biology letters

rsbl.royalsocietypublishing.org

Research

Cite this article: Madsen JG, Wang T, Beedholm K, Madsen PT. 2013 Detecting spring after a long winter: coma or slow vigilance in cold, hypoxic turtles? Biol Lett 9: 20130602.
http://dx.doi.org/10.1098/rsbl.2013.0602

Received: 2 July 2013
Accepted: 6 September 2013

Subject Areas:
behaviour, neuroscience

Keywords:
freshwater turtles, responsiveness, coma, evoked potential, hibernation, hypoxia

Author for correspondence:
Jesper G. Madsen
e-mail: jesperguldsmedmadsen@gmail.com

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2013.0602 or via http://rsbl.royalsocietypublishing.org.

Royal Society Publishing
Informing the science of the future

Neurobiology

Detecting spring after a long winter: coma or slow vigilance in cold, hypoxic turtles?

Jesper G. Madsen, Tobias Wang, Kristian Beedholm and Peter T. Madsen

Zoophysiology, Department of Bioscience, Aarhus University, Building 1131, 8000 Aarhus, Denmark

Many freshwater turtle species can spend the winter submerged in ice-covered lakes by lowering their metabolism, and it has been proposed that such severe metabolic depression render these turtles comatose. This raises the question of how they can detect the arrival of spring and respond in a sensible way to sensory information during hibernation. Using evoked potentials from cold or hypoxic turtles exposed to vibration and light, we show that hibernating turtles maintain neural responsiveness to light stimuli during prolonged hypoxia. Furthermore, turtles held under hibernation conditions for 14 days increase their activity when exposed to light or elevated temperatures, but not to vibration or increased oxygen. It is concluded that hibernating turtles are not comatose, but remain vigilant during overwintering in cold hypoxia, allowing them to respond to the coming of spring and to adjust their behaviour to specific sensory inputs.

1. Introduction

Freshwater turtles can survive an entire winter without oxygen, submerged at the bottom of frozen lakes; much attention has been devoted to understanding how these extraordinary reptiles maintain cardiovascular and nervous functions in complete anoxia at low temperatures [1,2]. Their anoxia tolerance critically depends on the ability to reduce energy expenditure while elevating anaerobic ATP production [3,4]. Cells within the central nervous system (CNS), particularly those involved in sensory functions, for instance vision, have a high ATP turnover [5]. The anoxia-tolerant turtles can decrease nervous activity, and hence reduce ATP consumption by rendering neuronal membranes less leaky (channel arrest) and by reducing the electrical activity (spike arrest) [6,7], which seem to account for an 80% reduction in electroencephalographic activity *in vivo* during anoxia [8]. This silencing of neuronal activity is primarily mediated by the release of the inhibitory neurotransmitter GABA and reduced activity of the AMPA and NMDA receptors for the excitatory neurotransmitter glutamate [1].

The marked reduction in the generation and propagation of action potentials and post-synaptic potentials, that is a state of self-induced anaesthesia, has led to the notion that freshwater turtles enter an unresponsive comatose state during winter hibernation [6,9–11], leaving a behavioural conundrum as to how the turtles sense the arrival of spring to resurface and resume normal functions. However, *in vivo* evoked retinal light responses of freshwater turtles are only marginally reduced in anoxia. [12]. The maintenance of retinal function and some vigilance during anoxia may allow turtles to use the photoperiod as a cue when spring arrives and also to engage sensible responses to environmental stressors.

Here, we investigate whether turtles indeed become comatose when anoxic at low temperatures by (i) measuring evoked potentials (EPs) in response to light and vibration stimuli and (ii) by measuring physical activity levels of cold hibernating turtles in response to different stimuli.

2. Material and methods

(a) Experimental animals

Trachemys scripta (0.15–0.5 kg) were purchased from Nasco (WI, USA) and kept in tanks (27°C) with access to a dry platform and a heating lamp allowing for behavioural thermoregulation. They were fed Tetra ReptoMin supplemented with vegetables, mussels and fish.

