

Habitat-mediated shifts and plasticity in the evaporative water loss rates of two congeneric pit vipers (Squamata, Viperidae, *Agkistrodon*)

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ABSTRACT

Question: Are increased rates of total evaporative water loss (TEWL) associated with evolutionary transitions from terrestrial to aquatic habitats? Do individuals acclimated to wet conditions demonstrate higher TEWL rates than those acclimated to dry conditions?

Organisms: Individuals of the snake species *Agkistrodon piscivorus* (Viperidae; semi-aquatic) and *Agkistrodon contortrix* (terrestrial) collected from the Savannah River Site, Aiken, SC, USA.

Methods: We held individuals in either wet or dry acclimation conditions for 10 days. We then measured TEWL of individuals in an environmental chamber and tested the effects of humidity acclimation and species on TEWL rate. The TEWL rate was evaluated in the context of hypothesized habitat transitions within *Agkistrodon*.

Results: The semi-aquatic *A. piscivorus* exhibited higher TEWL rates than *A. contortrix*, the species which represents the putatively ancestral condition (terrestriality). The higher TEWL rate in *A. piscivorus* is concordant with the evolutionary shift to aquatic habitats in this species. Additionally, snakes in wet acclimation treatments had higher TEWL rates than those in dry treatments, as predicted.

Keywords: habitat aridity, habitat transition, phylogeny, semi-aquatic, snakes.

INTRODUCTION

The proper maintenance of water balance is of great importance to all terrestrial vertebrates, particularly those in arid environments. Evaporative water loss is the main avenue of total water loss for a wide variety of taxa (Dmi'el and Zilber, 1971; Dawson, 1982; Jorgensen, 1997). Total evaporative water loss (TEWL) is chiefly composed of cutaneous and respiratory evaporative water loss (Mautz, 1982a; Tieleman and Williams, 2002). Previous studies have demonstrated that rates of TEWL are negatively correlated with habitat aridity in many vertebrate

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taxa, fitting adaptive predictions (Mautz, 1982a,b; Lahav and Dmi'el, 1996; Williams, 1996; Jorgensen, 1997; Dmi'el, 1998; Tieleman *et al.*, 2003; Williams *et al.*, 2004).

Recently, phylogenetic information has been incorporated into comparative evaporative water loss research (e.g. Williams, 1996; Tieleman *et al.*, 2003; Williams *et al.*, 2004), but few studies have examined the concordance between habitat shifts within a clade and change in TEWL rates. Dial and Grismer (1992) conducted a phylogenetic analysis of ecophysiological evolution in *Coleonyx* geckos (Eublepharidae), recovering four equally parsimonious hypotheses of derived-state acquisition. All hypotheses suggested that extant *Coleonyx* have evolved from a high humidity-dwelling, high-TEWL rate ancestor. Based on their preferred hypothesis, Dial and Grismer (1992) concluded that low TEWL rate has independently evolved twice within one clade of desert-inhabiting geckos. However, no studies on terrestrial vertebrates have examined the evolutionary consequences of a shift to habitats of increased humidity relative to the ancestral habitat of a clade. Thus, nothing is known in terrestrial vertebrates about the TEWL rate consequences of exploiting a novel, higher humidity habitat.

Additionally, short-term plasticity in TEWL rates has been documented for many taxa and may affect the outcome of TEWL studies (Kobayashi *et al.*, 1983; Kattan and Lillywhite, 1989; Williams and Tieleman, 2000; Tieleman and Williams, 2002; Haugen *et al.*, 2003). For the purposes of this study, we define plasticity as a change in an individual's TEWL rate in response to short-term changes in environmental conditions, such as acclimation to humidity. Green anoles (*Anolis carolinensis*), for example, acclimated to conditions of lowered humidity by exhibiting reduced rates of TEWL (Kobayashi *et al.*, 1983; Kattan and Lillywhite, 1989). Therefore, for a full understanding of the effects of habitat aridity on TEWL rate, studies must be able to discriminate between taxon-specific differences and differences resulting from plasticity in TEWL rate.

