

## Semelparous marsupials reduce sleep for sex

### Highlights

- During the breeding season, male, but not female, antechinus reduce their sleep
- One reproductive male more than halved his pre-breeding amount of sleep
- The magnitude of increased activity was predicted by the increase in testosterone
- Breeding antechinus show a decline of oxalic acid, a biomarker of sleep loss

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### In brief

Zaid et al. show that male, but not female, dusky antechinus—a small Australian marsupial that reproduces only once in its lifetime—reduces sleep during a 3-week breeding season. Wild agile antechinus might also be sleep deprived during the breeding season, as suggested by reduced levels of oxalic acid, a biomarker of sleep loss.

Article

# Semelparous marsupials reduce sleep for sex

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<https://doi.org/10.1016/j.cub.2023.12.064>

## SUMMARY

Sleep is a prominent, seemingly universal animal behavior. Although sleep maintains optimal waking performance, the biological drive to sleep may be incompatible with the life history of some species. In a multi-year study on semelparous marsupials in Australia, we provide the first direct evidence of ecological sleep restriction in a terrestrial mammal. Dusky (*Antechinus swainsonii*) and agile (*A. agilis*) antechinus have an unusual reproductive strategy characterized by the synchronous death of all males at the end of their only breeding season. Using accelerometry, electrophysiology, and metabolomics, we show that males, but not females, increase their activity during the breeding season by reducing sleep. In a trade-off between the neurophysiological requirements for sleep and evolutionary necessity for reproduction, strong sexual selection might drive males to sacrifice sleep to increase access to fertile females and ultimately maximize their fitness.

## INTRODUCTION

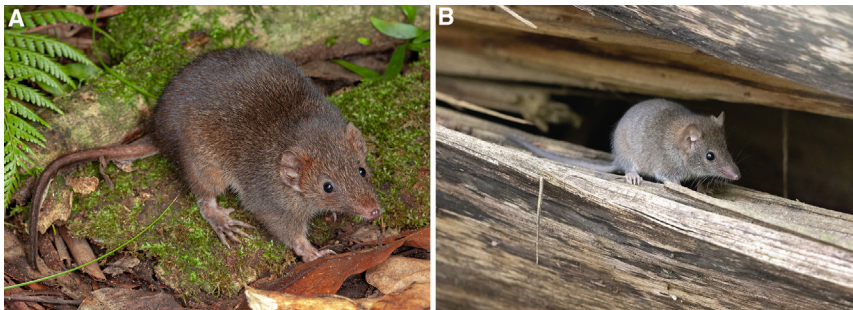
Sleep serves essential functions on a daily basis. Sleep is involved with neurogenesis,<sup>1,2</sup> synaptic scaling,<sup>3</sup> memory consolidation,<sup>4,5</sup> waste clearance from neuronal use,<sup>6</sup> and immune system and energy regulation.<sup>7–9</sup> In humans and animals, sleep restriction leads to neurobehavioral impairments, including deficits in attention, motivation, and memory, and an increase in emotional reactivity.<sup>10–12</sup> Moreover, given the evolutionary longevity<sup>13,14</sup> and ecological persistence of sleep,<sup>15</sup> sleep is thought to perform non-trivial functions that sustain waking performance.

However, increasing evidence reveals that some animals have evolved the ability to forgo sleep, seemingly without cost.<sup>16</sup> For example, based on the assumption that sustained trunk immobility reflects sleep, African elephant matriarchs (*Loxodonta africana*) sleep just 2 h most days and may go without any sleep for 46 h.<sup>17</sup> In a study on wild northern elephant seals (*Mirounga angustirostris*), brain activity measurements reveal that seals nap while diving, but for only 2 h per day for 7 months.<sup>18</sup> Another example of sleep loss comes from great frigatebirds (*Fregata minor*), which sacrifice sleep while engaging in long-distance foraging flights over the ocean.<sup>19</sup> Although the birds could sleep on the wing, they did so only 0.7 h per day, yet slept nearly 13 h per day on the nest. Consequently, great frigatebirds can sleep little for weeks, and likely months, but maintain the attention and cognitive faculties needed to find and consume prey.<sup>20</sup> Remarkably, some male pectoral sandpipers (*Calidris melanotos*) are active more than 95% of the time during their intense 3-week breeding season in the high Arctic, and those males

that sleep the least ultimately sire the most offspring.<sup>21</sup> In this way, sleep loss can be evolutionarily adaptive. This raises the possibility that other species with an extreme reproductive strategy might also greatly reduce sleep during the mating season.

Semelparity is a reproductive strategy characterized by one reproductive period in a lifetime.<sup>22</sup> Although semelparity has evolved multiple times, and is more widespread in invertebrates (e.g., mayflies [*Rhithrogena germanica*], cicadas [*Magicicada* spp.], redback spiders [*Latrodectus hasselti*], and giant Pacific octopus [*Enteroctopus dofleini*]), it is also found in some vertebrates, such as Pacific salmon (*Oncorhynchus* spp.). Interestingly, semelparity has also evolved in certain marsupial mammals, restricted to the males of some dasyurid and didelphis marsupials.<sup>23</sup> In semelparous marsupials, females are monoestrous and rear a single litter per year, and can survive to a second breeding season. In stark contrast, all males die synchronously within a population immediately after their first and only mating season.<sup>24</sup>

The physiological mechanism that leads to male death (known as “mating syndrome”) is thought to be triggered by a prolonged elevation of corticosteroids during their short and intense mating season,<sup>25,26</sup> exacerbated by a progressive rise in plasma testosterone. Increased corticosteroids, which inhibit immune function and inflammatory response of animals, play a crucial role in the cascade leading to fatal, stress-induced organ failure, pathogenic infections,<sup>27</sup> and parasite infestation.<sup>25,28</sup> Therefore, the hypothalamic feedback mechanisms that normally reduce corticosteroid levels are apparently lacking in males, but not females, of small semelparous marsupials. During the breeding season, males compete for access to as many females as possible to



**Figure 1. Image of dusky (*Antechinus swainsonii*) and agile (*A. agilis*) antechinus**

Photo credit: (A) dusky (*Antechinus swainsonii*) antechinus, Stephen Mahony and (B) agile (*A. agilis*) antechinus, Jono Dashper.

maximize reproductive success. Under these circumstances, the need for sleep might limit the time available to access receptive females, such that selection may favor males able to reduce sleep while maintaining the performance needed to forage, avoid predators, and secure mates.

Here, using a combination of behavioral analyses, electrophysiology, endocrinology, and metabolomics, we report on adaptive, sex-specific sleep restriction in two species of semelparous antechinus in both captive and wild environments. We studied 15 dusky antechinus (*A. swainsonii*; 10 males, [Figure 1A](#)) in an outdoor setting before and during the breeding season using back-mounted accelerometers; blood samples were collected to determine whether endocrine changes, such as testosterone, can predict sleep/wake patterns. Four males were studied in the laboratory for electrophysiological recordings of brain activity. We also collected blood samples from 38 wild agile antechinus (*A. agilis*; 20 males, [Figure 1B](#)) to see whether oxalic acid, a biomarker of sleep loss in eutherian mammals,<sup>29</sup> similarly decreases over the mating period in these marsupials. In doing so, we collected accelerometry, electrophysiological, and testosterone data for wild-caught dusky antechinus, and oxalic acid data for wild agile antechinus.

