may well continue to bind the IGF-1 receptor (as yet uncharacterized in reptiles) with equal affinity. Our analysis of the biochemical effects of individual amino acid changes suggests that the majority of reptile variants do not appear to exhibit crucial changes, and thus likely represent fully functional forms of IGF-1. However, four haplotypes (each found only once in a single individual) carry mutations that result in breakage of a disulfide bond, thus destroying the integrity of the IGF-1 protein structure (Table 5). If these haplotypes represent different copies of IGF-1, this suggests that these copies are most likely pseudogenes, as their protein product will be incapable of proper IGF-1 binding. The greatest potential for differential receptor binding affinity exists in aaB, aaE, aaF and aaK (as well as numerous unique variants found only once), which differ from aaC and other variants by a single proline (L57P), which may result in increased twisting. This is particularly of interest as aaB and aaC appear to be abundant within single individuals. Another variant that is widespread across species, aaD, may also exhibit differences in binding affinity, due to a mutation (I43T) resulting in a significant change in hydrophobicity. Whether or not the structural differences among any of the amino acid variants has functional ramifications, however, remains to be seen.

Evolutionary significance

It is important to note that whether or not the structure differs significantly among IGF-1 variants, fine-tuning of IGF-1 variant function may occur spatially or temporally. Elucidation of expression patterns of the different variants in different tissues or at different intervals during development would be necessary to establish whether any kind of
subfunctionalization has indeed occurred. It is also possible that these variants are selectively maintained not for functional disparity, but for increased volume of IGF-1 expression. Our work examining patterns of plasma IGF-1 in African house snakes (*L. fuliginosus*) has provided some evidence that IGF-1 peaks suddenly upon mating (Sparkman *et al.*, in prep). While regulatory changes in IGF-1 with respect to maturation and gestation have been described in a number of species, this mating-induced response has not been reported in any other group. Interestingly, mating-induced ovulation has been documented in both pythons (a basal group) and garter snakes, suggesting this may be a widespread phenomenon among snakes (Mendonca & Crews, 1990, Denardo & Autumn, 2001). This response may have evolved as an adaptation to the low densities which characterize many snake populations, allowing females to avoid unnecessary costs of ovulation when the odds of male presence are uncertain. Whatever the case, a peak in IGF-1 accompanying ovulation at mating may facilitate a rapid transition into reproductive mode required in such a situation (see Patthy 2008). And, in turn, having multiple copies of IGF-1 may allow a female to respond more quickly and flexibly to the mating stimulus, thus conferring a selective advantage.

There are a variety of scenarios such as this that might explain how diverse IGF-1 haplotypes within an individual continue to be maintained among extant non-avian reptile species. Most pertinent, however, are scenarios at the base of the reptile and squamate radiations that might explain why these duplications were maintained at the outset—especially in light of the fact that chickens, at least, appear to retain only a single copy of IGF-1. To understand potential ecological and physiological factors that would encourage the retention of IGF-1 variants, it may be helpful to consider general characteristics of
ancestral reptiles, in which the first IGF-1 duplication(s) putatively occurred.

Key innovations of the reptile lineage were made in the context of a transition from aquatic to terrestrial life. Reptiles (and birds nested within that lineage) carry several novel adaptations to terrestrial existence, including the evolution of thick, scaly skin (reducing evaporative water loss), internal fertilization (also shared with mammals), and shelled, amniotic eggs (Hickman et al. 2007). All of these characters continue to be shared among birds and other reptiles—crocodilians, turtles, tuatara, and squamates. However, the evolution of endothermy created an important physiological divide between birds and other reptiles. While ectothermic reptiles may have greater thermoregulatory capacity than amphibians, due to their superlative ability to absorb radiant heat energy (limited in amphibians due their thin-skinned susceptibility to evaporative water loss), they experience much larger fluctuations in metabolic rate than birds due to greater vulnerability to daily and seasonal changes in temperature. In addition, since the energetic requirements of ectothermy are much less demanding, reptiles may eat much less frequently, particularly when food is scarce. Among squamates, snakes are particularly known for their flexible metabolism (Secor & Diamond 1998; Anderson et al. 2005). Finally, the maintenance of traits such as indeterminate growth and reproduction (where organisms continue to grow throughout life, and increase reproductive output as they grow) in non-avian reptiles further sets them apart from birds, which like mammals, tend to cease growing after maturation and reproduce in relatively constant numbers during their reproductive years (and often show declines in reproductive output at late ages) (Martin 1995, Holmes et al. 2001).

