ORIGINAL ARTICLE

Inactivity Is Nycthemeral, Endogenously Generated, Homeostatically Regulated, and Melatonin Modulated in a Free-Living Platyhelminth Flatworm

Shauni Omond, BSc[1]; Linh M. T. Ly, BSc[1]; Russell Beaton[2]; Jonathan J. Storm, PhD[3]; Matthew W. Hale, PhD[2]; John A. Lesku, PhD[1]

[1]School of Life Sciences, La Trobe University, Melbourne 3086, Australia; [2]School of Psychology and Public Health, La Trobe University, Melbourne 3086, Australia; [3]Division of Natural Sciences & Engineering, University of South Carolina Upstate, Spartanburg 29301, SC

Introduction: Sleep either appeared once early in the evolution of animals, or at multiple instances over evolutionary time. Understanding whether sleep is a diagnostic trait for members of the kingdom Animalia has important implications for our understanding of the evolution of sleep and sleep functions. Unfortunately, knowledge on the phylogenetic breadth of sleep is restricted to vertebrates, a few arthropods and molluscs, and one species of nematode. There is a dearth of information on the other 30 or so animal phyla.

Aims and Methods: Here, we provide original data on a previously unstudied group of animals with respect to sleep: platyhelminth flatworms. These free-living animals are relatively simple, with a rudimentary central nervous system and absence of many other specialized physiological systems.

Results: Despite this simplicity, inactive flatworms appeared to be sleeping. Specifically, quiescence was organized in a circadian manner, occurring largely during the daytime. This basic rhythm persisted under constant darkness, suggesting that it was endogenously generated. Active flatworms responded more readily to stimulation, and flatworms recovered lost sleep by sleeping longer after a 3-hour period of inactivity deprivation. We were also able to increase inactivity in a dose-dependent manner with exposure to melatonin, a hormone that increases sleep in diurnal animals.

Conclusions: Taken together, these data expand our understanding of the phylogenetic extent of sleep and reinforce the idea that sleep evolved early in the evolutionary history of animals. However, additional studies on other types of animals are required for a comprehensive understanding of the origin(s) and evolution of sleep.

Keywords: evolution, function, *Girardia tigrina*, planarian, quiescence, sleep homeostasis, Turbellaria.

Statement of Significance

When did sleep first evolve? Did sleep appear multiple times? Do all animals sleep? Answers to these fundamental questions rely on an understanding of which animals sleep and identifying those that do not. Unfortunately, information on the presence (or absence) of sleep in the vast majority of animals, and even in the majority of animal "types" (or phyla), is lacking. Here, we present original data demonstrating the presence of sleep in platyhelminth flatworms using the accepted behavioral and pharmacological criteria for sleep. Although our results are consistent with the idea that sleep evolved early in the evolution of animals, much work remains on yet unstudied animal phyla.

INTRODUCTION

In most animals, sleep is a conspicuous behavioral state.[1,2] As a behavior, sleep is characterized by inactivity (or quiescence) that often occurs at certain times of the 24-hour day after the animal assumes a species-specific posture. Such nycthemeral organization reflects an endogenous circadian rhythm that is entrained by predictable changes in environmental factors,[3] including the day/night cycle.[4,5] While asleep, animals have decreased awareness of the local environment, such that the intensity of stimuli needed to evoke wakefulness (ie, the arousal threshold) is elevated. Unlike other quiescent states (eg, torpor or hibernation), sleep is rapidly reversible to wakefulness. Moreover, sleep is homeostatically regulated, meaning that (at least some) lost sleep may be recovered by sleeping longer and more deeply.[2] Finally, hypnotics and stimulants that promote sleep and wakefulness, respectively, in humans may be used to provide additional insight into whether immobility in other species reflects sleep or a different state altogether.[6–10]

All (or many) of these criteria have been tested on various animals, and all species studied have been found to sleep (Figure 1).[11,12] Most comparative studies of sleep have focussed on vertebrates, notably mammals[13–15] and birds,[16–19] and to a lesser extent, nonavian reptiles.[19–23] Larval and adult zebrafish (*Danio rerio*) sleep as well,[8,24] although little attention has been paid to fishes in general.[12] That said, there has long been an appreciation of the need for sleep studies in "simpler" animals.[13] Accordingly, early pioneering work on inactive invertebrates found equivalence between quiescence and sleep in various species of the phylum Arthropoda, including honey bees (*Apis mellifera*),[25] scorpions (*Heterometrus* and *Pandinus* spp.),[26] cockroaches (*Blaberus giganteus* and *Diploptera punctata*),[27,28] and more recently fruit flies (*Drosophila melanogaster*)[6,7,29] and crayfish (*Procambarus clarkia*).[30,31] Beyond arthropods, the presence of sleep has been demonstrated in at least four species of the phylum Mollusca: the common octopus (*Octopus vulgaris*),[32] common cuttlefish (*Sepia officinalis*),[33] great pond snail (*Lymnaea stagnalis*),[34] and California sea hare (*Aplysia californica*),[35] and also in the phylum Nematoda (*Caenorhabditis elegans*).[36] Preliminary data suggest that jellyfish (phylum Cnidaria) might also sleep,[37–39] although these early observations warrant further study.

Nevertheless, our understanding of the phylogenetic breadth of sleep remains far from complete (Figure 1). Indeed, of the approximately 1 million known arthropod species and 50,000 members of the Mollusca, convincing sleep-related data exist for only around one dozen species.[40] More generally, of the 36 recognized animal phyla, there is a complete absence of data for 31. Consequently, whether sleep evolved once early in the evolution of animals and has persisted—without exception—over evolutionary time or has evolved (and been secondarily lost) multiple times over the evolution of Animalia, are unresolved questions in comparative sleep research.

