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Author(s)	古澤, 千春
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**Sleep ecology of the brown trout:  
applying behavioral approach to a wild population**

(ブラウントラウトの睡眠生態学：  
野外個体群への行動アプローチの適用)

Chiharu Furusawa

A dissertation submitted to Division of Biosphere Science of  
Doctor of Philosophy in Graduate School of  
Environmental Science, Hokkaido University, Japan

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## **SUMMARY**

Sleep is an essential behavior for all animals. Numerous physiological and neurological studies have revealed multifaceted functions, such as energy conservation, recovery processes of body and brain, and maintenance of immune systems. However, the adaptive significance of sleep remains surprisingly unknown, largely due to a lack of ecological studies. While sleep has undoubtedly been shaped through natural selection, previous studies has mostly been conducted in laboratory settings, often isolating ecological factors. Consequently, these findings may detect captive-specific responses. Additionally, despite sleep being ubiquitous and homologous across animal kingdoms, there has been a taxonomic bias towards mammals and birds in previous studies. Because animals have undergone different evolutionary processes under ecological, physiological, and phylogenetic constraints, the adaptive significance of sleep may differ, even though sleep behaviors look similar. To comprehend the adaptive significance of sleep, it is important to clarify sleep ecology across a wide range of taxa in natural environments. While evaluating whether animals sleep was difficult, a behavioral approach can examine the presence of sleep in almost all animals. Therefore, applying a behavioral approach to wild populations should deepen the understanding of sleep ecology, particularly for previously overlooked animals.

In my doctoral dissertation, I examined sleep ecology in brown trout (Salmonidae) as a model species, by employing a behavioral approach. The remarkable variation in diel activity patterns in salmonid fishes suggests the potential for intraspecific variations in sleep patterns. This variation provides an opportunity to examine the factors driving these patterns and differences in fitness associated with them.

In Chapter 2, I verified whether brown trout meet behavioral criteria of sleep, including a typical resting posture, elevated arousal thresholds, and homeostatic regulation. As a result, brown trout exhibited quiescence as a typical resting posture and an elevated arousal threshold during quiescence. Nocturnal individuals also exhibited a rebound after quiescence deprivation, indicating a regulatory process by homeostasis. These results suggest the presence of sleep in brown trout. Furthermore, despite similar body sizes within the same population, diverse sleep patterns were observed, ranging from nocturnal to cathemeral. Therefore, brown trout could be a suitable model to examine sleep ecology and its adaptive significance.

In Chapter 3, I applied the behavioral approach to a wild population to evaluate sleep patterns in a natural environment. Since most studies have conducted mere observation for sleep-like posture, they have not distinguished sleeping and other

similar behaviors, such as resting. Based on Chapter 2, I measured arousal thresholds as an index to distinguish sleeping from resting in a wild population. As a result, the field method distinguished two behavioral states with different arousal thresholds (i.e., resting and sleeping). Habitat use patterns differed between these behavioral states. Sleeping individuals used safer habitat to predation compared to resting ones. Brown trout also slept when activity was not beneficial. Additionally, sleeping individuals were larger than resting one, suggesting a size-dependent sleep tactics. These results indicate that sleep has ecological functions associated with energy conservation and vulnerability to predation.

The presence of sleep has been widely recognized across the animal kingdom, ranging from jellyfish to human. However, how animals sleep in natural environments, or sleep studies in ecological perspectives remain largely unknown. This dissertation showed the potential to elucidate sleep ecology and its adaptive significance in natural populations, by applying a behavioral approach. Across the world, numerous naturalistic observations of sleep-like behaviors have been reported. Interestingly, these imply unique and diverse sleep strategies/tactics depending on taxa. Therefore, applying a behavioral approach to the wild would contribute a comprehensive understanding of the natural history, diversity, and adaptive significance of sleep in often-overlooked

animals.

# CHAPTER 1

## General Introduction



As humans sleep every day, sleep is an essential behavior for most animal life. Sleep often shows low responsiveness to ambient environments, accompanied by immobility. Therefore, during sleep, animals cannot engage in active behaviors such as foraging and reproduction. Surprisingly, this seemingly meaningless behavior is ubiquitous across animal kingdom, from jellyfish to humans (Campbell & Tobler, 1984; Nath et al., 2017). Not only is sleep behavior similar among animals, but neurochemical regulation processes are also preserved across a wide range of taxa (e.g., the neurotransmitter dopamine, mice: Dzirasa et al., 2006; fly: Kume et al., 2005; Andretic et al., 2005; melatonin: birds: Derégnaucourt et al., 2005; fish: Zhdanova et al., 2001; jellyfish: Nath et al., 2017). This suggests that sleep may be evolutionary homologous across animals (Anafi et al., 2019). Therefore, the ubiquity and homology of sleep imply that sleep serves important functions significant enough to overcome associated costs.

Sleep patterns exhibit high diversity across species, populations, and individuals (Campbell & Tobler, 1984). For instance, Africa elephants (*Loxodonta africana*) spends an average sleep time of only 2 hours (Gravett et al., 2017), while brown bats (*Myotis lucifugus*) allocate up to 20 h in a day for sleep (Brebbia & Pyne, 1972; Harding et al., 2022). Interestingly, chinstrap penguins attain 11 hours of sleep through more than 10000 times of micro sleep, each lasting only 4 seconds (Libourel et

al., 2023). Sleeping site selection also differs between species. In primates, diurnal species tend to sleep on trees, whereas nocturnal species use tree holes and nests (Pozzi et al., 2022). In reptiles, many arboreal species use unstable habitat for sleep, so that they can sense the vibrations caused by approaching predators (Mohanty et al., 2022). Sleep patterns can also significantly change within species, depending on environments. For example, some aquatic animals sleep for 9-10 hours/day at land, but only 0-1 hours/day during multiday trip at sea (Rattenborg et al., 2016; Kendall-Bar et al., 2023). Such incredible variations of sleep make the researchers convinced that sleeping behavior has been characterized under strong natural selection (Rattenborg et al., 2017; Lesku et al., 2019).

Numerous functions of sleep have been proposed mainly in physiological and neurological contexts using laboratory animals. For example, sleep functions in energy conservation by decreasing metabolism (mammals: Franken et al., 1992; fly: Stahl et al., 2017; fish: Kelly et al., 2022) and in the recovery process, such as the removal of harmful wastes products (Xie et al., 2013). Sleep is also strongly associated with cognition, such as learning and memory consolidation (Voster & Born, 2015; Pinheiro-da-Silva et al., 2017, 2018). Although sleep research in ecological contexts or natural environments has been limited (Rattenborg et al., 2017), it has been suggested that

immobility during sleep decreases the likelihood of encountering predators (Lima, 2005; Siegel, 2009). Some studies also proposed that sleep plays a role in suppressing parasite infections by maintaining immune systems (Preston et al., 2009; Opp, 2009; Imeri & Opp, 2009).

Sleep generally serves positive functions but also has negative aspects in ecological contexts. Notably, reduced level of consciousness provides several costs to animals. For instance, sleep state could be vulnerable to predation due to low responsiveness to the ambient environment. Additionally, the more animals sleep, the more they lose the opportunity for active behaviors, such as foraging and mating, potentially impacting their fitness. In fact, some males of pectoral sandpiper lose sleep during breeding season, as much as 3 weeks, which increases reproductive success without scarifying post-breeding survival (Lesku et al. 2012). Despite numerous proposed functions, how the costs and benefits of sleep link to fitness, or its adaptive significance, remains largely unknown. This is an important research gap to clarify one of the big enigmas in behavioral ecology: why do animals sleep?

To address the research gap, we need to tackle certain challenges. First, it is crucial to clarify sleep behaviors in wild animals. Since sleeping is highly vulnerable to predators or sudden environmental changes, animals usually sleep in specific safe places

(e.g. deep in the caves and tunnels, top of trees, protected nests; Pozzi et al., 2022; Mohanty et al., 2022) or times (e.g. midnight when visual predators cannot identify). Thus, it is highly difficult to find sleeping animals in the wild. In addition, many animals do not show specific postures that can be assumed as a sleeping behavior, especially for animals without eyelids, such as insects, fishes, amphibians: an immobile state can be either sleeping or just resting (e.g., Cirelli & Tononi, 2008; Kelly et al., 2022).

Second, past studies have predominantly focused on sleep patterns in the laboratory (Rattenborg et al., 2017). Importantly, sleep patterns may be significantly different between under safe-laboratory and risky-wild conditions. For instance, in captive animals tend to sleep much more compared to wild counterparts (e.g. the brown-throated three-toed sloth 9.6 hours in captivity vs. 15.8 hours in the wild, De Moura Filho et al. 1983, Rattenborg et al., 2008; African elephant, 3-6 hours in captivity v.s. 2.1 hours in the wild, Tobler, 1992, Gravett et al., 2017). These are probably due to the absence of ecological factors, such as predation and competition (Rattenborg et al., 2008; Voirin et al., 2014). Sleep research in laboratory animals may mislead the understanding of ecological functions and importance of sleep in natural environments.

Third, there is a need to alleviate taxonomic bias in sleep studies. Despite the

prevalence of sleep in the animal kingdom, previous studies predominantly focused on specific animals (Lesku et al., 2009; Lesku & Rattenborg, 2022). In the wild, mammals and birds constitute a large portion of previous studies (Rattenborg et al., 2017). Consequently, our ecological understanding of sleep remains limited. Importantly, present-day animals undergo distinct evolutionary processes under ecological, physiological, and phylogenetic constraints after diverging from common ancestral organisms. Even with similar sleep behavior, the adaptive significance of sleep could differ. Therefore, it is necessary to examine sleep ecology of a wide range of taxa, including species overlooked in previous studies.

A behavioral approach can tackle with these challenges. Sleep can be detected by measuring behavior and brain activity. In the behavioral approach, sleep is defined by behavioral criteria, such as stereotypic resting posture, elevated arousal threshold, and homeostatic regulation, which is often called as “behavioral sleep” (Piéron, 1913; Campbell & Tobler, 1984; Allada & Siegel, 2008). Alternatively, sleep state can also be identified by combining with the electroencephalogram (EEG) and the electromyogram (EMG) during behavioral sleep. This allows for the measurement of detailed sleep architecture, including REM (Rapid Eye Movement) and non-REM sleep (cf., Rattenborg et al., 2008). This electrophysiological approach, however, has great

disadvantages for applying to wild animal populations. For example, because the dataloggers for EEG and EMG are relatively large, these are applicable only to large animals (e.g., Rattenborg et al., 2008). Since the loggers must be implanted to animal brains, this approach is ethically unfeasible to some wild animals. In addition, animals may be removed the dataloggers by themselves or their moving (Rattenborg et al., 2017). The expense of the logger devices also limits the sample size. Consequently, behavioral approach is suitable to examine the sleep ecology in wild populations, especially small organisms such as insects and fishes, which have been overlooked in previous studies.

Salmonid fishes, one of the wild animals most intensively and extensively studied because of their economic importance (Criddle & Shimizu, 2014), are considered as good model to examine sleep in natural environments. They show remarkable variation in diel activity patterns among species, populations and even within individuals; this variation might be associated with sleeping (Reebs, 2002). For example, many salmonids become nocturnal when the water temperature decreases (Fraser et al., 1993). Social dominance also affects diel activity patterns, for example, subordinate individuals become more diurnal (Alanärä et al., 2001). However, researchers have focused only on active individuals and never tested if, when, and

where salmonids sleep, even though some salmonids show a typical resting posture considered as sleep (Keenleyside, 1962; Heggens et al., 1993; Roussel et al., 1999).

Variability at the species, population and individual levels combined with the resting posture enables us to investigate the sleep ecology in wild populations.

In my dissertation, I investigated the sleep ecology of brown trout by employing behavioral sleep approach. In Chapter 2, I confirmed if brown trout sleep in a laboratory setting based on behavioral criteria (i.e. typical sleeping posture, elevated arousal threshold, and homeostatic regulation). In Chapter 3, I applied the behavioral approach to a wild population. Firstly, I examined whether sleep can be distinguished from rest using behavioral approach. Secondly, I examined if habitat selection differs between sleeping and resting states. Third, I examined seasonal variation in sleep pattern to explore the adaptive function of sleep. In Chapter 4, I integrated the different chapters, critically evaluated the limitation of the study, and presented future perspectives (Chapter4).

## CHAPTER 2

### Behavioral sleep in brown trout



## **Abstract**

Sleep is a universal phenomenon reported in a wide variety of species, from jellyfish to humans, with varying patterns and functions across taxa. However, the adaptive significance of sleep remains largely unknown, especially in wild populations, due to the lack of adequate models to study the mystery of sleep. Salmonid fishes are good candidates since they are one of the most studied wild animals and show remarkable diel activity patterns within and among species. Here, for the first time, I show that a typical resting posture (contact with the riverbed) of the brown trout *Salmo trutta* meets the criteria of behavioural sleep: (1) a resting posture with behavioural quiescence, (2) elevated arousal thresholds and (3) homeostatic regulation as a response to sleep deprivation. I also found a remarkable individual variation in the sleep phenotypes (nocturnal, intermediate and cathemeral) even within the same population. It should be noted that homeostatic regulation was observed for the species with flexible diel activity. Because of their variability and flexibility, salmonids represent a promising candidate for an experimental model to clarify the advantages of sleep behaviour in wild populations.

## **Introduction**

Sleep is a periodical, reversible and recurrent state of reduced movement and sensory responsiveness to environmental stimuli regulated by homeostatic control (Allada & Siegel, 2008). Although animals seem vulnerable to threats and have disadvantages when asleep, it is ubiquitous in the animal kingdom, having been evolutionarily preserved and documented in a variety of species, from jellyfish to humans (Campbell & Tobler, 1984; Nath et al., 2017). Many functions of sleep have been proposed, such as memory consolidation, energy conservation, metabolic clearance, synaptic homeostasis and predator avoidance (Roth et al., 2010; Barone & Krieger, 2015). However, these hypotheses are mostly based on studies with laboratory animals, particularly mammals and birds (Aulsebrook et al., 2016). Surprisingly, little is known about the adaptive significance of sleep in wild populations (Aulsebrook et al., 2016; Rattenborg et al., 2017) largely because of the difficulty in finding sleeping animals in the wild (cf., van Hasselt et al., 2020). Since sleep patterns vary across species and populations (Campbell & Tobler, 1984; Rattenborg et al., 2017), a considerable amount of research is certainly needed to cover the wide range of all taxa to clarify the functions and adaptive significance of sleep identifying similarities and differences of its pattern.

The biggest challenge in sleep research in wild populations or animals other than

mammals and birds is the methodological verification of sleep. Sleep in animals with developed cerebral cortex is best described by examining electroencephalogram (EEG) and electromyogram (EMG) that show unique patterns (Yamazaki et al., 2020).

Techniques using subcutaneous electrodes can monitor the EEG or sleep states of animals constantly (Scriba et al., 2013), but the method is sensitive to animal motion and not suitable for normal life of wild animals (Rattenborg et al., 2017; but see Lesku et al., 2012). In addition, EEG cannot be monitored in species with diffuse nerve systems like jellyfish.