Agkistrodon piscivorus (Cottonmouth; Viperidae) and *Agkistrodon contortrix* (Copperhead; Viperidae) represent excellent species for examination of the effects of divergent habitats on TEWL rates. These congeners are closely related (Parkinson *et al.*, 2000, 2002) (Fig. 1)

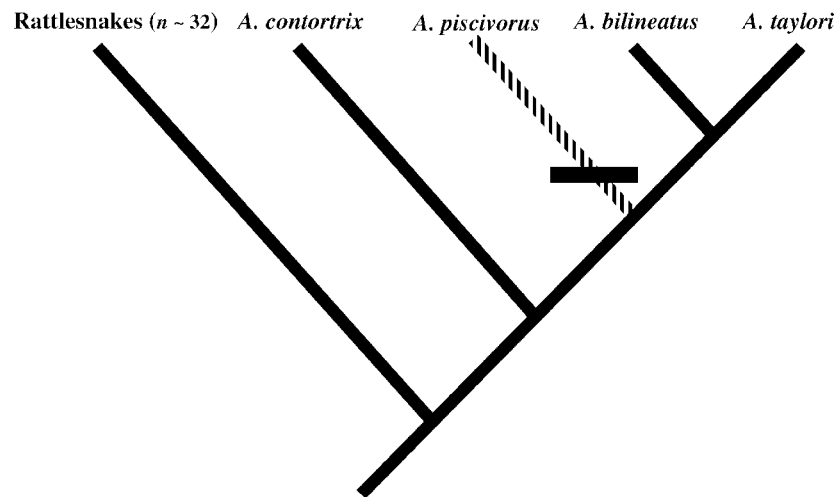


Fig. 1. Phylogeny of *Agkistrodon*, based on relationships presented by Parkinson *et al.* (2000, 2002). Solid branches indicate terrestrial habitat and the stippled branch indicates semi-aquatic habitat, with the bar representing the habitat shift by ancestral *A. piscivorus*. Rattlesnakes [*Crotalus*, *Sistrurus*: $n \sim 32$ (Place and Abramson, 2004)] represent the most likely sister clade to *Agkistrodon* (Parkinson *et al.*, 2002).

and sympatric across much of their geographic ranges, but typically occupy very different habitats (Gloyd and Conant, 1990). *Agkistrodon contortrix* is primarily a terrestrial species, whereas *A. piscivorus* is the most aquatic of all pit vipers (Gloyd and Conant, 1990; Ernst, 1992). Parsimony suggests that this shift to an aquatic habitat is derived within *A. piscivorus*, such that terrestriality in *A. contortrix* is the ancestral condition (Parkinson *et al.*, 2000, 2002). Therefore, we examined TEWL rates in these two species to determine whether the shift to aquatic habitats in *A. piscivorus* is associated with an increase in TEWL rate. We additionally examined plasticity in TEWL rate by exposing individuals to two humidity acclimation regimes, predicting that individuals subjected to wet acclimation environments would show higher TEWL rates than those subjected to dry environments. This design allowed us to distinguish between taxon-specific differences and those resulting from phenotypic plasticity.

METHODS

Animal collection and pre-trial maintenance

We obtained snakes primarily from the US Department of Energy's Savannah River Site in South Carolina, USA. *Agkistrodon contortrix* [$n = 10$; snout-vent length = 541 ± 23 mm, mass = 141 ± 18 g; mean \pm standard error (SE)] were captured primarily on roads. Individuals of *A. piscivorus* ($n = 9$; snout-vent length = 461 ± 28 mm, mass = 108 ± 13 g) were captured by hand in aquatic habitats. All snakes were collected in June and July 2002.

Before the acclimation treatments, we temporarily housed snakes outside for 2–17 days (under a pine forest canopy). Housing consisted of polyethylene containers (1 m high \times 1 m diameter) equipped with a sand and pine needle substrate, refugia and a large water bowl. Up to four *A. contortrix* were housed within a single container, while *A. piscivorus* were housed individually to prevent cannibalism (Ernst, 1992).

