Incorporating circadian and circannual rhythms into welfare assessments of zoo-housed animals

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Declaration

I wish to submit the thesis detailed above in according with the University of Stirling research degree regulations. I declare that the thesis embodies the results of my own research and was composed by me. Where appropriate I have acknowledged the nature and extent of work carried out in collaboration with others included in the thesis.

Signature: Kristine Gandia **Date: 15 December 2023**

Abstract

Zoos must conduct thorough welfare assessments to determine how well animals in their care are faring, and also understand the many factors which affect particular welfare states, so they can make appropriate interventions if needed. However, comprehensive welfare assessments require a lot of time and resources and there is no standard welfare assessment across zoos. In addition, an overarching factor that is not thoroughly understood is the effect that zoo environments have on welfare. This is particularly important when considering circadian rhythms. Circadian rhythms are adaptive physiological and behavioural changes that follow a 24-hr cycle. They are regulated by predictable external cues like light and temperature, which may be different in a captive environment. In this thesis I aim to address these issues by first collaborating with the Royal Zoological Society of Scotland Edinburgh Zoo to make their welfare assessment tool more efficient and reliable. I addressed efficiency by analysing concordance between assessors and determining whether the number of assessors could be reduced without losing reliability. I addressed reliability further by developing an evidence-based protocol for responding to behavioural and environmental questions that incorporate the assessment of daily rhythms and enclosure use. To demonstrate the importance of incorporating full circadian and circannual rhythms into the assessment of welfare and how circadian rhythms can illuminate the effects of the zoo environment, I conducted a study on giant pandas housed in zoos around the world, observing behavioural cycles across one year. Results indicate that giant pandas have clear circadian and circannual behavioural rhythms that are associated with life stage and sex, regulated by light, temperature and zoo specific external cues, and affected by latitudinal location. Overall, this thesis provides information and evidence on how zoos can take a holistic, evidenced-based approach to understanding the needs of their animals to consequently improve welfare and conservation efforts.

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“Until one has loved an animal, a part of one’s soul remains unawakened.”- Anatole France

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Chapter 1.

General Introduction: Current practices in animal welfare and the implications for circadian rhythms

Chapter 1. General Introduction: Current practices in animal welfare and the implications for circadian rhythms

In this thesis, my goal is to demonstrate a holistic approach to captive animal welfare assessments that incorporates circadian rhythms. The improvement of welfare for captive animals has been increasingly prioritised in recent decades. To promote and maintain a high standard of welfare for zoo-housed species, the welfare states of the animals must be assessed thoroughly. Welfare assessments then lead to action plans to either alleviate a negative welfare state or promote a positive one. The main difficulties with comprehensive welfare assessments are that they require a lot of time and resources and that a standardised assessment framework that captures all elements that affect welfare for captive species has not been established. In addition, an overarching factor that is not thoroughly understood is the effect that zoo environments have on welfare. Captive animals are often housed in areas of the world outside of their natural habitat ranges. Given that animals evolve to live in specific environments, it is important to understand how changes in the environment and zoo-specific external cues would affect welfare. This is particularly important when considering circadian rhythms. Circadian rhythms are physiological and behavioural changes that follow a 24-hr cycle that are regulated by predictable external cues like light. They are an adaptation to synchronise an animal to the environment in which they evolved and are present in most animals. These cycles have large implications for welfare as they regulate metabolic processes, behaviours, and even social structure. Given these properties of circadian rhythms, it is important to understand them, the factors which affect them, and how they are influenced by the zoo environment. In addition, accounting for them in welfare assessments can provide much needed context and evidence on the evolutionary needs of a species.

My thesis aims to present an evidence-based method for welfare assessment that accounts for circadian rhythms that can be applied across captive environments. It also takes an in-depth look at how investigating circadian rhythms can give details on what rhythms of behaviour are adaptive throughout life stages and between different sexes; as well as how the zoo environment and being housed outside of natural latitudes can affect animals. Overall, I present methods and information so that staff can better understand the needs of their species and appropriately provide for them, promoting positive welfare and increasing the likelihood of successful breeding and conservation. The remainder of this introduction will outline current welfare practices in zoos and the factors considered when assessing welfare, followed by information on circadian rhythms and how they can improve these efforts and make them more informative and effective.

1.1 Welfare assessment practices

1.1.1 Current views on welfare of captive animals

Over the last three decades, the importance of improving welfare standards for captive animals has been substantially increased (Tallo-Parra et al., 2023). This movement has been a result of the scientific community's developing understanding of sentience and pain perception in non-human animals. Sentience is the general capacity to feel; for instance, feeling hunger, satiety, pain, joy, boredom and excitement, though it does not require animals to reflect on feelings or understand what others may be feeling (Crump et al., 2022). Methodologies investigating whether animals are sentient generally look for two overarching criteria: (1) a central nervous system and other structures and psychoactive chemicals known to control pain responses, (2) physiological and/or behavioural responses to noxious stimuli, analgesics, and anaesthetics (Crump et al., 2022; Jones, 2013). By law, essentially all vertebrates are considered sentient in many countries, with cephalopods also considered sentient in the UK (Blattner, 2019)(Animal Welfare (Sentience) Act 2022). Much of the research relating welfare to sentience has been for vertebrates because they are the majority of animals involved in animal research (Browning & Veit, 2022) and used as livestock (Kumar et al., 2019), with the top five species researched on this topic being pigs, cows, sheep, chickens, and rats (Proctor et al., 2013). Despite this, research into sentience of other species and taxa has spiked in the last two decades -though still much more limited- that does suggest that for taxa such as fish, insects, spiders, crustaceans, annelids, nematodes, and molluscs there is evidence of nociceptors and nociceptive responses, suggesting the ability to experience pain (Jones, 2013). Many species which were once widely considered to not be sentient or experience pain like insects and fish, are now being found to have potential signs of sentience and pain. The likely reason much of this research is limited is because it is on taxa that do not benefit humans directly (Proctor et al., 2013). However, in zoos, these uncommon species are the majority of species housed and therefore it is important to make the assumption that all species are sentient and experience pain to elevate welfare practices.

The welfare movement progressed from wanting to stop animal cruelty, to stopping suffering, then to preventing suffering, and now in modern day to preventing negative welfare and promoting positive welfare (Harley & Clark, 2019). The Five Freedoms was the first highly influential welfare assessment framework that emerged to address increasing concerns of welfare for livestock focused on preventing suffering (Farm Animal Welfare Council Committee, 1979). The Five Freedoms were an internationally accepted standard for the welfare of animals, stating that animals must have freedom from (1) thirst and hunger, (2) discomfort, (3) pain, injury or disease, (4) fear and distress and (5) the freedom to express normal behaviour. The Five Freedoms model focuses on providing basic needs of survival to animals, emphasising the avoidance of negative welfare states. Though this model was highly influential for the field of animal welfare and formed the basis of legislation for the care of captive animals, it does not consider positive affective states and lacks detail on the variety of negative affective states, resulting in a model that does not provide a basis to determine the internal/external conditions and cognitive processes that would lead to those states (Mellor, 2016). With a better understanding of affective states and sentience in non-human animals, the field of animal welfare developed, and more emphasis was placed on providing animals opportunities for positive experiences rather than only trying to avoid negative states.

Positive affective states in animals can be the result of fulfilling a goal-directed behaviour, the anticipation of a reward, and the response to receiving a reward with either implicit or intrinsic value (Boissy et al., 2007; Wolfensohn et al., 2018). Including and identifying both positive and negative states in a welfare assessment results in a continuum of welfare outcomes from negative to positive since the two affective states are often described relative to each other on opposites ends of a spectrum, making them easily distinguishable (Spruijt et al., 2001). For instance, when assessing feeding, satiety would be described as a positive affective state and prolonged hunger as a negative affective state. The inclusion of positive states in welfare assessments provides a more in depth understanding of the animal's well-being, motivations, and personality; making the assessment more robust and reliable when used to determine management needs. The assessment of positive welfare states can be done through physiological indicators such as immunological assays or neuronal scanning techniques (Yeates & Main, 2008). However, more notably, certain positive affective states can also be assessed through behavioural markers such as play and social grooming (Yeates & Main, 2008). Since behavioural markers are often relatively easy to measure, this makes the inclusion of positive affective states in a welfare assessment practical and ultimately beneficial.

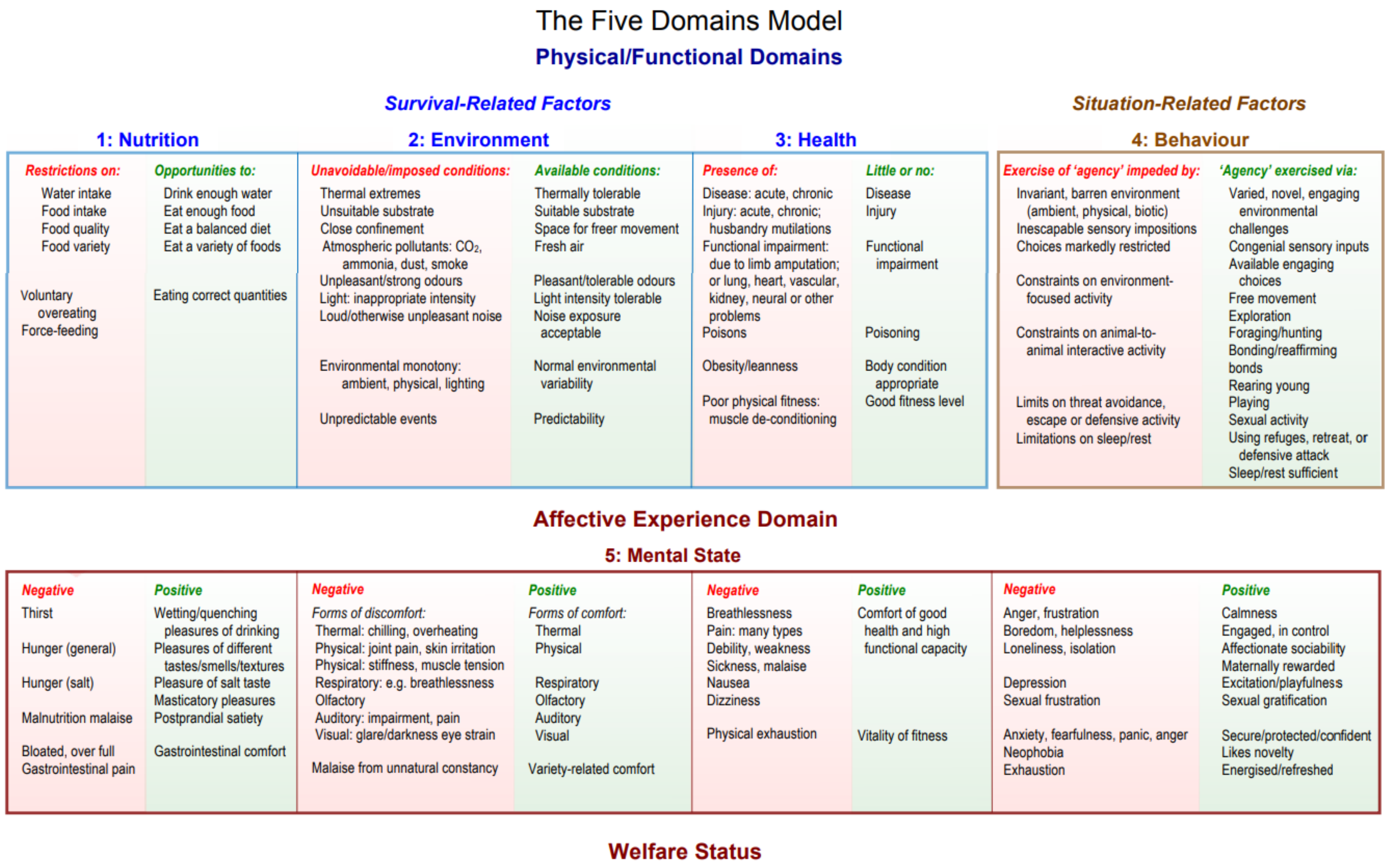
1.1.2 Five Domains model of welfare assessment

The Five Domains model for animal welfare assessment was developed as a response to the changing views on positive affective states within the field of animal welfare. The model is a framework for identifying positive and negative states in four physical/functional domains and one mental domain (Mellor & Beausoleil, 2015). The physical/functional domains include nutrition, environment, physical health, and behaviour. Importantly, the behaviour domain (now termed Behavioural Interaction) includes the influence on welfare of human interactions

(Mellor et al., 2020). This is essential to include for zoo animal welfare assessments since they regularly interact with zoo staff and visitors. Together, the state of these four domains influence the mental domain and indicate the overall welfare status of the individual animal. The negative and positive welfare states of the four physical/functional domains are reflected as positive and negative affective states in the final mental domain (Figure 1.1). In this way, the Five Domain model emphasises the importance of sentience (ability to feel) and accounts for all the factors that may result in changes to the affective state of an animal, putting the animal at the centre of a welfare assessment.

It is important to note that welfare goals cannot be to have animals in purely positive welfare states. Negative welfare states are necessary in certain instances as a motivation (motivated to avoid or end a negative affective state) (Spruijt et al., 2001; Yeates & Main, 2008). For instance, thirst and hunger are considered negative states, but experiencing short bursts of thirst and hunger will motivate an animal to eat and drink and lead to the positive affective states of satisfaction, satiety, and thirst quenching. Thirst and hunger are physiologically adaptive negative states that motivate an animal to seek sustenance. Positive affective states cannot exist without negative affective states because it is the continuum of affective states that allows for either end to feel positive or negative. If an animal is never hungry, it may not experience satisfaction from eating. Similar to experiencing cognitive challenges, overcoming the challenge of completing a cognitive task can be rewarding in itself, facilitate learning, and lead to positive welfare outcomes like exploring an environment more and displays of contentment (Clark, 2017). Therefore, the goal for captive animal welfare is to find a balance between necessary, but short lived, negative affective states that result in more, longer lasting, positive affective states.

The Five Domains framework, and other similar ones such as the Animal Welfare Assessment Grid (Justice et al., 2017) and the Animal Welfare Decision Tree (von Fersen et al., 2018), aim to assess multiple aspects of an animal's physiological health, physical surroundings and behaviours to inform on the welfare state of the animal. These assessments can be adapted for use with zoo animals and modified for each species/individual, making them good tools to reference when trying to establish a welfare assessment that is efficient and practical for the specific needs of the zoo. Chapter 2 provides further details on welfare assessment tools.



The Five Domains Model

Physical/Functional Domains

Survival-Related Factors

1: Nutrition

Restrictions on:	*Opportunities to:*
Water intake Food intake Food quality Food variety	Drink enough water Eat enough food Eat a balanced diet Eat a variety of foods
Voluntary overeating Force-feeding	Eating correct quantities

2: Environment

Unavoidable/imposed conditions:	*Available conditions:*
Thermal extremes	Thermally tolerable
Unsuitable substrate	Suitable substrate
Close confinement	Space for freer movement
Atmospheric pollutants: CO_2, ammonia, dust, smoke	Fresh air
Unpleasant/strong odours	Pleasant/tolerable odours
Light: inappropriate intensity	Light intensity tolerable
Loud/otherwise unpleasant noise	Noise exposure acceptable
Environmental monotony: ambient, physical, lighting	Normal environmental variability
Unpredictable events	Predictability

3: Health

Presence of:	*Little or no:*
Disease: acute, chronic	Disease
Injury: acute, chronic; husbandry mutilations	Injury
Functional impairment: due to limb amputation; or lung, heart, vascular, kidney, neural or other problems	Functional impairment
Poisons	Poisoning
Obesity/leanness	Body condition appropriate
Poor physical fitness: muscle de-conditioning	Good fitness level

Situation-Related Factors

4: Behaviour

Exercise of 'agency' impeded by:	*'Agency' exercised via:*
Invariant, barren environment (ambient, physical, biotic) Inescapable sensory impositions Choices markedly restricted	Varied, novel, engaging environmental challenges Congenial sensory inputs Available engaging choices
Constraints on environment-focused activity	Free movement Exploration
Constraints on animal-to-animal interactive activity	Foraging/hunting Bonding/reaffirming bonds Rearing young Playing Sexual activity
Limits on threat avoidance, escape or defensive activity Limitations on sleep/rest	Using refuges, retreat, or defensive attack Sleep/rest sufficient

Affective Experience Domain

5: Mental State

Negative	*Positive*
Thirst	Wetting/quenching pleasures of drinking
Hunger (general)	Pleasures of different tastes/smells/textures
Hunger (salt)	Pleasure of salt taste
	Masticatory pleasures
Malnutrition malaise	Postprandial satiety
Bloated, over full Gastrointestinal pain	Gastrointestinal comfort

Negative	*Positive*
Forms of discomfort:	*Forms of comfort:*
Thermal: chilling, overheating	Thermal
Physical: joint pain, skin irritation Physical: stiffness, muscle tension	Physical
Respiratory: e.g. breathlessness	Respiratory
Olfactory	Olfactory
Auditory: impairment, pain	Auditory
Visual: glare/darkness eye strain	Visual
Malaise from unnatural constancy	Variety-related comfort

Negative	*Positive*
Breathlessness Pain: many types Debility, weakness Sickness, malaise Nausea Dizziness	Comfort of good health and high functional capacity
Physical exhaustion	Vitality of fitness

Negative	*Positive*
Anger, frustration Boredom, helplessness Loneliness, isolation	Calmness Engaged, in control Affectionate sociability Maternally rewarded Excitation/playfulness Sexual gratification
Depression Sexual frustration	
Anxiety, fearfulness, panic, anger Neophobia Exhaustion	Secure/protected/confident Likes novelty Energised/refreshed

Welfare Status

Figure 1.1. From Mellor (2017), The Five Domains Model. The physical/functional domains outlined in blue (1-3; ”Nutrition”, ”Environment”, and “Health) mainly focus on the effects on welfare of internal factors. The final physical/functional domain (4, “Behaviour”, now termed “behavioural interaction” (Mellor et al., 2020)) focuses on the effects on welfare of external factors that impede or allow for agency/control. The negative and positive factors in the physical/functional domains correspond to negative and positive affective states mirrored in the “Mental state” domain (5). The overall affective experience of the animal in the “Mental state” domain equates to the welfare state of the animal.

1.1.4 The use of physiological markers in welfare assessments

Regular assessment of physiological markers can provide information on the health and welfare of an animal. Recently, advancements in the techniques to obtain physiological samples has made it easier for zoos to collect samples of urine, blood, and saliva. Ideally, these samples would be collected on a regular basis to analyse in conjunction with behaviour. However, the ability to collect samples is limited and often they are only analysed in relation to an event. For instance, studies have been conducted analysing the relationship with cortisol levels and events such as translocation and introduction of environmental enrichment (Hambrecht et al., 2020; J. Liu et al., 2006). These studies were able to make associations between cortisol levels and increased stereotypic behaviour in giant pandas (J. Liu et al., 2006) and determine the diurnal pattern of cortisol levels in African elephants which were higher in the morning compared to the afternoon (Hambrecht et al., 2020). Though these studies provide great amounts of information for specific events and physiological states, collecting and analysing samples throughout full diel and annual cycles can be beneficial in monitoring the physiological states of animals and supplementing behavioural observations for the analysis of welfare. In a study conducted on killer whales, blood samples were collected from 28 females throughout all stages of pregnancy and two months post-partum and several hormones assessed (Robeck et al., 2017). This study demonstrates how collecting longitudinal data of physiological markers can provide baselines of hormonal level patterns throughout normal pregnancy which other zoos housing killer whales can then use to assess the progression of pregnancy. Similar studies can be conducted across species to determine the diel and annual cycles of physiological markers that display adaptive rhythms. These baseline patterns can then be used in welfare assessments to determine if levels measured during an event or a point in time are within a normal range expected for that time of day, year, or life stage.

1.1.5 Implications of enclosure design on welfare

Around the world, zoos may have to meet both legislative and accreditation criteria. In the UK, the legislative framework is the Secretary of State’s Standards of Modern Zoo Practice (2012, currently under revision). Zoo accreditation requires that zoos follow guidelines on how animals are kept. One aspect of this legislation and accreditation guidelines is on enclosure design. Zoo associations tend to require that animals have appropriate enclosure sizes for the species and social group, have environmental conditions (e.g. temperature, humidity, light, etc.) that are comfortable throughout seasons or artificial conditions that suit, have shelters in outdoor enclosures that protect from different weather conditions, and to have furnishings and enrichment that allow for the natural behaviours of a species to be expressed and needs to be met (Caring for Wildlife: WAZA welfare strategy, 2023; EAZA Standards for the Accommodation and Care of Animals in Zoos and Aquaria, 2020; BIAZA Welfare Policy, 2023; AZA Accreditation Standards & Related Policies, 2024). These standards are meant to promote good welfare for the animals and tend to follow the philosophy of the Five Domains framework where creating a complex environment conducive to comfort and natural behaviours will contribute positively to an animal's affective state since the animal will have choice and be able to express natural behaviours which will prevent the animal from experiencing frustration and boredom. The difficulty in these standards is that they must also align with visitor expectations, education purposes, and be manageable by staff for husbandry and health care routines (de Azevedo et al., 2023; Hosey et al., 2013). In addition, to ensure the goals of the enclosure design are being met -to provide enough space and allow natural behaviours- there must be data evidencing the appropriateness of the enclosure (Brereton, 2020; Ross et al., 2009). Despite these difficulties in applying these high standards, they are necessary to promote good welfare for the animals.

In creating complex enclosures that are appropriate for the species and that can elicit natural behaviours, zoos must aim to understand an environment from their animal's perspective. This includes accounting for how the animal perceives stimuli (i.e. visible spectrum, olfactory capabilities, somatosensory capabilities, etc.), time, and the social world (de Azevedo et al., 2023). Enclosure use studies can provide information on the appropriateness of enrichment/enclosure features (Clark & Melfi, 2012; Liu et al., 2003), appropriateness of enclosures through time (Cuculescu-Santana et al., 2017; Rose et al., 2018), and appropriateness of social housing (including mixed-species housing) (Daoudi et al., 2017; Rendle et al., 2018). Taking an evidence-based approach to designing and updating enclosures is the only way to ensure that the features and design are achieving the intended welfare goals for the animals and is recommended by zoo associations (BIAZA Welfare Policy, 2023).

It has been suggested that increasing enclosure complexity can be more important than enclosure size, since providing more complex environments is associated with improved welfare (Hosey et al., 2013). Captive animals do not tend to use all of their provided space equally, but rather have certain preferred areas and features within their enclosures (Pines et al., 2007; Rose et al., 2018; Ross et al., 2009; Wheler & Fa, 1995). However, size and complexity have synergistic effects and must still be equally prioritised. For instance, in captive round island geckos (*Phelsuma guentheri*), it was found that the geckos generally avoided a glass substrate, but avoided it significantly more when inside a larger enclosure compared to a smaller enclosure, possibly indicating that space restriction might force geckos to occupy substrates that are not preferred (Wheler & Fa, 1995). Therefore, creating appropriately spacious and complex environments that can provide an array of retreats, shelters, and features that may be favoured is encouraged.

This goal becomes more complicated when considering both indoor and outdoor enclosures. Zoo association policies and welfare frameworks emphasise the importance of choice and control over being in indoor and outdoor enclosures, and zoo policies require that animals have suitable shelter from climatic conditions. However, these requirements fail to address a poorly understood factor on whether outdoor enclosures are suitable for species housed in zoos outside of their natural habitats that experience drastically different climatic conditions. Studies have addressed indoor vs outdoor use in relation to preference for a naturalistic environment/features or shelter (Pines et al., 2007; Ross et al., 2011) and in relation to the effects on stereotypic behaviours (Tan et al., 2013). However, a large gap of knowledge in the literature still exists on whether there are differences in use of outdoor enclosures for species housed at zoos that experience climatic conditions similar and dissimilar to those within the natural range of the species. This is a paramount issue to address given that animals synchronise to their environments and are adapted to certain climatic conditions (including their daily and seasonal changes) and also in considering whether outdoor enclosures can count as useable space when determining space requirements for species housed outside of their natural ranges.

1.1.3 Wild/natural behaviours and activity budgets in welfare assessments

Across welfare standards, it is said that good welfare, in part, comes from the ability to display natural behaviours (those displayed in the wild) (Farm Animal Welfare Council Committee, 1979; Mellor et al., 2020). In past studies, the discrepancy or similarity between activity budgets of wild and captive conspecifics were used to assess their welfare; interpreted as captive animals with dissimilar activity budgets to those in the wild have compromised welfare (Melfi & Feistner, 2002; Yamanashi & Hayashi, 2011). However, the comparison of behaviours between captive and wild conspecifics as an indication of welfare can be problematic or misleading (Howell & Cheyne, 2018; Veasey et al., 1996). The wild and captive environments are drastically different. In a captive environment space is limited, human proximity and interaction is constant, and there is less control over the environment and routines (Hosey, 2005). Animals show phenotypic plasticity in response to environmental changes (Whitman & Agrawal, 2009). Therefore, animals will attempt to adapt and modify behaviours to cope with their environment. Often, behavioural changes are adaptive, though sometimes they may be maladaptive. Behavioural adaptations to a captive environment might be perceived as maladaptive if strictly comparing the activity budgets of captive and wild animals (Howell & Cheyne, 2018). For instance, the amount of time spent foraging between a wild and captive animal may be different because it takes a longer time for the wild animal to obtain food than it does for the captive animal which is provided with food. In captivity, foraging for less time may be an adaptation to conserve energy. In addition, wild animals will experience more extreme hunger and frustration from not obtaining food that would lead to negative welfare states that would not be acceptable in a captive environment (Howell & Cheyne, 2018).

In addition to certain behaviours adaptively changing in length of occurrence in a captive environment, there may also be changes to the behaviours displayed that are still adaptive. Because of the plasticity in animal behaviour, individuals may find new behaviours that will achieve the same goal (Howell & Cheyne, 2018). For instance, in the wild, orangutans will build nests in trees to sleep in by gathering leaves and foliage. But in captivity, they might gather blankets and hay that have been provided to build nests on the ground. Though the process of building the nest is different, the same goal is achieved, and would therefore be considered a positive welfare outcome. The welfare benefit is the same because the behaviour itself is not important in relation to welfare, the goal of the behaviour is (Boissy et al., 2007; Veasey et al., 1996; Wolfensohn et al., 2018). Because of the plasticity of behaviour in response to the zoo environment, it is difficult to determine what healthy expression of natural behaviours looks like in a zoo environment.

Many studies have also assessed activity budgets as baselines within a single individual. For instance, studies comparing activity budgets before, during and after visitor presence (Sherwen & Hemsworth, 2019). This kind of assessment can provide insight into the animal’s affective state if activity budgets include behaviours that are specific to the individual such as retreating to a particular spot in an enclosure. If retreat only occurs in certain environmental conditions such as high visitor density, then one might conclude the animal was avoiding the stimulus and choosing preferred environmental conditions. However, assessing the adaptive benefits of the activity budgets can be difficult because as the behaviours assessed become more specific, they also become more variable between individuals, making it difficult to interpret what changes in behaviour simply indicate plasticity and which changes indicate a change in affective state.

Since activity budgets only provide information on the proportion of time spent displaying behaviours, they remove important context such as what time of day a behaviour is displayed and in what pattern. For instance, for a hypothetical individual, a behaviour like drinking can be found to be expressed within an average proportion of time suggesting it is a positive welfare indicator. However, for that species, most drinking is done during the hottest time of day, whereas this individual is drinking most of its water right after waking up at dawn. With this added context, the pattern of drinking would be considered a negative welfare indicator that needs further investigation. By integrating the rhythmicity of behaviour, we are better able to compare to natural behaviours, accounting for their evolutionarily adaptive qualities, and also create baselines that can be used at the individual and species level.

By establishing rhythms of behaviours displayed in captivity, the healthy expression of wild behaviours in the context of a zoo environment can be more easily determined. These rhythms can then be compared to wild rhythms. For example, in wild populations, activity budgets gained from radio collars have provided extensive information on the behavioural niche of species, seasonal energetic needs, and even shifts in needs during varying reproductive stages (Clark et al., 2017; Zhang et al., 2017). These activity budgets were broad, mainly describing active and inactive periods, but were able to provide information on evolutionarily beneficial activity. Therefore, in a captive environment, if the cycles of behaviour are compared, the welfare and needs of the individual can be assessed from a broad evolutionary perspective. To obtain 24-hr cycles in a captive environment, zoos can utilise cameras with night-time capabilities and motion sensors and radio collars to monitor movement, sleep and any disruptions to it, and behaviours of interest. These tools, in combination with keeper monitoring, can increase the amount of information considered when assessing the welfare of captive animals.

1.2 Circadian rhythms and their adaptive qualities

Circadian rhythms are cyclical changes in physiology (gene transcription, hormone secretion, and activity of peripheral tissues) and behaviour (activity, reproduction, social, etc.) that span across an ~24-hour period (Archer & Oster, 2015; Froy, 2011; Sukumaran et al., 2010; West & Bechtold, 2015). Circadian rhythms have endogenous and exogenous components (Aschoff, 1960). The endogenous component is the internal circadian clock which runs in constant conditions (i.e. constant light or constant dark) and is also known as the free-running clock (Froy, 2011; Golombek & Rosenstein, 2010; Vitaterna et al., 2001). This internal clock is a molecular circadian clock which operates through a transcription-translation feedback loop which takes ~24hr to complete (Patke et al., 2020). In unicellular organisms the circadian rhythm is generated from the cellular nature of the system (Vitaterna et al., 2001). In multi-cellular organisms, the circadian rhythm is generated by cells/cell clusters in certain regions of the organism (Vitaterna et al., 2001). In insects, these cell clusters can be found in certain brain regions like the optic and cerebral lobes, in non-mammalian vertebrates they can be found in the pineal gland, and in mammals, the structure is found in the hypothalamus and is called the suprachiasmatic nucleus (SCN) (Froy, 2011; Patke et al., 2020; Umemura & Yagita, 2020; Vitaterna et al., 2001). These regions are the central pacemakers which then regulate peripheral clocks in other regions of the organism like adipose tissues, metabolic systems or reproductive organs and eventually result in overt rhythms of behaviour (i.e. sleep/wake cycles, rhythmic social or maintenance behaviours).

The exogenous components of the circadian rhythm are the external/environmental cues, called zeitgebers, which entrain the central clock to be exactly 24 hr, synchronising the organism to its environment (Assefa et al., 2015). These zeitgebers can be the light/dark cycle, temperature and food intake (Figure 1.2) (Buhr et al., 2010; Chakraborty, 2020; Froy, 2011). Because of seasonal fluctuations of these zeitgebers, circadian rhythms will equally translate into circannual (yearly) rhythms (Golombek & Rosenstein, 2010; Meijer et al., 2007). Circannual rhythms also regulate physiological and behavioural changes on an annual scale as the shifts in light, temperature, and food availability adhere to changes in energetic homeostasis and initiate processes like reproductive cycles (Ikegami & Yoshimura, 2012), migration (Gwinner, 1996; Long & Stouffer, 2003; Wang et al., 2010), and hibernation (Hut et al., 2014). Pittendrigh (1960) hypothesised that the endogenous rhythm of species is slightly more or less than 24 hr (causing lagging or advancing overtime in relation to the earth’s rotational period) so that the endogenous clock must be continuously entrained to the outside environment. Constant detection of small deviation between exogenous and endogenous rhythms will continuously reset the endogenous clock to synchronise precisely with the exogenous factors. The optimal adaptive state of the circadian rhythm is for the central clock to be synchronised precisely with the environment, and for all of the internal clocks (central and peripheral) to be synchronised with each other.

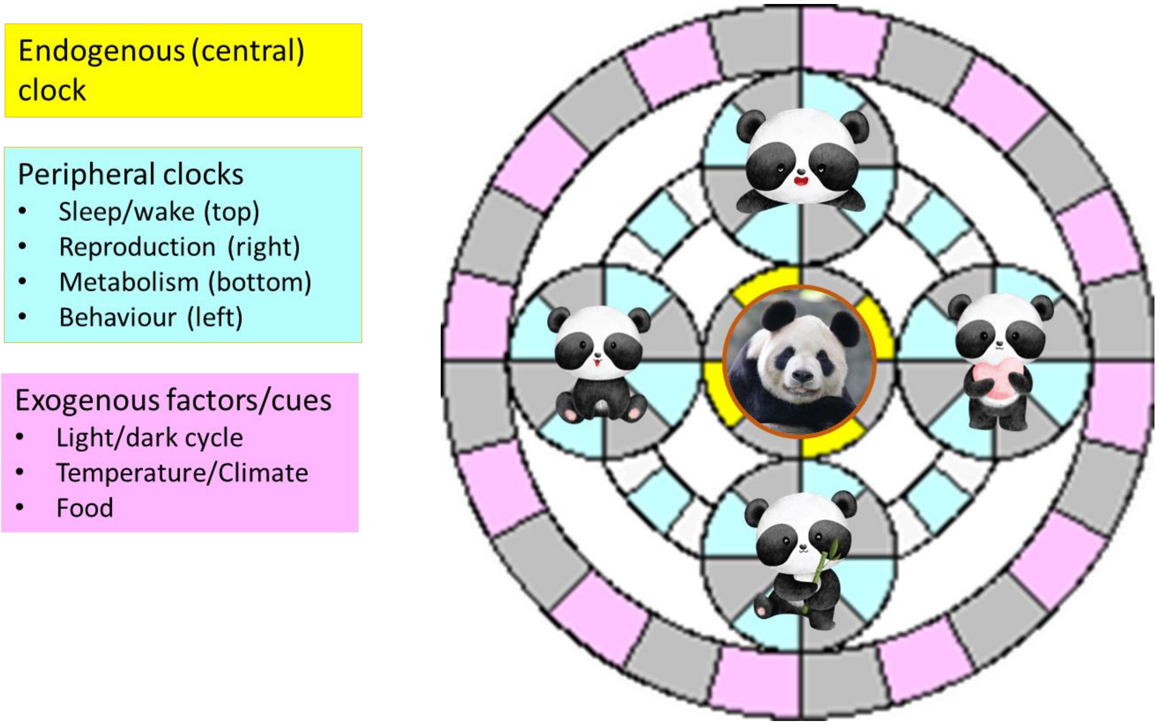


Figure 1.2. From Gandia (2024). This figure illustrates how circadian rhythms synchronise to the external environment and how the central circadian clock regulates peripheral clocks. The environmental cues (pink and grey) follow predictable patterns. For instance, pink can represent light and grey can represent dark. The endogenous central clock (yellow and grey circle) will also have a specific pattern that regulates the peripheral clocks (teal and grey circle) to synchronise to the external environment. The peripheral clocks listed under "peripheral clocks" correspond to the panda animations starting at the top and proceeding clockwise. The optimal synchrony between the different clocks and external cues would be for the grey portions in each circle to synchronise with the grey portions in the other circles and for the same to occur with the coloured portions of the circles. When grey portions are synchronizing with coloured portions, this represents desynchrony of the clocks which can result in detrimental effects for the animal.

Circadian rhythms have been recognised across the animal kingdom from birds, reptiles, mammals, amphibians, fish and arthropods, including insects (Froy, 2011; Siegel, 2008). The widespread evolution of circadian rhythms (including outside of the animal kingdom in unicellular organisms and plants) suggests an adaptive advantage to possessing one in an environment with cyclical changes (Froy, 2011; Patke et al., 2020). A study on cyanobacteria demonstrates this adaptive advantage of possessing a circadian rhythm in cyclical environments which is not present in constant environments (Woelfle et al., 2004). Woelfle et al. (2004) created competition between three strains of cyanobacteria growing together: wild-type and two strains with genetic mutations to clock genes which caused either a damped endogenous rhythm or arrhythmicity. In LD12:12 conditions, the wild type outcompeted the two strains with mutations to clock genes. However, in an environment with constant light, the arrhythmic strain significantly increased its population size while the wild-type saw no change in population size. These results suggest an advantage to possessing a circadian rhythm in a cyclical environment but a possible disadvantage to possessing a circadian rhythm in a constant environment.

The adaptive value in circadian rhythms and entrainment is that they allow species to synchronise to their environment and consequently anticipate changes to predictable cycles to then respond accordingly with physiological and behavioural changes to maintain homeostasis (Froy, 2011; Paschos, 2015; Patke et al., 2020; Rensing & Ruoff, 2002). In addition, circadian rhythms and proper entrainment to the exogenous factors also allow individuals to synchronise with conspecifics and heterospecifics to optimise social interactions or evade danger effectively. The latter is demonstrated in a study where chipmunks with a lesioned suprachiasmatic nucleus (preventing the ability to entrain to the exogenous factors) in a natural setting experienced higher mortality rates than chipmunks with intact suprachiasmatic nuclei, likely due to mistimed activity in the burrow at night attracting weasels (DeCoursey et al., 2000). Overall, endogenous clocks and the ability to synchronise with exogenous factors to synchronise all aspects of an organisms living experience seems to have an adaptive value to increase fitness and optimise energetic dynamics (Helfrich-Förster et al., 2020; Pilorz et al., 2020; Pittendrigh, 1960).

1.3 Circadian rhythms and welfare

Given the adaptive qualities of circadian rhythms, the importance of synchronising the endogenous clock to exogenous factors and the pervasive influence of circadian rhythms on all aspects of life for an organism, circadian rhythms have many implications for welfare. Berger (2011) provides arguments and examples on the importance of considering the temporal niche of a species in captivity on daily and annual scales and demonstrates how investigating circadian rhythms and the decoupling (desynchrony) of rhythms can identify stress states and welfare concerns for captive animals. Berger (2011) states that coupling and coordination between rhythms of different physiological and behavioural functions seems to be characteristic of a healthy and undisturbed animal. Since the circadian clock is responsible for synchronising all of these rhythms, it is said that all other rhythms are in a harmonic relationship with the 24hr period it follows. Therefore, investigating disruption to this harmonic quality of the circadian rhythm can provide insight into “stress” states since nearly all physiological and behavioural indicators of stress are under temporal regulation by the circadian clock (e.g. cortisol, activity levels, body temperature, heart rate, etc.).

The suggestion is to measure the Degrees of Functional Coupling (DFC): coupling between the behavioural rhythm of the individual and the diurnal environmental periodicity (0%= complete desynchrony between the 24hr environmental rhythm and behavioural rhythm, 100%= complete synchrony between these rhythms). In one example, with captive Mouflon (*Ovis orientalis musimon*), a significantly reduced DFC of activity was identified throughout illness and recovered gradually after treatment. In another example, the DFC of activity for a red deer (*Cervus elaphus*) was reduced to 0% (complete desynchrony) after it was trapped and its antlers sawn off (Berger et al., 2003). A final example, where the DFC of feeding and activity for a Przewalski's horse was significantly reduced for a month following translocation and introduction to a semi reserve demonstrates the broad applicability of this method in identifying potential disturbances and periods of stress for captive species and the sensitivity of the circadian system to stressors (Berger et al., 2003). The effects of decoupling/desynchrony of the circadian system has been studied across species. Detrimental effects such as metabolic disorders (Depner et al., 2014; Kolbe et al., 2019; Paschos, 2015), reduced immunity functioning (Irwin & Opp, 2017), reproductive dysregulation (Gamble et al., 2013; Mahoney, 2010), advanced aging (Manoogian & Panda, 2017; Welz & Benitah, 2020), and even reduced lifespan (Hurd & Ralph, 1998) have all been identified and demonstrate the broad implications for welfare in maintaining a harmonic circadian system. In considering this harmonic system, an analogy for circadian rhythms can be a musical, where the scene is the exogenous factor(s) which the conductor (central clock) is synchronizing to, to then orchestrate her musicians with several instruments (peripheral clocks) to harmonise. Similarly to when music is desynchronised with a scene, or instruments within the orchestra desynchronised with each other, when there is desynchrony between the central clock and the environment, the central clock and peripheral clocks, or between peripheral clocks, there can be detrimental effects to the animal.

Similarly to desynchrony being indicative of stress/negative affective states, synchrony of the circadian system can be linked to positive affective states. The classification of emotions through its components suggests that an emotion/affective state includes a behavioural component (a posture or activity), an autonomic component (visceral and endocrine responses), and a subjective component (emotional experience or feeling) (Boissy et al., 2007). The subjective, or emotional, component comes from awareness of internal (biological) states (Boissy et al., 2007; Jones, 2013). One difference between negative affective states and positive affective states is that positive affective states are expected to show temporal endogenous variation during the day, while negative states are not (Watson, 2000). This is because, as discussed earlier, positive affective states result from fulfilling a goal-directed behaviour, the anticipation of a reward, and the response to receiving a reward with either implicit or intrinsic value (Boissy et al., 2007; Wolfensohn et al., 2018). Positive affect is therefore the subjective experience and awareness of the activation of the biobehavioural reward system that operates under the principle of predictive homeostasis (Byrne & Murray, 2019; Moore-Ede, 1986). Therefore, this reward system has a temporal component which coordinates rhythmic engagement with a temporally varying environment to maintain homeostasis (Byrne & Murray, 2019). Negative affective states result from temporally random stressors. This temporal regulation of positive affect has been found in humans because of the ability to self-report affective states (Murray et al., 2002; Murray et al., 2009).

Murray et al. (2009) conducted a study with humans associating rhythms of positive affect with circadian rhythms under three conditions: naturalistic, constant environment with 30 hr of continuous wakefulness, and a forced desynchrony protocol where sleep/wake states were synchronised to a 28hr period. They found that in naturalistic conditions, 13% of variation in positive affect was explained by a 24hr sinusoid; in constant conditions, 25% of positive affect variance was explained by the rhythm of core body temperature (a strong indicator of the endogenous rhythm); and in forced desynchrony, positive affect synchronised with core body temperature with 24hr rhythmicity despite the 28hr routine. These results show the circadian control of positive affect and demonstrate the importance of considering circadian rhythmicity and synchrony when assessing positive affective states in non-human animals as well since the underlying principles of homeostasis, positive affective states and motivation, and biological rhythms would equally apply.

Circadian and circannual rhythms highlight how an animal is not separate from its environment, but both are intricately connected in one large feedback system. Therefore, in assessing animal welfare, understanding this connectivity is crucial to understand the factors that are evolutionarily adaptive and necessary to avoid excessive negative affective states and promote positive affective states by promoting overall synchrony of the circadian system.

1.4 Circadian rhythms and the zoo environment: Implications for welfare

However, in zoos, animals experience different environmental cues than they would experience in the wild; potentially from different climatic conditions and also new zoo-specific zeitgebers like visitors, keepers, and husbandry routines. The effects that these modified zeitgebers have on captive animals’ circadian (daily) and circannual (yearly) rhythmicity, and overall welfare, is largely unknown. This gap in knowledge is compounded by the fact that the rhythms of activity throughout the day and night and across the seasons is largely unknown for many captive animals since care of the animals is limited to the working day and seasonal changes in activity are not closely monitored (Brando & Buchanan-Smith, 2018). In relating circadian rhythms to the Five Domains, it is important to be aware of the various components of circadian rhythms of captive species, since circadian rhythms will be involved in temporally regulating and synchronising to many factors assessed in each domain (environment, nutrition, behavioural interactions, health, and the mental state) (Mellor et al., 2020). Understanding the circadian and circannual rhythms of behaviour in a species, and determining the zeitgebers that regulate these rhythms, can provide insight on the environmental, physiological, nutritional, and behavioural factors that are evolutionarily beneficial and necessary to a species. Investigating circadian rhythms for captive animals allows for a holistic approach in understanding an animal's needs that can then lead to effective, evidence-based welfare protocols and interventions.

1.4.1 Keeper control of feeding and thus, metabolic rhythms

Since peripheral tissues are regulated by the circadian clock, metabolism is one physiological process which follows a 24-hr cycle. By having a circadian rhythm of release, metabolic hormones can be regulated to be adaptive and promote optimal health. For instance, in a study by Stoelzel et al. (2020), rats were maintained on a high fat diet that developed into obesity, insulin resistance and reduced control of glucose by the ventromedial hypothalamus. Rats who were treated with dopamine agonists at times of natural circadian release experienced a reversal of the high fat diet effects and displayed reduced body weight, blood glucose levels, and plasma insulin levels compared to rats who received the same treatment but mis-aligned with natural circadian timing. This study demonstrates the adaptive quality of circadian timing and the importance of circadian synchrony in maintaining good physiological health and general welfare.

In a zoo environment, an adaptive metabolic rhythm would want to be promoted, as this would encourage optimal health in the animals. Zoo staff have a large influence on the regulation of the metabolic rhythm of an individual because feeding regimens are a strong zeitgeber that can supersede the central clock and regulate peripheral tissue clocks and activity (Froy, 2011). However, often, the feeding times and portion sizes given to zoo-housed species are different to those consumed by wild conspecifics. These altered feeding schedules have the potential to desynchronise metabolic processes with the central clock and alter activity cycles. For instance, a nocturnal mouse placed on a restricted feeding schedule during day hours will display food anticipatory activity and be more active around feeding times and less active during normal active hours at night (Carneiro & Araujo, 2012).

Out of phase feeding and activity has been associated with shifts in hypothalamic activity that are desynchronised with the central clock (Salgado-Delgado et al., 2010). The adverse effects of desynchronizing feeding and metabolic processes with the central clock have been well established (Paschos, 2015; West & Bechtold, 2015), and are linked to obesity and a variety of disorders involving insulin sensitivity and energetic balance (Depner et al., 2014). Being aware of the natural cycles of feeding for captive species and designing diets and feeding schedules in accordance with those cycles, can help zoo staff avoid the adverse effects of desynchrony and promote optimal health.

1.4.2 Encouraging and predicting healthy reproductive rhythms for more successful breeding efforts

Successful *ex situ* breeding is one of the ways in which zoos contribute to conservation efforts. Although some species are successfully bred in captivity, others present more difficulties. Since circadian rhythms regulate and influence many aspects of reproduction, both physiological and behavioural, defining these rhythms and determining the possible external factors that may regulate them (i.e. photoperiod or social cues) can help improve breeding efforts in captive environments. In humans, the circadian clock has been found to influence every stage of reproduction in females (Gamble et al., 2013). The circadian rhythm also determines the reproductive cycle for spontaneously and seasonally ovulating animals, with seasonal breeders being triggered to ovulate by certain daylengths signalling a particular time of year (Goldman, 1999). Day length also influences male reproduction, with males in several species showing development of testis and better sperm quality in association with exposure to a long photoperiod (Nunez Favre et al., 2012; Turek, 1972). Further details on the circadian control of reproduction can be found in Chapter 3.1.

Being aware of reproductive cycles and the environmental cues that trigger reproductive behaviours and physiological events can help zoo staff in not only predicting and identifying when their animals are ready to breed, but can also allow keepers to control the external factors that could increase the chances of successful breeding. Zeitgebers necessary for the initiation and success of mating can be mimicked in the captive environment increasing the likelihood that breeding individuals are synchronised in their behaviour and that individual physiological states are optimal.

1.4.3 Importance of sleep and the influence of the captive environment

In captivity, sleep is not often monitored (Anderson, 1998). Though there does not seem to be one consistent benefit to sleep, sleep can be seen as an adaptive state of inactivity

(Siegel, 2008). Siegel (2008) suggests that sleep has evolved to conserve energy and make behaviour more efficient in response to the surrounding environment. According to this hypothesis, should sleep be disrupted, either prolonged, shortened, or desynchronised, the efficiency of behaviours and energetic dynamics would be suboptimal and potentially detrimental to the organism. Because of this, monitoring the amount and cycles of sleep and rest can provide insight into the affective states of species.

In addition to providing information on welfare, monitoring sleep is also a way to determine whether the enclosure design is appropriate for the species. Caregivers are responsible for designing the environment of the animal and consequently, sleeping sites. Appropriate sleeping sites promote healthy sleep cycles and have the potential to improve general welfare. From research in primates, it is well known that appropriate sleeping sites are necessary for positive welfare. Captive orangutans build nests to sleep every night and show a preference for particular sites within enclosures (Bastian et al., 2018). A review on diurnal primate sleeping behaviour stated that primates choose their sleeping sites based on increased exposure to the sun, protection from wind and rain, and insect/parasite avoidance (Anderson, 1998). In elephants, floor substrate significantly influenced the amount of time elephants spent recumbent, an indication of sleep quality (Holdgate et al., 2016). In addition to influencing sleep, sleeping site appropriateness also influences social structure across species (Cui et al., 2006; Holdgate et al., 2016; Noser et al., 2003). These studies indicate how important it is to provide proper materials, sleeping sites, and choice for sleep to avoid disruption and desynchronisation of biological rhythms.

1.4.4 Altered and additional external cues in the zoo environment

The zoo environment is drastically different from wild environments. Though all attempts are made to create enclosures which closely mimic the wild, inevitably there are many differences. In zoos, animals experience limited space, consistent human interaction, human-controlled schedules, artificial lighting and temperature, and enclosure designs that do not always mimic natural habitats. These factors alter the external environment and the cyclical changes experienced by the animal and have the potential to regulate the circadian rhythm.

1.4.4.1 *Potential effects of latitude on circadian rhythmicity in the zoo environment*

The housing of species outside of natural latitudinal ranges in zoos around the world presents a concern when considering the latitudinal effects on circadian rhythms. Species are specially adapted to the seasonal cycles of light and temperature in the habitats in which they have evolved (Hut et al., 2013). In plants (the taxa from which endogenous clocks were discovered (de Mairan, 1729)), a wild plant with populations adapting across latitudes and a domesticated plant artificially selected for optimal yield across latitudes, both showed positive correlations between the circadian periodicity of leaf movement and latitude, suggesting an adaptive quality to synchronizing with the environment that increases fitness (Greenham et al., 2017). In insects, the critical photoperiod that initiates diapause displays a latitudinal cline across species, with species at higher latitudes having a longer critical photoperiod (Hut et al., 2013). Changes in temperature cycles with latitude can also have a large effect on organisms, as temperature often serves as a strong zeitgeber for species that are not homeothermic vertebrates (Rensing & Ruoff, 2002). Though temperature is a weak zeitgeber for most mammals, it still plays an important role in resetting circadian oscillators and enhancing internal circadian synchronisation (Buhr et al., 2010). Understanding the changes in the cycles of light and temperature across latitudes and their effects on organisms' circadian rhythms is important when assessing how an animal will respond to the external environment in a zoo (Figure 1.3). Further effects of latitude on circadian rhythmicity are discussed in Chapter 4.1.

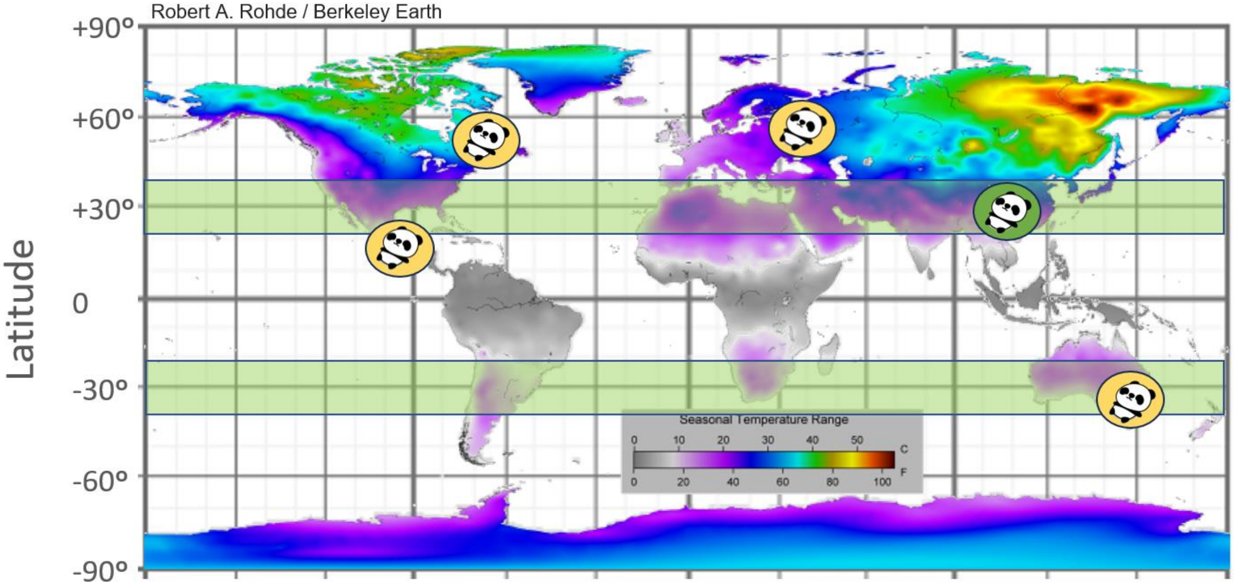


Figure 1.3. From Gandia (2024). This figure illustrates how animals in captivity may be housed in zoos around the globe that experience differing climatic conditions than their home range. The panda circled in green represents wild pandas in the historic natural range of pandas. The translucent green bars represent the latitude range of this home range across the globe, also reflected in the southern hemisphere. The pandas circled in yellow represent captive pandas housed in zoos around the world both within and outside of the latitudinal range for which they have evolved. The map displays the annual temperature range experienced around the globe in colour. Similar patterns would be seen for the differences in daylight between the summer and winter solstice across the globe, with latitudes closer to the poles experiencing larger changes in the amount of daylight throughout the year. This figure

demonstrates how animals housed outside of their natural latitudinal ranges may be exposed to different zeitgebers than their circadian clocks have evolved for when housed in zoos around the world.

1.4.4.2 *Expression of torpor and hibernation in the zoo environment*

Torpor and hibernation are states of dormancy in animals which are entered through sleep in an attempt to survive harsh environmental conditions like food scarcity, extreme dryness, or cold by conserving energy (Siegel, 2008). Torpor and hibernation are characterised by low body temperature, a greatly reduced metabolic speed, and decreased neuronal activity. Torpor can occur daily and is usually experienced in smaller bouts in comparison to hibernation, which is an extended form of dormancy (Geiser & Ruf, 1995). The daily and annual cycles of torpor and hibernation suggest a circadian control of dormancy. Across species, torpor and hibernation are induced by changes in food availability, ambient temperature, and photoperiod (Genin & Perret, 2003; Körtner & Geiser, 2000). These external factors have daily and annual cycles which are not always mirrored in captivity. Therefore, many species which hibernate or enter torpor are prevented from doing so in captive environments.

Species such as the American black bear do not always hibernate in captivity. However, American black bears which do not hibernate in captivity display the same insulin resistance in the winter as conspecifics who do hibernate in captivity and the wild (McCain et al., 2013). This can potentially lead to obesity or other metabolic issues in captive bears which do not hibernate, as they are still fed throughout the year despite changes in physiology that alter metabolism. Captivity can also alter aspects of torpor for species such as the mouse lemur. Changes in photoperiod and food availability affect the duration and depth of torpor (Genin & Perret, 2003). In a captive environment, humans control food, and light periods can differ from those experienced in the natural range due to latitudinal effects or artificial lighting. Should processes like hibernation and torpor be prevented or changed due to the altered zeitgebers in a captive environment, adjustments to husbandry and feeding regimens should be made to account for the physiological needs of the animal for that season or time of day.

1.4.4.3 *The effects of external cues triggering the onset of migration in a captive environment*

Migration is a seasonal event whereby an animal will travel long distances to reach different regions of the globe for reasons including mating, food and water availability, and the avoidance of harsh weather conditions. Much like other seasonal behaviours, migration is triggered within an animal by external cues that reach a certain triggering threshold at particular times of year. In migratory birds, this trigger is photoperiods of specific length depending on the season (Gwinner, 1996), which would equally trigger the motivation to migrate in captivity. The effects of the captive environment on migratory behaviour have been largely studied in birds. A specific behaviour called Zugunruhe, from German, which translates to "migratory restlessness", was first defined in birds inhibited from migrating. Zugunruhe involves birds displaying increased levels of activity and nocturnal migratory restlessness during periods which coincide with natural migratory behaviour (Gwinner & Czeschlik, 1978). This behaviour may indicate negative welfare states given the possible frustration of being motivated to migrate but being unable to.

Limited research has been conducted in other migratory species aside from birds investigating the occurrence of Zugunruhe. However, a study on Japanese eels (*Anguilla japonica*), which migrate to spawn, may indicate the importance of studying its occurrence across captive migratory species (Sudo & Tsukamoto, 2015). Sudo and Tsukamoto (2015) found that treating the eels with an androgen triggered increased locomotor activity, a sign of migratory restlessness. They also found that a decrease in water temperature which mimicked the change in water temperature in autumn during the onset of migration triggers a natural increase in the same androgen, suggesting that the seasonal external cue which triggers migration is water temperature (Sudo & Tsukamoto, 2015). In captivity, the expression of natural behaviours must be encouraged. Therefore, if several migratory species are displaying migratory restlessness, a behaviour indicating a physiological motivation to migrate with the inability to do so, it is an indication of negative welfare. More research must be done on how these effects can be minimised or even potentially how migratory behaviour can be mimicked in a captive environment.

1.4.4.4 *Husbandry routines as a potential zoo-specific zeitgeber*

The daily rhythms of husbandry routines follow a 24-hr cycle in a similar way that the circadian cycle does. Husbandry routines which include feeding, cleaning, and training sessions become a part of the external environment of the animal that is predictable in the same way light and temperature are. Therefore, in response to husbandry practices, animals may show predictable changes in their behaviour and physiology. Food anticipatory activity is one example of this response to human-controlled schedules and it has been shown to affect circadian rhythmicity and the affective states of animals (Anderson et al., 2015; Carneiro & Araujo, 2012; Waitt & Buchanan-Smith, 2001). Displaying food anticipatory activity may be a signal of circadian desynchrony in zoo animals if feeding times do not coincide with the natural circadian rhythm of feeding. Predictable feeding schedules also cause animals to display anticipatory behaviours which may signal stress should their frequency increase in response to the predictable schedule becoming delayed (Anderson et al., 2015; Waitt & Buchanan-Smith, 2001). For these reasons, in the zoo environment, the behaviours around feeding times and their cycles must be assessed together to contribute to the understanding of the welfare state of the animal.

