

# **Chronobiology of voluntary food intake in reindeer (*Rangifer tarandus tarandus*)**

**Leonardo Rescia**

*BIO-3950 Master's thesis in Biology  
Arctic Chronobiology and Physiology  
November 2018*





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**Supervisor**

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Cover photo by Leonardo Rescia

Photo of a female Norwegian reindeer (*Rangifer t. tarandus*)

## Acknowledgements

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A handwritten signature in black ink, consisting of a large, stylized 'R' or 'P' shape with a horizontal line crossing through it, and a small mark to the right.



## Abstract

In all organisms, biological rhythms arise from the interaction between endogenous clocks and a rhythmic environment. The timing of sunrise and sunset synchronize internal timing processes and inform the organism not only of the time of day, but also of the time of year. For animals living in polar regions biological timekeeping represents a challenge, given that the sun does not rise during the winter, nor set during the summer months. Due to the difficulty of assessing real time food intake in field studies, activity has so far been used as a proxy for feeding. Long term timing mechanisms such as seasonal rut, reproduction, appetite, antler growth and moult rely on circadian timing in order to sense changes in daylength indicating a change in season. In order to investigate this dilemma we analysed both, activity and food intake as parameters of rhythmic output. The aims of this study were to

- 1. Analyse the effect of photoperiod on the distribution of feeding and activity across season.**
- 2. Analyse ultradian and/or circadian patterns of activity.**
- 3. Use of simultaneous food intake and activity measurements to assess the validity of previous assumptions about activity as a proxy for food intake.**
- 4. Assess age differences in activity and feeding between reindeer calves and yearlings**

Locomotor activity and food intake in semi-domesticated Norwegian reindeer (*Rangifer tarandus tarandus*,  $n=8$ ) were repeatedly measured in Tromsø (69° 39' N, 18° 57' O, Norway) between November 2017 and August 2018. Half the animals were around a year of age, and half of them were only 6 months old at the start of the project.

For the first time food intake and activity were measured simultaneously, demonstrating that activity is not a good proxy for food intake. All animals displayed higher appetite and weight increase in the months prior to winter, preparing for the extreme cold and lack of food ahead. Overall activity and food intake were suppressed by darkness, a likely adaptation against nocturnal predators. Ultradian rhythms of activity prevailed in all animals both during day and night, throughout the year. Overall activity was lower in winter than summer, presumably as a strategy to conserve energy when resources are limited. Despite the dominance of the ultradian activity pattern

during the times of the midnight sun or constant darkness, periodograms revealed an underlying 24-hour rhythm indicating entrainment. At those times of the year with a rhythmic light dark signal, activity and feeding concentrated in the light part of the day with the biggest food intake occurring around civil twilight. We could demonstrate that timing of feeding is co-regulated by photoperiod and endogenous ultradian feed requirements. Contrary to Svalbard reindeer, mainland Norwegian reindeer synchronize feeding and activity bouts within the herd.

## **Keywords**

Chronobiology, reindeer, *Rangifer*, voluntary food intake, activity, photoperiod, circadian clock, ultradian rhythms, circadian rhythms



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# Introduction

## Biological rhythms

The daily rotation of the Earth around its own axis at a constant rate of one full revolution every 24 hours gives rise to highly predictable cycles of day and night. In combination with the Earth's axis tilt of  $23.5^\circ$ , this movement gives rise to the seasons. This exposes almost all life from unicellular organisms to mammals to daily and seasonal changes in abiotic variables such as light intensity and temperature, but also, consequently, biotic variables including food availability and predation risks. It is therefore beneficial for all organisms to keep in synchrony with these daily fluctuations in order to anticipate upcoming rhythmic changes in the environment. The tracking of time in biological systems is possible through endogenous clocks.

Biological rhythms in organisms occur with varying timescales. In this project we are mostly interested in daily and seasonal fluctuations of a variety of parameters.

## Circadian rhythms

Circadian rhythms are endogenously generated oscillations which persist in a constant environment with a period length ( $\tau$ ) of roughly 24 hours (*circadian*: Latin; 'circa' about + 'dies' a day). These oscillations are controlled by an inner circadian clock, which exerts changes in physiology or behaviour. Its mechanism of action is through a negative transcriptional-translational feedback loop in gene expression of clock genes, which are present in most cells of the body (Plautz *et al.*, 1997; Balsalobre *et al.*, 1998). This molecular feedback loop is synchronised or "entrained" daily by external cues called *Zeitgebers*, which in German means 'time giver'. Factors such as ambient temperature or food availability can be effective *Zeitgebers*, but the most reliable cue is light. As a result, circadian clocks produce daily cyclic changes in physiology and behaviour.

In order to be classified as an authentic circadian clock, a measurable cyclic variable must meet three criteria: (1), it must persist without environmental cues, thus demonstrating the endogenous nature of the clock; (2) it must be entrainable to the external environment through a *Zeitgeber*; (3) it must be temperature compensated,

i.e. able to keep a constant period length regardless of environmental temperature (Barrett & Takahashi, 1995).

Circadian clocks have great adaptative value for organisms which survive in variable but predictable environments, for example with fluctuations in temperature, food availability or light intensity follow a regularly recurring pattern. Therefore, circadian clocks help organisms to anticipate daily events which can be favourable to their survival and Darwinian fitness. For example a study by Ouyang *et al.* (1998) looked at survival rates of three strains of cyanobacteria *Synechococcus sp.* that only differed in their free-running period lengths, but had similar growth rates. Competition experiments between these strains demonstrated that resonance between free-running period and the length of the LD cycle was advantageous.

Another study that denotes the importance of the circadian system was performed by Spoelstra *et al.* (2016), who compared survival rates of wild mice (*Mus musculus domesticus*) against mice bearing a period-length mutation ( $\tau$  mutation in the protein casein kinase 1 $\epsilon$  producing gene) which reduced their free-running period in a dose dependent manner, leading to  $\tau < 24\text{h}$ . Mice which harboured the tau allele were more active during the day due to the mismatch between their endogenous clock ( $< 24\text{h}$ ) and their environment ( $T = 24\text{h}$ ). These mice had lower rates of survival that the authors ascribed directly to their mistimed activity rhythms.

### *The circadian system*

In mammals, the molecular machinery responsible for circadian oscillations is contained within most body cells (Brown & Azzi, 2013). In 1972, lesioning studies in the hypothalamic area of the brain of rats resulted in asynchrony of locomotor and drinking behaviour (Stephan and Zucker, 1972), identifying the role of a nucleus in the mammalian brain, namely the suprachiasmatic nucleus (SCN), the master clock required for the orchestration of the clocks in the rest of the body (peripheral clocks).

### *The master clock*

In mammals the suprachiasmatic nucleus (SCN) is found at the base of the hypothalamus (Gillette, 1991), and is composed of approximately 20,000 neurons placed in two clusters bilaterally above the optic chiasma (Reppert & Weaver, 2002).



Each neuron is capable of producing self-sustained synchronised oscillations close to 24h that persist indefinitely, unlike those in peripheral clocks (Reppert & Weaver, 2002). The SCN remains entrained or synchronised to the environment through photic information which is communicated through the retino-hypothalamic tract, a series of neurons directly connecting the eye retina with areas of the hypothalamus in the brain (Moore & Card, 1985). The SCN is responsible for the entrainment of oscillations in peripheral clocks.

### *Peripheral clocks*

The SCN can synchronise oscillations in the peripheral tissues through direct nerve signalling or indirect mechanisms such as chemical signals (Schibler *et al.*, 2016). However, other non-photic stimuli can also serve as Zeitgebers for oscillators in the periphery. A study by Damiola *et al.* (2000) demonstrated that mice fed during the day only (nocturnal animals) underwent a complete phase-shift in the cyclic secretion of hormones of the liver and kidney, although oscillations in SCN gene expression remained unaffected. This study highlights how a non-photic stimulus (food in this case) can serve as a Zeitgeber for peripheral clocks.

### Seasonal rhythms

The circadian clock is also an important component for seasonal timing. This was first studied by Erwin Bünning in plants, and is now referred to as the 'Bünning hypothesis' or external coincidence model (Pegoraro *et al.*, 2014). Increasing day length (photoperiod; *lat.* photo = light; period = duration) towards summer reaches the plants' photosensitive phase and stimulates physiological changes. This exposure to light during the photosensitive phase of the endogenous clock drives internal photochemical cascades leading to seasonal responses. Later on, Pittendrigh and Minis (1964) postulated that not one, but two coupled endogenous oscillators entrained by sunrise and sunset respectively, are critical for photoperiod dependent induction of seasonal responses. According to this internal coincidence model the phase-relationship between photoperiod and the two endogenous oscillations controls seasonal responses (Pittendrigh *et al.*, 1984).

Many studies have been carried out to determine whether photoperiodic responses are driven by external coincidence only or involve internal coincidence. Follet *et al.* (1974) measured levels of luteinizing hormone (LH) in blood plasma of white-crowned sparrows. LH is produced in the anterior pituitary gland and is involved in the seasonal stimulation of the reproductive organs, peaking in concentration when the animals are exposed to long-day photoperiods. Follet *et al.* took photosensitive sparrows and exposed them to 8 h of light followed by different periods of darkness in different trials before a second 8 h exposure to light. Levels of plasma LH oscillated with higher concentration peaks when the second light pulse coincided with a photosensitive period in the birds. This led to the understanding that LH secretion was regulated by an endogenous circadian clock which defines a period of photosensitivity, and that the timing between light pulses needs to coincide with the photoinductive phase of both endogenous oscillations in order to result in high levels of LH in blood which will ultimately drive photoperiodic responses.

It is then understood that circadian timing allows organisms to compare day length from one day to the next. The evidence obtained from the shortening or lengthening of the photoperiod over consecutive days (photoperiodic history) provides the organism with a sense of calendar information that can trigger endocrine and metabolic changes in physiology at specific times of the year (Hazlerigg & Wagner, 2006).

### *Melatonin*

In mammals, melatonin is secreted by the pineal gland through the following pathway: light information enters the body through photoreceptive cells in the retina; the signal is communicated to the suprachiasmatic nucleus (SCN) of the hypothalamus, the central oscillator that synchronises clocks in the rest of the body of mammals (West & Wood, 2018). Through an indirect neuronal pathway, the master clock then controls melatonin secretion from the pineal gland.

Melatonin is produced only in dim or the absence of light. Light exposure acutely suppresses the secretion of melatonin from the pineal gland and blood levels of the hormone fall fast due to its short half-life. The duration of melatonin present in blood thus accurately reflects photoperiod and time of year (Hazlerigg *et al.*, 2017).

### *Pituitary gland*

The highest concentration of melatonin receptors can be found in the most rostral portion of the pituitary known as the *pars tuberalis* (West & Wood, 2018). This tissue sits at the interface between the environmental photic information in the form of melatonin signal and those parts of the mediobasal hypothalamus controlling seasonal physiology (Reppert *et al.*, 1994). This signal further controls the release of thyroid stimulating hormone (TSH) which feeds back into the hypothalamus to drive seasonal output (West & Wood, 2018).

A study that explored the role of melatonin and the pituitary gland in seasonal processes was carried out by Lincoln and Richardson (1998) in sheep in which the hypothalamus was surgically isolated from the pituitary gland. The disconnection between the two led to abnormal cycles of seasonal processes such as body weight, food intake and reproduction.

Another study carried out by Woodfill *et al.* (1994) established that reproduction in sheep is controlled by an endogenous seasonal timer that is synchronised through the release of melatonin. The researchers administered melatonin to different groups of pinealectomized sheep mimicking the duration of melatonin in winter, spring, summer and autumn. They found that the only group that started reproductive neuroendocrine activation were the ones receiving melatonin for a duration that mimicked a summer photoperiod. This demonstrated clearly that photoperiod communicated to the organism through the melatonin signal influences seasonal life events.

Experiments in which part of the melatonin-producing pathway was disrupted have shown that animals become unresponsive to changing photoperiod and reproductive cycles were disrupted by e.g. amputation of the eyes in sheep (Legan & Karsch, 1983), lesions of the SCN (Scott *et al.*, 1995) or pinealectomy (Bittman *et al.*, 1983).

Bartness and Wade (1985) studied how photoperiod alters metabolism, leading to body weight changes in Syrian (*Mesocricetus auratus*) and Siberian (*Phodopus sungorus sungorus*) hamsters. They found that short days triggered fattening of Syrian hamsters, while making Siberian hamsters lose weight, and the opposite occurred under a long photoperiod. The amount and quality of food did not change between

trials, but the resting metabolic rate of the animals varied with photoperiod. This anticipatory endogenous adaptation to upcoming the seasonal changes in the environment is controlled by photoperiod.

## Arctic regions

The Arctic is composed of the region below the polar star *Polaris*. In ecological terms it is limited to the area enclosed by the 10°C isotherm, a circumpolar line connecting points with an average temperature < 10°C in July (Blix, 2005).

Above described studies emphasize the importance of photoperiod and a robust circadian clock as adaptive advantages in a rhythmic environment (Woodfill *et al.*, 1994; Ouyang *et al.*, 1998; Spoelstra *et al.*, 2016). However, polar regions present a photoperiodic dilemma to resident animals. The arctic is a highly seasonal environment with short periods of advantageous conditions for reproduction and growth. At the same time the Sun is above the horizon for up to 24 hours in summer and in winter does not rise at all, i.e. a reliable photic cue for the circadian system is lacking during large proportions of the year (See Fig. 1).

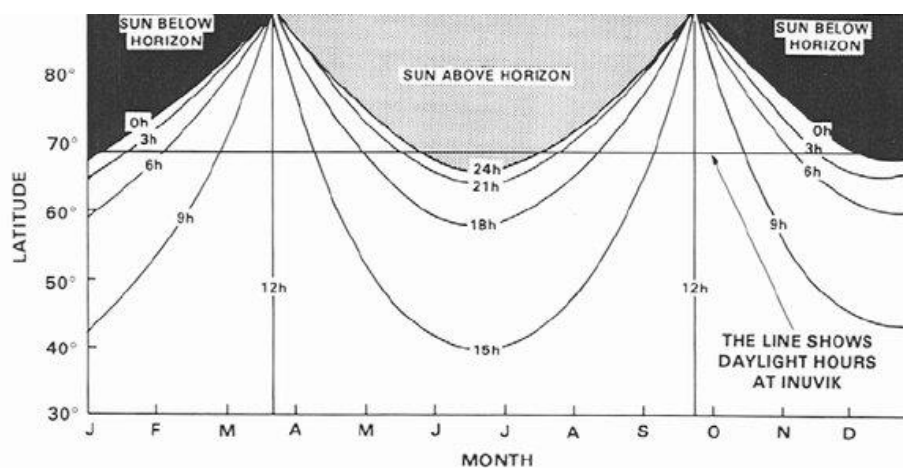


Figure 1. Duration of day and night at northern latitudes (90°-30°). (Burns, 1973)

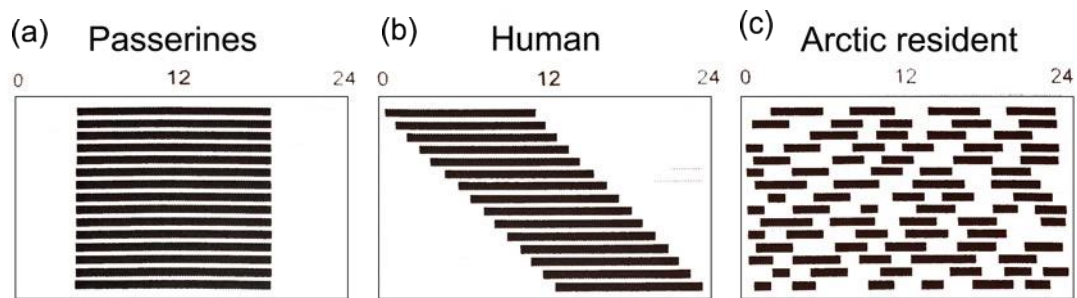
Studies of humans resident in polar regions revealed free-running rhythms with periods > 24 hours in winter (See Figure 2.) causing a desynchrony between their endogenous clocks and working schedules dictated by clock time. This led to decreased sleep efficiency, resulting in a series of sleep related disorders. This

suggests that compromised clock function brought about by polar conditions has a negative impact on human health (Arendt, 2012; Bhattacharyya *et al.*, 2008).

So how beneficial is it for organisms to maintain a circadian clock when they inhabit a constant environment? Several studies have demonstrated that some animals maintain rhythmicity in the periods of the year with constant light conditions (see *Figure 2.a*). An early study in nocturnal arctic rodents (ground squirrels, voles and lemmings) found that periodicity in the activity patterns was not lost over the summer period, remaining similar to that of the rest of the year, with the exception of lemmings, which maintained periodicity but had a phase shift from peak activity at night to the middle of the day (Swade & Pittendrigh, 1967). Similarly, three different species of bird (robins, thrush and redpoll) displayed rhythms in body temperature and sleep patterns synchronised to the lowest level of light intensity during the summer in Alaska (Karplus, 1952).

To the contrary, other animals seem to disengage their circadian clock during the Arctic summer and winter (See *Figure 2.c*) A study carried out by Stokkan *et al.* (1986) showed that during winter and summer Svalbard rock ptarmigan lose their circadian patterns of activity and food intake present for the rest of the year when a light-dark rhythm is available. In addition, Reierth *et al.* (1999) demonstrated a lack of rhythmic melatonin secretion during the arctic summer and winter. A recent study (Hawley *et al.*, 2017) revealed that Arctic charr in polar regions become arrhythmic during summer and winter, coinciding with periods of high and low availability of food. All these examples are understood to be evolutionary traits that allow these animals to take advantage of the arrhythmic environment they inhabit, for example to conserve energy in periods of starvation or to feed when the conditions to do so are optimal, and not to wait for a subjective day or night dictated by an internal clock.

These examples suggest that the adaptive value of a circadian clock in constant conditions does not offer any adaptive advantage and might be beneficial only during parts of the year. The different adaptations to constant conditions (Fig. 2) in polar regions may reflect differences in evolutionary histories and selection pressures.



**Figure 2. Overview of different strategies adopted by organisms to constant daylight represented as actograms.** Each actogram represents 15 consecutive days of activity data. Each row is a different day, and the x-axis is one day (24 hours). Black lines are active periods and white are periods of rest. (a) Passerine birds can detect small changes in light intensity and keep in synchrony with a 24-hour day in the arctic summer. (b) Humans display an endogenous free-running rhythm with a period length of ca. 25 hours leading to a phase delay in activity onset over time. (c) Svalbard ptarmigan uncouple circadian oscillations from behaviour and become arrhythmic. Image from van Oort (2008).

## The case of the reindeer

The reindeer (*Rangifer tarandus*) is a resident arctic herbivore, with a circumpolar distribution across latitudes 50 to 81° N through northern Scandinavia, North America, Greenland, and northern Asia (IUCN, 2018), covering up to 25% of the total landmass on Earth (van Oort, 2008). *Rangifer* is composed of 12 extant subspecies which vary in size and colouration. Reindeer are the only genus within cervids displaying female antler growth. Reindeer live roughly between 13 (males) and 17 (females) years, and become sexually mature between 1-3 years of age, depending on nutritional status (Bergerud, 2018).

The diet of reindeer varies through the year. Reindeer are ruminants described as intermediate feeders (Knott *et al.*, 2004) that feed on graminoids, forbs, shrubs and fungi during the summer. In winter reindeer use their hooves and antlers to uncover their feed from snow. They also feed on tree bark and lichens (Mathiesen *et al.*, 2000). Although the nutritional value of food material ingested in winter is lower than in summer (Bliss, 1962), previous studies on captive reindeer with unlimited access to pelleted food demonstrated that food intake is controlled by a seasonal variation of

appetite rather than food availability. Food intake varies with season with a peak in midsummer, and a nadir in winter (Mesteig *et al.*, 2000).

