

RESEARCH ARTICLE

Potential sources of intra-population variation in the overwintering strategy of painted turtle (*Chrysemys picta*) hatchlings

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ABSTRACT

Many temperate animals spend half their lives in a non-active, overwintering state, and multiple adaptations have evolved to enable winter survival. One notable vertebrate model is *Chrysemys picta*, whose hatchlings display dichotomous overwintering strategies: some hatchlings spend their first winter aquatically after nest emergence in the autumn, whereas others overwinter terrestrially within their natal nest with subsequent emergence in the spring. The occurrence of these strategies varies among populations and temporally within populations; however, factors that determine the strategy employed by a nest in nature are unknown. We examined potential factors that influence intra-population variation in the overwintering strategy of *C. picta* hatchlings over two winters in Algonquin Park, Ontario. We found that environmental factors may be a trigger for the hatchling overwintering strategy: autumn-emerging nests were sloped towards the water and were surrounded by a relatively higher percentage of bare ground compared with spring-emerging nests. Autumn-emerging hatchlings were also relatively smaller. Overwintering strategy was not associated with clutch oviposition sequence, or mammalian or avian predation attempts. Instead, autumn emergence from the nest was associated with the direct mortality threat of predation by sarcophagid fly larvae. Body condition and righting response, measured as proxies of hatchling fitness, did not differ between overwintering strategies. Costs and benefits of overwintering aquatically versus terrestrially in hatchling *C. picta* are largely unknown, and have the potential to affect population dynamics. Understanding winter survival has great implications for turtle ecology, thus we emphasize areas for future research on dichotomous overwintering strategies in temperate hatchling turtles.

KEY WORDS: Body condition, Fitness, Freeze tolerance, Maternal effects, Nest environment, Nest predators, Supercooling, Temperature

INTRODUCTION

Factors influencing population dynamics are well studied in animal ecology and conservation. Many environmental factors impact population dynamics because they directly influence reproduction and survival, especially during particular seasons (Aars and Ims, 2002). For temperate animals, winter is a severe energetic challenge that greatly influences both survival and the reproductive output of the subsequent year (Sendor and Simon, 2003). Winter adaptations include behavioural means of avoiding low temperatures (e.g.

migration, habitat selection), and physiological changes that permit survival at low temperatures (e.g. biochemical adjustments, reduced metabolic rate) (Marchand, 2013). Many species combine adaptations for an overwintering strategy that effectively promotes winter survival. Juvenile life-stages experience lower survival, particularly within the first year of life, than adults; this trend is consistent across juvenile endotherms, such as small mammals (Muchlinski, 1988; Sendor and Simon, 2003), birds (Peach et al., 1999) and rodents (Marchand, 2013); and ectotherms, such as salmonids (Huusko et al., 2007), amphibians (Tester and Breckenridge, 1964; Resetarits, 1986; McCaffery and Maxell, 2010), snakes (Viitanen, 1967; Parker and Brown, 1980; Macartney, 1985; Charland, 1989) and turtles (Ultsch, 2006). Overwintering survival greatly impacts juvenile recruitment and can, in turn, affect population dynamics. For example, in *Vipera vipera*, winter climate primarily affects population growth and decline through its effects on juvenile survival (Altwegg et al., 2005). Winter exerts substantial selection pressures on overwintering strategies that optimize survival.

Interestingly, some animals exhibit intraspecific variation in overwintering strategy, particularly with respect to the biochemical adjustments used to survive freezing temperatures. For example, many invertebrates vary in their ability to tolerate ice formation within body tissues (i.e. freeze tolerance) and to lower the temperature of cellular fluid below its freezing point (e.g. supercooling) (Baust et al., 1979; Baust and Lee, 1981; Tanaka, 1997; Lombardero et al., 2000). Also, *Hyla versicolor* differs in its capacity for freeze tolerance along a latitudinal gradient (Costanzo et al., 1992). Many turtles during the hatchling life stage (the first year of life) exhibit behavioural and physiological variation in overwintering strategies and can spend winter in either aquatic or terrestrial sites (Ultsch, 2006; Gibbons, 2013; Lovich et al., in press). Hatchlings that overwinter terrestrially either avoid freezing temperatures by burrowing below the frostline (e.g. *Terrepenne ornata*) (Ultsch, 2006), or remain within their nest cavity (above the frostline) and survive by either supercooling or freeze tolerance [e.g. *Chrysemys picta* (Schneider 1783), *Graptemys geographica* (Costanzo et al., 2008)]. Although an aquatic environment buffers turtles from freezing temperatures because of the high thermal buffering capacity of water (Costanzo et al., 1992), overwintering terrestrially within the nest is thought to be the preferred strategy for turtle hatchlings (Gibbons, 2013; Lovich et al., in press).

One field-based question that has yet to be answered is why, within a single turtle population, is the overwintering strategy variable? Two main hypotheses have been proposed to explain variability in hatchling turtle overwinter strategy; firstly, that overwintering in the nest is a passive response to environmental conditions that hamper autumn emergence, and secondly, that overwintering strategy is a plastic response to nest environmental factors and increases offspring fitness. The first hypothesis stems from the ideas that hatchlings may be (1) unable to emerge in the

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autumn because they cannot penetrate encrusted overlying soil, (2) clutches laid later in the season may not emerge because they require additional developmental time, and/or (3) autumn emergence is prevented by cool weather that reduces mobility of hatchlings (Obbard and Brooks, 1981; Tinkle et al., 1981; DePari, 1996; Blouin-Demers et al., 2000; Parren and Rice, 2004). The second hypothesis asserts that in-nest overwintering confers benefits that increase survival. Larger hatchling size confers greater overwintering survival (Mitchell et al., 2013), perhaps because larger hatchlings have more liver and carcass mass, the tissues used by hatchlings to support the energetic costs of overwintering (Muir et al., 2013). In contrast, Costanzo et al. (Costanzo et al., 2004) theorized that smaller hatchlings might supercool more readily, and thus survive lower sub-freezing temperatures, because the likelihood of lethal freezing increases with fluid volume. The nest environment also affects hatchling overwinter survival; for example, winter mortality is greatest when temperatures are lowest and soil moisture is highest (Storey et al., 1988; Costanzo et al., 2000; Costanzo et al., 2001; Costanzo et al., 2004). Autumn emergence could be to avoid a direct mortality threat; for instance, nest infestation by sarcophagid fly larvae (*Tripanurga importuna*) may stimulate emergence (Warkentin, 1995; Bolton et al., 2008; Spencer and Janzen, 2011). Finally, overwintering in-nest may provide hatchlings with additional time to develop in a lower risk environment (Costanzo et al., 2008).