A behavioural approach is widely used as a good alternative for sleep research (Campbell & Tobler, 1984; Hendricks et al., 2000b). Instead of the direct but invasive monitoring, researchers infer animal sleep based on three criteria for the lowered responsiveness, as well as physiological control; (1) stereotypic or species-specific resting postures with behavioural quiescence, (2) elevated arousal thresholds (i.e., increase in the time to respond to stimulus) and (3) homeostatic regulation (Campbell & Tobler, 1984; Allada & Siegel, 2008). Resting posture and elevated arousal thresholds directly measure responsiveness, whereas homeostatic regulation provides the evidence that the observed lowered responsiveness is physiologically controlled as the typical or rigorous sleep verified with neuroactivity monitoring. In the behavioural sleep

approach, homeostatic regulation is usually tested with the reactions when the observed sleep-like behaviour is deprived: if the sleep-like behaviour is controlled homeostatically researchers will find a rebound (e.g., extended sleep-like behaviours) after deprivation (e.g., Hendricks et al., 2000a; Zhdanova et al., 2001). It has been well-known that the characteristics of behavioural sleep match typical sleep by comparing the two methods in most studies (Cirelli & Tononi, 2008). Therefore, even animals without a brain or cortex can be confirmed if they are sleeping or not by behavioural approach (Campbell & Tobler, 1984; Nath et al., 2017).

Despite the greatest diversity among teleosts, behavioural sleep has been tested only in four groups of fishes (Zhdanova et al., 2001; Duboué et al., 2011; Shapiro & Hepburn, 1976; Kelly et al., 2021). All the four groups showed stereotypic resting posture with elevated arousal thresholds (e.g., Zhdanova et al., 2001), but homeostatic rebound was demonstrated only in zebrafish and cave fish (Zhdanova et al., 2001; Duboué et al., 2011). Therefore, physiological mechanisms and adaptive functions of sleep may differ among different fish taxa. Importantly, a recent neurophysiological study demonstrated a two-state of sleep in zebrafish, which may relate to REM and non-REM sleep (Leung et al., 2019). Because of the ancestral state and greatest diversity, sleep research in fishes is promising to investigate the origin and functions of sleep in

vertebrates.

I propose that salmonid fishes represent an excellent candidate for a model organism in animal sleep research. They are often in contact with riverbeds, and this quiescence state is accompanied by immobility and low responsiveness (Keenleyside, 1962; Roussel et al., 1999). This quiescence state has been considered a resting state (Keenleyside, 1962; Roussel et al., 1999; Larranaga & Steingrímsson 2015; Fingerle et al., 2016). Nonetheless, the possibility that this state is sleep cannot be ruled out. In this context, if salmonids are resting, they would escape when threats appear (e.g., a predator approaching). Thus, quantifying the arousal threshold is useful to verify the resting or sleep state (Hendricks et al., 2000b). Homeostatic regulation also provides strong evidence of sleep from a physiological perspective. Because many literatures reported a typical resting posture and variation in diel activity patterns in wild population of brown trout *Salmo trutta*, I experimentally examined if the typical resting posture meets the three criteria of behavioural sleep.

## **Materials and Methods**

### *Fish and laboratory conditions*

In March–April 2021 and January–April 2022, I collected wild brown trout parrs

(Appendix 1, table A1) from the Horonai River (42°40'N, 141°36'E; Hokkaido, Japan) by electrofishing (Model 12B, Smith-Root, Vancouver, WA, USA). I also collected individuals of similar size to avoid any confounding effects, such as ontogenetic or social status differences. Individual parrs (mean  $\pm$  SD = 148.8  $\pm$  6.2 mmFL) were transferred to separate aquariums (56  $\times$  25  $\times$  15 cm) tilted  $\sim$ 15° on the lower part under a 12:12 h light-dark cycle (lights on at 06:00; lights off at 18:00) in the laboratory at the Tomakomai Experimental Forest, Hokkaido University. Five aquariums were set in a row on a table and wood boards were placed between the aquariums to prevent visual interactions of individuals. The aquariums had a constant discharge of groundwater ( $\sim$ 132 cm<sup>3</sup>/s) and were supplied with pebble (5-64 mm) as a dominant substrate. The water temperature was maintained under 8 °C and was stable, i.e.,  $\pm$ 1 °C, within mean in individual series of the experiments (Table A1). Trout were not fed during the experimental periods. They were acclimated in the aquariums for two days before each experiment. After the experiments, fish were euthanised by an anaesthetic overdose and the sex was determined by inspecting the gonads.

#### *Behavioural assessment of activity rhythms*

After a preliminary experiment confirming the consistency of the activity rhythms of 5

brown trout for 7 consecutive days (Appendix 2, Figure A1), I recorded the activity of 43 brown trout for 48 h with video cameras (Everio GZ-L330, JVC KENWOOD Corporation, Kanagawa, Japan; Ltl-Acorn 5210A, LtlAcron Outdoors, GreenBay, Wisconsin, USA; MSP09-JP3004-2T, YESKAMO, Shenzhen, China). The behavioural states were documented at 1-min intervals for 30 min in the middle of each hour (ca. from minute 15 to minute 45 for each hour) and were classified as ‘swimming’ (off the bottom, moving fin) or ‘quiescence’ (continuously in contact with the bottom, with a motionless fin). The amount of activity in each hour was calculated from the proportion of the swimming state in the 30-min behavioural state observations. After this, each trout underwent the arousal thresholds or homeostatic regulation tests.

### *Arousal thresholds*

The arousal thresholds were measured as the response to a stimulus when the trout were at a swimming or a quiescence state ( $N = 19$ , the number of individuals in arousal thresholds experiment). A fish was considered quiescent when typical resting posture (i.e. sit on the bottom floor without mobility) lasted more than 1 minute. Three evenly-spaced mesh nets were set on the water surface and, depending on the fish position, one of the mesh nets was fallen remotely by using strained strings (electronic supplementary

material, movie S1). The time from the moment the mesh net touched the fish to the moment they responded by moving fin and body was quantified by a researcher who used a night vision goggle (NV200C, ZIYOUHU, Zhejiang, China). As the surface of the fish that was touched by the net might have affected their response time, I measured the proportion of the net that touched them to the fish's body size. This protocol was conducted four times per individual in random sequences (daytime: 08:00 and 14:00; night-time: 20:00 and 02:00).

#### *Homeostatic regulation of quiescence*

Because some nocturnal trout showed elevated arousal thresholds (see Results), I tested them for a compensatory increase (rebound) in quiescence when this state is disrupted. Quiescent trout during the light phase ( $N = 10$ ) were forced to swim by gently pushing their bodies using a plastic stick every 10 min for 6 h (electronic supplementary material, movie S2). I subsequently confirmed the rebound of quiescence in the dark phase, when nocturnal trout normally swim. Control experiments were also conducted without quiescence deprivation ( $N = 5$ ).

#### *Data analysis*



All statistical analyses were performed using R version 4.1.1 (R Core Team, 2021), and the significance level was set at 0.05. Using the “TSclust” package, activity rhythms were categorised by hierarchical time-series clustering analysis with Ward’s method and dynamic time warping as an index of time series dissimilarity (Montero & Vilar, 2014). To include the partial data due to complications with videos, I applied linear imputation using the “imputeTS” package (Moritz & Bartz-Beielstein, 2017). The stability of the clusters was assessed using the mean Jaccard similarities with 10,000 bootstraps (Henning, 2007). The data for arousal thresholds were analysed by removing the data that I could not measure by technical mistake and showed quiescence at less than 1 minute. I normalised the reaction time with log transformation and conducted a linear mixed model (LMM) and an Anova type II using the “lme4” (Bates et al., 2015) and “car” (Fox & Weisberg, 2019) to find differences in the arousal thresholds between behaviours (swimming or quiescence). The response variable was the reaction time and the explanatory variables were the behaviour, time (light or dark phase), activity rhythms (nocturnal, intermediate, cathemeral), the ratio of the net-over-fish surface and the interactions of behaviour with time and activity rhythms. Aquariums and individuals were included as random effects. To verify whether quiescence is homeostatically regulated, I compared the amount of activity during the dark phase between, before and

after subjecting trout to quiescence deprivation using the Wilcoxon signed-rank test.

### *Ethical Note*

No mortality or apparent injury was observed during electrofishing. Because social interactions by territoriality in salmonids cause a physiological stress, body damage and decrease in nutritional status (Ashley, 2007), social isolation of trout during our experiment served to improve welfare. Fish were not fed for 7 days at most, but even starvation for 14 days under low temperatures like this study should not affect stress levels and health in congeneric salmonids (Waagbø et al., 2017). In the quiescence deprivation experiment, trout did not show avoidance learning on a plastic stick or apparent darkening of body coloration, which is measured as stress index in salmonids (O'Connor et al., 1999; Watz et al., 2015) after the stimulus (electronic supplementary material, movie 2). After experiments, fish were placed into a bucket and were euthanized by an overdose of clove oil (2.0 g/L). This concentration was ten times as thick as usual one to anesthetize fish, which exceeds a lethal dose (Priborsky & Velisek, 2018). I placed fish in the solution for at least 10 minutes and confirmed immobility of the operculum and no response to physical stimulus. As a second method to make sure death of fish, I put the fish in a plastic bag with water and transferred it to a freezer (-

20°C). Fish were frozen rapidly in a freezer. A previous study revealed a species of salmonid fish lack cold responsive nociceptors (Ashley et al., 2006). They are not likely to feel pain by coldness because their habitat with very low temperature make it maladaptive to have cold nociceptors (Sneddon, 2019). All animal procedures were conducted according to Hokkaido University's animal experiments regulations and approved by the Committee for Animal Experiments in Field Science Center for Northern Biosphere (FSC) of Hokkaido University (ID30–2).

## **Results**

The activity rhythms of 43 brown trout were divided into three groups by hierarchical time-series cluster analysis (Figure 1a–b). The first group (mean Jaccard similarities: 0.79) was characterized by high levels of activity in the nighttime but low in the daytime (i.e. nocturnal). The second group (mean Jaccard similarities: 0.97) was high levels of activity regardless of time (i.e. cathemeral). The final group (mean Jaccard similarities: 0.67) was intermediate characteristics between nocturnal and cathemeral. Even though I used similar-sized individuals, cathemeral fish were significantly larger than those of the other groups, whereas sex and the condition factor did not affect the activity rhythms (Appendix 3; Figure A2).

Arousal thresholds were examined 62 times ( $N = 19$ ) as the reaction time to the stimulus. The reaction time showed a trimodal pattern (Figure 2, three peaks showed 3.5, 0 and -2 values, respectively). The upper and lower peaks occurred mainly at quiescence and swimming, respectively. The reaction time was significantly longer when at quiescence compared to swimming (LMM estimate: -2.19,  $t = -3.29$ ; Anova  $\chi^2 = 11.026$ ,  $df = 1$ ;  $P < 0.01$ ), whereas the other variables did not significantly affect the reaction time (Figure 1c; Table A2).

Quiescence deprivation in the light phase caused nocturnal trout to start swimming as soon as they were moved, whereas they switched from induced swimming to quiescence right after swimming. Quiescence deprivation significantly reduced the amount of activity in the following dark phase (Wilcoxon signed-rank test: deprivation  $V = 1$ ,  $P = 0.004$ ), contrary to the control trout that showed no significant changes in activity (Wilcoxon signed-rank test: control  $V = 12$ ,  $P = 0.31$ ; Figure 3).

## **Discussion**

Comparing the fitness of different phenotypes is one of the most appropriate approaches to clarifying the adaptive significance of a particular trait (Huntingford, 2012). Sleep is a highly species-specific trait with lower within-species variability (Campbell & Tobler,

1984). In this context, species that completely shift their diel activity or include individuals with different phenotypes are rather rare (Kronfeld-Schor & Dayan, 2003). Thus, our demonstration of behavioural sleep in brown trout is promising for the study of the adaptive functions of sleep in the wild.

Interestingly, our results showed multimodal peaks in the arousal thresholds, indicating different reaction levels to external stimulus depending on fish states. The upper modal group means very low reactions to stimulus and was observed only in quiescent individuals. This probably represents a typical sleeping in brown trout. The lower modal group reacted very quickly to the stimuli and was found mostly in swimming individuals. These individuals might have been wary of their surrounding environments and ready to escape. The middle modal group included a majority of individuals with both quiescence and swimming states. This should be a normal response of brown trout to the current stimuli (i.e. against a fallen object). However, even within the middle modal group, there was a slight difference between quiescence and swimming individuals. The difference may reflect the initial posture of the two states (i.e. sitting on the floor or swimming in the water column), rather than the two different sleep states recently demonstrated in zebrafish (Leung et al., 2019). Because sitting down on the river floor can save energy to hold in the water column against

water current, the quiescent state with the lower arousal threshold would represent a resting behaviour. Overall, this study offers the first quantitative evidence of a sleep state within quiescence, which is widely described in the wild (Bachman 1984; Roussel et al., 1999).

Homeostatic regulation of sleep can be considered as a physiological need for animals. However, recent studies suggest that sleep homeostasis is not always strictly controlled and needs for sleep differ among species, life stages, or ecological conditions (e.g., Lyamin et al., 2005; van Hasselt et al., 2020; Loftus et al., 2022). Among fish species, cavefish showed sleep rebound (Duboué et al., 2011), whereas sharks and tilapia did not (Shapiro and Hepburn, 1976; Kelly et al., 2021). Moreover, while sleep rebound was detected in zebrafish when sleep was deprived using electric and vibration shocks (Zhdanova et al., 2001; Yokogawa et al., 2007), deprivation by light exposure did not cause sleep rebound, suggesting that zebrafish have not evolved homeostatic regulation strongly (Yokogawa et al., 2007). Brown trout showed a clear sleep rebound, even though species that easily change diel activity should be tolerant to sleep deprivation. This indicates that sleep in brown trout is under strong physiological control and shifts in diel activity readily change their physiology. Or, rapid shifts of diel activity may incur some physiological costs. Although I could not sleep-deprive

cathemeral or intermediate individuals because of the short duration of quiescence, they may have different sleep patterns from nocturnal individuals.

Nocturnal, diurnal, or cathemeral activity patterns are characterised by the circadian rhythm. Sleep depth, which is related to the arousal thresholds (Rechtschaffen, et al., 1966), and sleep intensity, which is associated with the compensatory process for sleep deprivation (Samson et al., 2018), are associated with the circadian rhythm. Thus, it is crucial to understand these relationships. In honeybees, older bees (foragers) have stronger circadian rhythms and deeper sleep at night, whereas young bees exhibit no circadian rhythms (i.e., more cathemeral) and shallower sleep (Eban-Rothschild & Bloch 2008). Some lemur species with stronger circadian rhythm (diurnal species) had stronger sleep intensity than cathemeral species (Samson et al., 2018). In addition, younger people have both stronger circadian rhythms and sleep intensity than older people who show a more fragmented and shallower sleep pattern, while being more tolerant to sleep deprivation (Schmidt et al., 2012). In our study, only nocturnal trout had clear circadian rhythms and showed behavioural sleep, which suggests a similar relationship between the strength of circadian rhythms and sleep patterns. Because salmonids change their diel activity depending on ecological conditions (Fraser et al., 1993; Alanära et al., 2001), We can investigate how circadian rhythms mediate the

depth, intensity and duration of sleep to unravel its physiological control and adaptive function. Here, brown trout showed clear differences in activity rhythms, although no diurnal individuals were documented, probably due to the low water temperature (Fraser et al., 1993). I could examine the behavioural sleep in diurnal brown trout by controlling the temperature. Future studies in salmonids are needed to compare the depth, intensity and duration of sleep according to the activity rhythms and within the same individuals by manipulating ecological factors.

