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New perspectives on avian REM sleep

Gianina Ungurean

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Gianina Ungurean

Nouvelles perspectives sur le sommeil paradoxal des oiseaux

Devant le jury composé de :

Pr MAZZA Stéphanie, Professeure des Universités, Université Lyon 1, **Présidente**

Pr ADAMANTIDIS Antoine, Professeur, Université de Berne, Suisse, **Rapporteur**

Dr ATON Sara, Professeure Associée, Université du Michigan, États Unis, **Examinatrice**

Pr ARNULF Isabelle, Professeure des Universités, Praticien Hospitalier, Sorbonne Université, **Examinatrice**

Pr BLUMBERG Mark, Professeur, Université de l'Iowa, États-Unis, **Rapporteur**

Dr LIBOUREL Paul-Antoine, Ingénieur de Recherche, Centre de Recherche en Neurosciences de Lyon, **Co-directeur.rice de thèse**

Dr LUPPI Pierre-Hervé, Directeur de Recherche, Centre de Recherche en Neurosciences de Lyon, **Directeur de thèse**

Dr RATTENBORG Niels, Chercheur Institut Max Planck d'ornithologie, Allemagne, **Co-directeur de thèse**

*Drowsy birds at even gliding,
Round about their nests alight,
In among the branches hiding...
Dear, good night !*

*Silence through the forest creeping,
Lullaby the river sighs;
In the garden flowers sleeping...
Shut your eyes !*

*Glides the swan among the rushes
To its rest where moon light gleams,
And the angels whisper hushes...
Peaceful dreams !*

*O'er the sky stars without number,
On the earth a silver light;
All is harmony and slumber...
Dear, good night !*

Drowsy birds, Mihai Eminescu
English translation by Corneliu M. Popescu

Nouvelles perspectives sur le sommeil paradoxal des oiseaux

Résumé

Le sommeil est un comportement ubiquitaire que l'on retrouve chez tous les animaux étudiés à ce jour, de la méduse à l'homme. Chez les mammifères, le sommeil peut être divisé en deux états principaux sur la base de signatures électrophysiologiques cérébrales et du comportement : le sommeil lent et le sommeil paradoxal. Bien que des progrès importants aient été réalisés au cours des dernières décennies dans notre compréhension des mécanismes du sommeil, aucun consensus n'a été atteint concernant ses fonctions. À cet égard, les études comparatives peuvent non seulement donner un aperçu de l'évolution du sommeil, mais aussi révéler des principes généraux applicables à de nombreux groupes taxonomiques. Les oiseaux sont particulièrement intéressants pour les études comparatives car, bien qu'ils soient, au cours de l'évolution, plus étroitement liés aux crocodiles et autres reptiles non aviens qu'aux mammifères, ils présentent deux états de sommeil remarquablement similaires au sommeil lent et sommeil paradoxal des mammifères. Ainsi, la recherche sur les oiseaux pourrait compléter et approfondir notre compréhension de l'évolution et de la ou des fonctions du sommeil. Dans ce manuscrit, je présente les données de trois projets qui étudient les corrélats comportementaux, physiologiques et neurophysiologiques du sommeil chez les oiseaux, en mettant l'accent sur le sommeil paradoxal. Premièrement, nous avons enregistré la température corticale du cerveau chez des pigeons endormis afin de comprendre si, et comment elle varie en fonction des états de sommeil. Nous avons constaté que, comme chez les mammifères, la température corticale diminue pendant le sommeil lent et augmente pendant le sommeil paradoxal. Cependant, les vitesses de refroidissement et de réchauffement étaient plus lentes que chez les mammifères. Dans une seconde étude, aussi réalisée chez des oiseaux, nous avons mesurer les changements de taille de la pupille durant les différents états de vigilance. De manière surprenante, nous avons constaté que les changements de taille de la pupille chez les oiseaux sont opposés à ceux décrits chez les mammifères à la fois pendant l'éveil, le sommeil lent et le sommeil paradoxal. Grâce à des expériences pharmacologiques, nous avons également montré que les constriction de la pupille chez les oiseaux, contrairement aux mammifères, sont supportés par les récepteurs cholinergiques nicotiques. Le comportement pupillaire opposé est donc lié (en partie) au fait que, contrairement à l'iris des mammifères qui est contrôlé par des muscles lisses, l'iris aviaire est contrôlé par des muscles striés. Enfin, dans une troisième étude, nous avons réalisé pour la première fois des enregistrements IRMf chez des oiseaux endormis et obtenu une activité BOLD dépendante de l'état. Bien qu'encore en cours, ces données suggèrent que le sommeil paradoxal est associé à une large activation des structures corticales et sous-corticales, couvrant les voies visuelles, somatomotrices et les ganglions de la base. De manière inattendue, le sommeil lent était associé à une activité accrue du système ventriculaire, ce qui pourrait refléter une activité importante du système glymphatique par rapport au sommeil paradoxal. S'ils sont corrects, ces résultats suggèrent que le bénéfice des fonctions remplies par le sommeil paradoxal l'emporterait sur les coûts liés à la suspension temporaire du sommeil lent et notamment à son rôle dans l'élimination des déchets métaboliques par le flux glymphatique. Dans

l'ensemble, nos données montrent que les différentes composantes du sommeil paradoxal se manifestent de diverses manières à travers les groupes taxonomiques, avec des similarités fortes mais aussi des différences importantes. Ces variations constituent une riche ressource pour étudier l'évolution et les fonctions du sommeil paradoxal. Notamment, les caractéristiques partagées pourraient être liées à une fonction centrale du sommeil paradoxal, tandis que les divergences pourraient refléter des fonctions spécifiques à un taxon ou même des épiphénomènes résultant de la neurophysiologie spécifique d'un organisme. En embrassant cette diversité, les chercheurs obtiendront probablement une compréhension plus complète de ce qu'est et de ce que fait le sommeil paradoxal, une compréhension qui ne peut être obtenue en se concentrant exclusivement sur les mammifères.

Mots clés

sommeil, évolution, oiseaux, sommeil paradoxal, température du cerveau, pupillométrie, iris, IRMf, BOLD, mémoire

New perspectives on avian REM sleep

Summary

Sleep is a ubiquitous behavior found in all animals studied to date, from jellyfish to humans. In mammals, sleep can be divided into two major states, non-rapid eye movement (NREM) sleep and REM sleep based on brain electrophysiological signatures and behavior. Although important advances have been made in the past decades in our understanding of the mechanisms of sleep, a consensus has not been reached regarding its functions. In this respect, comparative studies may not only give insights into the evolution of sleep, but may reveal general principles applicable to many taxonomic groups. Birds are particularly interesting for comparative studies because, despite being evolutionarily more closely related to crocodiles and other non-avian reptiles than mammals, they exhibit two sleep states remarkably similar to mammalian NREM and REM sleep. Thus, research on birds could complement and further our understanding of the evolution and function(s) of sleep. In this manuscript I present data on three projects investigating behavioral, physiological, and neurophysiological correlates of sleep in birds, with a focus on REM sleep. First, we recorded cortical brain temperature in sleeping birds to understand whether and how it varies with sleep states. We found that as in mammals, cortical temperature decreases during NREM sleep and increases during REM sleep. However, the rates of cooling and warming were slower than in mammals. In a second study, we investigated state dependent changes in pupil size in birds. In mammals, the size of the pupil reflects the state of the brain during wakefulness and sleep. Surprisingly, we found that state-dependent changes in pupil size in birds are opposite those described in mammals during both wakefulness, NREM sleep, and REM sleep. Through pharmacological experiments, we also showed that pupil constrictions in birds, unlike mammals, are mediated via nicotinic cholinergic receptors. The opposite pupillary behavior is thus linked (in part) to the fact that, in contrast to the mammalian iris which is controlled by smooth muscles, the avian iris is controlled by striated muscles. Finally, in a third study, we conducted for the first time fMRI recordings in sleeping birds and obtained state-dependent BOLD activity. Although still in progress, this data suggests that REM sleep is associated with broad activation of cortical and subcortical structures, spanning visual and somatomotor pathways and the basal ganglia. Unexpectedly, NREM sleep was associated with enhanced activity in the ventricular system, which might reflect enhanced activity of the glymphatic system when compared to REM sleep. If correct, these results suggest that the benefits of expressing REM sleep outweigh the costs of temporarily suspending the proposed clearance of metabolic waste products via glymphatic flow during NREM sleep. Taken together, our data show that different components of REM sleep manifest in various ways across taxonomic groups, ranging from similar to opposite. This variation serves as a rich resource for investigating the evolution and functions of REM sleep. Notably, shared features are likely linked to the core function of REM sleep, whereas those that differ might reflect taxon-specific functions or even epiphenomenon resulting from the specific neurophysiology of an organism. Through embracing this diversity, researchers will likely obtain a more comprehensive understanding of what REM sleep is and does, that could not be obtained through focusing exclusively on mammals.

Keywords

sleep, evolution, avian, REM sleep, brain temperature, pupillometry, RIMs, fMRI, BOLD, memory

Noi perspective asupra somnului REM al păsărilor

Rezumat

Somnul este un comportament omniprezent, întâlnit la toate animalele studiate până în prezent, de la meduze la oameni. La mamifere, somnul poate fi împărțit în două faze majore, somnul cu mișcări oculare non-rapide (în limba engleză, rapid eye movement (REM) sleep) și somnul non-REM (NREM), pe baza semnăturilor electrofiziologice cerebrale și a comportamentului. Deși în ultimele decenii s-au făcut progrese importante în înțelegerea mecanismelor somnului, nu s-a ajuns la un consens în ceea ce privește funcțiile acestuia. În acest sens, studiile comparative nu numai că pot oferi informații despre evoluția somnului, dar pot dezvălui principii generale aplicabile la mai multe grupuri taxonomice. Păsările sunt deosebit de interesante pentru studiile comparative, deoarece, în ciuda faptului că sunt mai apropiate din punct de vedere evolutiv de crocodili și de alte reptile neaviare decât de mamifere, ele prezintă două stări de somn remarcabil de asemănătoare cu somnul NREM și REM al mamiferelor. Astfel, cercetările asupra păsărilor au potențialul de a completa și avansa înțelegerea noastră cu privire la evoluția și funcția sau funcțiile somnului. În acest manuscris, sunt prezentate trei proiecte care investighează corelatele comportamentale, fiziologice și neurofiziologice ale somnului la păsări, cu un accent pus pe somnul REM. În primul rând, am înregistrat temperatura corticală a creierului la păsările adormite pentru a înțelege dacă și cum aceasta variază în funcție de stările de somn. Am constatat că, la fel ca la mamifere, temperatura corticală scade în timpul somnului NREM și crește în timpul somnului REM. Cu toate acestea, ratele de răcire și încălzire au fost mai lente decât la mamifere. Într-un al doilea studiu, am investigat modificări ale dimensiunii pupilelor dependente de starea de vigilență și faza somnului la păsări. La mamifere, dimensiunea pupilei reflectă starea creierului în timpul stării de veghe și de somn. În mod surprinzător, am constatat că modificările dependente de stare ale dimensiunii pupilei la păsări sunt opuse celor descrise la mamifere atât în timpul stării de veghe, cât și în timpul somnului NREM și al somnului REM. Prin experimente farmacologice, am arătat, de asemenea, că la păsări, spre deosebire de mamifere, constricțiile pupilei sunt mediate prin intermediul receptorilor colinergici nicotinici. Comportamentul pupilar opus este astfel legat (parțial) de faptul că, spre deosebire de irisul mamiferelor, care este controlat de mușchii netezi, irisul păsărilor este controlat de mușchii striati. Într-un al treilea studiu, am efectuat pentru prima dată înregistrări RMNf la păsări adormite și am obținut o activitate BOLD dependentă de faza somnului. Deși sunt încă în curs de realizare, aceste date sugerează că somnul REM este asociat cu o activare amplă a structurilor corticale și subcorticale, extinzându-se în regiunile vizuale, somatomotorii și ganglionii bazali. În mod neașteptat, somnul NREM a fost asociat cu o activitate sporită în zonele sistemul ventricular, ceea ce ar putea reflecta o activitate sporită a sistemului glymphatic în comparație cu somnul REM. Dacă sunt corecte, aceste rezultate sugerează că beneficiile exprimării somnului REM depășesc costurile suspendării temporare a eliminării deșeurilor metabolice prin intermediul fluxului limfatic în timpul somnului NREM. Luate împreună, datele noastre arată că diferite componente ale somnului REM se manifestă în moduri diferite în cadrul grupurilor taxonomice, variind de la similar la opus. Această variație servește ca o resursă bogată pentru investigarea evoluției și funcțiilor somnului

REM. În special, caracteristicile comune sunt probabil legate de funcția de bază a somnului REM, în timp ce cele care diferă ar putea reflecta funcții specifice taxonului sau chiar epifenomene care rezultă din neurofiziologia specifică a unui organism. Prin îmbrățișarea acestei diversități, cercetătorii vor obține probabil o înțelegere mai cuprinzătoare a ceea ce este și face somnul REM, ce nu ar putea fi obținută prin studiul exclusiv al mamiferelor.

Cuvinte-cheie

somn, evoluție, păsări, somn REM, temperatura creierului, pupilometrie, iris, RMNf, BOLD, memorie

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General introduction

Sleep is ubiquitous – all animals carefully studied so far fulfill the five criteria currently used to define sleep behavior, namely quiescence, specific posture, reduced responsiveness, quick reversibility and homeostatic regulation (Piéron, 1913; Tobler, 1984; Deboer, 2015). Its widespread presence, the deleterious effects of sleep deprivation (Krause et al., 2017; Garbarino et al., 2021), and the fact that animals reduce and/or change their sleep architecture in response to ecological demands, but never completely forgo it (Lesku et al., 2012; Rattenborg et al., 2016; Lyamin et al., 2018; Ungurean et al., 2020b), underscores that sleep must fulfill important functions. Which those functions are remains, however, unresolved.

Proposed functions of sleep

The function or functions of sleep have been subject to debate and numerous theories have been elaborated. Sleep is a highly variable behavior when accounting for different species and taxonomic groups (Siegel, 2017; Campbell and Tobler, 1984; Lesku et al., 2009), but important differences in sleep duration can also be found within the same species (Geissmann et al., 2019). Due to the lack of a measurable variable (e.g. the amount of conserved energy) sufficient to explain most of the variation in sleeping times, some researchers suggested that sleep's main function is to immobilize animals and prevent them from behaving maladaptively or inefficiently in circumstances unfavorable for foraging, reproducing, or evading predators (Meddis, 1975; Siegel, 2009).

However, already since the time of ancient Greeks, sleep was thought to fulfill regenerative roles, and its beneficial effects are largely agreed upon. Sleep was suggested to promote immune function (Imeri and Opp, 2009; Opp, 2009), thermoregulation and energy management (Schmidt, 2014). Sleep certainly supports regenerative processes for the periphery, but such processes could also occur during physical rest while awake. The need for reduced responsiveness suggests that brain-related processes occurring during sleep cannot take place or are inefficient when the organism is engaged with the environment. Indeed, most prominent old and new theories posit that sleep is beneficial for the central nervous system. Awake behavior requires ongoing metabolic processes which produce molecules deleterious for the brain. Sleep was suggested to act as a time when housekeeping functions are fulfilled, such as clearance of waste products accumulated during wake (Hablitz and Nedergaard, 2021), and replenishment of depleted reserves and substances required for wake behavior (Mackiewicz et al., 2007). Sleep has also been linked to neuronal cellular repair functions; for example, during sleep the expression of DNA repair genes are upregulated

(Belleusi et al., 2016) and neuronal chromosome dynamics increases (Zada et al., 2019), thus facilitating the repair of DNA breaks accumulated during wakefulness.

Perhaps most intriguing discoveries highlight the role of sleep in high cognitive processes. This idea is opposite to the old, traditional view that sleep is a time of reduced activity for the brain as much as it is for the body. Converging evidence showed that offline brain activity beneficial for learning, memory consolidation, synaptic plasticity and synaptic homeostasis regulation (Vorster and Born, 2015; Rothschild, 2019; Klinzing et al., 2019; Puentes-Mestril and Aton, 2017; Navarro-Lobato and Genzel, 2019; Boyce et al., 2016; Cirelli and Tononi, 2021) occurs during sleep.

The presence of two sleep states, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) (described in detail below), each defined by a different set of neurological, physiological, and metabolic parameters intuitively implies that each fulfills a separate function, fostered by the respective state's environment. Indeed, in mammals, the neural oscillations characteristic of NREM sleep are thought to orchestrate memory transfer from the hippocampus to the cortex (Klinzing et al., 2019); and to correlate with cerebrospinal fluid (CSF) inflow, a process involved in brain clearance (Hablitz and Nedergaard, 2021; Fultz et al., 2019; Xie et al., 2013). REM sleep was linked to procedural, contextual, and emotional memory consolidation (Rasch and Born, 2013; Genzel et al., 2015; Wassing et al., 2019). In altricial mammals and birds REM sleep expression is more prevalent at birth and progressively declines throughout development (Roffwarg et al., 1966; Jouvet-Mounier et al., 1969; Frank and Heller, 1997; Scriba et al., 2013), while in precocial mammals which have adult-like sleep patterns at birth, REM sleep expression is higher *in utero* (Astic and Jouvet-Mounier, 1969; Ruckebusch, 1972; Ibuka, 1984; Astic et al., 1973). Due to its prevalence early in development, sleep (and particularly REM sleep) was linked to brain development and enhanced plasticity (Roffwarg et al., 1966; Blumberg, 2015; Bridi et al., 2015). Finally, the decrease in neuronal activity during NREM and the increase in REM sleep is associated with cooling and heating of the brain, respectively (Kawamura and Sawyer, 1965; Hayward and Baker, 1969; Csernai et al., 2019; Komagata et al., 2019). Although the exact mechanisms for the increase in brain temperature during REM sleep are not fully understood, this process is thought to result from the increased metabolism of active neurons during this state, or from higher blood inflow arriving from the warmer core, to support increased metabolic needs. It has been suggested that the core function of REM sleep, which follows NREM in the sleep cycle, is to warm up the brain and ensure an adaptive response when awakened (Wehr, 1992; Lyamin et al., 2018). Finally, NREM and REM sleep have also been suggested to fulfill complementary functions, or different aspects of the same function through complementary mechanisms (Vyazovskiy and Delogu, 2014; Tamaki et al., 2020).

Comparative perspective(s)

At a fundamental level, comparative approaches aim to understand which characteristics of sleep emerged early on during evolution, which have persisted and which have been lost. This approach relies on investigating and understanding the diversity of sleep phenotypes and how similarities or differences relate to the physiology and ecology of different species. Studies on dolphins and ducks have already inspired novel questions in the sleep research field. The finding that dolphins sleep unihemispherically (Mukhametov et al., 1977) changed the view that sleep is a global phenomenon capturing the whole brain, and set the ground for the discovery and further research on local sleep (Siclari and Tononi, 2017). The discovery of asymmetric sleep in ducks, expressed as a response to predation risk (Rattenborg et al., 1999), inspired research that led to the finding that humans also sleep less deeply with parts of the left hemisphere during the first night spent in a novel environment, apparently to monitor the surroundings for sounds at night (Tamaki et al., 2016). Although difficult to foresee, such findings can reveal overarching principles with unexpected fundamental and clinical applications.

As mammals express two types of sleep, another approach consists of determining which other taxonomic groups express the same states. Besides its fundamental value in understanding when they emerged during evolution (and ultimately with what purpose), identifying species expressing NREM and REM sleep could have applied value. If some species naturally express particularly high or low amounts of a sleep state, finding covarying physiological, anatomical or behavioral features might inform about what functions the sleep state serves. Some species also naturally exhibit changes in their sleep patterns that parallel pathological symptoms in humans. For instance, when exhibiting migratory restlessness in captivity, white crowned sparrows (*Zonotrichia leucophrys gambelii*) show short REM sleep latency, reduced NREM sleep and total sleep time (Rattenborg et al., 2004). The same behavioral patterns are also typical symptoms for depression and mania. Studying the mechanisms underlying this behavioral change, and potential mechanisms to cope with and combat deleterious effects could provide new research avenues for better understanding human diseases. Finally, if NREM and REM sleep is found in small animals, such as fish or flies, this would allow genetic manipulations and massive drug screenings in the search for treatments for human pathologies.

Determining which species have or don't have NREM and REM sleep

This approach, though, relies on determining which species have or do not have NREM and REM sleep. The discovery of the electroencephalogram (EEG) laid the technical ground for the discovery of NREM and REM sleep (Berger, 1929; Davis et al., 1937; Loomis et al., 1937; Aserinsky and Kleitman, 1953). Soon after, the brain electrical correlates became the main signatures of sleep, and the core of sleep research.

Although NREM and REM sleep are currently seen as two independent and in certain respects contrasting states, defining REM sleep and embracing its paradoxes was a strenuous process. In 1953, Aserinsky and Kleitman reported for the first-time recurrent periods of eye movement under closed eyelids, associated with low amplitude EEG and variable breathing rates in sleeping humans (Aserinsky and Kleitman, 1953). However, the study mainly focused on the association between eye movements and dreaming. In 1955 Dement and Chow discovered periods with activated, awake-like EEG cooccurring with bursts of eye movements in cats. The study was published only following numerous rejections (Dement, 1958) because the finding of awake-like brain activity did not fit with the common view that brain activity slows down during sleep. The previous reports, together with the findings of Michel Jouvet describing a paradoxical state with increased arousal thresholds, a lack of muscle tone, but accompanied by an awake-like cortical activity and pontine waves (Jouvet et al., 1959; Jouvet and Mounier, 1960) contributed to the definition of two sleep states and the revisiting the current theories of sleep (Dement, 1990). Similarly, a first report on REM sleep in birds was initially rejected in 1964 from the journal *Science* in part because of the short episodes of REM sleep which were not on par with previous findings in mammals. The study was finally published in the journal *Poultry Science* (Ookawa and Gotoh, 1964). The same year Michel Jouvet's group also reported REM sleep in chickens and pigeons (Klein et al., 1964).

As birds are evolutionarily more closely related to non-avian reptiles than mammals, the presence of REM sleep in birds and mammals raises the question of whether reptiles exhibit NREM and REM sleep. If they do, this would suggest that the two sleep states were present in their last common ancestor; if not, this indicates that NREM and REM sleep evolved independently in mammals and birds. Although a number of species have been recorded using similar techniques as those employed in mammals, the early studies on reptiles 'failed' to reliably identify NREM and REM sleep (Libourel and Herrel, 2016). For several decades the general consensus was that sleep evolved independently in mammals and birds, likely in relation to the independent evolution of homeothermy. The independent evolution scenario was called into question by recent reports (Shein-Idelson et al., 2016; Libourel et al., 2018; Leung et al., 2019; Iglesias et al., 2019; Medeiros et al., 2021). However, as I will discuss in the following section, it remains unclear whether the sleep states reported in bearded dragons and zebrafish larvae are homologous to mammalian and avian NREM and REM sleep.

In the following section, we will take a deeper, critical look into the latest advances in sleep research on animals throughout phylogeny and discuss their implications for understanding the evolution of sleep. This section is based largely on a review and a book chapter published previously (Ungurean et al., 2020b; Ungurean et al., 2021).

Overview over sleep patterns across taxa

Mammalian sleep

Sleep is most intensively studied, and its mechanisms are best understood, in mammals. During sleep, most mammals cycle through two types of sleep, defined by changes in behavior and neural activity: NREM and REM sleep. When compared to wakefulness, NREM sleep is characterized by slower and more regular respiratory and heart rates, reduced skeletal muscle tone and brain temperature, and a synchronous slow oscillation of membrane potentials in neocortical neurons (<1 Hz) generated by the alternance between depolarized ‘up-states’ with action potentials and hyperpolarized ‘down-states’ without, resulting in large, slow-waves on the EEG (Zepelin et al., 2005; Steriade et al., 1993) (Figure 1). Thalamocortical spindles, consisting of short bursts of faster waves (6–15 Hz depending on the species), are also hallmarks of NREM sleep (Klinzing et al., 2019; Diekelmann and Born, 2010; Voirin et al., 2014). Hippocampal sharp-wave ripples (80–140 Hz) also occur during NREM sleep, as well as during pauses in waking activity, such as grooming and feeding (Buzsáki, 2015). Conversely, REM sleep is characterized by irregular respiratory and heart rates, skeletal muscle twitching occurring on a background of muscle atonia, increased brain temperature, greatly diminished thermoregulatory responses, rapid eye movements under closed eyelids, and wake-like EEG activity (Peever and Fuller, 2017). In some mammals (e.g., rodents), a hippocampal theta rhythm (4–8 Hz) similar to that occurring in awake, ambulating animals occurs during REM sleep. The pattern of cycling between NREM and REM sleep changes throughout the sleep period, with larger amounts of NREM sleep and increased EEG slow wave activity (SWA; ~0.5–4.5 Hz power density) early on and larger amounts of REM sleep later in the sleep bout (Tobler, 2005). Since SWA increases following extended wakefulness and decreases during sleep, it is thought to reflect homeostatically regulated processes (Tononi and Cirelli, 2014). SWA correlates with arousal thresholds and therefore reflects sleep intensity and depth (Neckelmann and Ursin, 1993). Finally, the level of SWA increases (or decreases) locally in the mammalian cortex reflecting brain use (or disuse) during prior wakefulness (Huber et al., 2004; Huber et al., 2006).

Avian sleep

Non-REM Sleep

Avian NREM sleep, like mammalian NREM sleep, is characterized by high-amplitude, slow (peak at 2 Hz) EEG waves (Figure 1B). Slow-waves likely reflect the alternations of neuronal membrane potentials between depolarized up-states with action potentials and hyperpolarized down-states without. Similar to the mammalian neocortex, slow-waves travel through the avian cortex (hyperpallium) (Figure 2) (van der Meij et al., 2019a). Birds that spend most of the day awake show a decline in NREM sleep-related SWA; during sleep at night, suggestive of mammalian-like NREM sleep homeostasis

(Szymczak et al., 1996; Rattenborg et al., 2004). Accordingly, in most studies, short-term (6–8 h) sleep deprivation results in an increase in SWA during subsequent sleep (Martinez-Gonzalez et al., 2008; Jones et al., 2008; van Hasselt et al., 2019). However, a rebound in SWA was not found after 24 hours of sleep deprivation (Tobler and Borbély, 1988). Also, in the studies that varied the duration of sleep deprivation, a mammal-like dose-dependent positive relationship between sleep deprivation duration and the subsequent increase in SWA was not found (van Hasselt et al., 2019; van Hasselt et al., 2020).

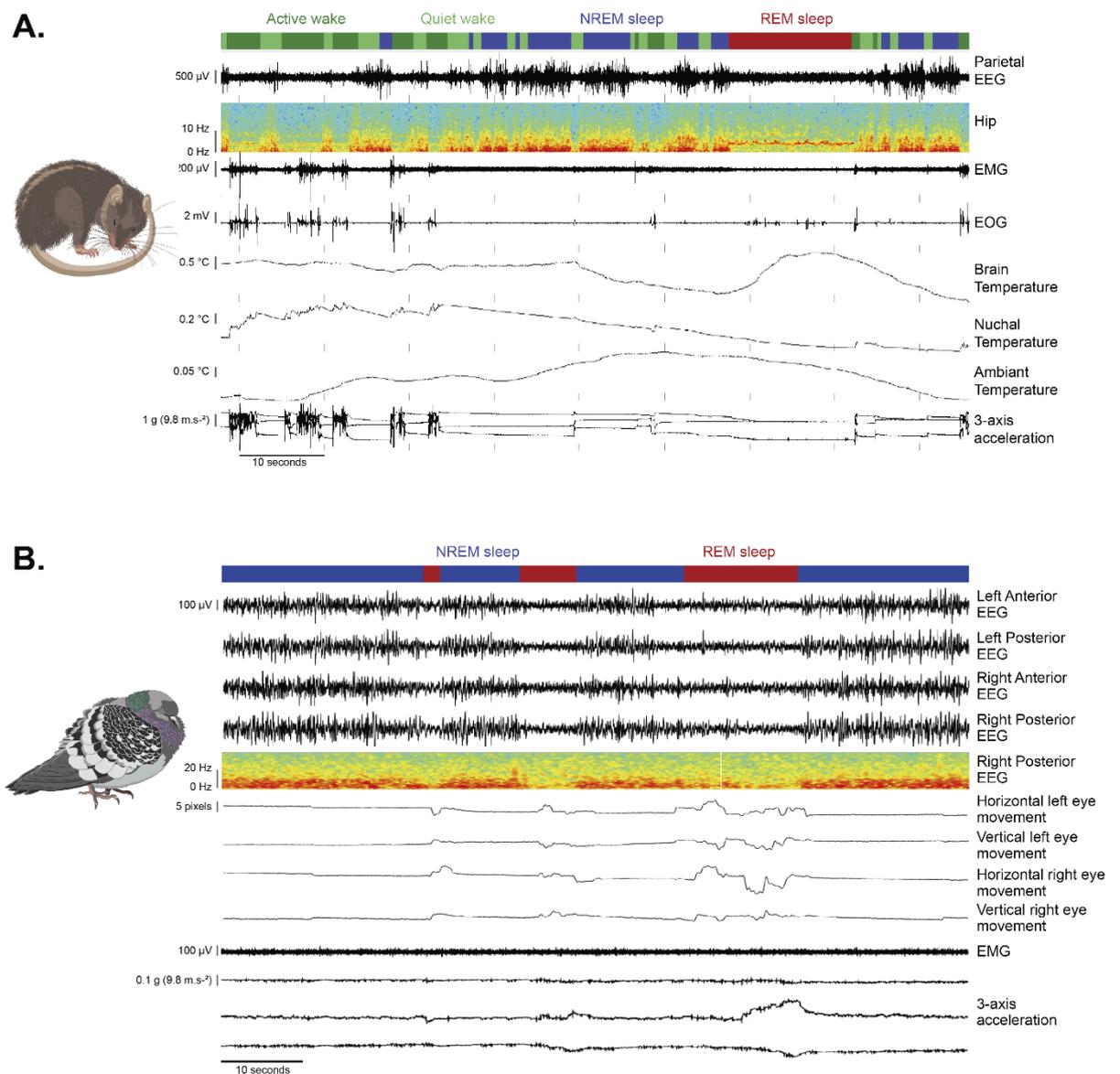


Figure 1. Main behavioral correlates of wakefulness and sleep in mammals and birds.

