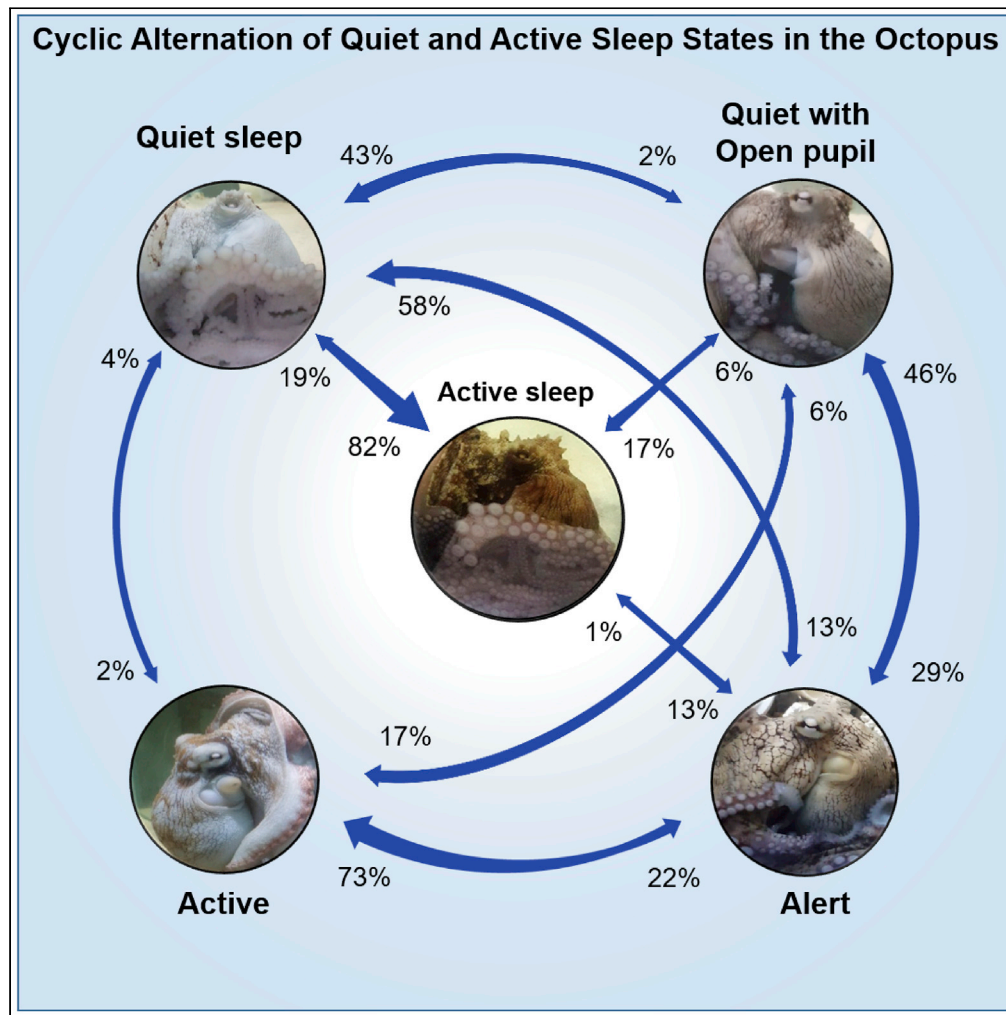


Article

Cyclic alternation of quiet and active sleep states in the octopus



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HIGHLIGHTS

Octopus has 'Quiet' and 'Active sleep', with different episode duration and periodicity

States differ on arousal thresholds, skin color and texture, and eye and mantle movement

The results suggest that octopus has a sleep cycle analogous to that of amniotes

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Article

Cyclic alternation of quiet and active sleep states in the octopus

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SUMMARY

Previous observations suggest the existence of 'Active sleep' in cephalopods. To investigate in detail the behavioral structure of cephalopod sleep, we video-recorded four adult specimens of *Octopus insularis* and quantified their distinct states and transitions. Changes in skin color and texture and movements of eyes and mantle were assessed using automated image processing tools, and arousal threshold was measured using sensory stimulation. Two distinct states unresponsive to stimulation occurred in tandem. The first was a 'Quiet sleep' state with uniformly pale skin, closed pupils, and long episode durations (median 415.2 s). The second was an 'Active sleep' state with dynamic skin patterns of color and texture, rapid eye movements, and short episode durations (median 40.8 s). 'Active sleep' was periodic (60% of recurrences between 26 and 39 min) and occurred mostly after 'Quiet sleep' (82% of transitions). These results suggest that cephalopods have an ultradian sleep cycle analogous to that of amniotes.

INTRODUCTION

Sleep is a well-studied behavior in amniotes such as mammals (Vanderwolf, 1969; Timo-laria et al., 1970; Delorme et al., 1964), birds (Low et al., 2008; Ayala-Guerrero et al., 1988), and some reptiles (Shein-Idelson et al., 2016; Libourel et al., 2018; Norimoto et al., 2020). Electrophysiological recordings in amniotes show distinct spectral profiles that comprise two major alternating sleep states, one quiet and another active (Gervasoni et al., 2004; Noda et al., 1969; Steriade et al., 1993). Much less is known about neurobiological rhythms in invertebrates because electrophysiological recordings remain very challenging in these animals, due to technical difficulties caused by a soft body, a rigid carapace, or life in the aquatic environment (Hendricks et al., 2000).

Despite these limitations, the study of invertebrate sleep has advanced using behavioral criteria originally developed to investigate mammalian sleep. These criteria comprise stereotyped or species-specific postures, maintenance of behavioral quiescence, elevated arousal threshold, state reversibility by sensory stimulation, responsiveness, and homeostatic regulation able to cause sleep rebound after deprivation (Hendricks et al., 2000; Campbell and Toblew, 1984; Greenspan et al., 2001; Meisel et al., 2011).

Among cephalopods, *Octopus vulgaris* (Cuvier, 1797) (Octopodidae: Cephalopod) meets all the criteria to define sleep (Meisel et al., 2011). For example, *Octopus vulgaris* specimens choose a preferred resting place and assume a typical posture of head lowered, arms curled around the body, motionless body except for sporadic shrinking and rapid, random movement of the suckers, pale body color, narrowed or completely closed eye pupils, and reduced ventilation rate (Meisel et al., 2011). Quiescent animals exposed to vibratory stimulation showed an elevated arousal threshold and state reversibility after intense stimulation. Furthermore, deprivation of the quiescence state led to a rest rebound (Meisel et al., 2011).

The sleep criteria mentioned earlier, except for the increase in the arousal threshold during quiescence, have also been met by *Sepia officinalis* (Linnaeus, 1758; Frank et al., 2012; Iglesias et al., 2019). Behavioral observations suggest the existence of a sleep state in which the animal is partially buried in the substrate with pupils closed and another sleep state with rapid chromatophore changes accompanied by

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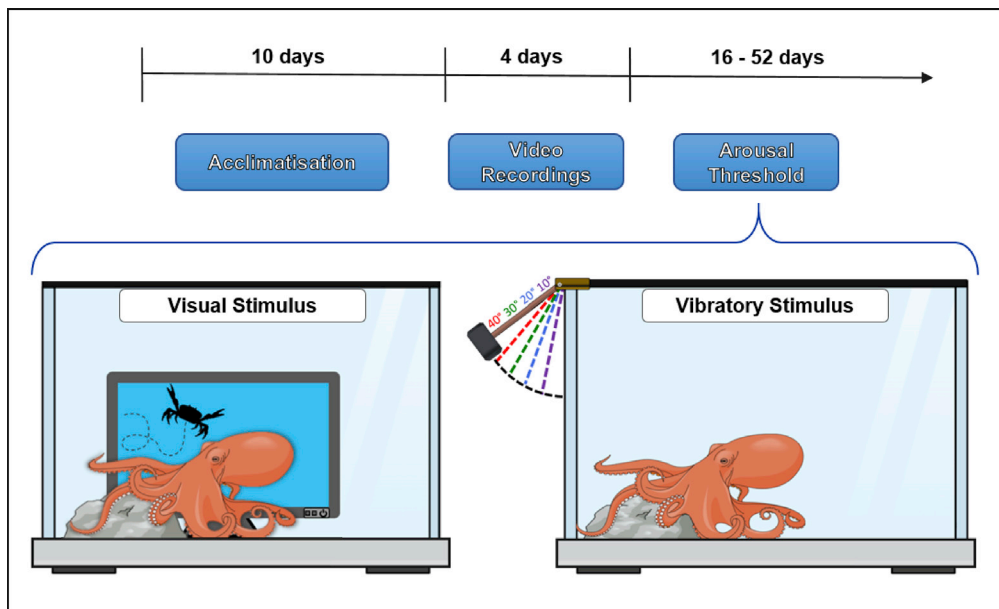


Figure 1. Experimental scheme

Animals were first subjected to acclimatization, followed by behavioral video recordings to assess the wake-sleep cycle and then a protracted period, during which the arousal threshold was measured. Visual stimulation was performed in three animals (octopuses 2, 3, and 4), whereas the vibratory stimulation test was performed in only one animal (octopus 3), which initially did not respond to the visual stimulation.

skin-texture changes, rapid eye movements, and arm twitching. Altogether, the behavioral evidence points to the existence of an ultradian rhythm (Frank et al., 2012; Iglesias et al., 2019).

Despite the mounting evidence that cephalopods display a Quiet/Active sleep cycle akin to amniotes, one can still argue that perhaps the specimens were not asleep but rather in a state of quiet alertness. This is a possibility because (1) responsiveness to stimulation was not tested in *Sepia officinalis* and (2) the studies of *Octopus vulgaris* investigated the quiescence state with pale body pattern, but not the 'Active sleep' state. Here we set out to address these gaps through a comprehensive behavioral quantification of all the sleep and waking states observed in *Octopus insularis* (Leite and Haimovici, 2008). Based on previous research (Meisel et al., 2011; Frank et al., 2012; Iglesias et al., 2019), we hypothesized that this species displays at least two consecutive quiescence states unresponsive to stimulation, comprising 'Quiet sleep' and 'Active sleep' states. To test this hypothesis, animals were video-recorded and systematically exposed to sensory stimuli during each state of interest to assess potential differences in the arousal threshold across the wake-sleep cycle (Figure 1).

RESULTS

Ethogram analysis reveals different quiescence states

The behaviors scored during the day were 'Active' state, 'Alert' state and five variations of the quiescence state, categorized as Quiet with open pupils ('QOP') (Figure 2A; Video S1), Quiet with closed pupil ('Quiet sleep') (Figure 2B; Video S2), Quiet with the 'half and half' skin pattern ('QHH') (Figure 2C; Video S3), Quiet with dynamic body pattern and movement of the eyes ('Active sleep') (Figure 2D; Video S4), and Quiet with only one eye movement ('QOEM') (Figure 2E; Video S5) (Table 1). For the behaviors recorded during the night, we observed the following types of quiescence states: 'QOP' (Video S6), 'Quiet sleep' (Video S7), 'Active sleep' (Video S8), and 'QOEM' (Video S9).

Among the quiescent states, 'QHH', 'Quiet sleep', and 'Active sleep' resembled sleep behavior. To further investigate this resemblance we performed the arousal threshold test during these states and during the 'Alert' state, which allowed for a comparison of differences in responsiveness to sensory stimulation.

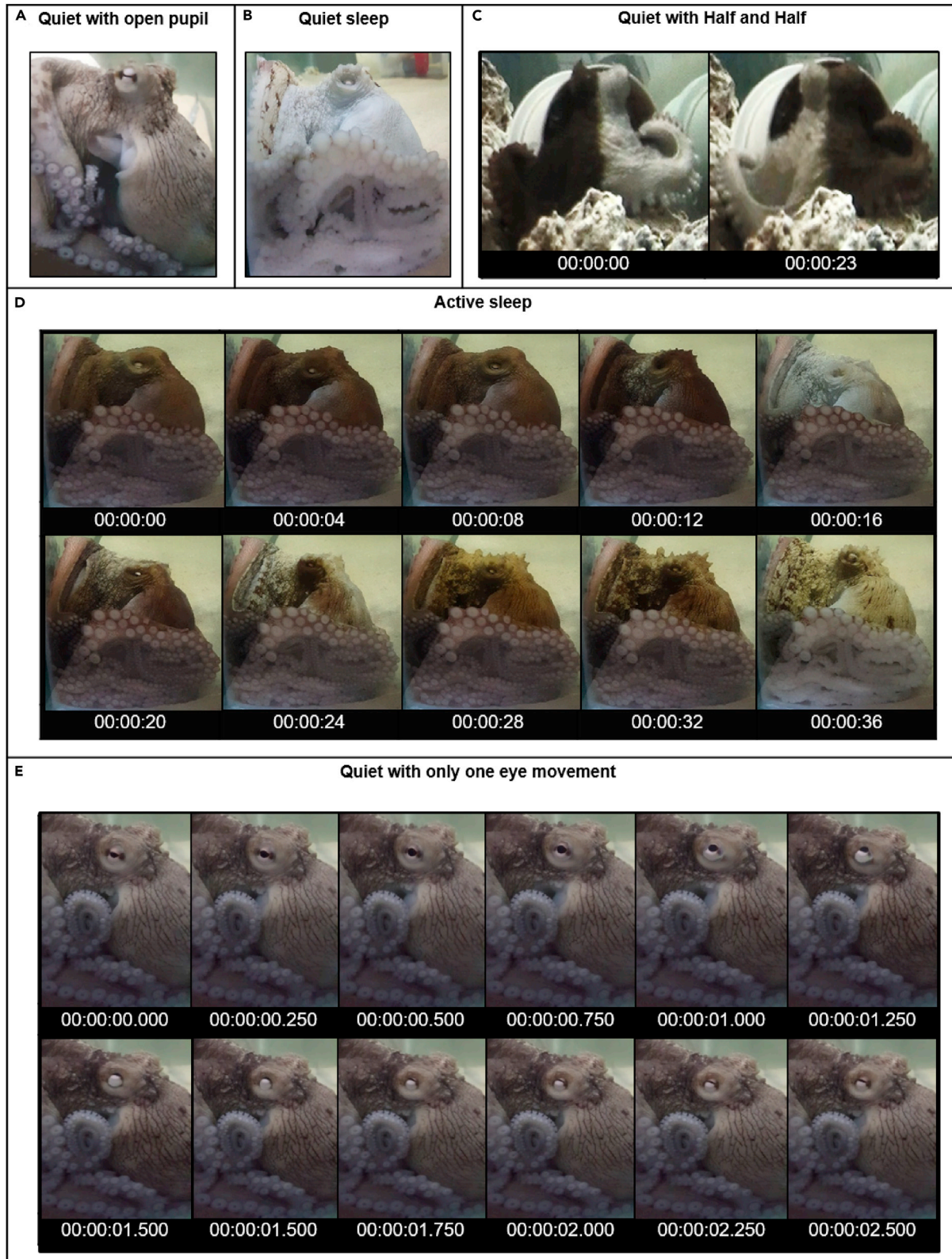


Figure 2. Behaviors observed during the different quiescence states

A total of five states were observed during quiescence: (A) Quiet state with open pupils; (B) 'Quiet sleep' with closed pupils; (C) Quiet with half and half skin pattern; (D) 'Active sleep' (quiet with dynamic pattern and eye movement); (E) Quiet with the movement of only one eye. See descriptions of each state in [Table 1](#).