### *Activity*

Reindeer are amongst the arctic residents in which the circadian clock has been observed to disengage in constant lighting conditions. Tyler *et al.* (2016) analysed activity data from free-ranging reindeer in northern Norway and Svalbard and noticed that circadian rhythmicity during the equinoxes gives way to ultradian rhythms of activity as the rhythmic photic environment disappears during the arctic winter or summer.

### *Molecular machinery*

It has been suggested that reindeer have weak circadian clocks at a molecular level (Lu *et al.*, 2010). Cultured reindeer skin fibroblasts containing transgenic gene promoters with a Luciferase reporter displayed arrhythmic bioluminescence, indicating the lack of a circadian molecular clockwork. A more recent *in silico* sequence analysis of specific clock components in reindeer demonstrated that core components of the circadian molecular machinery are significantly mutated and the resulting protein products might be non-functional. This study requires experimental confirmation, but may present a compelling narrative for the underlying mechanistic change which facilitated an evolutionary adaptation to the extreme photoperiodic conditions these animals encounter (Slåttebrekk, 2018).

### *Melatonin*

Several melatonin studies have been carried out in reindeer. Stokkan *et al.* (1994) found that although the hourly average melatonin concentrations in blood do not differ across the day during arctic summer and winter months, the small variation in respectively light or twilight intensity at midnight are enough to induce a minor rise of melatonin in the blood.

Hazlerigg *et al.* (2017) found that reindeer transferred from a natural light/dark cycle to constant darkness or constant light respond promptly by adapting their food intake, antler growth and fur moult rates, swiftly speeding through these seasonal processes to adjust according to the new photoperiodic cues. Moreover, Lu *et al.* (2010) showed

that melatonin secretion in reindeer is passively driven by exposure to light or dark instead of through endogenous mechanisms. During daylight, reindeer were exposed to short periods of darkness, causing a swift rise in plasma melatonin, with a followed exposure to light which suppressed melatonin secretion. This suggests that melatonin secretion is not under circadian control. These facts challenge the idea that circadian rhythms (abolished during constant light or constant darkness) are important for the regulation of seasonal processes.

## Activity and food intake

*Rangifer* is an intriguing species to study given the apparent flexibility in circadian organisation. Under constant lighting conditions activity becomes arrhythmic (van Oort *et al.*, 2007), as do melatonin secretion (Stokkan *et al.*, 1994) and the molecular clockwork (Lu *et al.*, 2010). Studies of seasonal voluntary food intake showed the adaptability of reindeer to the environment (Nilssen *et al.* 1984; Tyler *et al.*, 1999; Mesteig *et al.*, 2000; Stimmelmeyr, 2001), however circadian food intake measurements have not been reported so far.

Tyler *et al.* (2016) analysed activity data from Norwegian reindeer and inferred, based on other studies (Boertje, 1985), that > 85 % of the daily activity in reindeer is related to grazing, leading to the subjective understanding that feeding is also arrhythmic when the environmental conditions are so. This is in agreement with the idea that a robust circadian clock during the arctic summer and winter is disadvantageous as circadian organisation would be in contradiction to the transient feeding opportunities (van Oort *et al.*, 2007).

## Rhythmic properties of reindeer

All previous studies in reindeer suggest an apparently weak or disengaged circadian clock during periods of constant dark or constant light. However, seasonally timed processes such as rut, calving, antler growth, fattening or fur change require accurate synchronization to the annual change in photoperiod. The two factors appear to be under the control of a very weak circadian but strong seasonal system. It is commonly believed that circadian clocks convey Darwinian fitness to most organisms. However,



circadian rhythmicity appears to be of little advantage in an environment that is not rhythmic for months a year. Therefore, the plasticity of the circadian organization in reindeer can be understood as an evolutionary adaptation that allows the animals to ignore their circadian organization during periods of constant light conditions. At the same time, reindeer are highly susceptible to a light-dark rhythm when it is necessary, e.g. for the regulation of seasonal processes.

## **Aims**

Evidence to date indicates that reindeer have a weak circadian system. Current theory suggests that this trait has developed due to the specific selection pressures of living in the - at least transiently - arrhythmic arctic environment. It has been suggested that activity during the arctic summer and winter is largely driven by ruminal function (Tyler *et al.*, 2016). All previous work has inferred feeding behaviour from activity data. This study aims to challenge this association directly by testing if there is an interaction between photoperiod, voluntary feeding behaviour and activity.

This leads to my aims for this project:

- 1. Analyse the effect of photoperiod on the distribution of feeding and activity across season.**
- 2. Analyse ultradian and/or circadian patterns of activity.**
- 3. Use of simultaneous food intake and activity measurements to assess the validity of previous assumptions about activity as a proxy for food intake.**
- 4. Assess age differences in activity and feeding between reindeer calves and yearlings**

To answer the proposed research questions, high resolution measurements of activity and voluntary food intake in Norwegian reindeer (*R. t. tarandus*) were recorded under different natural photoperiods in northern Norway in individuals of different age.



## Materials and Methods

A total number of eight semi-domesticated Norwegian reindeer (*Rangifer tarandus tarandus*) were used in this study.

At the start of the feeding trials four reindeer were yearling females (the yearling group), namely animals with ID 2/16, 3/16, 5/16 and 6/16, all born in April 2016. The other four animals were between 5 and 6 months old female calves born in April-May 2017 (the calves group), namely animals with ID Gul/blå, a male, and Rød/hvit, Blå/hvit and Gul/rød, all females.

All experimental animals were bred and born in outdoor enclosures at the University of Tromsø (69° 39' N, 18° 57' O). The animals are kept in outdoor enclosures with semi-natural pastures and natural temperature and light conditions. They have access to water or snow, depending on the season, and are provided with *ad libitum* access to pelleted food (FK Reinfôr, Felleskjøpet, Norway) in addition to the naturally occurring pastures. The animals are occasionally provided with reindeer lichen (*Cladonia rangiferina*), which are highly digestible and helps to regulate and restore their gut flora (Mathiesen *et al.*, 2000). Lichens are low in protein but high in carbohydrates and are a favourite amongst reindeer. We therefore used lichen as a reward to reinforce calm behaviour in the presence of humans.

The two groups of animals that differed in age were kept separate at all times to avoid aggression and reduce social stress, but also with respect to room limitations in the experimental indoor facilities. Behaviour and physical condition such as posture, movement, pelage quality and excrement consistency were regularly assessed through visual inspection.

All procedures described in this project were approved by Mattilsynet (FOTS ID 13982).

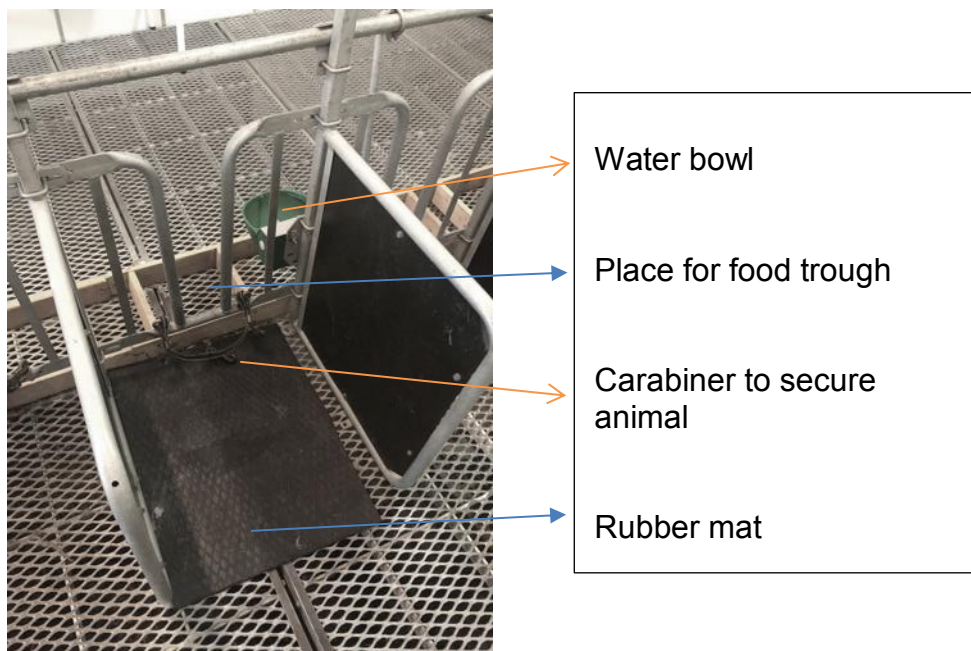
### Acclimation to experimental rooms

In order to gather food intake and activity data for this project, all experimental animals underwent short periods of 5 to 7 days in an indoor enclosure to accustom them to

being handled regularly and specially when in indoor enclosures. This acclimation allows feeding behaviour to be as natural and uninterrupted as possible, especially during the daily food and water renewal procedure and regular cleaning of the enclosure.

Taming took place in an indoor facility (L 9.3m x W 4.3m) fitted with individual stalls for ruminants, with free access to fresh water and pelleted food in troughs provided daily and *ad libitum*. Each stall (L 70 cm x W 65 cm) is delimited by metal railings on the front and sides. The stalls are open at the back, and have attachment points at the front, where the animals are secured by attaching a carabiner to their neck collars. On the front part, the railings have openings that allow the animals to put their heads through to reach the water and feed.

The animals were hitched up individually, positioned parallel to each other, allowing direct visual contact between them, with one free stall between them to allow better carer access and reduce social stress. The animals could stand up or lie down. The floor in the enclosure is a metal grid that allows depositions to fall through to a basement floor, facilitating the cleaning procedures, but each stall occupied by a reindeer had a rubber mat, where the animals could stand or lie down more comfortably (*Figure 3* and *Figure 4*).



*Figure 3. Individual stall for reindeer.* Top view of a single stall where reindeer were kept during the taming period. The green container is continuously refilled with fresh running water. The hitching method can be observed,

as well as the grid floor, the rubber mats utilised and the metallic structure of the stalls. The wooden structure secures the food bowl in place.

Electric lights in the taming room were set to follow on- and offset of civil twilight in Tromsø, whilst the tall windows allowed natural light to enter the room in addition. The intensity of the artificial lights in the room averaged 200 Lux in the area where the animals were hitched up, at the animal's head level.

The temperature in the room varied according to the local climate, given that air is free running from the outdoors and through the grid floor, however the animals produced body heat that contributed to an increase of a few degrees centigrade in ambient temperature.

The taming schedule consisted of 4 hours of human presence in the room with the animals: 2 hours in the morning, from 9am until 11am, and 2 hours in the afternoon, from 12pm to 2pm. General duties in the morning shift included cleaning of the room, weighing of leftover food pellets and renewal of food and washing of the water containers. The amount of refilled food was adjusted to individual needs to ensure the animals never ran out of pellets between feeding times.

In addition to this the animals were trained to accept human contact as a positive occurrence in their daily routine. Calm and/or interactive behaviour was rewarded with reindeer lichen (*Cladonia rangiferina*) as treats. The animals got used to being talked to and touched in various places of their body. Stroking consisted of general physical contact with the animals: touching between the ears, around the neck where the collars sit, the sides of the body, front and rear legs, and on the chin.

During the afternoon shift, the duties comprised primarily of human presence, talking to the animals, stroking and feeding of reindeer lichen.

In addition, walking the reindeer along the internal corridors of the animal facility was done with different purposes: to get the animals used to being transferred between different enclosures, to let them walk and stretch their legs, prevent boredom, and to allow them to feel comfortable in different indoor environments where they could look and smell novel rooms and situations, showing them that there is no danger in these environments. To do so, the animals also had to be trained to wear a halter around their head (*Figure 5*), In the beginning halter training was performed daily. First, halters were mounted and taken off again and the period of wearing was prolonged over time.

Later, the animals learned to walk with a lead rope attached to the halter with a carabiner and each animal was taken through the corridors for little walks on a daily basis. All reindeer were trained to have their weight taken regularly by walking onto a weighing platform and remaining still for a few seconds. The animals were then taken back to their stalls and the halters were removed.



*Figure 4. Taming room with reindeer.* The reindeer are placed with one empty stall in between animals. The food bowls are secured by the wooden structure on the floor, and fresh water is provided by the top metal railing. The animals are identified by the colours or writing on their ear tags. All members in the group of calves can be seen on this picture. Natural light enters the room through the windows at the back of the room, in addition to the electric neon lights on the ceiling.



*Figure 5. Halter and collars used during the experiments.* Although the animal in the picture is not one of the animals used in this experiment, it shows the type of halters and collars used during this project. Both halters and collars are made of hard-wearing nylon, and are adjustable to fit different size animals.

The taming process took place prior to taking the animals into the food measurement enclosure. This took place preceding food intake trials 1, 2 and 3 (see *Figure 9*), as the animals were not previously familiar with indoors environments, and a sudden change in their environment would have affected their food intake.

Moreover, the yearlings were taken into the taming room 2 additional times for 7 days each time during winter 2016 and spring 2017 prior to the beginning of the experiments.

During the first 2 taming sessions the animals took an average of 5 days to start feeding a regular amount of pelleted food, whilst it only took between 1 to 2 days to feed normally during last taming sessions.

## Voluntary food intake measurements (VFI)

The enclosure where food intake measurements took place is a restricted, partially roofed area, composed of a series of parallel-running corridors measuring from 1m to 1.5m wide, and 50m long each, separated by plastic-coated wire fences that allow the animals to move freely and to see and smell each other but preventing direct contact. The first 8 metres of the corridor are covered by a roof, where the food troughs and water bowls are kept. The floor is heated to prevent the water bowls from freezing during the winter months. The rest of the corridor length is open to the outdoors and does not have heating (*Figure 6 and*

*Figure 7*). This enclosure is internally known as “the Vifta”.



*Figure 6. Enclosures for food intake measurements.* Outside view of the enclosure where food intake is measured. The roofed area to the right extends 8m under the roof. To the left the corridors are closed at the point where grass grows, to prevent food intake other than the provided pellets. Other buildings belonging to the University of Tromsø can be seen close in the background.

Food was provided daily in troughs contained in purpose-built wooden boxes that were designed to fit a weighing scale suitable for outdoor use (*Figure 11*). The time of food refilling was logged every day, and generally took place between 10am and 1pm, depending on the feeding pattern of the previous days to avoid entering the room during a feeding bout, while ensuring the animals never run out of food.



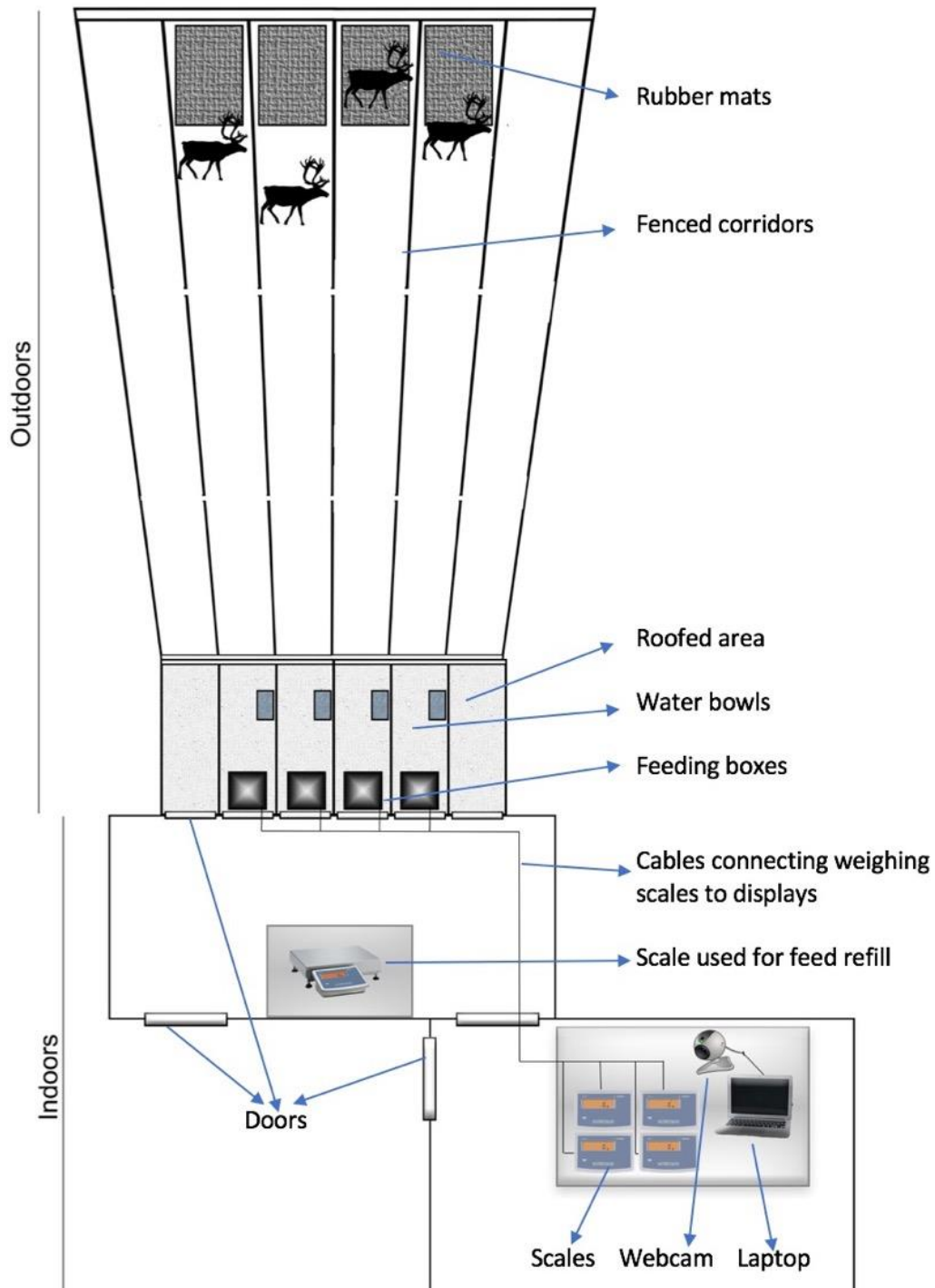
Cleaning of the enclosures with a power water hose was performed every 2 or 3 days, depending on food intake and deposition, and was carried out as fast as possible (averaging ca. 30 minutes) at the same time of feed refilling. Visual assessment of animal behaviour, general health and wellbeing was performed daily.

The food intake measurements took place in 4 different trials from September 2017 until July 2018 (*Table 1* and *Figure 9*).

Prior to trials 1 and 2, the animals underwent a week of taming, however, by the end of the year 2017, all animals were feeding a normal amount of pelleted food after only 1 or 2 days in the taming room. Further food intake measurement trials were therefore carried out by taking the animals straight from their outdoor enclosures into the experimental enclosures.

Each trial lasted between 7 and 14 days, depending on assessment of animal food intake and general behaviour. Once the reindeer were eating a normal amount of food consistently for at least 5 days, the trial was terminated and the animals were taken to their outdoor enclosure.

In the Vifta the reindeer were exposed to natural light exclusively and great care was taken that no electric lights were left illuminated in the corridors and rooms neighbouring the Vifta. However, some light pollution from the University campus and the city of Tromsø could not be avoided.