The reason for the divergent sleep phenotypes in brown trout is unknown. This was rather surprising, considering that fish size and experimental conditions were standardised. Still, cathemeral fish were significantly larger than nocturnal or intermediate trout, while having a non-significant tendency to a lower body condition (Appendix 3; Table A3; Figure A2). Such body shape is consistent with the characteristics of smolting individuals that migrate to the ocean in spring, eat more and exhibit longer, slimmer bodies compared to resident counterparts in some populations of brown trout and other salmonids (Hoar, 1976; Sundell et al., 1998; Ayer et al., 2018). This is considered an adaptive strategy, also known as compensatory growth since the size when entering the ocean significantly affects survival (Saloniemi et al., 2004; Jensen et al., 2022). Interestingly, several animals prioritize particular behaviours over



sleep for increasing fitness (e.g., Lesk et al., 2012; Rattenborg et al., 2016; Loftus et al., 2022), even though harmful consequences of sleep loss have been generally documented (Cirelli & Tononi, 2008). For instance, baboons did not appear to compensate for sleeping time sacrificed by socio-ecological conditions of sleeping sites, probably due to predation risk in less familiar locations and maintenance of social relationships (Loftus et al., 2022). Pectoral sandpipers males that sleep less time performed higher reproductive success in the breeding season, suggesting sleep loss could be an adaptive strategy under sexual selection (Lesk et al., 2012). Thus, if the ability to sustain consciousness has fitness advantages, emergence of cathemeral individuals in brown trout might be attributed to the advantage of growth rather than the cost associated with sleep loss.

Despite the gaps in sleep research, fish biologists, divers and aquarists have noticed typical resting postures or putative sleep in many fishes (e.g., parrot fish with their ‘mucus cocoon’) (Grutter et al., 2011). The behavioural sleep approach is a valuable tool in the research of sleep patterns and functions in fish, the most diverse vertebrate groups. In particular, the typical resting posture of brown trout or other salmonids can be easily observed underwater (Appendix 1) (Bachman, 1984; Roussel et al., 1999). Thus, once individual sleep phenotypes are determined, the function and

adaptive significance of sleep can be clarified in wild populations.

## **Appendix 1.** Experimental animals.

I chose the brown trout *Salmo trutta* as a model species. In the preliminary phase, I snorkelled in many rivers on Hokkaido Island (Japan) and found that brown trout exhibited the most distinct resting or putative sleeping behaviours among other species (e.g., rainbow trout, masu salmon, white-spotted char and Dolly Varden). Other researchers have reported the resting posture of brown trout, i.e., the motionless contact with the riverbed or substrates (i.e., quiescence) (e.g. Heggenes et al., 1993; Roussel et al., 1999). I further noticed that some quiescent brown trout could be touched by hand (i.e., high arousal thresholds) and others escaped before touching, leading to the hypothesis that the former were sleeping and the latter were resting.

Many studies have demonstrated the shift of diel activity patterns within and among salmonids (Reebs, 2002). In brown trout, the shift of diel activity rhythms has been well documented (Heggenes et al., 1993; Larranaga et al., 2019). The general pattern is that salmonids become nocturnal in winter or when in cold water (Reebs, 2002). During other seasons or when in non-cold water, individuals can change their diel activity rhythm at a high fish density (Fingerle et al., 2016), with abundant shelters for fish (Larranaga et al., 2015) or when they are subordinate (Alanära et al., 2001). Thus, if the diel activity rhythm correlates with the sleep rhythm and intensity, we can

compare the fitness of different sleep phenotypes to reveal the adaptive significance of sleep.

Brown trout in the Horonai River of the Tomakomai Experimental Forest were introduced in 1989 and no further introduction has been recorded in the last 20 years.

The population must be self-sustained because spawning activity and various size or age classes are observed, including occasional large silvery individuals (> 50 cm), which should be an anadromous form (C. Furusawa and R. Futamura, unpublished data).

Anadromy in introduced brown trout is also reported in other areas of Hokkaido (Goto et al., 2020).

## **Appendix 2.** Confirmation of individuals' consistency of activity rhythms.

From February 18 to February 25, 2021, I captured 5 brown trout (mean  $\pm$  SD = 153  $\pm$  8.3 mmFL) in Horonai River and assessed their diel activity rhythms for 7 days using the methods described in Material and Methods without acclimation. I did not feed the fish and ceased the experiment after 7 days. To test the periodicity of the diel activity, I conducted the chi-square periodogram analysis (Sokolove & Bushell, 1978) from day 2 to day 7 due to missing data because of video complications, using ActogramJ (Schmid et al., 2011). As a result, a significant periodicity, similar to the

nocturnal rhythm in this study, was indicated in three of five individuals for 6 days (Figure A1). Individuals with no periodicity also maintained consistently high activity, which corresponds to the cathemeral rhythm in this study (Figure A1). These findings suggest that the activity rhythms of brown trout have a certain consistency.

### **Appendix 3.** Biological and environmental factors affecting activity rhythms.

To evaluate the effects of biological (i.e., size, sex, condition factor) and environmental (i.e., water temperature) factors on activity rhythms, I conducted a multinomial logistic regression using the “nnet” package in R (Venables & Ripley, 2002). As for the index of the condition factor, I calculated residuals from the regression line between body size and body weight. The response variable was activity rhythms (cathemeral, nocturnal and intermediate), and the explanatory variables were body size, sex, condition factor, mean water temperature, and year. Body size significantly affected the activity rhythms (nocturnal: estimate -0.15,  $Z = -3.64$ ,  $P < 0.01$ ; intermediate: estimate -0.21,  $Z = -5.57$ ,  $P < 0.01$ ; table A3 and Figure A2a), with cathemeral fish being larger than individuals with other activity rhythms. Moreover, the median of the condition factor in cathemeral fish was lower than in the rest of the activity rhythms, although this was not statistically significant (cathemeral median: -0.03; nocturnal median: 0.005, intermediate median:

0.01; Figure A2b).

1 **Table A1.** Biological information of brown trout and the environmental factors in the experiments.

2

Experimental period	ID	Folk length (mm)	Body Weight (g)	Sex	Aquarium	Water Temperature (°C)	Light Intensity (lx)		Water inflow (cm <sup>3</sup> /L)
							Day	Night	
16-19 March 2021	1	156	42	m	1	4.67 ± 0.29	212.45 ± 1.38	0.02 ± 0.06	136.80
	2	156	39	f	3	4.67 ± 0.29	263.32 ± 37.24	0.44 ± 1.30	133.12
	3	154	36	f	4	4.67 ± 0.29	579.96 ± 14.16	0.30 ± 1.05	132.21
	4	150	34	m	5	4.67 ± 0.29	253.62 ± 12.87	0.08 ± 0.26	131.16
22-25 March 2021	5	149	37	f	1	4.70 ± 0.25	212.45 ± 1.38	0.02 ± 0.06	136.80
	6	153	39	f	2	4.70 ± 0.25	237.29 ± 10.23	0.19 ± 0.66	129.37
	7	160	46	f	3	4.70 ± 0.25	263.32 ± 37.24	0.44 ± 1.30	133.12
	8	156	38	m	4	4.70 ± 0.25	579.96 ± 14.16	0.30 ± 1.05	132.21
	9	155	40	m	5	4.70 ± 0.25	253.62 ± 12.87	0.08 ± 0.26	131.16
29 March -2 April 2021	10	144	33	f	1	5.90 ± 0.11	212.45 ± 1.38	0.02 ± 0.06	136.80
	11	146	33	f	2	5.90 ± 0.11	237.29 ± 10.23	0.19 ± 0.66	129.37
	12	149	34	f	3	5.90 ± 0.11	263.32 ± 37.24	0.44 ± 1.30	133.12
	13	143	31	f	4	5.90 ± 0.11	579.96 ± 14.16	0.30 ± 1.05	132.21
	14	145	34	f	5	5.90 ± 0.11	253.62 ± 12.87	0.08 ± 0.26	131.16
5-8 April 2021	15	158	42	f	1	6.39 ± 0.33	212.45 ± 1.38	0.02 ± 0.06	136.80
	16	147	33	f	2	6.39 ± 0.33	237.29 ± 10.23	0.19 ± 0.66	129.37

15-18 April 2021	17	141	33	f	3	$6.39 \pm 0.33$	$263.32 \pm 37.24$	$0.44 \pm 1.30$	133.12
	18	159	43	f	4	$6.39 \pm 0.33$	$579.96 \pm 14.16$	$0.30 \pm 1.05$	132.21
	19	144	30	f	5	$6.39 \pm 0.33$	$253.62 \pm 12.87$	$0.08 \pm 0.26$	131.16
	20	143	27	f	1	$6.70 \pm 0.13$	$212.45 \pm 1.38$	$0.02 \pm 0.06$	136.80
	21	143	33	f	2	$6.70 \pm 0.13$	$237.29 \pm 10.23$	$0.19 \pm 0.66$	129.37
	22	147	35	m	3	$6.70 \pm 0.13$	$263.32 \pm 37.24$	$0.44 \pm 1.30$	133.12
24-27 April 2021	23	144	33	m	5	$6.70 \pm 0.13$	$253.62 \pm 12.87$	$0.08 \pm 0.26$	131.16
	24	142	33	f	1	$7.41 \pm 0.12$	$212.45 \pm 1.38$	$0.02 \pm 0.06$	136.80
	25	150	39	m	2	$7.41 \pm 0.12$	$237.29 \pm 10.23$	$0.19 \pm 0.66$	129.37
	26	149	35	f	3	$7.41 \pm 0.12$	$263.32 \pm 37.24$	$0.44 \pm 1.30$	133.12
	27	141	32	m	4	$7.41 \pm 0.12$	$579.96 \pm 14.16$	$0.30 \pm 1.05$	132.21
	28	148	32	f	5	$7.41 \pm 0.12$	$253.62 \pm 12.87$	$0.08 \pm 0.26$	131.16
28-31 January 2022	29	155	41	f	1	$3.71 \pm 0.09$	$184.21 \pm 14.10$	$0.00 \pm 0.00$	133.80
	30	152	42	f	2	$3.71 \pm 0.09$	$327.73 \pm 9.25$	$0.00 \pm 0.00$	135.43
	31	157	48	f	3	$3.71 \pm 0.09$	$208.07 \pm 11.65$	$0.11 \pm 0.39$	128.93
	32	141	32	f	4	$3.71 \pm 0.09$	$225.23 \pm 15.48$	$0.13 \pm 0.46$	129.80
	33	146	34	f	5	$3.71 \pm 0.09$	$219.95 \pm 6.39$	$0.17 \pm 0.52$	133.51
	23-26 February 2022	34	147	38	f	1	$3.37 \pm 0.09$	$184.21 \pm 14.10$	$0.00 \pm 0.00$
35		142	29	m	2	$3.37 \pm 0.09$	$327.73 \pm 9.25$	$0.00 \pm 0.00$	135.43
36		138	33	f	3	$3.37 \pm 0.09$	$208.07 \pm 11.65$	$0.11 \pm 0.39$	128.93
37		151	37	f	4	$3.37 \pm 0.09$	$225.23 \pm 15.48$	$0.13 \pm 0.46$	129.80



30 March -2 April 2022	38	161	46	f	5	$3.37 \pm 0.09$	$219.95 \pm 6.39$	$0.17 \pm 0.52$	133.51
	39	150	42	f	1	$5.06 \pm 0.49$	$184.21 \pm 14.10$	$0.00 \pm 0.00$	133.80
	40	142	30	m	2	$5.06 \pm 0.49$	$327.73 \pm 9.25$	$0.00 \pm 0.00$	135.43
	41	141	33	f	3	$5.06 \pm 0.49$	$208.07 \pm 11.65$	$0.11 \pm 0.39$	128.93
	42	158	41	f	4	$5.06 \pm 0.49$	$225.23 \pm 15.48$	$0.13 \pm 0.46$	129.80
	43	146	37	m	5	$5.06 \pm 0.49$	$219.95 \pm 6.39$	$0.17 \pm 0.52$	133.51

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3

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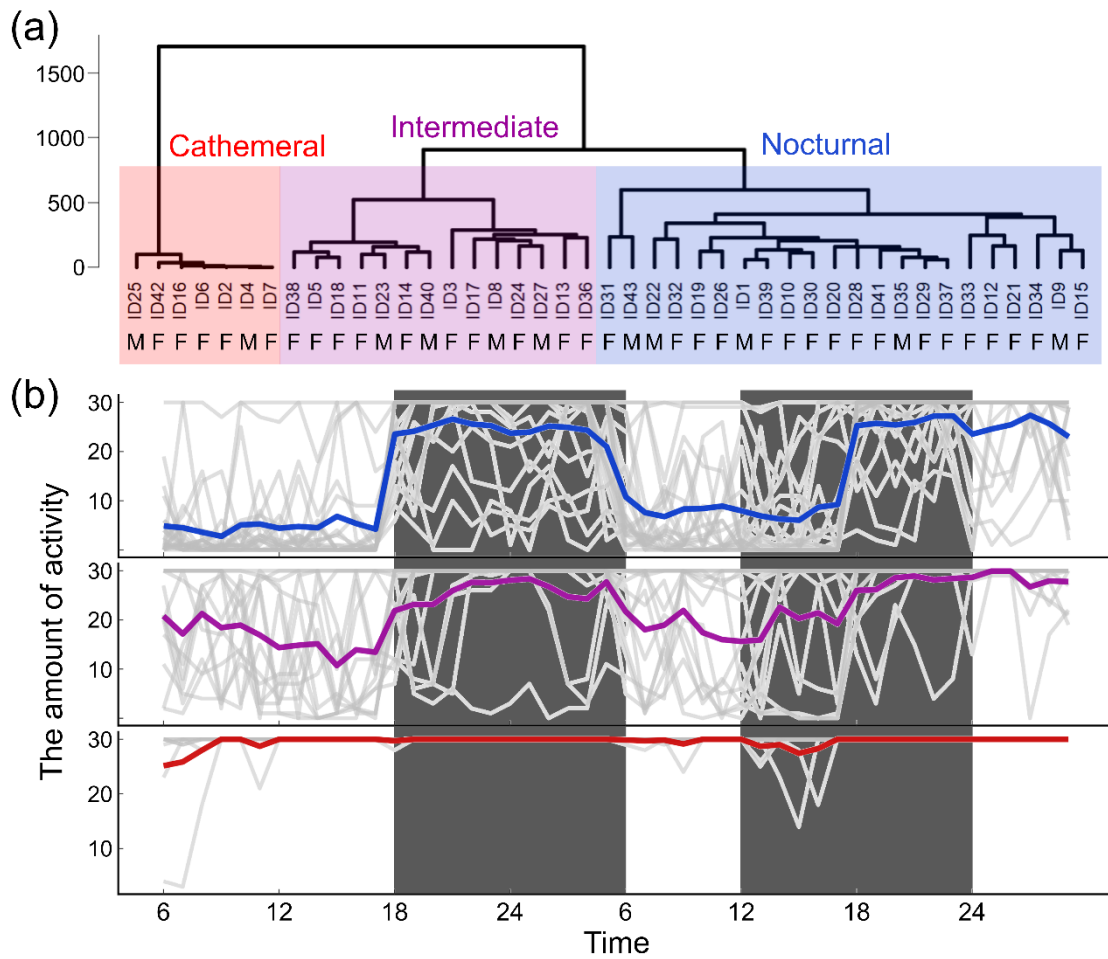
5

