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Cold-climate adaptation in the five-lined skink: A common-environment experiment

By

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Abstract

Cold-climate adaptation in the five-lined skink: A common-environment experiment

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Ectotherms in cold climates have unique obstacles, especially in winter. Reptiles at higher latitudes or elevations have differing strategies to survive harsher winters, but these differences (e.g. lowered metabolism and lower critical minimum temperatures) may be adaptations or the result of phenotypic plasticity that depends on environmental stimuli or an interactive effect of both. We collected five-lined skinks (*Plestiodon fasciatus*) from Texas and Minnesota to test for latitudinal differences in winter preparation in a common-environment experiment. We manipulated photoperiod and temperature to be either constant or decreasing, resulting in a 2x2 experiment. We asked three main questions: 1) Is there ecotypic variation between populations of lizards from different latitudes? 2) How do photoperiod and temperature independently or conjointly cue lizards to prepare for winter? 3) What variables can we measure to gauge whether lizards are preparing for winter? To determine how the lizards were preparing for winter, we measured several winter dependent variables. High-latitude lizards had greater oxygen consumption and smaller fat bodies. Lizards exposed to a decreasing photoperiod had greater oxygen consumption and lower blood glucose levels. Lizards exposed to a decreasing temperature had lower oxygen consumption, decreased food consumption, slower sprint speeds, lower critical minimum temperatures, and lower blood glucose levels. There was a significant interaction effect between photoperiod and latitude for oxygen consumption, meaning our high-latitude lizards responded more strongly to the photoperiod cue. This interaction is support for ecotypic variation between latitudes. More of our winter variables were influenced by temperature than photoperiod. Supercooling points and liver glycogen content were not affected by latitude, photoperiod, temperature, or sex. Our evidence of population differences in cold-climate traits could be proven adaptive in experiments that control for parental and developmental effects, but our data suggests adaptation in some variables.

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Chapter 1

Cold-climate adaptation in lizards: a review

Winter adaptations

Organisms face several challenges during winter including lack of food and exposure to low temperatures (Storey et al., 1992). To survive, organisms may rely on physiological adaptations such as storing lipids and carbohydrates, reducing metabolism, entering diapause (i.e. a period of suspended development), and increasing thermogenic capacity to produce extra heat (Merritt, 1986; Storey et al., 1992). They may also use behavioral adaptations such as burrowing in the snow and finding alternate food sources (Andreev, 1991). Organisms can also have morphological adaptations such as reduced appendages and insulative fur to stay warm during winter (Prestrud, 1991). Ectotherms have unique obstacles during winter as they cannot rely solely on internal heat production. They may have local adaptations or phenotypically plastic traits to survive. Specifically, this paper investigates several facets of overwinter survival in lizards.

Thermoregulation

Some ectotherms can produce metabolic heat, but they are most effective at controlling their body temperature through other forms of thermoregulation. Reptiles rely primarily on behavioral thermoregulation and can achieve their preferred body temperatures by accessing thermal heterogeneity of microhabitats (Hutchison and Maness, 1979). They can position their backs toward the sun, shuttle between sun and shade, flatten their body on a rock to gain heat by conduction, and stand on their toes to avoid hot substrates

(Huey, 1974; Hertz and Nevo, 1981). The timing of activity can also allow for precise thermoregulation. For example, *Sceloporus magister* may emerge from rock crevices earlier in the morning because the rocks warm quickly, allowing lizards to maximize heat absorption (McGinnis and Falkenstein, 1971). However, *Dipsosaurus dorsalis* may remain in burrows until midmorning when the ground reaches their preferred temperature. (McGinnis and Falkenstein, 1971). In addition, small lizards heat and cool faster than large lizards, necessitating different thermoregulatory strategies (Cowles and Bogert, 1944). In hot environments, smaller lizards tend to move into shade and cold refuges where they lose heat by both radiation and convection. Larger lizards may rely more on radiation and conduction; because of their larger body size, they have lower rates of heat exchange by convection. (Hertz and Nevo, 1981).

Physiological thermoregulation can be important as well (Hutchison and Maness, 1979). Reptiles can change heart rate, peripheral circulation (vasoconstriction and vasodilation), and spasmodic muscular contractions (like shivering in endotherms) to create heat. Some reptiles can produce extensive metabolic heat, but it is energetically costly (Hutchison and Maness, 1979). Even if endothermy is inefficient for thermoregulation in reptiles, it may be important for nest temperature regulation (Seebacher and Franklin, 2005). For example, American alligators produce enough metabolic heat to alter the sex ratios in their nestlings (Ewert and Nelson, 2003) and female *Python molurus* can maintain body temperatures above environmental temperatures while brooding (Van Mierop and Barnard, 1978). Cutaneous color changes also help some reptiles thermoregulate because darker colors absorb more solar radiation (Hertz and Nevo, 1981).

Overwinter survival

Even with effective thermoregulation, winter in many cold climates is too severe for reptiles to survive in an active state (Gregory, 1982). Therefore, reptiles hibernate underground (lizards; Burke and Ner, 2005; Rheubert and Trauth, 2013), underwater (aquatic turtles; Ultsch, 1989), or deep in rock crevices (lizards; Zani, 2005). The length of hibernation varies greatly depending on the species and the local climate (Gregory, 1982). For example, *Uta stansburiana* can be active year-round in the Colorado desert, but they have ~165 d of inactivity in eastern Oregon (Cowles, 1941; Zani, 2008). *Pantherophis obsoletus* may hibernate for 6 months in Kansas (Fitch, 1963) while *Thamnophis sirtalis* may hibernate for 8 months in Manitoba (Aleksiuk and Stewart, 1971).

Nevertheless, some lizards die overwinter due to exposure to low temperatures (Adolph and Porter, 1993). In particular, lower lethal limits can be influenced by environmental conditions such as moisture levels of hibernacula (Burke et al., 2002). Environmental ice is a nucleator and can lead to crystallization of body fluids across the skin of many species. Freeze-intolerant species cannot survive long after ice nucleation occurs so, even if lizards select suitable hibernacula, their exposure to environmental ice in any one winter can be lethal (Burke et al., 2002). Winter mortality is also body-size dependent; lizards that hatch earlier in the year and lizards that grow faster will be more likely to survive their first winter (Conover, 1992; Zani, 2008).

Although longer, colder winters at higher elevations or latitudes may seem worse for lizards in many ways, populations at these locations often have lower winter mortality rates (Wilson and Cooke, 2004; Zani, 2008). During cold winters, ectotherms have depressed metabolism and greater energy conservation (Pullin and Bale, 1989). During warmer winters, ectotherms have higher metabolism and are forced to use their energy stores faster and, consequently, winter mortality is higher (Wilson and Cooke, 2004; Zani, 2008). Adolph and Porter (1993) even found lower year-round mortality for high-latitude *Sceloporus undulatus* because activity periods are associated with higher mortality due to predation. So, in areas with longer inactive periods, overall mortality is often lower.

Environmental cues

Organisms may rely on changes in photoperiod, temperature, precipitation, or resource availability to cue them to the onset of winter. They may also undergo annual (free running) cycles entirely by internal, circannual rhythms, as shown by laboratory studies of animals in constant conditions (Gwinner and Dittami, 1990). If organisms are relying on external cues, photoperiod is the most reliable predictor of season (Farner, 1964; Heldmaier et al., 1989). Changing photoperiods affect mammals, birds, reptiles (Heldmaier et al., 1989), amphibians (Paniagua et al., 1990; Canavero and Arim, 2009), fish (Saunders et al., 1985), invertebrates (Kim and Song, 2000), and plants (Karssen, 1970; Wolf et al., 1990). Photoperiod may cue the initiation of hibernation and affect the energetic strategies by changing thermoregulatory behavior and lowering energy demands in small rodents (Heldmaier et al., 1989). For example, a cyclic photoperiod, rather than constant light or dark, resulted in greater cold tolerance in beet armyworms (Kim and Song, 2000). However, the specific cyclic photoperiod regime used had no effect on cold tolerance. Some birds and lizards rely on critical day lengths (Farner, 1964;

Licht, 1971). When the photoperiod drops below 13.5 hr, gonadal regression occurs in *Anolis carolinensis*, as the breeding season is over (Licht 1971). Hibernation is likely cued by a decreasing photoperiod in many lizards (Mayhew, 1965; Rismiller and Heldmaier, 1982).

Temperature is another important environmental cue. Since lizards are ectothermic, decreasing temperatures affect all aspects of behavior and physiology (Huey, 1982; Angilletta Jr. et al., 2002a). Lower metabolic rates are caused by reduced temperature (Mayhew, 1965). Temperature is also most likely to trigger spring activity in *Aspidoscelis sexlineata* (Etheridge et al., 1983). However, it is generally unknown whether behavioral and physiological changes are cued by the rate of decline or by a certain threshold value. Photoperiod and temperature can be linked because lizards may prefer higher temperatures in the light and colder temperatures in the dark (Rismiller and Heldmaier, 1982). *Lacerta viridis* preferred cooler temperatures during the daily scotoperiod and during hibernation than during the photoperiod and other times of the year (Rismiller and Heldmaier, 1982).