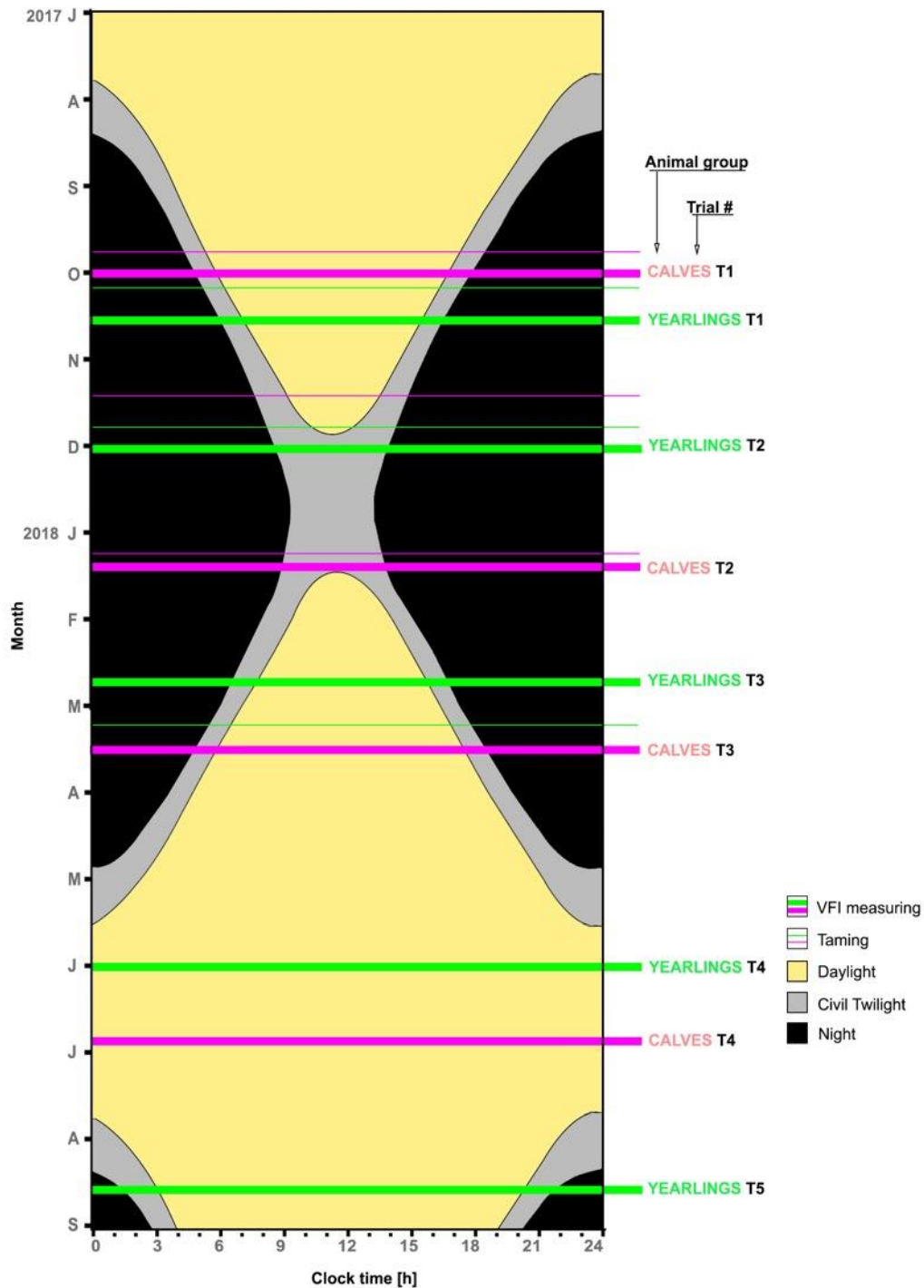
*Figure 7. 'Vifta', the individual enclosures used for food intake measurements.* The recording setup consisted of an outdoor area, with the fan type outdoor enclosure and the roofed area containing food bowls and water containers and an indoor section comprised of 2 rooms, one with a table top scale used to measure daily food renewal, and a second room where the scales from the outdoor feeding boxes were connected to a display. A laptop with a webcam was programmed to take a picture of the scales displays ca. every 20 minutes. Electronic instruments were kept indoors to avoid damage due to rain, heat or freezing. Note that the diagram is not to scale.



*Figure 8. Views of Vifta from different angles.* Left: view from the roofed area, with a feeding box at the forefront and the white water bowls further in the back. Top right: view from the outmost side of the corridor facing towards the roofed area where water and feed are located. Bottom right: outermost area of the enclosure with a view towards the fenced off pasture. Note the rubber mats on the floor which the animals like to lie down on.

## Trials

Below is an overview of the different VFI and taming trials carried out for this project.



*Figure 9. Overview of photoperiod regime in Tromsø with taming and food intake trial schedules.* The Y axis represents the months of the year, and the X axis clock time. The background colours denote lighting conditions day, with yellow for daylight, grey for civil twilight and black for night. Pink lines represent the group of Calves, with thin lines for taming sessions and thick lines for VFI measurements. Green lines respectively represent the group of yearlings.

The following table contains an overview of the VFI trials and the respective photoperiod they represent, although photoperiods DD and LL are not necessarily periods of constant illumination levels during the winter and summer in northern Norway, but these terms are only used to standardise chronobiological terminology.

*Table 1. Schedule of feeding trials.* Overview of the timing for the feeding trials carried out for both animal groups throughout the year. Photoperiod LD: Light/Dark, DD: constant Darkness; and LL: constant Light.

<i>Trial</i>	<i>Calves</i>	<i>Yearlings</i>	<i>Photoperiod</i>
1	Sept – Oct 2017	Oct 2017	<b>LD</b>
2	Jan 2018	Nov – Dec 2017	<b>DD</b>
3	March 2018	March 2018	<b>LD</b>
4	Jun – Jul 2018	Jun 2018	<b>LL</b>

A total of 4 feeding trials were carried out for the calves, and 5 for the yearlings (an extra trial in August), because of instrumentation problems during the summer trial, which needed to be repeated. The food intake measurements lasted for 7 to 14 days at a time. Each VFI trial was terminated once there were at least 5 consecutive days of normal food intake observed, to be able to use the data in statistical analyses.

### Setup for food intake measurements

To gather food intake measurements, wooden boxes were built to fit food troughs that sat on industrial weighing scales (Dini Argeo PBT6 weighing platform and Sartorius Midrics I electronic display (MIS1, Sartorius AG, Germany)). Each scale was connected to a display showing the amount of food left with a resolution of 2g.

The boxes were open at the top and had a cut out at the front part, where the animals can put the head through to reach their food. The troughs fitted closely to the sides of the wooden box and had wooden plinths right above the feeding troughs that prevented food spillage. The back part of the boxes had a hinged cut out wooden flap, that allowed retrieving the food troughs for refilling and cleaning (*Figure 10 and Figure 11*).

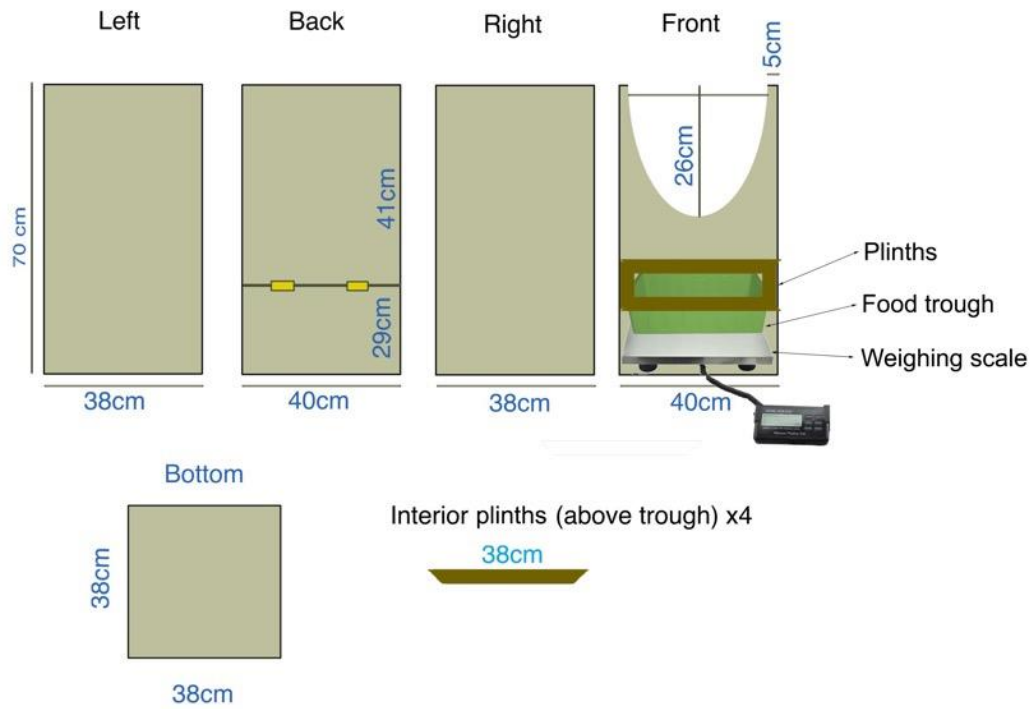


Figure 10. Diagram of the boxes used for food intake measurement. The feeding boxes contain a weighing scale and a food trough. The boxes were made of 15mm thick plywood.



Figure 11. Front view (left) and back view (right) of the feeding boxes built to fit a weighing scale (as seen on the right picture).

## Body weight recording

The weight of the reindeer was measured by walking them onto a platform scale (Sartorius Combics 2, Sartorius AG, Germany), as seen on *Figure 12*. Animal weight was taken every day during the taming process, and at the beginning and at the end of each food intake trial.

Body weight measurements commenced in October 2016 for the yearlings, both during taming sessions and before and after food intake measurements. Measurements for the calves commenced in September 2017.



*Figure 12.* Reindeer on the weighing platform, accompanied by the project Supervisor Dr. Gabi Wagner.

## Activity recording

All animals were equipped with Actiwatch® loggers (model: MotionWatch 8 – CamNtech, UK) taped to their collars. These loggers are small (36mm x 28.2mm x 9.4mm) and lightweight (9g including the battery, and excluding the straps), and powered by a CR2032 Lithium Coin Cell battery.

The Actiwatches are equipped with tri-axial accelerometers that measure omnidirectional motion with a sensitivity of 0.01 G and up to 8 G, and further converted to one plane values that are integrated over a sampling epoch to be recorded as activity count. Recordings are logged in a 4Mbits non-volatile memory, that allows internal storage of up to 120 days at an epoch length of 60 seconds.

Before mounting them on the collars of the reindeer, they were first set up with the specific animal ID, the start time, and the epoch length at 60 seconds. The Actiwatches were placed inside three watertight plastic bags, one inside the other, labelled with animal ID, and further tightly taped to the collars with duct tape (*Figure 13 (b)*).

The collars used were made of lightweight nylon straps, weighing 290g including the Actiwatches.

As the animals wore the collars for prolonged periods of time, the friction of these against their fur led to fur loss around the neck, as well as rubbing onto the skin when the animals were moulting from the winter to summer coat. To prevent this, soft synthetic furry collar covers were attached around the collars (*Figure 13 (a)*).

Once the collars were prepared, the animals were caught and equipped with the collars around their neck. For battery renewal and data collection, the animals were again caught. Every time the animals needed to be caught to either move between enclosures or to mount actiwatches on, the animal facility technicians Hans Lian, Hans Arne Solvang and Renate Thorvaldsen were helping to ensure a safe procedure, both for animals and personnel.





**Figure 13. Soft collar cover and taping of actiwatch onto neck collar.** (a) The furry collar covers prevented the collars from rubbing directly onto the skin, in particular when the animals were moulting from winter to summer coat. Both animals in the picture are members of the yearling group. (b) A collar with the actiwatch wrapped onto its outside with duct tape.

## Photoperiod

Photoperiodic data from the location where the experiments took place were extracted from [http://aa.usno.navy.mil/data/docs/RS\\_OneYear.php](http://aa.usno.navy.mil/data/docs/RS_OneYear.php), using the following coordinates: 69°40' N, 18°56' E. When referring to civil twilight, sometimes expressed as CvTw, it means the period of time from when the sun is right below the horizon, and up to a maximum of 6° below it. The amount of light given by the Sun's reflection on the atmosphere during civil twilight, is known to be able to cause photic responses on organisms, e.g. changes in melatonin production, which can inform the organism about the length of the day, and as a consequence, the development of the season (Reierth *et al*, 1999).

## Data handling

### Food intake measurements

The four scale displays were placed indoors close together to protect from the extreme temperatures in the Norwegian winter, and a computer was set to record a picture of the displays through a webcam every 20 minutes. All data were subsequently typed into Excel spreadsheets.

To calculate the amount of consumed food, the following formula was used:

$$VFI = W2 - W1$$

Where VFI= voluntary food intake

W1= Food trough weight at actual time

W2= Food trough weight at previous 20 minutes data point

Given that a data point at a specific time does not represent the amount of voluntary food intake exactly at that moment but the whole previous 20 minutes, the time prints were converted into bins, where each 20-minute period corresponds to a Bin, accounting for 3 bins per hours and 72 bins in a 24-hour day. As a result, 00:00:00 to 00:19:59 becomes bin 1, 00:20:00 to 00:39:59 corresponds to bin 2, etc.

Scale displays showing a higher weight than its previous observation and appearing as a negative value were handled in two different ways, depending on the value: values ranging from -2 to -12g were considered as fluctuations in moisture content in the feed (Mesteig *et al.*, 2000) and were zeroed. Values from -14g and lower were considered as the animals feeding and exerting pressure onto the scales and those data points were omitted.

## Animal weight

The weight of the animal was used to calculate the live body weight ( $l_{WB}$ ), being the mean between the weight of each animal at the beginning and at the end of each feeding trial, with the following formula:

$$l_{WB} = \frac{(W_B1 + W_B2)}{2}$$

Where  $l_{WB}$ = live body weight  
 $W_B1$ = body weight at the beginning of the trial  
 $W_B2$ = body weight at the end of the trial

The  $l_{WB}$  data was then used to calculate the specific voluntary food (sVFI) intake for each animal, expressed as 'g/kg  $W_B$ ' with the following formula:

$$sVFI \left[ \frac{g}{kg W_B} \right] = \frac{VFI}{l_{WB}}$$

Where  $sVFI$ = specific voluntary food intake  
 $VFI$ = voluntary food intake (g)  
 $l_{WB}$ = live body weight (kg)

## Defining a feeding bout and average meal size

VFI was binned into 20 min intervals and bin values below 0.25g/kg  $W_B$  were disregarded. Continuous feeding bins were treated as one feeding event or bout. Bins were allocated to the seasonally appropriate phase of the day (night, civil twilight or day) for analysis. Feeding bouts directly adjacent to or within civil twilight, were considered CvTw bouts. A bout that started in the civil twilight and prolonged over the following period (day or night), was also considered a CvTw bout.

To calculate the average meal size, the following formula was used:

$$aMs = \frac{\sum VFI}{n}$$

Where:  $aMs$ = average meal size  
 $VFI$ = voluntary food intake in a determined period of the day  
 $n$ = number of meals in the period

### *Activity and food intake*

To discriminate between locomotor activity representing VFI from the rest, the activity of each individual for a day was summed into 20-minute bins to match with VFI bins. Activity bins that corresponded to VFI bins  $> 0.25\text{g/kg } W_B$  were considered to be related to feeding. The remaining activity represents the portion of total daily activity not spent feeding.

### *Phase percent occupied by feeding*

The number of 20min-bins in each phase of the day (day, night, dawn and dusk) occupied with feeding (same threshold of  $< 0.025 \text{ g/kg } W_B$  was applied) were divided by the total number of bins in their corresponding phase, and further multiplied by 100 to finally obtain the % of each period that is occupied by food intake.

### **Activity**

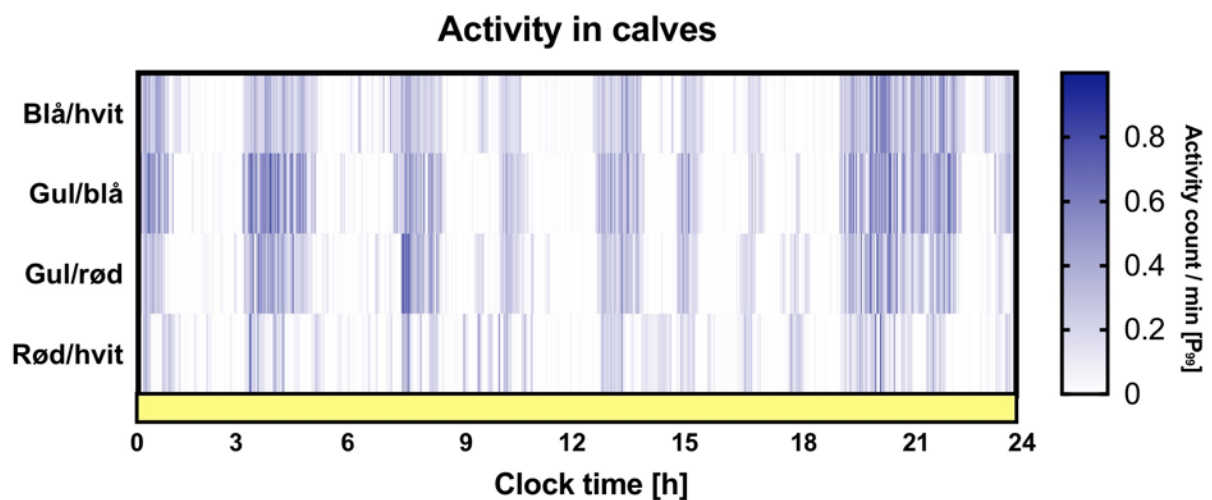
The data gathered from the Actiwatches were downloaded onto a computer using the software MotionWare (CamNtech, UK), and further handled in Excel. All activity data was normalised using a 99-percentile method ( $P_{99}$ ). This normalising method allows to eliminate differences in Actiwatch sensitivity that could emerge due to the positioning of the collar or tightness of it around the neck of the animal, and to ignore the 1% of values which are higher than the other 99% of values that might represent intense activity that could be associated with abnormal stressful situations such as when a person walks into the enclosures to refill the feed for the reindeer or to clean the enclosure, therefore this high end 1% values are considered outliers and discarded. The rest of values are divided by the highest value of the 99% of included values,

giving a final range between 0 and 1. This normalization allows comparison of activity between animals with different activity levels.

Actograms were created and analysed through chi-squared periodograms with the ImageJ plugin for ActogramJ (Schmid, Helfrich-Förster *et al.* 2011).

## Heatmaps

A representative heatmap of 4 calves is depicted in *Figure 14*. All individuals display similar patterns of activity, which is known as ‘herding effect’, apart from the animal at the bottom of the graph. The female Rød/hvit was sick at the time of measurement and not included in further analysis. Unless stated otherwise all figures and calculations are averages of activity or VFI from the 4 animals representing each group, yearlings or calves.



*Figure 14. Activity of 4 calves throughout a 24-hour day.* Each individual row represents a different calf. All measurements are from 3<sup>rd</sup> July 2018. The yellow bar at the bottom represents the photoperiod, which is constant daylight in summer. The graded bar on the right represents the normalised ( $P_{99}$ ) count of activity/minute, therefore, darker blue lines on each row denote higher levels of activity, and white periods represent rest.

## Phase angle calculations

The interim between the onset and offset of activity and VFI compared to a reference point are described as the phase angle differences ( $\Psi$ ), as an adaptation from Daan and Aschoff (1975). This allows to calculate the difference in time (m) between the initiation and cessation of activity and VFI bouts compared to civil twilight start and

end. As a result, bouts of activity or VFI starting before CvTw1 (during the night) are assigned negative values, and activity or VFI finishing after the end of CvTw2 (in the night) are assigned positive values. Data were analysed and averaged from three consecutive days. An activity bout was considered terminated when having at least 5 minutes with no activity at either end. For this analysis, feeding bouts were only considered if they occurred within the civil twilight or were directly adjacent to it.