## RESULTS

### Only males increase activity during the breeding season

We report a marked sexual dimorphism in both dusky (glmm  $z = 10.26$ ,  $p < 0.001$ ) and agile antechinus (glmm  $t_{120} = 15.34$ ,  $p < 0.001$ ), with males nearly double the mass of females (mean: dusky male =  $96.3 \pm 2.4$  g, female =  $55.3 \pm 1.9$  g; agile male =  $29.9 \pm 0.4$  g, female =  $18.2 \pm 0.5$  g). Both male and female dusky antechinus were more active at night, but could be active at any time of the 24-h day ([Figures 2](#) and [S1](#)). The level of activity was not different between males and females before the onset of the breeding season (glmm  $t_{14} = -0.058$ ,  $p = 0.95$ ; [Figure 3A](#)). During the mating period, however, only male antechinus increased activity (males: glmm  $z = 10.09$ ,  $p < 0.001$ ; females: glmm  $z = 0.89$ ,  $p = 0.37$ ), arising from increased night-time movement ([Figure 3B](#); [Table S1](#)). Indeed, one reproductive male more than doubled his pre-breeding activity level. Male restlessness was sustained into the post-breeding period. Overall, the magnitude of the activity increase was positively related to the magnitude of the increase in testosterone over the same period (glmm  $t_9 = 3.41$ ,  $p = 0.01$ ; [Figure 4](#)). The timing of male activity could vary within the pre-breeding, breeding, and post-breeding periods, depending on the timing of the full moon ([Figure 2](#)). During the

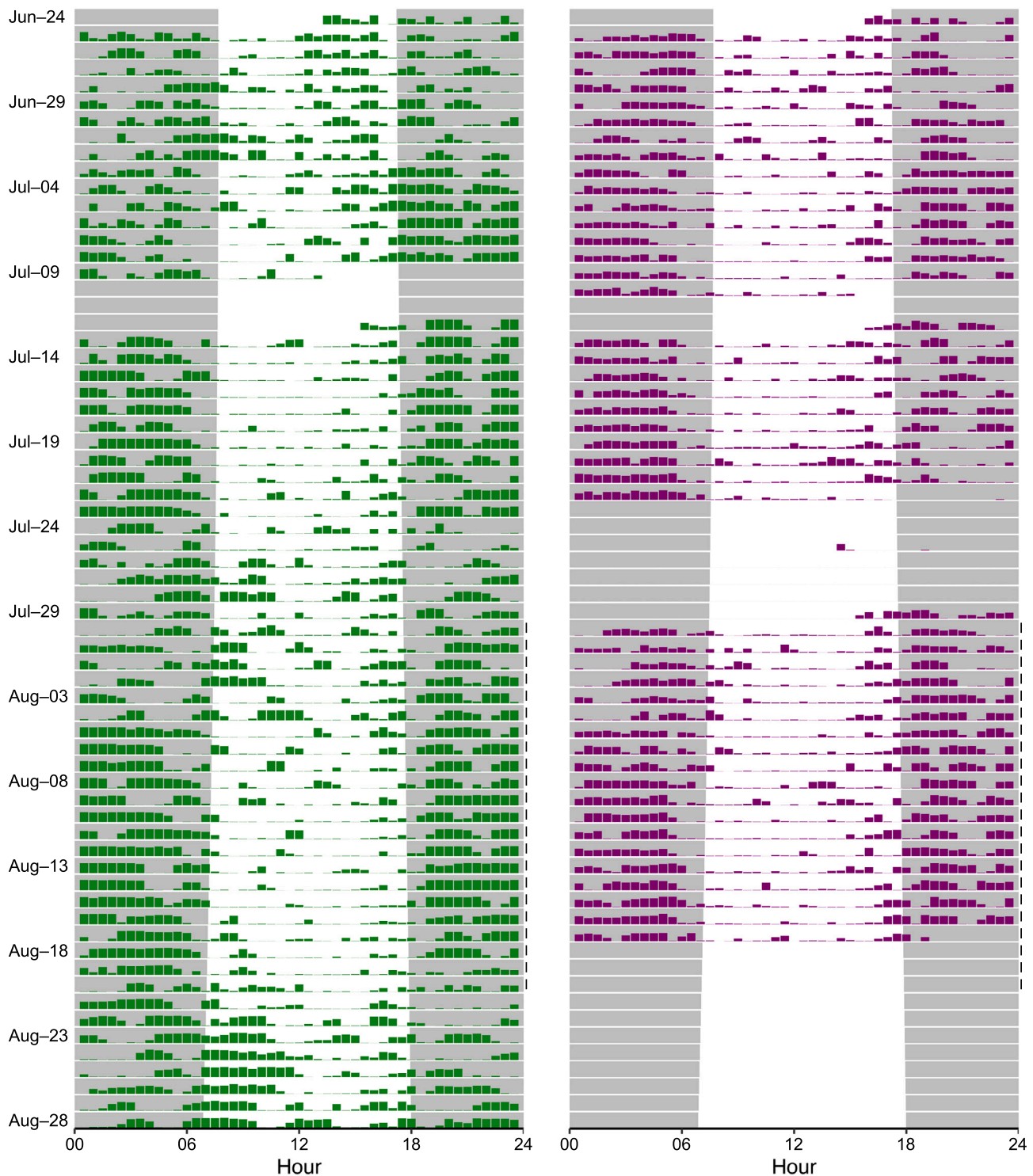
breeding season, some males shifted their peak activity to other times of the 24-h period ([Figure S2](#), see also [Figure S3](#)). Notably, the activity peaks of one male (E1\_17) shifted from early morning and late afternoon to a single night-time peak; two males (E4\_17 and E5\_18) delayed peak activity from late afternoon to the night; another shifted peak activity from the afternoon to the morning (E3-18).

### Restfulness is a proxy for sleep in dusky antechinus

To determine whether increased activity in reproductive males reflects less sleep, or simply less time spent immobile but awake, we performed two additional studies using electrophysiological and metabolomic analyses. First, we used the electroencephalogram (EEG) to measure how long it takes dusky antechinus to fall asleep.<sup>30</sup> Unlike other mammals, which show consolidated periods of sleep and wakefulness, dusky antechinus have upwards of 1,000 sleep episodes per day.<sup>30</sup> They do so by transitioning rapidly from active wakefulness to sleep ([Figure 5A](#)). Dusky antechinus typically spent fewer than 5 s in quiet wakefulness before engaging slow-wave sleep ([Figure 5B](#)). We also re-scored the electrophysiological signals using only the accelerometer (ACC) data to see whether activity alone is a good indicator of brain state. Indeed, the amount of sleep scored using both the EEG and ACC vs. the ACC alone were remarkably similar ([Figure 5C](#), no significant difference,  $t = 0.49$ ,  $p = 0.642$ ). If anything, the ACC-alone analysis slightly (but non-significantly) overestimated the true amount of sleep (mean = 2.6%). Thus, for these three reasons, ACC recordings can accurately measure sleep and wakefulness in dusky antechinus. This indicates that male dusky antechinus lost sleep during the breeding season.

### Declining oxalic acid levels suggest wild agile antechinus are sleep deprived

Next, to investigate whether wild male antechinus might also be sleep deprived during the breeding season, we measured the levels of oxalic acid—a biomarker of sleep loss in eutherian mammals—before and during the breeding season. Although we did not have accelerometry or electrophysiological data for wild agile antechinus, we observed the predicted decline in oxalic acid for males over the breeding season (males: glmm  $t_{17} = -2.61$ ,  $p = 0.021$ ; females: glmm  $t_{15} = -0.97$ ,  $p = 0.353$ ; [Figure 6](#)). However, the mean decline of oxalic acid was not significantly different between males and females (glmm  $t = -0.26$ ,  $p = 0.797$ ). This result (1) suggests that oxalic acid can be a useful indicator of sleep restriction for antechinus, (2) may hint that females are sleep deprived in the wild, perhaps owing to male harassment, and (3) provides evidence that a second species of antechinus are sleep deprived during the breeding season.

