BUOYANCY CONTROL IN COLD-SUBMERGED PAINTED TURTLES: IMPLICATIONS FOR OVERWINTERING PHYSIOLOGY AND BEHAVIOR

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Abstract: Western Painted Turtles (*Chrysemys picta bellii*) are the most anoxia-tolerant tetrapods known, capable of surviving anaerobically at 3°C for nearly 5 mo. In the northernmost latitudes of their range, adult painted turtles can experience winters lasting 6–7 mo. During this time, the pond surface is covered with ice and snow, denying the turtles access to air and trapping them in water that can become severely hypoxic or anoxic. Previous studies have reported that turtles overwinter near the bottom of ponds, either buried in mud or on top of the substrate, where oxygen levels are hypoxic or anoxic. The oxygen is depleted by other respiring biomass within the mud, but why the turtles are found on or within it is not understood. To better understand the overwintering behavior of painted turtles, we conducted a series of laboratory experiments to determine how a turtle's buoyancy is affected by temperature, depth, and access to air. Acclimating turtles to 3°C resulted in a large variation in turtle buoyancy, but preventing access to air caused all turtles to become negatively buoyant. We suggest that painted turtles overwinter in the mud or on the pond bottom not necessarily because of any particular behavioral adaptation, but simply because they become negatively buoyant while overwintering without access to air.

Key words: *Chrysemys picta*; Hypoxia; Lung volume; Specific gravity

Overwintering poses a severe challenge to sessile and nonmigratory organisms in extreme temperate climates. Ectothermic animals, including turtles, have evolved two basic strategies for surviving harsh winters: freeze tolerance or freeze avoidance (Storey, 1990). In the northern portions of their geographic range, painted turtles adopt both strategies in a developmentally dependent way, with nest-bound hatchlings utilizing freeze tolerance, whereas adults, which are freeze intolerant, use freeze avoidance, spending their winters underwater, where temperatures typically stay above freezing throughout winter (Ultsch, 2006). A trade-off associated with overwintering underwater is that the surface of ponds can freeze and trap turtles below the surface without access to air. Under these conditions, the turtle's oxygen (O_2) supply is limited to that which is dissolved in the water and can be taken up through extrapulmonary gas exchange (Jackson et al., 2004). Because O_2 is differentially dispersed throughout the water column (Bella, 1970), the location of an overwintering painted turtle within its pond has direct consequences for its overwintering physiology.

Turtles that overwinter in severely hypoxic or anoxic microenvironments must rely on anaerobic metabolism to support their energy needs. Dependence on anaerobic metabolism is possible because turtle metabolic rates are depressed during cooler winter months and metabolic demands are low (Ultsch and Jackson, 1982a). Anaerobic metabolism results in the production of lactic acid and the accelerated depletion of tissue glycogen stores, however, both of which likely determine overwintering survival of pond turtles (Warren et al., 2006). Because turtles can utilize extrapulmonary O_2 uptake to support their metabolic needs while submerged (Jackson et al., 2004), overwintering in oxygenated water could allow turtles to avoid many of the physiological challenges associated with overwintering in severely hypoxic or anoxic regions of the pond. Despite this potential advantage, overwintering painted turtles are almost always found either buried in the mud (Sexton, 1959; Ernst, 1972; Taylor and Nol, 1989) or on the surface of the pond bottom (St. Clair and Gregory, 1990; Crocker et al., 2000), where dissolved O_2 levels might be

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severely hypoxic or anoxic (Crawford, 1991; Rollinson et al., 2008).

It has generally been assumed that overwintering turtles actively choose to bury themselves, which would indicate that overwintering in or on the substratum provides a selective advantage that outweighs the challenges of anoxic overwintering (Ultsch and Jackson, 1985). This adaptationist view ignores the possibility that turtles overwinter on the pond bottom simply because they are a slave to their own body's density relative to water. Turtles regulate buoyancy by adjusting residual lung volume with reciprocal changes in the volume of water stored in the cloacal bursae and urinary bladder (Jackson, 1969). Regulating buoyancy affects the ease with which the turtle can move throughout the water column. A negatively buoyant animal would require more energy to stay in the shallows, whereas a positively buoyant animal would require more energy to remain at depth (Sato et al., 2013). No previous studies have considered how simple changes in a turtle's buoyancy might determine where a turtle overwinters.

We investigated how a turtle's buoyancy might be affected by various factors relevant to overwintering physiology (i.e., acclimation temperature, access to air, and depth). We provide a discussion of our findings in the context of a recent physiology-based hypothesis that explains why lung volume decreases in amphibians and reptiles when submerged underwater (Ultsch et al., 2004), as well as the implications for overwintering behavior and ecology in painted turtles.