(A) Raw signal traces over 90 s illustrating the correlates of different vigilance states in a rat. From top to bottom: hypnogram of the wake/sleep state scoring (active wake in dark green, quiet wake in light green, non-rapid eye movement (NREM) sleep in blue, and rapid eye movement (REM) sleep in red); parietal electroencephalogram (EEG); time frequency representation of the hippocampal local field potential; electromyogram (EMG) (high pass filter set at 10 Hz, order 2);

electrooculogram; brain temperature; nuchal temperature; ambient temperature; 3-axial accelerometry.

(B) Raw signal traces over 90 s illustrating the correlates of NREM and REM sleep in a pigeon. From top to bottom: hypnogram of sleep state scoring (NREM sleep in blue and REM sleep in red); left anterior and posterior hyperpallial EEG; right anterior and posterior hyperpallial EEG; time frequency representation of the right posterior hyperpallial EEG; left and right eye movements along horizontal and vertical axes defined relative to the plan of eyelid closure. Positive increases in signal values represent rostral and dorsal movements respectively; EMG (high pass filter set at 10 Hz, order 2); 3-axial accelerometry.

Panel A adapted from Massot et al., 2019. Panel B reproduced from Ungurean et al., 2020b.

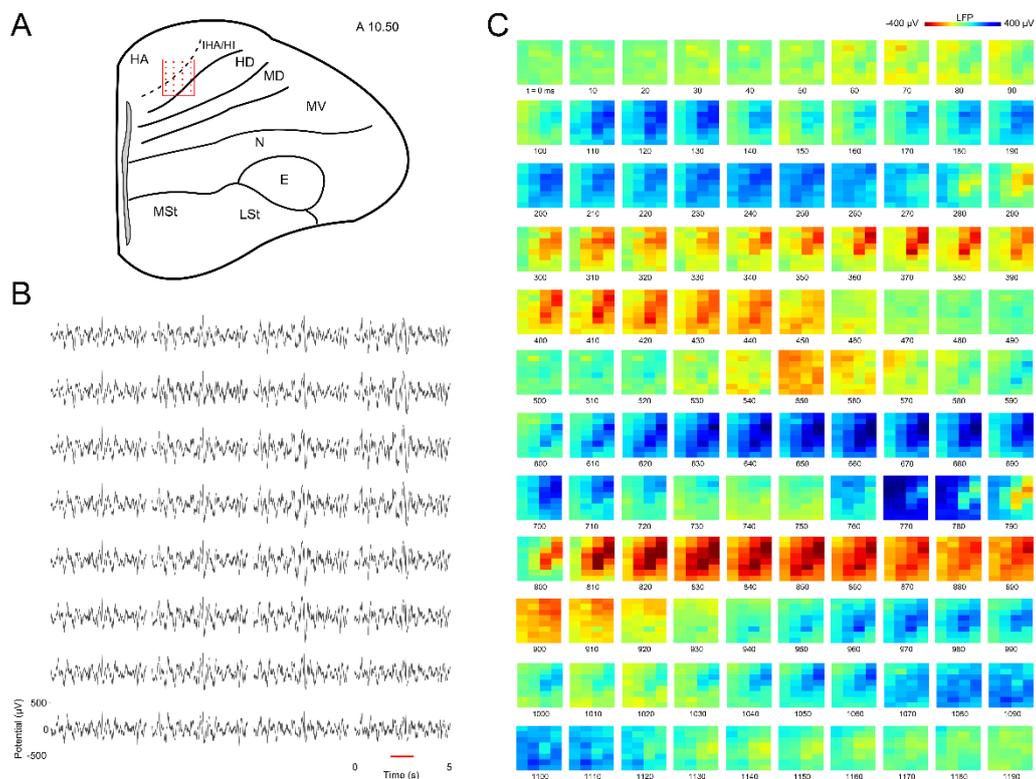


Figure 2. Traveling slow waves in the avian cortex (hyperpallium) during NREM sleep.

(A) Position of a 32-channel silicon electrode grid (red) in the hyperpallium of a pigeon (medial, left and dorsal, top). Input from the avian lateral geniculate nucleus (LGN) projects primarily to the interstitial part of hyperpallium apicale (IHA) and the hyperpallium intercalatum (HI). The underlying hyperpallium densocellulare (HD) receives relatively little input from the LGN. The hyperpallium overlies and is interconnected with the dorsal and ventral mesopallium (MD and MV) and nidopallium (N).

(B) Five-second example of local field potentials showing the spatial distribution of slow waves in the hyperpallium during NREM sleep.

(C) Propagating slow waves during NREM sleep: red underlined 1.2 s episode from panel (B) is visualized in a sequence of image plots where pixels represent electrode sites and electrical potential is coded in color. Both negative and positive potentials are largest in amplitude in the thalamic input layers. They also propagate most prominently within these layers and, to a lesser extent, the overlying hyperpallium apicale (HA).

E, entopallium; LSt, striatum lateral; MSt, striatum medial.

Reproduced from van der Meij et al., 2019a.

Nonetheless, as in mammals, SWA increases and decreases locally in the avian brain following local use and disuse, respectively, during prior wakefulness, possibly reflecting underlying changes in synaptic strength (Figure 3) (Lesku et al., 2011b). Although mammalian NREM sleep is associated with thalamocortical spindles, these rhythms have not been found in birds (van der Meij et al., 2019a). Hippocampal recordings during sleep have revealed sharp-wave ripples in some, but not all, avian species (Rattenborg et al., 2011; van der Meij et al., 2020; Payne et al., 2021). More research is needed to understand this diversity and the degree of homology between sharp-wave ripples reported in birds and mammals.

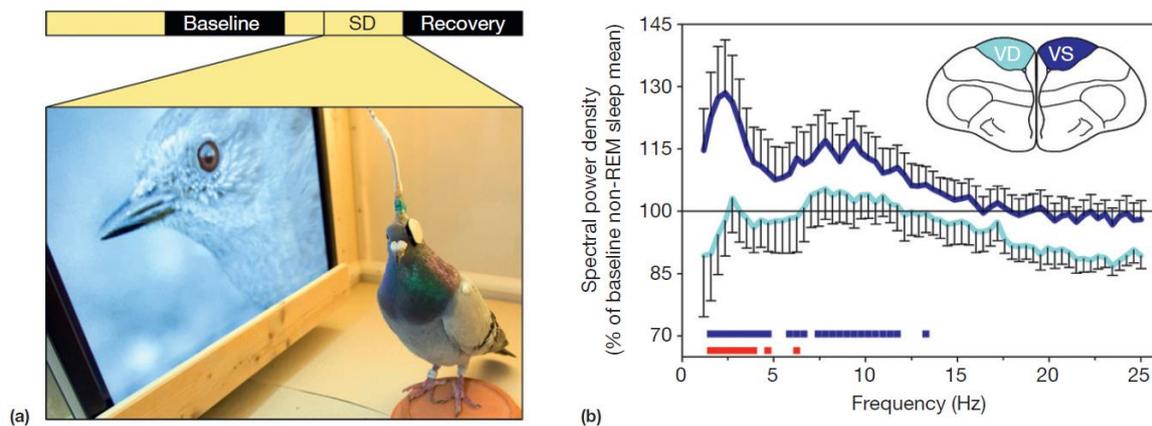


Figure 3. Avian local sleep homeostasis.

(A) Experimental design: a 12 h baseline night follows an undisturbed day; 4 h into the next day, pigeons were kept entirely awake while watching David Attenborough’s “The Life of Birds” with only one eye; the birds were then allowed to engage in recovery sleep.

(B) Spectral power density of NREM sleep during the first 3 h of the recovery night for the visually-stimulated hyperpallium (VS, dark blue) and visually-deprived hyperpallium (VD, light blue) expressed as a percent of the baseline night mean (100% line). Colored squares at the bottom of the plot reflect pairwise comparisons between power density of the VS hyperpallium during the recovery and baseline nights (dark blue), and the VS and VD hyperpallia during the recovery night (red). Note the significant <5 Hz inter-hyperpallial asymmetry in power density with greater power in the VS hyperpallium. Power density of the VD hyperpallium was not significantly different from baseline. Inset: drawing of a frontal view of a transverse section through the cerebrum of a pigeon highlighting the hyperpallium.

Modified from Lesku et al., 2011b.

REM Sleep

Many features of avian REM sleep largely overlap with those observed in mammals (Figure 1). As in mammals, the EEG during avian REM sleep resembles the high-frequency, low amplitude activity of an alert bird, but with bilateral eye closure, REMs, and twitches of the skeletal muscles. Gamma activity, a feature of mammalian REM sleep, has also been found in birds engaged in REM sleep (van der Meij et al., 2019a). As in mammals, thermoregulatory responses are reduced during REM sleep compared to wakefulness and NREM sleep (Heller et al., 1983). The incidence and duration of REM sleep episodes generally increase across the main sleep period in birds, a pattern also observed in mammals (Szymczak et al., 1993; Ayala-Guerrero et al., 2003; Low et al., 2008; Martinez-Gonzalez et al., 2008; Canavan and Margoliash, 2020). Furthermore, in most studies, REM sleep increases following sleep loss in both mammals and birds (Tobler and Borbély, 1988; Low et al., 2008; Martinez-Gonzalez et al., 2008; Newman et al., 2008 but see van Hasselt et al., 2019). Finally, REM sleep is highest in young and declines throughout ontogeny in altricial barn owls (*Tyto alba*), as in altricial mammals (Scriba et al., 2013).

Some other features of mammalian REM sleep are only partially expressed or have not been found in birds (Figure 2). Unlike mammals in which episodes of REM sleep can last several minutes, episodes of avian REM sleep rarely last longer than 10 s. Nonetheless, birds can exhibit over 750 episodes of REM sleep during a single night (Tisdale et al., 2018b; Canavan and Margoliash, 2020). Finally, in contrast to some mammals (e.g. rodents), REM sleep in birds does not appear to be accompanied by a hippocampal theta rhythm (Rattenborg et al., 2011).

The muscle atonia typical of mammalian REM sleep is rarely observed in birds. Nonetheless, birds often exhibit behavioral signs of reduced tone, such as head drooping, swaying, sliding of the wings off the side of the body, or the collapse of feathers held erect during preceding NREM sleep. A study of domestic geese suggests that birds are able to regulate muscle tone during REM sleep in a posture-dependent manner (Dewasmes et al., 1985). When geese enter REM sleep with their head fully supported on their back, atonia of the neck muscles is observed, but when they enter REM sleep with the head facing forward, unsupported, the head drops in a controlled manner and some muscle tone is maintained (Figure 4). Birds likely rely on this ability to partially or locally regulate muscle tone to balance while sleeping on one foot during REM sleep. Indeed, contrary to the popularly held view that perching birds rely on a passive perching mechanism in their foot to grasp a perch, a series of experiments in starlings indicates that perching birds actively hold on to the perch during sleep (Galton and Shepherd, 2012).

Neural signatures of sleep in non-avian reptiles

The presence of similar states in mammals and birds suggests that either these states were already present in their most recent common ancestor, or that they evolved independently in each lineage via convergent evolution. Since birds are a derived type of reptile, one would expect their closest living relatives (the non-avian reptiles) to show similar states if they were present in the common ancestor to mammals and birds. Unlike most studies on these endothermic animals which revealed largely consistent findings across a diverse array of species (Lesku et al., 2008b), early studies on reptiles reported highly variable results across species, and even contradictory results within the same species (Libourel and Herrel, 2016; Lesku et al., 2019). As the source of this variability remains uncertain, it has been difficult to reach a firm conclusion on how NREM and REM sleep evolved in mammals and birds. Recent work suggests some of this diversity may in fact reflect real, unexpected differences in the way sleep manifests across non-avian reptiles (Libourel et al., 2018).

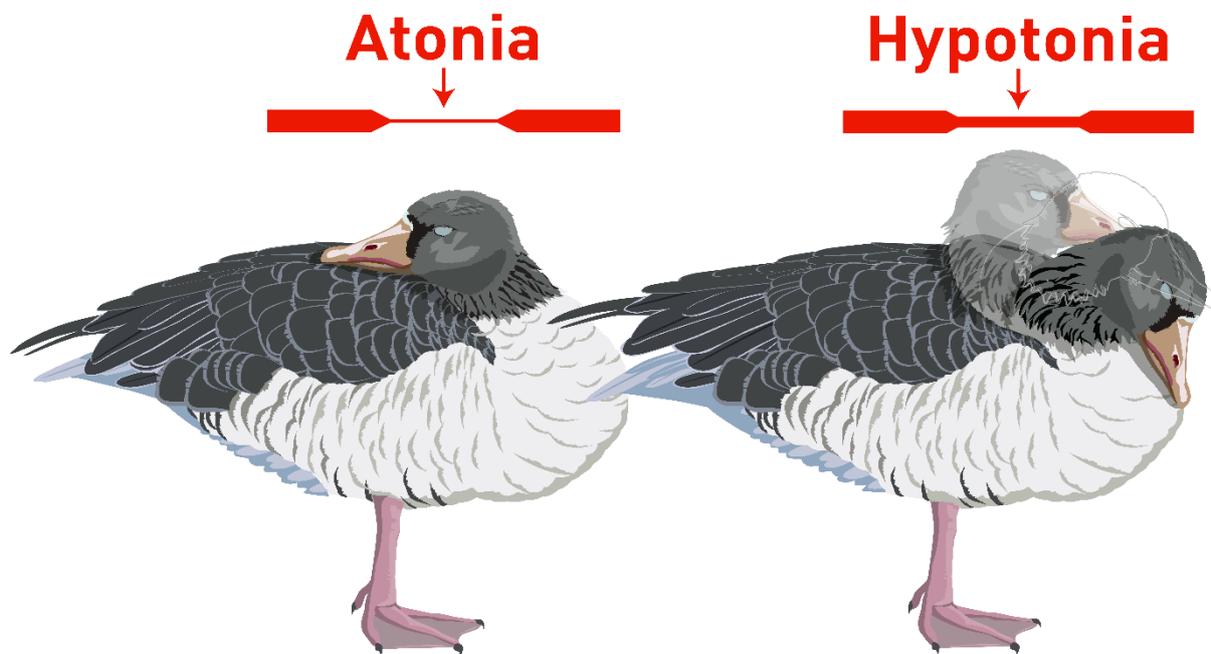


Figure 4. Posture-dependent regulation of muscle tone during REM sleep in geese. Along with many other birds, geese can engage in REM sleep while balancing on one foot. The head can face backward, supported on the birds back (left bird) or forward and unsupported (right bird). When the head is supported it usually remains still and the neck electromyogram (EMG) shows atonia. By contrast, when the head is unsupported it drops in a controlled manner and the neck EMG shows hypotonia. Birds only occasionally show behavioral signs of reduced tone in the muscles involved in holding the wings against the body and balancing on one foot. Illustration by Damond Kylo based on Dewasmes et al., 1985.

Reproduced from Blumberg et al., 2020.

Shein-Idelson et al. recently recorded the intracerebral activity of bearded dragons (*Pogona vitticeps*) during the dark period while the animals assumed a sleep posture with closed eyes (Shein-Idelson et al., 2016). They reported two distinct patterns of activity that alternated with an unprecedented regularity every 80 s (Figure 5A). The first state was characterized by frequent local field potential (LFP) depolarizations, which they named sharp-waves, based on their morphology. Most sharp-waves were accompanied by a burst of high (>70 Hz) frequency activity. The second state was characterized by LFP activity similar to wakefulness and was correlated with eye movements under closed eyelids.

The authors noted the similarities between the sharp-waves in dragons and the sharp-wave ripples recorded from the hippocampus in mammals during NREM sleep, as did earlier researchers who also found sharp-waves during sleep in a variety of reptiles (Hartse, 1994). However, the sharp-waves in dragons, and those described in earlier studies, were recorded from non-hippocampal brain regions that generate slow-waves in birds (Beckers et al., 2014; Lesku et al., 2011b; van der Meij et al., 2019a, b). Moreover, high-frequency activity is not unique to sharp-wave ripples, as it also occurs during the depolarized upstate of the slow oscillation in neuronal membrane potential that gives rise to EEG and LFP slow-waves in mammals (Grenier et al., 2001). Consequently, sharp-waves in dragons may reflect a phenomenon less comparable to mammalian hippocampal sharp-wave ripples, and instead be simply a variant (or evolutionary precursor) of slow-waves, with upstates shorter than those found in mammals and birds (Tisdale et al., 2018a). Interestingly, the latter suggests that mammals and birds independently evolved increased investment into upstates, and decreased investment into downstates, and the purported benefits of each: memory reactivation and neuronal rest, respectively (Tisdale et al., 2018a). In this respect, even if reptilian sharp-waves do not reflect the same phenomenon as mammalian sharp-wave ripples, they nevertheless may still be involved in memory consolidation. Reptilian sharp-waves might also support other unknown functions, unrelated to memory consolidation.

Viewed in isolation, the findings from bearded dragons suggest that two sleep states with features suggestive of NREM and REM sleep were present in the common ancestor to mammals and birds. However, several earlier studies failed to detect two distinct states in reptiles, including crocodylians, the closest living relatives to birds (Flanigan Jr et al., 1973; Warner and Huggins, 1978; Meglasson and Huggins, 1979). Although methodological differences might explain some of the differences between the earlier studies and the findings in the dragon, a recent study suggests that there may be real and unexpected diversity in the way sleep manifests across reptiles. Libourel and colleagues examined sleep in the bearded dragon and another lizard, the argentine tegu (*Salvator merianae*), using the same methods (Libourel et al., 2018). Importantly, they replicated the findings previously reported in the bearded dragon and found that the argentine tegu also exhibited two sleep states. However, the brain activity during these states differed markedly from that described in the dragon (Figure 5B). During the first

sleep state, no particular LFP frequency was dominant; sharp-waves occurred, but they were infrequent and had shorter durations when compared to the sharp-waves found in dragons. Conversely, the second sleep state was dominated by a 15 Hz oscillation not found during wakefulness that correlated with the occurrence of eye movements and a slight decrease in muscle tone compared to the first state. Unlike the regular cycling between sleep states observed across the night in dragons, switching between states in tegus was irregular, and the second state was more likely to occur at the beginning and end of the night.

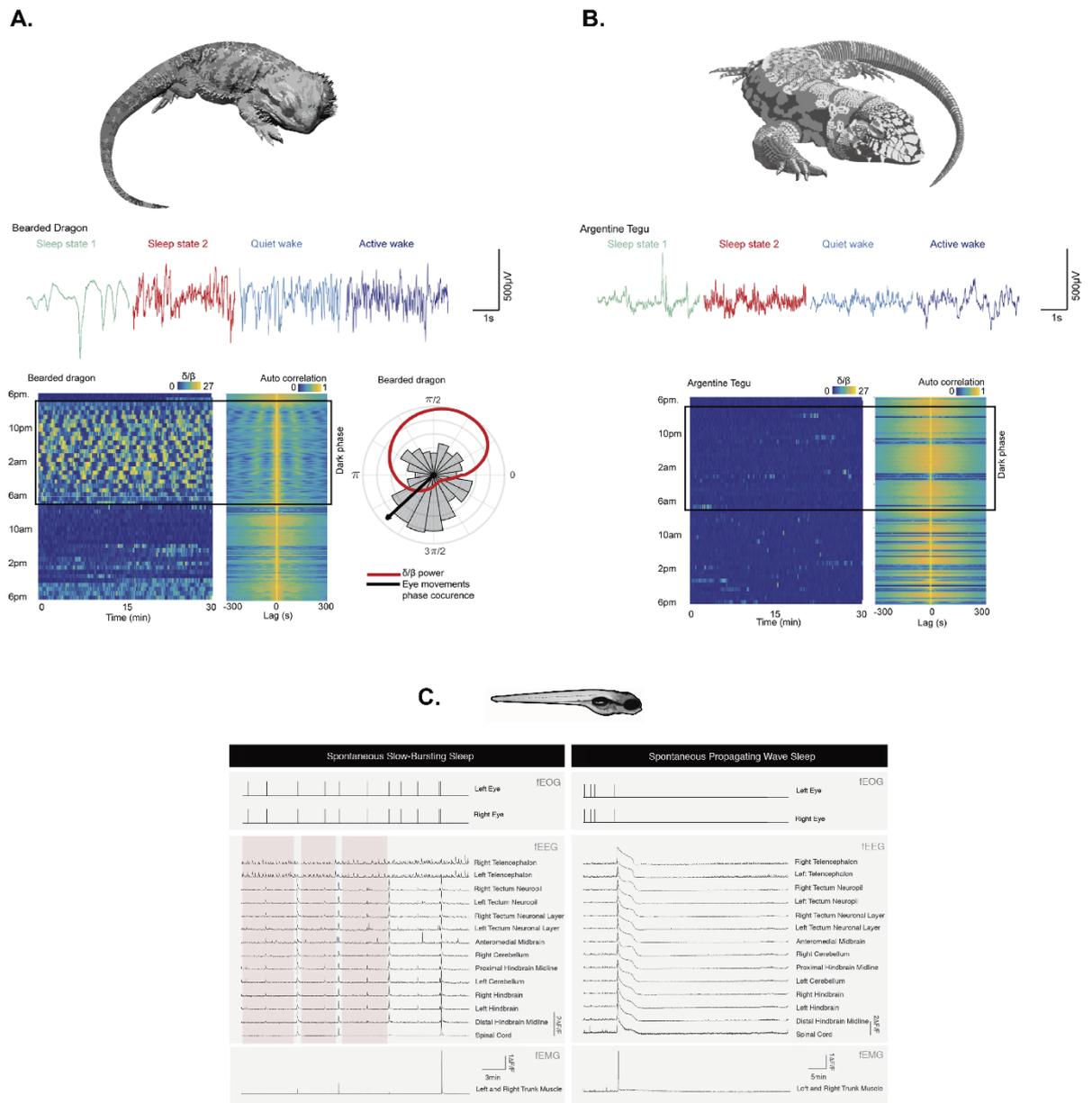


Figure 5. Comparative overview depicting the principal known neural and/or behavioral sleep characteristics in two reptile species and zebrafish larvae.

(A) Correlates of different vigilance states in the bearded dragon. Top: example of raw signals from one local field potential (LFP) channel recorded in the dorsal ventricular ridge (DVR) of a

bearded dragon during sleep state 1 (sharing similarities with mammalian NREM sleep) in turquoise, sleep state 2 (sharing similarities with mammalian REM sleep) in red, quiet wake in light blue, and active wake in dark blue. Bottom left: band power ratio ($\delta[0.5-4 \text{ Hz}]/\beta[11-30 \text{ Hz}]$). Each horizontal segment represents 30 min of the ratio computed with a 10-s window and a step of 0.1 s. The value of the ratio is color coded from 0 (blue) to 27 (yellow). Each line, from the top to bottom represents the changes in the ratio over 24 hour from 6 PM onwards. The dark rectangle indicates the dark period. Bottom center: normalized autocorrelation map of the δ/β ratio. The autocorrelation was computed within 600-s windows with a step of 1 s. Both figures reveal rhythmic cycling every 90 s between periods rich in δ frequencies (yellow) and periods rich in β frequencies (blue). This cycling takes place during the dark period, when the animal is lying on the floor with the eyes closed. Bottom right: The distribution of the eye movements within each δ - β cycle; the mean phase is represented with a black arrow. The red line represents the mean δ/β power ratio across the δ - β cycle.

(B) Neural correlates of different vigilance states in the argentine tegu. Top: example of raw signals recorded in the DVR of an argentine tegu using the same methodology and annotations as for the bearded dragon. Bottom: δ/β ratio (left) and autocorrelation (right) represented as for the bearded dragon. The two figures reveal the absence of a clear cycling pattern in the δ/β power ratio over the 24 hour.

(C) Polysomnographic recordings of the two sleep states spontaneously exhibited by zebrafish larvae (left – 30 min recordings depicting slow bursting sleep (SBS) activity; right – 60 min recordings depicting propagating wave sleep (PWS) activity). From top to bottom: eye movements; neural activity; muscular activity. Red boxes in the middle right panel show transient synchrony in the dorsal pallium, indicative of SBS, occurring between muscle twitches. The large amplitude signals in the middle left panel reflect the transient activation of the entire neural axis, followed by a period of neural silence. A large, prolonged contraction of the tail muscles occurs at the onset of neural axis activation.

Panels A and B reproduced from Libourel et al., 2018. Panel C adapted from Ref. Leung et al., 2019.

Collectively, these studies suggest that some lizards exhibit two sleep states, which share some similarities to NREM and REM sleep in mammals and birds. However, the large differences in terms of sleep neural signature and architecture in the two lizard species, suggests that some of the diverse findings in the older reptile literature may in fact be correct. As this earlier research failed to detect REM sleep in crocodylians, the closest living relatives to birds, and in the cortex and brainstem of sleeping turtles (Eiland et al., 2001), their sister group, it remains unclear whether REM-like sleep was present in the stem amniote or evolved independently several times. Further research on crocodylians and turtles is needed to determine if REM sleep was somehow missed in the earlier studies.

Two sleep states might also be present in other taxonomic groups, including invertebrates

Studies of amphibians and fishes can likewise shed light on the evolution of sleep states in vertebrates (Libourel and Herrel, 2016; Kelly et al., 2020). Amphibians remain

understudied; however, a new study using state-of-the-art imaging on young zebrafish (*Danio rerio*) reveals two sleep states in these aquatic animals (Leung et al., 2019). As larva, zebrafish are transparent, allowing for whole brain and body imaging. Researchers trained larvae to sleep while embedded in an agarose gel that allowed brain and muscle activity, and eye movements of transgenic fish to be recorded using light-sheet microscopy. Interestingly, the authors described two spontaneous states. The first state, named slow bursting sleep (SBS), was characterized by synchronized activation of the neurons in the dorsal pallium (a homologue of the mammalian neocortex) alternating with periods of neural silence (Figure 5C). Sleep deprived fish that were more likely to enter this state, showed stronger activations with a higher degree of synchrony, but longer intervals between successive activations, when compared to spontaneous sleep. In non-sleep deprived individuals, this state was associated with decreased muscle tone, isolated eye movements, and was interrupted by brief trunk muscle activity. The onset of the second sleep state, named propagating wave sleep (PWS), was characterized by eruptions of activity across the entire neuroaxis that lasted around five minutes, during which a wave of muscular activity propagated through the trunk for 10–15 s. This activation was followed by around 20 min of reduced brain activity. During PWS, eye movements were absent. The administration of hypnotics known to induce NREM and REM sleep in mammals induced two states with similar brain activity to those expressed spontaneously, albeit with different temporal dynamics.

The authors relate the two states to NREM and REM sleep, suggesting that they emerged at least 450 million years ago. However, in contrast to the slow oscillations between neuronal activity and neuronal quiescence occurring during NREM sleep in the mammalian neocortex, telencephalic neurons in the zebrafish oscillate at much lower frequencies. Moreover, an increase in neural activity spanning the entire brain network, followed by long-lasting neural suppression of activity has never been described in animals exhibiting REM sleep. The prolonged wave of muscular activity that propagates through the trunk during the onset of PWS is also unlike the brief twitches observed during REM sleep in mammals and birds. Consequently, whereas there are some general similarities between SBS and mammalian and avian NREM sleep, as well as NREM sleep-like state in reptiles, the link between PWS and REM sleep is less clear. Nonetheless, the brain-wide bursts of activity are certainly fascinating and warrant further study to determine their relationship with REM sleep or other processes.

Interestingly, two electrophysiological states have also been described in the fruit fly (*Drosophila melanogaster*) (Yap et al., 2017). LFP recordings revealed decreased activity during sleep compared to wakefulness, as in earlier recordings of the medial brain of *Drosophila* (Nitz et al., 2002; Bushey et al., 2015). However, unlike these other studies, which employed different methodologies, intermittent oscillations in the range of 7–10 Hz were observed during spontaneous sleep. These oscillations were most likely to occur during the first and last minutes of a sleep bout suggesting that they reflect a transitional sleep stage. In general terms, the presence of a change in brain activity is in

line with another arthropod, the crayfish (*Procambarus clarkii*), which also shows a sleep-related change in brain activity, albeit at different frequencies (Ramon et al., 2012). However, the study on drosophila is the first to electrophysiologically describe two sleep states in an invertebrate. Whether these two states are in any way homologous, or analogous, to NREM and REM sleep is unknown.