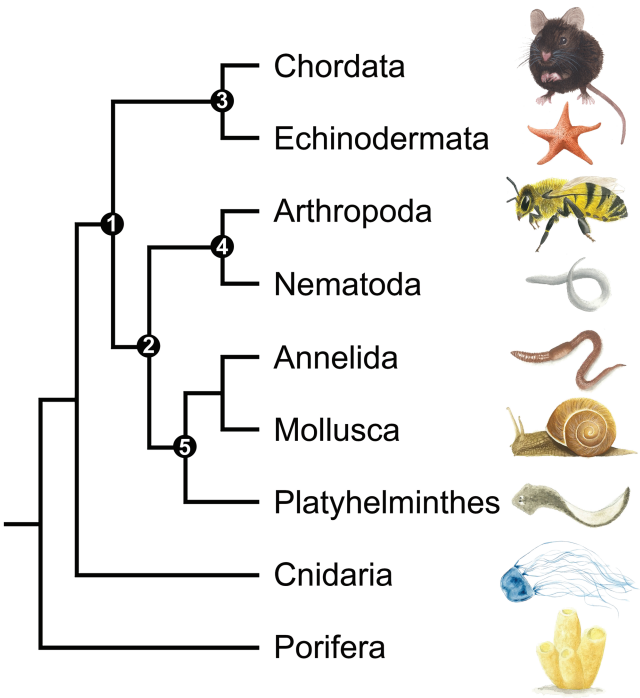

Figure 1—A simplified phylogenetic tree of the kingdom Animalia showing only the nine most species-rich phyla. Together, these phyla cover the majority of extant animals. Sleep has been demonstrated in at least one member of the Chordata (includes vertebrates), Arthropoda, Nematoda, and Mollusca. All other groups either lack sleep-related data entirely or have only preliminary evidence for sleep (Cnidaria). Numbered circles denote relevant evolutionary nodes. All descendants of (1) are members of the Bilateria (bilaterally symmetric animals), (2) protostomes, and (3) deuterostomes, (4) Ecdysozoans (molting animals), and (5) Spiralians. Evolutionary relationships were taken from Cannon et al.[43] Paintings reproduced, with permission, from Linh M. T. Ly.

Here, we provide original data on an unstudied animal phylum with respect to sleep: Platyhelminthes (flatworms). Flatworms are small, bilaterally symmetric, soft-bodied invertebrates that first appeared more than 800 million years ago.[41] They are "simple" in that they do not possess a central body cavity (coelom) nor do they have specialized circulatory or respiratory organs. Their digestive system (the gastrovascular cavity) contacts the environment through a single opening that serves both for the intake of food and excretion of waste. Their central nervous system is composed of a mass of cephalic ganglia in the head region and a pair of ventral nerve cords that extend the length of the body.[42] Two light-sensitive eyespots (ocelli) are located on the dorsal side of the head. It is noteworthy that from an evolutionary perspective, there are many different kinds of worm and not all are closely related. Some are members of the superphylum Ecdysozoa (Figure 1), which is composed of arthropods and also horsehair worms, priapulid worms, roundworms, and velvet worms. Others are of the Spiralian clade, a very diverse group of animals that includes bristle worms, earthworms, flatworms (this study), goblet worms, horseshoe worms, peanut worms, ribbon worms, tapeworms, and tubeworms, among other (nonworm) animals. Finally, there are (deuterostome) acorn worms, which are more closely related to vertebrates, including humans, than they are to any other (protostome) worm.[43] Consequently, the demonstration of the presence of sleep in nematode roundworms[36,44] does not mean that other kinds of worm necessarily also sleep nor does it negate the value of studying sleep in more distantly related worms, especially when there is a need for sleep data on most animal phyla.

METHODS

Free-living platyhelminth flatworms (*Girardia tigrina*) of the family Dugesiidae[45,46] were wild caught by Southern Biological (Melbourne, Australia) and kept in a temperature-controlled room maintained at 14 ± 1°C under a 12:12 light:dark (LD) photoperiod with lights off at 08:00 pm. Animals were group housed (20–30 individuals) in plastic dishes (170 × 125 × 70 mm high) containing commercially available spring water (40-mm deep). Flatworms were offered hard-boiled egg yolk for 120 minutes twice weekly; post feeding, animals were transferred to a container of clean spring water.

An activity recording system was custom designed and built for this study (Supplementary Figure 1). The frame of the system was an aluminum rectangular cuboid (805 × 600 × 600 mm high) with a wooden floor and ceiling. The top third of the two longer sides of the frame were aluminum panels. Three LED strip lights were affixed to these two sides to provide oblique, dim lighting (13 lux, comparable to twilight) to the testing area in the center of the floor; lights were computer controlled. Dim lighting was environmentally realistic as these flatworms inhabit turbid freshwater in nature. Within the frame was the infrastructure needed to video record flatworms during the day and night: a black-and-white video camera (A602f; Basler AG, Ahrensburg, Germany) and four infrared lights (950 nm), which were mounted equidistant from one another and from the camera in the center. This wavelength of light falls outside the visual range of flatworms.[47] Because the video camera was sensitive only to infrared light, the quality of lighting in the video files was unaffected by day/night changes in photoperiod. Finally, the testing area consisted of a white enamel bowl (150-mm diameter) positioned beneath the camera and set into a hole (144-mm diameter) in the plastic baseplate to ensure consistent positioning across trials. Two activity recording systems typically collected data concurrently and were used for all experiments. Flatworms were fed immediately before an experimental trial, unless stated otherwise.