Physiology

As ectotherms recognize environmental cues in autumn, their behavior and physiology will change (Huey, 1982; Heldmaier et al., 1989; Angilletta Jr. et al., 2002b). Reptiles at high latitudes or elevations may have localized mechanisms for surviving harsh winters (Adolph and Porter, 1993; Gutiérrez et al., 2010). Changes in metabolism, energy storage, critical minimum temperatures, supercooling ability, and freeze tolerance can occur in autumn and winter to increase overwinter survival.

Metabolism

Many studies show decreased metabolism when lizards are exposed to lower temperatures (Dawson, 1960; Mayhew, 1965; Prieto and Whitford, 1971; Sanders et al., 2015). However, there are also studies that demonstrate higher metabolism at low temperatures (Murrish and Vance, 1968; Watson, 2008). These opposing results can be explained by the study of different winter survival strategies. In autumn, some temperate or tropical lizards may increase their metabolism to remain active (Ragland et al., 1981; Gregory, 1982). At some point during autumn or winter, lizards from temperate climates may experience cold temperatures at which they can no longer remain active, so their metabolisms are depressed to save energy (Tsuji, 1988a). These strategies are referred to as compensation and inverse compensation, respectively (Ragland et al., 1981; Tsuji, 1988b; Ibargüengoytía et al., 2010). However, when acclimated to low temperatures, temperate lizards are more likely to have elevated metabolism while desert lizards have depressed metabolism (Al-Sadoon and Spellerberc, 1985). Although temperate lizards can acclimate to a wider range of temperatures, desert species reduce their metabolism when exposed to cold temperatures because they are unable to thermally acclimate (Al-Sadoon and Spellerberc, 1985).

Cold temperatures limit the ability of mitochondria in the body, which then limit activity of the organism (Pörtner, 2001). For compensation to occur, mitochondria increase aerobic capacity, which lowers low-temperature thresholds (Pörtner, 2001). Lizards in tropical areas do not need to adjust their metabolism seasonally the same way temperate lizards do because temperatures allow them to be active and food resources to be available year-round (Tsuji, 1988b).

Metabolic rates may not only be specific to a certain temperature, but may vary depending on the seasonal cycle. Patterson and Davies (1978) showed that *Lacerta vivipara* had the lowest metabolism when acclimated to 10ºC in December. In early March, the temperature was lowered to 5ºC and in late March the temperature was increased from 5 to 10ºC inducing metabolic rates similar to summer rates. So even though metabolism was measured at 10ºC both times, the winter metabolism was lower than the spring metabolism. This suggests that measurements of metabolism must be taken at the correct time of the seasonal cycle. Gatten et al. (1988) note that acclimation in a laboratory and acclimation in nature differ and can yield different metabolic rates.

The exponent for body mass in calculating metabolic rate of squamates is debated and differs among species. It ranges from 0.54 (Dawson and Bartholomew, 1956) to 0.8 (Andrews and Pough, 1985). The exponent for *Thamnophis* was found to be 0.59, which falls within this range as well (Bronikowski and Vleck, 2010). However, the metabolic rate of a small ground skink (*Lygosoma laterale*) was found to be 25% of what was expected based on the 0.54 exponent (Hudson and Bertram, 1966). So, the relationship between body mass and metabolic rates of reptiles might vary greatly depending on the species; an exponent of 0.54 to 0.8 might be appropriate.

Energy storage

During winter, reptiles can be inactive for long periods of time and must rely at least partially on stored energy to survive (Adolph and Porter, 1993). Lipids, glucose, and glycogen all have potentially crucial roles as energy sources throughout winter for hibernating reptiles (Dessauer, 1955a; Derickson, 1976; Zani et al., 2012).

Some researchers argue that lizards rely primarily on lipids as overwinter energy (Dessauer, 1955b; Mueller, 1969). These studies examined fat content at different points in the season to find cyclic patterns. However, other researchers say lipids are used primarily for reproduction (Hahn and Tinkle, 1965; Guillette and Sullivan, 1985; Méndez de la Cruz et al., 1988; Rocha, 1992). These studies point to the use of lipids for gonadal development and vitellogenesis. In lizards, lipids can be found in somatic fat bodies, tails, livers, eggs, or other tissues (Dessauer, 1955b). Researchers often measure fat bodies, however; more lipids were found in tails than fat bodies of *Plestiodon laticeps* during spring, summer and autumn (Vitt and Cooper, 1985). It is important to know that skinks with small fat bodies may have significant amounts of lipids stored in their tails or they might have little lipid storage so, analyzing tails, fat bodies, and other organs for lipids will give the best whole-body estimate of lipid content (Vitt and Cooper, 1985). As lipid composition in tissues varies, behavioral thermoregulation may differ as well. For example, lizards fed diets rich in saturated fatty acids had higher preferred body temperatures (Simandle et al., 2001).

Other researchers emphasize the importance of glycogen as overwinter energy (Dessauer, 1955a; Zani et al., 2012). Muscle glycogen is important for reptiles during exercise, but liver glycogen is more important during winter (Gleeson, 1991). Zani et al. (2012) found the amount of liver glycogen to be the most important determinant of overwinter survival in *Uta*. Glycogen is generally deposited in the liver during autumn (Dessauer, 1955a; Maggio and Dessauer, 1963; Moore, 1967). Throughout the winter, this liver glycogen is metabolized to glucose for use (Dessauer, 1955a; Moore, 1967). Voituron et al. (2002) saw an increase in blood glucose throughout the winter, however, they studied a freeze-tolerant lizard and winter survival mechanisms may differ from freeze-intolerant species. In freeze-tolerant lizards, glucose could act as a cryoprotectant to help organisms tolerate freezing without tissue damage. Lizards use more blood glucose and liver glucose during the winter because they do not have much freshly ingested food. Du et al. (2000) found that food consumption of *Plestiodon elegans* was decreased at lower temperatures, possibly due to decreased enzymatic activity at lower temperatures. Decreased appetite of several lizard species has been seen prior to hibernation (Dessauer, 1955a; Gregory, 1982).

Critical minimum temperature

Measuring an ectotherm's critical thermal minimum (CTMin) has is a common measure of cold tolerance. CTMin is considered equivalent to ecological death since it is the temperature at which locomotion stops and animals can no longer escape from predators or find food (Spellerberg and Spellerberg, 1972). The CTMin of insects (Gaston and Chown, 1999a), amphibians (Manis and Claussen, 1986) and reptiles (Spellerberg and Spellerberg, 1972) have all been measured by determining the temperature at which the animal loses the righting response. Loss is considered to be the inability of an animal to right itself within 30 sec when placed on its back (Spellerberg and Spellerberg, 1972; Wilson and Echternacht, 1987; Angilletta Jr. et al., 2002a).

Researchers have found that acclimation temperatures significantly affect CTMin of reptiles and amphibians (Brattstrom and Lawrence, 1962; Jacobson and Whitford, 1970; Huang et al., 2007). Kour and Hutchison (1970) acclimated four lizard species to 15, 25, and 35°C and found the resulting critical thermal minima to be 7.5, 7.8, and

9.6°C, respectively. Spellerberg and Spellerberg (1972) found that *Sphenomorphus tympanum* had a mean CTMin of 4.5°C in the winter and 6.1°C in the summer. These differing values emphasize the importance of appropriate acclimation temperatures when determining the CTMin of an organism.

CTMin varies not only by acclimation, but also by species. Most CTMin values of lizards are between 2ºC and 12°C (Kour and Hutchison, 1970; Spellerberg and Spellerberg, 1972; Hertz and Nevo, 1981; Brown, 1996; Gvoždík and Castilla, 2001; Angilletta Jr. et al., 2002a). Even within a genus, CTMin can vary widely. *Phrynosoma cornutum* has a CTMin of 2.75°C while *P. douglassii* has a CTMin of 9.46°C (Prieto and Whitford, 1971). When acclimated to 28ºC, *Eremias brenchleyi* has a CTMin of 3ºC while *E. multiocellata* has a CTMin of 9ºC (Li et al., 2009). The variety of CTMin seen in closely related species suggests that minimum temperatures evolve quickly or are phenotypically plastic. It is hard to tease apart genetic effects from plasticity because most studies are done with field-caught animals. Differences between field-caught individuals could be due to developmental effects or maternal effects. A common-environment experiment is needed to ensure differences seen are due to genetic differences.

Supercooling

Supercooling is a strategy used by many ectotherms when environmental temperatures are below freezing; they avoid ice formation at sub-freezing temperatures. Supercooling points are generally measured in laboratories as the temperature at which ice crystallization occurs and this may be significantly lower than in nature. In labs, supercooling points are measured by placing animals in a temperature-controlled chamber and cooling them at a constant rate (Costanzo et al., 1995). However, there are many ice-nucleating agents in nature that would cause freezing at higher temperatures (Costanzo et al., 1995; Burke et al., 2002).