Table 1. Description of the different quiescence states found in *Octopus insularis*

States	Description
Quiet with dynamic body pattern and eye movements (ACTIVE SLEEP)	The animal dynamically changes the skin color and texture and moves both eyes while contracting the suckers and the body, with muscular twitches.
Quiet with closed pupil (QUIET SLEEP)	Quiescence with the pupils of the eyes narrowed to a slit and pale body color. The animal can also sporadically display random movements of the suckers and arm tips without touching the environmental surfaces. However, these movements are much softer and slower than during the 'Active sleep' detailed above.
Quiet with open pupil (QOP)	Quiescence with generally pale body color, head lowered and motionless body, except for respiratory movements, and sporadic shrinking.
Quiet with the half and half body pattern (QHH)	The animal is head lowered and motionless and suddenly changes the skin pattern, which is generally pale during this quiescence state, to the 'half and half' skin pattern.
Quiet with only one eye movement (QOEM)	A cyclic one eye movement behavior, appearing pupil contraction and dilation together with exophthalmos.

Measuring arousal threshold

Visual stimulation test

The arousal response elicited by visual stimulation was analyzed in only three animals, because a few days after we finished the wake-sleep recordings of octopus 1, this animal began to show signs of decrease in health condition, such as less interest for food, so we excluded it from the arousal threshold tests.

These tests were performed when the octopuses were in the states 'Alert', Half and half ('QHH'), 'Quiet sleep' and 'Active sleep'. The behavioral states showed significant differences in time spent to react to the visual stimulation (Kruskal-Wallis $p = 1.30 \times 10^{-12}$). The highest latency was observed in the 'Active sleep' state (median 32.0 s, first quartile 22.5 s, third quartile 38.0 s), followed by the 'Quiet sleep' (median 6.0 s, first quartile 4.0 s, third quartile 9.0 s), 'QHH' (median 6.0 s, first quartile 4.0 s, third quartile 8.0 s), and finally the 'Alert' state with the shortest latency (median 4.0 s, first quartile 2.0 s, third quartile 6.0 s) (Figure 3A, upper panel).

The 'Active sleep' had the lowest number of stimulation trials with any reaction (highest number of stimuli classified as "absence of reaction"), followed by 'Quiet sleep', 'QHH' and the 'Alert' state (Figure 3A, bottom panel). Besides, the chi-square pairwise comparison showed significant differences regarding the absence of reaction between the states: 'Quiet sleep' and 'Alert' state (chi-squared test, $p = 6.11 \times 10^{-10}$), 'Quiet sleep' and 'QHH' (chi-squared test, $p = 3.51 \times 10^{-4}$), 'Alert state' and 'QHH' (chi-squared test, $p = 1.39 \times 10^{-2}$), 'Alert state' and 'Active sleep' (chi-squared test, $p = 2.20 \times 10^{-16}$), 'Active sleep' and 'QHH' (chi-squared test, $p = 1.02 \times 10^{-8}$). The only states that did not differ significantly from each other were 'Active sleep' and 'Quiet sleep' (chi-squared test, $p = 0.06$).

Vibratory stimulation test

The vibratory stimulation test was performed with one octopus ($n = 1$), and the results showed (Figure 3B) that in the 'Alert' and 'QHH' states, most responses were recorded at level 1. The 'Quiet sleep' state showed slower reactions, with a predominance of levels 2 and 4. The slowest reactions occurred during the 'Active sleep' state: during most trials, the animal responded only at level 5 or showed complete absence of reaction. The Pearson's chi-squared test showed that there were significant differences in response time across the behaviors ($p = 1.93 \times 10^{-4}$). There were more responses at levels 4 and 5 or "absence of reaction" for 'Quiet sleep' and 'Active sleep' states than for 'Alert' and 'QHH' states. Although the chi-squared pairwise-comparison showed that the 'Active sleep' responses differed significantly from those sampled during the 'Alert' state ($p = 5.52 \times 10^{-5}$) or QHH ($p = 1.70 \times 10^{-5}$), responses during 'Quiet sleep' were not significantly different from those sampled within the 'Alert' state ($p = 0.11$) or 'QHH' ($p = 0.08$).

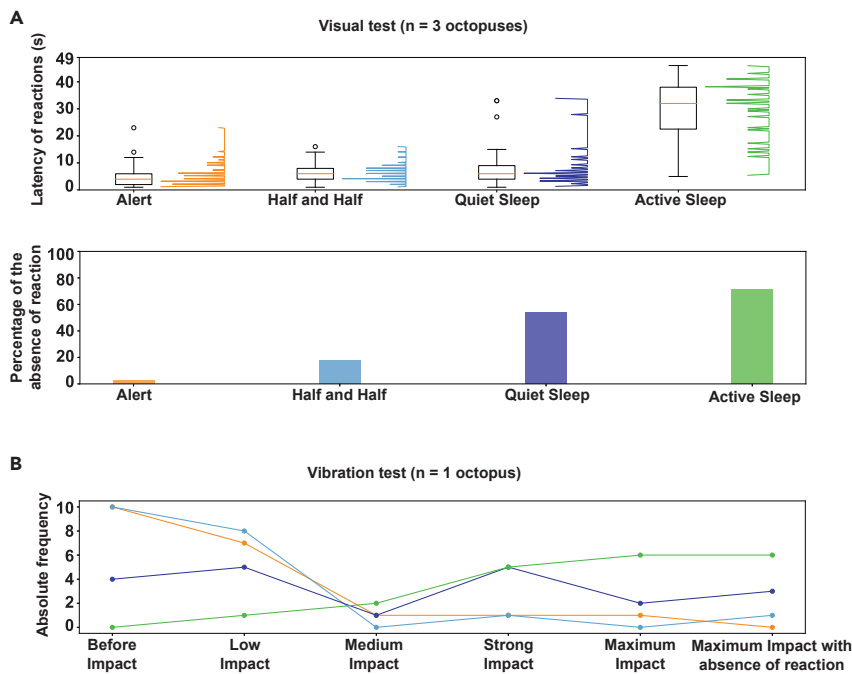


Figure 3. Reaction times after visual or vibratory stimulation

(A) The top panel shows the latency in seconds (y axis) of the animals' reaction on visual test when they were in the behaviors: 'Alert' state, 'Quiet with half and half' (QHH), 'Quiet sleep', and 'Active sleep' (x axis). The highest latency was during 'Active sleep' and the lowest was during the 'Alert state.' The bottom panel shows the percentage of absence of reaction for the same states. 'Active sleep' was the behavior with more absence of reaction, whereas the 'Alert' state had less absence of reaction.

(B) Frequency of animal's reaction on vibratory test (20 trials for each behavior). The lines represent the animals' behaviors: 'Alert' (orange), 'QHH' (light blue), 'Quiet sleep' (dark blue), and 'Active sleep' (green). On the x axis, the category "Before impact" comprises trials when the animal reacted even before the hammer's impact, i.e. in response to the hammer's movement. The inclination angles were 10° (low), 20° (medium), 30° (strong), and 40° (maximum impact). When the animal did not react even with the maximum impact, the trial was scored as "maximum Impact with absence of reaction." The 'Alert' state and 'QHH' showed the highest frequency of responses "before impact" (10 times), and this value decreased as the impact increased. The reaction frequency for these behaviors was below 2 times when the impact was maximum. 'Quiet sleep' showed little variation in frequency across different hammer impacts. Furthermore, it was more difficult to evoke a reaction when the animal was in 'Active sleep', so that the reaction frequency value increased as the hammer's angle increased.

Characterization of spontaneous behavioral alternation

The day-time behavioral states analyzed showed distinct distributions of episode durations: The 'Active sleep' (median 40.8 s, first quartile 25.8 s, third quartile 52.2 s) and the 'QHH' (median 10.2 s, first quartile 7.2 s, third quartile 13.8 s) states displayed much shorter durations than the 'Alert' state (median 2.83 min, first quartile 1.32 min, third quartile 6.9 min) and 'Quiet sleep' (median 6.92 min, first quartile 2.63 min, third quartile 15.04 min) states (Figure 4A). The 'Quiet sleep' state was the one with the most variation in duration. Kruskal-Wallis tests followed by Wilcoxon pairwise comparisons were conducted to determine whether there was any pattern of the duration of this state in relation to the states preceding or following it. These analyses also aimed to investigate the relationship between 'Active sleep' and 'Quiet sleep' episodes. The states that immediately preceded the 'Quiet sleep' state were 'Alert', 'QOP', 'QHH', and 'Active sleep', and the durations of 'Quiet sleep' episodes preceded by 'Active sleep' episodes were significantly shorter (Kruskal-Wallis $p = 4.91 \times 10^{-7}$) (Figure 5A).

The states immediately following the 'Quiet sleep' state were 'Alert', 'QHH', and 'Active sleep', and only the duration of the 'Quiet sleep' episodes immediately followed by 'Active sleep' episodes differed from the others by being significantly longer (Kruskal-Wallis $p = 1.18 \times 10^{-14}$) (Figure 5B).

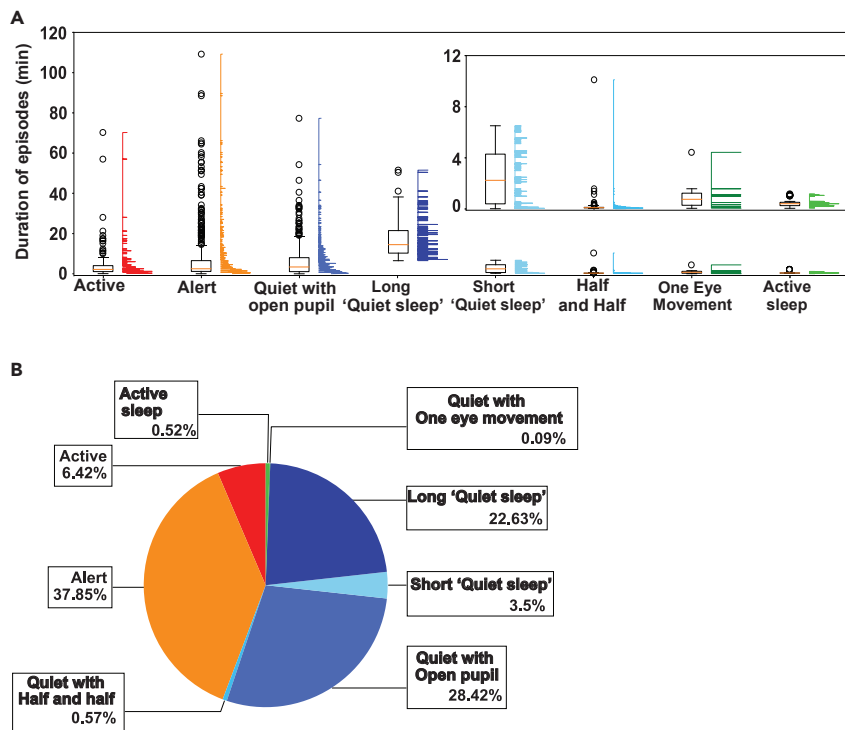


Figure 4. Characterization of the behaviors observed in *Octopus insularis*

Each color represents one behavior analyzed during the experiment.

(A) Boxplot and histogram of all animals' behavior observed through the video recordings during the 12 h of the light period. Data from the behaviors with shorter durations were zoomed in (top right inset).

(B) Pie chart showing the proportion of the total duration of each behavior for all animals.

The large variation in the duration of 'Quiet sleep' episodes prompted us to analyze more deeply the relationship between the durations of 'Quiet sleep' episodes and the likelihood of observing a neighboring 'Active sleep' episode. First, we looked for differences in the duration of 'Quiet sleep' episodes when they immediately preceded or did not immediately precede 'Active sleep' episodes: using Kernel Density Estimation, we plotted the durations distribution of the 'Quiet sleep' episodes immediately preceding 'Active sleep' episodes and of the 'Quiet sleep' episodes not immediately preceding 'Active sleep' episodes. These distributions show that the 'Quiet sleep' episodes that precede 'Active sleep' episodes are generally longer than those that do not precede 'Active sleep' episodes. The Kolmogorov-Smirnov test showed that these distributions were significantly different ($p = 1.36 \times 10^{-5}$). Figure S1A suggests that it is more likely that an octopus initiates an 'Active sleep' episode when they have been in a 'Quiet sleep' episode for a long time period. For this reason, we further sorted the 'Quiet sleep' episodes in two distinct categories: long and short. To classify these behaviors more accurately, we used a non-arbitrary parameter to split them into long and short 'Quiet sleep' episodes. Namely, we calculated the median time between (1) the mode of the distribution of 'Quiet sleep' episodes that occurred immediately before 'Active sleep' episodes and (2) the mode of other 'Quiet sleep' episodes that did not occur immediately before an 'Active sleep' episode (Figure S1A). In this way, short 'Quiet sleep' episodes had durations ≤ 6.51 min, whereas long 'Quiet sleep' episodes lasted >6.51 min.

Next, Kernel Density Estimation and the Kolmogorov-Smirnov test were used to compare the distribution of durations for 'Quiet sleep' episodes occurring immediately after 'Active sleep' episodes versus 'Quiet sleep' episodes occurring not immediately after 'Active sleep' episodes ($p = 0.22$). The distributions of the 'Active sleep' episodes immediately preceding 'Quiet sleep' episodes and not immediately preceding 'Quiet sleep' episodes were also compared ($p = 0.98$). Likewise, we further compared the 'Active sleep' episodes immediately succeeding 'Quiet sleep' episodes and not immediately succeeding 'Quiet sleep' episodes ($p = 0.27$). None of these comparisons showed significant differences (Figures S1B–S1D). These results indicate that there is a relationship between the duration of 'Quiet sleep' episodes and the

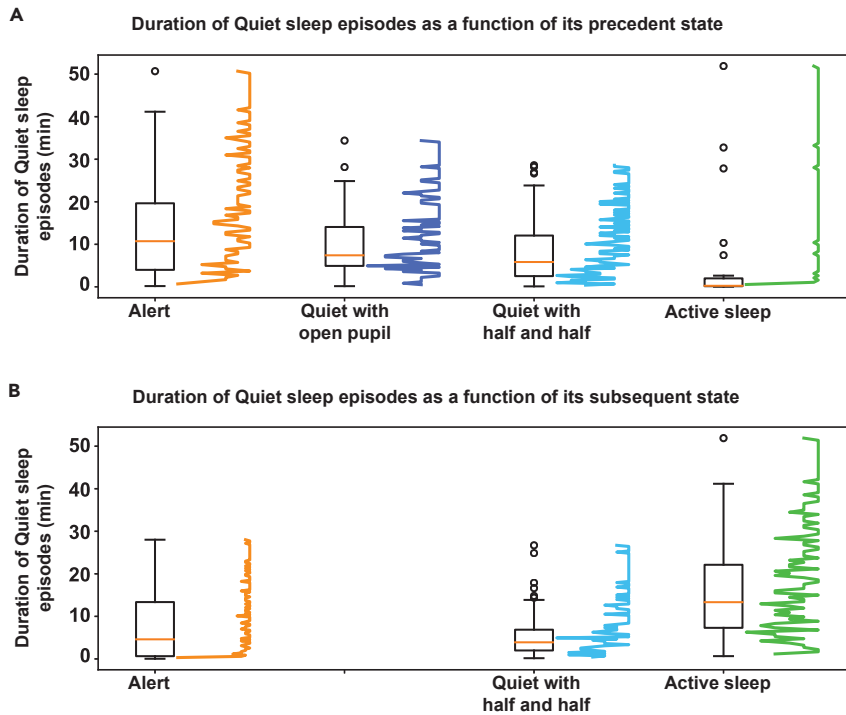


Figure 5. Duration of 'Quiet sleep' episodes as a function of precedent or subsequent states

States that occurred less than 10 times before or after 'Quiet sleep' were discarded from this analysis.