The purpose of our study was to elucidate why the overwintering strategy used by hatchlings varies within a population using the model species *C. picta*. *Chrysemys picta* is the most abundant, wide-ranging and well-researched turtle in North America; they range from British Columbia, Canada, easterly to Nova Scotia, Canada, and southerly to Louisiana, USA (Ernst and Lovich, 2009). Generally, a clutch of eggs is oviposited in the spring or summer, and the eggs hatch 89–99 days later (Ernst and Lovich, 2009). *Chrysemys picta* hatchlings may emerge from the nest in the autumn and are assumed to move to aquatic sites, or they remain in the nest throughout the winter and emerge the following spring (Hartweg, 1944; Cagle, 1954; Woolverton, 1963; Gibbons and Nelson, 1978; Churchill and Storey, 1992; Costanzo et al., 2008). The relative frequency of these two overwintering strategies varies temporally and geographically (Costanzo et al., 2008; Gibbons, 2013; Lovich et al., in press), but autumn emergence and subsequent assumed aquatic overwintering has been documented throughout the species' range (Finneran, 1948; Ernst, 1971; Christiansen and Galloway, 1984; St. Clair and Gregory, 1990; DePari, 1996; Rozycki, 1998; Waye and Gillies, 1999; Pappas et al., 2000; Costanzo et al., 2004; Carroll and Ultsch, 2007). In order to examine variation in overwintering strategy, our study had multiple objectives. (1) To

explore whether variation in overwintering strategy can be explained by nest environmental factors, concentrating on factors that have been highlighted in previous studies – heat units accumulated over incubation (Storey et al., 1988), vegetative cover, nest oxygen availability (Costanzo et al., 2001; Rafferty and Reina, 2012), oviposition date, and soil moisture (Costanzo et al., 2000; Costanzo et al., 2001), organic content (Costanzo et al., 1998) and texture qualities (Packard and Packard, 1997; Costanzo et al., 1998). (2) To determine whether overwintering strategy benefitted hatchlings. We tested whether mammalian and avian predators or predatory sarcophagid fly larvae triggered the emergence of hatchlings. We compared the body size of hatchlings before overwintering to determine whether hatchling size differed between strategies. Also, we compared proxies for hatchling fitness (body condition and righting response) prior to overwintering between strategies to determine whether hatchlings that stayed in-nest would benefit from a longer developmental time. (3) To examine the risks associated with overwintering aquatically versus terrestrially by comparing the winter temperature and available oxygen between marshes and nests. We predicted that winter temperatures in marshes would be higher than in nests, but that winter oxygen would be higher in nests than in marshes. (4) Finally, we undertook a preliminary investigation of maternal influence on overwintering strategy by examining whether the strategy was the same between multiple clutches from the same female (Friebele and Swarth, 2005).

RESULTS

Observational data

In 2010–2011, two nests emerged in autumn and 23 overwintered in-nest (of which five were excavated in the autumn). In 2011–2012, 16 nests emerged in autumn and 20 overwintered in-nest (of which six were excavated in autumn). Mean hatching success differed significantly between autumn- and spring-emerging clutches (Wald's z -statistic=322, $P=0.02$), with higher hatching success in autumn ($84\pm 5\%$; \pm s.e.m.) compared with spring-emerging clutches ($61\pm 6\%$). The overwintering success of hatched individuals in spring-emerging clutches was $67\pm 8\%$.

We found that, of the hatchlings tracked post-emergence, 45% (38 out of 85) reached water within 24 h post-release. The other turtles moved towards grass and woody brush piles away from the nearest water body (43%), and 12% were not found.

Hatchlings emerged from nests that accumulated more heat units (degree days, °D) over the incubation period than nests wherein hatchlings remained (Table 1). Hatchlings emerged from nests that were warmer over winter compared to nests in which hatchlings remained (Table 2; $t_{17}=2.11$, $P<0.01$). The lowest winter temperature within a nest was -4.9°C (Table 2). Nests from which hatchlings

Table 1. Environmental variables measured during the summer of 2011 that were included in the logistic regression model to examine whether environment influences the overwintering strategy used by painted turtle (*Chrysemys picta*) hatchlings

Variable	Description	Autumn emerging	Spring emerging
<i>date.laid</i>	Date of oviposition translated to date during the calendar year	166±8	165±8
<i>nest.depth</i>	Depth to bottom of nest cavity (cm)	9.6±1.6	10.0±1.3
<i>total.hatch</i>	Number of hatchlings within a clutch	7±2	6±2
<i>total.heat.units</i>	Heat units (°D) calculated using hourly trapezoid method	3958.3±414.5	3846.0±417.4
<i>avg.oxy</i>	Percent oxygen in the nest cavity measured in July, August and September	21.0±0.1	21.1±0.1
<i>slope</i>	Slope (deg) of the ground surface at the nest. Negative values indicate a downward slope towards water, and positive values indicate an upward slope towards water	-5.3±8.7	2.3±7.7
<i>soil.pc1</i>	First principal component for nest soil moisture and texture characteristics from PCA	-1.2±2.2	-0.3±2.1
<i>avg.bare.grnd</i>	Percent bare ground in 1 m ² quadrat around the nest measured in June, July, August and September	73.5±9.7	80.6±13.4

Mean \pm s.e.m. of each environmental variable for autumn- and spring-emerging nests (non-standardized values). PCA, principal component analysis.

Table 2. Thermal environment of painted turtle (*C. picta*) nests (N=19) from October to April 2010–2011 and 2011–2012

Thermal characteristics (°C)	Autumn emerging (N=6)	Spring emerging (N=13)
Mean temperature	2.82±0.16	1.91±0.13
Minimum temperature range	−4.84 to −2.33	−4.91 to 1.67
Maximum temperature range	20.68 to 38.27	15.78 to 25.42
Percentage of nests below −0.6°C	100	83
Percentage of nests below −4°C	100	31

Mean ± s.e.m. nest temperature, the range of the minimum and maximum temperatures, and the percentage of nests that spent time below −0.6°C (freezing point of body fluids for hatchling turtles) and −4°C (lower limit for freeze tolerance) summarized for autumn-emerging and spring-emerging nests.

emerged in autumn spent on average 500±142 h below −0.6°C (freezing point of body fluids) and 28±21 h below −4°C (the limit of freeze tolerance). Nests in which hatchlings overwintered spent on average 940±185 h below −0.6°C and 11±6 h below −4°C. The first principal component for soil characteristics did not differ between autumn- and spring-emerging nests ($t_{32}=1.74$, $P=0.09$). The nests contained low organic content (mean=2.1±0.3%) and consisted of predominately sandy soil (means: 22±2% gravel, 13±1% coarse sand, 39±2% sand and 17±1% fines).