Another human-controlled activity in zoo environments with a set schedule is training sessions. Because of the predictability of training, the sessions have the ability to regulate behavioural responses before and after sessions. For instance, bottlenose dolphins displayed changes in anticipatory, swimming, and play behaviour shortly before, shortly after, and in-between training sessions (Clegg et al., 2017). Though 24-hr cycles were not recorded to determine how this may affect the circadian rhythmicity of the dolphins, the study clearly depicts the ability for the sessions to regulate behavioural responses. Human-controlled schedules of animal activities must be assessed using a 24/7 approach to ensure that they do not affect the circadian rhythmicity of the species nor their general welfare.

1.4.4.5 *Zoo visitors as a zoo-specific external cue with the potential to desynchronise circadian rhythms*

Zoo visitors are a critical component for achieving the goals of zoos to raise awareness and increase education on species and fund their conservation. However, their habits and presence in zoos may have effects on the general welfare of the zoo-housed species and their circadian rhythms. One habit of visitors that can have direct effects on the circadian rhythms of zoo-housed species is unauthorised feeding. Since feeding is a strong zeitgeber, and zoo-housed animals are on strictly controlled diets with set schedules, providing food that is likely unhealthy and possibly toxic to the animal outside of this schedule can cause desynchrony between metabolic rhythms and the central clock.

Though most visitors do not provide animals with food outside of paid encounters, visitor presence still has an effect on the animals as an arousal stimulus that can potentially affect circadian rhythms (Mistlberger & Skene, 2004). Generally, visitors can have negative, positive, and neutral effects on zoo-housed animals (Hosey et al., 2023; Hosey, 2000; Sherwen & Hemsworth, 2019). The main negative effects include fear, avoidance, and changes in activity budgets. An altered activity budget, though more difficult to interpret as affecting welfare than clear fear responses, may be indicative of circadian desynchrony, but a 24-hr approach would need to be taken to determine this conclusively.

In addition to the negative effects having potential influence on circadian rhythmicity, positive effects may have them as well. Some positive effects discussed are increased stimulation and engagement with zoo visitors. These behaviours in response to zoo visitors can be detrimental should visitors be present during hours in which the species would normally be inactive. Stimulation and changes in frequencies of behaviours, those indicative of positive and negative welfare states, in response to visitors is of special concern when considering night-time events that are held in zoos across the world. These events are held late at night when zoos would normally be closed. For animals which generally have fixed daily and weekly routines, an added night-time event may be disruptive to their circadian rhythm. Events often include artificial light and noise from visitors. Light and noise can potentially disrupt the sleep of species which are normally inactive at night. For species which are normally active at night, visitor presence can increase fear, alertness, or alter behaviours. For all species, these disruptions can potentially alter the circadian rhythm and cause desynchrony of internal clocks.

One study investigating the effect of a month-long night-time lights event on western lowland gorillas (*G. gorilla gorilla*) found that most gorillas rested less during and after the event (Bastian et al., 2020). Behavioural measurements were taken during the hours of the event and during the daytime. Therefore, an alteration in the activity budgets during day-time hours outside of the event can be indicative of a disrupted circadian rhythm. Similarly, in a study investigating how a nighttime event affected spider monkeys (*Ateles geoffroyi*), it was found that the nighttime activity of the monkeys changed on nights of the event with the monkeys being significantly more active and resting more outdoors (in comparison to resting indoors) when compared to nights without the event (Proctor et al., 2020). For both studies, it is difficult to conclude whether these changes in activity demonstrated positive, neutral, or negative affective states in response to visitors. However, the results do clearly demonstrate the potential for zoo events to disrupt the circadian rhythm, though 24-hr observations would be needed to determine this conclusively. Taking a 24/7 approach to assess the welfare of species' before, during, and after events can provide insight into the extensive effects events may have on behaviour, physiology, and general welfare.

1.4.4.6 *Light pollution*

Many zoos are often in urbanised areas, presenting the risk for zoo-housed animals to be exposed to light pollution. Light pollution alters the natural cycle of the light/dark cycle and consequently effects the circadian rhythm. In a nocturnal mouse lemur, light pollution induced daily desynchronisation and caused a delay of onset/offset locomotor activity, decreases in locomotor activity at night, and increased overall core temperature (Le Tallec et al., 2013). In addition, exposure to light pollution also advanced oestrus in females since reproduction is induced by long photoperiods (Le Tallec et al., 2013). In captive blue tits, exposure to different coloured light at night changed the onset/offset of activity and part of their daily activity was moved into the night (De Jong et al., 2017). The change in onset/offset of behaviour are indicators of circadian desynchronisation and these studies provide evidence that it not only affects behaviour, but physiology as well. Therefore, creating zoo environments that eliminate the effects of light pollution are necessary for the general well-being of a species.

1.5 Current considerations of circadian rhythms in the zoo environment

1.5.1 Lighting: scheduling and light source

The effect of lighting on species activity cycles has been long understood in zoos, with many nocturnal enclosures maintained on an inverted light cycle. These practices are mainly implemented to ensure that visitors may view nocturnal species in active states. Though studies have observed the effects that the kind of light (i.e. wavelength and intensity) has on the behaviour of nocturnal, captive species (Frederick & Fernandes, 1994; Fuller et al., 2016), the main consideration when creating artificial light cycles remains as the length of day/night. In addition, the effects of lighting on non-nocturnal, captive species are rarely considered.

The wavelength of light is known to have effects on the behaviour and physiology of many species. Under blue light, nocturnal primates display less activity and lower melatonin levels when compared to red light exposure (Fuller et al., 2016). Similarly, laying hens display more explorative behaviours under green light and less aggressive behaviours under red light along with advanced reproduction (Huber-Eicher et al., 2013). A large factor in understanding which wavelengths will affect behaviour and physiology is knowing the wavelengths which are perceptible by the species. Awareness of the perception and effects of different coloured lights across species can help zoos in ensuring that lighting in enclosures is species appropriate and promotes positive welfare.

Light intensity and source also influence behaviour and physiology across species with varied circadian rhythmicities. Moonlight is one source of light that is rarely considered in the zoo environment but influences the behaviour of many species. Some species display higher activity in moonlight (Fernandez-Duque, 2003) while others display moonlight avoidance, likely in an attempt to avoid predation (Clarke et al., 1996). A study on African carnivores found that hunting activity increased with moonlight availability for diurnal carnivores but did not have an effect on the hunting activity of nocturnal carnivores (Cozzi et al., 2012). Mimicking moonlight in indoor enclosures or ensuring exposure to moonlight for outdoor enclosures can have large impacts on the behavioural repertoire of species, allowing species to display more naturalistic behaviours and consequently promoting positive welfare.

1.5.2 Temperature control and policies

Accredited zoos are required to follow specific guidelines on how to care for certain species. Part of these guidelines specify the temperature conditions in which the animals should be kept (Association of Zoos and Aquariums, European Association of Zoos and Aquaria). For ectotherms, they generally advise that animals be kept at quite specific temperatures for the day and night. However, for endotherms, they suggest a larger range of temperature in which the animal will be comfortable and only require that shelter from harsh weather is available. They may also suggest that a temperature-controlled room is provided, but do not require strict control of the temperature specific to the time of day. However, the daily cycles of temperature may be equally influential on the welfare of species as the range. For instance, Vera et al. (2023) conducted an experiment where they placed zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*) in tanks with gradient temperature to allow the fish choice on which temperature zone they wanted to occupy. They conducted 24-hr observations over several days and found that both the zebrafish and tilapia showed clear circadian rhythms of temperature preference, occupying sections with higher temperature during the second half of the light phase and preferring lower temperatures at the end of the dark phase (Vera et al., 2023). In relation to welfare, this experiment perfectly illustrates the idea that allowing choice and control over the environment is important. For ectotherms in particular, the ability to regulate body temperature to synchronise with physiological rhythms is necessary to maintain homeostasis. Though zoo guidelines for ectotherms specify temperatures for day and night, it may be enough to provide a variety of choices throughout the enclosure so that the animal can display agency in different preferred spots in the enclosure.

Daily cycles of temperature are particularly important for ectotherms and endothermic heterotherms, as they regulate torpor and consequently the arousal and rest periods which should be synchronised with the circadian rhythm as well behaviours related to thermoregulation (Abram et al., 2017; Körtner & Geiser, 2000; Turbill et al., 2008). Though temperature is a weak zeitgeber in endotherms, it may enhance the amplitude of the light/dark cycle signal when in-phase, thus enhancing the entrainment signal for the circadian rhythm (Rensing & Ruoff, 2002). Promoting synchronisation of the circadian rhythm for ectotherms and endotherms through temperature cycle regulation in captivity can enhance the welfare of species by allowing for the proper entrainment to the external environment and adaptive behavioural and physiological cycles.

1.5.3 Feeding regimens

In the past, when creating feeding regimens for zoo-housed species, the nutritional value of the food was prioritised over the appropriateness of the schedule and portion sizes for the species. Considering circadian rhythmicity and the effects of desynchronizing metabolic processes with the central clock, timing of meals and portion sizes are equally important as the nutritional value. Caregivers are usually limited to daylight hours for feeding, which is not ideal for animals who feed throughout the day, only at night, or very early in the morning (Brando & Buchanan-Smith, 2018; Gilbert-Norton et al., 2009). This results in the animals eating inappropriate amounts in one feeding session. For instance, wild lions are known to gorge feed and fast in the wild. However, in captivity, they are fed more often, usually each day, in smaller portions. When the diets of lions in captivity were changed to a gorge feeding/fasting regimen, the lions displayed more signs of satiety after meals, appetitive behaviour increased and digestibility of the food was improved (Altman et al., 2005; Höttges et al., 2019). Another study in sea bass divided sea bass into three groups: two of the groups were fed with automatic feeders that dispersed food three times a day, one with equal portion sizes each time (fixed automatic feeding) and the other with variable portion sizes that mimicked feeding in the wild (modulated automatic feeding), and the third group fed off of a self-feeder (Azzaydi et al., 1999). Results indicated that those on the modulated automatic feeding schedule displayed increased biomass and higher specific growth rate than those on the fixed automatic feeding schedule. Notably, fish on the modulated automatic feeding schedules seemed to display a pattern of appetite similar to that of the group on the self-feeders, whereas the appetite of the fish on the fixed automatic feeding schedule did not correspond with the schedule of feeding. These studies demonstrate the need to place equal importance on the nutritional value, portion sizes, and temporal dispersion of food when creating feeding regimens for captive animals. Zoos are beginning to take natural diel cycles of feeding into consideration when creating feeding regimens, but it needs to become a more widespread practice across species.

Though zoos are beginning to take into consideration the diel cycles of feeding when creating feeding regimens, the annual cycles of feeding are still rarely considered. In response to environmental changes, many species alter their diet with changes in energy expenditure dependent on the time of year (Fredriksson et al., 2006; Juáres et al., 2016; Tolo et al., 2008). Annual patterns of feeding persist in captivity, with pandas showing season specific preference for plant parts of the bamboo plant (Hansen et al., 2010). Accommodating for seasonal preference has implications for welfare as well. In Winter, sea bass normally display nocturnal feeding patterns. In an experiment where sea bass were fed with self-feeders, nocturnal automatic feeders, and diurnal automatic feeders from January to April, the self-feeding group switched from nocturnal to diurnal feeding by the end of the experiment. Interestingly, the specific growth rate of individuals on nocturnal feeders was highest in the first months and lowest in the last while the opposite was seen for the individuals on the diurnal feeders (Azzaydi et al., 2000). These studies demonstrate the importance of accommodating for seasonal preference in foods and feeding times in captivity to promote optimal health and welfare of the animal. Providing captive animals with the opportunity to display natural preference in feeding throughout the day and year can help to avoid desynchrony of the central clock and metabolic processes, and the associated adverse effects which even include reduced reproductive fitness (Xu et al., 2011).

1.6 Conclusion

Given the large influence that circadian rhythms have on behavioural and physiological processes, it is essential that within a captive environment, they are well understood and monitored across species. The welfare of a species can be difficult to assess within a captive environment if all the factors that influence their welfare are poorly understood or analysed separately. By observing circadian rhythmicity and welfare across the day, night, and seasons, we are encouraging that a more holistic approach be taken. Circadian rhythms provide information on the physiological, behavioural, and affective state of the animal by demonstrating how they are responding to their environment, both internal and external (Liu & Chang, 2017; Mistlberger & Skene, 2004; Paschos, 2015; Vitaterna et al., 2001). This analysis follows the five domains model of assessing welfare and incorporates the assessment of internal and external factors of nutrition, environment, health, and behaviour across diel and annual cycles to inform on the affective state of the animal (Mellor & Beausoleil, 2015; Mellor et al., 2020). For the nutritional domain, though the quality and quantity of food is emphasised, the feeding cycle is equally important as it influences the metabolic breakdown and nutrient intake of the food provided (Paschos, 2015). Ensuring that zeitgebers such as the light/dark cycle, feeding, and temperature have similar cycles to those found in the natural habitat of the species is directly related to the second domain, environment. The third domain, physical health, can be partially addressed by assessing circadian rhythms because determining whether an animal is displaying a proper circadian rhythm may indicate circadian synchrony and thus the avoidance of the adverse health effects of desynchrony (West & Bechtold, 2015). By ensuring proper zeitgebers in the captive environment, controlling for potential new zeitgebers like visitor presence and light pollution, and promoting circadian synchrony, captive animals may have the ability to display natural behaviours that have diel and annual cycles like proper sleep/wake cycles, hibernation, and reproduction, thus promoting positive states in the fourth domain, behaviour. By assessing circadian rhythms in the context of these four domains, insight can be gained for the fifth domain, the mental domain. Consequently, monitoring and assessing all aspects of a circadian rhythm can aid in providing information on the welfare status of a captive animal.

A main goal for zoos is conservation and given the persistent threats of habitat loss and population declines in species around the world, it is essential that research continues to find innovative ways to improve the reproductive success of species in captivity. Proper entrainment to external cues and the resulting circadian rhythm has direct effects on reproductive cycles of species. Therefore, monitoring the diel and annual cycles of activity in captive species can provide insight into mating behaviours and potential conditions that could improve mating and reproductive success. This holistic approach to assessing the needs of species does not only have the potential to improve conservation efforts but can also be utilised to promote positive welfare. The inclusion of night-time activities and annual cycles in assessing welfare facilitates the understanding of the needs of the species on a daily and annual basis. This information can be used to have an evidence-based approach when creating husbandry protocols, enclosure designs, and species management protocols to promote positive welfare.

To demonstrate the importance of a holistic approach to welfare and provide evidence on how circadian rhythms can enhance such an approach, my thesis describes the development and implementation of an evidence-based welfare assessment tool and then shows with giant pandas how circadian rhythms can inform on adaptive behavioural cycles and environmental conditions that may affect these cycles. In Chapter 2 I begin with demonstrating the process of making the RZSS Edinburgh zoo welfare assessment (based on the Five Domains) more efficient and reliable. I worked in collaboration with the RZSS curator, first providing evidence to justify delegating different categories of the assessment (nutrition, environment, health, husbandry, and behavioural interaction) to specialised staff for each category, reducing the number of assessors needed to complete the whole assessment without losing reliability. As the second portion of this study, I developed a protocol for an evidence-based approach to the behavioural interaction domain questions and some environment domain questions. This process is meant to be refined over time and eventually incorporate overnight assessments to account for full cycles. The finalised tool would then be a standardised welfare assessment that can be used by zoos across the globe to make it easier for comparison across zoos and to study the effects of environment and zoo location on welfare as a global community.

In Chapters 3 and 4, to elaborate on how these kinds of assessments could be used over time to eventually form baselines of circadian and circannual cycles, I present a study conducted throughout a full year on giant pandas, observing their behaviour on webcams across the world. Chapter 3 presents results on the effects that sex and life stage have on behavioural cycles and investigates possible adaptive energetic dynamics of the different cycles. This chapter highlights the importance of taking a 24/7 across the lifespan approach to welfare and demonstrates the differing needs throughout different life stages and the continuous nature of welfare. Chapter 4 investigates the effects of latitude and environmental factors (daylight and temperature) on the behavioural cycles of the giant pandas. This chapter highlights the importance of considering the environment as an extension of the animal and provides evidence suggesting that housing animals outside of their natural latitudinal ranges has effects on the adaptive rhythms of animals.

Overall, my thesis provides a holistic, evidence-based method that can be applied across captive environments so that staff can better understand the needs of their species and appropriately provide for them, promoting positive welfare and increasing the likelihood of successful breeding and conservation.

Chapter 2.

Creating an efficient and reliable welfare assessment protocol with Royal Zoological Society of Scotland (RZSS) Edinburgh Zoo

Chapter 2. Creating an efficient and reliable welfare assessment protocol with Royal Zoological Society of Scotland (RZSS) Edinburgh Zoo

Author contributions

Kristine M. Gandia (KMG), Hannah M. Buchanan-Smith (HMB-S), Jo Elliott (JE), and Sharon E. Kessler (SEK) conceptualised the project. KMG and HMB-S developed the methodology for the chapter. JE created the welfare assessment based on the BIAZA welfare toolkit and coordinated and curated responses from assessors in RZSS Edinburgh Zoo. KMG and HMB-S trained university students on behavioural observations, conducted reliability testing and organised students for data collection. KMG organised the data, analysed the data, and wrote the first draft of the chapter. KMG, HMB-S, SEK, and JE edited the chapter and approved the final version of the contents of the chapter.

Abstract

Currently, there is no accepted standard of welfare assessment across zoos. Comprehensive and regular welfare assessments are necessary for promoting positive welfare states in individual animals and identifying welfare risks in a timely manner. However, they require resources like time and appropriately trained staff that are often limited in zoos. In this study, we collaborated with RZSS Edinburgh Zoo to make their welfare assessment tool more efficient and evidence-based. Their welfare assessment tool follows the widely accepted Five Domains model for assessing welfare, based on the BIAZA toolkit which recommends multiple assessors, increasing the time required to complete the assessments. To make the tool more efficient, we provided evidence to justify reducing the number of assessors needed without losing reliability. To make it even more reliable, we made a protocol for an evidence-based approach to behavioural and some environmental domain questions. We created ethograms that included positive, neutral, and negative behavioural indicators of welfare that were aimed to directly answer the questions in the welfare assessment. These ethograms were made into *ZooMonitor* projects, where we could also address environmental domain questions and any other more specific welfare questions the zoo had about the particular species. With our training deliverables and available *ZooMonitor* projects, this protocol can be picked up and refined across zoos to create a standard of welfare assessment that provides reliable evidence of individuals' and groups' needs that will ultimately make welfare interventions more targeted, consistent and appropriate.

2.1 Introduction

Welfare assessments of zoo-housed species are necessary as an aspect of high standard care protocols. The welfare states of animals must be assessed and appropriate adjustments to their management must be made, if necessary, based on these assessments. Ideally, welfare assessments would be conducted regularly to monitor the affective state, physical health and general well-being of the animal and to create a comprehensive view of their individual needs, taking into consideration daily, annual and life cycles. However, because assessments of welfare must cover the full range of biological needs of the animal, they require much time, many resources and trained staff to conduct them. These factors become limitations for zoos in completing frequent assessments, especially considering the large number of individual animals and diversity of species in zoo collections. Though welfare assessments are not the only manner in which welfare concerns are brought up, with keepers being an important safeguard for flagging welfare concerns of the animals they are closely familiar with, there has still been increased importance being placed on conducting regular, comprehensive welfare assessments for captive animals. For example, the World Association of Zoos and Aquariums recently enforcing the regular assessment of welfare for their members (WAZA Code of Ethics, 2023). Therefore, these limitations must be addressed by the community and solutions developed. This project aims to address these issues by exploring ways to make the Royal Zoological Society of Scotland (RZSS) welfare assessment more efficient and reliable with an evidence-based approach that provides extensive insight into the welfare states of animals while minimising the resources and time commitment invested by zoo staff. RZSS Edinburgh Zoo is a member of BIAZA, which created a welfare toolkit modelled after the Five Domains (Harley & Clark, 2019; Mellor, 2017; Mellor & Beausoleil, 2015; Mellor et al., 2020), and which was used as the basis for their assessment tool.

Across the zoo and agricultural community, several welfare assessment frameworks have been proposed. The approaches assess resource-based indicators, animal-based indicators, or a mix of both (Blokhuis, 2008; Jones et al., 2022; Justice et al., 2017; Sherwen et al., 2018; Tallo-Parra et al., 2023; von Fersen et al., 2018). Resource-based indicators (inputs) are the resources provided by the institution like environmental design, nutrition plans, enrichment protocols, and veterinary care. Animal-based indicators (outputs) are the welfare indicators directly measured from the animal like behaviour and physical health. The welfare assessment frameworks aim to either assess the current welfare state of an animal or to identify potential risks. Most current welfare assessment frameworks use a mixture of both resource and animal-based indicators, with a varying balance between the two and a variety of different measures for both. Evaluating the strengths and weaknesses of these frameworks can give us direction in how to develop a tool that incorporates the different strengths and minimises weaknesses. One weakness across nearly all frameworks is the absence of accounting for daily, annual, and life cycles and the continuously changing needs of species across these cycles. Our aim through the development of this assessment tool is to take an evidence-based approach that addresses the continuous nature of welfare across the lifespan.

The framework proposed by Kagan et al. (2015), Universal Animal Welfare Framework, evaluates the institution. The framework assesses the institutional philosophy and policy, programmatic structure and resources, execution, and evaluation. This framework is good for identifying institution level risks to welfare (i.e. no training for staff on welfare philosophies, low standards for environmental design, no individual animal welfare protocols, etc.), and helps institutions identify areas where they can improve policy and implementation of high standard welfare practices. The advantage of this assessment is that it does not require much time or resources to evaluate the entire institution. The main limitation, which is recognised by those who developed it, is that institutional level practice does not always translate to optimal welfare for animals. Therefore, this framework would need to be followed up with more in-depth analysis of welfare at the animal level.

An assessment that addresses this gap is that proposed by Watters et al. (2019) in evaluating behaviour budgets. This approach is good for understanding animal outputs. However, Watters et al. (2021) recognises that other aspects of welfare must be addressed in conjunction with behavioural measures to contextualise the behaviours. When measuring only time budgets or the expression/non-expression of certain behaviours, much of the context of the behaviour is removed. As part of the movement towards measuring positive indicators of welfare through behaviour (Boissy et al., 2007; Yeates & Main, 2008), we must understand that certain behaviours are neutral and need context to be considered positive or negative. For instance, we can have a capuchin in a group whose time budget for feeding compares well to the average time spent feeding in the group. We would assume this is a positive indicator of welfare, but when investigated more closely, we see that feeding was occurring at times of day which would not be natural and could indicate some kind of metabolic desynchrony. Many behaviours like resting or grooming can be considered positive or negative depending on the conditions in which they are expressed, the timing of expression, and the proportion of time spent displaying the behaviour. Therefore, measuring only time budgets or whether a behaviour is or is not expressed often does not provide enough information to determine whether a behaviour is a positive or negative indicator of welfare. It also limits the interventions that can be implemented to try and promote positive behaviours because the details of expression and context are missing, preventing staff from recreating the conditions that may elicit the wanted behaviours.

Two popular frameworks that incorporate both resource and animal-based indicators into the welfare assessments are the Animal Welfare Assessment Grid (Justice et al., 2017) and the European Welfare Quality® project (Blokhuis, 2008). The AWAG was initially developed for animals involved in research but has been applied to captive wild animals in zoos. The Animal Welfare Assessment Grid uses caretaker ratings to assess physical health, procedural parameters, environmental comfort, and psychological well-being. The results are numerical and visual, making it easy to compare across individual animals/groups and across institutions. However, one limitation for this approach is that it mainly focuses on whether negative animal indicators are present and does not address positive welfare states.

Similar to the Animal Welfare Assessment Grid, the European Welfare Quality® project was initially developed to assess welfare of agricultural animals, but has been successfully applied to zoo animals as well (Clegg et al., 2015; Salas et al., 2018). The Welfare Quality® framework assesses four principles of welfare: whether there is good feeding, good housing, good health, and appropriate behaviour. Unlike the Animal Welfare Assessment Grid, this approach allows for the assessment of positive welfare states and emphasises the measurement of animal outputs. This approach is most similar to the frameworks based on the Five Domains and has strength in requiring reliable measures of species-specific welfare indicators based on literature.

The main framework based on the Five Domains was proposed by Sherwen et al. (2018). However, the assessment mainly included resource-based inputs and was limited on animal-based outputs. However, they found that training staff in risk assessment facilitated the recognition of more welfare risks and allowed for preventative measures to be taken. The results of the assessment led to interventions including plans for redesigning enclosures, changes to feeding regimes, and seeking more staff. Because the assessment was mostly resource-based, the interventions are based on the assumption that providing better and more relevant resources will result in better welfare, which cannot always be assumed since animals may respond in unpredictable ways to resources or have individual preferences and needs. However, the major strength in this assessment is the timescale. They were able to complete this assessment once a year for each species, an aspect of an assessment crucial to detecting welfare risks before they become major welfare concerns.

One important aspect of welfare that none of these frameworks address is the continuous nature of welfare. These frameworks do not apply the cyclical nature of the day, week and seasons to the approach of assessing welfare, nor address how welfare may be affected through different life stages. The final framework that has been proposed, which aims to address this, combining principles from the WQ and Five Domains, is the 24/7 across the lifespan approach to welfare assessment (Brando & Buchanan-Smith, 2018). This kind of approach emphasises taking a holistic approach to welfare that considers the natural history of an animal and how the context of a zoo environment affects natural cycles. The authors are clear it is not an animal welfare assessment though. The approach aims to map out and research whether the needs and wants of captive animals are being met, 24/7 across the lifespan. Though some frameworks encourage that rhythms be considered for aspects of welfare like the changing diet composition throughout the year, none go as in-depth to suggest that welfare is continuous on multiple time scales and should be assessed as such. This approach to welfare assessment is therefore unique and thorough in that it takes an evolutionary perspective to welfare assessment.

In collaboration with RZSS, the goal of this project was to develop their welfare assessment tool to be more reliable and efficient. Developing a welfare assessment tool goes beyond using a welfare assessment framework and requires testing the validity, reliability, and feasibility of the tool across the whole animal collection in a zoo. Jones et al. (2022) outlines a process for developing a welfare assessment tool (Figure 2.1), which we largely followed. This chapter will describe this process up until the step of piloting the tool, mainly focusing on the outcomes of this piloting step and describing the next steps in returning to the development step to then pilot again in the near future. Reaching the stage of tool implementation requires more piloting to finalise the tool which will go beyond this thesis.

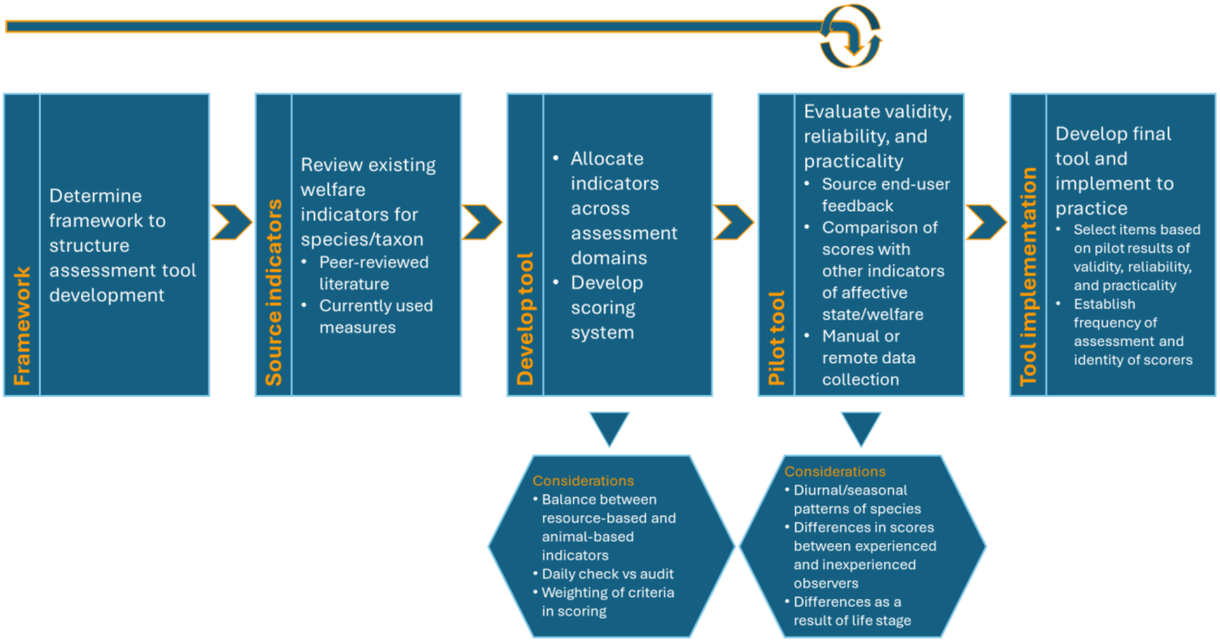


Figure 2.1. Modified from Jones et al. (2022) (Creative Commons Attribution CC BY 4.0 DEED, https://creativecommons.org/licenses/by/4.0/), a flowchart outlining the process of developing a welfare assessment for use in zoos and the considerations at each step. This chapter discusses the process of development up to piloting, and therefore does not cover tool implementation.

The initial step of deciding on a Framework (Figure 2.1) was achieved with the initial RZSS welfare assessment tool since it was modified from the BIAZA welfare toolkit that is based on the Five Domains. However, to further improve the tool, we aimed to address some of the major gaps and drawbacks of all the current frameworks and to incorporate their strengths. Table 2.1 summarises the strengths and drawbacks of the frameworks I described above which we assessed when considering which aspects of the frameworks we wanted to incorporate and which we wanted to minimise in the development of the tool. Ultimately, the strengths we wanted to incorporate were balance between resource-based and animal-based indicators of welfare, assessing positive and negative animal indicators, evidence-based approach to understanding animal outputs in the context of their environment and in response to changing external factors, sustainability and efficiency without being labour and resource intensive or compromising reliability, includes assessments by care staff familiar with the animals, and addresses cycles and the continuous nature of welfare (a few of these also being suggested for consideration under the ‘develop tool’ and ‘pilot tool’ steps by Jones et al. (2022), Figure 2.1).

Table 2.1. Summary of the strengths and drawbacks of the welfare assessment frameworks/processes considered for inclusion and exclusion in the welfare assessment tool developed.

	Universal Animal Welfare Framework (Kagan et al. 2015)	Evaluating behaviour budgets (Watters et al. 2019)	Animal Welfare Assessment Grid (Justice et al., 2017)	European Welfare Quality© project (Blokhuis, 2008)	Sherwen et al. 2018 Risk Assessment Based on Five Domains	24/7 Across the Lifespan Approach (Brando and Buchanan-Smith 2018)
Measures resource inputs	✓		✓	✓	✓	✓
Measures animal outputs		✓	✓	✓		✓
Strengths						
Identifies resource-based risks to welfare	✓				✓	
Efficient	✓		✓		✓	
Non-invasive	✓	✓	✓	✓	✓	✓
Centralises animal in welfare assessment		✓	✓	✓		✓
Considers assessment of caretakers familiar with individual animals		✓	✓	✓		✓
Easily interpretable results	✓		✓		✓	
Addresses both positive and negative welfare states		✓		✓		✓
Addresses the continuous nature of welfare 24/7 across the lifespan						✓
Weaknesses						
Resource and labour intensive		✓		✓		✓
Does not evaluate animal-based indicators	✓				✓	
Results can be misleading due to lack of information on other domains of welfare	✓	✓			✓	
Mainly focuses on negative animal indicators			✓			

Many aspects of an animal's welfare state (hormonal cycles, sleep quality, breeding, and general physiological health) are influenced or regulated by daily, annual and life cycles. Therefore, incorporating the assessment of behavioural rhythms solves the large concerns with using the expression of natural behaviours as an indication of positive welfare and the potential misinterpretation of time budgets. Rhythms can provide the context that activity budgets ignore by identifying whether behaviours are expressed in the appropriate context and at times of day, year, or life stage that are adaptive. Therefore, this allows us to more accurately interpret neutral behaviours as positive or negative. In addition, we want to address a major drawback with many of the frameworks of the disconnection between the assessment of the resource inputs and animal outputs. Our evidence-based approach will look at behaviours in response to their environment and towards specific environmental features through enclosure use data, allowing this approach to directly connect the resource inputs and assess whether the desired animal outputs are actually achieved in response to these environmental features and not simply assumed. This approach investigating natural rhythms in conjunction with their response to the environment would allow us to understand the contexts in which positive welfare states are achieved so that they can be promoted, and also the context in which negative welfare states occur so that targeted interventions can be proposed to more directly and effectively address welfare issues.

This study is a pilot study developing a holistic, highly informative, self-sustaining welfare assessment program that can be set up as a welfare monitoring program where behaviour is regularly recorded. The assessment program will have two components: (1) a welfare assessment tool which is the modified/improved RZSS assessment questionnaire (the questionnaire adapted from the BIAZA toolkit) and (2) an evidence-based protocol to the behaviour and environment questions. One of the biggest issues in developing a self-sustaining program that allows for in-depth assessments that can be conducted regularly is the time and resource constraints. Through this pilot we show how these assessments can be conducted at the group and individual level to demonstrate how quicker group assessments can be completed that can then lead to more in-depth analysis of individual welfare if concerns are flagged. In addition, we show a proof of concept of collaborating with university students to collect and analyse this data to reduce the labour of zoo staff. Ultimately, the goal is to create a welfare assessment program that has training materials and available ethograms that can be shared and applied across institutions.

A collaborative effort between zoos to build-up rhythms of behaviour to create baselines for comparison would also allow for the large gap in knowledge on what healthy behaviour within the context of a zoo is. This is important given that natural, wild behaviours are not always appropriate for comparison since the zoo environment is substantially different from the wild environment, resulting in behavioural responses that may be adaptive or maladaptive depending on whether the same goal or positive affective state can be achieved (Boissy et al., 2007; Howell & Cheyne, 2018). A standard of welfare assessment needs to be established in the zoo community with the increasing importance being placed on welfare. This project demonstrates the development of a holistic approach that provides detailed context and information on institutional and animal level welfare indicators that can be applied across species and across zoos.

2.2 Methods

In this section I describe the process of developing the welfare assessment tool in collaboration with RZSS Edinburgh Zoo. The BIAZA welfare toolkit which the RZSS welfare assessment is based on, encourages that multiple assessors from different departments (e.g. keeper, veterinarian, researcher) complete the assessment individually (Harley & Clark, 2019). This is suggested so that expert perspectives are gained for all kinds of questions. For instance, making sure to have an expert that understands veterinary records or someone who is very familiar with the animals and their daily behaviours and routines. The assessment process in RZSS followed these guidelines and there were always at least two assessors per species/individual animal. However, having each assessor complete the entire 50 question assessment is time consuming. Therefore, in my analysis, the first aim was to test concordance between assessors to determine whether it was necessary for all assessors to answer all questions or only a set of questions based on their expertise. The next aim was to assess concordance within each category of the assessment to analyse whether the assessment itself is reliable in addressing the general categories it aims to. The final aim was to develop an evidence-based approach for responding to behavioural and environmental category questions. These three efforts would ultimately be to make the welfare assessment process more reliable and efficient.

2.2.1 Ethics

We received ethical approval for this study from the University of Stirling Animal Welfare Ethics Review Body (protocol #3284 2490; 23/07/2021). In addition, we submitted a project proposal to RZSS Edinburgh Zoo and received approval (08/09/2021).

2.2.2 Initial consultation on current welfare assessment protocol

This project was a full collaboration between the University of Stirling and RZSS Edinburgh Zoo to ensure that the welfare assessment tool developed was fit for purpose for the zoo's needs, with a full understanding of the zoo's goals and limitations. To determine the best approach in making the welfare assessment more reliable and efficient, our first step was to have an open discussion in March 2021 as collaborators with the RZSS curator (JE), who oversees the collation of the data and information for the welfare assessments, to learn about the welfare protocol in place. These discussions would help us understand how the 'source indicators' and 'develop tool' step in Figure 2.1 were completed when RZSS was internally developing it, and also the areas where we would want to develop the tool further in a collaborative effort between RZSS and University of Stirling.

The welfare assessment approach developed by JE was based on the BIAZA welfare toolkit which follows the Five Domains approach to welfare assessment (Harley & Clark, 2019; Mellor et al., 2020). The assessment (Table A1) consisted of 50 questions in total split into five categories: (1) Nutrition; 4 questions, (2) Health; 9 questions, (3) Environment; 14 questions, (4) Husbandry; 9 questions, (5) Behaviour; 14 questions. These questions could be responded to with five possibilities: Yes, No, Unknown, N/A, or left blank. The RZSS process consisted of 2-4 assessors each responding to all 50 questions. Assessors for each assessment were a combination of the curator, species keepers, animal team leaders, and veterinarian but did not always include an assessor from each of these groups. The behaviour category was completed by conducting a 15-minute observation session and then responding to questions. The keeper would also use previous knowledge and awareness of their animals' behaviours to respond to questions in this category.

In our consultation, we were all in agreement that the questions covered all the key resource-based (input) factors that affect welfare, as well as key animal-based (output) factors and would be effective in accurately determining the welfare state of animals. The two issues that we decided to address to make the welfare assessment process more accurate and reliable were (1) the efficiency with which all 50 questions would be answered and (2) the amount of evidence used to address questions in the behaviour and environment categories. Below we discuss how we addressed these topics and the solutions we tested throughout this process.

The first area we addressed was the efficiency of the process. With multiple assessors needed to respond to all questions, the time investment to complete the process meant it may take years to assess the entire RZSS collection (in 2023 approx. 230 species, and approx. 2,700 animals – RZSS Edinburgh Zoo, RZSS High Wildlife Park). Though keepers continuously monitor the welfare of their animals through their care and flag welfare issues, infrequent full and comprehensive welfare assessments may mean that more complex or insidious welfare risks may slip under the radar until they have progressed to a more obvious stage of being a welfare issue. In addition, it would be difficult to assess positive welfare states and the conditions which promote them because conditions, life stages, and the needs of the group/individual might change significantly between assessments.

The second area addressed was the evidence which the behaviour responses are based on. Though a reasonable estimate may be gained from a 15-minute observation session, systematic data collection accounting for more full cycles of behaviour (i.e., daytime, nighttime, and seasonality) would help in adding more context to the observed behaviours and consequently result in more informative conclusions. In these instances, the keeper's long-term knowledge of displayed behaviours by the animals in their care can be incredibly helpful in directing data collectors towards behaviours to focus on, but relying entirely on keeper's recall could result in some inaccuracies due to the keepers having many animals in their care and a variety of responsibilities. In addition, several environment questions ask if certain environmental features are appropriate, but as in many zoos, evidence is not always collected on whether the resource inputs are achieving their intended purpose with the animal. Evidence of the use of space and environmental features can easily be recorded with the proposed methods without adding extra data collection aside from the behavioural data collection.

2.2.3 Addressing efficiency and validity of welfare assessment by analysing concordance of assessors and covariance of assessment questions

With all assessors responding to all 50 questions, including questions pertaining to categories outside of their expertise (e.g., veterinarians answering questions on husbandry), the process of collecting all responses can be lengthy. Therefore, we wanted to determine whether it would be appropriate to have assessors focus their efforts in areas of their expertise. So, veterinarians answering health questions, keepers answering husbandry, behaviour, and environment questions, and the curator collecting information for the nutrition category along with bringing general expertise to the husbandry, behaviour, and environment categories. JE also streamlined this process further by making it automated and allowing for questions to be answered on tablets and phones, thus removing the extra steps of transferring paper responses to electronic data sheets.

2.2.3.1 Assessor concordance to address efficiency

To decide whether it would be appropriate to delegate questions in this matter, I first had to determine if there was sufficient agreement between assessors on all questions to say that one assessor could provide representative responses. I did this by conducting concordance analyses between assessors' responses on the previously completed assessments. I used Fleiss Kappa concordance tests for each individual/group assessment to determine the level of agreement between assessors for each assessment.

2.2.3.2 Testing validity of assessment questions

We also wanted to determine whether the questions within each category were properly testing the overarching question of each category. Since in the assessment, a 'Yes' response was indicative of a positive welfare indicator and a 'No' response of a negative welfare indicator, we would expect covariance of responses within each category if the individual questions are all addressing the overarching question accurately. I tested this with Cronbach's alpha for responses across assessments but within each category, as well as for the overall assessment (all 50 questions). Cronbach's alpha is appropriate because it gives a measure of covariance relative to variance within these categories. A higher Cronbach's alpha is desired as it is indicative of covariance within these categories. In addition, to measuring the consistency within each category, we also conducted an omitted variable analysis where the Cronbach's alpha is recalculated when each question is omitted one at a time. When the Cronbach's alpha increases after omission, this suggests that the omitted variable tends to have a response that has high variance within the category and does not consistently covary with the other responses within the category.

2.2.4 Taking an evidence-based approach to behaviour and environment questions

2.2.4.1 Species selection

To determine whether the evidence-based approach to the behaviour questions is effective for assessing welfare across the species held in the RZSS Edinburgh Zoo collection, we wanted to choose a group of species that span across vertebrate taxa. In our selection, we also wanted to ensure that species which have limited information on their natural histories and behaviour are represented in the sample. This will help to avoid biasing the assessment towards species which have had extensive research on their behaviours and natural histories and ensure that the assessment can properly determine the welfare of species even when background information is limited, as is true for many species in zoo collections. In addition, choice of species was also determined by those which had a recent welfare assessment at the time of data collection, or an assessment that would occur simultaneously with behavioural data collection. With these criteria, we selected 10 species across four of the major taxa in vertebrates (fish, mammals, reptiles, and birds) (Table 2.2). These species were the yellow-breasted capuchin (*Sapajus xanthosternos*), brown capuchin (*Sapajus apella*), chimpanzee (*Pan troglodytes*), meerkat (*Suricata suricatta*), Nubian giraffe (*Giraffa camelopardalis camelopardalis*), eastern white pelican (*Pelecanus onocrotalus*), anemone fish (*Amphiprion ocellaris*), corn snake (*Pantherophis guttatus*), Taiwan beauty snake (*Orthriophis taeniurus*), and milk snake (*Lampropeltis triangulum*).

Whether the species was assessed at the individual or group level was determined by RZSS Edinburgh Zoo, and affected by ease of individual identification. Doing this allowed proof of concept on whether evidence could be reliably collected on the individual and group level depending on the needs of the zoo. To ensure that group and individual level observations were capturing the same data, we compared results between individual and group observations for the pelicans.

Table 2.2. Details of the species for which behavioural observations were completed.

Species	Taxa	Individual/Group	Other identifiers
Chimpanzee	Mammal	Group	2 groups separating mother and son through fission fusion groups
Yellow-breasted capuchin	Mammal	6 individuals	Female and male pairs in monkey house and 2 males housed individually
Brown capuchin	Mammal	6 individuals	3 males and 3 females. 3 from East group and 3 from West group. 2 from each rank: Low, Middle, High.
Meerkat	Mammal	2 groups	Male group indoors, female group outdoors
Nubian giraffe	Mammal	5 individuals	5 males housed together
Eastern white pelican	Bird	7 individuals and group	3 male/female pairs and 1 male
Anemone fish	Fish	4 individuals	Mating pair and 2 neutral individuals
Corn snake	Reptile	Individual	Male
Milk snake	Reptile	Individual	Male
Taiwan beauty snake	Reptile	Individual	Male

2.2.4.2 Ethogram development

In total, there were 19 questions that could be answered with systematic behavioural observations. These questions are listed in Tables 2.3 and 2.4. This includes all questions from the behaviour category and 5 questions from the environment category. In creating the ethograms, we wanted to ensure that each question could be answered with direct observations from the ethogram. Overall, the 14 behaviour questions are designed to ask about positive and negative behaviours and in general, species-typical behaviours. Ethograms within each taxa had general behaviours that would apply to most species within that taxa and then additional behaviours which might be more species-specific. For instance, locomotion, feeding/foraging and resting/sleeping were behaviours added to all mammal ethograms, but a behaviour like burrowing would be specific to the meerkat ethogram. Therefore, we were able to create skeletal ethograms within each taxa that could then be modified according to the species. In addition, we also added in behaviours or categories that were specific to welfare concerns that keepers may have flagged for their species and wanted more detailed data on. For instance, keepers may wish to know how alert an animal was of a predator which was housed nearby and in their line of sight.

We used the online application, *ZooMonitor* (Ross et al., 2016), to conduct our observations. The flexibility of the app allowed us to additionally address 5 questions in the environment domain which normally cannot be answered in high detail with traditional behavioural ethograms. These questions in general ask if the environmental features are appropriate, allow for species-specific behaviours, and are useful across different climatic conditions (Table 2.4). *ZooMonitor* has a useful feature where heat maps of the animal's location within the enclosure can be created. Enclosure maps were uploaded to the app that were either aerial views of the enclosure or renditions that indicated where key environmental features were. With this, we were able to track how often the animals were in specific areas of their enclosure and how much of the enclosure was actually used by the animal. In addition, heat maps could be created for specific behaviours. Therefore, we could determine where in the enclosure the animals were more likely to display specific behaviours like resting/sleeping or locomotion. We could also see enclosure use on days with specific weather conditions by adding a question at the start of the session on the weather conditions if they were housed outdoors or with access to the outdoors. All of these features allow for an evidence-based approach to the welfare assessment questions on whether the environment is appropriate and whether resource inputs are allowing for positive animal outputs, which would not be possible without the flexibility and user-friendly interface of *ZooMonitor*.

Table 2.3. Behavioural domain questions and colour-coding grouping them into over-arching questions being asked in the domain.

🟧 Is a positive behaviour being displayed?

🟩 Is an interaction positive?

🟨 Is a natural behaviour being displayed at the appropriate levels?

🟦 Is a negative behaviour being displayed?

	Behavioural domain questions
🟨	Performs appropriate levels of self-care behaviours (grooming, preening, drinking, resting, comfort activities)
🟩	Has mostly positive interactions with conspecifics or other animals
🟩	Has mostly positive or neutral interactions with staff/visitors
🟩	Responds appropriately to novel changes in the environment (interest in appropriate enrichment vs fear/aversion/apathy)
🟧	Can express choice and control over being in different (indoor/outdoor) areas (except for maintenance periods)
🟧	Exhibit appropriate territorial behaviour (patrolling, scent marking)
🟨	Exhibits appropriate foraging and feeding behaviours
🟧	Exhibits play behaviour (alone or socially)
🟨	Exhibit appropriate levels of rest and sleep
🟩	No evidence of dysfunctional social interactions
🟦	No evidence of abnormal or stereotypic behaviour
🟨	Exhibits reproductive behaviours as appropriate to the species and individual (courtship, mating, nest-building, incubating, birth, rearing etc.)
🟧	Exhibits species-specific behavioural needs (rooting, burrowing, climbing, perching, social grooming etc.)

Table 2.4. Select environmental domain questions and colour-coding grouping them into over-arching questions.

🟪 Are there appropriate shelters?

🟩 Is the enclosure and furnishings allowing for natural behaviours to be displayed?

	Select environmental domain questions
🟩	The size, shape and topography of the enclosure is appropriate for the species to exercise, explore and exhibit normal territorial behaviours
🟩	Substrates are suitable for the species (consider locomotion (abrasion, traction, support); resting (comfort, depth, cleanliness); foraging (depth, cleanliness); burrowing (will support tunnels, depth, secure))
🟪	Has appropriate shelters, retreats, visual barriers, off show areas from conspecifics and visitors
🟪	Has appropriate shade and shelter from weather/climate
🟪 🟩	Planting is appropriate for the species, providing shelter, shade, retreats, microclimate provision, feeding opportunities, and plants are not toxic and do not present an escape risk

🟩	Furnishings allow appropriate species-specific behavioural needs (climbing, swinging, jumping, perching, nesting, stretching, hiding, sleeping, flight, etc.)

2.2.4.3 Sustainability of data collection and analysis through collaboration with university students

One of the main goals of this study was to create a self-sustaining protocol to measure animal outputs continuously. Zoos have difficulty maintaining these kinds of programs because of the limited resources with staff and time (Brando & Buchanan-Smith, 2018). We aimed to help this by creating a program with the University of Stirling where students that are completing their theses, placements, or simply volunteering, could collect the empirical data on animal outputs. Collaborating with university students to collect and analyse data avoids having to require keepers to collect this data and for welfare staff to do the bulk of analysis. The time commitment for those engaging in behavioural data collection can be found in Table 2.5. Training students with different levels of experience and background in animal behaviour was a proof of concept on the ability to train people with no, to little experience in behavioural data collection with *ZooMonitor.* This approach can later be extended to the general public through vetting of volunteers for the data collection portion since *ZooMonitor* is user-friendly for non-experts. Though we did not train non-university students in this study, overtime, a range of projects with varying levels of difficulty can be made for continuous behavioural monitoring that would allow people with different skill levels to collect behavioural data.

2.2.4.3 Reliability testing

In total, 6 observers assisted in data collection across the different species. Students were writing dissertations or doing placements and were therefore assigned to one to three species to observe. Observers were trained on the ethograms and the use of *ZooMonitor* in-person (Table 2.5). To produce data that would be used for the welfare assessment, observers had to pass reliability testing. Since testing reliability purely from live observations results in many ethogram behaviours not being evaluated (Wark et al., 2021), we designed our reliability testing with two stages aiming to cover all ethogram behaviours as best as possible. The first stage was an ethogram quiz for which the observer had to receive >80%. The quizzes covered the definitions of each behaviour within the ethogram with a mixture of multiple choice, true/false, fill in the blank, definition, and scenario questions.

The second stage was live inter-observer reliability using the *ZooMonitor* projects in-person at the zoo. In the future, this stage can be completed with pre-recorded videos. Regardless of whether the project was for group or individual assessment, the observer would do reliability with an individual animal. The observer had to match 70% of observations with the lead investigator to pass. Observers were not allowed to begin data collection until they passed two or three in-person reliability tests depending on the difficulty of the ethogram. For simpler ethograms, like the pelican ethogram, three sessions had to be reliable. Observers were allowed unlimited attempts to pass, though reliability testing was ended if too many consecutive sessions were failed. In the future, when volunteers are incorporated, this same cut-off can be applied as to not require so much effort and time from zoo employees who are training volunteers.

Table 2.5. Estimated time commitment for each stage of behavioural data collection. The 3-day minimum for stage 4 (data collection) is an estimate of the least amount of time it would take to complete 2 sessions per hour (a minimum standard) of the working day. Ideally, this would be repeated several times throughout the year (at least once per season) and the 3 days of collection would be spread across 2 weeks.

Data collection stage	**Time commitment**
1. Familiarisation with *ZooMonitor* and chosen animal ethogram	1 day
2. In-person practice using *ZooMonitor*	2 days
3. Reliability testing a. Ethogram quiz (80% to pass) b. Video/In-person *ZooMonitor* reliability (70% reliability, 2-3 reliable sessions needed depending on difficulty of project)	Quiz: 1-3 days ZM: 2-5 days
4. Data collection for welfare assessment	3 days-1.5 weeks (dependent on species)

2.2.4.4 Data collection

Once reliability testing was passed, observers were allowed to collect data for use in the welfare assessments. Data collection was scheduled so that sessions were spread across the day, with the final spread of data being 2-4 sessions per hour during opening hours (10am-4 or 5pm depending on the season). This systematic data collection enabled us to gain an estimate of the daily cycles of behaviour and enclosure use which would remove bias if all sessions were recorded at one time of day. We also attempted to spread the sessions across two weeks minimum to best attempt to capture different weather conditions. The lengths of sessions per species and number of intervals per session can be seen in Table 2.6. This information shows how sessions do not need to be long to gain decent estimates of behaviour for groups and individuals. In the future, we would want to extend this data collection to the night, possibly employing cameras so that sessions can be completed by reviewing nighttime footage during working hours. This would allow an even more informative dataset that can be slowly built into baseline data that would emphasise sleeping behaviour as a crucial aspect of welfare.

To show how this approach can be done slowly over time, still capturing decent estimates of 24-hr cycles with fewer sessions at the beginning of building baselines, I present an example of data collection with giant panda behavioural data from Chapters 3 and 4. The example is meant to show how systematic data collection spanning across 24-hr periods can be informative and useful for capturing and understanding the adaptive circadian rhythmicity of a species. An overview of the methods we employed to make the RZSS welfare assessment more efficient and reliable can be found in Figure 2.2.

Table 2.6. Session lengths, number of intervals, and number of sessions completed for each species' *ZooMonitor* project.

Species	Type of observation	Session length	Session intervals	Sessions completed
Chimpanzee	Group	25 min	10	27 (11.25 hr)
Eastern white pelican	Group	10 min	15	24 (4 hr)
Meerkats	Group	10 min	10	37 (6.16 hr)
Yellow-breasted capuchins	Individual	10 min	20	40 (6.67 hr)
Brown capuchins	Individual	10 min	20	72 (12 hr)
Nubian giraffe	Individual	10 min	20	140 (23.33 hr)
Anemone fish	Individual	10 min	20	47 (7.83 hr)
Snakes	Individual	10 min	20	30 (5 hr)

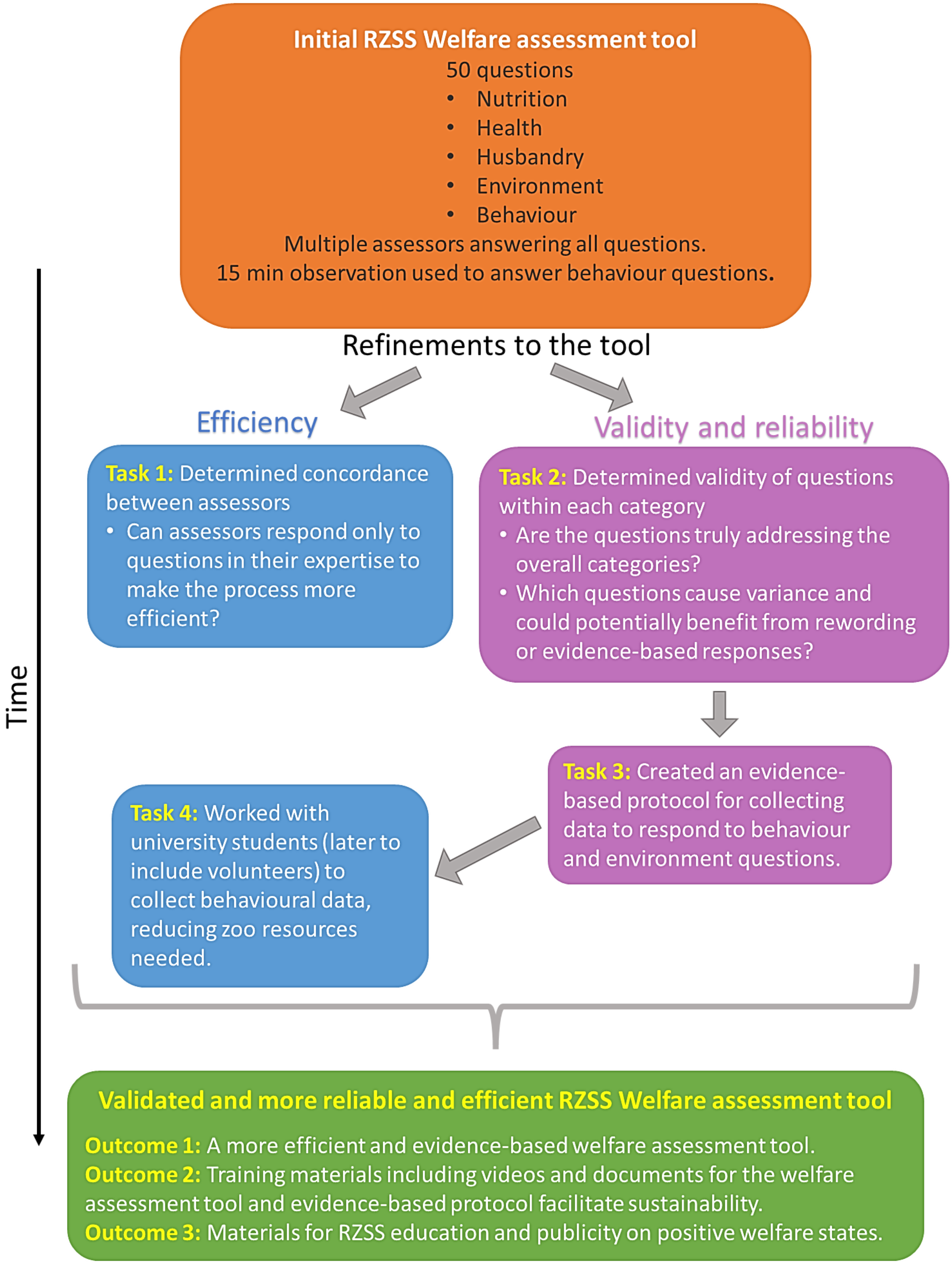


Figure 2.2. A diagram outlining the process of validating the RZSS Welfare assessment tool and making it more reliable and efficient (modified from Gandia et al. (2024)).

2.3 Results

The work we did with this project was pilot work to understand how a zoo-wide welfare assessment can be made more efficient and reliable. The process of doing this was trial and error. In this section we will go over the different outcomes of this piloting process (’pilot tool’ step in Figure 2.1) and discuss where we believe the assessment protocol could be readily applied and where some further improvement, identified through piloting, may be possible to make it more sustainable. Overall, our modifications to the assessment process did result in a more efficient and evidence-based approach with the only added effort from zoo staff being equivalent to what would be applied to other research conducted in the zoo.

