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

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Summary

Mammalian pupils respond to light[1, 2], and dilate with arousal, attention, cognitive workload and emotions[3], thus reflecting the state of the brain. Pupil size also varies during sleep, constricting during deep non-REM sleep[4-7] and dilating slightly during REM sleep[4-6]. Unlike mammals, anecdotal reports suggest that birds constrict their pupils during aroused states, such as courtship and aggression[8-10], 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[11-15], 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[1, 2]. 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[16]. 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[17, 18], and emotion[3], and is thought to play a role in social signaling[19, 20]. It has been suggested that in social interactions, pupil size and pupil mimicry are important for trust and bonding[21]. Pupil mimicry also occurs in non-human primates[22], and can extend to inter-specific interactions[23].

In birds, pupillary constrictions have been described during courtship and/or aggressive displays in species from several orders, including Charadriiformes, Passeriformes, and Psittaciformes[8-10, 24]. 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*)[25]. 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[26].

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[18, 27, 28], light non-REM sleep, and deep non-REM sleep [4-7]. During REM sleep, the pupils are mostly constricted[4-7], but can dilate from this constricted state[4-6]. 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 [6]). 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[6].

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[6], 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, $p = 1.2 \cdot 10^{-4}$), but this did not depend on the birds' sex (Figure 2F, linear mixed effect models, $p = 0.73$, Table S2).

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[29], 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[30]. 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[31, 32], RIMs associated with REM sleep might be a widespread behavior among birds.

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[15, 33, 34]. 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[11-14, 35, 36]. However, given the presence of some smooth muscle fibers in the adult avian iris musculature of several bird species[15, 37, 38], 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[36]. 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.

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[39], 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[40]. 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[41, 42], 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[43, 44]. 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[45]. 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[46].

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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.

Declaration of interests

The authors declare no competing interests.

Inclusion and Diversity

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science.

Main figure titles and legends

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, 3-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.

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 NREM sleep. The eye state is color-coded as in B. E. Average distribution of RIM durations. 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). Pigeon illustration by Yifan Pei.

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, eye and head movements (relative to the camera) in a sleeping Guira cuckoo. The eye state is color-coded as in B. In dark red are marked the periods with pupillary constriction, and in lilac are bouts with a stable dilated pupil. 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.

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 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.

STAR Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gianina Ungurean (gungurean@orn.mpg.de)

Materials availability

See the Key resources table for information about resources. This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- Custom MATLAB scripts used in this study will be shared on Gitlab (<https://gitlab.com/gianinau/pupil-behavior-birds>).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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[6]. 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[47].

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[48].

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 µl nicotinic cholinergic antagonist – rocuronium bromide, 10mg/mL; 100 µl muscarinic cholinergic antagonist – Tropicamide, 0.5%; or 100 µl 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[48-50]. 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[49], 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[51].

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[52].

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[53].