Acclimation treatments

Before testing TEWL rates, all individuals were assigned to one of two acclimation treatments (hereafter 'wet' and 'dry' treatments). Each acclimation treatment lasted for 10 days, following previous studies (e.g. Kobayashi *et al.*, 1983; Kattan and Lillywhite, 1989). For both treatments, snakes were housed individually in 11.5-litre polyethylene containers ($32 \times 24 \times 15$ cm) fitted with holes for ventilation, sphagnum moss as a substrate, and a small water bowl (filled daily; note that the water bowl was not large enough to allow a snake to lie in the water, and we never observed snakes attempting to put any portion of their body in the water during daily observations). To achieve a high relative humidity for the wet treatment, we misted containers daily with 75–80 ml of dechlorinated tap water. To create 'dry' conditions, we drilled additional holes in containers (to facilitate airflow) and these containers were not misted. Four HOBO data loggers (RH/TEMP H8, Onset Computer Corporation, Pocasset, MA, USA) were split evenly among each acclimation condition (i.e. two for each treatment) and monitored for 6 days. These data loggers indicated that wet conditions were constantly in the range of 95–100% relative humidity, and dry conditions in the range of 55–65% relative humidity. These values fall within the range of relative humidities recorded on the Savannah River Site during the active season of *A. contortrix* [mean daily low = 49.8%, mean daily high = 88.0% (Hunter, 2004)] and those experienced by aquatic-

foraging *A. piscivorus* (100% relative humidity). Throughout acclimation, we maintained all snakes at 23.5°C in a walk-in environmental chamber (M75, Environmental Growth Chambers, OH, USA) with a 14 h/10 h light/dark cycle. On day 2 of acclimation, we offered all snakes a pre-killed laboratory mouse (*Mus musculus*) with a mass equal to approximately 14% of the snake's body mass; TEWL measurements therefore were made on post-absorptive snakes (personal observation). We employed stratified random sampling to ensure that snakes of similar size (within species) were assigned to each treatment. We detected no differences in mass between treatments within each species (two-way ANOVA: $F_{1,15} = 0.06$, $P = 0.815$) or among species within each treatment (two-way ANOVA: $F_{1,15} = 2.18$, $P = 0.160$).

Evaporative water loss trials

We determined total evaporative water loss rates for *A. contortrix* and *A. piscivorus* using methods similar to Winne *et al.* (2001). Each snake was blotted dry and placed in a steel mesh cylinder. We constructed the cylinders to fit each individual's widest portion of the body in order to restrict movement [movement can increase TEWL (Dmi'el, 1972; Cohen, 1975)]. The combined mass of each cylinder and snake was determined to 0.01 g. We then immediately placed each cylinder/snake in an environmental chamber at 27°C, 50% relative humidity and constant darkness. Mass was recorded hourly for 6 h, at 8 h and every 6 h thereafter until 48 h; hourly mass loss was attributed to TEWL. Our methods did not allow the partitioning of cutaneous water loss from respiratory water loss; thus, we measured TEWL rate. After the 48 h measurement, we attempted to perform a repeated-measures design in which individuals were re-acclimated to the opposite acclimation environment. However, because of the high frequency of shedding during the second acclimation period (and thus loss of samples), we were unable to carry out the analysis due to insufficient power. All trials were conducted in June and July 2002, and began and ended at 09.00 h.

Statistical methods

Rates of total evaporative water loss were analysed as mean grams of mass lost per hour (mean value over 5 h; see below) and were \log_{10} -transformed to meet the normality assumption of analysis of covariance [ANCOVA (Sokal and Rohlf, 1995)]. Hours in which snakes excreted cloacal materials (i.e. liquid or fecal material) were excluded from the TEWL rate calculations. We also excluded the first hour of each trial, as we were unable to safely remove all surface moisture from the heads of these venomous snakes. After the first hour, snakes exhibited similar hourly TEWL throughout trials. Results from 48 h of data collection indicated that the first 6 h of data were sufficient to capture steady rates of TEWL. Thus, the 5 h of data after the first hour were used for subsequent analyses, as this interval resulted in the most reliable data (fewest excretions from cloaca). Preliminary analyses showed that TEWL rate did not differ between the sexes in either treatment (two-way ANCOVA, sex and treatment as independent variables, mass as covariate: *A. piscivorus*, $F_{1,4} = 0.19$, $P = 0.687$; *A. contortrix*, $F_{1,5} = 0.22$, $P = 0.658$); thus, the sexes were pooled in the final analysis.

We examined the effects of species and acclimation treatment on TEWL rate using a two-way ANCOVA, with species and treatment as independent variables and mass as a covariate [mass and TEWL rate exhibit negative allometry (Gans *et al.*, 1968; Cohen, 1975)]. Type III sums of squares was used, and the assumptions of parallelism and homogeneous variances were

verified for the analysis. We present least squares means with 95% confidence limits (Sokal and Rohlf, 1995). Statistical significance was set at $P < 0.05$. All analyses were computed using the JMP IN software package (Version 4.0.4, SAS Institute, Inc., Cary, NC, 2001).