Preliminary data revealed two main behavioral states: an active state where the body was elongated with the flatworm typically gliding forward, and an inactive state with the flatworm contracted and the animal immobile (Figure 2). An immobile flatworm could be transitioned into an active state with sufficient stimulation, including transferring them from one container to another. Exploring the temporal aspects of these behaviors formed the basis for our first experiments. Specifically, we measured the patterning of active and inactive states under a 12:12 LD photoperiod for 55 hours (n = 18 flatworms). Then, to determine whether this pattern persisted in the absence of photoperiodic cues, other flatworms were fed at 10:00 am (under normal lighting) and were then transferred to 0:24 dark:dark (DD) conditions at 12:30 pm; video recordings commenced at

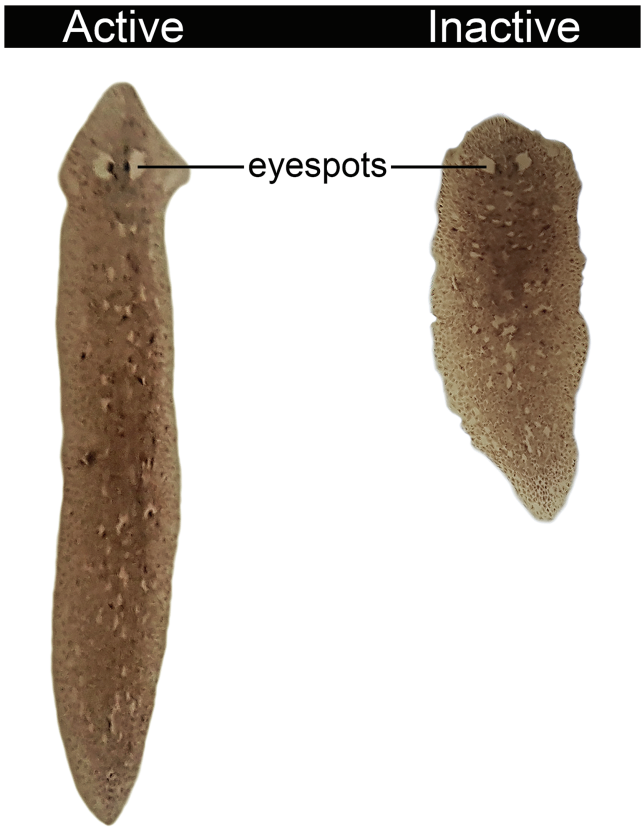

Figure 2—Photographs of our study species, *Girardia tigrina*, in its two states. An active animal glides on the substrate in exploratory behavior; while extended, they are approximately 10 mm in length. An inactive animal is contracted and immobile. Note the eyespots on the dorsal surface of the head. Photographs courtesy of Shauni Omond.

02:00 pm for the next 55 hours (n = 18). The activity recording system recorded a still image at the top of each minute. The resulting video was manually scored as "active" if the flatworm had moved in the intervening minute between adjacent stills and as "inactive" if it had not moved. Even modest movements, including those involving only parts of the animal (eg, lateral deflections of the head or tail) were counted as active.

Next, we sought to compare the responsiveness of active and inactive flatworms. Two eyespots on the dorsal surface of a flatworm's head are symmetrically located on either side of the midline (Figure 2). Flatworms are negatively phototaxic, meaning that they avoid light. Specifically, asymmetric light levels cause a flatworm to move in the direction of the lower light intensity.[48] We exploited this negative phototaxis and measured the latency to respond to an overhead light source. Flatworms were tested individually in a gray matte dish with 10 mL of spring water (n = 12) between 12:30 and 06:00 pm. Each flatworm was subjected to three trials in an active state and three in an inactive state (tested in a counter-balanced manner) with a minimum of 5 minutes between each trial. That is, a flatworm had to remain active or inactive for a full 5 minutes before proceeding to the next trial. Once this condition was met, an overhead light (ThruNite TN12, Shenzhen, China) shined down a tube of opaque paper and the time required for the flatworm to avoid the light (a lateral deflection) was recorded, up to a maximum of 30 seconds. Note, because negative phototaxis requires asymmetric light intensities to influence behavior, the head region (eyespots) of a flatworm could not be placed directly under the light. A pilot study showed that a flatworm tested in this manner would simply continue gliding straight through the light without any sign of deflection. Instead, the bright center of the light was focussed at the anterior-most end of the flatworm and a ring of dappled light haloed this point source. The eyespots were inside this more heterogeneous halo. The time taken between light on and a lateral deflection of the flatworm was recorded, such that an inactive animal that did not move or an active animal that continued through the beam of light, were scored as "no response."

However, these behavioral criteria alone are insufficient to determine whether some inactivity reflects sleep in flatworms. Immobility could reflect adaptive inactivity, while reduced responsiveness might suggest a lack of motivation to respond, rather than an increased arousal threshold. We conducted an experiment to determine whether this inactivity was homeostatically regulated (n = 16). For this experiment, we again used the activity recording system and a 12:12 LD photoperiod. Flatworms were individually pipetted into white enamel bowls (70-mm diameter) and an undisturbed (baseline) period of 33 hours was recorded (08:00 am–05:00 pm the following day). Starting at 05:00 pm on the second day, an inactivity deprivation (or forced locomotion) protocol commenced. This involved stimulating an immobile flatworm to move with combinations of (1) extracting and quickly expelling them back into the same (or different) bowl using a bulb pipette and (2) decanting the water out of the bowl with immediate replenishment. At 08:00 pm, the animals were able to behave freely and were recorded until 08:00 am the following day. Flatworm behavior in the resulting video was scored as "inactive," "active," or "stimulated" at the top of every second during the deprivation period and at the top of each minute for the baseline and recovery footage.

