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GIRAFFE'S URINE: FROM URINALYSIS TO PROTEOMICS AND METABOLOMICS

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ABSTRACT

This thesis focused on defining the urinary reference values and investigating the urinary proteome and metabolome of captive giraffes (*Giraffa camelopardalis*) by using a non-invasive sampling method. Urine samples were collected with a syringe from the ground, immediately after spontaneous voiding, by aspirating the upper part of samples, to avoid them to come into contact with the soil contaminants as much as possible.

To prove the sampling's reliability, cow urines were used and the results of both types of samples (those collected in sterile urine cups and those collected from the ground with a syringe) were compared. This experiment revealed no statistical differences in the variables investigated (urine total protein, uTP; urine creatinine, uCrea; urine protein:creatinine ratio, UPC; number of protein bands and band protein quantification detected by 1D-electrophoresis-SDS-PAGE), which proved the reliability of this sampling technique.

By establishing the urinary reference values as well as by identifying the urinary proteome, it was possible to obtain information about the renal function in giraffes for the first time. Just like other ruminants, urine of giraffes contains low quantity of proteins, the majority of which shows low molecular mass (MM). The proteins identified in their urines might act as a defence against microbes and play a role in the ability of giraffes to concentrate urine.

A first insight into the urinary metabolome allowed to identify and quantify 39 molecules; this provided some information on some physiological adaptations of giraffes and were influenced by sex and age.

LITERATURE REVIEW

1. URINE OF NON-DOMESTIC SPECIES

1.1. URINALYSIS

Urinalysis is a non-invasive test and a diagnostic tool that provides useful information for screening and detection of diseases (Haffey, et al., 2008; Parrah, et al., 2013). Moreover, it can give precious information about urinary tract, renal and non-urinary diseases (Parrah, et al., 2013; Callens & Bartges, 2015; Piech & Wycislo, 2019). When completed properly, this analysis could be an important and reliable tool for the detection of minor changes in kidney function, which might not be identified by blood biochemical values (Parrah, et al., 2013).

Urinary reference values reported in non-domestic species are scarce, albeit some data have been reported on exotic, wildlife, and zoo animals (Stacy & Hollinger, 2018; Alberton, et al., 2019), such as the okapi (Glatston & Smith, 1980), the wolf (Mech, et al., 1987), the desert mule deer (DelGiudice, et al., 1990), the moose (DelGiudice, et al., 1991), the koala (Canfield, et al., 1989; Vogelnest, 2015), the orangutan and the chimpanzee (Knott, 1997), the mountain gorilla (Sleeman & Mudakikwa, 1998), the dromedary camel (Gutierrez, et al., 2002), the rhinoceros (Haffey, et al., 2008), the Asian elephant (Wiedner, et al., 2009), the camel (El-Deeb & Buczinski, 2015), the giant panda (Burrell, et al., 2017), the dolphin (Jiménez-Zucchet, et al., 2019) or the sloth (Black, et al., 2019). Nevertheless, urinary reference values still have not been reported in giraffes and few studies have been published about their urine (Loskutoff, et al., 1986; Sullivan, et al., 2010).

Despite urinalysis being carried out by following the same protocol as in the domestic counterparts, species-specific anatomical characteristics should be pointed out for evaluating the health status from a clinical standpoint (Wiedner, et al., 2009; Stacy & Hollinger, 2018). For instance, elephants seem to have a moderate ability to concentrate urine; therefore, urine specific gravity should not be evaluated alone to determine hydration status or to discriminate between renal and prerenal azotemia (Wiedner, et al., 2009).

Urine samples might be collected thought different methods, for instance cystocentesis, manual expression of the bladder, spontaneous voiding as well as

directly from the floor or litter box (Sink & Weinstein, 2012; Callens & Bartges, 2015). However, when approaching non-domestic animals, it should be preferred to use non-invasive techniques (e.g. the training for collecting voided urine) that avoid sedation or anaesthesia as suggested or reported for giraffes (Sullivan, et al., 2010), apes (Murphy, 2015), cetaceans (Dold, 2015), giant pandas (Burrell, et al., 2017) and dolphins (Jiménez-Zucchet, et al., 2019). Nevertheless, various devices or manners have been adopted for the collection of samples in wildlife species: e.g. using clean plastic sheet (Knott, 1997), umbrella, bags, and first aid cotton; centrifuging the soil soaked in urine or pipetting leaves (Danish, et al., 2015); using a collecting vessel attached to a pole (Walzer & Stalder, 2015), to collect directly from the ground/floor (Glatston & Smith, 1980; Burrell, et al., 2017) or from snow (Mech, et al., 1987; DelGiudice, et al., 1988; DelGiudice, et al., 1991).

Irrespective of the collecting methods, it is important to consider preanalytical variables, referred both to the sampling methods and to the patients, when dealing with urinalysis, since these factors may influence the urine composition or the results of the analysis (Callens & Bartges, 2015). Preanalytical factors, especially the collecting methods and the conditions and time of storage, might affect the samples and thus could influence the results of the analysis: e.g., urines collected by spontaneous voiding or by cystocentesis could contain different other elements (e.g., spermatozoa, blood, epithelial cells, etc.) (Callens & Bartges, 2015). Urinalysis should be performed within 30 minutes or within 24 hours at the longest, after refrigerating the urine (Callens & Bartges, 2015; Piech & Wycislo, 2019).

Likewise, physiologic variables of patients and treatments or diagnoses should be carefully taken into consideration when interpreting the results (Callens & Bartges, 2015); these factors are particularly important for zoo species, considering that these animals come from different environments and they have different physiological needs (Hosey, et al., 2013a), which, in turn, should be reflected in a diversity of clinical findings.

Similarly, the analytical factor should also be controlled, so that variations can be reduced; consequently, the used methods should be monitored with quality control procedures, in order to limit analytical errors as much as possible (Friedrich, et al., 2012).

1.1.1. PHYSICAL AND CHEMICAL EVALUATION OF URINES

The physical examination of urine consists in the evaluation of colour and clarity of the samples; in fact, its turbidity and the presence of pigments can give important information about the health status of subjects, their diet or the medications they were given (Piech & Wycislo, 2019). The colour of the urine should be carefully considered when approaching the analysis, as the presence of certain pigments could affect the results of the chemical evaluation of the urines (Callens & Bartges, 2015). For instance, in domestic animals, normal urine colour, on visual inspection, ranges between yellow and amber and it should be transparent, albeit occasionally cloudy (Chew, et al., 2011a; Callens & Bartges, 2015). Conversely, red urine may be found in healthy marsupials (Vogelnest, 2015), and green–yellow urine in dolphins (Jiménez-Zucchet, et al., 2019). Likewise, the clarity of urine should be cautiously considered, since a cloudy sample might not be coupled with any pathologies, while a transparent sample could be found in a diseased animal (Parrah, et al., 2013).