Nest environment and overwinter strategy

The model that had the lowest small-sample-size corrected Akaike information criterion (AIC_c) score (30.36) included two environmental variables: nest slope and bare ground 1 m² around the nest [Nagelkerke's R^2 -squared value (R^2_N)=0.41; Table 3]. Hatchling painted turtles were more likely to emerge from nests surrounded by bare ground (β =−0.51, lower confidence limit LCL=−1.21, upper confidence limit UCL=0.03) and sloped towards water (β =−0.17, LCL=−0.39, UCL=−0.03).

Overwintering strategy did not differ significantly between first and second clutches ($P=0.59$). Of the first clutches, 30% emerged in the autumn and 70% overwintered in the nest, and similarly, 20% of second clutches emerged in the autumn and 80% overwintered in-nest.

Potential benefits to hatchlings

The number of mammalian and avian predation attempts did not affect the overwinter strategy ($P=0.95$). The mean number of predation attempts on a clutch was 0.3±0.2 per nest for autumn-emerging nests, and 0.3±0.1 per nest for clutches that overwintered

Table 4. Number of spring- and autumn-emerging painted turtle (*Chrysemys picta*) nests with sarcophagid fly larvae present in the nest cavity

Presence of sarcophagid fly larvae	Autumn-emerging nests (N=18)	Spring-emerging nests (N=43)
Present	9	10
Not present	9	33

Larvae presence was significantly related to hatchling overwintering strategy ($P=0.04$).

in-nest. Sarcophagid fly larvae nest infestation was significantly related to hatchling overwinter strategy ($P=0.04$); 50% of autumn-emerging nests were infested with fly larvae, whereas 23% of spring-emerging nests had evidence of fly larvae infestation (Table 4).

The body condition of hatchlings was not different among those that emerged in the autumn, those that were excavated in the autumn but would have overwintered in-nest and spring-emerging hatchlings ($F_{2,49}=1.52$, $P=0.47$). Hatchlings with a smaller carapace length were more likely to emerge from nests in autumn (Wald's z -statistic=2.35, $P=0.02$, $R^2_N=0.21$). In the autumn, hatchlings that emerged from nests had a mean carapace length of 26.21±0.25 mm, and hatchlings that would have overwintered but were excavated had a carapace length of 26.66±0.46 mm (Fig. 1). Spring-emerging hatchlings had shorter carapaces (mean=25.18±0.52 mm) post-overwintering when compared with hatchlings in the autumn pre-overwintering ($F_{2,48}=5.83$, $P<0.01$; Fig. 1). Hatchlings with a smaller mass were also more likely to emerge from nests in the autumn (Wald's z -statistic=1.94, $P=0.05$, $R^2_N=0.13$). In the autumn, hatchlings that had emerged from nests weighed 4.58±0.50 g, and hatchlings from excavated nests weighed 4.59±0.61 g. Mass was not different between autumn- and spring-emerging hatchlings ($F_{2,48}=2.59$, $P=0.09$); spring-emerging hatchlings had a mean mass of 4.24±0.52 g.

The latency period of hatchlings did not differ among sampling periods ($F_{2,49}=1.80$, $P=0.41$). The mean latency period of autumn-emerging hatchlings was 110±31 s, and that of hatchlings that were excavated in the autumn was 115±27 s. The mean latency period of spring-emerging hatchlings post-overwintering was 121±27 s. The second measurement in righting response, righting period, also did not differ among sampling categories ($F_{2,49}=0.40$, $P=0.82$). The mean righting period of autumn-emerging hatchlings was 13±7 s, and that of hatchlings that were excavated in autumn was 16±6 s, and of spring-emerging hatchlings was 26±9 s.

Table 3. Top 10 multiple logistic regression models that examined how environmental variables affect overwintering strategy in hatchling painted turtles (*C. picta*)

Model	k	LL	R^2_N	AIC _c	Δ AIC _c	w_i
avg.bare.grnd + nest.slope	2	−11.55	0.41	30.36	0	0.18
soil.pc1 + avg.bare.grnd + nest.slope	3	−10.29	0.51	30.81	0.45	0.14
avg.oxy + nest.slope	2	−11.93	0.38	31.12	0.76	0.12
nest.slope	1	−13.27	0.26	31.14	0.79	0.12
avg.bare.grnd + total.heat.units + nest.slope + date.laid	4	−8.92	0.60	31.38	1.02	0.11
avg.bare.grnd + avg.oxy + nest.slope	3	−10.82	0.47	31.86	1.5	0.08
soil.pc1 + nest.slope	2	−12.31	0.35	31.89	1.53	0.08
avg.bare.grnd + total.heat.units + nest.slope	3	−10.93	0.46	32.08	1.73	0.08
total.heat.units + nest.slope	2	−12.88	0.30	33.02	2.67	0.05
(null)	0	−15.75	0	33.68	3.32	0.03

The models were identified by their AIC_c scores. The model parameters, number of parameters (k), log-likelihood of parameters (LL), Nagelkerke's R^2 -squared (R^2_N), AIC_c, Δ AIC_c, and model weights (w_i) are all shown.

