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Drinking behaviour of llamas (*Lama glama*) and horses (*Equus caballus*) in response to saline drinking water

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SUMMARY

Drinking behaviour of llamas (*Lama glama*) and horses (*Equus caballus*) in response to saline drinking water

Saline drinking water can play an important role in the nutrition of domestic animals, either through the salinization of natural drinking water sources or the intentional application as part of rehydration therapy after exercises in horses. Whereas sodium (Na) constitutes an essential nutrient for several body functions in animals, excessive Na intake could affect feed and water consumption, and even cause death. Therefore, animals possess various strategies to prevent an excessive ingestion of Na and avoid detrimental effects on their health. However, there is still lack of knowledge on the sensitivity of llamas (*Lama glama*) and horses (*Equus caballus*) to varying NaCl concentrations in their drinking water. Using the example of two different digestive strategies, such as foregut fermenters (llamas) and hindgut fermenters (horses), the extent to which differences exist in their salt sensitivity in drinking water was investigated.

As a holistic approach, animal behaviour was examined in choice experiments, where the animals (n = 12 llamas, and n = 6 horses) could choose between different salt concentrations. The aims were to provide more insight into drinking behaviour of both species and to determine their behavioural adaptation strategies in discriminating and selecting different concentrations of saline water in choice tests. For both species, the same experimental design was applied which consisted of three phases: (1) a control for 1 week with only fresh water provided in two buckets, (2) a pairwise preference test for 3 weeks offering in two buckets the choice between fresh water and a saline solution with stepwise increasing NaCl concentration (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5%), and (3) a free-choice test for 3 weeks with six simultaneously provided buckets containing 0, 0.25, 0.5, 0.75, 1.0, or 1.25% NaCl, respectively. The body weight, body condition score and intake of water, feed and Na were recorded. Extensive 24-h video recordings were used to evaluate the duration and frequency of drinking behaviour.

The results demonstrated that both species possess a remarkable capacity to differentiate between saline water concentrations in two different experimental setups and to adjust their Na intake in self-selection. The typical reaction with higher Na intake was to avoid very high Na concentrations or to increase the total water intake. Both species had an outstanding interest in low-concentrated saline water without compromising their health or performance. The detailed analyses of llamas' drinking behaviour revealed their capacity for behavioural adaptation when more choice options

were offered by changing their drinking pattern with increased frequency of drinking and testing, while maintaining their diurnal rhythm of water intake.

KURZFASSUNG

Wasseraufnahmeverhalten von Lamas (*Lama glama*) und Pferden (*Equus caballus*) als Reaktion auf salzhaltiges Tränkewasser

Nutztiere können in verschiedenen Situationen mit salzhaltigem Tränkewasser in Berührung kommen, z. B. durch die Versalzung natürlicher Wasserquellen, zu welchen die Tiere Zugang haben, oder als Teil einer Rehydrierungstherapie bei Pferden nach intensivem Ausdauersport. Obwohl Natrium (Na) ein essentieller Mineralstoff für zahlreiche Körperfunktionen ist, kann eine übermäßige Na-Aufnahme die Futter- und Wasseraufnahme beeinträchtigen oder sogar zum Tod führen. Daher verfügen Nutztiere über verschiedene Strategien, um eine übermäßige Aufnahme von Na zu steuern und gesundheitsgefährdende Auswirkungen zu vermeiden. Es gibt jedoch nur wenige Erkenntnisse über die Empfindlichkeit von Lamas (*Lama glama*) und Pferden (*Equus caballus*) gegenüber verschiedenen NaCl-Konzentrationen im Tränkewasser.

Am Beispiel von zwei unterschiedlichen Ernährungstypen, dem Lama (Vormagenfermentierer) und dem Pferd (Dickdarmfermentierer) wurde untersucht, inwieweit Unterschiede in der Empfindlichkeit gegenüber Salz im Tränkewasser bei diesen bestehen (n = 12 Lamas und n = 6 Pferde). Als ganzheitliche Methode wurde das Tierverhalten in Wahlversuchen überprüft, wobei die Tiere zwischen verschiedenen Salzkonzentrationen wählen konnten. Das Ziel der Untersuchungen war es, mehr Einblicke in das Trinkverhalten der beiden Tierarten zu gewinnen und deren Verhaltensanpassungsstrategien bei der Unterscheidung und Auswahl verschiedener NaCl-Konzentrationen im Tränkewasser zu bestimmen. Die Versuche wurden für beide Tierarten in jeweils drei Phasen durchgeführt: (1) Kontrollphase (1 Woche), in welcher Süßwasser in zwei Eimern gleichzeitig angeboten wurde, (2) Paarweiser Präferenztest (3 Wochen), mit zwei Eimern in denen jeweils einer mit Süßwasser und ein weiterer mit einer Salzlösung angeboten wurde, deren NaCl-Konzentration schrittweise anstieg (0,25; 0,5; 0,75; 1,0; 1,25 oder 1,5%), und (3) Cafeteria-Test (3 Wochen), mit freier Auswahl aus sechs gleichzeitig bereitgestellten Eimern, die jeweils 0, 0,25; 0,5; 0,75; 1,0 oder 1,25 % NaCl enthielten. Das Körpergewicht und die Körperkondition, sowie die Aufnahmen von Wasser, Futter und Na wurden über alle Phasen hinweg aufgezeichnet. Umfangreiche 24-Stunden-Videoaufzeichnungen wurden außerdem zur Analyse des Trinkverhaltens der Lamas genutzt.

Die Ergebnisse der Experimente zeigten, dass Pferde und Lamas eine bemerkenswerte Fähigkeit zur Unterscheidung zwischen unterschiedlichen Salzwasserkonzentrationen in zwei verschiedenen Versuchsanordnungen besitzen und ihre Na-Aufnahme durch Selbstselektion anpassten. Die typische Reaktion auf eine höhere Na-Aufnahme bestand darin, entweder sehr hohe Na-Konzentrationen zu vermeiden oder die gesamte Tränkewasseraufnahme zu erhöhen. Außerdem zeigte sich, dass sowohl Pferde als auch Lamas ein außerordentliches Interesse an Wasser mit leicht erhöhten NaCl-Gehalten hatten, ohne dass dies ihre Gesundheit oder Leistung beeinträchtigte. Bei Lamas wurde beobachtet, dass diese ihr Trinkverhalten durch eine erhöhte Trink- und Testhäufigkeit anpassten, wenn mehr Wahlmöglichkeiten angeboten wurden, ohne jedoch ihren Tagesrhythmus der Wasseraufnahme zu verändern.

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ABBREVIATIONS

ADFom	Acid detergent fibre expressed exclusive of residual ash
ADL	Acid detergent lignin
aNDFom	Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash
ANOVA	Analysis of variance
BCS	Body condition score
BW	Body weight
CL	Crude fat (lipids)
CP	Crude protein
d	Day
DM	Dry matter
DMI	Dry matter intake
e.g.	exempli gratia
Fig.	Figure
GP	Gas production
h	Hour
i.e.	id est
LDT	Lower discrimination threshold
LS	Least squares
ME	Metabolizable energy
n.d.	Not detected (below detection limit)
PRT	Preference threshold
RET	Rejection threshold
SAC	South American camelids
SD	Standard deviation
SEM	Standard error of the means
TWDI	Total drinking water intake
TWI	Total water intake
UDT	Upper discrimination threshold

CHAPTER 1

General introduction

Domestic animals have used various adaptation strategies over the course of their domestication, in order to cope with specific environmental or nutritional requirements. These conditions may comprise the occurrence of stressors, such as extreme temperatures, and scarcity or salinization of drinking water. In order to withstand such conditions and additionally still maintain productivity, animals possess various stress response strategies, predominantly demonstrated by their physiology and behaviour.

The general process of water salinization describes the increasing concentration of total dissolved solids, as milligrams per litre water, mainly influenced by the concentration of cations (e.g. Ca^{2+}, Mg^{2+}, K^{+} or Na^{+}) and anions (e.g. HCO_3^{-}, CO_3^{2-}, SO_4^{2-} or Cl^{-}) in the water (Millero, 1974). With respect to the effect of NaCl in the drinking water of animals, different pathways need to be distinguished. Firstly, it could occur involuntarily, through salinization of ground water and soil (Pillsbury, 1981), which could either be a result of long-term geologic processes or anthropogenic activities, such as mining, irrigation or the usage of salt or salt-containing fertilizers (Böhrer et al., 1998; Foster et al., 2018; Flörke et al., 2019). Pillsbury (1981) investigated the increasing salinity of some North American rivers and described that a disturbed natural salt balance will consequently lead to accumulation of salts in the unsaturated zone or the drainage water. This effect is facilitated due to the diversion of freshwater, irrigation, evapotranspiration and salt accumulation in the soil, leading to an increased salinity in the downstream discharge water when the saline drainage flows back to the river (Pillsbury, 1981). However, global climate change, with its effects on increasing drought and flood events, as well as the rise in sea level, is projected to increase the drinking water salinization in long-term (Aragüés et al., 2015; Jeppesen et al., 2015). Especially in the water-scarce arid and semiarid zones (e.g. parts of South America, North America, the Middle East, Central Asia and Australia), with dilution capacities of rivers and lakes being lower and high usage of irrigation, salinization of drinking water poses a considerable challenge for livestock production (Williams, 2001; Vengosh, 2004; Foster et al., 2018). Secondly, salt stress in animals might also occur deliberately, through purposeful administration of saline water, which is administered with the aim to replace electrolyte and water losses via sweating, emerging due to excessive activities. During physical work or exercise, animals, such as horses or cattle used for sport, draft or transport, lose great amounts of sweat, which is required for their thermoregulation (McDowell and Weldy, 1967; Collier et al., 1982; Meyer et al., 1990; NRC, 2007). This sweat contains considerable amounts

of minerals, such as Na, K and Cl, whose amounts are highly correlated with the sweating rate and might increase during high environmental temperatures and increased perspiration rate, possibly resulting in significantly increased water and electrolyte requirements (Jenkinson and Mabon, 1973; Suttle, 2010), as shown in cattle under heat stress for K (Collier et al., 1982; Mallonée et al., 1985) or horses during exercising for Na (Coenen, 2005).

Na plays an important role as mineral in the nutrition of animals. It represents the major extracellular cation in their extracellular fluid and is therefore essential for maintaining normal osmotic pressure (Loly and Hopkinson, 2021). Furthermore it is also responsible for the maintenance of the acid-base balance, the blood pressure regulation and is required for the water regulation (Michell, 1989, 1995). For herbivores, additional Na is essential in their diet due to usually low occurrence of Na in soil and plants, whereas carnivores fulfil their Na requirements usually by feeding on other animals which possess high Na concentrations in their extracellular fluids (Yu et al., 1997; Ritz, 2012). When the intake of Na is lower than required, animals attempt to conserve more of it by reducing the Na excretion rate (French, 1955). In herbivores, a deficiency of Na in the diet could possibly result in increased appetite for Na salts (Dethier, 1977; McDowell, 1996; Liedtke et al., 2011), as shown for example in sheep (Denton and Sabine, 1961) and goats (Perry, 1984). Such natural craving behaviour is often indicated by the chewing and licking on wood, rocks, soil or bones (Hebert and Cowan, 1971; Perry, 1984). However, if salt deficiencies cannot be counterbalanced, animals could eventually become lethargic, lose appetite, develop growth deficiencies or have reduced milk yields (French, 1955; NRC, 2005).

For wild herbivores, especially ungulates, such as deer (Weeks and Kirkpatrick, 1976), elks (Dalke et al., 1965), goats (Hebert and Cowan, 1971) or moose (Botkin et al., 1973), but also rabbits or kangaroos (Blair-West et al., 1968; Denton, 1984), a migration behaviour from Na depleted areas towards salt sources emphasizes their strong salt seeking motivation. Interestingly, Rothman et al. (2006) even demonstrated that mountain gorillas, living in sub-humid and humid tropics, with Na levels in the staple forage being much lower than required, feed on decaying wood as an important source for Na.

The specific appetite for Na seems to be rather the result of an innate mechanism than of learning but could additionally be enhanced by experience (Fitzsimons, 1980). For herbivores, it was also demonstrated that Na depleted sheep can replace their Na deficit, when offered a saline solution with Na salts ($NaHCO_3$ or NaCl) within a few minutes

(Denton and Sabine, 1961). Interestingly, the sheep were able to accurately set the volume of a distinctive saline solution before the absorption of the water into the blood occurred. This led Denton and Sabine (1961) to the conclusion that there might be a central integration of taste-impulses from the tongue. These signal the concentration with pharyngo oesophageal proprioceptor impulses by metering volume swallowed. These receptors can serve to correct deficits and prevent against hypersalinity (Dethier, 1977). This mechanism can be explained by the need of wild herbivores to rapidly ingest water and salt to avoid predation by carnivorous predators (Liedtke et al., 2011). On the other side, animal species, which do also feed on meat, e.g. cats, dogs or rats, do also possess a certain appetite for Na, especially when depleted, though it is distinctively less pronounced than for herbivores (Carpenter, 1956).

Despite the occurrence of a certain appetite for NaCl in some animal species, voluntary ingestion of excessive amounts of Na could have intoxicating effects due to an increase in the NaCl concentration in their blood. Usually, animals can tolerate excessive intakes of NaCl through increased ingestion of drinking water to regain an isotonic state, which again might potentially increase the renal salt excretion through urine. However, this mechanism only works as long as continuous access to fresh water is provided, as shown e.g. for cattle (Weeth et al., 1960) or dogs (Kanter, 1953). Generally, increased salt ingestion via drinking water always leads to increased fresh water intake. However, this reaction is limited depending on the salt concentration, which cannot easily be balanced through increased fresh water intake. Also, if fresh water is not freely available, clinical signs such as a reduced appetite, weight loss, diarrhoea, incoordination, convulsions, oedemas, and eventually higher mortality, occurred more frequently in various animal species, such as cattle (Weeth and Haverland, 1961; Trueman and Clague, 1978; Nestor et al., 1988), sheep (Peirce, 1957) or even different poultry species (Krista et al., 1961).

Salt tolerance of an animal can be described as the ability to endure the ingestion of salt in the diet and could, for saline drinking water, be expressed as the maximum tolerable level of NaCl in the drinking water that can be safely ingested without pronounced adverse effects on health and production performance (NRC, 2005). However, tolerance does not necessarily include the voluntary acceptance or even preference of a certain NaCl concentration in the drinking water under normal physiological conditions. Sensitivity describes the sensory perception, such as vision, taste, smell, and touch, through receptor potentials. Based on that, the preference depends on the sensitivity of the receptors but does not necessarily correspond with the tolerance. If animals do not possess the adequate sensitivity, the preference could surpass the actual tolerance and

might be detrimental for the animals. The preference is expressed by the behaviour of animals, which, however, presupposes the possibility to choose. If salt is administered without the *ad libitum* access to fresh water, animals cannot choose freely according to their appetite or physiological requirements but rather ingest whatever is offered.

It is therefore advisable to identify the sensitivity of various animal species towards different NaCl concentrations in the drinking water under controlled conditions. These results might give more insights into the prospects of saline water as drinking source for animals in regions with naturally salinized water sources or as source of electrolytes for exercising animals. Nevertheless, there is lack of knowledge on the choice behaviour and preference for various NaCl concentrations in the drinking water of livestock and the animals' capacity to adjust their consumption according to physiological and nutritional requirements to avoid detrimental effects.

The sensitivity to Na depends on several factors. Whereas domestic animals often tolerate higher intakes of Na via the feed (Digby et al., 2011), the sensitivity towards Na in the drinking water seems different, usually resulting in lower Na intakes via drinking water (Goatcher and Church, 1970), probably due to different physiological responses (Masters et al., 2005) and the reduced taste enhancer effect (Ginane et al., 2011). Furthermore, additional factors, such as the individual animal, the different animal species (Goatcher and Church, 1970) and differences in their digestive system, the provided diet (Wilson and Dudzinski, 1973), the animals' age or physiological stage (Midkiff and Bernstein, 1983; Runa et al., 2019), might influence the discrimination behaviour towards saline solutions and have to be considered. For example, it was shown that ruminants are less sensitive to Na in their feed (Digby et al., 2011) than other species, such as poultry (Smith et al. 2000).

Preference tests could be an useful method to assess the choice behaviour of animals in situations where different saline solutions are offered at the same time, as reviewed for choice feeding in ruminant livestock by Meier et al. (2012). Whereas most choice experiments were conducted on the feeding behaviour of animals, especially ruminants, there are only a few studies regarding the discrimination behaviour of domestic animals in response to saline drinking water. Clearly, behavioural data and water intake rates could provide better understanding of drinking behaviour in response to saline drinking water.

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CHAPTER 2

Scope of the thesis

As outlined in Chapter 1, animals could be confronted with saline drinking water in various situations. Although there exist some data on few animal species about their tolerance and appetite towards saline drinking water, there is lack of knowledge on the sensitivity related to it. Moreover, there is a scarcity of data for animals with other digestive systems, like other foregut fermenters, such as llamas and alpacas, or hindgut fermenters, such as horses or rabbits. For the experiments of this study, llamas were chosen as representative for foregut fermenters due to their exposure to saline water in their natural environment, the high Andes. For hindgut fermenters, horses were chosen as example, due to their high Na losses via sweat during exercises. Against this background, the aim of the thesis was to evaluate the responses of llamas and horses towards variations of NaCl in their drinking water, which could assess their salt tolerance and their capacity to differentiate various NaCl concentrations in their drinking water. Furthermore, another intention was to provide more knowledge on the drinking behaviour and the diurnal rhythm under controlled stable conditions, including the evaluation of behavioural strategies of the animals in selecting different concentrations of saline water.

For both species, the same experimental design was applied. Prior to the confrontation with saline drinking water, the normal drinking behaviour of the animals was evaluated. Two different choice experiments were chosen as method to evaluate the taste discrimination behaviour towards saline drinking water in the llamas and horses. Firstly, a pairwise preference test with the choice between fresh and saline water with ascending NaCl concentrations was conducted. The differentiation between two offered solutions provides valuable data about the individual sensitivity and taste discrimination towards particular NaCl concentrations in the water. Secondly, a free-choice (cafeteria) test, including the simultaneous presentation of six buckets containing various NaCl concentrations, was conducted to determine the long-term intake of saline water. That experimental setup provided more information about the animals' capacity to adjust their ingestion of NaCl according to their nutrient requirements and to avoid possible impairments. While the pairwise preference test provided more short-term information, the second test revealed the long-term adjustments to saline drinking water.

The first (Chapter 3) and second (Chapter 4) manuscripts focus on the responses of horses and llamas related to nutritional aspects. This includes adaptive changes in their body condition, water intake, feed intake and sodium intake as reaction to the ingestion of saline drinking water. The experiments followed the same experimental design for

both animal species, which allows the post-experimental interspecific comparison. The aims of these experiments were to determine the preferred NaCl concentrations of horses and llamas in their drinking water. Additionally, the taste discrimination and individual sensitivity, as well as the long-term responses of the animals to saline water intake, were evaluated. The third manuscript (Chapter 5) provides more detailed results on the behavioural responses for the same experiment, based on individually-taken 24-h video recordings of all llamas. The diurnal drinking rhythm was examined and the decision making behaviour assessed when confronted with the choice between different NaCl concentrations in the drinking water to identify their preferred NaCl concentration. Finally, the General Discussion (Chapter 6) outlines the key results of the Chapters 3 to 5, and discusses them in a broader context. The results of both animal species are compared and various impact factors on their sensitivities to saline drinking water are discussed.

The manuscripts in Chapter 3 and 4 are published in scientific journals, whereas Chapter 5 was submitted for publishing in a scientific journal as part of this cumulative thesis. Each of these journals had different instructions and requirements for their layout and citation style. In order to be more consistent in appearance, the font was made uniform for the entire thesis.

CHAPTER 3

Sensitivity of ponies to sodium in the drinking water

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Abstract

Horses lose high amounts of Na through excessive sweating. These fluid losses can often not be replaced completely by voluntary water intake, requiring saline solutions as rehydration therapy to regain electrolyte balance. The experiment aimed to evaluate the sensitivity and tolerance of Shetland ponies towards different Na concentrations in their drinking water and contained three phases: (1) control: only fresh water provided, (2) pairwise-preference test: choice between fresh water and saline solution with stepwise increasing sodium chloride (NaCl) concentration (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5%), and (3) free-choice test: six simultaneously provided buckets containing NaCl concentrations of 0, 0.25, 0.5, 0.75, 1.0, or 1.25%. During the pairwise test, the ponies did not distinguish between fresh and 0.25% NaCl-water, but demonstrated clear preference for 0.5%, whereas > 0.75% NaCl was avoided/rejected. During the free-choice test, a pronounced preference of fresh over saline water was exhibited. The Na intake via salt lick was not reduced as response to higher Na intakes via water. The ponies exhibited a remarkable sensory discrimination capacity to detect different NaCl concentrations in their drinking water. The acceptance of solutions with low NaCl levels (0.25/0.5%) without adverse effects demonstrates potential as rehydration solution for voluntary intake.

1. Introduction

Sodium (Na) is an essential nutrient in animals and plays an important role in several body functions. It is not only the major extracellular cation, controlling the acid-base balance, osmotic regulation and the nutrient transport across cell membranes, but it is also critical for a regular function of muscles, the central nervous system and water metabolism (Rose, 1990; Johnson, 1995; Michell, 1995).

In horses, loss of Na via sweat plays an important role in their Na metabolism because they rely on sweating as major mechanism for heat dissipation during exercise (Meyer et al., 1990; Cohen et al., 1993; Coenen, 2005; McCutcheon & Geor, 2014). Horse sweat is hypertonic in relation to the plasma and contains considerable amounts of Na, ranging between 2.0 and 5.7 g/L (reviewed by Spooner et al., 2010) However, the sweat glands do not conserve Na, regardless of the horse's body stores (Lindner et al., 1983; Spooner et al., 2010). Sweat losses of around 1 L/100 kg body weight (BW) per hour during light exercise under temperate conditions (in Welsh ponies and crossbreeds, Meyer et al., 1990) may increase to 2 – 2.5 L/100 kg BW per hour when exercised in hot, humid conditions (NRC, 2007), thus leading to excessive Na losses. Accordingly, horses' daily maintenance Na requirement of 0.02 g/kg BW, may increase to about 3.1 g/kg BW due to exercise-induced Na losses via sweat (Coenen, 2005; NRC, 2007).

The level of plasma Na concentration plays a key role as primary stimulus for thirst and rehydration (Andersson, 1978; Fitzsimons, 1998). However, as sweating results in both water and electrolyte losses, the increases in plasma osmolality may not be high enough to trigger sufficient thirst (Butudom et al., 2002). Several studies have been conducted to counteract an incomplete voluntary replacement of lost body fluids in exercised horses. Investigations on forced hyperhydration included the application of oral electrolyte pastes or the administration of solutions via a nasogastric tube (Sosa León et al., 1997; Sosa León, 1998). However, non-voluntary Na intakes may cause adverse effects, as Zeyner et al. (2017) showed, that the provision of 100 g NaCl per day to horses in moderate work via the feed could also be associated with postprandial acidosis. Likewise, ulcer numbers and severity scores in the non-glandular stomach were significantly increased in horses under maintenance when repeatedly administered a commercial electrolyte supplement by dose syringe orally (Holbrook et al., 2005).

On the other hand, only few studies investigated the voluntary intake of saline water in horses as rehydration strategy during endurance exercise (Nyman et al., 1996; Butudom et al., 2002). Generally, horses have demonstrated their capacity to select their diets based on odour, taste or nutrients in choice-experiments (Goodwin et al., 2005a; Goodwin et al., 2005b; van den Berg et al., 2016a; Janczarek et al., 2018). With regard to solved electrolytes, Randall et al. (1978), in two-choice preference tests, showed that immature weaning foals discriminated different concentrations of sweet, salty, sour and bitter solutions, expressing a preference for water sweetened with sucrose and an aversion for water with sour taste. Saline water was consumed until 0.6% NaCl concentration, but further increases to 5% in NaCl concentration resulted in a strong rejection (Randall et al., 1978). Similar reactions to sour and sweet solutions were also described for mature horses of various breeds (Danel & Merkies, 2009; Merkies & Carson, 2011). When water with different salt concentrations (0.25 – 3.25% NaCl) was offered as sole drinking water, Somali donkeys only tolerated saline water until 1.0% NaCl, but decreased their BW when confronted with higher NaCl concentrations in their drinking water (Maloiy, 1970).

The effectiveness of the voluntary intake of saline water as rehydration strategy in exercised horses was shown in the study by Nyman et al. (1996). During endurance training (62 km ride), Arabian and crossbred horses had repeatedly access to either 1) tap water, 2) water after salt paste *per os* or 3) saline water (0.9% NaCl), respectively. Horses of the saline water treatment had the highest fluid intake and fastest compensation for their BW losses. Similarly, Butudom et al. (2002) evaluated in 2-year old Arabian geldings different initial rehydration solutions (tap water, 0.45% or 0.9% NaCl

solution) after a furosemide administration and treadmill exercise. The total amount of fluid imbibed during the 60 min recovery period was 11.4, 16.6 and 18.5 L for plain water, 0.45 and 0.9 NaCl concentrations, respectively. Both studies underline that the provision of saline water for voluntary intake as the initial rehydration after endurance exercise is superior to tap water only, which might rather inhibit thirst and delay recovery.