Finally, we also probed the underlying pharmacology modulating inactivity in flatworms using melatonin, a hormone that promotes sleep in diurnal animals.[8,49–51] Flatworms produce endogenous melatonin through the same biochemical pathways used by other animals.[52] Individual flatworms were placed into a bowl (70-mm diameter) for 40 minutes containing 20 mL of either: spring water (vehicle) or the vehicle with 0.1 µM, 1.0 µM, 10 µM, or 100 µM of melatonin (reconstituted from powder, Cat. No. M5250, Sigma-Aldrich Pty Ltd, Castle Hill, Australia) (n = 9 flatworms per treatment). As these dishes were much smaller than those used in other experiments, two or three flatworms were recorded simultaneously per system. Trials were conducted between 08:30 pm and 12:00 am. Video recordings were analyzed for total distance traveled (cm) using EthoVision XT 10 (Noldus Information Technology, Wageningen, The Netherlands).

Statistical Analyses

Data on the responsiveness of flatworms to an overhead light were not normally distributed, owing to a large number of nonresponses from inactive flatworms. Consequently, we used a nonparametric Wilcoxon signed ranks test to determine whether active flatworms responded more readily to the dappled halo of light than inactive animals. Data arising from the inactivity deprivation experiment

were analyzed using either paired *t* tests or simple linear regression, as specified below. Finally, the melatonin data were also non-normal, such that we used a nonparametric Kruskal-Wallis test to examine a main effect of concentration on flatworm activity, followed by post hoc Mann-Whitney *U* tests to determine which pairwise comparisons reached statistical significance.

RESULTS

We first sought to determine how activity and inactivity were organized over a 24-hour day. Flatworms were nocturnal, but there was a decrease in activity during the middle of the night (Figure 3, Supplementary Figure 2). This overall nycthemeral pattern appeared to be an endogenously generated circadian rhythm, as the basic pattern of decreased activity during the light phase persisted under constant darkness (Figure 3). Active flatworms reacted significantly more quickly (median response time: 7.9 ± 3.8 seconds) to a dappled halo of overhead light, relative to inactive flatworms, which did not respond (Wilcoxon signed ranks test: $Z = 2.371$, $p = .018$, Supplementary Table 1). This difference between the responses of active and inactive flatworms may reflect a difference in behavioral state (awake, asleep) or a difference in motivation to respond.

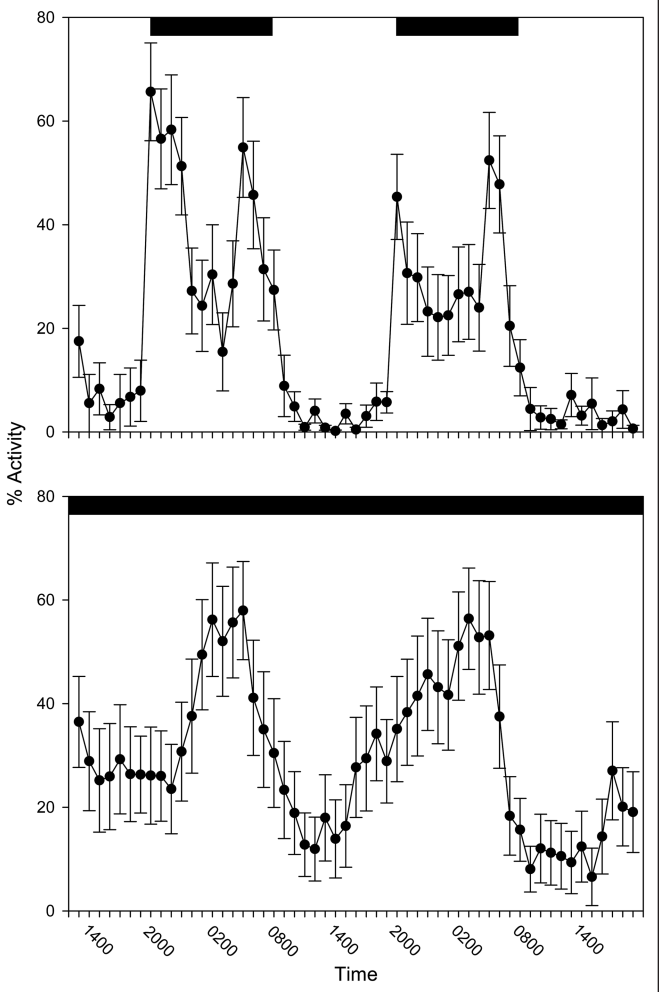

Figure 3—Flatworm activity patterns under a 12:12 light:dark photoperiod (top, *n* = 18) and constant darkness (bottom, *n* = 18). The percent time spent active (mean ± standard error of the mean) is plotted at the beginning of each hour. Black horizontal bars at the top of each plot denote the dark phase of the photoperiod.

To distinguish between these two possibilities, we then tested flatworms to see whether (1) inactivity was homeostatically regulated and (2) activity could be reduced in response to melatonin. Regarding the former, the flatworms experienced two undisturbed (baseline) days. Despite being significantly more active during the first hour on the first baseline day, these 2 days were broadly similar (Figure 4). At 05:00 pm on the second day, the flatworms were stimulated to move whenever they became quiescent. Immobile flatworms that were stimulated responded rapidly and switched to an active state. In practice, the amount of time spent encouraging a flatworm to move was low (minimum: 0.60% of the 3-hour treatment, maximum: 5.36%, mean ± standard error: 2.57 ± 0.09%) because a flatworm stimulated to move would continue to move for some time. By intermittently stimulating each flatworm, we were able to significantly increase the amount of activity relative to the same circadian time on the previous day (Figure 4). In contrast to the baseline night, when flatworms began their normal period of activity, flatworms that had been deprived of their daytime inactivity were significantly less active during the subsequent night when they were able to behave freely (Figure 4). The increase in quiescence observed after the 3-hour period of sustained locomotion, is unlikely to have arisen out of muscle fatigue given that the amount of activity induced during the treatment did not predict the amount of subsequent inactivity during either the entire recovery night (ß = −0.305, $p = .250$) or the first half of the recovery night (ß = −0.301, $p = .258$), when the magnitude