RESULTS

Rates of TEWL of *Agkistrodon piscivorus* ($n = 9$) were higher than those of *A. contortrix* ($n = 10$) (two-way ANCOVA: $F_{1,14} = 14.2597$, $P = 0.0020$; Fig. 2). In addition, snakes in the wet treatment ($n = 10$) had higher TEWL rates than snakes in the dry treatment ($n = 9$; two-way ANCOVA: $F_{1,14} = 9.3378$, $P = 0.0086$; Fig. 2). There was no significant interaction between species and treatment ($F_{1,14} = 0.4748$, $P = 0.5021$). *Agkistrodon piscivorus* in the wet treatment had a least squares mean TEWL rate of $153.0 \text{ mg} \cdot \text{h}^{-1}$ (95% confidence limits: $128.2\text{--}178.4 \text{ mg} \cdot \text{h}^{-1}$; $n = 5$), whereas those in the dry treatment had a mean of $108.4 \text{ mg} \cdot \text{h}^{-1}$ ($80.3\text{--}137.3 \text{ mg} \cdot \text{h}^{-1}$; $n = 4$). *Agkistrodon contortrix* in the wet treatment had a least squares mean TEWL rate of $97.1 \text{ mg} \cdot \text{h}^{-1}$ ($73.4\text{--}121.3 \text{ mg} \cdot \text{h}^{-1}$; $n = 5$), while those in the dry treatment had a mean rate of $70.5 \text{ mg} \cdot \text{h}^{-1}$ ($46.6\text{--}94.9 \text{ mg} \cdot \text{h}^{-1}$; $n = 5$).

DISCUSSION

Our results demonstrate that *A. piscivorus*, a semi-aquatic species, has increased rates of TEWL relative to its terrestrial congener *A. contortrix*. This is consistent with previous data on squamate reptiles that demonstrate a negative correlation between taxon-specific differences in TEWL rate and habitat aridity (Dial and Fitzpatrick, 1982; Mautz, 1982b; Dial and Grismer, 1992; Lahav and Dmi'el, 1996; Dmi'el, 1998). However, previous studies incorporating phylogeny have either (1) not inferred the direction of evolution or (2) shown that a low TEWL rate can evolve in

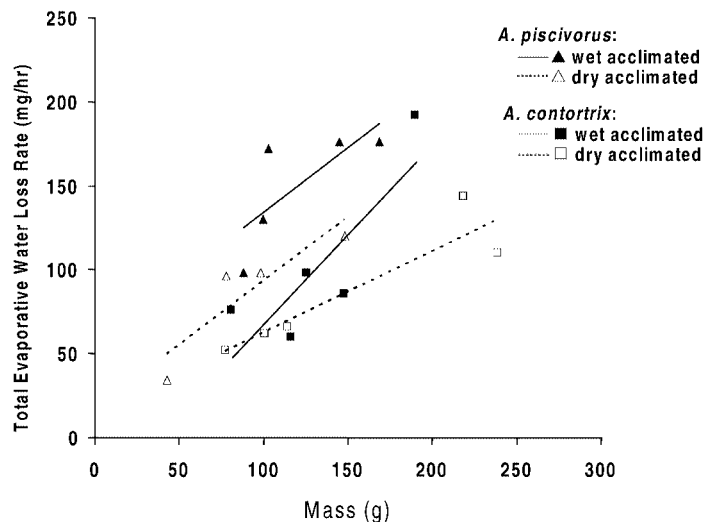


Fig. 2. Plot of initial body mass versus total evaporative water loss (TEWL) rate for *Agkistrodon piscivorus* and *A. contortrix*. After controlling for body mass (ANCOVA), TEWL rates were significantly higher in *A. piscivorus* ($n = 9$) than in *A. contortrix* ($n = 10$) ($P = 0.002$), and significantly higher for snakes in the wet acclimation treatment ($n = 10$) than those in the dry acclimation treatment ($n = 9$) ($P = 0.0086$). Best-fit lines were added to simplify viewing trends in the data.