However, knowledge on voluntarily accepted Na concentrations in horses is limited. Therefore, our study was designed to evaluate the responses of ponies towards variations of NaCl in their drinking water. The aims of the study were to determine the preferred NaCl concentration in the drinking water of horses, which might then be used as strategy for voluntary replacement of water and salt losses after endurance exercises. Taste discrimination and individual sensitivity of our ponies were evaluated in pairwise preference tests after Goatcher & Church (1970b), where fresh water and an alternative solution with ascending NaCl concentrations were offered simultaneously (Enke et al., 2022b). Subsequently, a free-choice test was performed (Raffa et al., 2002) for three weeks. Six water buckets with different NaCl concentrations (0 – 1.25%) were simultaneously offered to measure the animals' long-term responses to saline water intake and assess their preferred NaCl concentration in the drinking water. Overall, we hypothesized that our not exercised ponies would balance their total Na intake when non-saline fresh water is always optionally supplied.

2. Material and methods

The experiments conducted in this study were performed in accordance with the guidelines of the German animal ethics regulations and approved by the State Office for Consumer Protection and Food Safety of Lower Saxony, Germany (Ref. no.: 33.9-42502-04-16/2310).

2.1. Animals, housing and experimental design

Our experiment involved six non-pregnant, non-lactating Shetland pony mares (*Equus ferus caballus*), with an average age of 8.2 ± 3.7 years (range between 4 and 15 years) and initial BW of 131.5 ± 11.6 kg (range between 99 and 168 kg). The experiment was conducted from April to May 2019 at the experimental stable of the Department of Animal Sciences, University of Göttingen, Germany. Animals were purchased from local breeders. The ponies had no previous experience with choice tests or with saline water. The mares were not exercised throughout the study.

The animals were kept in an open housing system (Fig. 1). The ponies were randomly allocated to six individual pens (9.6 m²) littered with wood shavings and had permanent

access to an adjacent individual partly-roofed outdoor run (12 m²). Chopped hay (5 – 10 cm particle length) and water (fresh and saline) were offered for *ad libitum* consumption inside the pen in a trough or buckets, respectively. A standardized salt lick (K+S Minerals and Agriculture GmbH, Kassel, Germany) containing 57% Cl, 37% Na, 1.1% Ca, 0.6% Mg, 0% P, Mn_2O_3 (1,000 mg/kg), ZnO (1,000 mg/kg), $Ca(IO_3)_2$ (100 mg/kg), $CoCO_3$ (20 mg/kg) and Na_2SeO_3 (20 mg/kg) was placed in the feed bucket at free disposal. The drinking water was offered in buckets (each 10 L volume) placed in a wooden rack with openings for six buckets, numbered from 1 to 6, respectively (Fig. 1).

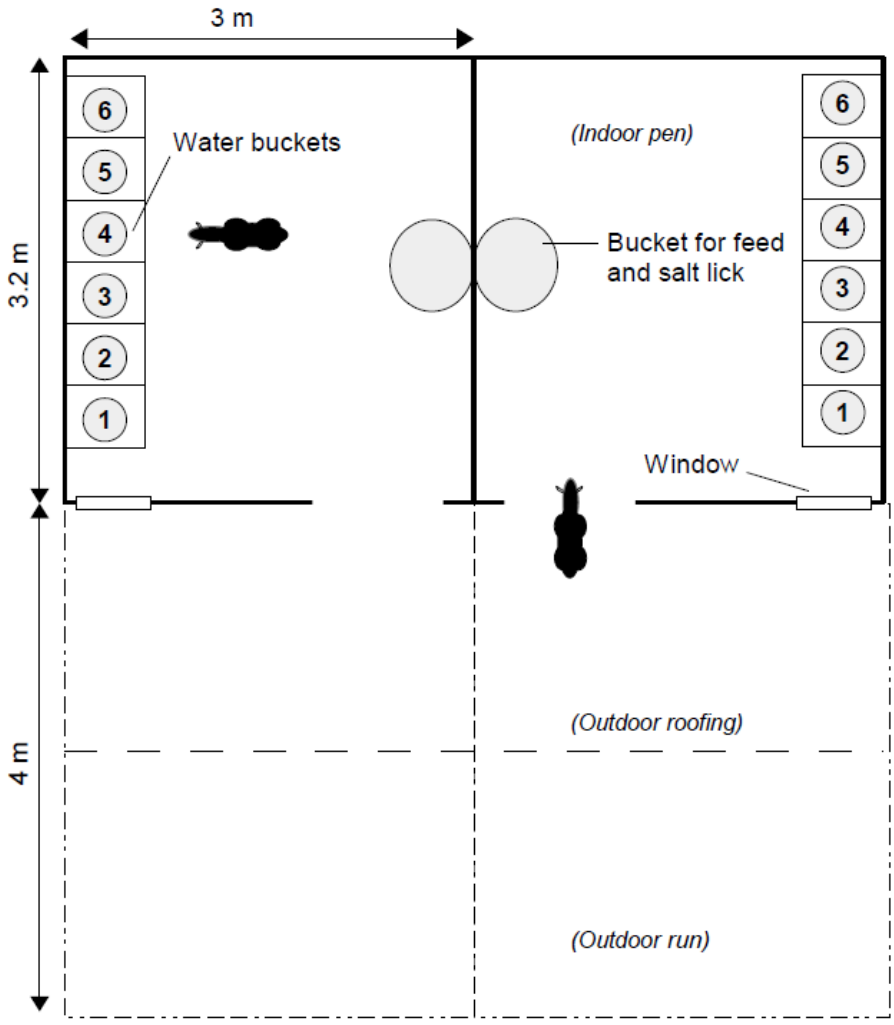

Fig. 1. Experimental barn: one room separated into two individual pens (for one animal each). The water buckets (1 – 6), as well as the feed and salt lick buckets, were placed inside the barn. In total, three rooms with each two pens in a mirror-inverted arrangement were used.

The housing climate was identical in all six pens. The average ambient temperature and relative humidity were measured with data loggers (iButton® temperature/humidity logger DS1923, Maxim Integrated Products, Inc., San José, CA, USA) every 30 minutes during the entire experiment and averaged 11.8 ± 0.13°C and 68.7 ± 0.5%, respectively.

Artificial light was provided for a lighting schedule with 14 h light and 8 h dark per day from 06:30 h to 20:30 h in addition to the natural light.

The experiment comprised three consecutive phases (Fig. 2). Phase 1 (two weeks) was a control phase for the habituation of the ponies. Only fresh water was offered in two adjacent buckets. The positions of the water buckets were changed across the six openings to accustom the ponies to the later experimental setup. The daily individual feed and water intake per animal were measured on four consecutive days per week.

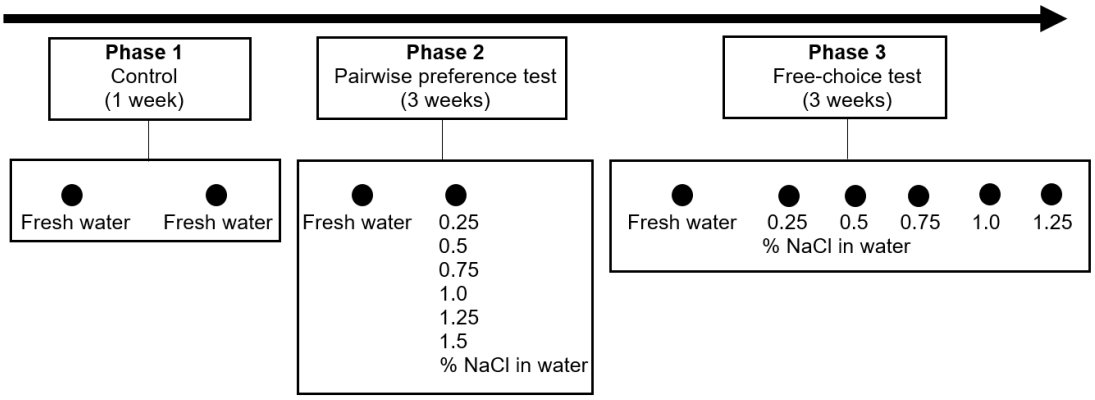

Fig. 2. Experimental design involving three phases. Phase 1: control, two buckets with fresh water were offered simultaneously. Phase 2: pairwise preference test, two buckets were offered, one containing fresh water and the other saline water with ascending salt (NaCl) concentration. Phase 3: free-choice test, six buckets were offered simultaneously with each different salt concentration in the offered water solution. The positions of the concentrations were changed randomly on a daily basis.

2.1.1. Pairwise preference test (phase 2)

In phase 2 (three weeks), a pairwise preference trial according to Bell (1959) and Goatcher & Church (1970a) was performed by offering the simultaneous choice between fresh and saline water. Na concentration of the test solution was gradually (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5% NaCl) increased to evaluate the sensitivity of the ponies. One bucket always contained fresh water, while the second bucket was filled with saline water in increasing concentrations. Each combination of concentrations was provided freely for two consecutive days, i.e. day one and two: choice between fresh water and 0.25% NaCl, day three and four: choice between fresh water and 0.5% NaCl, etc. Per week, two combinations were offered. The buckets were replenished daily and refilled when necessary to allow *ad libitum* water intake. The positions of the solutions were randomly changed each day to prevent preference for a particular bucket position (Meier et al., 2012). On the remaining three days of the week, only fresh water was offered.

2.1.2. Free-choice test (phase 3)

Subsequently, a free-choice experiment with a cafeteria-design was conducted for three weeks with the same animals. Six water buckets were simultaneously offered for four consecutive days per week in each pen, containing fresh water and five different concentrations of saline water (0.25, 0.5, 0.75, 1.0 and 1.25% NaCl). Each day, the concentrations were randomly distributed to the six positions to avoid sidedness of the ponies (Meier et al., 2012). On the remaining three days of the week, only fresh water was provided.

2.2. Measurements

2.2.1. Animal condition

The individual BW was recorded with a mobile scale (resolution: 0.2 kg, Salter Brecknell LS300, Smethwick, West Midlands, UK), once prior to the experiment (initial weight) and after each week of the experiment. It is expressed as BW (kg) and metabolic body size ($kg^{0.75}$). Additionally, body condition scores (BCS) were determined before and after each experimental phase (1 – 3) based on the procedure described by Carroll & Huntington (1988) for horses. The scale ranged from 0 = very poor to 5 = very fat, with 0.5 increments.

2.2.2. Water and salt intake

The average Na content of fresh water (tap water) was 7.55 mg/L. Salt with a 99.8% NaCl purity (Esco Siede-Speisesalz, Hannover, Germany) was used to prepare the exact salt concentrations of the different saline solutions. The concentrations were then validated with a refractometer (HI 96821; Hanna Instruments Inc., Woonsocket, RI, USA). The water buckets were refilled daily with the same amount of water for each animal (8 L) and refilled if necessary. The daily water intake was re-weighed after each day with a scale to the nearest 1 g (Sartorius CPA34000, Sartorius AG, Göttingen, Germany). A control bucket (8 L) was placed in the barn and re-weighed daily to assess the water lost by evaporation and correct the water intake. Total daily drinking water intake (TDWI) was defined as sum of consumed fresh and saline water. In addition, total daily water intake (TWI) was calculated from the sum of TDWI and water intake via the feed. TWDI and TWI were also expressed as function of BW to the power of 0.82 since water is used in evaporative cooling, body transport systems and metabolism (Wilson, 1989). The respective Na intake per animal was calculated from the intake of Na via fresh water, saline water and the salt lick. The daily Na intake from the salt lick was determined and averaged by re-weighing the salt lick before and after each week of the

experiment to the nearest 1 g using an electronic scale (Sartorius CPA34000, Sartorius AG). The ingestion of Na from the salt (NaCl) in the water was calculated based on the molecular weights. As the Na content of the provided hay was below the detection limit of the method (<0.2 g/kg DM, see below), Na from feed was set to zero.

2.3. Feed intake and analysis

Cut hay was offered to all animals every day at 08:00 h and refilled when necessary. The individual daily feed intake was calculated by reweighing the leftovers per day with an electronic scale to the nearest 1 g (Sartorius CPA34000, Sartorius AG). For the forage analyses, samples were taken weekly, kept at room temperature and ground prior to analysis (1 mm sieve) before being stored in airtight containers. The chemical analyses were performed according to to VDLUFA (2012), and method numbers were given in parentheses: DM (3.1), ash (8.1), crude fat (CL, 5.1.1, using a Soxtec 2055; Foss Analytical Systems, Hillerød, Denmark) and acid detergent lignin (ADL, 6.5.3). Crude protein (CP) was determined by Dumas combustion (4.1.2; Elementar VarioMAX CN, Langenselbold, Germany). The concentrations of fibre fractions, namely aNDFom (6.5.1; assayed with heatstable amylase) and ADFom (6.5.2), both expressed exclusive residual ash, were analyzed sequentially using an Ankom 220 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). For the analysis of the minerals in the forage, an aqueous extract using ion chromatography with conductivity detection (10.5.2, Dionex DX-100, Dionex, Sunnyvale, CA, USA) was used for the assay of Na and Ca. Additionally, Ca, Mg, P and K were analyzed by ashing and dissolving samples in HCl. Ca, Mg and K were measured by atomic absorption spectroscopy (Varian SpectrAA-300, Varian, Palo Alto, CA, USA), and P was measured photometrically. A Hohenheim gas test (25.1) was conducted to measure the 24-h *in vitro* gas production (GP, ml/200 mg DM). Subsequently, the metabolizable energy (ME) content was calculated by using equations for ruminants of GfE (2008), which are based on digestibility measurements on wether sheep: ME_r (MJ/kg DM) = 7.81 + 0.07559 × GP − 0.00384 × Ash + 0.00565 × CP + 0.01898 × CL − 0.00831 × ADFom (Ash, CP, CL, and ADFom are in g/kg DM and GP is in ml/200 mg DM). The ME content for horses (ME_h) was finally estimated according to Jansson (2013): ME_h (MJ/kg DM) = (1.12ME_r) – 1.1. The results are given in Table 1.

2.4. Statistical analysis

Data collected on a daily basis for each pony, were averaged across Na concentration, week or phase, respectively. Statistical analyses were performed using R 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). In general, linear mixed-effects

models were applied. However, when assumptions for parametric tests were not met, non-parametric Friedman's tests were used. For the parametric tests, the models were fitted with REML and the Satterthwaite's method was used for the approximation of the degrees of freedom. Analyses of variances (Anova) were conducted using Type III Wald F tests within the lme4 package. The model for the comparison of the BW, BCS, DMI, water and Na intakes between the three phases (phase 1: control, phase 2: pairwise preference test and phase 3: free-choice test) considered the phase (1, 2 or 3) as fixed and the animal as random effects. The model for comparisons of water intakes, Na intakes and the DMI during the pairwise preference test (phase 2) included the administered salt concentration in the saline water (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5%) as fixed and the animal as random effects. The model for comparisons of BW, total intakes of water, Na intake and DMI during the free-choice preference test (phase 3) included the fixed effect of weeks (1, 2 or 3) and the animal as random effect. Additionally, for the comparison of the water and Na intake during phase 3 per salt concentration, the model included the fixed effects of the offered salt concentration in the drinking water (0.0, 0.25, 0.5, 0.75, 1.0 or 1.25%), the bucket position (1 to 6), their interaction and the animal as random effect.

Table 1. Chemical composition (means ± SD) of the hay forage (g kg^{-1} DM, unless otherwise stated)[1].

Composition	Means ± SD
DM (g/kg)	919.9 ± 0.8
ME (MJ/kg DM)	8.0 ± 0.1
24-h gas production (g/200 mg DM)	40.4 ± 0.7
Crude ash	61.2 ± 1.1
Crude protein	71.8 ± 1.4
Crude lipids	16.8 ± 1.2
aNDFom	666.3 ± 5.4
ADFom	391.6 ± 4.4
ADL	60.5 ± 1.5
Calcium	4.7 ± 0.2
Phosphorus	1.8 ± 0.1
Sodium	n.d.
Magnesium	1.3 ± 0.1
Potassium	19.5 ± 0.3
Chloride	3.0 ± 0.1

[1]: Each value is based on at least two analytical replicates per week; n.d. = not detectable or below the detection limit of the method.

DM: dry matter value; ME: metabolizable energy; aNDFom: neutral detergent fibre assayed with heat-stable amylase and expressed exclusive of residual ash; ADFom: acid detergent fibre expressed exclusive of residual ash; ADL: acid detergent lignin.

In all models, the residual error was assumed to be normally distributed, independent and with a constant variance. These model assumptions were verified on the basis of residual plots and the Shapiro-Wilk normality test. The included Tukey-Kramer test was used to evaluate differences between groups. Statistical significance was pre-set at $p < 0.05$. The results are reported as least squares (LS) means (emmeans package) and their standard error (SE). To simplify, only LSmeans are given in the Tables and Figures, also where non parametric tests were used when assumptions for parametric tests were not met.

The Friedman's rank sum test of variance was calculated with the rstatix package followed by post-hoc pairwise comparisons using two-tailed Wilcoxon rank sum exact test to determine differences between phases, treatments or weeks. Significance was set at $p < 0.05$ and adjustments were made according to the false discovery rate method by Benjamini & Hochberg (1995).

For the pairwise preference test, the discrimination model proposed by Bell (1959) and Goatcher & Church (1970a) was used. For analyzing the proportion of the ingested saline water from the TDWI, weighted least squares of the TDWI were calculated based on the statistical models given before. Considering that a random total drinking water intake for two buckets should be 50% per bucket, a 95% confidence level resulted in a non-discrimination zone from 40% (lower discrimination threshold, LDT) to 60% (upper discrimination threshold, UDT). The rejection threshold (RET) was set at 20% intake, the preference threshold (PRT) at 80% intake. The reaction was defined as indifferent or random choice, when the proportional intake was between 40 and 60%.

3. Results

Table 2 summarizes the average results of the BW, BCS, the feed, water and Na intakes for each phase. This overview allows, in particular, the comparison of the saline water uptake between the pairwise preference tests (phase 2) and the free-choice system (phase 3). The ponies remained clinically healthy, without change in BW (140 kg ± 20.6 kg) or BCS (3.2 ± 0.67) over the course of the experiment ($p > 0.05$, Enke et al., 2022b).

3.1. Pairwise preference test (phase 2)

Compared with the control (Table 2, Table 3), DMI intake in phase 2 was by about 6% lower ($p = 0.015$), whereas TDWI was significantly higher ($p = 0.011$). However, over the course of phase 2, the DMI remained rather constant, with a daily mean of 64.2 ± 3.54 $g/kg^{0.75}$ and no significant changes as a result of the increased NaCl concentration in the saline water ($p = 0.121$; Tab. 3) . In both phases, large individual differences in TDWI

were observed ranging between 135.7 to 222.9 g/kg$^{0.82}$ during the control phase, and increased to between 153.9 and 299.9 g/kg$^{0.82}$ during phase 2.

TDWI increased with higher Na concentration in the test solution, but the increase was erratic and peaked at the choice with 0.5% Na, reaching 1.6 times of the TDWI during the control phase ($p < 0.001$). The TDWI abruptly decreased to the control level or even below when more than 1% NaCl was added to the test solution (Table 3).

Interestingly, the ratio of fresh to saline water intake changed with increasing NaCl in the saline water. While the ratio was 0.72 for 0.25%, it was lowest with 0.39 at 0.5% NaCl, i.e. the ponies ingested around 2.5 times more saline than fresh water. With higher NaCl concentrations in the test solution the ratio increased considerably in favour of fresh water, reaching 1.22, 1.7, 12.9 and 14.8 for 0.75, 1.0 and 1.5% NaCl, respectively.

Table 2. Mean (LSmeans) body weight and body condition, as well as daily feed, water and sodium intakes (g per day and corrected for metabolic body size) for the control phase (1), the pairwise preference test (2) and the free-choice test (3) in ponies (n = 6).

Trait	Phase			SEM	p-value
	1 (Control)	2 (Pairwise preference)	3 (Free-choice)		Phase effect
Body weight (kg)	134.7	136.5	135.5	13.0	0.271
BCS	2.9	3.0	3.1	0.33	0.130
Dry matter intake (g)	2674^{a}	2505^{b}	2544^{ab}	138.7	0.015
Dry matter intake (g/kg$^{0.75}$)	69.61^{a}	64.17^{b}	64.95^{b}	3.54	0.002
Fresh water intake (g)	9264^{a}	6683^{b}	5375^{c}	553	<0.001
Fresh water intake (g/kg$^{0.82}$)	171.4^{a}	119.9^{b}	96.4^{c}	7.72	0.002*
Saline water intake (g)	-	4540	4272	735	0.414*
Saline water intake (g/kg$^{0.82}$)	-	84.69	77.21	14.71	0.414*
Total drinking water intake (g)	9264^{a}	11223^{b}	9646^{a}	837	0.009*
Total drinking water intake (g/kg$^{0.82}$)	171.4^{a}	204.6^{b}	173.6^{a}	15.0	0.011*
Total water intake (g)	9489^{a}	11444^{b}	9869^{a}	847	0.009*
Total water intake (g/kg$^{0.82}$)	175.6^{a}	208.6^{b}	177.6^{a}	15.2	0.011*
Total water intake/dry matter intake	3.54^{a}	4.53^{b}	3.86^{a}	0.17	<0.001
Na Salt lick (g)	5.70	5.53	6.38	1.75	0.236
Na Salt lick (g/kg$^{0.75}$)	0.15	0.15	0.17	0.049	0.227
Na Fresh water (g)	0.070^{a}	0.050^{b}	0.041^{c}	0.004	<0.001
Na Fresh water (g/kg$^{0.75}$)	0.0018^{a}	0.0013^{b}	0.0008^{c}	0.0001	<0.001
Na Saline water (g)	-	10.97	7.48	1.84	0.103*
Na Saline water (g/kg$^{0.75}$)	-	0.29	0.19	0.05	0.103*
Na Total drinking water (g)	0.07^{a}	11.02^{b}	7.52^{b}	1.70	0.006*
Na Total drinking water (g/kg$^{0.75}$)	0.002^{a}	0.288^{b}	0.189^{b}	0.047	0.006*
Na Total (g)	5.77^{a}	16.55^{b}	13.90^{b}	2.88	0.009*
Na Total (g/kg$^{0.75}$)	0.15^{a}	0.43^{b}	0.36^{b}	0.08	0.009*

Total drinking water intake = total drinking water intake from fresh and saline water; total water intake = total drinking water intake + water from feed; Na Salt lick/Fresh water/Saline water/Total drinking water = sodium intake from salt lick, fresh water, saline water and total drinking water, respectively; Na Total = total sodium from fresh water, saline water and salt lick. Sodium content of hay was below the detection limit of the method. Phases: 1 = only fresh water offered in two buckets; 2 = fresh water was offered in one bucket, saline water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5% NaCl) in another bucket; 3 = six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.25% NaCl). ^{a,b,c} Means in the same row with different superscripts are significantly different at $p < 0.05$. * = non-parametric statistical tests were applied when requirements for parametric tests were not met.

Table 3. Pairwise preference test (phase 2). Effect of salt (NaCl) concentration in the saline water on mean (LSmeans) daily feed, drinking water and sodium intakes (g per day and corrected for metabolic body size) in ponies (n = 6).

	Salt concentration (% NaCl) in the saline water						SEM	p-value
Trait	0.25	0.5	0.75	1	1.25	1.5		Salt concentration effect
Dry matter intake (g)	2369	2562	2553	2544	2469	2537	146	0.120*
Dry matter intake (g/kg$^{0.75}$)	61.4	65.4	65.3	65.2	63.1	64.5	4.0	0.121*
Fresh water intake (g)	4498^{a}	4371^{a}	6436^{ab}	8060^{bc}	7754^{bc}	8982^{c}	729	<0.001
Fresh water intake (g/kg$^{0.82}$)	81.5^{a}	75.3^{a}	117.0^{ab}	144.9^{bc}	139.3^{bc}	161.3^{c}	10.3	<0.001
Saline water intake (g)	6017^{b}	10310^{a}	5285^{b}	4433^{b}	582^{c}	615^{c}	1119	<0.001
Saline water intake (g/kg$^{0.82}$)	113.2^{b}	191.8^{a}	96.1^{b}	85.3^{b}	10.8^{c}	10.9^{c}	23.5	<0.001
Total drinking water intake (g)	10515^{abc}	14681^{d}	11721^{bc}	12493^{cd}	8336^{a}	9597^{ab}	1150	<0.001
Total drinking water intake (g/kg$^{0.82}$)	194.7^{abc}	267.1^{d}	213.2^{bc}	230.3^{cd}	150.0^{a}	172.2^{ab}	22.9	<0.001
Total water intake (g)	10720^{abc}	14902^{d}	11944^{bc}	12716^{cd}	8560^{a}	9827^{ab}	1161	<0.001
Total water intake (g/kg$^{0.82}$)	198.5^{abc}	271.1^{d}	217.2^{bc}	234.3^{cd}	154.1^{a}	176.3^{ab}	23.1	<0.001
Total water intake/dry matter intake	4.48^{bc}	5.79^{a}	4.61^{bc}	4.94^{ab}	3.50^{d}	3.85^{cd}	0.29	<0.001
Na Salt lick (g)	4.80^{a}	4.80^{a}	5.14^{ab}	5.14^{ab}	6.65^{b}	6.65^{b}	1.86	0.001
Na Salt lick (g/kg$^{0.75}$)	0.13^{a}	0.13^{a}	0.14^{ab}	0.14^{ab}	0.17^{b}	0.17^{b}	0.05	0.002
Na Fresh water (g)	0.03^{a}	0.03^{a}	0.05^{ab}	0.06^{bc}	0.06^{bc}	0.07^{c}	0.01	<0.001
Na Fresh water (g/kg$^{0.75}$)	0.0009^{a}	0.0008^{a}	0.0012^{ab}	0.0015^{bc}	0.0015^{bc}	0.0017^{c}	0.0001	<0.001
Na Saline water (g)	5.95^{a}	20.32^{b}	15.60^{b}	17.44^{b}	2.86^{a}	3.63^{a}	3.2	<0.001
Na Saline water (g/kg$^{0.75}$)	0.16^{ab}	0.53^{c}	0.40^{bc}	0.47^{c}	0.07^{a}	0.09^{a}	0.09	<0.001
Na Total drinking water (g)	5.99^{a}	20.35^{b}	15.65^{b}	17.50^{b}	2.92^{a}	3.69^{a}	3.2	<0.001
Na Total drinking water (g/kg$^{0.75}$)	0.15^{ab}	0.5^{c}	0.4^{bc}	0.5^{c}	0.08^{a}	0.09^{a}	0.09	<0.001
Na Total (g)	10.79^{a}	25.15^{b}	20.79^{b}	22.63^{b}	9.57^{a}	10.35^{a}	4.52	<0.001
Na Total (g/kg$^{0.75}$)	0.29^{a}	0.66^{b}	0.54^{b}	0.61^{b}	0.25^{a}	0.27^{a}	0.13	<0.001

Total drinking water intake = total drinking water intake from fresh and saline water; total water intake = total drinking water intake + water from feed; Na Salt lick/Fresh water/Saline water/Total drinking water = sodium intake from salt lick, fresh water, saline water and total drinking water, respectively; Na Total = total sodium from fresh water, saline water and salt lick. Sodium content of hay was below the detection limit of the method. Phase 2 = pairwise preference test; water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5% NaCl) offered in one bucket in combination with another bucket of fresh water. ^{a,b,c,d} Means in the same row with different superscripts are significantly different at $p < 0.05$. * = non-parametric statistical tests were applied when requirements for parametric tests were not met.