One notable example in snakes associates dramatic genetic change with the evolution of metabolic flexibility: modern snakes (Alethinophidia) have duplicated mitochondrial
control regions and show evidence of having experienced accelerated rates of evolution in several mitochondrial genes critically involved in energetics (Kumazawa et al. 1998; Dong & Kumazawa 2005; Kumazawa 2004; Jian et al. 2007; Castoe et al. 2008). In a similar fashion, multiple copies of IGF-1 may have proved beneficial in the establishment of the physiological characteristics of early reptiles described above. The influence of IGF-1 on growth and reproduction has been well established across taxa, including reptiles. In mammals, plasma IGF-1 tends to increase during puberty, peak in adulthood, exhibit isolated peaks during gestation, and decline later in life (Roberts et al. 1990; Abbasi 1993; Holmes et al. 1997, Pollak 2000). In some populations of garter snakes, however, plasma IGF-1 increases with size/age with no sign of a decline (Sparkman et al. 2009). Thus, while the mechanistic relationship between IGF-1 and indeterminate growth and reproduction is unknown, it is possible that multiple copies of IGF-1 could foster a sustained or increasing demand for IGF-1 throughout life.

It is important to note in this history of evolutionary events, that the majority of fish also exhibit ectothermy (though perhaps not with the same degree of temperature/metabolic fluctuation in the aquatic as occurs in the terrestrial environment), as well as indeterminate growth and reproduction. However, the series of duplications described here does not appear to have occurred until after the colonization of land, so it is uncertain whether fish might also have found multiple copies useful for similar reasons (though tetraploid fish with two intact copies of insulin family hormones suggest this might be the case). And while the proliferation of IGF-1 is unique to non-avian reptiles, and squamates in particular, there may have been an interaction between ancestral traits shared with fish, and the novel traits shared with birds, that made multiple copies of IGF-1 distinctively advantageous to reptiles in the
CONCLUSION
This study documents a proliferation of IGF-1 mRNA diversity in non-avian reptiles, with multiple haplotypes present in single individuals—a phenomenon not reported in any other vertebrate group. As mutations in different haplotypes are scattered across the coding sequence, and do not reflect patterns in alternatively spliced forms of IGF-1 in other taxa, the evidence points towards multiple gene duplication events in the reptile lineage as the most plausible explanation. This conclusion is further supported by the simultaneous presence of two highly differentiated haplotype types, one distinctively squamate and the other distinctively bird/turtle-like, in individuals from two distantly related snake species. Further research targeting genomic DNA, however, is needed to definitively establish the existence of multiple IGF-1 gene copies. Furthermore, receptor binding affinity and the dynamics of developmental regulation among haplotypes are also promising avenues of research, and will allow us to evaluate the significance of multiple IGF-1 haplotypes for reptile physiology and life history.

ACKNOWLEDGEMENTS
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Sluimers, C.A., Bax, J.J., de Laat, J.A.P.M., Breuning, M.B., Romijn, J.A., & Wit,
### Table 1. GenBank accession numbers of non-reptile species used for maximum parsimony and Bayesian analyses. The ID indicated for each species is that used for the haplotype network (Fig. 3). ‘~’ indicates that a species was not used for the haplotype network.

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Table 2. Primer sequences for reptile IGF-1 mRNA sequencing. ‘F’ indicates a forward primer, while ‘R’ indicates a reverse primer. Two 151F primers were designed, one from the chicken (*Gallus gallus*) sequence, and one from the *T.marcianus* sequence (see Methods). Bold letters in 151F sequences indicate differences between chicken and snake-based primers.

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<tr>
<td>403R</td>
<td>5’-CAC TTC CTT TTG TGC TTT TGG CAT ATC-3’</td>
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<tr>
<td>353R</td>
<td>5’-CGC TGA GCA CGT ACA GAG CGT G-3’</td>
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<td>151F (chicken)</td>
<td>5’-GGC CCA GAA ACA CTG TGT GGT GCT GAG 3’</td>
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<tr>
<td>151F (snake)</td>
<td>5’-GGCCAA GAA ACA CTT TGT GGT GCT GAG 3’</td>
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Table 3. Chart of haplotypes (A) and amino acid variants (B) for fifteen reptile species. The number of individuals sampled, number of clones sequenced per species, and the number of variants found within a species are indicated in each table. The numbers in parentheses in the total number of haplotype/amino acid variant columns represent the range for those variables among individuals within a species (individuals for whom only a 1-2 clones were sequenced explain the low end of the ranges). The incidence of shared haplotypes/amino acid variants in any given species is indicated by an ‘x’. The ID for each species indicates the ID used for unique haplotypes in the haplotype network, and the variant IDs correspond to haplotype IDs of the shared haplotypes that code for those variants. aaI/J and aaL/M were coded for by two shared haplotypes (HapI/HapJ, and HapL/HapM, respectively). Two variants, aaO and aaP do not have corresponding haplotypes listed in the shared haplotype chart, as each of these was coded for by unique haplotypes that differed by one to two synonymous base pair substitutions.

