**Brain, Behavior and Evolution**

# Sleep-Related Electrophysiology and Behavior of Tinamous (*Eudromia elegans*): Tinamous Do Not Sleep Like Ostriches

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**Abstract**

The functions of slow wave sleep (SWS) and rapid eye movement (REM) sleep, distinct sleep substates present in both mammals and birds, remain unresolved. One approach to gaining insight into their function is to trace the evolution of these states through examining sleep in as many taxonomic groups as possible. The mammalian and avian clades are each composed of two extant groups, i.e., the monotremes (echidna and platypus) and therian (marsupial and eutherian [or placental]) mammals, and Palaeognaths (cassowaries, emus, kiwi, ostriches, rheas, and tinamous) and Neognaths (all other birds) among birds. Previous electrophysiological studies of monotremes and ostriches have identified a unique "mixed" sleep state combining features of SWS and REM sleep unlike the well-delineated sleep states observed in all therian mammals and Neognath birds. In the platypus this state is characterized by periods of REM sleep-related myoclonic twitching, relaxed skeletal musculature, and rapid eye movements, occurring in conjunction with SWS-related slow waves in the forebrain electroencephalogram (EEG). A similar mixed state was also observed in ostriches; although in addition to occurring during periods with EEG slow waves, reduced muscle tone and rapid eye movements also occurred in conjunction with EEG activation, a pattern typical of REM sleep in Neognath birds. Collectively, these studies suggested that REM sleep occurring exclusively as an integrated state with forebrain activation might have evolved independently in the therian and Neognath lineages. To test this hypothesis, we examined sleep in the elegant crested tinamou (*Eudromia elegans*), a small Palaeognath bird that more closely resembles Neognath birds in size and their ability to fly. A 24-h period was scored for sleep state based on electrophysiology and behavior. Unlike ostriches, but like all of the Neognath birds examined, all indicators of REM sleep usually occurred in conjunction with forebrain activation in tinamous. The absence of a mixed REM sleep state in tinamous calls into question the idea that this state is primitive among Palaeognath birds and therefore birds in general.

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## Introduction

Even though all organisms studied devote a large amount of time to sleep [Cirelli and Tononi, 2008], the function of this state remains actively debated. The failure to reach a consensus on "the" function of sleep may indicate that sleep serves more than one purpose. Once sleep evolved to perform an initial task, it likely took on many other functions over evolutionary time, as some biological processes may occur more efficiently in a quiescent animal [Mignot, 2008; Schmidt, 2014]. Indeed, the presence of two types of sleep, i.e., slow wave sleep (SWS) and rapid eye movement (REM) sleep, in mammals and birds suggests that sleep might serve more than one function [Vyazovskiy and Delogu, 2014]. One approach to revealing the purposes of SWS and REM sleep is to trace the evolution of the various neurophysiological traits that collectively define these states. As other biological traits that coevolved with these sleep traits may be functionally interrelated, this comparative approach can provide insight into the functions of sleep.

Mammalian and avian SWS and REM sleep have many features in common. In both groups SWS is associated with high-amplitude, low-frequency slow waves in the electroencephalogram (EEG) [Rattenborg et al., 2011; Lesku and Rattenborg, 2014]. In contrast, REM sleep is associated with an activated low-amplitude, high-frequency EEG pattern, as well as rapid eye movements, myoclonic twitching, and postural changes reflecting a relaxed skeletal muscle tone [Dewasmes et al., 1985]. In mammals and birds, the amount of REM sleep is highest in the young and it decreases over early ontogeny [Roffwarg et al., 1966; Jouvet-Mounier et al., 1970; Scriba et al., 2013]. SWS and REM sleep are both regulated in a similar manner in mammals and birds. The intensity of SWS, as indicated by the level of EEG slow wave activity (SWA, typically 0.5–4 Hz power), and the time spent in REM sleep both increase following sleep deprivation in mammals [Tobler, 1995] and birds [Jones et al., 2008; Martinez-Gonzalez et al., 2008; Rattenborg et al., 2009; Lesku et al., 2011b], indicating that both states are homeostatically regulated. In mammals, slow waves are generated by thalamocortical interactions [Steriade et al., 1993; Lemieux et al., 2014]. REM sleep-related cortical activation, reduction in muscle tone, and eye movements are all controlled by the brainstem [Siegel, 2011; Luppi et al., 2013; Weber et al., 2015] and its interactions with a network of other brain structures throughout the neuroaxis [Jego et al., 2013; Luppi et al., 2015; Peever and Fuller, 2016].

### Evolution of SWS and REM Sleep: Convergence or Inheritance?

Mammals, birds, and nonavian reptiles constitute the extant members of the amniote clade. Birds and nonavian reptiles are members of the Sauropsida whereas mammals are members of Synapsida, clades that last shared a common ancestor over 300 million years ago. Whether the presence of SWS and REM sleep in such evolutionarily distant groups is the result of convergent evolution or these states were present in, and inherited from, a common ancestor is still a subject of debate [Libourel and Herrel, 2015; Shein-Idelson et al., 2016]. Obviously, studies of the sleep patterns of nonavian reptiles and the anamniote out-group to amniotes (amphibians) are required to resolve this question. If SWS and REM sleep were inherited from the common ancestor to mammals and birds, then one would expect other members of the amniote clade (nonavian reptiles) to exhibit the same electrophysiological indicators of SWS and REM sleep observed in mammals and birds. Furthermore, if similar states were observed in anamniotes it would indicate that these states likely evolved even before the divergence of amniotes and anamniotes. On the other hand, if these two states evolved convergently in mammals and birds one would expect that SWS and REM sleep would either be absent or only exist in a primitive form in nonavian reptiles and amphibians. Finally, it is also conceivable that some, but not all, features of one or both states were present in the common amniote or anamniote ancestor. In this case, some but not all aspects of SWS and REM sleep may have evolved convergently in mammals and birds.