Other lines of evidence support the existence of two sleep states in invertebrates, with behavioral features suggestive of NREM and REM sleep. For instance, some insects twitch their antennae during rest, and fruit flies exhibit more leg and proboscis twitches when they are young (Dilley et al., 2018), similar to the frequent twitches observed in mammals during early development (Blumberg et al., 2013b). Perhaps even more convincing are the examples of cuttlefish (*Sepia officinalis*) and octopus (*Octopus insularis*). During wakefulness, the two mollusk species exhibit complex changes in skin color and pattern, mediated by chromatophores that are used to communicate with conspecifics and camouflage themselves from predators or prey. During sleep, they cycle through periods when they are quiescent with constricted pupils and mimic the substrate, and periods when their tentacles twitch, the eyes move and their color and patterning changes, resembling only fragments of the patterns commonly displayed during wakefulness (Medeiros et al., 2021; Iglesias et al., 2019; Frank et al., 2012). Interestingly, chromatophores are controlled by striated muscles, the very same type of muscle that twitches during mammalian REM sleep. These periods of activity are thus tantalizingly reminiscent of the twitching that occurs during REM sleep. Further research on cuttlefish, octopus, and other mollusks (Vorster and Born, 2015), is certainly warranted, as it might reveal whether NREM and REM sleep evolved very early in the evolution of animals, or independently multiple times.

The previous section highlights that many organisms exhibit two sleep states, which share some common features with the mammalian NREM and REM sleep. However, when carefully scrutinized, none shares the exact same definition with mammalian sleep. Interestingly, however, even within mammals and birds, the same rules agreed-upon in adult healthy individuals do not apply throughout ontogeny, wherein early on, NREM and REM sleep cannot be distinguished from electrophysiological recordings. Monotreme mammals and ostriches (*Struthio camelus*) are also hard to fit within the rules, as they exhibit sleep states with mixed features of both NREM and REM sleep (Allison et al., 1972; Siegel et al., 1996; Nicol et al., 2000; Siegel et al., 1999; Lesku et al., 2011b). In platypuses (*Ornithorhynchus anatinus*), behaviors typical of REM sleep, like eye movements and twitches and reduced muscle tone occur while their cortex shows slow wave activity, typical of NREM sleep (Siegel et al., 1999); while ostriches exhibit the typical EEG activation accompanying REM sleep behaviors in addition to mixed states similar to those observed in the platypus (Lesku et al., 2011a). The boundaries between NREM and REM sleep have also been blurred in adult mammals by recent studies showing that not all regions of the mammalian brain always switch from one state to

another at the same time, with the hippocampus often preceding the neocortex in transitioning to REM sleep (Emrick et al., 2016; Durán et al., 2018); local slow waves can occur in REM sleep (Funk et al., 2016); and twitches – behaviors typically associated with REM sleep – can occur during NREM sleep, in association with spindles in human infants (Sokoloff et al., 2021). Finally, patterns of sleep can occur locally during wakefulness (Nir et al., 2011; Vyazovskiy et al., 2011), suggesting that defining features of wake and sleep are also not necessarily mutually exclusive.

Thesis outline

In addition to furthering our knowledge about avian REM sleep, my doctoral work highlights the necessity to embrace the phenotypical diversity of sleep and adapt rigid mammalian-based definitions. The work is divided into three chapters (Chapter 2-4) and presents new findings on avian sleep which partially overlap, are completely opposite, or largely overlap with our current knowledge derived from mammalian studies.

In **Chapter 2** we present our findings that cortical brain temperature increases following REM sleep episodes in pigeons, but not in the Australian bearded dragon. Although avian and mammalian REM sleep share many common features, in birds the magnitude of the increase in cortical temperature is ten times smaller. These are the first recordings of cortical brain temperature using sensitive methods, and expand our understanding of the physiology of REM sleep in mammals and birds, and the REM sleep-like state in dragons.

Chapter 3 was inspired by the observation that pigeon's pupils are visible through the closed eyelid under infrared (IR) lighting. Thus, we systematically recorded for the first time the pupillary activity of awake and sleeping pigeons and showed that pupil behavior is opposite in birds compared to mammals. We also found that rapid pupillary constrictions and dilations, which we called rapid iris movements (RIMs) primarily occur during REM sleep and are controlled by striated muscles in the iris.

Finally, in **Chapter 4** we present preliminary data from functional magnetic resonance imaging (fMRI) recordings in sleeping pigeons. This experiment was initially designed with the aim of revealing the neural substrates underlying RIMs. In this chapter, however, we present preliminary data on REM and NREM sleep related activity. We found broad activation spanning the brainstem, midbrain, thalamus, and primary sensory, motor, and associative cortical (pallial) areas during REM sleep. Interestingly, NREM was associated with activation largely overlapping with non-brain areas, such as the ventricles. These results are consistent with previous finding on mammals, wherein REM sleep is characterized by broad activity in subcortical (subpallial) and cortical (pallial) areas, and NREM sleep is associated with increased CSF flow.

Box 1 – Anatomical organization of the avian brain and structural homologies

In an early comparative approach, Ludwig Edinger, considered the father of comparative neuroanatomy, linked behavioral observations in members of different taxa to the structural appearance of different brain areas. Following his observations, he proposed that structures with increased degrees of complexity have progressively evolved by addition over evolutionary time, as anatomical substrates for increasingly complex behavior and cognition (Edinger and Rand, 1908). ‘Lower’ organisms, such as fishes, were thought to have their brain dominated by old structures responsible for instinctive behavior, while mammals, and especially humans, were thought to have evolved a new, additional structure, the cortex, responsible for intelligent behavior. As in other non-mammalian organisms, the avian brain was thought to be dominated by basal ganglia (Figure 6).

However, converging data accumulated, suggesting that birds have high cognitive capabilities, in some species comparable or even surpassing those of mammals that depend on the cortex. This challenged the idea that bird brains are inferior to those of mammals. Several lines of evidence, including gene expression, connectivity, and embryology studies showed that a large part of the avian telencephalon is homologous to the neocortex (Figure 6, Jarvis et al., 2005). As such, an international consortium of neuroscientists revised the avian brain nomenclature. This resulted in numerous structures in the avian forebrain containing the suffix ‘pallium’ instead of ‘striatum’ in their names, reflecting the closer homology to the mammalian cortex, rather than basal ganglia.

Although the avian pallium does not occupy the outer-most layer of the cerebral hemispheres and although it is organized in apparently nuclear subdivisions – unlike the mammalian cortex which is organized in layers and functional columns – a ‘canonical’ mammalian-like pattern of connectivity, with column-like fibers spanning primary sensory areas, and long tangential laminar-like connecting columns to other associative and motor areas were recently described in birds (Stacho et al., 2020). These results further highlight the numerous shared features between the avian pallium and the mammalian cortex.

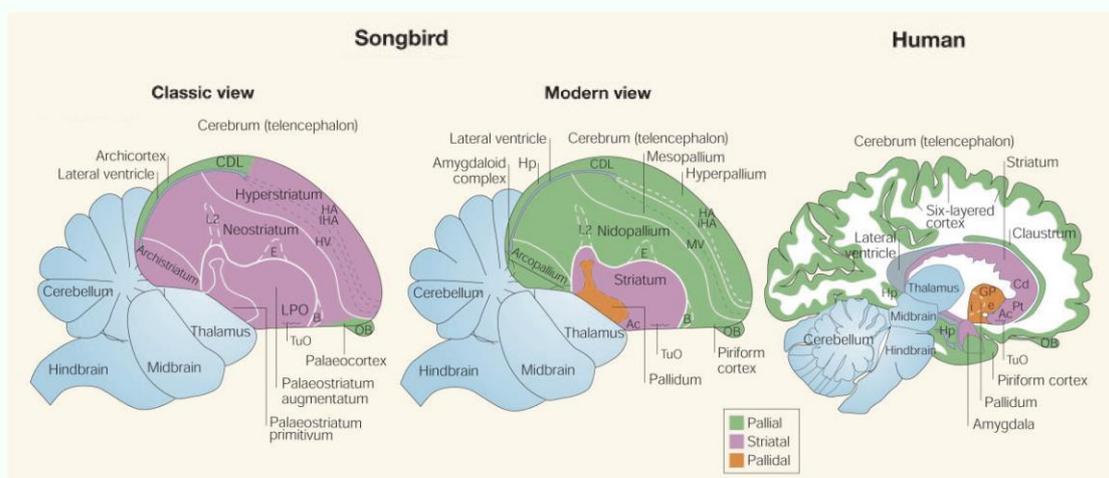


Figure 6. Avian and mammalian brain relationships.

A. Classic view of avian and mammalian brain relationships. Although past authors had different opinions about which brain regions are pallium versus subpallium, individual brain regions were

colored according to the meaning of the names given to those brain regions. Ac, accumbens; B, nucleus basalis; Cd, caudate nucleus; CDL, dorsal lateral corticoid area; E, ectostriatum; GP, globus pallidus (i, internal segment; e, external segment); HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; IHA, interstitial hyperstriatum accessorium; L2, field L2; LPO, lobus parolfactorius; OB, olfactory bulb; Pt, putamen; TuO, olfactory tubercle.

B. Modern consensus view of avian and mammalian brain relationships according to the conclusions of the Avian Brain Nomenclature Forum. Solid white lines are lamina (cell-sparse zones separating brain subdivisions). Large white areas in the human cerebrum are axon pathways called white matter. Dashed grey lines divide regions that differ by cell density or cell size; dashed white lines separate primary sensory neuron populations from adjacent regions. Abbreviations where different from b: E, entopallium; B, basorostralis; HA, hyperpallium apicale; Hp, hippocampus; IHA, interstitial hyperpallium apicale; MV, mesopallium ventrale.

Adapted from Jarvis et al., 2005.

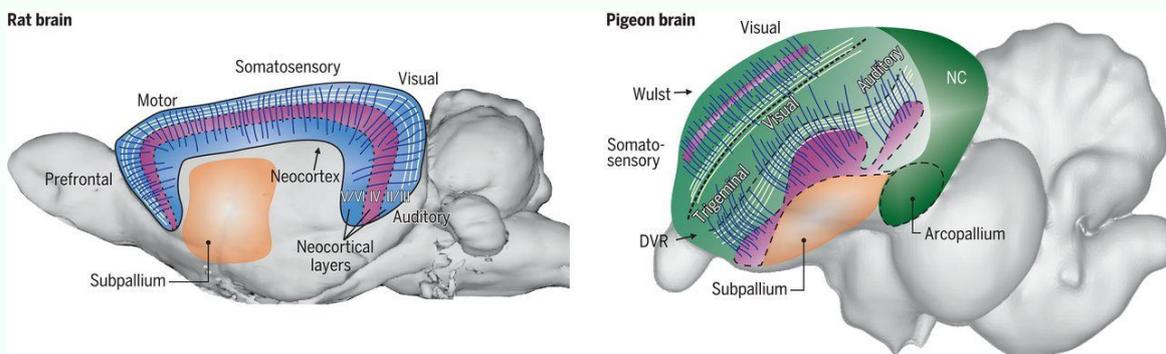


Figure 7. Fiber architectures of mammalian and avian forebrains.

Schematic drawings of a rat brain (left) and a pigeon brain (right) depict their overall pallial organization. The mammalian dorsal pallium harbors the six-layered neocortex with a granular input layer IV (purple) and supra- and infragranular layers II/III and V/VI, respectively (blue). The avian pallium comprises the Wulst and the DVR, which both, at first glance, display a nuclear organization. Their primary sensory input zones are shown in purple, comparable to layer IV. According to this study, both mammals and birds show an orthogonal fiber architecture constituted by radially (dark blue) and tangentially (white) oriented fibers. Tangential fibers associate distant pallial territories. Whereas this pattern dominates the whole mammalian neocortex, in birds, only the sensory DVR and the Wulst (light green) display such an architecture, and the associative and motor areas (dark green), as in the caudal DVR, are devoid of this cortex-like fiber architecture. NC, caudal nidopallium.

Reproduced from Stacho et al., 2020.

Summary of homologies

Below are presented areas which will be described later in this manuscript, their location in the avian brain, and their proposed homologues/analouges when possible. I acknowledge that debate still exists over one-to-one homologies between the mammalian cortical subdivisions and their avian counterparts. Nonetheless, connectivity and functional data is highlighted, to facilitate the interpretation of our results.

Hyperpallium – or hypertrophied pallium; also named Wulst, meaning bulge or swelling in German; appears as a protuberant area typically found along the dorsomedial surface of the cerebral hemispheres (Wild, 2009); has an apparent

layered structure, with four layers, of which one (interstitial hyperpallium apicale, IHA) receives rich thalamic projections from the avian homologous of the lateral geniculate nucleus (dorsal lateral geniculate nucleus, GLd), similar to the mammalian layer IV; divided into a rostral part and a caudal part, namely the rostral hyperpallium and the visual hyperpallium.

Rostral hyperpallium – represents the anterior extremity of the hyperpallium, located nearby the olfactory bulb; relatively small in size compared to the visual hyperpallium (Medina and Reiner, 2000); homologous to the mammalian somatomotor cortex.

Visual hyperpallium – situated dorsomedially, between the rostral hyperpallium and the hippocampal formation; occupies most of the hyperpallium; homologous to the mammalian primary visual area (V1).

Dorsal ventricular ridge (DVR) – although not found at the surface of the brain, but below the hyperpallium, this large area is of pallial nature, based on embryogenesis and expression of molecular markers; based on connectivity and function it is similar to the mammalian cortex; it is divided into two vast territories, the mesopallium (dorsal) and nidopallium (ventral).

Mesopallium – or middle pallium; area extending rostro-caudally below the hyperpallium and the hippocampal formation, and above the nidopallium; considered a higher order associative area; a one-to-one homology with a cortical mammalian sector has not been established (proposed homologies: (1) temporal neocortex (Butler et al., 2011), (2) neocortical layers 2 and 3 (Suzuki et al., 2012), (3) claustrum/insula and parts of the amygdala (Puelles et al., 2016)); nonetheless, known to be involved in various forms of learning, memory and cognition.

Nidopallium – or nested pallium; found below the mesopallium and extending around the dorsal border of the entopallium; is thought to be responsible for high executive functions; the caudo-lateral sector (nidopallium caudolaterale, NCL) is considered by some to be functionally analogous to the mammalian prefrontal cortex (PFC) (Güntürkün and Bugnyar, 2016).

Entopallium – structure situated deep in the avian forebrain, below the nidopallium; it is the primary target of the avian tectofugal visual pathway, receiving projections from the thalamic nucleus rotundus (nRT).

Hippocampal formation – located above the lateral ventricles, occupying the dorsal part and medial wall of the caudal telencephalon; although structurally different compared to the mammalian three-layered hippocampus, it plays a role in spatial memory (Rattenborg et al., 2011).

Arcopallium – arched pallium; situated in the ventral side of the caudo-lateral telencephalon; associative area receiving input from various sensory pallial regions, and projecting to motor areas; a one-to-one homology has not been established, but it is regarded as a major premotor area.

Pallial amygdala – situated dorsolaterally relative to and in proximity of the arcopallium; shares common features in terms of connectivity and function with the mammalian amygdala.

Basal ganglia – found at the base of the avian telencephalon; divided into a striatal and pallidal component; involved in motor learning and motor control.

Striatum – situated in the ventral part of the avian forebrain; is one of the component structures of the basal ganglia.

Globus pallidus – resides below the striatum; is one of the component structures of the basal ganglia; has similar connectivity with the mammalian globus pallidus.

Optic tectum – enlarged bilateral structure situated ventrolaterally below the forebrain; structure homologous to the superior colliculus.

Chapter 2

Comparative perspectives that challenge brain warming as the primary function of REM sleep

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Authors: Gianina Ungurean, Baptiste Barrillot, Dolores Martinez-Gonzalez, Paul-Antoine Libourel, Niels C. Rattenborg

Author contributions: G.U., P.-A.L., and N.C.R. designed the pigeon experiment. G.U. and D.M.-G. performed the pigeon surgeries. In consultation with P.-A.L. and N.C.R., G.U. conducted the experiment and analyzed the pigeon data. B.B. and P.-A.L. designed the bearded dragon experiment, performed the surgeries, conducted the experiment, and analyzed the data. G.U., B.B., P.-A.L., and N.C.R. contributed to writing the manuscript, and D.M.-G. provided comments.

Summary

Rapid eye movement (REM) sleep is a paradoxical state of wake-like brain activity occurring after non-REM (NREM) sleep in mammals and birds. In mammals, brain cooling during NREM sleep is followed by warming during REM sleep, potentially preparing the brain to perform adaptively upon awakening. If brain warming is the primary function of REM sleep, then it should occur in other animals with similar states. We measured cortical temperature in pigeons and bearded dragons, lizards that exhibit NREM-like and REM-like sleep with brain activity resembling wakefulness. In pigeons, cortical temperature decreased during NREM sleep and increased during REM sleep. However, brain temperature did not increase when dragons switched from NREM-like to REM-like sleep. Our findings indicate the brain warming is not a universal outcome of sleep states characterized by wake-like activity, challenging the hypothesis that their primary function is to warm the brain in preparation for wakefulness.

Keywords

REM sleep, NREM sleep, brain temperature, function, bird, reptile, mammal, EEG

Introduction

Sleep is a mysterious state of reduced environmental awareness found in animals ranging from jellyfish to humans (Joiner, 2016; Libourel and Herrel, 2016; Blumberg and Rattenborg, 2016; Nath et al., 2017; Anafi et al., 2019; Iglesias et al., 2019; Kelly et al., 2020). Sleep in mammals is composed of two states, non-rapid eye movement (NREM) sleep and REM sleep, typically distinguished from one another and wakefulness by changes in brain and muscle activity. NREM sleep is characterized by the slow alternation between periods of cortical neuronal silence (down-states) and periods with high firing rates (up-states) that collectively give rise to high voltage, slow waves (0.5 – 4 Hz) in the electroencephalogram (EEG) (Timofeev et al., 2001; Vyazovskiy et al., 2009). REM sleep is typically characterized by continuous high firing rates comparable to wakefulness (Evarts, 1962; Mukhametov and Strokova, 1977; Timofeev et al., 2001; Vyazovskiy et al., 2009; *reviewed in* Jones, 2016), resulting in low voltage, high frequency EEG activity. In addition, muscle tone is reduced during NREM sleep, when compared to wakefulness, and absent during REM sleep (Peever and Fuller, 2017). Nonetheless, brief muscle twitches occur during REM sleep resulting in movements, such as rapid eye movements. The patterns of brain activity that characterize NREM and REM sleep have been implicated in various forms of synaptic plasticity (Chauvette et al., 2012; Tononi and Cirelli, 2014; Boyce et al., 2016; Li et al., 2017; Klinzing et al., 2019), and twitching during REM sleep is thought to play a role in mapping the sensorimotor cortex during development and possibly adulthood (Dooley et al., 2020; Blumberg et al., 2020).

In addition to brain and muscle activity, other physiological processes also differ between NREM and REM sleep in mammals. Notably, in most mammals examined, cortical and sub-cortical brain temperature (T_{br}) decreases during NREM sleep and increases during REM sleep (Kawamura and Sawyer, 1965; Hayward and Baker, 1969; Kovalzon, 1973; Obál Jr et al., 1985; Wehr, 1992; Franken et al., 1992; Gao et al., 1995; Landolt et al., 1995; Csernai et al., 2019; Hoekstra et al., 2019; Komagata et al., 2019; see Hayward and Baker, 1968 and Hayward and Baker, 1969 for conflicting results in rhesus monkeys (*Macaca mulatta*)). Brain warming during REM sleep is thought to result from an increase in blood flow from the warmer body core to the brain to support this activity (Denoyer et al., 1991; Wehr, 1992; Parmeggiani, 2007; Pastukhov and Ekimova, 2012; Bergel et al., 2018). In this regard, brain warming might simply be a functionless biproduct of functions that require increased neuronal activity, such as brain development and other types of synaptic plasticity. Alternatively, it has been proposed that increased neuronal activity is the mechanism the sleeping brain employs to periodically warm itself (Wehr, 1992; Lyamin et al., 2018). According to this hypothesis, brain warming is beneficial because it counteracts cooling occurring during preceding NREM sleep, and thereby prepares the animal to awaken and rapidly interact adaptively with the environment (Wehr, 1992; Lyamin et al., 2018). The brain warming hypothesis remains largely untested, and it is unclear whether the brain warms during similar sleep states in other taxonomic groups.

Birds exhibit NREM and REM sleep characterized by changes in EEG activity similar to those observed in the mammalian cortex, despite differences in the organization of homologous neurons. In contrast to the laminar organization of neurons, with apical dendrites spanning the layers of the mammalian cortex, developmentally and functionally homologous ('cortical') regions of the avian brain (see also Box 1), such as the visual hyperpallium (homologue of the mammalian primary visual cortex) (Medina and Reiner, 2000; Güntürkün et al., 2017; Briscoe and Ragsdale, 2018; Stacho et al., 2020), are composed of nuclear structures consisting of densely packed stellate neurons (Olkowicz et al., 2016). Similar nuclear structures comprise the dorsal ventricular ridge (DVR), a large brain region only found in sauropsids (birds and non-avian reptiles), involved in performing cortex-like functions (Güntürkün et al., 2017). Despite this fundamental difference in cytoarchitecture, as in mammals, an increase in EEG and local field potential (LFP) slow waves (approximately 1-5 Hz with a peak at 2 Hz) in the hyperpallium distinguishes NREM sleep from wakefulness and REM sleep in birds (van der Meij et al., 2019a). As in mammals (Massimini et al., 2004; Huber et al., 2004), NREM slow waves travel through the hyperpallium (van der Meij et al., 2019a) and are homeostatically regulated in a local use dependent manner in pigeons (*Columba livia*) (Lesku et al., 2011b; Rattenborg et al., 2019). In addition to wake-like EEG activity, as in mammals, avian REM sleep is characterized by (at least partial) reductions in muscle tone (Dewasmes et al., 1985; Rattenborg et al., 2019), twitching, including rapid eye movements, suppressed thermoregulatory responses (Heller et al., 1983; Scriba et al., 2013), and its prevalence in young altricial birds (Scriba et al., 2013). One notable

difference between birds and mammals is the short duration (typically <10 s) of bouts of REM sleep in birds (Tisdale et al., 2018b).

Interestingly, NREM-like and REM-like sleep were recently described in bearded dragon (*Pogona vitticeps*) lizards (Shein-Idelson et al., 2016; Libourel et al., 2018; Norimoto et al., 2020). NREM-like sleep was characterized by high voltage, slow LFP ‘sharp-waves’ in the DVR, and REM-like sleep was characterized by LFP activity resembling wakefulness and eye movements occurring under closed eye lids. Although recent work has implicated the claustrum in the genesis of NREM sleep slow waves in mice and the slow ‘sharp-waves’ in bearded dragons, the claustrum appears to generate these field potentials via different mechanisms; in mice, the claustrum coordinates the synchronous inhibition of cortical neurons across several cortical areas, resulting in the down-state of slow waves (Narikiyo et al., 2020), whereas, in bearded dragons, bursts of neuronal activity (giving rise to LFP sharp waves) propagate as traveling waves of excitation from the claustrum to the rest of the pallial DVR (Norimoto et al., 2020; *see also* Tisdale et al., 2018b). Given this apparent difference and the fact that not all of the components that typically characterize REM sleep in mammals and birds have been established in bearded dragons, we refer to these states as *NREM-like* and *REM-like* sleep (Libourel and Barrillot, 2020).

As in mammals, during REM sleep in pigeons and REM-like sleep in bearded dragons, hyperpallial and DVR neurons, respectively, fire at an elevated rate when compared to preceding sleep (Shein-Idelson et al., 2016; van der Meij et al., 2019a), and therefore might cause T_{br} to increase via increased blood flow from the animal’s warmer core. However, few studies have examined sleep state-related changes in T_{br} in birds, and none have examined bearded dragons. In birds, four studies examined hypothalamic temperature and one measured temperature in the nidopallium, a structure in the DVR. Graf et al., 1987 present a 4-h plot of hypothalamic temperature occurring during wakefulness, NREM and REM sleep in pigeons recorded at 10°C ambient temperature, but hypothalamic temperature did not vary systematically with state. In rooks (*Corvus frugilegus*) recorded at 8 and 15°C, hypothalamic temperature declined during NREM sleep, but a significant increase was not detected during REM sleep (Szymczak et al., 1989). By contrast, Pastukhov et al., 2001 reported decreases and increases in hypothalamic temperature during NREM and REM sleep, respectively, in pigeons recorded at 21°C. It is unclear whether these differences are due to ambient temperature or the temporal resolution and sensitivity of the thermistors used in the respective studies. Hypothalamic temperature was also measured in sleeping ostriches (*Struthio camelus*), but the temporal resolution (once every 2 min) of the thermistor was too low to identify sleep state-related changes in temperature (Lesku et al., 2011a). In pigeons recorded at 22°C, temperature declined during NREM sleep, but a consistent change in temperature was not detected during REM sleep in the nidopallium (Van Twyver and Allison, 1972). Given that variations in T_{br} are smaller near the surface of the brain in chickens (*Gallus gallus domesticus*) (Aschoff et al., 1973), the thermistor may not have been sensitive enough to detect changes in temperature during REM sleep in the

nidopallium, a more superficial structure than the hypothalamus. Thus, apart from one study of hypothalamic temperature in pigeons, a consistent increase in temperature has not been detected during REM sleep in the birds examined. Moreover, the study reporting an increase in hypothalamic temperature during REM sleep did not characterize how temperature changed throughout and following bouts of REM sleep of different durations. It is also unknown whether temperature increases during REM sleep in ‘cortical’ portions of the avian brain, an apparent requisite for brain warming to have a positive impact on cognitive performance upon awakening (Wehr, 1992). Finally, it is unknown whether T_{br} varies with the type of sleep in bearded dragons. To address these questions, and thereby expand our understanding of T_{br} regulation during sleep in non-mammals, we examined sleep state-related changes in temperature in the visual hyperpallium of pigeons and DVR of bearded dragons.

Results

Across the night, pigeons experienced nearly one thousand (979.6 ± 100.4) bouts of REM sleep lasting 6 ± 0.6 s (range, 1 - 30.7 ± 7.1 s, $N = 7$). Figures 1A and B illustrate the relationship between brain state and changes in T_{br} on 90- and 10-min time scales. Decreases and increases in T_{br} were related to the occurrence of NREM and REM sleep, respectively, with the peak T_{br} associated with a bout of REM sleep occurring after the bout ended (Figure 1B). To characterize the precise time course of T_{br} change during bouts of NREM and REM sleep of varying durations (Figure 1C and 1D), we examined isolated bouts of each state. Longer bouts of NREM sleep resulted in greater decreases in T_{br} (linear mixed effect model, Table S1; β_0 (intercept) \pm SE = $-7.30 \cdot 10^{-3} \pm 8.70 \cdot 10^{-4}$, $p = 1.56 \cdot 10^{-4}$; β (slope) \pm SE = $3.11 \cdot 10^{-3} \pm 3.31 \cdot 10^{-4}$, $p = 8.55 \cdot 10^{-5}$) (Figures 1C and 2A) and longer bouts of REM sleep resulted in greater increases in T_{br} ($\beta_0 \pm$ SE = $7.86 \cdot 10^{-3} \pm 5.70 \cdot 10^{-4}$, $p = 6.48 \cdot 10^{-6}$; $\beta \pm$ SE = $3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$, $p = 1.04 \cdot 10^{-3}$) (Figures 1D and 2B). T_{br} peaked on average 9 s after a bout of REM sleep, regardless of bout duration ($\beta_0 \pm$ SE = 9.22 ± 1.93 , $p = 3.05 \cdot 10^{-3}$; $\beta \pm$ SE = $3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$, $p = 0.1$) (Figures 1D and 2C). This hysteresis accounts for the increase in T_{br} during short episodes of NREM sleep preceded by REM sleep (Figure 1B). Finally, we found that REM sleep was more likely to occur (density per 15 s) at lower T_{br} ($\beta_0 \pm$ SE = 2.18 ± 0.18 , $p = 2.21 \cdot 10^{-5}$; $\beta \pm$ SE = -0.50 ± 0.08 , $p = 1.02 \cdot 10^{-3}$) (Figure 2D).

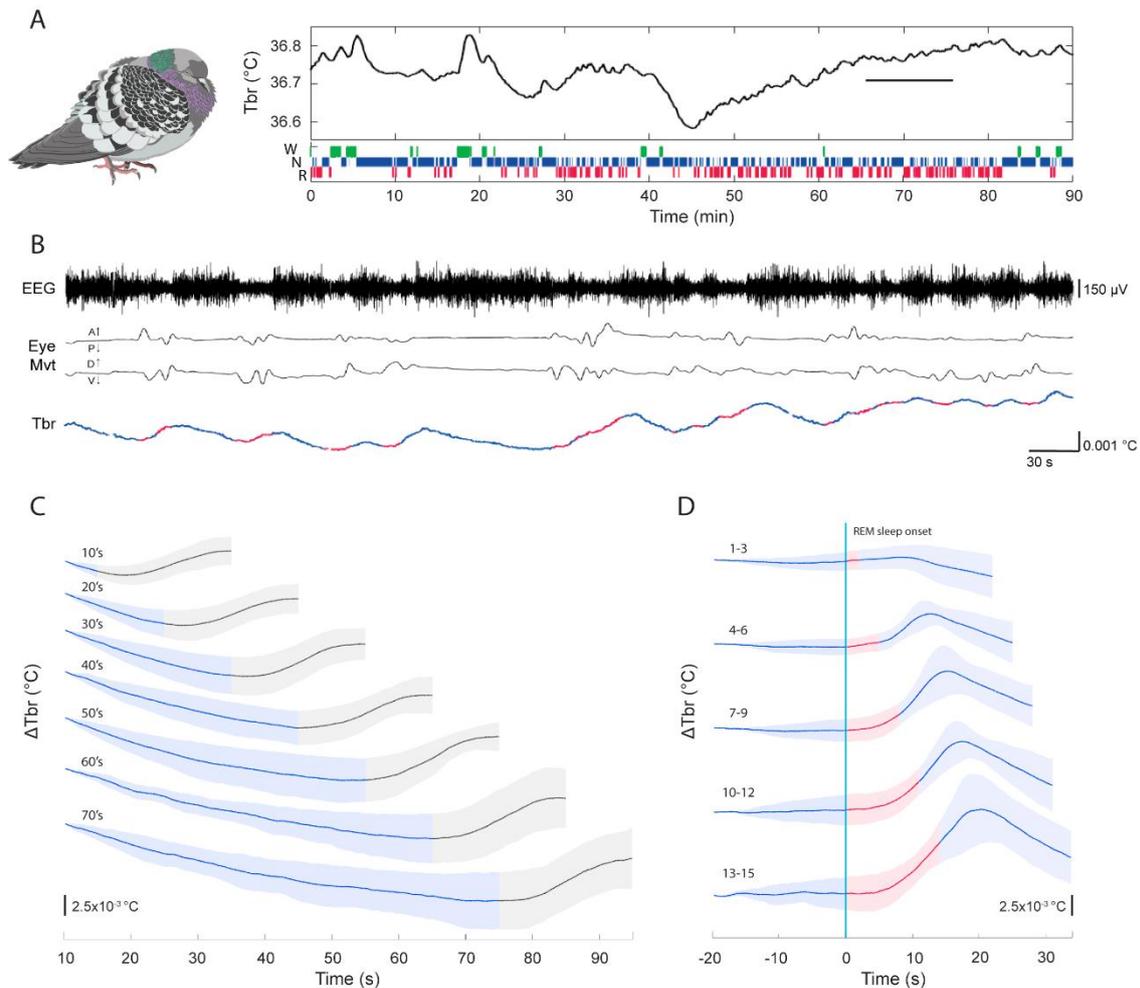


Figure 1. Brain temperature (T_{br}) during NREM and REM sleep in pigeons.

