

EXPLORING POTENTIAL INDEPENDENT LOSS OF CLOACAL BURSAE IN TURTLES OF THE FAMILY *GEOEMYDIDAE*

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Abstract.—Diamond-backed Terrapins (*Malaclemys terrapin*, Emydidae) and sea turtles (Cheloniidae and Dermochelyidae) lack cloacal bursae, which are paired sacs that branch off the cloaca. We predicted that only freshwater turtles would contain cloacal bursae. We tested this prediction in a phylogenetic analysis of the ecologically diverse clade Geoemydidae. This group includes species that use both freshwater and brackish water as well as fully terrestrial species, making it an ideal group to test our hypotheses regarding evolutionary losses and gains across this ecological spectrum. Five geoemydid species have been reported to use brackish environments. Through dissections, diceCT, and phylogenetic comparative methods, we determined that some species showed independent losses of cloacal bursae, but the losses did not follow the pattern that we originally predicted would occur. Habitat, whether terrestrial or brackish, does seem to influence whether a species has independently lost their cloacal bursae.

Key Words.—diceCT; evolutionary physiology; phylogenetic analysis; turtles.

INTRODUCTION

Cloacal bursae are paired sacs that branch off the cloaca of many freshwater turtle species (Fig. 1). Their main functions are gas exchange, buoyancy control, ion transport, and even water storage, with the latter function being potentially used both for wetting nesting substrates by females and as a defense against dehydration in both sexes (Peterson and Greenshields 2001). Sethna et al. (2022) revealed a phylogenetically independent loss of cloacal bursae in the emydid diamond-backed terrapin (*Malaclemys terrapin*), a brackish-water specialist that typically resides in brackish tidal wetlands. Because cloacal bursae allow for ion transport, their presence in species living in saltwater could result in dehydration or too much salt entering the body. Notably, sea turtles also do not have cloacal bursae (Jackson 2011).

Sethna et al. (2022) hypothesized that the brackish environment of the *M. terrapin* is the cause of this independent loss of cloacal bursae, and we hypothesized that similar situations may occur in other turtle species residing in brackish water. Similarly, fully terrestrial species nested in clades of predominantly freshwater species (e.g., Ornate Box Turtles, *Terrapene ornata*; Emydidae) also usually lack cloacal bursae (Dodd 2002). Our goal was to

test whether the absence of cloacal bursae was related to either the terrestrial or brackish environments of species, so we used the ecologically diverse family Geoemydidae to test our hypotheses. This clade contains turtle species in Asia and South America that primarily inhabit freshwater or terrestrial environments, but has some members speculated or confirmed to live in brackish habitats. We predict that only freshwater turtles will contain cloacal bursae.

MATERIALS AND METHODS

Morphological and ecological characters.—We used the phylogenetic tree of Testudines from Thomon et al. (2021) to map out the habitat of each species within the family. We used data from the Reptile Database (<http://www.reptile-database.org>) and the Red List of the International Union for Conservation of Nature (IUCN 2023) to determine the habitat of each species and then confirmed such in the primary literature, when possible. We analyzed 30 of the 71 recognized species in Geoemydidae (<http://www.reptile-database.org>) with specific efforts being made to include as many species known, or suspected, to use brackish water habitats. All the specimens used were adults.