Unfortunately, it is unclear whether nonavian reptiles exhibit mammalian/avian-like SWS or REM sleep [Libourel and Herrel, 2015]. A common (albeit not universal) electrophysiological correlate of behavioral sleep in reptiles is intermittent high-voltage sharp waves (HVS) in the reptilian cortex and the dorsal ventricular ridge [Hartse, 1994; Shein-Idelson et al., 2016]. Several investigators have suggested that HVS reflect neural activity comparable to that occurring in the mammalian hippocampus during SWS [Hartse, 1994; Shein-Idelson et al., 2016], whereas others have suggested that HVS are homologous in some respects to SWS-related slow waves [Rattenborg and Martinez-Gonzalez, 2014]. Despite these different opinions, most researchers agree that HVS occur during a state homologous to SWS in mammals and birds. In contrast, the existence of REM sleep in nonavian reptiles is more contentious. Behaviors suggestive of REM sleep, including eye movements under closed eyelids and myoclonic twitches, have been observed

across a variety of reptilian groups, in some cases accompanied by wake-like EEG activity [Tauber et al., 1966; Ayala-Guerrero, 1987; Ayala-Guerrero and Vargas Reyna, 1987; Ayala-Guerrero et al., 1988; Shein-Idelson et al., 2016; reviewed in Hartse, 1994; Libourel and Herrel, 2015]. However, because arousal thresholds were not assessed during these periods, it remains unclear whether they reflect REM sleep or partial awakenings [Hartse, 1994; Libourel and Herrel, 2015; Shein-Idelson et al., 2016]. In addition, several studies in lizards, turtles, and crocodilians have not found REM sleep [Hartse, 1994; Eiland et al., 2001; Libourel and Herrel, 2015]. Furthermore, the few electrophysiological and behavioral studies on sleep in amphibians and fish did not report REM sleep [Hartse, 1994; Árnason et al., 2015; Libourel and Herrel, 2015]. Given these incomplete and contradictory findings regarding REM sleep in nonmammalian/nonavian vertebrates, it is unclear whether REM sleep was present in the common amniote ancestor.

To gain insight into the evolution of SWS and REM sleep in mammals, several studies have also examined sleep in monotremes. Although monotremes are no more "basal" than therian mammals [Crisp and Crook, 2005], they retain a number of primitive traits, most notably egg laying [Warren et al., 2008], raising the possibility that they retain primitive sleep traits as well. Three studies have examined sleep in the echidna *Tachyglossus aculeatus*. The first two reported no behavioral (twitching or rapid eye movements) or EEG signs of REM sleep. Instead, the EEG only showed SWS-like slow waves [Allison et al., 1972; Siegel et al., 1996]. However, Siegel et al. [1996] observed an increased brainstem unit discharge variability, a pattern characteristic of REM sleep in eutherian (or placental) mammals, occurring in conjunction with cortical slow waves [Siegel et al., 1996], suggesting that aspects of SWS and REM sleep cooccur, but in different parts of the brain of sleeping echidnas. In contrast, another study of the echidna observed episodes of cortical activation occurring in conjunction with rapid eye movements during sleep and concluded that echidnas exhibit REM sleep, as in therian mammals [Nicol et al., 2000]. Nicol et al. [2000] demonstrated that the expression of REM sleep in echidnas is sensitive to ambient temperature and therefore suggested that unnaturally warm temperatures suppressed REM sleep in the earlier echidna studies.

Although the results from echidnas remain somewhat equivocal, the single study of the platypus (*Ornithorhynchus anatinus*) also suggests that monotremes exhibit a mixed sleep state combining features of SWS and REM sleep. During sleep in the platypus, frequent twitching of the bill, head, and eyes occurred in conjunction with cortical slow waves, suggesting that REM sleep-related activity was occurring at the level of the brainstem while the cortex generated EEG slow waves [Siegel et al., 1998]. Collectively, the results from monotremes, though unresolved in the echidna, suggest that at least some monotremes exhibit a mixed REM sleep state characterized by EEG slow waves in conjunction with brainstem activation.

Palaeognathae is a group of mostly large, flightless birds that includes ostriches, emus, rheas, and cassowaries, as well as the much smaller tinamous and kiwis. Palaeognathae and Neognathae diverged roughly 110 million years ago [Mitchell et al., 2014; Yonezawa et al., 2017]. The only electrophysiological study of sleep in a Palaeognath bird was conducted on ostriches (*Struthio camelus*). Interestingly, in addition to REM sleep with forebrain activation typical of Neognath birds [Amlaner and Ball, 1994; Roth et al., 2006], ostriches also showed a mixed sleep state similar to that observed in the platypus [Lesku et al., 2011a]. This state was characterized by periods of reduced muscle tone, a slowly falling head, and rapid eye movements under closed eyelids occurring during periods with EEG slow waves similar to those observed during preceding periods of SWS. The presence of a mixed sleep state in both monotremes and a Palaeognath bird suggested that this state, in which subcortical aspects of REM sleep are not exclusively associated with forebrain activation, may represent a precursor to REM sleep as observed in Neognath birds and Therian mammals [Lesku et al., 2011a].

The presence of a mixed REM sleep state in both monotremes and ostriches suggests that the evolution of REM sleep followed similar steps in mammals and birds. However, any evolutionary scenario based on monotremes and ostriches alone should be viewed as tentative given the small number of species examined and the unresolved controversy regarding the phenomenology of sleep in echidnas, as well as nonavian reptiles. Consequently, additional studies are needed to clarify the nature of sleep in monotremes and Palaeognath birds. In contrast to monotremes, which only consist of one species of platypus (family Ornithorhynchidae) and four species of echidna (family Tachyglossidae), there are six orders and about 60 species of Palaeognath birds.

Counter to all of the Neognath birds examined, ostriches exhibited a platypus-like sleep state. Although this may reflect a primitive sleep state, it is also possible that this form of sleep is somehow linked to other differences

between ostriches and Neognath birds, such as the ostrich's large size, flightlessness, and propensity to engage in SWS with its unusually long neck held upright in a periscopic manner and its eyes open. As a first step toward distinguishing between these alternatives, we chose to examine sleep in a species of tinamou. Tinamous, small "pheasant-like" birds, are the only Palaeognath capable of flight. Although the taxonomic relationship between tinamous and the flightless Palaeognath birds has long been the subject of debate, the most recent genetic analyses indicate that tinamous are nested within this group, being most closely related to the extinct moa, a large flightless ostrich-like bird [Harshman et al., 2008; Phillips et al., 2010; Mitchell et al., 2014; Yonezawa et al., 2017]. Furthermore, rather than reevolving an ability to fly, Mitchell et al. [2014] suggested that tinamous are the only members of this group to retain the ancestral ability to fly. Consequently, based on the retention of flightedness and a small body size, when compared to the other Palaeognath birds, tinamous may be more representative of the shared ancestor of this group.