Materials and Methods
Animal Husbandry

Twelve Western Painted Turtles (Chrysemys picta bellii; mean ±1 SE and range of mass = 217 ± 12 g and 166–291 g, respectively) of both sexes were purchased from a commercial vendor (Niles Biological, Sacramento, CA) and maintained in large (~500 L) flow-through tanks containing St. Louis municipal tap water with access to a 1.1 × 1.2–m basking ramp and a 60-Watt incandescent heat lamp placed 0.4 m above the ramp. Water temperature was 10–20°C and photoperiod was 12:12 h night:day until the subjects were used in experiments.

Turtles were offered ad libitum diets of Reptomin or chicken liver three times per week up until 48 h before experimentation. Subjects were housed for at least 60 d before experiments began. The same 12 turtles were used under all experimental conditions.

Experiment 1: Depth Effects on Buoyancy

To determine the effects of habitat depth on a turtle's scope for regulating its buoyancy, maximal specific gravity (SG; negative buoyancy) was first achieved by affixing Styrofoam floats (7.5 × 6.5 × 1.5 cm) to the carapace of each turtle ($n = 9$; subset of the 12). Subjects were then placed either in an aquarium (W × L × H = 26 × 51 × 31 cm) containing 25 cm of water, or a large cylindrical plastic basin (diameter = 53 cm) containing 64 cm of water. Twenty-four hours later, the specific gravity of the turtle was measured (described below). Achieving maximal SG ensured that the scope for the buoyancy compensation response would be the largest possible (i.e., SG values of subjects with floats were reference data, and not analyzed). We next removed the floats and determined minimal SG (positive buoyancy) by taping lead weights to the plastron so that each turtle's weight in water was doubled; we then returned the subjects to their respective tanks (25- or 64-cm depths). This procedure forced each turtle to increase its specific gravity so that it would become more buoyant. The doubled body weight was chosen from preliminary experiments, which showed this to be the maximum amount that these turtles could be weighted and still swim to the surface to breathe. We measured specific gravity for all subjects in each of 5 h after we had attached the lead weights. The scope for buoyancy compensation response was measured in the same nine turtles at both depths (25 cm and 64 cm). All acclimation and buoyancy measurements were conducted at water temperatures of 20°C (precision for weight measurements was ±0.01 g).

Specific gravity (dimensionless) was calculated as

$$SG = (\text{weight in air} + \text{weight in water})/\text{weight in air}.$$

Weight in water was determined by hanging the turtle from a wire that was attached to the bottom of a top-loading balance mounted

above the aquarium. The wire was attached to a hook that had been glued to the middle vertebral scute of the carapace with epoxy. Weight in air was measured by removing the turtles from the water and quickly weighing them on the same top-loading balance. SG values >1 indicate a tendency to sink, whereas SG values <1 indicate a tendency to float.

Experiment 2: Temperature Effects on Buoyancy

To determine the effects of cold-acclimation on turtle buoyancy, turtles ($n = 6$) were gradually acclimated to 3°C by decreasing the water temperature 2°C each day from 20°C for 8 d, and 1°C on the ninth day. A control group of separate turtles ($n = 6$) was maintained at a water temperature of 20°C for an identical period of time with access to air. Turtles did not have access to a basking platform and were not fed during this period. The turtles were held in identical coolers (W × L × H = 30 × 57 × 30 cm) with a water depth of 25 cm. The room was maintained on a 12:12 h light:dark photoperiod. Specific gravity was measured in both groups of turtles after 0, 5, 9, 11, 15, and 21 d from the start of cold acclimation. For the cold-acclimated turtles, the corresponding temperatures were 20°C and 10°C at 0 and 5 d, respectively. For all subsequent measurements (9, 11, 15, and 21 d), the cold-acclimated turtles were at 3°C. The sampling schedule was chosen to examine variation in SG at roughly the midpoint of the cold-acclimation (5 d from 20°C and 4 d from 3°C), at the earliest stage of cold-acclimation, and after a longer period of cold acclimation.

In this experiment, it was necessary to prevent each subject from expelling water it might have gained during the acclimation process. Therefore, we measured specific gravity in these turtles as in Experiment 1, with two differences:

(1) The cloaca was banded with a rubber O ring to prevent turtles from expelling water and changing their specific gravity prior to weighing in air.
(2) The turtles were prevented from breathing during weighing in water, which would also change SG during the weighing process.