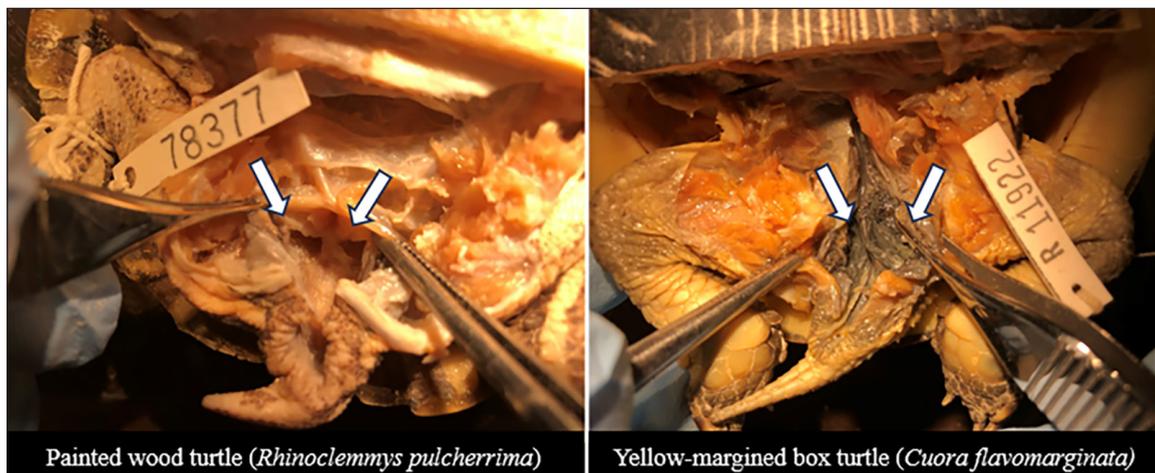


FIGURE 1. Dissections of (Left) a Painted Wood Turtle (*Rhinochlemmys pulcherrima*; NCSM 78377) with arrows indicating the paired openings of the cloacal bursae, and (Right) a Yellow-margined Box Turtle (*Cuora flavomarginata*; YPM 11922) with arrows indicating approximate location of where openings for cloacal bursae would be found if present. (Photographed by Megan Wright).

We performed dissections on specimens representing nine species (Appendix Table). We were limited in our dissection sampling due to the rarity of many of these species in museum collections and the destructive nature of dissections. Many of our loan requests were understandably denied by museum curators. To increase our sampling, we expanded our methodology to include diffusible iodine-based contrast-enhanced computed tomography (diceCT; Callahan et al 2021) in four additional species located at the University of Michigan Museum of Zoology (Appendix Table). We also were able to obtain scans from three species we had already dissected to confirm our diceCT methodology, including the Pond Slider (*Trachemys scripta*; Emydidae) as a species well known to have cloacal bursae to validate our technique (Fig. 2). We uploaded our CT data into 3DSlicer (Fedorov et al. 2012) and we constructed 3D renderings of the cloacal bursae. We included additional data for presence/absence of cloacal bursae from the review by Smith and James (1958). To resolve any taxonomic name differences between Smith and James (1958) and the current phylogenetic tree for Geoemydidae (Thomson et al. 2021), we used Reptile Database (<http://www.reptile-database.org>).

Phylogenetic analyses of cloacal bursae and habitat.—We performed all analyses using the phylogenetic tree of Testudines from Thomson et al. (2021), pruned to include only species for which we had data on presence/absence of cloacal bursae. We used an alpha level of 0.05, as is conventional, for all analyses described below (Fisher 1926). To measure

phylogenetic signal, we used the R package *phylosig* version 1.9–16 (Revell 2012) in R version 4.3.1 (R Core Team 2023). We calculated both Bloomberg’s K and Pagel’s λ for both cloacal bursae presence/absence and habitat type (freshwater, brackish, terrestrial, or some combination).. Because these tests are not designed for overlapping categorical variables, we scored mixed habitats (i.e., use of both freshwater and terrestrial) as a unique habitat type rather than as a combination of two other habitats. We were able to estimate phylogenetic signal with mixed habitat types scored as additive rather than unique habitats using binaryPGLM in package *ape* version 5.7–1 (Paradis and Schliep 2019) and *phyloglm* in package *phylolm* version 2.6.2 (Ho and Ané 2014); both packages allow traits to be coded as combinations of other traits in a manner that Bloomberg’s K and Pagel’s λ (as measured by *phylosig*) cannot accommodate. The function *binaryPGLMM* creates a phylogenetic Generalized Linear Mixed Model of the effect of predictor variables (e.g., habitat) on response variables (e.g., cloacal bursae) and measures phylogenetic signal as the scalar magnitude of the phylogenetic variance-covariance matrix of the model; it also returns the probability of no phylogenetic signal in a data set. The function *phyloglm* returns an alpha value that represents the correlation between phylogeny and the traits observed.