2.3.1 Modification 1: Justifying reducing assessors and delegating questions with concordance

2.3.1.1 Concordance between questions within each category of welfare assessment

Within each category of the assessment there seemed to be decent concordance (Table 2.7), suggesting that the covariance within each category was high and the categories were likely addressing the overarching theme well. The environment, husbandry and behaviour categories had very good internal consistency. The health and nutrition categories had an acceptable amount of internal consistency, though on the cusp of being questionable (Cortina, 1993). The omitted variable analysis revealed that 7 questions of the 50 in the assessment seem to cause variance within their respective categories (Table 2.8).

Table 2.7. Concordance for responses to questions within each category.

Category	Question range	# of questions	Cronbach's alpha
Nutrition	1-4	4	0.7865
Health	5-13	9	0.7190
Environment	14-27	14	0.9212
Husbandry	28-36	9	0.8798
Behaviour	37-50	14	0.9189
Whole assessment	1-50	50	0.9495

Table 2.8. Questions, that when omitted, resulted in higher Cronbach’s alpha for their category. The omitted variable column indicates the question number in the assessment (*Table A1*). The last column indicates the new Cronbach’s alpha for the category when the variable is omitted in comparison to the previous Cronbach’s alpha for the category seen in Tabe 4 (in brackets).

Omitted variable	Category	Question	Cronbach's alpha when omitted
2	Nutrition	A diet sheet is available for the species and is reviewed incorporating best practice guidelines for nutrition and evidence-based literature as available.	0.8935 [0.7865]
8	Health	Faeces are appropriately formed and normal for the species.	0.7312 [0.7190]
14	Environment	Temperature levels/gradients are within parameters appropriate for the species year-round (consider internal/external, seasonal, night-time, appropriate variation, choice, records, etc.).	0.9315 [0.9212]
15	Environment	Humidity levels/gradients are within parameters appropriate for the species year-round (consider internal/external, seasonal, night-time, appropriate variation, choice, records etc.).	0.9310 [0.9212]
16	Environment	Light levels, quality and photo period are within parameters appropriate for the species year-round (consider UV, photoperiod, flicker/glare, colour, internal/external, seasonal, night-time, appropriate variation, choice, records etc.).	0.9269 [0.9212]
33	Husbandry	Water feature is safe and of a depth/size/volume/gradient that allow species-specific natural behaviours.	0.8826 [0.8798]
45	Behaviour	Exhibits play behaviour (alone or socially).	0.9216 [0.9189]

For environment, the omitted variable analysis suggested that the environment questions that seemed to have lower covariance with the rest of the category's questions were exclusively questions that asked about whether different environmental conditions (temperature, humidity, and light) were appropriate year-round. These are factors which are difficult for zoos in general to measure year-round if a specific system to regulate these factors throughout the day and seasons is not in place. Because of this, a response to this question becomes more subjective. For instance, in a single assessment, an assessor might score "Yes" if no issues were encountered throughout the year, but another might score "Unknown" because there were no measurements, and a third might score "No" because the species had to be housed indoors for some days of the year due to more extreme weather conditions. The remaining questions in the environment category do not ask about the appropriateness of conditions year-round. Therefore, these 3 questions causing variance in the environment category highlights an issue that many zoos face in not having the resources to monitor environments year-round. Through the development of this project, future *ZooMonitor* projects can be made that could be used daily or weekly to record the temperature, humidity and light easily to provide clear evidence for the response to this question. These can be time-point surveys that can be incorporated into a routine that would only take less than 5 minutes to complete. If *ZooMonitor* is used, these results would automatically be downloaded into a database that can then be accessed by the welfare curator at the time of the welfare assessment to see the responses to these questions on a 24-hr and yearly scale to ensure that the conditions were appropriate throughout the day and seasons.

For husbandry, the omitted variable analysis revealed that the question on whether the water feature is safe and allowed for species/specific behaviours caused the most variance within the category. The difference was minimal, and the reason it likely did not covary with the other responses was because for 50% of assessments, it was not applicable. However, to improve the reliability of the response to this question in general, the data collected from the enclosure use can be used to determine how often they are around the water feature and what behaviours they display when they are around the water feature.

For the behaviour category, the only question which increased the Cronbach's alpha when omitted was on whether play behaviour is displayed. This is a rare behaviour, particularly with adult animals, and therefore may likely cause variance within the category because it is unlikely for the assessors which are not the animal keepers to catch it in the 15 min observation session done for the assessment. The keepers which know their animals, can recall having seen them play or not while other assessors would only have the ability to respond to the question from the 15 min observation. This behaviour is unlike mating/reproductive behaviours, the other behaviour specifically asked about in the assessment, because zoo staff do monitor mating/reproductive behaviours closely, and all relevant staff would be notified when these behaviours are displayed outside of the welfare assessment. This result of the omitted variable analysis showing play as causing variance highlights an area where the behavioural data collection would be useful and increase reliability of this response since assessors can reference evidence from more extensive behavioural observations to respond to it.

The two categories with lower, but still acceptable, Cronbach's alpha values were nutrition and health. For nutrition, the omitted variable analysis revealed that the question on whether a diet sheet was available caused the most variance within the category. However, it should be noted that since this category had only 4 questions, it is easier for one question to cause variance. Regardless, this question coming out in the omitted variable analysis shows how perhaps having assessors respond to questions in their area of expertise may be helpful. The welfare curator is responsible for collecting information from the literature on best practice guidelines for diet and nutrition and sharing that information with keepers. Therefore, their response to this question would be the most valuable and accurate.

For health, the question on whether faeces was healthy seemed to cause the most variance within the category. The improvement of the reliability and consistency of the responses to this question can also be achieved through the *ZooMonitor* data collection. This can be a part of a survey, or added as a question before a behavioural observation session. The data can then be accessed at the time of the welfare assessment.

Overall, there was good consistency and covariance within the different categories of the assessment with only a few questions that caused some variance. This data can help to target what areas of the assessment can be improved with an evidence-based approach to produce more consistent responses or questions that would be better addressed by the staff with the most accurate information. Sherwen et al. (2018) similarly produced a welfare assessment that assessed both resource inputs and animal outputs based on the Five Domains. However, the internal consistency of each category was not measured. This data demonstrates a method of validating assessments and highlighting areas that can be expanded or improved.

2.3.1.2 Concordance between assessors to justify reducing number of assessors

I analysed the agreement between assessors for 17 individual animals/groups. Each assessment was completed by 2-4 assessors. The average Fleiss Kappa value for agreement between assessors across assessments was 0.347 (Table 2.9), which is a fair amount of agreement between assessors, but not particularly strong (Landis & Koch, 1977). To understand where this agreement or disagreement between assessors stemmed from I also analysed the Fleiss Kappa values for each possible response in the assessment (Table 2.10). Each question had five possible responses: No, Yes, Not applicable, Unknown, or left blank. The only response with substantial agreement was ‘Not applicable’ (averaged K = 0.6659). The main responses of ‘Yes’ (averaged K= 0.3891) and ‘No’ (averaged K= 0.2717), both had fair agreement. The ‘Unknown’ (averaged K= 0.1111) and blank (averaged K= 0.0146) had poor and slight agreement, respectively.

Table 2.9. Average of the overall Fleiss Kappas for all assessments. Under kind of assessments, “All” is the combination of the individual and group assessments. The number of assessments is the number of individual animals/groups assessed. However, each individual/group had multiple assessors, so the number in parenthesis is the total number of assessments completed by individual assessors. The average number matched is the number of responses matched by all assessors in a single assessment.

Kind of assessments	Number of assessments	Average number of assessors	Average number matched	Average percent matched	Fleiss Kappa
All	17 (52)	3.06	23.94	47.88	0.3476

Individual	9 (27)	3	24.56	49.11	0.3498
Group	8 (25)	3.13	23.25	46.50	0.3450

Table 2.10. Average Fleiss Kappa for each possible response within the assessment.

Kind of assessments	No	Yes	Not applicable	Unknown	Blank
All	0.2717	0.3891	0.6659	0.1111	0.0146
Individual	0.2005	0.3920	0.7167	0.1099	-0.0178
Group	0.3517	0.3858	0.6087	0.1124	0.0523

These results indicate that most of the agreement between assessors came from questions which were not applicable to the individual/group. This could potentially skew the agreement to be higher with responses that are not relevant to the animal anyway. However, the poor agreement between unknown and blank responses would also skew the agreement to be lower.

The substantial disagreement on the unknown and blank answers suggests that when one assessor wrote unknown or left the response blank, there would be another assessor which did respond with yes or no. These results support the argument that it may be more effective to have assessors respond to questions where they have relevant or specialised knowledge, without losing reliability of accurately identifying the agreed response. This process may look like having veterinarians answer health category questions, or questions pertaining to physical health, keepers answering questions on behaviour and husbandry, and the welfare curator answering questions that require searching through the literature for best practice guidelines or species requirements. In this manner, there would be a smaller workload for each assessor, while maintaining the accuracy of responses to each category of questions. This would allow for a more efficient welfare assessment process, which is already resource intensive, and would mean more assessments can be completed in shorter timespans. This suggestion can be further justified by the fact that after all assessors complete the assessment, they have a consultation to create a consolidated assessment for that individual animal or group, which the welfare interventions are then based on. Therefore, for questions which are left blank or unknown for certain assessors, the consolidated assessment would still agree with the assessor which did provide a response.

The averaged agreement for the ‘Yes’ (averaged K= 0.3891) and ‘No’ (averaged K= 0.2717) responses was similar to the overall agreement between assessors across all assessments, which is a good sign that the assessors have decent agreement on definitive answers that is reflected across all assessments. However, the slight disparity in agreement between 'Yes' and 'No' responses may suggest that there is more agreement on when an animal is in good condition than when it is not. In the assessment, a 'Yes' response was indicative of a positive welfare state and a 'No' response of a negative welfare state. Therefore, these results may indicate that it is more difficult for assessors to identify and agree on a welfare concern. Therefore, although we do suggest that the number of questions each assessor answers is reduced by only answering questions they are specialised to answer, some questions may overlap in expertise between assessors and may still need multiple assessors to ensure welfare concerns are accurately spotted. The next step in making the assessment more efficient would be to determine which questions should be answered by specialised staff only and which questions overlap between assessors and would still benefit from multiple assessors.

2.3.2 Modification 2: Taking an evidence-based approach to behaviour and environment questions

2.3.2.1 Creating a sustainable observation protocol with university students

Overall, there was success in having several students collect reliable data on a range of animals without significant addition of work to the zoo staff. The curator was consulted at the start to decide which species needed to be observed and to coordinate between researchers and keepers for each species. The keepers were only involved in that they provided some context for the assessments at the start and for certain species updated the researchers on relevant information like housing rotation schedules. The researchers were responsible for collecting and analysing the data. This process of collaborating with university students is a proof of concept of suggestions that university research can greatly improve efforts to create knowledge bases and investigate in more detail welfare risks (Sherwen et al., 2018).

Zoos planning to extend this to a volunteer program would need to understand the time commitment and have a system in place ready to handle the initial higher workload in establishing the program. The data collection coordinator (the advisor at a university, researcher in a zoo, or welfare curator in a zoo) will have the responsibility of coordinating the observers, training them in behavioural data collection, and conducting reliability testing for them. To reduce the workload on one person, these steps can be managed between a team. The process of training spanned two to three weeks depending on the availability of students (Table 2.5). The most efficient way to complete training is to coordinate cohorts of observers to go through training together to avoid repeating the process for multiple observers individually. Each observer must go through at least two days of in-zoo practice to familiarise themselves with *ZooMonitor* and their study species. In these two days, the data collection coordinator should be working closely with the observers to provide feedback to ensure they are correctly identifying behaviours from the ethograms. Providing feedback will reduce the number of days they need training and more importantly reduce the amount of trials they would need to successfully pass reliability.

In the future, the reliability testing stage could be made more efficient for the data collection coordinator with reliability testing occurring through pre-set videos. Multiple long video recordings (at least two or three times the length of the observation session) of the animal in their enclosure can be collected, and the audio for the *ZooMonitor* beeps overlayed onto the video. This enable one video to be reused for several reliability sessions by moving the beep audio around the video. Then, the data collection coordinator would only need to do the session once themselves and send the video to the student (with timestamp of where *ZooMonitor* audio begins) to conduct a session of reliability. This would mean that the coordinator would not need to schedule extra sessions in-person with observers to conduct reliability, greatly reducing the effort of coordination and reducing the number of in-person days with the observers. Overtime, if many videos are collected, eventually the entire behavioural repertoire of the species can be captured and video compilations can be used to ensure all behaviours are tested (Wark et al., 2021). This method of reliability testing was used for the following chapters' reliability testing on pandas. If the reliability stage is made remote, then the training stage would not be as burdensome since it would only require coordinating the cohort for two days together in the zoo.

In our piloting, not all students were able to pass reliability testing, which meant their data was not useable by the zoo for welfare assessments. Therefore, procedures may need to be put in place where observers are switched to easier species if reliability is not reached after a certain number of sessions or, that observers are removed from the observation program. With university students, the latter may not be possible as the students may be required to produce theses or reports. Another solution would be to establish a vetting procedure before beginning the process with observers to ensure that they are likely to be able to complete the process.

Despite these drawbacks, much more empirical data was collected on the species than otherwise would have been possible with only the zoo's resources. Our results on training students with little to no background on behavioural observations and the species observed mimicked results for reliably training non-specialist students with varying levels of knowledge on elephant behaviour to reliably record behaviour (Webb et al., 2020). This reinforces the notion that non-specialist students and potentially volunteers can reliably collect data and greatly increase efforts to take an evidence-based approach to assessing welfare and understanding behaviour in zoos. In the future, as the program develops, certain steps will be made more efficient if there are files of reliability videos that would only need to be distributed.

2.3.2.2 Providing direct evidence for behavioural and environmental domain questions

2.3.2.2.1 Incorporating behavioural rhythms for greater and more reliable context of animal-based indicators

An important aspect of the behavioural data collected is how it addresses the rhythmicity of these behaviours throughout the day. Up to now, proposed frameworks for welfare assessments that evaluate animal outputs (Blokhuis, 2008; Greggor et al., 2018; Sherwen et al., 2018) only measure whether animals are displaying behaviours at all or evaluating time budgets of behaviours. However, analysing behaviour in this manner removes the context of whether behaviours are displayed at appropriate times of day or year. Timing and cycles of behaviour are adaptive and just assessing time budgets or whether certain behaviours are displayed or not may be misleading. For instance, in a time budget you may see that rest is occurring for the amount of time you'd expect for the species which would be scored as positive. However, if the animal is resting during times of the day that are not natural, then this would actually be an indication of negative welfare which would not be captured by just time budgets. Certain questions in the assessment ask if behaviours are being displayed at the appropriate levels. These questions could be answered with time budgets, but in the following section we will be demonstrating how addressing these questions with rhythms allows for more detailed conclusions to be drawn about social dynamics, interactions with the environment, and individual or group tendencies.

2.3.2.2.2 Addressing the 19 behavioural and environmental questions with evidence: Examples with individuals and groups

To demonstrate how a behavioural protocol could be applied to species across taxa, we conducted behavioural observations for several species (Table 2.2). The same welfare assessment (Table A1) was completed for each species at either the group or individual level. The data from the *ZooMonitor* projects were used to answer all the behavioural category questions and some of the environmental category questions (Tables 2.3 and 2.4 respectively) (19 questions total). Reports could then be created for each group or individual to assist in responding to the select 19 questions with figures generated either directly from *ZooMonitor* or within Excel from the downloaded data.

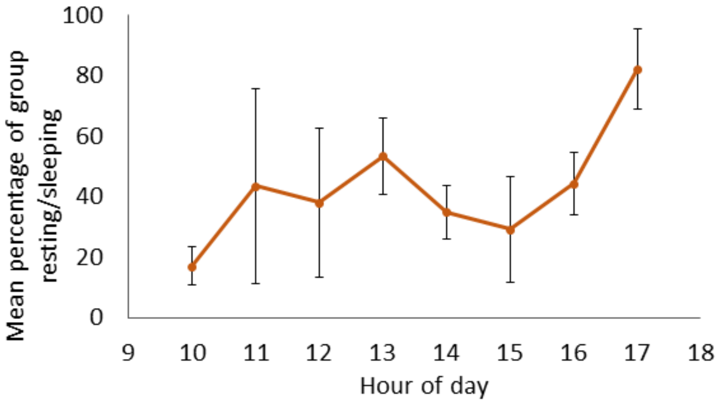
The remainder of this section will demonstrate how data from *ZooMonitor* can be presented to respond to the 19 questions by providing example data for one question from each of the 6 overarching questions mentioned in Tables 2.3 and 2.4. Each figure will have an example for two species, some with an individual and group to show how questions can be answered at the individual or group level. This aspect of the data collection is particularly important to demonstrate since many zoos would not have the time to do in-depth assessments of all individual animals but may need to conduct group assessments that could then lead to further, more meticulous assessments for any flagged individuals. From here, headings will indicate which overarching question is being addressed and which species are used as an example.

Is a natural behaviour being displayed at appropriate levels?: Pelican (group) and Yellow-breasted capuchin (individual)

Figure 2.3 provides data for the question on whether an appropriate amount of rest and sleep is shown for the eastern white pelican group and a yellow-breasted capuchin female. A few questions in the assessment ask if behaviours are displayed at the appropriate levels. Normal behaviours are well defined for captive wild animals, where any behaviour that is exhibited only in captivity or is obviously repetitive or stereotypic is considered abnormal (Hosey et al., 2013; Rose et al., 2017). However, determining what is an appropriate level is difficult without Best Practice Guidelines and detailed literature on behavioural rhythms/activity budgets, which is usually unavailable for most species, including pelicans. Overtime, this data collection can be used to create baselines of rhythms and levels of behaviours for an individual or group. However, in the time that these baselines are not built-up, we can gain an estimate of the groups resting/sleeping behaviour or compare between individuals. From the pelican data we can see that the group tends to rest/sleep throughout the day and in the late afternoon most of the group is resting/sleeping. For the group, we would answer ‘Yes’ given this data. For individual data collection, you can do additional analysis and determine the locations where resting is occurring and elevation in the case of capuchins. This kind of data provides a lot of context about resting/sleeping behaviour for an individual because it allows us to understand where the animal prefers to rest and how the environment might be affecting their rest. It can provide relevant information for welfare interventions if more resting sites want to be added so that the conditions match their preferred spot. For example, for this capuchin, which seems to prefer resting/sleeping on a flat elevated ledge in the outdoor area;

if staff wanted to encourage her to rest in other areas of the yard, they could add elevated, flat sleeping surfaces that provide protection and warmth.

(A) Eastern white pelican group



(B) Yellow-breasted capuchin individual female

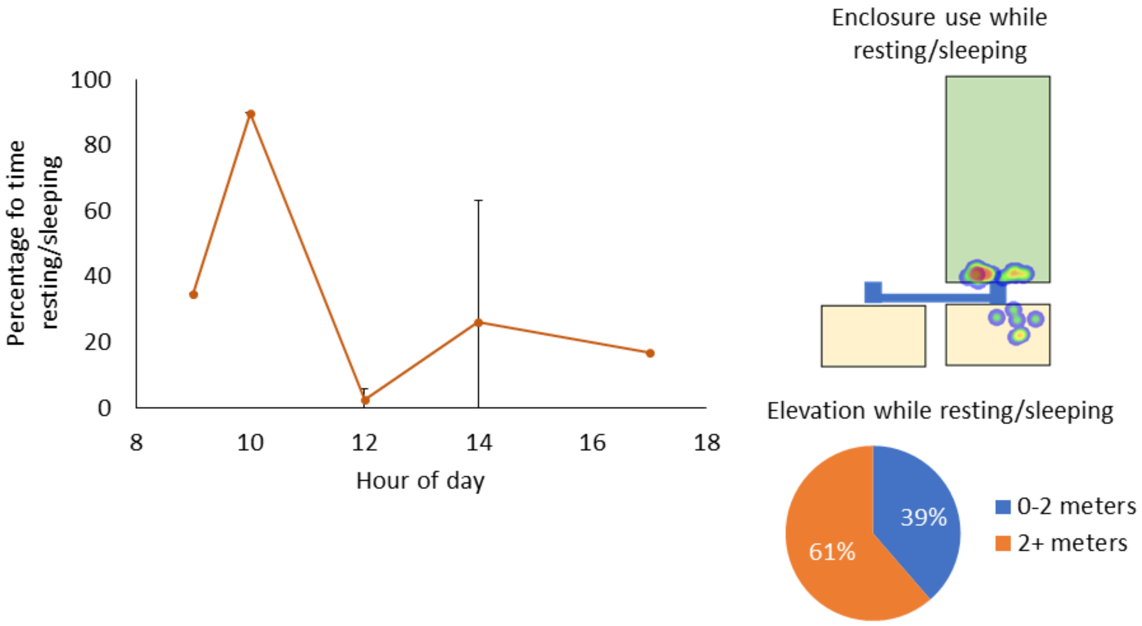
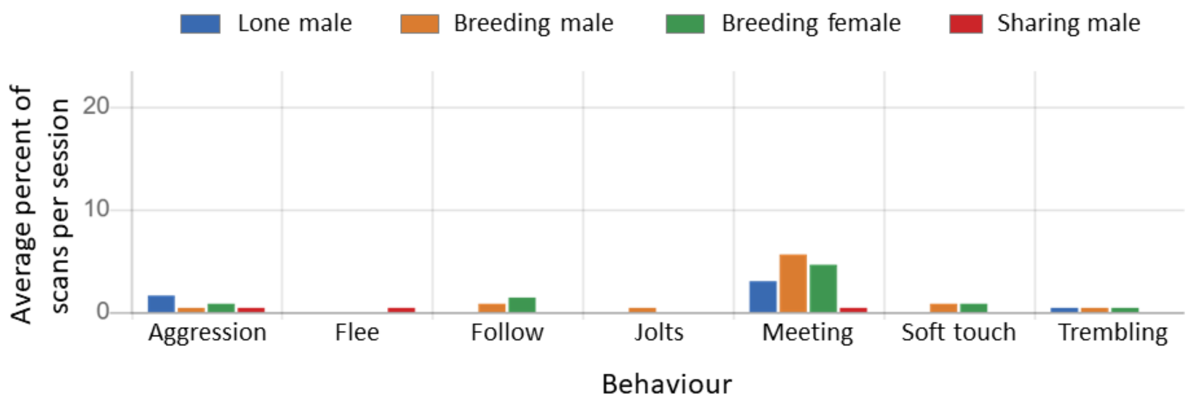


Figure 2.3. Data used to respond to behavioural category question “Exhibits appropriate levels of rest and sleep” from the over-arching question “Is a natural behaviour being displayed at appropriate levels?” for (A) eastern white pelican group (24 sessions, 4 hours) and (B) an individual yellow-breasted capuchin female (7 sessions, 1.17 hours). Bars in the graphs are standard deviations for the sessions averaged in the hour. Note that some points may not have standard deviations if there was only one session recorded in that hour. The capuchin data includes a heat map of her resting/sleeping locations, with the green area being outdoors and the beige area being indoors. The outdoor area she rested on was a ledge (2+ meters) next to the entrance to the indoor area. The capuchin’s data also includes the percent of intervals she was resting/sleeping at different elevations.

Is an interaction positive?: Anemone fish (individual) and chimpanzees (group)

The next example question is asking whether there are mostly positive interactions with conspecifics or other animals. In Figure 2.4 I show how this question can be addressed with either activity budgets or rhythms of the relevant behaviours. The anemone data (Figure 2.4a) is at the individual level and the chimpanzee (Figure 2.4b) at the group level. Anemone fish change sex based on dominance, with all individuals starting life as male and the largest male changing sex to female. In the zoo's reef tank there were four individuals and two anemones. There was a breeding pair on one anemone which will be referred to as 'breeding male' and 'breeding female', a submissive male which shared the anemone with the breeding pair which will be referred to as 'sharing male', and another submissive male who had his own anemone which will be referred to as 'lone male'. For the anemone fish we used a figure created within the *ZooMonitor* app which shows that most interactions are meeting and follow, with some aggression being shown mainly by the lone male. But overall, all individuals showed mostly positive interactions. For the chimpanzees, we show how we are able to compare the types of interactions between two groups of the same species (housed in two fission fusion subgroups separating a mother and a son). From the rhythms of the behaviours of interest (3 positive and 1 negative behaviour) we see that the mother's group does mostly show positive interactions with each other while the son's group seems to have a somewhat equal amount of aggression as positive behavioural interactions within the group. This data should prompt the welfare assessment team to figure out which chimps can be housed with the son that would not result in high levels of aggression.

(A) Anemone fish individuals



(B) Chimpanzee groups

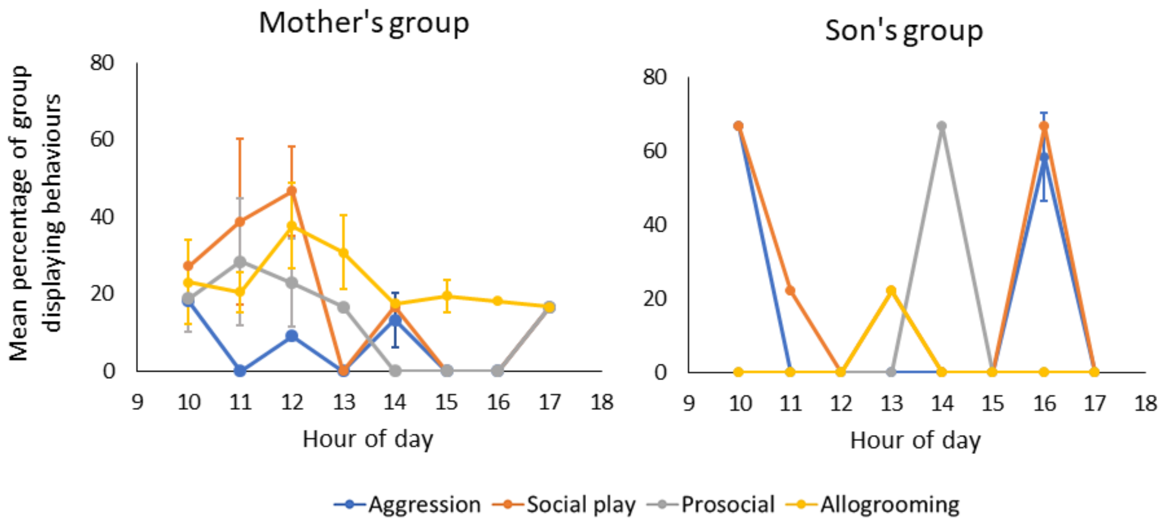
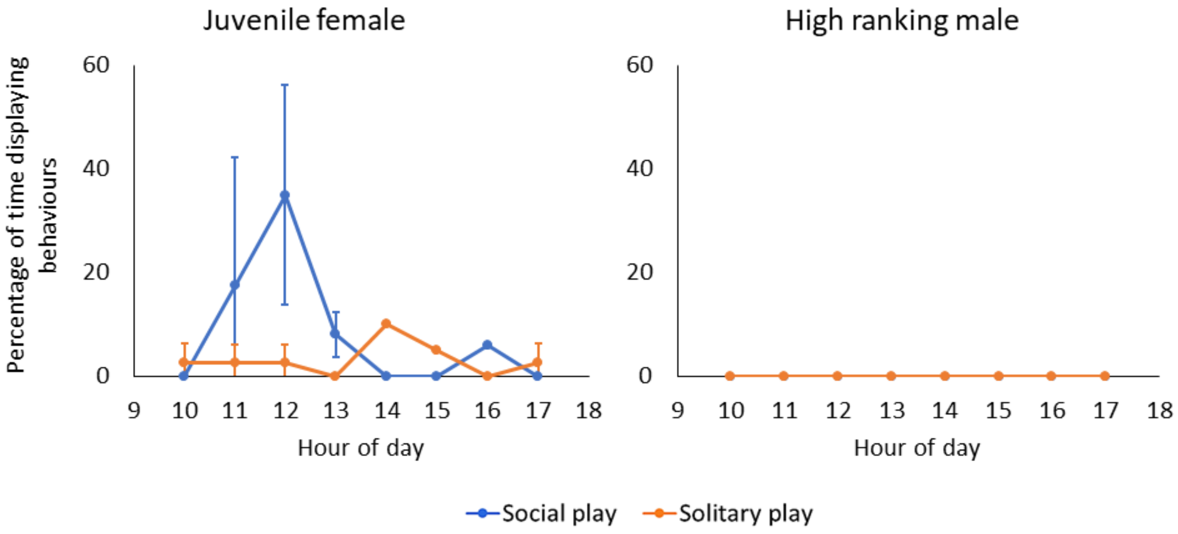


Figure 2.4. Data used to respond to behavioural category question ”Has mostly positive interactions with conspecifics or other animals” from the over-arching question “Is an interaction positive?” for (A) anemone fish (47 sessions, 11-12 sessions/individual) and (B) chimpanzees (16 sessions for mother’s group, 6.67 hours; 11 sessions for son’s group, 4.58 hours). For the anemone fish, positive/neutral behaviours are meeting, soft touch, and follow. The remaining behaviours are indications of negative interactions. The anemone figure is generated directly from *ZooMonitor*. These figures do not include measures of standard error. For chimpanzees, all listed behaviours are positive except for aggression. The bars are the standard deviation for the sessions averaged over each hour. Note that some points may not have standard deviations because only one session was recorded for that hour.

Is a positive behaviour being displayed?: Brown capuchins (individual) and meerkats (group)

The third example question is whether the animal exhibits play behaviour (Figure 2.5). We show example data with brown capuchin individuals and meerkat groups. Here I am demonstrating how having data on these kinds of behaviours provides lots of detail and context for comparison for a behaviour which could potentially be recalled by the keeper. For both species, we show the rhythms of behaviour for both social and solitary play since the rhythms may provide extra context. The brown capuchin data demonstrates how you can compare between adolescent and adult individuals for a behaviour like play where we may expect younger individuals to display more play behaviour (Figure 2.5a). The meerkat data (Figure 2.5b) shows how we can compare between sexes within the same species. The male and female meerkats were housed separately. Therefore, comparing this data between the groups can help the staff determine if the social housing situation affects the ability to display play. The ability to compare the ratio of solitary to social play and when these kinds of behaviours might be displayed in relation to other behaviours like feeding (for potential questions on resource availability and social consequences) can help us form more comprehensive pictures of social dynamics and the conditions which may promote positive welfare. Though keepers are very capable of tracking whether certain individuals exhibit behaviours like play, this level of detail can only be achieved with systematic evidence. Consequently, it allows for more appropriate directions to be taken when determining interventions if it is decided they are needed because outcomes can be better predicted based on the patterns and dynamics discovered from the data.

(A) Brown capuchin individuals



(B) Meerkat groups

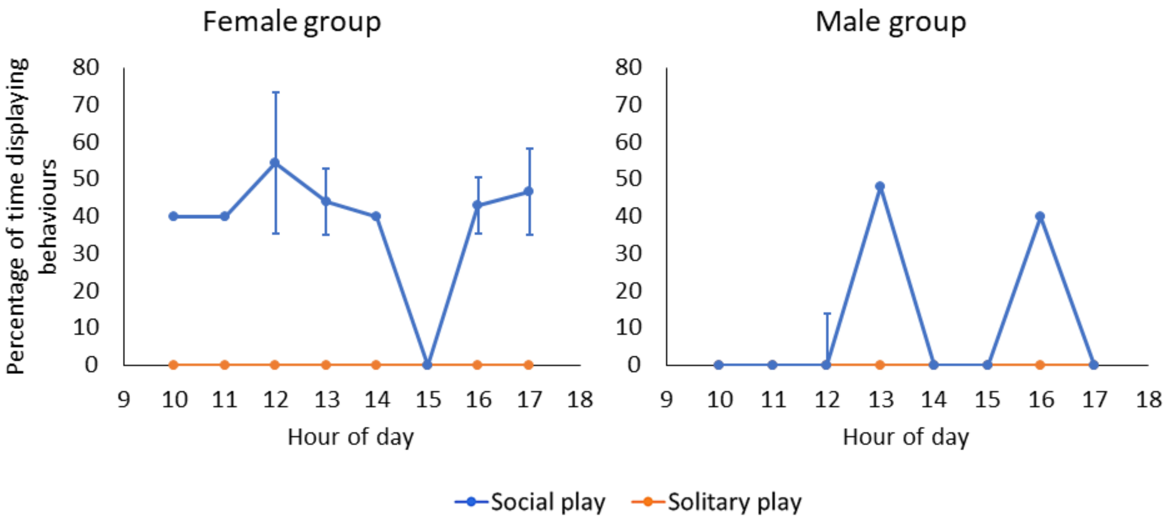


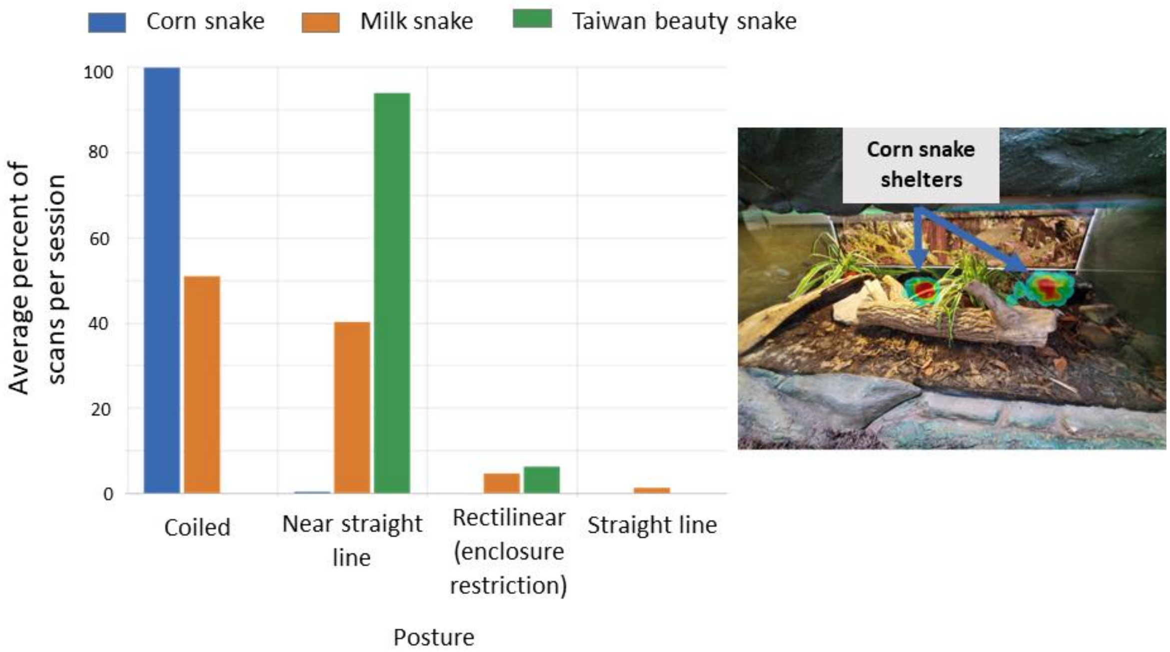
Figure 2.5. Data used to respond to behavioural category question “Exhibits play behaviour” for the over-arching question “Is a positive behaviour being displayed?” for (A) two individual brown capuchins (12 sessions each, 2 hours each) and (B) the female (25 sessions, 4.17 hours) and male (12 sessions, 2 hours) meerkat groups. All figures were generated within excel. The bars are the standard deviation for the sessions averaged over each hour. Note that some points may not have standard deviations because only one session was recorded for that hour. Results for the brown capuchins follow patterns we would expect with a younger individual showing more play behaviour and an older, high ranking individual not showing any. The peak in social play during midday can be investigated further to understand which play partner she prefers (if other young monkey) and whether the peak occurs during a time when older monkeys are napping. For the meerkats, the results show that the female group engage in social play more often. This does not indicate poor welfare for the male group, but

could be investigated further to determine if social play is less common between males because of the absence of females or because as a sex, they generally engage in less play.

Is a negative behaviour being displayed?: Snakes (individual) and anemone fish (individual)

Similar to whether play behaviour is displayed, there is a question on whether any abnormal behaviour is observed (Figure 2.6). In this figure I show how this kind of question of whether a behaviour is displayed or not (without asking about appropriate levels) can be answered with time budgets supplemented by maps to add important and informative context. In Figure 2.6a we see the proportion of time the three snakes spent in different postures. We also recorded other abnormal behaviours like interaction with transparent barrier, but these were not observed. However, with the snakes, I show that it is not only explicit behaviours that can be abnormal, but other species-specific aspects to behaviour as well. The rectilinear posture is when a snake takes the shape of the edges of their enclosure, indicating restriction by the enclosure. We see that two of three snakes do assume this position at times, which may mean that the size of the enclosure may not be appropriate. In addition, despite the corn snake not showing this behaviour, we can still say that the proportion of time spent in a coiled position (nearly 100%) may also be abnormal and a cause for concern. The map shows us that the snake was only ever observed in its two shelters (bowl and box), and was never seen outside of these hiding places which is why it was always in a coiled position. This data can lead to further investigation as to why the corn snake only stays in its shelters. For the anemone fish (Figure 2.6b), we see that the only individual who displays abnormal behaviour is the sharing male which shares an anemone with the breeding pair. With the heat map showing the location where the bobbing occurs, we see that it occurs exclusively just under the anemone. This might indicate that the fish is chased out of the anemone and displays this abnormal behaviour as close to the anemone as possible. These data are a good example of how enclosure data can be applied to questions outside of environmental questions to provide context if we want to understand the potential triggers for abnormal behaviours, allowing for targeted interventions to be tested.

(A) Snake individuals



(B) Anemone fish individuals

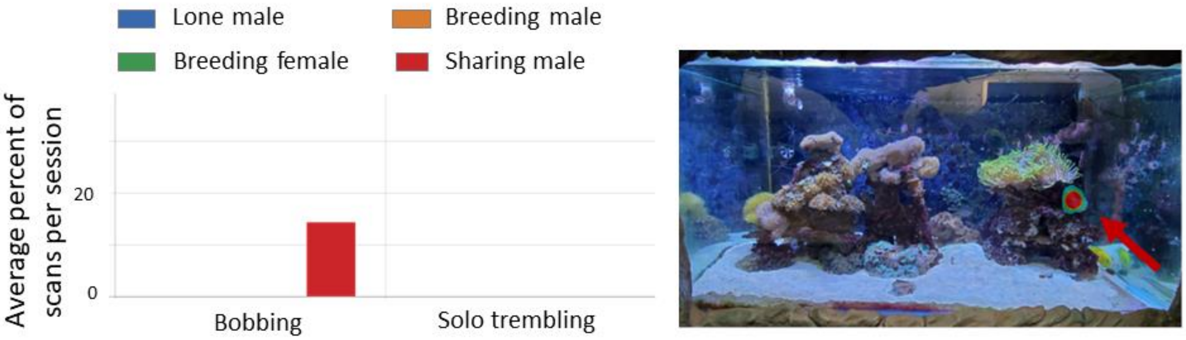
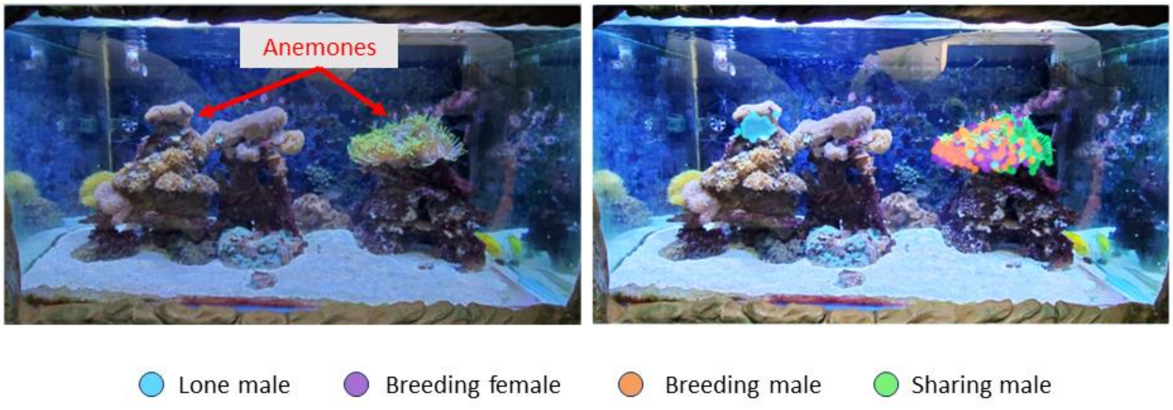


Figure 2.6. Data used to respond to behavioural category question “No evidence of abnormal behaviour” from the over-arching question “Is a negative behaviour being displayed?” for (A) snake individuals (11 sessions for corn snake, 1.83 hours; 8 sessions for milk snake, 1.33 hours; 11 sessions for Taiwan beauty snake, 1.83 hours) and (B) anemone fish individuals (11-12 sessions per individual, 1.83-2 hours per individual). Graphs for both the snakes and anemone fish are time budgets created directly within the *ZooMonitor* app. These figures do not include measures of standard error. These results demonstrate that if figures for rhythms are too time-consuming to create, using data quickly taken directly from *ZooMonitor*, but analysed together can be immensely more informative than assessing only time budgets or only heat maps. The snake behaviours (A) are postures, with rectilinear being the only posture that is inherently negative, whereas the other postures are neutral. The enclosure map pictured for the snakes is of only the corn snake enclosure displaying enclosure use for all sessions and behaviours. The shelters indicated in the map are a bowl (left arrow) with an opening which allowed the snake to coil inside and a plastic box shelter (right arrow). For the anemone fish (B) the figure displays time budgets of two abnormal behaviours. In the enclosure map, the arrow is indicating where bobbing was displayed for the sharing male.

Is the enclosure and furnishings allowing for natural behaviours to be displayed?: Anemone fish (individual) and giraffes (individual)

To show examples of using enclosure use data to directly answer environmental questions, I provide examples for the questions on whether enclosure features allow for species-specific behavioural needs (Figure 2.7) and whether the animals have appropriate shelter from weather/climate (Figure 2.8). I used anemone fish and giraffes to address the question on enclosure features allowing natural behaviours. In this approach I am assessing where in the enclosure specific behaviours tend to occur. By analysing the environmental features in this way, I am able to directly link the environment and animal to avoid assumptions on what behaviours certain environmental features may elicit. The data provides direct evidence that certain features do or do not elicit wanted, positive behaviours. For the anemone fish, the species-specific behaviour analysed in the figure is occupying the shelter (occupying the anemone). Though keepers would be able to tell us which fish occupy which anemone, looking at the individual enclosure use data for occupying the shelter shows us how the anemone is shared for the three individuals occupying the right anemone. We see that the dominant pair share most of the area of the larger anemone and that the sharing male only occupies the back portion of the anemone. This data, paired with the data in Figure 2.6b allows staff to determine whether there may be overcrowding, and whether adding another small anemone may be helpful in reducing the abnormal behaviour displayed by the sharing male. For the giraffes, the data shows how the giraffes do use most of their feeding stations to feed, but ignore one of the outdoor feeding stations which is closer to a visitor viewing area, and a few of the indoor feeding stations (may not be filled with food). These two examples demonstrate how using detailed enclosure use data provides a lot of information on whether enclosure features are used for their intended purposes. This level of detail of how often certain areas are used could not be determined from recollection or single session observations.

(A) Anemone fish individuals



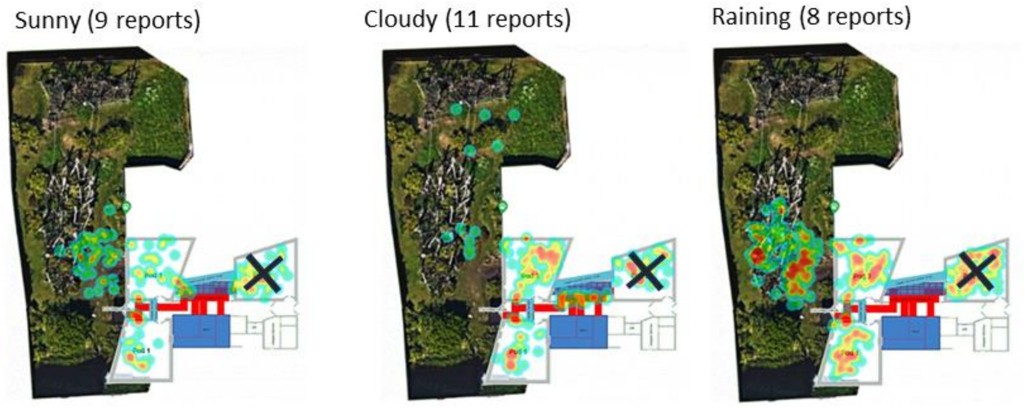
(B) Giraffe individuals

Figure 2.7. Data used to respond to the environment category question “Furnishings allow appropriate species-specific behavioural needs” from the over-arching question “Is the enclosure and furnishings allowing for natural behaviours to be displayed?” for (A) anemone fish individuals (11-12 sessions per individual, 1.83-2 hours per individual) and (B) male giraffe individuals (28 sessions per individual, 4.67 hours per individual). These data demonstrate how we can directly link behaviours to specific environmental features to determine if the features are eliciting the positive behaviours they are intended to elicit. For the anemone fish (A), the left photo indicates where the anemones are located. The anemone indicated by the left arrow is located behind the structure, so is not visible in the photo. The right photo illustrates enclosure use when displaying the behaviour of occupying the shelter. The distribution of the three fish on the right shelter is demonstrated with the breeding pair sharing most of the space on the anemone, and the sharing male occupying mainly the back of the anemone. For the giraffes (B), the left photo indicates where feeding stations are located, represented by green squares. The right photo shows enclosure use when displaying the behaviour of feeding for all giraffes.

Are there appropriate shelters?: Brown capuchins (individual) and chimpanzees (group)

The final example question asking whether there is shelter from weather conditions is shown with chimpanzees and brown capuchins. Though the previous question on species-specific behavioural needs can only be answered with enclosure data for individual assessments, questions on climate can be determined for individual and group observations. This is because it only requires an answer to a question at the start of the session to be answered on the kind of weather at the time of the session. For both the chimpanzees and capuchins, we see that the enclosure use is the same under cloudy and sunny conditions. However, for chimpanzees in rainy conditions (Figure 2.8a) and capuchins in windy with no rain conditions (Figure 2.8b), they seem to take shelter in their indoor enclosures. We also gain relevant information on general enclosure use and can see that the chimpanzees only seem to use one third of their outdoor enclosure, and mainly the area where they provision for food which is tossed from a balcony. Therefore, for the chimps, staff may want to further investigate if the outdoor enclosure is not being used because of the features, or because of the inability to seek warmer areas for a species which is used to warmer temperatures than those experienced in Scotland. This data can be further detailed by looking at enclosure use during specific temperatures. Whether climatic conditions are appropriate for certain species living outside of their natural ecosystems and latitudes is at the forefront of welfare questions across zoos. Because if the general temperature or temperature cycles are an issue, the space can ultimately be entirely useless regardless of the presence of appropriate features in outdoor enclosures. This kind of detailed data collection that allows for looking at how all these separate factors synchronously affect behaviour, linking the animal outputs to environmental inputs, can more easily help identify the causes of welfare concerns while also determining the external factors that may promote positive welfare states. With more detailed data, welfare interventions put in place based on the evidence are more likely to be effective.

(A) Chimpanzee groups

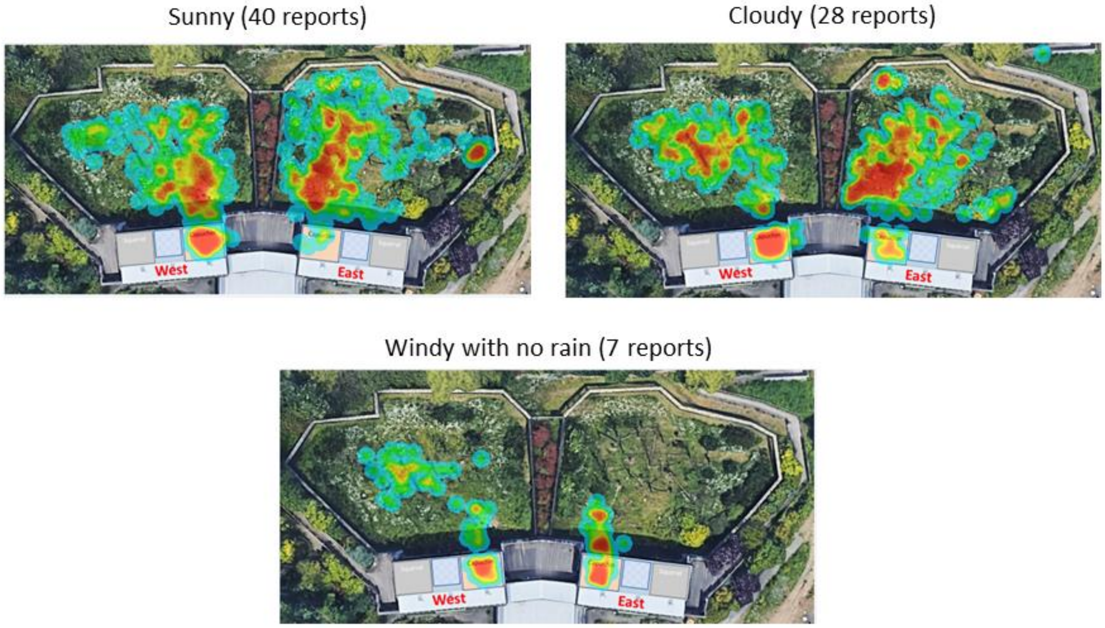


Sunny (9 reports)

Cloudy (11 reports)

Raining (8 reports)

(B) Brown capuchin individuals



Sunny (40 reports)

Cloudy (28 reports)

Windy with no rain (7 reports)

Figure 2.8. Data used to respond to the environment category question “Has appropriate shade/shelter from weather/climate” from the over-arching question “Is there appropriate shelter?” for (A) chimpanzee groups and (B) brown capuchin individuals. The ability to create heat maps for specified weather conditions provides very useful information on what environmental features may be fit for different kinds of weather. This information could confirm that certain features are providing appropriate shelter or direct staff towards features they may not realise could be enhanced to be fit for purpose. Each photo shows enclosure use for either both chimpanzee groups or all capuchin individuals during different weather conditions, with the number of sessions included in the heat map in parenthesis. For the chimpanzees, the black ‘X’ indicates an enclosure pod that does not have access to the outdoors.

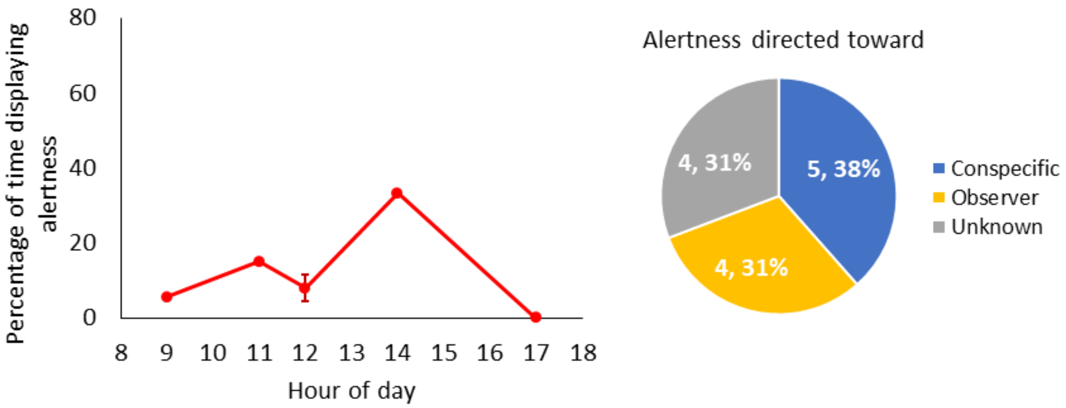
2.3.2.3 Addressing specific welfare risks/questions outside of welfare assessment

Although it is very important to conduct regular welfare assessments to assess welfare states of animals, welfare assessments are asking very general questions and are more of an overview on welfare. Many welfare risks and concerns are much more specific and nuanced than what would be asked in a general welfare assessment. In addition, welfare assessments are not the only way in which welfare risks are flagged. Keepers still monitor their animals daily and can flag an individual or group with a potential welfare risk for further investigation. With our *ZooMonitor* projects, we wanted to demonstrate how these more specific welfare questions can be asked in conjunction with the general questions for the welfare assessment without the need for extra projects or data collection.

Though all the ethograms were created to specifically answer the questions in the welfare assessment, we also consulted keepers on specific welfare concerns they might have about their animals so that we could incorporate them into the *ZooMonitor* project. In this way, we have one project that can be applied to the regular assessment but can also be used when looking at a more specific concern. To show how this works, I will present examples for yellow-breasted capuchins, anemone fish, and giraffes in which specific welfare questions which extended beyond the welfare assessment questions could be addressed without additional data collection.

In Figure 2.9, we can see an example with two male yellow-breasted capuchins. The general welfare assessment, *ZooMonitor* project was created with the enclosure use and all of the relevant positive, neutral, and negative behaviours. However, in this situation there were two male capuchins housed near lions, while another two males were housed elsewhere in the zoo away from any predators. The concern was whether being near to lions was causing any welfare concerns. To ask this question, we added a modifier to the alertness behaviour to specify to whom the alertness was directed; whether it was a conspecific, observer, keeper, heterospecific non-predator, heterospecific predator, or unknown. In comparing the monkeys housed near and away from a predator, we found that much more alertness was displayed for the monkeys near the predators and that it was often directed toward the predators. This example shows how a simple modifier can help address a more specific welfare question with detail without having to conduct entirely separate observations.

(A) Male capuchin housed away from predators



(B) Male capuchin housed near predator

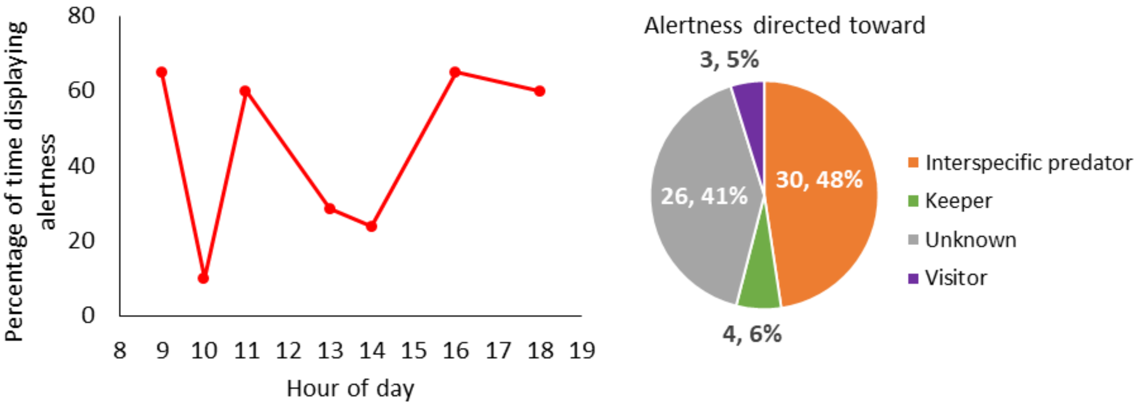


Figure 2.9. Percentage of time displaying alertness for (A) a male capuchin housed away from predators (6 sessions, 1 hour) and (B) another male capuchin housed near a lion (7 sessions, 1.17 hours). The bars are the standard deviation for the sessions averaged over each hour. Note that some points may not have standard deviations because only one session was recorded for that hour. The pie charts display the proportion of time the alertness was directed toward different entities. The numbers within the pie chart are the number of intervals (20 intervals per session) where alertness was recorded as directed toward the specified entity, followed by the percentage that represents from all alertness recordings.

The next example in Figure 2.10 is with anemone fish. In this example there was not a specific welfare concern that was being asked by keepers, but there were dynamics that would not have been asked by the welfare assessment that are important to understand. The breeding pair sometimes had eggs, so the question that was addressed serendipitously was how behaviours for all four fish, including the neutral fish, would change when eggs were present or absent. From the data on occupying the shelter, we can see that the breeding pair occupy the shelter much more often when eggs are absent. This was because when eggs were present they were displaying egg maintenance and nest defence related behaviours, which was expected. Interestingly, the two submissive males seemed to change in the amount of time they occupied their shelter as well depending on whether eggs were present or absent. The sharing male which shared the anemone with the breeding pair occupied the shelter less when eggs were absent, but the other more dominant neutral fish occupied its separate shelter more when eggs were absent. This could be because the more dominant neutral fish with its own anemone is more of a threat while eggs are present, so the female, who will protect the nest from predators, would aggress the neutral fish with its own anemone while eggs were present. Whereas when eggs were absent, the pair would occupy the shelter more and would not allow the neutral fish sharing their anemone much space. This example shows how data collection for the welfare assessment can still be used to ask questions beyond the assessment and gain a better understanding of the more nuanced dynamics of behaviour that are relevant to improving welfare.