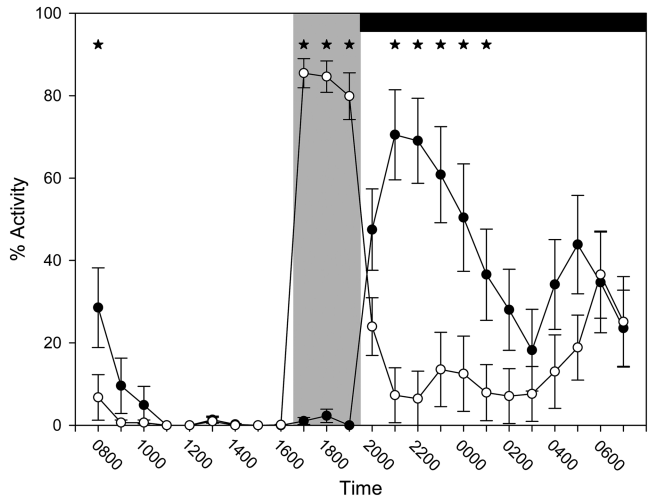

Figure 4—Effect of inactivity deprivation (or forced locomotion) on subsequent activity (*n* = 16). The first 24-hour period served as an undisturbed baseline (filled circles). The second 24-hour period (open circles) is divided into an additional 9-hour period of daytime baseline, prior to the start of inactivity deprivation (gray shading), followed by 12 hours of night-time recovery. The percent time spent active (mean ± standard error of the mean) is plotted at the beginning of each hour. A black horizontal bar at the top of the plot denotes the dark phase of the photoperiod. Stars reflect statistically significant differences between time points on the first and second 24-hour periods (paired *t* test, $p < .05$).

of the reduction in activity was most pronounced. Nor is the decrease in activity observed during the recovery night likely to be a response to stress induced during the inactivity deprivation procedure, as the amount of time stimulating flatworms during the treatment did not predict their level of inactivity during the first half of the recovery night (ß = 0.123, $p = .650$).

Finally, we exposed flatworms to solutions of varying concentrations of melatonin to see whether this hormone would decrease their level of activity. Accordingly, the total distance traveled by the flatworms depended on the concentration of melatonin (Kruskal-Wallis test: $\chi^2 = 13.688$, df = 4, $p = .008$). Specifically, we identified a *U*-shaped dose-response curve common in behavioral pharmacology (Figure 5). Activity was significantly lower when flatworms were exposed to 10 µM of melatonin relative to the vehicle-only group (Mann-Whitney *U* test: $U = 16.000$, $p = .031$); the reduction of activity observed at 1.0 µM of melatonin trended toward, but did not reach, significance ($U = 19.000$, $p = .058$).

DISCUSSION

Our results suggest that at least some of the inactivity observed in *G. tigrina* is sleep. Flatworms (1) engaged in spontaneous periods of quiescence, (2) typically accompanied with a contracted posture. (3) The temporal organization of sleep and wakefulness appeared to have been regulated (in part) by an endogenous circadian rhythm, as indicated by its persistence in the absence of photoperiodic cues. As in other animals, sleep in flatworms is (4) associated with an increased arousal threshold, yet is (5) rapidly reversible to an awake state and is (6) homeostatically regulated. Animals recovered sleep lost during the 3-hour deprivation by subsequently sleeping more. (7) Sleep can also be induced in a dose-dependent manner with exposure to melatonin. We elaborate on many of these points below.

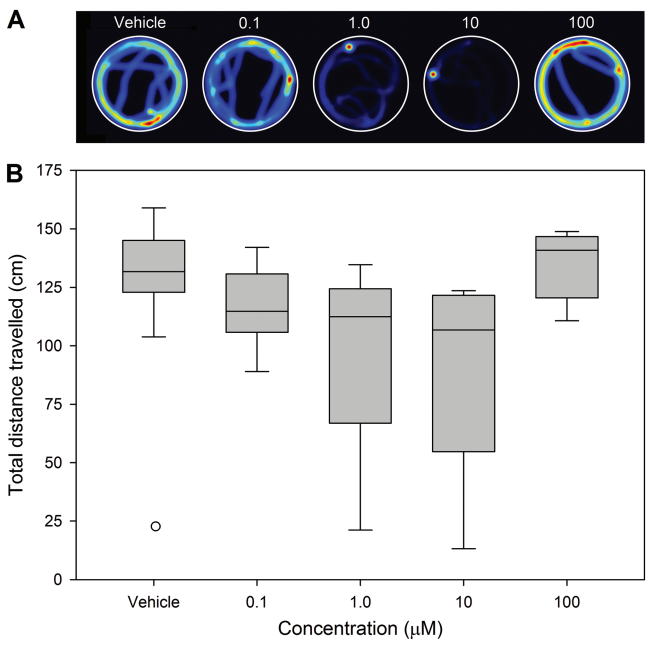

Figure 5—(A) Heat maps showing the representative response of individual flatworms to increasing concentrations of melatonin (µM). (B) Boxplot depicting group values of activity, measured as the total distance travelled in 40 minutes ($n = 9$ per concentration). The bottom and top edge of each box reflect first and third quartiles, respectively; the band inside the box is the median; the whiskers reflect minimum and maximum values; the single datapoint in the vehicle-only treatment is a statistical outlier, which was included in the statistical analyses.