Based on the relative intake of saline water, the responses of the ponies to ascending Na concentrations in the test solution are illustrated as ratio chart (Fig. 3). In their choices, ponies remained below the upper discrimination line at 0.25% NaCl, which can be interpreted as random choice or indifference. Their preferences for 0.5% NaCl concentration passed the upper discrimination line, indicating a moderate preference for the saline water. However, for 0.75% NaCl, a weak rejection was observed, while test solutions with 1.25 and 1.5% NaCl were strongly rejected. Differences between choices were significant for both 1.25 and 1.5% NaCl with the other pairwise tests ($p < 0.001$).

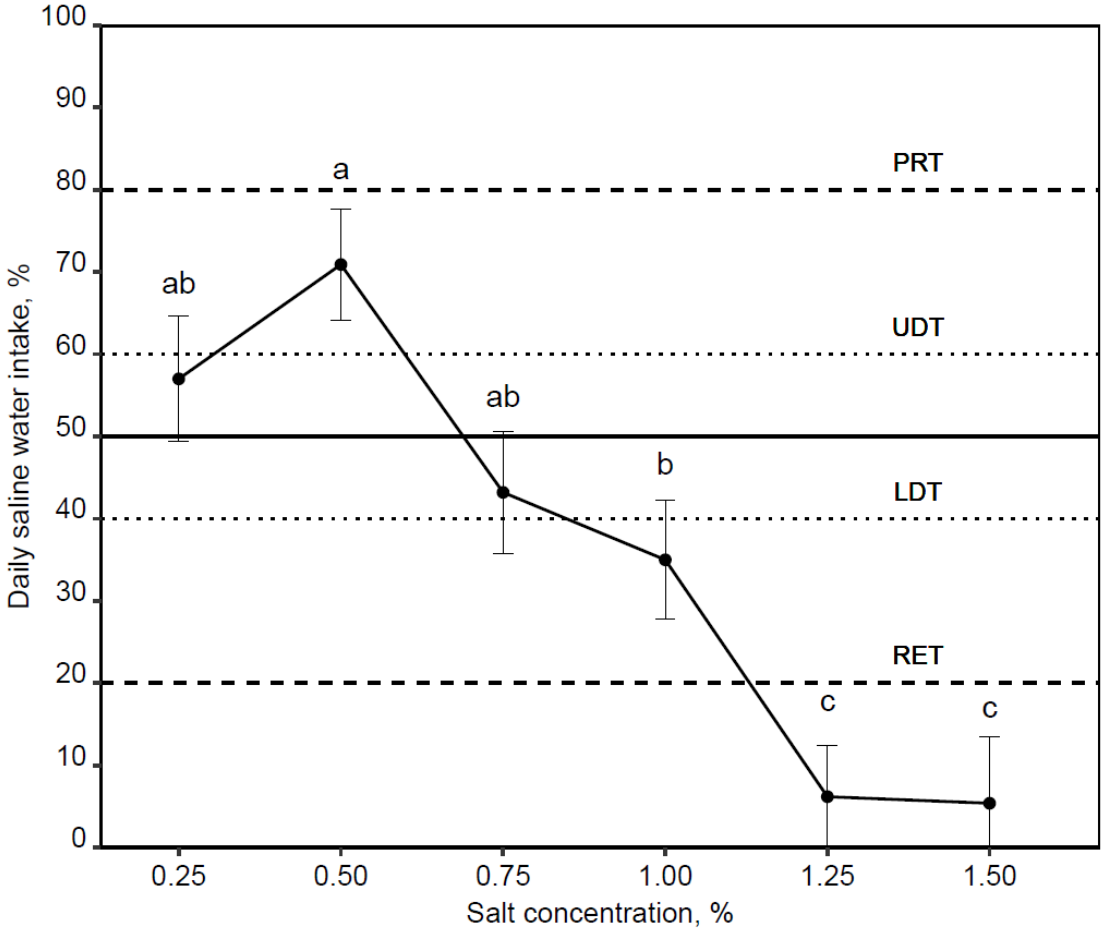

Fig. 3. Pairwise preference test (phase 2). Discrimination model (Goatcher & Church, 1970a) for the response of the ponies (n = 6) to ascending concentrations of NaCl in the saline water. The amount of ingested saline water is shown as a percentage ± SE of the total drinking water intake (TDWI) from both solutions. Lines: RET = rejection threshold; LDT = lower discrimination threshold; UDT = upper discrimination threshold; PRT = preference threshold. [a,b,c] Means in the same row with different superscripts are significantly different at $p < 0.05$.

We observed a 2.8 times higher total intake of Na across the pairwise preference tests, compared to the control phase (Table 2, Table 3). During the course of phase 2, the total Na intake was about twice as high at concentrations of 0.5, 0.75 and 1% NaCl compared

to the other pairwise test solutions ($p < 0.001$; Table 3). The distribution of the total Na intake from the saline water or the salt lick is of particular interest. Across phase 2, the ratio of Na ingestion through salt lick to Na through water was 0.52, but the share of Na which originated from either source changed with offered saline concentration and did not follow a linear pattern. Whereas the Na intake via saline water represented about 55% of the total Na intake at 0.25% NaCl, this share averaged 77% at concentrations of 0.5 and 1.0% NaCl and declined to about 33% with more than 1% NaCl in the saline water. The share of Na intake via the salt lick followed a U-shape, complementary to the intake by saline water, representing 44.8, 19.7, 25.9, 22.9, 68.0 and 62.9% of the total Na intake at concentrations of 0.25, 0.50, 0.75, 1.0, 1.25 and 1.5% NaCl, respectively (Table 3).

3.2. Free-choice preference test (phase 3)

During phase 3, the ponies had free simultaneous access to six buckets which were filled with fresh water and five different saline solutions (0.25 – 1.25%) for three consecutive weeks. The results are summarized as weekly means in Table 4.

During this phase, the ponies had a similar daily DMI as in phase 2, which was significantly lower compared to the control phase ($p < 0.001$) and fluctuated ($p = 0.001$) over the course of the three weeks (Tab. 4). The TDWI was significantly lower than during the pairwise preference test ($p < 0.011$), ranging between 142.3 to 211.1 g/kg$^{0.82}$ per day and pony, similar to the intake under control conditions. The position of the bucket had a significant effect ($p < 0.012$) on the water intakes of each saline solution. Interestingly, highest amounts of water were ingested from the buckets closest to the stable walls (1 and 6, see Fig. 2), with each 29.7 and 34.8 g/kg$^{0.82}$ per day. From the buckets in the middle (positions 2, 3, 4 and 5), less water was ingested being 20.6, 18.6, 18.5 and 17.1 g/kg$^{0.82}$ per day, respectively.

Table 4. Free-choice test (phase 3). Mean (LSmeans) daily feed, water and sodium intakes (g per day and corrected for metabolic body size) in ponies (n = 6).

	Week			SEM	p-value
Trait	1	2	3		Week effect
Dry matter intake (g)	2545[b]	2548[c]	2540[a]	146	<0.001
Dry matter intake (g/kg$^{0.75}$)	64.4[a]	65.6[c]	64.9[b]	3.53	<0.001
Fresh water intake (g)	4355[a]	5314[ab]	6457[b]	643	0.026
Fresh water intake (g/kg$^{0.82}$)	78.47[a]	95.78[ab]	114.92[b]	9.67	0.034
Saline water intake (g)	4776	4723	3357	808	0.248
Saline water intake (g/kg$^{0.82}$)	84.30	86.43	61.68	14.29	0.304
Total drinking water intake (g)	9130	10036	9813	828	0.115*
Total drinking water intake (g/kg$^{0.82}$)	162.8	182.2	176.6	11.1	0.115*
Total water intake (g)	9351	10255	10040	839	0.115*
Total water intake (g/kg$^{0.82}$)	166.7	186.2	180.7	11.3	0.115*
Total water intake/dry matter intake	3.63[a]	4.03[b]	3.94[ab]	0.20	0.026
Na Salt lick (g)	5.91[a]	6.32[a]	6.91[b]	1.75	<0.001
Na Salt lick (g/kg$^{0.75}$)	0.15[a]	0.17[b]	0.18[b]	0.05	<0.001
Na Fresh water (g)	0.03[a]	0.04[ab]	0.05[b]	0.005	0.026
Na Fresh water (g/kg$^{0.75}$)	0.0007[a]	0.0008[ab]	0.001[b]	0.0001	0.026
Na Saline water (g)	8.58	7.78	6.06	1.82	0.208
Na Saline water (g/kg$^{0.75}$)	0.21	0.20	0.16	0.04	0.289
Na Total drinking water (g)	8.62	7.83	6.11	1.81	0.210
Na Total drinking water (g/kg$^{0.75}$)	0.21	0.20	0.16	0.04	0.291
Na Total (g)	14.53	14.14	13.02	2.46	0.562
Na Total (g/kg$^{0.75}$)	0.36	0.37	0.34	0.07	0.705

Total drinking water intake = total drinking water intake from fresh and saline water; total water intake = total drinking water intake + water from feed; Na Salt lick/Fresh water/Saline water/Total drinking water = sodium intake from salt lick, fresh water, saline water and total drinking water, respectively; Na Total = total sodium from fresh water, saline water and salt lick. Sodium content of hay was below the detection limit of the method. Phase 3 = free-choice test, six water buckets were offered simultaneously with various NaCl concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.25% NaCl).

[a,b] Means in the same row with different superscripts are significantly different at $p < 0.05$. * = non-parametric statistical tests were applied when requirements for parametric tests were not met.

With regard to water fractions, the intake of fresh water was the lowest found in this study ($p < 0.002$), being 43.8 and 20.0% lower than during the control phase and pairwise preference test, respectively (Table 2). Fig. 4 shows how the ponies differentiated between the simultaneously offered solutions per week. The intake of fresh water was always the highest and increased with time. In week 1, the difference between intakes of fresh and saline water with 0.25% NaCl was not significant, whereas ponies increased their preference for fresh water significantly in weeks 2 and 3 ($p < 0.001$). Among the different saline solutions, clearer preferences and rejections emerged with increasing duration of the experiment. Drinking water with 0.25% NaCl was always the most ingested saline solution. While saline water with 0.5% NaCl was consumed to some extent, water with more than 0.5% NaCl was avoided and no significant differentiation between the low intakes of 0.75, 1.0 and 1.25% NaCl solutions was observed ($p < 0.001$). Across phase 3 the shares of TDWI for the different concentrations were 55.5, 26.9, 9.1, 3.0, 2.7 and 2.8% for fresh water and NaCl concentrations of 0.25, 0.50, 0.75, 1.0 and 1.5%, respectively. Overall, around 82.4% of the TDWI was ingested from salt concentrations below 0.5%.

Table 5. Free-choice preference test (phase 3). Mean (LSmeans) daily water and sodium intake through ingestion of respective drinking water with different salt concentration in ponies (n = 6).

	Salt concentration (% NaCl) in the drinking water						SEM	p-value
Trait	0	0.25	0.5	0.75	1	1.25		
Water intake (g)	5375^{a}	2590^{b}	883^{c}	287^{c}	255^{c}	269^{c}	228	<0.001
Water intake (g/kg$^{0.82}$)	77.5^{a}	37.3^{b}	12.7^{c}	4.1^{c}	3.7^{c}	3.9^{c}	3.3	<0.001
Na intake (g)	0.04^{a}	2.56^{c}	1.74^{bc}	0.85^{ab}	1.0^{b}	1.32^{b}	0.34	<0.001
Na intake (g/kg$^{0.75}$)	0.001^{a}	0.07^{c}	0.04^{bc}	0.02^{ab}	0.02^{ab}	0.03^{b}	0.01	<0.001

Water intake = water intake through ingestion of respective drinking water with increasing salt concentration; Na intake = Na intake through ingestion of respective drinking water with increasing salt concentration.

Phase 3 = free-choice preference test; six different water buckets with ascending salt concentrations (0%, 0.25%, 0.5%, 0.75%, 1% and 1.25% NaCl) offered simultaneously over three weeks. ^{a,b,c} Means in the same row with different superscripts are significantly different at $p < 0.05$.

Similar to phase 2, the daily total Na intake was increased by about 2.4 times, compared to the control phase (Table 2; $p = 0.009$). Interestingly, the ponies did not significantly ($p = 0.705$) change their total Na intake over the course of the free-choice experiment (Table 4). This was due to an opposite development of Na intakes from saline water or the salt lick. Thus, the share of ingested Na via saline solution to the total Na intake decreased and averaged 58.3, 54.1 and 47.1% in weeks 1, 2, and 3, respectively. At the same time the Na ingestion from the salt lick increased significantly by around 16.7% ($p < 0.001$). Table 5 summarizes the amount of Na intake through ingestion of the

respective test solutions. The Na intake from the fresh water was negligible. Most Na was ingested from the saline water with 0.25% NaCl, whereas less Na was consumed from higher concentrations of the provided solutions.

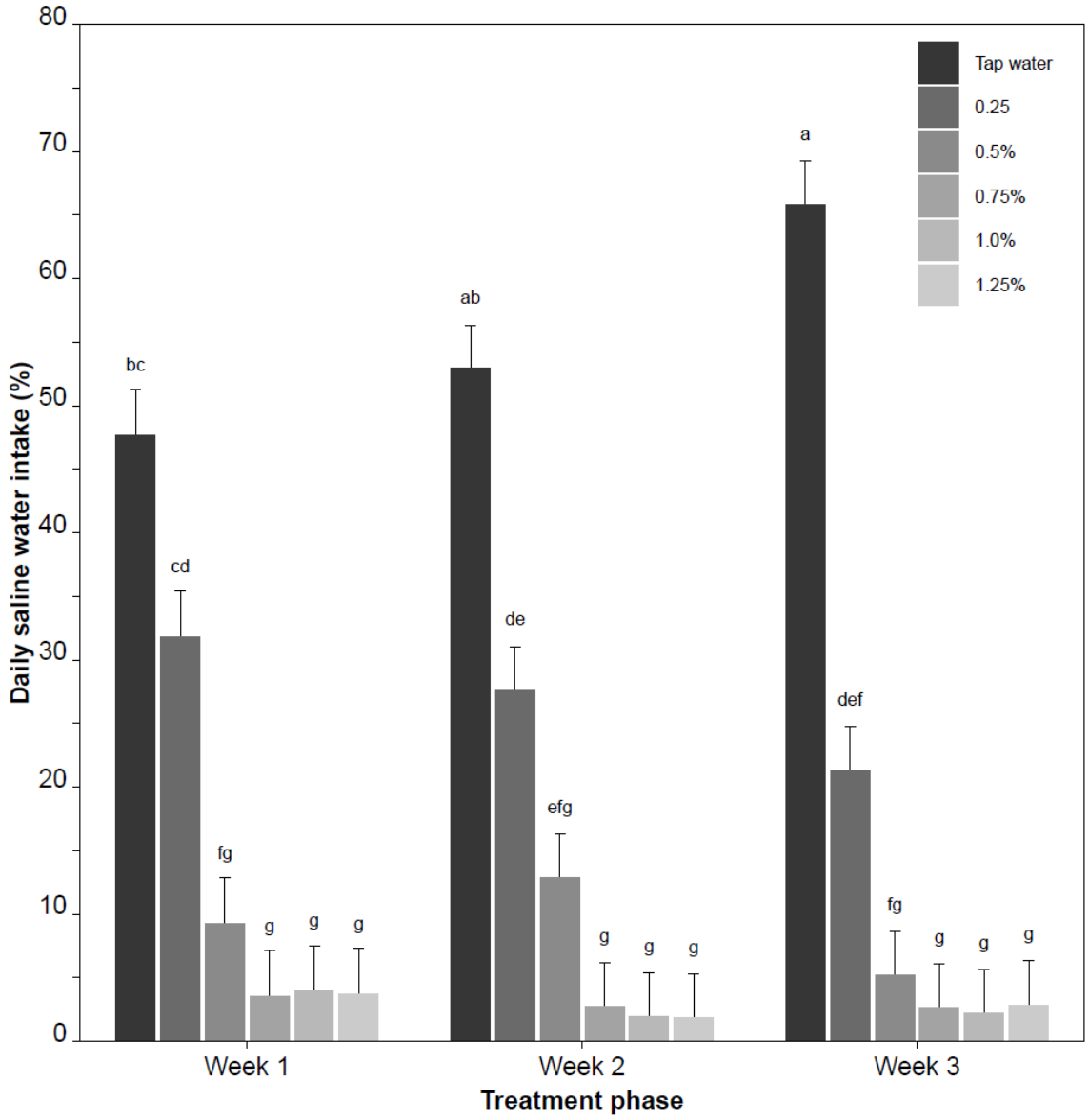

Fig. 4. Free-choice experiment (phase 3). Daily drinking water intake (% of total drinking water intake) for each salt concentration of ponies (n = 6), per treatment week. Simultaneous choice between six buckets with different salt concentrations. ^{a,b,c,d,e,f,g} Means in the same row with different superscripts are significantly different at $p < 0.05$ within the same week.

4. Discussion

4.1. Body condition and feed intake

During our experiment, BW and BCS were not affected by the ingestion of saline drinking water. Clinical signs for Na intoxication such as incoordination, convulsions, diarrhoea, colic, or oedemas (Wilson & Dudzinski, 1973; Sandals, 1978; Nestor et al., 1988; Holbrook et al., 2005) were not observed during the experiment. This is in agreement with the study of Schryver et al. (1987), where a diet containing 5% NaCl exerted no adverse effect on mature gelding ponies. Similarly, warmblood horses in moderate work receiving up to 100 g NaCl/day together with the concentrates remained healthy (Zeyner et al., 2017).

The DMI of our ponies was with 64.95 g/kg $BW^{0.75}$ below the maintenance recommendation of 90 – 110 g/kg $BW^{0.75}$ (GfE, 2014). However, similar DMI of 64.2 g/kg $BW^{0.75}$ (Cymbaluk, 1989) and 62.9 g/kg $BW^{0.75}$ (Moore-Colyer & Longland, 2000) were reported for non-exercised ponies under moderate temperatures. Compared with the control phase, the DMI of the ponies in our study declined by about 7% during the pairwise preference and the free-choice tests, when more Na was ingested. However, BW and BCS remained unchanged despite lower feed intake, suggesting that water retention might have contributed to the constant BW (Masters et al., 2005). In contrast, higher Na intake in the diet did not affect the DMI of ponies in the study of Schryver et al. (1987). The reactions of our ponies also disagree to results from ruminants, such as sheep or rusa deer (Grovum & Chapman, 1988; Kii & Dryden, 2005), where feed intake was increased with moderate Na intakes, possibly due to enhanced palatability of the feed.

4.2. Water intake

Under temperate conditions, the daily maintenance water requirement of adult horses is around 5 L/100 kg BW (Groenendyk et al., 1988; Cymbaluk, 1989) or 3 – 4 L/kg DMI (GfE, 2014), possibly increasing up to 15 L/100 kg BW or 7 L/kg DMI during exercise in hot climates (Frape, 2013). This corresponds with our control TDWI of 6.88 L/100 kg BW or 3.46 L/kg DMI. Overall, we observed a high individual variability in the TDWI with considerable fluctuations in the same animal, which has also been described in other studies as typical for horses (Randall et al., 1978; Schryver et al., 1987; Groenendyk et al., 1988).

During our experiment, great amounts of saline water were ingested when NaCl solutions were offered as alternative drinking water. Na is mainly excreted via renal excretion (urine), sweat and faeces. Thus, electrolyte imbalances due to excess Na intake can be corrected by e.g., higher water intakes and urination, as long as enough non-saline fresh

water is supplied (Sufit et al., 1985; Rose, 1990; GfE, 2014). These homeostatic responses have been shown in ruminants, such as cattle (Weeth et al., 1960), sheep (Wilson & Dudzinski, 1973) or red and fallow deer (Ru et al., 2005). In an experimental design comparable to this study, goats increased their TDWI from the control phase to about 36% (Runa et al., 2019a). In contrast, our ponies increased their TDWI only by about 19% in the pairwise preference test, when the highest Na intake was recorded. Interestingly, the TDWI dropped again during the free-choice test, when also the Na intake was slightly reduced. This suggests a link between water and total Na intake, as already shown by Jansson & Dahlborn (1999) for Standardbred geldings, with a salt lick as only Na source. However, in the study of Zeyner et al. (2017) water intake of warmblood mares was not significantly affected by salt addition of 50 or 100 g NaCl/day to the concentrates. At the same time, Na excretion via the urine was increased with Na supplementation, underlining the importance of regulation of renal Na excretion in horses (Lindner et al., 1983).

4.3. Sensitivity to sodium in the drinking water

During the pairwise preference tests, our ponies did not distinguish between fresh water and saline water with 0.25% NaCl. However, they showed a clear preference for water with 0.5% NaCl, whereas solutions with more than 0.75% were avoided or rejected. Our results agree with pairwise preference tests, where weaning foals showed indifferent responses until 0.63% NaCl in their drinking water (Randall et al., 1978). Interestingly, at 1.25% NaCl concentration, the immature horses still drank more than 20% from that solution, whereas in our experiment, drinking water with 1.25 or 1.5% NaCl was hardly consumed by our mature horses (<5% of the total TDWI). Similarly, Przewalski's horses *(Equus przewalskii)*, which are constantly confronted with saline water sources in their natural habitat around the Gobi desert, only chose water points with the lowest salt concentrations until 0.6% NaCl (Zhang et al., 2015). It is noteworthy, that Somali donkeys adapted to arid habitats only tolerated saline water until 1.0% NaCl as sole drinking water and lost BW when exposed to water with higher NaCl concentrations (Maloiy, 1970).

4.4. Long-term choice of saline water

The pairwise preference tests only allowed the evaluation of the short-term discrimination and sensitivity reactions of two days per choice option. In comparison, the free-choice test has shown to provide further information on the long-term reaction of animals to simultaneously offered choices (Raffa et al., 2002; Runa et al., 2019b). Despite changing positions of the Na concentrations, ponies quickly made specific choices. Their rather frequent drinking together with their well-developed spatial cognition (Wolff &

Hausberger, 1996) might have facilitated their task learning (McDonnell et al., 1999; Nyman & Dahlborn, 2001; Butudom et al., 2004).

Within the free-choice experiment, our ponies showed an interesting adaptation over the duration of three weeks. They developed a growing clear preference for fresh water, which amounted to about 47.7, 52.9 and 65.8% of the total water intake for week 1, 2 and 3, respectively. Interestingly, the mares increased their intake of fresh water by more than 2.1 L over the three weeks, whereas the saline water intake decreased by around 1.4 L. Thus, the intake of saline water was not only decreased and substituted by fresh water, but also over-compensated with additional fresh water intake. The ponies even reduced their intake of the 0.25% NaCl solution, which was not discriminated during the preceding two-choice preference test. This interesting observation suggests that some training of the discriminatory ability might have occurred due to the long-term exposure to saline water. Thus, thresholds for Na sensitivity of receptors are not constant and may be influenced by the Na status. For example, Na deficient sheep were shown to be more sensitive for changes in the Na concentration of their drinking water, the more severe their respective Na deficiency (Denton & Sabine, 1961).

4.5. Sodium intake

Common natural forages often contain Na concentrations lower than 0.1% of the DM (NRC (2007), which is also applicable to our study. Thus, the salt lick was the main source of Na during the control phase. During this phase, the ponies had a total Na intake of 0.15 g/kg$^{0.75}$ or 0.04 g/kg BW per day, being twice as high as the recommendations of 0.02 g/kg BW for the daily Na requirements in horses in maintenance under normal conditions (Coenen, 2005; NRC, 2007).