(A) Boxplot and histogram showing the duration in minutes (y axis) of 'Quiet sleep' episodes that occurred after the behaviors 'Alert', 'Quiet with open pupil' ('QOP'), 'Quiet with half and half' ('QHH'), and 'Active sleep' (x axis). The behaviors 'Alert', 'QOP', and 'QHH' occurred before long 'Quiet sleep' episodes, with medians 10.73, 7.41, and 5.84, respectively. 'Active sleep' episodes preceded the shortest 'Quiet sleep' episodes.

(B) Boxplot and histogram showing the duration in min (y axis) of 'Quiet sleep' episodes that occurred prior to the behaviors 'Alert', 'QHH', and 'Active sleep' (x axis). Long 'Quiet sleep' episodes (median value of 13.33 min) often led to 'Active sleep' episodes. 'Active sleep' was the only behavior that occurred after 'Quiet sleep' episodes with durations above 30 min. 'QHH' episodes were preceded by 'Quiet sleep' episodes of short duration (median 3.9 min), whereas 'Alert' episodes were preceded by 'Quiet sleep' episodes of intermediate duration (median 4.6 min).

subsequent occurrence of 'Active sleep' episodes, whereas the duration of 'Active sleep' episodes has no relationship with the occurrence of subsequent 'Quiet sleep' episodes. Importantly, short 'Quiet sleep' episodes are rarely followed by 'Active sleep' episodes.

The behaviors with the highest total time during the diurnal video recordings were the 'Alert' state (37.85%), 'QOP' (28.42%), and long 'Quiet sleep' (22.63%), whereas the ones with the lowest total time were 'Active' (6.42%), short 'Quiet sleep' (3.5%), 'QHH' body pattern (0.57%), 'Active sleep' (0.52%), and 'QOEM' (0.09%) (Figure 4B).

The behaviors with the highest frequencies per day were 'Alert' (35.56%) and 'QOP' (31.31%), whereas the behaviors 'QOEM' (0.50%) and 'Active sleep' (7.81%) had the lowest frequencies (Table S1).

There was a significant difference between the duration of almost all states observed. All quiescence behaviors differed except 'QOEM.' In addition, the 'Active', 'Alert', and 'QOP' states in which the octopus is possibly awake differed from all other quiescence states ('Quiet sleep', 'Active sleep', and 'QHH') except 'QOEM', as shown in Table S2.

Ultradian cyclicity of 'active sleep' and long 'quiet sleep' episodes

To analyze the cyclic pattern comprising the long 'Quiet sleep' and the 'Active sleep,' we excluded the feeding period because it interfered in the sleep cycle. From the total of 74 long 'Quiet sleep' intervals

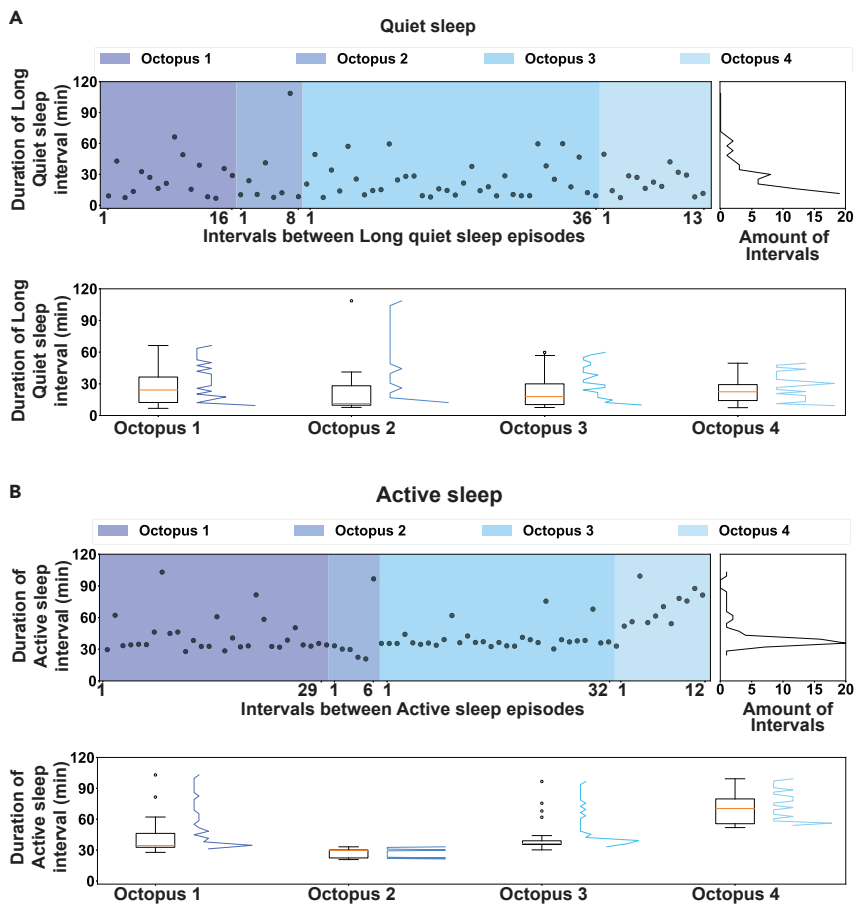


Figure 6. Ultradian Cyclicity of 'Active sleep' and long 'Quiet sleep' episodes

Each background color represents one individual octopus

(A) 'Quiet sleep' characterization: (Top panel) plots of intervals between long 'Quiet sleep' with histogram on the right; (Bottom panel) boxplot and histogram for the durations of intervals between long 'Quiet sleep' episodes for each octopus. (B) 'Active sleep' characterization: (Top panel) plots of intervals between 'Active sleep' with its histogram distributions on the right; (Bottom panel) boxplot and histogram for the 'Active sleep' interval durations of each octopus. For better visualization of the distribution, we excluded from the plot the data points 227.78 min and 150.45 min of the 'Active sleep' intervals and data point 173.47 min of the long 'Quiet sleep' intervals.

(time spent between the end of one episode and the beginning of the next episode of the same behavior), 60% had durations between 6.77 min and 25.48 min, i.e., with a wide range of durations (Figure 6A; Table S3 and Figure S2). From the total of 79 'Active sleep' intervals, 60% had duration between 29.58 min and 32.98 min, i.e. with a narrow range of durations (Figure 6B; Table S4).

A hypnogram was elaborated from the behavioral observation of one representative animal (octopus 3) for 200 continuous minutes in order to exemplify the ultradian cyclicity. Concomitantly with the hypnogram, we analyzed skin changes in color and texture, represented respectively by general skin color and localized skin patterning dynamics (Figure 7). Tracking of eyes and mantle movements was also performed for the same period (Figure S3). These parameters varied substantially across behaviors. For instance, during 'Quiet sleep' there was less variation of these parameters and during 'Active sleep' there was a more accentuated variation of the measures, whereas during the 'Alert' state we observed a constant and not extreme oscillation of skin color and texture, with mantle and eye movements.

General skin color dynamics across behavioral states

The pixel analysis of mean brightness values showed characteristic profiles of chromatophore color changes for each behavioral state (Figure 8A; Figure S4; Figure 7 second panel). When animals were in the 'Alert'

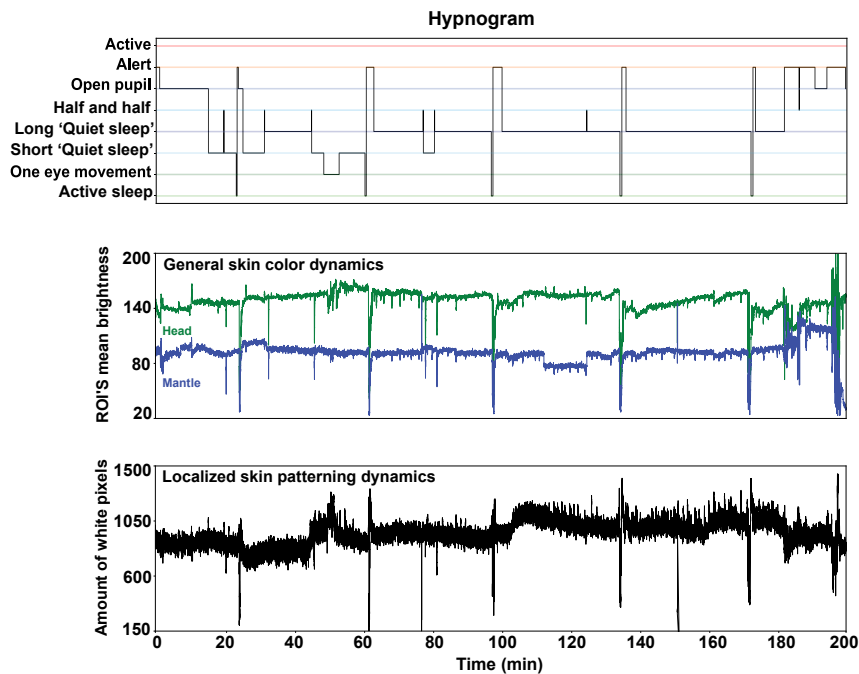


Figure 7. Hypnogram with corresponding measurements of general skin color dynamics and localized skin patterning dynamics

The figure depicts the behaviors of one representative animal (octopus 3) along 200 min of diurnal recordings, beginning at 06:36:50 and finishing at 09:56:50. The plot of general skin color dynamics depicts the mean color from two regions of interest delimited over the head and dorsal mantle (detailed in Figure 7). The plot of localized skin patterning dynamics shows changes in skin texture represented by the variation of the number of white pixels captured by the Canny algorithm along time. For both skin dynamic analyses, a marked variation of color and texture concomitant with the occurrence of 'Active sleep' can be observed.

state, brightness intensities recorded from the skin kept oscillating (mantle—median 0.56, first quartile 0.22, third quartile 1.25; head—median 0.75, first quartile 0.34, third quartile 1.59), whereas during the 'Quiet sleep' state there was very little variation in the measurements (mantle—median 0.21, first quartile 0.09, third quartile 0.47; head—median 0.25, first quartile 0.11, third quartile 0.61). However, during 'Active sleep' episodes there was a conspicuous and abrupt decrease in the measures, followed by intense brightness variation throughout the duration of the episode (mantle—median 2.09, first quartile 0.93, third quartile 3.94; head—median 2.8, first quartile 1.35, third quartile 5.14). This difference in color variation between these three states was confirmed by the statistical analyses for head and mantle (Kruskal-Wallis, head $p = 2.20 \times 10^{-16}$; mantle $p = 2.20 \times 10^{-16}$). The pairwise comparisons (Wilcoxon) with Bonferroni adjustment showed that in the head and mantle there were more variations in skin color during 'Active sleep' than during 'Quiet sleep' (head $p = 2.00 \times 10^{-16}$; mantle $p = 2.00 \times 10^{-16}$) and 'Alert' state (head $p = 2.00 \times 10^{-16}$; mantle $p = 2.00 \times 10^{-16}$). There was also more color variation during the 'Alert' state than during 'Quiet sleep' (head $p = 2.00 \times 10^{-16}$; mantle $p = 5.30 \times 10^{-12}$). These results are shown by the raw data and Gaussian filter of the number of white pixels over time (Figure 8A) and by the median of the variations shown by each octopus (Figure 8B). The other octopuses presented similar profiles, as shown in the Figure S4.

Localized skin patterning dynamics across behavioral states

The localized skin dynamics is caused by texture changes due to extension and retraction of the papillae and changes in color patterning within specific body regions. These dynamic changes in skin were investigated using the Canny algorithm, which captured edges in the image frames. The edges were visualized through white pixels that indicate the skin papillae's texture and the color patterning complexity (Figure 9A; Figure 7, third panel; Figure S5). There was a significant difference in variation of skin texture and complexity of color patterns across 'Quiet sleep' (median 1.00, first quartile 0.00, third quartile 3.00), 'Active sleep' (median 8.00, first quartile 3.00, third quartile 16.00), and the 'Alert' state (median 2.00, first quartile 1.00, third quartile 5.00) for all the octopuses (Kruskal-Wallis $p = 2.20 \times 10^{-16}$). The pairwise comparisons

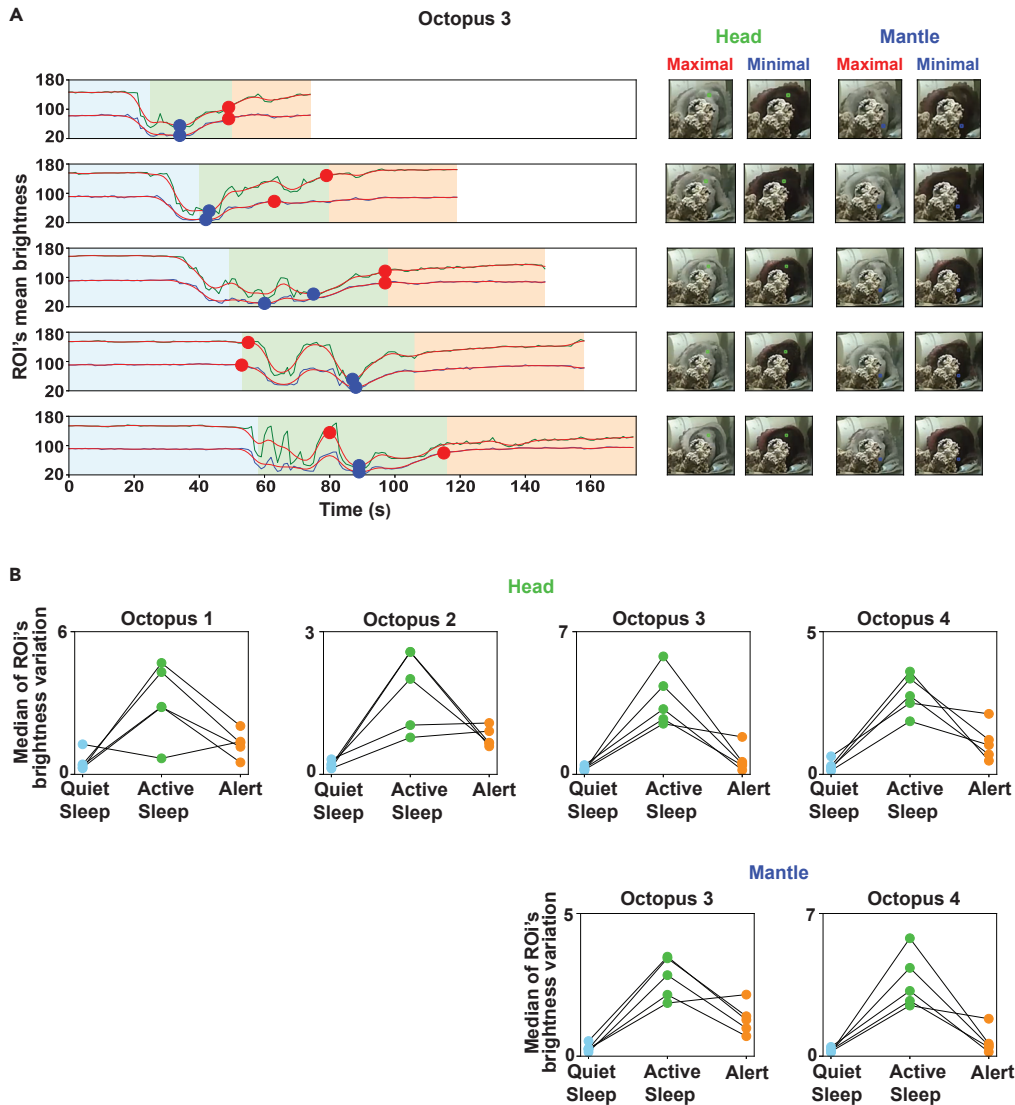


Figure 8. General skin color dynamics across 'Quiet sleep', 'Active sleep', and the 'Alert' state