## Methods

### Experimental Conditions

Three adult elegant crested tinamous (*Eudromia elegans*; birds 1 and 2, female; bird 3, male) were housed together under a 12 h:12 h light/dark cycle with a 15-min period of dim dawn/dusk lighting between the light and dark phases. During the light phase, light intensity levels in the enclosure (measured at the level of the birds) ranged from 275 to 340 lx. The enclosure was 4.2 × 3.4 m and included a small wire shelter lined with straw and a sand bath. The room had a slanted roof with a height of 2.0 m at the short end and 3.2 m at the high end. The birds were provided with ad libitum access to food and water. Because these birds seemed particularly responsive to environmental sounds, we played a radio at low volume during the light phase of the photoperiod to mask any noises from other parts of the animal holding facility. All housing and research methods reported herein were reviewed and approved by the Regierung von Oberbayern.

### Electrode Implantation

After establishing a surgical plane of anesthesia (1.5–2.0% isoflurane vaporized in 1.0 L/min $O_2$), the tinamous were implanted with two electrodes (gold-plated, round tipped, 0.5-mm diameter) to measure the EEG from the dura overlying the mesopallium and hyperpallium of the left hemisphere. As with other birds, the hyperpallium could be seen through the thin cranium, facilitating electrode placement; the mesopallial electrodes were placed 2.5–3.0 mm lateral to the hyperpallium [see Corfield et al., 2008]. One stainless-steel wire electrode was also glued over the supraorbital ridge to measure eye movements (electrooculogram; EOG) and another laid upon the nuchal (neck) muscle for the electromyogram (EMG). All electrodes shared a common reference over the

cerebellum. The electrodes were encased in Paladur® dental acrylic (Heraeus Kulzer, Hanau, Germany, http://www.heraeus-kulzer.com) and terminated at a connector plug on top of the head. This plug was connected to a logger (Neurologger 2A; www.vyssotski.ch/neurologger2) that has been used extensively to record the EEG in birds [Vyssotski et al., 2009; Lesku et al., 2012; Scriba et al., 2013; Rattenborg et al., 2016]. The Neurologger also recorded 3-dimensional acceleration using an accelerometer located on the top of the circuit board. The logger was configured to record EEG, EMG, EOG, and acceleration at 100 Hz. The enclosure was monitored with 5 video cameras fitted with IR light sources. Video was recorded throughout the entire 3-day experimental period.

### Sleep State Scoring, Eye Movements, and Spectral Analysis

The same 24-h period was visually scored using RemLogic (Embla RemLogic 3.4.0; Natus) for each tinamou. Scoring started at lights-off 48 h after handling the birds to attach the Neurologger. A careful initial review of all of the data revealed sleep states similar to those of Neognath birds and unlike those observed in ostriches. Consequently, to quantify the time spent in each state, criteria used in Neognath birds [Martinez-Gonzalez et al., 2008] were applied to the tinamous. The recordings were scored for wake, SWS, and REM sleep using 4-s epochs. To determine the state, first, EEG activity was visually examined for the presence of activation, i.e., a >50% reduction in amplitude from SWS levels. Second, behavior was evaluated using the EOG, EMG, acceleration, and video recordings. Epochs without both EEG activation and movement were scored as SWS. During SWS the eyes were usually open and eye movements were infrequent (see below). Epochs with activation were scored as wakefulness if they were associated with elevated EMG activity or rapid head movements, as reflected in the accelerometry and/or video recordings. Epochs with activation were scored as REM sleep if EMG activity remained unchanged or decreased from the preceding SWS level. REM sleep was also associated with frequent REM, eye closure (when visible on the videos), and in some cases the slow falling of the head, as revealed in the accelerometry and video recordings. Finally, large movements during active wakefulness (walking, feeding, and scanning) were often associated with large artifacts in the EEG. Such epochs were scored as wakefulness despite the absence of detectable EEG activation. For each state, all artifact-free epochs were analyzed with the fast Fourier transform (0.39-Hz bins) applied to Hamming-windowed data. Power for each frequency bin for each epoch of wakefulness, SWS, REM sleep was normalized as a percent of the mean power across all frequency bins for all artifact-free epochs of SWS occurring across the entire analysis period. Data for a given hour were plotted at the start of that hour. The average SWS-related SWA (0.78–3.9 Hz power) was calculated separately in 1-h time bins for each EEG channel across the 24-h experimental period. Bout durations were calculated by counting the number of successive epochs scored for each sleep state, such that a single epoch of another state terminated a bout. Reported values are either individual data or the mean ± SEM of all 3 birds.

In addition to scoring sleep states using the above criteria, we also examined eye movements more systematically. Eye movements were counted during each state. An eye movement was defined as a distinct deflection arising from the background activity. Given that the EOG recordings likely also detect electrical activity caused by eye opening and closing, as well as movements of the nictitating membrane, this method may have overestimated the

number of eye movements (saccades) occurring during each state. For the EMG channel, the average power density in the 9.8- to 50-Hz frequency bins was calculated for each state for the 12-h dark phase. The average power density for the EMG data was normalized as a percent of the average EMG power density for all epochs of SWS occurring during the dark phase.

## Results

High-quality recordings were obtained from all 3 tinamous. An awake tinamou was characterized by bilateral eye opening in conjunction with a high amount of movement including preening, feeding, and walking as revealed in the video and accelerometry recordings (Fig. 1a). Although the EEG during wake was mostly characterized by movement artifacts (mean % of epochs containing an artifact = $86.81 \pm 1.33\%$), activation was observed during pauses between large movements. The onset of sleep occurred quickly whenever the bird stopped moving. During SWS, the birds either remained standing motionless or sat down (Fig. 1a–d). The birds' head position varied somewhat depending on their body posture. When a bird was standing, its head was held erect, facing forward. When in a sitting position the head was usually oriented in the same direction but could also be drawn in towards the body with the back of the head supported against the body.

As with ostriches, the onset of REM sleep was characterized by rapid eye movements and a slow fall or rotation of the head and in some cases leaning of the whole body in a forward direction (Fig. 1c, d; online suppl. Video 1; see www.karger.com/doi/10.1159/000475590 for all online suppl. material). EMG activity (9.8–50 Hz power) was intermediate during SWS, lower during REM sleep (REM = $80.58 \pm 3.12\%$ of EMG activity during SWS), and higher during wake (wake = $721.04 \pm 196.23\%$ of EMG activity during SWS). REM sleep was readily distinguished from awakenings by the presence of increased EMG activity (Fig. 1b–d; online suppl. Video 1). Periods resembling the mixed sleep state reported in ostriches were not observed in any tinamou.