The two main behavioral states (active and inactive) observed in flatworms were organized in a nycthemeral manner. While inactivity occurred predominantly during the day, flatworms showed a multihour decrease in activity during the middle of the dark phase of the photoperiod. This midnight siesta is similar to the (midday) siesta seen in male fruit flies.[53] In *Drosophila*, siestas might reflect a mechanism to reduce activity at a time of the day when conditions are not optimal for being active (eg, owing to increased temperature or decreased resource availability[54]). The adaptive significance of siesta in flatworms is unclear, but the siesta per se may arise from prior sleep-wake history. Specifically, the siesta was not observed under constant darkness, perhaps because the flatworms delayed the increase in activity during the subjective night, relative to the abrupt increase seen under the baseline photoperiod. Consequently, if some inactivity reflects sleep, then flatworms housed in the absence of photoperiodic cues might have had a lower sleep pressure in the first third of the (subjective) night brought on by a delayed termination of the major sleep period.[55] Nevertheless, the main pattern of behavioral organization (ie, less activity during the subjective day) persisted in the absence of environmental *zeitgebers*, intimating an endogenous circadian rhythm.

In addition to these temporal organization-related data, we tried to assess whether inactive flatworms were less likely to respond to stimulation than active animals. Indeed, this was the case: the latency to respond to dappled light was shorter in flatworms already active. However, from these data alone, it is unclear whether inactive flatworms failed to detect the light because they were sleeping or rather that they processed the light but lacked the motivation to respond. That said, flatworms could revert to an active state quickly with sufficient stimulation, such as gentle handling or decanting water from their bowl. Indeed, we were able to exploit this response to probe for a sleep homeostasis-like response following inactivity deprivation.

Animals deprived of sleep-related quiescence subsequently sleep longer and more deeply.[2] Such homeostatic regulation has become one of the most important criteria in the behavioral identification of sleep.[2] Sleep homeostasis has been demonstrated in fruit flies,[29] the common octopus,[32] and California sea hare,[35] among other invertebrates[13,55] and many vertebrates.[2,56] Here, we observed sleep homeostasis in the flatworm, *G. tigrina*. Flatworms stimulated to move continuously during the last 3 hours of the day, a time when flatworms were otherwise almost completely inactive, showed a decrease in activity over at least 5 hours when allowed to behave freely. From this, it may appear as though flatworms overcompensated for lost sleep. The 2-hour difference of (seemingly) unaccounted inactivity could be explained if some of the quiescence was quiet wakefulness. For instance, not all immobility in fruit flies reflects sleep,[57] and this may also be the case in flatworms. Moreover, in conjunction with sleep loss, the flatworms may have experienced a more intense (or neurologically demanding) form

of wakefulness during the stimulation protocol, which further increased sleep pressure.[58,59] Note, the increase in quiescence observed during the recovery night is unlikely to have been caused by either (1) muscle fatigue or (2) stress because neither the amount of activity induced during the (sleep) deprivation procedure nor the amount of time spent stimulating flatworms predicted the level of subsequent inactivity.

We also provided data suggesting that flatworm sleep can be modulated using the hormone melatonin. Increasing concentrations of melatonin caused a dose-dependent decrease in activity. Evidence that melatonin might be soporific in a nocturnal animal would be, to our knowledge, unprecedented. Indeed, melatonin in many invertebrates is thought to be only a time-keeping molecule cued by photoperiod[52,60] and not one that also induces sleep.[50] Additionally, melatonin has been shown to be sleep-promoting only in diurnal animals that have peak melatonin levels during their major sleep period.[50] Conversely, in nocturnal animals, including flatworms,[52] the rise in melatonin coincides with spontaneous activity.

Competing interpretations of our melatonin results might be dose-dependent poisoning or neuromuscular impairment or cooling effects of melatonin that reduce activity.[61] However, these are unlikely. Specifically, while there is a dose-dependent decrease in activity over most concentrations of melatonin, which could reflect a dose-dependant increase in toxicity, possibly impairing neuromuscular function, none of the flatworms died during the experiment and activity levels under the highest concentration were comparable to baseline. The *U*-shaped dosage curve we observed is typical of pharmacological experiments, including hormones,[62,63] although we are unaware of such a curve having been reported previously for melatonin. As such, it is difficult to explain the specific mechanism responsible for activity levels at the highest melatonin dose to be similar to that of the baseline. Next, cooling is also unlikely. Animals can only offload heat to the environment when there is a thermal gradient between the animal and the surrounding media, with the animal having the higher temperature. Flatworms are ectothermic, putting them at thermal equilibrium with the temperature-controlled water. Not only would there be no thermal gradient to offload heat, but the flatworms are also dorsoventrally flattened, being only a fraction of a millimeter thick. This exceptional thinness gives rise to a high-surface area:volume ratio. Consequently, flatworms would be hard pressed to maintain a thermal gradient should they have the metabolic machinery to create one. Ultimately, we are left with our original interpretation: melatonin induces sleep in these nocturnal, ectothermic invertebrates. At the very least, we hope that these results will encourage more studies on the effects of melatonin on the physiology and behavior of nocturnal, ectothermic invertebrates.[60]

Understanding the evolutionary history of sleep can provide unique insight into the origin(s) and functions of sleep. Our comprehensive results provide the first data for sleep in the phylum Platyhelminthes. This finding reinforces the view that sleep appeared early in the evolution of animals: with the appearance of bilaterally symmetric animals (node 1 on Figure 1) and possibly even earlier.[37–39] Thus, the evolutionary age of sleep is at least 800 million years old.[41] Resolving which (if any) animals do not sleep may reveal the biological target(s) benefiting from sleep. For example, if jellyfish (phylum Cnidaria) were demonstrated to sleep, as per the study by Bedbrook et al.,[39] then its hypothetical absence in sponges (Porifera) might suggest that the evolutionary appearance of sleep was tied to the appearance of the nervous system. Similarly, an absence of sleep in jellyfish might suggest that simple neural tissues[64] are insufficient for sleep to manifest. Instead, sleep may be an emergent property of a more complex nervous system.[44] Notwithstanding, many animal phyla remain unstudied with respect to sleep, and even within studied clades (eg, arthropods), few species have been represented. Until more species of greater phylogenetic diversity have been observed sleeping, the full potential of the comparative approach as applied to sleep will not be realized, and it will remain unclear whether all animals sleep or if sleep evolved multiple times.