### (A)

<table>
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<th># clones</th>
<th>total # haps</th>
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SNAKES

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Table 4. Alignment of IGF-1 amino acid sequences for outgroup and reptile species. *Homo sapiens*, two representative fish (Fish1= *Siganus*, Fish2= *Salmo*), a frog (*Xenopus laevis*), and a conserved bird sequence (identical in several species of birds), followed by a variant present in two turtle species and two snake species (aaA), and additional shared squamate variants (aaB-aaP). Dots represent identity corresponding to *H. sapiens* residues. Amino acid residues 1-9 and 70 were not sequenced in reptiles. IGF-1 domains (B-C-A-D) are defined by arrows.

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Table 5. Identity, effect, and incidence of mutations within helical domains of squamate and turtle haplotypes. * indicates mutation occurred within the anti-human IGF-1 antibody binding region. Significant changes in hydrophobicity are indicated as ‘hydr’; ‘Gly’ represents a change to glycine, ‘Pro’ a change to proline. The incidence of a mutation is referenced either to the shared amino acid variant that carries it (Table 4) or, if unique, to the haplotype ID used in the haplotype network (Table 3, Fig. 3).

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<td>charge, hydr</td>
<td>T1A5</td>
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FIGURE LEGENDS

Figure 1. Cladogram of the fifteen reptile species from which IGF-1 liver mRNA sequences were obtained. Relationships are based on molecular phylogenies from Lawson 2005 & de Quiroz (2002), Vidal & Hedges (2005), Krenz et al. (2005) & Blair & Hedges (2005). Other major vertebrate groups are also shown as outgroup species.

Figure 2. 3D model of squamate IGF-1 (aaC in Table 4) threaded onto human IGF-1. The three disulfide bonds (constituted by six cysteine residues), and the location of the three α helices are indicated. The anti-human IGF-1 antibody binding region is shown in white.

Figure 3. IGF-1 haplotype network. Open circles are snake haplotypes, light grey are lizard, dark grey are turtles, and black are birds and other outgroup species (species key in Tables 1&3). Size reflects the frequency of haplotypes among species. Pie charts for shared haplotypes are divided according to the number of species within a group that carry that haplotype. Small black unlabelled nodes indicate mutations shared by all haplotypes derived from that node, but not present in the haplotype prior to that node. Each tick on connecting lines represents one base pair difference between haplotypes. Dark lines represent large numbers of mutations between groups: 29 base pair changes from squamate to bird/turtle/turtle-like, 31 to frog, 32 to mammal, and 42 to fish. Shared Hap IDs correspond to aa variant IDs, while unique haplotype IDs contain the species ID (T#, L# or S#--see Table 3), followed by the individual ID (A, B, C or D) and a number differentiating haplotypes within that individual.

Figure 4. Phylogram from maximum parsimony analysis. Based on one of 1000 equally parsimonious trees. Bootstrap support are values shown at key internal nodes. “Snakes & Lizards” is a collapsed clade consisting of multiple polytomies of squamate haplotypes.

Figure 5. Consensus tree from Bayesian analysis. Posterior probabilities are indicated at interior nodes. “Snakes & Lizards” is a collapsed clade consisting of multiple polytomies of squamate haplotypes.

Figure 6. Hypothetical phylogenetic history of IGF-1 gene duplications, represented by mapping a gene tree onto a species tree. Duplication events are depicted as black or gray boxes. The first duplication event is posited at the base of the reptile lineage. After this point, black lines represent IGF-1 copies that have high sequence identity among birds, turtles, and two snake species, while gray lines track the distinctively squamate IGF-1 copy. A second duplication event is also posited before the bird/turtle/squamate split, after the accumulation of eight mutations not seen in the distinctively squamate copy, but present in all descendant bird/turtle/snake copies. A third major duplication event is posited for the squamate IGF-1 copy after the accumulation of 15 mutations shared among all descendant copies. Loss of copies at the base of the bird and bird/turtle lineages are indicated by dashed lines. The exact number of duplicates generated at any point is uncertain, so only a limited number are depicted here.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
**SUPPLEMENTARY TABLE**