(A) T_{br} dynamics related to brain state across a 90-min period in a pigeon. Hypnogram (bottom plot) of brain state showing wakefulness (W, green), rapid eye movement (REM) sleep (R, red), and NREM sleep (N, blue). Note the frequent short bouts of REM sleep.

(B) Expanded 10-min period, demarcated by the bar in A, showing electroencephalogram (EEG) activity and T_{br} , both recorded from the hyperpallium, and eye movements during NREM and REM sleep, color coded as in A on the bottom T_{br} recording. Lower amplitude EEG activity and eye movements occur during REM sleep. Eye Mvt: left eye movements in the vertical (D, dorsal; V, ventral) and horizontal (A, anterior; P, posterior) planes determined from pupillometry.

(C) Longer bouts of NREM sleep are associated with greater brain cooling. Lines show the change in T_{br} (mean ± 1 standard deviation) relative to T_{br} 10 s after NREM sleep onset (0 °C) for NREM sleep bouts in 10 s duration categories (i.e. 10's = 10-19 s; 20's = 20-29 s; etc.). The 20 s after the middle of each category is plotted in grey, as it includes a mixture of the terminating state (wakefulness or REM sleep) and any subsequent states.

(D) Longer bouts of REM sleep are associated with greater brain warming. Lines show the change in T_{br} (mean ± 1 standard deviation) during 20 s of NREM sleep (blue) prior to REM sleep onset (vertical blue bar), during bouts of REM sleep (red) in 3 s duration categories (i.e. 1-3 s; 4-6 s; etc.), and during 20 s of NREM sleep following REM sleep offset, defined as the middle of each

duration category. For each duration category, T_{br} is plotted relative to T_{br} 20 s (0 °C) prior to REM sleep onset. Pigeon illustration by Damond Kyllö.

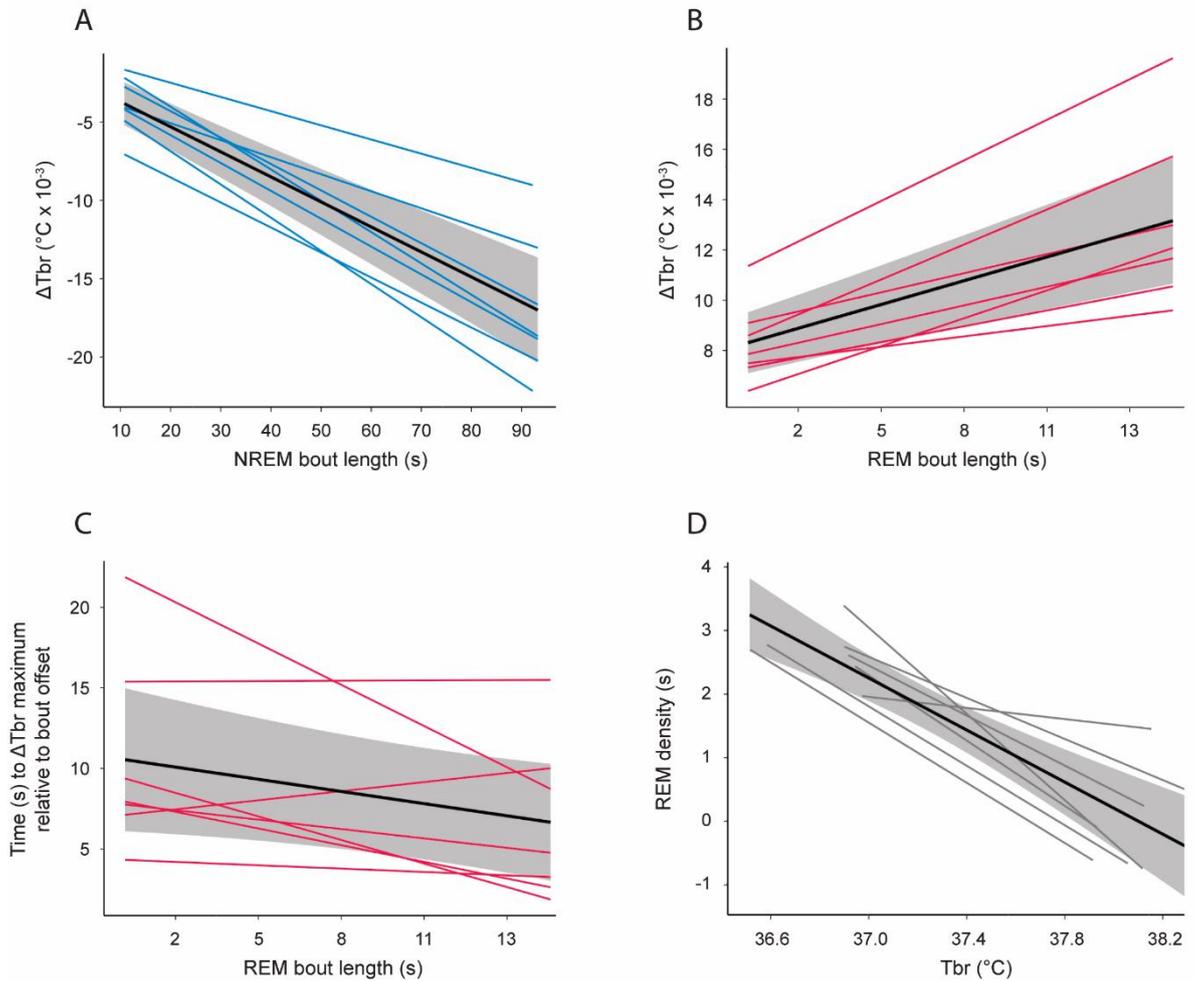


Figure 2. Relationship between (A) NREM and (B) REM sleep bout length and change in brain temperature (T_{br}) in pigeons. Y-axis values are the peak decreases and increases in T_{br} associated with bouts of NREM and REM sleep, respectively. (C) Relationship between REM sleep bout length and the time to the peak maximum T_{br} relative to the bout offset. (D) Relationship between REM sleep density (seconds per 15 s, see Methods) and T_{br} . Each plot shows the regressions for each pigeon (in blue and red for NREM and REM sleep, respectively) and the estimates (in black) with 95% confidence intervals (grey).

In bearded dragons, brain (DVR) activity alternated between NREM-like and REM-like sleep throughout most of the night (Figure 3A). The bearded dragons experienced 436 ± 133.1 bouts of REM-like sleep lasting 49 ± 12.8 s (range, $8.8 \pm 6.3 - 209.2 \pm 81.9$ s, $N = 4$). T_{br} either showed little change or decreased across the night (Figure S1). Despite exhibiting bouts of REM-like sleep that lasted on average 8.2 times longer than bouts of REM sleep in pigeons, average T_{br} did not increase when the bearded dragons switched from NREM-like to REM-like sleep (Figures 3B and S1).

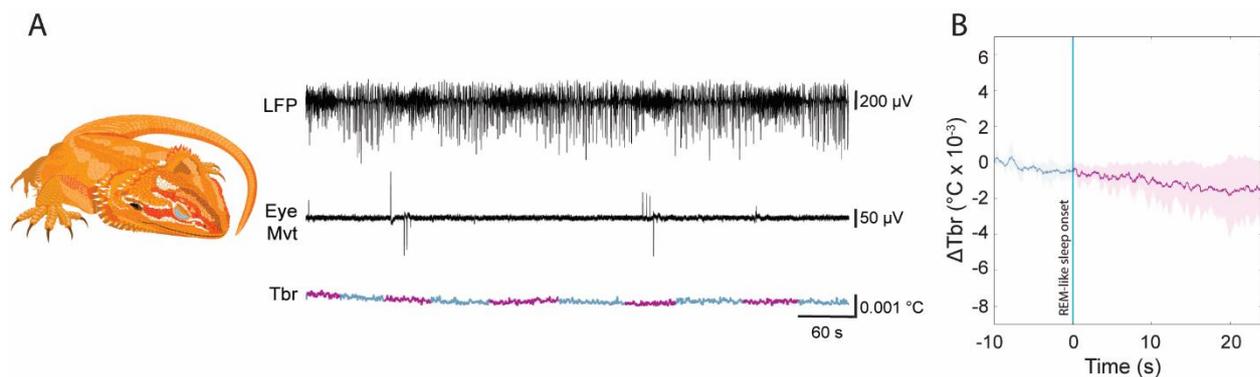


Figure 3. Brain temperature (T_{br}) during NREM-like and REM-like sleep in bearded dragons.

(A) 10-min local field potential (LFP) recording from the dorsal ventricular ridge (DVR) of a sleeping bearded dragon showing state-related changes in brain activity. NREM-like sleep is characterized by higher LFP amplitude and δ [0.5–4 Hz]/ β [11–30 Hz] ratio, when compared to REM-like sleep. The brain states are color coded (NREM-like sleep, cyan; REM-like sleep, magenta) in the bottom T_{br} recording. The electrooculogram (Eye Mvt) shows the association between eye movements and brain state.

(B) T_{br} recorded from the DVR of sleeping bearded dragons 10 s before and 30 s after the transition (vertical blue bar) between NREM-like and REM-like sleep. T_{br} values (mean \pm 1 standard deviation; see Figure S1 for individual data) are expressed relative to T_{br} 10 s before the transition. Note the absence of an increase in T_{br} following the transition between sleep states. The slight drop in T_{br} across the two states reflects the decline in T_{br} across the night (Figure S1). Bearded dragon illustration by Damond Kyylo.

Discussion

It has been proposed that brain warming during REM sleep enhances performance upon awaking (Wehr, 1992). For this to be true, warming should occur in sub-cortical and cortical structures, as adaptive performance likely depends on the entire brain. Our results demonstrate that ‘cortical’ warming occurs during REM sleep in pigeons, but not during a REM-like sleep in bearded dragons.

In pigeons, temperature in the visual hyperpallium decreased during NREM sleep and increased during REM sleep. Although this pattern is consistent with one study of hypothalamic temperature in pigeons, the average maximum increase was 10 times less in the hyperpallium (see Figure 1A in Pastukhov and Ekimova, 2012). The smaller change in temperature in the hyperpallium, the most dorsal part of the avian brain, is consistent with the observation that although temperature covaries throughout the brain in awake and sleeping chickens, it varies less near the dorsal surface (Aschoff et al., 1973). Also, the small magnitude of state-related changes in T_{br} might explain why a consistent increase in nidopallial temperature was not detected during REM sleep in the earlier study of pigeons (Van Twyver and Allison, 1972), as the average maximum

increase in T_{br} (0.01 °C) in the hyperpallium is equivalent to the minimum sensitivity of the thermistor used to measure temperature in the nidopallium.

Although the sleep-state dependent changes in T_{br} in pigeons are qualitatively similar to those observed in several mammals, the magnitude is much smaller. For example, the rate of cooling during NREM sleep and the rate of warming during REM sleep are approximately 10 - 20 and 5 - 12 times slower, respectively, than in rodents (Franken et al., 1992; Hoekstra et al., 2019). Moreover, the maximum average temperature increase associated with long (13 - 15 s) bouts of REM sleep, including the 9 s lag after REM sleep offset, is only 0.01 °C in the hyperpallium. The reasons for this difference are unknown, but might include insulation (feathers versus hair), the level of neuronal activity and associated blood flow in the respective states, and the regulation of body temperature and the temperature of blood reaching the brain (Porter and Witmer, 2016).

In contrast to the sleep state-related changes in T_{br} in pigeons, DVR temperature did not increase when bearded dragons switched from NREM-like to REM-like sleep. Based on research in mammals, the absence of brain warming might be due to blood flow to the brain. Either the increase in neuronal firing rate during REM-like sleep does not induce changes in blood flow or blood flow does increase, but a sufficient temperature gradient does not exist between the brain and body core for this flow to cause brain warming. In this regard, it would be important to know if dragons retain more heat acquired through basking and activity in the body than the head, due to the lower surface area to volume ratio of the body. It would also be informative to determine whether blood flow increases, as shown in rats (Bergel et al., 2018), during REM-like sleep in bearded dragons.

Our findings are consistent with the notion that sleep state-related changes in temperature are a trait unique to endotherms. However, more species of birds, as well as mammals, need to be examined to determine whether this is a general pattern. In addition, more reptiles need to be examined, especially given that the changes in brain activity that define sleep states in bearded dragons have not been found in all reptiles examined (Libourel and Herrel, 2016; Libourel et al., 2018). It would also be interesting to examine small and large reptiles, as T_{br} during sleep might depend on a reptile's size-dependent ability to retain core heat acquired through basking and physical activity (Tattersall, 2016). Investigating sleep and T_{br} in tegu lizards (*Salvator merianae*) that exhibit facultative endothermy during the reproductive season (Tattersall et al., 2016; Tattersall, 2016) or in Galapagos marine iguanas (*Amblyrhynchus cristatus*) that undergo periods of cooling while feeding at sea (Bartholomew, 1966; Butler et al., 2002), would also be informative. Finally, T_{br} should also be examined in other ectothermic animals, such as zebrafish and cephalopods, wherein REM-like sleep states have been described (Leung et al., 2019; Iglesias et al., 2019).

Our findings contribute to, but do not resolve, the debate over proposed homology between REM-like sleep in bearded dragons and REM sleep in mammals and birds

(Libourel and Barrillot, 2020). The interpretation of our findings depends on the emphasis given to specific components of REM sleep present in mammals and birds (Blumberg et al., 2020). If an increase in T_{br} is considered an essential feature of REM sleep, then our findings suggest that REM-like sleep in bearded dragons is not homologous to REM sleep in mammals and birds, and that REM sleep evolved independently in mammals and birds, perhaps in association with the independent evolution of endothermy. Alternatively, it is possible that the states are homologous and brain warming during REM sleep secondarily emerged independently in mammals and birds. Clearly, further studies are needed to define the essential components of REM sleep and their evolution (Blumberg et al., 2020; Libourel and Barrillot, 2020).

Brain warming might be an unimportant or even costly epiphenomenon of REM sleep in endotherms or an advantageous component of this state. According to the brain warming hypothesis, warming during REM sleep counteracts cooling during prior NREM sleep, and thereby prepares the animal to rapidly interact adaptively with its surroundings upon awakening (Wehr, 1992; Lyamin et al., 2018). The brain warming hypothesis is challenged by several lines of evidence (Gao et al., 1995; Lima et al., 2005; Ungurean and Rattenborg, 2018). Notably, mammals and birds sleeping in riskier situations suppress REM sleep (Lesku et al., 2008a; Gravett et al., 2017; Lyamin et al., 2018; Tisdale et al., 2018a; Tisdale et al., 2018b) which, according to the brain warming hypothesis, would render them less likely to respond adaptively to a threat upon awakening. Nonetheless, it is worth considering the implications that our findings have for this hypothesis. In pigeons, brain cooling during NREM sleep and warming (albeit small) during REM sleep, as well as the increased density of REM sleep when T_{br} is low, are consistent with the brain warming hypothesis. However, even though ecologically important behaviors, such as courtship song in male zebra finches (*Taeniopygia guttata*), can occur faster at naturally occurring higher brain temperatures (Aronov and Fee, 2012), behavioral tests are needed to determine whether the small increase in T_{br} during REM sleep in pigeons has an ecologically meaningful impact on performance upon awakening. Finally, regardless of whether REM-like sleep in bearded dragons is homologous to REM sleep in mammals and birds, the absence of brain warming suggests that the primary function (if one exists) of periods of increased neuronal activity during sleep is not to warm the brain. Other processes linked to increased neuronal activity, such as learning, memory consolidation, and brain development, are likely candidates for a shared function of increased neuronal activity during sleep.

Limitations of study

Our study has several limitations that should be taken into consideration. As we only studied pigeons and bearded dragons, more species need to be examined to determine whether our findings apply to other birds and reptiles. Additional studies are also needed to determine whether the relationship, or lack thereof, between brain state and T_{br} depends on ambient temperature and the animal's energetic status. It would also be important to test whether the small increase in T_{br} observed during REM sleep in

pigeons has an impact on performance upon awakening. Finally, additional research on bearded dragons is needed to establish the extent to which NREM-like and REM-like sleep are homologous to NREM and REM sleep in mammals and birds.

Methods

Pigeons

Surgical procedures

Adult domestic pigeons (*Columba livia*; Budapest variety; 1 female, 6 males) were surgically instrumented for recording the electroencephalogram (EEG) from both hemispheres, neck electromyogram (EMG), and eye movements under isoflurane anesthesia (1-3% in 0.5 LPM oxygen). The birds were administered an injection of Diazepam (2 mg/kg) into the breast muscle 15 min prior to surgery, an injection of Metamizol (150 mg/kg) into the breast muscle shortly before isoflurane anesthesia, 4 drops of Procaine (2%) for local analgesia prior to skin incision, and an intramuscular injection of Meloxicam (0.5 mg/kg) and Lidocaine gel to the wound at the end of the surgical procedure. The electrodes consisted of round-tipped gold pins (0.5 mm diameter) for the EEG and electrooculogram (EOG), and gold-plated electrode balls (1 mm diameter) for the EMG. The EEG electrodes were placed over the visual hyperpallium which is visible through a distinct window of thin bone on the surface of the cranium. Two EEG electrodes were placed on the dura overlying each (left and right) hyperpallium (2.5 and 3 mm lateral of the midline for the anterior and posterior electrode, respectively, and separated by 5 to 6 mm along the anterior-posterior axis). As part of another study, left and right pupil size and movements were detected through the closed eyelids via head-mounted micro camera modules (NanEye®, ams AG, 1 mm x 1 mm) connected to an image processing board (BAP Image Systems GmbH, AC62KUSB), and an infra-red LED (Kingbright®, 940 nm, 50 mA, 1.2 V) placed on the skull that illuminated the back of the eyes (Yüzgeç et al. 2016). For each hemisphere, a thermistor (Semitec®, 223Fμ3122-07U015, 0.28 mm diameter, thermal time constant of 20 ms, relative sensitivity 0.001°C) was inserted approximately 1 mm into the dorsal surface of the left and right hyperpallium, mid-way between the two EEG electrodes. Each thermistor was calibrated prior to implantation using a water bath and a digital thermometer.

Housing and recording

The pigeons were housed individually in an indoor aviary (1 m x 2 m x 2 m; 12 h:12 h light:dark photoperiod; 21°C) adjacent to, and in visual and auditory contact with, two other pigeons, each housed in similar aviaries. Food and water were provided *ad libitum*. After at least one week of post-surgical recovery and habituation to the recording equipment, the birds were recorded during the 12-h night while confined to their preferred sleeping perch. Electrophysiological data and head movements (accelerometry) were recorded at 256 Hz via a 2.6 g head-mounted transmitter. Images

from the micro cameras were recorded via a thin, light-weight tether that allowed the pigeon to freely move on its perch. The birds were disconnected from the tether and were free to move throughout their aviary once the lights came on in the morning.

Sleep scoring

The recordings were visually scored for wakefulness, NREM sleep, and REM sleep using standard criteria (Martinez-Gonzalez et al. 2008; Lesku et al. 2011b; Tisdale et al. 2018b). However, unlike previous studies which employed 4 s epochs, we used 1 s epochs to gain a more precise measure of the onset and offset of bouts of REM sleep. A bout of a given state was defined as one or more epochs of that state uninterrupted by an epoch of any other state; i.e. bouts could be as short as 1 s.

Eye movement tracking

The videos recordings of the birds' eyes were used to detect the pupil area through binary thresholding using a custom Matlab script. Variations in the position of the pupil area centroid were used as a proxy for eye movements. To correct for variation in camera position, the axes were rotated such that horizontal movements were along and vertical movements were perpendicular to the eyelid closure plane. Positive increases in signal values represent anterior and dorsal movements, respectively.

Brain temperature analysis

Although temperature was recorded from both hemispheres, in some birds, state-related changes in temperature were less evident in one hemisphere than the other, even though the changes in EEG activity were largely synchronous between the hemispheres. This variation likely reflects variation in the depth of the thermistor. Consequently, the thermistor that appeared the most responsive to state changes was selected for detailed analysis. Due to frequent state changes and the fact that temperature usually peaked after a bout of REM sleep, we took the following measures to isolate the relationship between sleep state and T_{br} . For NREM sleep, we analyzed temperature starting 10 s after the onset of a bout. For REM sleep, we selected for analysis the bouts that were preceded and followed by at least 20 s of NREM sleep to avoid the overlapping effect of REM sleep bouts occurring in close succession. For both states, changes in T_{br} were expressed relative to the temperature at the start of the analysis window. For Figure 1C, NREM sleep bouts were grouped into 10 s duration categories, and for Figure 1D, REM sleep bouts were grouped into 3 s categories. We only report data for categories that had at least 10 bouts that met the inclusion criteria for every bird. This resulted in seven categories for NREM sleep (10-19, 20-29, 30-39, 40-49, 50-59, 60-69, and 70-79 s) and five categories for REM sleep (1-3, 4-6, 7-9, 10-12, and 13-15 s). All reported values are the mean (\pm 1 standard deviation) of the individual bird means for each category.

Statistical analyses

On the non-grouped dataset shown in Figures 1C and D, we detected the timing and the values of the maximum decrease/increase in T_{br} for each T_{br} response curve associated with a bout of NREM/REM sleep. In the case of NREM sleep, the detection was limited to 10 s over the maximal length of the respective bout category to filter out potential effects of states occurring after the offset of a NREM sleep bout. We then tested whether longer NREM/REM sleep bouts were associated with higher decreases/increases in T_{br} using linear mixed effect models with the relative change in T_{br} (ΔT_{br}) as the response variable and the bout length as predictor. Individual identity was added as a random effect. A similar analysis was performed to test for a relationship between bout duration and the timing of the positive peaks relative to the offset of REM sleep bouts.

If one function of REM sleep is to warm the brain, then REM sleep should be expressed in higher proportions at lower T_{br} . We therefore tested for a relationship between T_{br} and the density of REM sleep during 15 s windows. For each bird, we divided the recording into 15 s windows. We then calculated the amount of REM sleep expressed in each 15 s window, and the average T_{br} for the first 7 s of the associated T_{br} data. The average for T_{br} was restricted to the first 7 s to account for the lag in response related to REM sleep bouts. The relationship was tested using a linear mixed effect model with REM sleep density as the response variable and T_{br} as predictor. Individual identity was added as a random effect. All statistical analyses were performed using RStudio. For mixed model analyses, we used the lme4 package (Bates et al. 2015). The denominator degrees of freedom were computed with Satterthwaite's method.

Bearded dragons

Surgical procedures

4 adult bearded dragons (*Pogona vitticeps*, 2 males, 2 females) were surgically instrumented for recording local field potentials (LFP) in the DVR, neck EMG, and eye movements under injectable ketamine (100 mg/kg) and medetomidine (200 μ g/kg) anesthesia. The LFP were recorded using a bundle of six 35- μ m diameter tungsten electrodes. The EMG was recorded using two stainless steel electrodes implanted in the neck muscles. The eye movements were recorded using one screw fixed in the skull on the supraorbital ridge of each eye. A calibrated thermistor (Semitec®, 223F μ 3122-07U015; or Micro-Betachip, GA100K6MCD1; the relative sensitivity of both sensors was 0.001°C, with a time constant of 20 ms) was inserted in the telencephalon 1 mm into the brain, targeting the DVR. The thermistor and LFP electrodes were positioned relative to the pineal eye (anterior-posterior (AP) 0 mm, medial-lateral (ML) 0 mm) and the surface of the skull (dorsal-ventral (DV) 0 mm). The thermistor positions were as follows: Dragon 1, AP -1 mm, ML 0 mm, DV -2.5 mm; Dragon 2, AP +1 mm, ML -1 mm, DV -2.5 mm; Dragon 3, AP -2 mm, ML -2 mm, DV -2.5 mm; and Dragon 4, AP +1 mm, ML +1mm, DV -2.5 mm. The LFP electrodes were placed bilaterally (AP -2 mm, ML +2 mm and -2 mm, and DV -5 mm).

Housing and recording

The lizards were housed individually in a glass terrarium (50 cm x 50 cm x 100 cm; 12 h:12 h light:dark photoperiod). Water was provided *ad libitum* and food (crickets, mealworms, and vegetables) was given every other day. After at least one week of post-surgical recovery and habituation to the recording equipment, the lizards were recorded for 3 to 7 days in their terrarium. Electrophysiological data and head movements (accelerometry) were recorded at 256 Hz via a 15g head-mounted transmitter (Massot et al. 2019). The animals were recorded at different room temperatures. Dragon 1 was recorded at a constant temperature (25°C), while the others were maintained with a more natural temperature gradient between a ‘hot spot’ (set at 40°C) and a ‘cold spot’ (25°C). Lizards housed with a gradient exhibited a natural decrease of their core temperature across the sleep period (Figure S1).

Sleep scoring

The recordings were visually scored for wakefulness and sleep using body position, head elevation, and eye state (open or closed). The animals were considered asleep when in a “sleeping” position (lying on the ground with hind and front limbs relaxed) with the head on the ground and both eyes closed for more than a minute. The two sleep states were scored using a method previously developed for lizards (Libourel et al. 2018).

Brain temperature analysis

To estimate the impact of sleep state on T_{br} , we examined T_{br} 10 s before the onset of REM-like sleep to 30 s after onset. All episodes of REM-like sleep occurring across the night were included in the analysis. The values plotted in Figure 2B are the mean of the nightly means (N = 5; one night for Dragons 1, 2, and 4, and two nights for Dragon 3); for individual bearded dragon data, see Figure S1.

Ethics statement

All experiments were conducted according to the 3R principles of animal experimentation and in accordance with the European Community Council Directive for the use of research animals. The protocols and procedures used were approved by the local ethics committee for animal experimentation (i.e., Government of Upper Bavaria for the pigeons and University Lyon 1 for the bearded dragons).

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Supplementary Material

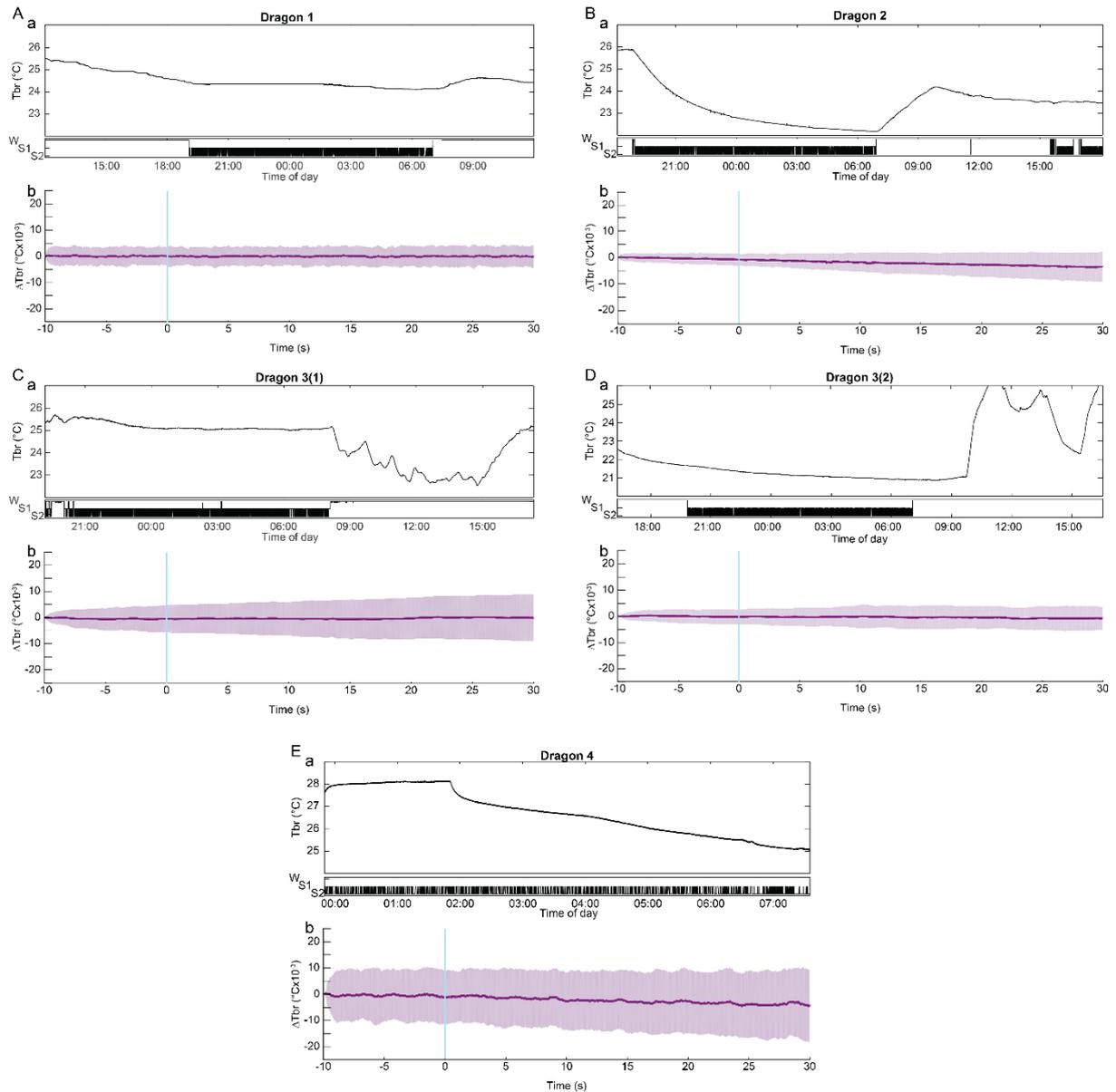


Figure S1. Brain temperature (T_{br}) across the main night-time sleep period for each bearded dragon (A-E; two nights (C and D) are shown for Dragon 3). For each individual, (a) shows T_{br} with a hypnogram (bottom) showing brain state (wakefulness, W; NREM-like sleep, S1; REM-like sleep, S2) and (b) shows T_{br} recorded from the dorsal ventricular ridge 10 s before and 30 s after the

transition (vertical blue bar) between NREM-like and REM-like sleep. T_{br} values (mean \pm 1 standard deviation) are expressed relative to T_{br} 10 s before the transition.