In the two-choice preference test the total Na intakes of the ponies exceeded the control intake by up to about three times and was more than five times higher than NRC recommendations (NRC, 2007). Since the Na intake during the control phase was already higher than recommended, it might be possible that the ponies were slightly Na depleted at the beginning of the study. However, the ponies continued ingesting excessive amounts of Na during the free-choice test, which cannot be explained with a possible Na deficit. Similarly, Schryver et al. (1987) observed a rather high and variable voluntary consumption of NaCl in horses ranging from 19 to 143 g of salt per day. Also Houpt et al. (1991) demonstrated in Standardbred mares an enormous appetite for salt from saline water (0.9% NaCl) and salt lick in response to a Na deficit through furosemide treatment. Animals ingested around 35% more Na than the control group. Since the

experiments were conducted for only six weeks, it is open to question whether the ponies would remain on the same high level of Na intake.

Against this background, the Na consumption via the salt lick attracts attention. Contrary to expectation, our ponies did not reduce their Na intake from the salt lick with increased Na ingestion from the saline test solutions. In both tests, Na consumption via the salt lick was even increased when less Na was ingested through the saline water. The high affinity of the ponies to the salt lick may be explained from different perspectives. 1) The previous saline water intake might have lowered the threshold for the sensitivity of Na receptors of the tongue. 2) Sensitivity of salt intake might differ depending on the source of intake, e.g. via feed or as solved electrolytes in the drinking water. Thus, studies in ruminants have revealed a higher salt tolerance from food than from drinking water (Wilson, 1966; Masters et al., 2005; Mdletshe et al., 2017). While sheep accepted salt concentrations of 5 – 20% in their diet without health problems, the prolonged consumption of saline water with 1.5 to 2% and greater caused severe health problems (Peirce, 1968; Digby et al., 2011). However, in a two-choice experiment with male yearling Hackney ponies, where diets with different NaCl concentrations from 0, 2 to 4% were offered, the diet containing less NaCl was always preferred by the ponies, implying a clear capacity to detect NaCl in the feed (Schryver et al., 1987). 3) Our ponies also differed remarkably from ruminants, which reduced their Na intake from the salt lick when provided with increased saline water concentrations (Smith et al., 1953; Runa et al., 2019a; Runa et al., 2019b). Thus, the ponies could have developed a habit to lick or even chew the salt lick. In this light, manipulation of the salt lick might originate from a behavioural motivation instead of a nutritional need and could be considered as redirected oral behaviour comparable to wood chewing (reviewed by Hothersall & Casey, 2012). Similarly, Schryver et al. (1987) suggested that not all voluntary consumption of their horses was related to the salt requirement, but also to habit and taste preference.

Surprisingly, the total drinking water ingestion during the free-choice test was similar to the control intake, although the ponies still consumed the double amount of Na compared to the control phase. According to the results from the previous pairwise test and those from rehydration therapy with saline water (Nyman et al., 1996; Butudom et al., 2002), a higher water intake induced by saline water ingestion was expected. Probably, reactions to short-term and long-term ingestion of saline water differ. Obviously, Na intake requires regulation by the animal because it is the major extracellular element influencing osmolarity of body fluids. However, some adaptation time seems comprehensible involving learning and physiological feedback from ingested Na. Horses have been shown to down-regulate their renal Na excretion in the case of low Na intake (Lindner et

al., 1983). On the other hand, horses were also able to significantly increase their concentration of urinary Na and Cl with higher NaCl supplementation in the feed (Zeyner et al., 2017). Thus, the present long-term exposure to NaCl ingestion might have enhanced the fine adjusting of voluntary daily Na intake.

4.6 Voluntary intake of saline water

Voluntary consumption of saline water can lead to increased total water intake. The question arises, which NaCl concentration in drinking water would be accepted by horses and induce the highest voluntary water intake to replace potential losses of water and electrolytes. In this context, the responses of the ponies to different NaCl concentrations in their drinking water can be used to derive recommendations for NaCl levels suitable for voluntary saline water intake. In the pairwise tests, our ponies did not differentiate between fresh and saline water of 0.25% NaCl. When higher NaCl concentrations were offered, they rejected solutions from 0.75% NaCl. This threshold is close to the 0.6% NaCl which was still accepted by weaning foals in the study of Randall et al. (1978). Similarly, in the free-choice situation, 81% of the ingested saline water was consumed from NaCl concentrations of 0.25 and 0.5%, whereas higher concentrations of 0.75, 1.0 or 1.25% NaCl were avoided. In our ponies, the highest total water intake was recorded for the pairwise choice between fresh water and 0.5% NaCl.

Voluntary intake of saline water as rehydration strategy could be hampered by neophobia (van den Berg et al., 2016b) or taste aversion of horses. Houpt et al. (1991) had to train mature horses to willingly drink a 0.9% NaCl solution. Although horses were Na depleted, they did not innately prefer saline to plain water. From our results, we suggest that a concentration of 0.25% NaCl could be readily accepted by naive horses as rehydration strategy. NaCl concentrations higher than 0.5% would require careful training for adaptation of the horses. In this context, it is worth noting the study of Zeyner et al. (2017) who showed that feeding of 100 g NaCl per day to horses in moderate work could also be associated with metabolic acidosis. Careful adaptation and control of Na intake should be used to avoid possible health impairments. Thus, the voluntary Na intake from saline water of our ponies was 273 to 438% higher than recommended to cover maintenance Na requirements. Further, it should be taken into consideration that additional excess Na intake could emerge from habitual Na overconsumption from the salt lick.

In conclusion, our ponies demonstrated remarkable sensory capacities to distinguish different concentrations of NaCl in aqueous solutions in two different choice tests. When offered in a pairwise choice or in a free-choice system, the ponies consistently avoided NaCl concentrations above 0.75%. The increasing preference of the ponies for plain over

saline water suggests that fine adjusting of voluntary Na intake requires some adaptation time. However, from the reactions of the ponies, we conclude that lower NaCl concentrations between 0.25 and 0.5% might be suitable for voluntary consumption of saline water.

Declaration of Competing Interests

The authors declare that they have no conflict of interest.

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CHAPTER 4

Preference and discrimination behaviour of llamas to saline drinking water

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Abstract

Global climate changes increase the risk of salinization of soil and water imposing possible health risks on livestock, especially in arid and semi-arid regions such as the Andes, the habitat of wild and domestic South American camelids (SAC), e.g. the llama (*Lama glama*). The aim of the present study was to evaluate the sensitivity and tolerance of llamas towards different NaCl concentrations in their drinking water. In total, 12 adult females with an average body weight (BW) of 140 kg ± 20.6 kg were kept under controlled conditions in individual pens. After a control phase (1 week) providing only fresh water, two choice tests were consecutively conducted: (1) a pairwise preference test (3 weeks) offering one bucket with fresh water and another with stepwise increasing NaCl concentration (0.25, 0.5, 0.75, 1.0, 1.25, 1.5%) and (2) a free-choice test (3 weeks) during which six buckets were simultaneously offered with NaCl concentrations of 0, 0.25, 0.5, 0.75, 1.0, and 1.25% NaCl. Chopped hay, water and a salt lick were provided for ad libitum intake. Records were kept on BW, body condition, feed, water and Na intake. Dry matter intake, total water and Na intakes increased during both choice tests ($P < 0.001$). Daily total Na intakes relative to metabolic body size ($g/kg^{0.75}$) averaged 0.04 ± 0.02, 0.19 ± 0.02 and 0.26 ± 0.02 during the control phase, the preference and the free-choice test, respectively. In the pairwise test, llamas showed a weak preference for saline water with 0.5 – 0.75% NaCl, and rejected water from 1.25% NaCl. During the free-choice system, llamas had a remarkable interest in saline water with shares from total drinking water intake for fresh water and concentrations of 0.25, 0.50, 0.75, 1.0 and 1.25% NaCl being 23.6, 21.2, 19.5, 13.8, 13.3 and 8.6%, respectively. Llamas demonstrated a similar capacity to differentiate between saline water concentrations in two different experimental setups and adjusted their Na intake in self-selection. The results suggest that their reactions to saline water are similar to those of goat breeds adapted to arid zones.

1. Introduction

Due to global climate change, salinization of ground water and soil is an increasing worldwide phenomenon (IPCC, 2015; The United Nations, 2015). Andean regions, the natural habitat of South-American camelids (SAC), are also confronted with the challenges of soil degradation and salinization (Oldeman et al., 1991; Nordt et al., 2004). In their native habitat, saline lakes naturally occur and SAC have been reported drinking from these water supplies (Franklin, 1983). With advancing climate change and land degradation, llamas will face increasing salinization of their natural water resources. Although Na is an essential nutrient for several body functions, such as the regulation of

the osmotic pressure, the acid–base balance, the water metabolism, the muscle and nerve functioning or the nutrient transport (Michell, 1995; Suttle, 2010), excessive salt intake may affect feed and water consumption of animals or even cause severe health problems (Peirce, 1957; Wilson, 1966; Wilson and Dudzinski, 1973; Trueman and Clague, 1978; McGregor, 2004b). Sheep have shown to tolerate high NaCl concentrations between 5% and 20% in their diet (Digby et al., 2011). However, there is a different sensitivity to ingestion of NaCl from either feed or drinking water, probably due to quicker absorption of dissolved nutrients (Wilson, 1966; Masters et al., 2005). Drinking water with NaCl concentrations between 1.0% and 1.3% was tolerated by sheep (Peirce, 1968). However, ingestion of saline water with a NaCl concentration of 2% or greater led to severe reduction in food intake, and possibly death in sheep, goats and cattle (Peirce, 1957; Weeth and Haverland, 1961; Wilson and Dudzinski, 1973; Hamilton and Webster, 1987; McGregor, 2004a). Camels (*Camelus* spp), the closest relatives of SAC, demonstrated a remarkable higher NaCl tolerance than sheep and goats and tolerated more than 1.5% salt in their drinking water (Abou Hussien et al., 1994; Assad and El-Sherif, 2002).

However, there has been no scientific investigation of the mineral nutrition of camelids (NRC, 2007; Fowler, 2010). Instead, the Na recommendations are extrapolated from the respective requirements of sheep, goats and cattle (van Saun, 2006b). There are anecdotal reports on guanacos (*Lama guanicoe*), the wild ancestor of the domestic llama *(Lama glama)*, drinking saline water (Darwin, 1844). However, no studies are available on responses to variations of NaCl in drinking water of llamas. For the study of taste discrimination in animals, different methods have been applied including electrophysiological methods (Bernard, 1964), recording of gustatory nerve impulses (Bell and Kitchell, 1966) or preference tests (Goatcher and Church, 1970b, 1970a). The principle approach in preference or choice tests is to provide a simultaneous choice of selections and then measure the animals' responses to each (Raffa et al., 2002). The choice may be associated with sensory components (e.g., smell, taste) and post-ingestive effects. Interestingly, herbivores can recognize Na directly by taste and show a specific appetite for Na when it is in short supply (Denton and Sabine, 1961).

The aim of the present study was to evaluate the responses of llamas towards different Na concentrations in their drinking water. Knowing the acceptability of water containing specific concentrations of NaCl could help to determine the overall salt tolerance (Wilson, 1966). Two different study designs were applied. First, a pairwise preference test (Goatcher and Church, 1970b) with the choice between fresh and saline water with ascending NaCl concentrations was conducted. This setup allows an exact

differentiation of the individual sensitivity and taste discrimination towards particular NaCl concentrations in the water. Second, a free-choice system offering simultaneously the choice between fresh water and water with different concentrations of NaCl to determine the long-term intake of saline water. In similar cafeteria designs, goats and sheep demonstrated their capacity to select a balanced diet according to their nutrient requirements (Fedele et al., 2002) and to avoid excessive intakes of saline water (Görgülü et al., 1996; Runa et al., 2019b).

2. Material and methods

The experiments conducted in this study were performed in accordance with the guidelines of the German animal ethics regulations and approved by the State Office of Lower Saxony, Germany for Consumer Protection and Food Safety, Germany (Ref. no.: 33.9-42502-04-16/2310).

2.1. Animals, housing and experimental design

The experiment was conducted at the Department of Animal Sciences, University of Goettingen, Germany from April 2017 to May 2017, and repeated from May 2018 to June 2018. In total, 12 non-pregnant, non-lactating llama dams were available for the experiment, six per repetition. All animals were born in Germany and belonged to the experimental station Relliehausen of Goettingen University. The animals had an initial average body weight (BW) of 140 kg ± 20.6 kg, ranging between 109 and 161 kg. The average age was 11 years ± 2.6 years (range between 5 and 14 years). Prior to the experiment, the llamas were used to drink fresh water with similar low Na concentration as the experimental tap water.

The animals were individually kept in an open housing system (Fig. 1). Three similar rooms of the experimental unit were subdivided into two individual pens (9.6 m^2) with straw as bedding material. An individual half-roofed outdoor run (12 m^2) with one feed trough was always freely accessible for each animal. Along one side of the pen a wooden rack was installed with openings for 6 water buckets numbered from 1 to 6, respectively. Chopped hay (5 – 10 cm particle length) and water (fresh and saline) were provided for *ad libitum* intake over the whole experimental period. Additionally, a salt lick (K+S Minerals and Agriculture GmbH, Kassel, Germany) containing 37% Na, 1.1% Ca, 0.6% Mg, 0% P, Mn_2O_3 (1000 mg/kg), ZnO (1000 mg/kg), $Ca(IO_3)_2$ (100 mg/kg), $CoCO_3$ (20 mg/kg) and Na_2SeO_3 (20 mg/kg) was placed in the feed trough at free disposal in each pen throughout the experimental period.

The environment was identical in all six pens during both repetitions. Ambient temperature and relative humidity were measured with data loggers (iButton® temperature/humidity logger DS1923, Maxim Integrated Products, Inc., San José, CA, USA), at a frequency of 30 min for each pen. In addition to the natural light, artificial light was provided for a constant lighting schedule with 14 h light and 8 h dark per day from 06:30 h to 20:30 h.

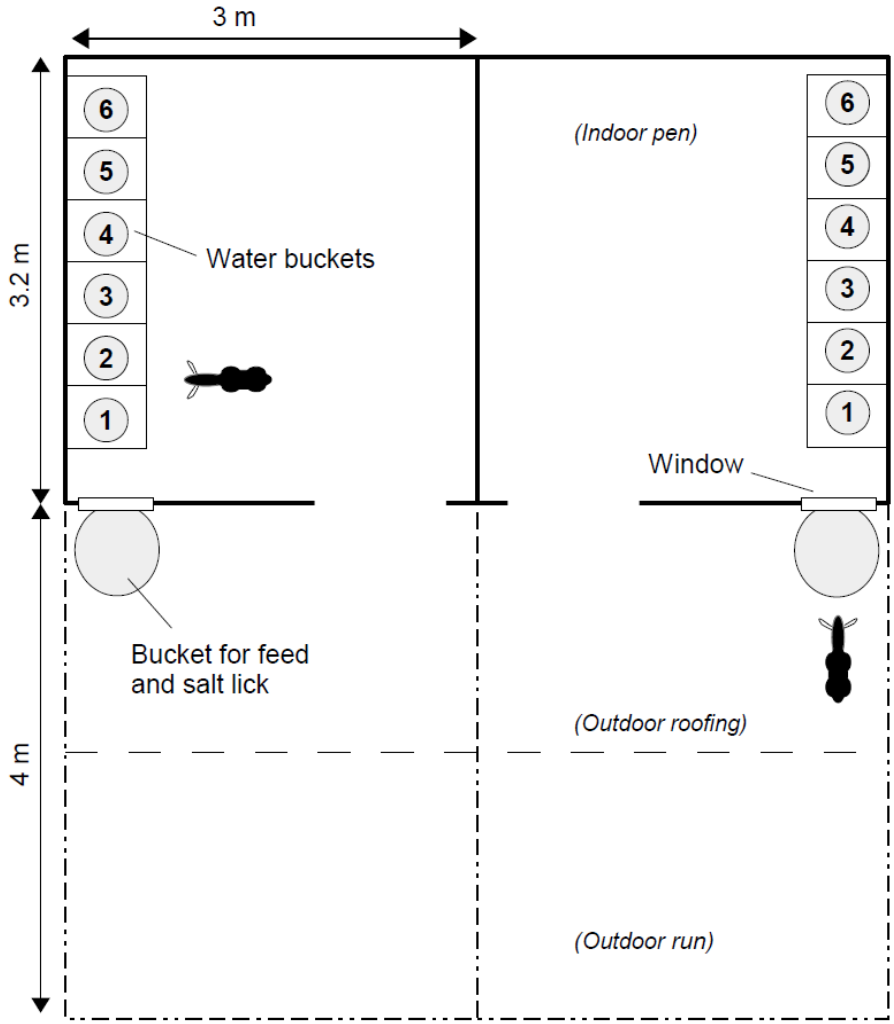

Fig. 1. Experimental barn: one room separated into two individual pens (for one animal each). The water buckets (1 – 6) were placed inside the barn. The feed/salt lick buckets were located in the outdoor run, accessible through an open gate. In total, three rooms with each two pens in a mirror-inverted arrangement were used.

In total, the experiment consisted of three consecutive phases (Fig. 2), which were each equally performed in the two repetitions: Phase 1 was the control phase (1 week) to allow acclimatization of the animals to the setup. Only fresh water was provided in two adjacent buckets. The buckets' positions were regularly changed across the six openings to accustom the animals to the later experimental setup. The individual feed and water intakes were measured on 4 consecutive days per week.

2.1.1. Pairwise preference test (phase 2)

In phase 2, the sensitivity of llamas towards different Na concentrations in their drinking water was tested for three weeks with a stepwise habituation. A pairwise preference trial according to Bell (1959) and Goatcher and Church (1970a) was conducted in order to evaluate the choice among different Na concentrations in the water. Two adjacent water buckets (10 L capacity) were daily filled. One bucket contained 5 L fresh water, while the second bucket was filled with 5 L saline water in increasing concentrations (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5% NaCl). Each combination of fresh and saline water was provided freely for two consecutive days, e.g., on day 1 and 2, animals could choose between fresh and 0.25% saline water, on day 3 and 4 choice between fresh and 0.5% saline water etc. Solutions were replenished every day. The position of the buckets was randomized to counteract possible sidedness of the animals (Meier et al., 2012). Each week, two combinations were offered. During the remaining 3 days of the week, only fresh water was offered.

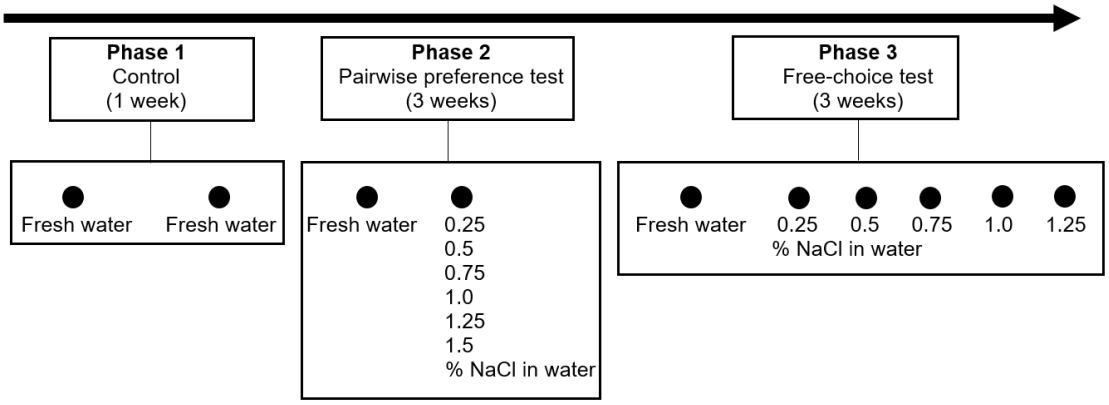

Fig. 2. Experimental design containing three phases. Phase 1: control, two buckets with fresh water were offered simultaneously. Phase 2: pairwise preference test, two buckets were offered with one containing test water with ascending salt (NaCl) concentration. Phase 3: free-choice test, six buckets were offered simultaneously with each different salt concentration in the test water solution. The bucket positions were changed randomly on a daily basis.

2.1.2. Free-choice test (phase 3)

A free-choice situation was offered for three weeks in phase 3, the week after finishing the pairwise preference test. Six identical water buckets filled with fresh or saline water with one of the five different concentrations (0.25, 0.5, 0.75, 1.0 and 1.25% NaCl) were offered simultaneously. Animals could choose freely between concentrations for five consecutive days per week. On the remaining two days of the week, only fresh water was provided. The buckets were again randomly allocated to the six possible positions to avoid sidedness.

2.2. Recorded traits

The BW of each animal was recorded with a mobile scale (resolution: 0.2 kg, Salter Brecknell LS300, Smethwick, West Midlands, UK) before the experiment and after both experimental phases and expressed as BW (kg) and metabolic body size ($kg^{0.75}$). At the same time, body condition scores (BCS) were assessed according to the procedure for llamas, described by van Saun (2006a). The scale ranged from 1 (emaciated) to 5 (grossly obese), with 0.5 increments.

The saline water was prepared with a salt of 99.8% NaCl purity (Esco Siede-Speisesalz, Hannover, Germany). In order to create the exact Na concentrations for the different test solutions, the salt water buckets were prepared using a stock solution of 10% NaCl purity. The exact Na concentration was then checked with a refractometer for NaCl measurements (HI 96821; Hanna Instruments Inc., Woonsocket, RI, USA). The average Na content of fresh water was 7.55 mg/L. The water buckets were refilled daily with the same amount of water for all animals (5 L) and the individual water intake per day was re-weighed at 08:30 h with a scale to the nearest 1 g (Sartorius CPA34000, Sartorius AG, Goettingen, Germany). Each bucket had an allocated number and location but a varying Na concentration in the water. A control bucket (5 L) was stationed in the barn and re-weighed daily to assess the water lost by evaporation and correct the water intake. Total daily drinking water intake (TDWI) was defined as sum of consumed fresh and saline water, corrected for the evaporative losses. In addition, total daily water intake (TWI) was calculated from the sum of TDWI and water intake via the feed. TWDI and TWI were also expressed as function of BW to the power of 0.82 since water is used in evaporative cooling, body transport systems and metabolism (Wilson, 1989).
The Na intake was calculated from the ingestion of Na originating from the fresh and test water, the feed, as well as the salt lick. The share of Na from the salt (NaCl) in the water was calculated based on the molecular weights. As the Na content of the provided feed (hay) was below the detection limit of the method (<0.2 g/kg dry matter (DM), see below), total Na intake was defined as the sum of intake from ingested water and salt lick.

The hay in both repetitions originated from the same batch. It was chopped to avoid wastage and offered daily for *ad libitum* intake at 08:00 h and refilled during the day when necessary. The individual feed intake was determined by re-weighing the leftovers per day to the nearest 1 g using an electronic scale (Sartorius CPA34000, Sartorius AG).

2.3. Chemical analyses

For chemical analyses, samples of the chopped hay were taken weekly. The samples were kept at room temperature and were ground prior to analysis (1 mm sieve) with a

mill and stored in airtight containers. Further analyses were performed according to VDLUFA (2012), and method numbers were given; DM (3.1), ash (8.1) and crude fat (CL, 5.1.1, using a Soxtec 2055; Foss Analytical Systems, Hillerød, Denmark). Crude protein (CP) was determined by Dumas combustion (4.1.2; Elementar VarioMAX CN, Langenselbold, Germany). The concentrations of aNDFom (6.5.1; assayed with heatstable amylase) and ADFom (6.5.2), both expressed exclusive residual ash, were analysed sequentially using an Ankom 220 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). A Hohenheim gas test (25.1) was conducted to measure the 24-h *in vitro* gas production (GP, ml/200 mg DM). The metabolizable energy (ME) content was calculated by using equations for ruminants of GfE (2008), which are based on digestibility measurements on wether sheep : ME (MJ/kg DM) = 7.81 + 0.07559 × GP − 0.00384 × Ash + 0.00565 × CP + 0.01898 × CL − 0.00831 × ADFom (Ash, CP, CL, and ADFom are in g/kg DM and GP is in ml/200 mg DM). The ME values of lower quality forages may be slightly higher (3-5 percent) for camelids than estimated for sheep (NRC, 2007). An aqueous extract using ion chromatography with conductivity detection (Dionex DX-100, Dionex, Sunnyvale, CA, USA) was used for the assay of Na and Cl. Additionally, Ca, Mg, P and K were analysed by ashing and dissolving samples in HCl. Ca, Mg and K were then measured by atomic absorption spectroscopy (Varian SpectrAA-300, Varian, Palo Alto, CA, USA), and P was measured photometrically. The results are illustrated in Table 1.

Table 1. Chemical composition (means ± SD) of the hay forage across both experimental repetitions (g/kg DM, unless otherwise stated)[1].

Composition	Means ± SD
DM (g/kg)	914.5 ± 2.7
ME (MJ/kg DM)	7.2 ± 0.1
24-h gas production (g/200 mg DM)	32.7 ± 0.7
Crude ash	71.7 ± 3.5
Crude protein	72.1 ± 3.6
Crude lipids	13.7 ± 0.6
aNDFom	663.8 ± 13.9
ADFom	414 ± 9.7
Calcium	7.2 ± 0.7
Phosphorus	1.3 ± 0.1
Sodium	n.d.
Magnesium	2.4 ± 0.2
Potassium	18.6 ± 0.8
Chlorid	3.1 ± 2.7

[1]: Each value is based on at least two analytical replicates per week; n.d. = not detectable or below the detection limit of the method.

DM: dry matter; ME: metabolizable energy; GP: 24-h gas production; aNDFom: neutral detergent fibre assayed with heat-stable amylase and expressed exclusive of residual ash; ADFom: acid detergent fibre expressed exclusive of residual ash.

2.4. Statistical Analyses

Data were recorded on a daily basis, averaged for each llama per phase and analysed across both repetitions. Statistical analyses were performed using R (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria) based on linear mixed-effects models. The Satterthwaite's method was used for the approximation of the degrees of freedom. Analyses of variances (Anova) were conducted using Type III Wald F tests within the lme4 package.