Costanzo and Lee (2013) say that small reptiles can often supercool from -8 to -18°C. Antifreeze proteins or antifreeze glycoproteins are found in some fish, plants, and invertebrates, but have not been found in terrestrial vertebrate ectotherms (Fletcher et al., 1982; Tomczak et al., 2001). Instead, to supercool, lizards rely primarily on dessication, clearing their guts, and sometimes on glucose to act as a cryoprotectant (Costanzo et al., 1995; Grenot et al., 2000).

Freeze-tolerance

Many amphibians and reptiles can tolerate freezing of tissues within their bodies. To do so, the fluid within cells is expelled into the extracelluar spaces so when the fluid freezes and expands, it does not break the cell membranes (Mazur, 1984; Storey, 1990). Successful freeze tolerance depends on the length of cold, the depth of cold, the proportion of body fluids frozen, and the number of freeze-thaw cycles (Costanzo et al., 1995). Many freeze-tolerant ectothermic species from slugs (*Arion* spp.) to painted turtles (*Chrysemys picta*) use glucose as a cryoprotectant (Storey and Storey, 1986; Churchill and Storey, 1991; Slotsbo et al., 2012) (For an explanation of glucose as a good cryoprotectant, see Costanzo et al. 1995, pg. 354). Other species may use glycerol, lactate, or amino acids as cryoprotectants as well (Storey and Storey, 1992). It is unknown for most species what percentage of body water can freeze without mortality.

Lacerta vivpara, can freeze 50% of its body water (Voituron et al., 2002), *Podarcis muralis* can freeze up to 28% (Claussen et al., 1990), *Chrysemys picta* can freeze 43-67% (Storey and Storey, 1992), and *Lithobates sylvaticus* can freeze 65-70% (Costanzo et al., 1993).

Storey and Storey (1992) suggest guidelines for defining a species as freeze tolerant because many animals can survive at least a short time with small percentages of body ice. They suggest that species should be able to freeze for 24 hr at -2.5ºC with 45-65% of body water frozen, ice nucleation should occur at relatively high sub-zero temperatures, and freeze tolerance should be necessary for overwinter survival.

Freeze tolerance is more physiologically stressful than supercooling, so freeze-intolerance is more common in vertebrate ectotherms (Costanzo and Lee, 2013). However, freeze tolerance, rather than supercooling, may be a better strategy for amphibians since the risk of inoculative freezing is more of a threat for organisms with permeable skin (Layne, 1991; Storey and Storey, 1992). To supercool, amphibians need to prevent dessication and inoculative freezing which is challenging in natural conditions (Storey and Storey, 1992). But lizards have tougher skin and scales which act as a barrier against ice nucleation so most can rely on supercooling (Halpern and Lowe, 1968). There are only two species of lizards known to survive substantial freezing of body water (Claussen et al., 1990; Voituron et al., 2002).

Body size differences

Bergmann's rule states that species and individuals within a species are larger in colder climates. This trend makes sense for endotherms, but the reverse may be true for ectotherms (Masaki, 1978). Differences in body sizes of ectotherms at different latitudes are likely genetic, at least for invertebrates (Mousseau, 1997). Some research suggests that lizards are smaller at higher latitudes while others show larger lizards at higher latitudes (Ashton et al., 2003; Cruz et al., 2005). Although smaller body sizes may be advantageous for ectotherms, as a higher surface-area-to volume ratio allows ectotherms to heat up quickly, they may simply be a consequence of a shorter growing season (Mousseau, 1997). Plains garter snakes (*Thamnophis radix*) were found to have similar growth rates throughout their range, but larger body sizes were observed at high latitudes (Tuttle and Gregory, 2012). High-latitude ectotherms have the possible advantages of longer day lengths during the growing season and reduced competition which may help them reach larger body sizes (Tuttle and Gregory, 2012).

Limits to distribution

Rapoport's rule states that species at higher latitudes will have greater latitudinal ranges (Gaston and Chown, 1999b). The climatic-variability hypothesis suggests that species at higher latitudes have wider thermal tolerances (Huey, 1978; Cruz et al., 2005). These hypotheses are supported by a study by Sunday et al. (2011) which examined thermal tolerance breadths of ectothermic species around the world. They found that increased climatic variability correlated with wider thermal tolerances. Pianka (1966) explained that low latitudes have more kinds of habitat, which leads to increased species richness and species diversity. The theory of climatic stability says that regions with more stable climates allow for more specialization and adaptions. However, species in the tropics are more range-limited than high-latitude species because they are more likely to encounter intolerable environmental conditions in areas adjacent to them (Janzen, 1967). Since species at low latitudes have more limited ranges and are more specialized, they may be impacted more by changes in climate (Tewksbury et al., 2008).

When examining range limitations for reptiles, lower temperatures are more limiting than high temperatures (Spellerberg and Spellerberg, 1972). Environmental minimum temperatures vary more with latitude than maximum temperatures, especially in the northern hemisphere where there is more land mass (Gaston and Chown, 1999b; Sunday et al., 2011). Critical or lethal temperatures reflect these environmental temperatures in that upper limits of species vary less across latitudes than lower limits (Addo-Bediako et al., 2000). In nature, it is more difficult for lizards to avoid their CTMin than their critical thermal maximum (CTMax) (Spellerberg, 1973). Lizards are only active near their upper lethal limits in when evading predators or defending territory (DeWitt, 1967).

Evolution of cold tolerance

It is often hard to tease apart adaptations and acclimation effects. To eliminate the possibility of phenotypic plasticity, researchers often use the F_1 generation to do common-environment experiments (Pulido et al., 2001; Lardies and Bozinovic, 2008). The evolution of cold tolerance is difficult to measure and is still largely up for debate. While lower lethal limits of insects decrease with increasing latitude (Addo-Bediako et al., 2000), most studies support the hypothesis that cold tolerance is evolutionarily conserved in vertebrates (Hertz and Nevo, 1981; Huey, 1982; Gvoždík and Castilla, 2001; Yang et al., 2007; Youssef et al., 2008; Michels-Boyce and Zani, 2015). Two studies on lizards do not support the hypothesis of evolutionary conservation of cold tolerance and demonstrate intraspecific differences in cold tolerance (Wilson and Echternacht, 1987; Weeks and Espinoza, 2013). However, lizards were held for 1-2 days in one study (Weeks and Espinoza, 2013) and lizards were tested the day of capture in the other (Wilson and Echternacht, 1987), so we cannot rule out acclimation effects in these cases. Phenotypic plasticity and acclimation can explain many differences in cold tolerance that we see but, acclimation can be costly so acclimation ability should be reduced at low latitudes and areas of favorable environmental conditions (Hoffmann, 1995).

Evolutionary pressure may be reduced if ectotherms are efficient thermoregulators (Gvoždík and Castilla, 2001). For example, lizards at higher latitudes may be more efficient thermoregulators because they have a decreased predation risk (Gutiérrez et al., 2010). Thermal physiology of reptiles probably evolves slowly due to a lack of variation in preferred temperatures and critical temperature limits (Huey, 1982). This lack of evolution is further explained by Fisher's fundamental theorem of natural selection which says that the rate of natural selection and evolution is equal to the genetic variation of the trait (Frank and Slatkin, 1992).

Summary

Lizards can be effective thermoregulators but, for many species in cold climates, they have to alter their behavior and physiology for some length of time during the winter. Lizards in warmer climates may stay active year-round, while lizards in colder climates may hibernate for six months of the year. Lizards use environmental cues such as photoperiod and temperature to determine the changing seasons. Temperature affects all aspects of behavior and physiology, but certain behavioral changes may be cued by changing photoperiods because photoperiod is the most reliable exogenous cue.

During autumn, lizards in cold climates make physiological changes to prepare for winter. They may increase their metabolism to stay active at colder temperatures or they may decrease their metabolism to save energy. Lizards rely on stored energy during winter when food resources are not available. So, they may store glycogen or lipids to use as overwinter energy. When lizards are acclimated to cold temperatures, they can have lower CTMin. Many small lizards can also supercool, or remain unfrozen at subzero temperatures. However, the supercooling point, or the freezing point, does not change based on acclimation. Some reptiles are freeze tolerant, but this strategy is uncommon in lizards. *Lacerta vivipara* is the only lizard species known to use freeze tolerance as a winter survival mechanism. Critical minimum temperatures of ectothermic species vary more across latitudes than critical maximum temperatures. But, within lizard species, cold tolerance rarely differs across latitudes. Cold tolerance may be evolutionarily conserved because lizards can avoid their CTMin and, therefore; there is a lack of evolutionary pressure.

Chapter 2

Introduction

Species near the equator or in stable environments are adapted to a narrow range of thermal environments while species that live at high latitudes experience a wider range of conditions (Pianka, 1966; Huey, 1978). Thermal limits may be less important for range determinations of endotherms if energy sources are available (Buckley et al., 2012). While some ectotherms can produce metabolic heat, it is energetically costly (Hutchison and Maness, 1979). Therefore, most ectotherms thermoregulate behaviorally by finding suitable microhabitats or by positioning their bodies to modulate heat transfer (Huey, 1982; Angilletta Jr. et al., 2002b). At low environmental temperatures, reptiles reduce activity for periods of time and, during winters at high-latitudes, they avoid freezing for several months in deep hibernacula (Gregory, 1982). Hibernation can affect cold-hardiness, metabolic rates, activity patterns, and energy storage of lizards.