Habitat-based models of cloacal bursae.—To separately estimate the effect of each habitat on cloacal bursae presence/absence, we used discrete

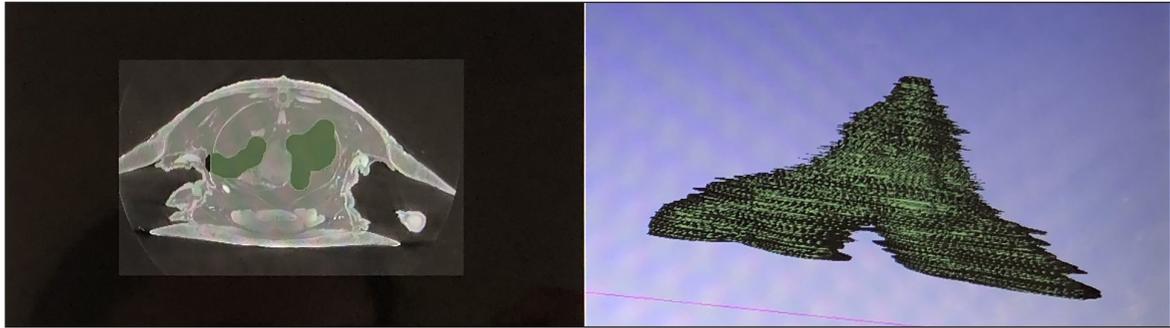


FIGURE 2. Example from the diceCT imaging of a Pond Slider (*Trachemys scripta*; Emydidae; UMMZ 210663). Both images are oriented where the posterior of the specimen is pointing towards the top. (Left) *in situ* image and (Right) digitally reconstructed model. (Photographed by Megan Wright).

character analysis in BayesTraits V4.0.0 (Pagel et al. 2004; Pagel and Meade 2006). We ran both discrete-independent and discrete-dependent models for each habitat. The discrete-independent model assumes that the evolution of the two characters is independent, while the discrete-dependent model assumes that the two characters are dependent on one another (i.e., if one is present the other will usually/always be present, or vice versa). For each model, we ran an MCMC analysis with a steppingstone sampler of 100 stones with 1,000 steps per stone and 10,000 iterations discarded as burn-in, for 1,010,000 iterations in total.

To estimate the effect of all habitats cumulatively on cloacal bursae presence/absence, we built a series of linear models in R. We used R because BayesTraits does not currently support both independent and dependent models of multi-state categorical variables. We built models using three different functions and packages: binaryPGLMM in package ape version 5.7–1 (Paradis and Schliep 2019), phyloglm in package phyloilm version 2.6.2 (Ho and Ané 2014), and glm in the base package stats version 3.6.2 (R Core Team 2023). We included all three models because binaryPGLMM and phyloglm perform similarly but treat binary traits differently (as a threshold evolution model vs. as a true binary trait) and because a non-phylogenetic generalized linear model may be appropriate in the absence of phylogenetic signal (Ives and Helmus 2011), as we observed in this case. We used an alpha value of 0.05 to evaluate the probability that the estimated effect sizes from each model were non-zero.