The model for the comparison of the BW, BCS, DMI, water intakes and Na intakes between the three phases (phase 1: control, phase 2: pairwise preference test and phase 3: free-choice test) included the following effects:

$$y_{ijk} = \mu + P_i + R_j + (P * R)_{ij} + A_k + e_{ijk}$$

where y_{ijk} is the observed value, μ is the overall mean, P_i is the fixed effect of the phase ($i = 1, 2, 3$), R_j is the fixed effect of the repetition ($j = 1, 2$), and $P * R$ the respective interaction. A_k is the random effect of the animal, and e_{ijk} the residual error (df = 5).

The model for comparisons of water intakes, Na intakes and the DMI during the pairwise preference test (phase 2) included the following effects:

$$y_{ijk} = \mu + R_i + S_j + (R * S)_{ij} + A_k + e_{ijk}$$

where y_{ijk} is the observed value, μ is the overall mean, R_i is the fixed effect of the repetition (i = 1, 2), S_j is the fixed effect of the administered NaCl concentration (%) in the test water (j = 0.25, 0.5, 0.75, 1.0, 1.25 or 1.5), and $\mathrm{P} * \mathrm{R}$ the respective interaction. A_k is the random effect of the animal, and e_{ijk} the residual error (df = 11).

The model for comparisons of water intakes, Na intakes and the DMI during the free-choice preference test (phase 3) was as follows:

$$y_{ijklm} = \mu + W_i + R_j + S_k + B_l + (R * S)_{jk} + (S * B)_{kl} + (W * S)_{ik} + A_m + e_{ijklm}$$

where y_{ijklm} is the observed value, W_i is the fixed effect of the week during the repetition (i = 1 to 3), R_j is the fixed effect of the repetition (j = 1 or 2), S_k is the fixed effect of the administered NaCl concentration in the test water (k = 0.0, 0.25, 0.5, 0.75, 1.0, 1.25 or 1.5), B_l the bucket position (l = 1 to 6) and the respective interactions $R * S$, $S * B$ and $W * S$. A_m is the random effect of the animal, and e_{ijklm} the residual error (df = 5).

The model assumptions were verified on the basis of residual plots and the Shapiro-Wilk normality test. A P-value of 0.05 was considered as significant. The results are reported as least squares (LS) means (emmeans package) and their standard error (SE). Repeated measurements were considered in the models. The included Tukey-Kramer test was used to evaluate differences between groups. Spearman rank correlation coefficients (r_s; confidence interval of 95%) were calculated with the included cor.test function between the BW and the total intakes of water and Na.

For the pairwise preference test, the discrimination model proposed by Bell (1959) and Goatcher and Church (1970a) was used. For analysing the proportion of the ingested test water from the TDWI, weighted least squares of the TDWI were calculated based on the statistical models given before. Considering that a random total drinking water intake for two buckets should be 50% per bucket, a 95% confidence level resulted in a non-discrimination zone from 40% (lower discrimination threshold, LDT) to 60% (upper discrimination threshold, UDT). The rejection threshold (RET) was set at 20% intake, the preference threshold (PRT) at 80% intake. The reaction was described as indifferent or random, when the proportional intake was between 40 and 60%.

3. Results

Table 2 summarizes the average results of the feed, water and Na intakes for each phase. This overview allows, in particular, the comparison of the saline water uptake between the pairwise preference tests (phase 2) and the free-choice system (phase 3).

Both repetitions followed the same experimental protocol. Average ambient temperature of the first repetition was lower (14°C ± 5.7°C) than the second (19°C ± 3.3°C). There were neither differences in the average BW or BCS of the animals between the repetitions, nor changes over the course of the experiment (Table 2). Further differences between repetitions were not detected. In general, the interactions between repetition and NaCl concentrations were not significant and results are shown across both repetitions. Overall, a weak positive correlation of was observed of the animals' total Na intake with the BW (r_s= 0.39, P < 0.001), whereas the TDWI did not correlate with the BW (r_s = 0.07; P = 0.08).

3.1. Pairwise preference test (phase 2)

During the control phase, when only fresh water was offered, drinking water intake averaged 3114 ± 246 g/d (repetition 1) and 3555 ± 246 g/d (repetition 2). We observed large individual differences in total drinking water intakes (TDWI) ranging between 40.6 and 95 g/$kg^{0.82}$ per day (phase 1) and 53.7 to 99.8 g/$^{kg0.82}$ per day (phase 2). The TDWI increased with incremented NaCl concentration during the pairwise preference test (P < 0.001). The lowest TDWI per $kg^{0.82}$ occurred when offering 0.25% NaCl concentration in the test water and peaked at 1.25% NaCl to 1.4 times the drinking water consumption of the control phase (Tables 2 and 3). The ratio of fresh to test water intake followed an interesting pattern (Table 3). The fresh water intake increased significantly with an increase in the NaCl concentration, reaching the maximum at 1.5% (P < 0.001, Table 3).

Based on the relative intake of saline water, the response of the llamas to ascending concentrations of Na in the test water is depicted in Fig. 3. The response was indifferent until 0.5 and 0.75%, where the llamas showed a weak preference for saline water. However, at 1% the preference declined and the llamas changed from a weak to moderate rejection of saline water at 1.25 and 1.5%, respectively. Within the ratio chart of the water intakes (Fig. 3), the saline water intake when offered 1.5% NaCl differed (P < 0.001) from the respective intakes of the pairwise tests with NaCl concentrations between 0.25 and 1%, respectively.

Table 2. Average (LSmeans) body weight and body condition, as well as daily feed, water and sodium intakes (g per day and corrected for metabolic body size) for the control phase (1), the pairwise preference test (phase 2) and the free-choice test (3) in llamas (n = 12), across both repetitions.

Trait	Phase			SEM	P-Value
	1 (Control)	2 (Pairwise preference)	3 (Free-choice)		Phase effect
Body weight (kg)	140.33	141.0	142.71	6.41	0.147
BCS	3.15	3.2	3.25	0.25	0.126
Dry matter intake (g)	1542[a]	1647[a]	1798[b]	67.3	<0.001
Dry matter intake (g/kg$^{0.75}$)	38.2[a]	40.6[a]	43.8[b]	1.75	<0.001
Fresh water intake (g)	3334[a]	2120[b]	912[c]	174	<0.001
Fresh water intake (g/kg$^{0.82}$)	59.5[a]	37.4[b]	16.2[c]	3.9	<0.001
Test water intake (g)	-	1917[a]	3662[b]	241	<0.001
Test water intake (g/kg$^{0.82}$)	-	33.5[a]	63.1[b]	4.1	<0.001
Total drinking water intake (g)	3334[a]	4037[b]	4574[c]	256	<0.001
Total drinking water intake (g/kg$^{0.82}$)	59.5[a]	70.9[b]	79.3[c]	5.3	<0.001
Total water intake (g)	3483[a]	4191[b]	4739[c]	261	<0.001
Total water intake (g/kg$^{0.82}$)	62.1[a]	73.6[b]	82.1[c]	5.4	<0.001
Total water intake/dry matter intake	2.29[a]	2.54[b]	2.64[b]	0.1	0.001
Na Salt lick (g)	1.8	1.5	1.4	0.5	0.458
Na Salt lick (g/kg$^{0.75}$)	0.04	0.04	0.03	0.01	0.404
Na Fresh water (g)	0.03[a]	0.02[b]	0.01[c]	0.001	<0.001
Na Fresh water (g/kg$^{0.75}$)	0.0006[a]	0.0004[b]	0.0002[c]	0.0001	<0.001
Na Test water (g)	-	6.2[a]	9.6[b]	0.9	<0.001
Na Test water (g/kg$^{0.75}$)	-	0.15[a]	0.23[b]	0.02	<0.001
Na Total drinking water (g)	0.03[a]	6.2[b]	9.6[c]	0.9	<0.001
Na Total drinking water (g/kg$^{0.75}$)	0.001[a]	0.15[b]	0.23[c]	0.02	<0.001
Na Total (g)	1.8[a]	7.7[b]	10.9[c]	1.1	<0.001
Na Total (g/kg$^{0.75}$)	0.04[a]	0.19[b]	0.26[c]	0.02	<0.001

Total drinking water intake = water intake from fresh and test water; Total water intake = total drinking water intake + water intake via feed; Na Salt lick/Fresh water/Test water/Total drinking water = Sodium intake from salt lick, fresh water, test water and total drinking water, respectively; Na Total = Total sodium from fresh and test water and salt lick. Phases: 1 = control phase, only fresh water offered in two buckets; 2 = pairwise preference, fresh water was offered in one bucket, test water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5% NaCl) in another bucket; Phase 3 = free-choice test, six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.25% NaCl). [a,b,c] Means in the same row with different superscripts are significantly different at $P < 0.05$. Sodium content of the hay was below the detection limit of the method.

Table 3. Pairwise preference test (phase 2). Effect of salt (NaCl) concentration (SC) in the test water on average daily feed, drinking water and sodium intakes (g per day and corrected for metabolic body size) in llamas (n = 12), across both repetitions.

	Salt concentration (% NaCl) in the test water						SEM	P-value
Trait	0.25	0.5	0.75	1	1.25	1.5		Salt concentration effect
Dry matter intake (g)	1616	1602	1665	1642	1722	1632	69	0.092
Dry matter intake (g/kg$^{0.75}$)	39.8	39.4	41.0	40.4	42.5	40.2	1.8	0.081
Fresh water intake (g)	1841^{a}	1390^{a}	1579^{a}	1772^{a}	2907^{b}	3230^{b}	260	<0.001
Fresh water intake (g/kg$^{0.82}$)	31.7^{a}	24.3^{a}	28.3^{a}	30.9^{a}	51.8^{b}	57.3^{b}	5.0	<0.001
Test water intake (g)	1626^{ab}	2329^{b}	2634^{b}	2050^{ab}	1768^{ab}	1099^{a}	355	0.003
Test water intake (g/kg$^{0.82}$)	29.4^{ab}	41.2^{a}	46.1^{a}	36.1^{ab}	30.1^{ab}	18.0^{b}	6.2	0.001
Total drinking water intake (g)	3467^{a}	3719^{ab}	4212^{ab}	3822^{ab}	4674^{b}	4329^{ab}	326	0.005
Total drinking water intake (g/kg$^{0.82}$)	61.1^{a}	65.5^{ab}	74.4^{ab}	67.0^{ab}	81.9^{b}	75.3^{ab}	6.4	0.006
Total water intake (g)	3620^{a}	3870^{ab}	4367^{ab}	3974^{ab}	4836^{b}	4482^{ab}	330	0.005
Total water intake (g/kg$^{0.82}$)	63.7^{a}	68.2^{ab}	77.1^{ab}	69.7^{ab}	84.7^{b}	78.0^{ab}	6.5	0.006
Total water intake/dry matter intake	2.2^{a}	2.4^{ab}	2.6^{ab}	2.4^{ab}	2.8^{b}	2.8^{b}	0.2	0.032
Na Salt lick (g)	1.7^{a}	1.7^{a}	1.6^{ab}	1.6^{ab}	1.3^{b}	1.3^{b}	0.5	<0.001
Na Salt lick (g/kg$^{0.75}$)	0.038^{a}	0.038^{a}	0.035^{ab}	0.035^{ab}	0.031^{b}	0.031^{b}	0.011	<0.001
Na Fresh water (g)	0.01^{a}	0.01^{a}	0.01^{a}	0.01^{a}	0.02^{b}	0.02^{b}	0.002	<0.001
Na Fresh water (g/kg$^{0.75}$)	0.0003^{a}	0.0003^{a}	0.0003^{a}	0.0003^{a}	0.0006^{b}	0.0006^{b}	0.0001	<0.001
Na Test water (g)	1.6^{a}	4.6^{ab}	7.8^{b}	8.1^{b}	8.7^{b}	6.5^{b}	1.3	<0.001
Na Test water (g/kg$^{0.75}$)	0.04^{a}	0.11^{ab}	0.19^{b}	0.20^{b}	0.21^{b}	0.15^{b}	0.03	<0.001
Na Total drinking water (g)	1.6^{a}	4.6^{ab}	7.8^{b}	8.1^{b}	8.7^{b}	6.5^{b}	1.3	<0.001
Na Total drinking water (g/kg$^{0.75}$)	0.04^{a}	0.11^{ab}	0.19^{b}	0.20^{b}	0.21^{b}	0.15^{b}	0.03	<0.001
Na Total (g)	3.3^{a}	6.3^{ab}	9.3^{b}	9.6^{b}	10.1^{b}	7.8^{b}	1.5	<0.001
Na Total (g/kg$^{0.75}$)	0.08^{a}	0.15^{ab}	0.23^{b}	0.24^{b}	0.24^{b}	0.18^{b}	0.03	<0.001

Total drinking water intake = water intake from fresh and test water; Total water intake = total drinking water intake + water intake via feed; Na Salt lick/Fresh water/Test water/Total drinking water = Sodium intake from salt lick, fresh water, test water and total drinking water, respectively; Na Total = Total sodium from fresh and test water and salt lick. Phase 2 = pairwise preference, fresh water was offered in one bucket, test water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5% NaCl) in another bucket. ^{a,b,c} Means in the same row with different superscripts are significantly different at P < 0.05. Sodium content of the hay was below the detection limit of the method.

The total Na intake was rather low in the control phase, due to the experimental setup with only fresh water in both buckets, and mainly originated from the salt lick. In comparison, total Na intake (per $kg^{0.82}$) during the pairwise preference test was 4.75 times higher than in the control phase ($P < 0.001$; Table 2). The total Na intake increased from 0.25 to 1.25% NaCl concentration in the test water ($P < 0.001$), and subsequently decreased at 1.5% (Table 3), in parallel to the intake of Na through the saline test water. In contrast, llamas reduced ($P < 0.001$) their Na intake through the salt lick with raised NaCl levels in the test water. The ratio of Na intake from the salt lick to the saline test water declined from 0.95 at NaCl concentration of 0.25% (i.e., almost same intake from both sources) to 0.15 at 1.25% NaCl in the test solution (most intake through saline test water).

3.2. Free-choice preference test (phase 3)

During phase 3, animals had free access for three consecutive weeks to five buckets with the same test solutions (except the 1.5% NaCl concentration) already offered during the pairwise preference tests in phase 2 and one bucket with fresh water. The results are summarized as weekly means in Table 4.

The DMI exceeded those during the previous phases and increased from the first to the remaining two weeks (Table 2 and 4, $P = 0.003$). The TDWI increased significantly over the different phases (Table 2), with the highest value during the free-choice test ($P < 0.001$). In the course of the pairwise preference test, the overall ratio of fresh water to test water intake was 1.12. During the free-choice system, this ratio dropped to 0.26, illustrating the large proportion of ingested saline test water. None of the water intake traits changed significantly over the three experimental weeks of phase 3 (Table 4). Additionally, the bucket position had no significant effect on the water intakes ($P = 0.21$).

While the TDWI remained similar across the three weeks, the composition of the ingested water changed. Fig. 4 depicts the differentiation of the llamas between the simultaneously offered solutions by experimental week. Across the free-choice phase, saline water with 1.5% NaCl was drunk the least. The proportional intakes for the different solutions were quite similar in week 1 and 2. The preferences roughly followed the same trend as in the pairwise test (Fig. 3) with highest intakes from fresh water and saline water of low concentrations up to 0.5% NaCl. This differentiation became more pronounced in the third week of the free-choice phase (Fig. 4). Across phase 3, the shares for the different concentrations from TDWI were 23.6, 21.2, 19.5, 13.8, 13.3 and 8.6% for fresh water and NaCl concentrations of 0.25, 0.50, 0.75, 1.0 and 1.5%,

respectively. Overall, only 21.9% of the TDWI was ingested from NaCl concentrations above 0.75%.

Table 4. Free-choice test (phase 3). Average daily feed, water and sodium intakes (g per day and corrected for metabolic body size) in llamas (n = 12), across both repetitions

	Week			SEM	P-Value
Trait	1	2	3		Week effect
Dry matter intake (g)	1716[a]	1818[b]	1833[b]	79.4	0.003
Dry matter intake (g/kg$^{0.75}$)	42.0[a]	44.5[b]	44.8[b]	1.8	0.007
Fresh water intake (g)	883	924	929	184	0.979
Fresh water intake (g/kg$^{0.82}$)	15.7	16.1	16.7	3.3	0.974
Test water intake (g)	3882	3496	3606	394	0.542
Test water intake (g/kg$^{0.82}$)	67.2	60.7	61.4	6.8	0.527
Total drinking water intake (g)	4766	4421	4535	335	0.505
Total drinking water intake (g/kg$^{0.82}$)	83.0	76.8	78.1	6.1	0.468
Total water intake (g)	4927	4592	4698	341	0.525
Total water intake (g/kg$^{0.82}$)	85.7	79.7	80.9	6.2	0.487
Total water intake/dry matter intake	2.9	2.5	2.5	0.1	0.057
Na Salt lick (g)	1.2	1.6	1.2	0.5	<0.001
Na Salt lick (g/kg$^{0.75}$)	0.03	0.04	0.03	0.01	<0.001
Na Fresh water (g)	0.007	0.007	0.007	0.001	0.979
Na Fresh water (g/kg$^{0.75}$)	0.0002	0.0002	0.0002	0.0001	0.975
Na Test water (g)	10.5	9.7	8.5	1.3	0.196
Na Test water (g/kg$^{0.75}$)	0.26	0.24	0.20	0.03	0.157
Na Total drinking water (g)	10.5	9.7	8.5	1.3	0.196
Na Total drinking water (g/kg$^{0.75}$)	0.26	0.24	0.2	0.03	0.157
Na Total (g)	11.7	11.3	9.7	1.6	0.164
Na Total (g/kg$^{0.75}$)	0.28	0.27	0.23	0.03	0.129

Total drinking water intake = water intake from fresh and test water; Total water intake = total drinking water intake + water intake via feed; Na Salt lick/Fresh water/Test water/Total drinking water = Sodium intake from salt lick, fresh water, test water and total drinking water, respectively; Na Total = Total sodium from fresh and test water and salt lick.

Phase 3 = free-choice test, six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, 1.25% NaCl). [a,b] Means in the same row with different superscripts are significantly different at P < 0.05. Sodium content of the hay was below the detection limit of the method.

In the free-choice test, the total Na intake surpassed ($P < 0.001$) the intakes during the control and the pairwise preference test (Table 2) The highest amount of ingested Na in week 1 of the free-choice test of 0.28 ± 0.03 g/kg$^{0.75}$ exceeded the control consumption by more than six times. Compared to the previous phase, the ratio of Na ingestion through salt lick to Na through water declined to 0.13. Similar to phase 2, the ingestion of saline water made the largest contribution to the total Na intake and averaged 92.9, 88.9 and 87% in weeks 1, 2, and 3, respectively. The Na intake from the fresh water was negligible. Table 5 summarizes the average daily water and sodium intakes through ingestion of the chosen test water. Water intake was highest from buckets with NaCl concentrations between 0 and 0.5% Na ($P<0.001$).

Table 5. Free-choice preference test (phase 3). Average (LSmeans) daily water and sodium intake through ingestion of respective test water with different salt concentration (g per day and corrected for metabolic body size) in llamas (n = 12), across both repetitions.

	Salt concentration (% NaCl) in the test water						SEM	P-Value
Trait	0	0.25	0.5	0.75	1	1.25		Salt concentration effect
Water intake (g)	912^{a}	942^{a}	916^{a}	712^{ab}	669^{ab}	423^{b}	98	<0.001
Water intake (g/kg$^{0.82}$)	16.2^{a}	16.7^{a}	15.8^{a}	12.3^{ab}	11.3^{ab}	7.0^{b}	1.8	<0.001
Na intake (g)	0.007^{a}	0.9^{ab}	1.8^{bc}	2.1^{c}	2.6^{c}	2.1^{c}	0.29	<0.001
Na intake (g/kg$^{0.75}$)	0.0002^{a}	0.02^{ab}	0.04^{bc}	0.05^{c}	0.06^{c}	0.05^{c}	0.007	<0.001

Water intake = through ingestion of respective test water with increasing salt concentration; Na intake = Na intake through ingestion of respective test water with increasing salt concentration. Phase 3 = free-choice preference test; six different water buckets with ascending salt concentrations (0, 0.25, 0.5, 0.75, 1 and 1.25% NaCl) offered simultaneously over three weeks. ^{a,b,c} Means in the same row with different superscripts are significantly different at $P < 0.05$.

4. Discussion

To the best of our knowledge, this is the first study that has been conducted on llamas by challenging them with saline water under controlled conditions. We used preference tests to evaluate the taste discrimination of concurrently offered choices of saline water. South American camelids (llamas, alpacas, guanacos and vicuña) and camels (*Camelus* spp.) are foregut fermenters with a three-compartmented stomach (Fowler 2010) which is not homologous with the rumen of true ruminants. Several studies underline the extremely efficient adaptation of camelids to conditions of poor vegetation, water scarcity or extreme climate conditions (Schmidt-Nielsen, 1964; Rübsamen and Engelhardt, 1975; Wensvoort et al., 2001). These characteristics make an interspecific comparison with ruminants interesting, in particular with goats who have shown a considerable water use efficiency and tolerance towards saline water (Silanikove, 2000; van Saun, 2006b; NRC, 2007).

4.1. Water intake

When only fresh water was offered (phase 1), our adult female llamas ingested 59.5 ± 5.3 g/kg$^{0.82}$ per day. A lower consumption of 42.5 ± 5.4 ml/kg$^{0.82}$ was recorded in adult castrated male llamas kept in pens under temperate climate and fed a hay diet (Rübsamen and Engelhardt, 1975). In comparison, Boer goats had higher intakes up to 93.1 g/kg$^{0.82}$ per day under temperate conditions and ad libitum access to chopped hay (Runa et al., 2019b; Runa et al., 2019a). However, llamas were able to reduce their renal water loss to a greater degree than goats (17 versus 29% of total water loss), indicating a high water efficiency.

During the preference tests, our llamas ingested considerable amounts of saline water. Obviously, Na intake needs to be regulated by the animal because it is the major extracellular element influencing osmolarity of body fluids. A key physiological response to high salt ingestion is to increase water intake to regain an isotonic state as shown e.g., for sheep (Peirce, 1957; Wilson and Dudzinski, 1973), goats (Runa et al., 2019a), rusa deer (Kii and Dryden, 2005) and red and fallow deer (Ru et al., 2005). Correspondingly, our llamas increased their drinking water consumption with higher Na intake and exceeded the fresh water intake of the control phase by 33% during the free-choice setup.

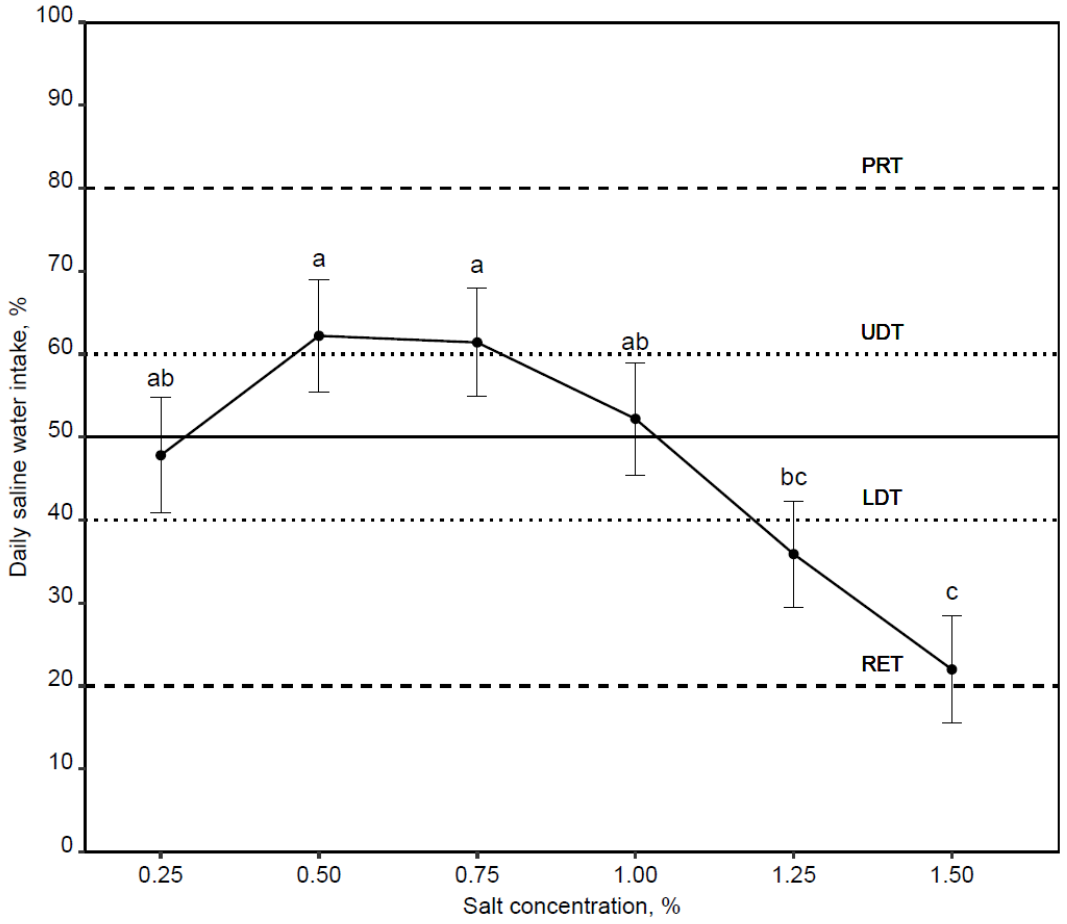

Fig. 3. Pairwise preference test (phase 2). Discrimination model (Goatcher and Church, 1970a) for the response of the llamas (n = 12) to ascending concentrations of NaCl in the test water. The amount of ingested saline water is shown as a % ± SE of the total drinking water intake (TDWI) from both buckets. Lines: RET = rejection threshold; LDT = lower discrimination threshold; UDT = upper discrimination threshold; PRT = preference threshold. [a,b,c] Means in the same row with different superscripts are significantly different at $P < 0.05$

However, this adaptive reaction is limited when only saline drinking water is available, as it increases salt load in the body fluids. In such cases, the animals have limited other adaptive responses to draw upon, beyond the excretion of increased amounts of salt via urine and a reduction of feed and water intake (Fowler, 2010). These physiological mechanisms were demonstrated in heifers who increased their water consumption by 52.8% when supplied drinking water containing 1% added NaCl (Weeth et al., 1960). However, water with 2% NaCl concentration impaired the animals' health and reduced their water consumption by 11% below the control level. In the present experimental setup, llamas always had access to fresh water. Apparently, increasing water intake to regulate the osmolarity of body fluids was effective and other adaptive responses such as reduction of feed intake were not activated.