The chemical evaluation of urines is generally performed with reagent strips, which are read by an automated dipstick reader or by visual inspection, and the results are interpreted following the graded scales reported by the manufacturer (Callens & Bartges, 2015; Piech & Wycislo, 2019). Urine strips allow the investigation of various urine parameters, such as pH, protein, glucose, bilirubin, ketones, occult blood, urine specific gravity or leukocytes (Callens & Bartges, 2015). However, the urine specific gravity and leukocytes are not reliable when measured with this device, and another type of testing for assessing them should be preferred (Piech & Wycislo, 2019). Indeed, dipstick test are generally designed for humans, thus various improper results might occur in veterinary practice (Defontis, et al., 2013). For instance, false protein dipstick positivity has been recorded when urine had an alkaline pH, as for dogs (Grauer, 2011), cows (Defontis, et al., 2013; Hermann, et al., 2019), Asian elephants (Wiedner, et al., 2009) and rhinoceroses (Haffey, et al., 2008). Urine dipstick leukocytes is specific, albeit not sensitive, for diagnosing pyuria in dogs, although it is not helpful in cats, judging by the high number of false positives (Reine & Langston, 2005). Furthermore, false dipstick positivity to ketones usually occurs in koalas (Vogelnest, 2015).

Therefore, on one hand, dipstick results should always be followed by further analysis, in order to confirm or to exclude positive outcomes. On the other hand,

knowing physiological peculiarities may help in the interpretation of the urinalysis in non-domestic animals.

1.1.2. URINE SPECIFIC GRAVITY

Urine specific gravity (USG) is the weight of the urine compared to that of an equal volume of water and it reflects both the total number of solutes and their weight (Chew, et al., 2011a). Different factors can affect USG in healthy animals; therefore, its interpretation is associated with clinical presentation, chemical analysis, and the hydration status of animals (Reine & Langston, 2005; Callens & Bartges, 2015). In domestic animals, the healthy range of urine specific gravity ranges between 1.025 - 1.045 (cattle), 1.015 - 1.040 (dog) and 1.036 - 1.060 (cat) (Parrah, et al., 2013).

In non-domestic animals, the USG values differ among species. For instance, in Asian elephants, the USG range is 1.007–1.025 (Wiedner, et al., 2009), while USG values between 1.020 and 1.050 have been reported in Bovidae and Antilocapridae (Wolfe, 2015), between 1.010 and 1.045 in macropods, and higher than 1.060 in koalas (Vogelnest, 2015). Furthermore, the USG range reported in clinically normal sloths is 1.020–1.031 (Black, et al., 2019) and different USG values have been reported for three species of captive rhinoceros: the greater one-horned rhinoceros (1.024 ± 0.00598), the Sumatran rhinoceros (1.015 ± 0.0069) and the African black rhinoceros (1.011 ± 0.0015) (Haffey, et al., 2008).

1.1.3. URINE MICROSCOPIC SEDIMENT EVALUATION

Microscopic examination of urine sediment begins with the centrifugation of the sample (Callens & Bartges, 2015). Then, few drops of the urine's supernatant are put on microscopic slides and the sediment is evaluated with a microscope at 10x and 40x objective magnifications (Callens & Bartges, 2015). Generally, the investigated components are biological (such as red and white blood cells, epithelial cells, casts, organisms - bacteria, fungi - crystals and mucus) and debris (such as artifacts or contaminants) (Reine & Langston, 2005; Chew, et al., 2011a).

However, some of these elements are not necessarily indicators of diseases, since they could be physiological findings in healthy animals: for instance, a quantity of up to 5 red and white blood cells is considered normal in urine composition, as well as crystals, the presence of which is frequently recorded in the urine of healthy dogs

and cats (Chew, et al., 2011a; Callens & Bartges, 2015). Squamous epithelial cells appear to be more common in urine samples collected by free-catch in dolphins (Jiménez-Zucchet, et al., 2019), while atypical struvite crystals seem to be usual in the elephant urine (Wiedner, et al., 2009).

The sediment examination should always be performed, either for the meaningful information it can provide or for the conditions which might not be diagnosed without this exam (Chew, et al., 2011a).

Urine sediment might have certain species-specific findings and, especially for uroliths, knowing the mineral composition is important for dissolving those existing or minimizing further growth (Osborne, et al., 2008). Various types of crystals are reported in literature in non-domestic species, both in healthy or diseased animals. For instance, cystinuria has been reported in captive maned wolves (Hammond, 2012). Urolithiasis was reported in cetaceans and, especially in bottlenose dolphins, it was associated with ammonium urate stones, calcium oxalate and struvite crystals (Dold, 2015; Jiménez-Zucchet, et al., 2019). Moreover, ammonium magnesium and amorphous phosphate crystals, and amorphous urate crystals have been reported in giant slots (Black, et al., 2019). Silica urolithiasis has been found in dromedary camels (Gutierrez, et al., 2002); calcium carbonate, struvite, calcium oxalate dihydrate, and amorphous phosphates in Asian elephants (Wiedner, et al., 2009), and mainly calcium carbonate in rhinoceros (Haffey, et al., 2008). Calcium carbonate uroliths have been frequently described in giraffes, too (Jones, et al., 2018).

1.2. URINE PROTEIN:CREATININE RATIO

The Urine protein:creatinine ratio (UPC) is one of the methods used for quantifying proteinuria and it is a useful tool for diagnosing a renal disease in cats and dogs (Grauer, 2011; Rossi, et al., 2016). This method is based on the assumption that, if the glomerular filtration rate remains stable, the protein and creatinine excretion rates reasonably remain constant during the day (Price, et al., 2005).

Proteinuria is considered as one of the most common abnormalities in routine urinalysis and could have various causes, which have allowed to categorize proteinuria on the basis of the site or the mechanism underlying it (Braun & Lefebvre, 2008; Grauer, 2011). However, the presence of proteins in the urine is not always associated with pathologies, since proteinuria could also have physiological causes and proteins

can be physiologically detected in the urine of normal dogs (Braun & Lefebvre, 2008; Grauer, 2011; Ferlizza, et al., 2020a). For instance, uromodulin – a protein produced by the healthy kidney - is the most abundant protein in normal canine urine (Devuyst, et al., 2017; Nkuipou-Kenfack, et al., 2017).

According to the International Renal Interest Society (IRIS), a non-proteinuric cat or dog should have a UPC value lower than 0.2 (IRIS, 2019), while different UPC values have been reported in domestic mammals, e.g. 0.04 - 0.25 in cattle (Hermann, et al., 2019) and <0.5 in horses (Schott & Esser, 2020).

Few studies have been published on the UPC in non-domestic species, e.g., sloths (0.1–0.6) (Black, et al., 2019) and felids (0.4 – 0.5) (Wack, 2008). As far as giraffes are concerned, their UPC value has not still been identified, albeit a urinary creatinine range of 32-628 mg/dL was published by Sullivan and colleagues (Sullivan, et al., 2010).

