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Animal behaviour

Energy conservation characterizes sleep in sharks

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Sharks represent the earliest group of jawed vertebrates and as such, they may provide original insight for understanding the evolution of sleep in more derived animals. Unfortunately, beyond a single behavioural investigation, very little is known about sleep in these ancient predators. As such, recordings of physiological indicators of sleep in sharks have never been reported. Reduced energy expenditure arising from sustained restfulness and lowered metabolic rate during sleep have given rise to the hypothesis that sleep plays an important role for energy conservation. To determine whether this idea applies also to sharks, we compared metabolic rates of draughtsboard sharks (*Cephaloscyllium isabellum*) during periods ostensibly thought to be sleep, along with restful and actively swimming sharks across a 24 h period. We also investigated behaviours that often characterize sleep in other animals, including eye closure and postural recumbency, to establish relationships between physiology and behaviour. Overall, lower metabolic rate and a flat body posture reflect sleep in draughtsboard sharks, whereas eye closure is a poorer indication of sleep. Our results support the idea for the conservation of energy as a function of sleep in these basal vertebrates.

1. Introduction

Sleep is a ubiquitous behaviour found across the animal kingdom, which is typically characterized by sustained immobility and reduced responsiveness [1]. Despite the vulnerability inherent with sleeping, its persistence across evolutionary time suggests it serves one or more core functions [2]. One hypothesis for such a core function is that sleep serves to conserve energy through enforcing restfulness and lowering metabolic rate relative to wakefulness [1,3,4]. Energy savings during sleep have been reported in diverse animals, including humans [5,6], cats [7], rats [8], birds [9] and fruit flies [10]. It is unknown, however, whether reduced energy expenditure also occurs in sleeping fishes.

Extant sharks represent the earliest group of jawed vertebrates and, therefore, may provide original insight into the evolution of sleep in vertebrates [11]. This rationale is particularly salient following the recent discovery of two sleep states in teleosts [12] and in at least two species of lizard [13,14] that in some respects resemble mammalian and avian non-rapid eye movement (non-REM) and REM sleep [15]. The existence of two sleep states in birds and mammals suggests that each state performs a different, but perhaps complementary, function. Any homology between the multiple sleep states observed in ectothermic vertebrates to that of endothermic vertebrates is unclear.

Recent studies have found that Port Jackson (*Heterodontus portusjacksoni*) and draughtsboard (*Cephaloscyllium isabellum*) sharks are nocturnal with a reduced responsiveness to stimulation while asleep [16,17]. However, as sleep is both a behavioural and physiological state involving multiple components, including changes in eye state, muscle tone, brain activity and metabolism [18], it is necessary to investigate as many sleep components as possible to fully characterize the sleep state, or states, in sharks [19].

Here, in draughtsboard sharks, we assessed changes in metabolic rate (mass-specific oxygen uptake rate or $\dot{M}O_2$), via intermittent-flow respirometry, and behaviours associated with sleep in other animals: eye state (open/closed), and body posture (upright/flat) over a 24 h period to determine whether sleep plays a role in energy conservation in sharks.

2. Material and methods

(a) Experimental animals and housing

Seven draughtsboard sharks (766–2705 g in weight) were collected from Hauraki Gulf, north-eastern New Zealand, and were housed in outdoor aquaria under natural light conditions. Animals were fed a diet of pilchards and held for a minimum of two weeks before the commencement of experiments. Food was withheld for at least 48 h prior to the start of experiments to ensure animals reached a post-absorptive state [20,21].

(b) Intermittent-flow respirometry system set-up

For detailed respirometry methods, see electronic supplementary material [22]. In short, the system comprised an acrylic respirometry chamber submerged within a reservoir tank of flow-through seawater held at constant temperature (17.5°C, 1 µm filtered, UV sterilized). Water was homogenized in the chamber by a pump drawing water from one end and expelling into the other, through a PVC tube [23]. A laptop computer, connected to a Firesting oxygen (O_2) meter with a contactless sensor spot (Pyroscience, Aachen, Germany) logged oxygen levels. $\dot{M}O_2$ measurement cycles were interspersed with flush cycles to ensure a high quality of water (per cent O_2 range 84–98%).

(c) Video recording set-up

Continuous, infrared illumination and overhead video recordings of animal behaviour during the 24 h measurement period were achieved following the methods detailed in Kelly *et al.* [16]. For further details on video recording set-up, see electronic supplementary material [22].