## Statistical analyses

### Periodograms

Chi squared periodograms were plotted to analyse rhythmicity on the activity data through the following formula:

$$Q_P = \frac{K \sum_{h=1}^P (\bar{X}_h - \bar{X})^2}{N^{-1} \sum_{i=1}^N (X_i - \bar{X})^2}$$

$P$  = set period

$K$  = number of sections with period  $P$

$h$  = hour in periods

$\bar{X}_h$  = Mean of hour  $h$

$i$  = time point of undivided recording

$X_i$  = value at time point  $i$

$\bar{X}$  = total mean

This formula computes the  $Q_P$  value for a period of time specified by the user (i.e. 1260 to 1620 minutes, which equals to a period from 21 to 27 hours). By doing this, the data is folded into sections of differing length which are then compared with each other following a chi-squared distribution. The largest  $Q_P$  value will be displayed as a peak with highest amplitude, corresponding to the true period length for the calculated time series (Refinetti *et al.*, 2007). All periodograms were calculated with a significance of  $p < 0.001$ .

Periodograms with a period length ( $\tau$ ) between 21 and 27 hours were created to analyse circadian rhythms, and  $\tau$  between 3 and 9 hours were selected to look for

ultradian rhythms. A total of 15 consecutive days of normalised ( $P_{99}$ ) were used in periodograms for the winter and summer seasons, however only 5 consecutive days were analysed for the spring, as the photoperiod changes very fast during this season, e.g. the days are 9 minutes longer every day, which represents over 1 hour longer every week.

All statistical analyses and graphs were created with Graphpad Prism 7.0d. Actograms and Chi-square periodograms were created with the plugin ActogramJ in ImageJ.





## Results

Measurements of body weight, activity and voluntary food intake were recorded in two groups of reindeer of different age, across different seasons in northern Norway. The objective was to determine differences between groups, and to analyse the influence of photoperiod on behavioural parameters.

### Body weight

The two groups of animals differed in age by one year. Bodyweight in the autumn of the animals' first year was similar in the two groups. A Mann-Whitney U-test indicated no body weight differences in autumn between yearlings weighed on 19.10.2016 (Mdn=57.6, n=2) and calves weighed on 26.9.2017 (Mdn=55.6, n=4),  $U=3$ ,  $p>0.05$ . From October 2017 the yearling group underwent a marked decrease of body weight during the winter months, confirmed by a paired t-test that showed significant body weight fluctuation in the animals between 7.10.2017 and 10.3.2018 ( $t_{(3)}=12.52$ ,  $p<0.05$ ). This decrease in body weight reached a plateau during spring, and was further followed by an increase in body weight noticeable towards the end of the summer. A further paired t-test comparing body weight of the yearlings on 10.3.2018 and 15.8.2018 confirmed this observation ( $t_{(3)}=9.68$ ,  $p<0.05$ ). The calves born in spring 2017 gained weight throughout the different seasons in the duration of the experiments. A repeated one-way ANOVA analysis confirmed this statistically significant increase in calf body weight across seasons from autumn (25.9.2017), to winter (12.1.2018) and to summer (5.7.2018):  $F_{(2,3)}= 22.52$ ,  $p<0.05$ .

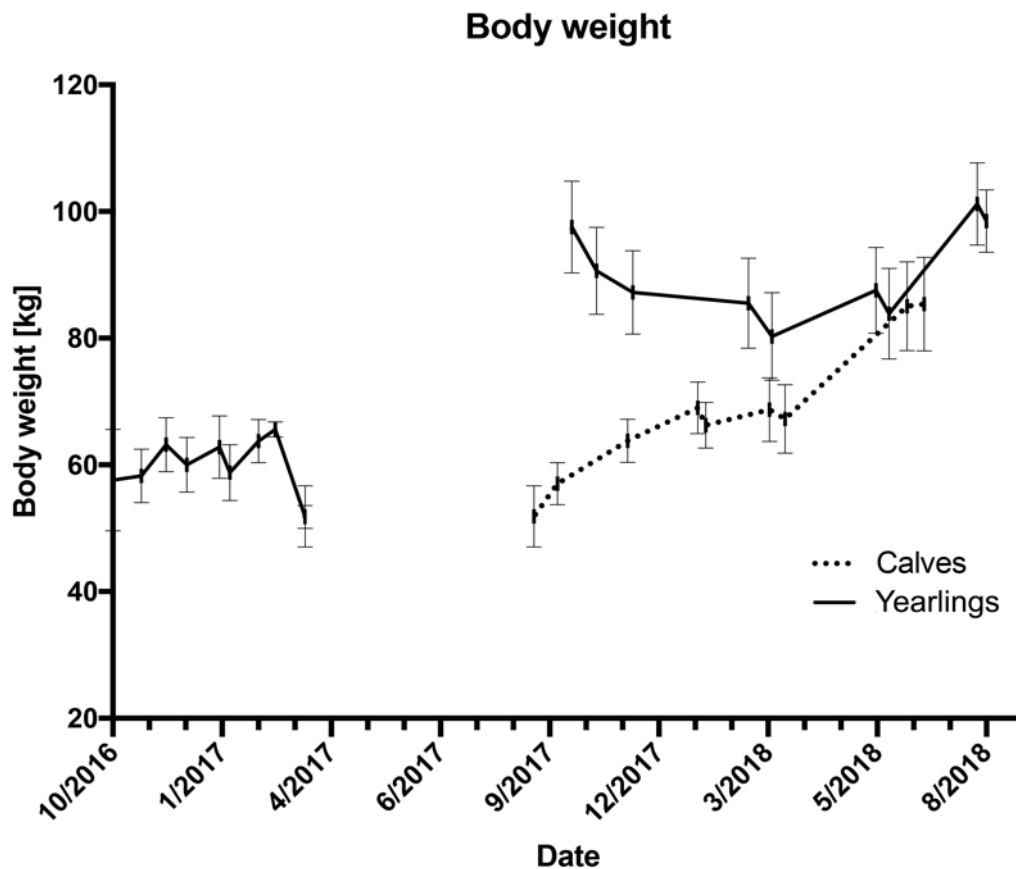


Figure 15. **Body weight of 2 groups of reindeer across the year.** The yearlings were born in spring 2016 and were roughly 1.5 years old at the beginning of the experiment. The calves were born during spring 2017. All reindeer were kept year-round at the animal facility of the University of Tromsø. Data are given as mean  $\pm$  SEM. Dotted lines represent the calves; continuous lines represent yearlings.

## Seasonal changes in activity and food intake

### Activity

The activity levels of the reindeer vary across seasons (2-way ANOVA  $F_{(2,66)} = 33.09$ ,  $p < 0.01$ ), and also between groups of animals ( $F_{(1,66)} = 6.751$ ,  $p < 0.05$ ; Figure 16). For actograms of yearling reindeer see AppendixFigure A, for calves see AppendixFigure B.

Tukey post-hoc tests (Table C) following 2-way ANOVA revealed that yearlings had overall higher activity levels in spring ( $p < 0.05$ ) and summer ( $p < 0.001$ ) compared to winter. Yearlings were significantly more active than calves only in the summer

( $p > 0.001$ ), while the level of activity was similar in the two groups in autumn and winter. The calves displayed no significant differences in activity levels across seasons.

## Food intake

Daily voluntary food intake (VFI) differed between groups ( $F_{(1,20)} = 27.35$ ,  $p < 0.001$ ) and between seasons ( $F_{(3,20)} = 43.45$ ,  $p < 0.001$ ) as confirmed by 2-way ANOVA tests. Results from Tukey post-hoc tests (*Table D*) showed that VFI only differed in autumn between the groups ( $p < 0.01$ ), while calves and yearlings displayed similar daily VFI for the rest of the year. VFI was significantly lower ( $p < 0.001$ ) in winter compared to summer in both, calves and yearlings.

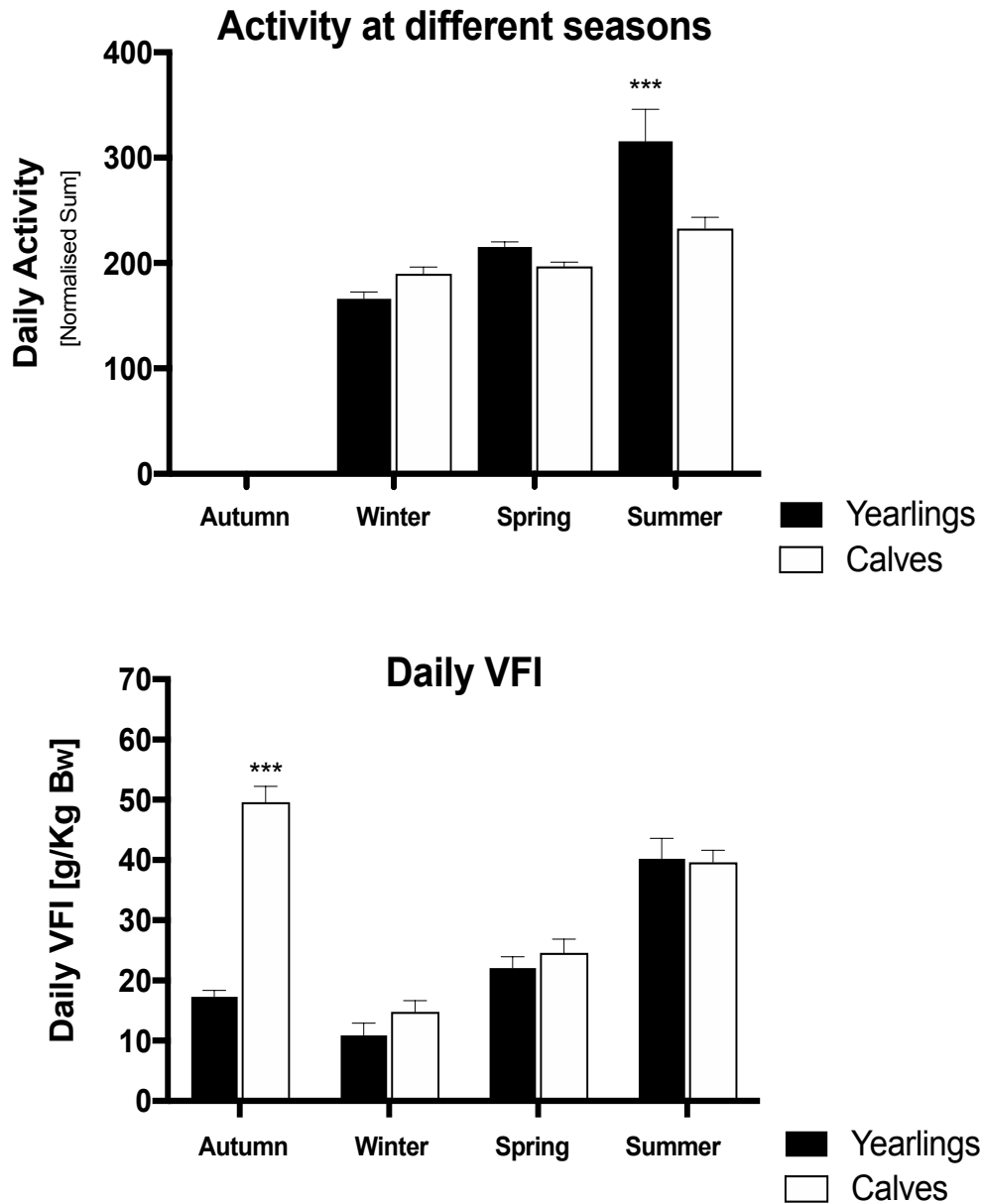


Figure 16. (a) Total daily activity of both groups of animals, yearlings and calves, across different seasons. Each bar represents the mean  $\pm$  SEM of 14 consecutive days of normalised activity for each season, and also the mean  $\pm$  SEM for the 4 animals in each group. For exact dates of measurements see Table 2. All measurements are taken when the animals occupy their usual outdoor enclosures. Activity measurements start winter 2017, and continue through to summer 2018. The black bars represent the yearlings, while the white bars represent the calves. (b) Total daily VFI of both yearlings and calves, across different seasons. Each bar represents mean  $\pm$  SEM of the 4 animals within each group, and of 3 consecutive days of food intake measurements. For exact dates see Table 2. Black bars represent yearlings, white bars represent calves.

The following table contains the specific dates selected for VFI and activity measurements.

**Table 2. Dates used for most graphs and analyses in this project.** The dates in the table have been specifically selected as good representations of each sampling, and because of matching dates for VFI and activity for analyses. The dates selected for measurements of food intake are from the end of each measurement trial. For the yearlings' summer measurements, animal with id: 2/16 is not included in measurements due to sickness Yearling autumn measurements do not include animal ID 5/16 due to a leg injury affecting appetite. Data for the calves during the winter period only contain data from one day, because of a technical problem with the weight recording system. Actiwatch recording started during November 2017, hence there are no activity recordings for autumn. summer activity for the yearling group only contains data from animal with id: 6/16 due to technical problems with the remaining actiwatches.

		Yearlings		Calves	
VFI	Autumn	23-25 Oct	2017	7-9 Oct	2017
	Winter	1-3 Dec	2017	12 Jan	2018
	Spring	5-7 Mar	2018	17-19 Mar	2018
	Summer	5-7 Jun	2018	30 Jun - 2 Jul	2018
Activity	Autumn	na		na	
	Winter	10-23 Dec	2017	10-23 Dec	2017
	Spring	19 Mar - 1 Apr	2018	25 Mar - 7 Apr	2018
	Summer	13-22 Jul	2018	13-22 Jul	2018

## Activity

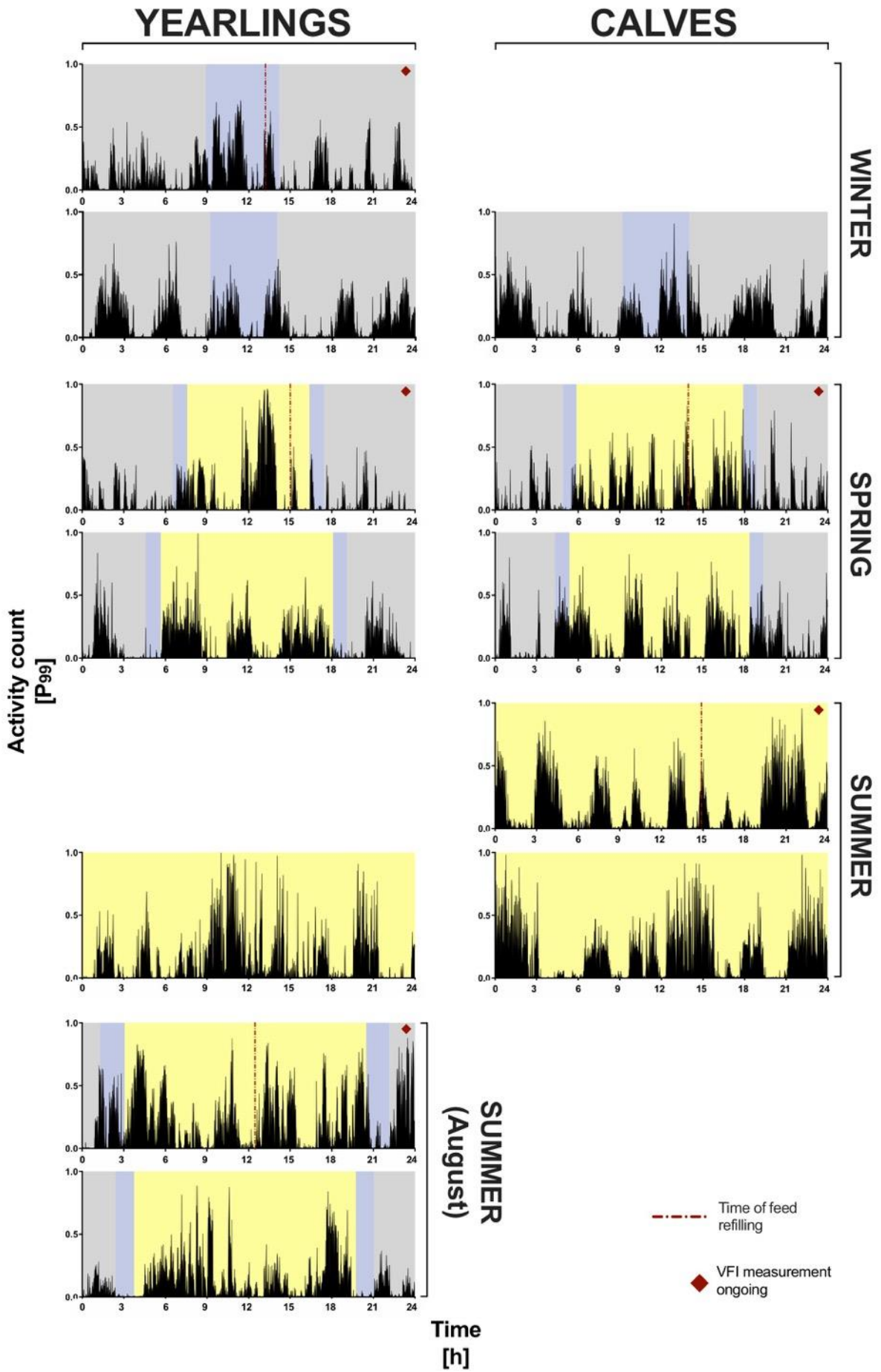
Activity measurements when the animals were housed in individual corridors for food intake measurements were comparable to those in the outdoor enclosures (*Figure 17*). Statistical tests found no significant differences between the conditions. The main activity bouts occur during the lighter phases of the day, i.e. civil twilight and day (2-way ANOVA tests below). Civil twilight is an important signal for the start or cessation of activity for both animal groups most of the time. During spring and summer (august) yearling activity bouts are regulated by the rise and set of the Sun.

During spring the average hourly activity of the calves was not significantly different between Vifta (the individual corridors) and outdoor enclosures (2-way ANOVA;  $F_{(1,2)}=13.82$ ,  $p<0.05$ ). Some additional differences were found between the phases of the day: the calves were significantly more active in the dusk compared to dawn ( $p<0.05$ ) when they were housed in the Vifta, but had similar high levels of activity during dawn and dusk when they were outdoors. The animals waited until sunrise to

commence the morning activity bout, but remained highly active throughout dusk. Although mean hourly activity was higher in the day

During winter no difference in the hourly activity levels of the yearlings between outdoors and hourly activity in Vifta (2-way ANOVA,  $F_{(1,3)}=3.624$ , ns) was found, but there were significant differences between hourly activity during the night and civil twilight ( $F_{(1,3)}=71.92$ ,  $p<0.05$ ). Upon further inspection through a Tukey post-hoc test, the hourly activity levels when the animals were in Vifta were significantly higher during twilight. Although the statistical tests showed no statistically significant differences when the animals were outdoors, the mean hourly activity levels in twilight were higher than in the night.

Hourly activity for the calves was not significantly different between night and civil twilight upon analysis with a t-test ( $p=ns$ ), however higher hourly activity values are observed during the hours of twilight (night=  $8.843 \pm 0.674$  SEM normalised activity,  $n=4$ ; twilight=  $11.25 \pm 0.8442$  SEM,  $n=4$ ).



*Figure 17. Normalised activity of captive Norwegian reindeer across 24 hours.* Each graph represents the Mean of the 4 animals in each group, apart from the summer graph for yearlings (n = 1 due to technical difficulties), and the summer graphs for the calves (n = 3 due to sickness). The shaded grey area represents night, blue civil twilight, and yellow day. The top graphs of each paired comparison marked with a red diamond are representations of activity when the animals are undergoing food intake measurements. Time of refilling of the feed in such cases is denoted with a vertical dotted dark red line. The Y axis is the amount of normalised activity / minute [P<sub>99</sub>], while the X axis represents the clock time [h].

## Periodicity

Activity rhythms with a period length close to 24 hours were found with significant peaks ( $p < 0.001$ ) in all seasons, and in both groups of animals, including during the time of the Midnight sun in summer and during the Polar night in winter (see *Figure 18 and Figure 19*).

Ultradian rhythms for activity with period lengths between 3 and 9 hours were also present and with significant peaks ( $p < 0.001$ ) in all seasons and in both groups of animals.