1.3. URINE PROTEOMICS

Proteomics is the study of the proteome, the entire set of proteins present in a tissue or in a biological fluid, e.g., urine (González-Buitrago, et al., 2007). Proteomics studies can lead to the discovery of biological markers of diseases, by comparing the protein patterns between healthy and pathological individuals (González-Buitrago, et al., 2007). Consequently, from a proteomics standpoint, urine – which contains proteins derived from the ultrafiltration of plasma and from the urinary tract – could provide useful biological markers, not only for kidney diseases but also for diseases affecting other organs (González-Buitrago, et al., 2007; Decramer, et al., 2008).

In physiological condition, the urinary proteins originate from the glomerular filtration whose slit ensures that the plasma proteins are present in low concentration (Braun & Lefebvre, 2008). Proteins with molecular mass (MM) above of 70 kDa are excluded from the filtrate; the albumin, whose MM is closest to the filtration threshold (average MM of 69 kDa), can be present in the ultrafiltrate and it is the first plasma protein to appear in case of glomerular disturbance (Braun & Lefebvre, 2008; Reece, 2015a). The proteins with low MM (< 40 kDa) are filtered by the glomeruli, but they are almost reabsorbed in the tubule (González-Buitrago, et al., 2007; Braun & Lefebvre, 2008). However, in physiological conditions, proteins with high molecular mass might be detected, i.e., uromodulin, which is a protein produced by the cells of

the renal thick ascending limb (TAL) of the loop of Henle and with a molecular mass of 80-90 kDa (Devuyst, et al., 2017; Wu, et al., 2018).

When a pathological condition interests one or both of these structures, a change occurs in the urinary proteome (Hokamp, et al., 2018). Thus, in urine, an abundance of proteins with a high and intermediate MM can be found when the glomeruli are involved; at the same time, an abundance of low MM proteins can be detected when tubules are affected (Hokamp, et al., 2018).

Various techniques have been used in proteomics studies. The first step in these assays is to separate the protein using different methods: gel-based proteomics (one-dimensional SDS-PAGE, two-dimensional gel electrophoresis), high-performance liquid-chromatography (HPLC) or capillary electrophoresis (CE) (González-Buitrago, et al., 2007; Isani, et al., 2018). After the separation, proteins are identified using Mass spectrometry (MS) or Tandem mass spectrometry (MS/MS) (González-Buitrago, et al., 2007). Another remarkably technology used in proteomics studies is the shotgun proteomics, which allows the identification of proteins from tandem mass spectrometry of their proteolytic peptides (Marcotte, 2007).

The application of these methodologies in veterinary medicine is relatively modern and has mainly focused on companion species. The urinary proteome of the dog has been widely investigated using different techniques (Forterre, et al., 2004; Giori, et al., 2011; Nabity, et al., 2011; Brandt, et al., 2014; Ferlizza, et al., 2020a) as well as that of the cat (Lemberger, et al., 2011; Ferlizza, et al., 2015; Maeda, et al., 2015; Giraldi, et al., 2020).

As far as farm animals are concerned, few data on the urine proteome have been reported and almost all of them were about cows (McDougall, 1965; Hwang & Lim, 1999; Pyo, et al., 2003; Simon, et al., 2008; Bathla, et al., 2015; Xu, et al., 2015; Rawat, et al., 2016; Isani, et al., 2018; Ferlizza, et al., 2020b), even though some studies have focused on sheep (McDougall, 1965; Palviainen, et al., 2012), horses (Scarpa, et al., 2007; Isani, et al., 2018), and goats (Ozgo, et al., 2009).

The literature on proteomics regarding the urine of non-domestic animals is fragmentary. Few studies have been published on big cats (McLean, et al., 2007), camels (Alhaider, et al., 2012) and California sea lions (Neely, et al., 2018).

1.4. URINE METABOLOMICS

Metabolomics is the quantitative measurement of metabolites in biological samples by using different approaches, such as 1H-nuclear magnetic resonance (NMR) and gas chromatography−mass spectrometry (GC-MS) (Sun, et al., 2015). Biofluids are used to study molecules which create a unique fingerprint (Zhang, et al., 2012). Moreover, since the metabolites are the end products of complex interactions occurring between the inside and the outside of the cell, the study of metabolites (via metabolomics) allows to determine interactions between genes and the environment (Goldansaz, et al., 2017).

Urine is an optimal biological matrix because it is effortlessly obtained, it is less complex than other body fluid (i.e., blood) and it has been successfully used in various medical fields, such as physiology or in the diagnosis of chronic diseases (Ryan, et al., 2012; Zhang, et al., 2012).

A quantity of papers about animal metabolomics that show how it can be used to help farmers, veterinarians, livestock researchers and the livestock industry have been published (Goldansaz, et al., 2017). Firstly, urine metabolomics could support the investigation of health status and the diagnosis of a disease (both in domestic and captive animals); secondly, it could be a suitable tool for investigating the physiological status of animals in the wild, since urinary metabolites are the final products of normal cellular processes (Ryan, et al., 2012; Zhang, et al., 2012).

Studies on urine metabolomics were reported in literature on veterinary species, such as dogs (Matsumoto, et al., 1995; Ferlizza, et al., 2020a), goats (Contreras-Jodar, et al., 2019), sheep (Li, et al., 2011), pigs (Merrifield, et al., 2011), horses (Escalona, et al., 2015; Zhu, et al., 2018) and cows (Sun, et al., 2015; Sun, et al., 2016; Ahamad, et al., 2017) as well as non-domestic animal, such as giant pandas (Zhu, et al., 2020a), yaks (*Bos grunniens*) (Zhu, et al., 2019) and camels (Ahamad, et al., 2017).

As far as giraffes are concerned, no study has focused on urinary metabolome, whereas few papers about fecal metabolites have been published (Wolf, et al., 2018; Mu, et al., 2019).

2. GIRAFFE

2.1. GIRAFFE AND ZOO ANIMAL WELFARE

Giraffe is a mammal part of the family Giraffidae, which includes only two members: the giraffe (*Giraffa camelopardalis*) and the okapi (*Okapia johnstoni*) (Bertelsen, 2015). Giraffe species is traditionally distinguished in nine subspecies, albeit some authors have suggested another taxonomic classification (Dagg, 2014a; Winter, et al., 2018).

Due to a population decline in the last years that has reduced the number of mature giraffes from 106 191 – 114 416 (1985) to 68 293 (2015), the species *Giraffa camelopardalis* was assessed as Vulnerable by the International Union for Conservation of Nature (IUCN), even though its nine subspecies have different trends (Bercovitch, et al., 2018; Fennessy, et al., 2018b; Fennessy & Marais, 2018; Fennessy, et al., 2018a; Marais, et al., 2018; Muller, et al., 2018; Muneza, et al., 2018; Wube, et al., 2018; Bolger, et al., 2019; Deacon & Tutchings, 2019) (Table 1).