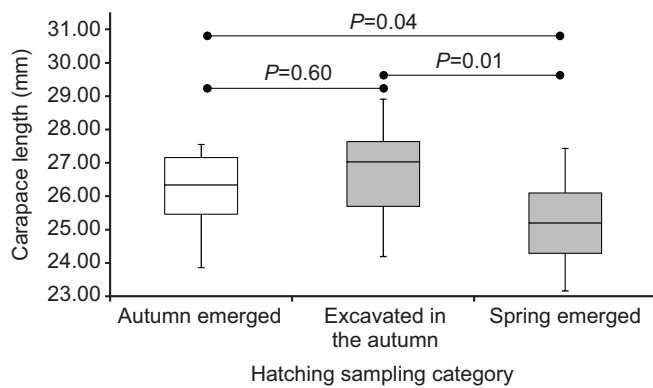


Fig. 1. Carapace lengths of hatchling painted turtles (*Chrysemys picta*) that emerged in the autumn (white box plot) and of hatchlings that overwintered in-nest (grey box plots) split into two groups: hatchlings excavated from nests in the autumn, and those that overwintered in-nest and were measured after natural emergence in the spring. The carapace length of hatchlings influenced overwintering strategy. In the autumn, small hatchlings were more likely to emerge from their nests (Wald's z -statistic=2.35, $P=0.02$, $R^2_N=0.21$). Post-overwintering, spring-emerging hatchlings were significantly smaller than hatchlings in the autumn preparing to overwinter in-nest ($F_{2,48}=5.83$, $P<0.01$; Tukey HSD values displayed on the figure). This indicates that overwintering had consequences that could impact hatchling survival. The boxes represent 25th and 75th quartiles, the line represents median and the whiskers represent minimum and maximum.

Maternal influence on overwintering strategy

From 2010–2012, there were six females for which we sampled two clutches during a nesting season. Out of these six double clutches, four (67%) exhibited the same overwintering strategy.

Comparison between aquatic and terrestrial overwintering sites

Winter temperatures differed between putative hibernation sites in marshes and nest cavities ($t_{53}=2.01$, $P<0.01$). The lowest marsh temperature ranged from -11.9 to 2.8°C , and the mean marsh temperature was $3.7\pm 0.2^\circ\text{C}$. The lowest in-nest temperature ranged from -4.9 to -1.7°C , and the mean temperature was $2.4\pm 0.1^\circ\text{C}$. The dissolved oxygen in marshes ranged from 2.1 to 95% air saturation from September 2011 until March 2012, and the mean dissolved oxygen was $37\pm 4\%$ air saturation. The available oxygen in nests over the same time period ranged from 20.6 to 21.2%, and the mean nest oxygen available during winter was $21.0\pm 0.01\%$.

DISCUSSION

Observational findings

Hatching success was higher in autumn-emerging nests than in spring-emerging nests, suggesting that conditions in the nest are not optimal for development and that overwinter strategy may be a passive response to a subpar nest environment. Winter survival in-nest depends on many factors, including nest temperature. In our study, nest temperatures fell below -4°C during winter (in nine out of 19 nests), and the overwintering success of spring-emerging clutches was $67\pm 8\%$.

Hatchlings remained overwinter in nests that were cooler than those from which hatchlings emerged. Also, clutches that overwintered in-nest experienced less time below the temperature limit of freeze tolerance ($<-4^\circ\text{C}$), below which they may have had to employ supercooling to survive. Owing to our field study limitations, the exact physiological mechanism that the hatchlings used to survive overwinter in our study is unknown. The four nests

in which the temperature fell below -4°C and in which hatchlings remained overwinter experienced 0–100% overwintering success. Perhaps hatchlings remain in nests that keep them cool, concomitant with a reduced metabolic rate overwinter, while still being at temperatures above the limit for freeze tolerance. Paterson et al. (Paterson et al., 2012) also found evidence that hatchling *Emydoidea blandingii* and *Glyptemys insculpta* selected overwintering sites with cooler temperatures than those in random plots. This begs the question, how might hatchlings in the autumn sense the future temperature conditions in their nest? Are hatchlings using another, closely related environmental variable to assess future nest temperatures?

Tracking hatchlings post-emergence in the autumn revealed that approximately half the hatchlings successfully reached aquatic sites within 24 h. The other hatchlings moved towards grass and woody brush piles away from the nearest water body and buried themselves 2–5 cm into the leaf litter. It is important to note that we tracked hatchlings for a limited time period (24 h), and their movements post-tracking are unknown. The natural, *in situ* post-emergence movements of hatchling *C. picta* have not been previously studied (Ultsch, 2006), and it has been assumed that all autumn-emerging hatchlings move directly towards water (Costanzo et al., 1995). Our findings suggest that there may be more variability in post-emergence movements. Warner and Mitchell (Warner and Mitchell, 2013) examined *C. picta* post-emergence movements within a small arena and found that hatchlings tended to move directly towards water. In contrast, Congdon et al. (Congdon et al., 2011) found that naïve hatchling *C. picta* within an arena oriented towards nearby, open, illuminated horizons regardless of whether the environment was aquatic or not. In our study, the other half of the tracked hatchlings buried themselves in terrestrial sites post-emergence in the autumn. Similarly, terrestrial overwintering after autumn emergence has been observed in *Malaclemys terrapin* (Muldoon and Burke, 2012), another species with dichotomous hatchling overwintering strategies (Baker et al., 2006). Interestingly, there was also evidence that autumn-emerging hatchlings utilized autumn to increase their energy reserve: one of the autumn-emerging hatchlings was observed actively foraging in water for aquatic insects less than 7 h post-emergence. Painted turtle hatchlings may emerge in the autumn to build up needed energy reserves for winter survival. Our findings highlight that hatchling post-emergence movements and overwintering behaviours remain largely unknown, even in a well-studied model species such as *C. picta*.

Nest environment and overwintering strategy

Our observational findings suggest that nest environmental factors may influence overwintering strategy, but these findings were not conclusively supported by our statistical modelling. We found some evidence that overwintering strategy is influenced by vegetation and the slope of the ground surface at the nest; although the effect strength of both environmental variables was weak; Nagelkerke's R -squared value ($R^2_N=0.41$) suggests that these two variables explain a little less than half of the variability in overwintering strategy. There were seven other models within two ΔAIC_c of the model with the lowest AIC_c , and these other models included additional environmental variables: average percent oxygen, oviposition date, total heat units and nest soil principal component 1 (Table 3). Of these eight models within two ΔAIC_c of each other, the one with the highest R^2_N value included vegetation and the slope of the nest, and also the oviposition date and the total heat units accumulated over incubation ($R^2_N=0.60$). Our limited sample size restricts the conclusions that we can

derive from our models because our statistical power was limited. But overall, the models suggest that environmental variables may affect overwintering strategy in painted turtles. In contrast, Friebele and Swarth (Friebele and Swarth, 2005) found that nest environment did not influence overwintering strategy in *Pseudemys rubriventris* hatchlings; however, they did not examine the same environmental variables used in our study.