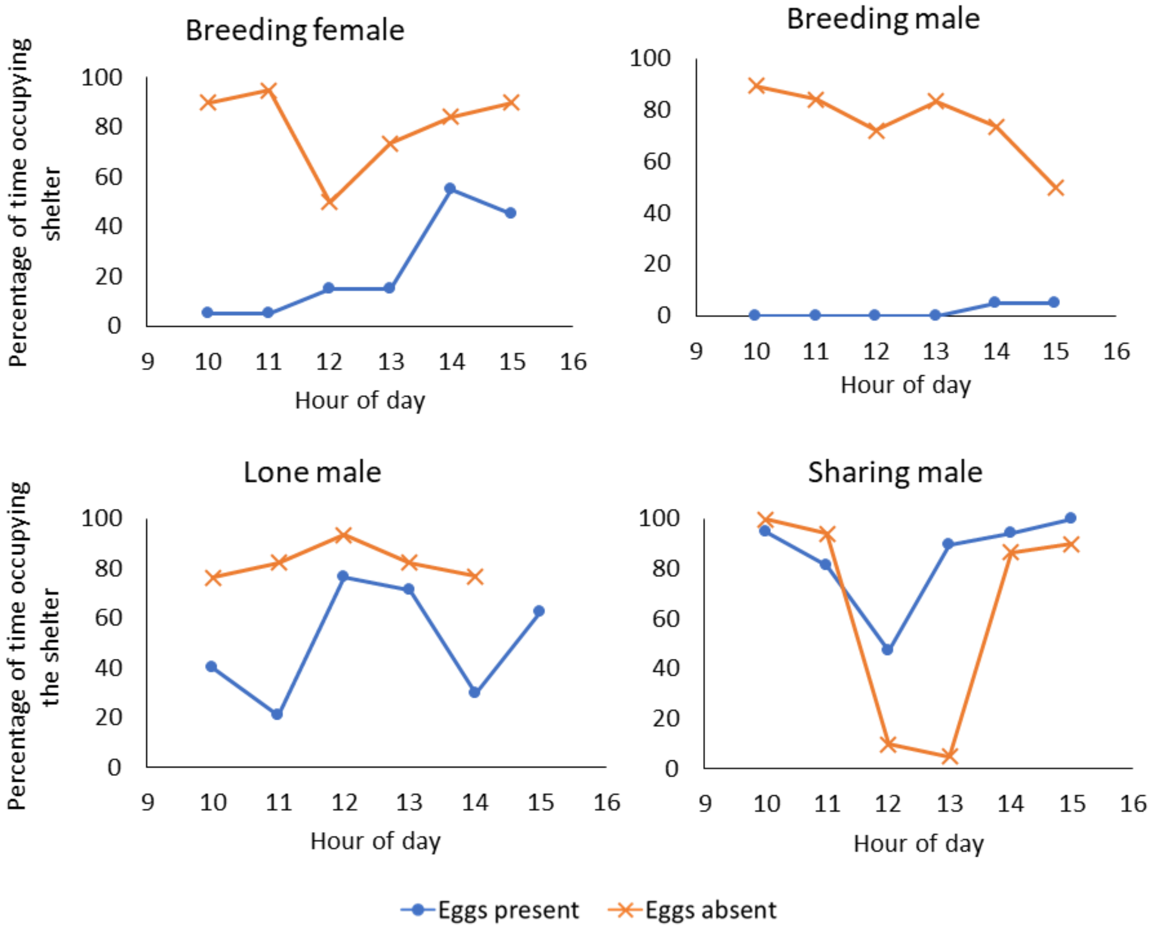


Figure 2.10. Percentage of time displaying occupying the shelter for the four individual anemone fish (11-12 sessions per individual, 1.83-2 hours per individual) when eggs were

present (orange line with x) and absent (blue line with dot). There are no standard deviations because only one session per hour was recorded for each condition. The breeding female and breeding male are the breeding pair producing and caring for the eggs. The lone male occupies a separate, smaller anemone and the sharing male shares the large anemone with the breeding pair.

The final example is with giraffes (Figure 2.11). Here, the keepers wanted to know the social dynamics between 5 bulls which were housed together. By adding in social modifiers for behaviours like follow, submissive yield, necking and fighting, we were able to understand the social dynamics and hierarchy of the group. However, as with any project that is done on individuals, extra training is needed for the observer to be able to distinguish between individuals.

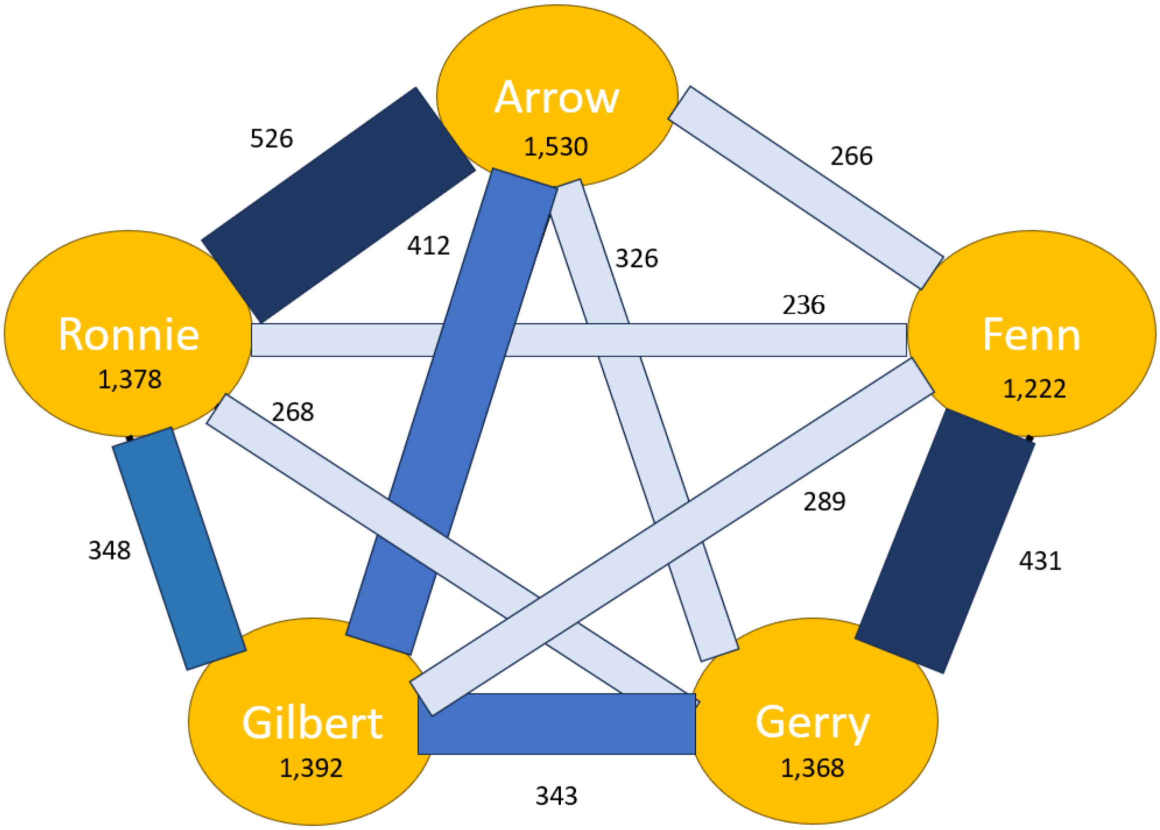


Figure 2.11. A sociogram of the five male giraffes housed together (28 sessions per individual, 4.67 hours per individual). Number of recordings where giraffes engaged in social behaviour or spent in proximity with one another. ‘Social behaviour’ includes ‘Approach’, ‘Follow’, ‘Necking’, and ‘Sparring’. Proximity data is gathered from all other behaviours, and was only included from the focal animal, as it cannot be certain that two giraffes in proximity to the focal are necessarily in proximity to one another. Numbers below the giraffe name are the total number of point samples in proximity/positive social behaviour, and the thickness and colour of the lines also represent these numbers (light to dark colour/thinner to thicker

line=fewer to more point samples). This figure and legend was created and written by the Master's placement student who collected the data.

These examples are meant to highlight the flexibility in using *ZooMonitor* and the amount of detail that can be drawn from these kinds of projects. Demonstrating how utilising this evidence-based approach can provide information for a general welfare assessment, but also to investigate in more detail individuals/groups that have been flagged for more specific welfare risks. Or even to investigate in retrospect dynamics that may be useful in exploring possible interventions that may be needed as in the case for the anemone fish. In the next steps of developing this welfare assessment tool, it would be helpful to compare the concordance between assessors for the responses to the behaviour and environment questions with only the single 15 minute session (and keeper knowledge) and then the responses with these data. If we found higher concordance when using an evidence-based approach, it would help support our idea of using an evidence-based approach as a way to increase reliability of welfare assessments.

2.3.3 Deliverables for sustainability and education

2.3.3.1 Training deliverables

To encourage sustainability of the modified animal welfare assessment tool a training presentation for the welfare assessment tool and collection of quantitative data was made and published on YouTube. The training presentation includes information on what welfare assessments are, an explanation of behavioural data collection and an introduction to *ZooMonitor* (ZooMonitor training presentation).

This will allow new research students and volunteers to collect the data in a consistent way, to inform future welfare assessments. It may also be useful to showcase RZSS to other zoos, to encourage wider uptake. In addition, a training video and documents have also been made to train on the use of *ZooMonitor* as an administrator.

Future development will include creating broader/simplified ethograms for general taxa that can be shared through *ZooMonitor* so data collection across institutions is consistent. We would also want to build a bank of video compilations of behaviours for several species to be used for training and reliability testing, reducing the workload for university advisors or welfare staff training volunteers in data collection. And finally, we want to develop Power BI projects for the shared ethograms that would allow quick figures with variation depicted (standard error) to be generated to respond directly to the welfare assessment questions, further reducing effort and the need for expertise in data management and analysis.

2.3.3.2 Education deliverables

Materials will be provided for RZSS education and publicity to promote information on welfare states of animals. Figure 2.12 is an example of how the results from the welfare assessments can be used as part of a full infographic describing the welfare assessment process in RZSS Edinburgh Zoo.

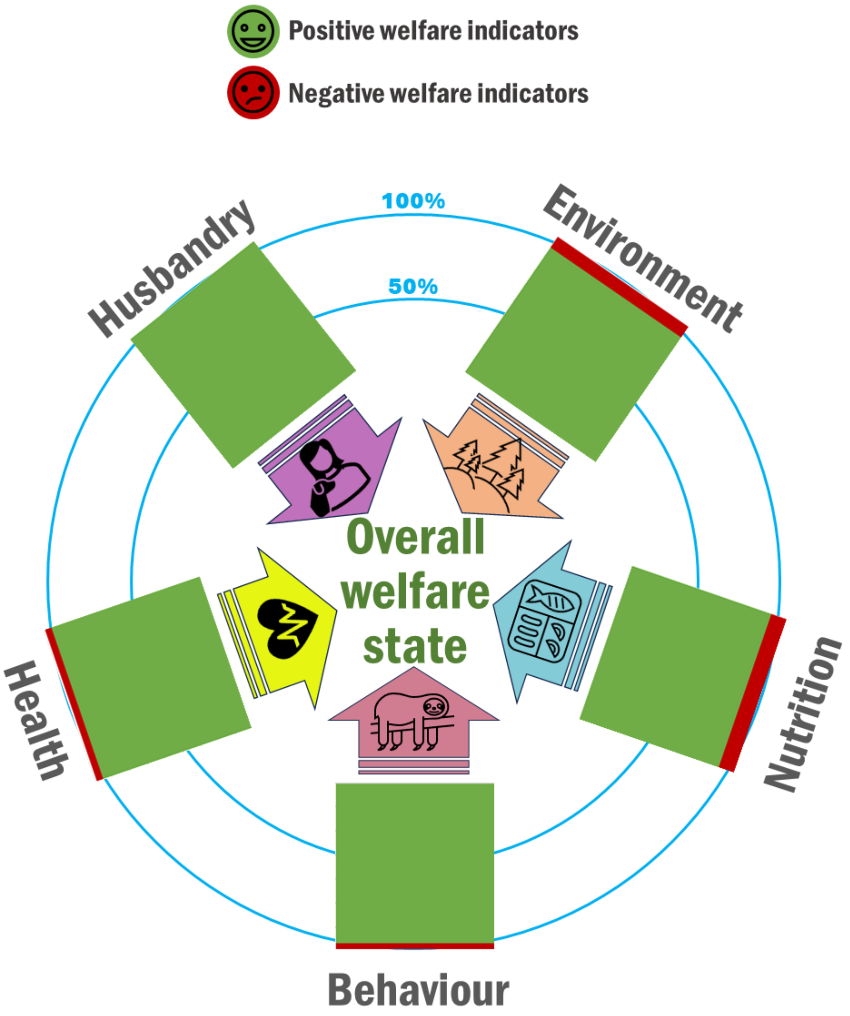


Figure 2.12. This figure can be used as part of a larger infographic detailing the welfare assessment process in RZSS Edinburgh Zoo. The figure describes the percentage of yes and no responses (positive welfare indicators and negative welfare indicators respectively) within each category of the welfare assessment for one of the animals assessed. The results pictured exclude the blank, unknown, and N/A responses. The figure shows how the results of the five categories in the assessment together inform on the overall welfare state of the animal. This kind of graphic can also be paired with data figures like those pictured in this chapter to detail how the behaviour and environment questions are responded to by showing

data for positive and negative behavioural indicators (Figures 2.3 and 2.4) and enclosure use (Figure 2.7 and 2.8).

2.4 Discussion

In this study I have described the process and results of the initial piloting of the modified RZSS welfare assessment tool and the evidence-based protocol to behaviour and environment questions. The outcomes of the piloting demonstrate that low concordance between assessors may be due to assessors limiting responses to areas relevant to their knowledge base, certain questions in the assessment cause variance that might be due to ambiguity in wording or lack of evidence-based responses, behavioural observations can be reliably conducted by observers with little to no experience, the assessment works at the individual and group level, and that taking an evidence-based approach to behaviour and environment questions provides detail and context that would not be possible without systematic quantitative data. The next steps in developing this welfare assessment tool will involve further modifying the assessment in accordance with what was found during piloting: delegating questions to staff with relevant expertise and knowledge, modifying the wording of certain questions for clarity, suggesting an evidence-based approach for other questions, developing more training and reliability testing materials to make the evidence-based protocol more sustainable, and assessing whether these changes improve reliability, validity, and feasibility.

2.4.1 Validity and reliability of welfare assessments applied to zoo animals

Reviews and articles covering the several welfare assessments available consistently emphasise the importance of having assessment tools that have their indicators validated and the reliability of the process assessed (Jones et al., 2022; Melfi, 2009; Tallo-Parra et al., 2023). Our piloting began the process of validating the tool in several ways (1) determining the validity of the assessment questions across several taxa, (2) determining the interobserver reliability for assessment questions, (3) determining the reliability of animal-indicators across several taxa, and (4) determining the feasibility of the entire process. Although many welfare assessment tools have been produced in the last decade following trends of placing increasing importance on animal welfare, most have not been validated, nor their reliability tested. This includes several welfare assessment tools and resources that are provided by zoo associations like EAZA within a welfare library such as the Detroit Zoological Society Individual Animal/Habitat Welfare Assessment, Wildlife Reserves Singapore: Animal Welfare Assessment, and the BIAZA Animal Welfare Toolkit.

The most validated assessments are the Welfare Quality© project and the Animal Welfare Assessment Grid, which like our assessment tool, evaluate resource inputs and animal outputs. The Welfare Quality© assessment has been tested for observer reliability in several domestic animals including pigs (Czycholl et al., 2016a, 2016b) and dairy goats (Vieira et al., 2018). For the pigs, inter-observer reliability was determined for a qualitative behaviour assessment (20 adjectives like “calm” or “tense” used to evaluate positive/negative emotions) and an assessment based on behavioural observations (30 minutes of observations with scan sampling every 2 minutes) (Czycholl et al., 2016a). The results suggested that the qualitative behavioural analysis did not produce good inter-observer reliability but the behaviour observations did. These findings support the shift towards pushing for an evidence-based approach to welfare assessment (Harley & Clark, 2019; Melfi, 2009; Whitham & Wielebnowski, 2013). In addition, it aligns with the next steps in our validation process to assess whether there is better inter-observer reliability in responses to the behaviour and environment questions when using the evidence-based protocol versus when assessors use only the one 15-minute observation session and recall.

The Animal Welfare Assessment Grid is a more evidence-based approach to welfare assessment. A thorough study determining the validity and reliability of the Animal Welfare Assessment Grid in assessing dog welfare found that it had good test-retest reliability, inter-rater reliability, construct validity and content validity (Malkani et al., 2022). However, an important limitation to consider with these studies validating the Welfare Quality© assessment and Animal Welfare Assessment Grid is that they were done with domestic animals since the original assessments were intended for agricultural and research animals, respectively. In addition, even within domestic species, the Welfare Quality© was considered to raise concerns for validity and reliability when applied to broilers (Tuyttens et al., 2015). Melfi (2009) highlights how many welfare indicators are validated with domestic animals that would not be closely related to the exotic animals housed in zoos and therefore may not be reliable when applied to zoo species. The Welfare Quality© assessment has been successfully adapted for use on bottle nose dolphins (Clegg et al., 2015), Dorcas gazelle (Salas et al., 2018), and proposed for the application to pygmy blue-tongue skinks (Benn et al., 2019). Though all of these studies used validated animal indicators, they still need further tests to determine their reliability. And again, a limitation is that these measures are only being assessed for specific species which does not necessarily generalise to the vast array of species in zoos.

In contrast, the Animal Welfare Assessment Grid was specifically adapted for use in zoos (Justice et al., 2017). However, it was not tested for reliability or validity until it was applied to gorillas (Brouwers & Duchateau, 2021), where it was determined to be feasible and reliable with good agreement between researcher and keeper assessors. However, the score sheets used included expected values which may have influenced scores and reduced objectivity. The more compelling test of reliability and validity for the Animal Welfare Assessment Grid in zoos was conducted in South Korea across 16 zoos (Ma et al., 2022). This study evaluated reliability across 11 species (birds, reptiles, and mammals) in 16 zoos, with each species being scored by a researcher, veterinarian, and zookeeper. They found good interobserver reliability and the process was very feasible, being completed in 14 days across all zoos. This validity and reliability analysis was most similar to the process we have conducted, evaluating reliability across several taxa with multiple assessors with different expertise. However, our validation and reliability testing process can be further improved once we finalise the tool and conduct piloting across several zoos in different countries. Though the piloting completed on our assessment to date is limited - with further piloting planned to continue - our assessment is still one of the few assessments which has had validity and reliability tested when compared to the many which are offered to zoos for adaptation. In addition, the strength in the modified RZSS welfare assessment tool and evidence-based approach compared to the Animal Welfare Assessment Grid is that it includes many more indicators of positive welfare with the evidence-based approach intended to be applied in a systematic way that allows for capturing rhythms of behaviour across the day, week, seasons, and life stages.

2.4.2 Creating baselines of behavioural cycles for later comparison

One of the main difficulties for creating valid and reliable zoo animal welfare assessments is the lack of literature on validated animal-based indicators for the broad number of species housed in zoos (Jones et al., 2022; Manteca et al., 2016; Tallo-Parra et al., 2023; Wolfensohn et al., 2018). As discussed, assessments with an evidence-based approach to animal-indicators tend to be more reliable than those taking a more subjective approach (Czycholl et al., 2016a; Manteca et al., 2016; Melfi, 2009). However, the issue still remains that baselines to gauge the quantitative data against are uncommon due to the limited research on the biology and behaviour of many species. This means that even from quantitative data, it can be difficult to assess welfare. The other main and related issue is that the zoo environment can make it difficult to determine how healthy behaviours are displayed because of the effects of the environment on behaviour and the adaptability of animals to different environments (Howell & Cheyne, 2018; Veasey et al., 1996). Howell and Cheyne

(2018) suggest that multiple environmental variables and animal indicators of welfare are measured in order to account for behaviour patterns in a captive setting. The RZSS welfare assessment tool and evidence-based protocol is designed to do exactly this. One of the strengths of this assessment is that it links the environment and the animal and provides evidence on how the environment may affect behaviours like we saw for the giraffe feeding stations and the anemone fish behaviours in relation to the anemone. This is unlike other welfare assessment tools that are designed to assess the environment and animal-based indicators separately (Blokhuis, 2008; Justice et al., 2017). The finalised tool will be designed to be flexible to be used to complete welfare assessments, but also to conduct research creating baselines on behavioural cycles and the environmental factors that may regulate or influence these cycles. In this manner, the welfare assessment process is self-improving because as more data is gathered, a better understanding of each species' biology, life history, and healthy expression of natural behaviours in a zoo context is developed.

Although we did not measure night-time behaviour in this study, it would be ideal for the next steps in development of this protocol to include night-time behaviour. Nocturnal behaviour would provide details that are often overlooked in behaviour and welfare assessments (Brando & Buchanan-Smith, 2018), but equally important to understand and consider for welfare. Excluding night-time activity is removing a large piece of the puzzle that influences the welfare of a species. Sleep is an evolutionarily necessary state and assessing sleep can provide insight into sleep quality, sleeping site appropriateness, and even social dynamics (Anderson, 1998; Cui et al., 2006; Harding et al., 2022; Holdgate et al., 2016) which would all contribute to the overall welfare state of an individual. However, other behaviours apart from sleep are also important to consider when investigating night-time behaviours. For the corn snake, activity budgets and enclosure use heat maps from data collected between 10am and 3pm indicated that the snake was hiding in its shelters 100% of the time. This could indicate a welfare concern if the snake is mostly inactive and not making use of its entire enclosure. However, corn snakes are crepuscular. Therefore, to definitively conclude whether this is a welfare concern, we would have to record behaviour at dawn and dusk to determine if the snake is more active and uses more of its enclosure during the hours we would expect. Similarly, with the anemone fish in our piloting, when there were eggs present, our daytime observations depicted the breeding male as the primary caretaker of the egg brood and the breeding female displayed guarding behaviours and few brood care behaviours. However, research investigating nocturnal brood care behaviour in anemone fish found that females display significantly more parental care at night and both sexes greatly increase these behaviours at night during hatching (Pacaro et al., 2023). During parental life stages it is important to understand the baselines for parental care to monitor the welfare of both the parents and the offspring. Ignoring night-time activities therefore removes much of the context that would allow us to properly monitor the progression of offspring rearing.

Given the importance of accounting for full behavioural cycles, when our welfare assessment tool and evidence-based protocol is finalised, the suggestion will be to collect data systematically to build complete baselines of behavioural cycles. For our initial piloting, our systematic approach involved conducting observations over a few weeks that eventually accumulated to an even spread of observations across the hours of the working day. This process can be expanded to collect data that would provide estimates of behavioural cycles over a full diel cycle, through each season, throughout a year, and eventually the lifespan. For instance, if data collection occurs for 4 days every season, then a session can be completed every hour for 6 hours, shifting that 6-hr window each day until one 24-hr estimate is established. This process can be repeated over the years, shifting by one month each year, which would result in one full year estimate of behavioural cycles within 4 years. Although these baselines would be built slowly overtime, Figure 2.13 shows how even beginning estimates with less detailed data can still be incredibly informative and representative of more robust data if collected systematically. Figure 2.13 depicts three graphs with circadian rhythm estimates using different amounts of data from one of the pandas in Chapter 3 and 4 (see methods for data collection in section 3.2 and 4.2 of thesis). Each graph uses increasingly more sessions to gain an estimate of a circadian rhythm in Summer. We can see that even with only 8 sessions- systematically collected to evenly span across one 24-hr period- we can gain an informative estimate of the patterns of activity cycles.

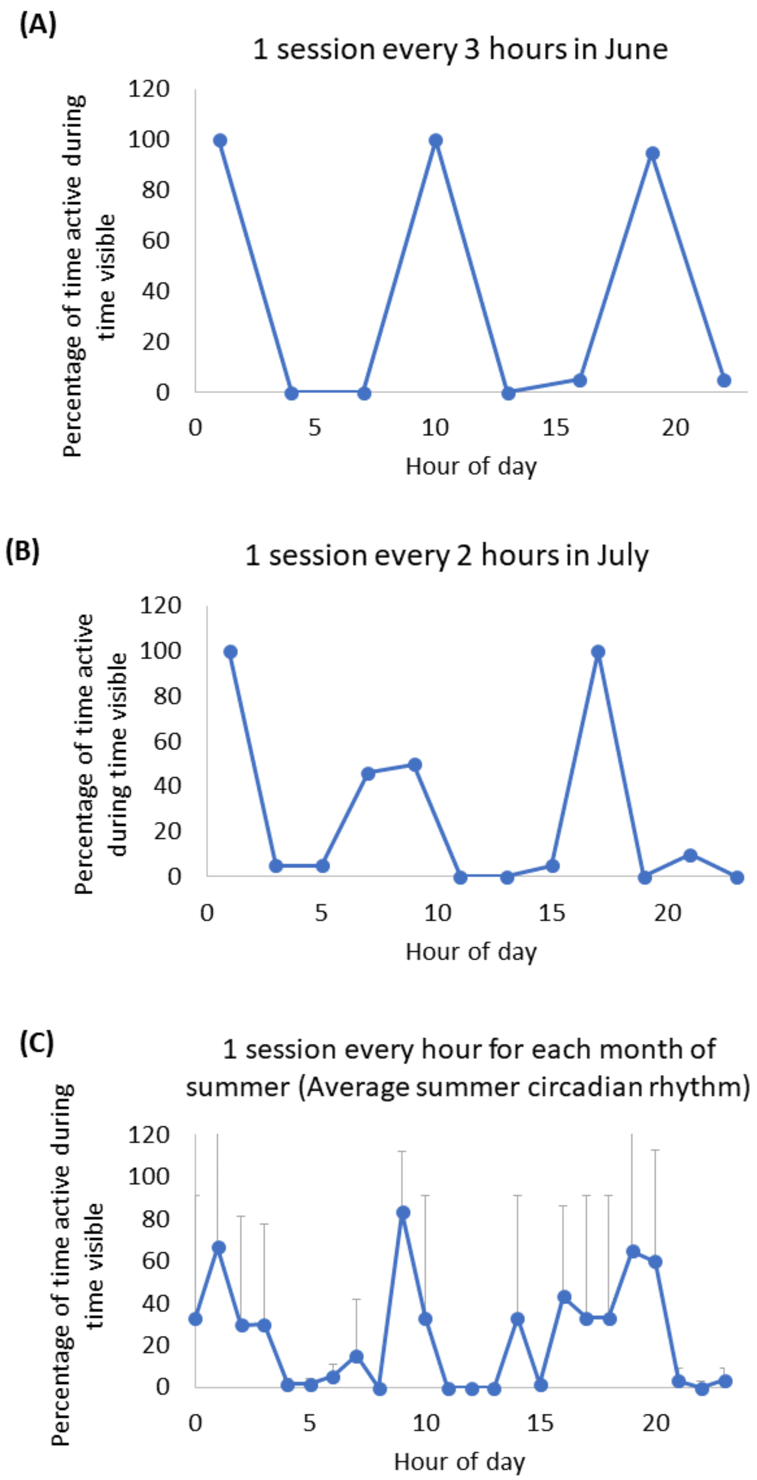


Figure 2.13. Depictions of circadian rhythm estimates of activity (any behaviour that is not resting/sleeping) over a Summer season for one giant panda (sub-adult female) using increasingly more sessions (each session is 10 minutes with 20 intervals) to form an estimate. (A) Estimate using 8 sessions total, systematically collected every 3 hours in the month of June. (B) Estimate using 12 sessions total, systematically collected every 2 hours in the month of July. (C) Estimate using 72 sessions total, with 24 sessions collected systematically every hour for each month of Summer (June, July, and August). The grey bars

are standard deviations (positive value only) in C. The progression in these estimates illustrates how a decent estimate can be gained by systematically collecting data to span evenly across a full 24-hr period. The three peaks in activity can be seen in all three graphs, demonstrating how important patterns can still be captured with less detailed data.

This systematic approach is practiced in the next data chapters on circadian and circannual cycles of giant pandas. These chapters demonstrate the potential power of baselines and the possible avenues for understanding and contextualizing behaviour when circadian and circannual rhythms are included in welfare assessments. With this method, zoos can build baselines of behavioural cycles that would be both individual and species-specific, accounting for day, week, seasonal, and life cycles; making it a powerful tool for comparison when welfare assessments occur. Individual and group data can be used long after an individual has died or a group has changed to further understand what conditions seem healthy and normal in different contexts. In addition to baselines for individuals and species within a zoo, baselines for species worldwide can be created through collaboration between multiple institutions adopting the same program and sharing ethograms. In this manner, the baselines can be built even quicker, and even more context can be understood about how the environment, different husbandry practices and zoo location may affect behaviour and welfare. These are all questions at the forefront of welfare science that would be more easily addressed if a standardised welfare assessment were established that could easily be picked up by institutions worldwide.

Having baselines for comparison would improve the validity of welfare assessment outcomes. They would also make the process more efficient by allowing the comparison of behavioural data collected by building up behavioural reference cycles for staff to compare their animal to at specific time points, similar to how typical growth trajectory is assessed. This use of baselines also makes the welfare assessment tool flexible in enabling certain categories like environment and behaviour to be assessed regularly with just timepoints being compared or more in-depth with a full assessment of the complete circadian and circannual rhythm of particular behaviours and the effects of environmental factors through those time periods.

2.5 Conclusion

This study demonstrates how the zoo community can begin to develop a welfare assessment program that is applicable across taxa and zoos. With this study we address several drawbacks of previous frameworks including lack of validity testing, lack of evidence-based approaches, disconnection between resource inputs and animal outputs, and the absence of a broader, continuous perspective of welfare. Our method of validating the assessment and determining areas that can be made more efficient and streamlined ensures that the welfare assessment process is more efficient while maintaining robustness.

Though the sustainability of the program is still being developed, I was able to demonstrate how collaborating with university students (and potentially volunteers at a later stage), with varying levels of experience in behavioural data collection was an effective way of gathering large amounts of data that detailed behaviour and welfare in ways that would not have been possible with only zoo resources and staff. I also highlight the ability to collect relevant and informative data on species which have limited literature available to reference for behaviour or life history traits. This evidence-based approach prevents the assumption that resource inputs are achieving the desired outputs and provides crucial details of behavioural expression and interactions with the environment that cannot be recalled by staff or estimated from single observation sessions.

This study mainly demonstrates that a systematic approach to assessing welfare can make the process more reliable and efficient. With welfare becoming increasingly important and mandatory to monitor for WAZA members, a program which can effectively flag risks and address serious welfare concerns by providing evidence to allow for targeted interventions needs to be established. Our approach is holistic, with a balanced focus on resource and animal-based measures, providing evidence that can not only lead to more reliable and effective interventions to promote positive welfare and prevent negative welfare, but also create baselines for what healthy rhythms and natural expression of behaviours looks like in zoo environments. This is particularly important to determine for species living in zoo environments with climatic conditions that do not match the conditions they evolved to and with exogenous, environmental factors whose cycles vary greatly from those experienced in the natural latitudes of the species (e.g. annual cycles of light and temperature). It is an approach that would greatly aid zoos in demonstrating their commitment to high welfare standards and practices, which would be beneficial for the animals, zoos and the public alike.

To demonstrate the importance of accounting for full circadian rhythms and using them in investigations to understand the needs of a species and how animals are affected by the zoo and natural environment, in the next two chapters I present a longitudinal study on giant pandas. These studies are complementary to the development of an evidence-based welfare assessment tool because they demonstrate the detail of information that can be gained from investigating the animal together with its environment through circadian rhythms. This information can then be used to inform on appropriate welfare interventions and enclosure designs that promote synchrony of circadian rhythms and the expression of natural, positive behaviours.

Chapter 3.

Understanding circadian and circannual behavioural cycles of captive giant pandas (*Ailuropoda melanoleuca*) can help to promote good welfare

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Chapter 3. Understanding circadian and circannual behavioural cycles of captive giant pandas (*Ailuropoda melanoleuca*) can help to promote good welfare

Author contributions

Conceptualisation, Kristine M. Gandia (KMG), Hannah M. Buchanan-Smith (HMB-S), Sharon E. Kessler (SEK) and Elisabeth S. Herrelko (ESH); methodology, KMG, HMB-S, and SEK; software, KMG; validation, KMG, HMB-S, SEK and ESH; formal analysis, KMG; investigation, KMH and HMB-S; data curation, KMG; writing—original draft preparation, KMG; writing—review and editing, KMG, HMB-S, SEK, and ESH; visualisation, KMG, HMB-S, SEK, and ESH; supervision, HMB-S and SEK; project administration, KMG and HMB-S. All authors have read and agreed to the published version of the manuscript.

Abstract

Circadian and circannual cycles of behaviour regulate many aspects of welfare including metabolism, breeding and behavioural interactions. In this study, we aim to demonstrate how determining circadian and circannual cycles systematically can provide insight into animals' needs and be part of an evidence-based approach to welfare assessment. We measured and analysed observational behavioural data of 13 zoo-housed giant pandas (*Ailuropoda melanoleuca*), across life stages and between sexes, each month for one year from live camera footage from six zoos across the world. Our results indicate that life stage was associated with changes in overall activity, feeding, locomotion, and pacing, and that sex influenced scent-anointing and anogenital rubbing. Overall, the circadian rhythms showed three peaks of activity, including a nocturnal peak, as seen in wild giant pandas. We also found associations between sexual-related, stereotypic/abnormal, and feeding behaviour possibly linked to the timing of migration of wild pandas, and elucidated the relationship between mother and cub, finding them concentrating maternal behaviours to mainly after closing hours. Understanding these cycle patterns can aid animal care staff in predicting changing needs throughout the day, year, and life cycle and pre-emptively provide for those needs to best avoid welfare concerns.

3.1. Introduction

Circadian rhythms have been recognised across the animal kingdom from birds, reptiles, mammals, amphibians, fish and arthropods, including insects (Froy, 2011; Siegel, 2008). The widespread evolution of circadian rhythms suggests an adaptive advantage to possessing one in an environment with cyclical changes; allowing species to anticipate changes in their environment, like temperature throughout a diel cycle or food availability through a circannual cycle, and respond accordingly to maintain homeostasis (Scheuerlein & Gwinner, 2002; Vera et al., 2023). In the wild, species exhibit cycles of activity throughout a 24-hour period and across the seasons. However, in captive environments, research identifying rhythms of activity throughout the day and night and across the seasons is limited to only a few species and is largely unknown within individual zoos as care of the animals is limited to the working day and seasonal changes in activity are not closely monitored (Brando & Buchanan-Smith, 2018). It is important to be aware of the various circadian rhythms of captive species, as many aspects of their welfare, including metabolism, breeding, and behaviours are regulated by their circadian clock (Froy, 2011; McCain et al., 2013; Mellor et al., 2020; Watts et al., 2016). Understanding the diel and annual cycles of behaviour and physiology of captive species can help in gaining a holistic view of their needs and thus inform zoo staff on measures to be taken to promote positive welfare. Our approach to assessing and addressing welfare follows the 5 domains model (Mellor et al., 2020) by incorporating the assessment of nutrition, environment, health, and behaviour on a diel and annual basis to inform an individual's welfare state.

Animals exhibit life history trade-offs expressed through physiological and behavioural trade-offs to maximise energy and fitness (Zera & Harshman, 2001). Energetic imbalances can lead to behavioural changes with implications for welfare (Dudde et al., 2018) or trigger physiological stressors (Anderson et al., 2011; Segner et al., 2012). Taking a closer look at cycles of specific behaviours can assist zoo staff in understanding the energetic needs of an animal. Energy maximisation occurs when an animal's fitness is optimised by maximizing net energy intake for a given time spent foraging (Schoener, 1971). Energy maximisation goes hand in hand with the circadian system since circadian clocks evolved as a means of synchronizing with the external environment in a way that is most conducive to fitness, which includes efficiently using energy and time (Froy, 2011). Across several species, one of the clear signs of aging is the inefficient use of energy that results from disruption of the central and peripheral circadian clocks (Manoogian & Panda, 2017; Terzibasi-Tozzini et al., 2017). Therefore, understanding the circadian and circannual cycles of behaviours involved in the conservation and expenditure of energy can assist zoos in deciding how to best provide for energetic needs of their animal throughout the lifespan, pre-emptively providing appropriate choices at appropriate times of day, year and life stages in preparation for changing needs.

Optimal foraging theory suggests that animals will choose the type of food, how much time is spent foraging and in which patches, and the speed at which to forage to maximise energy intake and minimise energy expenditure (Pyke et al., 1977). However, in captivity, zoo staff have nearly complete control of the way an animal will gain energy through food, controlling the kind of food, proportions, and feeding times. This means that zoo staff have a large influence on the regulation of the metabolic rhythm of an individual because feeding regimens are a strong entrainment cue that can supersede the central circadian clock and regulate peripheral tissue clocks and activity (Froy, 2011). Often, the feeding times and portion sizes given to zoo-housed species are different to those consumed by wild conspecifics (Altman et al., 2005; Brando & Buchanan-Smith, 2018; Orban et al., 2016). These altered feeding schedules have the potential to desynchronise metabolic processes with the central clock and alter activity cycles. For instance, a nocturnal mouse placed on a restricted feeding schedule during day hours will display food anticipatory activity (FAA) and be more active around feeding times and less active during normal active hours at night than when fed during the night (Carneiro & Araujo, 2012). Out of phase feeding and activity has been associated with shifts in hypothalamic activity that are desynchronised with the central clock (Salgado-Delgado et al., 2010). The adverse effects of desynchronizing feeding and metabolic processes with the central clock have been well established (Paschos, 2015; West & Bechtold, 2015), and are linked to obesity and a variety of disorders involving insulin sensitivity and energetic balance (Depner et al., 2014).

Aging increases the possibility of desynchrony because the circadian system ages causing decoupling of internal cell and/or tissue clocks and consequently overt rhythm changes (Froy, 2011; Liu & Chang, 2017). However, this outcome can be ameliorated, and associated diseases possibly prevented, by implementing a regular feeding schedule with appropriate kinds of food (Manoogian & Panda, 2017). This idea is demonstrated in a study by Cincotta et al. (Cincotta et al., 1993) where obese, aged rats were shown to possess differing circadian rhythms from those of younger, leaner rats. However, when the obese, aged rats were injected with metabolic hormones at the times in which the hormones peaked in young, lean rats, the obese, aged rats began displaying younger phenotypes. The obese, aged rats also showed a reversal of age-related insulin resistance and obesity. Being aware of the natural cycles of feeding for captive species, the effects of aging and designing diets and feeding schedules in accordance with that knowledge, can help zoo staff avoid the adverse effects of desynchrony and promote optimal health.

Breeding is a behaviour that zoos monitor closely to improve conservation efforts, however, successful breeding has been notoriously difficult in some species. In addition, the ability to perform natural sexual behaviours is necessary for animals to feel sexually gratified, contributing to positive affective states (Farm Animal Welfare Council Committee, 1979; Mellor et al., 2020). Since circadian rhythms regulate and influence many aspects of reproduction, both physiological and behavioural, understanding them can lead to improved breeding efforts and can help in understanding conditions that would allow these behaviours to be expressed. In humans, the circadian clock has been found to influence every stage of reproduction in females (Gamble et al., 2013). The circadian rhythm also determines the reproductive cycle for spontaneously and seasonally ovulating animals (Goldman, 1999). For spontaneously ovulating animals, the circadian rhythm optimises reproduction by ensuring that ovulation occurs at a time of day in which finding a mate and sexual receptivity is likely. For instance, in nocturnal rodents, ovulation occurs at night when encountering a mate is most likely (Goldman, 1999). For animals with a reproductive season, monitoring the time of year via the circadian clock (i.e. measuring day length) allows the animal to ovulate when environmental conditions are most favourable for gestation and rearing (Boden & Kennaway, 2006). Day length also influences male reproduction. In several species, male development of testis and better sperm quality are associated with exposure to a long photoperiod (Nunez Favre et al., 2012; Turek, 1972). However, prolonged exposure to a long photoperiod will then initiate a refractory period which is ended by exposure to a short photoperiod (Nunez Favre et al., 2012; Turek, 1972). Because of the broad influence circadian rhythms have on reproduction, when creating management and husbandry protocols, being aware of the circadian control of reproduction could potentially increase the rate of successful breeding attempts. This can be done by recognizing cycles of breeding, even cycles of behaviours that are not overtly related to mating like increased feeding or general activity, and providing resources to ensure that breeding pairs are synchronised in their behaviour and that individual physiological states are optimal. If breeding is successful, zoos must also understand the cycles of dynamics between parents and offspring so that they can adjust their husbandry care to provide the most appropriate resources to the parents so they may effectively raise their offspring.

In captivity, sleep is not often monitored (Anderson, 1998). Though there does not seem to be one consistent benefit to sleep, sleep can be seen as an adaptive state of inactivity

(Siegel, 2008) with several crucial benefits including homeostatic properties and improving cognitive functioning (Assefa et al., 2015; Pigarev & Pigareva, 2015), with prolonged sleep deprivation leading to unavoidable death. Siegel (Siegel, 2008) suggests that sleep has evolved to conserve energy and make behaviour more efficient in response to the surrounding environment. According to this hypothesis, should sleep be disrupted, either prolonged, shortened, or desynchronised, the efficiency of behaviours and energetic dynamics would be suboptimal and potentially detrimental to the organism. Because of this, monitoring the amount and cycles of sleep and rest can provide insight into the affective states of species and possibly indicate positive affective states or negative ones such as boredom and disease if analysed in the correct context (Fureix & Meagher, 2015). For instance, prolonged rest may indicate that an animal is ill and conserving energy to recover but can also be suggestive of a positive affective state in a species like the little brown bat which sleeps approximately 20 hours a day (Harding et al., 2022). Sleep health is paramount to general welfare, and therefore, more importance needs to be placed on promoting good sleep, which can only be done by understanding what a healthy sleep cycle is for individuals and species.

Migration is a seasonal event that has been widely studied in wild and captive bird species. In migratory birds, migration is triggered by photoperiods of specific length depending on the season (Gwinner, 1996). In preparation for migration, bird species will increase fat storages, activate or inactivate reproduction, and molt (Long & Stouffer, 2003; Moore et al., 1982). These changes also occur in captivity despite the inability of the birds to migrate. Migratory inhibition results in a behaviour called *Zugunruhe,* from German, which translates to “migratory restlessness.” Captive migratory birds will display increased levels of activity and nocturnal migratory restlessness during periods which coincide with natural migratory behaviour (Gwinner & Czeschlik, 1978). The freedom to perform natural behaviours is part of the gold standard for animal welfare (Farm Animal Welfare Council Committee, 1979). Therefore, the inability to migrate, despite being physiologically prepared, could indicate negative welfare states. In addition, the increased fat storages accumulated before the migration season may lead to metabolic disorders should they not be used for their intended purpose. This is not only a concern for migratory birds, but across species who migrate such as giant pandas, sea turtles, and certain bat species. However, despite the extensive knowledge of migratory restlessness in captive migratory birds, little is known of the effect of captivity on non-avian migratory species (Sudo & Tsukamoto, 2015). Investigating these effects by determining circadian cycles of behaviours associated with migration can lead to suggestions to adjust diets during migratory periods or provide opportunities for alternative exercise to promote positive welfare states.

Two of the main goals of reputable zoos are to promote positive welfare of their animals and conserve species. We aimed to investigate behaviours using a holistic approach that could provide insight into the animal's needs and potential welfare states or provide information on cycles of behaviour that can inform husbandry practices. To demonstrate this holistic approach and to investigate effects of life stage and sex on captive animal behaviour, we are investigating the circadian and circannual cycles of captive giant pandas, including a mother and cub pair as a case study. Giant pandas are seasonal breeders mating between March and April (Liu et al., 2002). Giant pandas are also a migratory species, following their food source of bamboo and migrating in Spring to feed on nutritious shoots (Wang et al., 2010). In the wild, giant pandas exhibit three peaks in activity throughout the diel cycle and a fluctuation in activity levels throughout the year, with a peak in June (Zhang et al., 2015). Giant pandas being such a specialised species, evolving over time to eat bamboo and adapting their behaviour for this food source, makes them a model species for this study as they would presumably need to have well balanced energy dynamics to sustain themselves. Giant pandas are also ideal for this study because they are a charismatic species and have large popularity worldwide. This means that they have many webcams for observation and can show proof of concept of how monitoring animals on cameras can be very informative while being non-invasive. In addition, pandas are a vulnerable species that are notoriously difficult to breed in captivity. Though large improvements in the captive breeding of pandas have been made, with mate choice being identified as a key factor in successful breeding (Martin-Wintle et al., 2015), further understanding of how pandas synchronise their breeding behaviour and raise offspring could greatly improve conservation efforts for successful breeding of captive pandas.

In this study, we recorded circadian cycles for one year for male and female captive giant pandas across life stages. We investigated the cycles of general activity and several more specific active behaviours including stereotypic/abnormal behaviours, sexual-related behaviours and maternal behaviours. These cycles were then compared to each other to infer possible synergistic dynamics between them and to determine possible associations with migration. We hypothesise that life stage and sex will have effects on circadian cycles. Our study cannot determine causality, but providing a holistic view of giant panda behaviour can give new insights on the needs of giant pandas and possibly inform on the husbandry and environmental factors that may promote the positive welfare states zoos want to see in their animals.

3.2. Materials and Methods

3.2.1. Ethics

We received ethical approval for this study from the University of Stirling Animal Welfare Ethics Review Body (protocol #2084 1591; 30/04/2020). The study also received endorsement from the Association of Zoos and Aquariums, Giant Panda Species Survival Plan (05/08/2020). In addition, we submitted research applications to all zoos involved and received approval from the participating zoo's administrations. We also sent a voluntary questionnaire to keepers asking information on (1) panda identities, (2) cameras, (3) enclosure design, (4) artificial lighting, (5) feeding husbandry and (6) mating behaviour. Three zoos provided some or all of the requested information. The identifying data for the zoos and pandas has been anonymised.

3.2.2. Study animal selection

The inclusion criteria for a giant panda were whether the panda was in a zoo that had a publicly accessible web camera or a surveillance system that we could be allowed access to. We selected 13 giant pandas (7 females, 6 males, including one mother and male cub) from six zoos (Table 3.1). We identified the pandas by sex and life stage. The life stages follow those determined by Hu (Hu, 2011) of cub (0-1.5 years), sub-adult (1.5-6 years), and adult (6+ years). Hu (Hu, 2011) also included a senior category (20+ years) which we excluded despite having senior pandas because of our limited sample size. The panda dam was categorised as being in a maternal life stage which we considered to be separate from adult. In addition, the two adults (male and female) from Zoo C could not be distinguished during observations, therefore, their data were combined and their sex labelled as unknown for analysis. All cameras were accessible 24 hours, however, cameras for 6 individuals did not have night-vision, so observations for these individuals were limited to daylight hours (Table 3.1). The remaining 7 individuals had data collected on a 24-hr basis.

Table 3.1. Information on the study animals and camera visibility. Within Zoo C, sub-adults could be distinguished from adults, but individuals could not be identified. Therefore, these individuals are listed as 5a, 5b, 6a, and 6b. Individuals with the same number had their data combined and for individuals 6a and 6b the sex was categorised as unknown in analysis. Mating opportunity refers to when pandas are normally given opportunities by keepers to breed naturally or through artificial methods.

Zoo	Panda	Sex	Life stage	Feeding schedule	Mating opportunity	Camera visibility
A	1	F	Adult	On average 9 (female) or 10 (male) times a day between 0700-1800h	Around March; either natural or artificial insemination	Daylight
	2	M	Adult			
B	3	F	Sub-adult	Unknown	Breeding pair, but unknown time and method	24 hours
	4	M	Sub-adult			
C	5a	F	Sub-adult	First bamboo feed by 0900h, second at 1200h, third between 1330-1430h, and final between 1530-1630h	None	Daylight
	5b	F	Sub-adult			
	6a	F	Adult		Post-reproductive	
	6b	M	Adult			
D	7	F	Sub-adult	First bamboo feed between 0745-0845, second before 1200, and final between 1300 and 1700	Around March; natural	24 hours
	8	M	Adult			
E	9	M	Adult	First bamboo feed between 0800-1000h, second around 1300h, and final around 1700-1800h	Castrated for medical reasons	24 hours
F	10	F	Maternal	Bamboo provided approximately 5x per day with first feeding at 0730h and final between 1330-1400h	N/A	24 hours
	11	M	Cub			

3.2.3. Behaviour observations

Behavioural observations were completed using the *ZooMonitor* application (Ross et al., 2016). The ethogram was designed to include most of the behavioural repertoire of giant pandas, covering behaviours that indicate positive, neutral and negative affective states (Table B1). The valences were based on principles that convey the importance of displaying natural behaviours at levels which are conducive to positive welfare (Bracke & Hopster, 2006; Mellor et al., 2020). Therefore, negative behaviours would be abnormal behaviours, ones displayed out of frustration, or high levels of aggression. Positive behaviours are those known to be associated with positive affective states like play, investigation, or sexual-related behaviours (Mistlberger & Skene, 2004) or natural behaviours that would be performed under natural conditions because they are pleasurable and promote biological functioning (Bracke & Hopster, 2006). We have defined neutral behaviours as those related to maintenance which are highly dependent on the context and levels at which they are displayed to be considered positive or negative like urinating/defecating, rest/sleep, and locomotion. Principles defining valence of behaviours will refer to species typical levels of the behaviours. However, these have generally not been established for most captive species. This study can help demonstrate a method for determining the species typical levels and cycles of behaviours to validate these baselines.

Data were collected through web cameras from December 2020-November 2021 for all pandas except the mother and cub. The mother was observed from December 2020-March 2022 and her cub was observed from March 2021-March 2022. The cub was aged 6 months when his observations began, providing an opportunity for a case study of how circadian rhythms develop in a cub. Focal sampling was completed for each giant panda using one 10-minute sessions with 30-second intervals in each hour to gain an estimate of behaviour in that hour. Each month, data collection began on the 10th day and continued through the end of the month until one daylight or one 24-hr cycle was recorded (dependent on the night-vision of the camera) for each panda. Observations were done in real-time. Observers were assigned at maximum 5 consecutive hours of observations, completing a maximum of three 10-minute sessions with a minimum of 30 minutes rest each hour. Observers would determine which pandas needed a session completed in the hour and scan cameras until they found a panda in view and would complete the 10-minute session for the panda in view. Observers were trained to observe all pandas except for the mother and cub which only the lead investigator observed. In addition, only the lead investigator had access to cameras at Zoo D and therefore these pandas were also only observed by the lead investigator. Between all 13 pandas, 2592 10-minute sessions (432 hours) were completed and used for analysis. A graphical representation of the sampling method can be seen in Figure 3.1.

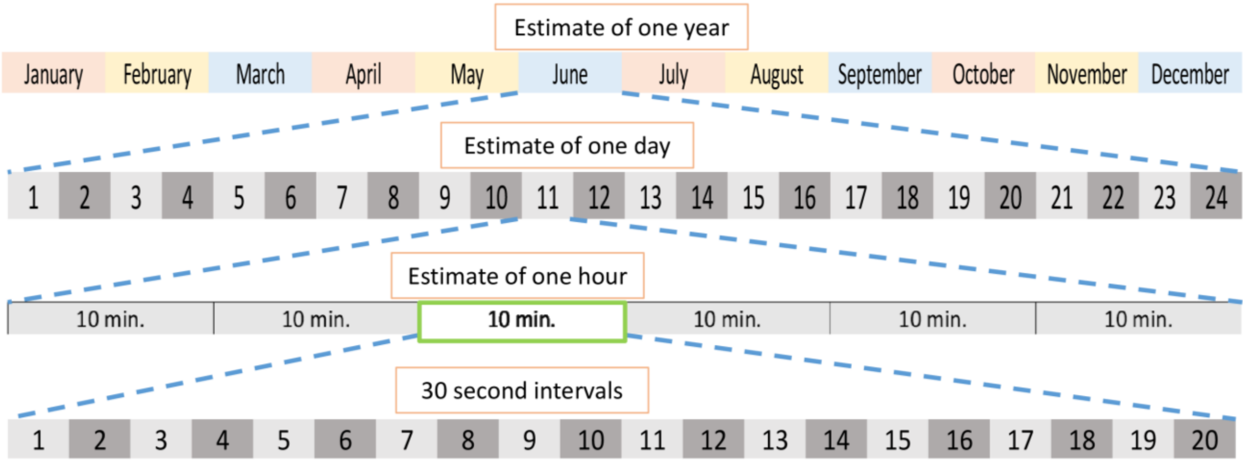

Figure 3.1. Representation of the sampling method to obtain estimates of circadian cycles and a circannual cycle. Each month, observations began on the 10th of the month and continued through the end of the month until one 10-minute session was completed for each hour of the day. For pandas observed on cameras without night vision, observations continued until a 10-minute session was completed for each daylight hour. Completion of 10-minute sessions depended on the availability of an observer.

Vocalisations were not recorded as some cameras did not have audio. Whether the panda was out of sight was also recorded, and a session was only saved if the panda was in sight for 60% of intervals (12/20 intervals) to provide representative data for analysis. Sessions with more than 8/20 out of sights were deleted and redone.

In total, 13 observers assisted in data collection throughout the data collection period. To produce data that would be used for analysis, observers had to pass reliability testing. Since testing reliability purely from live observations results in many ethogram behaviours not being evaluated (Wark et al., 2021), we designed our reliability testing with two stages aiming to cover all ethogram behaviours. The first stage was an ethogram quiz for which the observer had to receive >80%. The second stage was inter-observer reliability using a combination of compilations of short video clips of all behaviours listed in the ethogram and 10-minute recordings from the study pandas mimicking the way observation sessions would be completed using the web cameras and *ZooMonitor*. For the short video clips, beeps were placed at variable intervals so the full repertoire of pandas was covered, and indicated when to record a behaviour. For the 10-minute recordings, observers used *ZooMonitor* and the 30 second intervals to record. Observers had three attempts (each attempt had different videos or beeps were changed) to match at least 75% of the recordings from the lead investigator to pass this final stage. The 75% agreement threshold was based on those generally accepted for reliability (Graham et al., 2012). Each attempt contained 8-9 video clips with 4-5 beeps each, and 3-4 10-minute recordings with 30-second intervals.

3.2.4. Analyses

3.2.4.1. Variables

Our predictor variables were life stage (cub, sub-adult, adult, maternal), sex (male, female, unknown), hour of day, and season. Definitions of the behaviours we recorded are given in Table B1. We modelled activity, resting/sleeping, feeding, drinking, locomotion, pacing, bipedal standing, anogenital rubbing, and scent-anointing in separate models against the predictor variables. Activity was calculated by adding the counts of all behaviours except for resting/sleeping by 10-minute session. In addition, we refer to sexual-related behaviours as a category which includes anogenital rubbing, scent-anointing, show-interest and sexual behaviour (Table B1).

We also investigated the rhythms of maternal behaviours and proximity for the mother and cub. We determined the rhythms of nursing behaviour and non-nursing maternal behaviours (defined in Table B1). The proximity levels were in contact, proximate, and distant. Proximate was categorised as within 2 body lengths (back end to nose) of the focal panda and distant as greater than 2 body lengths away. Occasionally the pandas were separated by keepers, therefore, our analysis on proximity excludes this time since we aimed to investigate the choice in distance between a mother and cub throughout the cub life stage in captivity.

3.2.4.2 Zero-inflation negative binomial modelling and post hoc pairwise comparisons

It is common for ecological data to have a high amount of zero values resulting in zero-inflation that causes significant biases in analysis because the fit regression becomes flat (Fávero et al., 2021; Martin et al., 2005). These zeros are either ‘true zeros’ or ‘false zeros.’ With behavioural data, true zeros are observed from individuals that never display a behaviour or because a behaviour is not constantly displayed or rare. These zeros are also called structural zeros. False zeros occur from sampling error, if a behaviour is not displayed within the sampling period or if a behaviour is miscoded. These zeros are also known as sample zeros.

Within our data, each of our response variables had a very high percentage of zeros (between 45-98%). Our data will have both true/structural and false/sample zeros, but mainly zero-inflation due to true/structural zeros, which results in overdispersion (Martin et al., 2005). Therefore, we needed a model that worked for count rate data (counts of behaviour within a

10-minute session) and would account for zero-inflation due to a combination of factors, and the resulting overdispersion in the data. Negative binomial models are count models that have a parameter that allows for overdispersion (Lindén & Mäntyniemi, 2011; Martin et al., 2005). The most appropriate model considering the qualities of our data was the zero-inflation negative binomial (ZINB) mixture model. To conduct this analysis, we used the R package *glmmTMB* (Brooks et al., 2017). In *glmmTMB*, zero-inflated GLMMs have three components: a model for the conditional mean (Negative binomial in our study), a model for zero-inflation, and a dispersion model. The conditional mean and dispersion models analyse positive values using log links. The zero-inflation model describes the probability of observing a true/structural zero that is not generated by the conditional model. The values within the zero-inflation model are constrained between 0 and 1 by applying a logit link (Brooks et al., 2019; Brooks et al., 2017; Fávero et al., 2021). The overall fit of the ZINB mixture model is determined by all three components. Therefore, when interpreting the results, we must consider the results of all three models. The interpretation of the coefficients for the count model and zero-inflation model are different. A positive coefficient in the count model indicates an increase in the response with an increase in a continuous predictor or in the specified level of a categorical predictor. In contrast, a positive coefficient in the zero-inflation model indicates that a structural zero in the response is more likely with an increase in a continuous predictor or in the specified level of a categorical predictor.