(d) Experimental protocol

Animals were individually placed into the sealed respirometry chamber. Automated, intermittent-flow respirometry and video recordings began a minimum of 48 h later to allow each animal to acclimate to their new conditions before data collection began. Each protocol then lasted 24 h under a 12 : 12 light : dark photoperiod regime.

(e) Data analysis

Custom-written software calculated the gradient of the per cent O_2 decline and the associated residual sum of squares (R^2). $\dot{M}O_2$ ($mgO_2\,h^{-1}$) was then calculated from the decline in oxygen saturation. Metabolic rate and behaviour (eye states, posture and activity) data were manually scored second-by-second using the video recordings. Eye states were scored as open or closed and body postures of inactive sharks were scored as flat (lying flat on the bottom of the tank) or upright (sitting perched up on pectoral fins) (figure 1*a*). Activity states were scored as swimming, rest (inactive less than 5 min) or sleep (inactive more than 5 min); the latter has been shown to be associated with reduced responsiveness, and, therefore, a demonstrated reflection of sleep [17]. For details on statistical analyses used, see electronic supplementary material [22].

Table 1. Mixed effects model showing the effects of activity (swimming, rest and sleep) and photoperiod (12 h day and night) on residual $\dot{M}O_2$ values as calculated from a regression of $\dot{M}O_2$ and body mass underlying figure 1*d*. In each model, individual was set as a random effect; activity and photoperiod were treated as fixed effects.

effect	nominator d.f.; denominator d.f.	*F*-ratio	*p*-value
$\dot{M}O_2$			
activity	2; 21	15.38	<0.01
photoperiod	1; 21	25.08	<0.01
activity * photoperiod	1; 20	5.84	0.03

3. Results and discussion

(a) Sleeping sharks have a lower metabolic rate

Our previous studies showed that draughtsboard sharks are nocturnal [16,17]. Therefore, and unsurprisingly, swimming behaviour and mean $\dot{M}O_2$ levels of draughtsboard sharks with an $R^2 > 0.95$ (all activity states included) were significantly higher during the night ($t_{12} = 4.13$, $p < 0.01$) (figure 1*b*). However, from these data alone, it remained unclear whether restful sharks were sleeping sharks. To address this question, we sampled the $\dot{M}O_2$ data based on activity state (using the criteria of $R^2 > 0.8$ and bout length of greater than 90 s) to account for varying bout lengths found within each activity state. Shark mean $\dot{M}O_2$ levels were significantly lower during sleep (i.e. inactive for at least 5 min) and the highest during swimming (figure 1*c*).

These data were then parsed further to include the expression of each activity state during day and night to reveal whether sleeping animals consistently showed a lower metabolic rate while asleep. During the day, sharks never swam for more than 90 s (with an $R^2 > 0.8$), therefore, no daytime $\dot{M}O_2$ data were available for this state. The level of $\dot{M}O_2$ varied between activity states (swimming, rest and sleep) and between day and night (table 1), with $\dot{M}O_2$ level recordings again highest during night swimming (figure 1*d*). Metabolic rates were low and similar irrespective of whether sharks slept during the day or night. Variability in $\dot{M}O_2$ during night rest was similar to the variability observed during night swimming, suggesting that at least some night rest reflects quiet wakefulness. Accordingly, night rest $\dot{M}O_2$ was significantly higher than day rest (Tukey's *post hoc* test: $t_{21} = -5.06$, $p < 0.01$) and during sleep (day: $t_{20} = -7.18$, $p < 0.01$; night: $t_{20} = 4.97$, $p < 0.01$). Conversely, day rest $\dot{M}O_2$ was similar to $\dot{M}O_2$ during both day sleep ($t_{21} = 1.39$, $p = 0.73$) and night sleep ($t_{22} = -0.36$, $p = 0.99$), which might indicate that sharks fell asleep quicker during the day than during the night (to the effect that at least some day rest