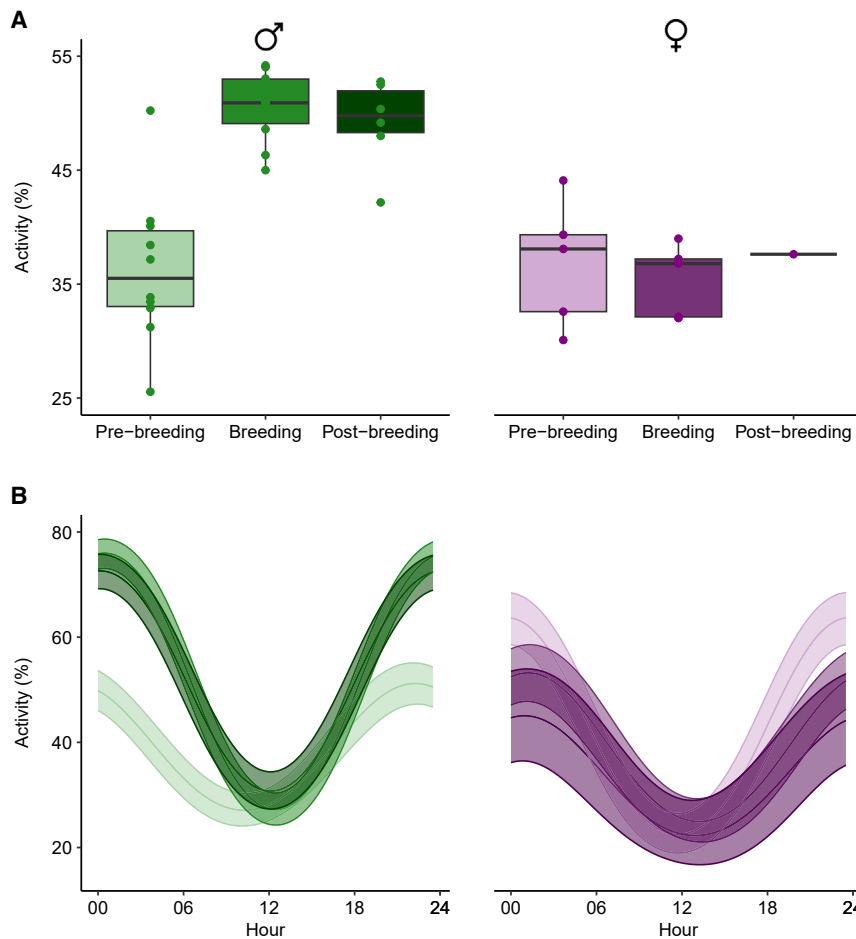


**Figure 2. Daily activity pattern of a male (green) and female (purple) dusky antechinus over a circa 2-month period**

Activity was recorded using a small and lightweight three-dimensional accelerometer mounted on the back.

Each row represents a 24-h day with bar height proportional to the amount of activity in 30-min intervals; gaps in the recording are instances where batteries were depleted or the logger came off the animal. Shaded areas along the actogram delineate night-time. A full moon occurring on Jul-28 and Aug-26 appears to be the reason for reduced night-time activity and augmented morning movement in the male. The breeding season (vertical dashed line) was estimated based on biological markers, including testosterone values and the presence of sperm in males and the presence of cornified epithelial cells in urine and the appearance of the pouch in females.

See also [Figure S1](#).



**Figure 3. Activity levels of dusky antechinus before, during, and after the breeding season**

(A) Boxplots show the median activity levels (line within each box), the upper and lower quartiles (top and bottom edge, respectively), and minimum and maximum values (whiskers) for males (green, n = 10, 10, 7) and females (purple, n = 5, 5, 1) before (light shade), during (darker shade), and after (darkest shade) the breeding season. Values on the y axis are the mean percentage of time spent active 4 days before, 4 days during, and 4 days after the breeding season; one outlier was removed from the male post-breeding box at 61% to improve visualization. (B) Daily activity patterns of males and females before, during, and after the breeding season. Values on the y axis are the mean proportion of activity calculated in 30-min epochs over a 24-h day averaged over 4 days before, 4 days during, and 4 days after the breeding season. The lines represent fitted glmm and shading represent 95% confidence intervals.

See also [Figures S2](#) and [S3](#) and [Table S1](#).

that the average time spent sleeping is between 15 and 17 h a day,<sup>33</sup> comparable to our observations. Taken altogether, the use of restfulness is a reliable proxy of sleep in antechinus.

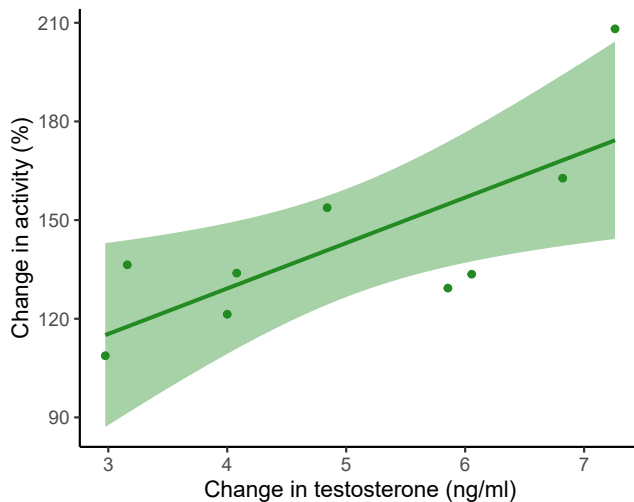
The activity of isolated male dusky antechinus increased in the absence of mating behavior and male-male agonistic encounters. Under wild conditions, such social interactions might have further increased male motivation and contributed to a greater degree of sleep restriction

## DISCUSSION

This study shows a marked increase in activity during the breeding season in male, but not female, dusky antechinus. Electrophysiological and metabolomic analyses indicate that captive dusky antechinus males, and wild agile antechinus males, are not only more active but also sleep less during this time. Reductions in sleep arise from reproductive pressure on males during their only breeding season and may be mediated by an increase in testosterone.

The inclusion of electrophysiological measurements was important as they can distinguish between quiet (restful) wakefulness and sleep. In other species, measures of behavior alone have not always been a reliable indicator of brain state. For example, ruminants and other large mammals can sleep while standing and with eyes open,<sup>31</sup> marine mammals can sleep while swimming<sup>32</sup> and diving,<sup>18</sup> and some seabirds can sleep while flying.<sup>19</sup> However, we found that dusky antechinus transitioned very rapidly from wakefulness to sleep, and the time spent quietly awake was negligible. Furthermore, non-breeding dusky antechinus spent an average of 15.3 h of the day inactive (asleep). Sleep data from eutherian species of similar body mass, such as collared lemming (*Dicrostonyx torquatus*), Heermann's kangaroo rat (*Dipodomys heermanni*), and Mexican volcano mouse (*Neotomodon alstoni*), showed

tion than we observed. Our finding that increased restlessness arises from reduced sleep is novel but complements studies on activity levels recorded in other dasyurids. For example, laboratory-housed male brown antechinus (*A. stuartii*) have higher activity during the breeding season compared to post-breeding,<sup>34</sup> contrary to our finding that males sustain high activity into the post-breeding period; in addition, wild male northern quoll (*Dasyurus hallucatus*) are more active than females at the end of the breeding season.<sup>35</sup> In semelparous species such as these, post-breeding activity is likely heavily influenced by the mating syndrome. Therefore, the reported changes in activity level in these other studies could reflect reduced activity in the post-breeding period rather than increased activity during breeding per se. Instead, our study is the first to compare male and female activity levels before, during, and after the breeding season, and to reliably relate restfulness with sleep using accelerometry, electrophysiology, and metabolomics. In this way, we were able to establish that males lose sleep during the breeding season, which provides important insight into their biological priorities. That said, we could not determine whether males recovered lost sleep by sleeping more intensely during the breeding season. In mammals, including antechinus (before the breeding season), slow-wave sleep is homeostatically regulated, such that extended periods of wakefulness lead to an increase in slow-wave sleep intensity, measured as low-frequency (< 4 Hz)



**Figure 4. The change in testosterone levels during the breeding season, relative to the pre-mating period, in male dusky antechinus predicts the magnitude of the increase in activity during the same period**  
Y axis is a 4-day average before and during the breeding season; the x axis is the difference between testosterone values taken at, or close to, the corresponding 4-day activity periods. Line represents fitted linear model and points are values for individual animals ( $n = 9$ ,  $p = 0.01$ ). Shaded area is the 95% confidence interval.

power density in the electroencephalogram (or slow-wave activity).<sup>30</sup> There is value in recording brain activity of males during the breeding season to look for compensatory increases in slow-wave activity in reproductive males.

We found a reduction in oxalic acid levels in wild agile antechinus over the breeding season. Oxalic acid is a metabolite that has been previously proposed as a cross-species biomarker of sleep loss.<sup>29</sup> In rats and humans, oxalic acid was markedly reduced following sleep loss and recovered to near baseline levels when animals were allowed to sleep freely. Similarly, a study on wild great tits (*Parus major*) found that oxalic acid decreased in response to increased night-time activity, suggesting they were sleep deprived.<sup>36</sup> Two other papers, also on great tits, found contrasting results. Juvenile<sup>37</sup> and adult<sup>38</sup> tits did not have lower oxalic acid under night lights; however, activity or electrophysiology were not measured, making it unclear whether the young birds were sleep deprived<sup>37</sup> and the dim lights (1.5 lux) may not have kept the birds awake.<sup>38</sup> Studies on tits aside, oxalic acid appears to be a biomarker of sleep loss in mammals.<sup>29</sup> In agreement with the sleep restriction observed in captive male dusky antechinus, wild agile antechinus showed compelling evidence for reduced sleep during the breeding season. The (non-significant) mean decline of oxalic acid in female antechinus might hint that females are sleep deprived in the wild, perhaps owing to male harassment.