For each behaviour ZINB mixture model, the zero-inflation model was the same as the conditional model. The categorical variables of season, life stage and sex were coded within the model using contrast sums. Therefore, each level of the variable was compared against a grand mean within the model which was the mean of the response variable means at each level of the categorical variable. In addition, Gandia et al. (2023b) determined that latitude, temperature and amount of daylight had an effect on behavioural cycles of giant pandas. Giant pandas at latitudes higher than the natural range displayed lower levels of activity and a more sporadic pattern of stereotypic/abnormal behaviours. Temperature and daylight had potentially regulatory effects on the patterns of activity. Therefore, although we did not include these variables in our models, our grouping variables were the individual panda nested within zoo to control for location effects. Zoos A, C and F are located at latitudes that match the natural range of giant pandas while Zoos B, D, and E are located at latitudes above the natural range towards the poles. Due to our limited sample size, we ran the models using Restricted maximum likelihood (REML) rather than Maximum likelihood. This is an iterative process and the final model that is presented is the one with the best estimation. We did not standardise for time out of sight by converting the counts to decimal rates per time in sight because the models require count data and because, among all sessions, the mean time in sight was 95.6% and the median was 100%.

In addition to models for all individuals and the selected behaviours, we also conducted ZINB mixture models for female vs. maternal female activity. This model only had hour, season and sex as predictor variables with sex having the levels of female or maternal female. Season was coded using contrast sums while sex was coded using the standard dummy coding where the female level served as a control. For the mother and cub pair, we also modelled nursing, maternal behaviours (all maternal behaviours except for nursing as described in Table B1), and proximity in separate ZINB mixture models against the predictors of hour and season. The models for nursing and proximity were mainly to determine whether these behaviours and proximities associated with a nursing mother would change overtime as the cub got closer to being weaned. Therefore, since the mother was observed for over a year, the season in these models was coded by year (Winter 20-21, Spring 21, Summer 21, Autumn 21, Winter 21-22, Spring 22), with Winter 20-21 only having the mother's data and Spring 22 only comprising of the month of March. Season was coded using contrast sums. The grouping variables of panda nested within zoo remained the same along with the use of REML.

The R program, *glmmTMB* allows for two kinds of negative binomial models, one which models the count data with linear regression and another which uses quadratic. The type of the regression chosen was dependent on which produced a better fit, determined by the AIC score and dispersion parameter. The kind of regression used for each variable is listed in Table 3.2.

Post hoc analysis of pairwise comparisons of estimated marginal means (least-squares means) were conducted on the season, sex, and life stage variables using the *emmeans* package in R (Lenth, 2023) to determine any significant differences between variable levels. Multiple comparisons was controlled for using the Tukey method. Test-wide alpha was set at 0.05.

3.2.4.3. Continuous wavelet transform and Wavelet coherence analysis

To extrapolate more information on mother and cub behavioural cycles throughout the year, the synchronisation between mother and cub, and how individuals synchronise sexual-related behaviours (all four sexual-related behaviours listed in Table B1), we used continuous wavelet transform and wavelet coherence analyses. Wavelet transform is a time series analysis where a signal is transformed into a wave with zero mean that is expanded and localised in both frequency and time. This allows for the detection of periodic patterns of a time series in both time and frequency domains while controlling for random background noise in the signal. Continuous wavelet transform is useful for analysing localised intermittent oscillations in a single time series, allowing for the identification of cycles within cycles (e.g. 8 hour cycle within a 24-hr cycle). This analysis is ideal for asking questions on how a cub's undeveloped circadian rhythm changes over time and the extent to which a mother's circadian rhythm is disrupted and recovered while having a dependent cub. The continuous wavelet analysis allows us to identify whether mother and cub display similar cycle lengths overall and whether the amplitude of these cycles is similar between the two.

With continuous wavelet transform we can also compare two wavelets and conduct a wavelet coherence analysis to determine how two time series are related to each other. With wavelet coherence analysis we can examine whether regions in time frequency space with similar high power have a sustained phase relationship, possibly suggesting a relationship between the signals (Grinsted et al., 2004). Essentially, we can determine areas of correlation between the two wavelets. This type of analysis can be used to determine when and to what extent mother and cub were synchronised in their behavioural cycles throughout the period of observation where both mother and cub were recorded (excluding first few months where only mother's behaviour was recorded). Wavelet coherence can also be used to determine whether breeding pairs in zoos are synchronizing their sexual-related behaviours. For our analysis, we conducted a wavelet coherence analysis between the mother and cub pair and for sexual related behaviours between breeding pairs with 24-hr data (2 pairs, Zoos B and D).

To conduct the analysis, we used the MATLAB Wavelet Toolbox developed by Grinsted et al. (Grinsted et al., 2004). Our data are non-stationary, so we used a continuous wavelet analysis with the Morlet wavelet and a scale resolution of 10 scales per octave, as suggested by Grinsted at al. (Grinsted et al., 2004), since these settings provide a good balance between time and frequency localisation. Our sampling period was set as $\Delta t=1$hr. We also decided to conduct continuous wavelet transform and wavelet coherence analysis because these analyses were used by Zhang et al. (Zhang et al., 2017) to address similar questions in wild giant pandas on activity data recorded using radio collars. We followed their analyses as closely as possible so that our data could be compared to the results found in wild pandas, including one mother panda. However, because of our sampling method, our behaviour signals are constructed of consecutive, representative 24-hr periods in each month. Therefore, we could only determine the patterns of circadian cycles and the annual cycle of those circadian cycles but could not infer anything about rhythms with a month-long period.

3.3 Results

Model summaries for each behaviour category can be seen in Table 3.2. Each of the behaviour category ZINB mixed models had significant coefficients in either the count model or the zero-inflation model, or both. The full results of significant coefficients from the general behaviour ZINB mixed models can be seen in Table 3.3. The full results of the significant coefficients from the female activity and maternal case study models can be seen in Table 3.4. Within these tables, coefficients with trends towards significance (α <0.1) are displayed. However, we will only discuss results that reached the α=0.05 level of significance below. We will describe the results for both components, addressing the count model, the zero-inflation model, and then the *post hoc* pairwise comparisons. The hour of the day was significant for all general behaviour ZINB mixture models within the count, zero-inflation, or both components (Table 3.3). For the models including only the mother and cub, we will only discuss pairwise comparisons for seasons below since we are mainly interested in the difference between seasons and hours, but pairwise comparisons of hour are not possible, however, the full list of significant coefficients for season and hour can be found in Table 3.4.

Table 3.2. Summary of ZINB models for activity, sexual-related behaviour, and stereotypic/abnormal behaviour. N/A appears for models which did not run (N/A across row), likely due to overparameterisation, or models for which the best fit was likely not found (N/A for AIC and BIC).

Behaviour category	Regression type	Iterations	AIC	BIC	Df(residual)	Dispersion parameter
Activity	Linear	58	13484.6	13631	2566	3.22
Resting/Sleeping	Quadratic	60	15680	15826.4	2566	3.7
Feeding	Linear	57	10541.2	10687.6	2566	2.56
Drinking	Linear	51	1456	1602.4	2566	0.238
Locomotion	Quadratic	49	4594.3	4740.7	2566	0.33
Pacing	Quadratic	54	2421.1	2567.5	2566	2
Bipedal standing	Quadratic	49	580.9	727.3	2566	0.33
Scent anoint	Quadratic	55	298	444.4	2566	2.52
Anogenital rubbing	Quadratic	40	434.9	546.2	2569	0.224
Female activity	Quadratic	52	6407.2	6493.8	1192	4.82
Maternal	N/A	N/A	N/A	N/A	N/A	N/A
Nursing	Quadratic	38	613.1	688.7	621	1.18
In contact	Quadratic	40	1722.6	1798.2	621	1.07
Proximate	Quadratic	41	2310.6	2386.2	621	1.67
Distant	Linear	38	N/A	N/A	621	1.12

Table 3.3. Summary of significant and potentially significant (p<0.1) coefficients from the ZINB models on general behaviours. In the conditional model (+) coefficients indicate the category level/variable is larger than the grand mean, and a (–) coefficient means it is smaller than the grand mean. For zero-inflation coefficients, (+) indicates the category level/variable is more likely to be a zero and (–) means it is less likely to be zero (i.e. more likely to be a positive integer). Variables with an asterisk did not reach the significance threshold but did show possible trends towards significance, with p<0.1.

	Conditional				**Zero-inflation**			
Behaviour	**Variable**	**Coefficient**	**Z-value**	**Pr (>|z|)**	**Variable**	Coefficient	Z-value	Pr (>|z|)
Activity	Summer	-0.057	-2.250	0.025	Summer	0.155	2.213	0.027
	Winter	0.063	-2.250	0.025	Autumn	0.213	2.850	0.004
	Cub	-0.105	-2	0.046	Spring	-0.320	-4.540	<0.001
	Maternal	0.118	2.390	0.017	Maternal	-0.481	-1.984	0.047
					Hour	-0.016	-2.410	0.016
Resting/sleeping	Spring	-0.064	-2.674	0.008	Spring	0.214	2.926	0.003
					Autumn	-0.314	-3.737	<0.001
					Summer	-0.209	-2.687	0.007
					Winter	0.310	4.213	<0.001
					Hour	0.020	2.815	0.005
					Adult	0.197	2.231	0.026
					Cub	-0.506	-3.797	<0.001
					Maternal	0.697	5.925	<0.001
					Sub-adult	-0.387	-4.628	<0.001
Feeding	Adult	0.177	2.590	0.010	Autumn	0.221	2.886	0.004
	Cub	-0.376	-3.370	<0.001	Winter	-0.189	-2.683	0.007
	Hour	0.007	2.460	0.014	Spring	-0.244	-3.534	<0.001
	Winter*	0.045	1.800	0.072	Summer	0.212	2.950	0.003
					Hour	-0.030	-4.565	<0.001
					Cub*	0.403	1.801	0.072
Drinking	Cub*	-2.094	-1.813	0.070	Spring	-0.536	-2.268	0.023
					Summer	0.532	2.345	0.019
					Hour*	-0.043	-1.761	0.078
					Male*	0.459	1.727	0.084
Locomotion	Winter	0.223	2.545	0.011	Spring	-1.040	-2.710	0.007
	Summer	-0.199	-2.163	0.031	Sub-adult	0.953	2.205	0.027
	Hour	-0.071	-5.932	<0.001	Hour	-0.494	-7.104	<0.001
	Adult*	-0.559	-1.774	0.076	Winter*	0.570	1.840	0.066
					Cub*	-0.869	-1.805	0.071
Pacing	Spring	0.251	2.460	0.014	Spring	-0.365	-2.937	0.003
	Maternal	1.546	1.999	0.046	Summer	0.298	1.970	0.049
	Hour	-0.041	-2.058	0.040	Hour	0.051	3.890	<0.001
	Cub*	-3.565	-1.750	0.080				

Bipedal standing	Spring	1.071	3.265	0.001	Winter	-1.373	-2.586	0.010
	Hour	0.400	5.028	<0.001	Autumn	1.383	1.916	0.055
					Hour	0.631	6.423	<0.001
Scent-anoint	Hour	0.397	4.822	<0.001	Hour	0.790	3.098	0.002
	Male	-1.477	-2.028	0.043	Summer*	-1.940	-1.710	0.087
Anogenital rubbing	Spring	1.054	2.510	0.012	Autumn	2.489	2.218	0.027
	Summer	-3.023	-4.047	<0.001	Summer	-5.262	-2.467	0.014
	Hour	0.221	3.406	0.001	Hour	0.412	3.406	<0.001
	Female*	0.973	1.823	0.068	Female	2.056	2.406	0.016
					Male	-2.680	-2.799	0.005
					Winter*	1.900	1.850	0.064

3.3.1. Activity

The statistical results are in Tables 3.3 and 3.4, and for activity graphically represented in Figure 3.2a. The count model for activity showed a slight decrease in activity in the Summer and a slight increase in the Winter when compared to the grand mean of all seasons. There were also lower levels of activity in the cub and higher in the mother when compared to the grand mean of all life stages. The zero-inflation model indicated that in the Autumn and Summer activity was more likely to be zero, while in the Spring pandas were more likely to show some level of activity. In addition, the mother's activity was less likely to be a zero. This result, paired with the mother's result in the count model, suggests that she was more consistently active and at higher levels than other individuals, which can also be seen in Figure 3.2a. Pairwise comparisons also revealed that the mother had significantly higher activity levels than sub-adults (0.165, z.ratio= 2.799, p= 0.026). Activity among all pandas was also lower in Summer compared to Winter (-0.120, z.ratio= -3.045, p= 0.012). For resting/sleeping, the count model revealed a slight decrease of rest/sleep in the Spring compared to the grand mean for all seasons. Within the zero-inflation model, all coefficients were significant except for either sex (Table 3.3). Post hoc analysis did not reveal any significant pairs.

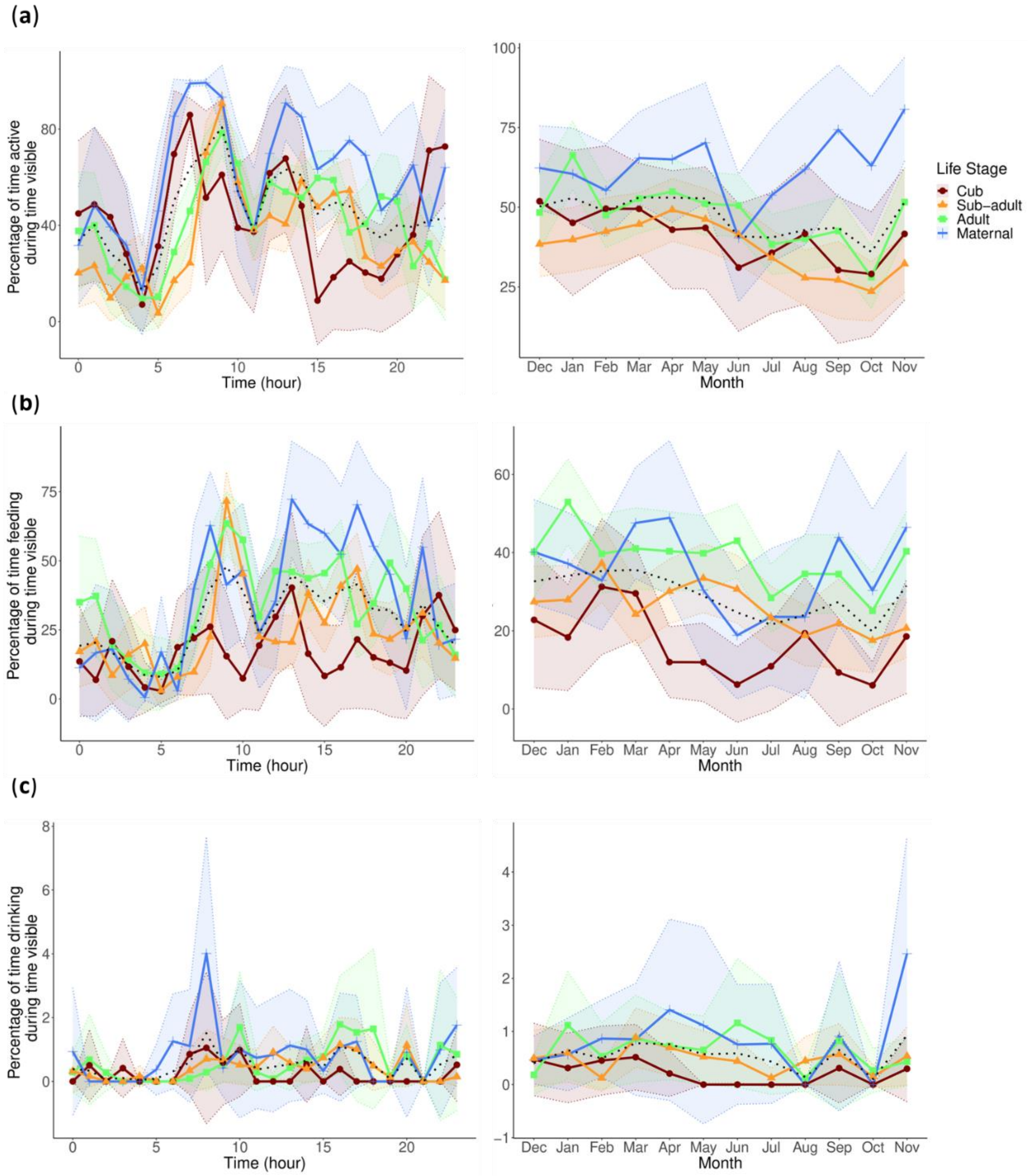

Figure 3.2. Circadian and circannual cycles of activity (a), feeding (b), and drinking (c) by life stage (cub, n=1 for 24 hours; subadult, n=5, 3 for 24 hours, 2 for daylight; adult, n=6, 2 for 24 hours, 4 for daylight; maternal, n=1 for 24 hours). Behaviours are displayed as estimated percentage of time active/displaying behaviour while in sight, controlling for time out of sight, and was averaged by hour (circadian) or month (circannual). The black dotted line represents the mean across all individuals. Scales are not standardised between circadian and circannual graphs. The shaded regions are the 95% confidence intervals for the activity.

Feeding accounted for 70.6% of active behaviour overall and consequently has similar circadian and circannual rhythmicity to overall activity (Figure 3.2b). The count model found higher levels of feeding in adults and lower levels in the cub compared to the grand mean of all life stages. The zero-inflation model indicated that feeding was more likely to be at zero in Autumn and Summer, and more likely to be a positive integer in Winter and Spring. In addition, the cub was more likely to display true zeros for feeding (does not include nursing behaviour). This result combined with the result from the count model for the cub indicates that the cub fed less often and likely at lower intensity than other individuals. Pairwise comparisons confirm this with the cub showing significantly less feeding behaviour than his mother (-0.525, z.ratio= -2.679, p= 0.037), sub-adults (-0.426, z.ratio= -2.709, p= 0.034), and adults (-0.553, z.ratio= -3.937, p<0.001), and can also be seen in his circadian and circannual cycle of feeding in Figure 3.2b.

For drinking, the count model and pairwise comparisons produced no significant coefficients or differences. However, the zero-inflation model indicated that it was more likely for drinking to be at the zero level during Summer and less likely during the Spring. The circadian cycle of drinking seems to show the largest peak among all individuals in the afternoon (Figure 3.2c).

For locomotion, the count model indicated an increase in locomotion in the Winter and a decrease in the Summer compared to the grand mean of all seasons. In addition to increased levels of locomotion in the Winter, the zero-inflation model also indicates that locomotion was more likely to be at zero levels in the Winter. These two results together may suggest that pandas have more intense but less frequent bouts of locomotion in the Winter. These results for the seasonal variation in locomotion are further supported by the pairwise comparison showing significantly more locomotion in Winter compared to Summer (0.421, z.ratio= 2.892, p= 0.02) and can be seen in Figure 3.3a. The zero-inflation model also suggests that locomotion is more likely to be a positive integer in the Spring. In addition, sub-adults are more likely to show true zeros for locomotion, while the cub is more likely to show some level of locomotion.

3.3.2 Stereotypic/Abnormal behaviours

We chose to analyse pacing and bipedal standing because these were the two most common stereotypic/abnormal behaviours, with pacing accounting for 89.3% of all stereotypic/abnormal behaviours and bipedal standing accounting for 4.9%.

Within the count model, we saw that the mother displayed more pacing compared to the grand mean across life stages. In addition, pacing increased in Spring compared to the grand mean of all seasons. The zero-inflation component also indicated that during Spring pandas were less likely to show true zeros for pacing, or rather, more likely to show a positive integer, which may partially account for the increased levels indicated in the count model as they were more consistently showing pacing. Conversely, in Summer, pandas were more likely to show true zeros for pacing. These results on the seasonality of pacing were further supported by *post hoc* pairwise comparisons showing that there was significantly more pacing in Spring compared to Summer (z ratio= 2.424, p= 0.0725). Also, despite being locomotor pacing, the circadian and circannual rhythm of pacing did not match that of locomotion (Figure 3.3).

The bipedal standing count model showed there was significantly more bipedal standing in Spring compared to the grand mean of all seasons. The zero-inflation model indicates that a true zero in bipedal standing is more likely in Autumn and less likely in Winter. In addition, pairwise comparisons indicate that bipedal standing in Spring is displayed at significantly higher levels than in Summer (1.699, z.ratio= 2.965, p= 0.016) and Winter (1.614, z.ratio= 3.287, p= 0.006). Although no results for life stage were significant for bipedal standing, we have displayed the life stages for comparison with pacing, as this behaviour was often done in conjunction with pacing. Bipedal standing also displays a similar early morning peak to pacing and increase in Spring (Figure 3.3c).

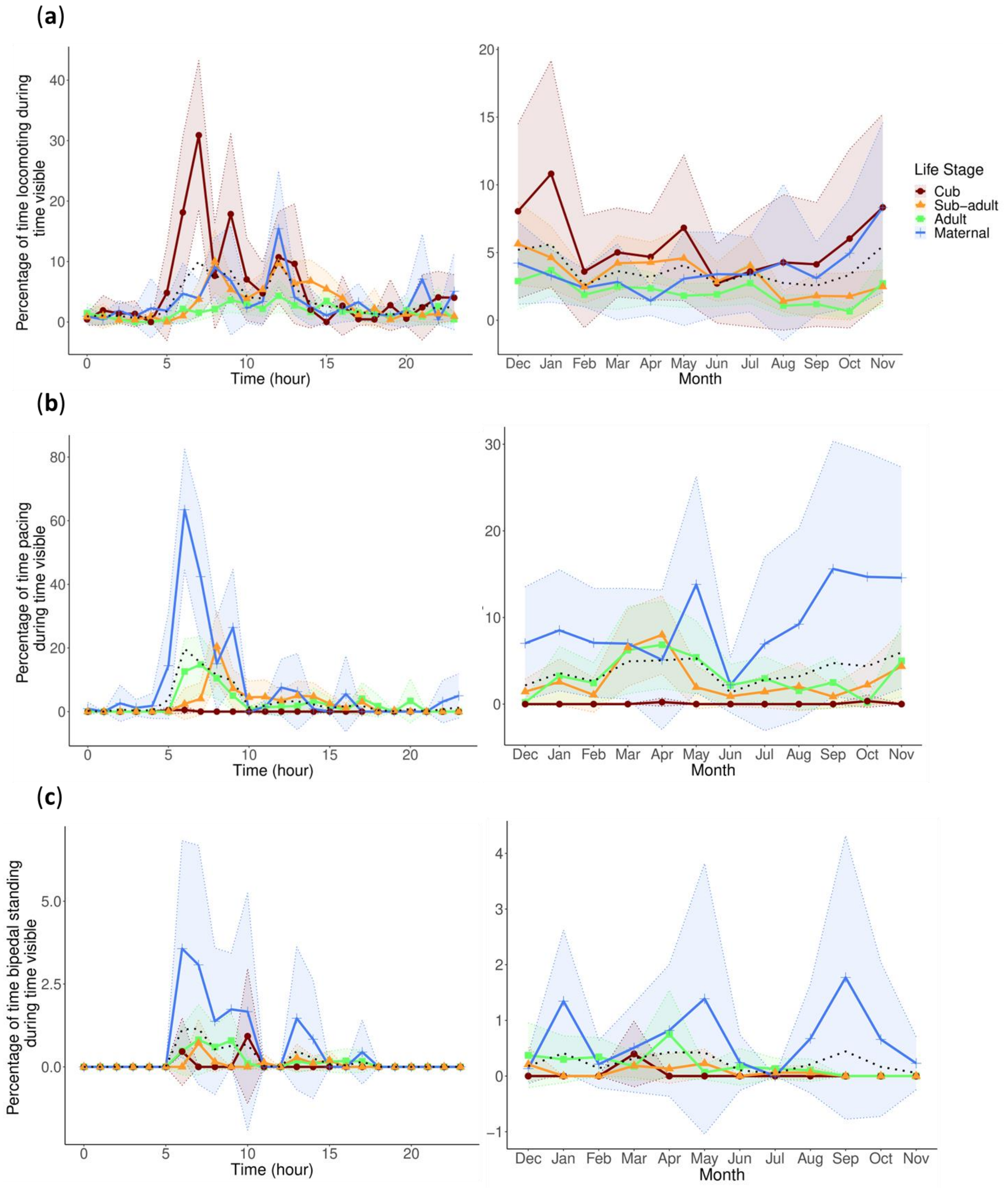

Figure 3.3. Circadian and circannual cycles of locomotion (a), pacing (b), and bipedal standing (c) across life stages (cub, n=1 for 24 hours; subadult, n=5, 3 for 24 hours, 2 for daylight hours; adult, n=6, 2 for 24 hours, 4 for daylight; maternal, n=1 for 24 hours). Behaviours are displayed as estimated percentage of time displaying behaviour while in sight, controlling for time out of sight, and was averaged by hour (circadian) or month (circannual). The black dotted line represents the mean across all individuals. Scales are not standardised between circadian and circannual graphs. The shaded regions are the 95% confidence intervals for the activity.

3.3.3 Sexual-related behaviours

During the study period, there was no mating witnessed. Therefore, our analysis consists of investigating the behaviours of scent-anointing and anogenital rubbing because they are thought to be ways of signalling home range occupation, competitive ability, and fitness (Bian et al., 2013; Charlton et al., 2020; White et al., 2002).

Within the count model for scent-anointing, males displayed a decreased level of scent-anointing compared to the grand mean observed across both males and females. Only the hour was significant within the zero-inflation model. *Post hoc* analysis revealed no significant differences between category levels. The circadian rhythm of scent-anointing reveals females scent-anointing at night when males are not scent-anointing (Figure 3.4b), however, it should be noted that those females are sub-adults.

Anogenital rubbing was modelled with only sex, season, and hour because adding age overparameterised the model. The count model suggested that anogenital rubbing significantly increases in the Spring and decreases in the Summer compared to the grand mean. The zero-inflation model also indicated that during Summer anogenital rubbing was less likely to be at the zero level, or rather pandas were more likely to show some level of anogenital rubbing. This result combined with the decrease in Summer seen in the count model may suggest that anogenital rubbing may occur more consistently in the Summer, but at low levels. The zero-inflation model also indicated that during Autumn, pandas are more likely to have true zeros for anogenital rubbing. In addition, females were more likely to have true zeros for anogenital rubbing while males were less likely. *Post hoc* pairwise comparisons showed that there was significantly less anogenital rubbing in Summer compared to Autumn ($z= -3.126$, $p= 0.01$), Spring ($z= -4.352$, $p<0.001$), and Winter ($z= -3.602$, $p= 0.002$). This decreased level of anogenital rubbing in Summer can be seen in Figure 3.4b. The circadian rhythm of anogenital rubbing seems to indicate that most anogenital rubbing occurs during daylight hours

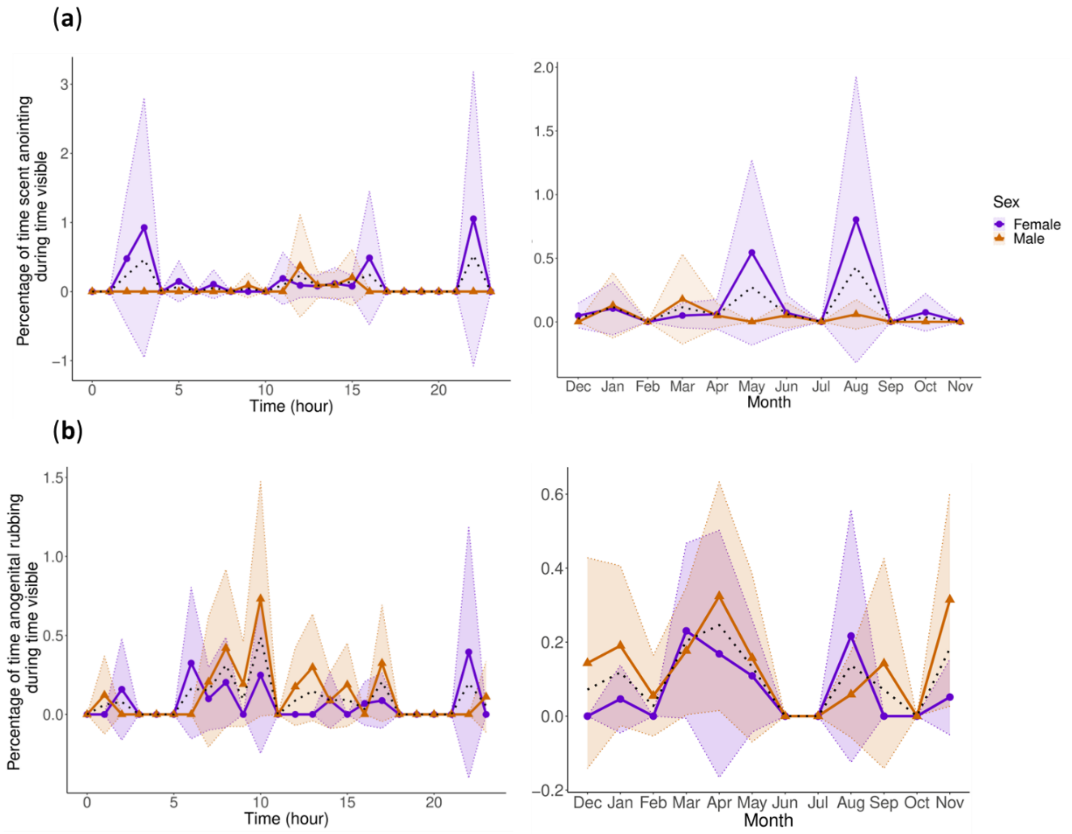

Figure 3.4. Circadian and circannual cycles of scent-anointing (a) and anogenital rubbing (b) by sex (male, n=5, 4 for 24 hours, 1 for daylight; female, n=6, 3 for 24 hours, 3 for daylight). Behaviours are displayed as estimated percentage of time active/displaying behaviour while in sight, controlling for time out of sight, and was averaged by hour (circadian) or month (circannual). The black dotted line represents the mean across all individuals. Scales are not standardised between circadian and circannual graphs. The shaded regions are the 95% confidence intervals for the activity.

To investigate to what extent a breeding pair synchronise their sexual-related behaviours (Table B1), we conducted a wavelet coherence analysis on two breeding pairs that had 24-hr data (Figure 3.5). Figure 3.5a and 3.5b present the scale-averaged wavelet spectrum for each individual, a smoothed signal averaged across all cycle lengths that, given our sampling method, essentially produces a circannual rhythm of circadian rhythms. Both females, despite being in different zoos, displayed similar patterns in their sexual-related behaviours, displaying three progressively more intense peaks through the year (small, medium, then large peak). Both females displayed a small peak in January and a medium sized peak in the mating season (March-May). Meanwhile, the males displayed more small/medium peaks throughout the year and one large peak. Both males displayed their largest peaks towards the end of Autumn or in Winter. The male from Zoo B showed consistent, but small peaks throughout the breeding season. These similarities between the females and males of the two zoos is interesting, however, more breeding pairs would have to be examined to determine if these kinds of patterns are consistent across females and males.

The two breeding pairs had different coherence patterns in their sexual-related behaviours. Coherence of cycle lengths greater than 24-hr cannot be interpreted because we only gained an estimate of one day in each month, so cycles greater than 24-hr in our data do not have real world equivalents. The pair from Zoo D had cycle lengths at 4h in phase in January, but during the mating season were out of phase in their 8h cycle lengths (Figure 3.5c). However, they had strong coherence across all cycle lengths in November, which can also be seen with their matching large peaks in their scale-averaged spectrums. The breeding pair from Zoo B did not have in phase sexual-related behaviours at any point in the year, but during the breeding season around April there was coherence in their 24-hr cycle lengths with the female signal leading and the male signal lagging, which then switches between August-September, with the male signal leading and the female signal lagging. There was also some coherence between November and December for cycle lengths between 8-16h.

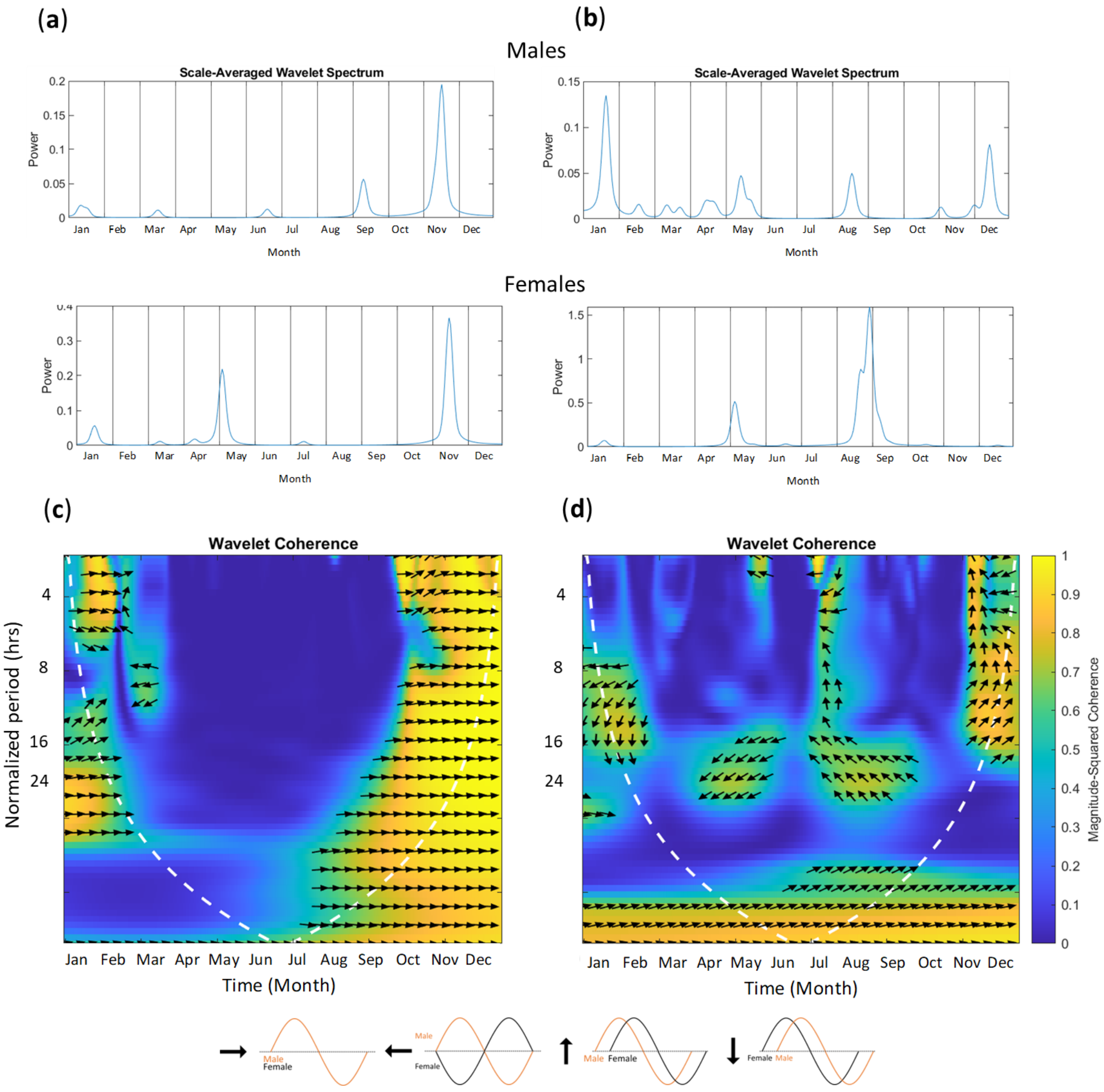

Figure 3.5. Wavelet coherence analyses of sexual-related behaviours between breeding pairs at Zoo D (c) and Zoo B (d). Zoo D has a sub-adult female and adult male and Zoo D has a male and female sub-adult pair. The dashed white line indicates a 5% significance level. The x axis is the time of year in months, the y-axis is the normalised frequency between the two signals, and the colour represents the strength of the correlation (yellow high, dark blue low, scale on right hand side). The kind of phase relationship between the signals are noted by the arrows (refer to arrow key). We have also displayed the scale-averaged wavelet spectrums for each individual panda at Zoo D (a) and Zoo B (b). These can be seen as smoothed circannual rhythms of the circadian rhythms of all sexual-related behaviour. The x-axis is the time in months which matches with the coherence figures, and the y-axis is the power (average magnitude across all period lengths for that time).

Table 3.4. Summary of significant and potentially significant (p<0.1) coefficients from the ZINB models of female activity, maternal behaviours, and mother and cub proximity. In the conditional model (+) coefficients indicate the category level/variable is larger than the grand mean, and a (-) coefficient means it is smaller than the grand mean. For zero-inflation coefficients, (+) indicates the category level/variable is more likely to be a zero and (–) means it is less likely to be zero (i.e. more likely to be a positive integer). Variables with an asterisk did not reach the significance threshold but did show possible trends towards significance, with p<0.1. "None" signifies that no coefficients reached significance thresholds in that model.

	Conditional				**Zero-inflation**			
Behaviour	Variable	Coefficient	Z-value	Pr (>|z|)	Variable	Coefficient	Z-value	Pr (>|z|)
Female activity	Maternal female*	0.105	1.750	0.08	Maternal female*	-0.684	-1.672	0.0945
Maternal behaviours	N/A				N/A			
Nursing	None				Hour	0.059	2.370	0.018
In contact	Spring 22	-0.481	-2.030	0.0420	Spring 22	-0.630	-1.995	0.046
	Summer 21	0.534	3.185	0.001				
Proximate	Autumn 21	-0.618	-4.473	<0.001	Spring 21	0.557	2.918	0.004
	Spring 22	0.448	2.968	0.003	Spring 22	-0.687	-2.398	0.016
	Summer 21	-0.594	-4.163	<0.001	Summer 21	0.415	2.065	0.039
					Hour	0.059	4.333	<0.001
					Winter 20-21*	0.436	1.802	0.072
Distant	Autumn 21	0.067	1.980	0.047	Autumn 21	-1.161	-3.802	<0.001
	Spring 21	0.091	2.740	0.006	Winter 20-21	0.640	2.802	0.005
	Spring 22	-0.161	-2.700	0.007	Hour	-0.044	-3.026	0.002
	Summer 21*	0.061	1.790	0.073				

3.3.4 Mother and cub behaviours

When modelling the activity of the maternal female against all other females, there was no significant difference between the maternal female and other females. However, given that this model was one individual compared to six, it is important to note that the difference showed a trend toward significance (0.105, z= 1.75, p= 0.08). The circadian and circannual rhythms also show the maternal female displaying consistently higher average activity levels than the other females, with some areas of no overlap between 95% confidence intervals (Figure 3.6).

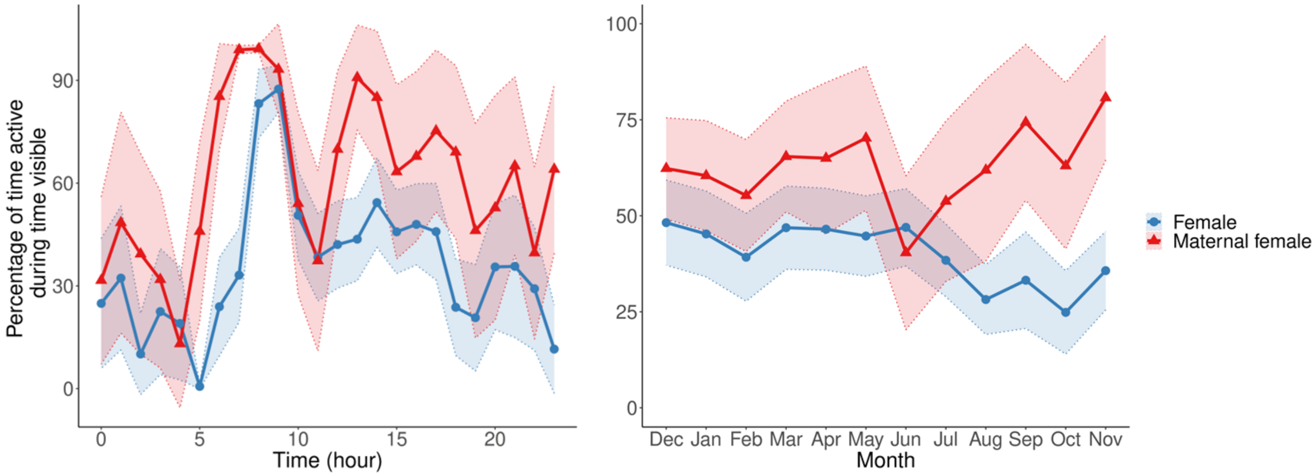

Figure 3.6. Circadian and circannual cycles of activity between the females (n=5, 2 for 24 hours, 3 for daylight) and the mother (n=1, for 24 hours). Behaviours are displayed as estimated percentage of time active while in sight, controlling for time out of sight, and was averaged by hour (circadian) or month (circannual). Scales are not standardised between circadian and circannual graphs. The shaded regions are the 95% confidence intervals for the activity.

When modelling the non-nursing maternal behaviours, the models would not run likely due to insufficient data. However, we still found it important to visualise the circadian and monthly rhythm of these behaviours (Figure 3.7a). For nursing behaviour, there were no significant differences between seasons, and only the hour was significant within the zero-inflation model. However, it is clear that within the circadian rhythms of both the general maternal and nursing behaviours that these behaviours were mostly displayed after zoo opening hours (Figure 3.7), with only one peak seen in the afternoon for nursing. These results are complemented by the circadian rhythm of proximity, where mother and cub spent the most time in contact or proximate after zoo hours and spent a larger proportion of time distant during hours when the zoo was open (Figure 3.8a).

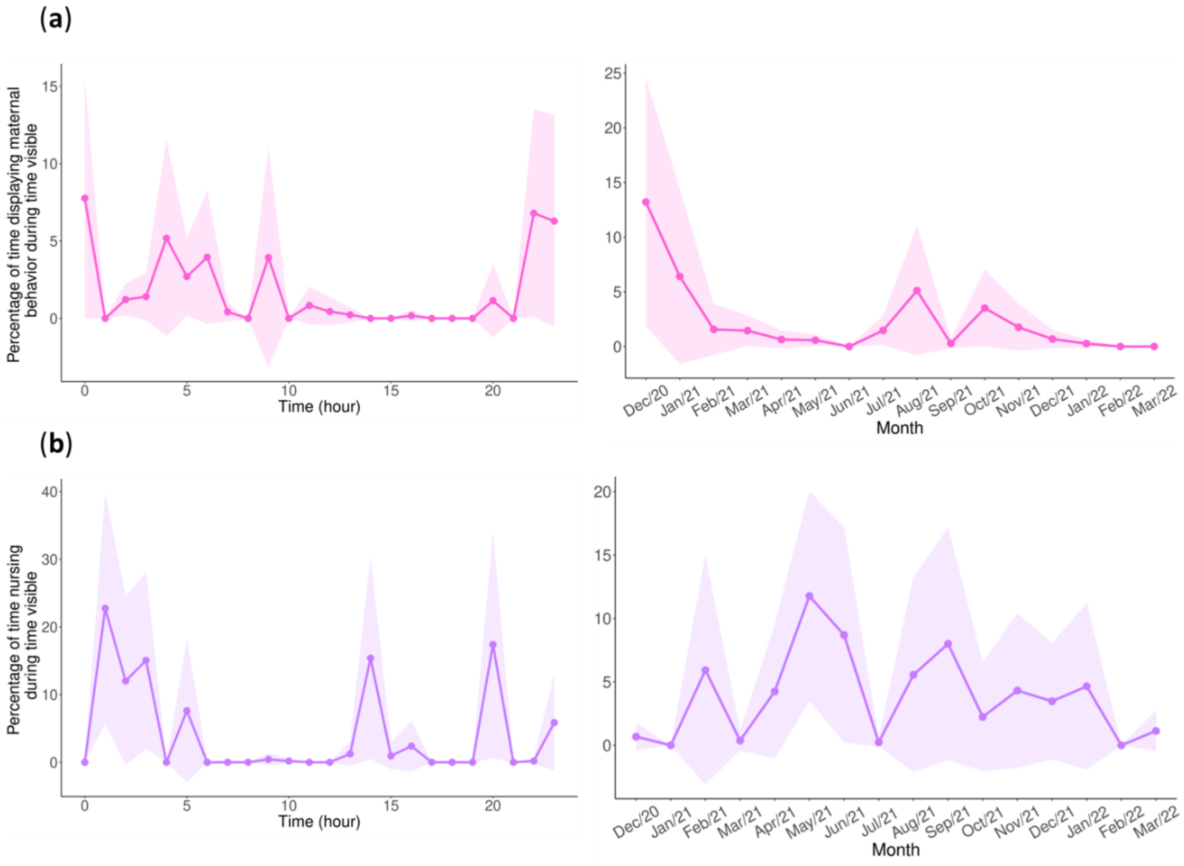

Figure 3.7. Circadian and circannual cycles of non-nursing maternal behaviour (a) and nursing (b) for mother and cub. Behaviours are displayed as estimated percentage of time displaying behaviour while in sight, controlling for time out of sight, and was averaged by hour (circadian) or month (circannual). Scales are not standardised between circadian and circannual graphs. The shaded regions are the 95% confidence intervals for the activity. Data were averaged between mother and cub.

The circannual rhythm of nursing did not show any changes over time, but it should be noted that the cub was not fully weaned (i.e. separated from his mother and eating independently) until 11 months after observations ended. Pairwise comparisons for the proximity of mother and cub indicated that they were significantly more in contact in Summer 21 compared to Autumn 21 (0.756, z.ratio= 2.857, p= 0.049) and Spring 22 (1.016, z.ratio= 3.141, p= 0.021). The pair were also proximate significantly less in Autumn 21 compared to Winter 20-21 (-0.722, z.ratio= -3.034, p= 0.029), Spring 21 (-0.742, z.ratio= -3.608, p= 0.004), Winter 21-22 (-1.155, z.ratio= -6.480, p<0.001), and Spring 22 (-1.066, z.ratio= -4.721, p<0.001). They were also proximate significantly less in Summer 21 compared to Winter 20-21 (-0.699, z.ratio= -2.865, p= 0.048), Spring 21 (-0.718, z.ratio= -3.452, p= 0.007), Winter 21-22 (-1.131, z.ratio= -6.23, p<0.001), and Spring 22(-1.042, z.ratio= -4.496, p<0.001). The model for distant displayed a model convergence warning which indicated that the best fitting model was likely not found, and therefore, AIC and BIC scores were not produced. However, pairwise comparisons indicate that the mother and cub spent significantly less time distant in Spring 22 compared to Spring 21 (-0.254, z.ratio= -3.281, p= 0.013), Summer 21 (-0.222, z.ratio= -2.867, p= 0.048), and Autumn 21 (-0.229, -2.952, p= 0.037). They also spent significantly more time distant in Spring 21 compared to Winter 21-22 (0.146, z.ratio= 2.763, p= 0.063). These results did not support our prediction that the mother and cub would gradually spend more time distant as more time passed.

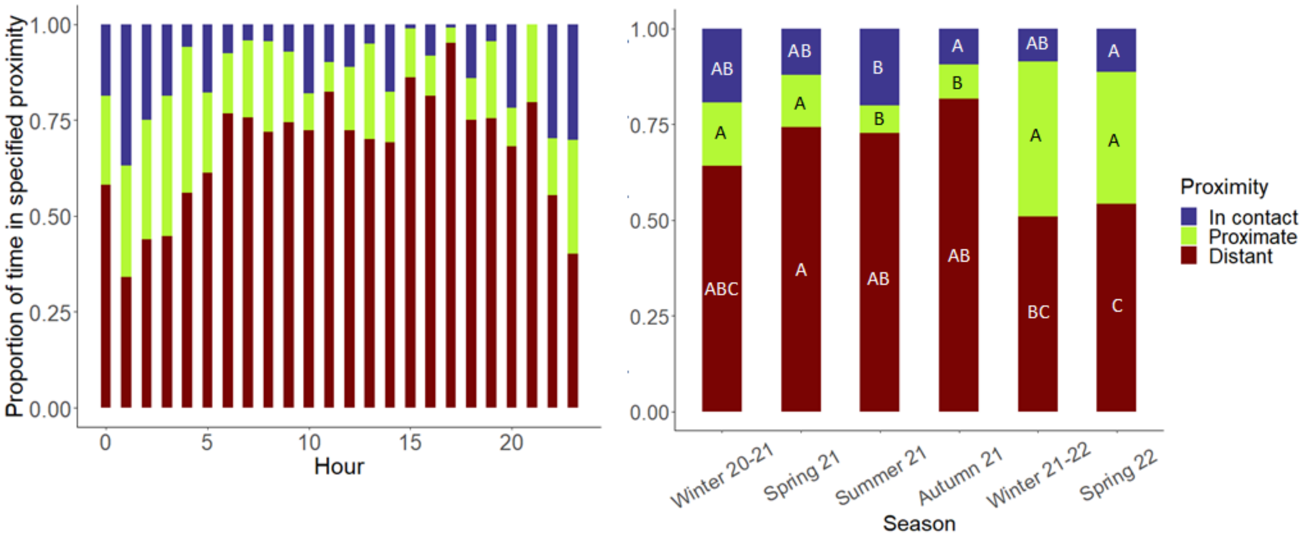

Figure 3.8. *Proximity (in contact, blue; proximate, green; distant, red) of mother and cub displayed as estimated proportions of time by hour, and season. The letters within the proximity categories represent significance. Within each proximity category, different letters represent a significant difference between seasons for that proximity. Seasons that share any letter for a proximity category are not significantly different. Time when mother and cub were separated by keepers was excluded so that only proximity when mother and cub had choice is displayed. Therefore, proportions of time are extended to 1 excluding keeper separation, which was only displayed during working hours of keepers.*

We compared the activity cycles of the mother (16 months of data) and cub (13 months of data) using continuous wavelet transform. We found that they displayed similar, clear cycle lengths of activity: ~3h, 5h, 8h, 12h, and 24h. The power of the cycles (a measure of the consistency of magnitude through time) was similar between the mother and cub for the 3h, 5h, and 8h cycles. However, the cub had a stronger 12h cycle and the mother had a stronger 24h cycle, with the mother showing increasing magnitude of her 24h cycle towards the end of the observation period (red square in Figure 3.9). In addition, we also conducted a wavelet coherence analysis to determine when activity for mother and cub were synchronised (Figure

3.10). We found that the mother and cub periodically had their 4-8h cycles synchronised throughout the observation period. Interestingly, their 24h cycles showed strong synchronisation between May-July 2021, which was not when the mother had a consistent and strong 24h cycle.

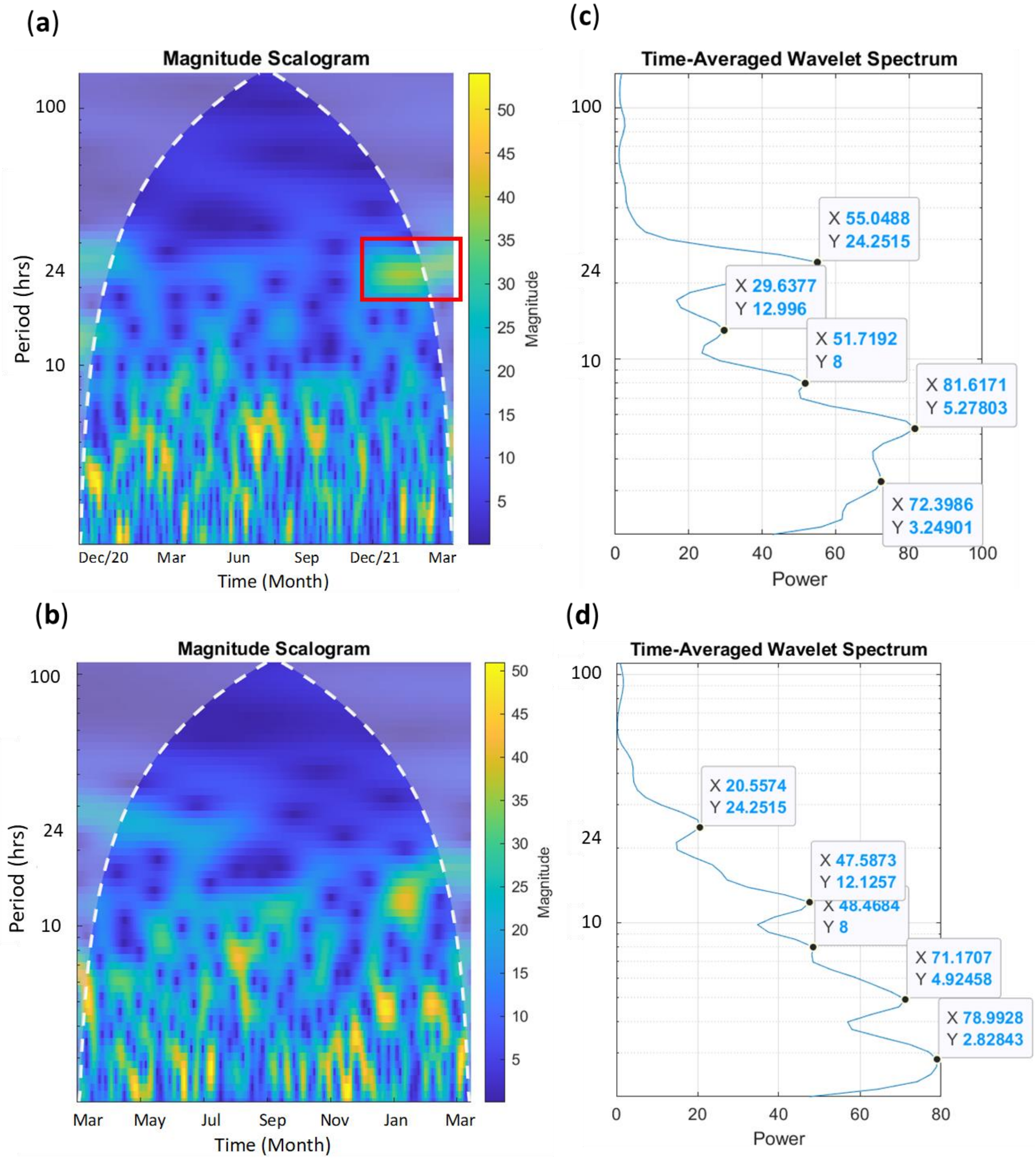

Figure 3.9. Continuous wavelet transform analysis of mother (a) and cub (b) overall activity. The x-axis represents the time in months across the observation period. Therefore, the mother has three more months of observations than the cub at the start. The y-axis is the period length in hours; periods above 24 hours do not have real-world equivalents given our

sampling method. The magnitude is represented with colour. The red square in the mother's wavelet highlights when her 24-hr period of activity was more consistent. Time-averaged wavelet spectrums are also presented for mother (c) and cub (d). These graphs represent the average magnitude of period lengths across the observation period. The x-axis is the power (magnitude over time) and the y-axis is the period length, which equates to the period lengths in the wavelet.

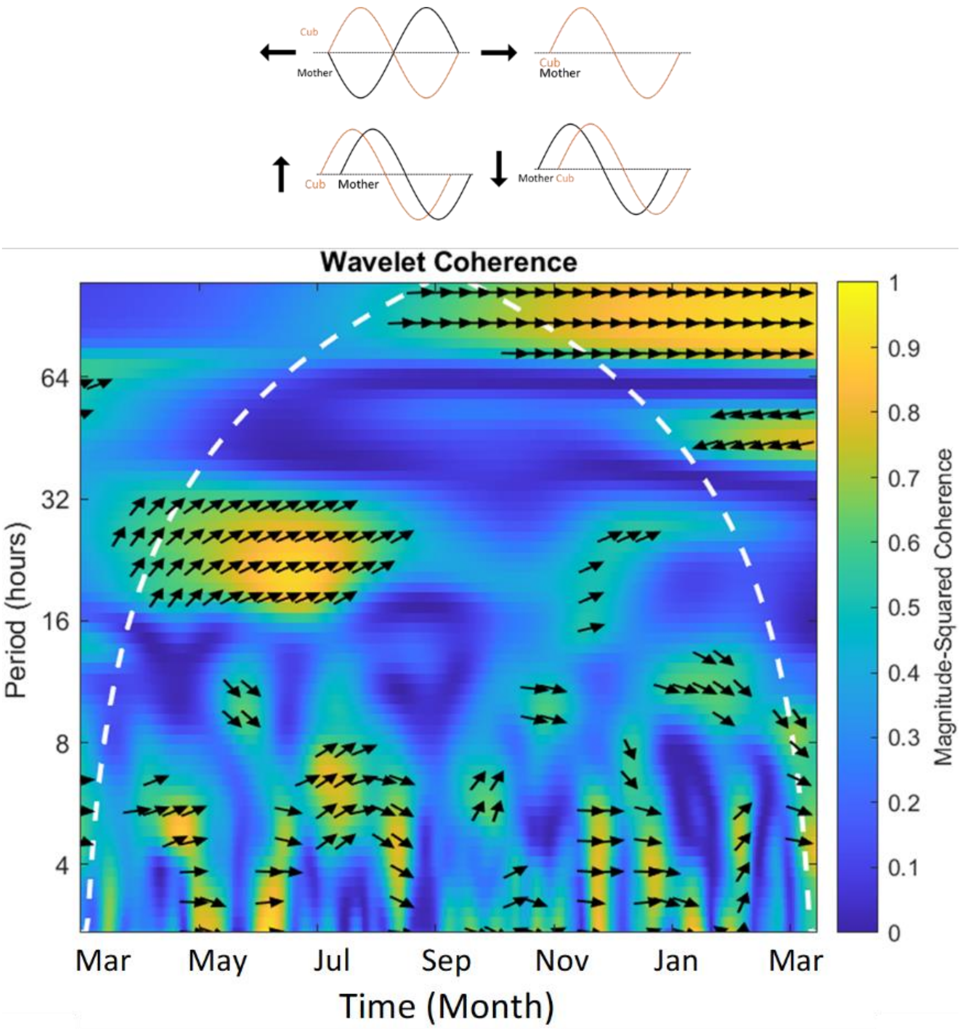

Figure 3.10. Wavelet coherence analysis of activity between the mother and cub pair. The x axis is the time of year in months, the y-axis is the normalised frequency between the two signals, and the colour represents the strength of the correlation (yellow high, dark blue low, scale on right hand side). The kind of phase relationship between the signals are noted by the arrows (refer to arrow key). Data presented in this wavelet excludes the data collected for the mother in Winter 20-21.