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SUPPLEMENTARY MATERIAL

Supplementary material is available at *SLEEP* online.

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SUBMISSION & CORRESPONDENCE INFORMATION

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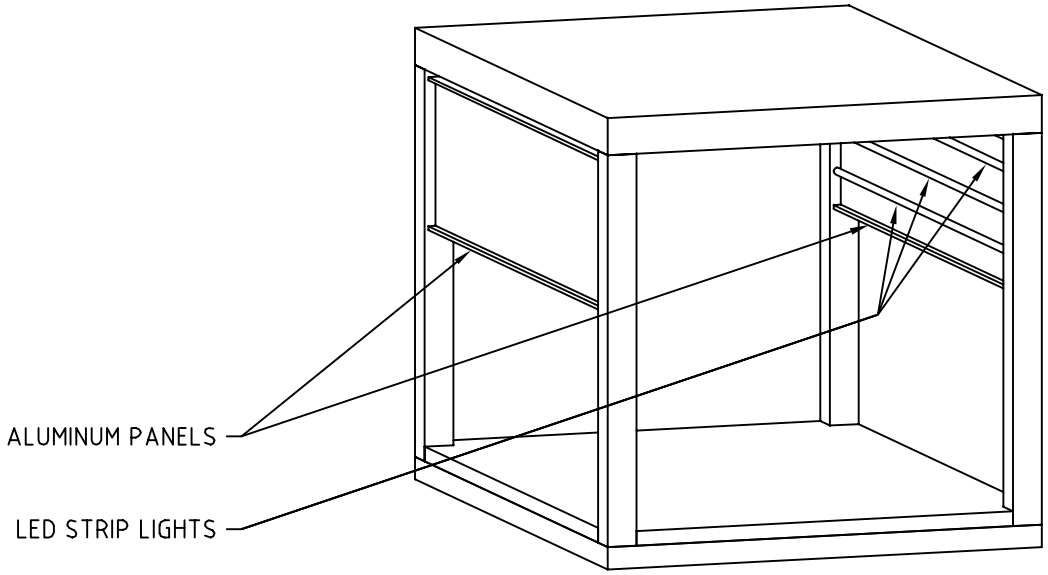
Address correspondence to: John A. Lesku, Kingsbury Drive, School of Life Sciences, La Trobe University, Melbourne 3086, Australia. Telephone: 61 (0)3 9479 1427; Fax: +61 (0)3 9479 1551; Email: j.lesku@latrobe.edu.au

DISCLOSURE STATEMENT

None declared.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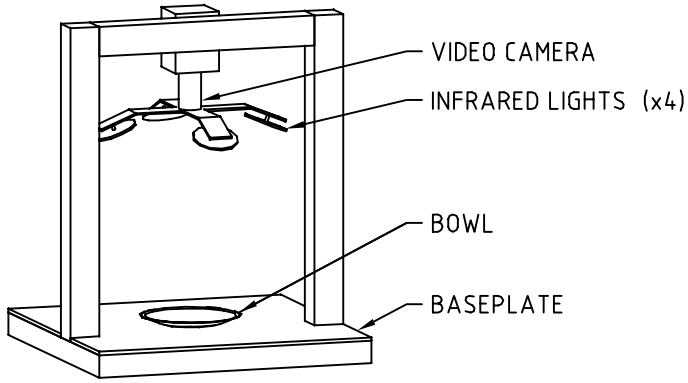
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BOWL
BASEPLATE