**Table S1.** Specimen details.

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APPENDIX. IGF-1 & Growth Rate: A Common Garden Experiment
Because IGF-1 modulates growth rate in mammals and birds, in addition to its pivotal role in stress resistance and aging, we sought to quantify how IGF-1 secretion impacts growth rate in T. elegans. Neonates from wild-caught dams born in August/September 2006 were bled at five different time points during their first two years of life (Table 1). The first two bleeds, during December 2006 and February 2007, did not reveal any significant relationships between plasma IGF-1 and growth rate or IGF-1 and feeding rate. Retrospective analysis revealed that snakes were experiencing high mortality and stress during this time due to food poisoning from their trout diet, which may have obscured any relationship.

We took blood samples again several months later, in July 2007, believing that the snakes had recovered and were now growing properly. **We found a negative relationship between IGF-1 and both growth and feeding rates over the previous two months (May - July), contrary to what we expected and to what has been reported in other species.**

Upon analysis of the growth data itself, it became clear that between May and July measurements, lakeshore snakes were not growing faster than meadow snakes, as had been previously documented (Bronikowski 2000). Uncertain how early-life stress and strong selection against large snakes, who ate most of the trout early in life, might have contrived these results, we did not bleed the snakes again until the following year.

We decided to bleed the 2006 cohort again in March 2008 before they transitioned from individual housing to group housing, where individual feeding rates could no longer be monitored. Even though we were aware that lakeshore snakes were still not growing faster than meadow snakes, we anticipated that IGF-1 would still be positively correlated with
growth rate regardless of ecotype, now that the snakes had had sufficient time to recover from early-life trauma. We tested whether individual growth rates during the preceding four months, along with feeding rates, related significantly to plasma IGF-1. No significant relationships (positive or negative) were found. This may be a result of the fact that growth rates were very low during this time (Fig. 1A). Furthermore, growth rates may have been fluctuating between measurement dates, as February/March feedings rates were significantly correlated to January feeding rates for meadow individuals, but not for lakeshore individuals.

Due to extraordinary colony history and health incidences, we switched our focus to the next year’s cohort of animals. These animals were born in the Fall of 2007. As of June 2008, lakeshore snakes were growing faster than meadow snakes (Fig. 1B). We bled these animals upon euthanization in November 2008. We also bled the surviving 2006 cohort at this time. Five-month growth rates were calculated from measurements in June and November 2008 for both cohorts. One-month growth rates were also calculated for the 2006 cohort between November 2008 and December 2008, for a more current index of growth.

Similar to all previous findings in the 2006 cohort, plasma IGF-1 was not predictive of either five-month or one-month growth rates (we could not relate feeding rate to IGF-1 in these animals, as they were group housed and individual feeding rate was unknown). The 2007 cohort showed a negative relationship between IGF-1 and five-month growth rate. An analysis of growth data revealed that while lakeshore snakes had been growing faster than meadow snakes in June, at some point over the summer meadow snakes began to grow faster than lakeshore snakes (Fig. 1B). This flip-flop was likely due to heat stress, which also resulted in high mortality, particularly in lakeshore snakes. Thus, a straightforward explanation of the negative correlation between IGF-1 and five-month growth is that growth
rates had changed dramatically between June and November. Because feeding is measured weekly (unlike size, which is measured quarterly), we were able to perform a more fine-scaled analysis of IGF-1 and feeding rate during the month preceding bleed date. **There was a significant positive relationship between plasma IGF-1 and one-month feeding rate in the 2007 cohort (Fig. 2A).**

The July 2007 data for the 2006 cohort (negative relationship between IGF-1 and two-month growth), and the November 2008 data for the 2007 cohort (positive relationship between IGF-1 and one-month feeding) carry the most weight—the former because relatively short-term rates of growth were known, and the latter because it provided (as far as feeding rate was concerned) the expected results. We have no *a priori* reason to believe that growth rates calculated between May and July 2007 did not accurately reflect the current growth rate for the 2006 cohort, but its negative correlation with IGF-1 runs counter to findings in any other species, and thus is very much open to question. The fact that growth has fluctuated a great deal in this cohort (Fig. 1A) suggests that at the time of bleeding in July, the snakes were just beginning to feel the effects of some sort of stressor, which was not picked up in subsequent growth data, as no measurements were made again until November 2007.