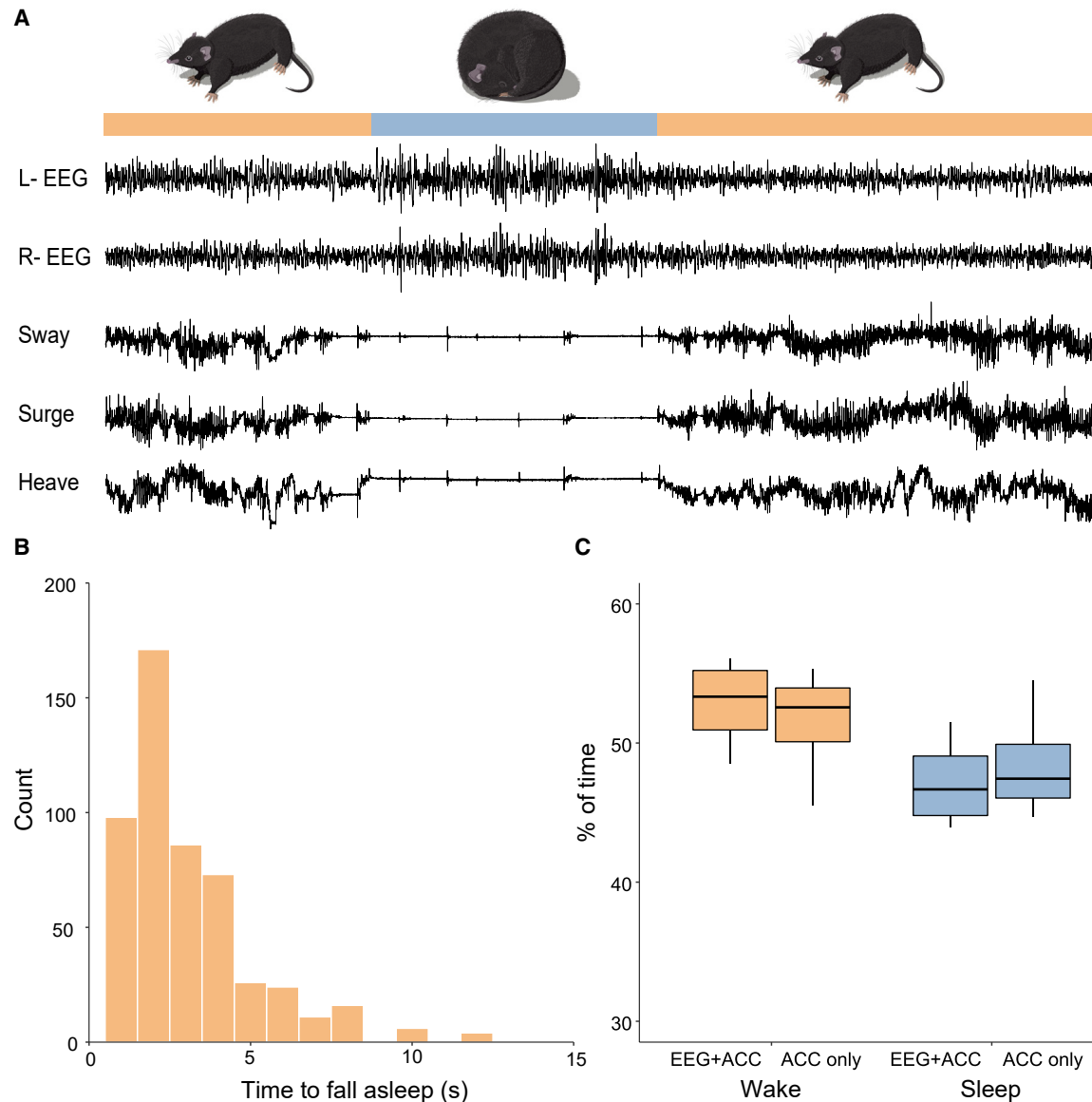
Sleep restriction in breeding male antechinus is likely to be an adaptive behavioral response driven by strong sexual selection. Male blue wildebeest (*Connochaetes taurinus*) have an exploded lek system and are more active during the rut.<sup>39</sup> Similarly, semelparous males, whose reproductive success depends on a single, short, and intense breeding season, rely on access to fertile females during a brief temporal window

to maximize their fitness. Therefore, short periods of highly synchronized estrus in females and promiscuous mating intensify male competition (contest and sperm competition), driving males to be more active and sacrifice sleep as a reproductive strategy to increase their access to females. Male antechinus have either a mechanism to thrive on less sleep during the 3-week mating period, or they accept the physiological costs of extended wakefulness in order to secure paternity shortly before they die.

The effect of polygynous semelparity on antechinus biology is reflected most conspicuously by a remarkable sexual size dimorphism in dusky and agile antechinus, with males almost double the mass of females. This observation is in agreement with previous observations in several semelparous species.<sup>40</sup> In polygynous species, male competition for access to females is intense and males are expected to exhibit traits, such as large size, that lead to competitive advantage. One of the most striking examples of sexual size dimorphism can be found in the southern elephant seal (*Mirounga leonina*), with adult males weighing 4–10 times as much as the adult females.<sup>41</sup> In ungulates, where males fight in pushing contests, and in some polygynous shore birds, where males compete on the ground and in the air, selection favors large body mass in males. The magnitude of the sexual size dimorphism in antechinus coupled with their semelparous nature is well fitted to their polygynous reproductive strategy.

Individual males varied in the magnitude of the increase in activity level from before and during the breeding season. The degree to which males increased activity correlated with the change of testosterone levels during the same time periods. Other mammals have shown a direct effect of testosterone in regulating activity levels. For example, in laboratory mice (*Mus musculus*) exogenous testosterone dramatically boosted physical activity,<sup>42</sup> while in rhesus monkeys (*Macaca mulatta*) and in wild male meadow voles (*Microtus pennsylvanicus*), activity level during the breeding season was correlated with plasma testosterone concentration.<sup>43,44</sup> In our study, it was not possible to directly measure reproductive success in captive or wild environments, but testosterone is an androgen hormone known to have a key role in mate attraction in several species.<sup>45–47</sup> Variation among individuals in testosterone is an indicator of reproductive condition, and perhaps of reproductive success as well. Accordingly, in pectoral sandpipers, males showed inter-individual variation of the amount of activity during the breeding season and males that were most active and slept the least, sired the most offspring.<sup>21</sup>

Our results also challenge popular hypotheses for the physiological trigger of post-breeding male die-off common to semelparous dasyurids. The cause of male die-off has been attributed to (1) the fatal effects of elevated corticosteroids<sup>25–28</sup> and, recently, (2) sleep deprivation.<sup>35</sup> Elevated corticosteroids are caused by a progressive rise of testosterone during the breeding season.<sup>28</sup> Such elevations have been measured in small semelparous marsupials, including antechinus.<sup>25,26</sup> However, the northern quoll, a larger semelparous species that shows a complete male die-off after the mating period, does not show elevated corticosteroid levels.<sup>48</sup> Elevated corticosteroids promote protein catabolism via gluconeogenesis.<sup>49</sup> The breakdown of proteins (muscle loss)



**Figure 5. Inactivity is a proxy for sleep in dusky antechinus**

(A) Representative traces of brain activity (left [L] and right [R] hemisphere electroencephalogram [EEG]) and head movements (accelerometer [ACC]) along the three cardinal axes of sway (lateral), surge (anterior-posterior), and heave (dorsoventral axis) during wakefulness (orange bar) transitioning into slow-wave sleep (blue bar) and again into wakefulness. Trace duration is 120 s.

(B) Histogram showing the frequency of the time taken to transition from wakefulness to sleep; antechinus fall asleep within seconds after the cessation of movement. One value of 32 s was recorded but not shown in the figure.