(b) Effects of temperature and anoxia on evoked potentials

EPs in response to vibration and light were measured using subcutaneous stainless steel electrodes in anaesthetized turtles (60 mg kg^{-1} ketamine and 4 mg kg^{-1} Rompun). The influence of body temperature (T_b) on the EPs was assessed as the turtles were cooled using a water bath (see the electronic supplementary materials), while EPs were recorded at 25, 23, 20, 18, 15, 12, 10, 8, 5 and 3°C over 126 ± 35 min. EPs were also recorded, as T_b was returned to 25°C. The effects of hypoxia on EPs were assessed at 20°C. Having established normoxic responses by repeated stimulations every 8 min, the turtles were rendered anoxic by mechanical ventilation of the lungs with 50 ml N_2 in combination with an anoxic atmosphere in the experimental chamber; this caused an immediate reduction in arterial PO_2 to less than 5 mmHg (figure 1). Light and vibration stimulations were repeated until the recorded EPs did not change. The turtles were then reoxygenated by mechanical ventilation with air (see the electronic supplementary material) while the peak-to-peak amplitude of the EP were measured. For each series of measurements, the amplitudes were normalized by dividing the values by the value of the measurement in normoxia or at starting temperature (figure 1).

(c) Spontaneous and evoked activity during hibernation in cold, anoxic water

Having established that anaesthetized anoxic turtles continue to exhibit EPs in response to light stimuli, we investigated whether a rise in temperature, oxygenation of the water, light and vibration would stimulate activity in non-anaesthetized hibernating turtles. Spontaneous activity was tracked with an infrared camera and scored before and after each stimulus, based on changes in relative illumination (see the electronic supplementary material). First, after 14 days in cold, anoxic water (PO_2 of the water was measured at 2.0 ± 0.5 mmHg; see the electronic supplementary materials), the turtles were filmed for a 2 h control period and then continuously exposed to one of the stimuli for 4.5 h. Activity before and during stimuli was normalized to the mean control period movement scores (see the electronic supplementary material).

3. Results

(a) Evoked potential studies of anaesthetized turtles

The EPs to both vibration and light decreased towards the noise level when T_b was reduced, and reappeared gradually when T_b returned towards 25°C (figure 1*a*). The EPs in response to vibration, however, recovered slower and EPs in response to light were higher upon rewarming (figure 1*a*,*b*). The EP responsiveness to vibration declined in anoxia and did not differ from the background noise after 1 h (figure 1*d*). By contrast, the EP responses to light persisted at normoxic levels throughout anoxia (figure 1*c*).

The evoked responses were evaluated using permutation tests to determine whether the median of the responses to the two stimuli differed significantly. These tests were performed separately for the data collected during cooling, reheating, hypoxia and reoxygenation (see the electronic supplementary materials). Tests with 10 000 iterations revealed significant differences between responses to light and vibration stimulation during reheating ($p < 0.01$), hypoxia ($p < 0.01$), reoxygenation, ($p < 0.05$) and for cooling ($p < 0.05$), indicating different degrees of neural shutdown for visual and vibrational senses.

(b) Behavioural responses of non-anaesthetized turtles

Figure 2*b–d* shows normalized movement data before and during exposures to light, oxygen and vibration. The data were tested (Wilcoxon signed rank) for significantly higher movement during stimulation compared with the control period. Movement increased as the temperature was elevated (figure 2*a*) and when the light was turned on (figure 2*b*), but significant changes in movements were not induced by reoxygenation of the water (figure 2*c*). There was a slight, but not significant, increase in movements to vibration stimuli (figure 2*d*). Heating caused a significant linear increase in movements with temperature ($p < 0.01$), showing that heating did increase activity.