Supplementary Table

Table S1. Summary table with the results of the linear mixed effect models. For the last four models the predictors were scaled upon fitting the model.

Category	Response	Predictor	Fixed effects			Random effects	
			Estimate \pm SE	t-val	p-val	Proportion (%) of variance explained by:	
						random intercept	random slope
Effect of bout length on ΔT_{br}	ΔT_{br}	<i>Intercept</i>	$-7.30 \cdot 10^{-3} \pm 8.70 \cdot 10^{-4}$	-8.39	$1.56 \cdot 10^{-4}$		
		NREM bout length	$3.11 \cdot 10^{-3} \pm 3.31 \cdot 10^{-4}$	-9.40	$8.55 \cdot 10^{-5}$	12.5	1.68
	ΔT_{br}	<i>Intercept</i>	$7.86 \cdot 10^{-3} \pm 5.70 \cdot 10^{-4}$	13.80	$6.48 \cdot 10^{-6}$		
		REM bout length	$3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$	6.04	$1.04 \cdot 10^{-3}$	2.80	0.02
Effect of bout length on the occurrence of min/max ΔT_{br} relative to bout offset	ΔT_{br} min occurrence	<i>Intercept</i>	-0.56 ± 0.55	-1.02	0.35		
		NREM bout length	-5.93 ± 0.79	-7.54	$2.77 \cdot 10^{-4}$	1.56	3.36
	ΔT_{br} max occurrence	<i>Intercept</i>	9.22 ± 1.93	4.79	$3.05 \cdot 10^{-3}$		
		REM bout length	$3.37 \cdot 10^{-4} \pm 5.59 \cdot 10^{-5}$	-1.98	0.1	6.33	0.30
Effect of T_{br} on the REM density	REM density	<i>Intercept</i>	2.18 ± 0.18	12.03	$2.21 \cdot 10^{-5}$		
		T_{br}	-0.50 ± 0.08	-5.99	$1.02 \cdot 10^{-3}$	1.85	0.34

Chapter 3

Pupillary behavior during wakefulness, non-REM sleep, and REM sleep in birds is opposite that of mammals

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Authors: Gianina Ungurean, Dolores Martinez-Gonzalez, Bertrand Massot, Paul-Antoine Libourel, Niels C. Rattenborg

Author contributions: Conceptualization, N.C.R.; Methodology, G.U., P.-A.L., and N.C.R.; Hardware, P.-A.L. and B.M.; Software, P.-A.L.; Investigation, G.U., D.M.-G., and N.C.R.; Formal Analysis, G.U., P.-A.L. and N.C.R.; Writing – Original Draft, G.U. and N.C.R.; Writing – Review & Editing, G.U., D.M.-G., P.-A.L. and N.C.R.; Resources, P.-A.L. and N.C.R.; Supervision, P.-A.L. and N.C.R.

Summary

Mammalian pupils respond to light (McDougal and Gamlin, 2015; Douglas, 2018), and dilate with arousal, attention, cognitive workload and emotions (Bradley et al., 2008), thus reflecting the state of the brain. Pupil size also varies during sleep, constricting during deep non-REM sleep (Berlucchi et al., 1964; Morrison and Pompeiano, 1970; Yüzgeç et al., 2018; Tsunematsu et al., 2020) and dilating slightly during REM sleep (Berlucchi et al., 1964; Morrison and Pompeiano, 1970; Yüzgeç et al., 2018). Unlike mammals, anecdotal reports suggest that birds constrict their pupils during aroused states, such as courtship and aggression (Hardy, 1963, 1974; Skeate, 1984), raising the possibility that pupillary behavior also differs between mammals and birds during sleep. Here, we measured pupil size in awake pigeons and used their translucent eyelid to investigate sleep state dependent changes in pupil size. Male pigeons constricted their pupils during courtship and other male-female interactions, but not while engaged in other waking behaviors. Unlike mice, the pupils were dilated during non-REM sleep, while over one thousand bursts of constriction and relaxation, which we call rapid iris movements (RIMs), occurred primarily during REM sleep. Consistent with the avian iris being composed largely of striated muscles (Pilar et al., 1987; Barsotti et al., 2010a, b; Barsotti et al., 2012; Coli et al., 2016), rather than smooth muscles, as in mammals, pharmacological experiments revealed that RIMs are mediated by nicotinic cholinergic receptors in the iris muscles. Despite receiving input from a parasympathetic nucleus, but consistent with its striated nature, the avian iris sphincter muscle behaves like skeletal muscles controlled by the somatic nervous system, constricting during courtship displays, relaxing during non-REM sleep, and twitching during REM sleep. We speculate that during wakefulness, pupillary constrictions are involved in social communication, whereas RIMs occurring during REM sleep might maintain the efficacy of this motor system and/or reflect the processing of associated memories.

Keywords

pigeon, cuckoo, avian, sleep, pupillometry, REM, twitches, iris, pupil

Results and discussion

In mammals and birds, the diameter of the pupil is under the control of the iris sphincter muscle which responds to changes in ambient light (i.e. the pupillary light reflex) via relatively well conserved neural mechanisms (McDougal and Gamlin, 2015; Douglas, 2018). Specifically, the Edinger-Westphal nucleus controls pupillary constrictions through its parasympathetic projections to the iris sphincter muscle via cholinergic neurons originating in the ciliary ganglion (Reiner et al., 1983). In addition to responding to light, in humans and other mammals, the pupil constricts when

focusing on near objects (accommodation) and dilates with arousal, attention, mental effort (Hess and Polt, 1964; McGinley et al., 2015), and emotion (Bradley et al., 2008), and is thought to play a role in social signalling (Hess, 1975; Kret, 2018). It has been suggested that in social interactions, pupil size and pupil mimicry are important for trust and bonding (Prochazkova et al., 2018). Pupil mimicry also occurs in non-human primates (Kret et al., 2014), and can extend to inter-specific interactions (Axelsson and Fawcett, 2021).

In birds, pupillary constrictions have been described during courtship and/or aggressive displays in species from several orders, including Charadriiformes, Passeriformes, and Psittaciformes (Hardy, 1963, 1974; Skeate, 1984; Jones, 2020). In parrots, rapid pupillary constrictions ('eye pinning'), occurring during seemingly positive and negative social interactions, are well-known among pet owners. In the only quantitative study, pupillary constrictions were associated with the utterance of preferred words in a pet Yellow-crowned Amazon (*Amazona ochrocephala*) (Gregory and Hopkins, 1974). Interestingly, the changes in pupil size reported in birds are opposite to those described in mammals, with constricted vs dilated pupils, respectively, associated with aroused states. However, the reports for birds are largely anecdotal and a systematic quantification is currently lacking.

Pupillary activity during courtship in pigeons

To determine the relationship between courtship behavior (Figure 1A) and pupil diameter, we measured the pupil size of freely-moving male pigeons using a custom-made, light-weight wireless video logger with a minicamera module (1 mm x 1 mm, CMOS sensor and lens) (Figure S1). The logger was mounted on the birds' back and the camera module was installed close to one eye using a head-mounted frame (Figure 1A). Prior to the experiment, the pigeons were progressively adapted to the equipment and the recording environment. On the recording day, males previously housed in same-sex aviaries were transferred to the recording arena and following baseline recordings were presented with a female. Generally, males quickly started to exhibit courtship displays typical for pigeons, consisting of stereotypical walking, bowing and cooing (Figure 1A). The pupils of quiet birds were dilated, but rapidly switched to a constricted state when the bird engaged in courtship behavior (Figure 1B) or when the female was approaching the male, for instance for allofeeding. Interestingly, the pupils were less constricted during copulation or when the bird was engaged in other motor behaviors, suggesting that pupillary constrictions are tightly coupled to male courtship displays (Figure 1C, Table S1). A specific link between pupillary constriction and courtship behavior might explain why pupil size increases when courtship in Indian peafowl (*Pavo cristatus*) is interrupted by the presentation of a predator (Yorzinski and Platt, 2014).

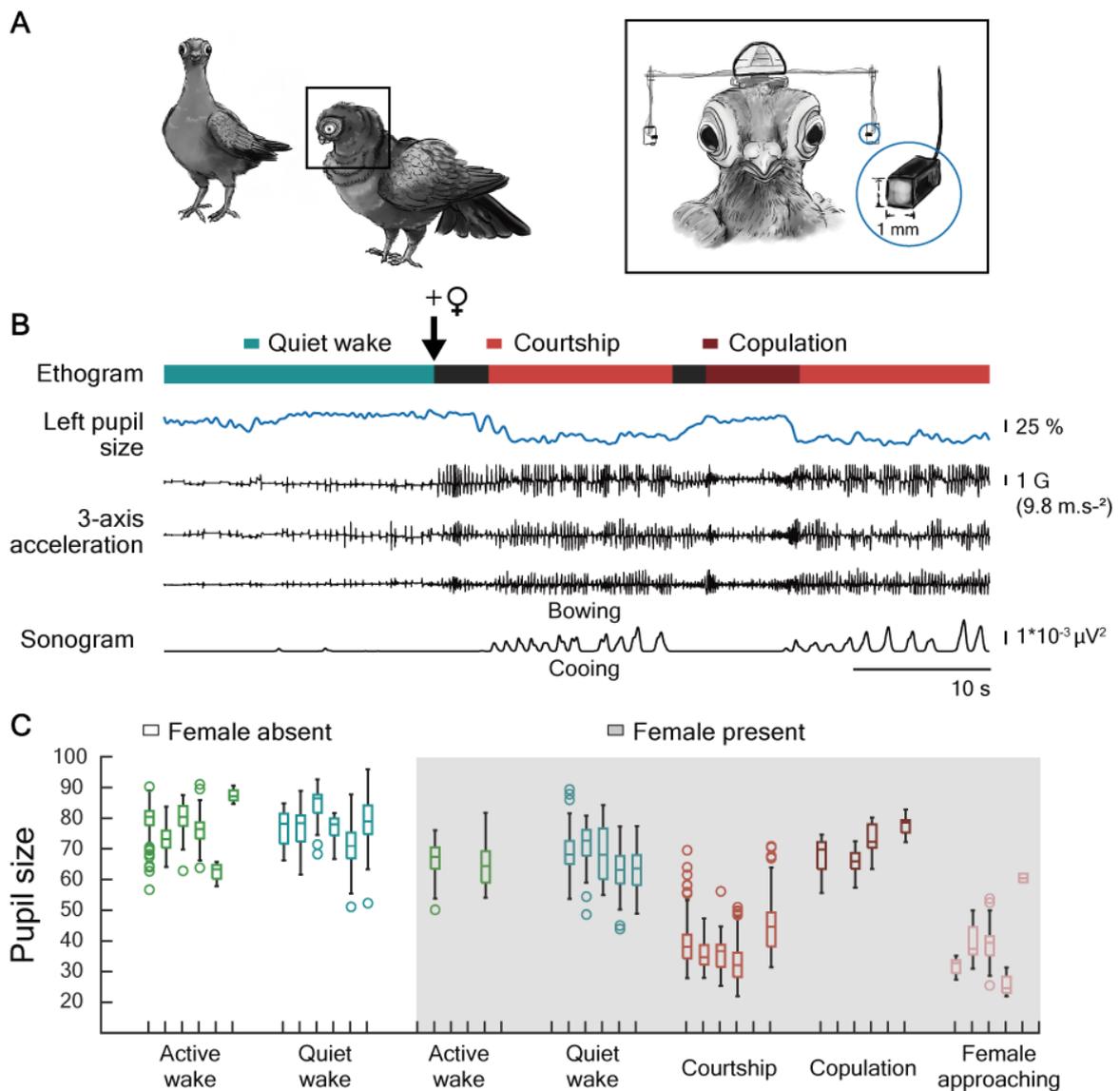


Figure 1. State-dependent changes in pupil size in awake pigeons.

(A) Left: male pigeons were recorded while alone or in the presence of a female. The males were equipped with minicamera modules installed near the eye via a head-mounted frame, connected to the processing board installed on the bird's back (see also Figure S1). Right: frontal view of the bird's head illustrating the head-mounted frame and the minicamera modules (blue circles).

(B) Example of raw recordings depicting, from top to bottom: the ethogram, left pupil size, three-axis head accelerometry, and the band power (200–600 Hz) of the sound recordings to visualize individual courtship calls. The arrow indicates when the female was introduced.

(C) Distribution of the average pupil size for 1 s epochs corresponding to each state (see also Table S1). Each boxplot represents one individual. Shaded in gray are the data for the behaviors that occurred when the female was present.

Pigeon illustrations by Yifan Pei.

Whole-night pupillometry recordings in sleeping birds

Only a few studies have investigated the relationship between pupil diameter and brain activity during sleep in mammals, and none have examined birds. In cats and mice,

the pupils are dilated during quiet aroused wakefulness and become progressively smaller as the animal transitions to drowsiness (Reimer et al., 2014; Vinck et al., 2015; McGinley et al., 2015), light non-REM sleep, and deep non-REM sleep (Berlucchi et al., 1964; Morrison and Pompeiano, 1970; Yüzgeç et al., 2018; Tsunematsu et al., 2020). During REM sleep, the pupils are mostly constricted (Berlucchi et al., 1964; Morrison and Pompeiano, 1970; Yüzgeç et al., 2018; Tsunematsu et al., 2020), but can dilate from this constricted state (Berlucchi et al., 1964; Morrison and Pompeiano, 1970; Yüzgeç et al., 2018). In cats, such dilations are associated with rapid eye movements and, in mice, where their time-course has been documented, they can last tens of seconds (see Figure 1 in Yüzgeç et al., 2018). In mice, sleep-related pupillary constrictions are mediated by the parasympathetic system through muscarinic cholinergic receptors and were shown to protect sleep by preventing light from reaching the retina and arousing the animal (Yüzgeç et al., 2018).

During previous experiments in our lab, we discovered that the pupils of pigeons are visible through their closed eyelids. This prompted us to investigate whether the differences in wake-related pupillary behavior observed between mammals and birds also manifest during sleep. We performed whole night recordings of pupil size using the IR-sensitive minicamera modules installed on a head-mounted frame, as in the previous experiment (Figure 2A, Figure S1). To increase the image contrast, and thereby facilitate the automated analysis of pupil size in the dark, we used infrared back-illumination pupillometry (iBIP), a technique developed in mice (Yüzgeç et al., 2018), wherein an IR light source placed above the rostral surface of the skull illuminated the back of the eyes. The IR frequency is outside the pigeons' visual spectrum, and thus invisible to the bird. With iBIP, the pupils appeared bright when the eyes were open, but could also be seen through the closed eyelid, albeit with lower signal intensity (Figure 2B). Thus, it was possible to detect eye state (open or closed) using the intensity of the signal, and to track pupil size and eye movements through the closed eyelids. During most of the time spent asleep, the pupils were dilated. However, the dilated state was frequently interrupted by fast constrictions and dilations (Figure 2CD), lasting 1.9 ± 0.2 s (Figure 2E), which we named rapid iris movements (RIMs). Most RIMs occurred in both eyes at the same time (Figure 2CD; Video S1). The pigeons experienced over one thousand RIMs per night (Figure 2F). When analyzing the data from three nights of recordings, we found that some birds consistently had more RIMs than others (repeatability = 0.64, $p = 1.2 \times 10^{-4}$), but this did not depend on the birds' sex (Figure 2F, linear mixed effect models, $p = 0.73$, Table S2).

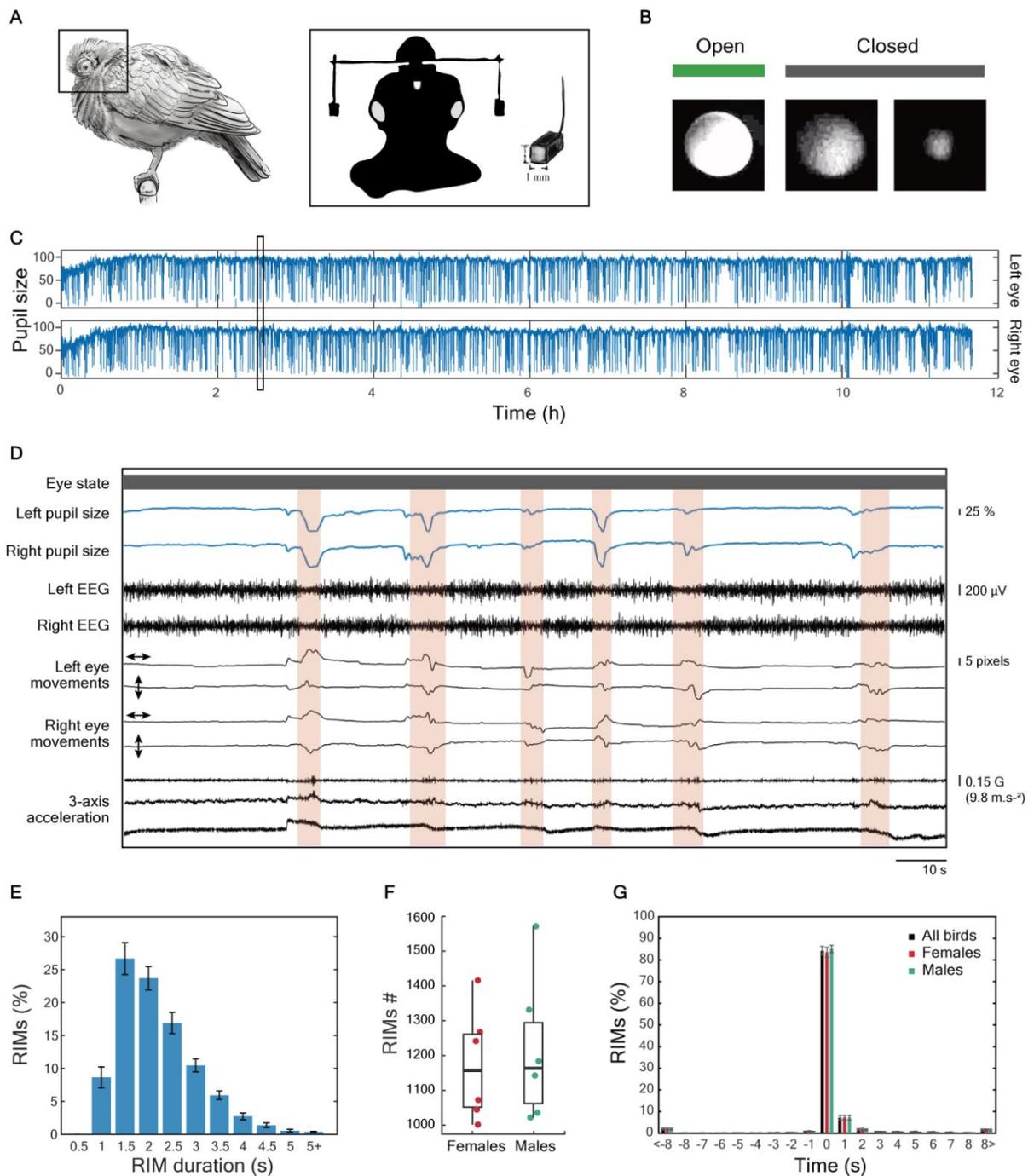


Figure 2. Behavioral characterization and EEG correlates of RIMs.

(A) Illustration of the experimental setup. The pigeons were recorded while sleeping in their favorite spot (left) while wearing an electrophysiology recording device and a head-mounted frame where the IR-sensitive minicamera modules were installed (see also Figure S1). An LED fixed above the rostral skull illuminated the back of the eye with invisible IR light, making the pupil appear bright (right).

(B) Example of pupil images obtained using infrared back-illumination pupillometry (iBIP) and the minicamera modules. Left: the eye is open, the median pixel intensity of the pupil area is high and the pupil is dilated in the dark; middle and right: the eye is closed and the signal intensity is lower; the pupil is, however, still visible through the closed eyelid, both when dilated and constricted.

(C) Example of raw recordings from both eyes in one bird during the 12 h dark period, where each downward peak demarcates a pupillary constriction (RIM).

(D) Expanded 180 s period, showing right and left hyperpallial EEG activity, eye movements, and head accelerometry associated with the pupillary activity. Note that the eye is closed during the entire period. Shaded in red are REM sleep bouts scored blind to pupillometry; non-shaded areas are bouts of non-REM sleep. The eye state is color-coded as in (B).

(E) Average distribution of RIM durations (mean \pm std).

(F) Number of RIMs per night grouped by sex. Each point represents the average of three nights of recordings for an individual (see also Table S2).

(G) Relationship between RIMs and bouts of REM sleep. The zero category represents all RIMs occurring during a REM sleep bout. Negative and positive numbers represent the time of occurrence before or after a bout of REM sleep (see also Video S1). Data presented as mean \pm std.

Pigeon illustration by Yifan Pei.

We next examined the temporal relationship between RIMs and sleep state. Sleep states were scored with 1-s epochs using EEG, head-mounted accelerometry, eye movements and eye state, while blind to pupil size. Non-REM sleep was defined by high-amplitude, low-frequency waves on the EEG, immobility and a lack of eye movements, and REM sleep by low-amplitude EEG activity, eye movements under closed lids, and other phasic events visible on the accelerometry, such as head movements and drops, and bill twitching (Video S1). We found that RIMs occurred mostly during REM sleep ($84.2 \pm 4.1\%$), or in the first second following the termination of a bout of REM sleep ($7.0 \pm 2.1\%$) (Figure 2G). These results show that, in pigeons, the pupils are dilated during non-REM sleep, while transient rapid bursts of pupillary constriction and dilation (RIMs) occur in tight temporal relation with REM sleep.

Are RIMs a behavior unique to pigeons, or do they occur in other birds? To address this question, we obtained video recordings of guira cuckoos (*Guira guira*) in a zoological park (Figure 3A). Like pigeons, guira cuckoos have translucent eyelids, brightly colored irides, and do not sleep with their heads tucked under feathers, allowing pupillometry through closed eyelids. Using DeepLabCut, a markerless video tracking algorithm based on deep neural networks (Mathis et al., 2018), we measured the size of the pupil, and movements of the eye, bill, head, and the tallest feather of the bird's crest. At night, we observed 1) quiet periods with closed eyelids, still head and eyes, and fluffed feathers, and 2) active periods with eye movements, head movements and drops, bill twitches and/or the collapse of feathers (Figure 3A and B, Video S2). Although no electrophysiological parameter was measured in guira cuckoos to unequivocally identify sleep states, in pigeons and other birds, these behaviors correspond to non-REM sleep and REM sleep, respectively (Rattenborg et al., 2021). As in pigeons, we observed fast pupillary constrictions (RIMs) during sleep co-occurring with phasic REM sleep behaviors, as shown by the higher variability of the eye, head, feather and beak position during automatically detected constricted versus dilated pupil states (Figure 3B). As guira cuckoos and pigeons are distantly related, having last shared a common ancestor 62-70 million years ago (Jarvis et al., 2014; Prum et al., 2015), RIMs associated with REM sleep might be a widespread behavior among birds.

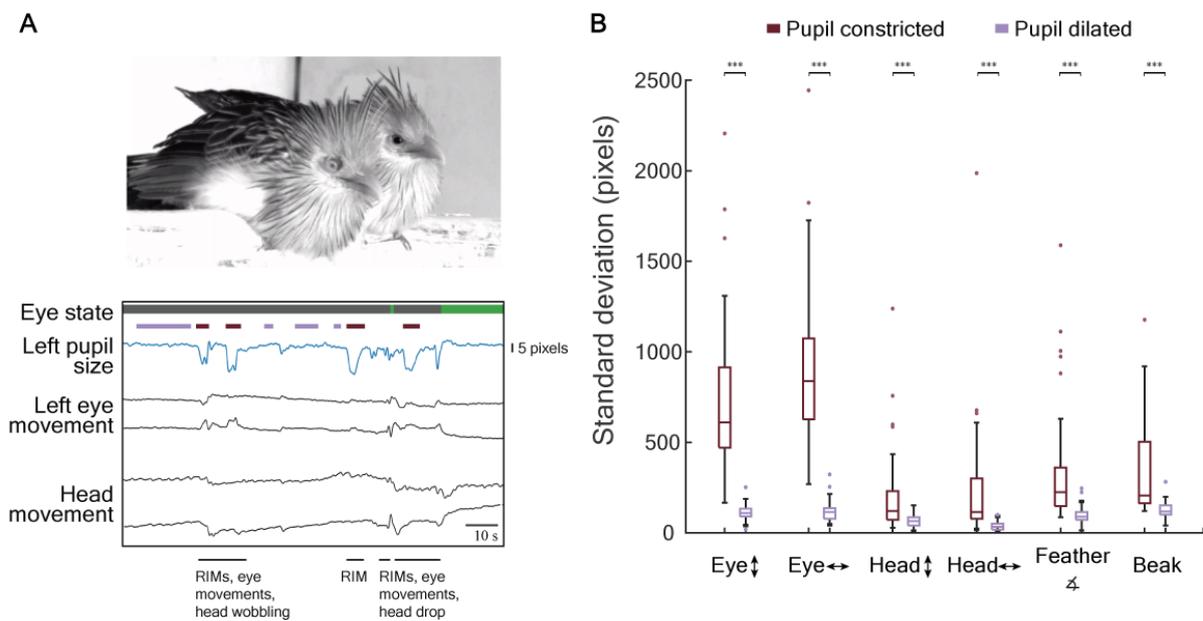


Figure 3. Sleep-state-dependent pupil dynamics in guira cuckoos.

(A) Top: two guira cuckoos during night recordings. Note the constricted pupil visible through the closed eyelid in the bird on the left and the dilated pupil in the bird with the open eye on the right. Bottom: 120 s of traces tracking pupil size and eye and head movements (relative to the camera) in a sleeping guira cuckoo. The eye state is color-coded as in

(B). The periods with pupillary constriction are marked in dark red and the bouts with a stable dilated pupil in lilac. (B) Signal variability in vertical and horizontal eye and head movements (relative to the camera), head feather angle, and movement of the upper beak during pupil dilation and RIMs. All the variables measured had higher variability, indicative of REM sleep, during RIMs compared to periods with dilated pupils (Student's t test, $p < 0.05$). See also Video S2.

Mechanisms mediating RIMs during sleep in birds

Although birds and mammals share a common parasympathetic pathway for pupillary constriction, the iris sphincter muscles controlling the size of the pupil are smooth in mammals, but mostly striated in birds (Pilar and Vaughan, 1971; Ferrari and Koch, 1984; Coli et al., 2016). Also, although the iris sphincter muscle receives cholinergic input from the Edinger-Westphal nucleus via the ciliary ganglion in mammals and birds, pupillary constrictions in response to light are mediated by muscarinic cholinergic receptors in mammals, but nicotinic cholinergic receptors in awake birds (Pilar et al., 1987; Barsotti et al., 2010a, b; Barsotti et al., 2012; Petritz et al., 2016; Susanti et al., 2021). However, given the presence of some smooth muscle fibers in the adult avian iris musculature of several bird species (Oliphant et al., 1983; Scapolo et al., 1988; Coli et al., 2016), we investigated whether the same mechanisms that mediate pupil constrictions during wakefulness mediate RIMs during sleep. By applying eye drops containing nicotinic cholinergic, muscarinic cholinergic, and adrenergic antagonists, we pharmacologically blocked the respective receptors in one

of the eyes, while the other received saline treatment. The treatment was administered when the lights turned off to minimize visual discomfort and stress. By comparing the number of RIMs during sleep in the first hour of the night between the treated and control eye we found a significant decrease only in the eye treated with a nicotinic cholinergic receptor antagonist (Student's t-test, $p = 2.5 \times 10^{-4}$, Figure 4A and B). This effect persisted for 2-4 h, a duration similar to that reported in awake pigeons treated with the same nicotinic cholinergic receptor antagonist (Susanti et al., 2021). Thus, both pupillary constrictions during wakefulness and RIMs during REM sleep are mediated via the contraction of striated muscle activated by nicotinic cholinergic receptors (Figure 4C).