As the nest slope towards water increased, hatchlings were more likely to emerge in autumn. One explanation for this relationship is that as the slope increases, it approaches the critical angle of repose, which is the steepest angle at which a material can be before it collapses (Graf, 1984; Jaeger et al., 1989). Thus, it would be beneficial for hatchlings to leave nests at steeper slopes that are unstable and could increase mortality. The angle of repose for sand is 35 deg, yet the steepest nest slope in our study was 22 deg so the risk of collapse was low. Furthermore, turtle nest collapse appears rare: sea turtle (*Chelonia mydas*) nest collapse, owing to sand textural characteristics, has been reported to cause hatchling mortality (Mann, 1977), and one *Glyptemys insculpta* nest, on a very steep slope, collapsed during incubation (J.L.R., personal observation). The risk of nest collapse may cause hatchlings to emerge from steeply-sloped nests, but it is more likely that the slope is related to other environment factors.

Steeper slopes may increase the temperature of nests because of enhanced solar radiation absorbance (Schwarzkopf and Brooks, 1987; Wood and Bjorndal, 2000). Warmer nests increase developmental rate and allow hatchlings to complete development by an earlier date. Once development is completed, hatchlings may need to emerge from nests because warm nests might reach lethally high temperatures, and hatchlings may experience high metabolic rates, causing them to quickly consume their yolk reserves and exit nests to forage prior to winter (Muir et al., 2013). Our models suggest that autumn-emerging hatchlings are from nests that accumulated more heat units over incubation (i.e. spent more time within optimal developmental temperatures) (Holt, 2000). Nest temperatures, stability and the slope of the nest may be inter-related variables, but how hatchlings can perceive nest slope or future temperature post-hatching while they are within the nest remains unknown.

Our models also suggest that hatchlings emerge from nests surrounded by bare ground. Less vegetation above a nest can result in lower temperatures during winter; without plants and leaf litter to trap an insulating layer of air between the snow and ground, nest temperatures become colder. Although it is logical to directly relate the nest environment to its effects on hatchlings, the environment may also affect the presence of organisms that in turn affect hatchlings (e.g. predatory fly larvae). Sarcophagid flies lay their eggs in sandy areas, and larvae burrow through sand into a nest (Bolton et al., 2008). Sarcophagid flies may avoid laying eggs in turtle nests with high levels of vegetation, and our study shows that hatchlings emerge from nests with sarcophagid larvae (discussed below). Are environmental factors directly triggering hatchling response, or indirectly affecting overwintering strategy by altering the presence of predatory flies?

The nest environment is complex, with many inter-related factors potentially influencing the development of hatchling turtles. More research is required to understand how hatchlings sense their nest environment. Our discussion of environmental factors that influence overwintering strategy is mainly speculative because of the weak effect the environmental variables we measured had on overwintering strategy, yet we hope our discussion inspires avenues of subsequent research.

Potential benefits to hatchlings

Autumn emergence appears to lower the risk of predation by sarcophagid fly larvae. Sarcophagid larvae opportunistically prey upon turtle eggs and hatchlings (Iverson and Perry, 1994; Smith, 2002; Bolton et al., 2008), and fly larvae cause direct mortality of hatchlings (Gillingwater, 2001). In contrast, disturbance by avian and mammalian predators did not appear to trigger hatchling emergence (but note that our nests were caged, and nest predator behaviour around uncaged and caged nests may differ) (but see Riley and Litzgus, 2013). Overall, variation in overwintering strategy may be related to the avoidance of a direct mortality threat from sarcophagid fly larvae. Overwintering in-nest is thought to be the *de facto* strategy (Gibbons, 2013), and perhaps predation by fly larvae has triggered a plastic response of autumn emergence and a subsequent alternative terrestrial or aquatic overwintering strategy.

Autumn-emerging hatchlings were smaller than hatchlings that overwintered in-nest. This finding is in contrast to the idea that smaller hatchlings should remain in-nest because they are likely to have increased supercooling abilities (Costanzo et al., 2004). Ice nucleation occurs when water molecules form ice in an organism (Rasmussen and Mackenzie, 1973), and the probability of ice nucleation increases with fluid volume (Vali, 1995). Our findings are supported by a recent study by Mitchell et al. (Mitchell et al., 2014) that found survival overwinter was higher for larger hatchlings. There may be a size threshold at which hatchlings leave the nest in the autumn in order to forage to build up energetic reserves prior to overwintering. Proxies for hatchling fitness measured in the autumn (body condition and righting response) did not appear to influence hatchling overwintering strategy, which contradicts the hypothesis that overwintering in-nest may be a *de facto* response to a lower developmental state (Costanzo et al., 2008).

Maternal influence on overwintering strategy

There may be some degree of parental influence on overwinter strategy, but overwintering strategy was not consistently associated with maternal identity. In our study, four out of six females' nest pairs exhibited the same overwinter strategy. In contrast, *P. rubriventris* nests that had been oviposited by the same female did not exhibit the same overwinter strategy (Friebele and Swarth, 2005); however, their sample size was small ($N=2$). Perhaps *C. picta* overwintering strategy is paternally influenced; if clutches oviposited by the same mother have different fathers, their overwintering strategy may differ. Paternity was unknown in our study; however, *C. picta* first and second clutches can share paternity 97.5% of the time (McGuire et al., 2011). Overall, the low sample size ($N=6$) limits the inferences we can make regarding genetic influence on overwintering strategy.

Comparison between aquatic and terrestrial overwintering environments

Putative aquatic overwintering sites had higher winter temperatures than terrestrial nests. In Indiana, aquatic overwintering sites also experienced higher temperatures than nests (Costanzo et al., 2008). Winter temperatures were colder but more stable in natal nests. Marshes experienced more fluctuations in winter temperatures thus exposing hatchlings to potentially deleterious environmental extremes. In our study, some aquatic overwintering sites reached lethal minimum temperatures, but others did not even reach freezing temperatures during winter (Table 2). Exposure to temperature extremes increases mortality either by exposure to freezing temperatures or as a result of increased metabolic rate causing

energy depletion and death (Greaves and Litzgus, 2007; Edge et al., 2009; Muir et al., 2013). Thus, in regards to temperature, natal nests pose less of a winter survival risk to hatchling *C. picta*.