4. Discussion

The anaesthetized turtles studied in our *in vitro* experiments retained neural responsiveness to light during severe hypoxia, while responsiveness to vibration under the same conditions was reduced. During cooling, responsiveness to both light and vibration were reduced to detection levels when reaching 3°C. Such severe reductions of responsiveness to stimuli in anaesthetized turtles in either cold or hypoxic conditions are consistent with these animals being endowed with mechanisms, such as channel and spike arrest, that drastically reduce CNS activity to reduce energy expenditure [7].

Despite this reduced neural activity, cold anoxic turtles *in vivo* still increase behavioural activity immediately when light stimulus was applied and gradually when heated. These immediate responses of non-anaesthetized turtles *in vivo* are remarkable, considering that metabolism of these animals is merely 0.5% of that in normoxia at 20°C [13,14], but nevertheless consistent with previous results showing maintained light sensitivity during anoxia [12,15]. We were not able to record electrical activity following light stimulation at 3°C, which probably reflect that the neural activity was below our experimental detection limit. The lack of electrical activity does not appear to be caused by the anaesthetics (see the electronic supplementary materials). In light of the *in vivo* results from hibernating turtles, it appears that the animals maintain nervous responsiveness to light, despite it being undetectable.

The maintenance of visual response in the absence of responses to vibrations suggests a differential shutdown of modalities within the turtle CNS, where energy conserving mechanisms seemingly are applied to a lesser extent in the neurons connected with the visual system. Combined with the faster recovery of light sensitivity and the behavioural responses to light demonstrated here, it is implied that vision is important for hibernating slider turtles.

The turtles also display increased activity when exposed to increasing temperatures. This is probably caused by the general