During sleep in all 3 birds, eye movements were more frequent during REM sleep (mean eye movements per min = $8.00 \pm 1.57$) than during SWS (mean eye movements per min = $0.10 \pm 0.03$). In addition, eye movements occurring during SWS generally occurred in isolation from other eye movements (mean eye movements per event = $1.30 \pm 0.09$), whereas during REM sleep they often occurred in groups (mean eye movements per event = $3.62 \pm 1.25$). The small deflections in the EOG channel during SWS (Fig. 1a, b) likely reflect brief oscillations of the eye, as previously described during wakefulness [Pettigrew et al., 1990] and SWS in other birds [Dewasmes et al., 1985; Tobler and Borbély, 1988]. In contrast to eye movements occurring during REM sleep, these eye movements were smaller and tended to occur at regular intervals [Dewasmes et al., 1985; Tobler and Borbély, 1988]. These small eye movements were not included in our quantification of eye movements.

Given that the birds were free to move around the large aviary, our ability to see both eyes simultaneously was limited. During periods when both eye were visible, bilateral eye opening occurred during wakefulness and SWS, and bilateral eye closure occurred during REM sleep. Additionally, in one tinamou which spent the entire night with the eye contralateral to the brain hemisphere from which we were recording facing the camera, entry into REM sleep was associated with eye closure and the return to SWS or brief arousals following REM sleep was associated with eye opening (online suppl. Video 1). The tinamou almost never closed this eye during SWS.

The time spent in wake, SWS and REM sleep was similar across individuals (Fig. 2a). Tinamous were diurnal, as wake comprised $10.94 \pm 1.75\%$ of the night and $63.95 \pm 2.45\%$ of the day. Conversely, SWS comprised $78.38 \pm 2.06\%$ of the night and $35.96 \pm 2.39\%$ of the day, and REM sleep made up $10.68 \pm 0.46\%$ of the night and $0.10 \pm 0.09\%$ of the day. The time spent in REM sleep increased from the first hour of the night until the peak in the second to last hour of the night, after which REM sleep decreased rapidly (Fig. 2a). The mean time spent in SWS decreased slightly and the time spent in wake remained constant across the night. The hourly time devoted to wake and SWS varied greatly across the light period (Fig. 2a).

The duration of state bouts varied across the recording period (Fig. 2b). The increase in REM sleep time throughout the night was due to an increase in both the duration and number of REM sleep bouts per hour (Fig. 2b, c). The mean REM sleep bout duration during the day ($5.61 \pm 3.49$ s) was shorter than during the night ($6.10 \pm 0.36$ s). There was an increase in REM sleep bout duration during the first hour of the day in 2 of the tinamous; however, as REM sleep made up a negligible percent of this hour, the increase is due to the presence of a few long bouts during the dark-to-light transition. The duration and number of SWS bouts decreased and increased, respectively, across the night (Fig. 2b, c). Wake bout durations were relatively constant across the night. Daytime SWS and wake bout durations were both variable.