### Periodograms for Yearlings

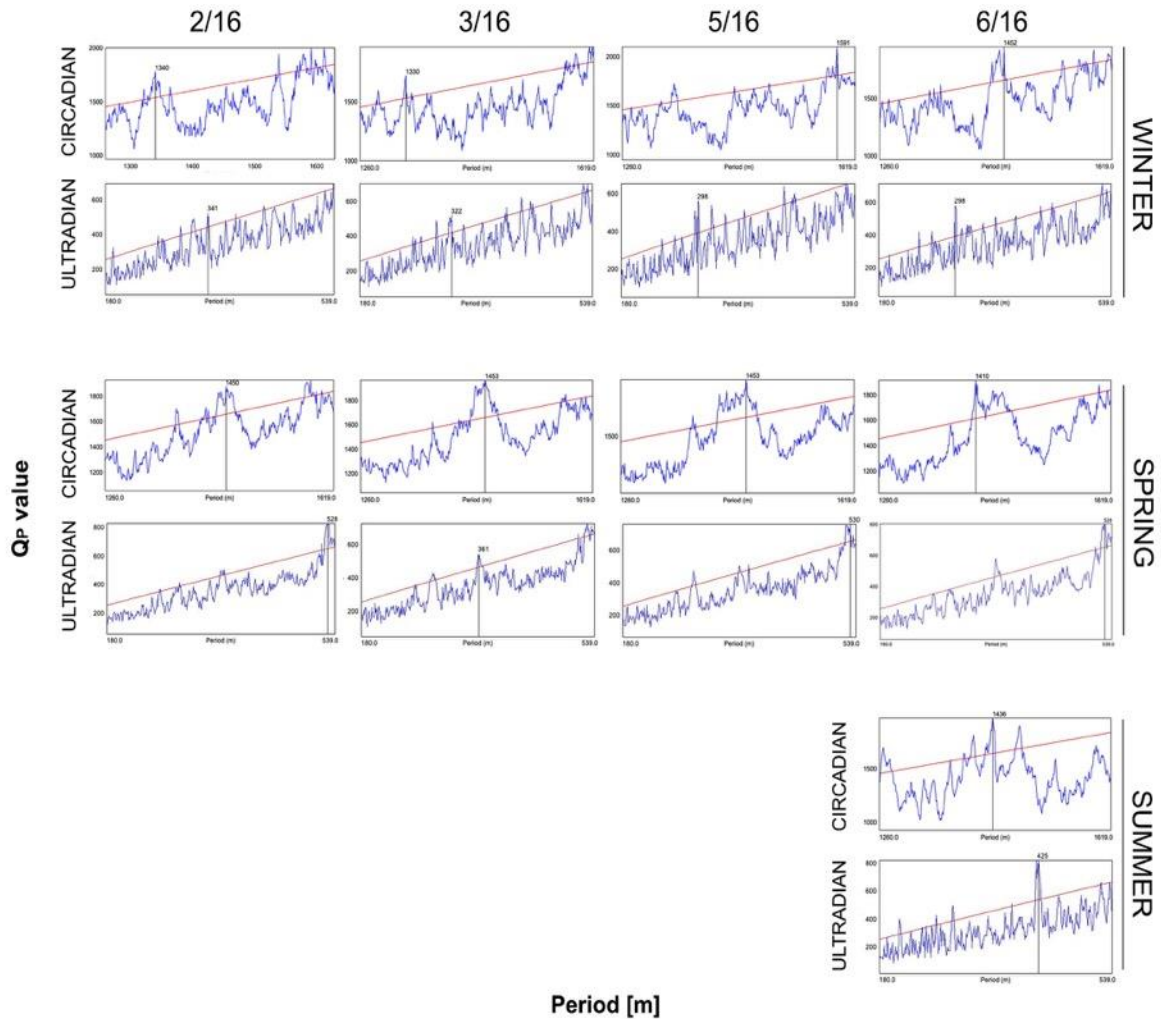
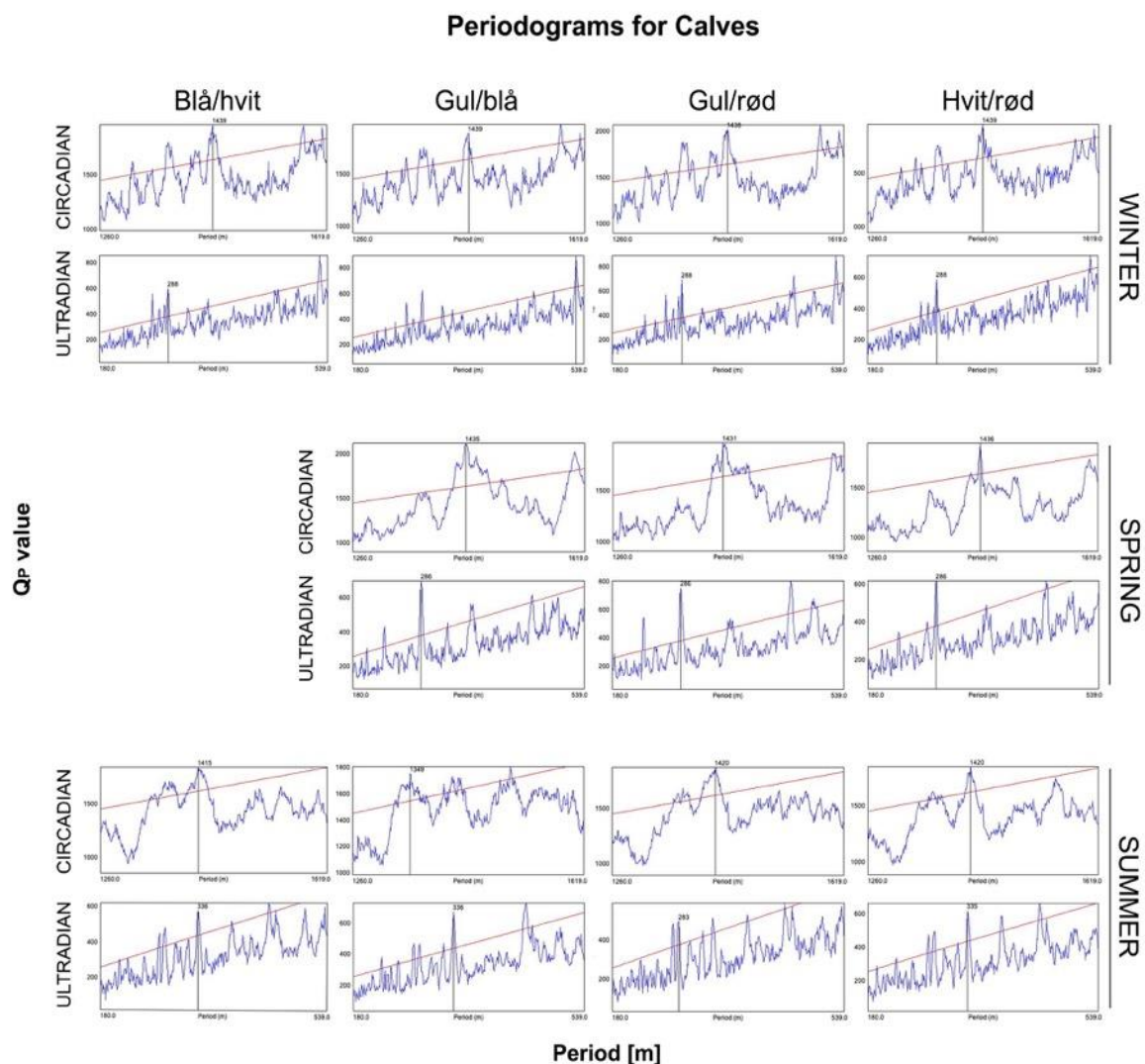


Figure 18. Periodograms for the group of yearling reindeer. Dates for the different periods can be found in Table 3. Winter and summer periods contain data from 15 consecutive days. The spring period contains only 7 days of data because of the rapid change in photoperiod near the equinox. Each column represents a different animal within the group. The X axis is the period of time (minutes), being the limits 1260 (21 hours) and 1620 minutes (27 hours) for the Circadian plots; and periods between 180 (3 hours) and 540 (9 hours) for the Ultradian plots. The Y axis is the Qp value. The red line across represents the level of significance of  $p \leq 0.001$ .

Table 3. Dates used in Periodograms. Dates for the spring period are shorter than winter and summer, given the rapid change in photoperiod from one day to the next. Data for the group of calves during the summer is limited to 8 days because of the availability of data.

	Yearlings		Calves	
Winter	5-20 Dec	2017	5-20 Dec	2017
Spring	24-29 Mar	2018	27 Mar - 2 Apr	2018
Summer	10-24 Jul	2018	11-18 Jul	2018



**Figure 19. Periodograms for the group of reindeer calves.** Dates for the different periods can be found in Table 3. Winter and summer periods contain data from 15 consecutive days. The spring period contains only 7 days of data because of the rapid change in photoperiod near the equinox. Each column represents a different animal within the group. The X axis is the period of time (minutes), being the limits 1260 (21 hours) and 1620 minutes (27 hours) for the Circadian plots; and periods between 180 (3 hours) and 540 (9 hours) for the Ultradian plots. The Y axis is the Qp value. The red line across represents the level of significance of  $p \leq 0.001$ .

## Voluntary food intake

### Distribution across the day

The distribution of the daily total food intake in the reindeer varied across seasons and between the yearlings and calves (Figure 20). The yearlings started feeding during

dawn (CvTw1) in spring and autumn, while the calves did not ( $p < 0.05$ ). During autumn, the calves fed more of their daily total during the day, compared to the yearlings., while yearlings fed more of their total during the night, compared to the calves ( $p < 0.05$ ). During winter, the distribution of VFI across night and civil twilight between calves and yearlings was also significantly different ( $p < 0.05$ ). The calves ate a higher percentage of their daily total during the night, compared to yearlings. During spring, the distribution of daily food was similar for calves and yearlings during the night, day and dusk. All results for multiple t-tests can be found in *AppendixTable E*.

### Seasonal variation in the number of daily meals

The average number of daily meals was different between groups ( $F_{(1,22)}=31.35$ ,  $p < 0.01$ ), and between seasons ( $F_{(3,22)}=24.75$ ,  $p < 0.01$ ), as confirmed by 2-way ANOVA tests (see *Figure 21*). Post-hoc analysis (included in *AppendixTable F*) revealed that calves had a more meals than yearlings during autumn and winter, while the values for both groups were similar in spring and summer.

Yearlings had the largest amount of meals in summer ( $11.4 \pm 0.6$  SEM meals/day,  $n=3$ ), and the lowest in winter ( $6.8 \pm 0.3$  SEM meals/day,  $n=3$ ;  $p < 0.001$ ).

The calves had a high number of meals in their first autumn ( $10.8 \pm 0.6$  SEM meals / day,  $n=4$ ), that was statistically lower ( $p < 0.05$ ) in winter ( $8.7 \pm 0.2$  SEM meals / day,  $n=4$ ). The summer number of meals ( $11.2 \pm 0.2$  SEM meals/day,  $n=4$ ) was significantly higher than winter, but not significantly higher than spring or autumn.

### Seasonal variation in the average size of daily meals

The average meal size varied in the groups of reindeer according to season (2-way ANOVA;  $F_{(3,22)}= 76.41$ ,  $p < 0.001$ ), but did not differ significantly between groups ( $F_{(1,22)}= 0.46$ , ns; see *Figure 21*).

Upon further inspection of the data through a Tukey post-hoc test, meal size in autumn was significantly larger in calves than in yearlings, and smaller during the spring ( $p < 0.05$ ; see *AppendixTable G*).

Yearlings ate in average larger meals during autumn ( $10.04 \pm 0.92$  SEM g/kg  $W_B$ ) and spring ( $11.32 \pm 1.04$  SEM g/kg  $W_B$ ), compared to winter ( $2.70 \pm 0.55$  SEM g/kg  $W_B$ ) and summer ( $2.30 \pm 0.09$  SEM g/kg  $W_B$ ), being the meals during the equinoxes up to 3 times bigger than during solstices ( $p < 0.05$ ; see *Appendix G*).

Similar to the yearlings, the calves consumed larger meals in autumn ( $14.80 \pm 0.83$  SEM g/kg  $W_B$ ) and in spring ( $7.57 \pm 0.91$  SEM g/kg  $W_B$ ), compared to winter ( $3.37 \pm 0.74$  SEM g/kg  $W_B$ ) and summer ( $2.80 \pm 0.38$  SEM g/kg  $W_B$ ), ( $p < 0.05$ , see *Appendix G*).

## Distribution of daily VFI

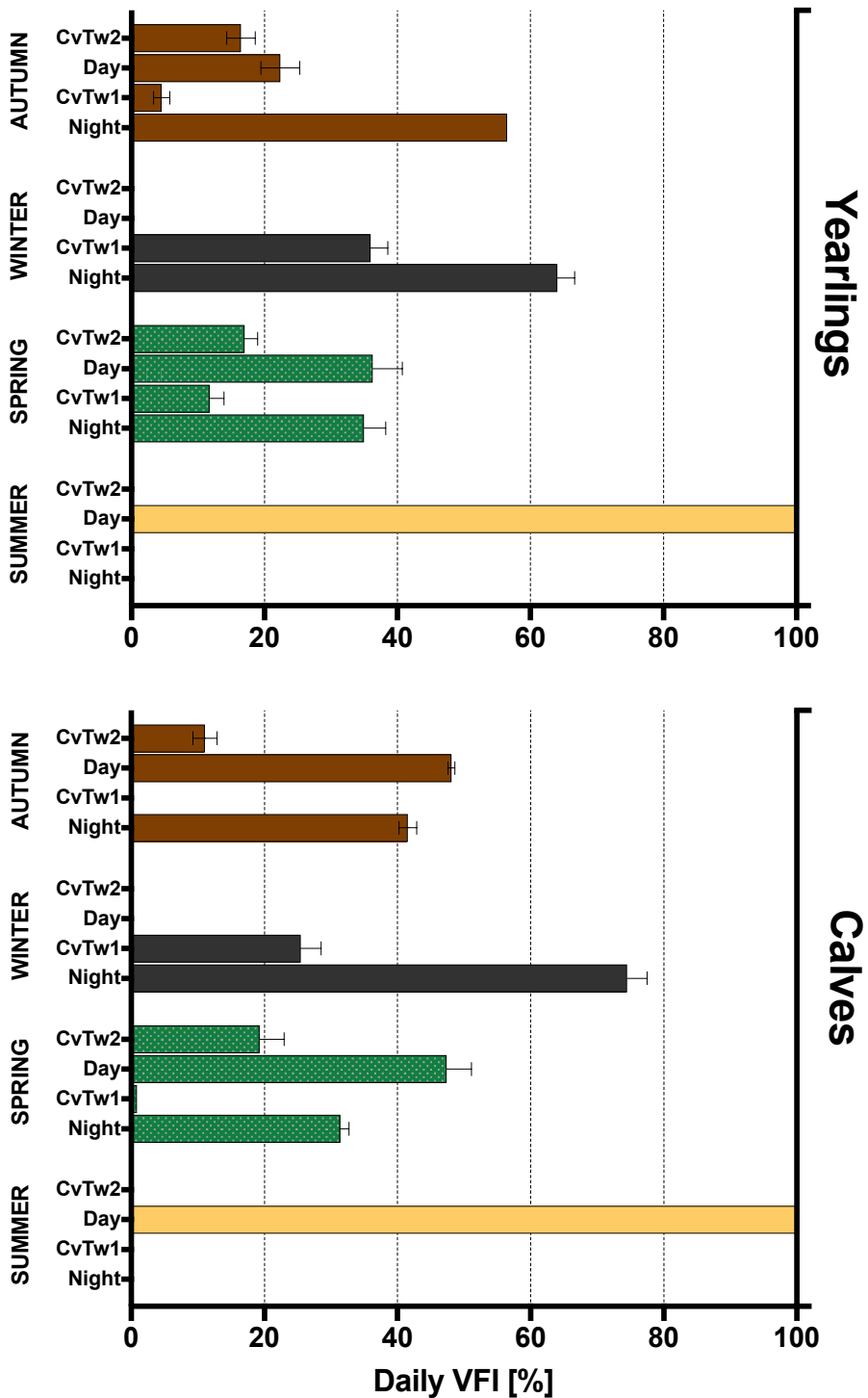


Figure 20. Distribution of daily food intake across the day and in different seasons. The average daily VFI of 3 days and 4 animals is plotted for each season and for each animal group, yearlings and calves. Descriptions of the dates are given in Table 2. The graph represents proportional food intake ( $n = 3$  days  $\pm$  SEM) distributed between the different phases of the day. The colours of the bars represent the season: brown for autumn, black for winter, green for spring and yellow for summer. CvTw1: civil twilight before sunrise; CvTw2: civil twilight after sunset.

## Number of meals vs meal size

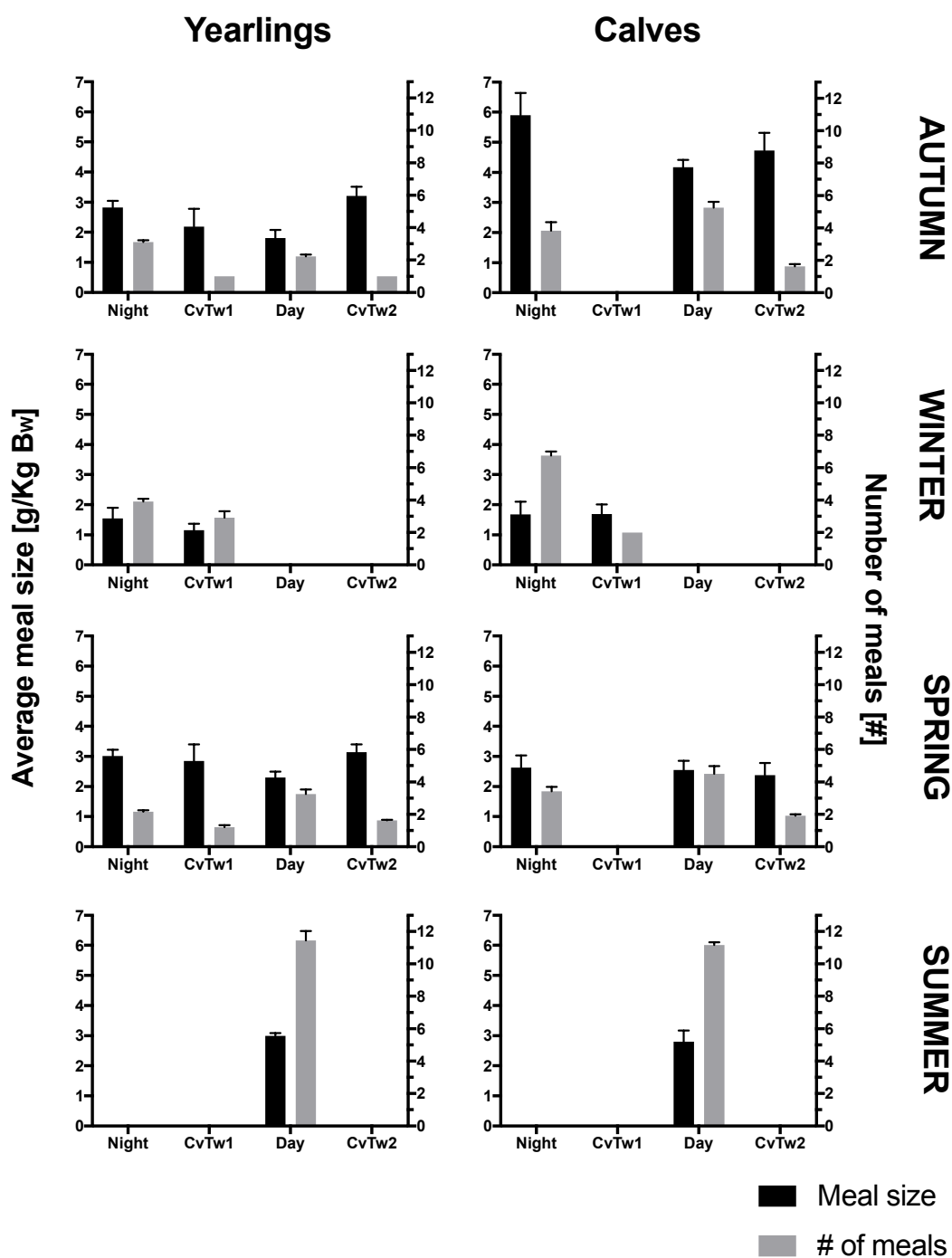


Figure 21. Meal size compared to the number of meals consumed across seasons. Each graph contains data from 3 consecutive days of food intake measurements in 4 animals per group, with the exception of the yearlings during the autumn sampling ( $n = 3$  due to sickness in one animal). For specific dates see Table 2. The left Y axis represents the average meal size in g/kg B<sub>w</sub>, plotted in back bars; and the right Y axis is the average number of meals consumed during each phase of the day (night, civil twilight or day), plotted in grey. CvTw1 is civil twilight at dawn, CvTw2 is the civil twilight at dusk. Graphs plotted on the left represent the yearlings, right hand side graphs are the calves.