At the time of sampling, the turtles were oriented so their head remained underwater while the band was placed over the tail. Without allowing the turtle's head above the surface, they were briefly placed in a 1-L plastic pail and transferred to the weighing aquarium, where the turtle was placed into a previously water-tared cage constructed from aluminum window screen that was suspended from the bottom hook of a top-loading balance placed above the aquarium. The turtle was prevented from breathing by ensuring that its head was always submerged in water throughout the weighing process. After weighing in water, turtles were weighed in air on a top-loading electronic balance and returned to their aquarium, after which the O-ring was removed. The water temperatures in the weighing chambers were the same as the turtle's acclimation temperature for each measurement.

Experiment 3: Cold Submergence Effects on Buoyancy

To test the hypothesis that preventing the turtles from having access to air while submerged in aerated water at 3°C affects buoyancy, a plastic screen was placed over the water surface in the 3°C cooler to prevent the turtles ($n = 6$) from breathing. This was intended to simulate the conditions a turtle would experience when the pond surface is frozen, preventing the turtle from aerial ventilation. The water was continuously bubbled with room air, resulting in a dissolved O_2 saturation of 82.8%. These saturation values are similar to those that might be found in the water column of some frozen ponds (Crawford, 1991). We chose to avoid full saturation of the water because it would have required vigorous aeration that would have affected the turtle's behavior. SG was measured before and after 31 d of submergence with the use of the methods described in Experiment 2.

Statistical Analysis

We used two-factor repeated-measures analyses of variance (RM-ANOVA) to test for the effects of time, treatment (depth or acclimation temperature), or time × treatment on specific gravity. Multiple post hoc comparisons were performed with the use of the

TABLE 1.—Summary of statistical analyses examining the influence of depth, temperature, and duration of submersion on buoyancy control in Western Painted Turtles (*Chrysemys picta bellii*).

Experiment	Analysis	Independent variable	*F* or *t* statistic	*P* value
1 (*n* = 9 per group)	Repeated measures	Time	15.453	<0.001
	Two-way ANOVA	Depth	5.188	0.052
		Time × depth	1.828	0.129
2 (*n* = 6 per group)	Repeated measures	Time	5.078	0.006
	Two-way ANOVA	Temperature treatment	2.743	0.129
		Time × temperature treatment	2.835	0.055
3 (*n* = 6)	Paired *t*-test	Time	−2.730	0.041

Student-Newman-Keuls tests. A paired *t* test was used to test for the effect of submergence time on specific gravity. The significance level for all analyses was α < 0.05. All computations were completed with the use of Sigmaplot v.12.0 (Systat Software, Inc., San Jose, CA).

RESULTS

Experiment 1: Depth Effects on Buoyancy

The SG of the turtles varied marginally as a function of the depth of the enclosure (Table 1, Fig. 1). After adjusting significance values for multiple pairwise comparisons, however, there was a difference only 1 h after adding the weights (*P* = 0.02), but not at any subsequent time (*P* ≥ 0.11). Therefore, we conclude that the depth of the environment does not appear to affect the buoyancy compensation response in an important way. Turtles fully compensated for the addition of weights and achieved maximal positive buoy-

ancy within 1 h at both depths. The 25-cm group had a minimum SG of 0.971 ± 0.015 and maximum of 1.018 ± 0.017. Individuals had an SG range of 0.908–1.063 when experimentally manipulated with lead weights. When placed into a water depth of 64 cm, the same subjects had a minimum SG of 0.962 ± 0.016 and maximum of 1.030 ± 0.017; SG values in this treatment ranged from 0.901 to 1.064.

Experiment 2: Temperature Effects on Buoyancy

Subject SG was affected by time in both groups (Table 1), with turtles at 15 and 21 d having a higher SG than they did at 9 and 11 d. However, there was no effect of temperature or a temperature × time interaction on the SG of the subjects (Table 1, Fig. 2), indicating that a turtle's buoyancy is independent of time and temperature. The cold-
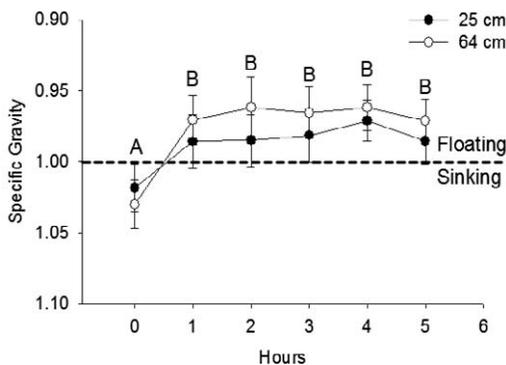