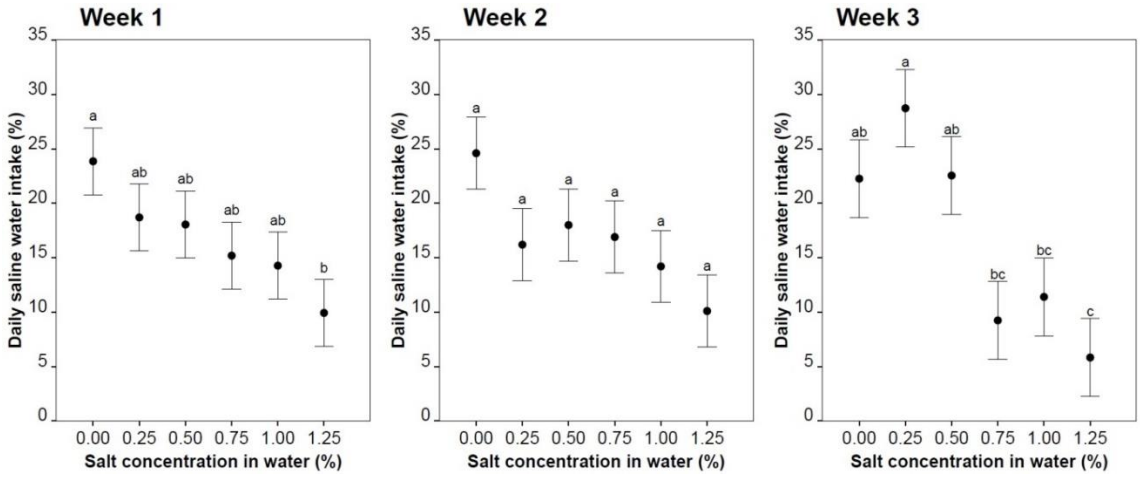

Fig. 4. Free-choice experiment (phase 3). Daily saline water intake (% of total drinking water intake; LS means and the+ir SE) for each salt concentration of llamas (n = 12), per treatment week. Simultaneous choice between 6 buckets with different salt concentrations. [a,b,c] Means in the same row with different superscripts are significantly different at $P < 0.05$ within the same week.

4.2. Sodium intake

The total Na intake increased over the course of the experiment. During the control phase, Na was mainly consumed via the salt lick. In contrast, during the pairwise preference and the free-choice test, the share of Na ingested from the water increased steadily at the concurrent expense of the intake via the salt lick. This suggests that llamas are able to adjust their Na intake from different sources. Similarly, Smith et al. (1953), demonstrated that cattle differentiated between various sources of salt. Goats, sheep and cattle demonstrated, in free-choice systems, their ability to adjust their diet to better meet physiological requirements (Kyriazakis and Oldham, 1993; Atwood et al., 2001; Fedele et al., 2002; Keskin et al., 2004) and to avoid excess Na intakes from saline water (Runa et al., 2019b).

There is a lack of scientific investigation of mineral nutrition in SAC and the actual Na requirement for llamas has not been defined (NRC, 2007). By extrapolation from studies of sheep, goats and cattle and adjustment for DMI, van Saun (2006b) calculated the Na requirement for llamas at 0.6 – 2.8 g/d, consistent with our Na intake of 1.8 ± 1.1 g/d during the control phase. However, during the pairwise preference and the free-choice test, the Na intake was more than four (7.7 ± 1.1 g/d) and six times (10.9 ± 1.1 g/d) higher, respectively, clearly exceeding the recommended intake of Na in other species (NRC, 2005, 2007). In a similar free-choice system with goats, Runa et al. (2019b) also showed that recommended amounts of Na were ingested during the control phase (0.8 ± 0.3 g/d), increasing to excessive intakes during the free-choice experiment (8.3 ± 0.3

g/d). In several herbivores, a natural craving behaviour for salt has been described and it is suggested from our results, that llamas also have a specific appetite for Na (Denton and Sabine, 1961; McDowell, 1996), increasing their Na intake when offered as free-choice. Because the free-choice test only lasted for three weeks, it is open to question whether the Na intake would remain on such a high level. It might be possible that the llamas' appetite for Na is rather relative than absolute and could be learned by experience, as stated for sheep (Suttle, 2010).

4.3. Dry matter intake, body weight and body condition

The DMI in adult llamas is usually around 1 – 1.5% of the BW (Johnson, 1994; van Saun, 2009; Fowler, 2010), consistent with our results of 1.1 – 1.3%. The present DMI was lowest during the control phase and significantly increased with higher intake of Na. This increase may be due to better palatability of feed (Grovum and Chapman, 1988). Similar reactions were observed for sheep (Peirce, 1957; Wilson, 1966; Wilson and Dudzinski, 1973), goats (Runa et al., 2019a), camels (Assad et al., 1997) and deer (Kii and Dryden, 2005; Ru et al., 2005) exposed to saline drinking water. In these studies, DMI usually increased until a NaCl concentration of 1.5% and declined as NaCl concentration increased. In heifers, only confronted with saline water, DMI was already reduced by 1.25% NaCl water (Weeth and Haverland, 1961).

In our experimental llamas, the BW and BCS were not negatively affected by the ingestion of saline water. Clinical signs indicative of Na intoxication such as abnormal behaviour, oedemas, weakness, anorexia, convulsions, or diarrhoea, were not observed (Weeth and Haverland, 1961; Trueman and Clague, 1978; Nestor et al., 1988). This agrees with studies on camels, the closest relatives to SAC, who demonstrated a remarkable high salt tolerance with regard to their productive performance (Abou Hussien et al., 1994; Assad et al., 1997; Assad and El-Sherif, 2002).

4.4. Sodium sensitivity and tolerance

In the pairwise test, our llamas showed a weak preference for saline water with 0.5 – 0.75% NaCl, a slight rejection of 1.25% and a stronger rejection at 1.5% NaCl water. In similar pairwise tests, cattle were shown to be very sensitive to NaCl in water, already rejecting saline water above 0.08%, while sheep revealed lower sensitivity, with indifferent reactions until 1.25% (Goatcher and Church, 1970b). Furthermore, Pygmy goats, exhibited a strong preference for water with 0.16 – 1.25% NaCl, whereas Saanen and Alpine goats already rejected 1.0% NaCl water (Goatcher and Church, 1970b). Boer goats showed a weak preference for saline water with 0.25 – 1.0% NaCl and an indifferent reaction for 1.25% in pairwise tests (Runa et al., 2019a).

However, it has been shown that thresholds for Na sensitivity are not constant. Thus, it was found for cattle and sheep that Na deficiency can cause an increased sensitivity to Na, allowing animals to detect minor changes in the water (Denton and Sabine, 1961; Bell and Kitchell, 1966; Bell and Sly, 1983). On the other hand, in a pairwise test, goats lowered their rejection threshold from 1.5 to 1.25% NaCl water after a stepwise adaptation to saline drinking water (Runa et al., 2019a). Thus, the Na regulation mechanisms are flexible depending on the total Na balance of the animal. The change in water choice might be explained by postingestive effects and/or the stimulation of receptors on the tongue, which in turn activate osmoreceptors in the hypothalamus (Ghanem et al., 2018).

In the present experimental designs, fresh water was always available. However, in the free-choice system llamas showed a remarkable preference for saline water of up to 76.43% of their TDWI. This high Na intake warrants further research into tolerance of llamas when only saline water would be available. Camels demonstrated a remarkably high NaCl tolerance and tolerated more than 1.5% NaCl in water, other than sheep and goats (Abou Hussien et al., 1994; Assad and El-Sherif, 2002). Interestingly, the salt load controlling mechanisms differ between species (Abou Hussien et al., 1994). While sheep and goats reduce high NaCl loads by increased water intake, increased glomerular filtration rate and renal salt excretion through urine (Potter, 1968; Dunson, 1974), camels protect themselves from salt stress by reducing water consumption per unit of metabolic body size (Abou Hussien et al., 1994; Assad and El-Sherif, 2002). In a comparative study camels, goats and sheep increased their total water intake by 130 ml/kg$^{0.82}$, 376 ml/kg$^{0.82}$ and 500 ml/kg$^{0.82}$, respectively, when exposed to water salinity of up to 1.7%. Apparently, the camel has evolved specific adaptive capacities (Wu et al., 2014). The kidneys are able to concentrate urine and conserve water by decreasing the glomerular filtration rate and increasing the tubular reabsorption of water (Siebert and Macfarlane, 1971). Salt content of camel urine may increase to twice the salt concentration of sea water (Fowler, 2010). This mechanism may partly explain the highly efficient water metabolism and high salt tolerance in drinking water found in camels (Schmidt-Nielsen and Schmidt-Nielsen, 1952; Schmidt-Nielsen et al., 1967; Siebert and Macfarlane, 1975). However, SAC seem not to possess the same capacity as their relatives, as llamas were not superior to goats in their ability to concentrate the urine (Rübsamen and Engelhardt, 1975).

4.5. Comparison of the testing methods

In the current study, llamas demonstrated their capacity to quickly adapt to different designs with varying options of fresh and saline drinking water in order to maintain an osmotic homeostasis. The pairwise preference setup for the taste response to test

solutions has already proven sensory discrimination capacities for goats, cattle and sheep (Bell, 1959; Goatcher and Church, 1970a; Runa et al., 2019a). However, these results only allow an assessment of the short-term discrimination and sensitivity reaction. In comparison, the method of free-choice tests additionally provides results for the evaluation of the long-term reaction when confronted to simultaneously offered water with different NaCl concentrations (Runa et al., 2019b).

Both tests revealed the challenge for llamas to differentiate between fresh water and water with low concentrations (until 0.5%) of NaCl. Nevertheless, the differentiation was more pronounced during the pairwise preference test. This observation might be explained from different perspectives. Llamas may have only limited sensory capacities to detect NaCl in aqueous solutions. However, there is a lack of information on taste receptors in llamas (NRC, 2007). In addition, as pointed out, thresholds for Na sensitivity may undergo changes.

It may be easier to distinguish two solutions (pairwise test) than six (free-choice test). In the free-choice test, the position of test solutions was daily changed. Thus, llamas were confronted with the dual task to detect a specific concentration (sensory input) and to remember the position of the respective bucket (learning task). Occasional video recordings revealed a large individual variation of drinking sessions per day. Frequently, animals had only one drinking session per day. This specific drinking behaviour may hamper learning and consumption decision of the animals (Langbein et al., 2007).

On the other hand, the low differentiation between low concentrations of NaCl in the free-choice test may indicate a generally higher tolerance of llamas for saline solutions. As llamas had already consumed high amounts of Na in the preceding pairwise test, the high saline water intake in the free-choice test cannot be explained by Na deficiency. It is noteworthy that goats in a similar free-choice system showed a clear preference for fresh water of up to about 60% of their total drinking water intake (Runa et al., 2019b), while in our llamas only 24% of fresh water was chosen. In addition, goats shifted their preference towards lower NaCl concentrations during the three weeks experimental phase of the cafeteria design (Runa et al., 2019b), whereas the choices remained quite similar in our llamas. Possibly the test duration was too short to allow the llamas full expression of their preferences. The capacity of SAC to tolerate long-term intake of saline water warrants further research also including physiological characteristics such as blood metabolites and blood or urine osmolality.

5. Conclusions

Llamas in the present showed a remarkable interest in low-concentrated saline water without compromising their health or performance. Their Na sensitivity and acceptance were comparable to goats. Camels, known to tolerate Na concentrations above 1.5% in their water, reduce their drinking water intake as reaction to salt, whereas our llamas were rather avoiding very high Na concentrations or increased their total water intake. Undeniably, llamas share some physiological features with camels with regard to their water efficiency, indicating an evolutionary adaptation to limited water supply. Previous reports suggest that their adaptive mechanisms are less developed and more similar to those of goats, particularly breeds, which originate from arid zones.

Declaration of Competing Interests

The authors declare that they have no conflict of interest.

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CHAPTER 5

Drinking behaviour of llamas (*Lama glama*) in choice tests for fresh or saline water

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Abstract

In the native region of South American camelids (SAC), the High Andes, llamas (*Lama glama*) face the dual confrontation with seasonal water scarcity and the risk of water salinization. The present aims are to provide more insight into drinking behaviour of llamas and their behavioural strategies in discriminating and selecting different concentrations of saline water in choice tests. Twelve adult non-pregnant female llamas with an average initial body weight of 140 kg ± 20.6 kg were kept under controlled conditions in individual pens. After a control phase (1 week) providing only fresh water, two choice tests were consecutively conducted: (1) a pairwise preference test (3 weeks) offering the choice between one bucket with fresh water and another with stepwise increasing NaCl concentration (0.25, 0.5, 0.75, 1.0, 1.25, or 1.5%) and (2) a free-choice test (3 weeks) offering six buckets simultaneously with concentrations of 0, 0.25, 0.5, 0.75, 1.0, and 1.25% NaCl. Chopped hay, water and a mineral lick were provided for *ad libitum* intake. Records were kept on body weight, body condition and daily drinking water intake. Individual 24-h video recordings (23-24 per animal) were analysed for drinking duration, frequency of drinking and testing, the latter being defined as drinking lasting maximum three seconds. Body weight and body condition remained constant. Overall, a low drinking frequency was observed, indicating an adaption to water scarcity. During the control phase, the pairwise preference and the free-choice test, the daily drinking duration ($p = 0.019$) and frequency ($p < 0.001$) increased significantly averaging 162, 238 and 263 s, and 3.71 to 5.17 and 7.60 bouts, respectively. Similarly, the testing-to-drinking frequency ratio increased ($p < 0.05$) averaging 0.26, 0.35, 0.79 in phase 1, 2 and 3. Most drinking bouts occurred during the daytime (83% of drinking bouts) and followed a biphasic rhythm with peaks in the morning and the evening. Llamas showed a remarkable tolerance of saline drinking water, similar to goats. Furthermore, llamas demonstrated their capacity for behavioural adaptation when more choice options were offered by changing their drinking pattern, while maintaining their diurnal rhythm of water intake. Decision-making was based on testing of solutions which increased considerably when more choice options were offered in the free choice test. Nevertheless, the llamas maintained a high intake of saline water. The low drinking frequency of the llamas might hamper their memory formation and discrimination learning when more than two samples are offered simultaneously.

1. Introduction

The llama (*Lama glama*) and the alpaca (*Vicugna pacos*) have been domesticated in South America 6,000–7,000 years ago from their wild ancestors, the guanaco (*Lama*

guanicoe) and the vicuna (*Vicugna vicugna*), respectively (Wheeler, 2012). In their largest distribution area, the High Andes, both domestic and wild South American camelids (SAC) are confronted with the twofold challenge of seasonal water scarcity and the increased risk of soil and water salinization due to global climate change (Oldeman et al., 1991; Nordt et al., 2004). Despite of the vital importance of water intake, only limited studies are available on drinking behaviour in SAC (for review see Gerken et al., 2019).

South American camelids ingest water by sucking in the water with the mouth slightly opened (Fowler, 2010). A labial cleft divides their upper lip and each side of the lip can be moved independently, whereas the lower lip is less mobile (Fowler, 2010). They cannot protrude the tongue to great extend (Fowler, 2010) and are apparently unable to lick (San Martin and Bryant, 1989).

With regard to adaptation of SAC to water scarcity, a low drinking frequency and water intake was already described in 1801 for two llamas imported from the Andes to the French menagerie of the French National Museum of Natural History, Paris. According to Lacépède and Cuvier (1801), the llamas did not drink at all when they had access to green grass and at all other times they drank very little. Apparently, the high percentage of water in fresh herbage can cover water requirements of grazing SAC to a large extent, resulting in seasonal fluctuations of water intake. Thus, female llamas kept in year-round outdoor housing in Germany ingested 9.17 L/d in summer (from drinking water, preformed water ingested in food and metabolic water), being twice as high as in winter with 4.22 L/d (Riek et al., 2017). This difference was mainly attributable to the increased ingestion of pasture in summer with a higher percentage of water compared to hay, fed in winter. Similarly, the water turnover rates in grazing llamas on pasture were found to be double that recorded indoors with hay feeding (Rübsamen and Engelhardt, 1975).

In wild vicunas, observed in the Andean regions of Southern Peru, a drinking bout consisted of a sequence of as many as five draughts with pauses between (Koford, 1957). Each draught had a duration of 5-15 s, then the vicuna raised the head and looked up. Vicunas drink once per day or eventually twice on very dry days, a period of 1–4.5 h intervening. Garrido et al. (1980) investigated the time budgets of wild guanacos (*Lama guanicoe*) in the faunistic Reserve of Cabo dos Bahías (Chubut Province, Argentina), based on scan sampling from sunrise to sunset (8-19:00 h). In the rainy season with abundant pasture, drinking behaviour was only recorded in 0-0.53% of all scans compared with 58-60% and 2.8-5.8% for grazing and browsing, respectively.

Very little information is available on diurnal distribution of drinking behaviour. Drinking activity of grazing guanacos in Patagónia (Argentine) peaked between 11-12 h (Garrido et al., 1980). In wild vicunas in Southern Peru drinking activity only occurred from 08:00 to 16.15 h, without any particular peak (Koford, 1957). In the study of Raggi et al. (1994), water intake during light and dark hours was recorded in five alpacas fed lucerne hay and kept in stables in Chile. The 24-h water intake averaged 2.9 l/animal with 0.9 l (31%) ingested during the dark period.

Concerning the response of SAC to saline water resources, there are only anecdotal reports on guanacos drinking water from saline lakes that naturally occur in the high Andes (Darwin, 1844; Franklin, 1983). Therefore, in a recent study, we investigated into the tolerance of llamas towards different concentrations of saline water (Enke et al., 2022b). In pairwise choice tests, llamas showed preference for saline water with 0.5 – 0.75% NaCl, but refused water with more than 1.25% NaCl. Further, llamas demonstrated their capacity to adjust their Na intake in a free-choice system with different NaCl solutions offered simultaneously. For further insight into their drinking behaviour during the choice tests, extensive 24-h video recordings where made and analysed in the current study. The present aims are (1) to provide more knowledge on drinking behaviour of llamas and its diurnal rhythm under controlled stable conditions and (2) to evaluate the behavioural strategies of the llamas in selecting different concentrations of saline water.

2. Materials and Methods

The experiments conducted in this study were performed in accordance with the guidelines of the German animal ethics regulations and approved by the State Office of Lower Saxony for Consumer Protection and Food Safety, Germany (Ref. no.: 33.9-42502-04-16/2310).

2.1. Animals and experimental design

The study was conducted at the Department of Animal Sciences, University of Göttingen, Germany from April to May 2017, with a repetition from May to June 2018. In both runs, six non-pregnant, non-lactating llama (*Lama glama*) dams were involved (total N=12). The llamas were born in Germany and belonged to the experimental station Relliehausen of Göttingen University. The initial body weight (BW) of the llamas before the start of the experiment was 140 kg ± 20.6 kg, ranging between 109 and 161 kg, the average age was 11 years ± 2.6 years, with a range between 5 and 14 years. The llamas had no previous experience with choice tests or with saline water.

The animals were kept in an open housing system with six individual pens, each 9.6 m² (Fig. 1) and had permanent access to an adjacent individual half roofed outdoor run (12 m²). Animals had visual contact with pen mates inside and outside the pen, but were not able to see their water choices. In each pen, six water buckets were mounted on the wall of each pen (distance from bottom of bucket to floor: 96 cm, bucket height: 23 cm) and numbered from 1 to 6, respectively (Fig. 1). The water buckets contained water (fresh or saline) for *ad libitum* intake. The feed trough was located in the half-roofed outdoor run. Animals were provided with hay (average dry matter content of 914.5 g/kg and CP content of 72.1 g/kg DM) for *ad libitum* intake. The hay in both repetitions originated from the same batch and was chopped (5 – 10 cm particle length) to avoid wastage. New feed was offered daily at 08:00 h and refilled during the day when necessary. Additionally, a mineral lick (K+S Minerals and Agriculture GmbH, Kassel, Germany) containing 37% Na, 1.1% Ca, 0.6% Mg, 0% P, Mn_2O_3 (1000 mg/kg), ZnO (1000 mg/kg), $Ca(IO_3)_2$ (100 mg/kg), $CoCO_3$ (20 mg/kg) and Na_2SeO_3 (20 mg/kg) was placed in the feed trough at free disposal in each pen throughout the experimental period. Straw was provided as bedding material.

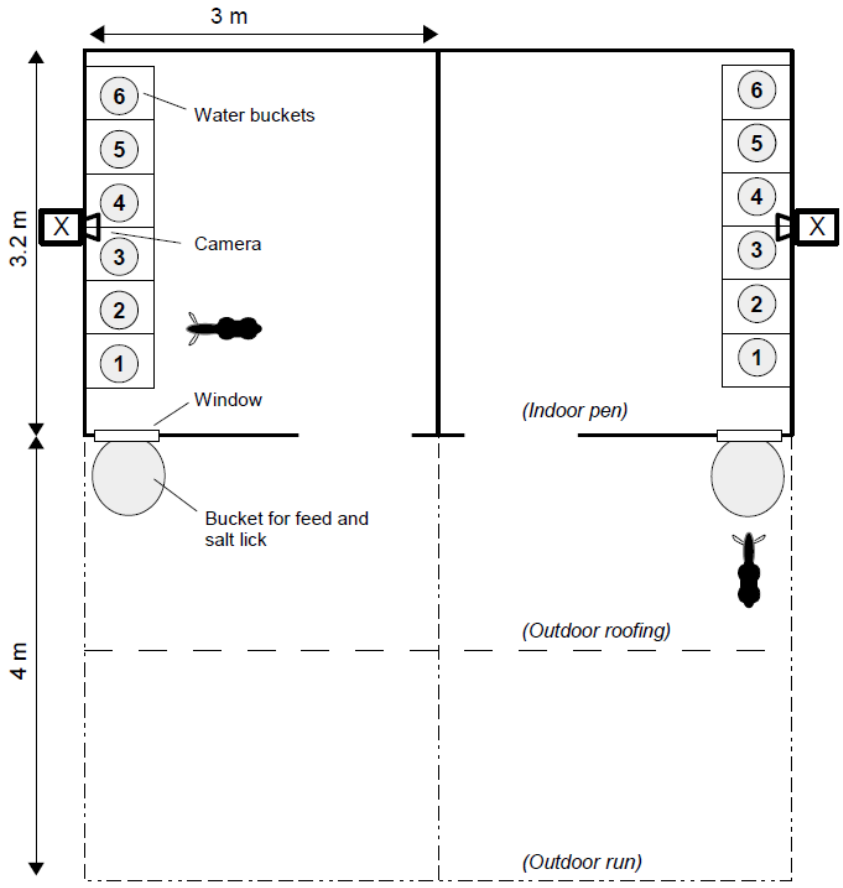

Fig. 1. Experimental stable. Each two pens were arranged mirror-inverted, each housing one llama. In total, six pens were used for the experiment. The water buckets (1 – 6) were placed inside the stable, whereas the buckets with feed and mineral lick were located in the outdoor run,

permanently accessible through an open door. Cameras were mounted above the water buckets in each pen.

The ambient temperature and relative humidity were measured with data loggers (iButton® temperature/humidity logger DS1923, Maxim Integrated Products, Inc., San José, CA, USA), at a frequency of 30 min for each pen. Similar lighting conditions of 14 h light and 8 h dark per day were ensured through artificial light inside the pens in addition to the natural light (lights on at 06:30 h and lights off at 20:30 h, each with a twilight phase of 15 min). The different saline solutions with their distinct NaCl concentrations were prepared with a salt of 99.8% NaCl purity (Esco Siede-Speisesalz, Hannover, Germany) mixed into a stock solution of 10% NaCl purity. The exact Na concentration was eventually verified with a refractometer for NaCl measurements (HI 96821; Hanna Instruments Inc., Woonsocket, RI, USA). The water buckets were refilled daily with the same amount of water for all animals (5 L) at 08:30 h.

2.1.1. Phase 1: control phase (1 week)

The experiment was performed twice and each repetition consisted of the same three consecutive phases as outlined in detail by (Enke et al., 2022b). The control phase allowed the acclimatization of the llamas to the experimental design and the observation of their normal drinking behaviour. Only fresh water was provided in two adjacent buckets. The buckets' positions were regularly changed across the six openings to accustom the animals to the later experimental setup.