## Seasonal distribution of time spent feeding according to phase of the day

The percentage of time spent feeding, according to the duration of each phase of the day differed on some occasions between groups and between seasons (*Figure 22*). Results from multiple t-tests can be found in *AppendixTable H*.

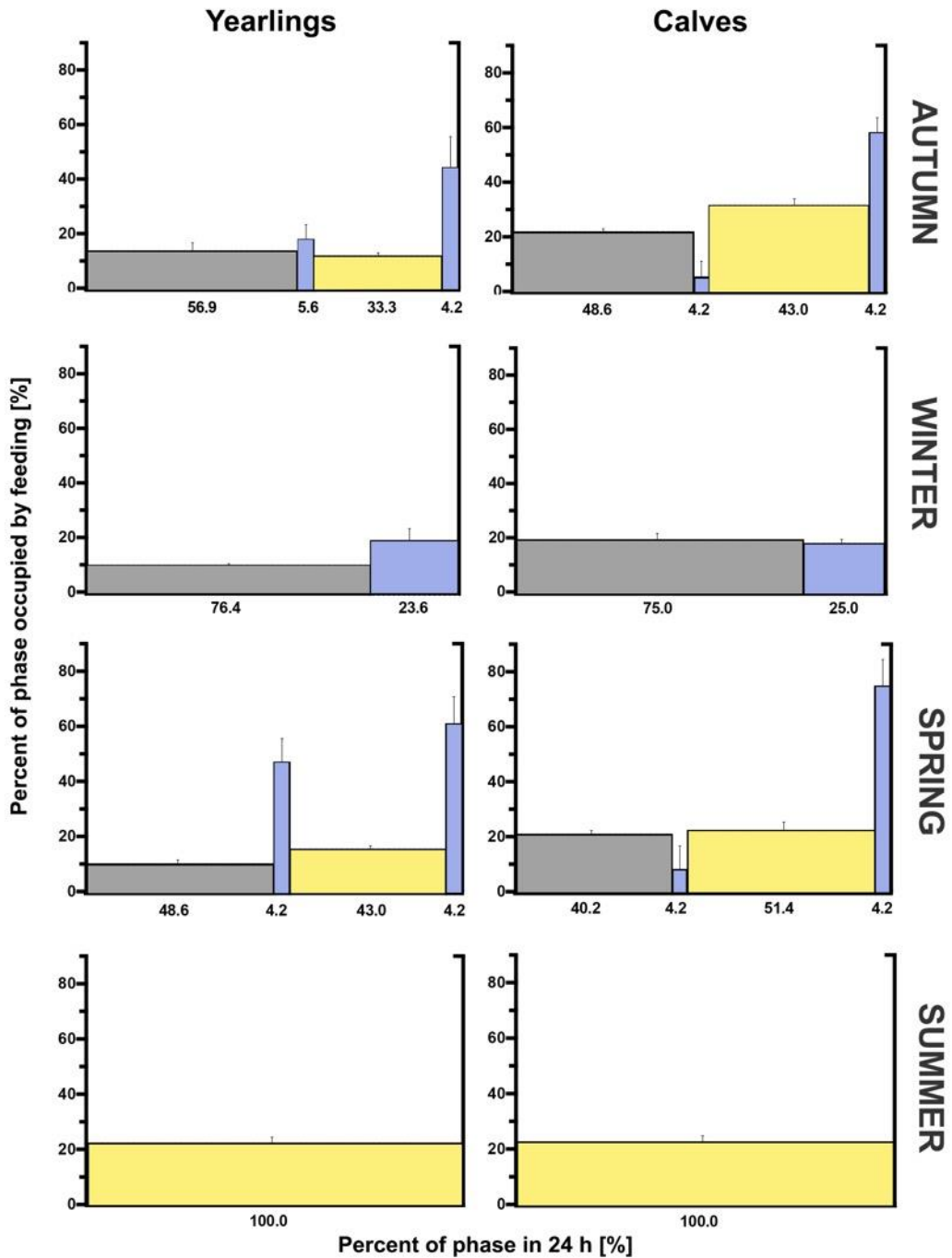
During autumn the calves spent more time feeding than the yearlings during both, day and night ( $p < 0.05$ ). Calves and yearlings spent  $22\% \pm \text{SEM}$  and  $11\% \pm \text{SEM}$  respectively of the night feeding, while the day was occupied with feeding in  $58\% \pm \text{SEM}$  and  $44\% \pm \text{SEM}$  respectively. The percentage of time spent feeding at dawn was similar in both groups (ns), with only a small percentage of its duration being occupied by feeding ( $6\% \pm \text{SEM}$  of the duration of dawn the calves, and  $19\% \pm \text{SEM}$  the yearlings). Contrary, dusk was occupied by feeding to a large percentage in both groups, with  $58\% \pm \text{SEM}$  of the time in calves, and  $44\% \pm \text{SEM}$  in the yearlings, and no significant differences between the two.

During winter, the percentage of the night phase occupied with feeding was slightly higher in the calves ( $19\% \pm \text{SEM}$ ) compared to the yearlings ( $10\% \pm \text{SEM}$ ) ( $p < 0.05$ ), while during civil twilight, yearling and calves spent similar time feeding ( $18\% \pm \text{SEM}$  the calves and  $19\% \pm \text{SEM}$  the yearlings).

In spring the percentage of time spent feeding during day and dusk were similar in both groups (ns), with a large part of the duration of dusk occupied by feeding ( $75\% \pm \text{SEM}$  in the calves and  $61\% \pm \text{SEM}$  in the yearlings). The day phase was occupied with feeding to  $23\% \pm \text{SEM}$  in the calves and  $16\% \pm \text{SEM}$  in the yearlings. No significant differences were found between those. However, dawn showed large differences, with the calves only feeding during  $8\% \pm \text{SEM}$  of the twilight while yearlings spent  $47\% \pm \text{SEM}$  of the duration of dawn feeding ( $p < 0.05$ ). On the other hand, calves spent more time feeding in the night ( $21\% \pm \text{SEM}$ ) compared to the yearlings ( $10\% \pm \text{SEM}$ ), ( $p < 0.05$ ).

Summer days were on average occupied by  $23\% \pm \text{SEM}$  of active time spent feeding in both groups.

## Phase percent occupied by feeding



**Figure 22. Phase percent occupied by feeding.** The X-axis is the % of the day that occupies each phase in a 24-hour day, represented by the width of the bars. The different colours represent the night in grey, civil twilight in blue, and daylight in yellow +SEM bars. The Y axis represents the % of each phase that is occupied by feeding behaviour. Each graph contains data from 3 days of food intake measurements, and from the 4 animals in each group, with the exception of the yearlings' summer graph (bottom left) which only contains data from animal with id: 6/16. For exact dates of measurements see *Table 2*.



## Correlations between activity and voluntary food intake

Measurements of VFI and activity were plotted together across a 24-hour period to assess their correlation across the different seasons (*Figure 23 (a)*).

During winter, the yearlings showed different average activity levels per hour between night ( $4.55$  activity counts/h  $\pm 0.18$  SEM) and civil twilight ( $10.15 \pm 0.71$  SEM) when the animals were in the individual enclosures (Vifta) (t-test,  $t_{(3)} = 7.24$ ,  $p < 0.05$ ). Contrary, hourly VFI levels did not differ between night and civil twilight (ns)

In spring, average hourly activity for the calves differed between phases of the day (One-way ANOVA,  $F_{(3,8)} = 22.78$ ,  $p < 0.05$ ). Dusk was the most active period ( $12.83$  activity counts/h  $\pm 1.58$  SEM) and dawn was the least active period ( $1.53 \pm 0.37$  SEM). The average hourly activity in the day ( $8.23 \pm 0.95$  SEM) and in the night ( $4.79 \pm 0.19$  SEM) were not significantly different. The average hourly VFI levels returned significant differences (One-way ANOVA,  $F_{(3,12)} = 5.86$ ,  $p < 0.05$ ). The significant differences resulted because there is no feeding at dawn. Night, day and dusk hourly VFI levels showed no significant differences between each other ( $0.77 \pm 0.19$ ,  $1.38 \pm 0.14$  and  $1.55 \pm 1.11$  SEM respectively, ns).

Mean values for hourly activity are following described for the yearlings in spring, although the low value of replicates ( $n=2$  because of equipment malfunction) does not allow for statistical comparisons. The mean highest values were recorded during dusk ( $\approx 17.28$ ) and dawn ( $\approx 13.84$ ), and the lowest in the nights ( $\approx 2.73$ ). The values in the day were  $\approx 7.05$ . In terms of VFI ( $n=4$ ), differences were found (One-way ANOVA,  $F_{(3,12)} = 7.05$ ,  $p < 0.05$ ). Further post-hoc revealed average hourly VFI at dusk ( $3.84 \pm 1.38$  SEM g/kg  $W_B$ ) to be higher than during the night ( $0.54 \pm 0.13$  SEM) and that during the day ( $0.67 \pm 0.36$  SEM) ( $p < 0.05$ ). Dawn average hourly VFI was  $2.50 \pm 1.90$  SEM g/kg  $W_B$ , although no significant differences were found between those (ns).

In August, the yearling group had differences in hourly activity level between the phases of the day (One-way ANOVA,  $F_{(3,12)} = 15.46$ ,  $p < 0.05$ ). The highest levels were recorded in the day ( $14.76$  activity counts/h  $\pm 0.51$  SEM), followed by dusk ( $12.04 \pm 1.06$  SEM). The lowest hourly activity was recorded in the dawn ( $6.11 \pm 0.60$  SEM), followed by the night ( $8.11 \pm 1.47$  SEM). Day values were significantly higher than the night and dawn ( $p < 0.05$ ). Hourly VFI levels were not significantly different across the different phases of the day (One-way ANOVA,  $F_{(3,12)} = 1.64$ , ns)

Further analyses of correlation between VFI and activity across the different seasons showed very low association between the two parameters, with correlation values ranging from  $r^2 = 0.002$  during the spring for the group of calves, to  $r^2 = 0.24$  being the highest correlation found in spring for the yearlings. Bouts of activity with no food intake occur in all seasons in both groups. Pearson's correlations returned low  $r^2$  values, demonstrating that only a very low percentage (up to 24% in the yearlings in spring) of daily activity is explained by feeding activity. All  $r^2$  and respective p values are shown in *Figure 23 (b)*, and were significant in all cases apart from summer (august) for yearlings.

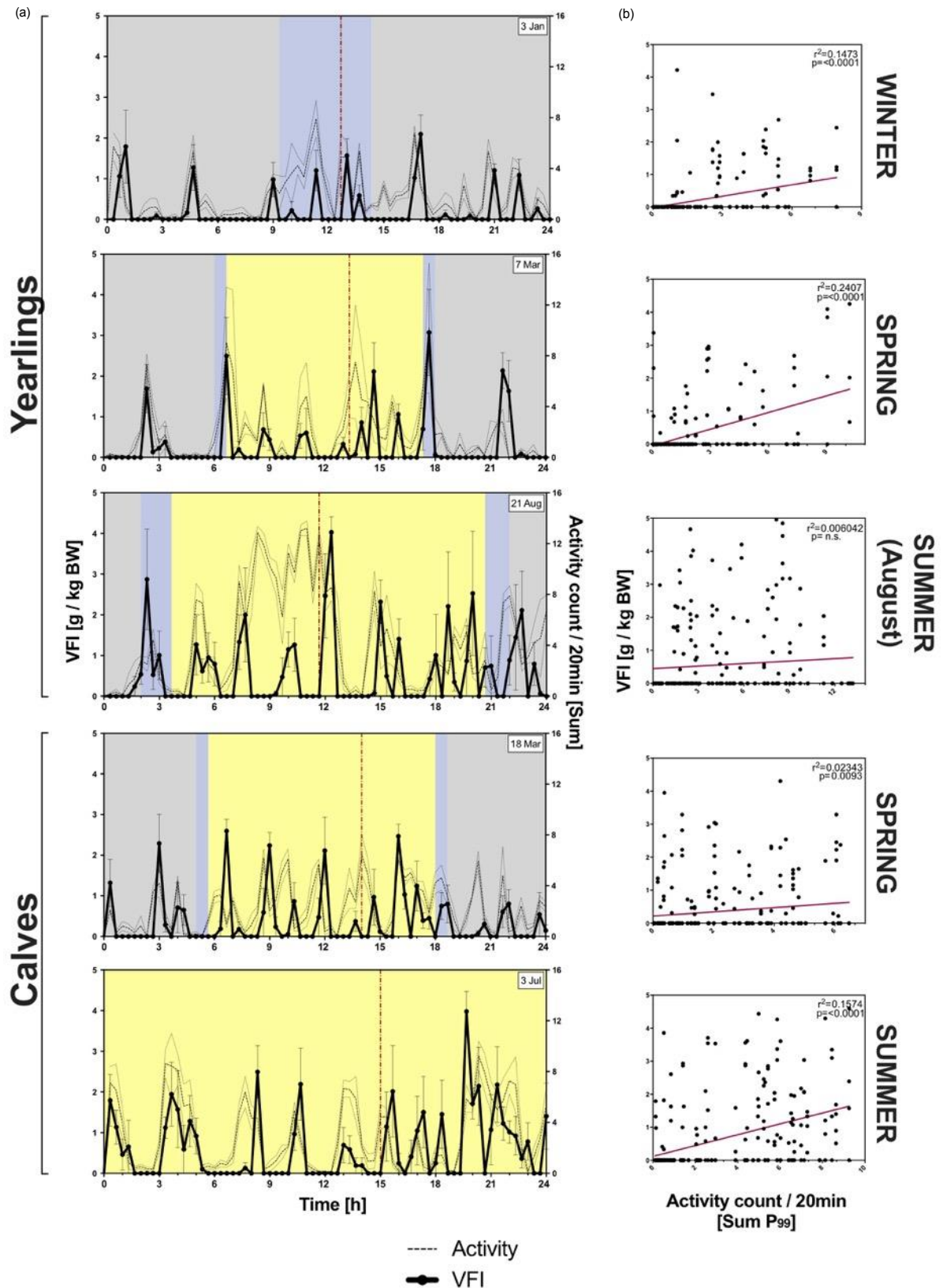


Figure 23. (a) VFI and activity across one representative day under different photoperiods. The colours on the graphs represent different photoperiods, being yellow the daylight, blue the civil twilights and grey the night. The left Y axis represents VFI (g/kg W<sub>B</sub>), and the right Y axis is the sum of activity in 20-minute bins, averaged between the animals in each group. Thick black lines represent mean VFI of the members of the group  $\pm$  SEM, and

the thin dotted lines represent activity mean of the members of the group, having thin outer and inner lines representing  $\pm$  SEM. The X axis is the Clock time (h). The specific date of measurement is written on the top right part of each graph. The horizontal dotted red lines represent the time of food refilling. In yearlings spring graph, activity n=2, because of equipment malfunction. (b). **Correlations between activity and VFI.** The X axis represents activity counts in 20-minute bins normalised to the 99 percentile, and the Y axis is VFI (g/kg W<sub>B</sub>). The correlations are for the same days as in the graphs to their left. The  $r^2$  value for each correlation is written in each graph, with a regression line plotted in red in each case.

Given the low correlation values between activity and VFI, the following percentages in *Table 4* have been calculated to determine the amount of activity that occurs during a day and does not explain feeding. The dates chosen for such calculations are the same dates as depicted in *Figure 23*.

*Table 4. Activity not explained by feeding.* The dates chosen for the calculations are representative dates which match those of *Figure 23*. The % depicted are the average of all the animals within the group (n=4).

<b>Average % of daily activity not explained by VFI</b>	
Yearlings winter 3 Jan	53.0 %
Yearlings spring 7 Mar	42.0 %
Yearlings summer 21 Aug	46.4 %
Calves spring 18 Mar	42.8 %
Calves summer 3 Jul	21.8 %

## Phase angle differences ( $\Psi$ )

The entraining diel rhythm provided by civil twilight was compared to endogenous rhythms in activity and voluntary food intake (see *Figure 24*).

The times of on- and offset for activity and VFI differ across seasons and between the two groups. Phase angle differences were only analysed for the winter and spring seasons since no activity data were available for autumn, and there is no civil twilight in summer. The data plotted are the average of three consecutive days and pooled from all animals within a group (n=4).

During winter, activity onset occurred on average 50 minutes before dawn. Feeding only commenced 20 minutes before twilight. Yearlings stopped being active circa 30 minutes after the offset of dusk, with no food intake associated. No corresponding data for activity and VFI were available for the group of calves because of instrumental problems.

During spring, activity of both yearlings and calves was in close synchrony with dawn, anticipating dawn by circa 10 minutes with active, but not feeding associated time.. Activity bouts linked to feeding always persisted until after dusk for both, yearlings and calves.

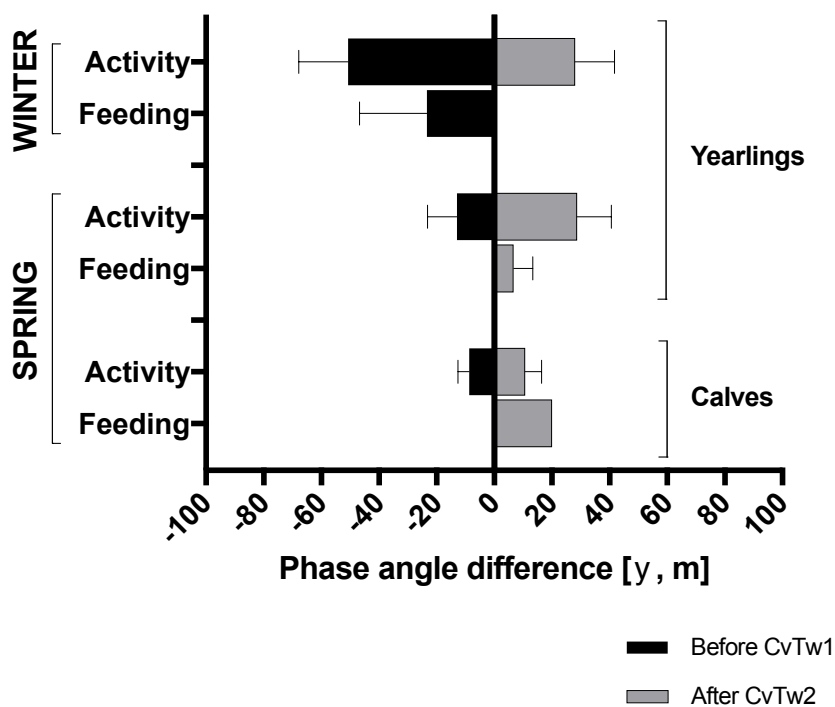


Figure 24. Phase angle difference ( $\Psi$ , min) for activity and for VFI bouts compared to the onset and offset of twilight. The data are from 3 consecutive days, and all animals in each group ( $n=4$ ). Exact dates can be found in Table 2. The abscissa value of zero represents the moment of onset and offset of civil twilight. Negative values (black bars) indicate activity or VFI bouts that start before civil twilight, during the night and prolong into the dawn. Positive values (grey bars) indicate activity or VFI that ends after the end of civil twilight, and prolong into the night. No winter data of both activity and VFI could be retrieved for the calves.