Our behavioral and pharmacological experiments show that birds and mammals have opposite pupillary activity during wakefulness, non-REM sleep and REM sleep. These differences are due (in part) to the fact that, unlike the mammalian iris, which is controlled by smooth muscles activated by muscarinic cholinergic receptors, avian pupillary constrictions are mediated through the contraction of striated iris muscles activated by nicotinic cholinergic receptors. Our findings are inconsistent with the proposed function of pupillary constriction occurring during mammalian sleep, wherein constriction of the pupils during deep non-REM sleep attenuates light reaching the retina and thereby prevents it from disrupting sleep. By contrast, in pigeons pupillary constricted states occupied only $5.4 \pm 0.7\%$ of the time asleep, while the rest of time the pupils were dilated, thereby potentially allowing visual input to reach the retina. Albeit filtered through the transparent eyelid, this might allow pigeons to detect approaching predators, especially while napping during the day. Finally, the fact that pigeons usually have their pupils dilated during sleep, suggests that the transitory pupillary constrictions (RIMs) occurring during REM sleep might serve functions other than protecting sleep.

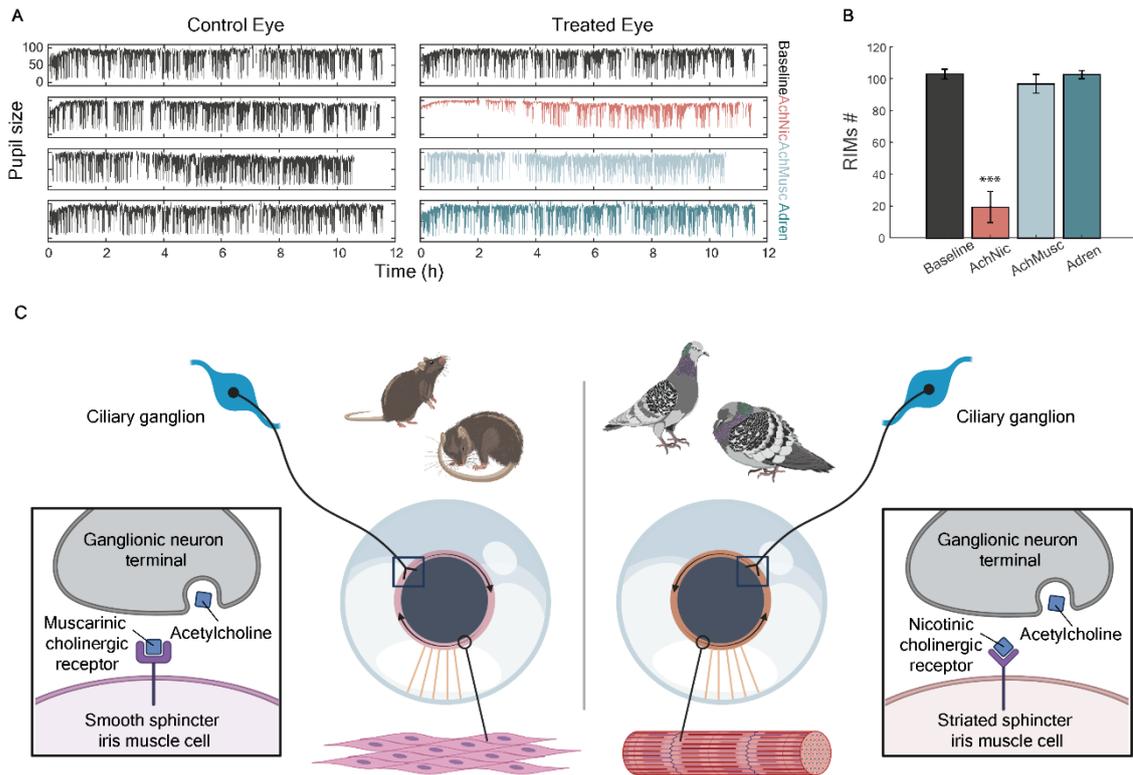


Figure 4. Nicotinic cholinergic receptors mediate pupillary constrictions in birds during wake and sleep

(A) Raw traces from one pigeon depicting the pupil dynamics during sleep in the control eye and the treated eye. For comparison, the first row shows the pupillary activity during a night without treatment.

(B) Bar plot with the number of RIMs (mean \pm std) during the first hour of recordings for each of the three treatments normalized per 100 RIMs in the control eye. For comparison, the same analysis was done for one recording without treatment per bird (“baseline” column). The eye treated with the nicotinic cholinergic antagonist shows significantly fewer RIMs, while the other two treatments had no significant effect.

(C) Comparative representation of the mechanisms underlying changes in pupil size during wake and sleep in mammals and birds. Both mammals and birds receive cholinergic terminals on the iris sphincter muscle from the ciliary ganglion neurons. In birds, unlike in mammals, the pupillary constrictions are mediated via nicotinic cholinergic receptors, typical of striated muscles.

Mouse and pigeon illustrations by Damond Kylo.

Are RIMs simply an epiphenomenon of brain activation occurring during REM sleep, or do they serve a function? Considering the co-occurrence of courtship displays with pupillary constrictions, the tight relationship between RIMs and bouts of REM sleep in birds, and growing evidence that REM sleep is involved in regulating emotional reactivity in mammals (Wassing et al., 2019), RIMs might reflect the activation of neural networks involved in processing information with ‘emotional’ content. Even though RIMs might be an epiphenomenon of such activity, they could be experimentally useful as markers of when the brain is activating these networks. Conversely, as proposed for other types of twitching occurring during REM sleep, RIMs, *per se*, might serve a

function. In mammals, REM sleep related twitches (generated in the brainstem) are most abundant during early infancy, and are thought to play a role in mapping the motor system by providing sensory feedback to the developing cortex (Blumberg et al., 2013a). In adult mammals, twitches might also recalibrate the sensorimotor system. Twitches of the wings, bill, eyes, and irides (RIMs) that occur during avian REM sleep might serve a similar function. Indeed, as with other motor displays (Byers et al., 2010; Barske et al., 2011), maintaining fine control of the iris musculature might be very important, especially if pupillary constrictions play a role in interspecific communication and mate choice.

If RIMs serve a function similar to that proposed for twitches in mammals, bidirectional communication should exist between pallial forebrain sensorimotor regions and the iris sphincter muscle. During wakefulness, constriction could be controlled by the sensorimotor region of the rostral hyperpallium through its indirect projections to the Edinger-Westphal nucleus via the reticular mesencephalic area (Gamlin and Reiner, 1991; Wild and Williams, 2000). However, it is unknown whether the reticular mesencephalic area or other brainstem regions initiate RIMs during REM sleep. In addition, muscle spindles, structures that provide sensory input about the contractile state of striated muscles, have not been found in the avian iris; although it cannot be ruled out that other stretch sensors provide this sensory feedback to the sensorimotor hyperpallium (Oksche, 1973). Ultimately, additional research is needed to understand the mechanisms and functions of RIMs occurring during REM sleep and pupillary constrictions during wakefulness, as well as the potential interrelationship between these behaviors.

Finally, our findings have broad implications for efforts to trace the evolution of sleep states. Traditionally, this is done through identifying which species (e.g. mammals, birds, reptiles, etc.) exhibit non-REM and REM sleep. A species is thought to exhibit these states if several neurological and physiological components (e.g., EEG, muscle tone, eye movements, respiration, etc.) vary together in the same manner as in mammals. In most respects, this is true for birds. However, our findings reveal that traits, such as pupillary behavior, can manifest in a diametrically opposite manner in different taxonomic groups; in this case, due (in part) to the different types of muscles controlling the iris. Consequently, our study highlights the diverse ways that sleep components can manifest and the need for adjusting rigid mammalian-based templates when studying the evolution of sleep (Blumberg et al., 2020).

Methods

Experimental Model and Subject Details

All experiments were conducted in accordance with German animal welfare guidelines and were approved by the local ethics committees for animal experimentation (Government of Upper Bavaria, permit number: ROB-55.1-2532.Vet_02-18-89).

Twelve adult domestic pigeons (*Columba livia*, Budapest variety; 6 females, 6 males, genetically sexed; weight 240.8 ± 14.4 g) were used in this study. Five were obtained from a local breeder and seven were from our breeding colony. All of the pigeons were drug and test naïve. Although we observed similar pupillary behavior in other varieties of domestic pigeons, the Budapest variety was particularly suitable for this study because of their large eyes relative to the size of the head, the brightly colored irides, translucent eyelids and the fact that, like other pigeon varieties, they do not tuck the head in the feathers while asleep. Birds were reared and housed in enriched colony aviaries. During the recording phase, the animals were housed individually in an indoor enriched aviary (1 m x 2 m x 2 m; 12 h:12 h light:dark photoperiod; 21°C) adjacent to, and in visual and auditory contact with, two other pigeons, each housed in similar aviaries. Food and water were provided *ad libitum*.

Method Details

Surgical procedures

Pigeons were surgically instrumented for recording the electroencephalogram (EEG) from both hemispheres, neck electromyogram (EMG), and eye movements under isoflurane anesthesia (1-3% in 0.5 LPM oxygen). The birds were administered an injection of Diazepam (2 mg/kg) into the breast muscle 15 min prior to surgery, an injection of Metamizol (150 mg/kg) into the breast muscle shortly before isoflurane anesthesia, 4 drops of Procaine (2%) for local analgesia prior to skin incision, and an intramuscular injection of Meloxicam (0.5 mg/kg) and Lidocaine gel to the wound at the end of the surgical procedure. The electrodes consisted of round-tipped gold pins (0.5 mm diameter) for the EEG and electrooculogram (EOG), and gold-plated electrode balls (1 mm diameter) for the EMG. The EEG electrodes were placed over the visual hyperpallium which is visible through a distinct window of thin bone on the surface of the cranium. Two EEG electrodes were placed on the dura overlying each (left and right) hyperpallium (2.5 and 3 mm lateral of the midline for the anterior and posterior electrode, respectively, and separated by 5 to 6 mm along the anterior-posterior axis). One EEG reference electrode was placed above the cerebellum. The EOG electrodes were placed in the orbital ridge bone over the eye and the EMG electrodes were placed in the neck muscles.

An infra-red LED (Kingbright®, 940 nm, 50 mA, 1.2 V) was placed rostrally on top of the skull to illuminate the back of the eyes (Yüzgeç et al., 2018). The LED was powered during the surgery and following recordings by a detachable light weight, flexible USB cable. The position of the IR light was customized for each individual for the best signal-to-noise ratio. After positioning, the LED was encased in dental cement.

Of twelve, seven pigeons (one female and six males) were implanted with a thermistor (Semitec®, 223Fμ3122-07U015, 0.28 mm diameter) inserted approximately 1 mm into the dorsal surface of the left and right hyperpallium, mid-way between the two EEG electrodes. The data was used in another study (Ungurean et al., 2020a).

Courtship experiments

Pigeons were recorded in an arena (1.8 m x 1 m x 0.5 m) equipped with a food dish, a water dish and a breeding box, placed symmetrically at each of its ends and identical to those used in the housing aviaries. The accelerometry was recorded at 256 Hz via a 2.6 g head-mounted transmitter within the range of ± 2 G along the three axes. To maximize the image acquisition frequency (14 fps), only the left eye was recorded using an IR-sensitive head-mounted minicamera module (NanEye®, ams AG, 1 mm x 1 mm) connected to a processing board (BAP Image Systems GmbH, AC62KMP) powered by a battery (19.6 g). The processing board and the battery were attached to the birds' back with a Velcro strip glued to trimmed feathers. In addition to the visible room lighting, four IR lamps (940 nm) were placed symmetrically on both sides of the recording area. Sound was recorded at 5 kHz with a lavalier microphone (Audio-technica®, ATR3350) placed close to the recording area and a custom-made Matlab script. To visualize the sound data at lower sampling rates, the power in the frequency range of the pigeon coos (200 - 600 Hz) was extracted. Two wide field cameras (Axis Communications®, M2026-LE Mk II) were placed above and laterally to view the entire arena.

The six male pigeons were progressively habituated to the recording equipment and experimental setup over three sessions lasting ten minutes each. On the experimental day, the individual was taken from the same-sex colony aviary, equipped, and placed in the recording arena. Following about five minutes of baseline recordings (289.3 ± 7.2 s), a female was introduced in the recording area for another ten minutes. Each individual was presented with an arbitrarily chosen different female.

Sleep experiments

Electrophysiological data and head movements (accelerometry) were recorded at 256 Hz via a 2.6 g head-mounted transmitter and a custom-made acquisition interface. The electrophysiological data were filtered between 0.1 Hz and 128 Hz digitalized between ± 0.128 mV on 16 bits. Images from the minicamera modules were recorded via a thin, light-weight tether that allowed the pigeon to freely move on its perch. The birds were disconnected from the tether and were free to move throughout their aviary once the lights came on in the morning. Two head-mounted minicamera modules (NanEye®, ams AG, 1 mm x 1 mm) connected to an image processing board (BAP Image Systems GmbH, AC62KUSB) were used to record the eyes at 10 fps. The pupils were visible when the animal had the eyes both open and closed. The signal allowed the detection of eye opening and closure, eye movements via tracking the center of the pupil, and the quantification of changes in pupil size.

After at least one week of post-surgical recovery, the pigeons were progressively habituated to the recording equipment. The birds were then recorded during the 12-h night while confined to their preferred elevated sleeping perch (Tisdale et al., 2018b).

Guira cuckoo sleep recordings

Adult Guira cuckoos of unspecified sex were recorded in the Bird Park of Villard les Dombes, in France. Six individuals were housed in an indoor aviary equipped with one sleeping perch. The birds were video recorded throughout the night at 20 fps using IR-sensitive cameras and an IR light (940 nm) source placed inside the aviary, close to the entrance, facing the sleeping perch. The video data was acquired using Streampix 8 software.

Pharmacological experiments

Six of the implanted pigeons (two females and four males) were used for this experiment. When the lights switched off, the treatment (12 μ l nicotinic cholinergic antagonist – rocuronium bromide, 10mg/mL; 100 μ l muscarinic cholinergic antagonist – Tropicamide, 0.5%; or 100 μ l adrenergic alpha-1 receptor antagonist – Dapiprazole, 0.5%), were administered to one of the eyes, while the other received the same amount of saline (NaCl solution, 0.9%, Ecolav®, B. Braun). Treatment sessions were separated by at least two days. The order of treatments and the side of the drugged eye were balanced and randomized.

Quantification and statistical analysis

Behavioral scoring in courtship experiment

The data was visually scored with 1-s epochs using accelerometry, sound and whole arena video recordings, while blind to pupil size. The states were defined as follows: active wake – pigeon engaged in motor behaviors other than courtship, copulation, or pecking; quiet wake – pigeon not engaged in motor behaviors other than head movements to scan the environment; courtship – pigeon actively displaying, with stereotypical walking, bowing, and cooing; copulation – the male mounted on the female, while flapping his wings; female approaching – the female was actively heading in the direction of the male, or was allofeeding or allopreening him; pecking – the pigeon was pecking near or at the food dish. Due to the low amount of data points, the pecking behavior was not included in the statistical analysis.

Sleep state scoring

The recordings were visually scored using a custom-made interface for wakefulness, non-REM sleep, and REM sleep using standard criteria (Martinez-Gonzalez et al., 2008; Lesku et al., 2011b; Tisdale et al., 2018b). Briefly, wake was characterized by low-amplitude, high-frequency EEG activity in combination with waking behaviors with open eyes; non-REM sleep by high-amplitude, low-frequency EEG activity, usually with bilateral eye closure and general quiescence; and REM sleep by an ‘activated’ low-amplitude, high-frequency EEG in combination with eye closure, eye movements, twitches, and head movements, including drops, as shown in the accelerometry recordings (Figure 2D). Because the amount of IR light emitted from the eye using iBIP is very low, additional IR light could not be used to acquire whole body videos in the dark, as this would reduce the contrast between the pupil and iris. Nonetheless, to illustrate typical REM sleep behavior, including pupillary constrictions, Video S1 was acquired with external IR lights, but without iBIP.

Unlike previous studies which employed 4-s epochs, we used 1-s epochs to gain a more precise measure of the onset and offset of bouts of REM sleep. A bout of a given state was defined as one or more epochs of that state uninterrupted by an epoch of any other state; i.e. bouts could be as short as 1 s. Consistent with previous studies (Martinez-Gonzalez et al., 2008), REM sleep represented 14.4 ± 2.6 % of the total night-time sleep time, and occurred in 950.1 ± 145.5 bouts, totaling 94.2 ± 16.8 minutes (Tobler and Borbély, 1988).

RIMs detection and quantification

Pupil size was estimated with a custom-made Matlab script which measured the bright pixel area of the pupil image. Pupillometry measurement artifacts were removed from the raw signal by manual scoring. The artifacts corresponded to periods of transition between eye opening and closure. Of note, during this time the pupil was dilated. The data was normalized between 0 and 100 using the 0.25% and 99% quantiles. Slow variations in the baseline were subtracted from the signal using a moving mean over 20 s. The data was smoothed with a moving mean over 700 ms, inverted, and the peaks with a minimum height of 15 and a minimum prominence of 1 were detected using 'findpeaks' Matlab function. Thus, a change in pupil size was identified as a RIM when the pupil area decreased below 85% of the corrected baseline; above 85% the pupil was considered dilated. RIMs were further classified as single and multiple peaks. A peak was considered single if the signal returned to baseline values before another peak occurred and multiple if it did not. Due to the overlapping nature of constriction bursts, the duration was calculated only for single peaks. The half-height duration of single peaks was also extracted using 'findpeaks' Matlab function.

RIM-REM analysis

All peaks detected from the pigeon data were tested to determine whether they occurred during a bout of REM sleep. If they did not, the nearest REM sleep bout start or end was detected and the timespan between the two was calculated.

From the cuckoo data, one video containing a single bird was cropped and down sampled for further analysis. The pupil size, beak movements, head and feather position were tracked using DeepLabCut. If, as in pigeons, RIMs cooccur with other REM sleep features, such as eye movements, head movements and drops, feather collapsing or beak movements, we expect to find higher variability in these signals during periods with pupil constrictions compared to periods with a stable dilated pupil. Thus, the signals corresponding to periods with closed eyes were manually scored, and then pupil constrictions were detected using the same methodology as for the pigeons. Periods with a quiet dilated pupil were automatically extracted by thresholding the moving std (5-s window) of the pupil signal. For each detected bout in the two conditions, the std of the signal corresponding to the other body parts was calculated.

Statistics

The linear mixed model analyses were performed in R using the lmer package. The other statistical tests were performed in Matlab. The level for statistical significance was set to $p < 0.05$.

In the courtship experiment, the pupil size data was averaged in 1-s bins corresponding to the scored epochs. To find the correlation between the pupil size and the bird's state, we modeled the courtship data using linear mixed effect models with the binned pupil size as the response variable, the state as predictor and the bird identity as a random effect. The state had seven categories: AW female absent, QW female absent, AW female present, QW female present, Courtship, Copulation, and Female Approaching. The data for the Pecking category was not included in the statistical model due to low sample size. A post-hoc test with the contrasts of interest was performed using the 'glht' function of the multcomp package, wherein the p-values were corrected for multiple comparisons (Bretz et al., 2010).

In the sleep experiments, to test for a differential expression of RIMs in females and males, we modeled data using linear mixed effect models, with the number of RIMs for each night as the response variable, the sex as predictor and the bird identity as a random effect. Next, we used the concept of repeatability as a measure of how consistent the expression of RIMs is within individuals, irrespective of their sex. More formally, repeatability reflects the proportion of variance accounted for by differences among individuals. The estimation of the behavior repeatability was computed using ‘rpt’ function from the rptR package (Nakagawa and Schielzeth, 2010).

In the cuckoo experiment, the distribution of the standard deviation during RIMs and during bouts with a stable dilated pupil were tested using a Student’s t-test. In pigeons, the effect of different pharmacological reagents compared to saline treatment were also tested using a Student’s t-test. For the baseline data (no treatment applied), one of the eyes was randomly considered as a reference.

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Supplementary Material

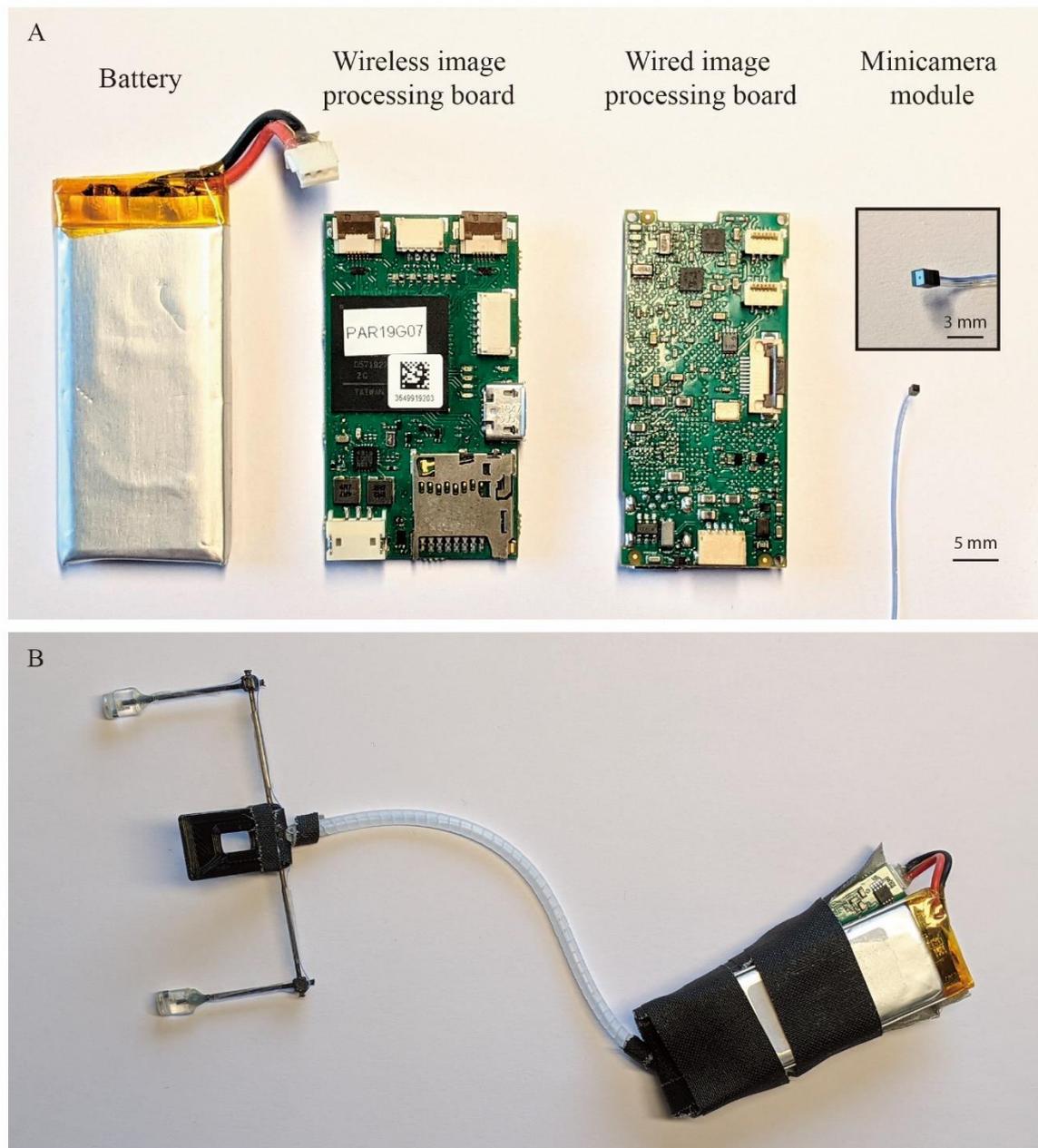


Figure S1. Equipment used for recording the eyes of freely moving and sleeping pigeons. Related to Figure 1 and 2.

(A) From left to right: battery used to power the wireless processing board; wireless processing board used for recordings in freely-moving animals; wired processing boards used for sleep recordings; minicamera module (CMOS sensor and lens) used for pupillometry and recording eye movements.

(B) Example of an assembled wireless system consisting of two minicamera modules installed on an adjustable frame. The cable connecting the cameras to the processing board are protected by a flexible cable wrap. The processing board and battery are covered by protective tape. For visualization purposes, only part of the electronic equipment was covered in the current figure.

Supplementary Tables

Table S1. Changes in pupil size (n=2752) predicted by state in the courtship experiment. Related to Figure 1.

The data was modelled using the state as predictor and the bird identity as a random effect. The contrast estimates were calculated and tested post-hoc. The ‘Estimate’ and ‘SE’ columns report the estimated difference between the focal state relative to the baseline state. The p-value tests whether these differences are significantly different than zero. Note the high and significant differences between courtship vs active wake, quiet wake and copulation (i.e. the pupil is more constricted by about 30% during copulation relative to the other states); no difference between courtship vs female approaching (i.e. both states have a similar effect); no difference between copulation vs quiet wake (i.e. the pupils are equally dilated in both states). The p-values were adjusted for multiple comparisons. Significant p-values are reported in bold. The proportion of variance explained by random effects (BirdID), calculated as its proportion relative to the total variance (Res and BirdID) after accounting for fixed effects (state) was 29.3%.

Focal state	Contrast		Estimate	SE	z	p
	Focal state	Baseline state				
Quiet Wake	Active Wake		1.24	0.47	2.62	0.06
Quiet Wake ♀	Active Wake ♀		0.02	0.83	0.03	1
Active Wake ♀	Active Wake		8.89	0.89	9.99	<0.001
Quiet Wake ♀	Quiet Wake		7.68	0.43	18.02	<0.001
Courtship ♀	Active Wake ♀		31.28	0.87	35.94	<0.001
Courtship ♀	Quiet Wake ♀		31.26	0.49	64.31	<0.001
Courtship ♀	Copulation ♀		30.44	0.9	33.91	<0.001
Courtship ♀	Female Approaching ♀		-1.61	0.77	-2.08	0.23
Copulation ♀	Quiet Wake ♀		0.82	0.94	0.87	0.94
Var(Res)*			42.94			
Var(BirdID)*			17.78			

♀ indicates female present

* Var(Res): residual variance. Var(BirdID): variance component of individuals (6 levels, i.e. 6 birds)

Table S2. Estimates of fixed (Bird sex) and random effects (Bird ID), and individual repeatability of RIM expression. Related to Figure 2.

No significant differences were found between males and females ($p_{\text{bird sex}} = 0.73$). A large proportion of the variance (65.8%) is explained by the random variable (Bird ID), after accounting for fixed effects (Bird sex). The number of RIMs expressed during one night is significantly repeatable for a given individual ($p_{\text{repeatability}} < 0.001$), thus some birds consistently expressed more RIMs while others consistently expressed less.

Predictor	Estimate	SE	p	95% CI
<i>Intercept</i>	1175.19	76.15		
Bird sex	38.92	107.39	0.73	
Var(Res)*	29310			
Var(BirdID)*	15260			
Repeatability†	0.64	0.16	<0.001	[0.24 0.84]

* Var(Res): residual variance. Var(BirdID): variance component of individuals (12 levels, i.e. 12 birds)

† repeatability was calculated without bird sex in the model, using the ‘rpt’ function from the ‘rptR’ package, with 1000 parametric bootstraps and 1000 permutations

Supplementary Videos

The supplementary videos are available on the page of the publication: <https://doi.org/10.1016/j.cub.2021.09.060>

Video S1. Sample video of a Budapest pigeon recorded under IR illumination during non-REM and REM sleep. Related to Figure 2.

From top to bottom: right and left side videos with whole body perspective and a zoom on the bird’s eye; hypnogram with non-REM and REM sleep color-coded in blue and red, respectively; right and left hyperpallial EEG signals; the black vertical line indicates the electrophysiological correlates of the current video frame. Note how the pupils are visible under closed eyelids and the pupillary constrictions (RIMs) occur during the REM sleep episode demarcated by typical eye movements, feathers collapsing and head movements, including a large drop.

Video S2. Sample videos of guira cuckoos recorded under IR illumination during sleep. Related to Figure 3.

The first example depicts two cuckoos during a period with prominent active behaviors, including eye, bill and feather movements, head movements and drops, and RIMs. The second example shows one cuckoo cycling through quiescent sleep periods with dilated pupils and periods with active behaviors. Although we did not categorize the data by sleep state for the analysis, the second example was labeled with the presumed ‘non-REM’ and ‘REM’ sleep states to help with the interpretation.

Chapter 4

Functional magnetic resonance imaging of the sleeping avian brain

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Authors: Gianina Ungurean*, Mehdi Behroozi*, Leonard Böger, Paul-Antoine Libourel, Onur Güntürkün, Niels C. Rattenborg

*Contributed equally

Author contributions: Conceptualization, G.U. and N.C.R.; Methodology, G.U., M.B., O.G, and N.C.R.; Hardware and software, P.-A.L.; Investigation, G.U., M.B. and L.B.; Formal Analysis, G.U. and M.B.; Writing – Original Draft, G.U. and N.C.R.; Writing – Review & Editing, G.U., M.B., P.-A.L. and N.C.R.; Resources, P.-A.L., O.G. and N.C.R.; Supervision, P.-A.L, O.G. and N.C.R.