More oxygen was available in nests than in marshes. The dissolved oxygen in marshes ranged from close to anaerobic (0% air saturation) to normoxic levels (above 80% air saturation); however, on average, marsh oxygen was close to hypoxic levels (below 30% air saturation). Winter oxygen concentration in nests was consistently similar to the oxygen concentration of air (21.0% oxygen); thus, gas exchange is probably not a challenge to terrestrially overwintering hatchling turtles that breathe with their lungs. In contrast, gas exchange in aquatic sites presents a challenge during winter, especially because hatchlings have a lower anoxia tolerance compared to adults (Packard and Packard, 2004; Reese et al., 2004), and must rely on less-efficient cutaneous routes of gas exchange. Lower anoxia tolerance in hatchlings may itself be a driving factor in the prevalence of terrestrial overwintering by hatchling turtles (Gibbons, 2013).

Conclusion

The overwintering strategy of hatchling *C. picta* may be influenced by nest environmental factors. Smaller hatchlings were more likely to emerge from the nest in the autumn, which may indicate that they use the autumn to increase energy reserves prior to overwintering. Hatchlings were significantly smaller post-overwintering, which indicates that overwintering terrestrially may influence future survival. Yet, the fitness consequences of dichotomous overwintering strategies are unknown, and more research is needed to understand the costs of overwintering aquatically versus terrestrially for hatchling turtles. Most notably, sarcophagid fly larvae may be stimulating autumn emergence, such that hatchling overwintering strategy is associated with predator avoidance. Our study was a first step in elucidating the factors that cause within-population variation in a turtle species with a dichotomous hatchling overwinter strategy.

MATERIALS AND METHODS

All work involving animals was performed under an approved Laurentian University Animal Care Committee protocol (animal use protocol number 2008-12-02) and was authorized by permits from the Ontario Ministry of Natural Resources.

Study area

The two study sites are in Algonquin Provincial Park, Ontario, Canada. The western site is located along the Highway 60 corridor west of the Wildlife Research Station (WRS, 45°35'N, 78°30'W). The eastern site is 60 km northeast of the first (45°87'N, -77°77'W). The habitat at both sites consists of a mosaic of water bodies within forest (Edge et al., 2010). Elevations at the western site are higher (ca. 585 m above sea level) than at the eastern site (ca. 150 m above sea level), and consequently the first site experiences a colder and wetter climate (Ontario Ministry of Natural Resources, 1998). Nest data were pooled from both sites to address our study objectives.

Field sampling

Nest site monitoring

Monitoring of nesting sites began when females started exhibiting nesting behaviours, and occurred from 20 May to 20 June in 2010, and from 5 June to 4 July in 2011. Nest sites were searched by researchers on foot from dawn (~05:00) to around 10:00, and in the afternoon from dusk (~17:00) until nesting activity ceased. Both first and second clutches were sampled; this allowed examination of maternal effects and determination of whether overwinter strategy depends on oviposition date. Nest site monitoring ceased when 3 days elapsed continuously without nesting behaviours.

Nests were excavated after females completed oviposition. As eggs were removed, they were numbered using a pencil to ensure they were returned to the nest in the same order and orientation, and then placed in moist vermiculite in a plastic bin. After eggs were removed, nest cavities were filled with excavated nest cavity soil to prevent desiccation. Eggs were transported in a vehicle to a field lab at the western site, and measured in the field at the eastern site. The mass and dimensions of the eggs were measured for a long-term study (R. J. Brooks, University of Guelph, and J. D. Litzgus, Laurentian University). Measuring occurred within 24 h post-oviposition, prior to the vitelline membrane adhering to the shell surface (Yntema, 1968; Rafferty and Reina, 2012), ensuring no trauma to developing embryos (Samson et al., 2007).

Eggs were reburied in the original nest cavity, with a waterproofed temperature data logger that recorded the temperature hourly in the centre. Data loggers were either an iButton® (accuracy of ±1°C or 0.5°C; Thermochron DS1921G; Dallas Semiconductor, Sunnyvale, CA), or a HOBO Stowaway® (accuracy of ±0.2°C; TidbiT TBI32-05+37; Onset Computer Corp., Bourne, MA). Data logger types and the waterproofing methods did not differ in temperature readings (Roznik and Alford, 2012) (our data: $F_{3,2480}=2.01$, $P=0.94$). Also, in 2011, a golf Wiffle® ball (4 cm diameter, Wiffle Ball Inc., Shelton, CT, USA) with 30 cm of tubing (Tygon®, R-3603, Fisher Scientific, Whitby, ON, Canada) extending into the middle of the Wiffle® ball was buried in the centre of the nest cavity with tubing extending out of the ground. A two-way stop cock closed the tube from the environment. This system was used to measure oxygen in the nest (see below). Finally, each nest was covered by a wire cage to prevent depredation. Cages were made of 1 cm mesh hardware cloth, and were open-bottom cubes with dimensions of 30×30×40 cm with 8 cm flaps (Riley and Litzgus, 2013).

Nest environment monitoring

In 2010, once per month from oviposition to October, and on the day of emergence, environmental variables were measured. In 2011, environmental variables were measured bi-weekly from oviposition to October, on the day of emergence and monthly during winter (October to April). Environmental measurements were collected on vegetation cover, soil moisture, nest slope and oxygen. Vegetation cover was estimated by placing a 1 m² quadrat on the ground with the nest in the middle; the percentage of bare ground, herbaceous plants, woody plants and leaf litter within the quadrat were visually estimated (Wilson, 1998). Soil samples (~150 g) were collected 30 cm away from the nest at the same depth; the sample was held in a sealed glass bottle until the soil moisture was measured. The slope of the ground above the nest was measured using a level with a rotating vial (Fatmax® Xtreme Torpedo Level, 43-609, Stanley Tools Canada, Oakville, ON, Canada) angled towards the closest water body.

Nest oxygen was measured by drawing air out of the nest through the Wiffle® ball and tube system, and then through an oxygen sensor (S102 Flow-through Oxygen Sensor, Qubit Systems Inc., Kingston, ON, Canada) using a direct current air pump (Garrett et al., 2010). The oxygen sensor was calibrated using ambient air prior to each measurement. Using the volume of the average nest cavity (32 cm³), Wiffle® ball (17 cm³) and tubing (4 cm³), and the rate at which the direct current air pump pulled oxygen from the nest (176 cm³ min⁻¹), we estimated that the time for oxygen in the nest to reach the sensor was ~20 s. Thus, the oxygen measurement at 30 s was used in analysis, as we wanted to examine steady-state values.