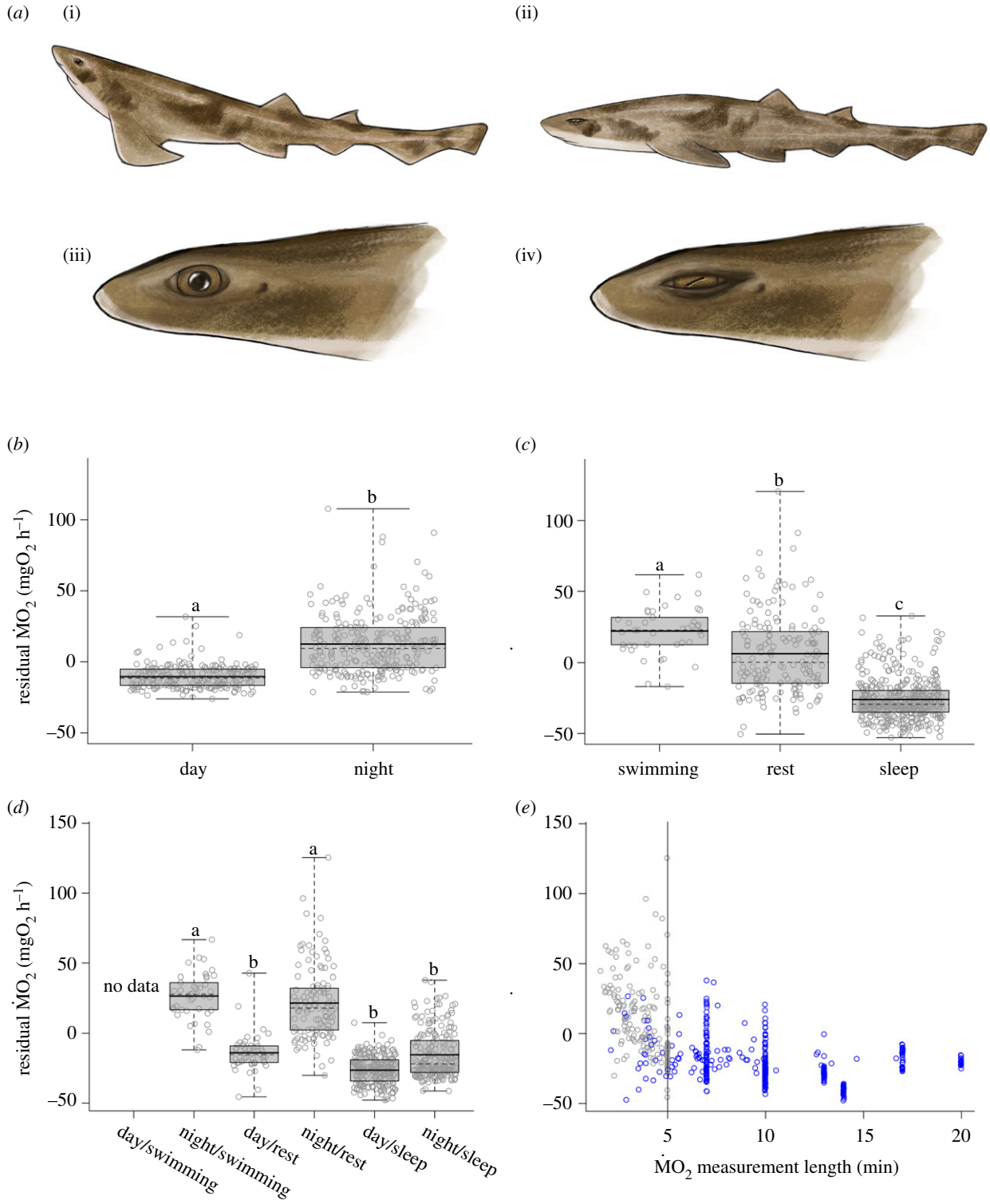

Figure 1. (*a*) Drawings of upright (i) and flat (ii) body postures, and open (iii) and closed (iv) eyes used to score behavioural data from video recordings. (*b*) Box plot of day and night residual $\dot{M}O_2$ values (i.e. controlling for body mass) from intermittent-flow respirometry measure periods (all activity states included) over a 24 h period (L : D 12 : 12). (*c*) Box plot of residual $\dot{M}O_2$ values across three activity states (irrespective of photoperiod) using subsampled data points from all measure periods with an applied criteria of an $R^2 > 0.8$ and a length of greater than 90 s. (*d*) Box plot of the residual $\dot{M}O_2$ values in (*c*), but partitioned by photoperiod (day, night). (*e*) Regression of subsampled residual $\dot{M}O_2$ values against subsample duration (blue indicates sleep; grey denotes rest); all data fit the criteria of an $R^2 > 0.8$ and a length of greater than 90 s; vertical line indicates 5 min of inactivity. For (*b*,*c*,*d*), solid black lines indicate means; dotted lines denote medians; edges of boxes represent quartiles; whiskers reflect maximum and minimum values; grey circles represent individual samples (random *x*-axis dispersal); significant pairwise contrasts are denoted by the letters a, b and c.

should actually be considered to be day sleep). Cube-root transformed residual $\dot{M}O_2$ values did not vary with changes in measured rest or sleep duration (figure 1*e*). However, for a given measurement period, the cube-root of residual $\dot{M}O_2$ during sleep was between 0.19 and 1.07 less than that observed during rest. These data, therefore, reinforce the results found by Kelly *et al*. [17] that sharks restful for at least 5 min were asleep. Thus, not only do sleeping sharks have reduced responsiveness to stimulation, they also have a lower metabolic rate.