Table 1 Giraffe subspecies, IUCN classification, number, trend and corresponding references. [1] Marais, et al., 2018; [2] Fennessy & Marais, 2018; [3] Wube, et al., 2018; [4] Deacon & Tutchings, 2019; [5] Fennessy, et al., 2018a; [6] Muneza, et al., 2018; [7] Fennessy, et al., 2018b; [8] Bercovitch, et al., 2018; [9] Bolger, et al., 2019.

Subspecies	IUCN Classification	Number	Trend	References
G. c. angolensis	Least Concerned	10 173*	↑	[1]
G. c. antiquorum	Critically Endangered	1 400*	↓	[2]
G. c. camelopardalis	Critically Endangered	455*	↓	[3]
G. c. giraffa	Least Concerned	21 053 – 26 919§	↑	[4]
G. c. peralta	Vulnerable	425*	↑	[5]
G. c. reticulata	Endangered	11 048*	↓	[6]
G. c. rothschildi	Near Threatened	> 1 468*	↑	[7]
G. c. thornicrofti	Vulnerable	420*	-	[8]
G. c. tippelskirchi	Endangered	35 000§	↓	[9]

G. c.: *Giraffa camelopardalis*; * Mature individuals; § Total population.

Consequently, due to their reduction in recent years, various actions have been taken to protect giraffe populations, for instance the establishment of Giraffe and Okapi Specialist Group (GOSG)[1] or the inclusion of giraffe (*Giraffa camelopardalis*) in

[1] https://www.giraffidsg.org/

Appendix II of the Cites (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (CITES, 2019; CITES, 26/11/2019).

Furthermore, scientists and foundations – as, for instance, Save the Giraffes[2], Giraffe Conservation Foundation[3], Giraffe Conservation Alliance[4], Save Giraffe Now[5] or World Giraffe Foundation[6] - have been striving to save giraffes from extinction, and have been carrying out projects for the conservation of this species throughout Africa.

Among the entities who are working to protect giraffes and generally the endangered species, zoos should not be forgotten. Indeed, zoological parks play an important role in defending threatened species by organizing *ex situ* activities as well as *in situ* conservation projects (Tribe & Booth, 2003). Zoos can breed captive endangered animals and might reintroduce them in the wild or they can take care of campaigns about the conservation of biodiversity; furthermore, zoos might support research aiming at broadening knowledge on these endangered species (Hosey, et al., 2013b). Indeed, gathering biological or behavioural knowledge about the species is also useful to improve the way the animals in captivity are managed (Kersey & Dehnhard, 2014).

According to the Council of the European Union, zoos need to achieve different aims when hosting wild species, e.g., research, education and veterinary care (The Council of the European Union, 1999). They must pursue all these intents with a commitment to ensure high standards of animal welfare, an important aim for modern zoos (Kagan & Veasey, 2010; Paul-Murphy & Molter, 2019).

Firstly, it is important to bear in mind that welfare is strictly coupled with the individual: the same events may impact the animals in different ways; the animals, in turn – as they come from various backgrounds – could have or not have the abilities to face a particular situation (Hosey, et al., 2013a; Paul-Murphy & Molter, 2019). Defining and assessing animal welfare are two fundamental values that have to be reached in zoos in order to reduce sources of stress and to establish sensitive indicators of welfare (Kagan & Veasey, 2010; Paul-Murphy & Molter, 2019).

[2] http://savethegiraffes.com/
[3] https://giraffeconservation.org/
[4] http://www.giraffealliance.org/
[5] https://savegiraffesnow.org
[6] http://www.worldgiraffefoundation.org/

Consequently, while it is fundamental to guarantee a good quality of life for animals in captivity, it is vital to know the physiological and behavioural needs of a species and each animal's personality, considering that specimens hosted in zoos come from different environments (Kagan & Veasey, 2010; Hosey, et al., 2013a). For instance, giraffes – which are adapted to living in tropical climates - require heated stables (from 18° C to 24° C) when they are housed in moderate climates areas (Bertelsen, 2015). As a matter of fact, hypothermia is one of the causes of giraffes' death, due to their inability to maintain the body temperature when exposed to cold weather for long (Gage, 2019). Besides, female giraffes might particularly prefer to spend time with some specific individuals than other ones; hence, this preference should be taken into account when managing giraffes (Gage, 2019). Giraffes are also considered browsers that mainly eat foliage; as a consequence, when in captivity, they should be encouraged to use their tongues and play with enrichments that might help implementing their species-specific behaviours and avoid oral stereotypies (Valdes & Schlegel, 2012; Gage, 2019).

Nevertheless, species housed in captivity are faced with various sources of stress, which include the routine husbandry as veterinary examination (Morgan & Tromborg, 2007). In zoos, veterinary care should focus on preventing diseases mainly by planning parasite control, husbandry techniques, treatments and daily animals' observations in an accurate manner, in order to gather signs of illness or pain as soon as possible (Meehan, 2015; Murphy, 2015). Zoo staff have to guarantee animal health, but the process could be controversial. In fact, veterinary practices, such as the record of temperature, could be quite impracticable on zoo animals, hence other methods are used for checking health status, e.g., body condition score and gait scoring (Hosey, et al., 2013c). From a veterinary standpoint, the diagnosis of diseases, which would require testing to confirm the suspected diagnosis, is important for zoo animals and a key point for pursuing their welfare (Hosey, et al., 2013c). Moreover, when it is necessary to perform laboratory analysis, the collection of biological samples usually involves sedation or anaesthesia and – since they are generally conducted together with other veterinary procedures which require immobilization - they are not performed routinely (Hosey, et al., 2013c). However, each procedure must be carefully considered and its costs and benefits need to be weighed out every time anaesthesia seems to be the only manner to perform those procedures (Hosey, et al., 2013c).

Considering all this, various strategies can be chosen to reduce the stress as much as possible and to assess the health status of animals, including training or biological sampling with non-invasive techniques (Bechert, 2012; Gage, 2019). For instance, due to the giraffe's predisposition to have anaesthetic-related complications, some zoos where these mammals are hosted have successfully trained them to accept medical treatment (e.g., hoof trimming) or biological samples collection (e.g., blood or urine) (Sullivan, et al., 2010; Bertelsen, 2015; Gage, 2019). Yet, the training of zoo animals features both pros and cons: although this practice has been positively used to reduce the stress associated with handling (for examples routine veterinary procedure or husbandry), it shows several disadvantages, including the need of a competent staff (Hosey, et al., 2013d). In fact, giraffes are particularly prone to panic and are nervous, big animals, thus every change or innovation should be introduced slowly, in order to help them cope with their fear so that they won't panic or hurt themselves or the staff (Dagg, 2014b). Conversely, the collection of biological samples with non-invasive techniques might be a useful tool to reduce the stress related to capture and anaesthesia (Bechert, 2012) and it does not require special zoo's facilites. For instance, urine and faeces, which are routinely excreted, might be collected with minimal, or none, contact with the animals; urines in particular might be sampled from the floor repeatedly or extracted from natural substrates, as reported in various species (Sullivan, et al., 2010; Bechert, 2012; Kersey & Dehnhard, 2014; Burrell, et al., 2017).