(A) The left panel shows changes in skin color represented by the variation of the ROI's mean brightness (y axis) along 174 s (x axis) for five transitions across 'Quiet sleep' (blue zone), 'Active sleep' (green zone), and the 'Alert' state (orange zone) from octopus 3. The intervals analyzed for 'Quiet sleep' and the 'Alert' state had the same duration of the 'Active sleep' episode that occurred between them. The examples are organized from the shortest 'Active sleep' episode, on the top, to the longest, on the bottom. The green line indicates the head's color and the blue line indicates the mantle's color. Both signals are well followed by the Gaussian filter (red line). All the behavioral transitions show a similar profile, with little value variation during 'Quiet sleep' and the 'Alert' state, and with a marked variation during 'Active sleep', so that both extreme values (highlighted by the blue and red dots) occurred during 'Active sleep'. The right panel shows a picture of the octopus with the ROI's selected for head (green) and mantle (blue) when the animal color achieved its maximum value (most pale) and minimum value (most dark).

(B) Scatterplots showing the medians of delta (500 ms) Gaussian values for the color analysis of each behavior per octopus ($n = 4$). The top panel shows results for the head, and the bottom panel shows results for the mantle. Note that the mantle was properly visible in two animals only, so octopuses 1 and 2 were not included in the mantle color analyses. The color variation in all animals followed a general profile in which the highest color variation occurred during 'Active sleep'.

(Wilcoxon) with Bonferroni adjustment showed that there was significantly more variation in skin texture during 'Active sleep' than during 'Quiet sleep' ($p = 2.00 \times 10^{-16}$) and 'Alert' state ($p = 2.00 \times 10^{-16}$). There was also significantly more variation during the 'Alert' state than during 'Quiet sleep' ($p = 3.40 \times 10^{-16}$), as shown by the raw data and Gaussian filter of the number of white pixels over time (Figure 9A) and by the

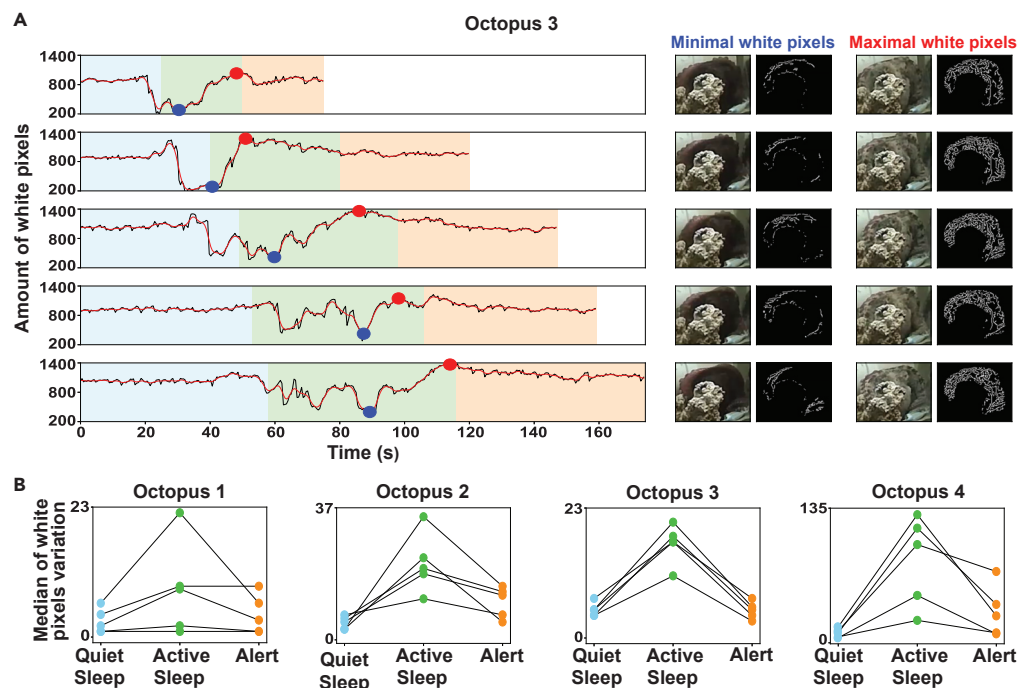


Figure 9. Localized skin patterning dynamics across 'Quiet sleep', 'Active sleep', and the 'Alert' state

(A) The left panel shows changes in skin texture represented by the variation of the number of white pixels (y axis) along 174 s (x axis) for five transitions across 'Quiet sleep' (blue zone), 'Active sleep' (green zone), and the 'Alert' state (orange zone) from octopus 3. The intervals analyzed for 'Quiet sleep' and Alert had the same duration of the 'Active sleep' that occurred between them. The examples are organized from the shortest 'Active sleep' episode, on the top, to the longest, on the bottom. The red line is a Gaussian filter and follows closely the real data, represented by the black line. All the behavioral transitions show a similar profile, with little variation during 'Quiet sleep' and the 'Alert' state, and with a marked variation during 'Active sleep', so that both extreme values occurred during 'Active sleep' and all the highest values occurred after the lowest values. The top value among the five sampled transitions is on the last line, having 1,367 white pixels and the lowest value is on the first line, with 281 white pixels. The right panel shows the moment during 'Active sleep' when the animal displayed minimum body pattern complexity, represented by the minimal number of white pixels captured by the Canny edges algorithm (blue dots on the graphs and minimal white pixels column); the maximal count of white pixels are represented by the red dots on the graphs and maximum white pixels (red) column.

(B) Scatterplots showing the medians of delta (500 ms) Gaussian values for the behavioral analysis of each octopus ($n = 4$). The median values of 'Active sleep' are mostly higher than during other behaviors (except for one transition in octopus 1, when the value did not change across behaviors).

median of the variations shown by each octopus (Figure 9B). The octopuses 1, 2, and 4 presented similar profiles, as shown in the Figure S5.

Eye movements across behavioral states

For each octopus we analyzed eye movements for five periods comprising a natural sequence of 'Quiet sleep', 'Active sleep', and the 'Alert' state. The octopuses showed significant differences in eye movements across states (Kruskal-Wallis $p = 2.20 \times 10^{-16}$). Besides, pairwise comparisons (Wilcoxon) with Bonferroni adjustment show that the increase rate during 'Active sleep' (median 0.56, first quartile 0.32, third quartile 1.32) was significant in comparison with 'Quiet sleep' (median 0.36, first quartile 0.21, third quartile 0.79) ($p = 2.00 \times 10^{-16}$) and 'Alert' (median 0.44, first quartile 0.25, third quartile 1.02) ($p = 5.20 \times 10^{-15}$). There was also a significant increase in eye movements during the 'Alert' state in comparison with 'Quiet sleep' ($p = 8.60 \times 10^{-7}$) (Figure 10).

Mantle ventilation movements across behavioral states

The octopuses 3 and 4 showed significant differences in mantle movement across 'Quiet sleep' (median 0.70, first quartile 0.29, third quartile 4.67), 'Active sleep' (median 1.12, first quartile 0.41, third quartile 4.60), and the 'Alert' state (median 0.67, first quartile 0.28, third quartile 3.23) (Kruskal-Wallis $p = 1.43 \times 10^{-8}$). The pairwise

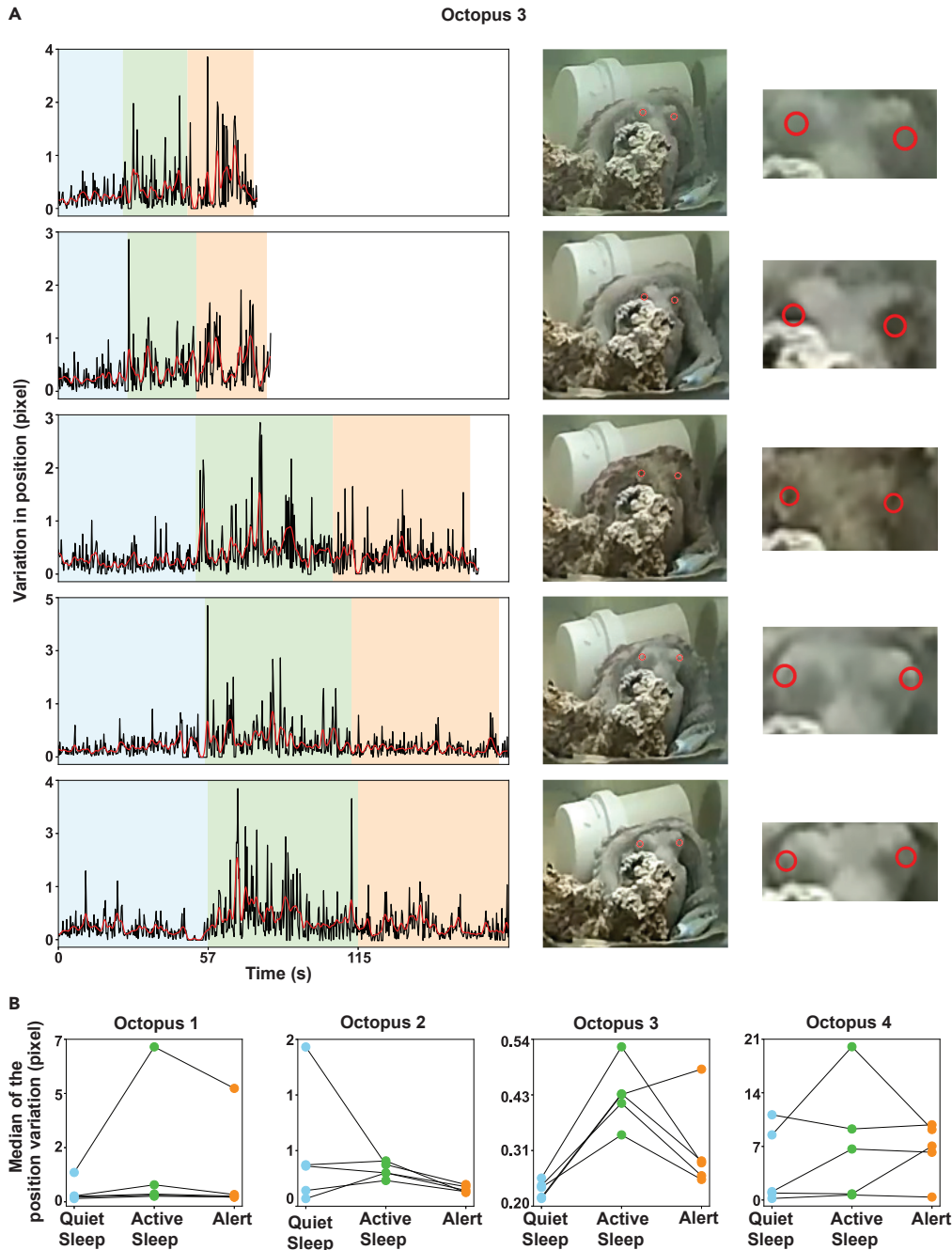


Figure 10. Eye movements across 'Quiet sleep', 'Active sleep', and the 'Alert' state

(A) The left panel shows the sum of the position variation for the left and right eyes (y axis) over time (x axis) for five transitions between 'Quiet sleep' (blue zone), 'Active sleep' (green zone), and the 'Alert' state (orange zone) for octopus 3. The intervals analyzed for 'Quiet sleep' and the 'Alert' state had the same duration of the 'Active sleep' that occurred between them. The examples are organized from the shortest 'Active sleep', on top, to the longest, on the bottom. The red line on the graph is a Gaussian filter and follows closely the real data, represented by the black line. The right panels display an example of the region where marks were placed on each eye to perform the tracking, with zoomed-in images on the right.

(B) Scatterplots show the medians of delta (300 ms) Gaussian values for the behavioral analysis of each octopus (n = 4).

comparison (Wilcoxon) with Bonferroni adjustment shows that the increased rate during 'Active sleep' was significant in comparison with 'Quiet sleep' ($p = 2.90 \times 10^{-5}$) and the 'Alert' state ($p = 6.50 \times 10^{-12}$). Besides, the increase in mantle movements during 'Quiet sleep' in comparison with the 'Alert' state was also significant ($p = 0.04$). Figure 11 shows in detail for octopus 3 the variation of mantle position over time for the behavioral sequences comprising 'Quiet sleep', 'Active sleep', and the 'Alert' state (Figures 11A) and the scatterplot with the medians of these transitions for both octopuses (Figures 11B).

Behavioral transitions show a characteristic pattern

To quantify comprehensively how specimens of *Octopus insularis* transit between specific behavioral states, the entire dataset recorded during daytime was subjected to graph analysis. The graphs of behavioral transitions show that some of the states were more correlated with each other and that there was a pattern in the sequence of their transitions (Figure 12). 'Active sleep' occurred after 'Quiet sleep' 82% of the times, with 18% coming from short 'Quiet sleep' episodes and 64% from long 'Quiet sleep' episodes. The behaviors with the highest probability to occur after long 'Quiet sleep' were 'Active sleep' (57%), the 'Alert' state (24%), and 'QHH' (16%). Furthermore, 'QHH' was more strongly correlated with quiet behaviors, with 50% of the inputs coming from 'QOP' and 45% of all short 'Quiet sleep' outputs leading to 'QHH'. The 'Quiet sleep' episodes were mostly preceded by 'QHH', the 'Alert' state, and 'QOP', but almost never by 'Active sleep', with significant differences between their frequencies (chi-squared test, $p = 2.20 \times 10^{-16}$). When this occurred the 'Quiet sleep' episodes were almost invariably short (chi-squared test, $p = 4.27 \times 10^{-3}$).