To know when to prepare for winter and hibernate, lizards could be using photoperiod, temperature, resource availability, precipitation, or endogenous cues (Mayhew, 1965; Seebacher and Franklin, 2005). Photoperiod is the most reliable predictor of season (Farner, 1964; Heldmaier et al., 1989), but reptiles probably use temperature cues as well, because autumn conditions vary spatially (Etheridge et al., 1983). The shortening of photoperiods cue winter dormancy in lizards whereas decreases in temperature slow their metabolic rate (Mayhew 1965). Rismiller and Heldmaier (1982) found that photoperiod not only affects lizard activities such as hibernation, but it also causes a change in preferred body temperature in *Lacerta viridis*. Although researchers seem to agree that decreasing photoperiod and/or temperature cues lizards to become inactive, little is known about the associated physiological changes.

Cold tolerance may be defined as the physical limits to an organism's survival such as the lowest temperature, the greatest duration of exposure to low temperature, the greatest number of thermal cycles, or maximal rates of cooling. One common way to test cold tolerance is to measure the critical thermal minimum (CTMin), the temperature at which lizards lose locomotive functions (e.g. Angilletta Jr. et al., 2002; Weeks and Espinoza, 2013). Another way to test cold tolerance is to determine the lower lethal limit (e.g. Costanzo et al., 2001; Michels-Boyce and Zani, 2015). For freeze-intolerant species, this lower lethal limit is the temperature of ice nucleation, or the supercooling point (SCP).

Reptiles overwintering in places such as pond bottoms, in trees, or in below-ground hibernacula above frost depth may experience temperatures below 0ºC and must either supercool or tolerate freezing to survive (Costanzo et al., 1995; Costanzo and Lee, 2013). Supercooling is a common mechanism used by organisms that are unable to survive ice formation in their tissues. Crystallization and tissue damage is prevented by increasing solute concentration within cells or by dehydrating the body to decrease freezing temperatures (Costanzo and Lee, 2013).

During autumn, many lizard species show decreased metabolism when exposed to low temperatures (Dawson, 1960; Mayhew, 1965; Prieto and Whitford, 1971; Sanders et al., 2015), but in some cases lizards show greater metabolic rates at low temperatures (Murrish and Vance, 1968; Watson, 2008). These opposing results can be explained by considering alternative strategies for surviving winter. Lizards from warmer climates may not need to hibernate for long or at all so, when they experience low temperatures, they increase their metabolism to remain active (Ragland et al., 1981). However, lizards from temperate climates need to hibernate longer to conserve energy; during autumn, their metabolism is depressed to save energy (Tsuji, 1988a). These strategies are referred to as compensation and inverse compensation, respectively (Ragland et al., 1981; Tsuji, 1988b; Ibargüengoytía et al., 2010).

As metabolism decreases, food consumption and body condition likely decrease as well. In support of this, studies show that lizards at cold temperatures are less likely to attack prey, more likely to have failed attacks, and more likely to consume smaller prey (Van Damme et al., 1991; Du et al., 2000). Temperature affects the physiology and behavior of ectotherms so, at colder temperatures, overall performance decreases as well (Angilletta Jr. et al., 2002b). Sprint speed is a good measure of whole-body performance (Watson and Formanowicz, 2012) and it is ecologically relevant because an organism's performance may determine whether it can catch prey or escape from predators (Van Berkum, 1985).

Liver glycogen, blood glucose, and lipids stored in fat bodies can all be metabolized to provide energy during winter. Liver glycogen content is crucial for overwinter survival; Zani et al. (2012) found insufficient liver glycogen levels, rather than stored lipids or hydration state, to be the primary cause of death in overwintering *Uta*. In anoles (*Anolis carolinensis*), circulating glucose is converted to liver glycogen in autumn so blood glucose levels drop before winter (Dessauer, 1953). The conversion of blood glucose to liver glycogen during autumn suggests blood glucose levels would be low throughout winter. However, blood glucose could increase periodically during the winter when the glycogen is metabolized to glucose (Moore, 1967). The trend of an increase in blood glucose and a decrease of liver glycogen throughout winter has been documented in lizards (see Gregory, 1982). Stored lipids are another important source of energy for lizards during periods of inactivity (Derickson, 1976; Adolph and Porter, 1993). Some studies report the mobilization of lipids from fat bodies as a source of overwinter energy (Dessauer, 1953; Avery, 1970) while others show that fat bodies are primarily used for vitellogenesis and provisioning of embryos in the spring (Hahn and Tinkle, 1965; Méndez de la Cruz et al., 1988).

Physiological changes in autumn may differ among species and populations at different latitudes. The climatic-variability hypothesis suggests that species at higher latitudes have wider thermal tolerances (Cruz et al. 2003; Huey 1978; Slobodkin and Sanders 1969; Pielou 1975). Sunday et al. (2011) compared ectothermic species globally and found that, as predicted, species at higher latitudes and in more variable climates generally have greater cold tolerances (lower critical temperatures and lethal limits). However, they generalized critical temperatures for each species and did not examine tolerances intraspecifically. Weeks and Espinoza (2013) examined populations of one species of lizard and found that CTMins of geckos in Argentina are correlated with winter temperatures. That is, lizards from populations that naturally experience colder winters have lower CTMins than those from populations in milder climates.

However, there is also significant support that thermal physiological traits, such as CTMin, are generally evolutionarily conserved in vertebrate ectotherms (Huey, 1982). This generalization is supported by research showing that some lizards and frogs have similar critical or lethal thermal limits among populations in different conditions (Manis and Claussen, 1986; Yang et al., 2007; Michels-Boyce and Zani, 2015). If local conditions do not influence critical temperatures, acclimation effects and large genetic distances (caused by long times since divergence and by divergent selection) might cause differences in critical temperatures (Yang et al., 2007).

We used five-lined skinks (*Plestiodon fasciatus*) from high- and low-latitude populations to estimate ecotypic differences in responses to autumnal cues. *P. fasciatus* has one of the most extensive geographical ranges of North American lizards extending from Florida and Texas (Conant 1975) north into Canada (Howes and Lougheed, 2004). This range provides a wide environmental gradient within which ecotypic variation may have evolved. Their abundance and wide-spread range make them an ideal organism for the study of adaptation to cold climates. We compared populations differing significantly in latitude to detect local adaptations in physiological mechanisms for surviving winter. By bringing lizards from two latitudes together in controlled laboratory settings, side-by-side manipulative experiments allowed us to detect ecotypic differences in reaction norms of several winter dependent variables. Differences in response variables may provide evidence for adaptive responses to cold stress. We examined the effects of constant and decreasing photoperiod as well as constant and decreasing temperature on each dependent variable, for a total of four treatment groups.

Methods

We collected *P. fasciatus* during the spring and summer of 2016 from high latitudes (Minnesota and Wisconsin) and from low latitudes (Texas). Lizards were captured by hand and transported to the laboratory. We housed each skink in an individual 4.4-L terrarium (13 x 14 x 24cm) floored with a peat moss substrate (1 cm depth), and supplied water *ad libitum*. THG heat tape was used to establish a thermogradient in each terrarium from 20-32 ± 1°C. Lizards were fed crickets three times per week and misted daily.

We performed a 2x2 experiment with manipulations of photoperiod and temperature (Table 1). An environmental chamber in the animal-care facility was divided in half with a light-proof partition of two layers of black plastic. The lights in one half were programmed to mirror the sunrise and sunset times of 45.4º N (type of light bulbs and use of redundant lighting to prevent accidental darkness during the light cycle). The other half of the room also mirrored sunrise and sunset times of 45.4º N until the summer solstice. From June 20, 2016 to the end of the experiment, we kept the photoperiod constant in the second half of the room. Lizards from each latitude were split equally between the photoperiods. The ambient temperature was maintained at a constant 21 ± 1°C in both sides of the chamber, creating two temperature-constant (T_C) treatments: photoperiod decreasing (P_DT_C) and photoperiod constant (P_CT_C).

A second environmental chamber equipped with refrigeration was also divided in half and lit with the same two photoperiod treatments as in the warm chamber and initially maintained at 21°C. Half of the animals from each of the first two treatments were randomly assigned to the cold chamber and were moved there on September 12, 2016. The temperature decreased gradually from 21°C to 11°C from September 19 to November 21 at a rate of 1°C per week. In this way, two additional treatments were created: P_DT_D and P_CT_D. Lizards from each latitude were equally divided among the four treatment groups.

Oxygen consumption

In January, we measured oxygen consumption (respiration rates) of all lizards. Lizards were fasted for 48 hr prior to respiration trials. Within 20 min of illumination in the morning, lizards were placed individually in 250-ml biochambers with a Vernier oxygen gas sensor (O2-BTA) in the grommet. We placed soda lime packets in each chamber to absorb carbon dioxide gas. The gas sensors sampled the biochamber air every 15 min for 7 hr. The first hour of data was discarded to allow time for lizards to approach standard metabolic rate. Oxygen concentrations were graphed and the slope of the regression line was recorded. Only lines with an r^2 value >0.9 were accepted. The slopes, representing the average oxygen consumption rate, were corrected for body mass by dividing by $\text{mass}^{(0.54)}$ (Dawson and Bartholomew, 1956).