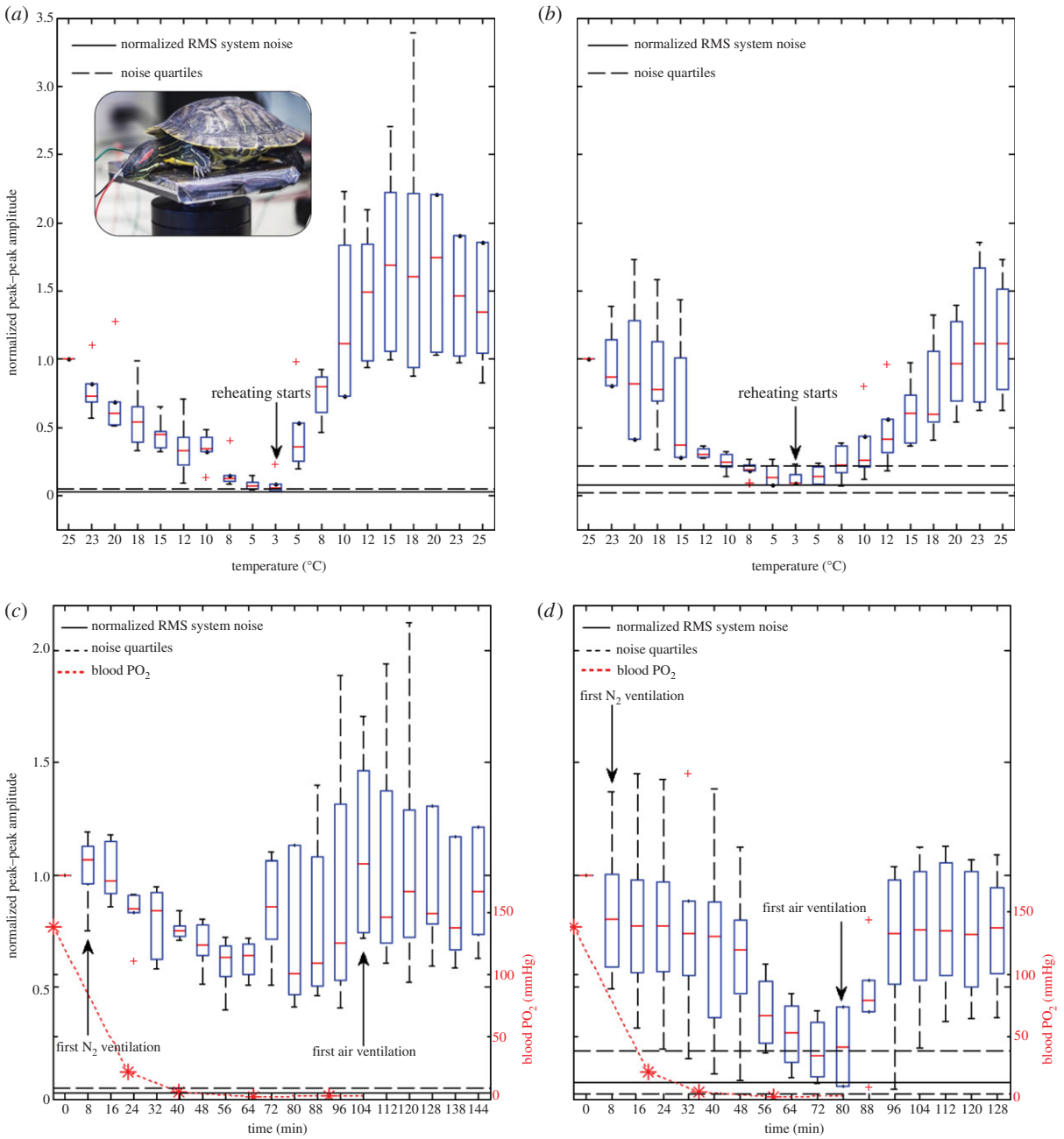

Figure 1. Box plots of normalized peak–peak amplitude for EP experiments. Red lines represent the median response from six turtles at a given temperature, blue boxes show the lower and upper quartile values and whiskers are the most extreme values within 1.5 times the interquartile range. Red crosses are outliers. (*a*,*b*) Light and vibration responsiveness during cooling and reheating. (*c*,*d*) Light and vibration responsiveness during hypoxia and returning normoxia. Hypoxia–reoxygenation turtles were ventilated with N_2 every 8 min during blood sampling and stimulation experiments. Red traces in (*c*,*d*) blood PO_2 measurements of a turtle being ventilated with N_2 with units shown on the right *y*-axis. In (*a*), a turtle is shown on top of the shaker with electrodes placed (Photo courtesy of Jesper Rais).

positive effect of temperature on virtually all biological processes in ectothermic animals. With an increased metabolic rate, the time period the turtles can stay submerged is shortened, increasing the need for the animals to breathe. However, during natural conditions such an increase in activity would be advantageous, as increased water temperature would only occur when the ice cover had melted, allowing the turtles to surface. Likewise, when the ice cover melts, light levels in the water will rise. For the turtles, this would be a direct indication that spring has arrived and that it is possible to surface. Whether the animals actually use increasing light levels during spring is, to our knowledge, unknown. This study has demonstrated that they have the ability to use increased light as a seasonal cue. It would be interesting to monitor turtle activity during spring arrival and examine whether there is any correlation between light, temperature and turtle activity during this period. Some studies have been carried out using radio telemetry to record the animals' activity during hibernation [16]. However, these studies do not attempt to correlate environmental changes, such as increasing light and temperature, with the turtles' activity.

In conclusion, we show that freshwater turtles do not enter a state of unresponsive coma when overwintering in cold, hypoxic water, but that they, in a state of slow vigilance, retain some modalities and appropriate motor functions during winter hibernation.