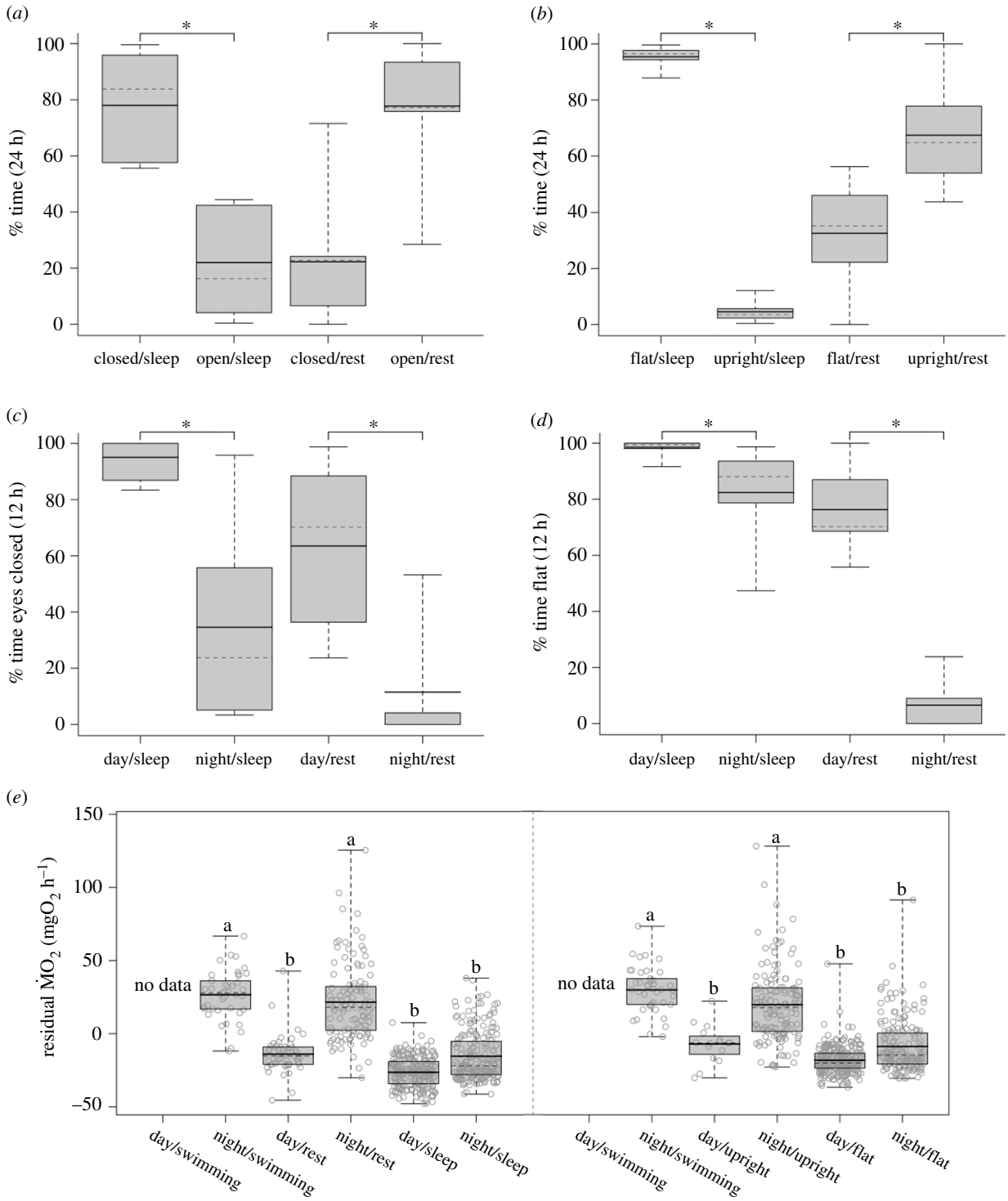

Figure 2. (*a*) Box plot of the per cent time animals were observed with open and closed eyes, and (*b*) in flat and upright postures between sleeping and rest states. (*c*) Box plot showing the per cent of time animals were observed with closed eyes and (*d*) a flat body posture during sleeping and resting states throughout the (12 h) day and night. (*e*) Comparative boxplots of residual $\dot{M}O_2$ values across (left) activity states and (right) posture, partitioned by photoperiod, using subsampled data points from all measure periods. For all panels, solid black lines indicate means; dotted lines denote median; edges of boxes represent quartiles; whiskers reflect maximum and minimum values. For (*a–d*), significant pairwise contrasts are denoted by asterisks. For (*e*), grey circles represent individual samples (random *x*-axis dispersal); letters a and b denote pairwise contrasts.

(b) Recumbent posture is a better indicator of sleep than eye closure

While swimming, sharks always had their eyes open. When resting, the eyes were also more likely to be open ($z_{6,13} = 161.40$, $p < 0.01$) (figure 2*a*). Conversely, during sleep, the eyes were most often closed ($z_{6,13} = 353.30$, $p < 0.01$). Postural changes were also associated with sleep as sleeping animals adopted a flat body posture ($z_{6,13} = 456.60$, $p < 0.01$) (figure 2*b*) whereas resting animals sat upright ($z_{6,13} = 158.50$, $p < 0.01$). This might, at first, suggest that closed eyes and a flat posture reflect sleep, and both are behaviours commonly associated with mammalian sleep [24]. However, upon separating states by photoperiod, we found that eye closure was more common during day sleep ($z_{6,12} = 241.74$, $p < 0.01$) and day rest ($z_{6,11} = 121.09$, $p < 0.01$) (figure 2*c*), a behavioural pattern that has also been observed in the large-spotted dogfish (*Scyliorhinus stellaris*) [25]. However,

animals that were inactive for more than 5 min (i.e. asleep) during the night had eyes open in approximately 38% of all cases. Taken together, this suggests that eye closure is more likely associated with an external factor, such as the presence of light rather than sleep. Similarly, the proportion of flat body posture was significantly higher during rest ($z_{6,11} = 122.49$, $p < 0.01$) and sleep states ($z_{6,12} = 83.33$, $p < 0.01$) throughout the day (figure 2*d*). This supports our $\dot{M}O_2$ data that animals inactive for at