3.4 Discussion

Our results show the holistic perspective we gain on behaviour when observing circadian and circannual cycles. Our hypotheses that life stage and sex would influence these cycles was supported as we found significant coefficients and/or differences between life stages for overall activity, resting/sleeping, feeding, locomotion, and pacing, and significant coefficients by sex for scent-anointing and anogenital rubbing. There were no differences among life stages or by sex for drinking nor bipedal standing. However, all behaviours displayed daily and seasonal patterns. Understanding these cycle patterns can aid animal care staff in predicting changing needs throughout the day, year, and life cycle and pre-emptively provide for those needs to best avoid welfare concerns. Though the sample size in this study is relatively small, we believe that the conclusions are still meaningful and can be built upon in future studies.

3.4.1 Holistic view of active and inactive behaviour provides insight into energy and behavioural dynamics

Investigating the cycle of overall activity is a simple way of gaining information and context on an animal's routine, circadian clock health, and sleep health. In relation to routine, it was clear that our study pandas all followed a similar activity cycle with three peaks in activity: largest in the early morning, second spread throughout the afternoon, and a third in the middle of the night. An earlier study investigating the cycles of activity in captive pandas at the Wolong nature reserve found two peaks of activity in the daytime, similar to our results of the daytime peaks (Mainka & Zhang, 1994). However, in a study completed on 5 wild pandas fitted with radio collars collecting 24-hr data, it was discovered that giant pandas have three peaks including some nocturnal activity (Zhang et al., 2015). Therefore, demonstrating that captive pandas follow similar rhythms to their wild counterparts. The inclusion of nocturnal activity provides a complete picture of these cycles and allows us to infer with more surety what a homeostatic cycle looks like in pandas.

In addition to this similar result in the circadian rhythm, we also found very similar results in the circannual rhythm when compared to wild pandas. Zhang et al. (Zhang et al., 2015) found that activity peaked in Spring, reduced in Summer and Autumn, then increased again in Winter. Our models and figures illustrated very similar cycles, where the highest activity across individuals was seen in Spring. These similar results between giant pandas in the wild and captivity depict the strength of circadian clocks and indicate the importance of considering them when understanding behaviours, and consequently, the needs of a species. Since circadian clocks are an adaptive mechanism for synchronizing the internal environment with the external environment to optimise functionality and energy, they inherently play a large role in their welfare.

Resting/sleeping behaviour is important to investigate when determining the welfare state of an animal because it can be one of the clearest indicators of circadian system health. Our results indicate that pandas rest/sleep slightly less in the Spring, perhaps in correlation to the migratory or mating period, though further investigation would be needed. They also generally seemed to rest/sleep at midday, and then again at night, mixed with nocturnal activity. The adults and maternal female tended to be more likely to display zero rest/sleep while the cub and sub-adults were more likely to display positive integers. This could indicate that adults have more sporadic rest/sleep when compared to sub-adults, which can be supported by the larger variability in sleep seen at night in adults. Though definitive conclusions cannot be drawn, the sleep/wake cycle is known to be disrupted with aging as a result of general aging of the circadian system resulting in desynchrony with the external environment and decoupling of internal circadian clocks, often resulting in irregular sleep or arousal thresholds (Liu & Chang, 2017; Pilorz et al., 2020; Terzibasi-Tozzini et al., 2017). In addition, sleep is crucial to the functioning of innate immunity, as many cycles of immune responses and DNA repair are dependent on sleep (Gaddameedhi et al., 2011; Irwin & Opp, 2017). Therefore, tracking sleep in captive environments is important to assess the overall health of an individual, especially older individuals, and could assist in identifying early symptoms of underlying disorders that result in circadian dysregulation.

All zoo animals have a need for a suitable diet, which is usually entirely provided by zoo staff. In order to provide appropriate food for a species, we have to understand their metabolic and energetic needs and how these cyclically change throughout the day, seasons, and lifespan. Investigating the rhythms of more specific major active behaviours, including feeding, and inactive behaviours can help in this understanding. Giant pandas are peculiar in that they have very low energy expenditure relative to other species (Nie et al., 2015). This is likely an evolutionary trait because of their diet of bamboo which their gut is not well adapted to obtain high levels of nutrition from since they are incapable of ruminating to draw more nutrients. Therefore, pandas must eat large amounts of bamboo to compensate, spending higher proportions of time foraging and feeding. We found that pandas spend 70.6% of their active time feeding, with adults showing significantly higher proportions of their time feeding compared to the grand mean and the cub showing lower proportions. Nie et al. (Nie et al., 2015) found a significant effect of body mass on the energy expenditure of pandas, with larger body mass coinciding with higher daily energy expenditure. With presumably larger body masses, adults would need to feed more often to maintain their weight given their higher energy expenditure. The cub spending significantly less time feeding would be due to his smaller body mass and the fact that he was nursing throughout the observation period which was not coded as feeding. This kind of data can be used to determine the amount of food needed for different life stages, and could also be used as indicators of a transition into new life stages. The cycles of feeding will be explained in the context of migration and in relation to stereotypic/abnormal behaviours later.

Locomotion is an activity that depletes energy more than other activities. Therefore, according to optimal foraging theory, this behaviour would have to occur in an efficient manner given the low- quality bamboo food source of pandas. Our results suggest that locomotion is an activity limited to the day, with very low levels occurring at night. Mainly, locomotion seems to reach its highest level at midday and then steadily decreases into the late afternoon. This may be to conserve energy and avoid displaying an energetically taxing behaviour in the hotter times of day. We also found that locomotion significantly increases at colder temperatures in the Winter. Since a panda’s pelage is able to efficiently retain body heat, it would be an ideal time to increase locomotion. In addition to these results, it is known that wild pandas show a very low mean movement speed (Nie et al., 2015), further maximizing their energy.

The circadian and circannual cycles of drinking have not previously been determined in giant pandas. The circadian rhythm of drinking seems to have several peaks throughout the day and night that match the peaks seen in activity, with the largest peak of drinking among all individuals in the later afternoon. This peak could be in response to completing most of the energy expenditure of the day that occurs mostly in the first half of the day. We also found drinking was more likely to be at the zero level during Summer and more likely to be a positive integer during Spring. These shifts in drinking coincide with the seasonal decreases and increases in activity in the Summer and Spring, respectively. Like many other organs, the renal system has a circadian clock that regulates water retention and the homeostasis of electrolytes (Firsov et al., 2012). Having a baseline for comparison for individual or species level drinking rhythms could help in early identification of issues related to water retention and homeostasis if a change in the amount or timing of drinking occurs. These rhythms could be paired with the rhythms of urination and defecation. Our sampling combined urination and defecation, making it difficult to conduct meaningful comparisons with drinking.

We investigated the rhythms of scent-anointing and anogenital rubbing as sexual-related behaviours because they are associated with signalling home range occupation, competitive ability, and fitness (Bian et al., 2013; Charlton et al., 2020; White et al., 2002). The circadian rhythm of scent-anointing displayed males concentrating their scent-anointing to daylight hours, while females showed two large peaks of scent-anointing in the night in addition to lower frequencies in the daylight. However, it should be noted that these large peaks were displayed by sub-adult females, and it is unknown whether they would continue displaying this behaviour once they reached sexual maturity. Our results were contrary to a previous finding that male giant pandas scent anoint more often than females (Charlton et al., 2020). It is important to highlight, however, that two of the six males in our sample likely had reduced sexual competitiveness because one adult male in our sample is castrated for medical reasons and another is a cub. Male pandas in the study by Charlton et al. (Charlton et al., 2020) were seen to anoint preferentially with strong odours, likely as an olfactory signal of competitiveness to other males (Charlton et al., 2020).

The seasonal cycle of scent-anointing that we found may support the competitive signalling hypothesis, since we recorded males scent-anointing mainly in January and March leading into the mating season while the females show a similar peak in January, but have their largest peaks in May and August. Though again, important to note that the peak in August by females was entirely by sub-adult females, so it may be an indication of sexual immaturity. (Charlton et al., 2020; Ma et al., 2021)

Anogenital rubbing and scent-anointing have similar functions and are presumed to be related to each other. They are often displayed in response to each other in carnivores, including other bears and giant pandas (Charlton et al., 2020; Rieger, 1979). Indeed, we did see similarity in the cycles of anogenital rubbing and scent-anointing and their frequencies. Anogenital rubbing occurred at significantly higher levels in the Spring compared to the grand mean, and can be seen increasing in both males and females, supporting the hypothesis that it is related to sexual signalling since its highest levels are shown in the breeding season (D. Liu et al., 2006). Both signals also showed decreases in the Summer, though levels were also less likely to be zero in Summer like in scent-anointing; suggesting that both behaviours are shown more consistently but at lower intensities in the Summer. We did not find significantly higher or lower averages of anogenital rubbing by sex, however, females were more likely to display true zeros for anogenital rubbing while males are more likely to display some level of anogenital rubbing. These are similar to results in other studies on giant pandas that found males scent mark more often than females (Charlton et al., 2020; White et al., 2002), and could be an indication of male-male competition.

3.4.2 Relationships between migratory, sexual-related, feeding, and stereotypic/abnormal behavioural cycles

Migration is an inherent behaviour in many species. (Gwinner, 1996; Gwinner & Czeschlik, 1978)The phenomenon of migratory restlessness has mainly been studied in birds. It occurs in species that are both obligate migratory birds (Agatsuma & Ramenofsky, 2006) and facultative migratory birds (Watts et al., 2016). However, migratory restlessness in non-avian captive species has not been given much investigation. Though migratory restlessness has not been investigated, similar questions have been asked of captive carnivores with large home-ranges. It has been found that stereotypic pacing frequency was positively correlated with home-range size and average chase distance of captive carnivores (Clubb & Mason, 2003; Kroshko et al., 2016). It could be that in non-avian migratory species, migratory restlessness is expressed through stereotypic pacing or other stereotypic/abnormal behaviours.

Giant pandas are a migratory species, their migration patterns have been investigated in the wild and evidence suggests that the main motivation for migration is following the emergence of nutritious bamboo shoots, though there are also effects of solar radiation and habitat preference (Liu et al., 2011; Liu et al., 2002; Zhang et al., 2015; Zhang et al., 2017; Zhang et al., 2018). Wild pandas initiate migration from middle April to early June, migrating within several days to their Summer range and returning over several weeks from early September to October (Liu et al., 2002). The fast migration at the start of Spring coincides with the emergence of bamboo shoots across the elevational gradient, with shoots at higher elevations showing a gradient delay in emergence (Zhang et al., 2018). Importantly, the initial period of migration also coincides with the breeding season in Spring. The same study used GPS collars and were also able to note that despite the elevational migration pattern across the pandas, individual paths were distinct, and associated these different pathways with the possibility that pandas were also seeking mates.

In captivity, pandas will presumably have two concurrent motivations to migrate, to find emerging bamboo shoots and to breed. Our investigation into the circadian and circannual rhythms of feeding, sexual-related behaviours, and stereotypic/abnormal behaviours can help begin to elucidate the connections between these behaviours and migration in captive giant pandas. The most common stereotypic/abnormal behaviours recorded in our study were locomotor pacing and bipedal standing. The circadian and circannual rhythms of these two behaviours were very similar. This is not surprising as bipedal standing was often incorporated into pacing behaviour. The circadian rhythms both showed clear peaks in the early morning hours and decreased levels throughout the rest of the day, with hardly any display of either behaviour through the night. For the circannual rhythms of pacing and bipedal standing, the pairwise comparisons for both behaviours indicated that there were increased levels in Spring, the time of initial migration and the breeding season, that were significantly higher than in Summer, respectfully.

The circannual rhythms of pacing and bipedal standing closely resemble that of anogenital rubbing, with the peaks for all three behaviours being in Spring. Anogenital rubbing and handstand urination was also commonly incorporated into pacing. With anogenital-rubbing being the behaviour more closely linked to sexual signalling, it is likely not coincidental that these behaviours were displayed together, and could be an indication of their intent when incorporated into pacing. Previous studies have also found a relationship between stereotypic behaviour and sexual-related behaviour in pandas with one study finding that captive males show more locomotive stereotypies than females and that the behaviour is correlated with reproductive performance (Martin et al., 2020). Gandia et al. (2023b) also conducted wavelet coherence analysis of stereotypic/abnormal and sexual-related behaviour signals and found coherence between the cycles. Interestingly, wild male pandas show increased locomotion in the mating season when compared to females (Liu et al., 2002), but our results on locomotion display very different results on the circannual rhythm of locomotion, with the highest levels being in the Winter. This disparity between wild and captive locomotor activity could suggest that captive pandas replace locomotion with locomotor pacing to fulfil the unmet need of migration and longer travel periods during the mating season.

Although the circannual rhythms of anogenital rubbing and stereotypic/abnormal behaviour were similar, the circadian rhythms were quite dissimilar, which leaves a gap in the explanation for the drivers behind the daily rhythmicity of stereotypic/abnormal behaviour. One explanation for the clear rhythmicity of an early morning peak in both pacing and bipedal standing (an anticipatory behaviour displayed at keeper doors where pandas will stand on hind legs to peek through windows into keeper areas) could be feeding anticipatory activity (FAA). Pandas across all the zoos studied were left with bamboo overnight and would receive fresh bamboo in the mornings when keepers arrived. Predictable feeding schedules cause animals to display anticipatory behaviours which may signal stress should their frequency increase in response to the predictable schedule becoming delayed (Anderson et al., 2015; Bassett & Buchanan-Smith, 2007; Waitt & Buchanan-Smith, 2001). Since stereotypic/abnormal behaviours may be a sign of anticipation (Waitt & Buchanan-Smith, 2001), the early morning peak in feeding behaviour and both pacing and bipedal standing, which do not coincide with sexual behaviour in the pandas, may indicate anticipation in relation to keeper and fresh food arrival.

This result is similar to that found in the study investigating rhythms of activity in captive pandas at the Wolong reserve where pandas were seen to have two peaks of daytime activity coinciding perfectly with feeding times (Mainka & Zhang, 1994). Additionally, in a study on captive brown bears, the bears were placed on a reversed feeding regimen, being fed during dark hours, resulting in their feeding schedule completely switching to being nocturnal, evidencing FAA (Ware et al., 2012). Displaying FAA may be a signal of circadian desynchrony in zoo animals if feeding times do not coincide with the natural circadian rhythm of feeding, and should be monitored in relation to welfare. It is especially important to consider since not just feeding time, but diet composition can override and desynchronise the central circadian clock, with chronic effects leading to premature aging (de Goede et al., 2018; Kolbe et al., 2019; Manoogian & Panda, 2017; Takasu et al., 2012). Pandas display a seasonal preference for plant parts in both the wild and in captivity, with a strong, natural inclination towards shoots in the Spring for wild individuals. One possible way of easing FAA could be to provide pandas with appropriate amounts of their seasonal preferences taking into account the wild and innate rhythms of preference for plant parts, and providing enough of these parts to sustain pandas while keepers are not present.

Our results may indicate a combined effect of anticipation for feeding and breeding opportunities. Zoo staff can use the cycles and paired behaviours to infer the needs that the panda is wanting to express. Pacing paired with bipedal standing can be an indication of food anticipatory activity, while pacing combined with anogenital rubbing can be a desire for breeding opportunities. Observing full cycles and comparing them all together allows for new associations between behaviours to be made. Though the associations between stereotypic/abnormal behaviours, feeding, and mating cannot be generalised to all species, the method in which these associations are investigated can. Circadian and circannual rhythms provide a fuller picture of behaviour and investigating them provides new insights into possible motivations behind behaviours.

3.4.3 Case study of mother and cub

Previous studies on giant pandas have investigated cycles of maternal activity levels in the wild (Zhang et al., 2017) and in captivity (Mainka & Zhang, 1994). These studies provided insight into the circadian and circannual cycles of maternal female activity, and allowed for comparison to other adults and females, but were not able to observe maternal behaviours in detail, and for the captive study, were not able to capture nighttime activity. Our study is able to expand on the knowledge of maternal behaviour in captive pandas to assist zoos in understanding mother and cub dynamics to best provide for their care.

Our results did not show a significant difference in the maternal female's activity compared to other females, which is a similar result to what was found by Mainka and Zhang (Mainka & Zhang, 1994) in maternal females whose daytime activity cycles across four seasons did not find a significant difference either. However, our results did show the maternal female displaying consistently higher activity levels than the other females in both her circadian and circannual rhythm. Though not significant, this increased activity could be a signal of maternal care since the mother must sustain herself as well as display other maternal behaviours which the non-maternal females do not display.

Circadian rhythms of new mammalian mothers are often disrupted in response to having an infant which is still developing a circadian rhythm and nursing. Although most mammal newborns, aside from cetaceans, have increased sleep and reduced mobility after birth associated with requirements for brain and body development (Siegel, 2008), mothers must still be increasingly attentive to their newborn, displaying new behaviours only associated with care of offspring. In our study, we found that the cub did indeed show reduced activity compared to other life stages, showing more rest and less feeding, a result also found in cubs in Wolong reserve (Mainka & Zhang, 1994).

The full circadian cycle of mother and cub behaviour, including maternal and nursing behaviours, had not been investigated previously in captive pandas. This was an opportunity to investigate how the circadian rhythm of a cub develops in captivity, how a mother's circadian rhythm changes or not as a result of having a nursing cub, and how levels of nursing and other maternal behaviours shift over the first years of a cub's life. In mammals, circadian rhythms begin to develop slowly before birth, largely emerging in different cell types at stages of cellular differentiation (Umemura & Yagita, 2020). After birth, there has been evidence in rats and humans that the circadian rhythm is partially entrained by the light/dark cycle and partially by the mother's cycle (Ohta et al., 2002; Thomas et al., 2016; Tsai et al., 2011). In rats, it has also been found that certain clock genes shift after weaning, including in peripheral organs like the liver (Honma, 2020; Ohta et al., 2002). These studies suggest that a nursing mother can likely have effects in the development of an infant's circadian rhythm and that nursing behaviour itself plays an important role in regulating shifts. Though we did not measure causality, we did see evidence of the mother and cub displaying similar cycle lengths of behaviour in their wavelet transforms (Figure 3.9). The main difference was seen in the mother presenting a 24-hr cycle with increased magnitude toward the end of observations while the cub had a 24-hr cycle with low magnitudes throughout the study. This is consistent with predictions that the cub would not have a consistent circadian rhythm in the first stage of life and that the mother would potentially have a deregulated circadian rhythm while nursing and would slowly regain a consistent circadian cycle. This may be further supported by the synchronisation in their 24-hr cycles when the mother did not have a strong 24-hr cycle, potentially indicating that the mother was synchronizing to an irregular 24-hr cycle that the cub possessed.

Our results showing that mother and cub did not have changes in levels of nursing throughout the course of observation is expected as the cub was still dependent by the time observations ended. Keepers began creating independence by gradually housing mother and cub separately beginning 2 months after observations ended. Occasional nursing was seen by keepers until the separation process was completed and mother and cub were housed entirely separate 11 months after the study concluded. However, another study on a giant panda’s maternal behaviour found a gradual decrease in non-nursing maternal behaviour toward her cub up to 150 days after birth (Zhang et al., 2000). We also saw this pattern over time, though firm conclusions cannot be drawn because of the inability to model these behaviours. We expected that proximity between mother and cub would match this result and decline overtime as was also seen in the study by Zhang (Zhang et al., 2000). However, the proximity data across the seasons did not show an increasing proportion of time spent distant between mother and cub, but rather an increase in proximity towards the end of the observation period. Without data on “approach” and “leave” we are unable to determine who is regulating the proximity. Interestingly, nursing and other maternal behaviours were mainly observed in the night. The circadian cycle of proximity matched this where mother and cub were proximate and in contact mainly after working hours. Further investigation should be done to determine if this is a natural cycle of nursing behaviour in pandas, or whether this was only observed because nighttime allowed for undisturbed interactions between mother and cub in the absence of zoo visitors and staff. Giant pandas are notoriously difficult to breed, and even following a live birth, cubs may be rejected in both the wild and captivity (Zhang et al., 2000). Therefore, understanding the rhythms of maternal behaviours can assist staff in providing the proper environment and privacy at the right times of day and across the time nursing, helping giant panda mothers successfully raise their offspring.

3.5 Conclusion

Our study demonstrates a holistic approach to assessing animal behaviour, their needs, and welfare indicators through the use of circadian and circannual rhythms. Circadian and circannual rhythms provide a fuller view of what a healthy, adaptive cycle of behaviour is, and since circadian clocks regulate internal, physiological clocks, the rhythms can also provide insight into the physiological state of the animal. Using this approach to study captive giant panda behaviour allowed us to understand possible energetic dynamics between behaviours like locomotion, drinking, feeding and resting. It is pleasing to report the natural cycle of three activity peaks in the captive giant pandas, similar to their wild counterparts. These results also demonstrate how this method can be used to determine species typical levels of behaviours to validate valences applied to behaviours in relation to welfare. In addition, with this broad view, we were also able to highlight some possible links between the timing of migration in the wild and stereotypic/abnormal behaviour, sexual-related behaviours, and feeding behaviours in captivity, suggested this is how thwarted migration may manifest. We also expanded on knowledge of how a mother and cub interact in captivity, adding important context on the kinds of maternal behaviours displayed and the times of day that are more likely to show mother and cub interacting. Exploring sex and life stage in concordance with rhythms adds another layer of context. Investigating all these variables together, life stage, sex, time of day, and time of year is more informative than investigating any of them individually because connecting them allows for new associations and predictable changes in rhythm, by sex, or life stage to be identified.

Circadian clocks are evolutionarily adaptive and are at the basis of biological functioning in living organisms. This makes circadian rhythms equally relevant in wild individuals as in captive ones, since the rhythms persist in captive environments and regulate many processes affecting welfare. We must be conscious of circadian rhythms as a way to educate ourselves on the evolutionary needs of an animal. This method can be modified in accordance with each zoos questions to investigate welfare states of animals. The insight gained from these kinds of analyses on animal behaviour can be used by zoos to modify their practices of care in accordance with the biological needs of a species and also allows them to predict what resources or special care might be needed across the day, year, and lifespan.

Chapter 4.

Latitudinal and zoo specific zeitgebers influence circadian and circannual rhythmicity of behaviour in captive giant pandas (*Ailuropoda melanoleuca*)

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Chapter 4. Latitudinal and zoo specific zeitgebers influence circadian and circannual rhythmicity of behaviour in captive giant pandas (*Ailuropoda melanoleuca*)

Author contributions

KMG, SEK and HMB-S contributed to conception and design of the study. KMG collected the data, organised the database, and performed the statistical analysis. KMG wrote the first draft of the manuscript, SEK and HMB-S restructured and edited sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Abstract

The circadian clock influences many aspects of animal welfare including metabolism, breeding, and behaviour. In most species, circadian clocks are internal clocks regulated by external environmental cues called zeitgebers. The most common zeitgebers are light/dark cycles, food, and temperature. However, within captive environments, animals can be housed at latitudes with different light/dark cycles than their natural habitat and most other zeitgebers are controlled by humans. The effects that modified zeitgebers have on captive animals' circadian and circannual rhythmicity is largely unknown. To explore this and potential welfare implications, we measured and analysed observational behavioural data of zoo-housed giant pandas for one year utilizing live camera footage from five zoos across the world. The worldwide distribution of the zoos gives us the unique opportunity to investigate how housing giant pandas within and outside of their natural latitudinal range can affect circadian rhythmicity and behaviour. Focal sampling was completed for eleven giant pandas each month for 12 consecutive months to gain an estimate of one circannual cycle. Within each month, we estimated one daylight or 24-hr cycle of activity/behaviour by conducting 10-minute observation sessions systemically each hour the pandas were visible. Zero-inflated negative binomial mixture models found that latitude is associated with activity levels, with pandas housed outside of their natural latitudinal range displaying less activity than those within their latitudinal range. Amount of daylight, temperature minimum, and temperature range were also associated with activity cycles, potentially acting as zeitgebers. An association between sexual-related and stereotypic behavioural cycles was found, with the circannual cycles fluctuating in synchrony throughout several points in a year. These results indicate that changes to common zeitgebers and environmental conditions can influence circadian and circannual cycles. The widespread evolution of circadian rhythms suggests an

adaptive advantage to possessing one in an environment with cyclical changes, allowing species to anticipate changes in their environment and respond accordingly. Therefore, although animals are highly adaptive, creating a captive environment that mimics the environmental conditions for which the animal has evolved can encourage naturalistic cycles that ultimately aid in promoting positive welfare states and increasing chances of successful breeding and conservation.

4.1 Introduction

Providing zoo environments that promote good animal welfare is important for conservation, education, and from an ethical perspective. With the increased importance placed on captive animal welfare and the recent enforcement of welfare evaluations in zoos (World Association of Zoos and Aquariums), holistic and informative ways of evaluating welfare must be explored. One factor that could potentially influence the welfare of animals within zoos is the monitoring and management of their circadian rhythms. Circadian rhythms are the inherent, natural cycles of physical, mental, and behavioural processes that repeat roughly every 24 hours. Circadian rhythms exist across the animal kingdom from birds, reptiles, mammals, amphibians, fish and arthropods, including insects (Froy, 2011; Siegel, 2008). The widespread evolution of circadian rhythms suggests an adaptive advantage to possessing one in an environment with cyclical changes, allowing species to anticipate changes in their environment and respond accordingly to maintain homeostasis. In the wild, species exhibit cycles of activity throughout a 24-hr period and across the seasons. However, in captive environments, the rhythm of activity throughout the day and night and across the seasons is largely unknown as care of the animals is limited to the working day and seasonal changes in activity are not closely monitored (Brando & Buchanan-Smith, 2018). In addition, the effect of latitude on circadian rhythmicity of captive animals is not well understood. It is important to be aware of the circadian rhythms of captive species, as many aspects of their welfare, including metabolism, breeding, and behaviours, including interactions with their environment, are regulated by their circadian clock (Mellor, 2017).

Circadian rhythms operate on an approximately 24-hr endogenous clock which can be entrained by external cues called zeitgebers, such as the light/dark cycle, feeding, and temperature (Buhr et al., 2010; West & Bechtold, 2015). The circadian clock regulates the clocks of peripheral tissues (liver, adipose tissue, digestive tissue, etc.) and determines the rhythms of physiological processes and behaviours (Archer & Oster, 2015; Froy, 2011; Gaddameedhi et al., 2011; West & Bechtold, 2015). Since the circadian rhythm is entrained by light, food, and temperature, natural, seasonal fluctuations in these factors cause seasonality in the circadian rhythm. Understanding the diel and annual cycles of behaviour and physiology of captive species can help in gaining a view of their needs and thus inform zoo staff on measures to be taken to promote positive welfare. This approach to assessing and addressing welfare follows the 5 domains model (Mellor et al., 2020) by incorporating the assessment of nutrition, environment, health, and behaviour on a diel and annual basis to interpret the affective states of species.

Species are specially adapted to the seasonal cycles of light and temperature in the habitats in which they have evolved. Intra and inter-species latitudinal clines in biological rhythmicity have been observed (Greenham et al., 2017; Hut et al., 2013; Paolucci et al., 2019). In shore birds, a latitudinal cline for incubation bout length was seen across 30 species (Bulla et al., 2016). In the parasitoid wasp *Nasonia vitripennis,* the critical photoperiod that initiates diapause (a period of suspended development) displays a latitudinal cline across populations, with populations at higher latitudes having a longer critical photoperiod (Paolucci et al., 2013). These studies demonstrate that latitude has an evolutionary effect on rhythmicity, further evidenced by latitudinal clines in the genes that regulate these rhythms (Costa et al., 1992; Helfrich-Förster et al., 2020; Hut et al., 2013). The adaptive nature of these latitudinal clines should be considered in captive settings where these adaptations are challenged by differing climatic conditions.

Changes in temperature cycles with latitude can also have a large effect on organisms, as temperature often serves as a strong zeitgeber for species that are not homeothermic vertebrates (Rensing & Ruoff, 2002). Daily cycles of temperature are particularly important for ectotherms and endothermic heterotherms, as they regulate torpor and consequently the arousal and rest periods which should be synchronised with the circadian rhythm as well as behaviours related to thermoregulation (Abram et al., 2017; Körtner & Geiser, 2000; Turbill et al., 2008). For instance, Magnone et al. (2005) found that a clock gene, Per2, was regulated by ambient temperature changes in the ruin lizard (*Podarcis sicula*). Although temperature is a weaker zeitgeber in endotherms when compared to daylight, it may enhance the amplitude of the light/dark cycle signal when in-phase, thus enhancing the entrainment signal for the circadian rhythm (Rensing & Ruoff, 2002). In addition, temperature can also be of more consequence for mammalian species which experience extreme cycles of daily temperature such as the camel (*Camelus dromedarius*), for which body temperature cycles were entrained by the ambient temperature cycles in constant light or dark conditions (El Allali et al., 2013). Understanding the changes in the cycles of light and temperature across latitudes and their effects on organisms' circadian rhythms is important when assessing how an animal will respond to the external environment in a zoo.

A question that has not been thoroughly addressed in animal welfare research, but is of growing concern in the field, is how well captive animals can adapt to climatic and latitudinal conditions outside of their natural ranges. Because latitude establishes the extent of the plasticity of the circadian rhythm, the effects of the frequent transfer of species between zoos around the world and across latitudes must be studied and considered. Proper entrainment of the circadian clock to the external environment synchronises animals with their environment and conspecifics, resulting in adaptive physiological, behavioural, and social functioning. In Pallas cats, photoperiod stimulates gonadal activity in both males and females, synchronizing their physiological readiness to reproduce (Brown et al., 2002). However, exposure to artificial light outside of the breeding season through zoo events also stimulates gonadal activity and a subsequent refractory period which has negative effects on the ability to breed during the breeding season (Brown et al., 2002). Improper entrainment to the external environment can result in disruption or desynchrony of the circadian rhythm, which is linked to adverse health across species including reduced reproductive success, metabolic disorders, and even shortened lifespan (Hurd & Ralph, 1998; Irwin & Opp, 2017; West & Bechtold, 2015). In the nocturnal mouse lemur, exposure to light pollution changed the circadian rhythmicity of their core temperature, showing general increases in core temperature, and changed the rhythmicity of locomotor activity, with delays in the onset/offset and reduced locomotor activity during the night (Le Tallec et al., 2013).

Animals which experience seasonality may be better suited to adapt to varied latitudes as they naturally must adapt to seasonal changes. Conversely, animals within a habitat with little seasonality do not need to adjust to seasonal changes, as external cues maintain a relatively constant rhythm throughout the year. Therefore, when these animals are relocated to an area with extreme seasonality, it may be difficult for them to adapt to seasonal changes, potentially resulting in desynchrony of the circadian rhythm throughout the year or in specific seasons. To promote circadian synchrony within a zoo, it may be useful to first understand the effects that latitude has on the circadian rhythms of zoo-housed species and then mitigate the effects of extreme changes in seasonality for animals which do not experience seasonality, and to mimic certain aspects of seasonality for animals which do normally experience seasonality.

In addition to latitudinal effects on circadian rhythmicity, the zoo environment can also be influential as it is significantly different from wild environments. Though attempts are made to create enclosures which closely mimic the wild, inevitably there are many differences. In zoos, animals experience limited space, regular human presence, human-controlled schedules, artificial lighting and temperature, and enclosure designs that do not always mimic natural habitats. These factors alter the external environment and the cyclical changes experienced by the animal and have the potential to regulate the circadian rhythm. The daily rhythms of husbandry routines follow a 24-hr cycle in a similar way that the circadian cycle does. Husbandry routines which include feeding, cleaning, and training sessions become a part of the external environment of the animal that is predictable in the same way light and temperature are. Therefore, in response to husbandry practices, animals may show predictable changes in their behaviour and physiology.

Another ongoing question and a main goal for zoos is how to improve conservation efforts. Given the persistent threats of habitat loss and population declines in species around the world, it is essential that research continues to find innovative ways to improve the reproductive success of species in captivity. Since circadian rhythms regulate and influence many aspects of reproduction, both physiological and behavioural, understanding them can lead to improved breeding efforts. In humans, the circadian clock has been found to influence every stage of reproduction in females (Gamble et al., 2013). The circadian rhythm also determines the reproductive cycle for spontaneously and seasonally ovulating animals (Goldman, 1999). Proper entrainment to external cues and the resulting circadian rhythm has direct effects on reproductive cycles of species. Therefore, monitoring the diel and annual cycles of activity in captive species can provide insight into mating behaviours and conditions that could improve mating and reproductive success.

The welfare of captive species is paramount. However, to promote positive welfare, the welfare state of the animal and the factors affecting welfare must be well understood. The widely accepted Five Domains framework for assessing welfare addresses four physical/functional domains (nutrition, environment, physical health, and behaviour) and a mental domain (Mellor et al., 2020). Circadian/circannual rhythms influence each of these domains. Therefore, monitoring and assessing rhythmicity becomes a holistic approach to assessing welfare. This holistic approach to assessing the needs of species does not only have the potential to improve conservation efforts, but can also be utilised to promote positive welfare. The inclusion of night-time activities and annual cycles in assessing welfare facilitates the understanding of the needs of the species on a daily and annual basis. This information can be used as an evidence-based approach when creating husbandry protocols, enclosure designs, and species management protocols to promote positive welfare.

To demonstrate this holistic approach and to investigate climatic/latitudinal effects on captive animal behaviour, we are investigating the circadian and circannual cycles of captive giant pandas as a case study. Giant pandas have a historic natural latitudinal range of 26°N-42°N (Loucks et al., 2001). They are seasonal breeders mating between March and April (Liu et al., 2002). Females have a single, annual oestrus period with mating receptivity lasting only 1-3 days (Schaller, 1985). The mating strategies involve females and males synchronizing multimodal signalling behaviour around the oestrus cycle with social/sexual behaviours increasing during this period (Owen et al., 2013) and mate choice increasing the likelihood of successful breeding (Martin-Wintle et al., 2015). Therefore, although hormonal data was not available for this study, we can still demonstrate how understanding the circadian rhythmicity of these sexual-related behaviours can aid zoo staff in identifying when their pandas are receptive for breeding and if behaviours are desynchronised with oestrus if zoos then pair the behavioural data with hormonal data.

Giant pandas are also a migratory species, following their food source of bamboo with a preference for nutritious shoots that emerge in Spring (Wang et al., 2010). They initiate migration from middle April to early June (coinciding with the breeding season), migrating within several days to their Summer range and returning over several weeks from early September to October (Liu et al., 2002). In the wild, giant pandas exhibit three peaks in activity throughout the diel cycle and a fluctuation in activity levels throughout the year, with a peak in June (Zhang et al., 2015). Giant pandas being such a specialised species, evolving over time to eat bamboo and adapting their behaviour for this food source, makes them an ideal animal for this study as they would presumably need to be well synchronised to their environment to sustain their lifestyle. Giant pandas are also ideal for this study because they are a charismatic species and have large popularity worldwide. This means that they have many available webcams for observation across different latitudes that allow us to study latitudinal effects on cycles and show a proof of concept of how monitoring animals on cameras can be very informative while being non-invasive. In addition, pandas are a vulnerable species that are notoriously difficult to breed in captivity. Though large improvements in the captive breeding of pandas have been made, with mate choice being identified as a key factor in successful breeding (Martin-Wintle et al., 2015), further understanding of how pandas synchronise their breeding behaviour could greatly improve conservation efforts for successful breeding of captive pandas.

In this study, we recorded circadian cycles for one year for captive giant pandas at latitudes that are matched and mismatched to their natural latitudinal range. We investigated the latitudinal effects on behavioural cycles and how the information from these cycles can be used to understand the environmental, behavioural and social needs of pandas. We hypothesise that there will be differences in behavioural cycles across latitudes due to the differences in the cycles of the external zeitgebers they are exposed to. We also hypothesise that zoo environments will have additional zoo specific zeitgebers like husbandry routines that may synchronise with behavioural cycles. Our study cannot determine causality, but finding relationships between behavioural cycles and external factors can help inform zoos on how they can better provide for the needs of their animals, and can give insight on the husbandry and environmental factors that may promote the positive welfare states they want to see in their animals.

4.2 Materials and Methods

4.2.1 Ethics

We received ethical approval for this study from the University of Stirling Animal Welfare Ethics Review Body (protocol #2084 1591; 30/04/2020). The study also received support from the Association of Zoos and Aquariums, Giant Panda Species Survival Plan (05/08/2020). In addition, we submitted research applications to all zoos involved and received approval from the participating zoo's administrations. We also sent a voluntary questionnaire to keepers asking information on (1) panda identities, (2) cameras, (3) enclosure design, (4) artificial lighting, (5) feeding husbandry and (6) breeding opportunities. Three zoos provided some or all of the requested information. The identifying data for the zoos and pandas has been anonymised.

4.2.2 Study animal selection

The inclusion criteria for a giant panda was whether the panda was in a zoo that had a publicly accessible web camera or a surveillance system that we could be allowed access to. If the zoos approved the use of their cameras and the pandas were consistently in view, the pandas remained in the study. As a charismatic species with large public interest, giant panda web cameras are very popular, making the species ideal for this study because we were able to select individuals from around the world. We selected 11 giant pandas (6 females, 5 males) from five zoos (Table 4.1). We identified whether a panda was housed in a latitude within or outside of the giant panda historic natural latitudinal range (26°N-42°N)(Loucks et al., 2001), with the southern hemisphere's mirrored latitudes (26°S-42°S) counting as a matched latitude. Zoos A and C are located at latitudes that match the natural range of giant pandas while Zoos B, D, and E are located at latitudes above the natural range towards the poles. There were no zoos located outside of the natural range towards the equator. We also identified the pandas by sex and age group, with sub-adults being individuals aged 6 years or younger. This age was decided based on the life stage in which giant pandas reach sexual maturity (D. Liu et al., 2006). All cameras were accessible 24 hours, however, cameras for 6 individuals did not have night-vision, so observations for these individuals were limited to daylight hours (Table 4.1). The remaining 5 individuals had data collected on a 24-hr basis.

Table 4.1. Information on the zoo latitude, study animals, husbandry, indoor lighting and temperature. Within Zoo C, sub-adults could be distinguished from adults, but individuals could not be identified. Therefore, these individuals are listed as 5a, 5b, 6a, and 6b. Individuals with the same number had their data combined and the sex was omitted in analysis for individuals 6a and 6b. Zoo E also housed a female panda whose data could not be collected for the study because her footage was not accessible. Zoos A and C are located at latitudes that match the natural range of giant pandas while Zoos B, D, and E are located at latitudes above the natural range towards the poles. Breeding opportunity refers to when pandas are normally given opportunities by keepers to breed naturally or through artificial methods.

Zoo	**Zoo Latitude**	**Panda**	**Sex**	**Age group**	**Breeding opportunity**	**Camera visibility**	**Indoor lighting**
A	Match	1	F	Adult	Around March; either natural or artificial insemination	Daylight	Unknown
		2	M	Adult			
B	Mismatch	3	F	Sub-adult	Breeding pair, but unknown timing and method	24 hours	Unknown
		4	M	Sub-adult			
C	Match	5a	F	Sub-adult	None	Daylight	Natural + Artificial Turned on when needed
		5b	F	Sub-adult			
		6a	F	Adult	Post-reproductive		
		6b	M	Adult			
D	Mismatch	7	F	Sub-adult	Around March; natural	24 hours	Natural + Artificial Scheduled with dimming mimicking light in Chengdu, China
		8	M	Adult			
E	Mismatch	9	M	Adult	Castrated for medical reasons	24 hours	Natural + artificial Turned on/off at beginning and end of day

4.2.3 Behaviour observations

Behavioural observations were completed using the *ZooMonitor* application (Ross et al., 2016). The ethogram was designed to include most of the behavioural repertoire of giant pandas, covering behaviours that indicate positive, neutral and negative affective states (Table B1).

All observations were made through web cameras from December 2020 – November 2021. Focal sampling was completed for each giant panda using 10-minute sessions with 30-second intervals every hour to gain an estimate of behaviour in that hour. Each month, data collection began on the 10th day and continued through the end of the month until one daylight or one 24-hr cycle was recorded (dependent on the night-vision of the camera) for each panda. The 10-minute sessions were completed depending on the availability of an observer and the presence of the giant panda on camera. A graphical representation of the sampling method can be seen in Figure 4.1.

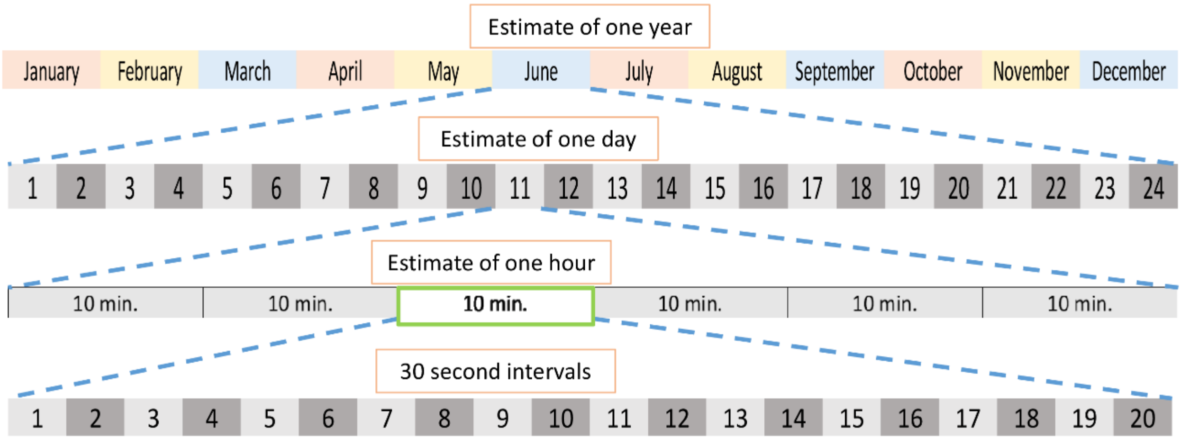


Figure 4.1. Representation of the sampling method to obtain estimates of circadian cycles and a circannual cycle. Each month, observations began on the 10th of the month and continued through the end of the month until one 10-minute session was completed for each hour of the day. For pandas observed on cameras without night vision, observations continued until a 10-minute session was completed for each daylight hour. Completion of 10-minute sessions depended on the availability of an observer and the visibility of the panda on camera. Figure reproduced from Gandia et al. (2023a).

Along with behaviour, we also recorded the following at each interval: whether the panda was in their indoor or outdoor enclosure and whether the camera was displaying in colour or black and white (due to ambient light becoming dark). Vocalisations were not recorded as some cameras did not have audio. Whether the panda was out of sight was also recorded, and a session was only saved if the panda was in sight for 60% of intervals (12/20 intervals) to provide representative data for analysis. Sessions with more than 8/20 out of sights were deleted and redone.

In total, 13 observers assisted in data collection throughout the data collection period. To produce data that would be used for analysis, observers had to pass reliability testing. Since testing reliability purely from live observations results in many ethogram behaviours not being evaluated (Wark et al., 2021), we designed our reliability testing with two stages aiming to cover all ethogram behaviours. The first stage was an ethogram quiz for which the observer had to receive >80%. The second stage was inter-observer reliability using a combination of compilations of short video clips of all behaviours listed in the ethogram and 10-minute recordings from the study pandas mimicking the way observation sessions would be completed using the web cameras and *ZooMonitor*. For the short video clips, beeps were placed at variable intervals so the full repertoire of pandas was covered, and indicated when to record a behaviour. For the 10-minute recordings, observers used *ZooMonitor* and the 30 second intervals to record. Observers had three attempts (each attempt had different videos or beeps were changed) to match at least 75% of the recordings from the lead investigator to pass this final stage. The 75% agreement threshold was based on those generally accepted for reliability (Graham et al., 2012). Each attempt contained 8-9 video clips with 4-5 beeps each, and 3-4 10-minute recordings with 30-second intervals.

4.2.4 Analyses

4.2.4.1 Variables

Two of the main goals of zoos are to conserve species and promote positive welfare of their animals. Therefore, in order to investigate ways in which zoos can further these goals, we focused our analysis on general activity, sexual-related behaviour, and stereotypic/abnormal behaviour. Determining how zeitgebers potentially regulate or synchronise with these behaviour categories can help us understand how to create environments that are more conducive to the expression of positive and sexual-related behaviour. Each behaviour category- activity, sexual-related, and stereotypic/abnormal- was calculated by adding the counts by 10-minute session of respective behaviours listed in Table 4.2. We modelled activity, sexual-related behaviour, and stereotypic/abnormal behaviour in separate models against several predictor variables. The activity category (all behaviours except for resting/sleeping) is used since circadian rhythms are often described as patterns of active/inactive states. The predictor variables were zoo latitude as described in Table 4.1

(categorical with two levels: match and mismatch), temperature minimum, temperature range, amount of daylight in hours, season, and hour of the day. Temperature measures and daylight were recorded from a weather website (timeanddate.com). The location of the zoo was searched and the maximum/minimum temperature and number of daylight hours recorded from the same date as the observation. In addition, age group and sex were included in the model as controls.

Table 4.2. Behaviours combined for the behaviour categories analysed. See Table B1 for full list and definition of behaviours.

Behaviour Category	Combined behaviours
Activity	All behaviours except for resting/sleeping.
Sexual-related	Anogenital rubbing, sexual, show interest, and scent-anoint.
Stereotypic/Abnormal	Pace, bipedal standing, self-mutilation, cage climb, regurgitation, pirouette, and head-toss.

4.2.4.2 Zero-inflation Negative binomial modelling

It is common for ecological data to have a high amount of zero values resulting in zero-inflation that causes significant biases in analysis because the fit regression becomes flat (Fávero et al., 2021; Martin et al., 2005). These zeros are either ‘true zeros’ or ‘false zeros.’ With behavioural data, true zeros are observed from individuals that never display a behaviour or because a behaviour is not constantly displayed or rare. These zeros are also called structural zeros. False zeros occur from sampling error, if a behaviour is not displayed within the sampling period or if a behaviour is miscoded. These zeros are also known as sample zeros.

Within our data, each of our response variables had a very high percentage of zeros: activity, 46.3%; sexual-related, 97.2%; stereotypic/abnormal, 91.9%. Our data will have both true/structural and false/sample zeros, but mainly zero-inflation due to true/structural zeros, which results in overdispersion (Martin et al., 2005). Therefore, we needed a model that worked for count rate data (counts of behaviour within a 10-minute session) and would account for zero-inflation and the resulting overdispersion. Negative binomial models are count models that have a parameter that allows for overdispersion (Lindén & Mäntyniemi, 2011; Martin et al., 2005). The most appropriate model considering the qualities of our data was the zero-inflation negative binomial (ZINB) mixture model. To conduct this analysis, we used the R package *glmmTMB* (Brooks et al., 2017). In *glmmTMB*, zero-inflated GLMMs have three components: a model for the conditional mean (Negative binomial in our study), a model for zero-inflation, and a dispersion model. The conditional mean and dispersion models analyse positive values using log links. The zero-inflation model describes the probability of observing a true/structural zero that is not generated by the conditional model. The values within the zero-inflation model are constrained between 0 and 1 by applying a logit link (Brooks et al., 2019; Brooks et al., 2017; Fávero et al., 2021). The overall fit of the ZINB mixture model is determined by all three components. Therefore, when interpreting the results, we must consider the results of all three models. The interpretation of the coefficients for the count model and zero-inflation model are different. A positive coefficient in the count model indicates an increase in the response with an increase in a continuous predictor or in that level of the categorical predictor compared to the other levels. In contrast, a positive coefficient in the zero-inflation model indicates that a structural zero in the response is more likely with an increase in a continuous predictor or in that level of the categorical predictor compared to the other levels.

For each behaviour category ZINB mixture model, the zero-inflation model was the same as the conditional model which included pandas nested within zoos. The categorical variables of season, age group and sex were coded within the model using contrast sums. Therefore, each level of the variable was compared against a grand mean within the model which was the mean of the response variable means at each level of the categorical variable. Due to our limited sample size, we ran the models using Restricted maximum likelihood rather than Maximum likelihood. This is an iterative process and the final model that is presented is the one with the best estimation. We did not standardise for time out of sight by converting the counts to decimal rates per time in sight because the models require count data and because, among all sessions, the mean time in sight was 95.6% and the median was 100%. Post hoc analysis of pairwise comparisons of estimated marginal means (least-squares means) were conducted on the season variable using the R package *emmeans* (Lenth, 2023) to determine any significant differences between variable levels. Multiple comparisons were controlled for using the Tukey method. Test-wide alpha was set at 0.05.

For the stereotypic/abnormal behaviour ZINB model we added sexual-related behaviours as a predictor because a study on 101 captive giant pandas by Martin et al. (2020) found an association between stereotypic behaviour and reproductive performance. However, since stereotypic/abnormal behaviours are not seen in wild giant pandas, and therefore would not predict sexual behaviour in the wild, we did not include stereotypic/abnormal behaviours as a predictor in the sexual-related behaviour ZINB model. In addition, *ggplot* allows for two kinds of negative binomial models, one which models the count data with linear regression and another which uses quadratic. For the stereotypic/abnormal behaviour model we used the quadratic negative binomial because it reduced the dispersion 10-fold, implying a better fit.

4.2.4.3 Wavelet coherence analysis

In order to extrapolate more information on how behaviours and zeitgebers synchronise/desynchronise throughout the year, we used continuous wavelet transform coherence analyses. Wavelet transform is a time series analysis where a signal is transformed into a wave with zero mean that is expanded and localised in both frequency and time. This allows for the detection of periodic patterns of a time series in both time and frequency domains while controlling for random background noise in the signal. Wavelet transform is useful for analysing localised intermittent oscillations in a single time series, but we can also use a wavelet coherence analysis to determine how two time series are related to each other. With wavelet coherence analysis we can examine whether regions in time frequency space with similar high power have a sustained phase relationship, possibly suggesting a relationship between the signals (Grinsted et al., 2004). Essentially, we can determine areas of correlation between the two wavelets.

This type of analysis is ideal for our questions addressing the relationships between zeitgebers and behaviours. It allows us to identify times of the year in which the zeitgeber and behaviour are correlated and determine the kind of phase relationships and the implications for how the zeitgeber potentially regulates or synchronises with that behaviour. We can also determine whether these relationships would change based on the latitudinal location. For our analysis, we conducted a wavelet coherence analysis for the continuous predictor variables that came out significant in the ZINB models so that we could extrapolate more information on the kind of relationship between the predictor and respective behaviour category.

To conduct the analysis, we used the MATLAB Wavelet Toolbox developed by Grinsted et al. (2004). Our data are non-stationary, so we used a continuous wavelet analysis with the Morlet wavelet and a scale resolution of 10 scales per octave, as suggested by Grinsted at al. (2004), since these settings provide a good balance between time and frequency localisation. Our sampling period was set as Δt=1hr. We also decided to conduct continuous wavelet transform and wavelet coherence analysis because these analyses were used by Zhang et al. (2017) to address similar questions in wild giant pandas on activity data recorded using radio collars. We followed their analyses as closely as possible so that our data could be compared to the results found in wild pandas. However, because of our sampling method, our behaviour signals are constructed of consecutive, representative 24-hr periods in each month. Therefore, we could only determine the patterns of circadian cycles and the annual cycle of those circadian cycles but could not infer anything about rhythms with a month-long period.

4.2.4.4 Indoor vs outdoor activity levels and budgets

Most captive animals have both indoor and outdoor enclosures. Zeitgebers tend to be controlled for indoor enclosures (i.e., temperature and artificial lighting). However, for outdoor enclosures, the zeitgebers are those for the latitude which the zoo is located. Because of this, we wanted to examine how time outdoors is associated with activity levels. To provide more context to this relationship, we determined the activity budgets while indoors versus outdoors and looked at the difference between the latitude groups since pandas at mismatched latitudes would presumably experience outdoor external factors for which they are not adapted to. We conducted Welch's paired sample t-tests to compare the levels of locomotion, resting/sleeping, feeding, and stereotypic/abnormal behaviour between the indoor and outdoor enclosures within each latitude category (matched/mismatched).

4.3 Results

Model summaries for the three behaviour categories can be seen in Table 4.3. Each behaviour category did indeed display overdispersion. Each of the behaviour category ZINB mixed models had significant coefficients in either the count model or the zero-inflation model, or both. Below we will describe the results for both components, addressing the count model and then the zero-inflation model. Our interpretation of the data in the Figures is usually based on both visual inspection of the overlapping confidence intervals combined with which variables came out as significant in the models.

Table 4.3. Summary of ZINB models for activity, sexual-related behaviour, and stereotypic/abnormal behaviour. N/A is indicated where a score was not produced because of model convergence.

Behaviour category	Regression type	Iterations	AIC	BIC	Df(residual)	Dispersion parameter
Activity	Linear	87	9884.4	10045.9	1921	3.59
Sexual-related	Linear	49	N/A	N/A	1921	1.62
Stereotypic/Abnormal	Quadratic	65	1979.4	2152.0	1920	1.14

4.3.1 ZINB model results for activity

Within the count model, the negative coefficients for temperature minimum (-0.007, z=-1.970, p=0.049) and temperature range (-0.014, z=-2.955, p=0.003) suggest a decrease in activity with an increase in both the temperature minimum and temperature range for the day. The trend toward significance for the positive coefficient for daylight (0.017, z=1.828, p=0.068) indicates a potential, slight increase in activity with an increase in amount of daylight. A trend toward significance was also seen for activity at mismatched latitudes (-0.139, z= -1.834, p= 0.067) and could suggest a potential, slight decrease in activity at mismatched latitudes compared to activity displayed at matched latitudes.

Within the zero-inflation component, latitude was significant, with the positive mismatch coefficient (1.058, z=6.082, p<0.001) suggesting that pandas at a mismatched latitude are more likely to have a true/structural zero for activity when compared to pandas at matched latitudes. In other words, pandas at mismatched latitudes generally show more sporadic activity than pandas in matched latitudes. The negative coefficient for Spring (-0.402, z=-2.052, p=0.04) indicates that during this season, it is less likely for a panda to display a true/structural zero in activity. This means that during Spring pandas are more likely to be consistently active. Conversely, the positive coefficient for Autumn (0.220, z=2.104, p=0.035) suggests that pandas are more likely to show a true zero in activity during Autumn. Hour of day was also significant (-0.018, z=-2.265, p=0.024). Post hoc comparisons did not reveal any significant differences in activity between seasons.

Figure 4.2 depicts the circadian and circannual cycles of activity by latitude. We found similar circadian patterns of activity between the matched and mismatched groups despite their latitudinal difference. Likewise, the circannual cycle of activity between the two groups are very similar and only diverge between December and March, which includes part of the breeding season in March and April (Liu et al., 2002).

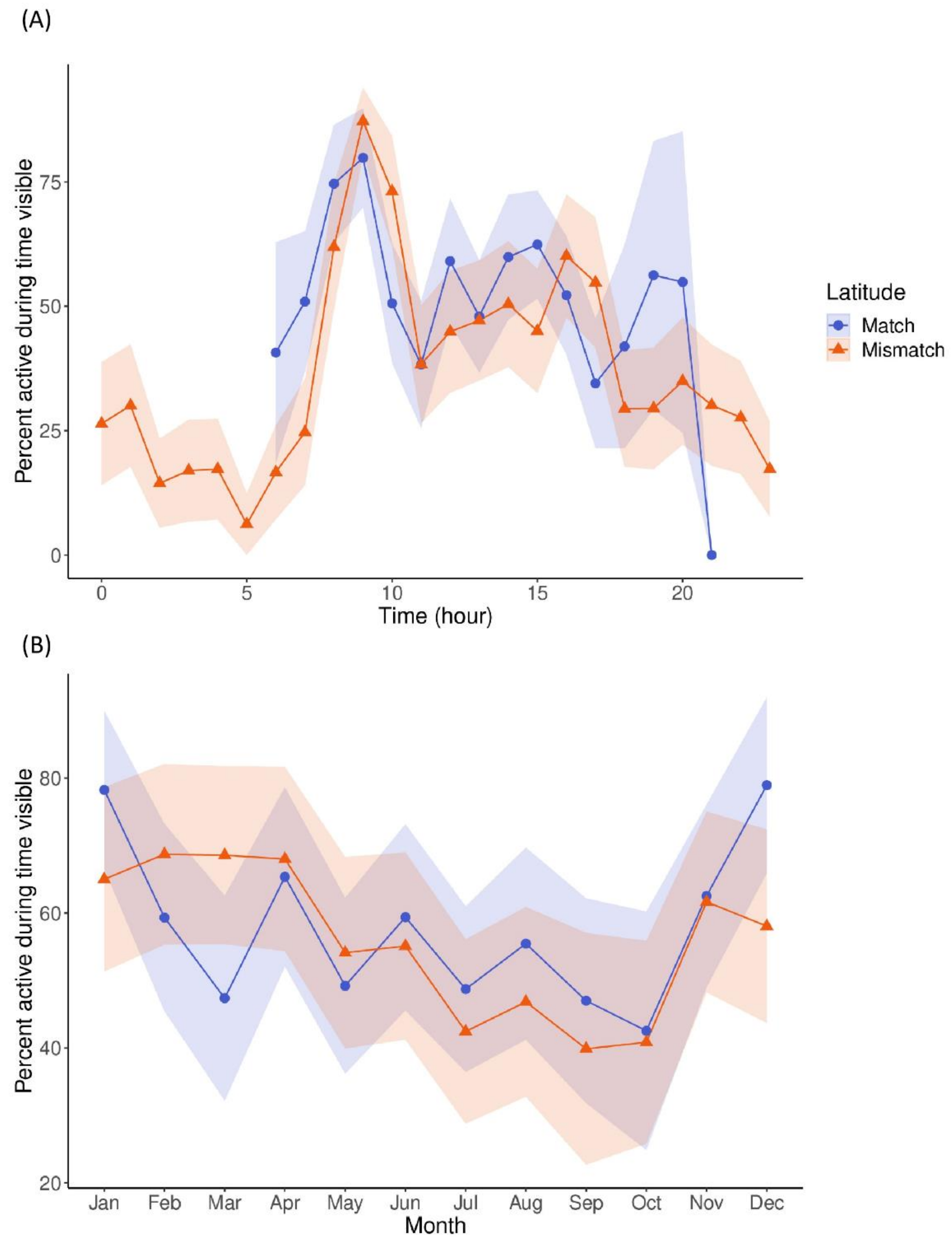


Figure 4.2. Line plots displaying the circadian cycles (A) and circannual cycles (B) of activity for pandas in matched (blue, n=6) and mismatched (orange, n=5) latitudes. Activity is displayed as percentage of time active while in sight, controlling for time out of sight, and was averaged by hour (A) or month (B). The shaded regions are the 95% confidence intervals for the activity. The circadian cycle for pandas at matched latitudes is only for daylight hours, as these pandas had cameras without night vision, and therefore could not be recorded. The circannual cycles displayed are from a 9-hour daylight subset of data so that it could be comparable between the two latitude categories.