**Fig. 1.** Recordings showing electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and accelerometry patterns typical of wakefulness (green bar), slow wave sleep (SWS) (blue bar), and rapid eye movement (REM) sleep (red bar). **a** This example starts with a period of SWS characterized by high-amplitude slow waves, low EMG activity, and the absence of movements as reflected in the accelerometer recordings. The bird then switches to wakefulness characterized by EEG activation, elevated EMG activity, and large movements in all acceleration channels. Note that the large potentials in the EEG channels (asterisks) are associated with deflections in the EMG and accelerometry channels and therefore likely reflect movement artifacts. **b** This plot shows a period of SWS punctuated by a short arousal. Notice the sharp increase in amplitude in the EMG, which coincides with a sharp jerking movement indicated by the deflections in all of the accelerometry channels. The arousal is followed by a short period of activation in both EEG channels. **c** This example shows bouts of REM sleep interrupted by a short bout of SWS. The second bout is followed by a brief awakening characterized by a sharp increase in the EMG activity channel and a brief EEG activation following the movement. Notice the slight slow movements of the head and rapid twitches reflected in the accelerometry channels, the presence of small deflections indicating rapid eye movements in the EOG channel, and activation in both EEG channels during the REM sleep bouts. This plot also contains a second brief arousal toward the end of the recording. **d** This example contains two further REM sleep bouts. The first is characterized by a pronounced example of head dropping as indicated by the large movements in the surge accelerometry channel. Both REM sleep bouts are followed by brief arousals. Each example lasts 60 s. EEG, EOG, and EMG channels are all filtered (high-pass filter set at 1 Hz; low-pass filter set at 35 Hz) and scaled similarly across examples. The accelerometry channels are scaled the same in all examples. Vertical bars on the right of the EEG and EOG traces denote 100 μV. Bars on the right of accelerometry channels denote 500 milli-g-forces and for the EMG channel the bars denote 20 μV.
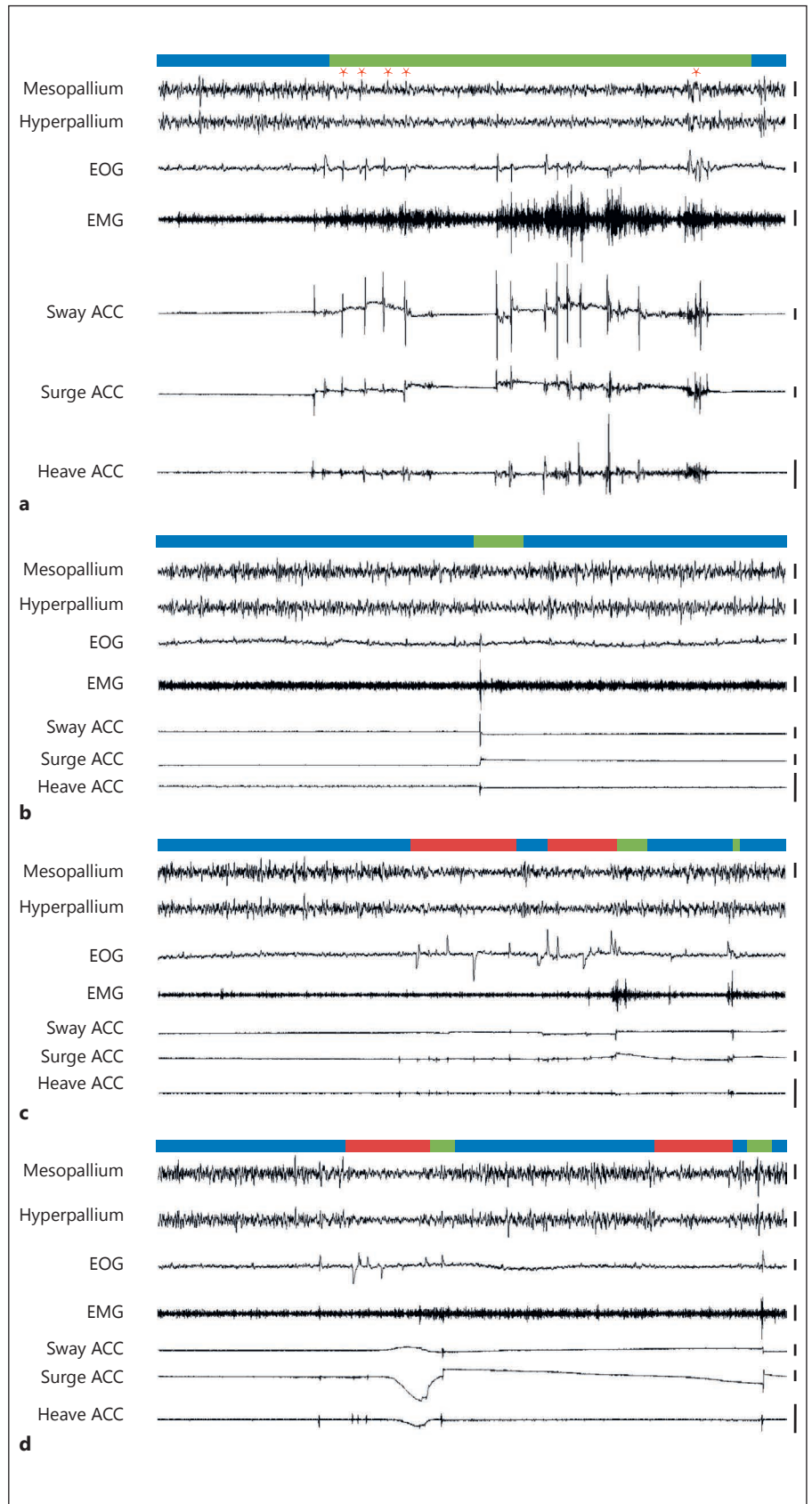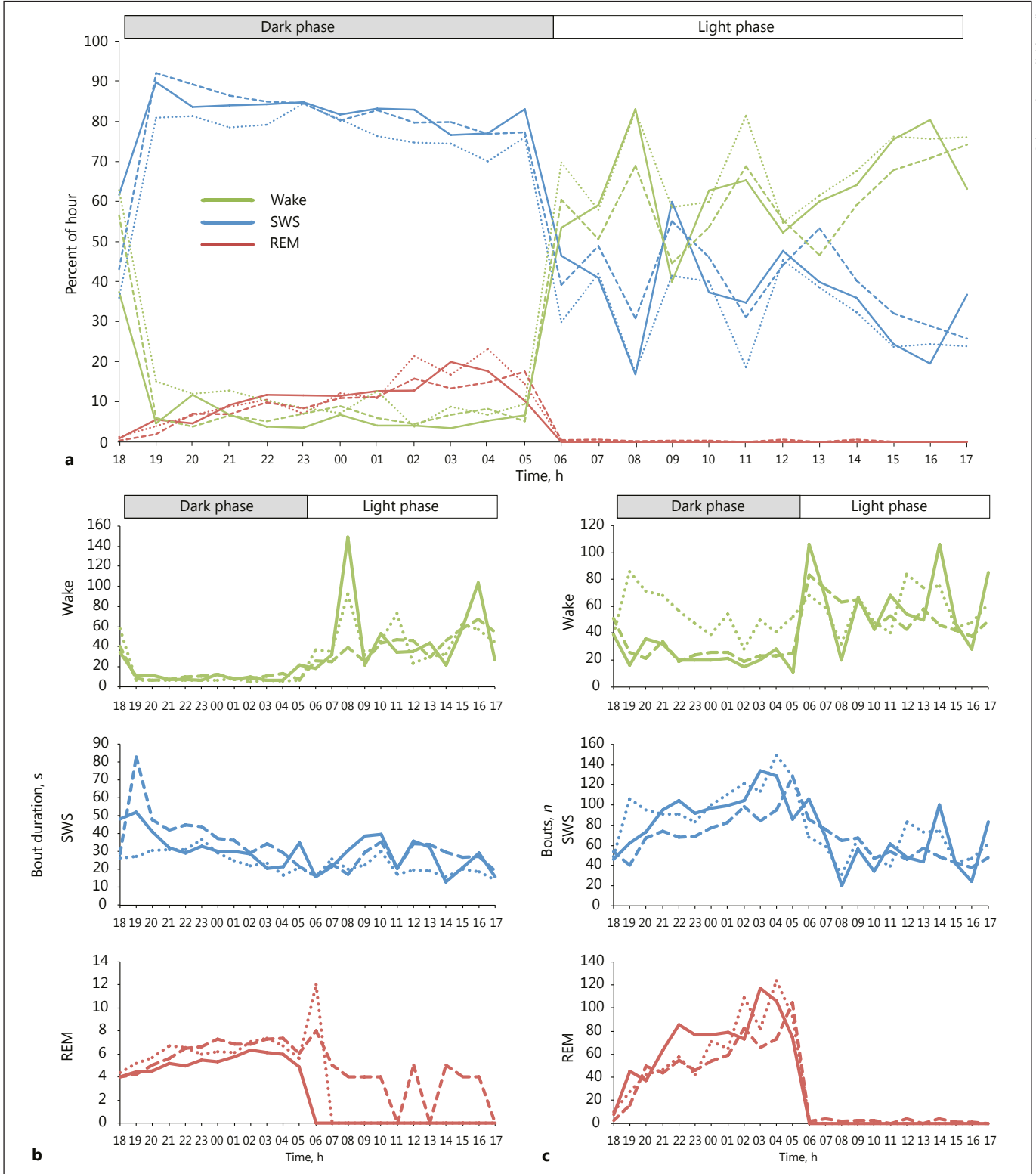
**Fig. 2. a** Timing of sleep/wake states across the 24-h recording. The percent of each hour devoted to wake, slow wave sleep (SWS), and rapid-eye movement (REM) sleep is plotted for each bird. Wake, SWS, and REM sleep bout duration (**b**) and number (**c**) of bouts for individual birds. The dark and light phases are indicated in the bar above the plot. Data for birds 1, 2, and 3 are indicated by solid, dotted, and dashed lines, respectively.
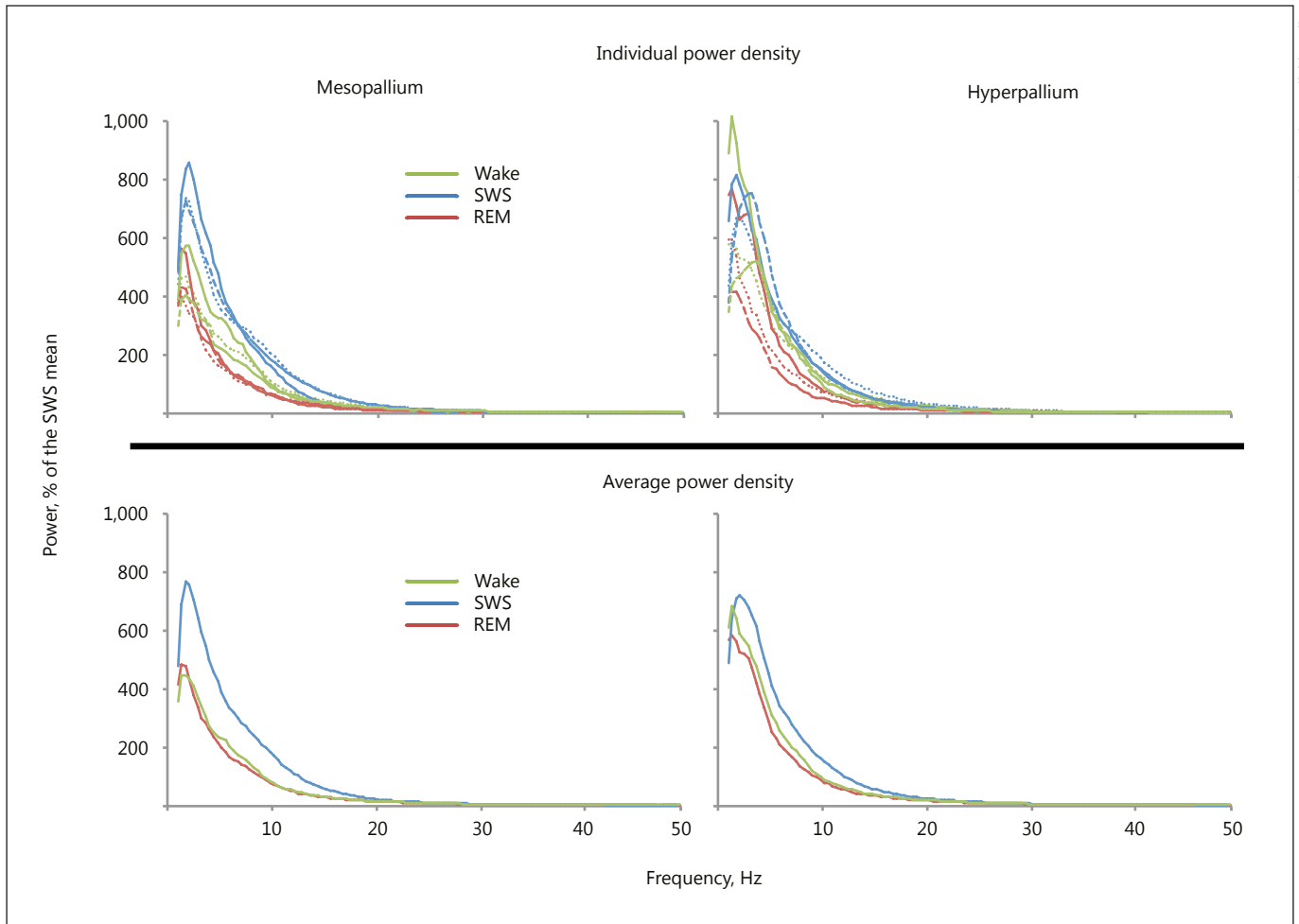
**Fig. 3.** Normalized power for wake, slow wave sleep (SWS), and rapid-eye movement (REM) sleep for both electroencephalogram channels for individual birds, as well as mean power for each electroencephalogram channel. Data for birds 1, 2, and 3 are indicated by solid, dotted, and dashed lines, respectively.