**Table A2.** Anova results of the LMM analysis for the reaction time.

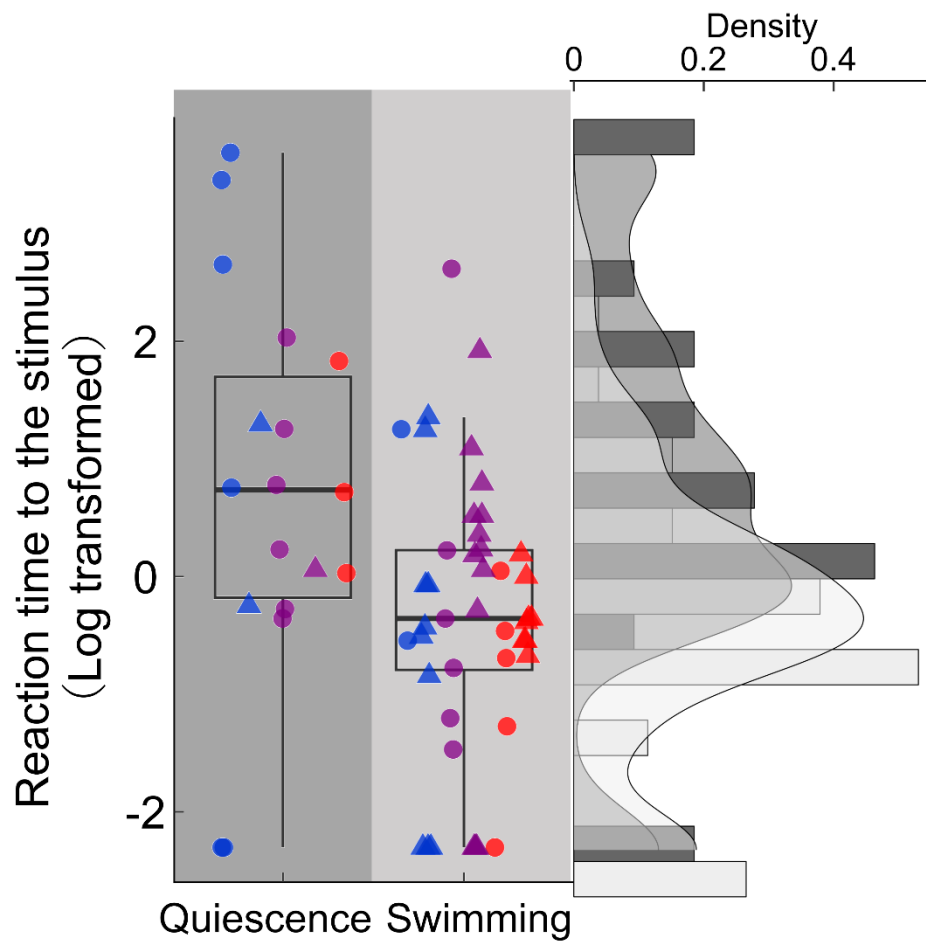
Fixed effects	$\chi^2$	<i>df</i>	<i>P</i> -value
Behaviour	11.026	1	<b>&lt; 0.01</b>
Time	0.209	1	0.65
Activity rhythms	0.335	2	0.85
The ratio of net-to-fish surface	0.037	1	0.85
Behaviour * Time	0.007	1	0.93
Behaviour * activity rhythms	3.256	2	0.20

**Table A3.** Summary of multinomial logistic regression analysis for biological and environmental factors affecting activity rhythms.

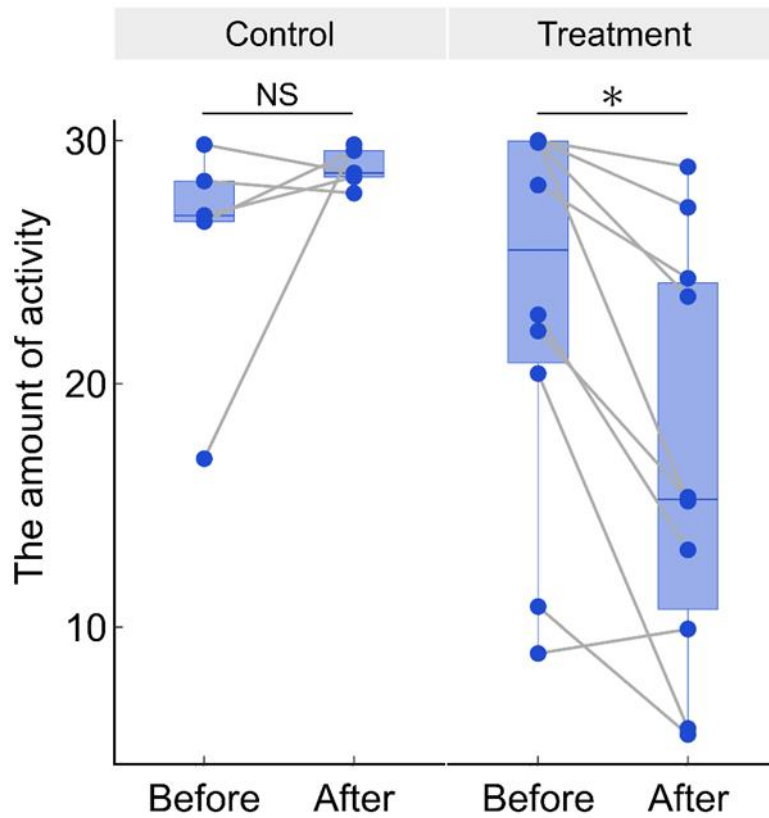
Fixed effects	Estimate	Z-value	P-value
<b>Nocturnal</b>			
Intercept	23.558	3.61	< <b>0.01</b>
Body size	-0.154	-3.66	< <b>0.01</b>
Sex: male	-0.293	-0.26	0.79
Condition factor	1.553	0.18	0.86
Water temperature	0.074	0.160	0.87
Year: 2022	1.973	1.24	0.21
<b>Intermediate</b>			
Intercept	35.802	6.17	< <b>0.01</b>
Body size	-0.220	-5.74	< <b>0.01</b>
Sex: male	-0.014	-0.01	0.99
Condition factor	6.251	0.69	0.49
Water temperature	-0.333	-0.69	0.49
Year: 2022	-0.494	-0.28	0.78



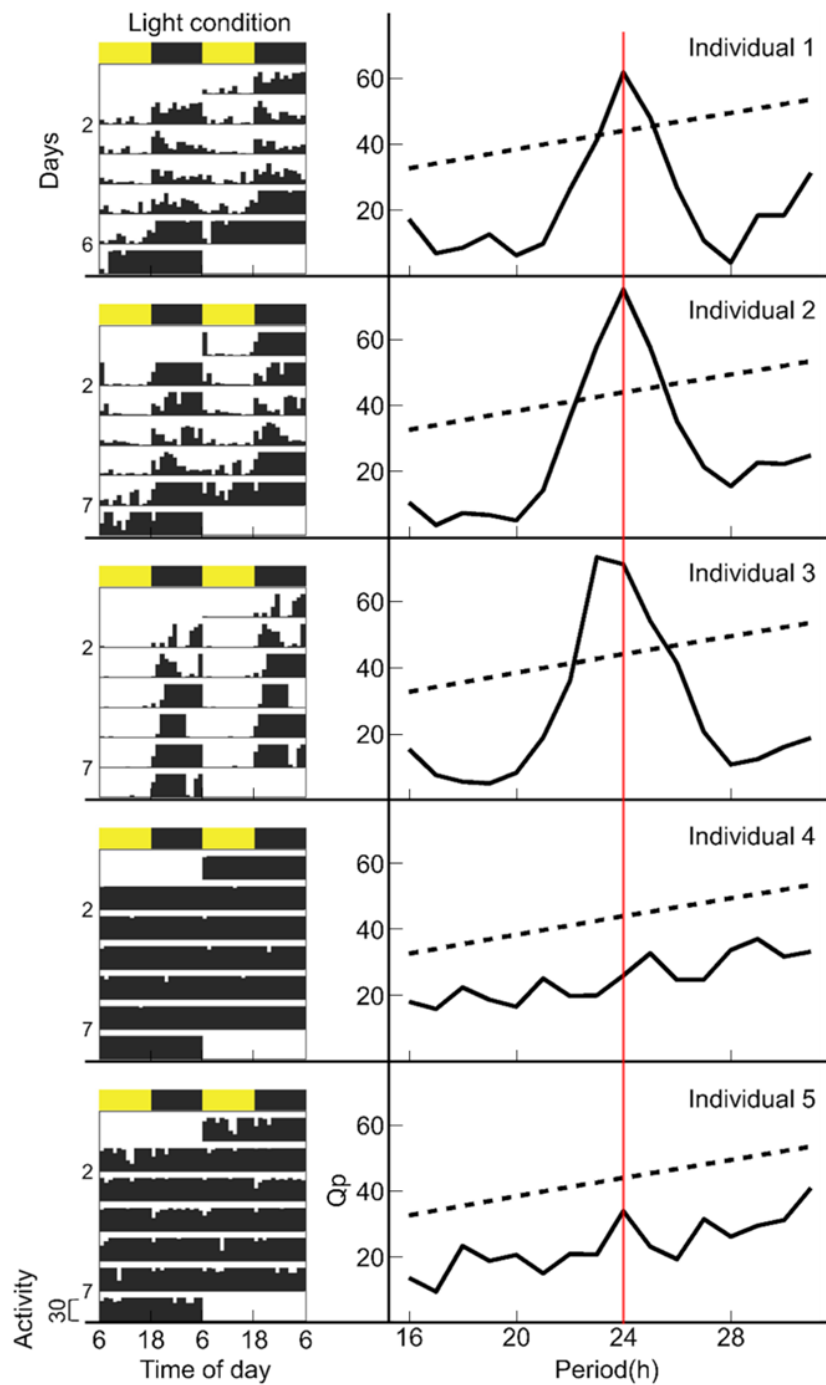
**Figure 1.** Intraspecific variations of activity rhythms. (a) Hierarchical time-series clustering of activity rhythms. Individual ID and sex were shown in each tip (F: female, M: male). Cathemeral, nocturnal, and the intermediate activity rhythms were statistically supported (details in the text). (b) Activity rhythms of each cluster (upper panel: nocturnal, middle panel: intermediate, lower panel: cathemeral). Light grey lines and coloured lines represent individual values and means, respectively. Coloured backgrounds indicate photoperiods (white: light phase; dark grey: dark phase).



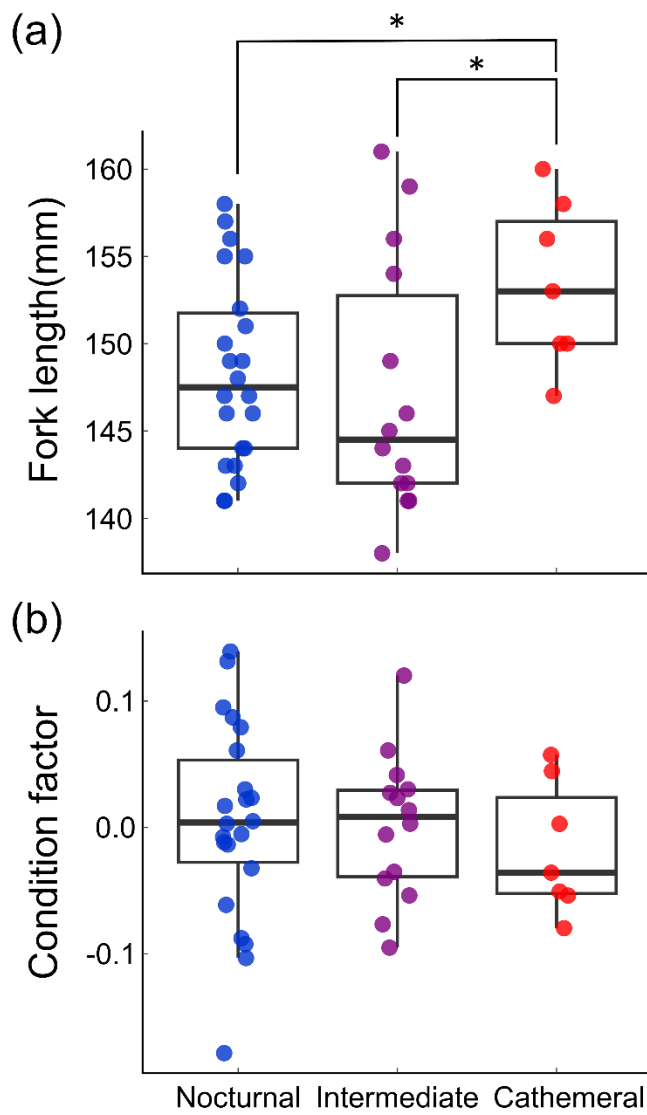
**Figure 2.** The arousal thresholds (i.e., reaction time in second) in swimming and quiescence states. Dark grey and light grey backgrounds and bars show quiescence and swimming, respectively. Coloured plots indicate activity rhythms (blue: nocturnal, purple: intermediate, red: cathemeral). Circle and triangle plots represent day and night, respectively. The boxplots show the median (the internal lines) and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.



**Figure 3.** The amount of activity in nocturnal trout before and after quiescence deprivation. An asterisk represents a significant difference (Wilcoxon signed-rank test: deprivation  $V = 1$ ,  $P = 0.004$ ) between before and after quiescence deprivation. The boxplots show the median (the internal lines) and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range and the circles are the data points.



**Figure A1.** Double-plotted actograms (left) and periodograms (right) of brown trout's activity in captivity. The black bars in the actograms indicate the amount of activity. In the three top graphs of the periodograms, the significant peaks (at approximately 24 h) are shown by a red line. The dotted line represents a confidence level of 95%.



**Figure A2.** Fork length (a) and condition factor (b) against the different activity rhythms. Asterisks indicate statistically significant differences ( $P < 0.01$ ).



## CHAPTER 3

Application of behavioral approach to the  
wild

## **Abstract**

Sleep is a ubiquitous behavior across the animal kingdom and should have important adaptive functions. However, sleep ecology in wild animals remains poorly understood due to the challenge of identifying sleeping individuals. They are usually cryptic and difficult to distinguish from other behaviors, such as resting. Despite their similar appearance, sleep state is more vulnerable to predation than rest due to reduced consciousness. Thus, location and time would vary between these behavioral states. To test this hypothesis, I applied behavioral approach to a wild population of brown trout. I previously confirmed in a lab experiment that sleeping trout significantly reduced the responsiveness to the physical stimulus (increased arousal threshold). Thus, sleep and rest should be distinguished by if and how many times I can touch the individuals. I also examined the diel and seasonal patterns of sleeping in brown trout. Previous studies have shown that many salmonids become nocturnal in winter, but these have not distinguished if observed individuals were active, resting, or sleeping. Underwater surveys in summer and winter separated sleep from rest in the wild brown trout population. Importantly, sleeping individuals used microhabitats with slower water currents, and more cover, compared to resting fish. This suggests that sleep site is less vulnerable from predators and more efficient for saving energy. Moreover, sleeping

individuals were significantly larger than sleeping fish, suggesting that the costs of losing opportunity (e.g. gain foods) are smaller for larger individuals. In other words, smaller individuals probably need to grow at the expense of sleep. Only less than 10 % of observed trout were identified as sleep. This confirmed that the previous studies not distinguishing awake and sleep are valid to evaluate diel sleeping patterns. In addition, like previous studies, I also found that brown trout tended to sleep more in winter especially at daytime compared to summer. Because resource availability is low and predation risk is high at daytime in winter, they should sleep at the timing when the gains from activities are comparatively low, the pattern called as an adaptive inactive state. Overall, this study underscored the importance of distinguishing sleep from other quiescent behaviors to understand the adaptive functions of sleep in natural environments.