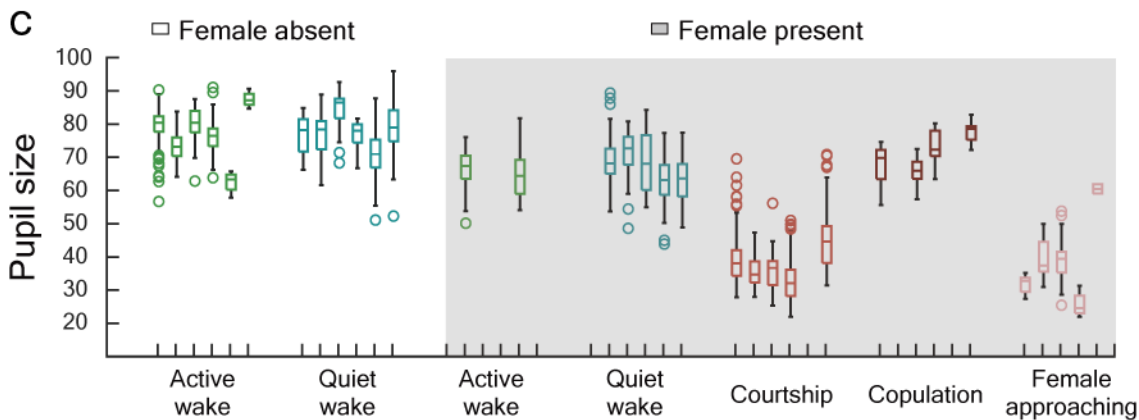
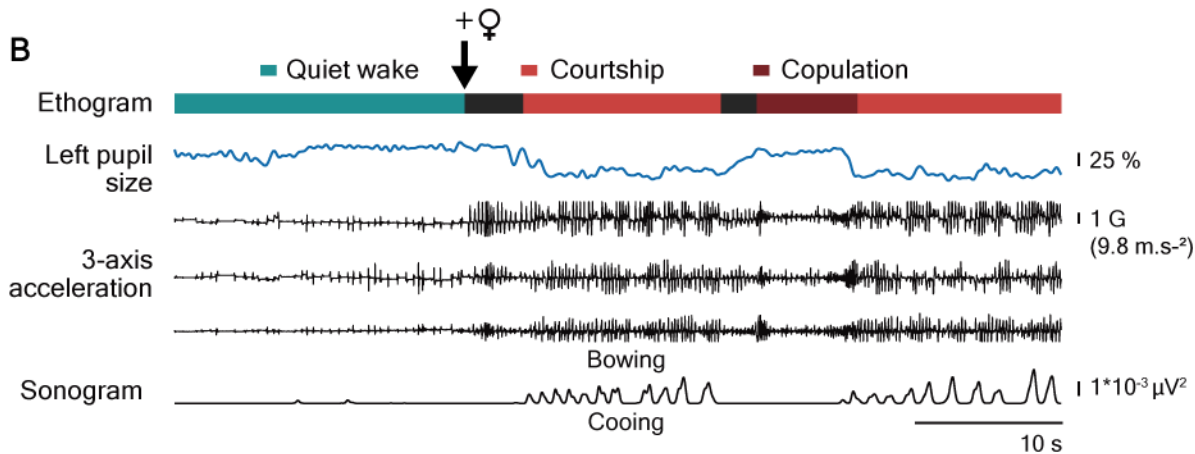
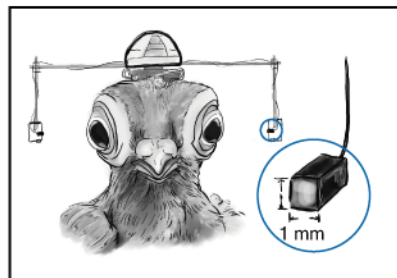
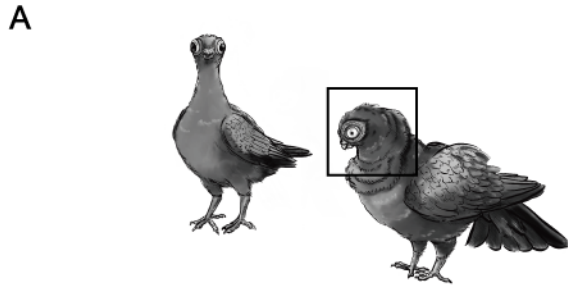
In the cuckoo experiment, the distribution of the standard deviation during RIMs and during bouts with a stable dilated pupil were tested using a Student's t-test. In pigeons, the effect of different pharmacological reagents compared to saline treatment were also tested using a Student's t-test. For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