4.3.2 Activity wavelet coherence analyses

Since temperature range and temperature minimum were significant, and daylight near significance in the count model for activity, and latitude near significance in the count model and significant in the zero-inflation component, we also analysed these three zeitgeber measures by latitude using wavelet coherence (Figure 4.4). Matched and mismatched latitudes had different circannual cycles of these zeitgeber measures (Figure 4.3). There was a much larger change in daylight throughout the year at the mismatched latitudes (Δhr≈15) when compared to matched latitudes (Δhr≈5). The temperature minimum was consistently lower throughout the year at mismatched latitudes compared to the temperature minimum at matched latitudes. The temperature range throughout the year between the latitudes seemed to be completely out of phase with each other.

The wavelet coherence analyses (Figure 4.4) show how the phase relationships between activity and the three zeitgebers differed between the latitude locations. For the coherence between daylight and activity, both latitudes showed similar coherence in Spring at the normalised cycle lengths of 8-16hrs with the activity signal leading, but in Autumn, matched latitudes showed a lagging activity signal, while mismatched latitudes did not show much coherence at all. In the coherence analysis for activity and temperature minimum, the two latitude groups displayed some similar coherence between May and June around the 8hr cycle length with the signals being nearly in phase with each other, and a small similarity from September to October with activity shifting from leading to nearly out of phase. However, around the 1-day cycle length, the matched latitude displayed a consistent phase relationship that was not present for mismatched latitudes between April and August, with the activity signal leading. The coherence analyses for activity and temperature showed no similarities between the latitudes. Coherence occurred at different cycle lengths and times of year, or with differing phase relationships.

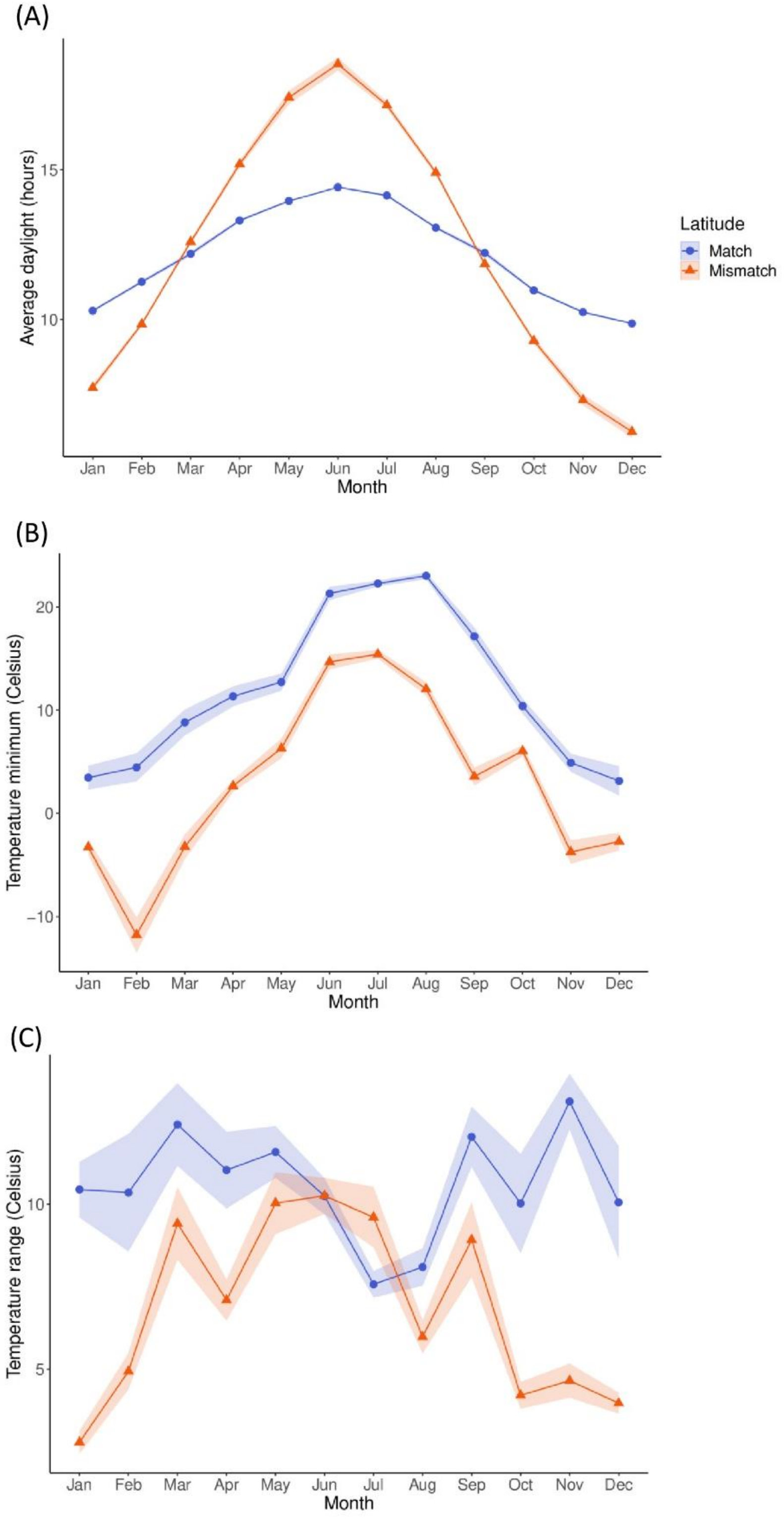
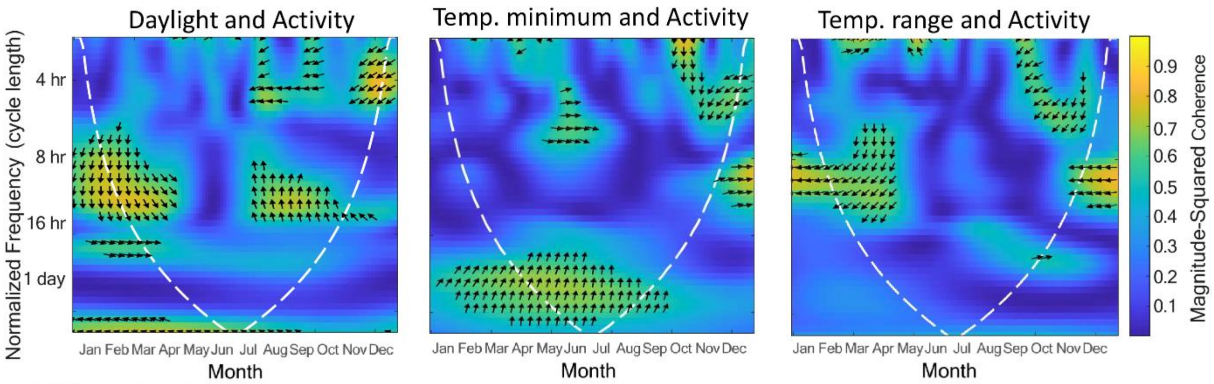


Figure 4.3. Annual cycles of average daylight (A), temperature minimum (B), and temperature range (C) between the zoos at matched (blue, n=6) and mismatched (orange, n=5) latitudes. The measurements were averaged by month. The shaded regions are the 95% confidence intervals of the measurements.

(A) Matched latitudes



(B) Mismatched latitudes

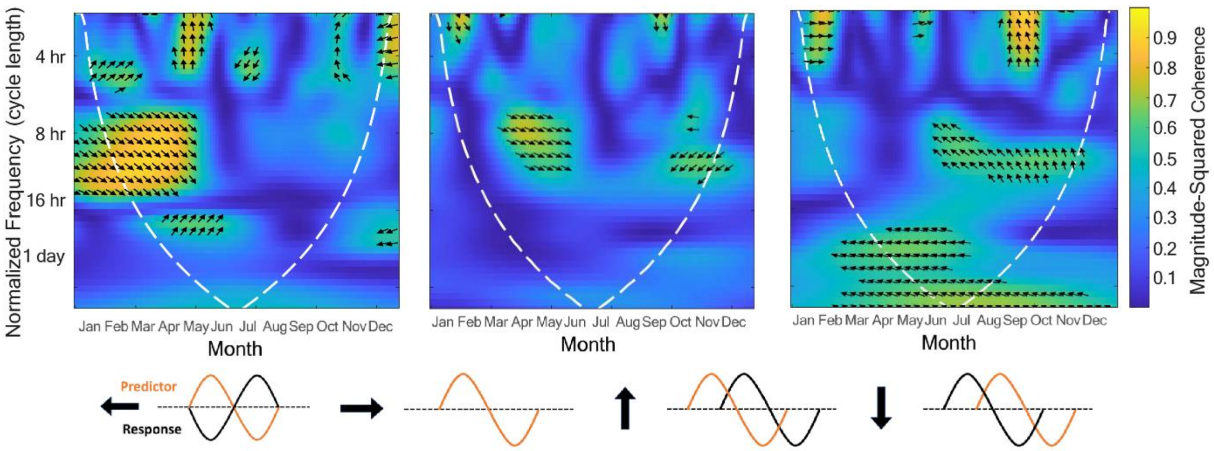


Figure 4.4. Wavelet coherence analyses between activity and daylight, temperature minimum, and temperature range, respectively. The top row shows these coherence analyses for pandas in matched latitudes (n=6), and the bottom row for pandas in mismatched latitudes (n=5). The data entered for the analyses were a 9-hour subset of daylight data so that comparisons can be made between the two latitude categories. The dashed white line indicates a 5% significance level. The x axis is the time of year in months, the y-axis is the normalised frequency between the two signals, and the colour represents the strength of the correlation (yellow high, dark blue low, scale on right hand side). The kind of phase relationship between the signals are noted by the arrows (refer to arrow key).

4.3.3 Outdoor vs indoor activity

When comparing the circadian cycles of activity levels and percentage of time outdoors, we found that activity levels coincide almost perfectly with the amount of time outdoors (Figure 4.5). For the activity budget comparison indoors vs outdoors and by latitude, we found that pandas at matched latitudes displayed very similar activity budgets both indoors and outdoors with no significant differences in locomotor, resting/sleeping, feeding and stereotypic/abnormal behaviour between the indoor and outdoor enclosures (Figure 4.6).

Meanwhile, pandas at mismatched latitudes displayed a significantly higher proportion of locomotor activity in the outdoor enclosures (t=-2.157, df=56, p=0.035).

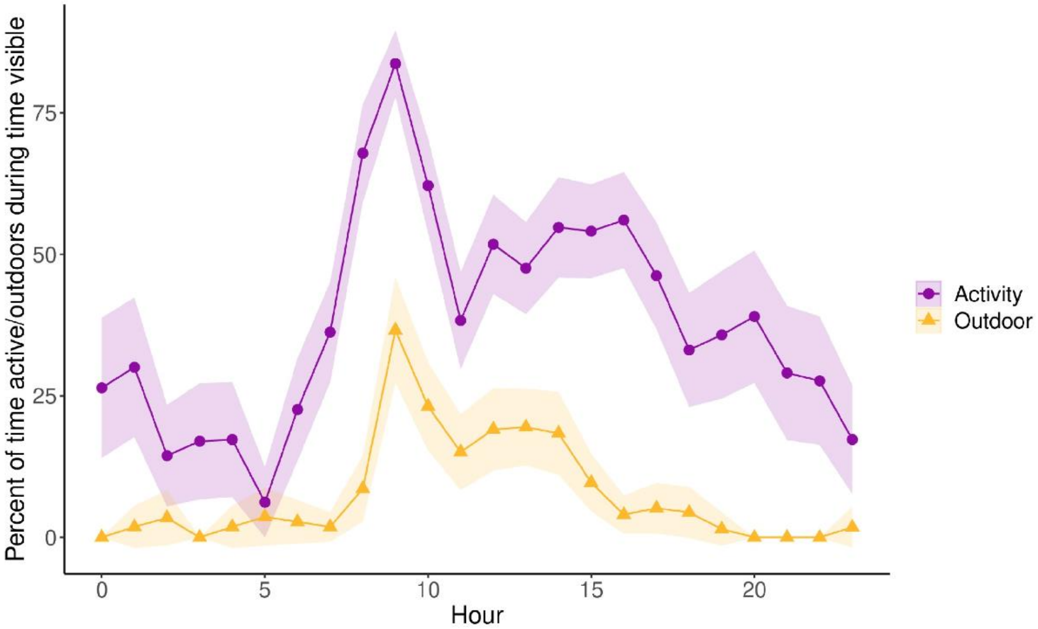


Figure 4.5. Percentage of time active (purple) and outdoors (yellow) throughout the circadian cycle for all pandas (n=11). Measurements are displayed as percentage of time while in sight, controlling for time out of sight, and averaged by hour. Shaded regions are 95% confidence intervals. The percentage outdoors is opposite to the percentage of time indoors. Therefore, a measurement of 0% time outdoors represents 100% time indoors and vice versa.

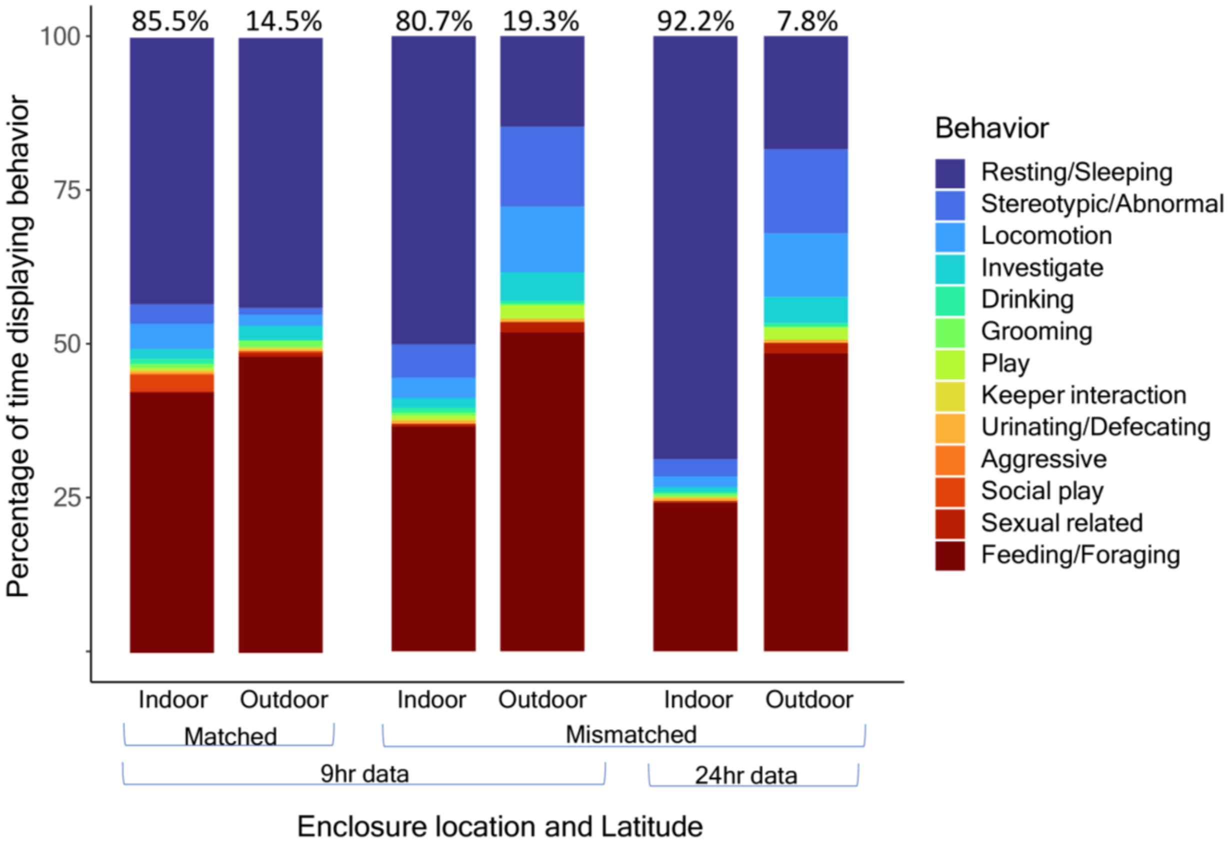


Figure 4.6. Activity budgets in percentage for indoor and outdoor enclosure space compared between pandas at matched (n=6) and mismatched (n=4) latitudes. Panda 9 is excluded from this analysis because cameras were fixed on the indoor enclosure most of the year. Data are proportions of time represented as percentages out of 100%, not counts. For pandas in mismatched latitudes, activity budgets are further compared between the 9-hour daylight subset of data, for comparison with the matched latitudes, and the full 24 hours of data. The colour indicates the behaviour. Percentages at the top of the bars indicate the total percentage of time spent indoors or outdoors.

4.3.4 ZINB model results for sexual-related and stereotypic/abnormal behaviour

The model for sexual-related behaviour displayed a model convergence warning, which indicated that the best fitting model was likely not found, and therefore, the AIC and BIC scores were not produced. However, the model was run several times and produced the same values within the count and zero-inflation model. In the count model for sexual-related behaviour, the positive coefficient for Spring (1.634, z=3.157, p=0.002) suggests an increase in sexual-related behaviour during this season compared to the grand mean of all seasons. This increase coincides with the breeding season. Conversely, the negative coefficient for Autumn (-1.412, z=-2.158, p=0.031) and potentially significant negative coefficient for Winter

(-1.105, z=-1.843, p=0.065) suggest decreases in these seasons compared to the grand mean for all seasons. Post hoc comparisons confirm these patterns showing Spring having significantly higher sexual-related behaviours than Autumn (3.046, z.ratio=2.908, p=0.019) and Winter (2.857, z.ratio=2.857, p=0.022). Hour of day (0.273, z=5.761, p<0.001) was also significant. Within the zero-inflation component, the hour of day coefficient (0.612, z=4.392, p<0.001) was also significant. The positive coefficients for adults (1.438, z=2.307, p=0.021) and females (2.579, z=2.885, p=0.004) suggest that these groups are more likely to show true zeros for sexual-related behaviour.

In the count model for stereotypic/abnormal behaviour, the positive coefficient for Spring (0.494, z=2.876, p=0.004) indicates an increase in stereotypic/abnormal behaviour compared to the grand mean for all seasons, coinciding with the breeding season. However, for Winter (-0.587, z=-2.384, p=0.017), stereotypic/abnormal behaviour decreased in comparison to the grand mean of all seasons. Post hoc comparisons confirm this result with stereotypic/abnormal behaviour being significantly higher in Spring compared to Winter (1.082, z.ratio=3.080, p=0.011). In addition, sexual-related behaviour showed a decrease as stereotypic/abnormal behaviour increased (-0.506, z=-4.381, p<0.001). Together, these results follow predictions that stereotypic/abnormal behaviours are related to sexual-related behaviours since they increase in the breeding season and show an inverse relationship to sexual-related behaviours. The latitude mismatch coefficient (1.127, z=1.845, p=0.065) trended toward significance in the count model, suggesting potentially higher levels of stereotypic behaviours seen for pandas at mismatched latitudes compared to those at matched latitudes. The coefficient for males (-0.398, z=-2.173, p=0.030) was also significant in the count model. Within the zero-inflation component, the positive latitude mismatch coefficient (1.251, z=2.640, p=0.008) indicates that those pandas located in mismatched latitudes are more likely to have a structural zero for stereotypic behaviour, perhaps displaying them more sporadically but at higher levels, as the count model would suggest. The negative coefficients for Spring (-0.436, z=-2.345, p=0.019) suggests that pandas are generally more likely to show positive values for stereotypic/abnormal behaviour in the Spring compared to other seasons. The negative coefficient for sexual-related behaviour (-2.622, z=-4.500, p<0.001) follows predictions that with an increase in sexual-related behaviour, it becomes more likely that the panda will display some level of stereotypic/abnormal behaviour. Hour was also significant within the zero-inflation model (0.034, z=1.993, p=0.046).

Since sexual-related behaviours can be expressed differently between sexes, and at least one sex came out significant for both the sexual-related and stereotypic/abnormal behaviours, we displayed the circadian rhythms of these behaviours by sex (Figure 4.7). The circadian rhythms of sexual-related behaviours were different between the sexes, with most individuals concentrating sexual-related behaviours to daylight hours, but females also showing larger peaks in the night. However, it should be noted that these peaks were displayed by sub-adult females, and therefore, it is unknown if these are patterns true to all females, or a sign of sexual immaturity. In contrast, the circadian rhythm for stereotypic behaviours were quite similar between the sexes, with both sexes displaying a relatively high peak in the morning and smaller peaks throughout the day, without many stereotypic behaviours in the night hours. Interestingly, although the circadian rhythms for these two behaviours were not similar, the circannual rhythms followed a very similar pattern (Figure 4.8). Sexual-related behaviours are quite rare, so although the scale is nearly 10× smaller than that of stereotypic/abnormal behaviours, these two behaviours seem to fluctuate in-synchrony throughout the year.

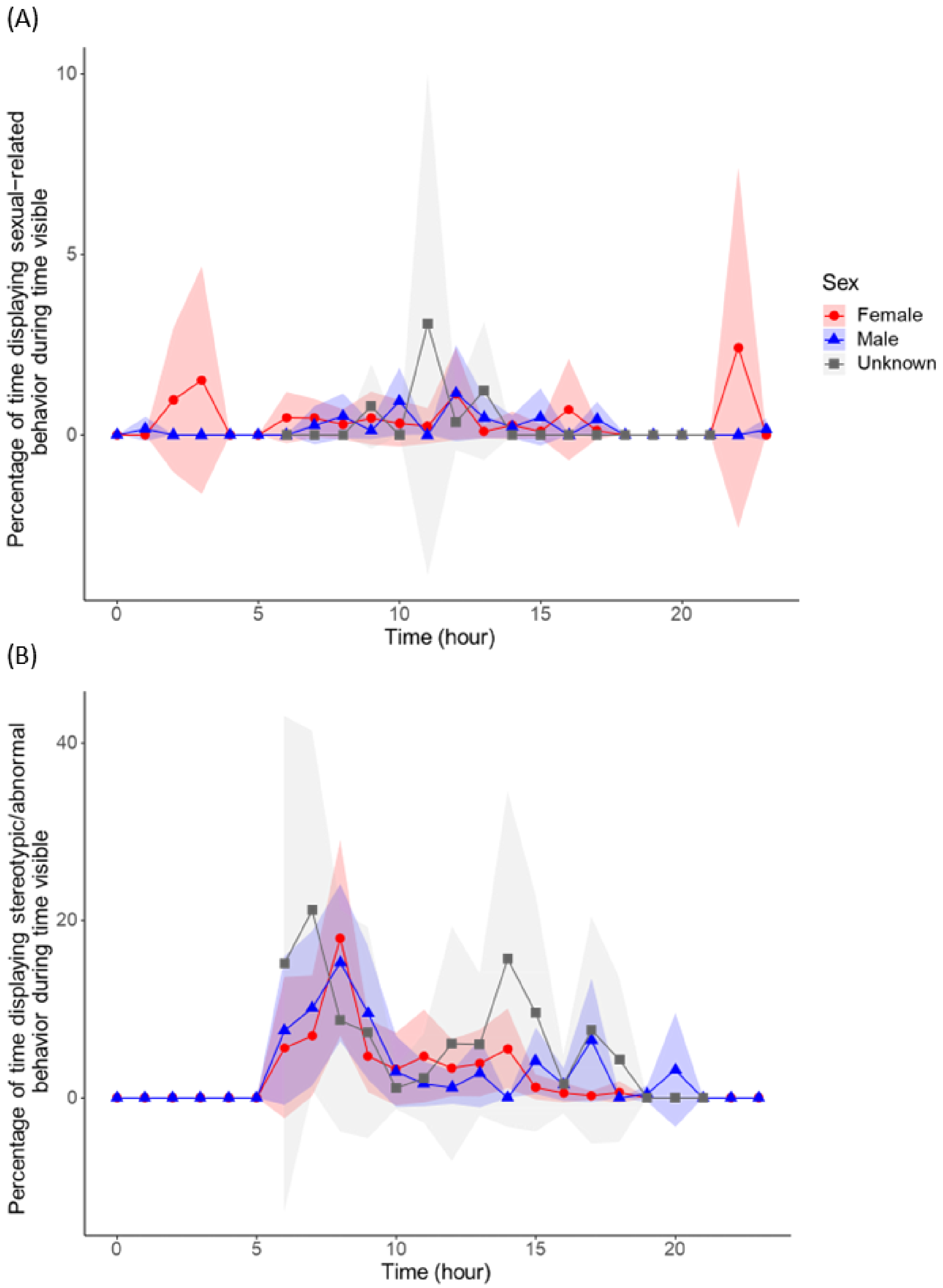


Figure 4.7. Circadian cycles of females (red, n=5), males (blue, n=4), and unknown sex (n=2) for sexual-related (A) and stereotypic/abnormal (B) behaviour. Behaviours are percentages of time in sight, controlling for time out of sight, and averaged by hour. Shaded regions are the 95% confidence intervals.

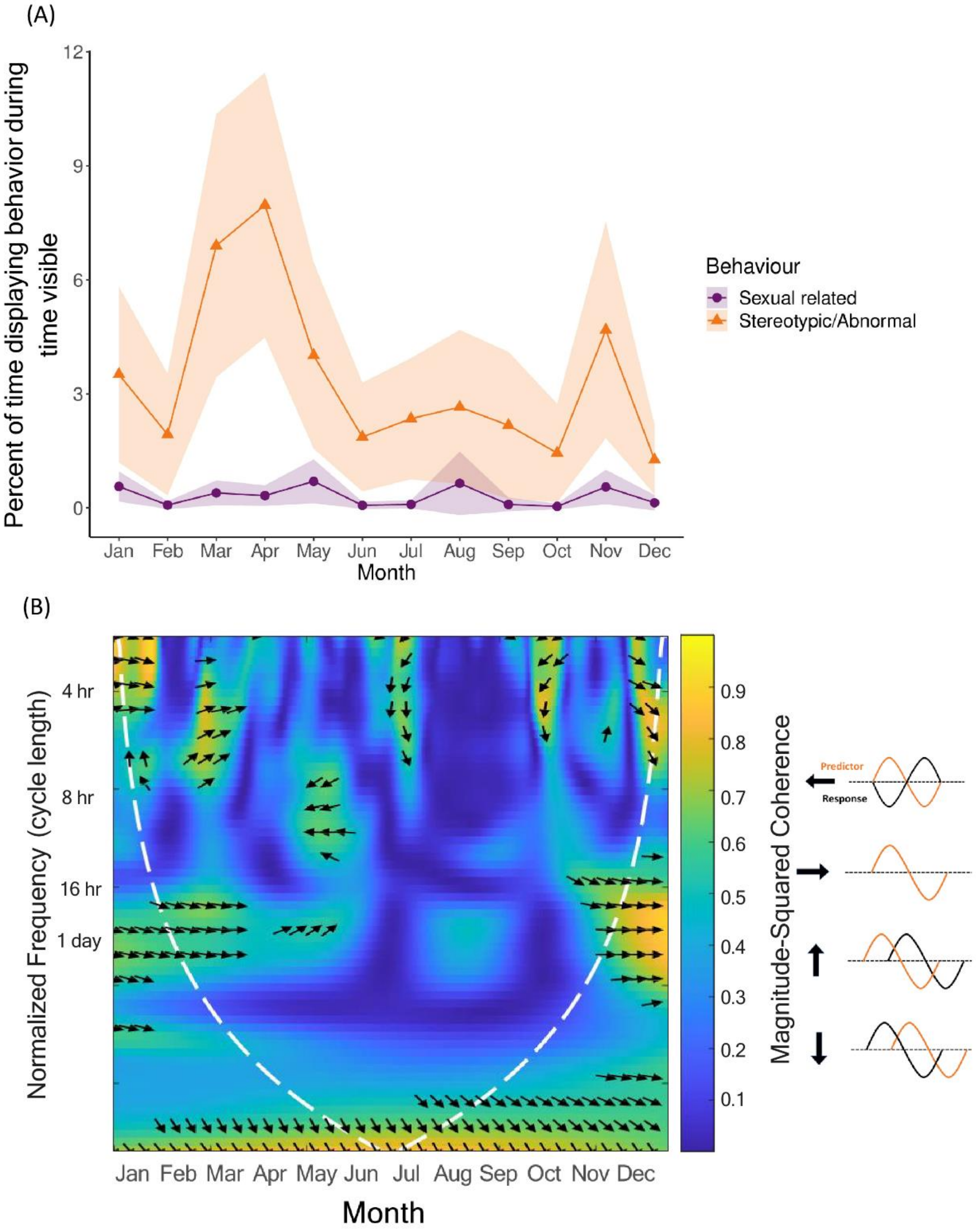


Figure 4.8. Relationship between sexual-related (purple) and stereotypic/abnormal (orange) behaviours for all pandas (n=11). Circannual cycles (A) for the behaviours are percentages of time in sight, controlling for time out of sight, and averaged by hour. Shaded regions are the 95% confidence intervals. The data for the wavelet coherence (B) are the averaged signals for all pandas using the full 24-hr dataset for those individuals who had it. The x axis is the time of year in months, the y-axis is the normalised frequency between the two signals, and the colour represents the strength of the correlation (yellow high, dark blue low, scale on right hand side). The kind of phase relationship between the signals are noted by the arrows (refer to arrow key).

4.3.5 Wavelet coherence for sexual-related and stereotypic/abnormal behaviour

To extrapolate more information about this relationship between sexual-related behaviour and stereotypic/abnormal behaviour, we conducted a wavelet coherence analysis for the two behaviour categories (Figure 4.8). We found that between the coinciding peaks of these two behaviour categories, there was coherence. The two behaviour signals were in phase with each other between January and March around the 4hr and 1-day cycle lengths. Around July and October, when both signals were increasing after dipping, there was coherence at the 4hr cycle length with the stereotypic/abnormal signal leading. Then, between November and December, when both signals were fading, there was also coherence at the 4hr and 1-day cycle lengths with the stereotypic/abnormal signal leading.

Since a relationship between stereotypic/abnormal behaviours and sexual-related behaviours was established, and the motivation to display sexual-related behaviours is presumably tied to opportunities to mate, which vary amongst zoos, we examined the circannual cycles of stereotypic/abnormal behaviours by zoo in line plots (Figure 4.9). Interestingly, all pandas except for the castrated male in Zoo E, regardless of sexual maturity or mating opportunity, displayed the highest peak in stereotypic/abnormal behaviour in the Spring during the mating season. This reinforces the notion that the display of stereotypic/abnormal behaviours may be linked to hormonal fluctuations for breeding, though a larger sample size, especially of castrated individuals, and hormone analyses would still be more informative.

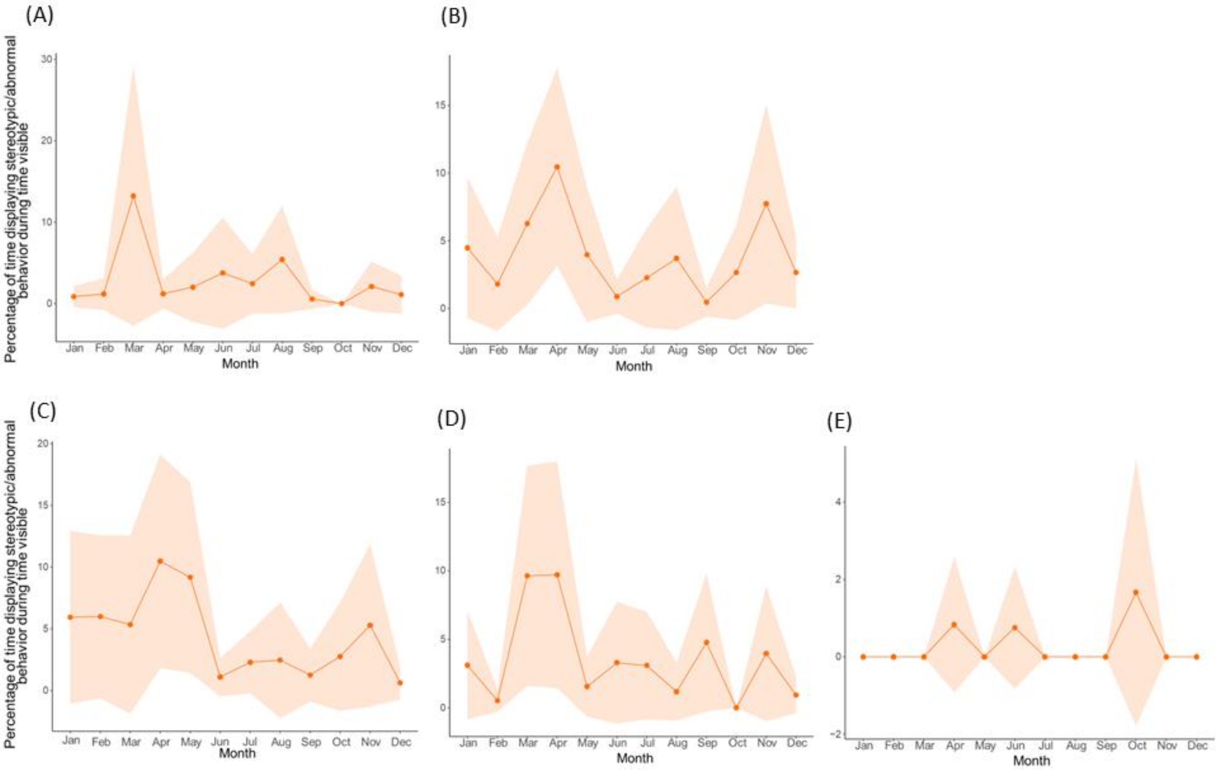


Figure 4.9. Circannual cycles of the percentage of time in sight displaying stereotypic/abnormal behaviour by zoo (lettering following Table 4.1) with data averaged by month. A and C are zoos at matched latitudes. B and D are zoos at mismatched latitudes. E is a castrated individual (medical reasons) at a mismatched latitude. The shaded regions are 95% confidence intervals.

4.4 Discussion

From our results, we found clear relationships and coherence between circadian/circannual cycles and zeitgebers, both natural and zoo specific. Our hypothesis that behaviour cycles will change depending on the latitude match/mismatch and the corresponding zeitgebers was supported by the differences we found by latitude for activity and stereotypic cycles. Our second hypothesis that zoos will have additional zeitgebers that animals entrain to was also supported by the differences in indoor/outdoor activity budgets and the peak of activity and stereotypic behaviour in the early morning hours that could potentially indicate anticipatory behaviour for keepers and fresh food arriving. We have also demonstrated how circadian and circannual cycles can be used to provide a fuller picture of behaviours and their potential regulators, allowing zoos to better manage them and promote positive behaviours by providing a conducive environment and husbandry schedules.

4.4.1 Differences in latitudinal zeitgebers influence activity cycles

Our data on activity showed clear relationships with the external zeitgebers of daylight, temperature minimum, and temperature range, with some differences in coherence and phase relationships between the latitudes. There was also a general finding that pandas at mismatched latitudes were more likely to display inactivity in comparison to pandas at matched latitudes. The difference in the cycles and levels of activity were expected because the zeitgeber cycles themselves differed between matched and mismatched latitudes. Therefore, if animals entrain to their external zeitgebers, we expect the activity cycles to shift in accordance with the changes in those zeitgeber cycles.

Despite this, when looking at the averaged circadian cycles of activity between matched and mismatched latitudes, there were no clear differences. The circadian rhythm is an endogenous rhythm that persists in constant conditions (i.e. constant light and temperature) (de Mairan, 1729; Golombek & Rosenstein, 2010). Since endogenous clocks are ~24-hr (slightly longer or shorter), when allowed to free-run (i.e., under constant conditions), the cycle will drift or display phase delays or advances. Therefore, organisms need daily entrainment to external zeitgebers to be exactly 24-hr (Froy, 2011). The pandas displaying similar circadian rhythms despite being at different latitudes with varying photic and non-photic zeitgebers, demonstrates how strong this endogenous cycle can be. What may be occurring is that the circadian cycle of activity will follow a phase pattern throughout the year, but simply needs to be entrained daily to be exactly 24-hr. What might shift as the natural zeitgeber fluctuates throughout the year is the amplitude of that phase pattern, not the phase pattern of the circadian cycle itself.

This was demonstrated in the circannual rhythm of activity as the activity levels shifted through the year. Between pandas at matched and mismatched latitudes, the circannual rhythms of activity remained very similar between the groups through most of the year but diverged between December and March. This finding, along with our wavelet coherence analyses between activity and daylight, temperature minimum, and temperature range (Figure 4.4) show how entrainment might change depending on the rhythm of the external zeitgeber. Changes in annual activity levels for species are triggered by a shift in an external cycle. The external zeitgeber must reach a certain threshold in its signal that is recognised by the animal’s internal clock as an indication of the time of year (e.g. daylight reducing to six hours as an indication of Winter) and thus trigger the activity appropriate for that time of year. This is seen across species for behaviours like torpor and hibernation, which are induced by changes in food availability, ambient temperature, and photoperiod (Genin & Perret, 2003; Körtner & Geiser, 2000), or migration in birds which is triggered by photoperiods of specific length depending on the season (Gwinner, 1996). If the external zeitgeber is not shifting to the proper threshold, it will not trigger the coinciding shift in activity.

The discussion of potential zeitgebers that influence giant panda activity would not be complete without an understanding of their adaptation to bamboo as a food source. Giant pandas are peculiar in that they have a very low energy expenditure relative to other mammals (Nie et al., 2015). This is a trait that is likely to have evolved because of the inability to digest bamboo fully, to extract the most nutrients. Therefore, pandas must eat large amounts of bamboo to compensate, spending higher proportions of time foraging and feeding than other bear species. We found that pandas spend the majority (70.6%) of their active time feeding. Understanding their feeding ecology is important because their migration patterns have been investigated in the wild, and evidence suggests that their main motivation for migration is to follow the emergence of nutritious bamboo shoots, though there are also effects of solar radiation and habitat preference (Liu et al., 2011; Liu et al., 2002; Zhang et al., 2015; Zhang et al., 2017; Zhang et al., 2018). Wild pandas initiate migration from mid-April to early June, migrating within several days to their Summer range and returning over several weeks from early September to October (Liu et al., 2002). The fast migration at the start of the Spring coincides with the emergence of bamboo shoots across an elevational gradient, with shoots at higher elevations showing a gradient delay in emergence (Zhang et al., 2018). The ecological factors influencing migration could mean that although we discuss the effects of daylight and temperature on activity below, food availability may also be a zeitgeber in the wild that affects activity and migratory behaviour in particular.

Importantly, the initial period of migration also coincides with the breeding season in the Spring. Zhang et al. (2018) used GPS collars and were also able to note that despite the elevational migration pattern across the pandas, the individual paths were distinct, and they were associated with the possibility that pandas were also seeking mates. The synchrony between migratory and breeding behaviours could be linked to the high protein and high amino acid quality of shoots resulting in weight gain (Wang et al., 2017) potentially being beneficial leading into the breeding season, though the increase in culm consumption (plant part with highest caloric absorption) during the summer is still not well understood (Hansen et al., 2010). Ultimately, pandas show seasonal preferences for plant parts in the wild (Liu et al., 2002; Zhang et al., 2015; Zhang et al., 2018) and captivity (Hansen et al., 2010; Wang et al., 2017), and with food availability being a potentially strong zeitgeber for migration, further investigation beyond this study would need to be done to understand its potential as a zeitgeber.

4.4.1.1 Activity and daylight

In our coherence analysis between activity and daylight, the strongest zeitgeber for mammals (Golombek & Rosenstein, 2010), we saw similar coherence and phase relationships between the latitude groups between March and June, but a seemingly missing coherence between August and October for those pandas at mismatched latitudes when compared to pandas at matched latitudes.

The known migratory activity in March/April coincides with the coherence we see in the wavelet coherence for activity and daylight for pandas in both latitudinal locations. This can indicate that daylight may be one of the external zeitgebers that trigger this migratory behaviour. The difference we see in the coherence for pandas at mismatched latitudes between August and October around the period of returning migration could be because the daylight cue in the Autumn is not at the proper threshold. The amount of daylight at matched and mismatched latitudes are nearly identical in March and September, however, between October and February, the amount of daylight at mismatched latitudes is much less than at matched latitudes, and much more between April and August. With daylight being a strong indicator of time of year, it may be that pandas at mismatched latitudes receive a daylight signal in the Autumn that is not recognised by their internal clock, or does not reach the proper threshold, and therefore does not trigger a shift in activity. This may be why we see activity levels between latitudes diverge between November and April. These differences in activity levels and synchronisation with the daylight zeitgeber between the latitude locations does not necessarily indicate poor welfare for either location, but supports the argument that animals in a captive environment will respond to external cues with shifts in their activity. Therefore, this should be known by staff who house these animals, so they can be aware of when to expect changes in activity and how to modify the environment accordingly. For migratory species, allowing access to more or larger areas during periods of natural migration may be a solution.

4.4.1.2 Activity and temperature

Though temperature is a weak zeitgeber for most endotherms, it still plays an important role in resetting circadian oscillators and enhancing internal circadian synchronisation (Buhr et al., 2010). Refinetti (2010) found that mice respond to temperature cycles as a zeitgeber, though not as efficiently as they do to daylight. These findings indicate that although temperature is not as strong a zeitgeber as daylight, it is still important to consider how it may influence the behavioural cycles of a captive animal. Indeed, in our study, we did find a relationship between activity and temperature measures with activity decreasing as both temperature minimum and temperature range increase. Previous research on pandas also suggests that temperature is an important external cue for activity. Liu et al. (2011) investigated solar radiation in relation to the monthly distribution of pandas in the Foping Nature Reserve. Solar radiation is the intensity of sunlight and therefore directly related to temperature, with higher solar radiation resulting in higher temperatures. They found that pandas begin migrating in Spring when the solar radiation changes from low to high and migrate back when the radiation shifts from high to low, indicating a potential regulatory effect of solar radiation on migratory patterns. They also found that the panda tracking locations had a much higher minimum solar radiation level than the minimum solar radiation for the whole of the nature reserve. This indicates that the pandas use solar radiation minimums to select their habitat. Zhang et al. (2015) expanded on this finding and discovered that pandas choose areas with lower solar radiation in warmer months and higher solar radiation in cooler months (Zhang et al., 2015). In addition, Zhang et al. (2017) conducted wavelet coherence analyses between activity and solar radiation and temperature. They found coherence with activity at the 1-day cycle length throughout much of the year for both solar radiation and temperature, indicating that pandas synchronise their daily activity cycles with daily temperature cycles.

Similarly, in our coherence analyses with temperature, we found coherence between temperature minimum and activity at the 1-day cycle length during warmer months for pandas at matched latitudes but not mismatched latitudes. This would be the season in which the temperature minimum reaches its highest point at both latitude locations. However, in the mismatched latitudes, the temperature minimum in summer was reaching temperatures similar to temperature minimums in Spring and Autumn for the matched latitudes. Therefore, it could be that this is another cue that does not reach the proper threshold in mismatched latitudes to synchronise with activity levels. However, despite the absence of coherence between temperature minimum and activity during warmer months for pandas at mismatched latitudes, activity levels were similar for summer between the latitude groups. Interestingly, for the temperature range and activity coherence, we found coherence at the 1-day cycle length in summer for pandas at mismatched latitudes but not at matched latitudes. The temperature ranges between the latitude locations were similar from May-September and quite dissimilar from October-April. Therefore, further investigation could determine if during warmer months, pandas will initially synchronise their activity levels to temperature minimums, but if that cue is not reaching the proper threshold, they will use temperature range as a similar cue. It would also be beneficial to investigate how temperature affects their choice in habitat in a captive environment, and whether captive pandas, like their wild counterparts, will choose cooler areas in the summer and warmer areas in the winter. In a zoo environment, this habitat selection can be the choice between an indoor and outdoor enclosure. Understanding these relationships between activity, temperature, and habitat choice can aid zoos in determining appropriate temperature conditions so that they can provide the proper choices at the right times of year and promote circadian synchrony.

4.4.2 Zoo specific zeitgebers can potentially entrain behaviour cycles

With temperature and lighting being clear zeitgebers that regulate activity, it was important to investigate how the climate controlled indoor enclosures in zoos influenced activity. We found that the circadian cycle of activity fluctuated in synchrony with the percentage of time spent outdoors. This can be due to several factors. One explanation may be that pandas prefer to rest indoors where the climate is controlled. Another explanation may have to do with the fact keepers will often move food to the outdoors to lure the pandas outdoors so that they may clean their indoor enclosures. This means that they would often be feeding (the most common active behaviour) while outdoors. To investigate further, we looked at activity budgets while indoors and outdoors and examined the latitudinal differences since pandas at mismatched latitudes would presumably be experiencing natural zeitgebers outdoors that they may not be adapted to. Pandas at matched latitudes had very similar indoor/outdoor activity budgets, while pandas at mismatched showed a significantly higher proportion of locomotive activity outdoors.

The cause of the difference in locomotor activity between indoor and outdoor enclosures in pandas at mismatched latitudes but not matched latitudes could not be determined but may be due to preferences for increasing locomotion in colder temperatures (outdoor temperatures at mismatched latitudes towards the poles would be cooler) to decrease energy expenditure (Nie et al. 2015) or due to enclosure design. Further investigation with larger sample sizes and clearer information on schedules for when pandas have access to indoor/outdoor enclosures would be needed to determine if differences in temperatures and lighting have significant effects on activity budgets. However, our findings do provide some evidence that the climate control for indoor enclosures at mismatched latitudes expose the animals to a different set of zeitgebers than those experienced in outdoor enclosures. As mentioned in the discussion on temperature minimum, if a captive animal chooses their habitat based on temperature, then it would be ideal for zoos to understand the temperatures they prefer at different times of year and ensure that they do provide their animal with these choices at the appropriate times of year within the indoor enclosures. Zoos often attempt to mimic the specific species' natural climate conditions within their indoor enclosures. However, if this cannot be done, it may be easier to house animals within their natural latitudinal range. Zoos may also want to consider whether only an indoor enclosure with appropriate conditions is adequate for their animal, or if an outdoor area with zeitgebers and climate they are evolved for is also needed.

Aside from the added zeitgebers of the controlled climates of indoor enclosures, husbandry routines which include feeding, cleaning, and training sessions become a part of the external environment of the animal that is predictable (Bassett & Buchanan-Smith, 2007) in the same way light and temperature are, and cause arousal, making them a potential non-photic cue. Therefore, in response to husbandry practices, animals may show predictable changes in their behaviour and physiology. Our results may indicate some form of anticipatory behaviour for feeding or husbandry practices since there is a clear peak of activity in the early morning hours when keepers arrive. Pandas across all the zoos studied were left with bamboo overnight and would receive fresh bamboo in the mornings when keepers arrived. This prediction of anticipatory behaviour could be further supported by the same peak in stereotypic/abnormal behaviour in the early morning-which does not coincide with the circadian cycle of sexual-related behaviours- because predictable feeding schedules also cause animals to display anticipatory behaviours which may signal stress should their frequency increase in response to the predictable schedule becoming delayed (Anderson et al., 2015; Waitt & Buchanan-Smith, 2001).

4.4.3 Determining circadian and circannual cycles of wanted and unwanted behaviours can help zoos manage them

Stereotypic/abnormal and sexual-related behaviours are of major concern for zoos, with the former behaviours being unwanted and the latter being crucial to the goal of conservation of species. Therefore, understanding these behaviours and the factors that may regulate them are of extreme importance to zoo staff and have many implications for the goals of zoos. Our findings on the relationship between sexual-related and stereotypic/abnormal behaviours in giant pandas provided further evidence on the previously determined relationship by Martin et al. (2020). Their results found seasonal differences in the amount of stereotypic behaviour displayed along with sex differences across those seasons. Our results support the findings that there is a strong relationship between sexual-related behaviour and stereotypic/abnormal behaviour. We also have similar results indicating that stereotypic/abnormal behaviour and sexual-related behaviour vary across seasons, with pandas showing significantly less stereotypic/abnormal behaviours in the Winter, and increased sexual-related behaviour in the

Spring, as expected with the breeding season. However, we did not find sex differences in the amount of stereotypic behaviours displayed.

Our results were able to illuminate how this relationship between sexual-related and stereotypic behaviour is expressed. On a daily scale, the cycles of stereotypic/abnormal and sexual-related behaviours do not synchronise. However, on an annual scale, the cycles do fluctuate in synchrony. The circadian cycle of sexual-related behaviour shows adults concentrating their sexual-related behaviour very efficiently to daylight hours, while sub-adults display these behaviours more randomly throughout the day/night. These age group differences could potentially indicate that the time of day in which sexual-related behaviour is displayed is a mark of sexual maturity. There may be a benefit to concentrating sexual-related behaviours to daylight hours so that mating pairs are synchronised in their behaviour, increasing the chances of successful mating. Synchrony of reproductive behaviours between reproductive individuals is an adaptive benefit to having a circadian rhythm. For instance, in nocturnal rodents, ovulation occurs at night when encountering a mate is most likely (Goldman, 1999). For animals with a reproductive season, monitoring the time of year via the circadian clock (i.e. measuring day length) allows the animal to ovulate when environmental conditions are most favourable for gestation and rearing (Boden & Kennaway, 2006). Further investigation would need to be done to determine if this concentration of sexual-related behaviour to daylight hours is regulated by hormonal cycles that develop when a giant panda reaches sexual maturity. However, the information on the behavioural cycles alone can help zoos understand the optimal time of day to provide their pandas with mating opportunities.

The synchronised fluctuation and coherence between the circannual cycles of sexual-related and stereotypic/abnormal behaviours was a very informative finding. The wavelet coherence displayed that the stereotypic/abnormal signal are in phase around the breeding season, and outside of the breeding season, the stereotypic/abnormal signal leads. These phase relationships may indicate that captive pandas express stereotypic behaviours out of frustration for not being able to express sexual-related behaviour, displaying an increase in stereotypic/abnormal behaviours with sexual-related behaviours as a way to fulfil the unmet needs. This prediction is further supported by the fact that for our study pandas, the most common stereotypic behaviour was pacing. This makes sense with it being related to sexual behaviours because pandas migrate during the mating season, with wild males showing increased locomotion in the mating season when compared to females (Liu et al., 2002), and captive males showing more locomotive stereotypies than females and the behaviour correlating with reproductive performance (Martin et al., 2020). Also, our study pandas tended to incorporate anogenital rubbing and handstand urination into their pacing or bipedal standing. Anogenital rubbing and handstand urination are sexual-related behaviours because scent marking is used as a chemical signal of home range occupation and potentially fitness, with males scent marking more often than females (Charlton et al., 2020; White et al., 2002). Bipedal standing (done at keeper doors) would be related to anticipatory behaviour with keepers as mentioned before.

Further evidence of this relationship between stereotypic/abnormal and sexual-related behaviours can be seen from our finding that pandas at mismatched latitudes are less likely to display any level of stereotypic/abnormal behaviour when compared to pandas at matched latitudes. Since Spring migration and the breeding season occur at the same time, and we found that daylight is a potentially strong zeitgeber that synchronises with activity for pandas at matched latitudes at the same times of year that they are likely to be migrating back and forth, we would want to investigate further whether the same synchrony that is missing in the mismatched latitudes would also be a missed threshold for mating behaviour in mismatched latitudes. Although our study did not find any relationship between daylight, temperature minimum or temperature range with sexual-related behaviours, the behaviours are extremely rare and therefore a larger sample size with higher resolution signals would help us explore the potential zeitgebers for sexual-related behaviour. However, since sexual-related and stereotypic/abnormal behaviours are related, and temperature range showed a significant relationship with activity, and a trend toward significance with stereotypic/abnormal, this cue could merit further investigation. Interestingly, for two of the zoos, temperature range fluctuated in synchrony with stereotypic/abnormal behaviour. In addition, though only a sample of one, there was an interesting result where the castrated individual displayed the least, and almost no amount of stereotypic/abnormal behaviours. This could support the theory that stereotypic/abnormal behaviours are instigated by an unmet desire to display sexual-related behaviours, so when that desire is reduced from castration, so are the levels of stereotypic/abnormal behaviour.

Our results elucidating the relationship between sexual-related and stereotypic/abnormal behaviours is a demonstration of how zoos can use information about circadian and circannual cycles of behaviour to understand and manage these behaviours. Although this relationship cannot be generalised to all species, the manner in which we determined the relationship and understood it can be extended to other species to determine the triggers for stereotypic/abnormal behaviours so that they may be reduced, and to promote sexual-related behaviours. For giant pandas, the expression of stereotypic/abnormal behaviour with anogenital rubbing and handstand urination can be an indication to keepers that the pandas would like a mating opportunity. In addition, since sexual-related and stereotypic/abnormal behaviour is displayed approximately every three to four months in our study pandas, we would recommend that zoos consider allowing mating opportunities outside of the breeding season because they may actually mate at different times of year if allowed.

4.5 Conclusion

Given the large influence that circadian rhythms have on behavioural and physiological processes, it is essential that within a captive environment, they are well understood across species. The welfare of a species can be difficult to assess if all the factors that influence their welfare are poorly understood or analysed separately. By assessing rhythmicity across the day, night, and seasons, we are contextualizing behavioural responses on a broader scale and understanding both internal and external factors which may influence their behaviour and ultimately welfare. Individual organisms do not exist in a vacuum of conditions, their continued existence depends on their ability to properly synchronise their internal environment with the external one in order to anticipate changes and respond accordingly through physiological or behavioural changes (Golombek & Rosenstein, 2010). The optimal external conditions for an organism to live in are the conditions for which their circadian rhythm has evolved. Therefore, in captive environments, we want to attempt to provide these optimal conditions to our animals as well as understand how conditions which they would not experience in the wild can affect them at individual, group and species level.

Through this study we have demonstrated a holistic approach to identify complex interactions between an animal and their external environment. Zoos can take this approach of systematically monitoring behaviour, even if slowly over time, to understand their animals' needs and apply the information to construct appropriate enclosures and husbandry practices/schedules, taking into account how those needs change cyclically over time. As a community, zoos can also use this approach and information to conduct further research to address questions at the forefront of captive animal welfare on whether species should be housed outside of their natural climatic and latitudinal conditions. Our results demonstrate that there are clear relationships between the cycles of external factors and behaviour. Therefore, it would benefit zoos to investigate these relationships further so that they may provide their animals with appropriate choices, enhance conservation efforts, promote circadian synchronicity, and consequently improve welfare.

Chapter 5.

Discussion

Chapter 5. Discussion

5.1 Thesis results summary

This thesis took the novel approach of demonstrating a holistic, evidence-based welfare assessment method that incorporates circadian rhythms to understand and account for evolutionarily adaptive rhythms. In Chapter 2, making the RZSS welfare assessment more reliable and efficient, I found that the assessment could likely benefit from assessors answering questions from areas of their expertise to make the process more efficient. I also demonstrated the amount of context and information that could be gained from taking an evidence-based approach to behaviour and environment questions by assessing them in conjunction with each other and accounting for daily rhythms. The finalised tool is meant to provide a standardised welfare assessment tool that can be used across captive animal institutions, making it easier to compare welfare and welfare practices across the globe. In Chapters 3 and 4, the benefits of assessing circadian rhythms as a standard practice were investigated with giant pandas as an example species. I found that captive pandas housed outside of their natural latitudinal range display less activity than those within their latitudinal range. Amount of daylight, temperature minimum, and temperature range were also associated with activity cycles, potentially acting as zeitgebers. The results also indicate that life stage was associated with changes in overall activity, feeding, locomotion, and stereotypic pacing, and that sex influenced scent anointing and anogenital rubbing. Overall, the circadian rhythms showed three peaks of activity, including a nocturnal peak, as seen in wild giant pandas. I also found associations between sexual-related, stereotypical/abnormal, and feeding behaviour, which are possibly linked to the timing of migration of wild pandas. I elucidated the relationship between a mother and cub, finding that they concentrate maternal behaviours to mainly after zoo closing hours. These results clearly demonstrate the amount of information that can be gained from investigating circadian rhythms in a captive setting. These kinds of results have implications for welfare in that the information gained can be used to design enclosures conducive to circadian synchrony and adjust for the changing needs of a species across the lifespan. This thesis on a more holistic approach to welfare assessments and investigating circadian rhythmicity in captive species as a standard begins to demonstrate how we can apply a welfare standard that is inherently self-improving because as we learn more about species from the data collected, the better we can apply welfare interventions.