RESULTS

Morphological and ecological characters.—Wariss et al. (2012) noted that Spot-legged Wood

Turtle (*Rhinoclemmys punctularia*) individuals were found in coastal-plain lake, flooded forest *igapó*, interdunal lake, and tidal channel habitats, some of which contain brackish water. Mantziou and Rifai (2014) documented that the Balkan Terrapin (*Mauremys rivulata*) can reside in freshwater habitats, including rivers, seasonal ponds, and lakes, but also that they can live in brackish coastal lagoons, irrigation canals, and reservoirs. Ernst et al. (2000) reported that the Painted Terrapin (*Batagur borneoensis*) and the Northern River Terrapin (*Batagur baska*) live in tidal, brackish estuaries. The Indian Roofed Turtle (*Pangshura tecta*) is known to use brackish environments (IUCN 2023; Vattakaven et al. 2016). Thus, we had five geoemydid species known to reside in brackish habitats with which to test our hypothesis of independent loss of cloacal bursae in such habitats (Fig. 3, Table 1).

Among the species we sampled, four species appear to have independently lost their cloacal bursae: *R. punctularia* (freshwater, brackish, and terrestrial), the Asian Leaf Turtle (*Cyclemys dentata*; freshwater), the Keeled Box Turtle (*Cuora mouhoti*; terrestrial), and the Yellow-margined Box Turtle (*Cuora flavomarginata*; terrestrial). The brackish species *M. rivulata* and *P. tecta* retain cloacal bursae. The following terrestrial-only species also retain the structures: the Indochinese Box Turtle (*Cuora galbinifrons*), the Black-breasted Leaf Turtle (*Geoemyda spengleri*), the Furrowed Wood Turtle (*Rhinoclemmys areolata*), the Painted Wood Turtle (*R. pulcherrima*), and the Mexican Spotted Wood Turtle (*R. rubida*).

Phylogenetic signal.—We found no evidence of phylogenetic signal in distribution of cloacal bursae presence/absence across the tree as measured by Bloomberg's K ($K = 0.400$ $P = 0.370$) and Pagel's λ