## **Introduction**

Sleep typically exhibits low responsiveness to ambient environments, making animals vulnerable to certain threats such as predation or abrupt environmental change. In addition, sleep generally constitutes a large proportion of time (20-40% of life), which reduces the time for other activities, such as foraging and mating (i.e. opportunity loss, Roth et al., 2010). Therefore, animals should adopt proper sleep strategies to minimize these ecological costs (Lima, 2005). Classical behavioral observations and recently developed technologies have thus far illuminated a variety of different sleep strategies (Amlaner & Ball., 1983; Rattenborg et al., 2016; Pozzi et al., 2022; Mohanty et al., 2022; Rattenborg, 2023), primary focusing on mammals and birds. For example, primates use top of trees, tree holes, and nests as sleep sites to avoid predators and attain stable environments (Pozzi et al., 2022). Some birds reduce sleep time during breeding season to compensate opportunity loss (Lesku et al., 2012). Giraffe adopt a guarding system that some group members are vigilant as sentinel even if other members are likely to sleep (Burger et al., 2020). However, our understanding of sleep ecology in wild animals remains limited, due to taxonomic bias in previous studies.

One of the biggest obstacles for sleep ecology is the difficulty in identifying sleeping individuals. Because wild animals sleep in cryptic sites such as tree holes and

tunnels (e.g., Pozzi et al., 2022), it is difficult to find sleeping individuals in natural environments. In addition, many animals show immobile behaviors, such as resting and freezing, which are often difficult to separate from sleep. In fact, many studies have not distinguished sleeping from other similar behaviors and treated them as a resting behavior (e.g., Mohanty et al., 2022). However, these states are apparently different ecologically and physiologically, for example, sleeping individuals are much more vulnerable to approaching predators compared to resting ones. Surprisingly few studies have addressed the differences of resting and sleeping in natural populations, which sheds new lights on the ecological functions and roles of sleep.

Behavioral sleep is a useful tool for detecting the presence of sleep in wild animals (e.g., Singhal et al., 2007; Ferretti et al., 2019). Sleep is defined by behavioral criteria, such as stereotypic resting posture and an elevated arousal threshold (called as “behavioral sleep”) (Piéron, 1913; Campbell & Tobler, 1984; Allada & Siegel, 2008). This is easy to use compared with electrophysiological approach that measures brain activity. In particular, arousal thresholds serve as an index to distinguish between sleep and rest (Piéron, 1913; Cirelli & Tononi, 2008). Even with similar immobile states, resting individuals respond to approaching potential threats immediately, whereas sleeping individuals cannot notice because of high arousal thresholds. A field method

using arousal threshold should be useful to distinguish between these similar behaviors in wild animals.

Salmonid fishes are a good model to examine sleep ecology because they show considerable variations in diel activity rhythms at species, population, and individual levels (Reebs, 2002). For example, salmonids become nocturnal when and where ambient temperature is low, like in winter or in spring-fed streams (Heggenes et al., 1993; Fraser et al., 1993). Social interaction also affects diel activity patterns, such that subordinates become more active during daytime when predation risk is higher (Alanära et al., 2001). Individual variations and plasticity are particularly important to link the sleep strategies to fitness component (e.g., Lesku et al., 2012; Steinmeyer et al., 2013). Even though diel activity patterns should strongly tie to sleep, no studies have investigated the sleep behavior in salmonids. In a laboratory setting, I confirmed the sleeping behavior of brown trout, using behavioral approach (Chapter 2, Furusawa & Koizumi, 2024). Because sleeping trout significantly reduced the responsiveness to the physical stimulus (Chapter 2), sleep and rest should be distinguished by if and how many times I can touch the individuals during underwater observations.

The aims of this chapter are to apply behavioral approach to wild brown trout and evaluate sleep patterns and tactics. More specifically, considering the decreased

consciousness during sleep, which make it more vulnerable than rest, I examined whether trout select safer sites for sleeping compared to swimming and resting. In addition, I explored the diel and seasonal pattern of sleeping in wild brown trout. While previous studies have suggested that many salmonids become nocturnal in winter, the results are based only on observed individuals – they did not distinguish if the observed individuals were active, resting, or sleeping. Importantly, some authors reported that many individuals exhibit a quiescence state during the nighttime (e.g., Roussel et al., 1999; Jakober et al., 2000), a stereotypic resting or sleeping posture of salmonids (Chapter 2, Furusawa and Koizumi, 2024). This implies that some observable individuals are sleeping, potentially making the interpretation of previous results on diel activity pattern biased. As far as I know, this is the first study to assess the diel sleeping pattern in wild fish populations.

## **Materials and methods**

### *Underwater surveys*

During the winter (January to February) and summer (August) 2022, field surveys were conducted in a 150m section of the Horonai River, a small spring-fed stream at Tomakomai experimental forest (42°43'N, 141°36'E) in Hokkaido, Japan. Brown trout,

introduced to this river in 1989, are dominant species around the study section. They are probably self-sustained given the observed spawning activity and various size or age classes (C. Furusawa and R. Futamura personal observation). Underwater observation was carried out at six different times (0300, 0700, 1100, 1500, 1900, and 2300) of the day in counterbalanced sequences. The observer, wearing dry suits, entered at the lower end of the study section and crawled slowly upward with a zigzag fashion. In winter, the surveys were divided into three subsections (each 50 m), and the observations were conducted sequentially from the lower end. Upon encountering fish, the observer recorded total lengths, behavioral states, arousal thresholds, and holding positions and depths. Total length was estimated to the nearest 1 cm using the distance between landmarks (e.g. stones, woods) measured with a ruler.

Behavioral state was categorized into swimming (off the bottom, with moving fins) as behavioral awakening and quiescence (continuously in contact with the bottom, with motionless fins), which is a typical resting posture in salmonid fish (e.g. Roussel et al., 1999). The quiescence state was further divided into resting and swimming using arousal thresholds (Chapter 2; Furusawa & Koizumi, 2024). In this field survey, arousal thresholds were evaluated by gently pressing 1 cm on the side of the body using a plastic stick. When fish showed a flight reaction (i.e., escaping before pressing,



maintaining slowly the distance, and the onset of sudden swimming), I categorized it as resting. When fish showed no such flight reaction, the state was categorized as sleeping. When the observer approached swimming trout as behaviorally awakening state, they always showed flight reaction before touching (C. Furusawa, personal observation). When fish were sleeping, pressing was repeated until they showed a flight reaction to evaluate the depth of sleep (see Chapter 2).

#### *Measuring environmental variables*

Physical environmental factors (depth, water velocity, and cover) at a holding point were measured after underwater observation. Depth and water velocity were measured using a ruler and an electromagnetic velocity meter (LP-30, KENEK Corporation, Japan), respectively. The presence of cover at a holding point was recorded, such as water plants and undercut banks. Water temperature was also measured every hour using a TidbiT v2 temperature logger (Onset Computer Corporation, Bourne, MA, USA) during the study periods.

#### *Statistical analysis*

To clarify the differences in the holding points among behavioral states (i.e. swimming,

resting and sleeping), I constructed linear mixed models (LMM) and general linear mixed models (GLMM) with binomial distribution using the “lme4” package (Bates et al., 2015). The response variable was each environmental factor (i.e. depth, velocity and cover) or fish total length. Velocity and total length were Box-cox transformed to normalize the distributions using “car” package (Fox & Weisberg, 2019). The normality of the distribution of residuals was assessed using a QQ plot. Cover was set as a binary response variable, representing absence (0) or presence (1), for the GLMM. Explanatory variables were the behavioral states (swimming, resting and sleeping). Random factor was the timing of survey. Furthermore, to investigate the diel and seasonal sleeping pattern of brown trout, I used Fisher's exact tests and Pearson's chi-square tests among different behavior categories (i.e. swim, rest, and sleep) and daytime/nighttime in summer and winter, respectively. The Fisher's and Pearson's tests were selected depending on sample size. All statistical analyses were performed using R version 4.3.1 (R Core Team, 2023), and the significance level was set to 0.05.

## **Results**

During the study periods, mean water temperature in winter and summer were 3.32°C (range, 1.26 to 4.89) and 12.8°C (range, 11.0 to 14.7), respectively (Figure 1).

Underwater observation using the arousal threshold categorized 514 fish behaviors into 187 swimming (36.3%), 289 resting (56.2%), and 38 sleeping (7.3%) states. Even within the sleep state, arousal thresholds greatly varied: many individuals did not wake up even though I pressed them more than 10 times (Figure 2).

The results of LMMs showed significant differences among behavioral states (Table 1; Figure 3). Sleeping individuals used the focal sites with more abundant cover compared to swimming and resting individuals (GLMM: summer, swimming vs sleeping estimate = -2.18,  $z = -2.86$ ,  $P = 0.0117$ , resting vs sleeping estimate = -2.13,  $z = -2.87$ ,  $P = 0.0116$ ; winter, swimming vs sleeping estimate = -2.28,  $z = -3.45$ ,  $P = 0.0116$ , resting vs sleeping estimate = -2.72,  $z = -4.24$ ,  $P = 0.0001$ ; Table 1; Figure 3). They also used the sites with lower water velocity than others (LMM: summer, swimming vs sleeping estimate = 1.08,  $t = 5.67$ ,  $P < 0.0001$ , resting vs sleeping estimate = 0.638,  $t = 3.42$ ,  $P = 0.0021$ ; winter, swimming vs sleeping estimate = 0.785,  $t = 6.56$ ,  $P < 0.0001$ , resting vs sleeping estimate = 0.185,  $t = 1.66$ ,  $P = 0.223$ , but median of sleeping was lower than resting; Table 1; Figure 3). On the other hand, site use for water depth changed between summer and winter. In winter, sleeping and resting individuals used shallower sites, whereas sleeping and swimming individuals used deeper sites in summer (LMM: depth, swimming vs resting estimate = 9.13,  $t = 4.9$ ,  $P < 0.0001$ ,

swimming vs sleeping estimate = 9.8,  $t = 3.27$ ,  $P = 0.0035$ , Table 1; Figure 3).

Additionally, body size of sleeping and swimming individuals was significantly larger than resting individuals (LMM: summer, swimming vs resting estimate = 0.0317,  $t = 2.69$ ,  $P = 0.0208$ , resting vs sleeping estimate = -0.0854,  $t = -3.38$ ,  $P = 0.0024$ ; winter, swimming vs resting estimate = 0.0413,  $t = 4.21$ ,  $P = 0.0001$ , resting vs sleeping estimate = -0.0585,  $t = -3.82$ ,  $P = 0.0005$ ; Table 1; Figure 4).

Behavioral states of brown trout were significantly different between daytime and nighttime in both season (summer, Fisher's exact test  $P < 0.01$ ; winter, Pearson's chi-square test,  $\chi^2 = 23.7$ ,  $P < 0.01$ ; Figure 5). For sleep state, more trout slept in winter (6.86-16.0%) compared to summer (3.23-5.88%), and trout slept more at daytime in winter (daytime: 16.0% vs. nighttime: 6.86%), but this trend was not observed in summer (5.88% vs. 3.23%). On the other hand, brown trout were more active (i.e. swimming) in summer particularly at daytime (69.9%) compared to winter (21.6-43.2%) (Figure 5).

## **Discussion**

Sleep behaviors in wild animals are often reported without measuring arousal thresholds (e.g., Mohanty et al., 2022; Pickholtz et al., 2023; Pozzi et al., 2022). However, mere

observation cannot distinguish whether animals are sleeping or resting in many cases, which may bias the interpretations of the results. This study applied behavioral approach to wild brown trout, as previously confirmed in a laboratory experiment (Chapter 2). Even though apparent postures were the same, habitat use was significantly different between sleep and rest (Figure 3). This suggests that decreased consciousness to ambient environment resulted in different site selection. Furthermore, I found that sleeping individuals were larger than resting one, suggesting a size-dependent sleep tactics. I also examined the diel and seasonal variation in sleep timing, particularly focusing on if observed individuals were sleeping. Indeed, some individuals were sleeping, but the proportion was less than 10%. The overall sleeping pattern was that brown trout sleep more in winter particularly at daytime compare to summer, which was consistent with previous studies on the diel activity (not sleeping) patterns of salmonids.

Based on the laboratory experiment for behavioral sleep in brown trout (Chapter 2), I assumed that sleeping state is distinguishable from resting state by whether the observer touches the individual repeatedly. This boundary also has an ecological implication. Animals in natural environments need to quickly react to approaching predators for survival. If animals are touched by predators, they are at a high risk of being killed. Swimming individuals always showed a flight reaction to the

observer's approach. Therefore, the absence of a flight reaction to the observer, a potential predator, indicates a state of decreased consciousness, a characteristic of typical sleep. However, it is still difficult to strictly separate the sleeping and resting states by the arousal thresholds used in this study. I assumed that the individuals that were touched twice or more were sleeping, but some individuals touched only once might have included sleep state because the response to the stimulus was somewhat lowered. In fact, different levels of sleeping have been reported in many animals (Tobler & Neuner-Jehle, 1992; Dewasmes & Loos, 2002; Eban-Rothschild & Bloch, 2008). Considering the potential sleeping individuals for the category where individuals were touched once, I reanalyzed the data treating this category as a sleep state, but the results were essentially the same (Appendix 1). Thus, the main conclusion was unchanged despite the ambiguity. Further studies are needed for the transitions from resting to sleeping, or vice versa.

Not only the transition from resting to sleeping, I observed a large variation in arousal thresholds even within the sleep state (Figure 2). Arousal thresholds measurements are associated with sleep depth (e.g., Neckelmann & Ursin, 1993; Dewasmes & Loos, 2002) and different stages of sleep have been well documented in mammals, birds, and invertebrates (e.g., Tobler & Neuner-Jehle, 1992; Dewasmes &

Loos, 2002; Eban-Rothschild & Bloch, 2008). A recent study identified that zebrafish exhibits two stages of sleep, possibly related to REM sleep and non-REM sleep (Leung et al., 2019). Interestingly, the variation of arousal thresholds can be seen as a bimodal pattern with the number of pressing only once and more than 10 times as modes (Figure 2). I also found a multi-modal arousal threshold in the laboratory experiment with some individuals showing remarkably high thresholds (Chapter 2). These results suggest that wild brown trout might also have shallow and deep sleep states like non-REM sleep. To understand sleep architecture in fish, future studies are needed to examine the meaning of the variation in arousal thresholds.

Because animals cannot escape during sleep, sleep site selection is important to cope with predation. This study showed that cover and water velocity significantly differed among behavioral states (Figure 3; Table 1), identifying more abundant cover and lower water current as important factors for sleep sites of brown trout (Figure 3; Table 1). Abundant cover is particularly important for stream-dwelling fish to avoid detection by avian predators (Penaluna et al., 2016). In addition, not only does low velocity make it easier to maintain a resting posture, but it could also function in the early detection of predators by lateral line systems. Fish can detect approaching predators using their lateral line systems (McHenry et al., 2009; Stewart et al. 2013;

2014), and this sensor is masked by flowing environments (Feitl et al., 2010; Diamond et al., 2016). For water depth, I did not find a clear pattern of sleep site selection – it changed between summer and winter. This indicates that cover and velocity are more important than water depth. On the other hand, swimming individuals generally used deeper sites. Visible predators, such as birds, often detect prey through movement, making swimming individuals more susceptible to detection (Misslin, 2003). Therefore, swimming individuals might have preferred deeper sites to reduce predation risk.