2.1.2. Phase 2: Pairwise preference test (3 weeks)

During the pairwise preference test, the reactions of the llamas towards different Na concentrations in their drinking water was tested for three weeks with a stepwise habituation. A pairwise preference trial according to Bell (1959) and Goatcher and Church (1970) was conducted in order to evaluate the choice among different Na concentrations in the water. Two adjacent water buckets (10 L capacity) were daily filled with each 5 L solution. One bucket contained fresh water, while the second bucket was filled with saline water in increasing concentrations (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5% NaCl). Each combination of fresh and saline water was provided freely for two consecutive days, e.g., on day 1 and 2 animals had the choice between fresh and 0.25% saline water, on day 3 and 4, they choose between fresh and 0.5% saline water etc. Each week, two combinations were tested. During the remaining 3 days of the week, only fresh water was offered. Both buckets and their containing solutions were replenished every day. The position of the buckets was randomized daily to counteract side effects (Meier et al., 2012).

2.1.3. Phase 3: Free-choice test (3 weeks)

During the free-choice test, six identical water buckets were filled with fresh or saline water with one of the five different concentrations (0.25, 0.5, 0.75, 1.0 or 1.25% NaCl) and offered simultaneously. Each day, the solutions were replenished and allocated in a new random order to the six possible positions to avoid sidedness. Animals could choose freely for five consecutive days per week. On the remaining two days of the week, only fresh water was provided.

2.2. Data collection

Individual BW was recorded with a mobile scale (resolution: 0.2 kg, Salter Brecknell LS300, Smethwick, West Midlands, UK) before the experiment and after each experimental phase. At the same time, body condition scores (BCS) were assessed according to the procedure for llamas, described by van Saun (2006a), with a scale ranging from 1 (emaciated) to 5 (grossly obese), with 0.5 increments. Amounts of drinking water intakes were recorded daily from both fresh and saline water by reweighing the buckets to the nearest 1 g (Sartorius CPA34000, Sartorius AG, Göttingen, Germany) (Enke et al., 2022b).

High-resolution cameras (Dallmeier DF4820HD-DN/IR, 720 p, 12.5 fps; Dallmeier Electronic, Regensburg, Germany) with automatic day/night switching were used for video recording the drinking behaviour of the llamas. The integrated infrared illumination also allowed the 24-h recording during the dark period. In each pen, one camera was mounted above the six water buckets (Fig. 1).

In the control phase, individual videos (24 h) were recorded on one day in the first repetition and two days in the second repetition. During the preference test (phase 2), drinking behaviour was recorded once for each combination. In the free choice system, videos were taken on at least one day per each week and animal. For each animal, 11 (first repetition) or 14 (second repetition) video recordings were analysed by continuous observation, resulting in a total of 150 videos. Daytime was defined as the period between 06:30 and 20:30 h, whereas the remaining time, between 20:30 and 06:30 h, was considered as night.

Drinking behaviour of our llamas was defined as follows:

Drinking: the animal placed its head into the bucket for longer than three seconds. In general, contractions of the upper neck muscles were clearly visible suggesting swallowing of water. Llamas frequently raised the head and interrupted water intake, swallowed and masticated while looking around and then continued drinking. When the

animal lifted its head and interrupted drinking from the same bucket for less than 30 s, this was considered as continuation of the same drinking bout. However, when the animal moved its head to another bucket and continued drinking from the new bucket for longer than three seconds, this was recorded as new drinking bout. Drinking frequency was defined as number of drinking bouts. Duration (s) of a single drinking bout was determined including the time of short-term interruptions of drinking.

Testing: When the drinking behaviour lasted maximum three seconds, this activity was defined as testing without significant intake of water. The frequency of testing was used as indicator of the animals' strategy to discriminate between different salt concentrations within the choice experiments.

2.3. Statistical analyses

The behavioural data were recorded on a daily basis, averaged for each llama per phase and analysed across both repetitions. The following traits were considered: drinking water intake on the respective day with video recording (L/24 h), drinking frequency (number of drinking bouts/24 h), testing frequency (number of testing/24 h), duration of a single drinking bout (s), and total drinking duration (s/24 h). Additionally, the drinking water intake per drinking bout, the drinking water intake per s of drinking, as well as the ratio of testing/drinking bouts were computed and analysed. For evaluation of the diurnal drinking rhythm, the frequency of drinking bouts was summarized per hour across all animals and expressed per phase as hourly percentage of total drinking bouts.

Statistical analyses were performed using R (version 4.1.2 "Bird Hippie"; R Foundation for Statistical Computing, Vienna, Austria) based on linear mixed-effects models (R package: lmerTest). The models were fit by REML and the t-tests used the Satterthwaite's method for approximation of the degrees of freedom. Analyses of variances (ANOVA) were conducted using Type III Wald F tests. Repeated measurements were weighted and considered in the models.

For the analysis across the three phases (phase 1: control, phase 2: pairwise preference test and phase 3: free-choice test), our model considered the repetition (1 or 2) and the phases (1, 2 or 3) as fixed and the animal as random effects. For the analysis of the diurnal drinking rhythm, the hour of the drinking bout (0 – 23) was added as additional fixed effect, and its interaction with the phases (1, 2 or 3) was included. For the comparison of traits within phase 2, the pairwise preference test, the model considered the repetition (1 or 2) and NaCl concentration of the drinking water (0, 0.25, 0.5, 0.75, 1, 1.25 or 1.5%) as fixed and the animal as random effects. For the analysis of the drinking behaviour during the free-choice test (phase 3), the repetition (1 or 2), as well as the

week (1, 2 or 3) were considered as fixed effect, whereas the animal was included as random effect. For the analysis of the drinking behaviour per offered salt concentration during the free-choice test (phase 3), the repetition (1 or 2), as well as the NaCl concentration of the drinking water (0, 0.25, 0.5, 0.75, 1 or 1.25%), were considered as fixed effect, whereas the individual animal was set as random effect. For the analysis of the bucket position effect, differences among the drinking frequency from different drinking buckets were tested with a chi-square test ($p < 0.05$) using the R function chisq.test.

For all models, it was assumed that the residual error was normally distributed, independent and with a constant variance, which was verified on the basis of residual plots and the Shapiro-Wilk normality test. A p-value of 0.05 was considered as significant. The results are reported as weighted least squares (LS) means (R package: emmeans) and their standard error (SE), which includes the Tukey-Kramer test that was used to evaluate differences between groups.

3. Results

The obtained results are reported across both repetitions (Table 1). Overall, the mean ambient temperature of the first repetition was lower (14°C ± 5.7°C) than in the second repetition (19°C ± 3.3°C). The llamas appeared clinically healthy, without significant change in their BW or BCS over the course of the experiment ($p > 0.05$).

3.1. Water intake, drinking bouts and duration

Across repetitions and phases, we observed large individual differences in drinking behaviour with total daily drinking duration ranging between 40 and 1095 s, while frequencies of drinking bouts and testing per day ranged between 1 to 26 and 0 to 15 times, respectively. Across all phases, lamas ingested between 968 and 8896 g drinking water per day. The average time lapse between drinking activities was 2.9, 2.2 and 1.6 h for the control, pairwise preference test and free-choice test, respectively.

Overall, behavioural traits recorded were rather similar in both control and the pairwise test, while most significant differences existed to the free-choice phase (Table 1). When the choice between fresh and saline water was offered (phase 2 and 3), llamas readily ingested saline water. The total drinking duration increased from the control phase (162 s) to the pairwise preference and free-choice phase by more than 46 and 62%, respectively ($p = 0.019$, Table 1, 2, 3) and paralleled the concurrent increase in total drinking water intake.

Interestingly, the total drinking duration of fresh water intake dropped over the course of the experiment, whereas the time spent ingesting saline water increased concurrently by 86% from the pairwise preference to the free-choice phase ($p < 0.001$; Table 1). Within the free-choice test, the total drinking time remained constant from the first to the second week, but increased sharply in the third week ($p = 0.02$, Table 3), as the animals drank more saline water. Across the three weeks of the free-choice experiment, llamas spent only around 18% of their total drinking time with ingestion of fresh water. The mean duration of a single drinking bout remained unchanged during the control and pairwise preference phase, but decreased significantly in the free-choice phase ($p = 0.003$) (Table 1). At the same time, the drinking frequency exceeded twice the control frequency during the latter. Within the pairwise preference tests, llamas drank more frequently when higher

Table 1. Overview of the weighted average (LSmeans) daily drinking water intakes, drinking durations and frequencies, as well as the testing frequency, for the control phase (1), the pairwise preference test (2) and the free-choice test (3) in llamas (n = 12), based on 24-h video recordings, across both repetitions.

Trait	Phase			SEM	P-value
	Control (1)	Pairwise preference (2)	Free-choice (3)		(Phase effect)
Total 24-h video recordings (n)	18	72	60		
Drinking water intake (l)					
Total (l)	3.05a	3.74b	4.58c	0.27	<0.001
Fresh water (l)	3.05c	2.07b	0.81a	0.21	<0.001
Saline water (l)	-	1.67a	3.77b	0.29	<0.001
Drinking water intake/drinking bout (l/bout)					
Total (l)	1.06	1.11	0.79	0.12	0.051
Fresh water (l)	1.06b	1.09b	0.62a	0.12	0.009
Saline water (l)	-	0.84	0.75	0.13	0.531
Drinking water intake/drinking duration (l/s)					
Total (l/s)	0.024	0.024	0.022	0.004	0.830
Fresh water (l/s)	0.024	0.024	0.019	0.004	0.378
Saline water (l/s)	-	0.019	0.022	0.003	0.280
Drinking duration (s)					
Total (s)	162.2a	237.6ab	262.5b	37.6	0.019
Fresh water (s)	162.2c	121.1b	45.8a	21.9	<0.001
Saline water (s)	-	116.5a	216.7b	25.9	<0.001
Duration of single bout (s)					
Total (s)	51.0b	51.5b	37.6a	4.21	0.003
Fresh water (s)	51.0b	49.2b	37.5a	3.24	0.024
Saline water (s)	-	38.9	35.4	4.0	0.444
Drinking frequency (n)					
Total (n)	3.71a	5.17a	7.60b	0.79	<0.001
Fresh water (n)	3.71b	2.64b	1.08a	0.41	<0.001
Saline water (n)	-	2.53a	6.51b	0.67	<0.001
Testing frequency (n)					
Total (n)	1.04a	1.97a	5.33b	0.61	<0.001
Fresh water (n)	1.04	1.08	0.86	0.33	0.828
Saline water (n)	-	0.89a	4.47b	0.52	<0.001
Testing frequency/drinking frequency	0.26a	0.35a	0.79b	0.13	0.005

Drinking water intake = water intake from total, saline or fresh water on the respective days with video recording. Phases: 1 = control phase, only fresh water offered in two buckets; 2 = pairwise preference, fresh water was offered in one bucket, test water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25, or 1.5% NaCl) in another bucket; Phase 3 = free-choice test, six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, or 1.25% NaCl). [a,b,c] Means in the same row without a common superscript are significantly different at $P < 0.05$.

Table 2. Pairwise preference test (phase 2). Modification of daily weighted average (LSmeans) drinking water intake, drinking durations, frequency of drinking bouts and testing in llamas (n = 12) when offered the choice between fresh and saline water with different NaCl concentration, by salt concentration in the saline water, based on 24-h video recordings, across both repetitions.

	Salt concentration (% NaCl) in the test water						SEM	P-value
Trait	0.25	0.5	0.75	1	1.25	1.5		(Salt concentration effect)
Drinking water intake (l)	2.5a	3.69ab	3.58ab	4.37b	4.34b	3.97b	0.36	<0.001
Drinking water intake/drinking duration (l/s)	0.03b	0.027ab	0.025ab	0.023ab	0.023ab	0.018a	0.005	0.046
Drinking duration (s)								
Total (s)	115.2a	177.4ab	199.2ab	244.4abc	319.9bc	369c	56.4	<0.001
Fresh water (s)	61.7a	65a	58.2a	103ab	188.1bc	250.6c	33.6	<0.001
Saline water (s)	53.5	112.4	141	141.4	131.8	118.8	37.5	0.321
Duration of single bout (s)	51.4	53.9	55.1	50.8	46.1	51.6	5.7	0.838
Drinking frequency (n)	2.3a	3.5ab	3.9ab	5.7abc	8.3c	7.3bc	1.29	0.001
Testing frequency (n)	0.5a	1.6ab	1.5ab	1.8ab	3.7b	2.8ab	0.74	0.018
Testing frequency/drinking frequency	0.16	0.48	0.43	0.26	0.42	0.4	0.13	0.307

Pairwise preference test (phase 2) = fresh water was offered in one bucket, saline water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25 or 1.5% NaCl) in another bucket. [a,b,c] Means in the same row without a common superscript are significantly different at $P < 0.05$.

NaCl concentrations in the saline water were offered until 1.25% NaCl (p = 0.001, Table 2). It is noteworthy, that the water intake per drinking duration remained the same in all phases, which means that the ingestion intensity was constant for both fresh and saline water.

3.2. Testing frequency

Testing frequency was very low in the control phase and increased with the number of choices offered by 5.13 times the control in the free-choice phase (p < 0.001, Table 1). Interestingly, the llamas tested the fresh water equally often, while the testing frequency of saline water increased very strongly from the pairwise preference to the free-choice phase (Table 4). Within the pairwise preference test, the testing behaviour followed a similar pattern as the drinking, also peaking at the choice between fresh and 1.25% NaCl water (Table 2). The testing-to-drinking frequency ratio was more than doubled for the free choice phase, suggesting a remarkable change in drinking pattern of the llamas (p = 0.005) (Table 1, Table 4). However, frequency of testing was not related to the NaCl content of the ingested solution. Table 5 illustrates exemplarily for one animal the change of the drinking behaviour from the control over the 2nd to the 3rd phase. As can be seen in the example, testing frequency also mirrors the number of shifts between buckets as part of the decision process.

Table 3. Free-choice test (phase 3). Modification of daily weighted average (LSmeans) drinking water intake, drinking durations, frequency of drinking bouts and testing in llamas (n = 12) when offered the simultaneous choice between fresh and saline water with different NaCl concentrations, by experimental week, based on 24-h video recordings, across both repetitions.

	Week of the experiment			SEM	P-value
Trait	1	2	3		(Week effect)
Drinking water intake (l)	4.82	4.38	4.55	0.42	0.631
Drinking water intake/drinking duration (l/s)	0.025	0.024	0.020	0.004	0.427
Drinking duration (s)					
Total (s)	230a	210.7a	346.8b	46.2	0.02
Fresh water (s)	36.1	45.1	56.0	20.9	0.778
Saline water (s)	193.8ab	165.5a	290.8b	34.9	<0.001
Duration of single bout (s)	32.2	38.7	41.9	3.73	0.057
Drinking frequency (n)	7.67ab	6.08a	9.04b	1.15	0.025
Testing frequency (n)	6.5b	3.54a	5.96b	0.897	0.009
Testing frequency/drinking frequency	0.9	0.68	0.88	0.23	0.693

Free-choice test (phase 3) = six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, or 1.25% NaCl). [a,b] Means in the same row without a common superscript are significantly different at P < 0.05.

3.3. Effect of bucket position

During the pairwise preference phase, the llamas chose the left bucket in 47.8% of their drinking activities compared to 52.2% for the right one, with no significant side preference ($p = 0.757$). Under the assumption of random choice during the free-choice phase, all drinking activities should be equally distributed across the six buckets. However, in our llamas, the average drinking percentage per bucket differed ($p < 0.001$) from the expected value of 16.6%, being 12.9, 15.3, 12.8, 24.2, 17.9, and 16.9% for the bucket positions 1 to 6, respectively. From week one to week three of the free-choice phase, the llamas established an increasing preference for bucket position 4 (19.7 versus 29.3 %).

Table 4. Free-choice test (phase 3). Average weighted (LSmeans) water intakes, durations and frequencies, as well as the testing frequency of the respective test water with different salt concentrations (0 – 1.25% NaCl) in llamas (n = 12), based on 24-h video recordings, across both repetitions.

	Salt concentration (% NaCl) in the test water						SEM	P-Value
Trait	0	0.25	0.5	0.75	1	1.25		(Salt concen-tration effect)
Drinking water intake (l)	0.81	0.90	0.95	0.66	0.81	0.45	0.16	0.278
Drinking water intake/drinking duration (l/s)	0.029 b	0.025ab	0.021ab	0.025a b	0.015ab	0.014 a	0.004	0.021
Drinking duration (s)	45.8	57.8	48.0	29.5	44.6	33.9	10.1	0.258
Duration of single bout (s)	43.2	39.8	31.6	33.9	36.9	30.6	5.1	0.357
Drinking frequency (n)	1.08	1.54	1.49	0.88	1.24	1.22	0.23	0.091
Testing frequency (n)	0.86	0.97	0.64	0.86	0.51	0.99	0.17	0.131
Testing frequency/drinking frequency	0.61	0.44	0.32	0.54	0.28	0.61	0.11	0.081

Free-choice test (phase 3) = six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, or 1.25% NaCl). [a,b] Means in the same row without a common superscript are significantly different at $P < 0.05$.

3.4. Diurnal rhythm

The diurnal distribution of average frequency of drinking bouts reveals a clear biphasic rhythm (Fig. 2) with mean peaks in the morning between 09:00 and 12:00 h (31.9% of all drinking bouts) and in the evening between 17:00 and 21:00 h (31.8% of all drinking bouts). Apparently, there was a link between the daily provision of fresh feed (08:00 h) and water (08:30 h) and the subsequent occurrence of drinking activities as the llamas increased their drinking frequency starting at 09:00 h by around 24 times, compared to 1 h before. Overall, the hourly maximum proportion of 12.6% of the daily drinking bouts

occurred between 10:00 and 11:00 h, i.e., 2 h after feed provision, the minimum (0.1%) between 07:00 and 08:00 h. During the control phase, most of the daily drinking bouts (3.2, 85%) took place during the day, compared to 0.5 (15%) during the night. In parallel, total drinking time during daytime (06:30 to 20:30 h) averaged 137 s. In the night (20:30 to 06:30 h), the llamas drank considerably less, on average for only 25 s ($p < 0.001$). Interestingly, similar diurnal drinking patterns were observed for the three experimental phases without any marked differences due to the different exposure of llamas to NaCl in the drinking water (Fig. 2).

Table 5. Examples of sequence of drinking behaviour in llama dam 'Raya' during one drinking bout, by phase.

Time	NaCl content of chosen bucket (%)	Behaviour	Duration (sec)	Bucket position
Phase 1				
08:41:09	0	Testing	2	Right
08:41:22	0	Drinking	22	Left
Phase 2				
20:16:42	0	Testing	1	Right
20:16:45	1.5	Drinking	10	Left
20:16:57	0	Testing	1	Right
20:17:05	1.5	Drinking	68	Left
Phase 3				
12:22:21	1.25	Testing	1	3
12:22:23	0	Testing	1	4
12:22:25	0.25	Testing	1	5
12:22:29	0.5	Testing	2	6
12:22:35	0.25	Testing	1	5
12:22:37	1.25	Testing	1	3
12:22:48	0.25	Drinking	9	5
12:23:04	0	Testing	3	4
12:23:17	1.25	Drinking	35	3

Phases: 1 = control phase, only fresh water offered in two buckets; 2 = pairwise preference, fresh water was offered in one bucket, test water with ascending salt concentrations (0.25, 0.5, 0.75, 1.0, 1.25, or 1.5% NaCl) in another bucket; Phase 3 = free-choice test, six water buckets were offered simultaneously with various salt concentrations (0, 0.25, 0.5, 0.75, 1.0, or 1.25% NaCl)

4. Discussion

4.1. Drinking behaviour and water intake

The drinking behaviour of our llamas was very comparable to that described for wild vicunas (Koford, 1957). The frequent lifting of the head during drinking to look around may be part of the llamas' vigilance behaviour. Similar to earlier reports in vicunas (Koford, 1957) and llamas (Lacépède and Cuvier, 1801), drinking frequency was found to be low, but showed high individual variation. Time budgets for drinking were

comparable to those of wild guanacos, where drinking was recorded for 0–0.53% of all scans during daytime (08:00 – 19:00 h; Garrido et al., 1980). Regarding small ruminants, the drinking behaviour of our llamas might be best compared with that of goats. This applies, e.g., to low time budgets for drinking of 0.10–0.19% per 24 h and the daily drinking frequency of 3.6–8.1 bouts found in Boer goats kept indoors with water provided *ad libitum* (Al-Ramamneh et al., 2010). In contrast, sheep had drinking shares of 0.15–0.32% per 24 h (Das et al., 1999), with daily drinking frequencies of 13.3–17.4 bouts (Al-Ramamneh et al., 2010).

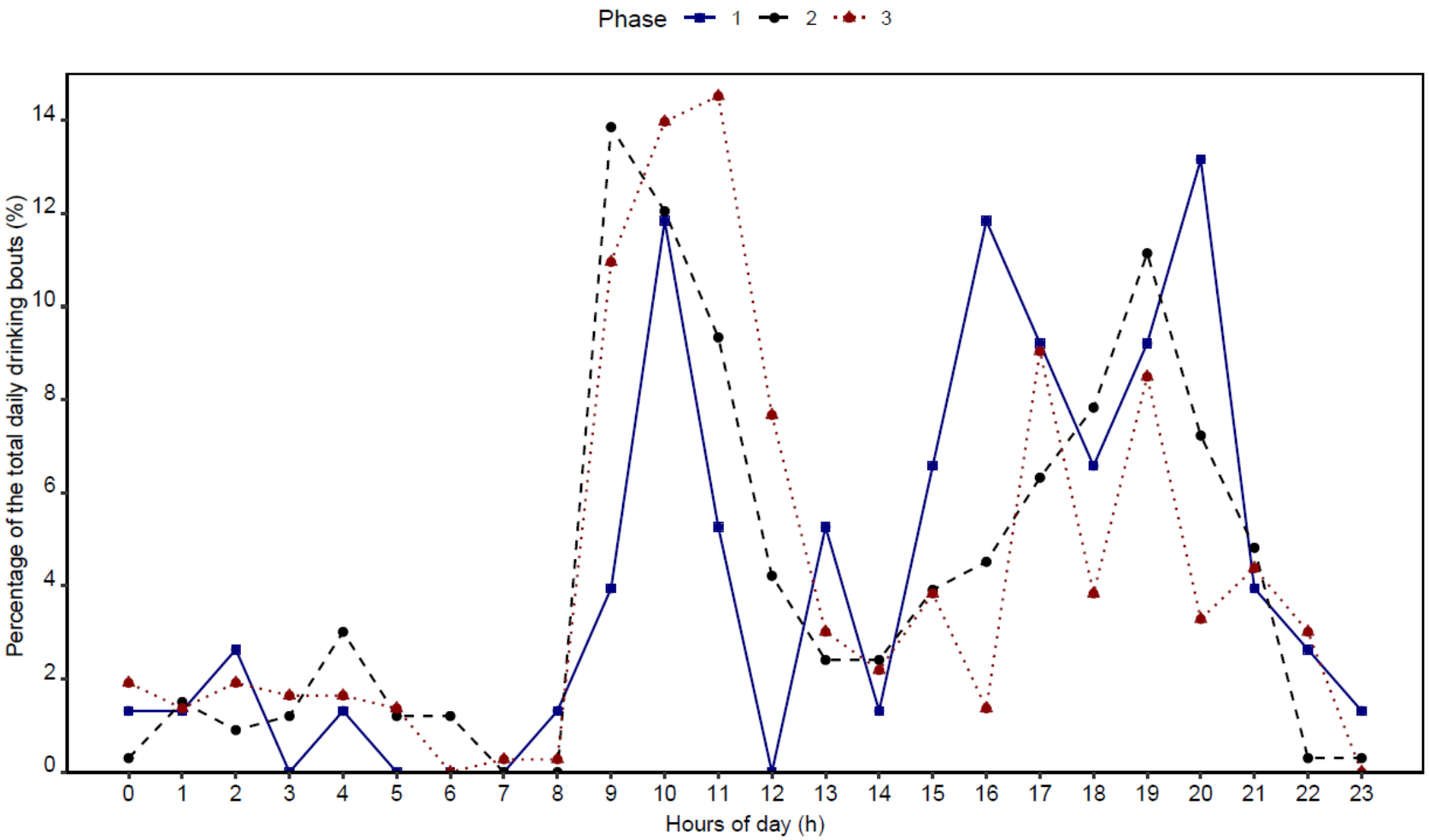

Fig. 2. Diurnal drinking rhythm based on frequency of drinking bouts expressed as hourly percentage (%) of average total drinking bouts, by phase. Phase 1: control, two buckets with fresh water were offered simultaneously. Phase 2: pairwise preference test, two buckets were offered with one containing fresh water and the other with saline water in increasing NaCl concentration (0.25 – 1.5%). Phase 3: free-choice test, six buckets were offered simultaneously with six different NaCl concentrations in the drinking water (0 – 1.25%).

Llamas readily ingested saline water in the choice tests. However, Na intake needs to be regulated by the animal because it is the major extracellular element influencing osmolarity of body fluids. As a key physiological response to high salt ingestion, our llamas increased their water intake to regain an isotonic state (Enke et al., 2022b). Similar adaptive responses were shown by e.g., sheep (Peirce, 1957; Wilson and Dudzinski, 1973), goats (Runa et al., 2019a), rusa deer (Kii and Dryden, 2005) and red and fallow deer (Ru et al., 2005), when exposed to saline water. Correspondingly, llamas increased their total drinking time per day in the choice tests. Interestingly, the drinking water intake per drinking duration was not affected, instead the llamas increased their drinking frequency. However, single drinking bouts where significantly shorter from saline than from fresh water in both choice tests. Moreover, in the free choice phase, also drinking of fresh water was shorter, suggesting splitting of larger bouts in favour of shifting between solutions during the decision process.