## Discussion

The main objective of this project was to assess the influence of diverse Arctic photoperiods on activity and feeding behaviour and the validity of activity data usage as a proxy for food intake in norwegian mainland reindeer. In order to do this, high-resolution measurements of voluntary food intake and locomotor activity were recorded simultaneously in two groups of reindeer differing in age.

This project investigated the distribution of locomotor activity and voluntary food intake of yearling reindeer (> 1 year) and calves (< 1 year) that inhabit a sub-Arctic area in northern Norway where photoperiod has extremes of constant light in summer and continuous dark in winter. Measurements of locomotor activity and voluntary food intake were carried out on both groups of animals

*This is the first study combining long term measurements of locomotor activity and voluntary food intake across seasons in Norwegian reindeer (*Rangifer t. tarandus*) and thus offers new insights into the chronobiology of both parameters.*

Due to a herding effect (van Oort *et al.*, 2007), individuals displayed low parameter variation and the relatively low sample size of four reindeer per group was thus sufficient to demonstrate daily and seasonal patterns of activity and voluntary food intake.

## Body weight

Yearlings and calves had comparable body weight at the beginning of their respective first autumn.

Many animals accumulate body fat during the summer and lose it gradually over winter, e.g. reindeer (Tyler *et al.*, 1999), groundhog (*Marmota monax*, Concannon *et al.*, 2001), homing pigeons (Sargisson *et al.*, 2007) and Svalbard ptarmigan (*Lagopus muta*, Mortensen *et al.*, 1983). These annual fluctuations in body weight are believed to be the result of the seasonal control of lipogenesis and lipolysis (Concannon *et al.*, 2001), or of changes in voluntary food intake (Tyler *et al.*, 1999).

Annual body weight fluctuations in reindeer are seen as an adaptation to the approaching season. Male reindeer accumulate fat towards the end of summer to supplement the rather poor quality winter forage, offering coverage against starvation (Tyler & Blix, 1990). Moreover, they can focus their attention on reproduction in autumn. It is also advantageous for females to accumulate fat at the end of summer, as this confers greater reproductive fitness given that they will be able to invest in the growing foetus and further lactate when their calves are born the following spring (Adamczewski *et al.*, 1987).

It has previously been demonstrated that reindeer have increased microbial activity and increased surface area of the rumen mucosa in winter to cope with seasonal forage variation (Mathiesen *et al.*, 2000). This additional seasonal adaptation of the digestive system allows them to maximise nutritional benefit from the poor-quality winter forage.

The results obtained in this project showed an expected decrease in body weight of yearlings during the winter months, followed by a weight increase starting in spring, in accordance with the previously mentioned studies. On the other hand, the calves showed steady body weight increase throughout the study. Studies on caribou (Adamczewski *et al.*, 1987) and cattle (Berg & Butterfield, 1976) showed that calves grow very rapidly during their first summer and autumn, and that most of the material accumulated is lean body mass rather than fat. Reports in cattle and sheep nutrition showed that the amount of digestible protein necessary for lean body growth without fattening is higher in younger animals (Armsby, 1921) than their older counterparts. The steady increase in body weight of the calves during their first year is probably the result of just such an increase in lean body mass.

## **Distribution of activity and voluntary food intake across the year**

Two groups of reindeer differing in age by one year were studied and we can demonstrate that *age influences activity and voluntary food intake*.



## Activity

The activity measurements taken in Vifta displayed similar patterns of distribution, length and number of bouts compared to measurements taken outdoors, in similar seasons (i.e. winter in vifta vs winter outdoors), however the intensity of recordings from outdoors was higher. This can be explained by the animals having greater space availability to move through and to reach their food troughs, taking also into consideration the different terrain inclinations outdoors, which are not present in Vifta. The adaptability of reindeer to the contrasting conditions during the arctic year are reflected in the varying activity displayed across the year.

The activity levels of the calves were relatively similar across the year, but the yearling group displayed large differences between seasons. The yearlings displayed significantly higher activity levels during the summer compared to spring and winter. Hagemoen and Reimers (2002) attributed similar results to the animals being harassed by insects, however, the calves studied in this project did not reflect this. Therefore, insect harassment is not considered to be influencing higher activity levels in summer in the group of yearlings. Higher activity levels during summer have previously been reported for different deer species: Svalbard reindeer (Loe *et al.*, 2007; van Oort *et al.*, 2007), red deer (Georgii, 1981), moose and roe deer (Cederlund, 1989). The lowest activity levels recorded for the yearlings are those in winter. According to Loe *et al.* (2007) the low winter activity levels are due to the longer times necessary to digest the low plant quality feed.

## Voluntary food intake

Previous studies in Svalbard and Norwegian reindeer have shown that feeding changes with seasons, with increased voluntary food intake during the summer months compared to winter (Nilssen *et al.*, 1984; Mesteig *et al.*, 2000). Moreover, the same results are obtained when the animals are fed *ad libitum* (Stimmelmeyr, 2001; Tyler *et al.*, 1999).

In this project voluntary food intake varied across seasons. The yearlings showed the lowest VFI levels in winter, increasing in spring and with the highest values in the summer. The calves showed the highest levels in their first autumn, lowering in winter

and then increasing through spring and winter. The high peak of VFI observed in the first autumn for the calves could be attributable to metabolism related to body size, where smaller animals have higher energy demand than larger counterparts, resulting in increased food intake (Allison, 1985).

The results obtained in this project are similar to those found in the studies mentioned above, given that all animals were provided with *ad lib* food, and still seasonal differences in voluntary food intake are noticeable.

## **Effect of photoperiod on activity and food intake**

*Photoperiod plays an important role in the synchronization of the circadian clock of reindeer.* Both, activity and food intake measured in this study, were influenced by photoperiod.

### **Activity distribution across a day**

It was originally inferred by Erriksson *et al.* (1981) that circadian rhythms in Scandinavian reindeer (*Rangifer tarandus L.*) serve to keep the members of a herd in synchrony.

The results obtained in this project from measurements of activity on the two different groups of reindeer showed a clear synchronization between members in each group. They are active during the day at similar times and with a similar intensity of locomotor activity, similar to results obtained by Collins and Smith (1989). Moreover, the synchronization between individuals seems to be stronger during winter, perhaps due to the lack of an entraining LD cycle, and the strengthening effect of the social group, in accordance with van Oort *et al.*, (2007 & 2008).

During winter, activity was distributed in even bouts across the clock, with no higher peaks of activity during civil twilight compared to the night, although the onset and offset of civil twilight served as a Zeitgeber for the start and end of activity bouts. In periods with day and night as in spring or late summer/autumn, nocturnal activity was reduced compared to the day. Civil twilight initiates the morning activity bout and ends the evening bout. However, the onset of night did not entirely stop activity. Colman *et al.* (2001) stated that reindeer are more active during the night than other ruminants.

This can be due to the gregarious nature of Scandinavian reindeer, where food availability might play a role, as well as the need to maintain stable gut microbiota (Maier & White, 1998), given the poor forage quality.

Summer activity bouts were longer lasting than the rest of the year, with intense activity occurring around the clock.

Although a previous study by Colman *et al.* (2001) reported no increase of activity related to sunset or sunrise, the results obtained in this study suggest a close relationship between the two, in agreement with Erriksson *et al.* (1981) in Scandinavian reindeer, and with other cervid species (Cederlund, 1989; Georgii, 1981; Green & Bear, 1990). In the data analysed in this project it is particularly noticeable the onset of activity synchronised with dawn. The contradictory statements could be due to the fact that Colman *et al.* (1981) examined sunset/sunrise, but not civil twilight as applied by the other studies.

It is worth mentioning that the experiments for this project were carried out in an area with anthropogenic disturbances that could not be abolished, like light and noise pollution from surrounding buildings and building sites and aeroplanes flying above. This could have affected the activity patterns in reindeer.

### Activity in individual corridors

The animals for this study were usually kept in outdoor semi-natural enclosures, where they have access to natural pastures depending on snow cover, and also are provided with a daily ration of pelleted food. For food intake measurements the reindeer were housed in individual corridors (Vifta) within sight of each other. In these corridors the animals were provided daily with *ad libitum* pelleted food. Activity under both conditions was comparable in the number and duration of activity bouts, but differing in amplitude. This could be explained by the space availability in their outdoor enclosures, where the distances to reach feeding patches or to roam are larger, as well as the differences in the terrain which are not present in their individual corridors.

Although the animals had no choice of food and less room to roam in the Vifta, the patterns of activity were not compromised. Paired t-tests confirmed this. This

corroborates the usefulness of the single housing facility at UiT for comparative studies in the future.

## **Behavioural responses to photoperiod**

*The studied reindeer showed increased activity and voluntary food intake with lengthening of photoperiod. Higher levels of both parameters were generally centred around civil twilight when it was present.*

### **Time spend feeding**

During autumn and spring, the time of the civil twilight associated with sunset is an important period of the day for the reindeer to feed, as it is a response meant to fill the rumen and support a steady release of nutrients during the night (Gregorini, 2012) when predatory risk is increased and well as it is the need for vigilance (Tyler *et al.*, 2016).

The results obtained in this study showed that the reindeer were active and feeding for a large part of the duration of dusk. According to Gregorini (2012), dusk is the time when pasture presents its maximum nutritional quality (Mayland *et al.*, 2005). In the present study, reindeer were always offered the same type of pelleted feed but still showed high feeding activity during dusk. Therefore, a preferential selection of dusk feeding associated with increased dusk forage quality is not represented in this study, but an endogenously driven need to fill the rumen for the night ahead.

The group of calves did not feed during dawn in spring or autumn, but waited until sunrise. The yearlings fed during dawn but spent less time on feeding than during dusk. This is in accordance with Gregorini (2012) who suggests that, although the forage presents its lowest nutritional value at dawn, hunger after the nocturnal fast is elevated and makes the animals seek food. On the other hand, a recent study (Johansen *et al.*, 2018) demonstrated that plants require specific light wavelengths for photosynthesis, which account for two thirds of the day in the Arctic summer. As a consequence, the nutritional value of the forage vary during the day.

In both, yearlings and calves, only 20% of the active time was occupied with feeding during the summer months. Skogland (1984) reported wild reindeer to feed up to 49% of the time in summer. Mårell *et al.* (2002) reported grazing and browsing in reindeer

during 79% of the time in summer. The difference in results can be attributable to the rest of activities associated with processing of the food e.g. search for food, mastication, regurgitation, rumination (Cederlund, 1989) that have not been measured in this project. Some of the grazing related activities such as mastication and rumination are not represented in the results from this project, given that the amount of locomotor motion associated with such activities is too small and the accelerometry devices are not as sensitive.

During winter, both calves and yearlings spent ca. 20% of the duration of civil twilight feeding ( $CvTw \cong 6$  h). Only 10% of the night phase was occupied by feeding in the group of yearlings, while the calves spent 20% of the night feeding. Skogland (1984) reported that grazing in wild reindeer lasted up to 44% of the time in winter, however those observations were carried out in southern Norway, and only during the light phase of the day which represents a maximum of 12 hours, therefore, data for the night is missing.

Day feeding prevailed during autumn and spring in the group of calves in accordance with their diurnal behaviour (Erriksson *et al.*, 1981). The yearlings also spent more time feeding in the day compared to the night during spring (LD 8:14, +2h CvTw), but less so in autumn (LD 10:12, +2h CvTw). This could be due to the night being longer than the day in autumn, driving rumen fill decrease and as a consequence increasing hunger and forcing them to feed at night.

## Meal number vs meal size

The group of calves had a higher number of meals and higher average meal size during autumn and winter, compared to yearlings. Autumn in particular was characterised by both, a larger number of meals and larger meal size compared to the yearlings, especially in the size of nocturnal feeds.

During summer both yearlings and calves had similar number and average size of meals.

According to Loe *et al.* (2007) the amount of time spent feeding at night should increase with body size in African ungulates. This statement refers to the fact that most African predators are nocturnal, and smaller prey animals are more vulnerable to predation. In this project opposite results to those of Loe *et al.* (2007) are observed. Although

reindeer predators are recognised in the area (wolverine, lynx and golden eagle; Jernsletten & Klovov, 2002), the reindeer used in this project have been bred and born in fenced facilities at the University of Tromsø and therefore have never been exposed to such threat. This might be one of the reasons why calves feed more frequently at night compared to yearlings. Moreover, the greater night feeding observed in calves could be due to the higher metabolism in the calves and constant need to refuel energy while building lean body mass (Adamczewski *et al.*, 1987; Allison, 1985; Berg & Butterfield, 1976). Previous studies have not differentiated age groups in their observations and this project demonstrates clearly that activity and feeding requirements is not equal across ages in reindeer in a herd.

## **Correlations between activity and voluntary food intake**

### VFI vs activity

The first noticeable thing from inspecting locomotor and voluntary food intake data simultaneously is that although many activity peaks correspond with food intake, not all do. Moreover, the intensity of activity does not reflect the amount of food intake. The spring season for the yearling group is characterised by the main bouts of food intake associated with the onset and offset of civil twilight, displaying most of the activity during the day with many small feeding bouts, and having shorter but more intense bouts of feeding during the night with reduced locomotor activity. The situation for the calves is slightly different. Food intake bouts are distributed throughout day and night, with no significant peaks during twilight. As a consequence, it is difficult to generalise the behaviour of a group of reindeer, given that it changes with season and age of the individuals.

The winter season is composed of short but intense isolated peaks of food intake during the night, although extra activity seems to concentrate during the lighter phase of the day, during civil twilight.

Summer displays ample bouts of activity and long feeding times in calves.

## Periodicity

*Periodograms confirmed the presence of circadian and ultradian patterns of activity in reindeer of different age, and across different seasons.*

### Circadian rhythms

Activity rhythms with a period length close to 24 hours were found in all animals and in all seasons including periods of constant light in summer and constant dark in winter, all with a significance of  $p < 0.001$  (see *Figure 18* & *Figure 19*). During winter, the yearlings had significant peaks of recurrence that deviated the most from 24h, with a range from 22 to 26 hours, while the calves had period lengths of exact 24 hours. During summer all animals kept a period length close to 24 h. It is worth mentioning that the animals were not free-roaming in a wild natural setting, and external factors such as surrounding buildings with anthropogenic noise and artificial lights, as well as aircrafts flying above could have conferred periodicity and a sense of timing to the reindeer, perhaps overruling innate photoperiodic responses. Moreover, these animals were regularly fed a ration of pelleted food, to allow them to meet their energetic demands. Attempts were made to keep the timing of food refilling as irregular as possible, although certain level of periodicity could not be avoided, one of the reasons being that if the animals were left without food refilling for more than ca. a day, they could run out of food.

This means that despite periods of constant light or constant darkness in summer and winter we cannot exclude the presence of entraining factors such as human activity, artificial light and maybe even regular feeding times. Just as the measurements for this study ended, Arnold *et al.* (2018) published an article on Svalbard reindeer in their natural environment. The authors of this study, too, found clear indication of synchronized activity and rumen temperature. Since the animals studied by Arnold *et al.* were not exposed to human activity or artificial light, this study corroborates our findings.

*The results obtained in this project denote that there is a functioning circadian clock throughout the year in Norwegian reindeer. A 24-hour period length is evident in all seasons, including constant light in the arctic summer and constant dark in the arctic*

winter. It is true that although the conditions seem constant during those periods, the light intensity of the Sun in summer or the luminance during twilight in winter are not constant, and might serve as a Zeitgeber to reindeer.

## Ultradian rhythms

Ultradian rhythms were found in all animals and all seasons. Both yearlings and calves displayed ultradian rhythmicity with similar period lengths, close to 5h during the winter. During spring and summer, the rhythms were of 4.8 and 5.4 hours respectively for calves, and 8.1 and 7 hours for the yearlings. It is clear that the age of the animals has an effect on behavioural clock output. Both calves and yearlings showed shorter ultradian period lengths in winter, compared to summer. This could be due to the shorter time spent feeding during winter that might not be sufficient to satiate the animals' hunger for a long time, hence the need to feed at shorter intervals compared to summer, reflecting this in shorter ultradian rhythms. Based on a model developed by Tyler *et al.* (2016), the trade-off between increased risk of attack by predators at night and hunger, can be attributed to the animals having shorter bouts of activity/feeding during constant darkness in winter by requiring a higher number of cycles to meet their energetic demands.

On the other hand, this might seem contradictory to the idea that reindeer retain their rumen fill for longer time in winter, given the poorer nutritional quality of the food (Loe *et al.*, 2007), and therefore should result in longer lasting ultradian rhythms.

Based on the results obtained in this project, one reason why shorter ultradian cycles are observed in winter can be attributed to the fact that during winter the animals almost exclusively feed on pelleted food, while in summer the easy availability of fresh pastures might lead to increased digestion times, extending the length of the ultradian rhythms. According to Pinares-Patiño *et al.* (2011), sheep digestion rates on pelleted food are higher than those measured on pastures.

Given the fact that the previous observations in free-ranging reindeer (Arnold *et al.*, 2018) reported shorter lasting ultradian longer rhythms in winter, compared to summer, the difference in duration could lay in the influence of pelleted feed, affecting digestion rates and consecutively hunger and behaviour. As a result, digestive processes in reindeer during the arctic summer and arctic winter seem to have great impact over



the regulation of activity, revealing only weak endogenous circadian control, in agreement with van Oort *et al.* (2007).

*Overall, the presence of ultradian rhythms during constant darkness in winter and constant light in summer was confirmed in all animals.* This was done via visual assessment in actograms that depicted free-running rhythms over long periods, and ultimately through periodograms.

## **Phase angle differences**

The diel rhythms of activity onset and VFI onset followed the pattern described by Tyler *et al.* (2016). The phase angle differences between onset of activity and onset of twilight were greater in winter than in spring. In the evening, offset of activity had a similar difference of ca.30 minutes compared to dusk offset, both in winter and in spring. However, activity and VFI were not always displayed simultaneously. It is noticeable how phase angle differences between activity and twilight, and between VFI and twilight are rarely of equal length.

## **Is activity a good proxy for food intake?**

### **Activity not explained by VFI**

Although VFI is always accompanied by locomotor activity, the opposite does not hold true.

Activities associated with grazing in ruminants were previously mentioned e.g. seeking for food, ruminating, etc. (Cederlund, 1989). However, there is an ample of other behaviours that are not linked to grazing and still represent an important part of the daily activity.

Penned calves and juvenile cattle show play behaviour as an important component of their social interactions (Jensen *et al.*, 1998). Cleaning of the antlers' velvet and rutting behaviour at the end of the summer are also reflected in locomotor activity logged by actiwatches, but again would not be associated with feeding. On the contrary, feeding in male reindeer during the rut is highly reduced (Tyler & Blix, 1990). During this time

males can lose much of their fat reserves in order to focus their attention on their group of females without the distraction of feeding.

All in all, the percentages of activity not representing feeding calculated in the results section can symbolise a series of other behaviours that are accompanied by movement of the animal.

*The use of activity as a measure of VFI will likely lead to an overestimation of the latter.*

## Summary

To date daily food intake has not been measured accurately in Rangifer. Logging activity data along with high-definition food intake measurements have helped towards filling this knowledge gap, allowing parallel analysis of these, two parameters.

Activity and voluntary food intake varied across the different seasons, displaying maximum values for both parameters in the summer months, and the lowest values in the winter.