Previous studies in mammals and reptiles have also documented sleep sites selection for specialized predator avoidance (Mohanty et al., 2022; Pozzi et al., 2022). For instance, diurnal primates use cliffs and tall trees for sleep sites to spot terrestrial predators (Pozzi et al., 2022). Many arboreal lizards sleep on unstable perch to detect predators and be able to escape from them (Mohanty et al., 2022). Thus, the difference in habitat use between resting and sleeping in brown trout should result from different responsiveness to predators.

This study also revealed that sleeping individuals were larger than resting ones (Figure 4). Body size is crucial for salmonids in determining their competitive ability (Nakano & Furukawa-Tanaka, 1994). Dominant individuals, being larger and possessing higher competitive ability, monopolize the most valuable periods and places,



while subordinates expand their activity and/or shift activity timing to compensate for growth (Alanära et al., 2001; Fingerle et al., 2016). Consequently, competition for prey leads to intraspecific variations in activity rhythms (Alanära et al., 2001; Brännäs, 2008). Additionally, the relatively lower growth demands in larger individuals could contribute to the diverse activity rhythms. Several studies have reported that larger fish often show a preference for nocturnal activity (Imre & Boisclair, 2004; Breau et al., 2007). This could be explained by their adoption of a tactic to minimize risk with low foraging efficiency (Fraser & Metcalfe, 1997; Elliot, 2011; Watz et al., 2014), because their size has reached the critical size for survival and reproduction (Clark, 1974). These suggest that larger individuals are active only during the most valuable periods. Allocating sleep to less valuable periods might be an adaptive tactic for energy conservation.

For the diel sleeping pattern, brown trout slept more in winter particularly at daytime. On the other hand, brown trout were most active at daytime in summer (i.e. highest proportion of swimming state). This pattern is consistent with the previous studies that based on the observed individuals in natural streams (Heggenes et al., 1993; Valdimarsson et al., 1997), as well as laboratory experiments (Fraser et al., 1993). This confirmed that the diel activity patterns inferred from observable individuals can be

translated to the diel sleeping patterns. Because detecting or confirming sleeping animals in natural conditions is generally difficult, it is useful to know their sleeping patterns can be inferred only from active or observable individuals.

Why do brown trout sleep at daytime in winter? Because winter is the season when foods are scarce and temperature is low, expected growth rate or benefit of foraging is low (Conover, 1992; Metcalfe et al., 1999; Huusko et al., 2007). In addition, swimming speeds of poikilothermic fishes decrease with decreasing ambient temperature and, therefore, the predation risk of visual homeothermic predators, such as mammals and birds, become higher in winter (Metcalf et al., 1999). In line with sleep ecology, this pattern can be explained by the adaptive inactive state, where animals should sleep at the timing when the gains from activities are comparatively low, thereby reducing energy expenditure (Siegel 2009). Furthermore, Siegel (2009) proposed that reduced activity during sleep decreases risk of injury and detection from predators (Meddis, 1975; Siegel, 2009). Another theoretical study has also suggested that sleeping when predators are active is an adaptive strategy for prey, if sleep site is safe (Acerbi & Nunn, 2011). Since sleep site was considered safer to predators in brown trout (Figure 3), decreased activity and crypsis by sleep would be beneficial for predator avoidance.

In this study, we revealed sleep ecology in wild brown trout. Previous studies

focused only on “active” individuals that the researchers could observe at certain periods of time (e.g., Fraser et al., 1993; Heggenes et al., 1993), but as shown in the present study, even some observable individuals are sleeping. Thus, it is important to distinguish different behavioral states to deepen our understanding of ecological importance of sleep. Furthermore, considering differences in ecological functions resulting from spatiotemporal patterns between resting and sleeping, future studies are necessary to understand how sleep may impact fitness.

**Appendix 1.** The analysis treating the flight reaction at the moment of touch (arousal threshold: 1) as a sleep state.

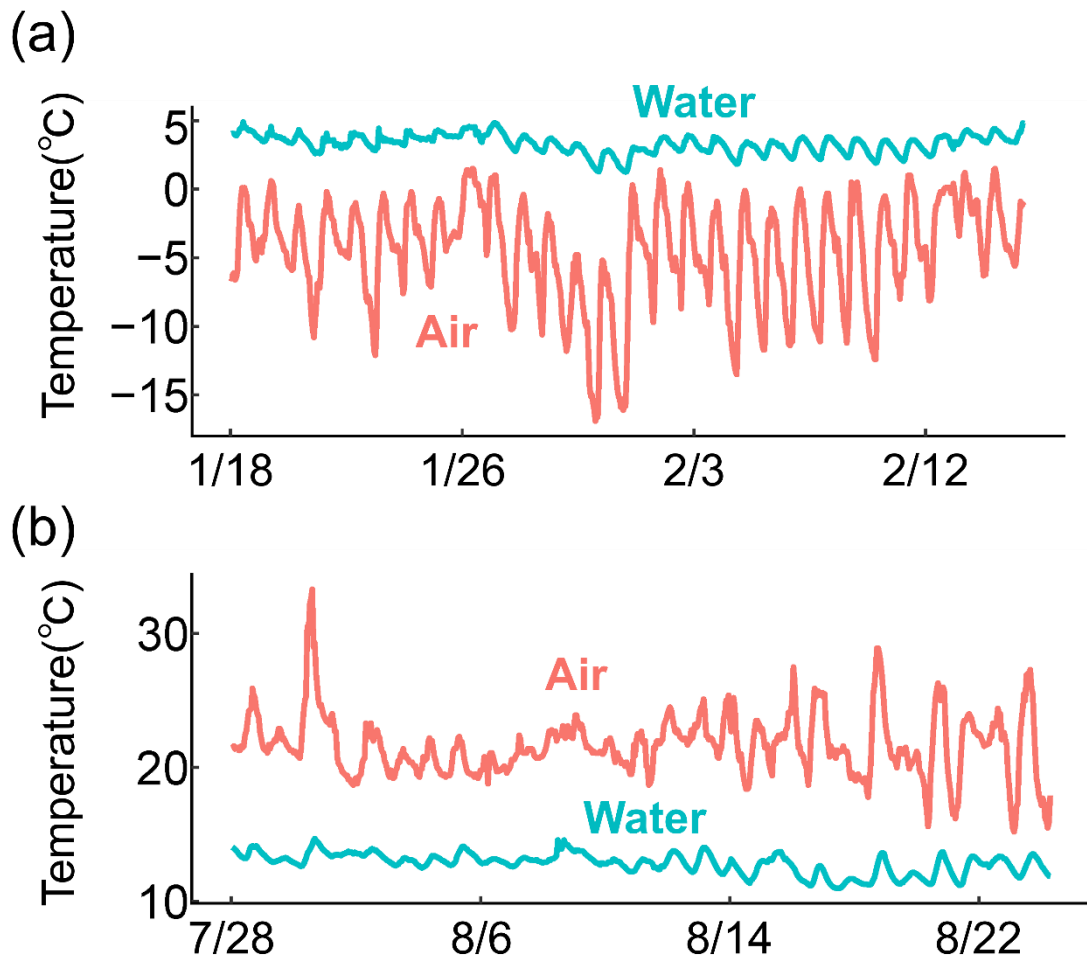
To investigate how the boundary to distinguish sleep from rest influences the results presented in Chapter 3, I reanalyzed the same LMMs treating the flight reaction at the moment of touch (arousal threshold: 1) as a sleep state (originally as a rest state). The results showed that all the significant variables were unchanged, except that sleeping individuals used habitats with lower water velocity than resting ones in winter (Table A1). This even enhanced the discussion that sleeping individuals used safer or more energetically efficient habitats compared to resting individuals.

**Table A1.** Re-analysis for comparison of site selection among behaviors using linear mixed models (LMM) and generalized linear mixed models (GLMM), treating the flight reaction at the moment of touch (arousal threshold: 1) as sleep.

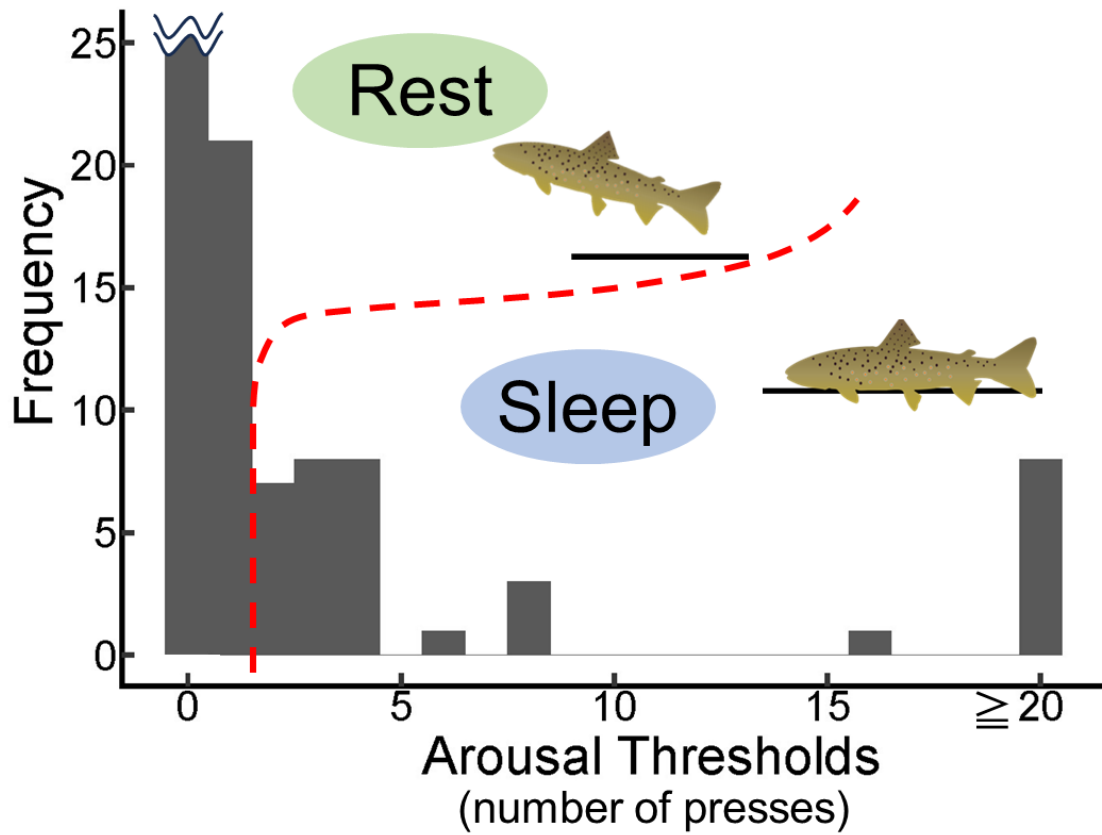
Season	Response variables	Explanatory variables	Estimate	Statistical value	P-value	
Summer	Depth	<i>Behavior</i>				
		Swim vs Rest	7.84	t = 3.04	0.0075	
		Swim vs Sleep	1.89	t = 0.389	0.92	
		Rest vs Sleep	-5.94	t = -1.27	0.416	
		Velocity	<i>Behavior</i>			
			Swim vs Rest	0.448	t = 5.12	<.0001
	Swim vs Sleep		0.855	t = 5.16	<.0001	
		Rest vs Sleep	0.407	t = 2.54	0.0316	
		Cover	<i>Behavior</i>			
			Swim vs Rest	-0.0693	z = -0.207	0.977
	Swim vs Sleep		-1.47	z = -2.43	0.04	
		Rest vs Sleep	-1.4	z = -2.41	0.0421	
		Total length	<i>Behavior</i>			
			Swim vs Rest	0.0312	t = 2.62	0.0256
	Swim vs Sleep		-0.0295	t = -1.31	0.389	
	Rest vs Sleep	-0.0607	t = -2.8	0.0153		
	Winter	Depth	<i>Behavior</i>			
			Swim vs Rest	8.97	t = 4.75	<.0001
Swim vs Sleep			10.1	t = 3.96	0.0003	
	Rest vs Sleep	1.14	t = 0.489	0.877		
	Velocity	<i>Behavior</i>				
		Swim vs Rest	0.574	t = 7.77	<.0001	
Swim vs Sleep		0.812	t = 8.01	<.0001		
	Rest vs Sleep	0.237	t = 2.58	0.0282		
	Cover	<i>Behavior</i>				
		Swim vs Rest	0.538	z = 1.8	0.168	
Swim vs Sleep		-1.45	z = -3.31	0.0027		
	Rest vs Sleep	-1.99	z = -4.87	<.0001		
	Total length	<i>Behavior</i>				
		Swim vs Rest	0.0435	t = 4.37	0.0001	
Swim vs Sleep		-0.0038	t = -0.275	0.959		
	Rest vs Sleep	-0.0473	t = -3.77	0.0006		

**Table 1.** Summary of the results of linear mixed models (LMM) and generalized linear mixed models (GLMM) for comparison of site selection among behaviors.