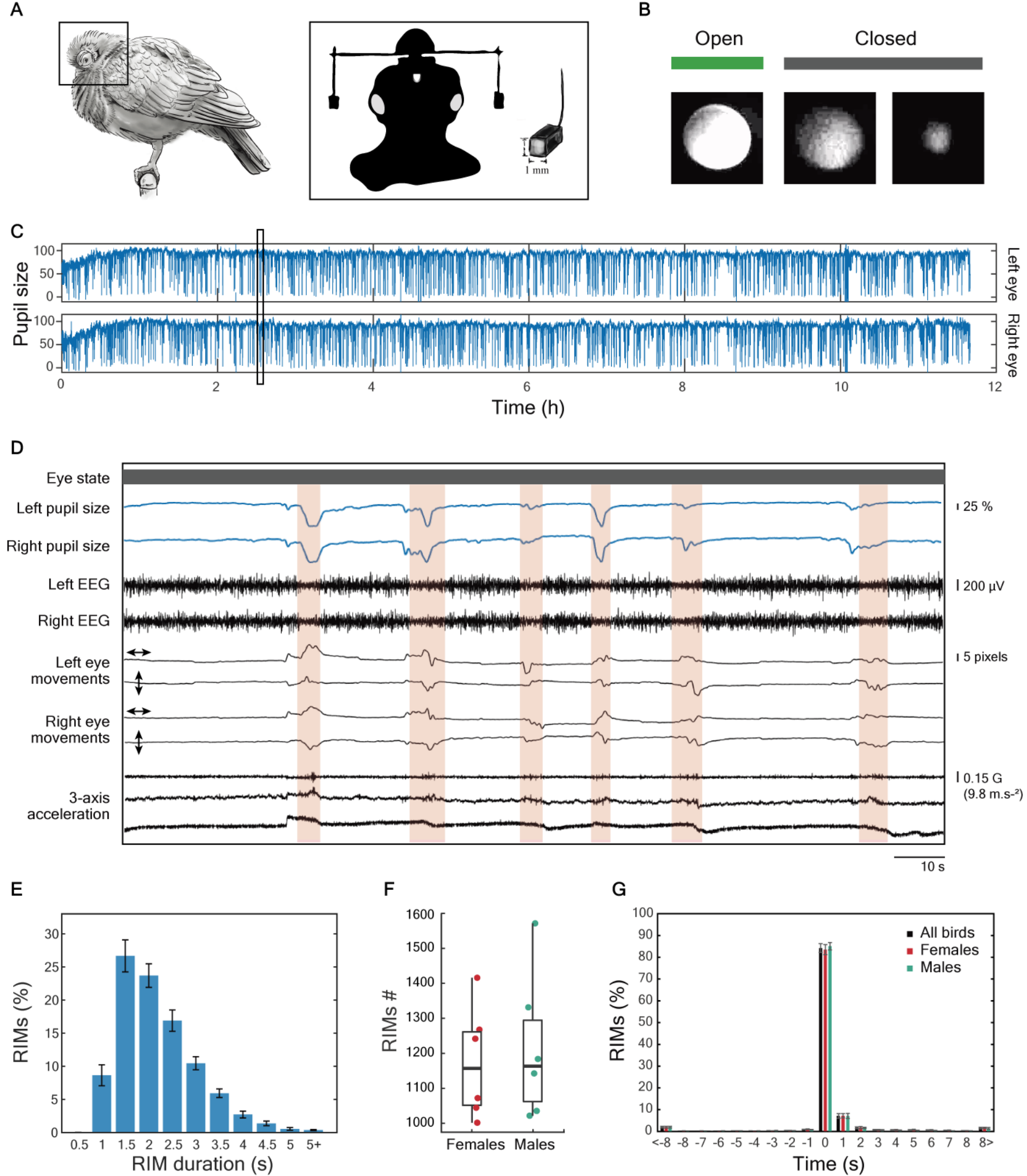
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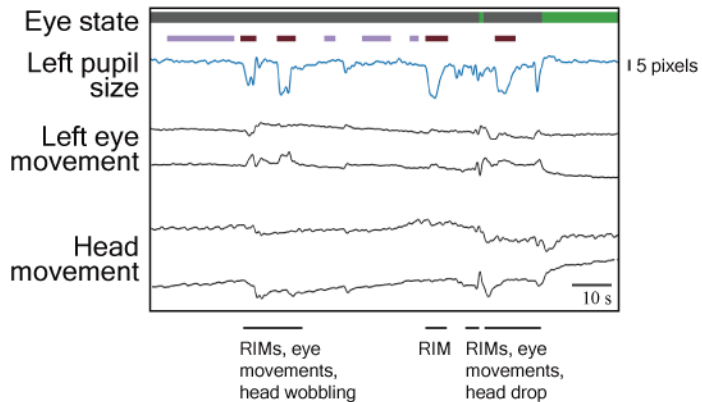
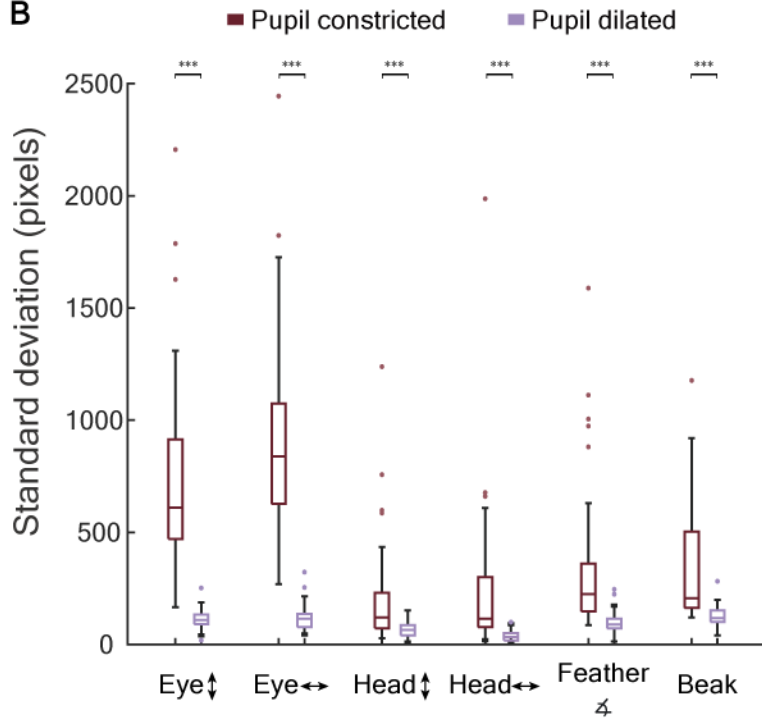
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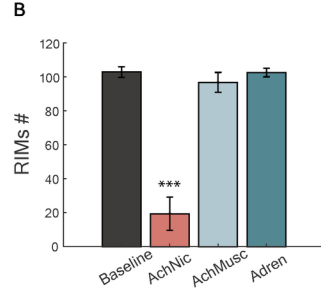
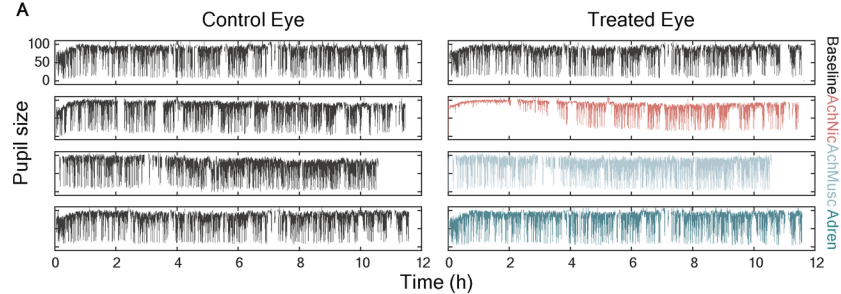