DISCUSSION

In the present work, we showed that *Octopus insularis* displays two different quiescence states that fulfill the behavioral criteria for sleep, namely 'Quiet sleep' and 'Active sleep'. We characterized these states with regard to general body skin color dynamics, local skin patterning dynamics, eye and mantle movements, episode duration, episode periodicity, transition probabilities, and arousal thresholds. Altogether, the results point to a cyclic-state dynamics in which the behavioral sequence 'Quiet sleep' to 'Active sleep' to 'Alert' state is prevalent.

'Quiet sleep' has already been observed in *Octopus vulgaris* (Meisel et al., 2011), but 'Active sleep' has not yet been described in the literature for the octopus. However, this 'Active sleep' is similar to the behavior named REM-like sleep in *Sepia officinalis* (Iglesias et al., 2019), by analogy with rapid-eye movement (REM) sleep in mammals. In both species there were eye movements, dynamic chromatophore patterning, and a sudden simultaneous darkening of the mantle and head chromatophores at state onset (Frank et al., 2012; Iglesias et al., 2019). Another similarity between the 'Active sleep' here described and the REM-like state described in *Sepia officinalis* (Iglesias et al., 2019) is the presence of an ultradian rhythm for both behaviors, with a characteristic cycle. The periodicity of REM-like sleep in *Sepia officinalis* was 36.34 ± 1.46 min, whereas in *Octopus insularis* 60% of the intervals between 'Active sleep' episodes had durations between 29.58 min and 32.98 min. Importantly, an ultradian sleep rhythm is also observed in mammals (Trachsel et al., 1991), birds (Walker and Berger, 1972), and in the reptile bearded dragon *Pogona vitticeps* (Ahl, 1926; Norimoto et al., 2020).

Regarding this periodicity, octopus 4 differed a bit from the others with regard to durations of the intervals between consecutive 'Active sleep' episodes. Reptiles, such as the bearded dragon, can change the period of neural oscillations according to the environment's temperature, so that an increase in temperature leads to a decrease in periodicity (Shein-Idelson et al., 2016). However, considering that during this study the water temperature was kept identical for all animals and that octopus 4 was the female with the highest body weight and probably the most mature animal assessed (Lima et al., 2014), it is possible that *Octopus insularis* specimens undergo changes in their sleep cycles as they age.

During quiescence animals also presented the behavior 'QHH', which was also observed in *O. vulgaris* during resting periods (Meisel et al., 2011). For this reason, we included this state in the arousal threshold experiment. The visual latency test performed with *Octopus insularis* showed a significant and gradual increase in arousal threshold from the 'Alert' state to QHH, 'Quiet sleep', and 'Active sleep'. These results strongly suggest the existence of different sleep states in octopuses.

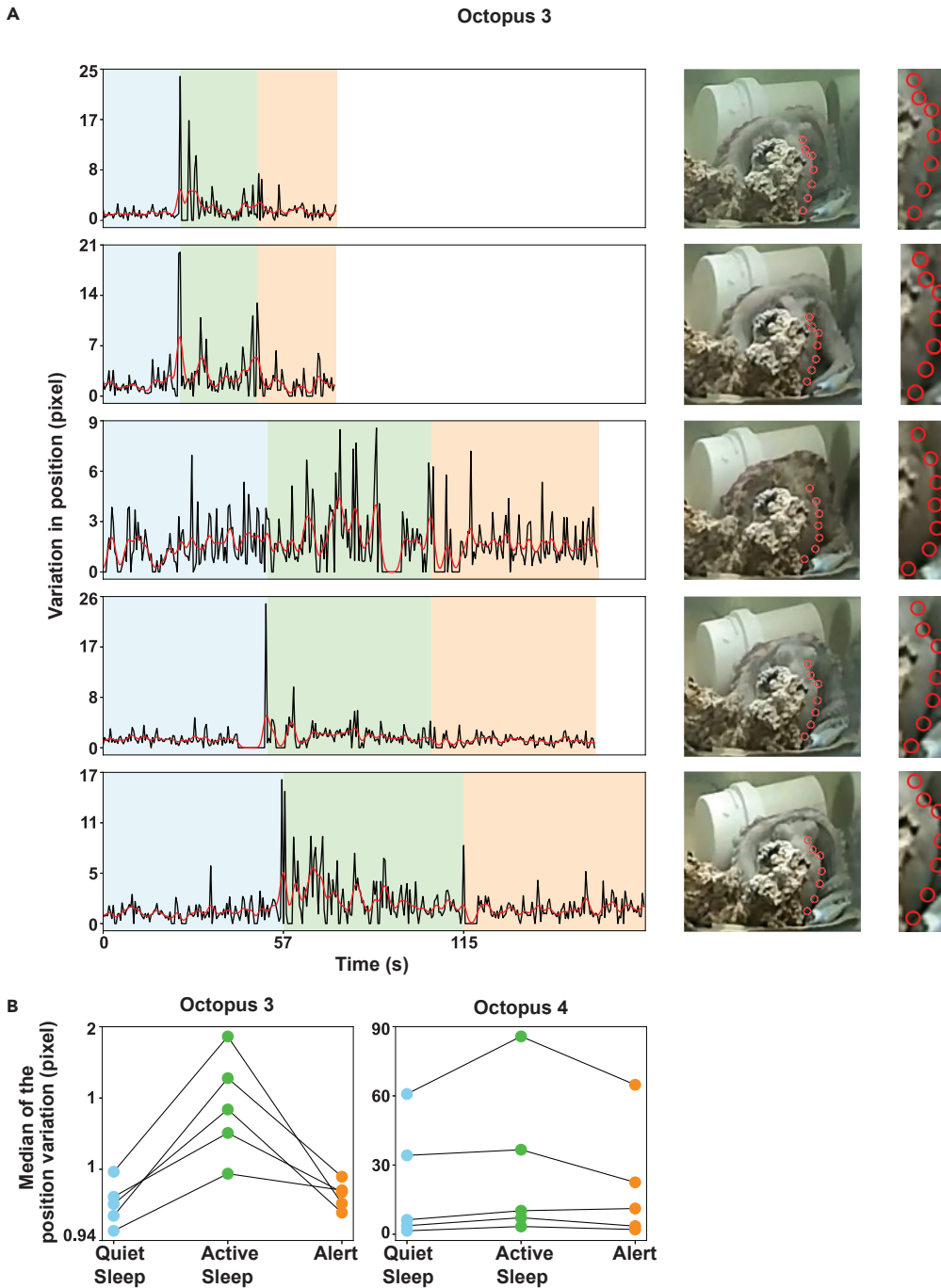


Figure 11. Mantle ventilation movements across 'Quiet sleep', 'Active sleep', and the 'Alert' state

(A) The left panel is showing sum of the position variation of seven different spots chosen in the mantle for the tracking (y axis) along the time (x axis) for five transitions between 'Quiet sleep' (blue zone), 'Active sleep' (green zone), and the 'Alert' state (orange zone) from octopus 3. The intervals analyzed for 'Quiet sleep' and the 'Alert' state had the same duration of the 'Active sleep' that occurred between them. The five examples are organized from the shortest 'Active sleep' episode, on top, to the longest episode, on the bottom. The red line on the graph is a Gaussian filter and follows closely the real data, represented by the black line. The right panels display an example of the region where seven marks (red circles) were placed on the mantle to perform the tracking, with zoomed-in images on the right.

(B) Scatterplots showing the medians of delta (500 ms) Gaussian values for the behavioral analysis of each octopus (n = 2).

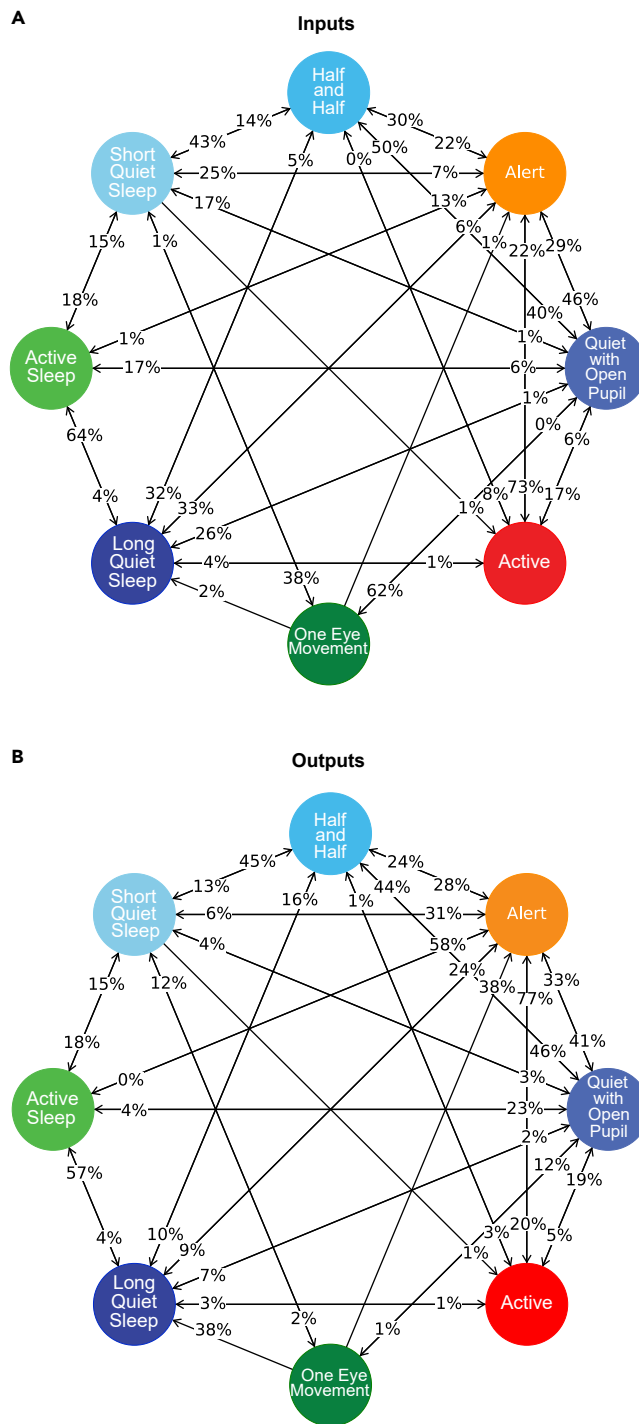


Figure 12. Proportions of transitions between behaviors

Graphs show the proportions of behavioral transitions that occurred along the 180 h and 49 min of video recordings ($n = 4$ octopuses). Each node of the graph represents one behavior described on the ethogram and the edges indicate transitions.

(A) Percentage of the total inputs for each behavior.

(B) Percentage of total outputs for each behavior.

We also hypothesized that the “half and half” body pattern is related to a rest behavior, something also reported for *Octopus vulgaris*, including an alternation of the body half that gets dark a few minutes later (Figure 2C) (Meisel et al., 2011). However, other authors have reported that this behavior is related to intraspecific interactions with other cephalopods, such as in the squid *Sepioteuthis sepioidea* (Blainville, 1823; Mather, 2016). In our experiment, it is possible that the octopuses were seeing their own reflection in the aquarium wall and the ‘QHH’ is a response to environmental stimuli rather than a resting behavior. Nevertheless, in agreement with our hypothesis, the arousal threshold during ‘QHH’ was significantly higher in comparison with the ‘Alert’ state, and the peaks occurred during periods of quiescence. Thus, this state may be related to a lighter sleep state preceding or interspersing the ‘Quiet sleep’.

The ‘Quiet state with only one eye movement’ (‘QOEM’), which has never been reported for any cephalopod species, also seems to be a rest behavior. However, further studies must investigate whether this unusual type of eye movement can also occur with both eyes simultaneously, whether it can also be observed in the natural environment, and what are the physiological processes underlying it. The fact that this behavior also occurred during the night, with lights off, indicates that it is not triggered as a response of the reflexive glass tank (Video S9).

Our study demonstrates that quiescent states shown by specimens of *Octopus insularis* fit most of the behavioral criteria for sleep. The existence in cephalopods of at least two different sleep states within an ultradian wake-sleep cycle contrasts with the apparent existence of a single sleep state in other mollusks, such as in *Aplysia californica* (Cooper, 1863; Vorster and Born, 2017) and *Lymnaea stagnalis* (Linnaeus, 1758; Stephenson and Lewis, 2011). If extended to multiple species of non-cephalopod mollusks, this difference suggests an independent evolution of ‘Active sleep’ in cephalopods and amniotes.

One of the main interests in the field is to establish parallels between the ‘Quiet sleep’ and ‘Active sleep’ states presented here and the different physiological responses found in the mammalian non-REM (NREM) and REM sleep states. Given that the ‘Active sleep’ state has not been described for octopuses before, it is necessary to investigate whether the similarity of this state with REM sleep goes beyond the behavioral similarities observed in this work, such as elevated arousal threshold, eye movements, and body twitches (Aserinsky and Kleitman, 1955; Dement, 1958; Dillon and Webb, 1965). The similarities between the ‘Quiet sleep’ of octopuses and NREM sleep in vertebrates, such as behavioral quiescence, with only brief and minimal movements (Muzet et al., 1972), and increased arousal threshold (Neckelmann and Ursin, 1993), prompt the need for further investigation on whether octopuses and mammals undergo similar physiological processes. Recordings of brain activity during ‘Quiet sleep’ and ‘Active sleep’ can be used to search for the occurrence of typical neural oscillations found in mammals (Steriade et al., 1993, Hobson and Mccarley, 1971), but there is of course a major limitation in this comparison, because the vertebrate and cephalopod brains are not homologous and differ substantially in tissue organization. Furthermore, given that it is very challenging to successfully place electrodes in the octopus brain, (Brown et al., 2006) remains the only published electrophysiological study of octopus’ sleep. However, although it shows interesting findings in four specimens of *O. vulgaris*, such as increase in neural activity during quiescence, the video recordings were sampled using time lapse. For this reason, we can speculate that ‘Active sleep’, which has a brief episode duration, could have been severely undersampled. Furthermore, it is possible that during such increase in brain activity the animal was actually undergoing a short ‘Active sleep’ episode as described here, which may have been missed in the video record. Therefore, we recommend further studies in this field to assess brain electrophysiology in continuous behavioral recordings.

As proposed by (Reiter et al., 2018), it is reasonable that chromatophore expansion could serve as a proxy for motor neuron activity, because each chromatophore is controlled by a small number of motor neurons, and each motor neuron controls a small number of chromatophores (Reed, 1995). Therefore, the skin patterning computational analyses can be useful to understand neural activity of these animals. The analysis of skin color and texture dynamics revealed differences in chromatophore pattern and papillae exposure across episodes of the ‘Alert state’, ‘Quiet sleep’, and ‘Active sleep’. During the ‘Alert’ state there were oscillations, whereas during ‘Quiet sleep’ they remained relatively constant and during the ‘Active sleep’ they change abruptly, in accordance with the dynamic body pattern. Furthermore, the onset of ‘Active sleep’ episodes was characterized by a sudden and simultaneous darkening of the mantle and head, as described for what has been called REM-like state in *Sepia officinalis* (Iglesias et al., 2019). In addition, we also found a moderate increase in eye movements and ventilation rate during ‘Active sleep’.