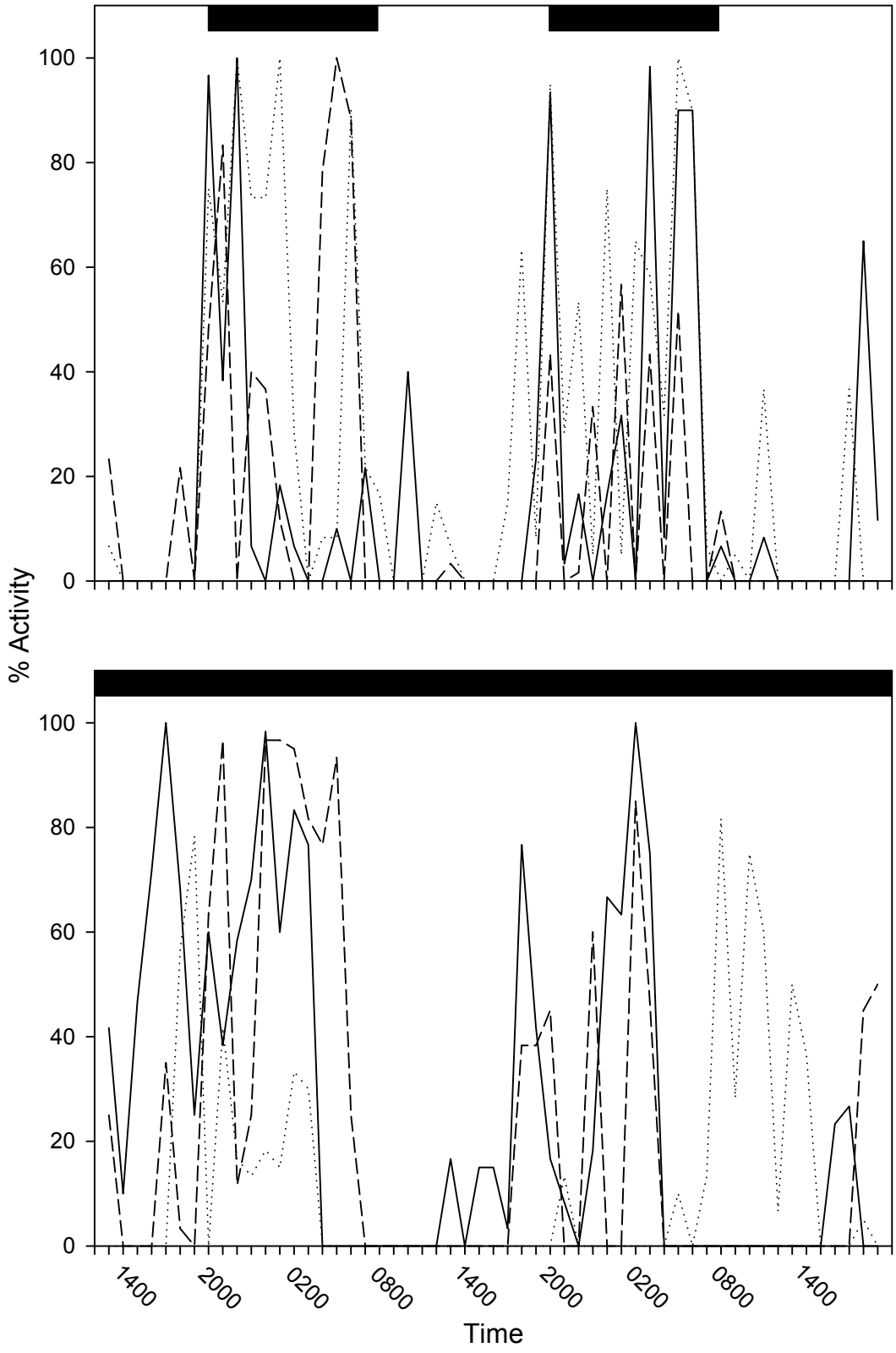
% Activity
100
80
60
40
20
0
1400
2000
0200
0800
1400
2000
0200
0800
1400
Time

Table S1. Raw data for the arousal threshold experiment.

Worm ID (1-12)	Trial (1-3)	State (Active, Inactive)	Response? (Yes, No)	Time to Respond (s) (Max.: 30; "No Response": 31 s)	Median Response Time (s) (Per flatworm across three trials)
1	1	Active	No	31.0	7.5
1	2	Active	Yes	0.2	.
1	3	Active	Yes	7.5	.
1	1	Inactive	No	31.0	31.0
1	2	Inactive	No	31.0	.
1	3	Inactive	No	31.0	.
2	1	Active	Yes	4.3	4.7
2	2	Active	Yes	5.2	.
2	3	Active	Yes	4.7	.
2	1	Inactive	No	31.0	31.0
2	2	Inactive	No	31.0	.
2	3	Inactive	No	31.0	.
3	1	Inactive	No	31.0	31.0
3	2	Inactive	No	31.0	.
3	3	Inactive	No	31.0	.
3	1	Active	No	31.0	31.0
3	2	Active	Yes	14.2	.
3	3	Active	No	31.0	.
4	1	Inactive	No	31.0	31.0
4	2	Inactive	No	31.0	.
4	3	Inactive	No	31.0	.
4	1	Active	No	31.0	4.0
4	2	Active	Yes	4.0	.
4	3	Active	Yes	2.8	.
5	1	Inactive	No	31.0	31.0
5	2	Inactive	No	31.0	.
5	3	Inactive	No	31.0	.
5	1	Active	Yes	8.3	8.3
5	2	Active	Yes	4.5	.
5	3	Active	Yes	11.5	.
6	1	Active	Yes	2.9	4.7
6	2	Active	Yes	4.7	.
6	3	Active	No	31.0	.
6	1	Inactive	No	31.0	31.0
6	2	Inactive	No	31.0	.
6	3	Inactive	No	31.0	.
7	1	Active	Yes	3.0	31.0
7	2	Active	No	31.0	.
7	3	Active	No	31.0	.
7	1	Inactive	No	31.0	31.0
7	2	Inactive	No	31.0	.
7	3	Inactive	No	31.0	.
8	1	Inactive	No	31.0	31.0
8	2	Inactive	No	31.0	.
8	3	Inactive	No	31.0	.
8	1	Active	No	31.0	5.7
8	2	Active	Yes	1.0	.
8	3	Active	Yes	5.7	.
9	1	Inactive	No	31.0	31.0
9	2	Inactive	No	31.0	.
9	3	Inactive	No	31.0	.

9	1	Active	Yes	1.6	31.0
9	2	Active	No	31.0	.
9	3	Active	No	31.0	.
10	1	Inactive	No	31.0	31.0
10	2	Inactive	No	31.0	.
10	3	Inactive	No	31.0	.
10	1	Active	No	31.0	31.0
10	2	Active	No	31.0	.
10	3	Active	No	31.0	.
11	1	Active	Yes	2.8	2.8
11	2	Active	Yes	5.2	.
11	3	Active	Yes	2.4	.
11	1	Inactive	No	31.0	31.0
11	2	Inactive	No	31.0	.
11	3	Inactive	No	31.0	.
12	1	Active	No	31.0	31.0
12	2	Active	No	31.0	.
12	3	Active	No	31.0	.
12	1	Inactive	No	31.0	31.0
12	2	Inactive	No	31.0	.
12	3	Inactive	No	31.0	.