Food consumption

We fed lizards crickets three times per week and recorded the number consumed. Mass consumed was calculated as number of crickets times mean cricket mass and consumption rates were expressed per lizard body mass.

Sprint speeds

A racetrack (2.5-m length, 9-cm width) was constructed with a substrate of two layers of rubber non-adhesive shelf liner. Test runs on sand, pebbles, fabric, cardboard, and rubber shelf liner showed the most natural and fastest runs on rubber shelf liner. Lizards were placed in one end and chased to the other end by hand. All lizards were tested at the temperature they were acclimated to; T_C lizards were tested at 21ºC and treatments and

T_D lizards were tested at 13ºC. Over 2 d, at least two trials were conducted with each lizard. The track was marked every 0.5 m and the run was video recorded. The speed of each 0.5-m interval was determined in Avidemux2.6. Sprint speed was considered the fastest 0.5-m interval. Speeds were size-corrected by dividing by body mass$^{(0.33)}$ (Huey and Hertz, 1982).

Critical minimum temperature

We determined the CTMin of each lizard beginning January 28, 2017. Four open-top containers were submerged 3.5 cm in a circulating bath containing anti-freeze solution. During each trial, one lizard was placed in each container. Cloacal temperatures were monitored through ultrafine thermocouples connected to a data collector with USB interface. The initial temperature was 13°C and the bath was decreased 1°C per 20 min. Every 10 min, we inverted each lizard to observe their righting response. In the absence of righting, we lightly stroked the lower abdomen with a brush to stimulate the righting reflex. We measured CTMin as the body temperature at which the lizard did not right itself within 30 sec (Simandle et al., 2001; Cruz et al., 2005; Weeks and Espinoza, 2013).

Supercooling point

We continued cooling 4 lizards from each treatment group (2 high-latitudes and 2 low-latitude lizards, n=16) by the methods described above to determine SCPs. The SCP was recorded as the lowest temperature reached before ice formation, as detected by the sudden increase in cloacal temperatures resulting from the exothermic nucleation. Freezing was lethal in all but one lizard.

Dissection

The four hatchling lizards were not sacrificed due to their small size and were donated to the education collection. Adult lizards not killed by freezing were euthanized by decapitation (n=11). Blood was collected from the trunk of decapitated lizards and the concentration of glucose was determined using a portable digital glucometer (CVS/pharmacy Advanced Glucose Meter). We dissected all adult lizards, whether they were sacrificed by freezing or decapitation. We removed and weighed the fat bodies. Fat body mass was divided by total lizard mass. The liver was removed and stored at -80ºC until analysis of glycogen content.

Liver glycogen

The liver glycogen protocol was from Irwin and Lee (2003) and personal communication with Peter Zani. Each liver sample was weighed and homogenized in 0.6 N $HClO_4$. We incubated 50 µl of the mixture with 0.77 M $KHCO_3$ and amyloglucosidase in a 0.15 M acetate buffer. After incubation, additional $HClO_4$ and $KHCO_3$ were added (final concentrations 0.16 M $HClO_4$ and 0.33 M $KHCO_3$) and the mixture was centrifuged. The supernatant of this solution included the glucose plus glycogen in the liver sample.

The remaining homogenized mixture was centrifuged for 10 mins at 16000 RCF and the supernatant was mixed with $KHCO_3$ (final concentration 0.14 M). This mixture was centrifuged again and the supernatant of this solution included only the free glucose in the liver sample. We added 10 µl of each supernatant to a multi-well plate and then 200 µl enzyme-color reagent to each well. The plates were incubated in darkness for 45 min and then read on a spectrophotometer at 450 nm. Standards of 0, 0.13, 0.37, and 0.47

mg glucose/mL were included on each plate. The range of liver glycogen concentration was previously unknown in five-lined skinks. We adjusted the liver sample concentrations and concentrations of standards until the liver values fell within the range of the standards. Our liver samples were highly variable and occasionally glycogen concentrations fell above the 0.47 mg standard. We graphed the known standards and used the regression equation of this line to determine concentrations of the unknown samples. The concentrations were then converted, square-root transformed, and analyzed.

Body condition

We weighed each lizard every 2 weeks. We compared body mass of each lizard on August 8th to weights at euthanasia. For each time period, the natural log of the SVL was plotted against the natural log of the mass. To calculate a scaled mass index of condition (M_i), methods of Ibargüengoytía et al. (2016) were followed (Eqn 1).

M_i=Individual mass x [average SVL of sample/individual SVL] x slope of the regression line. Eqn (1).

Statistical methods

Data were analyzed using ANOVAs. For food consumption, we used a repeated measures ANOVA. If variables violated the assumptions of an ANOVA, we log-transformed or square-root transformed the data to correct non-normality or inequality of variance. We used transformations to permit the detection of interactive effects; such interactions are not visible in non-parametric tests. We log-transformed the data for oxygen consumption. We used square root transformations for fat body mass and liver glycogen data. Sprint speed and CTMin data still violated ANOVA assumptions after transformation, so we used Mann-Whitney U tests. We applied a Bonferroni correction whenever multiple comparisons were planned, so only p-values < 0.0125 were considered significant. All dependent variables were tested against photoperiod, temperature, latitude, and sex. We also looked for correlations between liver glycogen levels and food consumption, blood glucose levels and food consumption, and fat body mass and food consumption. Means are presented as mean ± SE for all variables.

Results

Lizard collection

We collected 6 adult females, 4 adult males, and 4 hatchlings (sex unknown) from Minnesota and Wisconsin (n=14). The mean body mass was 6.7 ± 0.43 g for adults and 2.4 ± 0.37 g for hatchlings. The SVL was 64.1 ± 1.46 mm for adults and 47.3 ± 3.40 mm for hatchlings. These high-latitude lizards were collected between June 26 and August 1, 2016. We captured two lizards from Interstate State Park in MN (45.3947 N, 92.6678 W), six from Vicksburg County Park (44.6633 N, -95.2303 W), one from Cold Springs Wildlife Management Area (44.6094 N, -95.1425 W), and four from Interstate State Park in WI (45.3984 N, 92.6450 W). From Texas, we collected 4 adult females and 13 adult males (n=17). The mean body mass was 8.9 ± 0.67 g and the mean SVL was 67.2 ± 1.32 mm. All but three of the Texas animals were collected in March-April 2016. The final three were added to treatment groups on October 12. All lizards from Texas were collected from either Tucker Woods in Nacogdoches (31.6299 N, 94.6447 W) or on private property north of Nacogdoches (31.7012 N, 94.6797 W).

Latitude effects

Latitude had a significant effect on fat body mass ($F_{(1,24)}$=8.85, p=0.007; Fig. 1; Table 2). Low-latitude lizards had larger fat bodies with the mean weight-corrected fat pad sizes of 0.017 ± 0.003 g. High-latitude lizards had weight-corrected fat pad sizes of 0.0061 ± 0.001g. Fat body mass was not correlated with food consumption (r^2=0.07, p=0.19). Latitude also had a significant effect on oxygen consumption ($F_{(1,26)}$=18.19, p<0.001; Fig. 1; Table 2). High-latitude lizards had 61% greater oxygen consumption.

Sex effects

Sex had a significant effect on the change in body condition ($F_{(2,23)}$=4.26, p=0.027; Fig. 2; Table 2). The mean increase in the body condition index of females was 0.20 ± 0.092 while the mean for males was 0.064 ± 0.055. Both means were positive so lizards overall improved their body conditions in the lab, but females had a greater increase in body condition.

Photoperiod effects

Photoperiod had a significant effect on oxygen consumption ($F_{(1,26)}$=14.52, p=0.001; Table 2). P_D lizards had 64.5% greater oxygen consumption. There was also a significant interaction between latitude and photoperiod on oxygen consumption ($F_{(1,26)}$=4.49, p=0.044). P_C lizards had higher blood glucose levels of 120.83 ± 10.35 mg/dL. P_D lizards had mean blood glucose levels of 91.17 ± 10.19 mg/dL ($F_{(1,8)}$=6.24, p=0.037; Table 2; Fig. 3).

Temperature effects

Oxygen consumption was also affected by temperature ($F_{(1,26)}$=64.75, p<0.001; Table 2). T_C lizards had 96.1% greater oxygen consumption. Food consumption was affected by temperature as well ($F_{(1,5)}$=15.01, p=0.012; Table 2; Fig. 4). Of the 19 total weeks of differential temperature treatment, T_D lizards consumed less food than T_C lizards during the final 11 weeks. Blood glucose levels were positively correlated with food consumption (r^2=0.35, p=0.045; Fig. 3), so glucose levels were affected by decreasing temperatures as well ($F_{(1,8)}$=6.10, p=0.039). T_C lizards had mean blood glucose levels of 120.67 ± 10.34 mg/dL. T_D lizards had mean blood glucose levels of 91.33 ± 10.29 mg/dL. Sprint speeds were faster at higher temperatures (U=2.0, p<0.001; Fig. 4). T_D lizards sprinted slower at 0.28 ± 0.01 m/s. T_C lizards sprinted at 0.78 ± 0.03 m/s. T_D lizards had a mean CTMin of -4.0 ± 0.29ºC while T_C lizards had a mean CTMin of 0.06 ± 0.18ºC (U=0, p<0.001; Fig. 4).