5.2 Responding to a shifting perspective on welfare in zoos

Finding evidence of sentience or the ability to experience pain has been the driver for the massive shift in the last few decades to improve welfare standards of captive species (Browning & Veit, 2022; Jones, 2013; Kumar et al., 2019; Proctor et al., 2013). Zoos and aquariums are unique among captive animal organisations in that they house many vertebrate and invertebrate species which do not have extensive research done on their ability to experience pain (physical or psychological), possess sentience, their natural behaviours and their physiology. However, in choosing to keep animals in captivity, zoos have ethical and moral responsibilities to uphold high standards of welfare (Zoo Licensing Act 1981, Standards of Modern Zoo Practice, 2012). Therefore, if we are equating the need for welfare with evidence of sentience, it has been suggested that assuming sentience in mammals, birds, reptiles, amphibians, fish and cephalopods is wise and that other taxa should be given welfare consideration that meet their physiological and ecological needs (Harley & Clark, 2019). However, I believe it is safer for zoos to assume that all animals possess sentience and can experience pain. Essentially, zoos should assume “sentient until proven non-sentient” rather than “non-sentient until proven sentient”, because following the philosophy of the latter is more difficult to ethically recover from should an animal be discovered to be sentient and welfare standards were not prioritised, than in the former if high welfare standards were upheld and it is discovered the animal is non-sentient. Therefore, zoos have a unique difficulty in applying the same standard of care across diverse taxa, because you need an in-depth understanding of the natural history, physiology and general needs of a species in order to provide for those needs. However, this difficulty cannot be an excuse to reduce welfare standards for lesser-known species, but rather motivation to understand their needs better.

Modern zoos have four main goals: conservation, education, research and recreation and also prioritise upholding high welfare standards of care (Greenwell et al., 2023). Conservation is often at the forefront of these goals since it addresses a global crisis that we face in the middle of the ongoing Anthropocene extinction (Beer et al., 2023; Turvey & Crees, 2019). However, all four goals to conserve, educate, research and provide recreation, along with providing high standards of welfare, are interconnected and cannot be achieved independently, but require prioritisation of each for all to succeed. To conserve species, zoos need collective support of the vision of conservation from the public and high/consistent visitor numbers to fund their conservation research and efforts. For zoos to gain public backing for conservation, visitors need to understand the reasoning for conservation, be invested in the survival of species, and have positive perceptions of zoos so that they are willing to visit (De la Fuente et al., 2017; Dove & Byrne, 2014). Our welfare assessment tool

(Chapter 2) could help in improving not just welfare, but conservation and education. The tool can make welfare intervention decisions more appropriate by taking an evidence-based approach, which consequently allows for more natural behaviours to be displayed by captive animals for release and improved breeding efforts for conservation, and finally educates the public on welfare practices and inadvertently animal behaviour by involving them in data collection.

Dove and Byrne (2014) found that zoo visitors do indeed have a sound understanding of the human impact on wildlife populations despite having misconceptions about biodiversity and ecosystems. Zoos are then responsible to educate visitors beyond the base knowledge they gain in every-day society to help them understand biodiversity, the human connection to nature, and the need to conserve species for the benefit of all living beings, including humans. Most zoo and aquarium associations require members to measure the effectiveness of their conservation and educational programs to maintain accreditation. And indeed, research does suggest that zoos improve visitors' knowledge of conservation and the importance of maintaining a healthy ecosystem through biodiversity, but research is limited on how this education changes visitor's future actions in implementing pro-environment and conservation behaviours in their daily lives (Learmonth et al., 2021). One of the main barriers for visitors in changing post-visit behaviour is that they do not know how to contribute to conservation beyond donating money (Nygren & Ojalammi, 2018). In addition, Stoinski et al. (2002) found that a conservation opportunity was 20 times more effective on-site than off-site to encourage conservation-related behaviour. Therefore, it may be beneficial for zoos to provide as many *in situ* conservation opportunities for visitors (Godinez & Fernandez, 2019) and find ways to motivate visitors to return to the zoo often.

To encourage zoo visitors to return frequently, they must have positive perceptions of zoos and their goals, and particularly of animal welfare. Increased public concern in animal welfare across different sectors (farm, laboratory, and captive wild animals) has created political pressure and driven policy and legislative change directed at improving care of animals (De la Fuente et al., 2017). In particular, zoo visitors' perceptions of zoos and animal welfare is greatly influenced by whether they witness animals displaying positive or negative behaviours (Godinez & Fernandez, 2019). Godinez et al. (2013) conducted a study investigating the correlation between visitors' categorisation of jaguar behaviour being stereotypic or species-typical and their perception of the jaguars' well-being, the exhibit quality, and the quality of their experience. They found that visitors who correctly categorised stereotypic behaviours as abnormal (roughly half of the visitors), generally rated the well-being of the jaguar, exhibit quality, and their own experience lower than those visitors who categorised the stereotypic behaviour as a normal active behaviour. This study aptly demonstrates how the public can be astute in recognizing welfare concerns and how this would negatively affect their experience at a zoo and very likely their willingness to return to a zoo. Therefore, zoos must find ways to improve public perception of welfare while acknowledging that visitors can recognise welfare concerns.

Another study by Warsaw and Sayers (2020) can address this and may direct us towards possible solutions to help improve public perceptions on welfare. Warsaw and Sayers (2020) investigated how awareness of accreditation influenced perception of zoo animal welfare. They provided scenarios that pertained to each domain of the five domains and sequentially provided more context, asking what the visitor's perception of the welfare state of the animal was after each sequential piece of information and after informing them of the zoo being accredited. For instance, a tiger that is pacing and then providing information that the tiger would be fed in 10 minutes, or otters that are huddled together for warmth and then providing more information that they are huddled on a heated rock. Warsaw and Sayers (2020) found that the perception of the welfare state was increasingly more positive with more context and that visitors were generally assured of the welfare of the animal once they were informed of accreditation. Though the latter result was not as prominent for visitors who initially rated the scenario as depicting a negative welfare state. It is important to note that these studies investigating zoo-visitor perceptions are biased towards people already willing to visit zoos, and very little is known about how the perception of non-visitors would change since they are already unwilling to visit zoos. However, this study provides two potential directions for improving public perception of welfare: informing visitors of accreditation and providing transparency by educating visitors on welfare programs and protocols to inform them of the efforts to improve welfare in detail.

Once fully developed, our welfare assessment tool (Chapter 2) can be used in implementing these two strategies to improve perceptions of welfare for visitors. Visitors can be informed that the welfare assessment itself is done in accordance with accrediting associations to assure visitors that the protocol is reviewed and/or enforced by associations composed of experts in animal care. In addition, our training materials used to recruit students and volunteers to collect behavioural data can also be used to educate the public and be transparent on welfare practices. Those who decide to volunteer and collect data could also inform visitors of what they are doing, demonstrating transparency of welfare practices, and explain to the visitors the different behaviours being measured and provide examples of interventions that can be implemented to improve welfare based on the data. By informing visitors of these welfare assessments, we are educating them on the species behaviours, the connectedness of the animals to the environment which may be paralleled to humans and our connection to the environment, and simultaneously providing transparency. Providing transparency is likely one of the more important aspects in improving public perception of zoo animal welfare because it helps the public trust that zoos are not attempting to hide the welfare states of their animals. If the public perceives zoos as trying hide the welfare states of their animals, they may assume the worst about welfare practices and why zoos would not be open about them. However, if zoos are open about their practices, it allows them to shift the perspective for visitors, making it known that zoos are aware that welfare is not perfect, but demonstrate that great efforts are being made to improve welfare practices. This approach of being open about these practices and allowing the public to participate in improving the welfare of animals can also show people that zoos take the concerns of the public seriously and want to include them in improving these efforts. One way to achieve this would be for zoo staff who give talks to visitors to communicate findings of welfare studies being conducted in the zoo.

Aside from welfare having an indirect effect on conservation efforts through its influence on public perception of zoos, welfare also has more direct effects on conservation. Greggor et al. (2018) argue that improving welfare directly improves conservation by promoting natural behaviours that can lead to more successful reintroductions and breeding attempts *in situ*. They follow the ‘Opportunities to Thrive’ principles where animals are given the opportunity to have a strategically presented, well-balanced diet, self-maintain, for optimal health, to express species-typical behaviour, and to have choice and control. Greggor et al. (2018) mention that providing naturalistic environments and diets, and promoting natural behaviours, will allow the animal to develop necessary survival skills for reintroduction while equally improving welfare for the animal while in captivity. In relation to breeding, having good welfare and natural breeding opportunities that allow for the display of species-typical breeding behaviours (i.e. providing birds with nesting materials to display natural nest-building behaviours), can help zoos understand when their animals are ready to breed. In addition, giving animals the opportunity to care for offspring instead of human-rearing, improves welfare for the offspring's long-term coping ability and for the parent by reducing stress from removal of offspring and allows parents to develop rearing skills and bonds with their offspring, which would improve breeding efforts in the long-term ex-situ and in-situ should the individuals be released (Porton & Niebruegge, 2006).

These kinds of practices follow the highly regarded “One Plan Approach” to conservation, coined and promoted by the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Conservation Planning Specialist Group (CPSG), in which it is suggested that species conservation planning should include all populations of a species (*in situ* and *ex situ*) and be developed by all responsible parties to create management strategies and conservation actions for a single conservation plan across all populations (Byers et al., 2013; Traylor-Holzer et al., 2019). Developing approaches like this would address the interconnectedness of the three goals for modern zoos to conserve, educate, and provide high standards of welfare to animals.

A large part of what may make the ex *situ* portion of the plans effective is having evidence-based approaches to assessing conservation success and welfare. A shift towards emphasizing an evidence-based approach to zoo management has been increasing in the last decades (Binding et al., 2020; Greggor et al., 2018; Melfi, 2009; Wolfensohn et al., 2018). It has been clearly recognised that having evidence to base conservation and welfare decisions on makes the efforts more targeted, relevant, and effective. However, it can be difficult to implement sustainable evidence-based systems due to limited resources and time. Our demonstration of using university students provides a possible solution to this issue. By outsourcing data collection to the university students, and potentially public volunteers in the future, we demonstrated how similar programs can enable zoos to collect a lot more evidence without a lot of increased effort within the zoo. Though our approach needs to be modified to be more efficient through developing more training/reliability testing materials, it provides a starting point for an evidence-based approach to welfare assessment.

An evidence-based approach which can be implemented across institutions would be most beneficial since it would allow for direct comparisons between zoos on the success of conservation and welfare programs. The welfare assessment tool proposed in this thesis (Chapter 2) can begin to address the welfare portion of a One Plan Approach to conservation. By taking an evidence-based approach to behaviour and environment, information on how the environment is influencing the animal’s ability to display natural behaviours and circadian rhythms can be measured. This information can address conservation questions that determine if animals are developing the necessary skills and behavioural repertoires for reintroduction and successful breeding and rearing of offspring. By incorporating circadian rhythms, we inherently assess how well an animal is adapting to an environment and whether the environment and husbandry practices are conducive to these adaptive rhythms. It is paramount that zoos establish an evidence-based approach to welfare management since ineffective welfare interventions can be more easily avoided without trial and error and wasted resources and time. Taking an evidence-based approach prioritises the animal in welfare programs by focusing on their needs and the proven interventions that improve their welfare.

5.3 Redefining what healthy and natural behaviours look like in a zoo

Though zoos want to encourage naturalistic/wild behaviours as a means of promoting positive welfare and ensuring animals which are reintroduced are adapted for life in the wild, all wild behaviours are not signs of good welfare and some have no way of being expressed in a zoo (Howell & Cheyne, 2018; Veasey et al., 1996). Some wild behaviours such as evading predators or stress responses are not ones we want to promote in a captive environment. Others such as migration and chase hunting have no way of being fully expressed in a captive environment, despite efforts with enrichment devices. In addition, zoo environments are very different from wild environments and have different cues that would never be experienced in the wild like husbandry practices, zoo events, regular human interaction and limited space. Animals are influenced by their environment and may display phenotypic plasticity in response to it, including changes to physiology, behaviour, life history, and even morphology that may be adaptive or maladaptive (Whitman & Agrawal, 2009). Adaptive, plastic behaviours in a zoo environment can look like spending less time foraging and more time playing, while maladaptive behaviours can be abnormal repetitive behaviours that do not subsist (Howell & Cheyne, 2018). Because of all of this, a difficult question to answer in welfare science is how the context of a captive environment changes the healthy expression of wild behaviours and what standard we should be comparing to when assessing behaviour in reference to welfare.

To answer this question, we must understand which behaviours are evolutionarily necessary and must be expressed in the same way as in the wild to have good welfare and which behaviours are more plastic and can be replaced with alternative behaviours and still be adaptive. To tease these apart we must take a broad perspective and understand that behaviour is a final step in a continuous and interconnected chain of events: genetic expression → physiological processes → mental states and processes → behaviour. Each step influences any of the other steps and they are all also influenced by the external environment. In addition, all steps will be expressed in different ways depending on the life stage and sex of the individual animal. Therefore, in order to tackle the complex question of what constitutes healthy expression of wild behaviours in a zoo environment, we need to account for the interplay of all these steps in the expression of behaviour and recognise that living beings are not separate from their environment.

Circadian rhythms can be used to tackle this complex question by addressing all of these factors simultaneously. Circadian rhythms inherently give us a hint into what is evolutionarily adaptive since they persist in environments outside of natural habitats and in the absence of external cues (Froy, 2011). In addition, genetic expression, physiological processes, behaviours, and even mental states regulated by hormones are regulated by circadian rhythms. By discovering what the natural, adaptive rhythms of all of these processes are and understanding the necessary synchrony between them and the external factors that regulate them, we can better understand the adaptive ranges of species and what changes to these rhythms and synchrony are still within a healthy range of expression.

The results from the giant pandas (Chapters 3 and 4) demonstrate how we can use circadian rhythms to begin to uncover what may be healthy ranges of expression of behaviours in response to external factors, at different life stages, and for pandas of different sexes. By investigating the expression of these rhythms of giant pandas across the globe, we were able to obtain a sample that could indicate the effects of housing pandas at latitudes with more extreme seasons than their natural habitat range. In addition, trying to match the analysis of the data to a similar study done on wild giant pandas gave us insight on which circadian rhythms tend to persist in a captive environment, which are more plastic, and which ones, when stifled, result in maladaptive behaviours. This approach maintains that wild behaviours can be a standard to compare to, but is more practical and less restrictive by accounting for plasticity and trying to determine the adaptive range of these rhythms. Zoos can use this technique of assessing all of these factors simultaneously to compare to the wild to discern what may be healthy behaviour or not. By investigating circadian rhythms, we are linking the animal to their environment and understanding how and why animals display certain behaviours. Contextualizing these behaviours allows us to replicate the conditions in which they occur to try and promote positive welfare for all species.

The most striking similarity between the wild and captive pandas we investigated were the three peaks in activity, including one peak in the night. The fact that this rhythm persists regardless of the setting and external factors tells us that it is a rhythm that is likely necessary to express to maintain homeostasis. Therefore, in a zoo setting we would want to provide all the necessary resources (i.e. sufficient bamboo for these active periods which are 70% feeding) for the animal to display this rhythm in a healthy way. However, if we have strong, stable rhythms, this means that other rhythms which may be more plastic could potentially desynchronise from the stable ones. Therefore, we would have to determine what regulates these other rhythms so that we may promote circadian synchrony of all internal clocks. In the case of the pandas, it seems that the rhythms of migration and/or mating behaviours are stifled and replaced with abnormal/stereotypic behaviours. Pacing can be adaptive or maladaptive depending on whether the same goal is achieved. However, with the pandas, it may be maladaptive if they are not able to expend as much energy as they want to release during migration or gain the proper storages from nutritious shoots that they are seeking through migration, nor display the mating behaviours and interact the way they want to. Assessing all of these behaviours against the more persistent rhythms of diel and annual activity and comparing to the wild rhythms of activity gives us a broad perspective to understand what behavioural rhythms may be adaptive and how we can try and promote these synchronous rhythms through the resources and external cues provided to the animals.

This thesis demonstrates how assessing wild behaviour is still very important in contextualizing what healthy behaviours are when displayed in captivity. Therefore, it would be beneficial to the entire zoo community for more research to be conducted that investigates these kinds of factors as circadian rhythms in captivity and the wild. By conducting research that is directly comparable between the wild and captive environments because of similar or identical methods, we can gain insight more quickly on not just what constitutes healthy behaviours in a captive environment, but also what elements of a wild environment are necessary to live a healthy life; meaning that it will be easier to translate *in situ* conservation programs to successful *ex situ* programs. This approach to research would follow the goals of creating One Plan approaches for conservation.

Though the research on pandas provides a good starting point for zoos in conducting this kind of research across all species, it is still limited in the rhythms and the external factors that regulate them that we assessed. Since circadian rhythms regulate gene expression and many physiological processes, and since there are several other external factors other than light and temperature that influence these rhythms, future research on this topic should expand on the variables analysed to gain further insight.

Many genes are under circadian regulation and the proper timing of gene transcription is known to be crucial in preventing or managing metabolic disorders like obesity and diabetes, in preventing inflammatory diseases and autoimmune disorders, and preventing cancer (Sukumaran et al., 2010). Therefore, detecting abnormal or desynchronised rhythmicity of gene expression is a way to detect risks to health and/or manage disease. In a zoo setting, however, these measurements are likely not possible due to the complexity in measuring them and the more limited knowledge on genes for most species housed in zoos. However, the hormones which these genes produce can be much more easily measured with similar outcomes. Zoos often do measure hormone levels of animals, but measuring the circadian rhythmicity of these hormones can be more informative and would likely enable zoos to detect risks much easier if they see that the circadian rhythmicity of a hormone is abnormal or desynchronised from other rhythms. Hormones can provide insight into metabolic health, sleep health, and mating capability or readiness (Challet, 2015; Goldman, 1999; Ikegami & Yoshimura, 2012). Creating baselines of hormonal rhythms within a zoo setting would be immensely helpful to detect risks quickly by comparing the levels in a point in time to the expected levels for that life stage, time of year, or time of day.

Collecting and analysing samples of a variety of hormones throughout full diel and annual cycles can be beneficial in monitoring the physiological states of animals and supplementing behavioural observations for the analysis of welfare. One study that demonstrates the ability to monitor physiological markers in this way was conducted with bottlenose dolphins (Funasaka, 2011). Investigators were able to collect blood samples every 3 hrs throughout 24-hr periods during the Spring equinox, Winter solstice, and Summer solstice. They established clear diel cycles of cortisol, testosterone, and rectal temperatures and saw seasonal changes in cortisol levels in relation to water temperature. Cortisol is a highly studied hormone due to its relation to stress, but can be misleading if not paired with other data (Broom, 2017). This study on bottlenose dolphins demonstrates how determining the rhythmicity of cortisol in relation to water temperature can provide needed context in understanding the potential source of the possible stress. If implemented across species, these kinds of investigations can provide a holistic view of physiological states of animals in relation to their external environment to be used in the analysis of welfare and subsequent care of the animals. Should invasive sample collection be difficult, technology such as collars that measure heart rate and body temperature or thermal cameras can be used to monitor more basic physiological indicators that may still provide sufficient information on the physiological state of an animal when paired with behavioural data (Stewart et al., 2005; Stewart et al., 2017).

Other possible zeitgebers or external cues that would be important to include in investigations would be food availability, zoo events, and lunar cycles. Lunar cycles are particularly important for marine animals, but also influence land animals (Chakraborty, 2020). Lunar cycles regulate reproductive cycles in many marine species (Chakraborty, 2020; Takemura et al., 2004), and therefore, since an aspect of welfare is for animals to have the ability to display natural breeding behaviours, zoos should understand the lunar cycle effects to their marine species to provide conditions, such as artificial moonlight (following the lunar cycle) for indoor facilities, that allow these natural behaviours. A study on nocturnal and diurnal spiny mice (*Acomys cahirinus* and *Acomys russatus*) demonstrates how studying behaviour, hormones and a zeitgeber together can be quite informative in determining effects to welfare because it ties together the environment, physiological indicators and behavioural indicators (addressing multiple domains from the Five Domains simultaneously) (Gutman et al., 2011). Nocturnal mice risk predation from owls when foraging, and more significantly on full moon nights due to greater visibility. Gutman et al. (2011) found that the nocturnal and diurnal spiny mice (which evolved to be diurnal circa 0.3-0.5 million years ago) both exhibit increased cortisol metabolite levels and reduced foraging on full moon nights when compared to new moon nights. The results suggest that the full moon may act as an engrained cue, since the diurnal mouse also exhibited similar effects despite not being at risk of owl predation due to their diurnal activity, and that the threat of predation changed behaviour and hormone levels in response to this environmental cue. This study highlights the importance of investigating what behaviours and rhythms are evolutionarily engrained but not obvious. Conducting similar studies for the benefit of captive species would help in illuminating what behaviours may be less obvious adaptations that persist in captive environments.

In relation to the giant pandas, since we were unable to determine the zeitgebers that might regulate breeding (Chapter 4), follow-up studies can include investigating the rhythmicity of breeding hormones, in both males and females, in relation to different environmental cues to determine what may be regulating the subsequent behaviours. In addition, since the main motivation to migrate is the emergence of bamboo shoots, and possible links between bamboo shoots and physiological preparation for the breeding season have been proposed, an important investigation would be to determine if shoot availability acts as a zeitgeber for breeding hormones and/or behaviours. The results could guide zoos on how to design their enclosures and which cues to provide to promote healthy breeding behaviours at the appropriate times of year. In addition, with baselines of rhythmicity of sexual hormones for not just females, but males as well, zoos can investigate whether the interventions are achieving the desired responses from the pair by comparing hormone levels and behaviours to the baseline to determine if both individuals are ready and willing to mate. These kinds of approaches can be taken with all zoo animals where we determine the rhythmicity of physiological and behavioural indicators of welfare in response to predictable environmental cues so that a broader and more comprehensive picture is formed of what constitutes adaptive synchrony between these rhythms and the multiple factors that influence welfare. In this way, zoos can better determine the most conducive environments for their animals to display positive affective states.

This approach can be combined with our proposed approach to welfare assessments to gain data on species populations around the world in zoos. By extending this kind of research to all species and encouraging collaborations between zoos to conduct similar studies investigating circadian rhythms, we can begin to understand what baselines of behaviours look like in captive environments by having broad samples that cover all life stages with a variety of external factors both latitudinal and zoo specific. A concerted effort with zoos around the world would help to determine more quickly what constitutes healthy behaviours in a captive environment.

5.4 Rethinking how and where we house captive species

In understanding what form healthy behaviours and rhythms present themselves in captive environments and the many factors which have a synergistic effect on welfare, as a zoo community, we must begin to reconsider how, and possibly where, certain species are housed. In the past few decades, zoos have made incredible improvements to the standards of care and the environments they provide to their species (Beer et al., 2023). However, the process of improvement is never-ending, and zoos have a responsibility to update their practices with updated research and shifting public perceptions. Given the strong public opinions on zoos currently, zoos and zoo associations have a great opportunity to shift the public's perception in a drastic way by responding with evidence-based changes to their practices, even if gradually. The goal of this thesis is to provide a starting point for ways to gather evidence and then implement change that account for the evolutionary needs of species, taking a 24/7 across the lifespan approach (Brando & Buchanan-Smith, 2018). The hope is that the studies provide insight on how to promote adaptive, synchronised rhythms and positive affective states.

The overall suggestion from this thesis is that zoos make the best efforts to create environments that have evidence showing they are conducive to positive welfare and enable synchrony of circadian rhythms. The first suggestion to achieve these changes is that zoos begin to build indoor enclosures that better mimic natural habitats. Zoo association welfare policies tend to require that environmental conditions (e.g. temperature, humidity, light, etc.) are comfortable throughout seasons or that there are artificial conditions that suit (Caring for Wildlife: WAZA welfare strategy, 2023; EAZA Standards for the Accommodation and Care of Animals in Zoos and Aquaria, 2020; BIAZA Welfare Policy, 2023; AZA Accreditation Standards & Related Policies, 2024). However, they do not require that the seasonality of these factors are mimicked. Best practice guidelines for accredited institutions will sometimes suggest that seasonality of either lighting, temperature, humidity, and/or food types are mimicked with the wild environment (AZA Animal Care Manuals: Penguin; EAZA Best Practice Guidelines: Midwife toads, Orangutan, Manatee), however, this is not true for all species that have guidelines. More importantly, best practice guidelines are only available for a small portion of captive species. In addition, indoor enclosures are often built to be easily manageable and conveniently maintained to provide a clean shelter; often resulting in enclosures which are far from natural, incorporating a lot of concrete and fewer elements than you would find in outdoor enclosures. My suggestion is that zoos, as standard practice, try to create indoor enclosures with environmental conditions that mimic the rhythmicity you would find in the species' natural habitat. This would include, but would not be limited to, mimicking light cycles with dimming dawn and dusk that changes throughout the year in accordance with the natural rhythms, natural cycles of temperature on a 24-hr and annual scale, lunar cycles, and humidity cycles. Providing naturalistic cycles, especially light (the strongest entraining cue for many species), could help entrain animals to an environment that more closely resembles the rhythms their internal clocks are adapted to synchronise to.

Although mimicking rhythms of natural environmental cues could very likely help, zoos would still need to account for zoo-specific zeitgebers. We encourage zoos to investigate which zoo-specific cues, like visitor presence and husbandry practices, highly influence the rhythmicity of their animals to then try to mediate the response to these cues, or at least illicit behavioural responses at times that would be natural. For instance, if a zoo has public feeding of giraffes, the zoo would want to limit these feedings to when giraffes would show feeding behaviours. Another example is if it is discovered that husbandry practices are a zeitgeber due to arousal, then keepers may want to find schedules that would mimic when the animal naturally becomes more active or conduct husbandry practices at times that a natural cue would cause the same level of arousal.

The final main issue that we suggest zoos investigate more thoroughly is indoor and outdoor enclosure use and the factors that may affect this, in particular, environmental factors that may affect outdoor enclosure use. Best practice guidelines include space requirements for species (all AZA Animal Care Guidelines and EAZA Best Practice Guidelines). However, just requiring a certain amount of space is not enough, there needs to be evidence that all the space is actually useable. This question has become a topic of research. However, in a review article, Brereton (2020) notes that much of the research gathering evidence on enclosure use is biased toward mammals, in particular carnivores and apes. This would suggest that enclosure use for birds, amphibians, and reptiles are not equally prioritised. From our welfare assessment data examples (Chapter 2), we see that space use for the anemone fish should be considered in relation to overcrowding and for the milk and Taiwan beauty snakes, evidence that an enclosure restrictive posture is sometimes displayed. These results highlight the importance of taking an evidence-based approach to enclosure use for all species.

For species with indoor and outdoor enclosures, we are also strongly encouraging zoos to investigate whether outdoor environmental conditions are within an adaptive range that is suitable for the species. The combined results from the giant panda research and the welfare assessments demonstrate that outdoor enclosures are often not used in the ways we would expect, likely linked to environmental conditions. The giant pandas at mismatched latitudes (see Chapter 4, Figures 4.5 and 4.6) used outdoor enclosures significantly less; and across all the pandas, outdoor enclosures were more likely to be used when the temperature range for the day was larger and less likely to be used when the amount of daylight was longer (Unpublished data, Table C1, Figures C4 and C5). These results could suggest that pandas which are housed at latitudes which experience more extreme conditions, are less likely to use outdoor enclosures because the environmental conditions, which cannot be controlled, are not suitable for what they are adapted to. From the welfare assessments, we also saw chimpanzees not using their entire enclosures and the chimpanzees and brown capuchins preferring indoor enclosures during rain and windy conditions. The chimpanzee data was collected in the Summer, but future research would want to compare the enclosure use data between different seasons. Since chimpanzees are from savanna woodlands, grassland-forest mosaics, and tropical moist forests, Winter conditions in Scotland may not be in their adaptive range or at the very least, may not be comfortable, forcing them indoors.

Zoos should place increasing importance on developing methods to gather data to enable evidence-based approaches to designing indoor enclosures to mimic wild, adaptive conditions that regulate circadian rhythms appropriately. These kinds of changes can include matching the rhythms of temperature, lighting, and perhaps even weather (e.g. seasonal fake snow), to help regulate their circadian clocks to a natural rhythm by providing them with a predictable environment that their body has evolved to recognise. By investing in state-of-the-art indoor enclosures, this would also engage visitors more and can educate them on why the zoo goes to great lengths to mimic many conditions of their natural environment in so much detail. This level of care could also help public perception of welfare.

However, for species with both indoor and outdoor enclosures, should they not use their outdoor enclosure because of environmental conditions, making enclosure size requirements irrelevant, as a worldwide zoo community perhaps we should begin to rethink which species should be housed in certain parts of the world. One solution may be for species which cannot be solely housed indoors (most zoo-housed species), to only be housed in zoos at latitudes that match their natural habitats. Though many other climatic factors may change by location within a latitude range, by at least keeping animals within their natural latitudinal range, we can be sure that the conditions and rhythms are much more likely to be in an adaptive range. Though a more drastic suggestion, this solution would still allow for species to be housed around the world. In addition, it can be very engaging for visitors to learn about local species or species that occupy their own latitude range. Learning about why this practice would be implemented can also help humans connect more with the animals and nature since visitors can be educated on circadian rhythms by also being taught about their own circadian rhythms.

5.5 Conclusion

Modern zoos face a lot of pressure with the major shift in public opinion on welfare and the need to conserve species being paramount in the midst of climate change. How zoos respond to this pressure can either bring zoos into a new era of how we keep captive animals and conserve species or potentially bring an end to captive animal institutions. To keep up with modern research and public opinion, zoos can begin to take more holistic approaches to welfare and conservation. These changes should be the inclusion of more evidence-based approaches that address *in situ* and *ex situ* practices simultaneously. For instance, more research investigating and comparing circadian rhythms in the wild and captivity can help improve *ex situ* welfare by providing evidence on environments that are conducive to positive welfare, but also inform *in situ* conservation practices by helping us understand the ecology of species and their evolutionary balance with their environment. With welfare in particular, a broader perspective must be taken on how we address it by seeing animals as intrinsically connected to their environment and seeing welfare as a continuous attribute of an animal that changes throughout the lifespan. In this way, we are better able to predict the changing needs of an individual, accounting for their environment as an extension of the animal, to provide the highest standard of care.

Appendix A

Table A1. The full 50 question welfare assessment developed by JE that is based on the BIAZA Welfare Toolbox.

Question number	Domain	Question
1	Nutrition	Clean fresh water is available on demand in a species appropriate manner to all animals
2	Nutrition	A diet sheet is available for the species and is reviewed incorporating best practice guidelines for nutrition and evidence-based literature as available
3	Nutrition	An appropriate quantity of high quality, nutritious food is available to all species/individuals and life stages (include browse and/or graze where appropriate)
4	Nutrition	Food is presented appropriately for the species/individual (consider timing, method, position in enclosure, social interactions, variation)
5	Health	Body condition score is within normal limits
6	Health	Regular weights are taken and recorded
7	Health	Animals appear to be in good physical condition (consider plumage/pelage/scales/scutes, skin, teeth, feet/hooves/claws, horns/antlers, eyes, nostrils etc.)
8	Health	Faeces are appropriately formed and normal for the species
9	Health	Animals have good muscle tone and fitness level with no evidence of weakness/debility
10	Health	Species/individuals are subject to preventative medicine regime (consider import testing, quarantine, vaccinations, parasitology, bacteriology etc)
11	Health	Animals are free from signs of injury or pain (consider lameness, flinching, wincing, husbandry mutilations, current treatment etc)
12	Health	Animals are free from chronic or recurring acute disease/injury/impairment (and note remedial actions in place if not)
13	Health	Veterinary care can be provided in a way which minimises distress (consider operant conditioning, nets, darting, crush cages, separation areas)
14	Environment	Temperature levels/gradients are within parameters appropriate for the species year round (consider internal/external, seasonal, night-time, appropriate variation, choice, records, etc.)
15	Environment	Humidity levels/gradients are within parameters appropriate for the species year round (consider internal/external, seasonal, night-time, appropriate variation, choice, records etc.)
16	Environment	Light levels, quality and photo period are within parameters appropriate for the species year-round (consider UV, photoperiod, flicker/glare, colour, internal/external, seasonal, night-time, appropriate variation, choice, records etc.)
17	Environment	Noise and vibration levels are within parameters appropriate for the species year round (consider internal/external, seasonal, night-time, appropriate variation, choice, etc.)
18	Environment	Air quality is high and free from pollutants, heavy dust, aversive odours and is well ventilated

19	Environment	Perimeter is secure (prevent escapes, excludes predators) and safe with no risk of harm (e.g., electric fences)
20	Environment	The size, shape and topography of the enclosure is appropriate for the species to exercise, explore and exhibit normal territorial behaviours
21	Environment	Substrates are suitable for the species (consider locomotion (abrasion, traction, support); resting (comfort, depth, cleanliness); foraging (depth, cleanliness); burrowing (will support tunnels, depth, secure))
22	Environment	Surfaces drain well with no pooling of faecal matter, urine or rainwater (unless this is appropriate to the species)
23	Environment	Has appropriate shelters, retreats, visual barriers, off show areas from conspecifics and visitors
24	Environment	Has appropriate shade and shelter from weather/climate
25	Environment	Planting is appropriate for the species, providing shelter, shade, retreats, microclimate provision, feeding opportunities, and plants are not toxic and do not present an escape risk
26	Environment	Furnishings allow appropriate species-specific behavioural needs (climbing, swinging, jumping, perching, nesting, stretching, hiding, sleeping, flight, etc.)
27	Environment	There is evidence of changes/variability to the environment and furnishing (enrichment)
28	Husbandry	The animal has free choice over where it spends its time within the enclosure
29	Husbandry	The enclosure is clean and hygienic to the appropriate level for the species
30	Husbandry	The enclosure is not over-stocked
31	Husbandry	Social grouping is appropriate
32	Husbandry	There is evidence of a pest control programme within the enclosure and no evidence (live animals, faeces etc) of pest species within the enclosure
33	Husbandry	Water feature is safe and of a depth/size/volume/gradient that allow species-specific natural behaviours
34	Husbandry	Life support systems are fully operational and regularly maintained
35	Husbandry	Water flow and movement allows sufficient oxygenation
36	Husbandry	Water testing is carried out on a routine basis and checked against acceptable ranges
37	Behaviour	Animals can be observed approaching and moving away from conspecifics freely
38	Behaviour	Performs appropriate levels of self-care behaviours (grooming, preening, drinking, resting, comfort activities)
39	Behaviour	Has mostly positive interactions with conspecifics or other animals
40	Behaviour	Has mostly positive or neutral interactions with staff/visitors
41	Behaviour	Responds appropriately to novel changes in the environment (interest in appropriate enrichment vs fear/aversion/apathy)
42	Behaviour	Can express choice and control over being in different (indoor/outdoor) areas (except for maintenance periods)
43	Behaviour	Exhibit appropriate territorial behaviour (patrolling, scent marking)

44	Behaviour	Exhibits appropriate foraging and feeding behaviours
45	Behaviour	Exhibits play behaviour (alone or socially)
46	Behaviour	Exhibit appropriate levels of rest and sleep
47	Behaviour	No evidence of dysfunctional social interactions
48	Behaviour	No evidence of abnormal or stereotypic behaviour
49	Behaviour	Exhibits reproductive behaviours as appropriate to the species and individual (courtship, mating, nest-building, incubating, birth, rearing etc.)
50	Behaviour	Exhibits species-specific behavioural needs (rooting, burrowing, climbing, perching, social grooming etc.)

Appendix B

Table B1. Panda ethogram used to code behaviour, and references from the literature from which the definitions were adapted. Our interpretation of welfare valence is when the giant panda performed the behaviour at its species' typical levels for captive individuals. Reproduced from Gandia et al. (2023a).

Behaviour	Welfare valence	Description	Reference
		Solitary	
Resting/ Sleeping	Neutral	Inactivity (lying, sitting or standing on all four), either awake or asleep. If standing, the panda should not be investigating or seem as though attention is on anything specific. Shifting between resting positions still counts as resting as long as it does not involve locomotion or becoming alert.	(Ryan & Litchfield, 2020)
Locomotion	Neutral	Directional travel at any speed. Only includes short bouts of travel with a seemingly intended goal or long bouts of travel with no evidence of a stereotypic or repetitive pattern. Includes climbing.	
Feeding/ Foraging	Positive	The manipulation or eating of food/water provided by keepers or foraging for plants growing in enclosure. Includes food search activity, moving around enclosure, sniffing ground or air.	
Play	Positive	Playful running, gymnastics, interacting with objects (paw or mouth manipulation of objects – includes enrichment items). Includes playing in water (for example splashing).	
Investigate	Positive	Panda places nose within 5 cm of inedible object and sniffs or licks the object.	(Swaisgood et al., 2001)
Anogenital rubbing (sexual-related)	Positive	Applying pressure to the hind area below the tail by moving the hind quarters in a back-and-forth motion around or up and down on the surface of an object or on the wall. Can be performed in four positions: squat, reverse (backing up to a vertical surface), leg cock (against a vertical surface with one leg raised), and a full handstand (both hindfeet elevated off the ground with body fully extended).	(Liu et al., 2005; White et al., 2002)
Scent anointing (sexual-related)	Positive	Rubbing face, head, neck, and/or shoulders on object or wall in fluid movements or by rolling on it, making contact with head, neck, shoulders, and upper back. Can use paws to try and spread odour.	(Bian et al., 2013; Charlton et al., 2020)
Urinating/ Defecating	Neutral	Assuming a squat, leg-cock, handstand, or standing posture on the wall or ground to excrete urine or faeces.	(Liu et al., 2005)
Grooming	Positive	Scratching and licking of the pelage.	
Drinking	Neutral	Placing mouth at surface of pools or spouts and ingesting water.	
Keeper interaction	Depends	Training or any interaction where keeper is intentionally trying to gain and maintain the attention of the panda. Can occur at any location in enclosure where keeper is visible to the panda.	
		Social	
Aggressive	Negative	Animal forcefully swats with forepaws, lunges towards other individual, grapples, or bites with force.	(Owen et al., 2013)
Show interest (sexual-related)	Positive	Animal responds to the other party by sniffing the other participant, pushing or pulling at the fence between, or swaying or locomoting back and forth with proximity of the other animal which must be clearly in view, even if through a mesh barrier.	

Sexual (sexual-related)	Positive	Female presents anogenital region to male. Male sniffs or licks anogenital region and/or nudges or paws at anogenital region through wire mesh. Note that the female must first display “sexual” behaviour before the male responds with “sexual” behaviour.	
Social play	Positive	Nonaggressive chasing, wrestling, inhibited biting or pawing at other individual. There should not be attempts to escape and individuals can alternate between subordinate and dominant positions.	
Stereotypic/Abnormal			
Pace	Negative	Stereotypic pacing (back and forth, or perimeter locomotion, in a repetitive sustained pattern, tracing the same route at least 3 times consecutively) or quasi-stereotypic pacing (same as stereotypic pacing, except animal need not take the same path 3 or more times in a row. Any pacing in which a predictable pattern emerges. There may be variations in the routine or the animal may alternate between a limited number of travel paths).	Panda project PDX Wildlife*
Bipedal standing	Negative	Standing on hind legs and looking through glass, fence, or outside enclosure seemingly in anticipation of something.	(Liu et al., 2003)
Self-mutilation	Negative	Self-inflicted physical harm, such as biting or chewing the tail or leg, or hitting the head against a wall.	
Cage climb	Negative	Stands bipedally and sways or makes climbing motions, as if attempting to escape.	(Swaisgood et al., 2005)
Regurgitation	Negative	Vomits and reingests vomit repeatedly.	
Pirouette	Negative	Stands on hind legs and spins body at least 90 degrees.	
Head-toss	Negative	Swings upward or to the side in a swinging movement.	

* https://www.pdxwildlife.com/become-a-citisen-scientist/ (accessed 1 May 2020).

Appendix C

Table C1. Significant results from a zero inflation negative binomial model (see methodological detail in Chapter 4) for outdoor activity with the predictors being latitude location (match/mismatch), activity, daylight, temperature minimum, and temperature range. The model includes all pandas from zoos A-D. The male from Zoo E is excluded due to his camera being fixed on the indoor enclosure for most of the year. In the conditional model (+) coefficients indicate the category level/variable is larger than the grand mean, and a (-) coefficient means it is smaller than the grand mean. For zero-inflation coefficients, (+) indicates the category level/variable is more likely to be a zero and (–) means it is less likely to be zero (i.e. more likely to be a positive integer).

Conditional				**Zero-inflation**			
Variable	Coefficient	Z-value	Pr (>|z|)	Variable	Coefficient	Z-value	Pr (>|z|)
Mismatch	-0.385	-3.143	0.002	Activity	-0.090	-8.697	<0.001
				Daylight	0.094	2.206	0.027
				Temperature range	-0.083	-3.573	<0.001

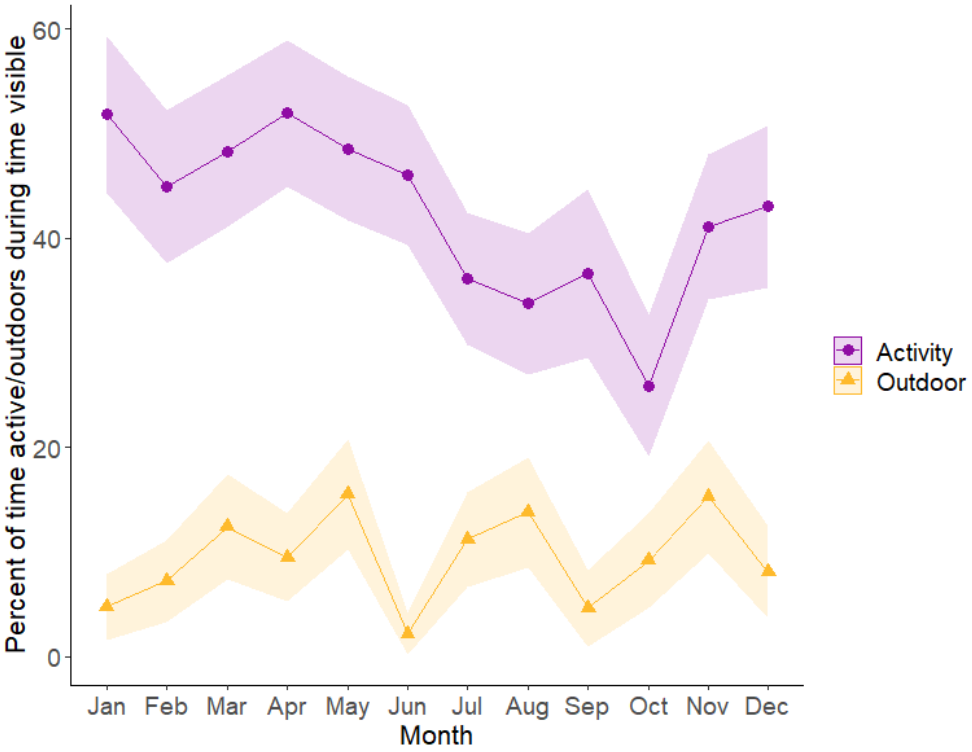


Figure C1. Percentage of time active (purple) and outdoors (yellow) throughout the circadian cycle for all pandas in zoos A-D (n=10). The male from Zoo E is excluded due to his camera being fixed on the indoor enclosure for most of the year. Measurements are displayed as percentage of time while in sight, controlling for time out of sight, and averaged by month.

Shaded regions are 95% confidence intervals. The percentage outdoors is opposite to the percentage of time indoors. Therefore, a measurement of 0% time outdoors represents 100% time indoors and vice versa. These results must be paired with those of Figure 4.5 to see that the association between activity and percentage of time outdoors (result in Table C1) is likely due to the similar circadian rhythmicity between these two signals and not the circannual rhythms, as we see in this figure.

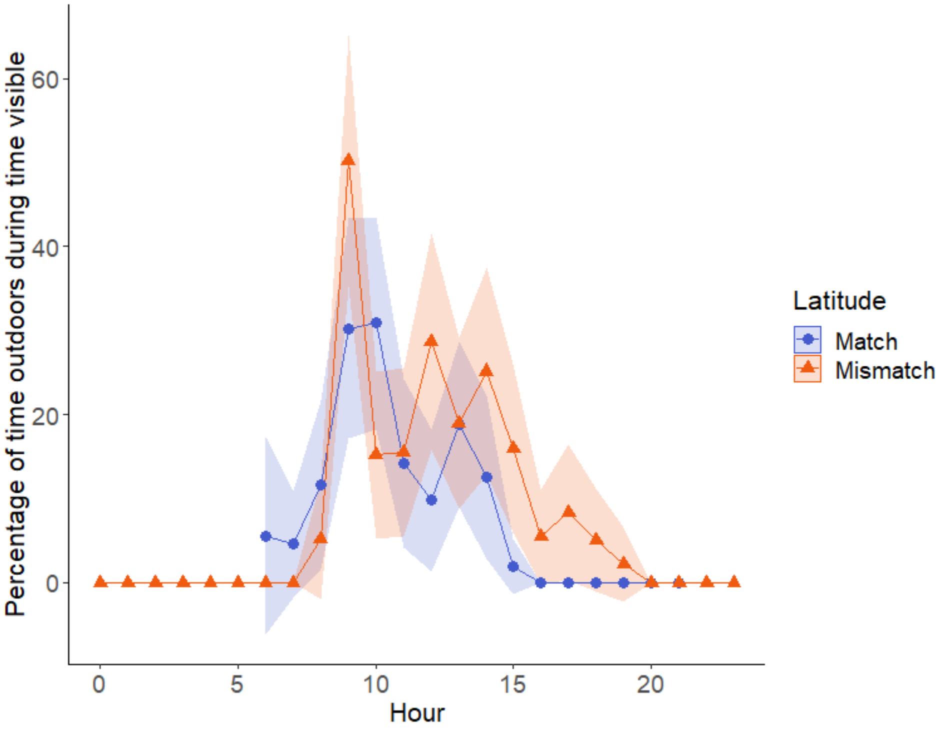


Figure C2. Circadian rhythms of percentage of time spent outdoors between pandas at matched latitudes (n=6) and pandas at mismatched latitudes (n=4). The male from Zoo E is excluded due to his camera being fixed on the indoor enclosure for most of the year. Measurements are displayed as percentage of time while in sight, controlling for time out of sight, and averaged by hour. Shaded regions are 95% confidence intervals. The percentage outdoors is opposite to the percentage of time indoors. Therefore, a measurement of 0% time outdoors represents 100% time indoors and vice versa. In this figure we cannot see the difference in activity between the pandas at matched and mismatched zoos which the model found (Table C1).

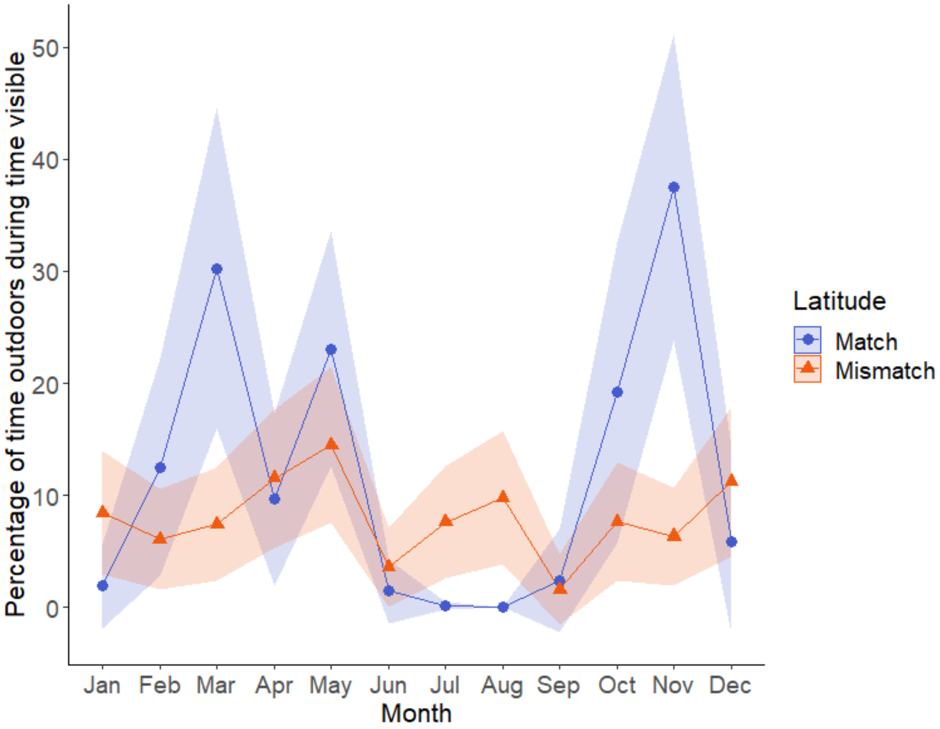


Figure C3. Circannual rhythms of percentage of time spent outdoors between pandas at matched latitudes (n=6) and pandas at mismatched latitudes (n=4). The male from Zoo E is excluded due to his camera being fixed on the indoor enclosure for most of the year. Measurements are displayed as percentage of time while in sight, controlling for time out of sight, and averaged by month. Shaded regions are 95% confidence intervals. The percentage outdoors is opposite to the percentage of time indoors. Therefore, a measurement of 0% time outdoors represents 100% time indoors and vice versa. In this figure we can see the difference in activity between the pandas at matched and mismatched zoos which the model found (Table C1), which shows pandas at mismatched latitudes spending significantly less time outdoors than pandas at matched zoos. This figure suggests the difference is on an annual scale and not a daily scale (Figure C2).

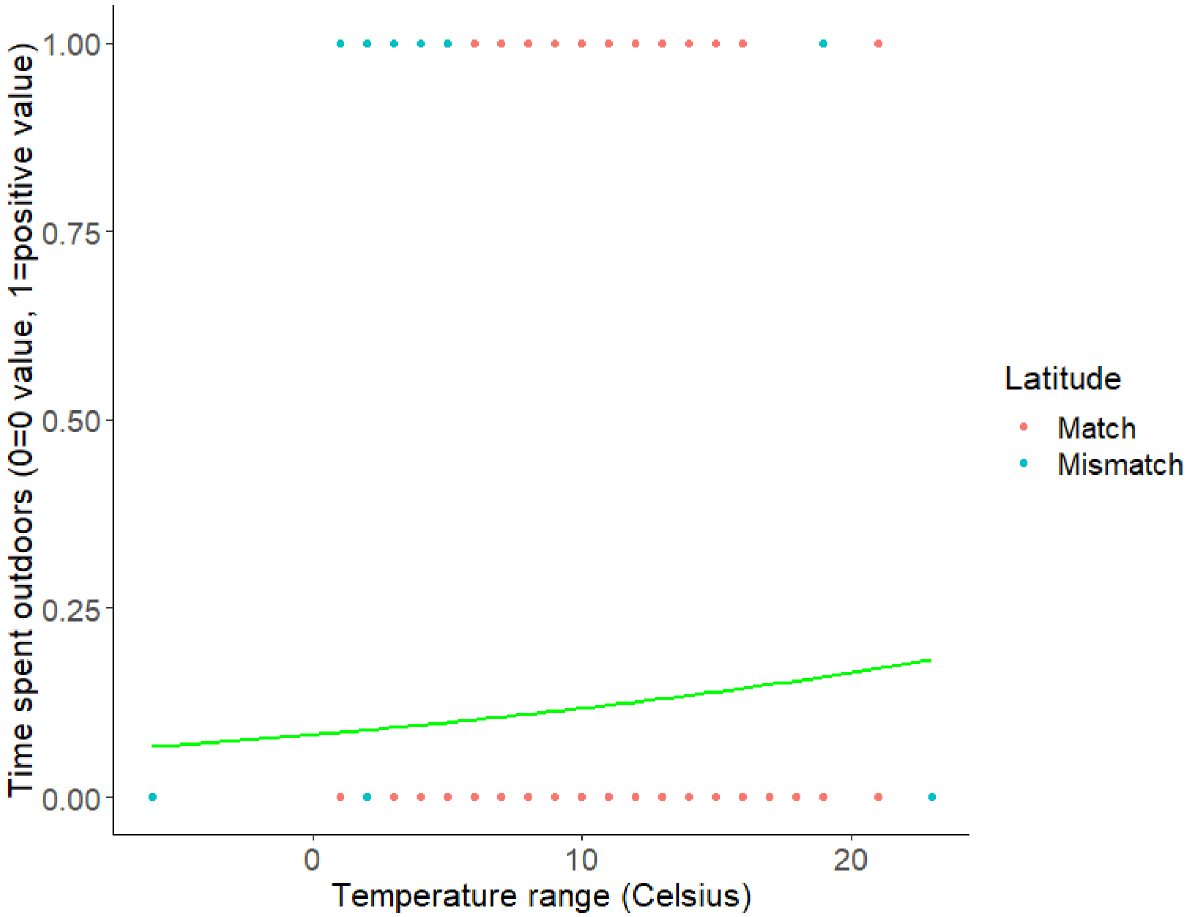


Figure C4. Logistic regression (green line) between temperature range and time spent outdoors since temperature range was significant in the zero-inflation portion of the ZINB model (Table C1) for time spent outdoors. Since the zero-inflation portion of the model transforms data into 0 (0 values) and 1 (any positive value), the data is displayed as 0's and 1's in the same manner. Data is displayed for all pandas in zoos A-D (n=10), excluding the male from Zoo E due to his camera being fixed on the indoor enclosure for most of the year. Data points are distinguished between pandas at matched latitudes (red) and pandas at mismatched latitudes (blue). Data points heavily overlap due to the integer quality of the temperature range variable. The results indicate that giant pandas are more likely to spend time outdoors when the temperature range is higher.

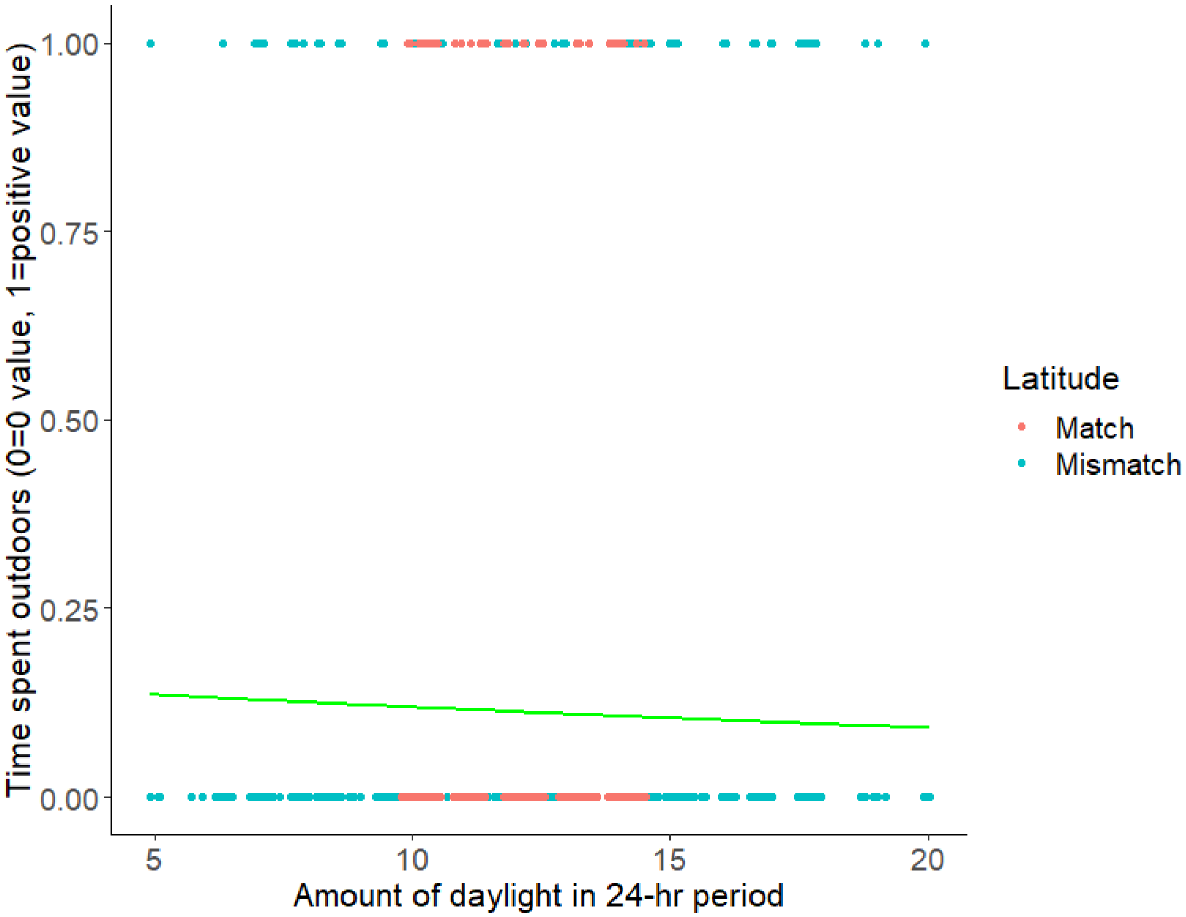


Figure C5. Logistic regression (green line) between amount of daylight and time spent outdoors since daylight was significant in the zero-inflation portion of the ZINB model (Table C1) for time spent outdoors. Since the zero-inflation portion of the model transforms data into 0 (0 values) and 1 (any positive value), the data is displayed as 0's and 1's in the same manner. Data is displayed for all pandas in zoos A-D (n=10), excluding the male from Zoo E due to his camera being fixed on the indoor enclosure for most of the year. Data points are distinguished between pandas at matched latitudes (red) and pandas at mismatched latitudes (blue). The results indicate that giant pandas are less likely to spend time outdoors when the amount of daylight in a 24-hr period is longer.

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