Corticosterone levels in the 2006 cohort (at birth, and at two years of age) were an order of magnitude higher than those of similarly-sized juveniles in the field, and adult female levels were also elevated in the lab (Robert *et al.* 2008, Sparkman unpublished data). Such high levels of physiological stress may be problematic for measurement of other physiological variables, and indicate that *T.*elegans may not adapt easily to the lab environment. In this case, independently occurring stressors (related to food, temperature, or housing), whether operating directly through suppressive effects of corticosterone or not,
resulted in highly plastic individual growth rates. As any conclusions regarding the relationship between IGF-1 and growth rate must be accompanied by robust estimates of actual growth rate at the time of bleeding, this plasticity prevented us from achieving our goal.

LITERATURE CITED
Table 1. Details regarding date of blood sampling, assay results, and relevant circumstances surrounding each assay of plasma IGF-1 in neonate *Thamnophis elegans* from two different cohorts.

<table>
<thead>
<tr>
<th>Date</th>
<th>Correlation: IGF-1 &amp; growth rate</th>
<th>Correlation: IGF-1 &amp; feeding rate</th>
<th>2006 Cohort Notes</th>
<th>2007 Cohort Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>December 2006</td>
<td>none</td>
<td>none</td>
<td><em>trout poisoning</em> --period of high mortality; feeding rates uninformative, growth rates may be fluctuating</td>
<td>~</td>
</tr>
<tr>
<td>January 2007</td>
<td>none</td>
<td>none</td>
<td><em>trout poisoning</em> --period of high mortality; feeding rates uninformative, growth rates may be fluctuating</td>
<td>~</td>
</tr>
<tr>
<td>July 2007</td>
<td>negative</td>
<td>negative</td>
<td>growth and feeding rates available between May and July but growth rates may have been fluctuating during this time, possibly due to heat stress</td>
<td>~</td>
</tr>
<tr>
<td>March 2008</td>
<td>none</td>
<td>none</td>
<td>only have growth rates over a 4-month period (November-March); growth rates were very low during this time, and feeding rates were fluctuating</td>
<td>~</td>
</tr>
</tbody>
</table>
| November 2008 | 2006 Cohort: none  
2007 Cohort: negative | 2006 Cohort: n/a  
2007 Cohort: positive |  
no current individual feeding data due to group housing; previous measurement was in June, and growth rates were suppressed compared to the previous summer--this was likely due to heat stress as there was also high mortality (particularly in lakeshore snakes) in July/August; post-assay growth rates were very low between November and December | positive correlation between IGF-1 and October feeding, but have no current growth measures, which is important, as individual growth rates shifted dramatically over the summer--possibly due to heat stress |
Figure 1. Growth rate variation over time in two *T. elegans* cohorts. Repeated measures analysis of growth rate for the 2006 (A) and 2007 (B) cohorts was performed with ecotype, sex, and ecotype*sex as fixed effects, birth size as a covariate, and litter nested within ecotype as a random effect. For the 2006 cohort (A) there were no significant effects. For the 2007 cohort (B), there were significant effects of time*eco (*P* < 0.0001) and birth size (*P* = 0.0412). Standard errors of the mean are shown.
Figure 2. Relationship between plasma IGF-1 and one-month feeding rate (A) and five-month growth rate (B) in the 2007 cohort. Scores are corrected for other effects in an ANCOVA model with ecotype, sex, and sex*ecotype as fixed effects, one-month feeding rate, five-month growth rate, and birth size as the covariates, and litter nested within ecotype as a random effect. Only one-month feeding rate and five-month growth rate were significant effects (shown). Note that if five-month feeding rate is included in the model instead of one-month feeding rate, it is a non-significant effect. The relationship between one-month feeding rate and plasma IGF-1 remains significant whether or not five-month growth rate is in the model. Dotted line=meadow; solid line=lakeshore.
CONCLUSION

Summary
The study of life-history evolution is a complex endeavour and requires a diversity of approaches. In this dissertation, I documented the nature of age-specific reproduction in two life-history ecotypes of a long-lived vertebrate, and evaluated its ramifications for evolutionary theories of senescence. I also showed how immune defense and endocrinology of growth may explain certain aspects of life-history trade-offs. Finally, I provided evidence that the metabolic context in which life history is manifest may be unique in non-avian reptiles, by demonstrating the existence of multiple IGF-1 mRNA haplotypes in squamates and turtles.