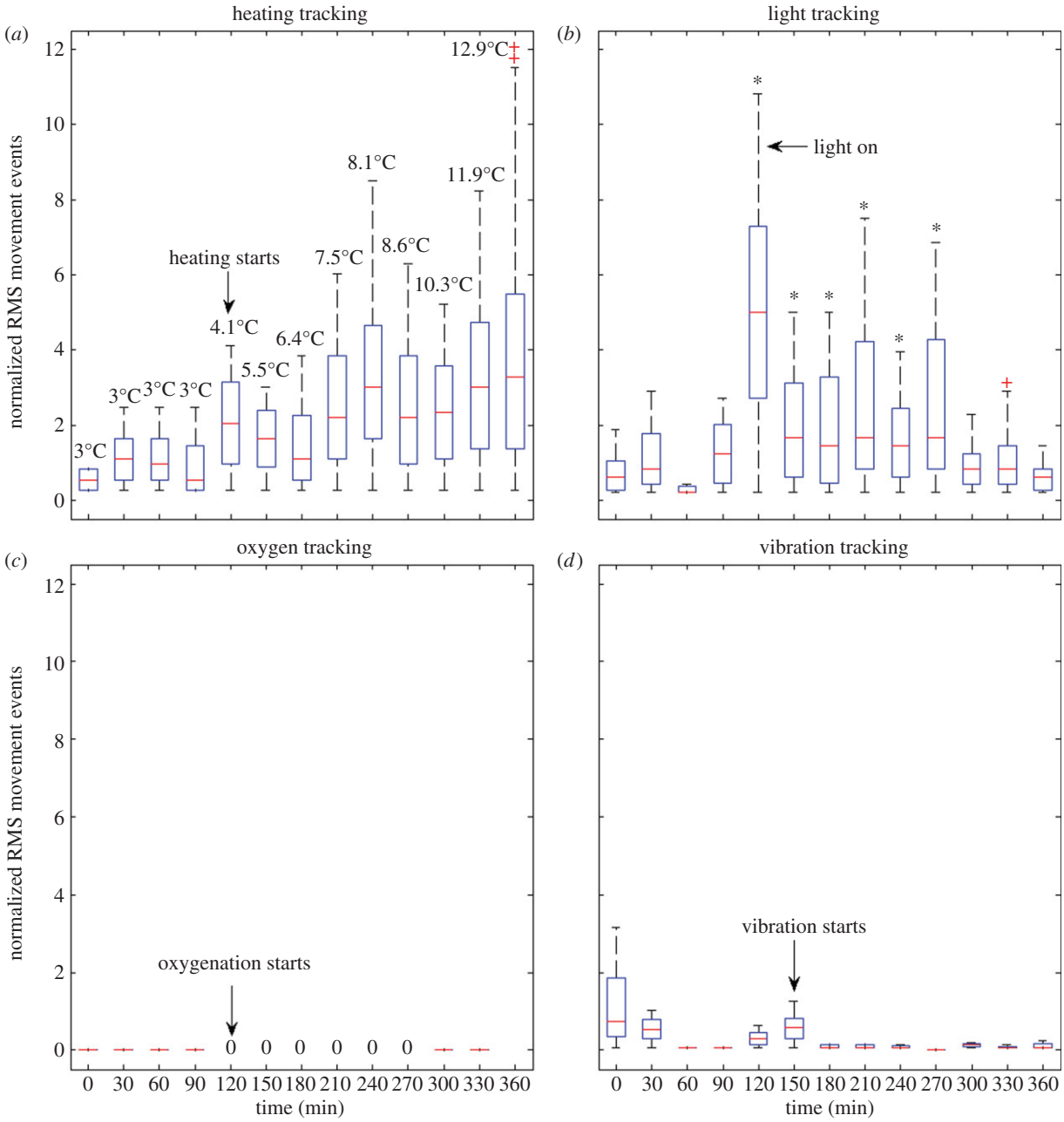

Figure 2. Normalized movement events of cold anoxic turtles. Each box represents 30 min; boxes 0–120 min are control periods where no stimulus is applied. The remaining bars represent movements during stimuli exposure. (*a–d*) Turtle movement during different stimulus application. For (*b–d*), asterisks denote significantly higher movement events than in the control period. Zeroes denote no detectable movement during that period.