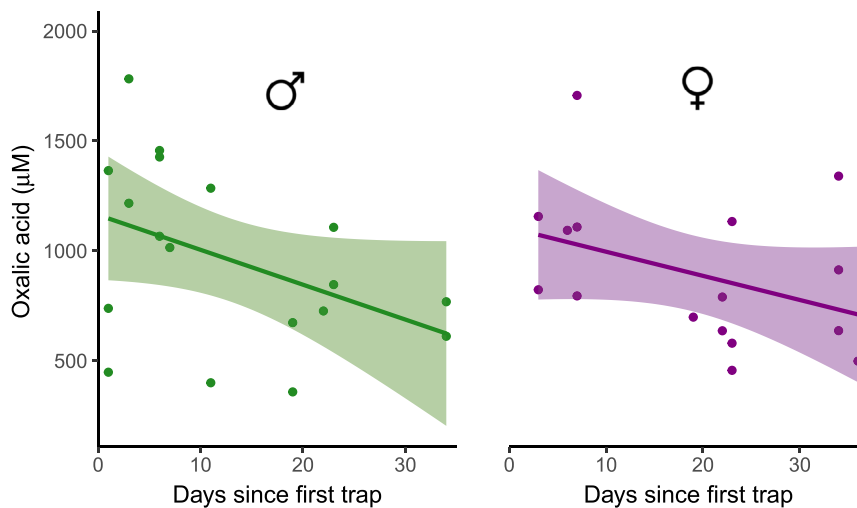
(C) Percentage of time spent awake or asleep scored using both the EEG and ACC (left) or the ACC alone (right); boxplots show the median activity levels (line within each box), the upper and lower quartiles (top and bottom edges, respectively), and minimum and maximum values (whiskers). Sleep scored using only the ACC was not different from that using the EEG.

Artwork by Damond Kyllö.

may allow small mammals, with a limited ability to store energy as fat, to sustain high waking performance. Therefore, instead of triggering male death, increased corticosteroids could be an adaptive advantage in small semelparous marsupials to sustain mating efforts.<sup>50</sup>

A second explanation for male die-off has been attributed to sleep deprivation.<sup>35</sup> Laboratory-bred rats chronically sleep deprived for weeks ultimately die.<sup>51</sup> The rats develop lesions

on their extremities and lose fur and body mass, despite actually eating more. Post-breeding antechinus, with skin lesions, superficially resemble these sleep-deprived rats. However, despite all males losing sleep during the breeding season, the magnitude of sleep lost was never as extreme as that for rats. Furthermore, although eight of our ten male dusky antechinus survived beyond the breeding season (such males become sterile<sup>52</sup>), two individuals died synchronously at the end of the breeding season, but



**Figure 6. Oxalic acid level of male and female wild agile antechinus over a 37-day period that includes the breeding season**

Lines represent fitted generalized linear model and points are values for individual animals. Shaded areas are 95% confidence intervals. The level of oxalic acid was not different between male and female agile antechinus ( $t = -0.26$ ,  $p = 0.797$ ), even while the mean decline of oxalic acid reached significance for males (green,  $n = 18$ ,  $p = 0.021$ ), but not females (purple,  $n = 16$ ,  $p = 0.353$ ).

these were not the ones that lost the most sleep. Thus, sleep loss is unlikely to be the cause of male die-off in semelparous dasyurids. Instead, there appears to be an unknown trigger from the natural environment as the cause of this synchronous “programmed” death.

In their single, short, and intense breeding season, male antechinus sacrifice sleep to maximize their fitness. The retention of some sleep suggests that sleep serves essential restorative functions. In the trade-off between the need to reproduce and the need to sleep, strong sexual selection has led to the evolution of costly morphological, physiological, and behavioral traits that enable males to maintain high performance on less sleep. These results build on growing evidence for remarkable and adaptive plasticity in the amount and timing of sleep across a diversity of species, contributing to the ongoing debate over the functions of sleep and its relationship to health and longevity in animals and humans.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **METHOD DETAILS**
  - Ethics statement
  - Model species and their natural habitat
  - Antechinus trapping
  - Experimental set-up
  - Dusky antechinus naturalistic enclosures and husbandry
  - Measurements of daily activity pattern in dusky antechinus
  - Inactivity as proxy of sleep

- Blood sampling and measurement of oxalic acid and testosterone
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2023.12.064>.

## ACKNOWLEDGMENTS

We would like to acknowledge Dr Jack Pascoe, Shayne Neal, and the entire team at the Conservation Ecology Centre for space and help during the months of field work in Cape Otway. We would like to thank our volunteers for helping in the field, especially Brayden Redwood, Dylan Lee, Owen Lishmund, Brook Zanco, Jamie Willey, Lita Trimmings, Francesca Leonard, Morgan Humphrey, Rebecca New, and Saul Avery. We also thank Jan Bruggink for help with the assays, and Carlo Catoni for technical support with the accelerometers.

This research was funded by the Australian Research Council Discovery Early Career Researcher Award (DE140101075, J.A.L.), Australian Research Council Discovery Project (DP170101003, J.A.L.), Groningen Institute for Evolutionary Life Sciences (P.M.), and La Trobe University Department of Environment, Ecology and Evolution (E.Z.).

## AUTHOR CONTRIBUTIONS

Conceptualization, E.Z. and J.A.L.; methodology, E.Z., J.A.L., P.M., and A.L.V.; investigation, E.Z., F.W.R., and R.D.J.; writing – original draft, E.Z.; writing – review and editing, E.Z., J.A.L., P.M., F.W.R., M.V., R.D.J., and ALV; visualization, E.Z., J.A.L., F.W.R., and M.V.; supervision: J.A.L. and P.M.; project administration: E.Z. and J.A.L.; funding acquisition: E.Z., J.A.L., and P.M.

## DECLARATION OF INTERESTS

Authors declare no competing interests.