2.2. KIDNEYS AND URINE VALUES OF GIRAFFES

There are only few studies focusing on giraffe's urinary system and urinalysis (Loskutoff, et al., 1986; Wolfe, et al., 2000; Maluf, 2002; Osborne, et al., 2008; Sullivan, et al., 2010; Sullivan, et al., 2013; Damkjær, et al., 2015). Giraffe's kidney was described in depth by Maluf (2002), which pointed out particular characters in this mammal's organ and the giraffe's renal physiology has been defined recently by Damkjær and colleagues (2015). The main features of giraffe's kidneys are reported in Table 2.

Table 2 Main features of giraffe's kidneys and corresponding references. [1]: (Maluf, 2002); [2]: (Damkjær, et al., 2015); [3]: (Veldhuis, et al., 2020).

Features	***Giraffa camelopardalis***	**References**
General		
Shape	C-shape	[1]
Kidneys vs body mass (%)	0.21(adult) - 0.54 (young)	[1]
External feature	No external lobation; superficial furrow (young)	[1]
Renal capsule		
Thickness (mm)	0.20 - 0.75	[1] [2]
Architecture	Robust and almost inextensible, without perforation by blood vessels	[1]
Burst pressure[a]	600-650 mmHg	[2]
Cortex		
vs parenchymal renal mass (%)	63	[1]
Cortex thickness	11-24 mm	[1]
Columna renalis	None	[1]
Medullary rays	Ascending and descending hairpin loop of Henle and collecting ducts. Medullary rays split the cortex with proximal tubules in thin elongated bundles	[1] [2]
Mass of cortex vs Mass of medulla	1.7:1.0	[1]
Medulla		
vs renal parenchyma (%)	4 (Inner medulla)	[1]
Architecture	Crest-type and C-curvature	[1]
Relative medullary thickness (RTM)	2.84	[3]
Inner medulla (IM)	Pale. Includes: interstitium, blood capillaries, collecting ducts, thin medullary hairpin loops. Exposed to urine in the pelvis via the pelvic extensions	[1] [2]
Outer medulla (OM)	9 times heavier than IM	[1]
Outer stripe of OM	Thin (0 – 2000 μm thick). Probably rearranged	[1] [2]
Inner stripe of OM	Broad. Exposed to urine in the pelvis via the pelvic extensions	[2]
Medullary Processes	Mainly composed by inner stripe of OM. Single layer of cuboidal epithelium and, under this layer, presence of α-smooth muscle actin	[1] [2]
Vascular processes	Transitional epithelium. Contain interlobar veins and arteries, fibrous tissue*	[1] [2]
Renal pelvis		
Architecture	C-curvature; deep extension into the inner stripe of OM	[1]
Dimension (mm)	Long: 147 (121, in young), wide: 11	[1]
Wall	Transitional epithelium	[1]

[a] Maximal pressure sustained by renal capsule [2].

* In some part of the vascular processes, the interlobar blood vessels were located in a less cellular connective tissue; the latter seems similar to the mucoid, mesenchymal connective tissue reported in the human umbilical cord and in the human vitreous body of the eyeball [2].

During times of drought, African herbivores – such as giraffes - have developed different adaptations (i.e., ecological, physiological and behavioural) to reduce water loss and, consequently, to conserve body water (Kihwele, et al., 2020). Indeed, it has been demonstrated that animals living in arid and semiarid regions are generally able to highly concentrate their urine (Al-kahtani, et al., 2004).

Considering that even in semi-desert areas giraffes seldom drink water, it seems that this mammal has developed adaptations to withstand drought and to be able to make do with the quantity of water supplied by vegetation for surviving (Dagg, 2014c). However, the water drinking habits of giraffes are still unknown, as some animals have been seen to drink often, while others rarely drink at all (Dagg, 2014c).

2.2.1. ANATOMY

Despite their state of hypertension, the kidneys of giraffes are surprisingly similar to the kidneys of the other mammals and they do not display signs of hypertension-related damage (Maluf, 2002; Damkjær, et al., 2015).

However, some peculiarities have been detected in their kidneys' anatomy, leading to suggest a correlation between these findings and the unusual biomechanical properties (Damkjær, et al., 2015). Namely, a mesenchymal connective tissue similar to those found in umbilical cord and vitreous body of the eyeball in humans has been found in some part of vascular processes (Damkjær, et al., 2015).

Moreover, two of the three structures responsible for the production of concentrated urines - i.e., renal pelvis, the relative medullary thickness and the cortical tubules (Abdalla, 2020) - reveal some peculiarities in giraffes.

As far as renal pelvis is concerned, it extends deeply into the inner stripe of the outer medulla, almost reaching the corticomedullary border and its walls are lined by transitional epithelium (Maluf, 2002). Indeed, the presence of specialized pelvic fornices is considered one of the factors responsible for the urine concentrating ability (Al-kahtani, et al., 2004). Interestingly, it seems that giraffe urine (from the area cribrosa of the crest) would pass between the vascular and medullary processes (Maluf, 2002). The author advanced two hypotheses for explaining this facility. The first supposition proposes that urea in the pelvic extensions could diffuse into the medullary processes, from where it is carried to rumen in order to be hydrolysed into

ammonia by microorganisms, this way contributing to the anabolism of amino acids and proteins (Maluf, 2002). Indeed, it has been pointed out that, when the diet of animals was low-protein, the renal pelvis in sheep provided the urea recycling; in fact, an increase of the recycling of urea guarantees the reduction of its urinary lost, due to a significant reduction of the filtered load (Cirio & Boivin, 1990). The second hypothesis is that the pelvic extensions allow the urea's deposit in the renal *interstitium* for concentrating the urine, without the intervention of the antidiuretic hormone (ADH) (Maluf, 2002). Indeed, even in sheep, it has been demonstrated that the renal pelvis plays an important role in the final concentration of urine, suggesting that it causes the diffusion of solutes through the thin pelvic epithelium to the close tissue of the outer medulla (Faix, et al., 1996). Likewise, a complex structure of the renal pelvis has been found even in the dromedary camel, where the main cavity of the pelvis presents three dimensional extensions, which arguably play a role in urine concentration and water conservation (Abdalla, 2020). Interestingly, the walls of the renal pelvis lined by transitional epithelium have been also reported in this latter species, where these walls are impermeable to water and urea (Abdalla, 2020).