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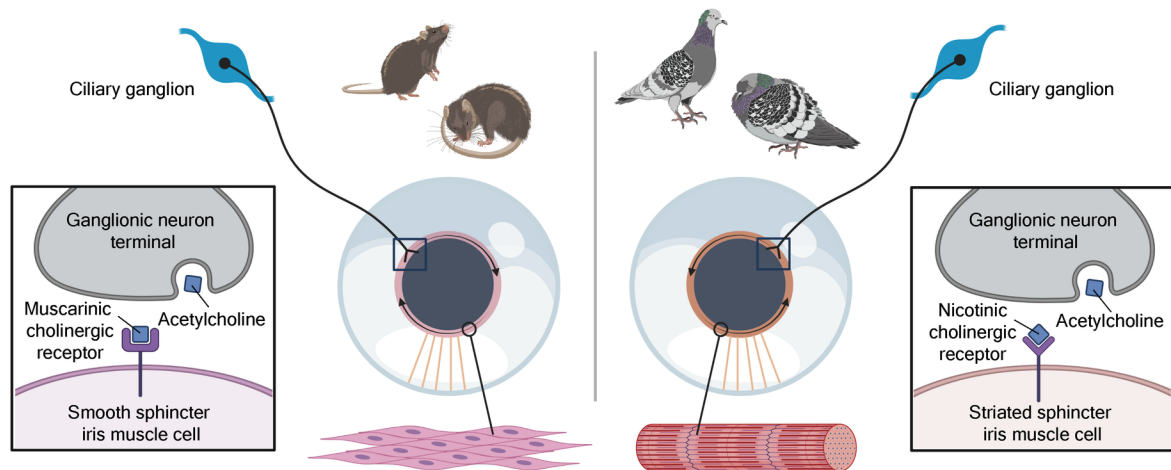




A**B**



C



Mammals

Birds

Aroused wake



Relaxed wake



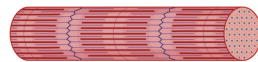
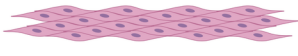
Deep non-REM sleep



REM sleep



Smooth iris
sphincter muscle



Striated iris
sphincter muscle