Monitoring nest predator interactions

Nests were surveyed daily during nesting and hatching seasons for depredation attempts, and weekly in July and August (Burke et al., 1998; Kolbe and Janzen, 2002). A 'depredation attempt' was recorded if substrate had been cleared away from around the nest cage, and/or the nest cage had been unearthed. After recording a depredation attempt, the soil was replaced so that multiple attempts could be recorded (Riley and Litzgus, 2014).

Hatching season monitoring

Hatchlings were collected and sampled during three periods: (1) natural autumn emergence, (2) excavated in late autumn from nests in which they would have remained overwinter and (3) natural spring emergence. The demarcations of each period are described below.

The known incubation duration for painted turtles (Ernst and Lovich, 2009) was used to estimate the emergence time, at which time daily monitoring for autumn emergence began. When a nest emerged, hatchlings and un-hatched eggs were collected, and the presence or absence of sarcophagid fly larvae within the nest was recorded (Chidester, 1915; Bolton et al., 2008). Data loggers were left in nests to record winter temperatures (see below).

A proportion of the un-emerged nests ($N=11$ out of 61, five nests in 2010 and six in 2011) were excavated in late autumn, and the hatchlings and un-hatched eggs were collected. Excavation occurred after the daily mean air temperature dropped below 5°C for three consecutive days. In 2010, nests were excavated from 1 to 4 October, and in 2011 nests were excavated from 2 to 7 October. These hatchlings, if not excavated, would have been likely to overwinter in-nest as temperatures had become too cool for emergence. These hatchlings were used in comparisons of body size and condition, as well as righting response (see below) between autumn-emerging hatchlings and hatchlings preparing to overwinter in-nest.

In spring 2011, nests were monitored daily for spring emergence after mean air temperatures rose above freezing consistently (late April to early May). In spring 2012, nests were excavated from 6 to 10 April. Hatchlings and un-hatched eggs were collected, and probable cause and time of death for any dead hatchlings was noted – whether death occurred during incubation and/or overwinter and was due to freezing and/or energy depletion or destruction by roots and/or fly larvae. Hatchlings that died during winter due to freezing and/or energy depletion had little trauma and were not decomposed. Hatchlings that died during incubation because of plant roots were covered in root masses, and hatchlings that died because of fly larvae predation consisted of only skin and scutes. Hatchlings that died during incubation also were more decomposed (e.g. scutes flaking off, body parts rotten).

Hatchling variables measured

Hatchling midline carapace and plastron lengths were measured to the nearest 0.01 mm (3148, Traceable Digital Calipers, Control Company, Friendswood, TX, USA). Hatchling mass was measured using a digital scale (SP202, Scout Pro, Ohaus Corporation, Pine Brook, NJ, USA) to the nearest 0.1 g.

Each hatchling underwent a righting test, which consisted of placing each hatchling on its carapace on a cloth-covered board (30×15 cm) and waiting for the turtle to flip over onto its plastron. Two variables were measured: (1) latency period, which is the time from placement until the first righting attempt, and (2) righting period, which is time from the first righting attempt until successful righting (Rasmussen and Litzgus, 2010; Riley and Litzgus, 2013). The temperature in the lab was recorded for each trial (ranging from 17 to 27°C). Each trial was recorded with a digital camera (Photosmart R742, Hewlett-Packard Development Company, Mississauga, ON, Canada), and latency period and righting period were scored from the videos. Turtles that cannot right themselves are more likely to be predated, to desiccate and/or drown (Finkler and Claussen, 1997). Thus, righting ability is considered to represent a hatchling's future survival (Freedberg et al., 2004; Delmas et al., 2007). Hatchlings were processed within 24 h and released at their nests.

During release, we examined whether hatchling *C. picta* moved to aquatic overwintering sites after autumn emergence (Costanzo et al., 1995). In 2011, before release, autumn-emerging hatchlings ($N=85$) were dusted with ultraviolet light-activated fluorescent powder (UV Phosphorus Powder, Singapore). We avoided contact with eyes, mouth, nostrils and cloaca (Stapp et al., 1994). At night, hatchling trails were tracked using hand-held UV lamps (Raytech Raytector 5, Model R5-FLS-2, Middletown, CT, USA), and we recorded whether trails led towards or away from water during this first 24 h.

Winter environmental monitoring

Nest and aquatic overwintering environments were monitored from October 2011 to April 2012. Nest oxygen (using the method described above) was measured monthly. Temperature loggers recorded data hourly at a nest depth of 10 cm. Aquatic overwintering environment was monitored over the same period; firstly, by measuring dissolved oxygen content in the marshes (with

overwintering adult turtles) adjacent to nests. Dissolved oxygen was measured at a standardized water depth of 60 cm using a dissolved oxygen meter (accuracy of ±2% air saturation; YSI 556 MPS, YSI Inc., Yellow Springs, OH, USA). Data loggers recorded hourly temperatures at a depth of 10 cm within the marsh substrate at putative hatchling overwintering sites and some known adult overwintering sites. Previous observations indicate that turtle hatchlings overwinter at a substrate depth of 2 to 15 cm within water bodies (Ultsch et al., 2007; Paterson et al., 2012). In total, 44 data loggers were placed in 12 marshes; from each marsh, 2–5 data loggers were retrieved in April 2012.

Soil analyses

Soil samples were used for several analyses: grain size, moisture and organic content. Approximately 5 g of soil that had been collected at oviposition and each environmental measurement period was weighed and then dried in an oven at 65°C within 24 h post-collection. Dried samples were weighed and the mass lost was recorded as soil moisture content. The remaining soil samples were transported to Laurentian University, Sudbury, ON, Canada, and air-dried. For organic content analysis, empty crucibles were first weighed and then dried samples were sieved using a number 25 sieve, and ~5 g of soil was placed into each crucible. Crucibles were then re-weighed and placed in a muffle furnace. The muffle furnace program consisted of increasing the temperature by 0.7°C min⁻¹ to a temperature of 150°C, then the temperature remained at 150°C for half an hour. The temperature was then increased by 0.3°C min⁻¹ to 450°C where it remained for 2 h. After 30 h had elapsed, crucibles were removed, cooled in a desiccator and weighed a final time. The weight lost was recorded as organic content (Hughes et al., 2009).