4.2. Diurnal rhythm of drinking behaviour

The diurnal distribution of drinking frequency characterizes llamas as light active. Similarly, when recording activity and feeding behaviour of alpacas by automatic devices, Scheibe et al. (1991) described the pattern of daily activity as light active with ultradian modulation. Our llamas showed most of their drinking bouts during the day (83%), which compares with alpacas housed in stables that performed 69% of their water intake during the daytime (Raggi et al., 1994). For wild vicunas, Koford (1957) observed drinking activity only between 08:00 to 16.15 h, which is probably due to the separation of resting and watering places. Similar low nocturnal drinking activity was described for goats and sheep (Das et al., 1999; Al-Ramamneh et al., 2012; Salama et al., 2021).

In the investigation of Scheibe et al. (1991), alpacas on pasture began their activities in the early morning. Interestingly, although the light turned on at 06:30 h, our llamas did not start drinking with light onset. This is in contrast to sheep, whose drinking activity peaked after sunrise or the first hour of the light period (Hunsaker and Wolynetz, 1979; Das et al., 1999; Al-Ramamneh et al., 2012). In the current study, onset of drinking was mainly triggered by the provision of fresh feed and water in the morning. A close link between foraging and subsequent drinking was also described for vicunas (Koford, 1957), camels (Abdel-Rahman and Mosaad, 2005), goats (Rossi et al., 1999; Al-Ramamneh et al., 2012), and sheep (Das et al., 1999; Al-Ramamneh et al., 2012). Thus, as Scheibe et al. (1991) pointed out, additional food distribution may play an important role in modulation of activity rhythms. A second peak in llamas' drinking activity was present prior to the resting time in the night, similar to patterns of feeding activity in

alpacas (Scheibe et al., 1991). It is noteworthy, that despite changes in the drinking frequency between the three phases, the present diurnal biphasic rhythm remained similar.

4.3. Discrimination and decision-making

The present choice tests allow deeper insight into decision-making in the llamas regarding nutrient intake. Choice tests have been used to assess the willingness of animals to choose between different feeds (Meier et al., 2012). Feeds offer a broad range of sensory stimuli such as odour, colour, taste, and even tactile cues which facilitate the discrimination (Provenza et al., 1996). Scherer et al. (2019) showed that in goats offered different silages, recognition of the feeds and decision-making processes were based on the smell of the food. For most mammals, olfaction is the principal chemosensory modality (Lledo et al., 2005). However, NaCl concentration of water solutions can probably only be differentiated by taste, thus requiring a different decision approach of the animals.

During the choice tests, the lamas were confronted with the dual task to detect a specific concentration (sensory input) and to remember the position of the respective bucket (learning task). Thus, the decision-making was more challenging for the free-choice test involving 6 options. Our llamas showed an interesting behavioural approach by increasing their testing frequency. As testing was most frequent during the free choice phase with six options, testing could be influenced by number of options only. However, llamas also tested significantly more per drinking bout in the free choice situation, indicating that this behaviour was used for deliberate discrimination between NaCl concentrations. This assumption is underlined by the llamas' behaviour during the pairwise preference test, where the testing reached the highest values when choices between fresh water and water with 1.25 or 1.5% NaCl were offered, respectively. As outlined by Enke et al. (2022b), llamas showed a weak preference for saline water with 0.5–0.75% NaCl, but rejected drinking water from 1.25% NaCl. Possibly, the testing is especially used for the rejection of high NaCl concentrations. This assumption is underlined by the lowest testing frequency found for the saline solution with 0.25%, which the llamas were apparently unable to differentiate from fresh water (Enke et al., 2022b).

Different than assumed, in the free choice situation, frequency of testing was not related to the Na content of the ingested solution. In addition, the doubled testing-to-drinking frequency ratio might indicate that animals were undecided in their choices. Thus, despite the high testing rate, the total drinking duration spent on saline water remained high with no reduction of total Na intake (Enke et al., 2022b). On the other hand, the

increasing position preference might indicate that our llamas modified their decision-making behaviour because the offered saline concentrations were below 1.5% and probably equally acceptable and tolerated.

The high ingestion of saline water might be explained in different ways.

(1) In the semiarid high Andes, salinity of natural water resources may increase temporality, thus favouring animals with high saline tolerance by natural selection.

(2) Herbivores have evolved a specific appetite for sodium (Denton and Sabine, 1961) and the hedonic taste value of saline solution may override normal regulatory mechanisms. This hedonic value depends on the mineral status of the animals and may be characterized as labile (Ginane et al., 2011). However, our animals were not Na-deprived as already in the pairwise preference test their Na intake was more than four times (10.9 ± 1.1 g/d) higher (Enke et al., 2022b) than calculated Na-requirements (van Saun, 2006b).

(3) It has been shown that a dehydrated (for four days) guanaco drank 9 L of water within 8 min (Mario and Morrison, 1963). Generally, it is know that camelids have small and elliptical erythrocytes, which can withstand volume increase up to 240% during rapid water absorption without rupture (Yagil et al., 1974; Smith et al., 1979; Fowler, 2010). In other animals, such an amount of water would cause severe osmotic problems, also suggesting that the anatomy of the camelids' forestomach differs considerably from that of ruminants (Fowler, 2010). This fact may play an important role in slow absorption of drinking water, allowing osmotic equilibrium to be established. Thus, post-ingestive feedback mechanisms could take effect with a delay, compared to, e.g. monogastric animals. This could explain why our llamas did not down-regulate their Na-ingestion during the free choice, whereas ponies in a similar set up quickly shifted to a preference of fresh water comprising 56% of their water intake (Enke et al., 2022a).

(4) The present decision making requires a complex coordination between sensory taste input, memorizing of NaCl concentration and position, and post-ingestive feedback. Given the paramount importance of olfaction in mammals, there might be differences in memory formation between sensory inputs from taste receptors compared to those evoked by smell receptors. The low drinking frequency of the llamas might hamper their memory formation and discrimination learning when more than two samples are offered simultaneously (Langbein et al., 2007). Zahorik et al. (1990) and Houpt et al. (1990), in taste-aversion trials, showed that ruminants and horses can build aversions to specific food when they are associated with a negative consequence, e.g. illness induced by

apomorphine. Interestingly, the animals were only able to learn to avoid the food, when illness occurred shortly after feeding, indicating a narrow time window for memory formation based on post-ingestive feedback.

5. Conclusions

Llamas exhibited a low drinking frequency indicating adaption to water scarcity. The interspecies comparison to goats revealed similarities in their tolerance of saline drinking water. Llamas demonstrated their capacity for behavioural adaptation when more choice options were offered by changing their drinking pattern with increased frequency of drinking and testing, while maintaining their diurnal rhythm of water intake. Decision-making was based on testing of solutions which increased considerably when more choice options were offered. As saline solutions can probably only be discriminated through the taste sense, further research on taste perception in llamas would be of interest. Such studies should also consider the interplay between memory formation based on taste signals and the association with osmotic regulatory mechanisms.

Declaration of Competing Interests

The authors declare that they have no conflict of interest.

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CHAPTER 6

General discussion

The present thesis focused on the drinking behaviour of horses and llamas, and their responses to saline drinking water. It was investigated, whether both species possess a sensitivity for NaCl in their drinking water and whether they can differentiate minor changes in the NaCl concentration of their drinking water. Additionally, it was examined whether both species possess strategies to self-balance their Na intake, in order to avoid shortage or overdosing. The results provided more information about the NaCl concentration which is voluntarily tolerated by these species. For llamas, these responses were compared with the normal drinking behaviour, which was, for the first time, described under controlled housing conditions.

In the presented studies, both species expressed different behavioural responses in their sensitivity to saline drinking water. Primary reactions on higher NaCl intake, such as increased total drinking water intake (Peirce, 1957; Wilson and Dudzinski, 1973; Runa et al., 2019) were displayed in both llamas and horses (Chapter 2 and 3). From the results shown, both species possess the capacity to differentiate solutions with differing NaCl concentrations. However, llamas demonstrated a more distinctive acceptance, or even preference, for drinking water with moderate NaCl concentrations (0.25 – 0.75%) than horses. Horses, on the other side, rejected saline solutions above 0.75% to a much higher extent than llamas. The horses' preference for fresh water, and the concurrent avoidance of saline water, increased remarkably over time during the free-choice experiment. This could be explained by the fact that the fine-adjustment of the Na intake might take some time. The llamas, on the other hand, showed almost no differentiation between fresh water and saline solutions with 0.25 or 0.5% NaCl over the duration of the free-choice experiment.

In llamas, high relative intakes of saline water were observed during both experimental setups. Presumably, llamas possess a higher acceptance, if not even preference, for saline solutions, especially with lower NaCl concentrations. Alternatively, the llamas might have also been unable to accurately differentiate fresh water and saline solutions with lower salt concentrations (up to 0.5% NaCl). The unusually high intakes of Na without any visible reduction over time could support this suggestion. Only the testing behaviour (i.e., short drinking bout of maximum three seconds) could be an indicator for their capacity to differentiate between various NaCl concentrations in the drinking water and adjust their salt intake according to their requirements (Chapter 4). However, although testing increased when confronted with higher NaCl concentrations as

alternative to fresh water, the testing rate remained rather unchanged within the long-term free-choice test. Future research with longer experimental duration might provide more insights if llamas simply cannot differentiate exactly low-saline solutions from fresh water or possibly even possess far more tolerance towards high Na intakes than previously known. The rather unknown requirements for minerals in llamas, which are currently still based on the extrapolation from the requirements of sheep, goats and cattle, complicate a conclusion about whether or not the animals are putting themselves in a critical health situation in the long-term due to their high Na intake levels. Besides, llamas could also possess Na elimination strategies, comparable to camels, which are not fully discovered yet (Chapter 3). However, in the current study, llamas demonstrated capacities more similar to goats.

Impact of domestication on sensitivity to saline drinking water

In general, there are various factors affecting the sensitivity of animals to salts in the drinking water, which would require more attention to identify. Differences in the origin and domestication of animal species could play an important role for their response to saline drinking water. Wild horses (*Equus ferus*) originate from the Western part of the Eurasian steppe, characterized by dry, grassy plains, and distinct seasonal changes between dry summer and cold winter, where their domestication also occurred around 6,000 years ago (Formozov, 1966; Warmuth et al., 2012). The natural existence of salt and also concurrently occurring water scarcity in that region (Groisman et al., 2018; Struck et al., 2022), could be an explanation for their increased sweating rate, which could serve as alternative pathway to the Na excretion via urine, in order to reduce fluid losses. Since their domestication, horses spread over the entire planet and were since then selectively bred for human purposes. Both these facts could be an explanation for the reduced interest of saline solutions with higher NaCl concentrations and their respective long-term down-regulation in horses. Llamas, on the other side, were domesticated 6,000 – 7,000 years ago from the wild guanaco (*Lama guanicoe*) in the Puna ecosystem of the Andean region (Pires-Ferreira et al., 1976; Wheeler, 1995, 2012). However, their exact origin is still not completely clear (Diaz-Maroto et al., 2021). Indeed, it was only 3,800 years ago when llamas were moved into lower elevations and only 1,400 years ago llamas were kept on the northern coasts of Peru and Ecuador (Shimada and Shimada, 1985; Stahl, 1988). Although llamas were an important factor for the Inca expansion, their numbers dropped drastically during the 16th century after the Spanish conquest, through the introduction of previously unknown animal diseases, and the displacement through new exotic livestock species, such as sheep, goats, cattle and pigs (Wheeler, 2012; Metcalf et al., 2014; Vilá and Arzamendia, 2022). Only after that, the

population of llamas gradually recovered and husbandry beyond South America started in the 19th century (Vilá and Arzamendia, 2022). These facts suggest a lower domestication intensity compared to the horse and emphasize their adaptation to the Andean regions. The relatively high acceptance and preference for saline drinking water in comparison to horses, could therefore be explained with their much closer proximity to their wild ancestors, which originate from arid regions with salinity in natural drinking water sources (Oldeman et al., 1991; Nordt et al., 2004). Overall, it seems that the animals' various origins and intensities in their domestication and utilization could play an essential role in their adaptation to extreme environments, such as salinity, and their sensitivity to saline drinking water.

The digestive system and its interaction with excessive salinity in the drinking water

On the other side, the differences in the digestive system of animals might be an important factor for the interplay between animal species and saline drinking water, as well as their respective response mechanisms. However, the exact role of Na in the digestive tract of animals and its implication on the microbial community and its activity is still not fully investigated. Results exist mainly for ruminants, especially on the implications of NaCl on the rumen and its microbiota (Bailey, 1961; Rogers et al., 1979; Valtorta et al., 2008). In the ruminal fluid, volatile fatty acids and Na are the main agents for maintaining the ruminal osmolality. Sodium is the most abundant cation inside the ruminal fluid (Bergen, 1972; Bennink et al., 1978) and due to its origin from saliva, the Na concentration in the rumen is proportional to that from saliva (Bailey, 1961). It is known that the osmotic pressure in the rumen increases due to higher intake of NaCl, whereas the ruminal pH level drops coincidentally, which is normally between 5.5–6.8, depending on the diet (Hogan, 1961; Bergen, 1972; Potter et al., 1972). The increasing osmotic pressure could also lead to a higher ruminal water flow, with increased dilution rate of the rumen fluid, which could partially explain the increased water consumption (Rogers et al., 1979; López et al., 1994). Similarly, Rogers and Davis (1982) also showed that infusion with NaCl can affect the concentrations of volatile fatty acids by reducing the molar percentage of rumen propionate and increasing the molar percentage of acetate.

It is stated that osmotic effects in the rumen wall have an important function on the feed intake (Carter and Grovum, 1990). However, osmoreceptors could not be clearly demonstrated and it is also unclear if these mechanisms also work when the source of salinization is the drinking water and fresh water as alternative is unavailable. Bennink

et al. (1978) and Valtorta et al. (2008) demonstrated the remarkable ruminal buffering capacity in cattle by feeding diets with varying Na concentrations or offering saline water, which both did not influence the osmotic pressure and microbiome in the animals. Obviously, whereas ruminal microbes could withstand short-term changes in their ruminal fluid tonicity, the continuous increased salt intake is rather unfavourable for the microorganism population and metabolism when the tonicity in the rumen is beyond their physiological levels (Carter and Grovum, 1990; Attia-Ismail, 2008). It was even reported for cattle and sheep that increasing rumen osmotic pressure via intraruminal infusion of hypertonic solutions from usually around 250–280 mOsmol/kg to 400 mOsmol/kg could inhibit the cellulose digestion and decrease rumen turnover times, which could explain the observed reduction in the DMI (Bergen, 1972; Rogers et al., 1979). It was also shown that the occurring elevated rumen dilution has an additional negative impact on the population of the ruminal protozoa (Warner and Stacy, 1965).

Horses and llamas possess different digestive systems, which could have an effect on the animals' interaction with saline solutions and the tolerance to it. Generally, the administration of NaCl could also have harmful effects on animals, possibly even causing severe symptoms, such as gastric ulcers or cancer (Tsugane, 2005), or even death (Peirce, 1957). Therefore, it is important to identify the effect of NaCl on the digestive tract and its potential as buffer in different animal species. The equine gastrointestinal tract is characterized by its hindgut fermentation with extended fermentation compartments in the large intestine, both in the caecum and colon (Stevens and Hume, 1998). The hindgut possesses the highest microbial activity with fibre-fermenting bacteria which mainly rely on cellulose and hemicellulose (Santos et al., 2011; Dicks et al., 2014). The significance of the fermentation in the hindgut is still not fully understood and only few experiments have focused on the contribution of the hindgut microbial population to the buffering capacity towards the excessive intake of NaCl, especially via the drinking water. It is known that the oral administration via syringe of hypertonic electrolyte solutions is associated with exacerbation of gastric ulcers in the equine gastric mucosa (Holbrook et al., 2005). Additionally, there is evidence that oral doses of 100 g NaCl per day, provided in loose form during feeding, could potentially induce a mild metabolic acidosis (Zeyner et al., 2017). As for donkeys, it is stated by Kasirer-Izraely et al. (1994) that their hind gut constitutes a water reservoir which helps to maintain the osmotic balance, similar to the rumen. However, the precise effect on the equine gastrointestinal tract, especially the hindgut and its microbiota, has not yet been revealed.

Although there are some similarities between their digestive systems to that of ruminants, there are several distinctive differences in the camelids' anatomy, taxonomy, behaviour and physiology, as reviewed by Fowler (2008). Just as ruminants, camelids are foregut fermenters with multiple compartments in their stomach allowing to digest and utilize plant fibre. However, ruminants such as cattle, goats or sheep, have four compartments (rumen, reticulum, omasum and abomasum), whereas camelids only possess three (C-1, C-2 and C-3), with the C-1 being comparable to the rumen in ruminants as the main location for fermentative digestion and the stratification of contents (Vallenas et al., 1971; Wang et al., 2000). It was also shown by Woodall and Skinner (1993), that the shorter length of the large intestine in camelids, especially alpacas, could be related to the water metabolism and the camelids' better adaptation to water scarcity, compared to ruminants (Pérez et al., 2016). Another important factor is the ability of camelids to drink excessive amounts of water within shortest time, without adverse effect on their health, due to their small and elliptical erythrocytes (Yagil et al., 1974; Fowler, 2010).

Surprisingly, despite these distinct adaptation of camels to harsh conditions in arid and semi-arid regions and their capacity to utilize low-quality forage (Shawket and El Shaer, 2016), there is a lack of knowledge on the mode of action of NaCl in their digestive system. It was shown that salt-tolerant halophytes play an important role as forage for camels, which are able to digest plants that are apparently indigestible for goats and sheep (Payne and Wilson, 1999; El Shaer, 2010). Hence, camels must possess mechanisms to cope with high salinity in their diet and protect their gastrointestinal tract (Schmidt-Nielsen et al., 1967; Siebert and Macfarlane, 1975). Apparently, llamas are less adapted to salinity than camels. In addition, there exist no data on the effect of excessive NaCl intake and its impact on the gastrointestinal tract in South American Camelids. Furthermore, it is still unknown which role the fermentation compartments, especially the C-1, play and how their buffering capacity to NaCl can be described. However, due to some similarities to the ruminants' digestive tract, it seems likely that similar mechanisms might exist as in ruminants.

Experimental methods for the evaluation of salt sensitivity and tolerance in animals

Despite the factors discussed above, the assessment of the animals' sensitivity and tolerance can be achieved in various ways. Depending on the mode of its administration, the animals' tolerance and sensitivity to NaCl can vary widely. Considerable differences were observed when NaCl was either offered via the drinking water or the feed of animals. Although sheep can tolerate NaCl concentrations up to 20% in their feed (as

reviewed by Digby et al., 2011), NaCl concentrations above 2% in their drinking water might already cause reduced feed intake and possibly death (Peirce, 1957; Wilson and Dudzinski, 1973). It was concluded that quicker absorption of dissolved nutrients from the drinking water leads to different sensitivity of NaCl from either feed or drinking water (Wilson, 1966; Masters et al., 2005). However, the method of NaCl administration also plays a remarkable role. Experimental setups could include the administration of saline solutions through the rumen fistula (Wilson and Dudzinski, 1973) or as enforced oral administration (Holbrook et al., 2005; Zeyner et al., 2017). Furthermore, various studies evaluated the acceptance of animals to saline drinking water without any fresh water as alternative (Peirce, 1957; Weeth and Haverland, 1961; Maloiy, 1970; Nyman et al., 1996; Ru et al., 2005). These results provide insight into the overall tolerance towards different levels of NaCl in saline solutions, indicating the exact NaCl concentration which poses a risk to the animals' health. However, these outcomes do not supply information about the actual preference for or discrimination of saline solutions. The main physiological response to NaCl intake is the rise in the fresh water intake to equilibrate the high salt intake by increasing the renal excretion via urine (Peirce, 1957; Wilson and Dudzinski, 1973; Runa et al., 2019). However, if access to fresh water is not provided, animals are forced to ingest saline water with possible metabolic consequences, which cannot be counteracted through fresh water intake.

Preference trials, which include fresh water as alternative to saline drinking water, turned out to be a better alternative to evaluate the animals' willingness in drinking different saline solutions in a choice situation. These tests are extensively discussed in Chapters 3, 4 and 5. For the current thesis, the pairwise preference tests provided insights into the short-term sensitivity to saline solutions, but were especially helpful due to their previous application in other species, allowing an interspecific comparison. Moreover, the short-term presentation of pairs with different NaCl concentrations allows many comparisons within short time. However, the short duration of each pairwise arrangement with a saline solution and fresh water, could also be a major weakness of this method. The differentiation of saline solutions poses a notable challenge to animals. However, studies on mechanisms of tolerance or preferences are hampered due to lack of knowledge whether some animal species might possess sensory capacities to identify NaCl solutions by smell (NRC, 2007) and the fact, that crystalline NaCl appears to be odourless (DeMeo, 1994). Obviously, it was shown in the current studies that horses and llamas reduced their intake of unfavourable saline solutions when offered over longer periods. It could be concluded that the pairwise preference test with ascending concentrations of NaCl in saline water provided more insights regarding the overall

discrimination or preference, which could easily be assessed by the model for response thresholds developed by Bell (1959) and Goatcher and Church (1970a). In comparison, the free-choice test provided valuable data about the expression of the sensitivity and the adaptation to multiple options of saline water.

Generally, it is known that vertebrates, such as ruminants, camels or horses, possess lingual receptors (Bell and Kitchell, 1966; Eerdunchaolu et al., 2001; Kikut-Ligaj and Trzcielińska-Lorych, 2015), allowing them to differentiate basic tastes (sweet, bitter, salty, sour and umami) via their taste buds, which are located in the gustatory papillae on the tongue (Goatcher and Church, 1970b; Randall et al., 1978; Ginane et al., 2011). Their interaction with the taste stimuli initiates the transmission of a signal to the brain (Ginane et al., 2011). The horses and llamas in the current studies (Chapter 3, 4 and 5) both demonstrated remarkable sensory discrimination capacity to detect different NaCl concentrations in aqueous solutions. Both exhibited rejection of higher NaCl concentrations, which found its expression during the free-choice experiment, where both species were able to select the appropriate saline water intake. Especially the llamas demonstrated noteworthy capacities as response to saline drinking water, such as their pronounced testing behaviour.

Nevertheless, it should be mentioned that for both species, the choice tests led to an excessive intake of Na, which was only reduced slightly over the entire free-choice experiment. It seems that for both species, the adaptation requires some time for accurately adjusting the Na intake and develop a clear preference which fulfils physiological and nutritional requirements. In other preference tests where silages were offered to goats, a relationship was observed between the feed intake in the first three minutes of video recording and the feed intake over three hours, indicating a high predictive power of the initial feed intake for the latter preference (Scherer et al., 2019). That could imply that the decision-making process already occurs in the first minutes of feeding. Whereas this setup would allow to obtain data more efficiently than during 24h-recordings, it seems unlikely that horses or llamas would make their decision between different NaCl solutions at that early stage, considering the time required to adjust their Na intakes. In addition, drinking is less frequently elicited in short-term choice experiments than feeding. However, in water deprived animals, there would be a higher probability that drinking occurs in the first three minutes after water provision. But it seems that postingestive feedbacks are required in animals to establish their preference for saline solutions, whereas in feeding experiments the decision-making processes occured at a much earlier stage due to the smell emitted by the silages (Scherer et al., 2019). As saline solutions presumably have no odour, the animals' decision might not

rely on their olfactory capacities as in the case of feed. Overall, the results of the current thesis confirm that mammals are generally attracted to lower concentrations of NaCl. On the one hand, this is reflected by frequently choosing a salty solution over a salt-free one, but on the other hand by rejecting highly concentrated saline solutions, even during salt deprivation (Oka et al., 2013). Possibly, mammals possess sensing cells in the tongue which are less sensitive to lower saline concentrations but eventually cause a complete aversion to solutions high in NaCl to prevent negative effects on the animals' health.

Conclusions

This thesis provides valuable insights into the drinking behaviour of llamas and horses in relation to their response to saline drinking water. For the first time, the drinking behaviour of llamas was analysed and its diurnal rhythm outlined. Furthermore, both species have demonstrated their capacity to differentiate various NaCl concentrations in the drinking water and adjusted their Na intake over time. However, differences in their taste discrimination and individual sensitivity were observed, which became more pronounced during the free-choice test, where llamas demonstrated more tolerant long-term responses to saline water intake than horses. It was also shown that both species can balance their salt intake from different concentrations over longer time periods. However, the exact regulation mechanisms are still unknown. Further studies should also consider longer time periods to identify whether the observed initial trend to decrease the excessive Na intake will become more significant. Also, it would be advisable to control the mineral status of the animals, since that might play an important role in their Na appetite. It would also be interesting to gain more insights into the detection mechanisms of high NaCl concentrations, especially on those leading to an aversive reaction and rejection of saline water. There is still a lack of knowledge about how breeding and domestication influenced the Na tolerance and sensitivity of various animal species, as well as the impact of the specific digestive systems. However, both animal species demonstrated a certain adaptation capacity to a potential water salinization of fresh water sources, which generally might increase in some regions over the course of global climate change.

Finally, the current thesis delivered more insights into the attraction of NaCl for animals that do not experience a NaCl deficit, which is still poorly understood. The present results demonstrate the potential of saline drinking water of low NaCl concentration as drinking source for llamas and horses along with more insights in how animals perceive the sensory world.

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