FIG. 1.—The effects of depth (25 vs. 64 cm) on the buoyancy compensation response of Western Painted Turtles (*Chrysemys picta bellii*; *n* = 9 per group) at 20°C. Plotted values represent means ±1 SE; differing letters indicate significant differences between time points for all groups.
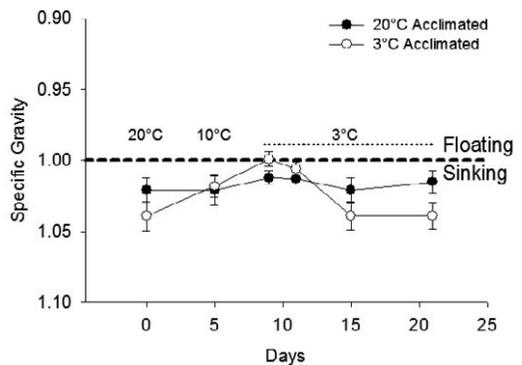

FIG. 2.—The change in specific gravity (SG) of Western Painted Turtles (*Chrysemys picta bellii*; *n* = 6 per group) during acclimation to either 20 or 3°C over a 21-d period. The temperatures above the dashed line (SG = 1.00) indicate the temperature of the 3°C–acclimating group at the time SG was measured. Plotted values represent means ±1 SE.
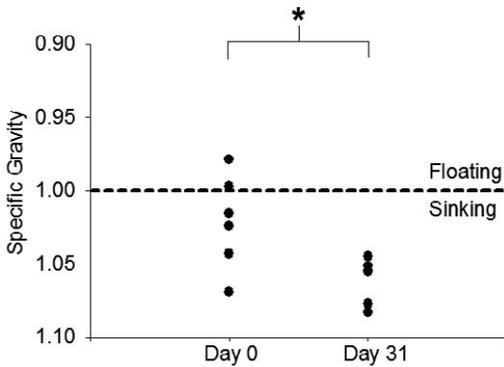
FIG. 3.—Values for specific gravity of Western Painted Turtles (*Chrysemys picta bellii*; $n = 6$ per group) before and after 31 d of normoxic submergence at 3°C. Asterisks indicate a difference in mean values between time periods (paired *t*-test, $P < 0.05$)

acclimated group started at 0 d and ended at 21 d with identical mean values for SG (1.039 ± 0.009), despite fluctuations including a low mean SG at day 9 (0.999 ± 0.005). Individual SG values had a range of 1.016–1.075 at 0 d, and a range of 1.004–1.069 at 21 d. Both cold and warm acclimated groups had SG values >1 most of the time, demonstrating a near-constant tendency to sink in water.

Experiment 3: Cold Submergence Effects on Buoyancy

When the subjects were submerged in aerated water at 3°C for 31 d, the mean value for SG was greater at the end of that period (Table 1, Fig. 3). Turtles started at 0 d with a mean SG of 1.021 ± 0.013 and ended at 31 d with a mean of 1.061 ± 0.006. When allowed access to air, the subjects showed more variability in buoyancy.

DISCUSSION

The results of this study are novel and directly relevant to the ecology of aquatic pond turtles living in extreme temperature environments. We have shown that buoyancy in Western Painted Turtles is entirely dependent on whether the turtle has access to air. Overwintering turtles of this species that do not have access to air should, therefore, become negatively buoyant and, as a consequence, sink to the pond bottom. Whether or not a turtle becomes buried in the mud or

simply settles on the surface of the mud would depend on the length of time the turtle remains inactive on the pond bottom and the physical characteristics of the substrate. Therefore, well-aerated regions of the pond would be relatively unavailable to turtles without the aid of powered swimming and most turtles will likely experience some degree of environmental hypoxia for most of the winter.

Our findings that painted turtles compensate their buoyancy within 1 h are similar to those of Peterson and Gomez (2008) showing compensation within 4 h. We further demonstrated that this species can compensate its buoyancy at two different depths, indicating that individuals prefer to adjust buoyancy, rather than use powered swimming, to navigate through the water column to surface and breathe. At both depths, the response was always maximal, which means the turtles do not make themselves more buoyant just because they are weighted in deeper water. Simply acclimating the subjects to 3°C did not affect their buoyancy, which indicates that, if given the option, turtles will likely breathe air even though they can utilize extrapulmonary (i.e., cutaneous) gas exchange during submergence at cold temperatures (Jackson et al., 2004). Pulmonary gas exchange is presumably more advantageous for turtles than nonpulmonary means because it ensures a finer control over the pH, and O_2 and CO_2 contents of the blood, as well as buoyancy, the latter of which affects the cost of locomotion in water in aquatic animals (Sato et al., 2013).