Received: September 28, 2023

Revised: November 16, 2023

Accepted: December 19, 2023

Published: January 25, 2024



REFERENCES

- Guzman-Marin, R., Suntsova, N., Methippara, M., Greiffenstein, R., Szymusiak, R., and McGinty, D. (2005). Sleep deprivation suppresses neurogenesis in the adult hippocampus of rats. *Eur. J. Neurosci.* *22*, 2111–2116.
- Meerlo, P., Mistlberger, R.E., Jacobs, B.L., Heller, H.C., and McGinty, D. (2009). New neurons in the adult brain: the role of sleep and consequences of sleep loss. *Sleep Med. Rev.* *13*, 187–194.
- Tononi, G., and Cirelli, C. (2014). Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. *Neuron* *81*, 12–34.
- Diekelmann, S., Wilhelm, I., and Born, J. (2009). The whats and whens of sleep-dependent memory consolidation. *Sleep Med. Rev.* *13*, 309–321.
- Raven, F., Van der Zee, E.A., Meerlo, P., and Havekes, R. (2018). The role of sleep in regulating structural plasticity and synaptic strength: implications for memory and cognitive function. *Sleep Med. Rev.* *39*, 3–11.
- Xie, L., Kang, H., Xu, Q., Chen, M.J., Liao, Y., Thiyagarajan, M., O'Donnell, J., Christensen, D.J., Nicholson, C., Liff, J.J., et al. (2013). Sleep drives metabolite clearance from the adult brain. *Science* *342*, 373–377.
- Lesku, J.A., and Schmidt, M.H. (2022). Energetic costs and benefits of sleep. *Curr. Biol.* *32*, R656–R661.
- Imeri, L., and Opp, M.R. (2009). How (and why) the immune system makes us sleep. *Nat. Rev. Neurosci.* *10*, 199–210.
- Siegel, J.M. (2009). Sleep viewed as a state of adaptive inactivity. *Nat. Rev. Neurosci.* *10*, 747–753.
- Klein, B.A., Klein, A., Wray, M.K., Mueller, U.G., and Seeley, T.D. (2010). Sleep deprivation impairs precision of waggle dance signalling in honey bees. *Proc. Natl. Acad. Sci. USA* *107*, 22705–22709.
- Kreutzmann, J.C., Havekes, R., Abel, T., and Meerlo, P. (2015). Sleep deprivation and hippocampal vulnerability: changes in neuronal plasticity, neurogenesis and cognitive function. *Neuroscience* *309*, 173–190.
- Johnsson, R.D., Connelly, F., Gaviraghi Mussoi, J., Vyssotski, A.L., Cain, K.E., Roth, T.C., and Lesku, J.A. (2022). Sleep loss impairs cognitive performance and alters song output in Australian magpies. *Sci. Rep.* *12*, 6645.
- Nath, R.D., Bedbrook, C.N., Abrams, M.J., Basinger, T., Bois, J.S., Prober, D.A., Sternberg, P.W., Gradinaru, V., and Goentoro, L. (2017). The jellyfish *Cassiopea* exhibits a sleep-like state. *Curr. Biol.* *27*, 2984–2990.e3.
- Rattenborg, N.C., and Ungurean, G. (2023). The evolution and diversification of sleep. *Trends Ecol. Evol.* *38*, 156–170.
- Lima, S.L., Rattenborg, N.C., Lesku, J.A., and Amlaner, C.J. (2005). Sleeping under the risk of predation. *Anim. Behav.* *70*, 723–736.
- Lesku, J.A., and Rattenborg, N.C. (2022). The missing cost of ecological sleep loss. *Sleep Adv.* *3*, zpac036.
- Gravett, N., Bhagwandin, A., Sutcliffe, R., Landen, K., Chase, M.J., Lyamin, O.I., Siegel, J.M., and Manger, P.R. (2017). Inactivity/sleep in two wild free-roaming African elephant matriarchs – Does large body size make elephants the shortest mammalian sleepers? *PLoS One* *12*, e0171903.
- Kendall-Bar, J.M., Williams, T.M., Mukherji, R., Lozano, D.A., Pitman, J.K., Holser, R.R., Keates, T., Beltran, R.S., Robinson, P.W., Crocker, D.E., et al. (2023). Brain activity of diving seals reveals short sleep cycles at depth. *Science* *380*, 260–265.
- Rattenborg, N.C., Voirin, B., Cruz, S.M., Tisdale, R., Dell'Omo, G., Lipp, H.P., Wikelski, M., and Vyssotski, A.L. (2016). Evidence that birds sleep in mid-flight. *Nat. Commun.* *7*, 12468.
- Weimerskirch, H., Bishop, C., Jeanniard-du-Dot, T., Prudor, A., and Sachs, G. (2016). Frigate birds track atmospheric conditions over months-long transoceanic flights. *Science* *353*, 74–78.
- Lesku, J.A., Rattenborg, N.C., Valcu, M., Vyssotski, A.L., Kuhn, S., Kuemmeth, F., Heidrich, W., and Kempenaers, B. (2012). Adaptive sleep loss in polygynous pectoral sandpipers. *Science* *337*, 1654–1658.
- Bonnet, X. (2011). The evolution of semelparity. In *Reproductive Biology and Phylogeny of Snakes*, R.D. Aldridge, and D.M. Sever, eds. (Science Publishers Inc.), pp. 645–672.
- Nowak, R.M. (2018). Order Dasyuromorphia. In *Walker's Mammals of the World: Monotremes, Marsupials, Afrotherians, Xenarthrans, and Sundatherians* (Baltimore: Johns Hopkins University Press), pp. 111–177.
- Bradley, A.J. (2003). Stress, hormones and mortality in small carnivorous marsupials. In *Predators with Pouches: The Biology of Carnivorous Marsupials*, M. Jones, C. Dickman, and M. Archer, eds. (CSIRO Publishing Melbourne), pp. 254–267.
- Bradley, A.J., McDonald, I.R., and Lee, A.K. (1980). Stress and mortality in a small marsupial (*Antechinus stuartii*). *Gen. Comp. Endocrinol.* *40*, 188–200.
- McDonald, I.R., Lee, A.K., Than, K.A., and Martin, R.W. (1986). Failure of glucocorticoid feedback in males of a population of small marsupials (*Antechinus swainsonii*) during the period of mating. *J. Endocrinol.* *108*, 63–68.
- Lee, A.K., Bradley, A.J., and Braithwaite, R.W. (1977). Corticosteroid levels and male mortality in *Antechinus stuartii*. In *The Biology of Marsupials*, B. Stonehouse, and D. Gilmore, eds. (Palgrave), pp. 209–220.
- Naylor, R., Richardson, S.J., and McAllan, B.M. (2008). Boom and bust: a review of the physiology of the marsupial genus *Antechinus*. *J. Comp. Physiol. B* *178*, 545–562.
- Weljie, A.M., Meerlo, P., Goel, N., Sengupta, A., Kayser, M.S., Abel, T., Birnbaum, M.J., Dinges, D.F., and Sehgal, A. (2015). Oxalic acid and diacylglycerol 36:3 are cross-species markers of sleep debt. *Proc. Natl. Acad. Sci. USA* *112*, 2569–2574.
- Zaid, E., Vyssotski, A.L., and Lesku, J.A. (2022). Sleep architecture and regulation of male dusky antechinus, an Australian marsupial. *Sleep* *45*, zsc114.
- Ruckebusch, Y. (1972). The relevance of drowsiness in the circadian cycle of farm animals. *Anim. Behav.* *20*, 637–643.
- Lyamin, O.I., Manger, P.R., Ridgway, S.H., Mukhametov, L.M., and Siegel, J.M. (2008). Cetacean sleep: an unusual form of mammalian sleep. *Neurosci. Biobehav. Rev.* *32*, 1451–1484.
- Lesku, J.A., Roth, T.C., Rattenborg, N.C., Amlaner, C.J., and Lima, S.L. (2008). Phylogenetics and the correlates of mammalian sleep: a reappraisal. *Sleep Med. Rev.* *12*, 229–244.
- Körtner, G., and Geiser, F. (1995). Body temperature rhythms and activity in reproductive antechinus (Marsupialia). *Physiol. Behav.* *58*, 31–36.
- Gaschk, J.L., Del Simone, K., Wilson, R.S., and Clemente, C.J. (2023). Resting disparity in quoll semelparity: examining the sex-linked behaviours of wild roaming northern quolls (*Dasyurus hallucatus*) during breeding season. *R. Soc. Open Sci.* *10*, 221180.
- Ouyang, J.Q., de Jong, M., van Grunsven, R.H.A., Matson, K.D., Haussmann, M.F., Meerlo, P., Visser, M.E., and Spoelstra, K. (2017). Restless roosts: light pollution affects behaviour, sleep, and physiology in a free-living songbird. *Glob. Chang. Biol.* *23*, 4987–4994.
- Raap, T., Pinxten, R., and Eens, M. (2018). Artificial light at night causes an unexpected increase in oxalate in developing male songbirds. *Conserv. Physiol.* *6*, coy005.
- Ulgezen, Z.N., Käpylä, T., Meerlo, P., Spoelstra, K., Visser, M.E., and Dominoni, D.M. (2019). The preference and costs of sleeping under light at night in forest and urban great tits. *Proc. Biol. Sci.* *286*, 20190872.
- Malungo, I.B., Gravett, N., Ganswindt, A., and Manger, P.R. (2023). Male blue wildebeest increase activity during the rut, but not at the expense of rest. *J. Comp. Physiol. B*, 1–14.
- Lee, A., Woolley, P., and Braithwaite, R. (1982). Life history strategies of dasyurid marsupials. In *Carnivorous Marsupials*, M. Archer, ed. (Royal Society of NSW), pp. 1–11.
- Ralls, K., and Mesnick, S. (2009). Sexual Dimorphism. In *Encyclopedia of Marine Mammals*, W.F. Perrin, B. Würsig, and J.G.M. Theewissen, eds. (Academic Press), pp. 1005–1011.