Season	Response variables	Explanatory variables	Estimate	Statistical value	P-value
Summer	Depth	Behavior			
		Swim vs Rest	8.2	t = 3.22	0.0042
		Swim vs Sleep	-4.05	t = -0.723	0.75
	Velocity	Rest vs Sleep	-12.25	t = -2.24	0.0669
		Behavior			
		Swim vs Rest	0.442	t = 5.14	<.0001
		Swim vs Sleep	1.079	t = 5.67	<.0001
		Rest vs Sleep	0.638	t = 3.42	0.0021
		Behavior			
	Cover	Swim vs Rest	-0.0514	z = -0.154	0.987
		Swim vs Sleep	-2.1785	z = -2.86	0.0117
		Rest vs Sleep	-2.1271	z = -2.87	0.0116
	Total length	Behavior			
		Swim vs Rest	0.0317	t = 2.69	0.0208
		Swim vs Sleep	-0.0537	t = -2.08	0.0963
Winter	Depth	Rest vs Sleep	-0.0854	t = -3.38	0.0024
		Behavior			
		Swim vs Rest	9.134	t = 4.9	<.0001
	Velocity	Swim vs Sleep	9.798	t = 3.27	0.0035
		Rest vs Sleep	0.664	t = 0.235	0.97
		Behavior			
		Swim vs Rest	0.6	t = 8.17	<.0001
		Swim vs Sleep	0.785	t = 6.56	<.0001
		Rest vs Sleep	0.185	t = 1.66	0.223
	Cover	Behavior			
		Swim vs Rest	0.442	z = 1.52	0.282
		Swim vs Sleep	-2.278	z = -3.45	0.0016
	Total length	Rest vs Sleep	-2.721	z = -4.24	0.0001
		Behavior			
		Swim vs Rest	0.0413	t = 4.21	0.0001
	Swim vs Sleep	-0.0171	t = -1.05	0.545	
	Rest vs Sleep	-0.0585	t = -3.82	0.0005	

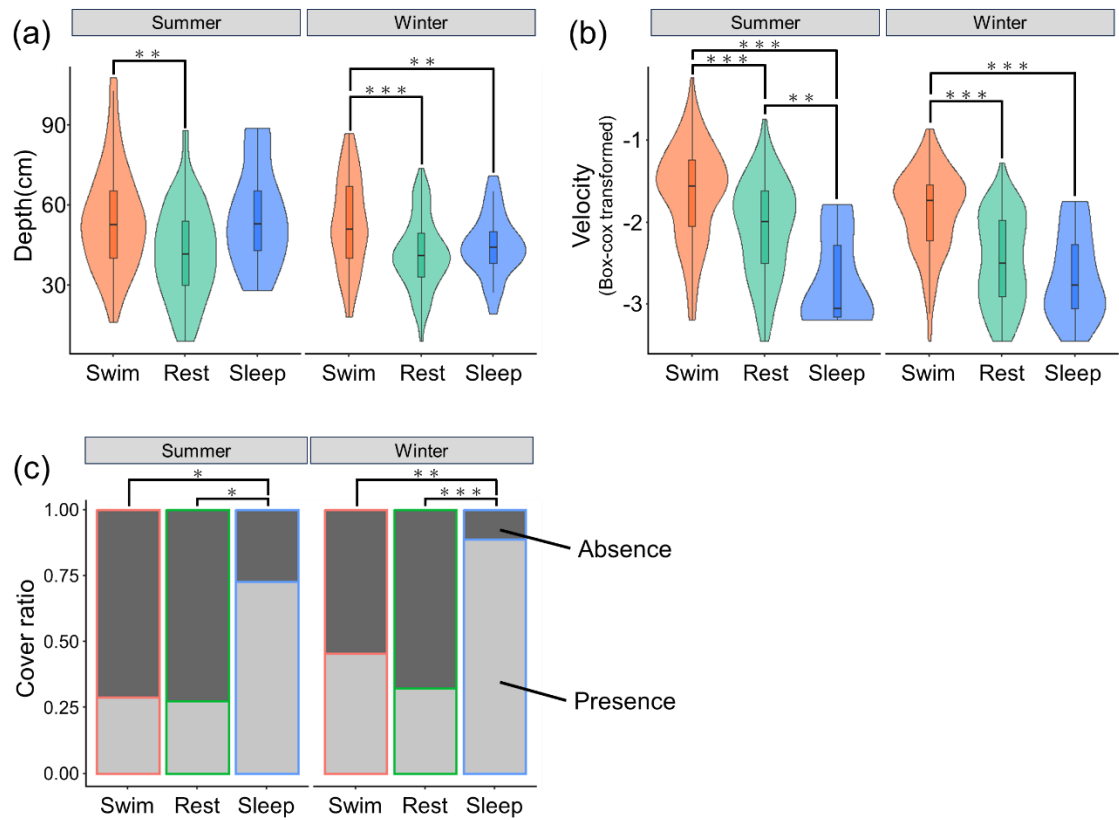


**Figure 1.** Water temperature and air temperature in winter (a) and summer (b) during the study period.

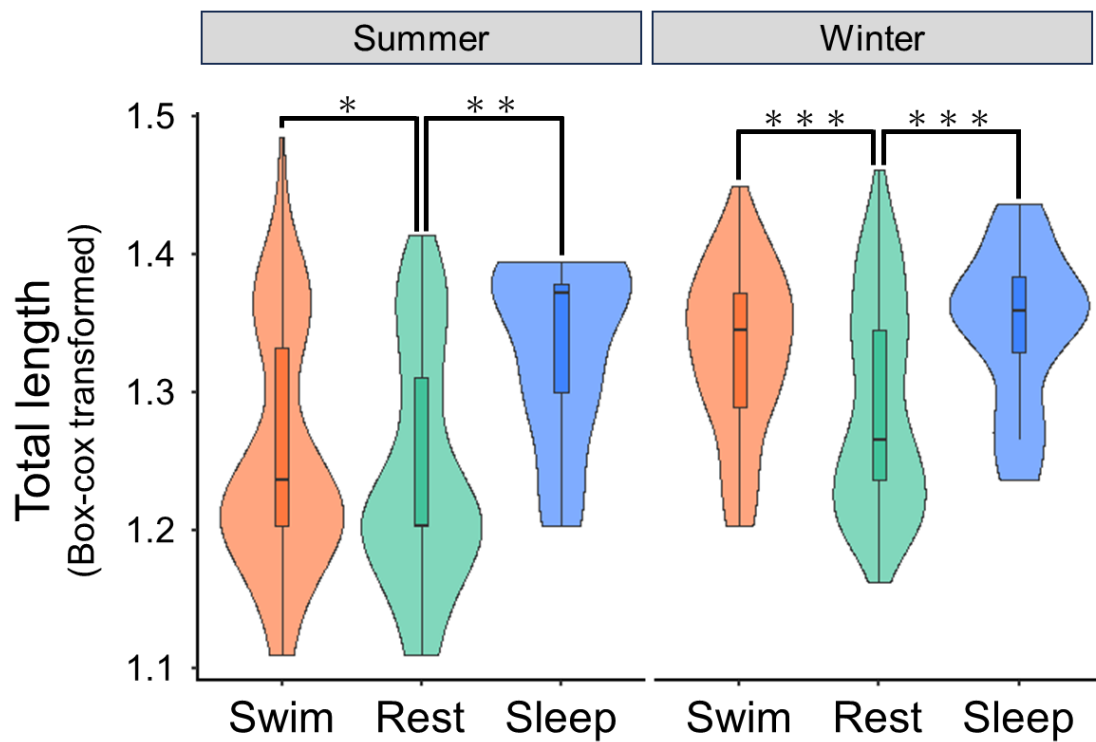


**Figure 2.** A variation in arousal thresholds. The red line indicates the boundary to distinguish sleep from rest.

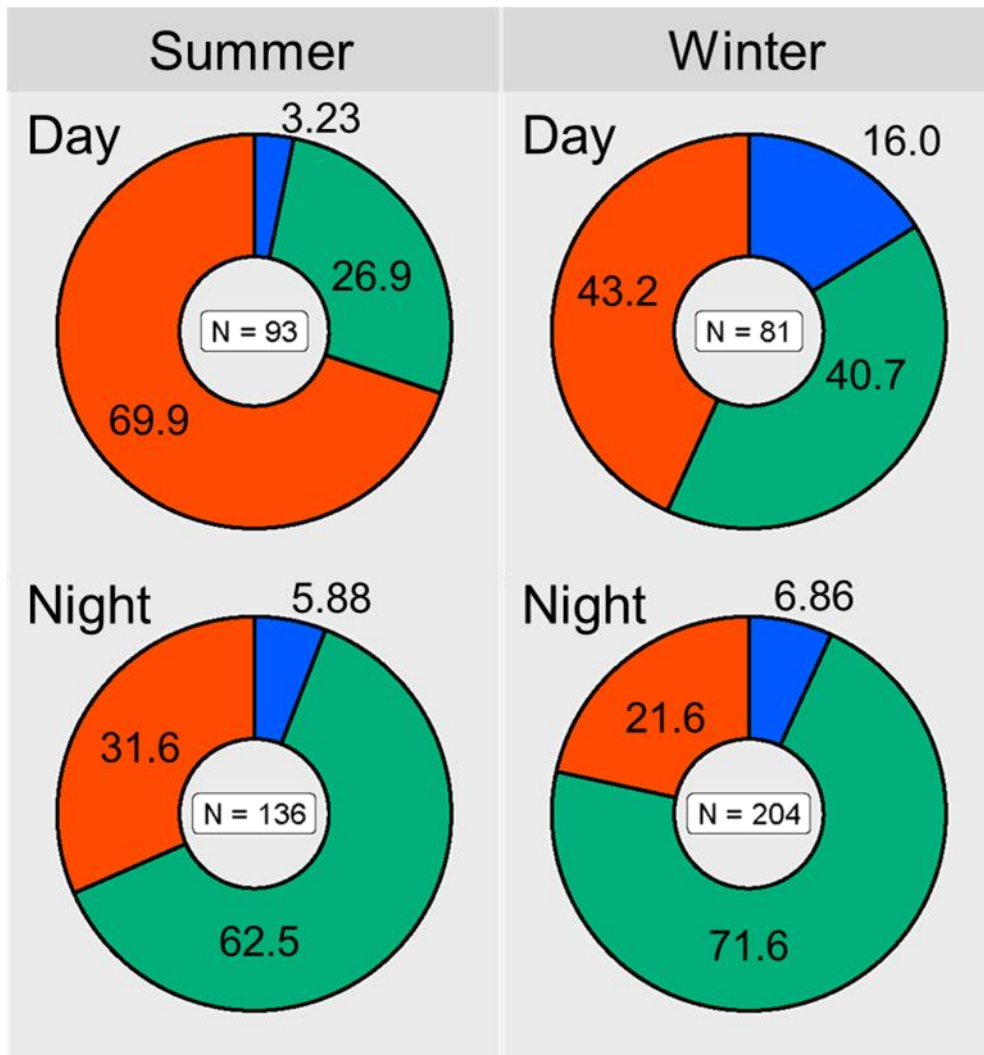




**Figure 3.** The differences in environmental factors (a, depth; b, water velocity; c, cover) among behavioral states (i.e. swim, rest, and sleep) in summer and winter. The asterisks show significant differences as follows: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. The boxplots show the median (the internal lines) and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.



**Figure 4.** The differences in body size among behavioral states (i.e. swim, rest, and sleep) in summer and winter. The asterisks show significant differences as follows: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The boxplots show the median (the internal lines) and 25th and 75th percentiles; the whiskers indicate the values within 1.5 times the interquartile range.



**Figure 5.** The ratio of behavioral states (i.e. swim, rest, and sleep) during the daytime and the nighttime, in summer and winter. The numbers and colors indicate the ratio (%) and the states of each behavior (red: swim; green: rest; blue: sleep).

# CHAPTER 4

## General discussion

Because of the essential roles of sleep in our life, numerous studies have investigated the sleep patterns, architectures, neurological and physiological functions, and genetic mechanisms of sleep, highlighting its importance and necessity for animals (Keene & Duboué et al., 2018; Lakhiani et al., 2023). However, these studies are mostly focused on laboratory animals, especially model species, such as mice, rats, or macaques. A vast variety of sleep pattern and architecture across the animal kingdom undoubtedly indicate strong natural selection acting on sleep behavior in wild populations (Lesku et al., 2019). Our understanding of costs and benefits of sleep in natural environments and how sleep patterns have been shaped by natural selection is far behind to the vast knowledges of physiological, neurological, and genetic aspects of sleep (Roth et al., 2010). This also means that ecological studies have considerable potential to advance sleep biology with the collaboration of previous proximate approaches (Roth et al., 2010; Aulsebrook et al., 2016; Rattenborg et al., 2017).

To fill this gap, there is a need to identify sleep behavior and examine sleep ecology in wild animals, which poses challenges due to cryptic of sleep. Moreover, past studies have often focused on specific taxa, especially mammals and birds (Lesku et al., 2009; Rattenborg et al., 2017; Lesku & Rattenborg, 2022), leading to a significant taxonomic bias. Therefore, clarifying sleep ecology in overlooked animals, such as fish

and insects, which constitute large proportions of diversity among vertebrates and invertebrates, is crucial. Salmonid fishes were considered as excellent candidates for examining sleep, because they exhibit large intraspecific variation in sleep (Chapter 2), possibly linked to flexible diel activity influenced by ecological contexts (Reebs, 2002). This flexibility allows researchers to investigate when, where and why animals sleep in wild populations, which ultimately leads to the deep understanding for the ecological and evolutionary functions and significance of sleep.

In Chapter 2 of my doctoral dissertation, I verified the presence of sleep in brown trout based on behavioral criteria (resting posture, an elevated arousal threshold, and homeostatic regulation) in the laboratory. In Chapter 3, I applied the behavioral approach to explore sleep ecology in the wild brown trout. My field method successfully distinguished sleep from rest and revealed new aspects of sleep ecology, including habitat use, size dependency, and diel and seasonal sleeping pattern. In this general discussion, I integrated the different chapters, critically evaluated the limitation of the study, and presented future perspectives.

### **Characteristics of sleep in brown trout**

This is the third case study that verified behavioral sleep among fishes using three

criteria (i.e. typical quiescence posture, elevated arousal threshold, and rebound after sleep deprivation) followed by zebrafish (*Danio rerio*) and Mexican tetra (*Astyanax mexicanus*). Also, this is the first study that assessed sleep pattern in a wild fish population. Thus, my doctoral dissertation filled an important taxonomic gap in sleep research.

Animals may adopt either monophasic sleep (i.e. the majority of sleep concentrate on a single episode of sleep per day) or polyphasic sleep (i.e. partitioning sleep time into multiple episodes of sleep per day) (Capellini et al., 2008). One episode of sleep is from the start to the end of sleep, which is also called as “bout”. Small animals tend to have polyphasic sleep, whereas large animals tend to have monophasic sleep (Capellini et al., 2008). Small animals cannot preserve much energy, so they cannot sleep with a long bout (Capellini et al., 2008). Moreover, if animals are prey with high predation risks, sleep partition or use of safe sleep habitat probably function as an anti-predator strategy (Lima, 2005; Lesku et al., 2009). Zebrafish mainly sleep at nighttime and their sleep is highly fragmented (Sorribes et al., 2013). A bout continues just 20 seconds, and they sleep again right after short awaking (Sorribes et al., 2013). This is probably an adaptation to notice predators as soon as possible. Mexican tetra has a longer sleep bout than zebrafish (day:  $5.49 \pm 1.2$  min; night:  $42.41 \pm 13.1$  min)

(Duboué et al., 2011). Interestingly, a cave, blind population of Mexican tetra have shorter bout length than normal populations (Duboué et al., 2011), which is considered to be an adaptation to seek scarce food resources in cave environments, at the expense of sleep (Table 1; Jaggard et al., 2017). I observed that nocturnal brown trout usually showed no activity during the 30 min observations with the quiescence on the bottom (Chapter 2). Thus, it is likely that the sleep bout continues more than 30 min. Moreover, nocturnal trout seem to sleep throughout the daytime and rarely sleep during the nighttime (Chapter 2). Therefore, brown trout, especially nocturnal individuals, should adopt monophasic sleep. This should be reasonable because salmonids are often top predators in river communities and sleeping sites are safe (Chapter 3). Comparing different species of fishes will confirm if the monophasic/polyphasic tactics follows the patterns reported in some mammals (Capellini et al., 2008).

I found a remarkable variation of sleep pattern for the brown trout even under the same experimental setting and similar body size (Chapter 2). Most notably, I found the individuals that did not sleep (i.e., cathemeral), at least one week (Appendix 2 in Chapter 2). In a field survey, I also observed active individuals in the daytime of winter when the activity is least beneficial (Chapter 3). Although I did not monitor individual sleep pattern, this indicates the presence of cathemeral individuals in natural



environments. Since homeostatic rebound was observed (Chapter 2), sleepless should incur some physiological costs. Thus, the potential existence of cathemeral individuals suggests a large benefit of not being sleep. Smolt or pre-smolt juveniles of salmonids become active during food-limited winter to grow bigger before migrating to ocean, where the survival is size-dependent (Saloniemi et al., 2004; Jensen et al., 2022). The cathemeral brown trout I observed may be such pre-smolt individuals. Lesku et al. (2012) reported an adaptive sleep loss of male pectoral sandpiper over the 3-weeks breeding period without apparent reduction of survival. The sleepless duration, growth, and survival of cathemeral brown trout will clarify the reason why some individuals do not sleep.