Sieve analysis was used to determine soil grain size. The sieve sizes used were numbers 8, 16, 30, 50, 100 and 200. Sieves were weighed individually and placed in a stack. Approximately 50 g of an air-dried soil sample was placed at the top of the stack and put on a sieve shaker for 10 min. Sieves were individually weighed again to determine the amount of soil retained in each (Soil Survey Division Staff, 1993; Hughes et al., 2009). The percentage of soil in each sieve out of the total amount of soil in the stack was calculated, and the Canadian Soil Survey Committee system was used to classify soil types: gravel (≥2 mm grain diameter, percentage in a number 8 sieve), coarse sand (0.5–2 mm grain diameter, percentage in number 16 and 30 sieves), sand (0.15–0.5 mm grain diameter, percentage in number 50, 100 and 200 sieves) and fines (≤0.075 mm grain diameter, percentage that had gone through the number 200 sieve).

Data handling and analyses

Summary statistics

To assess whether oviposition date affected overwinter strategy, nesting dates were coded in annual numeric sequence (Wilimovsky, 1990). Hatching success (%) was calculated as the number of hatched eggs divided by the number of eggs laid. Hatchlings that were depredated by fly larvae or roots during incubation were included as hatched in this calculation. Overwintering success (%) was calculated as the number of hatchlings alive after winter divided by the number of live hatchlings in the nest pre-overwintering. Hatching and overwintering success were compared between hatchlings from autumn- and spring-emerging nests using a Mann–Whitney–Wilcoxon test (Gotelli and Ellison, 2004).

Nest temperature data were used to calculate total heat units (HU): a variable that relates both mean nest temperature and variation to embryonic development (Holt, 2000). Degree days (°D) are the number of heat units accumulated over 24 h above a threshold temperature (T_0). Below the threshold temperature, no development takes place, but above it development occurs (Holt, 2000). The threshold temperature (T_0) for painted turtles is 14°C (Les et al., 2007). The equation uses hourly temperatures ($T_0, T_1, T_2, \dots, T_{23}$) to calculate heat units above a threshold temperature (T_0 ; Holt, 2000).

$$HU = \left[\left((T_0 + T_1) / 2 \right) + \left((T_1 + T_2) / 2 \right) + \dots + \left((T_{22} + T_{23}) / 2 \right) \right] / (24 - T_0). \quad (1)$$

Mean, minimum and maximum nest temperatures, as well as the amount of time a nest spent below -0.6°C (freezing point of body fluids for hatchlings) (Packard and Packard, 2004) and -4°C (lower limit for freeze

tolerance) (Storey et al., 1988) from October until April was calculated to understand the nest environment to which hatchlings were exposed.

Statistical analyses

All statistical analyses were performed in R (R Development Core Team, 2012). All data are shown as means \pm 1 s.e.m. A significance level of $\alpha=0.05$ and 95% confidence limits were utilized for all tests.

Environmental variables measured throughout incubation were averaged for each nest. A principal component analysis (PCA) was used to transform six soil variables (percentage of soil within each soil texture class, organic content and moisture) into one principal component that summarized 54% of variation within these variables (Manly, 2005). The maximal multivariate logistic regression model examined the relationship between turtle overwinter strategy (a binary response variable: spring=0 or autumn emergence=1) and eight environmental variables (Table 1) considering main effects only (owing to our low sample size; $N=22$ nests), and was fit using generalized linear models (glm package) (Logan, 2010). Variables were standardized (by subtracting each variable by its mean divided by 1 s.d.) for use in the models. Model ranking with AIC_c and averaging was completed using the R packages glmulti (Calcagno and de Mazancourt, 2010) and MuMIn (Barton, 2009). To assess model fit, we used R^2_N (Nagelkerke, 1991), which is fitted by maximum likelihoods and generalizes traditional linear regression R^2 to situations where residual variance is not adequately defined (Nakagawa and Schielzeth, 2013). R^2_N is not sensitive to the number of variables in the model, so AIC_c is used to identify the model that accounts for the most variation in the data with the fewest variables.

Data on clutch oviposition sequence (i.e. first or second clutch for an individual mother) are count data ($N=61$); thus, a Fisher's Exact test was used to examine relationships between oviposition sequence and overwinter strategy (Gotelli and Ellison, 2004).

Predator presence, hatchling size, body condition and righting response were analyzed to determine whether overwintering strategy might provide benefits to hatchlings ($N=61$ nests). Mammalian and avian predation attempts and sarcophagid fly larvae presence data are count data, so data were analyzed using Fisher's Exact tests (Gotelli and Ellison, 2004). Hatchling variables were averaged across hatchlings for each clutch (Davy et al., 2014). Size, body condition and righting response were compared among hatchling sampling periods: autumn-emerging hatchlings, hatchlings that were excavated in the autumn and those that emerged in the spring. Assumptions of normality and heterogeneity of variance were verified, and data were transformed for normality as needed (see below). Carapace length and mass were compared among hatchling sampling periods using an ANOVA and an *a posteriori* Tukey honest significant difference (HSD) test that adjusted for multiple comparisons in order to identify differences among sample means (Logan, 2010). A logistic regression, fit using glm (Logan, 2010), examined the relationship between overwintering strategy (a binary response variable: spring=0, or autumn emergence=1) and hatchling size (carapace length and mass). To examine body condition, a linear mixed effects model (LMEM) of mass against the fixed effects of carapace length and hatchling sampling period, and the random effect of mother's identity (to control for genetic effects) was used (Garcia-Berthou, 2001; Litzgus et al., 2008; Riley and Litzgus, 2013). Righting response variables (latency period and righting period) were transformed using log ($y+1$) to ensure normality. Latency period and righting period were compared among hatchling sampling periods using LMEMs that included the fixed covariate of trial temperature, which is linearly related to ectotherm performance (Hutchison et al., 1966), and the random effect of mother's identity. LMEMs were performed using the R package lme4 (Bates et al., 2014). If a significant interaction was found in the LMEMs, it was reported; if no significant interaction was found, only main effects were tested and reported.

To determine risks associated with aquatic versus terrestrial overwintering, winter temperatures were compared between marshes and nests using an unequal variance Student's *t*-test. Nest temperature data were averaged from October 2011 until April 2012. Oxygen in-nest and dissolved oxygen in marshes was averaged from September 2011 until April 2012. Oxygen data were not compared statistically, as oxygen was measured in

different media (water in marshes versus air in nests). Instead, oxygen data are described and compared qualitatively.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.L.R., G.J.T. and J.D.L. conceived and designed the experiments. J.L.R. performed the experiments and analyzed the data. J.L.R., G.J.T. and J.D.L. wrote the manuscript.

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