42. Jardí, F., Laurent, M.R., Kim, N., Khalil, R., De Bundel, D., Van Eeckhaut, A., Van Helleputte, L., Deboel, L., Dubois, V., Schollaert, D., and Decallonne, B. (2018). Testosterone boosts physical activity in male mice via dopaminergic pathways. *Sci. Rep.* **8**, 1–14.
43. Gordon, T.P., Rose, R.M., Grady, C.L., and Berstein, I.S. (1979). Effects of increased testosterone secretion on the behaviour of adult male rhesus living in a social group. *Folia Primatol.* **32**, 149–160.
44. Perrot-Sinal, T.S., Innes, D., Kavaliers, M., and Ossenkopp, K.P. (1998). Plasma testosterone levels are related to various aspects of locomotor activity in wild-caught male meadow voles (*Microtus pennsylvanicus*). *Physiol. Behav.* **64**, 31–36.
45. McDonald, P.G., Buttemer, W.A., and Astheimer, L.B. (2001). The influence of testosterone on territorial defence and parental behaviour in male free-living rufous whistlers, *Pachycephala rufiventris*. *Horm. Behav.* **39**, 185–194.
46. Vitousek, M.N., Rubenstein, D.R., Nelson, K.N., and Wikelski, M. (2008). Are hotshots always hot? A longitudinal study of hormones, behaviour, and reproductive success in male marine iguanas. *Gen. Comp. Endocrinol.* **157**, 227–232.
47. Preston, B.T., Stevenson, I.R., Lincoln, G.A., Monfort, S.L., Pilkington, J.G., and Wilson, K. (2012). Testes size, testosterone production and reproductive behaviour in a natural mammalian mating system. *J. Anim. Ecol.* **81**, 296–305.
48. Oakwood, M., Bradley, A.J., and Cockburn, A. (2001). Semelparity in a large marsupial. *Proc. Biol. Sci.* **268**, 407–411.
49. Romero, M.L., and Butler, L.K. (2007). Endocrinology of stress. *Int. J. Comp. Psychol.* **20**, 89–95.
50. Lee, A.K., and Cockburn, A. (1985). *Evolutionary Ecology of Marsupials* (Cambridge University Press).
51. Everson, C.A., Bergmann, B.M., and Rechtschaffen, A. (1989). Sleep deprivation in the rat: III. Total sleep deprivation. *Sleep* **12**, 13–21.
52. Woolley, P.A. (1966). *Reproductive biology of Antechinus stuartii* Macleay (Marsupialia: Dasyuridae) (The Australian National University).
53. Allocca, G., Ma, S., Martelli, D., Cerri, M., Del Vecchio, F., Bastianini, S., Zoccoli, G., Amici, R., Morairty, S.R., Aulsebrook, A.E., et al. (2019). Validation of ‘Somnivore’, a machine learning algorithm for automated scoring and analysis of polysomnography data. *Front. Neurosci.* **13**, 207.
54. Brooks, M., Kristensen, K., Benthem, K., Magnusson, A., Berg, C., Nielsen, A., Skaug, H., Mächler, M., and Bolker, B. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modelling. *R J.* **9**, 378–400.
55. Ermer, T., Nazzari, L., Tio, M.C., Waikar, S., Aronson, P.S., and Knauf, F. (2023). Oxalate homeostasis. *Nat. Rev. Nephrol.* **19**, 123–138.
56. Book, A.S., Starzyk, K.B., and Quinsey, V.L. (2001). The relationship between testosterone and aggression: a meta-analysis. *Aggress. Violent Behav.* **6**, 579–599.
57. Pinheiro, J.C., and Bates, D.M. (2000). *Mixed-effects models in S and S-PLUS* (Springer).

## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Biological samples</b>		
Dusky antechinus urine samples	Collected from wild animals	N/A
Dusky antechinus blood samples	Collected from wild animals	N/A
Agile antechinus blood samples	Collected from wild animals	N/A
<b>Critical commercial assays</b>		
Oxalic acid colorimetric assay kit	BioVision Inc., USA	<a href="https://www.biovision.com/">https://www.biovision.com/</a>
Enzyme-linked immunosorbent assay (ELISA) kit for detection of testosterone	MP Biomedicals Germany GmbH, Germany	<a href="https://www.mpbio.com">https://www.mpbio.com</a>
<b>Deposited data</b>		
Analyzed data	This paper	Available on request
<b>Experimental models: Organisms/strains</b>		
Dusky antechinus ( <i>Antechinus swainsonii</i> )	Wild-caught	N/A
Agile antechinus ( <i>Antechinus agilis</i> )	Wild-caught	N/A
<b>Software and algorithms</b>		
Axy4 – Axy manager	Techno Smart, Italy	<a href="https://www.technosmart.eu">https://www.technosmart.eu</a>
RemLogic 3.4.4	Embla Systems, USA	<a href="https://neuro.natus.com/products-services/embla-remlogic-software">https://neuro.natus.com/products-services/embla-remlogic-software</a>
Delimit	Delimit Software Inc., Canada	<a href="https://delimitsoftware.com/index.html">https://delimitsoftware.com/index.html</a>
Machine-learning algorithm Somnivre™	Allocca et al. <sup>53</sup>	<a href="https://doi.org/10.17632/tzjkyrvh.1">https://doi.org/10.17632/tzjkyrvh.1</a>
Neurologger 2A downloader	Evolocus, USA	<a href="https://www.vyssotski.ch/neurologger2.html">https://www.vyssotski.ch/neurologger2.html</a>
R Studio version 1.4.1	R Development Core Team 2013, Austria	<a href="https://www.R-project.org/">https://www.R-project.org/</a>
glmmTMB	Brooks et al. <sup>54</sup>	<a href="https://CRAN.R-project.org/package=glmmTMB">https://CRAN.R-project.org/package=glmmTMB</a>
<b>Other</b>		
Neurologger 2A	Evolocus, USA	<a href="https://www.vyssotski.ch/neurologger2.html">https://www.vyssotski.ch/neurologger2.html</a>

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Erika Zaid ([erikazaid@gmail.com](mailto:erikazaid@gmail.com)).

#### Materials availability

This study did not generate new unique reagents.

#### Data and code availability

All data reported in this paper will be shared by the [lead contact](#) upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

### METHOD DETAILS

#### Ethics statement

This study was approved by the La Trobe University Animal Ethics Committee (AEC 16-54) and the Victoria Government Department of Environment, Land, Water and Planning and National Parks (Permit n. 10007988).

### Model species and their natural habitat

The study was conducted on two species of dasyurids: dusky and agile antechinus. Animals were trapped and studied in the Great Otway National Park (38°45'S, 143°32'E) in the south-west of Victoria, Australia. The topography within the study area alternates between steep slopes and wet gullies. On the slopes, mountain ash (*Eucalyptus regnans*), mountain grey gum (*E. cypellocarpa*) and messmate stringybark (*Eucalyptus obliqua*) establish a tall canopy (40–60 m), with myrtle beech (*Northofagus cunninghamii*) and blackwood (*Acacia melanoxylon*) in the gullies. The midstorey consists of a variety of small trees and shrubs including *Acacia* spp., hazel pomaderris (*Pomaderris aspera*), sweet bursaria (*Bursaria spinosa*) and an abundant fern flora dominated by tree ferns (*Cyathea australis*, *Dicksonia antarctica*). This complex, multitiered vegetation characterise suitable habitat for dusky and agile antechinus.

### Antechinus trapping

Antechinus were trapped using Elliott traps (10 × 10 × 30 cm) baited with a mixture of rolled oats, peanut butter, honey and bacon. Dry leaves or impermeable cotton were added for insulation; plastic bags were fitted around traps to ensure captured animals remained dry. Dusky antechinus were trapped at four different localities. Between 30 and 150 traps, placed at 5 m intervals were set in the morning and checked twice a day at dawn and dusk. Traps were left onsite for a maximum of four consecutive nights and then were moved to a different location. Agile antechinus were trapped at three different localities using Elliott traps. Between 20 and 40 traps, placed at 5 m intervals were set in the afternoon and collected the following morning. Traps then were moved to a different location in the same locality for an additional night if the number of animals trapped were not sufficient. Traps in which native rodents (*Rattus fuscipes* and *Mastacomys fuscus*) were captured were replaced with clean traps and repositioned. Captured antechinus were examined to confirm species and to determine sex and weight. Agile antechinus were handled immediately on site and released, but dusky antechinus were transported to the outdoor enclosures located at the Conservation Ecology Centre, Cape Otway (38°49'S, 143°31'E) for study.

### Experimental set-up

The study on dusky antechinus was conducted during austral winter in 2017 and 2018. A total of 15 animals (10 males) were trapped between May and June and their daily activity pattern was measured continuously before and during the breeding season. Starting two weeks after capture, a blood sample was taken every (*circa*) 15 days for physiological analysis. At the same time, we recorded mass, male testis size (length and width), female pouch appearance, and signs of fur loss, parasite load and skin lacerations. To determine the timing of reproduction, a urine sample was collected twice a week from each individual and examined to look for changes in the number of cornified epithelial cells, which are indicative of estrus cycling in females, or an increase in the number of sperm in males. The study on agile antechinus was conducted between July and September 2018. A total of 38 (20 males) animals were trapped in the wild, a blood sample was taken at the site of trapping and each animal was released shortly thereafter.