No effects

In the analysis of variance on liver glycogen content and SCP, there were no significant effects (Fig. 5). Liver glycogen was also not correlated with food consumption (r^2<0.01, p=0.92). SCP ranged from -8.55 to -15.80ºC with a mean of -12.69 ± 0.54ºC. Ice nucleation in the body of the lizards was lethal in all but one instance; one male from Texas experienced ice nucleation at -9.33ºC. We removed him from the cold-water bath 38 min after ice nucleation, and within 5 min he began flexing his trunk and moving limbs. He remained at room temperature and made a full recovery within 1.5 hours.

Discussion

Overview

We hypothesized that lizards from different latitudes respond differently to the same environmental cues. These differences would then support the hypothesis that there are ecotypic variations between populations from different latitudes. We also hypothesized that lizards are using different environmental cues to make changes in preparation for winter. To test these hypotheses, we collected lizards from two different latitudes, brought them into the lab and exposed lizards from each latitude to different photoperiod and temperature regimes. We then measured a suite of physiological response variables to look for differences. We found few differences between populations. Oxygen consumption is the only variable that suggests ecotypic variation in our lizards. However, as predicted, lizards use photoperiod and temperature as cues for different physiological changes. Temperature was more important than photoperiod for most of the variables we measured.

Latitude effects

Our high-latitude lizards had greater oxygen consumption, which could be explained by compensation. Watson (2008) compared three skink species in the genus *Plestiodon* and found that *P. fasciatus* (five-lined skinks) had the highest respiration at all three temperatures measured. The higher respiration rates support Watson's hypothesis that *P. fasciatus* would have higher metabolism at lower temperatures because of its northern range and compatibility with cold climates. This trend could also exist within populations of *P. fasciatus*. Our high-latitude lizards live in colder climates so they could have adapted higher metabolic rates to stay active for as much of the year as possible. Longer growing seasons allow lizards to achieve larger body sizes, which are associated with higher overwinter survival (Zani, 2008). Therefore, *P. fasciatus* from higher latitudes could have increased metabolism to increase the length of their growing season, which would lead to greater overwinter survival.

We predicted that high-latitude lizards would store more energy and therefore have larger fat bodies than low-latitude lizards because high-latitude lizards experience longer winters and have longer periods of inactivity. Our results show the opposite of what we predicted. It is likely that *P. fasciatus* use lipids in fat pads primarily for gonadal development and reproduction, like many other lizards, and not as a source of overwinter energy (Guillette and Sullivan, 1985; Méndez de la Cruz et al., 1988). Therefore, low-latitude lizards may have larger fat bodies because they breed earlier or invest more into reproduction.

Sex effects

For the body condition index comparison, we subtracted the final body condition of all lizards in January 2017 from their body condition in August 2016. Females probably increased body condition more because several females caught were post-reproductive and their body conditions were poor after staying with their eggs for 4-8 weeks. So, it was intuitive that females in the lab would improve their body conditions more significantly than males.

Photoperiod effects

Our P_D lizards had greater oxygen consumption. This suggests that *P. fasciatus* elevate metabolism to stay active longer in autumn until the temperature falls below some threshold; i.e. they compensate. Since our T_D lizards had decreased metabolic rates, this temperature threshold is higher than 11ºC. The interaction between photoperiod and latitude on oxygen consumption suggests that high and low-latitude lizards respond differently to the same photoperiod cues. Our high-latitude lizards responded more strongly to the P_D cue; they increased oxygen consumption more than did low-latitude lizards. This interaction suggests ecotypic variation in respiration rates between latitudes. P_D lizards had lower blood glucose concentrations which could be due to lizards preparing for winter by converting blood glucose into stored glycogen (Dessauer, 1953). However, we did not see an associated increase in liver glycogen in P_D or T_D lizards to support this.

Temperature effects

The T_C lizards had 96.1% greater oxygen consumption than T_D lizards. Our results are in agreement with other studies that found lizards had decreased metabolism when exposed to lower temperatures (Dawson and Bartholomew, 1956; Mayhew, 1965; Prieto and Whitford, 1971; Sanders et al., 2015). However, our differences were greater than differences between oxygen consumption at similar temperatures in *Uta* (54.5% difference; Dawson and Bartholomew, 1956) and *Phrynosoma* (29.2% difference; Prieto and Whitford, 1971).

As predicted, T_D lizards decreased their food consumption over time. We first saw a significant difference between temperature treatments the week of November 7, 2016, 8 wk after the temperature treatment started. At this time, the refrigerated chamber was 13ºC. During the last 4 weeks, T_C lizards consumed 33.16 ± 1.62 mg/g/day while T_D lizards consumed 6.44 ± 2.57 mg/g/day. Lizards were at 21 and 11ºC respectively, but the consumption rates are comparable to *Uta stansburiana* who consumed 0 to 50 mg/g/day between 20 and 28ºC (Waldschmidt et al., 1986). T_D lizards also had lower blood glucose concentrations. Blood glucose levels were correlated with food consumption, so the lower blood glucose levels could have been because of lizards consuming fewer crickets.

T_D lizards were slower. Cold temperatures slow muscle contractions (Rall and Woledge, 1990; Swoap et al., 1993) which leads to slower sprint speeds (Ibargüengoytía et al., 2007; Watson and Formanowicz, 2012). Five-lined skinks were previously found to run 0.7 to 1.3 m/s between 15 and 30ºC (Watson and Formanowicz, 2012). At 20ºC, their mean sprint speed was around 0.7 m/s while our lizards at 21ºC had a mean of 0.78 ± 0.03 and a maximum of 0.93 m/s.

T_D lizards had lower CTMin, which supports our hypothesis. It is well known that cold acclimation lowers the CTMin of ectotherms, but the actual CTMin values were previously unknown for five-lined skinks (Brattstrom, 1968; Kour and Hutchison, 1970; Spellerberg and Spellerberg, 1972). Fitch (1954) noted that at 1.8ºC, five-lined skinks could not move normally and estimated their CTMin to be around 1.4ºC. The CTMin of five-lined skinks has also been estimated to be 2.5ºC (Brattstrom, 1965) and 7.7ºC (Youssef et al., 2008). These estimates were made by observing congeneric species and by the behavior of five-lined skinks at higher temperatures. Our results indicate that even if individuals are not cold acclimated, their CTMin is below these estimates at 0.06 ± 0.18ºC. We found a mean CTMin of -4.00 ± 0.29ºC for cold-acclimated lizards meaning that these lizards are far more cold-hardy than previously thought. Acclimation can be costly, so acclimation ability should be reduced at low latitudes; however, we did not see this latitudinal trend (Hoffmann, 1995).

No effects

SCP of five-lined skinks were previously unknown. It was known that they could supercool to at least -5ºC and that they were not freeze-tolerant (Fitch, 1954). We did not find any effects of latitude or treatment group, but our mean SCP of -12.69 ± 0.54ºC falls within the normal supercooling range for small reptiles (-8 to -18ºC; Costanzo and Lee, 2013). Freeze avoidance seems to be the most common winter strategy in lizards, although it is unknown if many species can tolerate freezing. Both lizard species currently known to survive freezing are in the Lacertidae family (Claussen et al., 1990; Voituron et al., 2002). We had one lizard survive ice nucleation but the percentage of body water frozen is unknown. Five-lined skinks can potentially tolerant limited ice formation in the body, but it is unlikely that this ability is ecologically relevant. Further studies are needed to determine the prevalence and depth of freeze tolerance in these lizards.

The lack of higher liver glycogen levels in T_D or P_D lizards is likely due to these lizards using blood glucose and liver glycogen as a source of overwinter energy. Liver glycogen is important for overwinter survival of *Uta* and other lizards (Dessauer, 1955b; Zani et al., 2012), so it is likely important for five-lined skinks as well. We might not see differences in our lizards due to large variation in liver glycogen levels and small sample sizes.

Evolution of cold tolerance

It makes sense that lizards experiencing harsher winter conditions would be more cold tolerant. We see this overall trend when looking at mean thermal tolerances of ectotherms worldwide, but the trends are unclear between closely related species or intraspecifically (Sunday et al., 2011). There was no difference in CTMin or SCP between our high- and low-latitude lizards. These results agree with the findings of many other researchers, further supporting the hypothesis that cold tolerance is evolutionarily conserved, meaning that cold tolerance has strong phylogenetic inertia (Hertz and Nevo, 1981; Huey, 1982; Gvoždík and Castilla, 2001; Yang et al., 2007; Michels-Boyce and Zani, 2015; but see Wilson and Echternacht, 1987; Weeks and Espinoza, 2013). Youssef et al. (2008) specifically suggested that thermal biology of the *Plestiodon* genus is evolutionarily conserved.