The experiments were performed with permission from the Danish Inspectorate for Animal Experimentation within the Danish Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food administration (journal number 2006/561-1192).

Acknowledgements. We greatly appreciate helpful critique from Göran Nilsson and Donald C. Jackson.

Data accessibility. Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9mg16.

Funding statement. This study was supported by the Danish Natural Science Research Council grants to P.T.M. and T.W.

References

1. Buck LT, Hogg DWR, Rodgers-Garlick C, Parmenter ME. 2012 Oxygen sensitive synaptic neurotransmission in anoxia-tolerant turtle cerebrocortex. In *Arterial chemoreception: from molecules to systems* (ed. C. A. Nurse). *Adv. Exp. Med. Biol.* **758**, 71–79. (doi:10.1007/978-94-007-4584-1_10)
2. Jackson DC. 2002 Hibernating without oxygen: physiological adaptations of the painted turtle. *J. Physiol.* **543**, 731–737. (doi:10.1113/jphysiol.2002.024729)
3. Lutz PL, Nilsson GE. 1997 Contrasting strategies for anoxic brain survival: glycolysis up or down. *J. Exp. Biol.* **200**, 411–419.
4. Staples JF, Buck LT. 2009 Matching cellular metabolic supply and demand in energy-stressed animals. *Comp. Biochem. Physiol.* **153A**, 95–105.
5. Wong-Riley MTT. 2010 Energy metabolism of the visual system. *Eye Brain* **2**, 99–116. (doi:10.2147/EB.S9078)

6. Hylland P, Milton S, Pek M, Nilsson GE, Lutz PL. 1997 Brain Na^+/K^+-ATPase activity in two anoxia tolerant vertebrates: crucian carp and freshwater turtles. *Neurosci. Lett.* **235**, 89–92. (doi:10.1016/S0304-3940(97)00727-1)
7. Hochachka PW. 1986 Strategies against hypoxia and hypothermia. *Science* **231**, 234–241. (doi:10.1126/science.2417316)
8. Fernandes JA, Lutz PL, Tannenbaum A, Todorov AT, Liebovitch L, Vertes R. 1997 Electroencephalogram activity in the anoxic turtle brain. *Am. J. Physiol.* **273**, R911–R919.
9. Nilsson GE, Lutz PL. 2004 Anoxia tolerant brains. *J. Cereb. Blood Flow Met.* **24**, 475–486. (doi:10.1097/00004647-200405000-00001)
10. Nilsson GE. 2001 Surviving anoxia with the brain turned on. *News Physiol. Sci.* **16**, 217–221.
11. Nilsson GE. 2010 *Respiratory physiology of vertebrates: life with and without oxygen.* Cambridge, UK: Cambridge University Press.
12. Stensløkken KO, Milton SL, Lutz PL, Sundin L, Gillian MCR, Stecyk JAW, Nilsson GE. 2008 Effect of anoxia on the electroretinogram of three anoxia-tolerant vertebrates. *Comp. Biochem. Physiol.* **150A**, 395–403.
13. Herbert CV, Jackson DC. 1985 Temperature effects on the response to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid–base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiol. Zool.* **58**, 670–681.
14. Jackson DC. 2000 Living without oxygen: lessons from the freshwater turtle. *Comp. Biochem. Physiol.* **125**, 299–315. (doi:10.1016/S1095-6433(00)00160-4)
15. Rábl K, Reglödi D, Bánvölgyi T, Somoyvári-Vigh A, Lengvári I, Gábriel R, Arimura A. 2002 PACAP inhibits anoxia-induced changes in physiological responses in horizontal cells in the turtle brain. *Reg. Pept.* **109**, 71–74. (doi:10.1016/S0167-0115(02)00189-1)
16. Ultsch GR. 2006 The ecology of overwintering among turtles: where turtles overwinter and its consequences. *Biol. Rev.* **81**, 339–367. (doi:10.1017/S1464793106007032)