Photoperiod is an important stimulus for the distribution of daily activity and voluntary food intake. The highest values for both measurements were mostly concentrated around the onset and offset of civil twilight, true to the crepuscular nature of reindeer. Light periods promote activity and VFI, while darkness reduce both.

Ultradian and circadian rhythms were found in all animals and across the different seasons, denoting the uninterrupted functioning the biological clock, although periods of constant light in the arctic summer and constant dark in the polar winter showed a weakening effect on the clock output.

Activity and voluntary food intake are not always a synonym of each other, therefore deducing one from the other might lead to the misinterpretation of the data.

Yearlings and calves had different approaches in terms of energy acquisition and conservation, where the calves tended to feed higher amounts and be less active than their older counterparts, pointing to differences in their metabolism.

## Outlook

This project provides novel information about the chronobiology of voluntary food intake and activity in Norwegian reindeer (*Rangifer t. tarandus*).

Differences were found between animals of different age, therefore this might be important to consider in future studies. Moreover, dissimilarities between animals of different sex and reproductive status are also important factors to be considered.

Locomotor activity has not proven to be a good proxy for food intake, therefore, future studies should consider them separately.

The UiT facility with individual corridors has proven to be satisfactory for comparative studies in reindeer.

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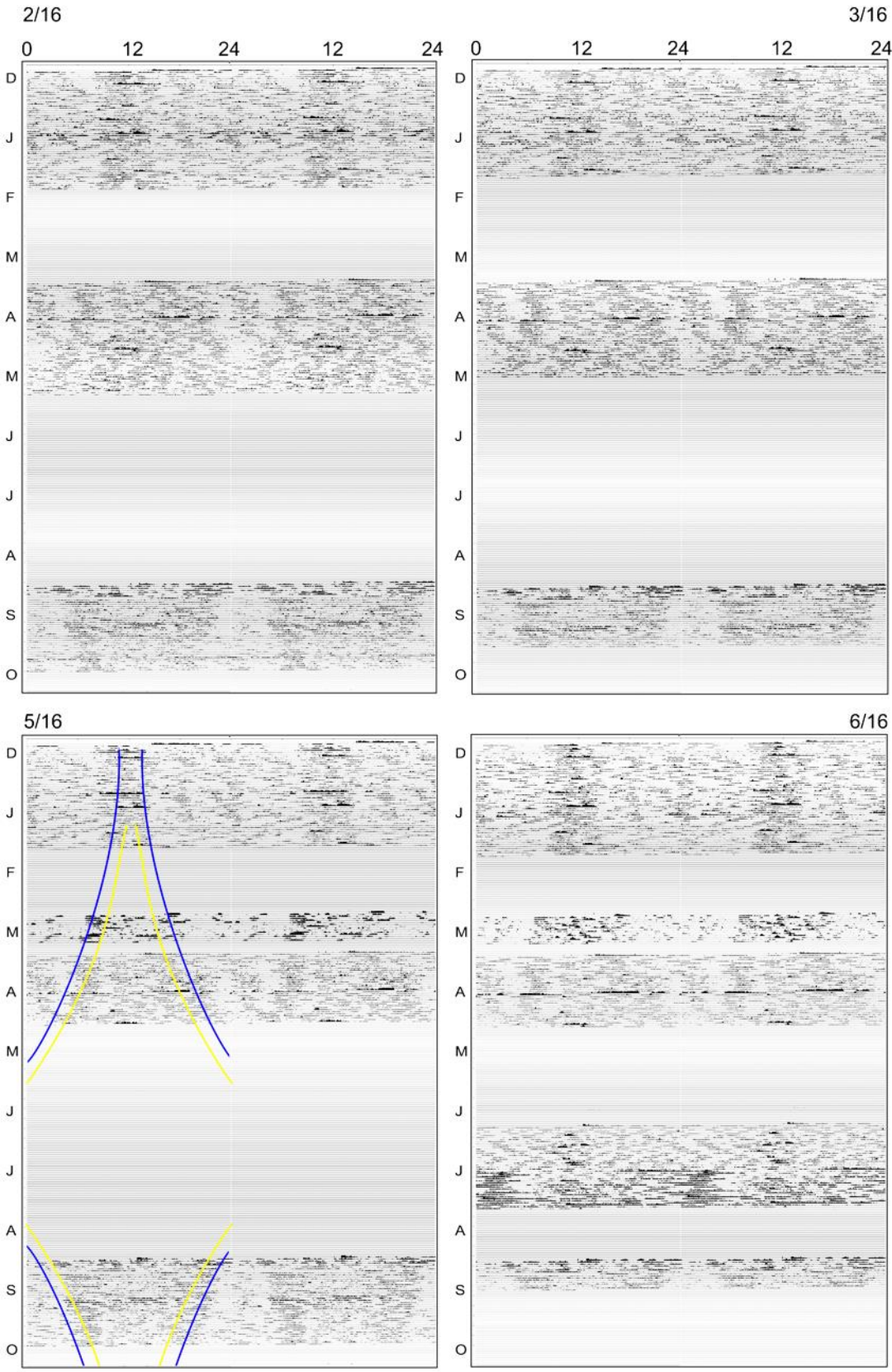


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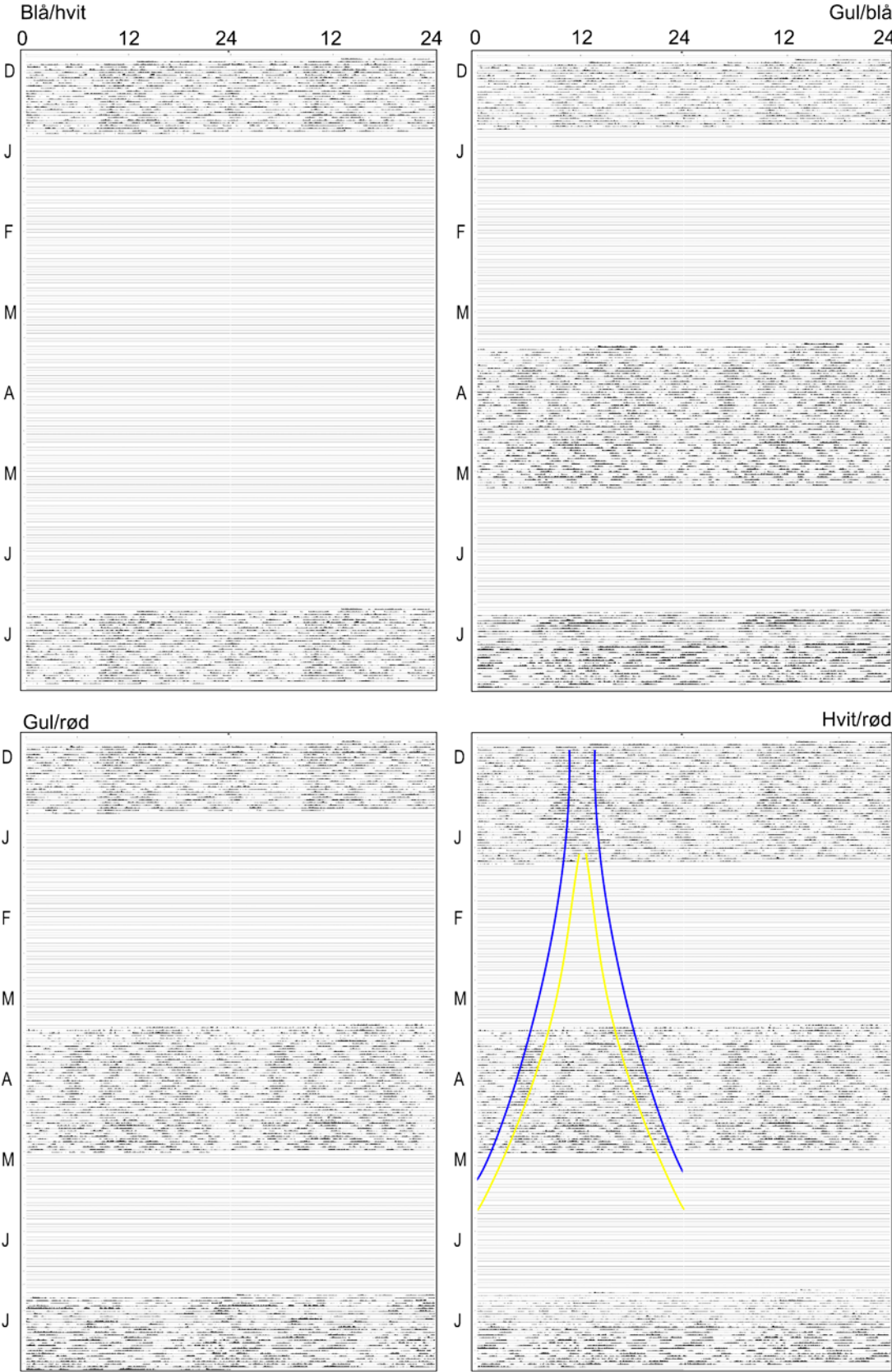
# Appendix

## Appendix A. Actograms for reindeer yearlings.



*Figure A. Actograms for reindeer yearlings.* Measurements lasted from December 2017 to October 2018, as represented by the letters of the months on the Y axis. The actograms are double-plotted for easier visualisation, clock time is given on the top X axis. Each actogram represents a different member in the group of yearlings, named above each graph. Data are normalised to 99 percentile. The yellow lines on the bottom left graph represent sunrise and sunset; the blue lines represent onset and offset of civil twilight.

# Appendix B. Actograms for reindeer calves



*Figure B. Actograms of reindeer calves.* Measurements lasted from December 2017 to July 2018, as represented by the letters of the months on the Y axis. The actograms are double-plotted for easier visualisation, clock time is given on the top X axis. Each actogram represents a different member in the group of calves, named above each graph. Data are normalised to 99 percentile. The yellow lines on the bottom right graph represent sunrise and sunset; the blue lines represent onset and offset of civil twilight.

## Appendix C. Post-hoc: Activity across seasons

*Table C. Tukey post-hoc tests comparing activity levels between yearlings and calves, and between different seasons.* Activity values are the average daily sums of 5 to 15 consecutive days (depending on season) of normalised activity data. Exact dates are given in Table 2. Data are pooled from all animals within each group. Exact dates are given in *Table 2*.

Tukey Post-hoc tests comparing Activity between groups of animals and between seasons						
	Mean 1	Mean 2	SE of diff.	q	df	P value
<b>Yearling vs Calves</b>						
Winter vs Winter	166.1	190	15.66	1.526	66	ns
Spring vs Spring	215.3	196.8	17.15	1.078	66	ns
Summer vs Summer	315.3	232.7	18.53	4.458	66	<b>0.001</b>
<b>Yearlings</b>						
Winter vs. Spring	166.1	215.3	15.66	3.142	66	<b>0.037</b>
Winter vs. Summer	166.1	315.3	17.15	8.697	66	<b>&lt;0.001</b>
Spring vs. Summer	215.3	315.3	17.15	5.829	66	<b>&lt;0.001</b>
<b>Calves</b>						
Winter vs. Spring	190	196.8	17.15	0.3964	66	ns
Winter vs. Summer	190	232.7	17.15	2.489	66	ns
Spring vs. Summer	196.8	232.7	18.53	1.937	66	ns

## Appendix D. Post-hoc: VFI across seasons

Table D. Tukey post-hoc tests comparing daily VFI levels between yearlings and calves, and between different seasons. Values are given in average daily g/kg  $W_B$  and data is pooled from all animals within each group. Dates corresponding to measurements are contained in Table 2.

Tukey post-hoc comparisons in VFI between groups and between seasons						
	Mean 1	Mean 2	SE of diff.	q	df	P value
<b>Yearling vs Calves</b>						
Autumn vs Autumn	17.27	49.61	3.99	11.46	20	<b>&lt;0.0001</b>
Winter vs Winter	10.9	14.79	3.257	1.687	20	ns
Spring vs Spring	22.07	24.57	3.257	1.089	20	ns
Summer vs Summer	40.25	39.61	3.99	0.2265	20	ns
<b>Yearlings</b>						
Autumn vs. Winter	17.27	10.9	3.99	2.259	20	ns
Autumn vs. Spring	17.27	22.07	3.99	1.699	20	ns
Autumn vs. Summer	17.27	40.25	3.99	8.144	20	<b>0.0003</b>
Winter vs. Spring	10.9	22.07	3.257	4.848	20	<b>0.0445</b>
Winter vs. Summer	10.9	40.25	3.257	12.74	20	<b>&lt;0.0001</b>
Spring vs. Summer	22.07	40.25	3.257	7.894	20	<b>0.0004</b>
<b>Calves</b>						
Autumn vs. Winter	49.61	14.79	3.257	15.12	20	<b>&lt;0.0001</b>
Autumn vs. Spring	49.61	24.57	3.257	10.87	20	<b>&lt;0.0001</b>
Autumn vs. Summer	49.61	39.61	3.99	3.545	20	ns
Winter vs. Spring	14.79	24.57	3.257	4.249	20	ns
Winter vs. Summer	14.79	39.61	3.99	8.799	20	<b>0.0001</b>
Spring vs. Summer	24.57	39.61	3.99	5.33	20	<b>0.0218</b>



## Appendix E. Multiple t-tests: daily distribution VFI across seasons

*Table E. Multiple t-test comparison between yearlings and calves on distribution of VFI across the day.* Values are given in % of total daily VFI consumed in each phase of the day. Values are from 3 consecutive days and pooled from all animals within each group. Exact days can be found in *Table 2*.

Multiple t-tests % VFI across different day phases						
Season	Phase	Mean Calves	Mean Yearlings	SE of diff.	t	P value
<b>Autumn</b>	Night	41.56	56.5	1.686	8.862	<b>&lt;0.001</b>
	CvTw1	0	4.581	1.021	4.487	<b>0.006</b>
	Day	48.14	22.41	2.515	10.23	<b>&lt;0.001</b>
	CvTw2	11.08	16.51	2.801	1.938	ns
<b>Winter</b>	Night	74.5	64.04	4.008	2.611	<b>0.040</b>
	CvTw1	25.5	35.96	4.008	2.611	<b>0.040</b>
<b>Spring</b>	Night	31.44	34.96	3.562	0.9869	ns
	CvTw1	0.8457	11.78	2.309	4.738	<b>&lt;0.001</b>
	Day	47.39	36.27	5.828	1.909	ns
	CvTw2	19.32	16.99	4.148	0.5625	ns

## Appendix F. Post-hoc: number of meals across seasons

*Table F. Tukey post-hoc tests comparing number of meals between yearlings and calves and across seasons.* Values given are average number of meals during a period of 3 days of food intake measurements, and from the 4 animals in each group, with the exception of the yearlings during the autumn sampling (n = 3 due to sickness in one animal). For specific dates see *Table 2*. A meal is defined as uninterrupted feeding that terminates when there is a 20-minute gap without feeding.

Tukey post-hoc comparisons in number of meals between groups and between seasons						
	Mean 1	Mean 2	SE of diff.	q	df	P value
<b>Yearling vs Calves</b>						
Autumn vs Autumn	7.333	10.71	0.6113	7.807	22	<b>0.000</b>
Winter vs Winter	6.833	8.75	0.566	4.789	22	<b>0.045</b>
Spring vs Spring	8.25	9.833	0.566	3.956	22	ns
Summer vs Summer	11.44	11.17	0.6113	0.6426	22	ns
<b>Yearlings</b>						
Autumn vs. Winter	7.333	6.833	0.6113	1.157	22	ns
Autumn vs. Spring	7.333	8.25	0.6113	2.121	22	ns
Autumn vs. Summer	7.333	11.44	0.6535	8.896	22	<b>&lt;0.001</b>
Winter vs. Spring	6.833	8.25	0.566	3.54	22	ns
Winter vs. Summer	6.833	11.44	0.6113	10.67	22	<b>&lt;0.001</b>
Spring vs. Summer	8.25	11.44	0.6113	7.39	22	<b>0.001</b>
<b>Calves</b>						
Autumn vs. Winter	10.71	8.75	0.566	4.893	22	<b>0.039</b>
Autumn vs. Spring	10.71	9.833	0.566	2.186	22	ns
Autumn vs. Summer	10.71	11.17	0.566	1.145	22	ns
Winter vs. Spring	8.75	9.833	0.566	2.707	22	ns
Winter vs. Summer	8.75	11.17	0.566	6.038	22	<b>0.006</b>
Spring vs. Summer	9.833	11.17	0.566	3.332	22	ns

## Appendix G. Post-hoc: meal size across seasons

*Table G. Tukey post-hoc tests comparing average meal size between yearlings and calves and across seasons.* Values given are average meal size during a period of 3 days of food intake measurements, and n=4 in each group, with the exception of the yearlings during the autumn sampling (n = 3 due to sickness in one animal). For specific dates see *Table 2*. A meal is defined as uninterrupted feeding that terminates when there is a 20-minute gap without feeding.

Tukey post-hoc comparisons in average meals size between groups and between seasons						
	Mean 1	Mean 2	SE of diff.	q	df	P value
<b>Yearling vs Calves</b>						
Autumn vs Autumn	10.04	14.8	1.13	5.954	22	<b>0.007</b>
Winter vs Winter	2.696	3.365	1.046	0.9042	22	ns
Spring vs Spring	11.32	7.567	1.046	5.073	22	<b>0.029</b>
Summer vs Summer	2.998	2.796	1.13	0.253	22	ns
<b>Yearlings</b>						
Autumn vs. Winter	10.04	2.696	1.13	9.195	22	<b>&lt;0.001</b>
Autumn vs. Spring	10.04	11.32	1.13	1.598	22	ns
Autumn vs. Summer	10.04	2.998	1.208	8.248	22	<b>&lt;0.001</b>
Winter vs. Spring	2.696	11.32	1.046	11.66	22	<b>&lt;0.001</b>
Winter vs. Summer	2.696	2.998	1.13	0.3774	22	ns
Spring vs. Summer	11.32	2.998	1.13	10.42	22	<b>&lt;0.001</b>
<b>Calves</b>						
Autumn vs. Winter	14.8	3.365	1.046	15.46	22	<b>&lt;0.001</b>
Autumn vs. Spring	14.8	7.567	1.046	9.779	22	<b>&lt;0.001</b>
Autumn vs. Summer	14.8	2.796	1.046	16.23	22	<b>&lt;0.001</b>
Winter vs. Spring	3.365	7.567	1.046	5.68	22	<b>0.011</b>
Winter vs. Summer	3.365	2.796	1.046	0.7698	22	ns
Spring vs. Summer	7.567	2.796	1.046	6.45	22	<b>0.003</b>

## Appendix H. Multiple t-tests: percent of time spent feeding across seasons

*Table H. Multiple t-test comparisons between yearlings and calves on percent of time spent feeding in each phase of the day.* Values are expressed as % of the duration of each phase of the day that the animals spend feeding. Values are from 3 consecutive days and pooled from all animals within each group (n=4), apart from summer, which only has n=3 because one animal was sick. Exact dates can be found in *Table 2*.

Multiple t-tests % of each day phase spent feeding						
Season	Phase	Mean Calves	Mean Yearlings	SE of diff.	t	P value
<b>Autumn</b>	Night	22.02	11.11	1.108	9.845	<b>0.000</b>
	CvTw1	5.556	18.52	9.034	1.435	ns
	Day	31.72	12.04	2.784	7.071	<b>0.001</b>
	CvTw2	58.33	44.44	11.23	1.237	ns
<b>Winter</b>	Night	19.44	10	2.239	4.219	<b>0.006</b>
	CvTw1	18.06	19.12	4.413	0.2407	ns
<b>Spring</b>	Night	21.09	10.24	1.742	6.232	<b>0.001</b>
	CvTw1	8.333	47.22	11.79	3.3	<b>0.016</b>
	Day	22.51	15.59	3.046	2.272	ns
	CvTw2	75	61.11	13.51	1.028	ns
<b>Summer</b>	Day	22.8	22.53	2.853	0.09467	ns