However, our results suggest that other winter variables may be adapted. The greater oxygen consumption of high-latitude lizards shows that lizards from different environmental conditions can respond differently to the same environmental cues. So even though cold tolerance may not be adapted, other winter survival mechanisms could be. Our study used field-caught specimens so we cannot rule out the possibility of phenotypic plasticity. Future studies using F_1 specimens are needed to tease apart the genetic component.

Summary

There is little variation between five-lined skinks from our high- and our low-latitude populations. Even though the populations are separated by about 1610 km and 13º latitude, they respond similarly to temperature and photoperiod cues. There was no difference in cold tolerance whether looking at CTMin or SCP. The only variable that suggests ecotypic variation between our latitudes is oxygen consumption. The high-latitude lizards responded more dramatically to the photoperiod cue, demonstrating the only variable in which lizards responded differently to the same external cue.

As predicted, we had response variables that were affected by photoperiod, temperature, both cues, and neither cue. Most of the variables were affected by temperature. Blood glucose levels and oxygen consumption were affected by both photoperiod and temperature. SCP, fat body mass, liver glycogen content, and body condition change were not affected by photoperiod or temperature. So, five-lined skinks are using different autumnal cues for various physiological changes.

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Appendix

Table 1. Experimental design. Treatment effects will indicate whether response variables require temperature cues, photoperiod cues, or both.

		Photoperiod treatment	
		Decrease	Constant
Temperature treatment	Decrease	Caused by both/either	Caused by temperature
	Constant	Caused by photoperiod	Control

Table 2. Summary ANOVA table.

Variable	Source	SS	df	Mean square	F	P
Blood glucose	Temp	2581.3	1	2581.3	6.10	0.039
	Photo	2640.3	1	2640.3	6.24	0.037
	Error	3383.3	8	422.9		
	Total	143800	12			
Oxygen consumption	Latitude	0.46	1	0.46	18.19	<0.001
	Temp	1.63	1	1.63	64.75	<0.001
	Photo	0.36	1	0.36	14.52	0.001
	Latitude*Photo	0.11	1	0.11	4.49	0.044
	Error	0.65	26	0.03		
	Total	9.76	31			
Body condition index	Sex	0.41	2	0.20	4.26	0.027
	Error	1.10	23	0.05		
	Total	2.18	26			
Food consumption	Time	3.15	1	3.15	237.81	<0.001
	Temperature	0.62	1	0.62	15.01	0.012
	Temp*Time	0.51	1	0.51	62.38	0.001
	Error	0.19	5	0.04		
Fat body mass	Latitude	0.01	1	0.10	8.85	0.007
	Error	0.04	24	0.00		
	Total	0.33	26			

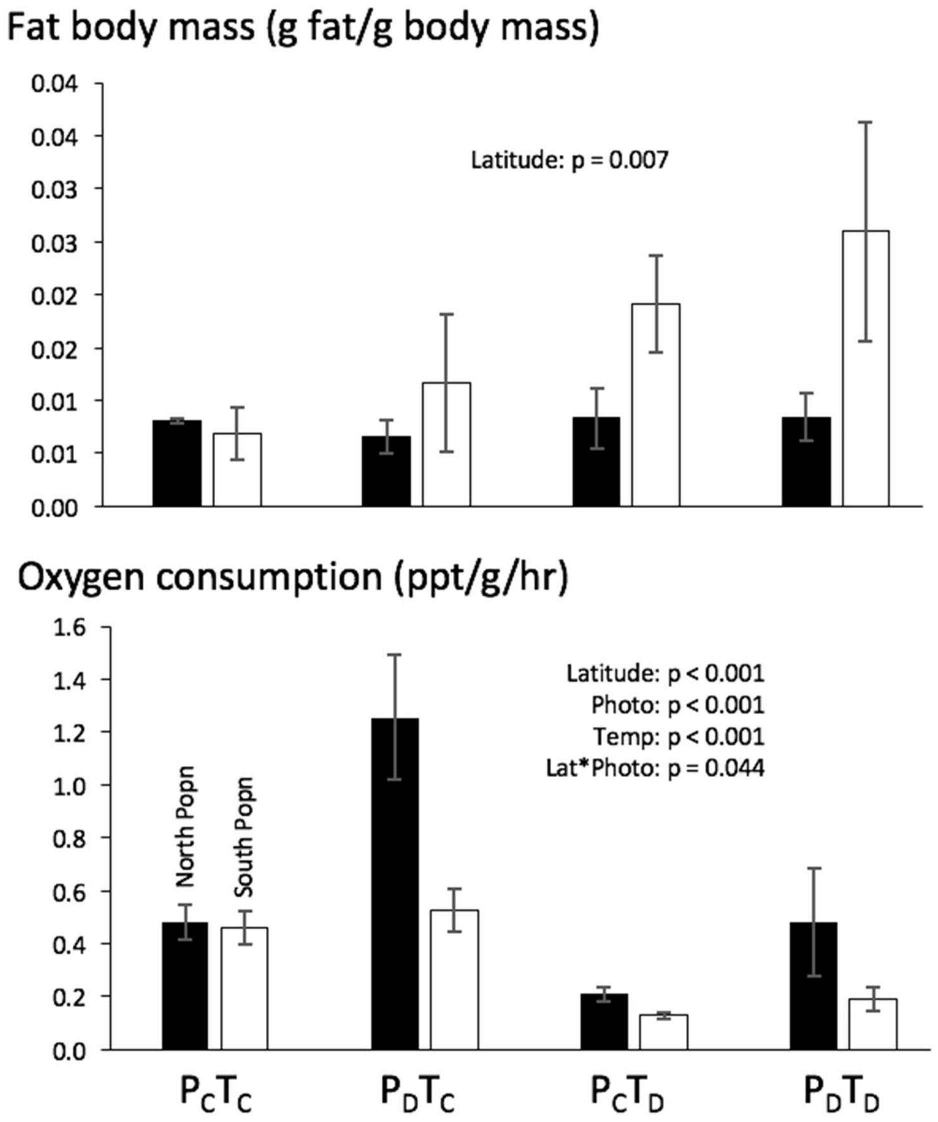

Figure 1. Effects of latitude. Upper: Low-latitude lizards had larger fat bodies. Lower: High-latitude lizards had greater oxygen consumption. Error bars are ± SE.

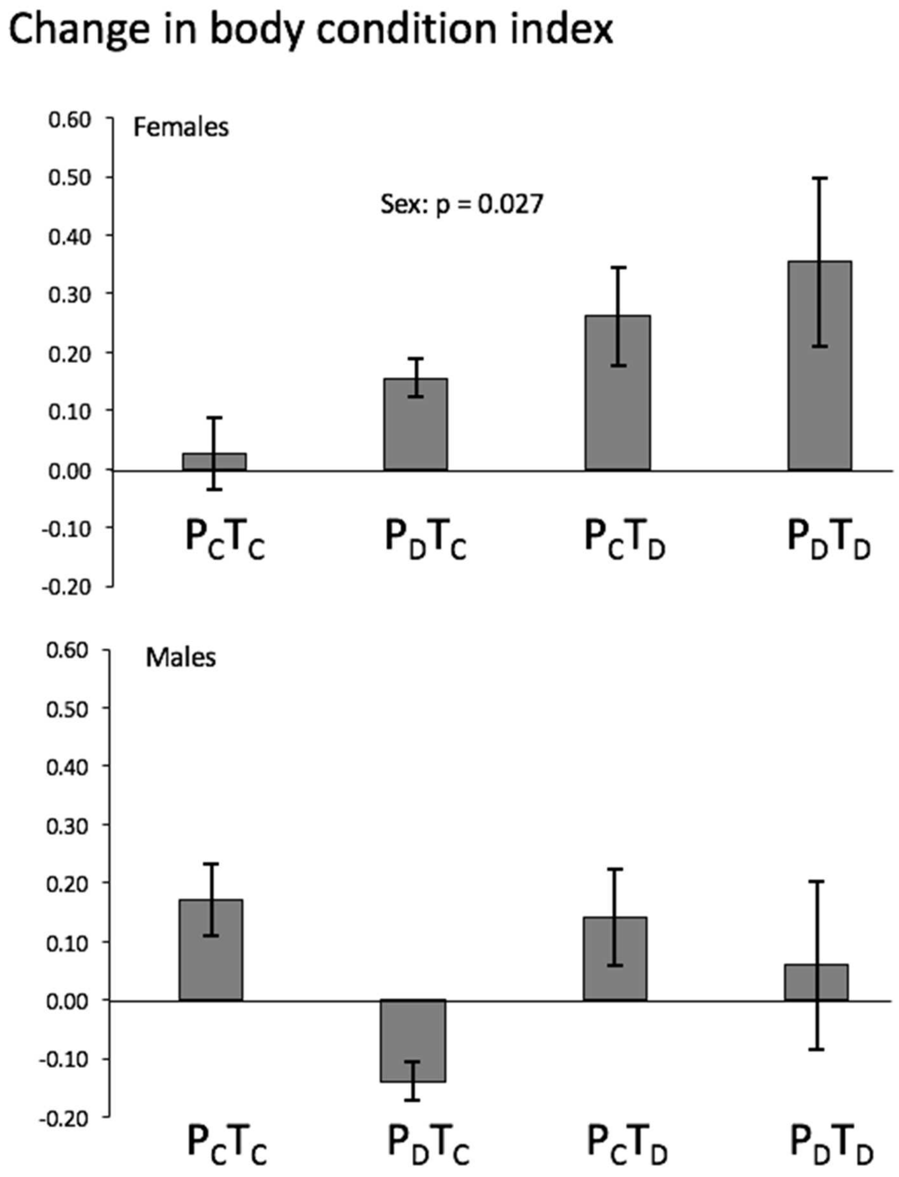

Figure 2. Effect of sex. Females improved their body condition in the lab more than males.