Senescence & the garter snake
In Chapter 1 we presented 20 years of data on age-specific reproduction demonstrating that neither fast-growth lakeshore nor slow-growth meadow Thamnophis elegans from the Eagle Lake region show signs of reproductive senescence in the field. In fact, both ecotypes show increasing reproductive output with age up to the latest ages, with lakeshore snakes increasing reproduction with age at a significantly more rapid rate than meadow snakes. Continued and increasing reproductive output throughout life has been previously reported in other indeterminately growing fish, reptiles, and various invertebrate species (Finch 1990). However, this phenomenon has rarely been examined in the context of differing strengths of extrinsic mortality (Reznick et al. 2004 being the notable exception). Williams’ (1957) Antagonistic Pleiotropy (AP) theory of aging suggests that sources of extrinsic mortality select for alleles with fitness benefits early in life that incur a cost later in life, and that high extrinsic mortality will result in faster senescence. Lakeshore T.elegans
experience higher rates of juvenile mortality, and much-reduced adult survivorship than meadow snakes (Bronikowski & Arnold 1999, unpublished data). The former is most likely due to higher rates of extrinsic mortality, while the latter is potentially due to a combination of both extrinsic and intrinsic factors. However, contrary to the AP prediction, we found that lakeshore snakes suffer no apparent costs to fast growth, early maturation, and high reproductive output, at least on the reproductive axis.

While we found no evidence of reproductive senescence, other measures are also relevant to evaluating the presence (or absence) of somatic senescence in *T.elegans*. In Chapters 2 and 3, we presented data on plasma IGF-1 and innate immunity in snakes spanning the juvenile-adult size range. IGF-1 showed a relationship to size/age that was remarkably plastic and varied both by ecotype and year. At least once over the three year sampling period, however, lakeshore and meadow snakes both exhibited a positive linear relationship between IGF-1 and size, a pattern very different to that seen in mammals, where IGF-1 peaks at puberty, and declines thereafter (Pollak 2000). Our study of innate immunity does open the possibility that *T.elegans* may experience immunosenescence, as there is a significant quadratic fit between natural antibody/complement-mediated lysis titers and body size, with the slope of the curve declining shortly after maturity. Interestingly, these decline in immune function with age appeared to be driven by lakeshore snakes. However, as the sample-size of large snakes was quite small, more in-depth sampling targeting the oldest (largest) age-groups must be undertaken to substantiate this finding. In other physiological studies, lakeshore snakes have shown less efficient mitochondrial metabolism as well as less efficient cellular repair mechanisms than meadow snakes (Bronikowski 2008, unpublished data). Thus there is some evidence that they have sacrificed self-maintenance for fast
growth, as optimality theories would suggest; however, a significant cost has yet to be demonstrated.

Long-term demographic analyses on *T. elegans* survivorship are currently being assembled for publication, and may shed light on whether fast-growth lakeshore snakes show higher mortality rates with age than slow-growth meadow snakes (Bronikowski & Arnold, unpublished data), indicative of faster senescence. If this proves to be the case, it will stand in support of the AP theory of aging. If not, however, it may suggest that lakeshore snakes suffer no intrinsic cost to their fast lives. This would not necessarily be imply that these snakes are “Darwinian demons” (Law 1979), but that trade-offs in this system exist on an ecological rather than physiological level. The importance of ecological trade-offs has been expounded by Reznick *et al.* (2000), with reference to Spitze *et al.*’s (1991a, 1991b) report of waterfleas (*Daphnia pulex*) exposed to predation exhibiting decreased age at maturity, increased size at maturation, and increased fecundity, at no apparent cost. All of these traits, contrary to expectation, were positively genetically correlated. The fact that “superfleas” have not taken over the world, these researchers propose, may be due to genetic by environment (GXE) interactions, whereby the combination of traits that has the highest fitness in one environment may have reduced fitness in another. The cost in this case could be that when resources are abundant, increased allocation to costly feeding machinery may be beneficial, but when resources are scarce, it becomes a liability. In the Eagle Lake garter snakes, a similar situation may be in effect, where lakeshore snakes transplanted to a meadow environment could be at a significant disadvantage in the face of limited food availability and cooler temperatures, to which meadow snakes appear to have adapted (Bronikowski & Arnold 1999, Bronikowski 2000).
A modeling approach based on our age-specific reproduction data could be used to predict how the higher rate of increase in age-specific reproduction in lakeshore than in meadow snakes might affect the mutation/selection balance under varying parameters for extrinsic mortality in the two habitats. Realistic scenarios for extrinsic mortality at different sizes/ages would be fostered by field studies quantifying aspects of avian predation pressure in the two habitats (Sparkman, Billings, Bronikowski & Arnold, in prep). Using this method, we could determine whether—if extrinsic mortality is set at a higher level in lakeshore than in meadow habitats—the increase in fecundity with age shown by lakeshore snakes could change the mutation/selection balance so as to offset senescence. The result may be that senescence is predicted to happen at equal rates in both ecotypes, or even that meadow snakes should exhibit the faster rate. If neither show senescence on any axis, suggesting either that they do not senesce or they are able to delay senescence to a degree that it becomes irrelevant in the wild, this may be a moot point as far as this system goes. Nevertheless, it may be useful for other studies examining the evolution of life histories in indeterminately growing species that do senesce.