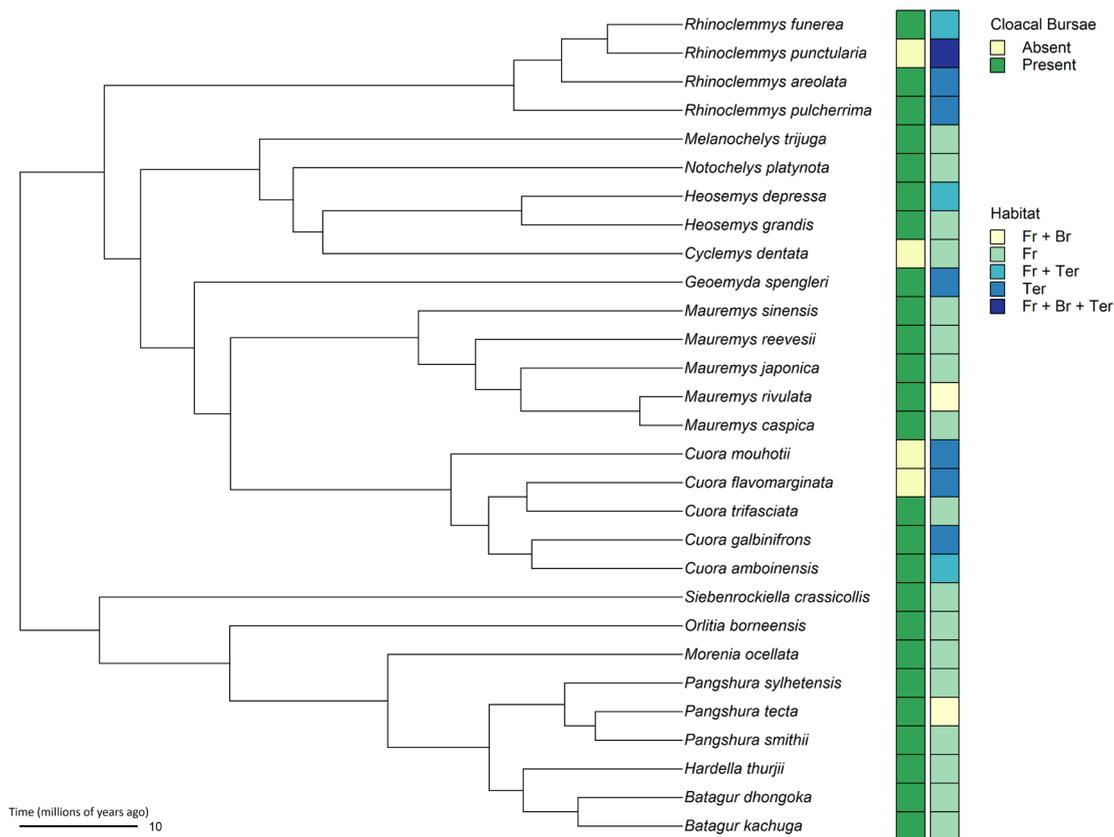


FIGURE 3. The presence or absence of cloacal bursae and habitat of each species in relation to the phylogenetic tree of Geoemydidae. The presence or absence of cloacal bursae (first column) was determined through dissection, diceCT analyses, or data from Smith and James (1958). The habitat of each species (second column) found on this phylogenetic tree by Thomson et al. (2021) was determined using the Reptile Database (<http://www.reptile-database.org>) and the Red List of the International Union for Conservation of Nature (IUCN 2023). The tree was trimmed to the sample dataset and does not include all of the species within Geoemydidae. Branch lengths indicate estimated time since divergence between species according to the reconstructions done by Thompson et al. (2021). The abbreviations Fr = freshwater, Br = brackish water, and Ter = terrestrial.

($\lambda < 0.001$, $P = 1.0$). We also found no phylogenetic signal in habitat distribution across the tree as measured by either Bloomberg's K ($K = 0.522$, $P = 0.065$) or Pagel's λ ($\lambda = 0.512$, $P = 0.082$); however, binaryPGLMM estimated the phylogenetic signal of habitat and cloacal bursae to be 2.270, with a probability of 0.215, or 21.5%, indicating that the null hypothesis (no phylogenetic signal) is supported. Similarly, phyloglm estimated the phylogenetic correlation of habitat and cloacal bursae as 0.069, on a scale where 0 indicates strong phylogenetic signal and 1 indicates the signal expected under Brownian motion. The discrepancy in significance between these methods of estimating phylogenetic signal can be attributed to the different methods of coding mixed habitats; as stated previously, mixed habitats were treated as unique habitats by Bloomberg's K

and Pagel's λ but were interpreted as combinations of single habitats by binaryPGLMM and phyloglm. The independent and dependent models of cloacal bursae/habitat evolution had very similar log marginal likelihood estimates for all three habitats. The freshwater independent and dependent models had log marginal likelihood values of -30.205 and -29.882, respectively. The terrestrial independent and dependent models had log marginal likelihoods of -33.546 and -32.852, while the brackish models had log marginal likelihoods of -24.011 and -24.084.

Additive models of cloacal bursae and habitat.—All three models of the effects of habitat on condition of cloacal bursae showed the same general trends (Table 2). Using binaryPGLMM, our model showed a generally negative effect of terrestrial and brackish

TABLE 1. Comparison of presence/absence of cloacal bursae and habitat type of studied species. Data on cloacal bursae from this study found by dissection are marked by asterisk (*), data found by diceCT are marked by a caret (^), and remaining data are from Smith and James (1958). Abbreviations are FW = freshwater, BW = brackish water, and T = terrestrial, U = unknown sex, and symbols are ♀ = females and ♂ = males.