Conclusions

Despite the great evolutionary distance between vertebrates and invertebrates, the sleep behavior of *Octopus insularis* shares many features with the sleep of amniotes, including the ultradian cyclic pattern observed for the 'Active sleep' state. The recent evidence of 'Active Sleep' in *Drosophila* (Tainton-Heap et al., 2021) suggests strong selection pressure across evolution for an alternation between 'Quiet' and 'Active' sleep states. The occurrence of the non-REM/REM alternation in mammals, birds, and in some reptiles, such as in the bearded dragon and in the argentine tegu *Salvator merianae* (Duméril and Bibron, 1839; Libourel et al., 2018; Shein-Idelson et al., 2016), points to a common origin of the wake-sleep cycle in these groups of animals, which share a common ancestor (Libourel et al., 2018). However, considering that cephalopods split from vertebrates more than 500 million years ago (Vitti, 2013; Shu et al., 2001), it is likely that the sleep behaviors observed here, despite their similarity to those found in amniotes, are analogous rather than homologous to these states.

By the same token, the fact that analogous nervous systems such as the cephalopod brain and the vertebrate brain evolved similar behavioral sequences across the wake-sleep cycle is strongly suggestive of convergent evolution. Cephalopods have evolved *de novo* neural structures termed lobes, including the vertical lobe that is involved in long-term memory and shares some functional features with the mammalian hippocampus (Gutnick et al., 2016; Nixon and Young, 2003). Indeed, cephalopod evolution seems to have converged with vertebrates with regard to the neural mechanisms underlying learning (Gutnick et al., 2016; Shomrat et al., 2015; Hochner et al., 2003). It remains to be investigated whether the physiological functions of sleep, in this far-evolving taxon, also resemble the functions performed in amniotes, such as metabolic detoxification (Xie et al., 2013; Hablitz et al., 2019) and cognitive processing (Boyce et al., 2016; Blanco et al., 2015).

Limitations of the study

A more accurate analysis of the behavior 'QHH' would involve estimating the arousal threshold using the visual stimulus to selectively affect only the dark side or the pale side separately. We tried to do it, but it proved very difficult to have the animal stay long enough in the same position, to ensure that it was really seeing the monitor with only one eye. Furthermore, even if we could establish beyond doubt that just one eye was able to see the monitor, the other eye still could be stimulated by reflections on the aquarium's glass.

If 'QHH' is in fact a sleep state, it may be akin to the unihemispheric sleep observed in bird and aquatic mammals (Rattenborg et al., 1999, 2000, 2016; Ridgway et al., 2006; Lyamin et al., 2002; Mascetti, 2016). Unihemispheric sleep occurs during slow wave sleep (SWS), and the eye corresponding to the sleeping hemisphere remains closed. However, another limitation of this study was that we were not able to compare the pupil contractions recorded from the pale and dark sides during the 'QHH'. Pupil size was difficult to measure because (1) 'QHH' is a behavior with reduced frequency and very short duration; (2) we were often able to observe only one of the eyes because of the camera position; (3) commonly the eye from the dark side also gets dark, making it difficult to distinguish the pupil from the rest of the eye; and (4) given the asymmetries in skin color, we could not assume that both eyes had their pupils equally open or closed.

For a deeper understanding of 'Active sleep' it will be important to quantify more precisely the occurrence of body twitches. Because the twitches are distributed all over the octopus' body, variations of focus and body position made these twitches difficult to quantify here. Another caveat of the present study is that we did not assess sleep rebound. This hallmark of sleep has been documented in *Octopus vulgaris* (Brown et al., 2006; Meisel et al., 2011), so it is quite likely that *Octopus insularis* also has a sleep homeostatic regulation.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Sidarta Ribeiro (sidartaribeiro@neuro.ufrn.br).

Materials availability

Our study did not generate new unique reagents.

Data and code availability

Original materials, data and code have been deposited in OSF at https://osf.io/f6jyu?view_only=c8341a1535ad472d908f7a5b629e99a3.

METHODS

All methods can be found in the accompanying [transparent methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2021.102223>.

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AUTHOR CONTRIBUTIONS

SLSM, SR, and TSL conceived and designed the experiments and literature search; SLSM and SS secured laboratory space and funds for the research; SLSM and SR wrote the paper and PHL contributed with the writing; WB and EBS contributed with study design and data interpretation; SLSM and MMMP performed the experiments and collected and analyzed the data; SLSM, MMMP, and FDL performed specimen collection in the field; FDL, SLSM, PHL, and IGM performed the statistical analysis; SLSM, MMMP, PHL, JBCO, IGM, and SR prepared figures and/or tables; SR, TSL, FDL, WB, EBS, and SS reviewed the paper and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

**Cyclic alternation of quiet
and active sleep states in the octopus**

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SUPPLEMENTAL INFORMATION

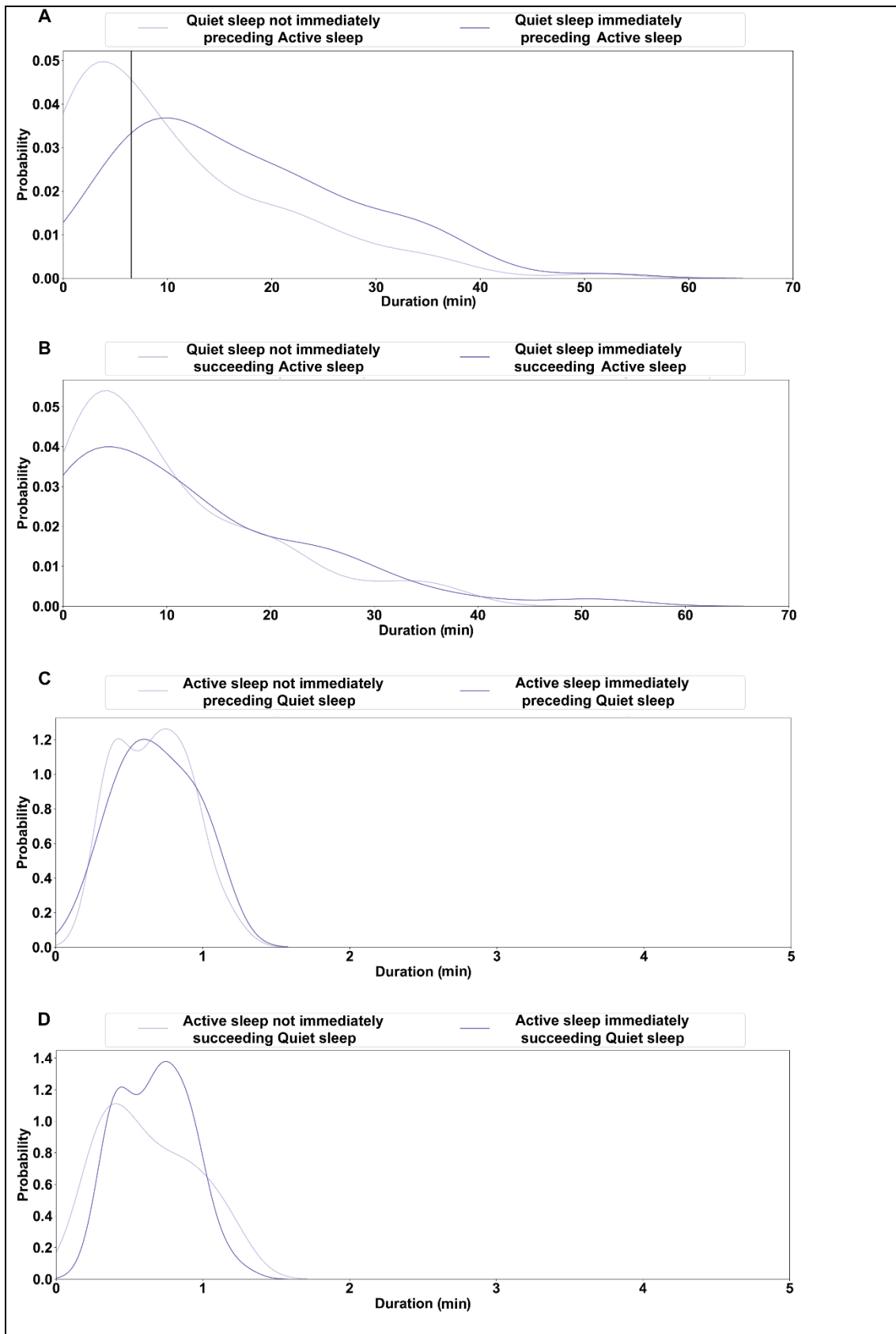
Resource Availability

Lead Contact: Sidarta Ribeiro

Data and Code Availability: Original data and code have been deposited in OSF at https://osf.io/f6jyu/?view_only=c8341a1535ad472d908f7a5b629e99a3

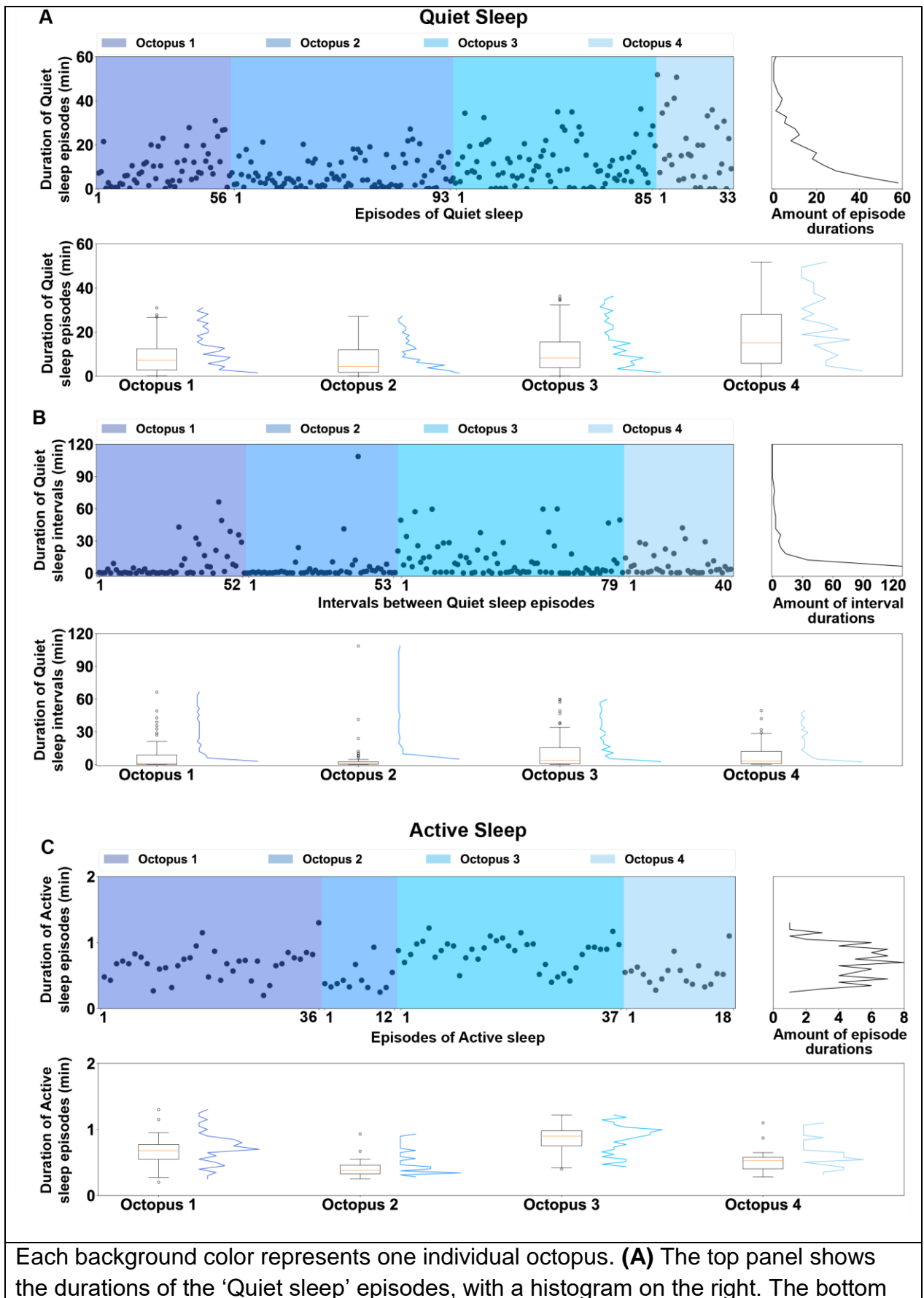
Supplemental Figures

Supplementary Figure S1. Duration distributions of 'Quiet sleep' and 'Active sleep' episodes, according to the states that either preceded or succeeded them, Related to Figure 5.