As far as relative medullary thickness (RTM) is concerned, this value is estimated by measuring the depth of the medulla from the cortico-medullary junction to its innermost depth, which projects into the renal pelvis (Reece, 2015b). It is the ratio between the medullary thickness (MT) and kidney volume (KS), where kidney volume is calculated as cube root of the product of the three-size dimension, i.e. $(\text{length} \times \text{breadth} \times \text{width})^{1/3}$ (Al-kahtani, et al., 2004; Kihwele, et al., 2020). RTM is considered a structural index for quantifying the relative length of the longest loops of Henle and a better predictor of urine concentrating ability (Al-kahtani, et al., 2004; Reece, 2015b). Recently, this index has been used with other functional traits for quantifying water requirements of African ungulates, included giraffes, observing that herbivores reduce water loss throughout simultaneous and various pathways, including urine (Kihwele, et al., 2020). Authors reported the RTM of giraffes (Table 2) and they stated this mammal as a low water dependent species (Kihwele, et al., 2020; Veldhuis, et al., 2020). Surprising, RTM of giraffes (2.84) is closer to the human (RTM = 3) than dromedary camels (RTM = 7.89) (Reece, 2015b; Kihwele, et al., 2020; Veldhuis, et al., 2020).

As far as cortical tubules are concerned, the cortical proximal tubules between the medullary rays were arranged into thin elongated bundles in giraffes (Damkjær, et al., 2015).

2.2.2. PHYSIOLOGY

As far as renal physiology is concerned, the most intriguing inquiry about giraffes is how this species could cope with its hypertension (twice than other mammals) without experiencing kidney damage (Damkjær, et al., 2015).

This issue was investigated recently and it has been found out that giraffes had lower values of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) compared to other mammals with a similar size; however, the filtration fraction is similar to other mammals (Damkjær, et al., 2015). The authors postulated that these findings might be related to some unique features of giraffe, i.e., a robust renal capsule and a valve structure at the junction between the renal vein and the vena cava (Damkjær, et al., 2015). Indeed, when comparing the giraffe renal capsule to the cow, it was discovered that this structure was twofold thicker and had fivefold larger collagen content per unit area, allowing the animal to withstand high intrarenal pressures (Damkjær, et al., 2015). Moreover, the authors found that the valve function was intermittent, leading them to speculate that this structure, by maintaining a high renal venous pressure, might support a high renal interstitial pressure (Damkjær, et al., 2015). Consequently, these unique characteristics seem to be responsible for the very high interstitial hydrostatic pressure within the Bowman capsule and the resulting lower glomerular filtration rate; these unique adaptations reduce the pressure gradient across the glomerular membrane and would protect giraffe kidney against hypertension (Damkjær, et al., 2015).

Additionally, the presence of high concentration (and high variation) of the hormone arginine vasopressin (AVP) was detected in the giraffe's plasma; nevertheless, the same authors concluded that more studies are needed to confirm these findings (Damkjær, et al., 2015). Vasopressin, or antidiuretic hormone (ADH), is a hormone secreted by the supraoptic nuclei of the posterior pituitary, whose target cells are the cortical collecting tubules and medullary collecting ducts; the latter increase their permeability for water, depending on the amount of vasopressin (Reece, 2015b). In the kidney, the AVP regulates the transporters aquaporins (AQPs), which

are involved in urine concentration (especially the AQP isoforms 1, 2, and 3) (Nawata & Pannabecker, 2018). Aquaporins are a family of membrane proteins (about 30 kDa) that act as water channels, which have been reported in Bactrian camels, cattle and goats (Elfers, et al., 2014; Wang, et al., 2018). In fact, it has been found that, compared with cattle, Bactrian camel – a species living in the desert and semi-desert area – strongly expresses the aquaporins 2, 3 and 4 in the renal medulla, leading the authors to conclude that some differences in water transport occur between these two species (Wang, et al., 2018). In goats, it has been reported that a reduction of the diet's nitrogen intake is responsible for the rise of the plasma AVP concentration, which in turn causes an aquaporin 2 and urea transporter (UT-A1) expression increase and optimises the absorption of urea in young ruminants (Elfers, et al., 2014).

In conclusion, considering giraffe's kidney peculiarities and the habitat where this mammal lives – dry seasons and changes in the compositions of food available for animals (Dagg, 2014d) – it is reasonable to believe that giraffes, even though not yet demonstrated, might possess analogous urine concentration mechanisms to deal with the reduction in water availability and in food protein intake. However, further studies are required to corroborate this hypothesis.

2.2.3. URINE

The clinical biochemistry of giraffe urine is still an almost unexplored topic and studies are scarce. Investigation on giraffe's urine has focused on urinary steroid (Loskutoff, et al., 1986), nutrient concentration (Sullivan, et al., 2010) and urolithiasis (Wolfe, et al., 2000; Sullivan, et al., 2013). In particular, urolithiasis has been reported in literature and it is a severe problem in giraffes, which could cause the death of the animals, and seems to have a nutritional origin (Wolfe, et al., 2000; Maluf, 2002; Osborne, et al., 2008; Sullivan, et al., 2010; Valdes & Schlegel, 2012; Sullivan, et al., 2013; Bertelsen, 2015). Male animals appear to be more affected by urolithiasis and the sigmoid urethral flexure is one the most common tract involved (Sullivan, et al., 2013; Jones, et al., 2018). Calcium carbonate uroliths are frequently described in giraffes, where a high intake of dietary phosphorus and concentrate seems to be a contributing factor (Jones, et al., 2018), although in literature various uroliths (e.g. struvite) have been reported (Wolfe, et al., 2000; Maluf, 2002; Osborne, et al., 2008; Sullivan, et al., 2010). Notably, kidney stones have been reported even in wild giraffes during the dry

season and these phenomena might be related to a dietary imbalance of calcium and phosphorous, which drive the animals to display pica behaviour (Dagg, 2014d). In fact, leaves – one of main components of the giraffe's diet - are rich in calcium, whilst soil are rich in phosphorous (Dagg, 2014d).

3. AIMS AND OBJECTIVES

Given this scenario and, especially, considering giraffes' predispositions, their particular and unique physiology and the lack of data about the urinary parameters, the aims of this study were to establish the reliability of urine sampling from the ground, define urinary reference values, and study the urinary proteome and metabolome.

In particular, three experimental studies were carried out:

1. **Experiment 1**: to set up a useful and reliable non-invasive urine sampling method from the ground. To pursue this objective, a preliminary study on cattle was performed, comparing the results obtained from urines collected in sterile cups with those obtained from the same samples aspirated from the ground. The data are part of the paper:

   Fasoli S., Ferlizza E., Andreani G., Sandri C., Dondi F., Isani G. Noninvasive sampling method for urinalysis and urine protein profile in captive giraffes, Journal of Veterinary Diagnostic Investigation, 2021, *33(1)*, 25-34; doi: 10.1177/1040638720975370.