Species	Cloacal Bursae (Y/N)	Habitat	Sex
Black Wood Turtle (<i>Rhinoclemmys funerea</i>)	Y*	FW, T	♀
Spot-legged Wood Turtle (<i>R. punctularia</i>)	N^	FW, BW, T	♀
Furrowed Wood Turtle (<i>R. areolata</i>)	Y^	T	♀
Painted Wood Turtle (<i>R. pulcherrima</i>)	Y*	T	♀
Mexican spotted Wood Turtle (<i>R. rubida</i>)	Y^	T	♀
Indian Black Turtle (<i>Melanochelys trijuga</i>)	Y	FW	U
Malayan Flat-shelled Turtle (<i>Notochelys platynota</i>)	Y	FW	♀
Arakan Forest Turtle (<i>Heosemys depressa</i>)	Y	FW, T	U
Giant Asian Pond Turtle (<i>H. grandis</i>)	Y	FW	U
Asian Leaf Turtle (<i>Cyclemys dentata</i>)	N*	FW	♀
Black-breasted Leaf Turtle (<i>Geoemyda spengleri</i>)	Y*	T	♀ & ♂
Chinese Stripe-necked Turtle (<i>Mauremys sinensis</i>)	Y	FW	♀
Chinese Pond Turtle (<i>M. reevesii</i>)	Y	FW	♂
Japanese Pond Turtle (<i>M. japonica</i>)	Y	FW	U
Balkan Terrapin (<i>Mauremys rivulata</i>)	Y	FW, BW	U
Caspian Turtle (<i>Mauremys caspica</i>)	Y*^	FW	♀
Keeled Box Turtle (<i>Cuora mouhotii</i>)	N	T	♀
Yellow-margined Box Turtle (<i>C. flavomarginata</i>)	N*	T	♀
Golden Coin Turtle (<i>C. trifasciata</i>)	Y*	FW	♀
Indochinese Box Turtle (<i>C. galbinifrons</i>)	Y*	T	♀
Amboina Box Turtle (<i>C. amboinensis</i>)	Y	FW, T	U
Black Marsh Turtle (<i>Siebenrockiella crassicollis</i>)	Y	FW	U
Malaysian Giant Turtle (<i>Orlitia borneensis</i>)	Y	FW	♂
Burmese Eyed Turtle (<i>Morenia ocellata</i>)	Y	FW	U
Assam Roofed Turtle (<i>Pangshura sylhetensis</i>)	Y	FW	U
Indian Roofed Turtle (<i>P. tecta</i>)	Y	FW, BW	U
Brown Roofed Turtle (<i>P. smithii</i>)	Y*^	FW	♀
Crowned River Turtle (<i>Hardella thurjii</i>)	Y^	FW	♂
Three-striped Roofed Turtle (<i>Batagur dhongoka</i>)	Y	FW	U
Red-crowned Roofed Turtle (<i>Batagur kachuga</i>)	Y	FW	U

habitats on bursae presence and a weaker positive effect of freshwater habitats on the presence of cloacal bursae. Our model, constructed using phyloglm, showed a similar negative effect of terrestrial and brackish habitats on bursae presence and positive effect of freshwater habitats, and it had an AIC value of 29.532. The model made using glm showed the same trend and had an AIC value of 27.282, comparable to the AIC of the model made using phyloglm despite ignoring the underlying phylogeny.

DISCUSSION

We predicted that geoemydid turtles residing in brackish environments would have independently lost their cloacal bursae, as was seen in the brackish emydid *M. terrapin* (Sethna et al. 2022), but this prediction was not supported by our data. Though the brackish-inhabiting *R. punctularia* did independently lose cloacal bursae, both *M. rivulata* and *P. tecta* still possess cloacal bursae despite partially residing in brackish environments. Three other species also

TABLE 2. Estimated effect sizes of freshwater, terrestrial, and brackish habitats on the presence of cloacal bursae under three different models. Abbreviations are B = regression coefficient, SE = standard error, and P = P -value of null hypothesis that $B = 0$.