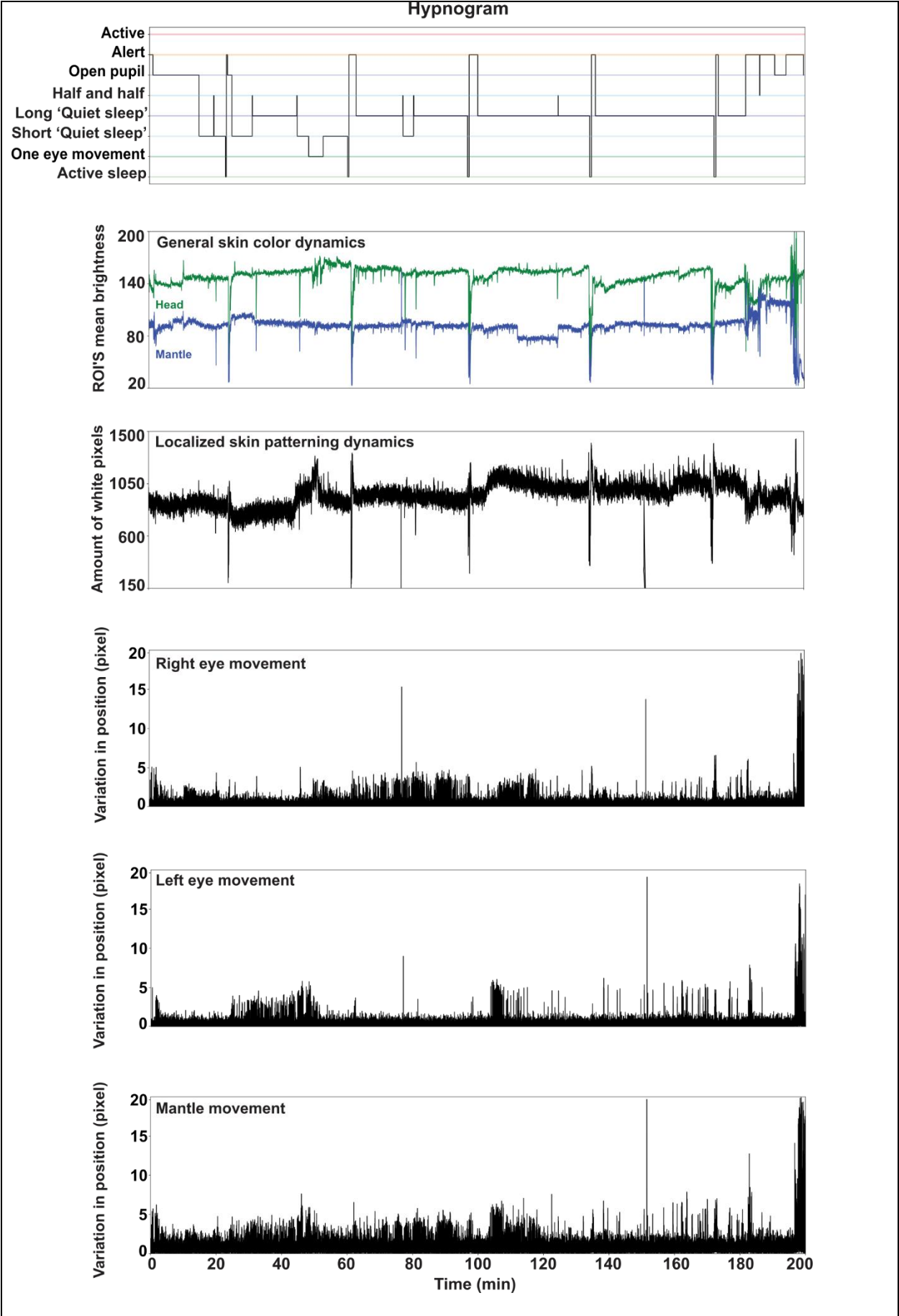
Kernel Density Estimation was used to compare the distributions of durations of: **(A)** 'Quiet sleep' immediately and not immediately preceding 'Active sleep' (black vertical line indicates the average of modes from two distributions). **(B)** 'Quiet sleep' immediately succeeding and not immediately succeeding 'Active sleep'. **(C)** 'Active sleep' immediately and not immediately preceding 'Quiet sleep'. **(D)** 'Active sleep' immediately and not immediately succeeding 'Quiet sleep'. The x-axis displays durations in min, and the y-axis displays the probability of a given duration to occur.

Supplementary Figure S2. Episode duration and intervals between episodes for 'Quiet sleep' (long and short) and 'Active sleep', Related to figure 6.



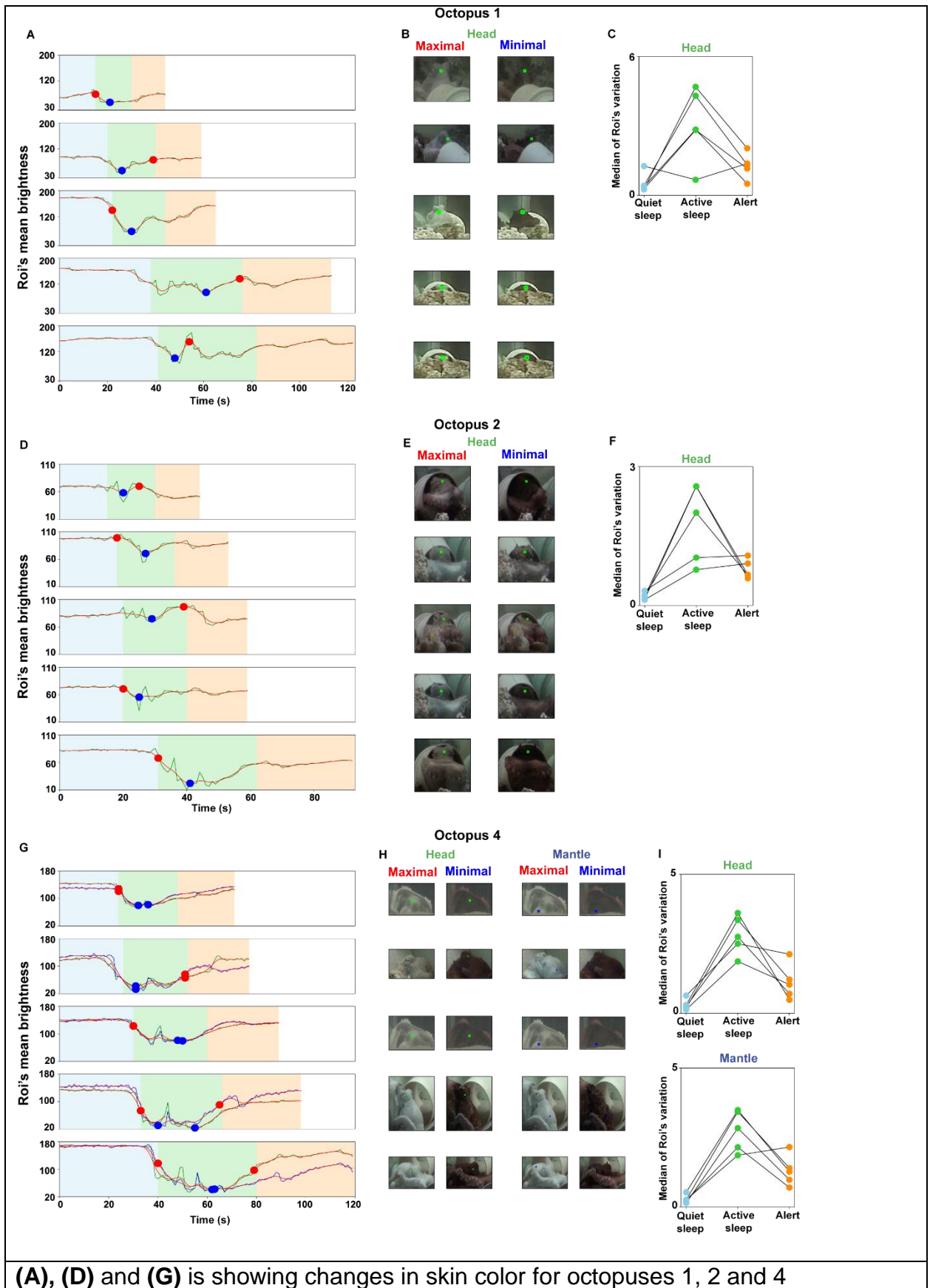
panel shows the bloxpot and histogram of these durations for each octopus. **(B)** The top panel shows the duration of the intervals between all the 'Quiet sleep' episodes, with a histogram on the right. The bottom panel shows the bloxpot and histogram of these intervals durations for each octopus. **(C)** Top panel represents the durations of each 'Active sleep' episode, with histograms on the right. The bottom panel shows the boxplot and histogram of 'Active sleep' episodes durations for each octopus.

Supplementary Figure S3. Hypnogram with measurements of general skin color dynamics, localized skin patterning dynamics, eye and mantle movements, Related to figure 7.



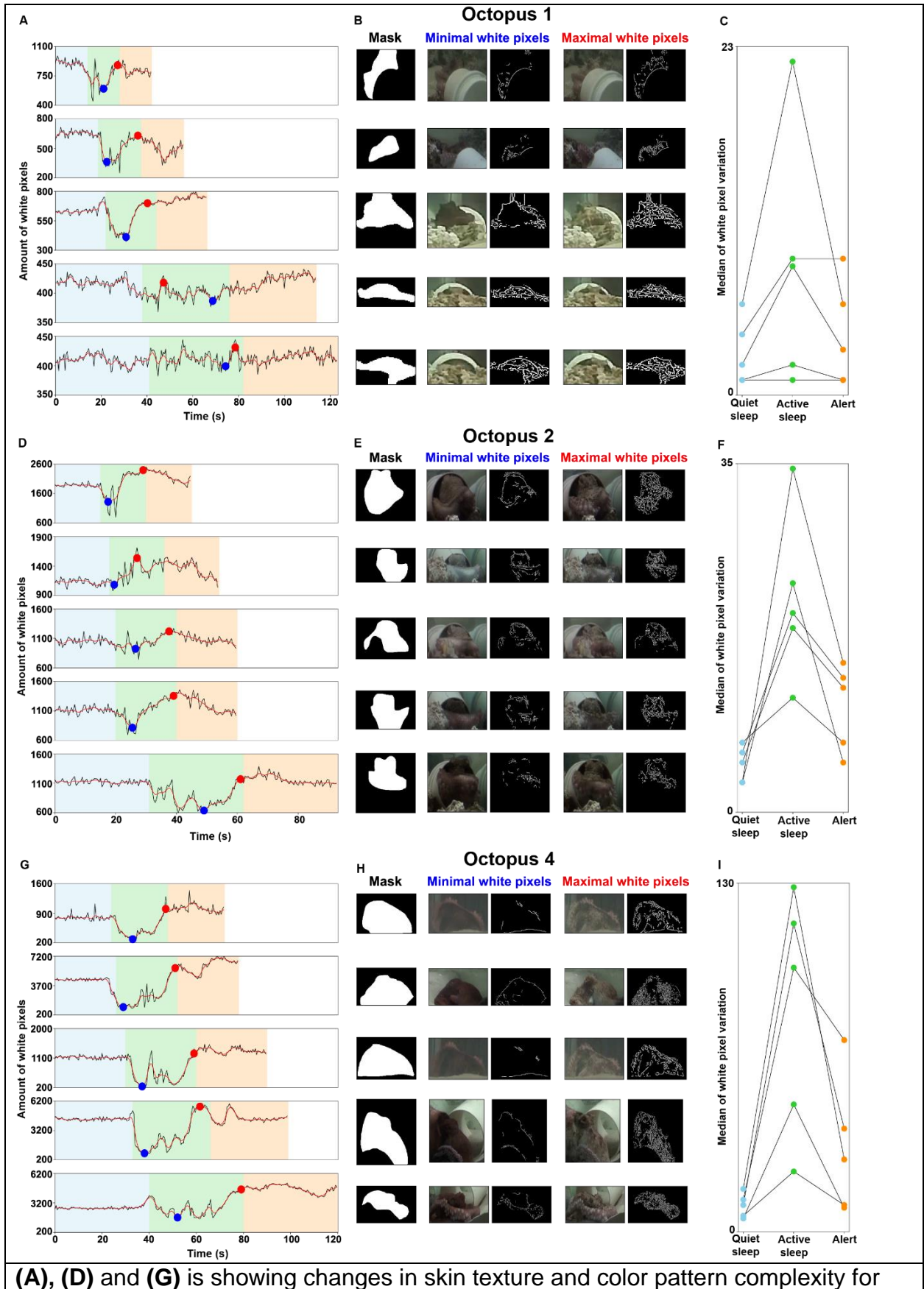
The figure depicts the behaviors of one representative animal (octopus 3) along 200 min of diurnal recordings, beginning at 06:36:50 and finishing at 09:56:50. The plot of general skin color dynamics depicts the mean color from two regions of interest delimited over the head and dorsal mantle (detailed in **Figure 8**). The plot of localized skin patterning dynamics shows changes in skin texture represented by the variation of the number of white pixels captured by the Canny algorithm along time. For both skin dynamic analyses, a marked variation of color and texture concomitant with the occurrence of 'Active sleep' can be observed. The eye position plots show the movements tracked for the right and left eyes. The mantle position plot shows mantle movements represented by the sum of seven different regions of the mantle (detailed in **Figure 11**).

Supplementary Figure S4. General skin color dynamics across ‘Quiet sleep’, ‘Active sleep’ and the ‘Alert’ state, Related to Figure 8.



respectively, represented by the variation of the ROI's mean color (y-axis) along time (x-axis) of five transitions among 'Quiet sleep' (blue zone), 'Active sleep' (green zone) and 'Alert' state (orange zone). The intervals analyzed for 'Quiet sleep' and the 'Alert' state had the same duration of the 'Active sleep' that occurred between them. Besides, the 5 examples are organized from the shortest 'Active sleep' episode, on top, to the longest episode, on the bottom. The green line represents the head's color and the blue line the mantle's color. The lines are well followed by the Gaussian filter (red line). All the behavior transitions have a similar profile, with little value variation during 'Quiet sleep' and the 'Alert' state, and with a marked variation during 'Active sleep', which comprised both extreme values (highlighted for 'Quiet sleep' and the 'Alert' state). **(B)**, **(E)** and **(H)** are showing the picture of the octopuses with the ROI's selected for head (green) and mantle (blue) when the animal color achieved its maximum value (more pale) and minimum value (more dark). **(C)**, **(F)** and **(I)** The scatterplots show the medians of delta (500 ms) Gaussian values for the color analysis of each behavior for the region of head (octopuses 1, 2 and 4) and mantle (octopus 4).

Supplementary Figure S5. Local skin dynamics across the states ‘Quiet sleep’, ‘Active sleep’ and the ‘Alert’ state for octopuses 1, 2 and 4, Related to figure 9.



octopuses 1, 2 and 4 respectively, which is represented by the variation of the number of white pixels (y-axis) along time (x-axis) of five transitions among 'Quiet sleep' (blue zone), 'Active sleep' (green zone) and the 'Alert' state (orange zone). The intervals analyzed for 'Quiet sleep' and 'Alert' had the same duration of the 'Active sleep' that occurred between them. Besides, the 5 examples are organized from the shortest 'Active sleep', on top, to the longest, on the bottom. The red line on the graph is a Gaussian filter and follows closely the real data, represented by the black line. All the behavior transitions have a similar graph profile, with little values variation during 'Quiet sleep' and 'Alert' state and with a marked variation on 'Active sleep', so that both extreme values (blue and red dots) occurred during 'Active sleep' and all the highest values occurring after the lowest values. **(B)**, **(E)** and **(H)** show the rectangular area cropped from the analyzed frames from octopuses 1, 2 and 4, respectively, including: (1) the mask demonstrated by the white regions which delimited the animal body, excluding arms and background; (2) the moment on 'Active sleep' when the animal is with lowest and highest body pattern complexity (texture and color), represented by the minimal and maximum number of white pixels captured by the Canny borders algorithm (blue and red dots on the graphs). **(C)**, **(F)** and **(I)** represents the scatterplot graphs of octopuses 1, 2 and 4 respectively, showing the medians of delta (500 ms) Gaussian values for skin dynamics analysis. The median values of 'Active sleep' are mostly higher than other behaviors (excepted one transition on octopus 1 where the value does not change across the behavior).

Supplemental tables

Supplemental Table S1. Average of the absolute frequency per day and median duration of the behavioral states, Related to Figure 4.

States	Average frequency/day	Median Duration (min)
Active	10.00	2.32
Alert	35.56	2.83
Quiet with Open Pupil	31.31	3.61
Quiet sleep	17.00	6.92
Quiet with Half and Half	27.44	0.17
Active sleep	7.81	0.68
Quiet with Only One Eye Movement	0.50	0.92

Supplemental Table S2. P values for comparisons between states of the duration of the states observed. The values in bold indicate significant differences (Kruskal-Wallis test), Related to figure 4.