In the laboratory experiment conducted during winter, I detected nocturnal, cathemeral, and its intermediate sleep patterns. In the field observation, brown trout became more diurnal in summer, which is also consistent to the previous studies that based on diel activity rhythms (i.e. not based on sleep pattern). Thus, brown trout showed highly variable sleep patterns even in the same population. This is particularly useful to investigate the costs and benefits of sleep in terms of fitness components, such as survival or growth. For example, we can test if individuals with less sleep have higher growth rates but lower survival. Although monitoring of sleep patterns in each

individual is still challenging but not impossible by using the behavioral approach that I developed in Chapter 3.

I also showed a potential use of behavioral approach for assessing the depth of sleep. For mammals and birds, which can be equipped with EEG devices, different levels of sleep and its respective roles are widely recognized, especially for REM and non-REM states (e.g., Rattenborg et al., 2008). It has been challenging for small animals but a recent study succeeded in measuring sleep signatures of zebrafish by coupling with brain activity recording and behavioral assessment (Leung et al., 2019).

Importantly, the researchers found at least two different sleep states, possibly equivalent to REM and non-REM sleep (Leung et al., 2019). For the behavioral approach, I found that some brown trout exhibited remarkably high arousal thresholds, in addition to moderate levels of thresholds, both in the laboratory and field (Chapter 2 & 3).

Although I did not find the difference in habitat use between the high vs. moderate threshold individuals (results not shown), probably because of limited sample size, the behavioral approach may greatly advance our knowledges for the roles of sleep depth.

### **Sleep from ecological perspectives**

Past studies have proposed many benefits and functions of sleep, such as removal of

harmful wastes products, learning and memory consolidation, and energy conservation by decreasing metabolism (Franken et al., 1992; Stahl et al., 2017; Xie et al., 2013; Vorster & Born, 2015; Pinheiro-da-Silva et al., 2017, 2018). The biggest cost of sleep is considered as an opportunity loss (Roth et al., 2010) – indeed, humans spend about one-third of our life for sleeping, significantly reducing active times. However, these are mostly based on laboratory studies using model animals to understand humans' sleep, partly because of applied aspects, such as sleeping disorder (e. g., Akintomide et al., 2011). Many sleep researchers consider that brain-related functions are highly important (Roth et al., 2010; Krueger et al., 2016), and this is probably true for humans and other mammals and birds that have high cognitions. However, the fact that every animal, even those without brain, sleeps poses a big question why animals sleep: for body recovery and energy conservation, resting may be enough. As I have repeated, there is a large gap between laboratory researchers who study model organisms to examine proximate mechanisms and field researchers who study wild animals to explore ultimate factors.

In wild animals, predation is another large cost because the sleep is an unconsciousness state. Thus, many animals adopt counterbracing strategy or tactics, especially for sleep site selection (e.g., Mohanty et al., 2022; Pozzi et al., 2022; Rattenborg, 2023). I also showed brown trout use safer habitat when they sleep

compared to when they rest. Even though sleep and rest look very similar, these are indeed different. The temporal sleep pattern was also consistent with the adaptive inactive state, in which animals should sleep when the benefits of being active are comparatively small (Siegel, 2009). This is probably one of few adaptive significance of sleep in ecological contexts.

The new finding of my dissertation is size-dependent sleep behavior. Growth is crucial, especially for smaller individuals, to buffer predation risk and overwinter safely (e.g., Breau et al., 2007, Huusko et al., 2007). Therefore, sleep, despite its costs such as vulnerability and foraging opportunity loss, might impose more disadvantages on smaller individuals than the loss of several physiological benefits. Some studies have suggested that higher benefits from awakening, more than the costs of sleep deprivation, drive the evolution of the ability to dispense with sleep (Lesku et al., 2012; Rattenborg et al., 2016). For instance, male pectoral sandpipers that hardly sleep obtained high reproductive success, suggesting that sleep loss can be an adaptive strategy.

Surprisingly, harmful effects of sleep loss were not likely in such animals (Lesku et al., 2012; Loftus et al., 2022). Larger individuals, on the other hand, may invest in sleep and attain physiological benefits, due to decreased predation risk and lower growth demands. As discussed in the previous section, field monitoring of individuals with

different sleep patterns, particularly focusing on body size, is promising to clarify the adaptive significance of sleep in natural environments.

### **Limitation of the study**

The biggest limitation of the present study is the undetected individuals. I initially thought, among other reasons, one of the advantages for salmonids in sleep ecology is the habitat being concentrated on very limited areas (i.e. within streams), which enables individual tracking possible. For mammals, birds, insects, or any other organisms, it is highly difficult to detect a large proportion of individuals in a population. For fishes, we can collect 60-90% of individuals relatively easily by using electric fisher (e.g., Wildman & Neumann, 2003). However, even though I observed approximately fifty behavioral states in each field survey (Chapter 3), there were many individuals that I could not observe. I estimated the undetected individuals using 2, 3-pass removal population estimates with an electric fisher (Appendix 1; Table A1). As a result, 60-70% of individuals were not observed by underwater surveys (Appendix 1; Table A2). I further examined where were the undetected individuals using a PIT-tag system (Appendix 2; Table A3). I managed to detect 7 individuals and, importantly, all the individuals were sleeping, hiding under covers, such as undercut bank, woody debris, or

course rocks. Because of many structures in streams, I believe that most undetected individuals were sleeping in such cryptic manners. Therefore, although I showed the sleeping individuals used safer habitats (Chapter 3), the result seems rather conservative: individuals would use covers much more than expected. Then, I also recalculated the temporal habitat use of Chapter 3 (Figure 1). The tendency was unchanged (i.e. sleep more in winter particularly at daytime), but the proportion of sleep individuals increased considerably. More than 60% of individuals are considered to sleep at any given periods, indicating a large sleep time a day. Although challenging, future research should develop the method to detect hiding individuals within streams.

If such frequent, long sleep is a characteristic of salmonids, this has a great implication in sleep biology. It is noteworthy that most trout may not engage in rest but in sleep. While animals can recover from fatigue and conserve energy during rest, sleep plays important physiological functions that they cannot execute during the waking state (Roth et al, 2010). Especially, the deep sleep state relates to the consolidation of memory and learning (Roth et al., 2010), clearance of harmful metabolites in several animals (mammals: Xie et al., 2013; birds: Ungurean et al., 2023; fly: van Alphen et al., 2021). Salmonid fishes, including brown trout, are required to have good spatial memory to their habitat to feed and mate efficiently, as well as homing (Dittman &

Quinn, 1996; Rodríguez et al., 2021). They also form social structures or dominance hierarchies (Nakano & Furukawa-Tanaka, 1994), and some salmonids can use individual recognition and transitive inference as a complex cognitive process (White & Gowan, 2013). This ability is considered beneficial to increase fitness in such narrow community by memorizing/learning competitors and avoiding unnecessary competition. It has been well-documented that sleep deprivation negatively affects cognitive performance in many animals, including fishes (Vorster & Born, 2015; Pinheiro-da-Silva et al., 2017, 2018). Therefore, brown trout might sleep to maintain brain functions that play crucial roles in survival and reproduction.

### **Future prospects**

Across the world, there are many reports and naturalistic observations of sleep-like behaviors in animals (e.g., Kelly et al., 2020ab; Mohanty et al., 2022). These reports imply unique and diverse sleep strategies/tactics depending on taxa (e.g., Win, 1955; Mohanty et al., 2022; Pickholtz et al., 2023). Although the presence of sleep has been verified throughout the animal kingdom, studies examined sleep in natural environments are very limited. Therefore, it is noteworthy that behavioral sleep framework is a useful tool to clarify the diversity, natural history, and ecological

importance of sleep in often-overlooked animals.



**Appendix 1.** Estimation of population size in study section.

To estimate the population size in a study section, I conducted double- and triple-electrofishing in winter (February 18th, 2022) and summer (July 9th, 2022), respectively, with block nets set at the upper and lower ends of the capture area to prevent fish from escaping. Additionally, in winter, I captured fish every three divided study sections. Fish were captured three weeks prior to and three days after underwater observation in summer and winter, respectively. The population size was calculated using the removal method with the program CAPTURE (White et al., 1982; available at [www.mbr-pwrc.usgs.gov/software/capture.html](http://www.mbr-pwrc.usgs.gov/software/capture.html)). Using population size data, I calculated detection rates in a section, time, and season separately as follows:

Detection rate = the number of observations / estimated population size.

## **Appendix 2.** Exploring undetected individuals.

Field surveys were conducted in a 350 m area, including the study section. Fish capture using an electric shocker was carried out on May 2, 2023. Captured fish were anesthetized using a clove oil bath and surgically implanted with a PIT tag (12.0 mm × 2.12 mm, Oregon RFID, Inc) into the body cavity. On September 18, 2023, the observer initially crawled upstream and confirmed the absence of fish in the water column. Subsequently, the observer identified the locations of hidden fish by sweeping a portable PIT-antenna (Oregon RFID Portland). The shelters covering fish were then gently removed and observed. The arousal thresholds of the found fish were measured using the same method employed in this study. As a result, I managed to observe seven hidden fish, exhibiting a resting posture with elevated arousal thresholds (i.e., a typical sleep state) (Table A3).

**Table A1.** The population estimation in the study area.

Season	Range(m)	Pass	Captured individuals	Population size
<i>Winter</i>				
	0-50	1	33	
		2	9	51
	50-100	1	78	
		2	11	100
	100-150	1	28	
		2	4	36
				(sum, 186)
<i>Summer</i>				
	0-150	1	111	
	0-150	2	39	
	0-150	3	24	188

**Table A2.** Detection rate by underwater observation in this study.

Season	Section(m)	Time	Detected individuals	Estimated population size	Detection rate (%)
<i>Winter</i>					
	0-50	3:00 AM	18	51	35.3
		7:00 AM	3	51	5.88
		11:00 AM	6	51	11.8
		3:00 PM	6	51	11.8
		7:00 PM	24	51	47.1
		11:00 PM	16	51	31.4
	50-100	3:00 AM	26	100	26
		7:00 AM	15	100	15
		11:00 AM	17	100	17
		3:00 PM	15	100	15
		7:00 PM	22	100	22
		11:00 PM	30	100	30
	100-150	3:00 AM	20	36	55.6
		7:00 AM	7	36	19.4
		11:00 AM	7	36	19.4
		3:00 PM	5	36	13.9
		7:00 PM	20	36	55.6
		11:00 PM	28	36	77.8
<i>Summer</i>					
	0-150	3:00 AM	40	188	21.3
		7:00 AM	29	188	15.4
		11:00 AM	23	188	12.2
		3:00 PM	41	188	21.8
		7:00 PM	44	188	23.4
		11:00 PM	52	188	27.7

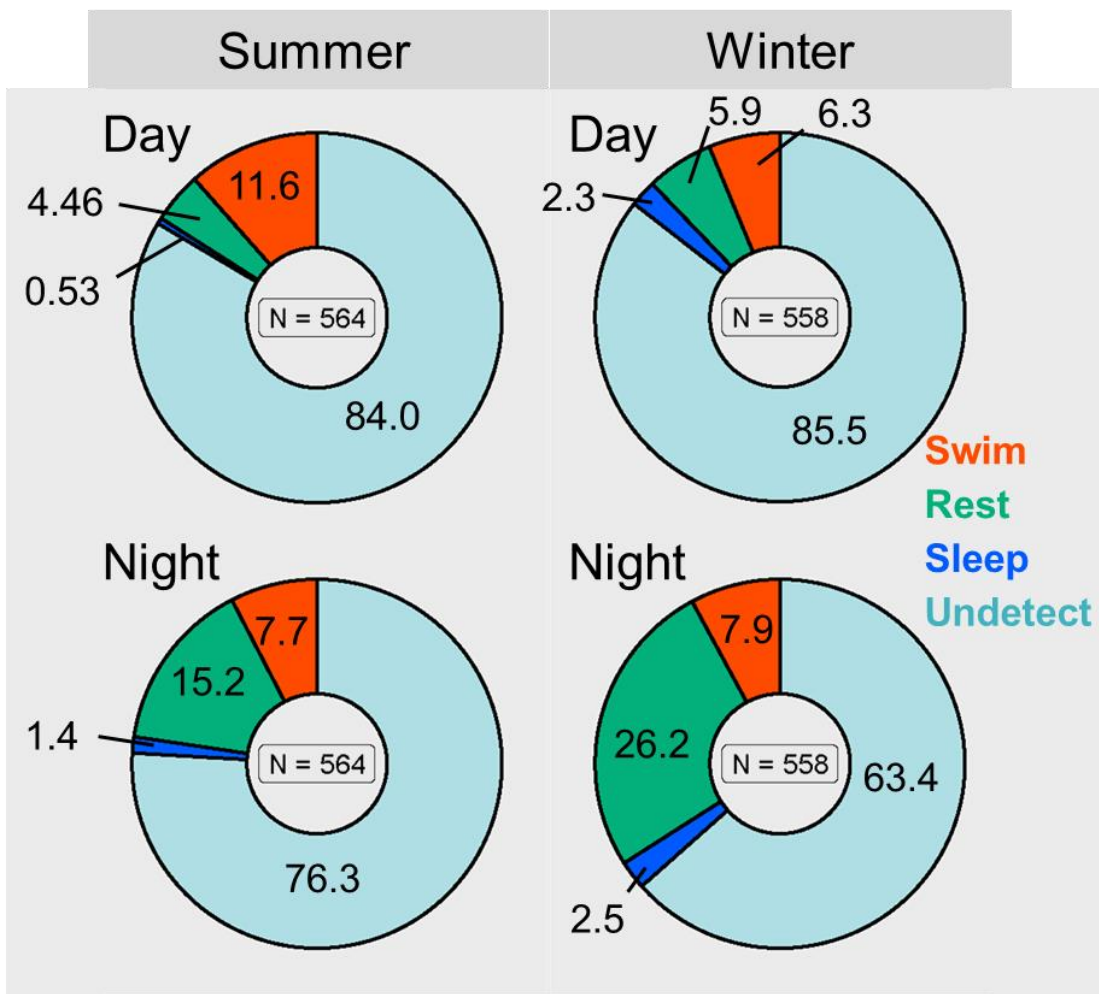
**Table A3.** Information about individuals undetected by underwater observation.

Date	Total length (cm)	Behavior	Arousal thresholds	Shelter
2023/9/18	13	quiescence	2 ≧	In underwater roots
2023/9/18	13	quiescence	2 ≧	In underwater roots
2023/9/18	16	quiescence	2 ≧	Under the rock
2023/9/18	12	quiescence	2 ≧	Undercut bank
2023/9/18	14	quiescence	2 ≧	Undercut bank
2023/9/18	16	quiescence	2 ≧	Dead branch
2023/9/18	13	quiescence	2 ≧	In underwater roots

**Table 1.** Sleep traits in fish.

Species	Total Sleep Time (min)	The number of bouts (mean±SD)	The bout length(min) (mean±SD)	
			Day	Night
Mexican tetra				
Surface	819.3 ± 68.6	47.71 ± 6.3	5.49 ± 1.2	42.41 ± 13.1
Pachón	242.7 ± 76.3	12.61 ± 5.5	2.07 ± 1.3	3.85 ± 1.6
Tinaja	107.4 ± 13.7	24.61 ± 2.7	1.52 ± 0.1	2.20 ± 0.2
Molino	136.88 ± 38.5	30.94 ± 7.3	1.2 ± 0.3	1.2 ± 0.3

Modified the figures in Duboué et al., (2011)



**Figure 1.** The ratio of behavior in the daytime and the nighttime, including undetected individuals estimated by population size.

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