Habitat	binaryPGLMM			phyloglm			glm		
	B	SE	P	B	SE	P	B	SE	P
Freshwater	0.931	1.842	0.613	0.633	1.456	0.664	0.871	1.666	0.601
Terrestrial	-1.384	1.926	0.472	-1.311	1.485	0.377	-1.575	1.660	0.343
Brackish	-2.522	1.838	0.170	-1.764	1.402	0.208	-2.068	1.675	0.217

exhibited independent loss of their cloacal bursae: *C. mouhotii* and *C. flavomarginata*, both terrestrial species, and *C. dentata*, a freshwater species. This repeated independent loss of cloacal bursae is strongly contrary to the principle of parsimony that has long been used in the design of phylogenetic methods, such as ancestral state reconstruction (Swofford and Madisson 1992). This unusual rate of independent losses may explain our mixed phylogenetic signal results: that neither cloacal bursae nor habitat had significant phylogenetic signal when tested separately but did have a weak phylogenetic signal when the two traits were considered together. The discrepancy in significance between these methods of estimating phylogenetic signal can be attributed to the different methods of coding mixed habitats; as stated previously, mixed habitats were treated as unique habitats by Bloomberg's K and Pagel's λ but were interpreted as combinations of single habitats by binaryPGLMM and phyloglm. The low degree of phylogenetic signal may also be attributable to our broad sampling approach, which took one or a few species from each genus but did not provide full coverage of any genera.

The independent loss of cloacal bursae in one brackish and two terrestrial species aligns with expectations from known losses in brackish, marine, and some terrestrial species. Nevertheless, our broader comparisons of independent and dependent models showed no significant effect of any single habitat on gain/loss of cloacal bursae (as would be shown by a difference in log marginal likelihoods greater than two units). Though all three linear models of the effect of habitat on cloacal bursae presence/absence produced results that were similar but not strictly significant, the negative trend between brackish and terrestrial habitats and cloacal bursae and positive trend between freshwater habitats and cloacal bursae was suggestive in all three. Our models show that habitat is not the only deciding factor in whether cloacal bursae will be maintained or lost, but that habitat does suggest whether a species will have an independent loss of its cloacal bursae or not.

Cloacal bursae are used for multiple functions including gas exchange, buoyancy control, ion transport, and water storage (Peterson and Greenshields 2001), although Jørgensen (1998) emphasized that not all turtles that have them appear to use them in the same manner. Similar to terrestrial emyids (e.g., *Terrapene ornata*), we found that two terrestrial geoemydid species had lost their cloacal bursae. Terrestrial species evidently do not need cloacal bursae for their main functions, which all involve water in some capacity. Therefore, independently losing them would potentially help individuals save the energy and resources spent to make and upkeep this structure, increasing their fitness.

Ion transport can represent a physiological challenge in marine species and relatively few tetrapods tend to live in these habitats because they face significant obstacles to their osmoregulation (Butler et al. 2021). This would explain why sea turtles also do not have cloacal bursae. The most surprising result from our analyses was the independent loss of cloacal bursae in a freshwater species, *C. dentata*, despite our models and the literature showing that cloacal bursae is much more common in freshwater species. It is worth noting that the emydid *M. terrapin* is an obligate, brackish-water specialist, and none of our geoemydid species known to use brackish water appear to be so specialized. Perhaps they retain the bursae and simply do not use them while using brackish water habitats. An additional challenge to our study was the relatively sparse information on the basic natural histories of many geoemydid species, which we attempted to resolve by being cautious of our labelling of habitats for species. It is evident that some species use multiple habitats through ontogeny and/or season, but the extent to which this influences the cloacal bursae is beyond the scope of this project.

Cloacal bursae remain understudied yet are evidently important to the physiological ecology of some species, and our results show that they are evolutionarily labile. Our results also point to a possible relationship between habitat and the use of

these structures. Future research could investigate the structure and function of cloacal bursae between and within species as they relate to habitat use. Even for species with cloacal bursae, the environmental conditions under which the individuals actually use them are unknown. Outstanding questions concerning these structures include ones related to ontogenetic development, sexual dimorphism, and seasonal and inter-individual variation in size and structure.