	Active	Alert	QOP	QHH	Quiet sleep	Active sleep
Alert	0.28	-	-	-	-	-
Quiet with Open Pupil	0.09	1	-	-	-	-
Quiet with Half and Half	0.00	0	0	-	-	-
Quiet sleep	0	0	0	0	-	-
Active sleep	0	0	0	0	0	-
Quiet with Only One Eye Movement	0.33	0.12	0.16	0.01	0.01	1

Supplemental Table S3. Cyclicity of long ‘Quiet sleep’ episodes, for a total of 142 episodes analyzed, Related to Figure 6.

Number of Intervals	Percent	Episodes
74	100%	6.77, 7.42, 7.43, 7.58, 7.67, 8.0, 8.13, 8.2, 8.35, 9.08, 9.12, 9.2, 9.23, 9.25, 9.25, 9.88, 10.13, 10.22, 10.45, 10.5, 11.48, 12.12, 12.25, 13.42, 13.85, 14.17, 14.17, 14.25, 14.4, 15.15, 15.47, 15.93, 16.35, 16.4, 17.85, 17.93, 18.3, 20.6, 21.28, 21.57, 22.45, 23.83, 24.58, 25.22, 25.48, 27.05, 27.07, 28.07, 28.37, 28.55, 28.58, 28.85, 29.27, 32.02, 32.63, 34.15, 35.63, 37.67, 38.22, 38.9, 41.2, 42.15, 42.85, 46.73, 49.07, 49.32, 49.52, 57.2, 59.55, 59.62, 59.77, 66.3, 108.62, 173.47.
45	60%	6.77, 7.42, 7.43, 7.58, 7.67, 8.0, 8.13, 8.2, 8.35, 9.08, 9.12, 9.2, 9.23, 9.25, 9.25, 9.88, 10.13, 10.22, 10.45, 10.5, 11.48, 12.12, 12.25, 13.42, 13.85, 14.17, 14.17, 14.25, 14.4, 15.15, 15.47, 15.93, 16.35, 16.4, 17.85, 17.93, 18.3, 20.6, 21.28, 21.57, 22.45, 23.83, 24.58, 25.22, 25.48.

Supplemental Table S4. Cyclicity of ‘Active sleep’ episodes, for a total of 79 intervals analyzed, Related to Figure 6.

Number of Intervals	Percent	Episodes
79	100%	20.83, 22.42, 27.83, 28.45, 29.58, 29.7, 30.17, 30.27, 31.97, 32.23, 32.55, 32.62, 32.67, 32.75, 32.92, 32.93, 32.98, 33.17, 33.23, 33.23, 33.38, 33.77, 34.05, 34.1, 34.15, 34.38, 34.6, 34.75, 35.45, 35.48, 35.48, 35.52, 35.85, 36.0, 36.05, 36.18, 36.32, 36.4, 36.45, 36.92, 36.97, 37.12, 37.95, 38.32, 38.32, 38.62, 39.02, 39.05, 39.15, 40.67, 41.25, 42.57, 44.13, 44.95, 46.27, 46.28, 50.4, 51.9, 54.22, 55.25, 56.05, 58.35, 60.77, 61.48, 61.95, 62.15, 68.0, 70.38, 75.53, 75.7, 78.15, 81.32, 81.52, 87.55, 96.68, 99.28, 103.03, 227.79, 150.45.
47	61%	29.58, 33.38, 34.15, 34.75, 34.38, 27.83, 38.32, 32.67, 32.75, 28.45, 32.23, 33.17, 32.62, 31.97, 38.62, 34.1, 32.93, 35.48, 34.05, 33.23, 30.17, 29.7, 35.52, 35.48, 35.45, 36.05, 34.6, 35.85, 33.77, 39.15, 36.18, 36.45, 37.12, 32.55, 36.4, 33.23, 32.92, 39.05, 36.32, 30.27, 39.02, 36.97, 37.95, 38.32, 36, 36.92, 32.98.

Transparent Methods

Animals

The general guidelines for care and husbandry of the octopuses during this study were in accordance with the Directive 2010/63/E.U. Brazil's animal welfare regulation does not include cephalopods, so this study did not require a protocol or approval number. The capture of specimens was approved by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO) through the Permanent License for the collection of zoological material number 33754-1.

Four specimens of *Octopus insularis*, a tropical species from Western Atlantic (Leite et al., 2008, Lima et al., 2017) one male (155g) and three females (300g, 450g, 535g) were collected by S.L.S.M. and M.M.M.P. at the Buzios Beach (Latitude 6°0'14.29"S, Longitude 35° 6'21.01"W) between March 2018 and February 2019. The animals were brought to the laboratory at the Federal University of Rio Grande do Norte, where they were acclimatized for 10 days before the experiments began. The octopuses were kept individually in a 1.0 x 0.7 x 0.6 meters glass tank, which was part of a closed circulation system including a sump comprising protein skimmers, passive filters, and a de-nitrification tank to filter the water.

The animals were provided with a semi-natural enriched environment, including sand, stones, and PVC pipes, with a water temperature at 25 °C. In natural environments, these animals are commonly found in the temperature range from 24 °C to 29 °C (Longo et al., 2015, Leite et al., 2009). S.L.S.M. and M.M.M.P. fed each octopus twice a day (9:00 and 17:00) with defrosted crabs or shrimps. A 12h/12h cycle light/dark schedule was established using a timer, with lights on at 5:50 and off at 17:50. At the end of the studies, the animals were anesthetized with MgCl₂ 3.5% in seawater and euthanized by de-cerebration.

According to their weight, we numbered the octopuses from the lightest (octopus 1 - male, 155g) to the heaviest (octopus 4 - female, 535g).

Video Recording and Ethogram

Two HD cameras (Sony-Handycam HDR-CX550 and Intelbras IC3) were placed at the aquarium's opposite sides to record behaviors in colored videos. Each animal was video recorded during the 12 h of light period (with few interruptions due to camera adjustments), for four days. Light-time recordings were chosen because the *Octopus vulgaris* from Bermuda is mostly inactive during the day in its natural environment (Mather, 1988), and recent studies suggest that the common octopus found in this region is actually the *O. insularis* (Lima et al., 2017). Furthermore, according to field research developed in Brazil, *O. insularis* has crepuscular habits, being less active during the day (Leite, personal communication). From the visual inspection of the videos, an ethogram was created for the behaviors displayed by each animal. From a total of 192h of recordings, 11h and 11min were discarded due to memory card limitations and feeding periods, when the dynamics of spontaneous behavioral transitions was disturbed. The ethogram with the sequence of behaviors and each behavior's duration was elaborated from the visual inspection of 180h and 49min of video recordings (n = 4 specimens, 48 h from octopus 1, 46h and 46min from octopus 2, 47h and 32min from octopus 3, 38h and 30min from octopus 4).

The behaviors were initially classified within the categories Active, Alert, and Quiet, as described in (Brown et al., 2006). We then investigated behavioral changes during the Quiescence state that could be similar to 'Quiet with closed pupil' or to 'Quiet with the half and half skin pattern' ('QHH') observed in *Octopus vulgaris* (Brown et al., 2006, Meisel et al., 2011), as well as the 'Active sleep' observed in *Sepia*

officinalis (Frank et al., 2012, Iglesias et al., 2019), and searched for any other quiescence state not yet described in cephalopods. The duration and frequency of these behaviors were assessed. We also asked whether there was a sequential pattern in their occurrence. For comparison, we tried to record all the animals during the night. However, the camera's infrared light has a short-range through the water column, and hours of recording were lost when animals moved away from the focus. Despite this difficulty, we recorded 114 h from one animal (octopus 3), which tended to stay quiet in the aquarium corner, maintaining its position relative to the camera placed at a close distance.

Measurements of arousal threshold

Octopuses 2, 3, and 4 were visually stimulated to quantify the arousal threshold across distinct behavioral states. The visual stimulation (detailed below) was performed when the animal was in the 'Alert' state and during the different states initially categorized within the Quiescence state (**Table 1**).

Octopus 3 initially failed to respond to the visual stimulus even when awake and alert. Thus, in the case of this animal, we decided to apply a vibratory stimulus (details below) to confirm the lack of sensory responses across the same behavioral states investigated by using the visual stimulus. Two weeks after the vibratory stimulation test, we attempted the visual stimulation again with the same octopus and were then able to elicit arousal responses. Inspections of the video recordings suggest that octopus 3 was still in the process of habituation when first subjected to the visual stimulation test, which led to a freezing behavior during the trials. We kept this animal in the study because it was in good health, as indicated by key physiological

parameters (Fiorito et al., 2015). Octopuses commonly display such intraspecific behavioral variation (Mather and Anderson, 1993).

The behaviors considered as reactions to sensory stimulation were changes in body pattern (color and texture) and sudden alterations in posture. In the visual stimulation case, reactions also included orienting the head to look at the screen and attempts to attack the crabs moving on the screen (see below). The visual and vibratory stimuli were mild, near the perceptual threshold, to properly discriminate the different states' levels of responsiveness.

During these sensory stimulation tests, each octopus was kept alone in the experimental room. Simultaneously, the behavior was recorded through a camera that transmitted video signals in real-time to a monitor in the next room, where a researcher (either S.L.S.M. or M.M.M.P.) supervised the experiment. Both tests occurred between 8:00 and 16:30.

Visual stimulation test to quantify arousal threshold

A computer screen (25.8 x 14.5 cm, pixel resolution 1366 x 768, maximum illuminance of 71 lux, average illuminance of 32 lux) was placed in contact with the wall of the aquarium as close as possible to the octopus, so that the animal could easily visualize it (**Figure 1**, Visual stimulation). When the animal was in one of the behavioral states of interest (**Table 1**), a 50 s video showing live crabs moving was initiated remotely. The time taken by the octopus to react to the stimulus was assessed by video inspection. When the animals failed to show any reaction throughout the 50 s, the trial was scored as 'absence of reaction'.

Vibratory stimulation test to quantify arousal threshold

A rubber hammer was attached to the aquarium's upper corner and tied to a nylon wire passed to the experimenter's room through a window. This wire had four marks so that pulling it up to one of these marks would set the hammer at the angles of 10°, 20°, 30°, and 40° respectively, to hit the aquarium wall with four different, increasing intensities (**Figure 1**, Vibratory stimulation). The hammer was triggered when the animal was in each of the behaviors of interest mentioned above. The hammer was only triggered to strike with a more vigorous intensity when the animal failed to respond to the previous (weaker) stimulus. Whenever the animal elicited a behavioral response before the hammer hit with the smallest intensity (presumably due to visual perception of the hammer's movement), the arousal threshold was recorded as level 1. If the behavioral response was elicited after the hit with the angle of 10°, the arousal threshold was recorded as level 2, and so on for the other angles. When the animal failed to react to all intensities (up to the 40° angle), the trial was considered as 'Maximum Impact with an absence of reaction'. This procedure was video recorded for subsequent analysis.

Behavioral transition graphs

Behavioral transition graphs were elaborated from the ethogram using Python version 3.6.9 (Rossum and Drake Jr., 2011) to quantify the sequential order of the behaviors observed in the video recordings (n=4, a total of 180h and 49min of recordings during the light phase, over four days). The directed graphs were used to characterize all the behavioral transitions observed and to determine whether there are typical behavioral sequences across the wake-sleep cycle.

Quantification of general skin color dynamics

We used pixel color intensity values to compare chromatophore changes across behavioral states and assess whether each behavior has specific characteristics and different variation rates. The original videos were recorded at the rate of 29.97 frames/s in the RGB (Red, Green and Blue) color system. Therefore, to measure pixel color intensities we used brightness (i.e., amount of light), which is represented by range of integer values (grayscale), going from 0 (black color, absence of brightness) to 255 (white color, highest brightness). This range of values came from the conversion of the three RGB values, present in the original image (video frame), to grayscale. For the conversion from RGB to grayscale, we used the function *cvtColor()* from the OpenCv library (Bradski and Kaehler, 2008). We delimited two square regions of interest (ROIs; 5x5, a total of 25 pixels) located on the octopus' body, over the dorsal mantle and the head. These regions were chosen because 1) they contain small and more numerous chromatophores with multiple innervations in comparison with other areas, thus providing a reliable sample of the highly complex changes in whole-body color (Leite and Mather, 2008) and 2) they remain almost in a static position when the octopus displays the sleep-like behaviors of interest. The choice of dimension was based on a previous study of *Sepia officinalis* (Iglesias et al., 2019). The mean brightness of the region of interest, ranging from 0 (black) to 255 (white), was calculated in each video frame for each behavior of interest.

Quantification of local skin patterning dynamics across behavioral states

Localized skin dynamics, caused by texture changes due to extension and retraction of the papillae and changes in color patterning within specific body regions, were investigated using the Canny algorithm for edge detection (Green, 2002). To

execute the Canny algorithm more precisely and faster within each ROI, we carried out two steps: (1) every video frame was cropped to obtain a smaller rectangular region delimitating exactly where the animal was located; (2) a customized mask was applied to restrict the ROI further to include the arms but not the arm tips and suckers, which were prone to movement artifacts (**Figure S5**). Edge changes across frames of interest were calculated as the difference in the number of white pixels generated by the Canny algorithm. We smoothed this profile using a Gaussian filter ($\sigma = 2$), with a frame-to-frame interval of 500 ms.

Quantification of eye and mantle movements

Eye and mantle movements were tracked using DeepLabCut (Mathis et al., 2018), with two marks (one for each eye) and seven marks for the mantle. Then, the sum of the position variation of the left and right eye and the mean values for the seven marks on the mantle were used for the analysis. A 50 layers deep convolutional neural network (ResNet-50) was employed for automatic marking, using 30 frames per video for manual marking (to produce train and test datasets) and a training fraction of 70%. To remove artifacts, coordinates $>$ or $<$ 2 SD from the mean were replaced by the average value of the segment considered. Eye and mantle movements across frames of interest were calculated using a Gaussian-smoothed time series ($\sigma = 2$) of the Euclidean distances between X-Y coordinate pairs, with a frame-to-frame interval of 300 ms. After performing a parametrical analysis, this interval was chosen to search for the time interval that maximized differences across states (data not shown). Eye movements were quantified in all animals, but mantle movements were quantified in only two animals (octopuses 3 and 4) because the other two animals

preferred using the PVC pipe as a shelter, so the mantle was not visible during their quiescence behaviors.

Statistical Analysis

The assumptions of normality of the distribution and homogeneity of the variance were examined with the Kolmogorov–Smirnov and Levene tests (Zar, 2007). Since none of the datasets met the parametric assumptions, non-parametric tests for repeated measures were conducted. To verify whether there were significant differences in episode duration, texture and color skin dynamics, eye and mantle movement across behavioral states, the Kruskal-Wallis test was used. Pairwise comparisons between states of interest were performed using the Wilcoxon signed-ranks test, with Bonferroni correction for multiple comparisons. The same statistical tests were carried out to analyze response latencies after visual stimulation across behavioral states and a chi-squared test to compare the states' absence of reaction. In the case of the vibratory stimulation, which was performed only in the animal that was initially unresponsive to visual stimulation, chi-square tests were performed to verify differences in the number of observations at each intensity level across behavioral states. All statistical analyses were conducted using the software R version 3.3.2 or Python version 3.6.9.

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