When the cold-acclimated painted turtles were prevented from surfacing to breathe, all of the turtles became negatively buoyant and sank over the subsequent 31 d. There are two possible explanations for the buoyancy change, both of which require the turtles to decrease their lung volumes and probably increase the volumes of either their bladder or cloacal bursae (Jackson, 1969, 1971). First, the subjects might have simply exhaled while underwater and, without the ability to surface and breathe, were unable to neutralize their buoyancy. Captive turtles exhale during escape diving (personal observations), presumably to facilitate descent. The second explanation is based on studies of lung

collapse in both amphibians and reptiles (Ultsch and Jackson, 1982a,b; Ultsch et al., 2004). The proposed mechanism is as follows: O_2 in the lungs is consumed by the tissues, but because the rate of CO_2 production is low and its solubility in water is high, the metabolically produced CO_2 is simply lost to the ambient water by extrapulmonary means, presumably across the skin (Jackson et al., 2004). The result is a decrease in lung volume and, perhaps most importantly, an increase in the partial pressure of lung nitrogen (N_2) to a level above that in the surrounding water. This partial pressure gradient favors the diffusion of N_2 from the lung into the blood and then by bulk flow to a gas-exchange surface, presumably the skin (Jackson et al., 2004), whence it diffuses into the surrounding water. As N_2 leaves the lung over time, the lung gradually collapses even further, thereby decreasing its buoyancy even more.

Regardless of the exact mechanism for the buoyancy change, our observations have important implications for overwintering ecology and physiology in painted turtles and probably other pond turtles occurring in temperate regions. During winter, pond surfaces in the northern portions of their range freeze, and the turtles are prevented from breathing air. Within 31 d of ice formation, all of the turtles should have sunk to the bottom of the pond, either because they exhaled or because they are respiring while submerged in water with a nitrogen gas tension less than 1 atm. Exceptions would occur if the turtles are actively swimming, or being held off the bottom by an object such as a rock or tree branch. Therefore, most painted turtles should experience whatever conditions exist at the pond bottom. Previous limnological studies (Crawford, 1991; Rollinson et al., 2008) have shown that the O_2 levels on the pond bottoms are considerably hypoxic or even anoxic, even when O_2 is present closer to the pond surface. Therefore, most dissolved O_2 in an overwintering turtle's environment is unavailable to them without continuous, powered swimming.

Although this study focused on painted turtles, the aforementioned mechanisms are likely to affect any turtle species that overwinters in ponds that freeze over, especially basking turtles such as Spotted Turtles (*Clemmys guttata*), sliders (*Trachemys* spp.), Bog Turtles (*Glyptemys muhlenbergii*), and Blanding's Turtles (*Emydoidea blandingii*). All of these species are found in temperate regions with severe winters, and live in ponds that are likely to become hypoxic or anoxic at the pond bottom or within the substrate during winter (Ultsch, 2006). The ranges of these turtles do not extend as far north as painted turtles, so they are likely to experience shorter periods of ice cover and would be subjected to shorter periods of severe hypoxia and anoxia. Less anoxia-tolerant basking species, such as map turtles (*Graptemys* spp.) and Wood Turtles (*Glyptemys insculpta*), choose overwintering habitats whose surfaces do not freeze or have higher dissolved O_2 levels, such as rivers or streams (Ultsch, 2006). Regardless of hypoxia tolerance or overwintering habitat, we would expect all of these species to show a buoyancy response similar to painted turtles, as the only requirements for the buoyancy change are a skin that is permeable to gas (Jackson et al., 2004), cold temperatures, and a partial pressure of N_2 less than 1 atm.

In summary, we have shown that the buoyancy compensation response in Western Painted Turtles is independent of depth, and that buoyancy is unaffected by cold acclimation alone. However, when cold-acclimated turtles are submerged in aerated water, they will always develop the tendency to sink, forcing them to overwinter on the pond bottom where they are least likely to have access to dissolved O_2 for extrapulmonary uptake. Although sinking allows turtles to avoid the energetic consequences of remaining in the water column through active swimming, they will be forced to rely on anaerobic metabolism, at which point, survival will depend on their anaerobic energy stores (glycogen) and ability to tolerate lactic acidosis (Warren and Jackson, 2008). Therefore, it is not surprising that the turtle species found in ponds with the longest winters is also the most anoxia tolerant (Ultsch et al., 1985).

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