least 5 min are sleeping. The fact that animals engaged in rest (inactive less than 5 min) during the day spent more time flat also supports the idea that some daytime rest might represent sleep. This might suggest that animals fell asleep faster during the day. It is important to note that night and day $\dot{M}O_2$ data partitioned by posture showed a similar pattern to the data partitioned by activity (figure 2*e*). This suggests that both the amount of time spent inactive and body posture are good predictors for sleep in this species.

4. Conclusion

The collection of metabolic data via intermittent-flow respirometry in marine fishes, including sharks, is well explored [23,26–31]. Until now, however, no work had directly investigated the metabolic rates of sleeping fishes *per se*. This study highlights that, like in many vertebrates [1], sleep in sharks is associated with reduced metabolic rate. Thus, the hypothesis that sleep is important for energy conservation [3,4] is supported by this study in a primitive vertebrate. By doing so, we have provided the first physiological evidence of sleep in sharks and find support for our published (behavioural) report on sleep in draughtsboard sharks [17]. Sleep is largely unstudied in this diverse group of cartilaginous fishes and future research should focus on other physiological indicators of sleep, such as changes in brain activity, for a more complete portrait of sleep in these vertebrates.

Data accessibility. Datasets and electronic supplementary material are available at datadryad.org. https://doi.org/10.5061/dryad.m37pvmd2z. The data are provided in the electronic supplementary material [22].

Authors' contributions. M.L.K.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, writing—original draft and writing—review and editing; S.P.Collins: data curation, formal analysis, investigation, methodology, software, visualization, writing—original draft and writing—review and editing; J.A.L.: formal analysis, supervision, visualization, writing—original draft and writing—review and editing; J.M.H.: supervision and writing—original draft; S.P.Collin: funding acquisition, resources, supervision and writing—original draft; C.A.R.: formal analysis, funding acquisition, resources, supervision, visualization, writing—original draft and writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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Competing interests. We declare we have no competing interests.