2. **Experiment 2**: to establish the urinalysis reference values in captive giraffes and to study the urinary proteome. To pursue this objective, urines were subjected to routine urinalysis, proteome separation using SDS-PAGE and proteins identification using mass spectrometry. This latter investigation has been performed in collaboration with Dr. Elisa Bellei from the University of Modena and Reggio Emilia (Italy). The data are reported in the paper:

   Fasoli S., Andreani G., Dondi F., Ferlizza E., Bellei E., Isani G. Urinary reference values and first insight into the urinary proteome of captive giraffes. Animals 2020, *10*, 1696; doi:10.3390/ani10091696.

3. **Experiment 3**: to study the urinary metabolome of captive giraffes by Proton Nuclear Magnetic Resonance Spectroscopy. This study was performed in collaboration with Dr. Luca Laghi and Dr. Chenglin Zhu from the Department of Agro-Food Science and Technology (University of Bologna, Cesena, Italy). The data are reported in the paper:

   Zhu C., Fasoli S., Isani G., Laghi L. First Insights into the Urinary Metabolome of Captive Giraffes by Proton Nuclear Magnetic Resonance Spectroscopy. Metabolites 2020, *10*, 157; doi:10.3390/metabo10040157.

EXPERIMENT 1

URINE COLLECTION FROM THE GROUND[7]

[7] Part of the following data is included in the paper: Fasoli S, Ferlizza E, Andreani G, Sandri C, Dondi F, Isani G. Noninvasive sampling method for urinalysis and urine protein profile in captive giraffes. J Vet Diagn Invest. 2021 Jan;33(1):25-34. doi: 10.1177/1040638720975370.

INTRODUCTION

The study of threatened species, or more generally of non-domestic animals, withholds more difficulties compared to the research on domestic species, due to the inherent limitations of obtaining clinical and laboratory data on the former (Kersey & Dehnhard, 2014). Firstly, the number of wild species is lower than that of their domestic counterpart, this way limiting the access to animals (Kersey & Dehnhard, 2014). Secondly, an accurate diagnosis of wild animals in captivity might be feasible only after invasive procedures (Hosey, et al., 2013c). Finally, the invasive procedures could perturbate the results of the medical tests, and this is particularly evident in the field of endocrinology (Kersey & Dehnhard, 2014).

The use of non-invasive techniques to assess the health status of wild animals prevents animal immobilization (Bechert, 2012), therefore, these procedures should be preferred when routinely monitoring the health status of these animals.

Urine can be considered an excellent biological sample, as it can give information about renal and urinary tract disorders and non-urinary diseases (Parrah, et al., 2013; Piech & Wycislo, 2019). Moreover, this biofluid can be collected using non-invasive approach (Bechert, 2012). Different urine sampling methods have been reported (e.g., from leaves, first aid cotton, clean plastic sheets as well as from concrete flooring or extracted from natural substrates, such as sand and snow) and non-invasive techniques have been applied in various non-domestic mammalian species such as chimpanzees, macaques, orangutans, okapi, giant pandas and wolves (Glatston & Smith, 1980; Mech, et al., 1987; DelGiudice, et al., 1988; Knott, 1997; Thompson, et al., 2009; Danish, et al., 2015; Burrell, et al., 2017). Although the main advantage of sampling the urine from the ground/floor is to minimize the stress of animals (Bechert, 2012), the procedure shows some disadvantages such as the presence of debris contamination or bacteria due to the unsterile collection method (Burrell, et al., 2017).

Since urine can be collected in adequate amounts repeatedly and non-invasively, this way avoiding stress to the animals (Kersey & Dehnhard, 2014; Kurien, et al., 2004; Parrah, et al., 2013; Piech & Wycislo, 2019), this sampling technique could also be useful for giraffes. In fact, giraffes are particularly liable to stress and panic and this type of temperament makes even the training a tricky process for animal taming

(Dagg, 2014b). Moreover, this species is particularly prone to show anesthetic-related complications and subsequent death (Gage, 2019), this way limiting the access to their biological samples.

Given this scenario, the aim of this experiment was to establish if the collection of samples from the ground could be a reliable method, at the same time evaluating the possible pre-analytical interference from ground contaminants. To reach this aim, voided urines from 10 cows were collected by free-catch sampling and compared to a syringe sample from the ground. Urines from cattle were used to achieve this issue given the feasibility of sampling the urines in sterile urine cups in these domestic animals, a technique quite inapplicable to giraffes.

MATERIAL AND METHODS

1. URINE COLLECTION

Urines (~ 20 mL) from 10 cows were collected in sterile urine cups (Pic Solution, Safe Sterile Box 100 mL) in June 2019. Cows were located in a dairy farm near Verona (Italy) and the sampling was performed after milking, while they were eating.

Each sample was divided in 2 aliquots (~ 10 mL): 1 of these 2 aliquots was thrown to unpaved ground and 5 mL of urine were collected with a syringe, whereas the other 1 aliquot was used as a control sample. To minimize the soil contamination, only the upper part of the samples was aspirated. The resulting 20 samples were subjected to complete urinalysis.

A further test was performed on 1 urine sample collected from one giraffe in November 2019. Namely, it was possible to collect 2 aliquots from one giraffe: one at the start of spontaneous voiding (in sterile urine cup) and another one at the end of the voiding (with a syringe from the ground). Urine sampling from this animal was done during daily husbandry activities. These 2 aliquots were subjected to complete urinalysis.

2. URINALYSIS

Physical examination of the 20 urine samples obtained from cows was performed by visual inspection of colour and clarity. These analyses were performed within 3 hours from the urine collection, following the guidelines reported in dogs and cats (Callens & Bartges, 2015; Piech & Wycislo, 2019).

The chemical evaluation was performed by visual inspection and with semi-quantitative dipstick test (Combur10 Test, Roche Diagnostic, Basel, CH), putting one drop of urine on each net and comparing the colour change of the strip with reference after 60 seconds (Table 3). Urine specific gravity was assessed by a refractometer (Giorgio Bormac, 41012 Modena, Italy).

Table 3 Scales of Combur10 Test Urine Strips.