### Dusky antechinus naturalistic enclosures and husbandry

Each animal was individually housed outdoors in Cape Otway in a circular, polyethylene enclosure (diameter = 200 cm, height = 70 cm). Each enclosure was fitted with a sturdy metal mesh roof and the floor was covered with 10 cm of sand, a dense cover of vegetation, leaf litter, natural debris and logs as enrichment materials in an attempt to best simulate their natural environment. Twice a week a little amount of excrements was moved between enclosures to provide sensory cues of conspecific. Each animal had a wooden nest box (30 × 19 × 22 cm) with dry eucalypt leaves as bedding material. Dry eucalyptus leaves were also scattered throughout the enclosure to allow animals to build their own nest. Clean water was provided *ad libitum* and food (Wombaroo Small Carnivore Mix, Wombaroo Food Products, South Australia, Australia) was provided every day in the afternoon. Wombaroo is a nutritionally balanced live-food substitute for carnivorous marsupials (75% powder 25% water). The amount of food provided was based on each individual's body weight and adjusted to ensure the right amount of food was always available. Additionally, two (annelid) earthworms, crickets (*Acheta domesticus*) or mealworms (*Tenebrio molitor*) were provided three times a week.

### Measurements of daily activity pattern in dusky antechinus

After two weeks in the outdoor enclosure, each individual was fitted with a small (19 × 10 × 7 mm), lightweight (2.5–2.8 g) accelerometer data logger that records body movements along the three cardinal axes at 10 Hz as well as body temperature (accelerometer models: Axy3 and Axy4, TechnoSmart spa, Italy). Accelerometer data loggers were secured to a collar made of a nylon safe-ty (tm) cable tie (118 mm × 3 mm) with superglue and a strip of electrical tape. To reduce the risk of abrasion, collars were covered with a layer of (smooth) heat-shrink tubing. Each data logger was waterproofed with transparent shrinking tube before it was secured to the collar (total weight 3 g). The time and date of logger activation and deactivation were recorded. The logger was replaced every 15–30 days or sooner if the collar had detached from the animal. This activity data was complemented with behavioural observations using a motion sensor camera (Reconyx HC500 hyperfire, Reconyx, Holmen, USA). Moreover, in the second field season, a miniature, digital and high definition camera with infrared night vision (AHD cameras) was placed in each nest box for at least 48 h to observe the animal.

### Inactivity as proxy of sleep

To test whether measures of activity and inactivity are reliable proxies of wakefulness and sleep, respectively, we used electrophysiological data obtained from our previous study on dusky antechinus.<sup>30</sup> We quantified the time each animal (n = 4) spent in quiet

wakefulness before falling asleep by measuring each transition from wakefulness to sleep. Furthermore, to quantify the potential discrepancy between estimates of sleep derived from combined EEG, EMG, and accelerometry (ACC) data and those derived from accelerometry data alone, we re-scored the baseline recording of each animal using the ACC alone (i.e., while hiding the EEG and EMG traces). Recordings were manually scored by the same observer (E.Z.) using RemLogic (RemLogic v. 3.4.4, Natus Neuro, USA). Dusky antechinus were individually housed in the laboratory under a naturalistic photoperiod in enclosure (129 × 85 × 43 cm high) with natural vegetation and ground litter. Animals had a surgery to implant EEG and EMG electrodes, with bioelectric signals recorded with the Neurologger 2A that included a head-mounted 3-dimensional accelerometer.<sup>30</sup>

### Blood sampling and measurement of oxalic acid and testosterone

We assessed two physiological components that could play an important role in relation to changes in activity levels and sleep patterns over the breeding season: oxalic acid and testosterone. Oxalic acid (C<sub>2</sub>H<sub>2</sub>O<sub>4</sub>) is a strong dicarboxylic acid that accumulates in many plant tissues and plays a role in regulating pH, osmosis, and calcium storage. In animals, oxalate is either absorbed from dietary intake or produced from glycolate metabolism in the liver.<sup>55</sup> Oxalic acid has recently been proposed as a cross-species biomarker of sleep loss.<sup>29</sup> Testosterone is an androgen hormone. In non-human animals, testosterone causes an increase in aggressive and territorial behaviours, as demonstrated by correlational and experimental studies.<sup>56</sup> In antechinus, testosterone is thought to play an important role in the physiological cascade that leads to the death of all the males at the end of the breeding season.<sup>28</sup>

To measure oxalic acid from wild agile antechinus and testosterone from captive dusky antechinus, a blood sample (< 250 μL) was collected every two weeks by puncturing the saphenous vein. Blood was kept on ice, centrifuged at 1500 × g for 10 min within 4 h and then stored at –20°C during the field season (up to 2 months). Samples were then moved to a –80°C freezer until analysis at the University of Groningen, The Netherlands (2 months later). The level of oxalic acid in peripheral plasma was quantified from 3 μL of sample volume. Oxalic acid concentration was determined from a standard curve of oxalate (BioVision Inc., USA) and absorbance was measured at 450 nm with a spectrophotometer (ELISA reader). We ran two plates and repeated samples from the same individual were always analysed within the same plate. The mean intra-plate coefficient of variability (CV) was 7.24%. A control plasma sample with a known amount of oxalic acid was added to each plate. Testosterone concentrations were determined by using a solid phase enzyme-linked immunosorbent assay (ELISA) kit (MP Biomedicals Germany GmbH, Germany). Ten micro litres of each sample was added to individual wells on the assay plate alongside a standard curve with six points ranging from 0 ng/mL to 25 ng/mL and a control plasma sample with a known amount of testosterone. The samples were added randomly within and across plates but an individual's repeated samples were always included on the same plate. The plate was read at 450 nm on a multi-well spectrophotometer within 5 min the completion of the assay. The mean intra-plate CV was 8.08%.

### QUANTIFICATION AND STATISTICAL ANALYSIS

We assessed the presence of sexual dimorphism in body mass in both species. For dusky antechinus, we used a generalised linear mixed model (glmm) with Gaussian error distribution where the dependent variable was body mass and animal ID was a random intercept to account for repeated measurements taken of individuals; in agile antechinus we used a generalised linear model (glm) with body mass as the dependent variable. To quantify activity and inactivity for dusky antechinus, we used the supervised machine-learning algorithm Somnivore and scored data in 10-s epochs. Somnivore has been validated for use on humans, mice, rats, and other species.<sup>53</sup> We then tested whether there was a sex-specific change in activity from before to during the breeding season. For each individual, we calculated the percentage of time active for 4 days before, 4 days during, and 4 days after the breeding season. First, we compared pre-breeding activity levels between males and females using a glm. We then tested for differences between pre-, during, and post-breeding season activity levels separately for each sex using a glmm, with beta distribution and a logit link where the dependent variable was percentage of time active and animal ID was included as a random intercept. Finally, to test how this change was reflected in the daily pattern of activity we modelled the percentage of time active (in 30-min time bins) as a function of time period, sex and time of day (Table S1) using a glmm with a beta distribution and a logit link. A two-way interaction between the explanatory variables (sex, period and time of day) was included. The time of day, being a circular variable, was converted to radians before being included in the glmm. It was then incorporated into the model as the sine and cosine of time.<sup>57</sup> Animal ID was included as a random intercept. In the beta distribution glmm on percentage of time active, the time of day was included as a random slope. We also tested whether the change in activity in males can be predicted by change in testosterone over the breeding season using a glm with delta activity (difference in activity before and during the breeding season) as the dependent variable and delta testosterone (difference in testosterone level before and during the breeding season) as the explanatory variable. To determine whether activity alone is a good indicator of brain state we assessed whether EEG + ACC scoring was similar to that obtained using the ACC alone using a glm with activity as the dependent variable and a Gaussian error distribution. Finally, we tested whether a sex-dependent change in oxalic acid level occurred before and during the breeding season in agile antechinus. We used a glm with oxalic acid as a dependent variable and forest (i.e., trapping location) as a fixed factor to account for any potential difference between forests and breeding period. All analyses were performed in the R statistical environment (R Development Core Team 2013) and glmm models were fitted with glmmTMB.<sup>54</sup>