In general, the normalized EEG power for each state and brain region was similar across birds (Fig. 3). However, for unexplained (probably technical) reasons this pattern was different in the hyperpallium for bird 1. Aside from this channel, the power spectra were similar to those described in other birds [Martinez-Gonzalez et al., 2008; Scriba et al., 2013].

The mean normalized SWS-related SWA was higher during the day (746.88 ± 83.24%) than during the night (627.18 ± 33.44%; Fig. 4); however, SWA changed little within each phase. Individual day and night values for SWS-related SWA were as follows: bird 1 (day, 909.57%; night, 692.16%), bird 2 (day, 696.13%; night, 581.12%), and bird 3 (day, 634.93%; night, 608.23%). Thus, when compared to the night, SWS occurred in shorter and

more intense (based on SWA) bouts during the day (Fig. 2c, 4). This difference was particularly clear in bird 1 and least pronounced in bird 3.

## Discussion

The aim of this study was to gain insight into the evolution of avian sleep by determining whether the unusual mixed sleep state described in ostriches [Lesku et al., 2011a] is present in another Palaeognath bird, i.e., the elegant crested tinamou. If an ostrich-like mixed sleep state exists in tinamous, this would strengthen the idea that it reflects a primitive trait among Palaeognath birds. Alternatively, the presence of Neognath bird-like sleep states
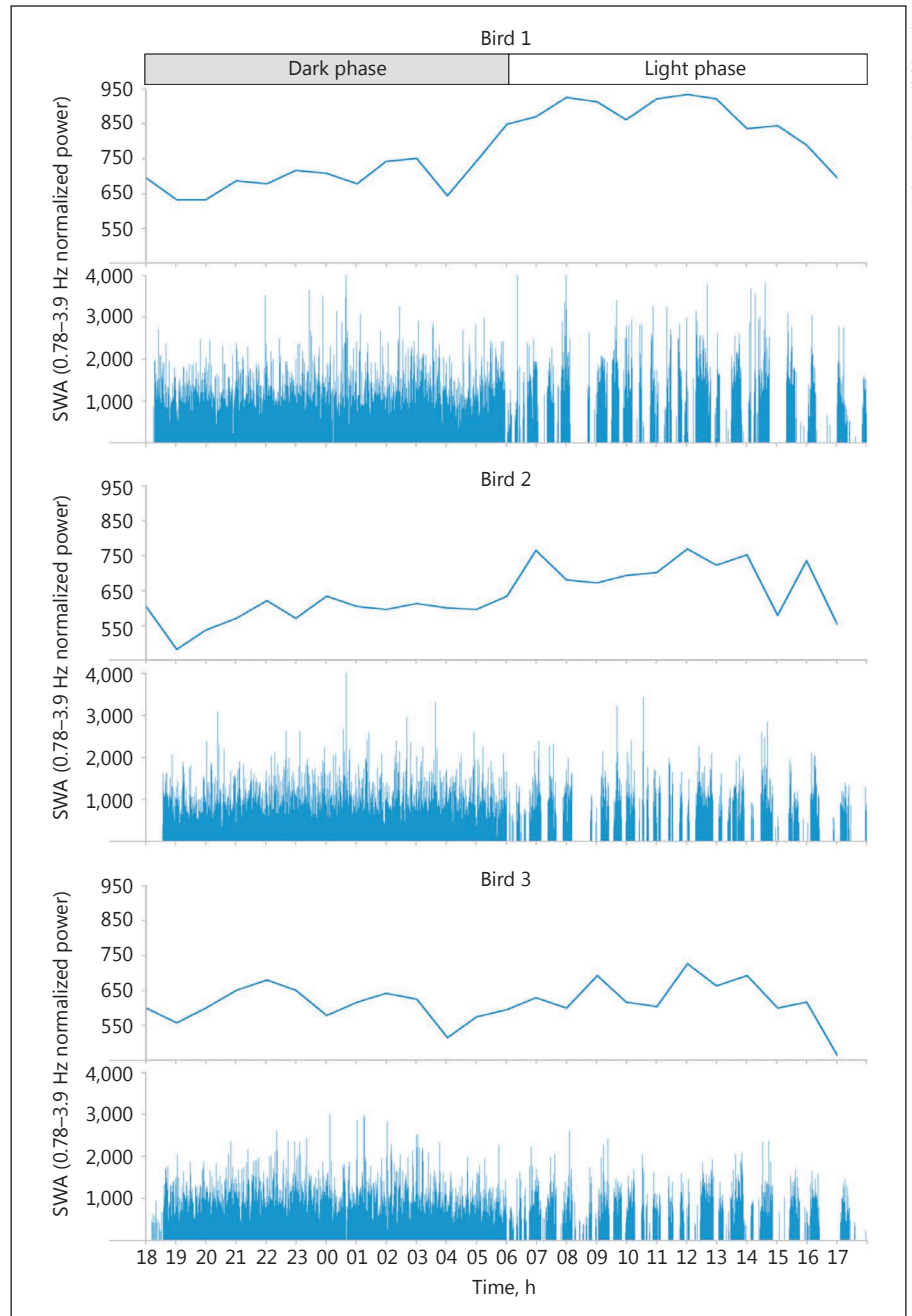
**Fig. 4.** Normalized electroencephalogram slow wave activity (SWA; 0.78–3.9 Hz of power) during slow wave sleep (SWS) recorded from the mesopallium of each bird. The top plots show hourly SWA and the bottom plots show SWA for individual 4-s SWS epochs. Epochs of SWS containing an artifact were assigned a value of zero, as were epochs scored as rapid eye movement sleep or wake.

in tinamous would increase the likelihood that the mixed sleep state in ostriches is tied to some specific aspect of their biology. Our findings support the latter scenario.

As in Neognath birds, REM sleep in ostriches is characterized by eye closure, rapid eye movements, and reduced muscle tone, with the latter being reflected as reduced EMG activity and/or drooping of the head. However, unlike Neognath birds wherein these aspects of REM

sleep are associated with EEG activation, they were not always associated with EEG activation in ostriches. Instead, as in the platypus, they also occurred with SWS-like EEG slow waves. In tinamous, eye closure, rapid eye movements, and a reduced muscle tone were usually associated with EEG activation, as in Neognath birds. Eye movements occurring in conjunction with EEG slow waves occurred very rarely as compared to eye move-

ments occurring during REM-related activation. Furthermore, eye movements occurring during REM-related activation tended to occur in groups, whereas those that occurred during EEG slow waves tended to be isolated eye movements. Also, the duration of REM sleep episodes and the overall amount of REM sleep were lower in tinamous than in ostriches [Lesku et al., 2011a] and comparable to values for Neognath birds [Amlaner and Ball, 1994; Roth et al., 2006; Martinez-Gonzalez et al., 2008]. Despite these differences, the average hourly amount of REM sleep increased across the night in tinamous and ostriches, as in several Neognath birds. As in some Neognath birds [Low et al., 2008; Martinez-Gonzalez et al., 2008], this increase in REM sleep was mediated by an increase in the number and duration of REM sleep bouts in tinamous.