Abstract

Sleep is a behavior encountered across all species and taxonomic groups studied so far and is thought to fulfill important functions, although a consensus over what those functions are and what their relative importance is has not been reached. Most studies done on taxa other than mammals mainly employ EEG to study sleep, and do not capture activity occurring below the brain surface. Birds, like mammals, express NREM and REM sleep, defined by similar behavioral, physiological and electrophysiological correlates. In this study, for the first time, we used fMRI to image whole-brain activity of sleeping birds. Unlike mammals, pigeons readily slept in the noisy scanner and frequently cycled through both NREM and REM sleep. During REM sleep, extended regions of the visual system, basal ganglia and somatomotor pathways were activated, similar to previous reports on humans. Unexpectedly, NREM sleep was correlated with increased fMRI signal in regions with CSF, which likely reflect increased CSF flow. We propose that the activation found in REM sleep relates to its memory function, and is incompatible with CSF clearance.

Introduction

Both birds and mammals engage in two sleep states, rapid eye movement (REM) and non-REM (NREM) sleep during which the behavioral, physiological and neurophysiological correlates vary in a similar manner between the two sleep stages in both taxa (Ungurean et al., 2020b). Notably, the EEG patterns characteristic of avian NREM and REM sleep are strikingly similar to those of mammals: while in NREM sleep the EEG is dominated by slow waves (0.5-4 Hz), generated by synchronized oscillations of neuronal membrane potentials between depolarized and polarized states, and REM sleep is associated with activated wake-like activity. However, EEG measurements primarily capture the electrical activity of areas situated close to the brain surface. In birds, EEG signals are further limited by the fact that several cortical (pallial) areas are nested deep inside the brain (Box 1). The electrophysiological activity of such avian structures can be accessed only with depth electrodes, but the spatial resolution of this method is necessarily limited by the number of electrodes that can be placed in the brain.

Metabolic-based methods have proven useful as an alternative for recording whole-brain activity. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) are two widely used imaging methods which measure glucose metabolism and the hemodynamic response respectively, as markers of neural activity (Hyder and Rothman, 2017). Although the temporal resolution is much slower compared to EEG, both methods can be used to map activity throughout the brain, including activity correlated with sleep states and sleep behavior.

Indeed, several studies on human subjects have correlated PET and fMRI activity with NREM or REM sleep, or with various phasic electrophysiological or behavioral activity occurring during the two sleep states. REM sleep was associated with broad metabolic activity of various cortical, thalamic and midbrain areas. More specifically, the activation spanned the mesopontine tegmentum and midbrain nuclei, thalamus, basal forebrain and diencephalic structures, limbic structures in the medio-temporal lobe, including amygdala and hippocampus, medial prefrontal cortex, occipito-temporal

visual cortex, and anterior cingulate cortex (Maquet et al., 1996; Braun et al., 1997; Nofzinger et al., 1997; Maquet, 2000; Nir and Tononi, 2010). The activations were related, among others, to (re)activation of brain networks involved in awake experiences, emotional memory processing and memory consolidation (Maquet et al., 2000; Maquet, 2000).

Conversely, NREM sleep was associated with a global decrease in the cerebral blood flow compared to wakefulness. The most prominent decreases in activity were found in subcortical (brainstem, thalamus, basal ganglia, basal forebrain) and cortical (prefrontal cortex, anterior cingulate cortex, and precuneus) regions. Besides the slow waves, in humans and other mammals, spindles (11-16 Hz oscillations) are also a hallmark of NREM sleep. Despite the global decreases in blood flow, activity time-locked to slow waves and spindles revealed transient increases in blood-oxygen-level-dependent (BOLD) signal in humans. Spindles were found to positively correlate with activity in the lateral and posterior thalamus, anterior cingulate cortex, insula and superior temporal gyrus. Slow waves were associated with increased responses in the inferior and medial frontal gyrus, parahippocampal gyrus, precuneus, posterior cingulate cortex, ponto-mesencephalic tegmentum, and cerebellum. These activation patterns were suggested to reflect areas involved in the generation and/or modulation of NREM oscillations, memory consolidation and sensorimotor processing during this sleep state (Dang-Vu et al., 2010).

Although fMRI continues to be an increasingly popular neuroscience technique, and has been used to investigate different aspects of the brain-wide activation during sleep (Fukunaga et al., 2006; Dang-Vu et al., 2008; Horowitz et al., 2008; Boly et al., 2012; Tagliazucchi and Laufs, 2014; Mitra et al., 2015; Stevner et al., 2019; Logothetis et al., 2012), few studies on humans (Wehrle et al., 2007; Hong et al., 2009; Sterpenich et al., 2014) and none on non-human mammals or other taxa examined physiological (non-anesthetized) REM sleep. In birds, most of the sleep studies used EEG recordings. Only recently, van der Meij and colleagues used depth probes to record the hyperpallium (homologue of the mammalian primary visual cortex) and the thalamus in naturally sleeping birds. They found that slow waves propagate in a similar manner in the avian and mammalian cortex (van der Meij et al., 2019a). However, oscillations in the spindle range (11-16 Hz) were not found in the hyperpallium or thalamus. The same study reported bursts of gamma activity during REM sleep confined to the thalamic input layers of the hyperpallium. Interestingly, previous studies did not find hippocampal theta activity during REM sleep in birds (Rattenborg et al., 2011; Payne et al., 2021). Taken together, these lines of evidence suggest that some electrophysiological processes, which have previously been shown to play a role in memory consolidation, are similar in mammals and birds, while others are different or absent in birds.

Which functional pathways are active in REM and NREM sleep in birds? How similar are they with the ones previously described in mammals? And how do similarities and differences in the metabolic-based recordings relate to similarities and differences previously reported using electrophysiological techniques? We aimed to tackle these questions using fMRI on sleeping pigeons.

Results

Pigeons engage in NREM-REM sleep cycle in fMRI conditions

In this study, we adapted a habituation protocol previously used for imaging awake birds (Behroozi et al., 2020) to acquire sleep data (Figure 1A). Briefly, pigeons previously implanted with an MRI compatible head fixation pedestal were habituated to restraint in a mock MRI machine for progressively increasing amounts of time. The habituation was done in the dark and in the absence of other stimuli, aside from the noise of the machine. Infrared (IR) illumination and IR-sensitive video cameras were used to monitor sleep behavior. Additionally, six birds were implanted with removable EEG electrodes to monitor brain state. At the end of the training phase, the birds were cycling through both NREM and REM sleep, as identified through the behavioral and EEG recordings (Figure 1B, left). After training and one week prior to fMRI recordings, the EEG electrodes were removed. When placed in the fMRI scanner, the birds continued to exhibit sleep behavior, including bouts with eye and bill movements and/or pupillary constrictions (RIMs) characteristic of REM sleep (Figure 1B, right).

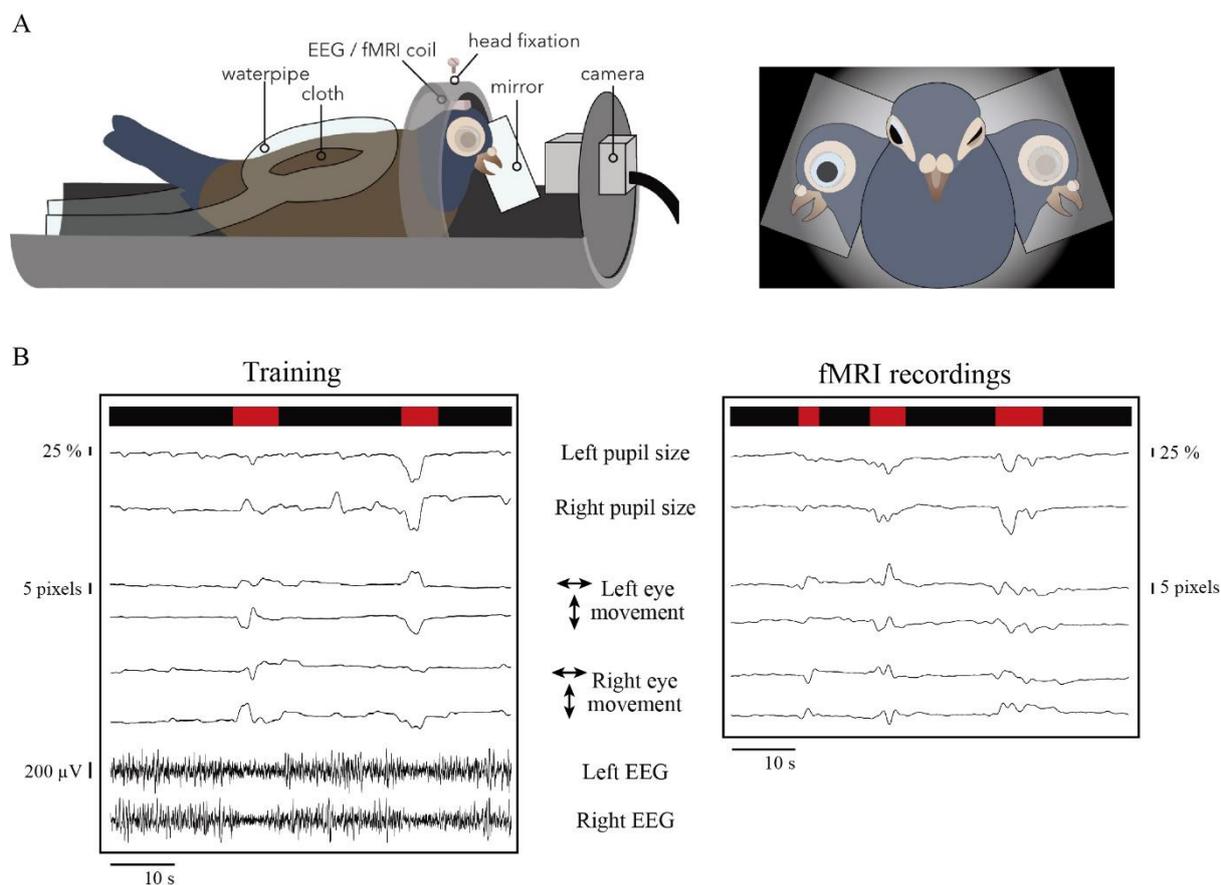


Figure 1. Pigeons cycle through NREM and REM sleep in fMRI recording conditions.

(A) Schematic illustration of the fMRI setup. The pigeons are installed in the system in supine position, with their head fixed to limit motion artifacts (left). An infrared (IR) camera is positioned frontally to record the bird's behavior. Two mirrors, installed on each side of the bird's head, provide information about the state of the eye, of the pupil and bill movements (right).

(B) At the end of their training phase, pigeons cycle through NREM and REM sleep, identifiable from the EEG recordings. As in naturally sleeping birds, NREM sleep is associated with behavioral quiescence and dilated pupils, while REM sleep is associated with eye and bill movements, and pupillary constrictions (left). The same behavioral patterns are observed during the recordings in the fMRI machine (right).

Pigeon illustrations by Leonard Böger.

The data was manually scored using the video recordings. To make the scoring process more efficient, actimetry signals were calculated for the eyes, irides, and bill region and visualized together with the video recordings. NREM sleep was defined by bilateral eye closure, stable breathing (as visible in the videos and bill actimetry signals), immobility of the eyes and absence of other bill movements than those related to breathing. REM sleep was characterized by eye closure accompanied by movements of the eyes, bill, and/or the collapse of head feathers held erect during preceding NREM sleep. As described in the previous chapter, rapid constrictions and dilations of the iris which we named RIMs are closely associated with REM sleep. Thus, RIMs were also used to identify REM bouts and their absence to confirm NREM sleep.

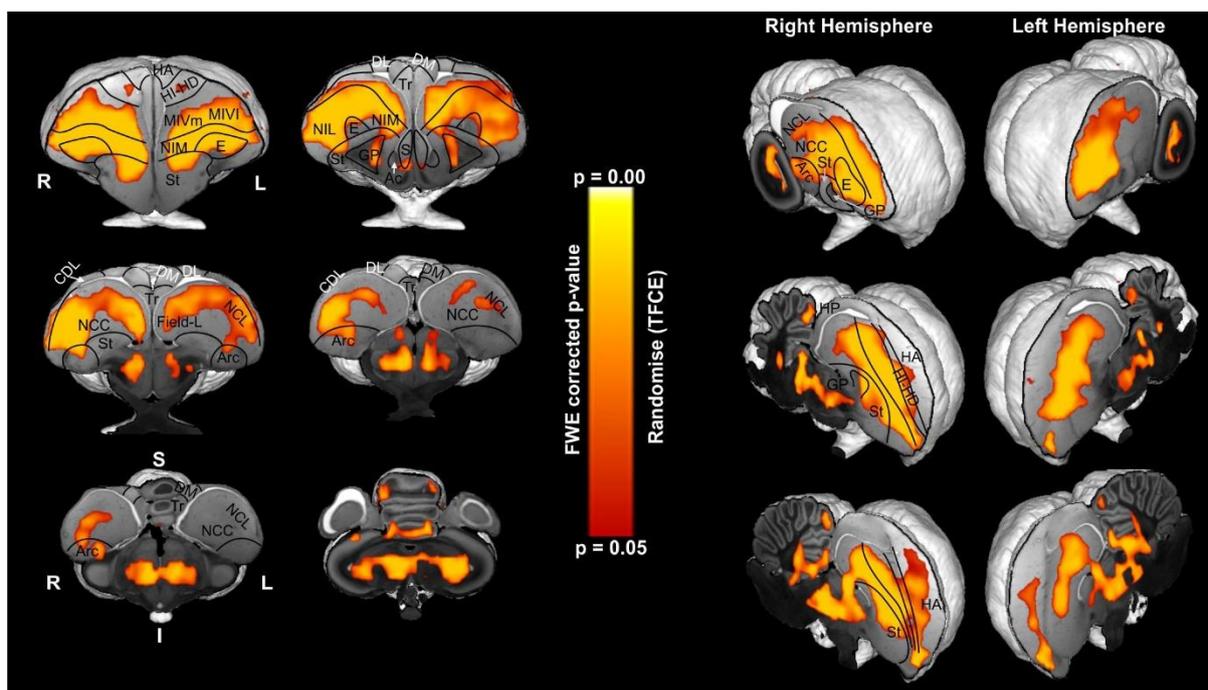


Figure 2. fMRI activity temporally related to REM bouts. The coronal (left) and sagittal (right) sections depict the activation clusters spanning cortical (visual and sensorimotor hyperpallium, mesopallium, nidopallium, entopallium and right arcopallium) and subcortical (striatum, globus pallidus, thalamus, the core of the optic lobes and cerebellum) areas.

BOLD signal patterns during REM sleep

To reveal the activity patterns associated with REM sleep behavior, we used the REM vs NREM sleep contrast in the first level analysis. The estimated parameters of brain activation were then entered into a second-level group analysis using FSL-randomize nonparametric permutation tests tool (Jenkinson et al., 2012) with 5000 permutations

and threshold free cluster enhancement (TFCE) to correct for multiple comparisons. Following this preliminary analysis, we found significant clusters of activation in numerous brain regions. From cortical (pallial) to subcortical (subpallial), significant clusters of activity were found in the somatomotor region of the (anterior) hyperpallium, extended areas of the visual hyperpallium, mesopallium, and nidopallium, most of the entopallium, the right arcopallium and pallial amygdala, the antero-medial striatum adjacent to the entopallium and medial striatum, the medio-ventral nucleus rotundus, extended areas in the core of the optic lobes, and antero-medial areas of cerebellum lobules (Figure 1, see also Box 1). Notable areas that did not show significant activation in REM sleep, compared to NREM sleep, were the hippocampal formation and the optic tectum.

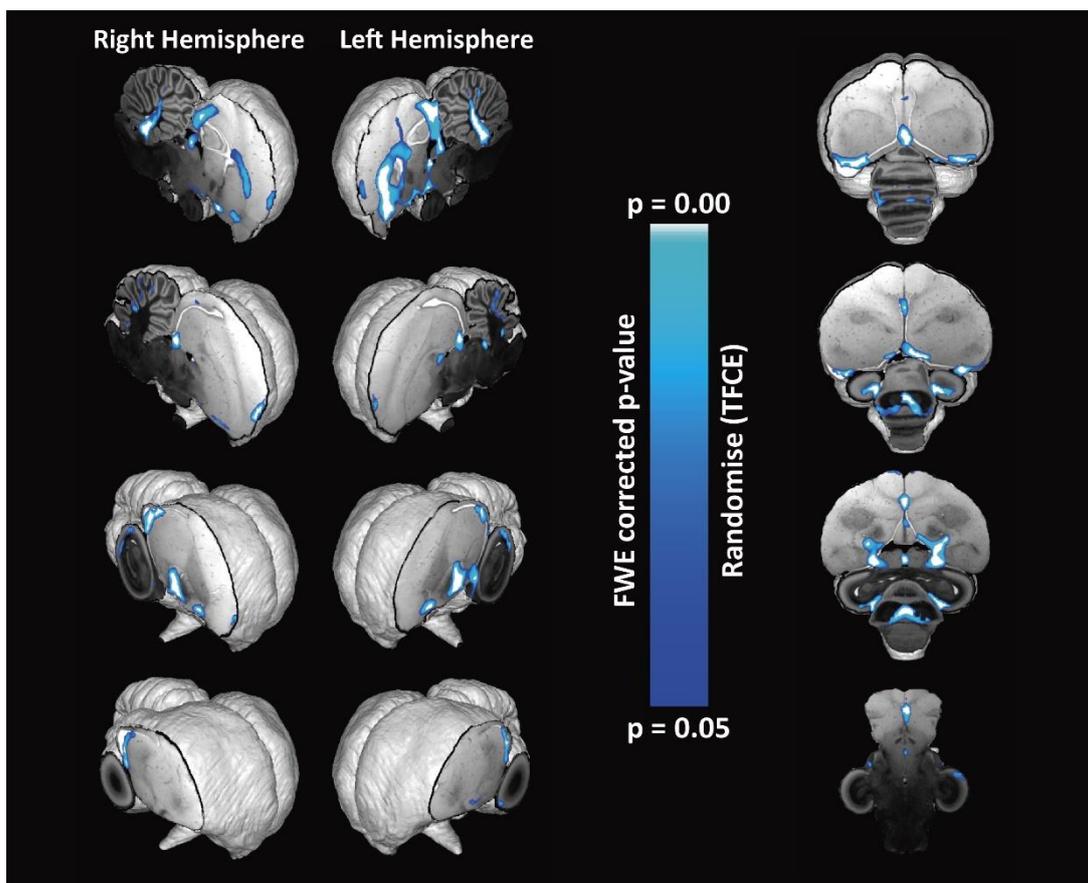


Figure 3. fMRI activity temporally related to NREM bouts. The activation clusters are concentrated in CSF areas. The activity extends anteroposteriorly (see sagittal sections) and mediolaterally (see sagittal and horizontal sections), along the ventricles. The activity found in the striatum overlaps with a region penetrated by major blood vessels (see third sagittal cut in the right hemisphere).

Signal patterns during NREM sleep

Unexpectedly, most of the significant activation clusters associated with NREM sleep were located in non-brain areas or close to the brain border. The signal overlapped with numerous regions of the avian brain ventricular system (Mestres and Rascher, 1994). It extended along the lateral ventricles, first medially along the antero-posterior axis, then

bifurcated laterally as it reached the posterior side of the forebrain, englobing the caudal nidopallium. Clusters of activation were also found in the fourth ventricle (primarily the cerebellar recess), the mesencephalic tectal ventricles, and regionally in the third ventricle, near the lateral ventricle. The activation was not continuous throughout the entire ventricular system, lacking signal in the brainstem area of the fourth ventricle. Other activated areas were found in the interhemispheric fissure, the areas between the optic tectum and the cerebellum and in the rostral HA layer of the hyperpallium, near the brain surface (Figure 3). The clusters of activation present in brain areas were located in the striatum.

Discussion

Methodological considerations

Our current data serves, first, as a proof of concept for fMRI data acquisition in sleeping birds. After habituation to the setup and experimental conditions, pigeons readily cycled through both NREM and REM sleep. Actually, in our recordings it was difficult to identify clear epochs of wakefulness, as pigeons closed their eyes soon after being installed in the scanner. From our previous EEG recordings in freely moving animals we observed that immobility, even during eye opening, is often accompanied by synchronized EEG activity, indicative of drowsiness. Thus, we currently lack a comparison between wakefulness and NREM and REM sleep, which could be informative from a comparative perspective, as some of the previous studies (Maquet et al., 1996; Nofzinger et al., 1997) reported regions activated in each sleep state in relation to quiet wakefulness (i.e. awake, in the absence of any cognitive task). Indeed, the type of contrast used influences the results, as previously found in PET studies (Maquet, 2000), and is important for a correct interpretation of the data. Wakefulness in birds could be obtained by i) repeating the experiment during the subjective day of the animals, when the sleep pressure is lower and the chance of obtaining spontaneous wakefulness is higher; ii) recording animals trained to report readiness to engage in a task; iii) awakening the birds using different types of sensory stimuli and extracting the common activated areas. In all cases, EEG recordings are needed to confirm the wakefulness state. It is, however, important to note that although at EEG level both wakefulness and REM sleep appear similar, quiet wakefulness is a fundamentally uncontrolled heterogenous state, as it depends on the mental and cognitive state of the individual or stress levels, even in studies of humans (Christoff et al., 2009; Tagliazucchi and Laufs, 2014).

REM sleep activation

The REM-NREM sleep contrast in our fMRI study revealed that some of the activity found in REM sleep in birds is similar to the activity previously described in mammals, namely the activation of visual areas, basal ganglia and motor regions.

As in mammals, the avian visual system consists of two ascending pathways from the retina, the thalamofugal and the tectofugal, equivalent of the geniculostriate and colliculo-pulvinar-cortical pathways. The avian thalamofugal neurons connect the retina to the dorsal lateral geniculate nucleus (GLd, homologous with the LGN), and to the thalamorecipient layer (IHA) of the hyperpallium (homologous with the primary visual

cortex). The tectofugal pathway runs from the retina to the optic tectum, the nucleus rotundus (nRT) in the thalamus, to reach the entopallium in the avian forebrain. The information is then relayed to higher associative areas in the mesopallium and nidopallium. In our preliminary results, we found activation in regions of the hyperpallium, including IHA, the thalamic input layer, suggesting that parts of the thalamofugal visual pathway is activated during REM sleep. Additionally, the extended activation clusters in the core of the optic tectum, in the medial nRT, in the entopallium, mesopallium and nidopallium suggest a strong activation of the tectofugal pathway. Unlike in mammals wherein most of the visual processing occurs through the thalamofugal pathway, in birds the tectofugal pathway plays the major role. As REM sleep has been suggested to be involved in the processing and consolidation of different memory traces, including visual memories (Maquet et al., 2000; Sterpenich et al., 2014), our results might reflect reactivation of neuronal ensembles recruited during wake behaviors, for memory processing and consolidation.

As in mammals, the optic tectum receives input from structures of the basal ganglia system. The basal ganglia in birds has a similar functional connectivity as in mammals, with indirect and direct pathways, which functionally inhibit or promote movement, respectively. Two direct pathways corresponding to the mammalian striatopallidal and cortico-striatal-thalamo-cortical circuitries also exist in birds. The first pathway runs from the striatum, to the substantia nigra pars reticulata (SNr), and the brainstem; while the second connects the striatum to the internal globus pallidus (GPi), the motor thalamus, and the cortex (Reiner, 2016). In addition, the avian striatum receives input from all major cortical areas, as in mammals. The activation of the various cortical areas, as well as partial activation of the striatum, globus pallidus and thalamus indicates that the basal ganglia pathways are activated during REM sleep. Our results are in line with previous PET studies on humans, wherein the basal ganglia showed strong decreases in activity during NREM sleep and increases during REM sleep (Braun et al., 1997). Lesion and electrophysiological studies indicate a role of the basal ganglia in the sleep-wake cycle (Lazarus et al., 2013). Due to its role in motivation, habit formation and reward behaviors and the cholinergic innervation of the striatum, a neuromodulator characteristic of REM sleep, the basal ganglia were suggested to play a role in the memory processing function of REM sleep (Hasegawa et al., 2020). In birds, the basal ganglia are involved in various forms of learning (Xiao et al., 2018; Gao et al., 2019). As some basal ganglia pathways are more segregated and even specialized in songbirds (Doupe et al., 2005), research investigating the activity of avian basal ganglia during sleep could shed new light on the role of the basal ganglia in memory processing.

The major motor output of the avian basal ganglia, however, is via striatal projections to the pretectum, subthalamic nucleus (SNr), tectum and the brainstem. This pathway is not well defined in mammals, but is present in birds, reptiles and amphibians. Based on its input to the tectum, this pathway was long suggested to be involved in head and eye movements in all three taxa (Reiner et al., 1998; Reiner, 2007), although this remains to be demonstrated. If true, the activation of the striatum and pretectal nuclei, could be related to the phasic eye saccades occurring during REM sleep. This hypothesis is also supported by activation of cerebellar lobes involved in different aspects of eye movements in both mammals (Kheradmand and Zee, 2011) and birds (Wylie, 2013; Wylie et al., 2018).

The activation of the rostral pallium, a somatomotor area where the body is represented, is similar to the previously reported activation of the human motor cortex. In mammals, it is thought that the strong inhibition of motoneurons inducing paralysis in the skeletal muscles prevents motor output (Valencia Garcia et al., 2018). This hypothesis is based on the observation that patients suffering of REM sleep behavior disorder, a disease characterized by loss of muscular atonia due to neurodegeneration of brainstem neurons, exhibit strong and complex motor behaviors during REM sleep (Arnulf, 2012). Complex behaviors were thought to be generated cortically and to reflect dream enactment. Alternatively, based on the fact that cats and rats continue to exhibit twitches after the cortex was disconnected from the brainstem (Villablanca, 1966; Kreider and Blumberg, 2000); and that in infant rats the activity of the motor cortex follows rather than precedes twitching behavior (Tiriac et al., 2014), it has been also suggested that the activation of the motor cortex is not necessarily related to motor output (Blumberg and Plumeau, 2016). Conversely, the sensorimotor cortical activity could reflect feedback from twitches generated subcortically, which contribute to the development and maintenance of the sensorimotor cortical maps (Blumberg et al., 2020). Our results add further support for the second hypothesis. Birds engage in REM sleep while standing, and thus, need to maintain tone in the postural muscles to prevent them from falling. Additionally, when present, muscle atonia manifests only locally and transiently, as suggested by slow head drops, without systematic reductions in neck muscle tone (Rattenborg et al., 2019). If muscle atonia is necessary for suppressing motor behavior generated cortically during REM sleep, then the avian motor cortex should remain inactive during REM sleep to avoid potentially dangerous behavior. Thus, the activity of the somatomotor cortex and other motor cortical areas could be due (in part) to subcortically generated twitches observed during REM sleep, like eye, iris, bill and wing movements. The activation of motor systems might also be linked to other processes, like synaptic changes related to motor learning, as shown in mice (Li et al., 2017).

Interestingly, we found that only the right pallial amygdala was more active during REM sleep than NREM sleep in pigeons. In birds, the pallial amygdala is a brain region with similar connectivity as the mammalian amygdala, and with activity correlated to social and mating interactions (Güntürkün et al., 2017). It has been suggested that REM sleep specific activation of the amygdala is responsible for the emotional component of dreams and is involved in emotional memory processing (Deliens et al., 2014; Genzel et al., 2015). The unilateral activation of the amygdala in our results could be related to lateralized input prior to the recording sessions, which we did not measure. Scanning the surroundings preferentially with one of the eyes was reported in several bird species; while chicken chicks decrease their behavioral fear response more following lesions in the right arcopallium compared to left-side lesions (Leliveld et al., 2013), suggesting a behavioral and brain lateralization in processing ‘emotional’ information. Interestingly, several lines of evidence indicate that in mammals normal amygdala activity during wakefulness involves lateralized and/or asymmetric responses depending on the valence of the stimulus (positive versus negative), type of response (fear, stress, pain, happiness, etc), type of stimulus (visual, auditory), the delivery side of the stimulus (left or right), or subject’s sex (Allen et al., 2021; Sergerie et al., 2008; Blair et al., 2005; Frühholz et al., 2015; Cahill et al., 2004). Thus, our results raise the question whether altering unilaterally amygdala activity during wakefulness in mammals would also reveal lateralized reactivation during REM sleep, as suggested in pigeons. However, since the avian pallial amygdala neighbors the arcopallium, a

premotor area, further studies are needed to establish the specificity of this activation to regions involved in aroused behavior.

The broad activation in the pons, thalamus and cortex (pallium) bears some resemblance with the mammalian ponto-geniculo-occipital (PGO) waves. PGO waves have been initially described in cats (Jouvet and Michel, 1959), where they are most prominent in the pons, lateral geniculate nucleus (LGN) and the occipital cortex. PGO waves occur during wakefulness, NREM, but mostly during REM sleep. Waves with similar characteristics have also been described in the pons and LGN of nonhuman primates (Cohen and Feldman, 1968; Vuillon-Cacciuttolo and Seri, 1978), in the pons (P-waves) and cerebellum of rats (Kaufman and Morrison, 1981; Marks et al., 1980; Farber et al., 1980), and in the pons, thalamus and cortex of humans (Ioannides et al., 2004; Fernández-Mendoza et al., 2009; Lim et al., 2007; Gott et al., 2017). PGO waves are currently seen as a widespread mammalian feature of REM sleep and were suggested to play a role in sensorimotor integration, learning, and development. In humans, before their presence being confirmed with depth electrodes in patients with Parkinson disease (Lim et al., 2007; Fernández-Mendoza et al., 2009), PET and fMRI studies were the first to propose their existence, reporting activity in the key PGO areas (Peigneux et al., 2001). Our results raise the question whether PGO waves also exist in birds, although they were not found in the electrophysiological studies done on birds (van der Meij et al., 2019a, but see Sugihara and Gotoh, 1973). Further studies are needed, including electrophysiological recordings in the pons, to firmly conclude whether PGO waves are present or not in birds.