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MEGAN WRIGHT is currently a Ph.D. candidate at the University of Georgia, Athens, USA, in the Cellular Biology Department. She received her B.S. in Biology from the Georgia Institute of Technology, Atlanta, USA, where she studied cloacal bursae and surfacing behaviors in Diamond-backed Terrapins (*Malaclemys terrapin*) at Zoo Atlanta, GEORGIA, USA. This informed her research on cloacal bursae in geoemydid turtles and how their presence/absence is related to habitat. Megan maintains her love of herpetology through hiking and trips with other herpers. (Photographed by Anna Iouchmanov).



SAGE BABISH is currently an M.S. student at the University of Nevada, Reno, USA, working on their degree in Ecology, Evolution, and Conservation Biology. Sage received their B.S. in Biology from the Georgia Institute of Technology, Atlanta, USA, where they worked on a range of herpetological studies, including phylogenetics and animal behavior. Their research interests now center on population genetics and local adaptation, and they are currently studying local adaptation and TTX resistance in the Sierra Garter Snake (*Thamnophis couchii*). (Photographed by Michelle A. Werdann).



LILY GOWENS received her B.S. in Biology from the Georgia Institute of Technology, Atlanta, USA, and is fascinated by the evolutionary mechanisms involved in how organisms adapt to their ever-changing environment. Her undergraduate research experiences probed these ideas across developmental and behavioral genetics, morphology, and field ecology, and for her undergraduate thesis, she investigated the response of North American bird communities to climate change. Lily is also a scientific illustrator and enjoys using these skills to visually communicate research about the natural world. (Photographed by Sidharth Srinivasan).



JOSEPH R. MENDELSON III is the Director of Research at Zoo Atlanta, Georgia, USA, and an Adjunct Professor at the Georgia Institute of Technology, Atlanta, USA. He has been studying various aspects of the systematics, morphology, behavior, biogeography, and natural history of amphibians and non-avian reptiles since the 1980s, focusing especially on anurans and snakes in Central America and the North American deserts. (Photographed by R. Andrew Odum).

APPENDIX TABLE. Details of specimens examined by dissection and diceCT. Museum acronyms are from Sabaj et al. (2020).

Species Name	Method Used	Museum Information
Asian Leaf Turtle (<i>Cyclemys dentata</i>)	Dissection	YPM 11778
Yellow-margined Box Turtle (<i>Cuora flavomarginata</i>)	Dissection	YPM 11922
Indochinese Box Turtle (<i>Cuora galbinifrons</i>)	Dissection	UTA-JRM 5147
Golden Coin Turtle (<i>Cuora trifasciata</i>)	Dissection	MVZ 23932
Black-breasted Leaf Turtle (<i>Geoemyda spengleri</i>)	Dissection	ASUZC 17292, MVZ 234643
Brown Roofed Turtle (<i>Pangshura smithii</i>)	Dissection, diceCT	MVZ 248393, UMMZ 227972
Caspian Turtle (<i>Mauremys capsica</i>)	Dissection, diceCT	MVZ 237439, UMMZ 65474
Black Wood Turtle (<i>Rhinoclemmys funerea</i>)	Dissection	UF 116451
Painted Wood Turtle (<i>Rhinoclemmys pulcherrima</i>)	Dissection	NCSM 78377
Furrowed Wood Turtle (<i>Rhinoclemmys areolata</i>)	diceCT	UMMZ 129703
Spot-legged Wood Turtle (<i>Rhinoclemmys punctularia</i>)	diceCT	UMMZ 142647
Mexican spotted Wood Turtle (<i>Rhinoclemmys rubida</i>)	diceCT	UMMZ 82257
Crowned River Turtle (<i>Hardella thurjii</i>)	diceCT	UMMZ 227976
Pond Slider (<i>Trachemys scripta</i>)	diceCT	UMMZ 210663