The absence of an ostrich-like mixed sleep state in tinamous challenges the idea that this type of sleep is a primitive trait for Palaeognath birds. Identifying the ancestral state in Palaeognaths will require studying additional species (e.g., other tinamou species, cassowaries, kiwis, emus, and rheas). If these birds exhibit sleep states like those observed in Neognath birds and the elegant crested tinamou, this would suggest that REM sleep with forebrain activation is a primitive trait for all extant birds. Alternatively, if all flightless Palaeognath birds sleep like ostriches this would seemingly suggest that the mixed sleep state is a primitive trait for Palaeognaths, with tinamous independently evolving Neognath-like sleep states. However, recent studies on Palaeognath evolution suggest another scenario. Even though the fact that tinamous are nested within a group of flightless birds seemingly suggests that they reacquired flight, recent genetic and biogeographic evidence suggests that tinamous actually retained the ancestral ability to fly, and each of the other extant Palaeognath birds independently evolved flightlessness [Mitchell et al., 2014; Yonezawa et al., 2017]. In this case, if the mixed sleep state found in ostriches is somehow linked to physiological changes associated with flightlessness in Palaeognath birds [Withers et al., 1987; Li et al., 2014; Eliason et al., 2016] it is possibile that tinamous retain the primitive sleep patterns for Palaeognath birds and therefore birds in general. Ultimately, in addition to examining other Palaeognath birds, further studies aimed at clarifying the nature of sleep in crocodilians, the closest reptilian relatives to birds, are needed to tease apart the evolution of avian REM sleep.

Although the results from tinamous do not rule out the possibility that the mixed sleep state observed in ostriches is a primitive trait among Palaeognath birds, they do cause us to reconsider whether this state reflects an idiosynchro-

ny of sleep in ostriches. Engaging in SWS with their eyes open is unlikely to be linked to the mixed sleep state, as tinamous and some Neognath birds also sleep in this manner but do not show the mixed sleep state [Berger and Walker, 1972; Šušić and Kovačević, 1973; Tobler and Borbély, 1988; Rattenborg et al., 2001]. However, in contrast to tinamous, which often pull their head into their body during sleep, ostriches sleep with their unusually long neck held fully extended and perpendicular to the ground. Consequently, it is conceivable that the maintenance of this unusual sleep posture is largely incompatible with a fully integrated brainstem and forebrain REM sleep state. Although dissociated states of this nature have not been described before in birds, there is precedence for some aspects of REM sleep to depend on a bird's posture. Notably, when geese sleep with their head fully supported on their back they show mammalian-like nuchal (neck) EMG atonia, whereas when they sleep with their head facing forwards and unsupported some muscle tone is maintained during REM sleep [Dewasmes et al., 1985]. As a result, rather than dropping in a free fall, the head droops in a slow and controlled manner when geese enter REM sleep. Although the association between other brainstem mediated aspects of REM sleep (rapid eye movements) and EEG activation is maintained when geese sleep with their head held forward, the dependence of atonia on head posture supports the notion that the unusual posture of ostriches might contribute to an even more pronounced dissociation between brainstem and forebrain aspects of sleep. If this is correct, then ostriches sleeping with their heads laid out on the ground, as observed by Immelmann [1959], would be expected to exhibit a fully integrated REM sleep state like that observed in tinamous and Neognath birds. Unfortunately, this less vigilant sleep posture was not observed in the only electrophysiological study of sleep in ostriches [Lesku et al., 2011a]. Consequently, additional studies of ostriches and other long necked Palaeognath birds, such as emus, are needed to test this hypothesis. However, even if the mixed state observed in ostriches is the result of a behavioral artifact, this still does not explain the presence of a mixed state in the platypus, which sleeps with their head resting on the ground [Siegel et al., 1996].

In addition to the differences in the basic characteristics of REM sleep between ostriches and tinamous, there were also large differences in the duration of REM sleep episodes and the overall amount of REM sleep. The ostriches spent 26.3 ± 1.3% of the 24-h total sleep time in REM sleep. REM bouts lasted an average of 27 ± 7 s, with a maximum duration of 5 min when calculated using behavioral criteria [i.e., eye closure and head drooping;

Lesku et al., 2011a]. This is the most REM sleep and the longest REM sleep bout duration reported for any bird by a substantial margin [Amlaner and Ball, 1994; Roth et al., 2006]. In tinamous, the mean REM sleep bout duration was 6 s, the maximum REM sleep bout duration was 28 s for the total 24-h recording, and the REM sleep as a percent of the total sleep time was only 8.65 ± 0.59%. In contrast to ostriches, these values from tinamous more closely resemble those reported for all other birds [Roth et al., 2006; Martinez-Gonzalez et al., 2008]. Interestingly, the platypus also exhibits more REM sleep than any other mammal if REM sleep is calculated using behavioral criteria similar to those employed in ostriches [Siegel et al., 1999; Siegel, 2005; Lesku et al., 2006, 2008].

Although the focus of our study was on REM sleep, it also revealed aspects of SWS regulation. As in mammals, SWS-related SWA increases following a period of sleep loss and decreases with time spent asleep, suggesting that SWA reflects homeostatically regulated sleep processes [Jones et al., 2008; Martinez-Gonzalez et al., 2008; Rattenborg et al., 2009; Lesku et al., 2011b]. In songbirds, this pattern is evident during sleep at night following an undisturbed day comprised largely of wakefulness [Szymczak et al., 1996; Rattenborg et al., 2004; Jones et al., 2008]. In contrast, in pigeons, SWA at night following an undisturbed day is relatively constant [Tobler and Borbély, 1988; Martinez-Gonzalez et al., 2008]. Nonetheless, the SWA at night increases in pigeons if daytime napping is prevented [Martinez-Gonzalez et al., 2008]. As in undisturbed pigeons, the SWA during SWS at night was relatively constant in tinamous. Interestingly, although SWS-related SWA varied little within the light or dark phases, SWA was higher during daytime naps than during SWS at night. The higher level of SWA during the light phase may reflect a homeostatic response to the periods of extended wake, which only occurred during this time; however, sleep deprivation studies are needed to confirm the homeostatic regulation of SWA in tinamous.

## Conclusions

Tinamous sleep more like Neognath birds than the more-closely related (Palaeognath) ostrich. Unlike the ostrich, which exhibits a mixed sleep state, combining features of REM sleep and SWS like that observed in the platypus, in tinamous SWS and REM sleep were clearly delineated states. Although these findings do not rule out the possibility that the avian ancestor to extant birds exhibited a mixed ostrich-like sleep state, they do complicate this initial interpretation based only on data from ostriches. Notably, the findings from tinamous raise the possibility that the mixed sleep state observed in ostriches reflects some aspect of their biology or behavior, such as sleeping with their neck and head held high off the ground with eyes open. Studies of ostriches and other large Palaeognath birds sleeping with their heads resting on the ground or on their back are needed to test this hypothesis. Such studies would provide a better understanding of the phylogenetic, physiological, and behavioral factors influencing the expression of REM sleep in birds.

## Disclosure Statement

The authors declare no conflict of interests.

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