**Immunity & the garter snake**

In Chapter 2, we supported the ecoimmunological pace-of-life prediction that fast-living organisms will invest more in constitutive innate immunity than slow-living organisms. Furthermore, we provided evidence that lakeshore snakes not only grow faster, but also develop immune function faster than meadow snakes. Differential investment in constitutive innate immunity between the ecotypes has been confirmed in neonates of wild-caught dams raised in a common environment, suggesting that it is not merely a plastic
response to parasitism or food availability in the two habitats (Palacios, Sparkman &
Bronikowski, in prep).

We also tested the other side of the pace-of-life hypothesis, that predicts that slow-
living organisms will invest more in acquired immunity than fast-living organisms, using
lymphocyte abundance as a measure of acquired immunity. No difference in lymphocyte
abundance was evident between the two ecotypes. As lymphocyte abundance can be difficult
to interpret, however, and is not a preferred measure of acquired immunity, my collaborator
Gaby Palacios and I also attempted to test this hypothesis by assaying specific antibody
response to a challenge from a foreign antigen. We inoculated both juvenile and female adult
*T.elegans* of both ecotypes with either rat erythrocytes or sheep erythrocytes, two antigens
commonly used for immunological challenges that have elicited a response in other reptiles,
including snakes (e.g., Hussein *et al.* 1979; El Deeb *et al.* 1980; El Ridi *et al.* 1981; Saad *et
al.* 1986; Leceta & Zapata 1986). However, neither juvenile nor adult *T.elegans* showed
increased levels of specific antibodies in response after one to two months, even upon
injection with a booster shot after one month. Interestingly, Madsen *et al.* (2007) had similar
difficulty with diphtheria and tetanus antigens in pythons. As early studies in other reptile
species have been able to elicit a response using these antigens, these results are perplexing,
and worthy of investigation in their own right. However, *T.elegans* should be tested with
other commonly used antigens, such as diphtheria-tetanus, and keyhole-limpet hemocyanin
(KLH), to see if these can be used to compare acquired immunity in lakeshore versus
meadow snakes. Another measure of acquired immunity (which we did not attempt), *in vitro*
lymphocyte proliferation, could also be useful for this purpose.
In addition to assays of acquired immunity, further investigation should be made into the diversity and abundance of parasite fauna (other than trematodes) in meadow and lakeshore habitats that have an affinity for garter snakes. Quantification of relevant parasite abundance is important both to establish their relationship to measures of immune defense, and to understand better the force of extrinsic mortality being exerted by parasites, and whether it differs between the two habitats. This is clearly no small task, and one that will be difficult to accomplish completely. However, there is much more that can be done. One strategy could be to quantify internal macroparasites in collections of preserved specimens of snakes from the field. Another potential strategy is to use PCR screening to identify/quantify bacterial parasites (e.g., Hellgren et al. 2004; Reullier et al. 2006).

As previously mentioned, two of the three measures of innate immunity (natural antibodies and complement-mediated lysis, but not bactericidal competence) suggested that *T. elegans*, particularly lakeshore snakes, may exhibit immunosenescence. The fact that all three measures did not show the same pattern (bactericidal competence showed a strong linear increase with age/size) affirms that measurement of multiple components of immune defense is crucial. Future study of immune defense in older, larger snakes of both ecotypes will be of great interest to determine whether one or both present robust evidence of immunosenescence.