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Energy conservation characterizes sleep in sharks

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Supplementary Material

Materials and Methods

Experimental animals and housing

Seven adult draughtboard sharks (*Cephaloscyllium isabellum*; 4 females, 3 males; 766-2705 g in weight; 350-720 mm in length) were obtained in 2018 from a commercial fisherman of the Leigh coast, northern New Zealand. At the Leigh Marine Laboratory, they were held in a 1200 L tank with flow-through 200 μm filtered seawater (hourly water changes) at ambient temperature (17-18 °C range) and fed pilchards every three days. Animals were held on a 12:12 light:dark cycle and allowed to acclimate for at least two weeks before experiments. Experiments were approved by the University of Auckland Animal Ethics committee (approval no. 001983).

Intermittent-flow respirometry setup

The custom-built respirometer held one shark at a time and consisted of a sealed acrylic chamber (external length: 870 mm, width: 320 mm, depth: 170 mm; capacity: 38 L) with tubing between it and two small submersible pumps (eHeim compact 1000, Stuttgart, Germany). The chamber was large enough to facilitate free movement and swimming of sharks. The first (circulation) pump was connected to the chamber at opposite ends and was designed to constantly mix seawater internally for accurate oxygen saturation measures. The combined

volume of the chamber, circulation pump and tubing was 42.7 L. The second (flush) pump was only connected to the chamber at one end and was designed to flush fresh, clean seawater from the main experimental tank on demand through the chamber via activation of an on/off relay unit (Cleware USB-Switch C, Hollingstedt, Germany). Seawater was flushed out of the chamber via a tube port at the opposite end. The flush outflow ran to a level above the reservoir water level to avoid any water exchange while the pump was off (Svendsen et al., 2016A). The two pumps ran in a crossflow design ensuring a high degree of mixing in the chambers when both were running (Svendsen et al., 2016A). Oxygen saturation of the water inside the chambers was measured continuously (at 1 Hz) using a sensor spot (OXSP5, Pyroscience, Aachen, Germany) placed on the inside of the chamber lid that was monitored and recorded by a FireSting O_2 meter (Pyroscience, Aachen, Germany). The O_2 meter was calibrated to air-saturated water from the experimental tank before each trial.

Oxygen consumption rate ($\dot{M}O_2$) was measured using intermittent stop-flow respirometry (Steffensen, 1989), where a respirometry cycle involved a period with the chamber 'open' to the surrounding water when the flush pump was on, and 'closed' when off. The open period is also referred to as the 'flush' phase, where metabolites are expelled, and oxygen saturation is replenished. This ensures oxygen saturation in the chambers always returned to > 92%. Following closure, oxygen saturation in the chamber declines linearly when fish respire at a constant rate, % O_2 never fell below 84% (Svendsen et al., 2016A). Oxygen measured by the sensor spots, however, is delayed behind chamber average and the recorded change in oxygen saturation is initially concave down in the closed phase. Closed periods were thus split into a short 'wait' and 'measurement' phase with oxygen saturation data only gathered from the latter when the decline was linear. Respirometry cycle (measure, flush, wait) lengths varied between individual sharks, and day and night due to variations in mass and

activity levels, respectively, with the following ranges: measure day 600-1200 s, night 420-780 s; flush: day and night 240-300 s; wait: day and night 180 s.

The FireSting O_2 meter was connected to a laptop computer with customized respirometry software (LeighResp, John Atkins design) that automated the respirometer cycle and logged oxygen saturation and calculated the change of oxygen saturation decrease per unit time ($\frac{\Delta\%\text{sat}}{\Delta t}$) for each measurement phase. $\dot{M}O_2$ in units of milligrams of oxygen consumed per hour (mg O_2 h^{-1}), was then calculated using the equation:

$$\dot{M}O_2 = \frac{\Delta\%\text{sat}}{\Delta t} a V_{RE}$$

where, a is the solubility coefficient of oxygen (mg O_2 $\%sat^{-1}$ L^{-1}) in seawater (35 ppt salinity, 17.5°C temperature, ~100 kPa atmospheric pressure; Loligo Systems, Viborg, Denmark) and V_{RE} is the effective volume of the respirometry chamber (L, chamber minus shark volume estimated as body mass assuming equal density as seawater).

Video recording setup

Video recordings of animal behaviour during the 24-h measurement period were achieved following the methods detailed in Kelly et al. (2020). Two further IR-sensitive cameras, fitted with custom-built waterproof housings, were submerged within the reservoir tank and placed centrally along the left and right (long) sides of the respirometry chamber (placed 400 mm back from the chamber), in order to record shark lateral posturing and eye states.