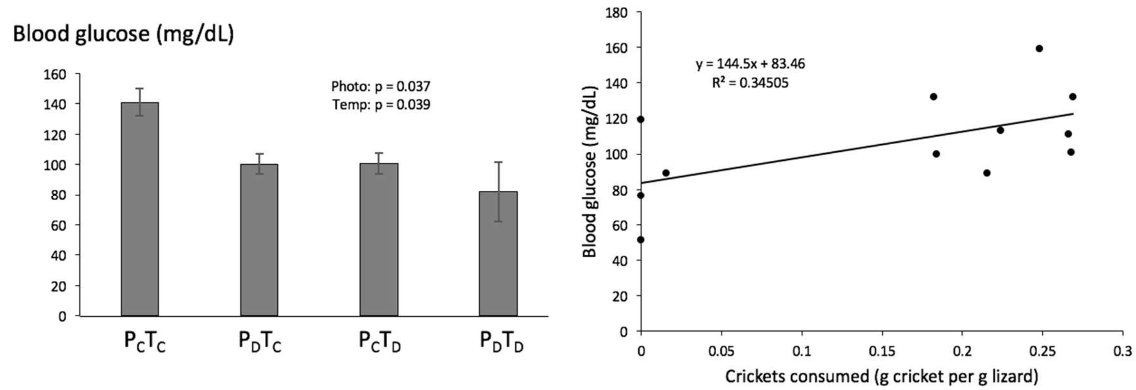

Figure 3. Effects of photoperiod. Left: P_D lizards had lower blood glucose. Right: Correlation between blood glucose concentration and cricket consumption. Greater food consumption is positively correlated with blood glucose levels. There is also a significant effect of oxygen consumption (see Fig. 1).

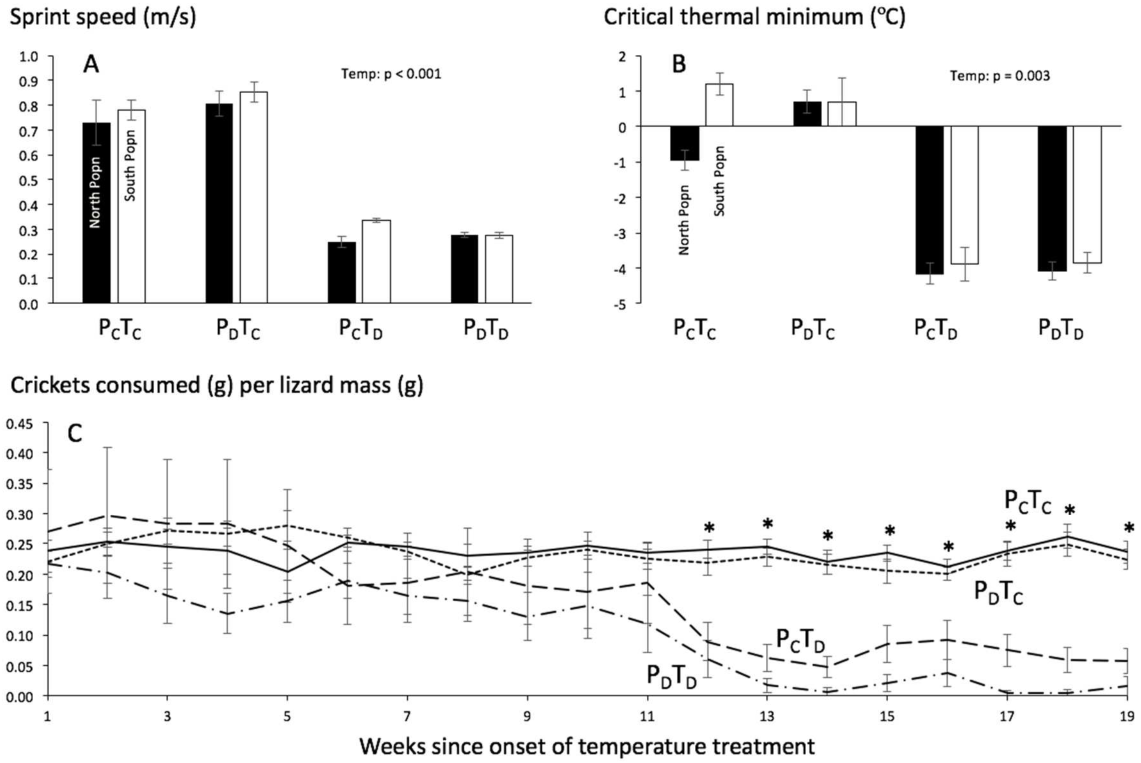

Figure 4. Effects of temperature. A: T_D lizards were slower. B: T_D lizards had lower CTMin. C: Food consumption of lizards in each treatment group over time. Lizards from both latitudes are pooled. T_D lizards have significantly lower food consumption ($p<0.05$) starting at week 12, as denoted by asterisks. There are no differences in photoperiod treatment. There are also significant effects on oxygen consumption (see Fig. 1) and glucose (see Fig. 2).

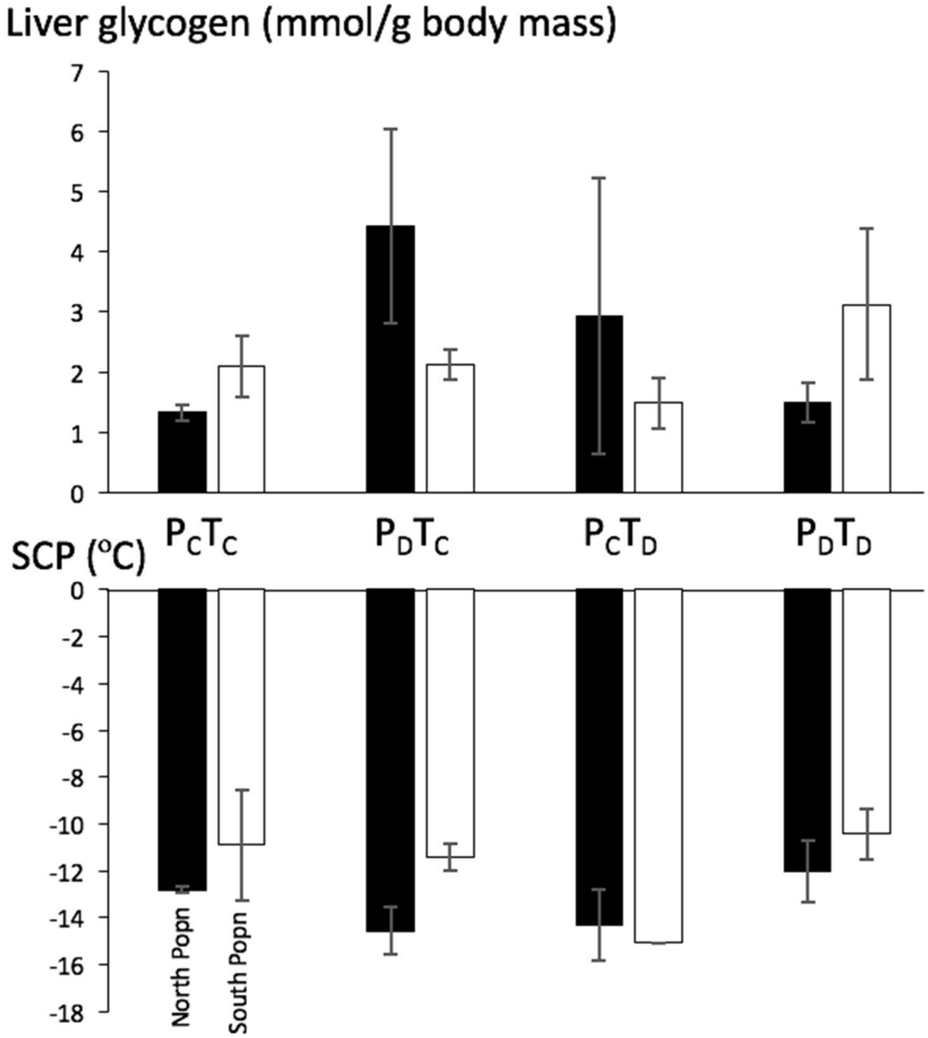

Figure 5. There were no significant effects of latitude, sex, photoperiod, or temperature on liver glycogen content (upper) or supercooling point (lower).