response to evolutionary transitions from humid to arid habitats (e.g. Dial and Grismer, 1992). Our results indicate that a higher TEWL rate can result from evolutionary transitions in the opposite direction, from terrestrial to aquatic habitats. Further studies of *Agkistrodon* that partition cutaneous and respiratory water loss would be helpful in elucidating the physiological mechanism(s) and possible evolutionary causes for the observed species differences in TEWL rate. For example, previous research has demonstrated that differences in TEWL rates among snake species are due primarily to cutaneous water loss (Prange and Schmidt-Nielsen, 1969; Dmi'el, 1972, 1985; Lahav and Dmi'el, 1996). Cutaneous water loss, in turn, is primarily regulated by intra-epidermal lipids (Roberts and Lillywhite, 1980, 1983). If production of these lipids is energetically expensive, then we would expect selection to reduce the energy allocated to maintenance of a lipid barrier in species exploiting novel humid habitats, where desiccation is unlikely (Cohen, 1975). Alternatively, or additionally, the relaxation of a selective pressure to maintain effective barriers to water loss (Cohen, 1975) may allow increased TEWL to aid in evaporative cooling (Tracy, 1976; DeNardo *et al.*, 2004).

Interspecific comparisons of other Viperidae species have also demonstrated a negative correlation between TEWL rate and habitat aridity (Dmi'el, 1972, 1998; Lahav and Dmi'el, 1996). However, Dmi'el (2001, p. 65) suggested that an 'effect of taxonomic status' on TEWL rate may occur in reptilian genera or families. Among four snake species in the Middle East, for example, two species of vipers had much lower rates of TEWL than two Colubridae species of equal mass, regardless of the habitat aridity experienced by the species (Dmi'el, 1972). Additionally, colubrids from a study with identical methods (Lahav and Dmi'el, 1996) all had higher TEWL rates than did vipers in the study of Dmi'el (1972). Although we found clear evidence of species divergence in TEWL rate in the genus *Agkistrodon*, species differences were not as large as other interspecific comparisons of southeastern US taxa (e.g. Winne *et al.*, 2001). Interspecific variation in TEWL rate among vipers thus appears to be low relative to other taxa, but may vary via changes that are consistent with adaptive predictions, such as those observed in the current study and other TEWL studies (e.g. Dmi'el, 1998). More TEWL research is needed to quantitatively assess the role of phylogeny in the evolution of TEWL rate within the Viperidae.

Phenotypically plastic responses to short-term humidity acclimation also influenced TEWL rate in the present study. Wet-acclimated individuals of both species exhibited higher TEWL rates than those acclimated to dry conditions, as predicted. Additionally, the lack of statistical significance of the species \times treatment interaction indicates that plasticity in response to acclimation environments was similar in both species. Thus, our results corroborate those of other studies suggesting a role for short-term (i.e. non-developmental) phenotypic plasticity in squamate TEWL rates (Kobayashi *et al.*, 1983; Kattan and Lillywhite, 1989). Further research on humidity acclimation in TEWL rate is necessary to elucidate the mechanism(s) that may be operating to influence individual responses to environments of contrasting humidity.

Our results contradict those of Dunson and Freda (1985), who observed no difference in water permeability of shed skins of *A. piscivorus* and *A. contortrix*. Three differences in design may partly explain the discrepancy between the two studies. First, whereas we measured TEWL from whole animals, Dunson and Freda examined water permeability of shed skins. Second, Dunson and Freda used a smaller sample size (*A. piscivorus*, $n = 6$; *A. contortrix*, $n = 2$), which probably reduced the power of their analyses. Third, Dunson and Freda did not regulate humidity in laboratory holding facilities, and we have shown that water loss (and thus permeability) can be plastic in short-term holding conditions.

In summary, we observed taxon-specific differences in TEWL rate between *A. piscivorus* and *A. contortrix* in the manner predicted. We also found that intraspecific differences in these species can result from short-term humidity acclimation. Evaporative water loss rates were higher in the species exploiting a novel aquatic habitat (*A. piscivorus*) than in a terrestrial congener (*A. contortrix*). While the ancestral habitat type in *Agkistrodon* is unambiguous, future studies should examine TEWL rate in the other two species of *Agkistrodon* (*A. bilineatus*, *A. taylori*) and the rattlesnake outgroup to infer the likely ancestral state of TEWL rate in *Agkistrodon*. By including more species, the patterns of concordance between habitat shifts and TEWL rate within this clade could be more broadly interpreted (Garland and Adolph, 1994).

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