Experimental protocol

All trials were carried out in a respirometer held in a larger 1500 L experimental tank (1900 mm diameter, 600 mm depth) supplied continuously with flow-through 1 μm filtered, UV treated (ClearTec UV-C 11W, Pond One, Australia) seawater at 17.5 °C. Animals were individually placed into the sealed respirometry chamber 24 h after last feeding. Automated, intermittent-flow respirometry and video recordings began 48 h later to allow each animal to acclimate to their new conditions and reach a post absorptive state before data collection began. Each protocol then lasted 24 h under a 12:12 light:dark photoperiod regime. Three respirometry cycles were run after each shark was removed to measure background changes in O_2 (Svendsen et al., 2016A), but these levels were found to be negligible (slopes non-significantly different from zero, $p > 0.05$). Body mass recordings of sharks were taken before and after each protocol via a digital bench scale (Wedderburn WS20110K, Auckland, New Zealand). The respirometry chamber was scrubbed with dilute sodium hypochlorite then thoroughly rinsed with fresh water following the end of each experimental protocol.

Data analysis

Custom-written software calculated the gradient of the % O_2 decline and the associated residual sum of squares (R^2). $\dot{M}O_2$ was then calculated from the decline in oxygen saturation as described above. All metabolic analyses were carried out on residual values from regressions of $\dot{M}O_2$ against body mass (Packard and Boardman, 1988, 1999; Lesku et al., 2009). The first metabolic analysis compared rates during day and night. We regressed the mean day and night $\dot{M}O_2$ for each shark against body mass (regression I) and then used a paired Student's t-test on the residuals (accounting for repeated measures) to compare day versus night. We visualized this with residuals of all $\dot{M}O_2$ values calculated with respect to regression I (Figure 1b). Secondly, we compared differences in residual $\dot{M}O_2$ between activity states (irrespective of

photoperiod) (Figure S1). As bouts of each activity state varied greatly in length and R^2 within each measure period, we subsampled data points from all measure periods and applied strict criteria in order to standardize the quality of the data while retaining a large sample size. Each data point met the criteria of an $R^2 > 0.80$ and a length of > 90 s (in order to clean the data of false $\dot{M}O_2$ values due to the inherent variability in the measurement of O_2 saturation; 631 of 1452 cycles retained). We recognize that metabolic data with an $R^2 > 0.95$ is typically considered the most reliable (Svendsen et al., 2016A; 2016B). When we applied a criteria of $R^2 > 0.95$ to our subsampling method, we obtained data that was qualitatively similar to that which we obtained with an $R^2 > 0.80$. Importantly, however, the $R^2 > 0.80$ data included a much larger sample size which was more amenable to statistical analysis. Similarly, the time criteria of > 90 s maximized sample size (increased with decreasing critical value) while maintaining quality in $\dot{M}O_2$ values (increased with increasing critical value). We, therefore, regressed the mean swimming, rest and sleep $\dot{M}O_2$ for each shark against body mass (regression II). The residuals were then used to compare the three activity states with a mixed effects model (individual as random effect; activity as a fixed effect). We conducted Tukey's *post hoc* tests to determine where significance was reached. We visualized this with residuals of all $\dot{M}O_2$ values calculated with respect to regression II (Figure 1c). Thirdly, activity $\dot{M}O_2$ data was further partitioned by photoperiod. Not all combinations of activity and photoperiod were included as no sharks engaged in swimming during the day with $\dot{M}O_2$ values that met selection criteria. We, therefore, regressed the mean $\dot{M}O_2$ values from the five combinations (night/swimming, day/rest, night/rest, day/sleep, night/sleep) for each shark against body mass (regression III). We used a mixed effects model (individual as a random effect; activity and photoperiod as fixed effects) to determine whether there was a significant difference in residual $\dot{M}O_2$ across the activity and photoperiod combinations. Tukey's *post hoc* tests again determined where significance was reached. We visualized this with residuals of all $\dot{M}O_2$