Finally, the lack of activation in the hippocampal formation during REM sleep, in contrast to the activation previously reported in humans, could be related to the lack of hippocampal theta activity in birds (Sugihara and Gotoh, 1973; Rattenborg et al., 2011; Canavan and Margoliash, 2020; Payne et al., 2021). Alternatively, this result might indicate a similar level of activity in the hippocampus in both NREM and REM sleep, or that potential differences in activity result in no net metabolic change between the two states.

NREM sleep activation

Our finding of significant activation clusters in non-brain areas during NREM sleep was unexpected. Such activity was reported once before in humans (Hong et al., 2009) and is likely related to the recent discovery that CSF flow is enhanced during NREM sleep. Increases in CSF flow during sleep are thought to remove potentially damaging metabolic waste products from the brain. During wakefulness, increased neuronal activity leads to the accumulation of metabolic waste products, such as amyloid- β , implicated in Alzheimer's disease (Kang et al., 2009; Winer et al., 2020). During NREM sleep, cerebral spinal fluid (CSF) flows into the paravascular space along arteries penetrating the brain and enters the parenchyma (facilitated by the astrocytic water channel aquaporin) (Ulv Larsen et al., 2020), where it mixes with the interstitial fluid, and then drains via venous paravascular spaces, removing waste in the process (Xie et al., 2013). A potential mechanistic link between this CSF flow and slow waves was revealed in a recent fMRI study of humans. Ultra-slow oscillations (0.05 Hz) in the amplitude of EEG slow waves were coupled to and preceded blood oxygen level-dependent and CSF oscillations in the brain and fourth ventricle, respectively (Fultz et

al., 2019). It is thought that reduced neuronal activity during slow wave off-states reduces oxygen consumption, and thereby cerebral blood flow, volume and pressure, leading to increased CSF flow through the brain. If correct, then CSF flow should diminish or stop during REM sleep, a state characterized by activated wake-like EEG activity without slow waves; however, this has so far not been established (Hablitz and Nedergaard, 2021).

The increase in CSF flow during NREM sleep in pigeons provides comparative support for the waste removal hypothesis for NREM sleep first proposed in mammals. In addition, the absence or attenuation of flow in REM sleep suggests that NREM sleep-related slow waves play a role in CSF flow. In comparison to the on/off bursting pattern of neuronal activity during NREM sleep, the continuous wake-like activity and associated metabolic demand for an influx of oxygenated blood (Bergel et al., 2018; Turner et al., 2020) might hydraulically impede CSF flow by increasing intracerebral pressure during REM sleep. Additionally, if reduced neuronal activity during slow wave off-states indeed reduces cerebral blood flow and increases CSF flow, the activation clusters in the striatum – which appear to be located in the areas of major blood vessels penetrating the brain – might be related to enhanced CSF flow along the perivascular space of contracted vessels.

Although both NREM and REM sleep were shown to be individually involved in different types of memory consolidation (Boyce et al., 2016; Klinzing et al., 2019), and might fulfill complementary roles in this process (Vyazovskiy and Delogu, 2014; Tamaki et al., 2020), their relative contribution is still poorly understood. Interestingly, our findings suggest that only the memory processing steps occurring during NREM are compatible with CSF flow and waste removal. Indeed, in addition to impeding CSF flow, brain activation during REM sleep likely produces new metabolic waste products. In this case, despite complementing some NREM functions, REM sleep competes with other NREM functions. Conversely, the alternation between the two sleep states and the oscillations in slow wave amplitude during NREM sleep might facilitate waste clearance by increasing CSF mixing and diffusion in the parenchyma (Fultz et al., 2019; Tuura et al., 2021). Further investigation of such dynamics and potential tradeoffs between NREM and REM sleep might inform our understanding of the mechanisms and functions of the NREM/REM sleep cycle and sleep, in general.

If the signal recorded in the periventricular space is indeed related to CSF movement, it is reasonable to expect it throughout the entire ventricular system. The lack of signal in the anterior part of the fourth ventricle is likely explained by a combination of the following reasons: (i) the anterior region of the fourth ventricle is a constrained area; (ii) this area is located deep in the brain, making signal detection more difficult compared to more superficial constrained areas, like the lateral ventricles; (iii) the CSF flow/molecular exchange could be more enhanced in localized areas. Nonetheless, it is interesting to note that similar discontinuities are also apparent in humans (Hong et al., 2009).

Although the source of the signal found in the ventricular system remains unknown, several lines of evidence support the idea that the signal relates to a difference in CSF dynamics (flow, production or/and composition) during NREM compared to REM sleep. Previously, CSF flow was measured with fMRI using contrast agents injected in brain CSF areas (Iliff et al., 2012; Lee et al., 2015; Ringstad et al., 2017; Absinta et al., 2017) or fast acquisition sequences (Fultz et al., 2019). In the latter case, the signal

corresponds to ‘fresh’ fluid, which has not experienced radiofrequency pulses entering the first imaging plane. However, CSF flow was also extracted from the first imaging plane following relatively slower acquisition paradigms and without contrast agents (Han et al., 2021). The fact that the CSF signal in our data did not lose intensity through saturation along successive acquisition planes could be explained by the fact that we used interleaved slice acquisitions, which would allow signal desaturation. An enhancement in the fMRI CSF signal has also been demonstrated after oxygen enrichment, for example through oxygen (100%) inhalation (Losert et al., 2002; Anzai et al., 2004; Zaharchuk et al., 2005; Mehemed et al., 2014). Thus, the data might also reflect higher oxygen concentrations in the CSF flowing inside the brain during NREM sleep, which through its paramagnetic properties ‘dilutes’ other diamagnetic molecules dampening the fMRI signal. To robustly demonstrate that the NREM sleep related signal is due to CSF flow, it would be necessary in the future to repeat the sleep behavioral paradigm while imaging the brain using fast acquisition sequences. This protocol would also allow to calculate the speed of the flow and compare it with the current data available in mammals. Invasive methods using tracers injected in the CSF could also be used, since our study shows that birds can be trained to sleep head-fixed. Using magnetic resonance spectroscopy (MRS) and comparing the emission spectra in CSF regions during NREM and REM sleep could also unravel potential differences in CSF composition between the two states, and shed light on the origin of this signal.

Methods

Surgical procedure

The birds were surgically implanted with a plastic pedestal prior to the fMRI experiments, as described in Behroozi et al., 2020. Briefly, the birds were induced using a ketamine/xylazine mix (70% ketamine, 30% xylazine) administered intramuscularly in the breast muscle. The birds were maintained under anesthesia using isoflurane. The head was fixed in the stereotactic frame. After skin incision, plastic screws screwed anteriorly and posteriorly in the porous cavities of the skull and a plastic pedestal was fixed on top of the bird’s skull using dental cement. In addition to the plastic pedestal, six birds were implanted epidurally over the hyperpallium with wire electrodes, soldered to a connector. The electrodes and connector were embedded in biocompatible skin glue, covered by a thin layer of dental cement. After the habituation phase, the birds were lightly anesthetized using isoflurane, and the dental cement layer, followed by removal of the EEG electrodes. The implant was then covered again with a smooth layer of dental cement.

Habituation procedure and electrophysiology recordings

To reduce stress and induce the birds to engage in sleep behavior, the pigeons were habituated to the fMRI setup. The pigeons were wrapped in a cloth, then head-fixed in a mock fMRI restrainer for progressively increasing amounts of time, from 10 to 120 minutes. A recording of the scanner noise, with increasing sound levels, was also played during the training sessions. The habituation was done in the dark, and in the absence of stimuli other than the sound. All birds were recorded during this phase using IR lighting and IR-sensitive cameras, at 30 fps. The EEG signal of the six birds implanted with electrophysiology electrodes was additionally recorded at 256 Hz using the Oneiros recording system.

Data acquisition

All MRI data collection was carried out in a Bruker BioSpec small animal MRI scanner system (7T horizontal bore, 70/30 USR, Germany) at Ruhr University Bochum, Bochum, Germany. We used an 80 mm transmit quadrature birdcage resonator in combination with a single-loop 20-mm surface coil to collect resting-state and anatomical data. By positioning the ring surface coil around the head, it was possible to reduce the artefacts related to the different sources of motion, such as different body part movements. The Bruker ParaVision 6 software was used to acquire the MR data. Since changes in respiration may affect BOLD signals and the brain connectivity maps, respiration waveform was measured using a small pneumatic pillow placed under the pigeon's chest muscles (Small Animal Instruments, Inc. Model 1025T monitoring and gating system) during all resting-state measurements.

To localize the correct position of pigeons' brain within the scanner bore, a series of scout images were measured at the beginning of each scanning session. Three runs (horizontal, coronal, and sagittal) were acquired using multi slice rapid acquisition (RARE) with the following parameters: TR = 4 s, TE_{eff} = 40.37 ms, RARE factor = 8, no average, acquisition matrix = 128 × 128, FOV = 32 × 32 mm, spatial resolution = 0.25 × 0.25 mm², slice thickness = 1 mm, number of slices = 20 horizontal, 17 sagittal, and 15 coronal. Based on these images, 11 slices were defined to cover the entire telencephalon, optic lobes, midbrain and cerebellum.

Resting-State fMRI (rsfMRI) data were acquired using a single-shot multi-slice RARE sequence adapted from Behroozi et al. (2018, 2020), with the following parameters: TR = 4000 ms, TE_{eff} = 41.58 ms, partial Fourier transform accelerator = 1.53, encoding matrix = 64 × 42, acquisition matrix = 64 × 64, FOV = 30 × 30 mm², in-plane spatial resolution = 0.47 × 0.47 mm², radio-frequency pulse flip angles for excitation and refocusing = 90°/180°, slice thickness = 1 mm, no slice distance, slice order = interleaved, excitation and refocusing pulse form = scanner vendor gauss512, receiver band width = 50,000 Hz. Two saturation slices were positioned manually to saturate most of the signal from the eyes to avoid brain image corruption due to eye movements. Each run of the rsfMRI recordings included a minimum of 1000 volumes. To check the reproducibility and stability of the results, rsfMRI data were recorded twice in different days.

For spatial normalization, high-resolution T2-weighted image from all animals were acquired using a RARE sequence with the following parameters: TR = 2000 ms, TE_{eff} = 50.72 ms, RARE factor = 16, number of averages = 1, FOV = 25 × 25 × 15 mm³, matrix size = 128 × 128 × 64, spatial resolution = 0.2 × 0.2 × 0.23 mm³. Total scanning time was 17 min.

To represent the results at high-resolution anatomical images, one of the animals was deeply anesthetized with equithesin and transcranial perfused with a phosphate-buffered saline solution (PBS, 0.12M), followed by a mixture of paraformaldehyde (PFA 4%) and Dotarem® (1%). High-resolution 3D anatomical data was collected using a T2*-weighted sequence using a FLASH sequence with a flip angle of 15°, a spectral bandwidth of 50 kHz, a number of mean values of 64, a TR of 9.47 ms, a TE of 4.738 ms and a scan repetition time of 5,000 ms. The images have a field of view of 25*25*20 mm³ and an isotropic spatial resolution of 0.05 mm in all three directions. The recording time was ~ 20 h. The behavior of the animals was simultaneously recorded using an

MRI-compatible video camera (12M-i, MRC Systems, Heidelberg, Germany; B/W) with an LED-light (IR) (outside the pigeon's visible spectrum) incorporated. The videos were acquired at 30 fps. All recordings were synchronized using a TTL signal.

Behavioral data analysis

The sleep states were manually scored using the video recordings into three states, wakefulness, NREM and REM sleep according to the following criteria: awake when the bird had the eyes completely open and eye movements were present; NREM sleep when the eyes were closed, the pupils dilated, and in absence of eye or other movements except for breathing; REM sleep when the eyes were closed and other phasic behaviors occurred (eye, bill, and iris movements) and/or feathers collapsed. To make the scoring process more efficient, actimetry signals (global changes in pixel values) from the eye and bill region were also used along with the videos.

MRI data processing

All fMRI data processing was performed using tools from FMRIB Software Library (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL>, version 5.0.9), the Analysis of Functional NeuroImages (AFNI, version 20.0.09 <https://afni.nimh.nih.gov/>), and Advanced Normalization Tools (ANTs, <http://stnava.github.io/ANTs/>) software. A standard pipeline was used to pre-process the rsfMRI data. Prior to any preprocessing steps, the DICOM images were converted to 4D NIFTI data, the voxel size was upsampled by factor of 10, and first 10 volumes were discarded to ensure longitudinal magnetization reached steady state. Later, the following standard data preprocessing steps were applied: (i) motion correction using MCFLIRT (FSL's intra-modal motion correction tool), (ii) slice time correction (interleaved acquisitions), (iii) removal of non-brain tissue from functional data (using a BET and manual cleaning), (iv) spatial smoothing (using a Gaussian kernel of FWHM = 8 mm, after upscaling), (v) global intensity normalization by a single multiplicative factor for each scan run (for group analysis), (vi) high-pass temporal filtering (with cutoff at 100 s), and (vii) registration of the data to the high-resolution anatomical images using affine linear registration (12 degrees of freedom). A population-based template was generated using `antsMultivariateTemplateConstruction.sh` script (Avants et al., 2008). After analyzing individual subjects, the results were normalized to the population-based template using FMRIB's Nonlinear Image Registration Tool (FNIRT) (Andersson et al., 2007) for group analysis. For visualizing the results, the group results were non-linearly warped to the high-resolution post-mortem anatomical image. 3D MRI images were visualized using the Mango software (<http://ric.uthscsa.edu/mango/>, version 4.1).

Statistics and reproducibility

Whole-brain statistical analysis was carried out using FEAT (FMRI Expert Analysis Tool), a part of FSL. The GLM contained the two regressors, the NREM and REM sleep blocks. We used our estimated pigeon hemodynamic response function (HRF, Behroozi et al., 2020) to be convolved with the mentioned regressors. In addition, six scan-to-scan estimated motion parameters were added as nuisance regressors to account for any residual effects of animal movement. Contrasts of interest from first-level analyses were then taken to the higher-level analysis using the nonparametric FSL's Randomise function (Winkler et al., 2014). Statistical significance was assessed using permutation testing with 5,000 permutations, Threshold-Free Cluster Enhancement (TFCE), and a family-wise error (FWE) corrected cluster p-value of $p = 0.05$.

The experiment was run in two independent sessions. A two-sample paired t test (implemented in FSL) was used to compare two sessions of the experiment. For each session, an independent GLM was modelled. The results were compared using a two-sample paired t test in FSL. Since there was no significant difference between the two sessions of each run, we modelled both sessions as one GLM model.

General discussion

Our results advance our knowledge of avian sleep, and sleep neurophysiology in general. We showed that some correlates of REM sleep vary in a similar, partially similar or opposite manner in birds compared to mammals. Our work has implications for understanding the evolution of sleep through comparative approaches, implications for studies on social communication, and methodological implications for studying pupillometry during wake and sleep and employing fMRI methodology for sleep studies on birds.

The evolution and function of sleep examined through comparative approaches

Sleep is a diverse behavior and even birds, the taxonomic group closest to mammals in terms of NREM and REM sleep correlates, exhibit important differences in terms of sleep phenomenology. However, both similarities and differences can be as informative in understanding the mechanisms and functions of sleep. Indeed, mammal-based theories for the functions of NREM and REM sleep that fail to explain this diversity are likely missing the big picture.

Multiple potential roles for RIMs: reflecting ‘emotional’ memory replay and consolidation, mapping and maintaining the motor system, maintaining a healthy eye environment, but not protecting sleep

The study on the pupillary activity during wake and sleep shows that the avian pupil behaves oppositely throughout all vigilance states, a finding reflecting not only a particularity of birds’ behavior, but one with potentially important implications for understanding avian and mammalian sleep. In mammals, growing lines of evidence indicate that REM sleep plays a role in emotional memory processing (Wassing et al., 2019; Genzel et al., 2015). The association between pupil constrictions and courtship displays, and its reoccurrence during REM sleep periods raises the question of whether RIMs reflect reactivations of neural networks involved in awake (courtship) behavior. If this is the case, it would be interesting to identify the neural substrates underlying RIMs using whole brain imaging methods, and to further record the respective areas in behaving and sleeping animals using electrophysiological methods. The reoccurrence of similar firing patterns or oscillatory frequencies could indicate that related memories are replayed and consolidated. Comparing the similarities and differences between oscillations and the mechanisms underlying memory consolidation processes with those

described in mammals, might reveal overarching principles not readily apparent through mammalian studies alone.

Although the adult avian iris sphincter muscles are mostly striated (Coli et al., 2016; Pilar and Vaughan, 1971; Ferrari and Koch, 1984), chicks' iris muscles transition from predominantly smooth early during development to striated as they mature (Pilar et al., 1987; Volpe et al., 1993; Link and Nishi, 1998). Such major changes in the iris musculature likely require subsequent somatomotor mapping. Further, if pupillary constrictions are an important component of social signaling, play a role in mate choice, or carry various forms of information (see next section) during awake behaviors in adult birds, it would be essential to develop, adapt and maintain fine control of the iris muscles. Like other twitches of skeletal muscles occurring on a background of muscle atonia (Blumberg et al., 2013a), RIMs might contribute to sensorimotor mapping by conveying sensory feedback to the brain. Investigating whether RIMs, like other twitches, occur more frequently early in development and their relationship with the smooth to striated transition could shed light on their potential twitch-like function. As this system requires sensory receptors and an ascending sensory pathway, it will be also important to establish whether stretch receptors exist in the avian iris muscle and their neural connections. Suppressing RIMs in one eye using nicotinic cholinergic antagonists and performing sleep fMRI recordings could also inform about the existence of sensory feedback from the pupil.

Conversely/additionally, RIMs could fulfill functions related to the maintenance of a healthy eye environment. Birds have large eyes with thick retinæ, abundant in densely packed neurons, favorable for increased visual acuity (Meyer, 1977). Although metabolically highly active, avian retinæ are devoid of inner blood vessels. To regulate the composition of the vitreous body, birds have evolved a highly vascularized fan-shaped structure, called the pecten, that protrudes into the vitreous humor and supplies oxygen and nutrients. Fast oscillatory eye movements cause the pecten to mix and diffuse the molecules in the vitreous body (Pettigrew et al., 1990). The eye environment is also regulated through fluid flow. In mammals, fluid can either exit the eye through the pupil and the anterior chamber, or through transport by retinal astrocytic cells along the optic nerve, from where it drains along the lymphatic vessels (Mogensen et al., 2021). In mice, the ocular glymphatic fluid exchange is driven by the higher intraocular versus cranial pressure and is enhanced by light-induced pupillary constrictions (Wang et al., 2020). Similar to the fast oscillatory eye movements that propel the pecten during both sleep and wake in birds, RIMs might act as another motor component active during REM sleep, which maintains the ocular-cranial pressure gradient and facilitates glymphatic waste removal, and/or facilitates fluid movement via the anterior chamber. Further studies on the mechanisms of avian ocular clearance and activation of motor systems during sleep could contribute to better understanding sleep physiology and pathology.

RIMs do not appear to serve a visual gating function, like that proposed for pupil constriction in mice (Yüzgeç et al., 2018), as they occupy a very small proportion of the time spent asleep ($5.4\% \pm 0.7\%$). Rather, the time spent asleep with dilated pupils might allow pigeons and cuckoos to process some visual information and detect approaching predators. Although many bird species can tuck their head while asleep, and/or have feathers covering their eyelids, completely dilated pupils might allow enough light through to detect ecologically meaningful visual input, such as the shadow of a looming predator. Birds which usually tuck their heads, can also sleep with the head untucked like pigeons (Ferretti et al., 2019) and might use this posture to increase the potential for changes in light to reach the retina through their dilated pupils.

Implications of the fMRI study

The preliminary results on metabolic activity related to NREM and REM sleep in birds revealed overlaps and differences with the previous studies on mammals. In birds, REM sleep was associated with activation across large subcortical (subpallial) and cortical (pallial) areas. This pattern is consistent with our brain temperature study, wherein REM sleep is followed by brain temperature increases, likely due to an inflow of warm blood from the core to support the increased neuronal activity. Interestingly, no brain temperature variations were found in the bearded dragon following REM-like bouts with increased neural activity. In the future it would be informative to assess whether the lack of temperature increases is linked to the absence of a temperature differential between the core and the brain in reptiles, or whether it reflects the absence of increased blood flow during REM sleep. The activation of the visual system during avian REM sleep is consistent with the visual nature of birds, while the lack of activity in the hippocampus could be related to the lack of a theta rhythm in birds. As the oscillatory activity of sleeping birds differs from mammals, it has been suggested that they have evolved different mechanisms for memory processing and consolidation (van der Meij et al., 2020). Future fMRI-based studies could shed a light on the mechanisms of memory consolidation in birds.

If the NREM activations found in CSF areas are indeed related to CSF flow during sleep, it suggests that brain clearance is a function of sleep that was conserved, or evolved independently in both mammals and birds. In mammals, norepinephrine levels and slow wave amplitude were suggested to play major roles in CSF flow regulation. Awake mice show increased CSF flow after norepinephrine antagonist treatment (Xie et al., 2013); and peaks in slow wave ultra-slow oscillations (0.05 Hz) in the amplitude of EEG slow waves precede blood CSF peaks in the human brain (Fultz et al., 2019). In the future, it would be interesting to investigate whether similar relationship exists in birds. In mammals, norepinephrine levels are high during wakefulness, lower in NREM sleep and further decrease in REM sleep (Aston-Jones and Bloom, 1981). Consequently, if norepinephrine is a major driver, then CSF flow should reach maximum levels during REM sleep. If the neurochemistry of wakefulness and sleep is conserved between

mammals and birds, the suppression of CSF flow during REM sleep suggests that low levels of norepinephrine are not a major CSF flow driver. Rather, the fact that CSF activity is suppressed during REM sleep, a state characterized by low amplitude, high frequency EEG activity, indicates that CSF flow is somehow linked to slow wave activity. During NREM sleep, the off-states of the slow oscillation in neuronal membrane potentials likely reduce metabolic demands and, therefore, the flow of blood into the brain, as suggested by brain cooling during REM sleep in pigeons. Due to reduced blood flow, the diameter of the blood vessels likely decreases, thereby increasing the perivascular space around the vessels and facilitating CSF flow. By contrast, increased neuronal activity and metabolic demands during REM sleep, likely increases blood flow (as reflected in the increase in brain temperature) and vessel diameter, thereby impeding CSF flow. Interestingly, synchronized on and off periods of neural activity, like those observed during NREM sleep, are a conserved feature in other species, like non-avian reptiles and fish. It would be thus interesting to study whether a similar relationship exists in those species. Such results would imply that brain clearance is a conserved function that evolved early on in relation to a sleep state with synchronized neural activity.

Implications for studies on social communication

The opposite pupillary dynamics reported in our study is in part due to the fact that pupillary constrictions occurring in wakefulness and REM sleep are mediated in birds via nicotinic cholinergic receptors typical of striated muscles. However, differences in the muscle type do not fully explain our results; differences in brain connectivity and signaling to the iris via the Edinger-Westphal nucleus likely also play a role. The striated aspect of the avian iris and the strong constrictions observed in numerous species have long raised the question of whether birds can voluntarily control the size of their pupils, but no scientific investigation has addressed this question. Pupillary constrictions during wakefulness could be controlled by cortical (pallial) motor regions, like the rostral hyperpallium and arcopallium, both having descending projections to the Edinger-Westphal nucleus through the mesencephalic reticular formation (Gamlin and Reiner, 1991; Wild and Williams, 2000; Zeier and Karten, 1971). In the future, it would be interesting to attempt to train birds to constrict their pupils in exchange for a reward. This experimental paradigm would allow us to investigate which brain areas have correlated activity, and potentially exert control over the pupillary behavior. Training a bird to constrict one or both pupils could also be used to examine motor reactivations during sleep, aspects of the laterality of this behavior, and the associated neural correlates.

Our data also suggests that pupillary constrictions are tightly coupled to courtship displays and might be an important component of these behaviors. Research on avian courtship displays has focused mainly on the vocal and behavioral components. Interestingly, although pupillary constrictions have been anecdotally reported in several

species, prior to our study they had not been systematically quantified, likely due to the difficulty of reliably measuring pupil size in a bird engaged in complex behaviors. By presenting a novel method for measuring pupil size in freely behaving birds, our study opens the door to further investigation into the role pupillary behavior plays in courtship and other forms of social communication, such as aggression. For example, in the future, it would be interesting to assess whether the pupillary activity works in a binary, bimodal fashion (constricted-dilated), or whether it carries multiple types of information. It would also be interesting to examine how pupil behavior between individuals correlates with their interactions, and compare these findings with previous studies on humans wherein pupil mimicry positively correlates with trust and is thought to be involved in social bonding (Prochazkova et al., 2018; Kret and De Dreu, 2017).

In birds and other species, courtship displays contain multiple components exhibited in the same, or multiple sensory modalities. An example is the pigeon, wherein the stereotypical walking, bowing of the head, cooing and pupillary constrictions occur simultaneously. Recent research has shown that the outcome of a multimodal/multisensory display is not simply the sum of outcomes for each individual component (Mitoyen et al., 2019). Thus, it would be interesting to understand how pupillary activity integrates in complex courtship displays. One possibility is to use eye drops that inhibit pupil constrictions in one or both eyes to investigate changes in the behavioral responses of females to courtship. Additionally, the female pupillary activity could serve as a finer measurement for courtship performance evaluation.

Birds have a large palette of iris colorations (Oliphant et al., 1992). The iris brightness, besides making the eyes more or less conspicuous, impacts the contrast with the black pupil and the visibility of its size. Our observations have so far been biased towards species with bright pupils, but it would be interesting to investigate how the pupil behaves during wakefulness and sleep in birds with dark irises.

Finally, many bird species execute complex motor behaviors as part of their courtship. Interestingly, some species which also constrict their pupils during courtship, like the greater bird of paradise (*Paradisaea apoda*), rehearse and perfect their display alone, or in the presence of other males during their development, for periods that can last years. It would be thus interesting to investigate in these species how the pupil behaves in the absence of a female and how pupil constrictions change throughout development.

Methodological implications

Through this work we have demonstrated the feasibility of combined accelerometry, electrophysiological (including brain temperature), and pupil recordings in freely moving birds. As the animals adapted well to wearing the device and engaged in courtship behaviors when presented with a female, this setup could be used in other semi-natural conditions to record pupillometry, eye state and movement, gaze direction

or food intake. The main advantages of the minicamera technology compared to the currently existing ones are the reduced size of the sensors (1 mm x 1 mm), reduced weight and reduced obstruction of the visual field. The impact on the visual field could be reduced further by combining translucent IR reflective mirrors and minicamera modules placed outside the animal's visual field (Wallace et al., 2013). The reduced weight of the processing board, makes it also suitable for recordings on small animals.

The sleep fMRI paradigm is only at its beginnings and could be used in the future for a wide range of experiments, including investigating the neural correlates of various phasic activities during REM sleep or changes in connectivity dynamics during sleep. The fMRI setup has been successfully used on awake pigeons engaged in color discrimination tasks (Behroozi et al., 2020), thus paradigms involving a task during awake periods followed by sleep could be envisioned as a way to study memory consolidation and replay. Finally, the fMRI setup could allow studying sleep ontogeny through longitudinal studies.

Conclusion

Taken together, partial differences in sleep features, such as the length REM sleep episodes, magnitude of sleep state dependent brain temperature changes, or muscle tone regulation, or even species-specific features, like the RIMs in birds and a theta rhythm in mammals suggests that REM sleep does not manifest as a predefined and rigid set of neurophysiological parameters, even in two taxonomic groups with the most similar sleep states. The diversity of the findings complicates attempts to understand the evolution and function of sleep.

Although making researchers lose sleep at night, this diversity can also provide a rich source of information. The features currently used to characterize NREM and REM sleep may have evolved at different times throughout evolution and may have changed to best serve the functions related to an animals' ecology and biology. Thus, shared components of different sleep states across taxonomic groups might reveal traits that evolved early on, were conserved throughout evolution and are linked to shared, core function(s) of sleep. Differences and species-specific features might not reflect only species-specific adaptations, but could reveal overarching principles and serve as models for better understanding mammalian sleep. Examples drawn from my dissertation work are the RIMs and fMRI activity. The data presented in this manuscript only provides the very first descriptions of awake pupil constrictions and RIMs occurring in REM sleep; and state-dependent fMRI brain activity. Our results open more new questions than they answer. It will be the task of future research to advance these findings and find if and how RIMs are involved in 'emotional' memory consolidation, in mapping the motor system, in maintaining a healthy eye environment, or in other functions not considered so far; which brain areas contribute to RIMs and other phasic

REM sleep activity; how memories are processed and consolidated at a systems level; and whether the glymphatic activity is indeed reduced or suppressed during REM sleep.

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Acknowledgements

Have I ever imagined that I will be studying avian sleep? Certainly not. But somehow, I ended up doing it, and along the way a completely new world opened to me. First, a world of people fascinated, aware and observant of most living creatures, especially the feathered ones. This made me realize that I was more disconnected from nature than I thought, despite loving it. Secondly, I discovered the field of comparative (sleep) neuroscience. Now I find it absolutely essential to understand how various processes, including sleep, work in a variety of organisms. Looking back on it, I now think I am fortunate to have had the chance to break out of the traditional research model and study birds.

My PhD was an amazing professional experience. Carrying research in a resourceful place, such as Max Planck, means cutting-edge infrastructure, a very good platform for networking and developing collaborations, and opportunities to be creative. But most of all, it means freedom of thinking, and I am very glad I got to experience this.

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