**IGF-1 & the garter snake**

Our study of plasma IGF-1 in free-ranging *T. elegans* revealed significant distinctions in lifetime IGF-1 profiles between the two ecotypes. In all three years of study, fast-growth lakeshore gravid females had significantly higher levels of IGF-1 than slow-growth meadow females. Furthermore, lakeshore non-gravid adults consistently exhibited a positive
correlation between IGF-1 and body size, while in non-gravid meadow adults this relationship was different each year—there was no relationship the first (wet) year, a negative relationship the second (dry) year, and a positive relationship during the third (restricted to a brief, early wet period) year. We suggest that the variability in the relationship between IGF-1 and body size in meadow snakes is due to variability in amphibian prey availability in different years, as IGF-1 responds plastically to changes in nutrition. We also report significantly higher levels of IGF-1 in shedding snakes. As lakeshore snakes grow faster, and therefore shed more frequently, they may have more frequent peaks in IGF-1 than meadow snakes. Overall, we conclude that IGF-1 may have facilitated the evolution of high-reproductive performance in lakeshore snakes, and that these animals (whether primarily due to genetic or environmentally-based differences in plasma IGF-1) are likely to have higher lifetime levels of IGF-1 than meadow snakes. Thus we provide a foundation for determining whether higher levels of IGF-1 activity in lakeshore snakes makes them less resistant to oxidative stress over the long term, and thus produces a trade-off with lifespan.

A complication of studying a non-model, free-living species is that very little information may be available on basic genetics and physiology. This is certainly the case for snakes. We found exciting evidence that IGF-1 is unique in snakes and other reptiles in that it is expressed in multiple haplotypes. Whether these haplotypes represent the existence of multiple IGF-1 genes and exhibit functional differences, has yet to be determined. It has been suggested that the shift from multiple insulin-like ligands with one receptor in invertebrates, to three main ligands (insulin, IGF-1, IGF-2) with independent receptors in vertebrates may have opened up the possibility for evolution to proceed along different
trajectories. In other words, traits tightly correlated via a single receptor, may have become uncoupled as the receptors diversified into separate pathways (Tater 2003). Though the existence of multiple copies of IGF-1 in non-avian reptiles is not as dramatic as the change seen between invertebrates and vertebrates, it still creates the potential for IGF-1 to be regulated on a spatially and temporally diverse manner, which may well have far-reaching implications for reptile physiology.

To fully understand the activity of any one hormone, it is important to consider not only the hormone itself (and all its forms), but also its binding proteins and receptors, as well as important molecular agents in the signaling pathways downstream. Selection may result in changes in one or all of these components to influence the phenotypic end-result of their cumulative activity. Thus, though plasma IGF-1 has been associated with higher growth rate in other study systems, this measure should not stand alone. Not only should the IGF-1 receptor be considered, but also the multiple binding proteins that serve to increase its half-life in the blood and may either facilitate or inhibit IGF-1 movement and receptor binding (Duan 2005). Members of the IGF-1 signal transduction pathway, such as p66<sup>shc</sup>, which has known effects on stress resistance and lifespan in mammals, are also of interest here (Pelicci <i>et al.</i> 1992; Migliaccio <i>et al.</i> 1999).

Other growth and metabolic hormones, such as the hyroid hormones, triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>), and corticosterone, may also contribute to our understanding of the physiological trade-offs that mould life-history strategies. With the help of a collaborator, I measured T<sub>3</sub> and found no differences between the two <i>T.elegans</i> ecotypes, but T<sub>4</sub> should also be examined (Sparkman, MacKenzie & Jacques, unpublished data). Robert <i>et al.</i> (2008) found higher levels of corticosterone in meadow gravid than in lakeshore gravid females in
the field, and Gaby Palacios and I found the same pattern in both gravid and non-gravid
snakes in another year (Sparkman & Palacios, in prep). We are still exploring the
physiological ramifications and adaptiveness of these differences for the two ecotypes.

Closing remarks
In this dissertation, I have emphasized how unique qualities of long-lived,
indeterminately growing organisms (snakes in particular) can refine and, in some cases
transform, our ideas regarding how life histories evolve. I have also addressed the ways in
which immune and endocrine mechanisms may underlie trade-offs in both indeterminately
growing and determinately growing species in a natural, ecological setting. In my research I
employed a combination of field and lab-based strategies that spoke to the difficulties of
studying wild animals in the lab, where some measures (e.g., innate immunity) may provide
compelling support to field studies, while others (e.g., IGF-1) may add more confusion than
clarification. All in all, this dissertation reaffirms the importance of long-term research on
long-lived species. Such pursuits act both as a source for invaluable long-term data, as well
as a rich context in which to ask questions in the short-term. I am immensely grateful for the
privilege of working with these animals (from whom we will always have much more to
learn), and the people that study them.

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