values calculated with respect to regression III (Figure 1d). A mixed effects model (individual as random effect; activity and activity length as fixed effects) was used to distinguish whether rest and sleep duration affected rest and sleeping residual $\dot{M}O_2$ values calculated from regression III. All rest and sleeping residual $\dot{M}O_2$ values were used in this analysis and needed cube-root transformation to satisfy the normality assumption of the model. We visualized these data with a scatter-plot of back-transformed residual $\dot{M}O_2$ values (Figure 1e). Generalized linear mixed effects models with binomial distribution, in which individual was set as random effect, were used to compare differences in posture and eye state between activity states and photoperiods (Figure 2a-d). Finally, respirometry cycles were subsampled according to posture (flat, upright, swimming) and the same criteria as above was applied to clean the data (612 of 1393 cycles retained). As day/swimming was, again, not represented by any $\dot{M}O_2$ values, we regressed the mean $\dot{M}O_2$ values from the five represented posture/photoperiod combinations for each shark against body mass (regression IV). To obtain a normal distribution of residuals, a log10-log10 transformation was needed for regression IV. We also combined the five available crosses of posture and photoperiod to form a single variable (levels of night/swimming, day/upright, night/upright, day/flat, night/flat) to perform a mixed effects model (individual as a random effect; posture and photoperiod as fixed effects) on the residual $\dot{M}O_2$ values. Again, Tukey's *post hoc* tests determined where significance was reached. To calculate residuals of $\dot{M}O_2$ values for visualization, values were firstly back-transformed (Figure 2e). All analyses were conducted in R (v 1.0.143), using lme4 package for mixed effects models.

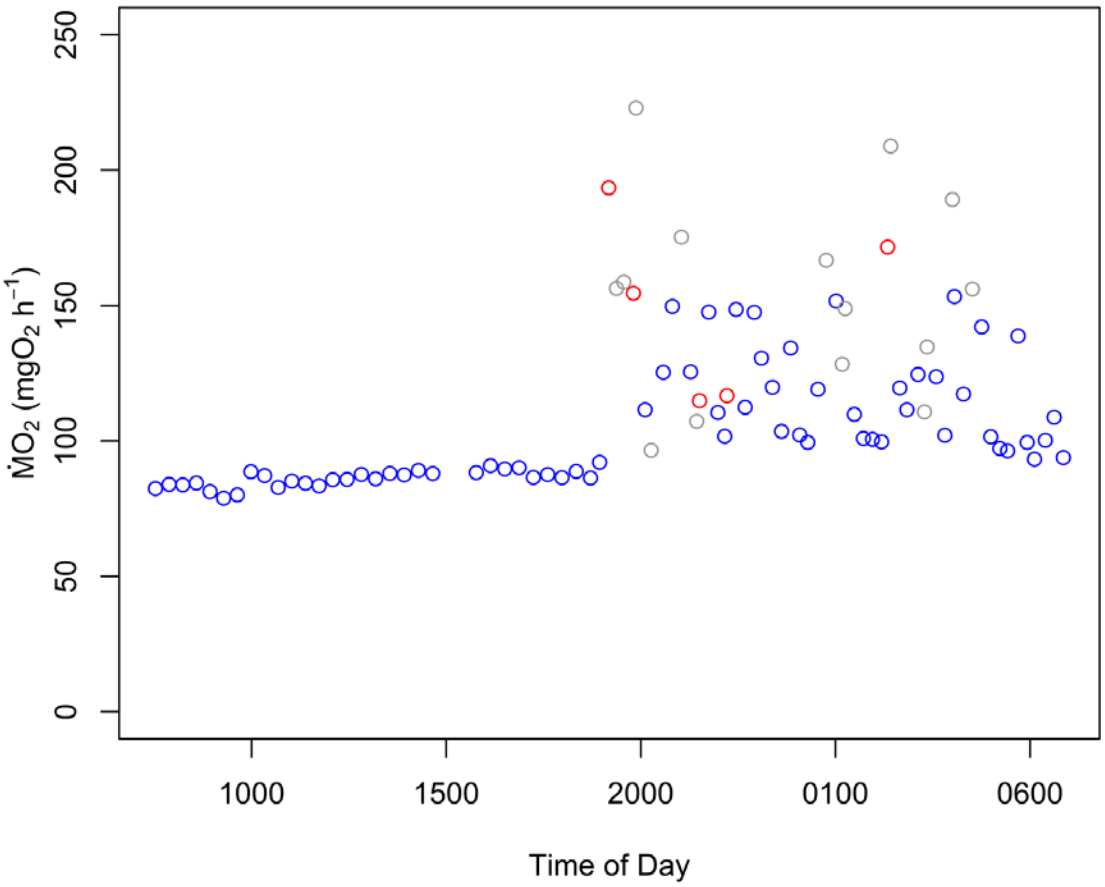

Figure S1. Time series of $\dot{M}O_2$ levels across three activity states (blue indicates sleep; grey denotes rest; red reflects swimming) using subsampled data points from intermittent-flow respirometry for a single shark over a 24-h period (L:D 12:12). Only values that satisfied applied criteria of an $R^2 > 0.80$ and a length of > 90 s were used.

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