	Negative	**+**	**++**	**+++**	**++++**
Protein (mg/dL)	0	30	100	500	2000
Blood (RBC/µL)	0	10	25	50	250
Leukocytes (WBC/µL)	0	~10-25	~75	~500	-
Bilirubin (mg/dL)	0	1	3	5	-
Glucose (mg/dL)	0	50	100	>300	-
Ketones (mg/dL)	0	15	50	150	-
pH			5 - 9		

The urine samples were centrifuged at 1,500 × *g* for 10 min and the supernatants underwent microscopic sediment analysis (Ferlizza, et al., 2015). The latter was performed by resuspending 50 µL of urine sediment and subsequently placing it on two 26 x 76 mm microscope slides covered by a 20 mm^2 glass coverslip. The urine sediments that were both unstained and stained with fuchsine solution (Samson Reagenz; Dr. Grogg Chemie AG, Stettlen-Deisswil, CH) were examined under low-power field (100x) and high-power field (400x). The evaluation of the components of the urine sediments was performed according the Table 4. Supernatants were stored for 1 month at -80°C for further analyses.

Table 4 Guidelines used to perform the microscopic sediment analysis.

Variable	**Negative**	**+**	**++**	**+++**
Cells				
Epithelial cells	<5 element	5-10 cells	11-50 cells	> 50 cells
Red blood cells	<5 element	5-10 cells	11-50 cells	> 50 cells
Leukocytes	<5 element	5-10 cells	11-50 cells	> 50 cells
Sperm	<5 element	5-10 cells	11-50 cells	> 50 cells
Casts	<5 element	1- 4 casts	5-10 casts	11-20 casts
Crystals	<5 element	1- 4 crystals	5-10 crystals	11-20 crystals
Soil contaminants				
Pollen	<5 element	5-10 elements	11-50 elements	> 50 elements
Vegetable fibres	<5 element	5-10 elements	11-50 elements	> 50 elements
Mold spores	<5 element	5-10 elements	11-50 elements	> 50 elements

3. URINE TOTAL PROTEIN AND URINE CREATININE

Urine total protein (uTP) and urine creatinine (uCrea) were obtained using commercial kits (Urinary/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus-Beckman Coulter, Brea, California 92821-6232, USA) and were performed using an automated chemistry analyser (AU 400, Olympus-Beckman Coulter, Brea, California 92821-6232, USA). The urine protein:creatinine ratio (UPC) was calculated through the formula:

$$\text{UPC} = \frac{\text{uTP (mg/dL)}}{\text{uCrea (mg/dL)}}$$

The calibration of both methods was performed in accordance with the manufacturer instructions for urine using standard materials (Urinary/CSF Protein Calibrator; Urine Calibrator; Beckman Coulter, Brea, CA, USA) and the controls were done on a daily basis using a commercially available quality control solution (Liquichek, Urine Chemistry Control, Bio-Rad Laboratories, Irvine, CA, USA).

3.1. URINE TOTAL PROTEIN[8]

The pyrogallol red-molybdate method, (Olympus System Reagent - Urinary/CSF protein, OSR 6170) was used to determine the urine total protein (uTP). The Urinary/CSF protein reagent is specific for the determination of total proteins in human urine and cerebrospinal fluid (CSF). At the basis of this method, there is the absorbance change occurring when the red pyrogallol-molybdate complex binds the basic amino groups of proteins. In fact, the red pyrogallol-molybdate complex has a maximum absorbance at 470 nm whilst the blue-purple complex, which forms when proteins are present in the sample, has a maximum absorbance at 600 nm. The absorbance of the latter is directly proportional to the protein concentration in the sample.

Linearity: it is linear for concentrations between 4 and 200 mg/dL.

Reagents: pyrogallol red 47 µM, sodium molybdate 320 µM, succinic acid 50 mM, sodium benzoate 3.5 mM, sodium oxalate 1.0 mM, methanol 0.8% and detergent.

[8]https://www.beckmancoulter.com/wsrportal/techdocs?docname=/cis/BAOSR6x70/%25%25/EN_URINARY-CSF%20PROTEIN.pdf

3.2. URINE CREATININE[9]

The concentration of urinary creatinine was determined using a kinetic modification of the Jaffe procedure (Olympus System Reagent - Creatinine, OSR 6178). At the basis of this method is the formation of a yellow-orange complex, a by-product of creatinine and picric acid in an alkaline environment. The resulting change in the rate of absorbance at 520/800 nm is directly proportional to the concentration of the complex, which in turn is related to the concentration of creatinine.

Linearity: it is linear for urinary concentrations between 1 and 300 mg/dL.

Reagents: sodium hydroxide 120 mmol/L, picric acid 2.9 mmol/L, preservatives.

4. 1D-SDS-PAGE ELECTROPHORESIS

All urine supernatants were subjected to sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Urine proteins were separated using an electrophoresis system (NuPAGE, Thermo Fisher Scientific, Waltham, Massachusetts, USA) on precast 4-12% polyacrylamide gel in reducing conditions with MES buffer (2-[N-morpholino-ethanesulfonic acid]) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing SDS.

For each sample, 2 μg of proteins were loaded and electrophoresis was carried out and gels were stained with SilverQuest™ Staining Kit (SilverQuest Thermo Fisher Scientific, Waltham, Massachusetts, USA). After staining, the gels were digitalized using a densitometer (ChemidocMP, BioRad, Hercules, California, USA) and the pherograms were obtained using commercial software (ImageLab, BioRad, Hercules, California, USA).

The qualitative evaluation of protein profiles was performed by visual inspection of pherograms and by the calculation of the total number of bands in each sample.

The quantification of the protein bands was performed by the ImageLab software (ImageLab, BioRad, Hercules, California, USA), which determines the volume of each protein band through the analysis of the pixel values in the digital

[9]https://www.beckmancoulter.com/wsrportal/techdocs?docname=/cis/BAOSR6x78A/%25%25/EN_CREATININE.pdf

image. The volume is calculated as the sum of all the pixel intensities within the band boundaries. Each band's volume was subsequently compared to the entire volume of the lane and the corresponding value calculated as percentage. The micrograms of each band were calculated using the following formula:

$$X\ \mu g = \frac{\%\ \text{band} \times \text{sample}\ \mu g\ \text{loaded}}{100}$$

5. STATISTICAL ANALYSIS

Statistical analysis was performed using commercially available software [MedCalc Statistical Software v.19.0.7 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020), 2019; RStudio-1.2.1335 Statistical and R, R version 3.4.3].

Before performing the comparison between the data obtained from the two sampling methods, the normal distribution was tested and $p > 0.05$ was considered indicative of normal distribution. The Wilcoxon test for paired samples was performed comparing the free-catch urine samples and the urine collected from the ground, this way evaluating the impact of the sampling method on urinalysis in cattle (dipstick, USG, uTP, uCrea and UPC).

Likewise, on SDS-PAGE results, the total number of bands of urine samples collected by free catch and of those collected from the ground were compared using the Wilcoxon test for paired samples. Moreover, to evaluate the quantitative differences of the protein profiles between the two collecting methods (free-catch and from the ground), a linear model was performed. The band values were expressed as protein percentage or μg. A $p \leq 0.05$ was considered indicative of differences between the two sampling methods.

RESULTS

1. COWS

Bovine urines were collected both from the ground and in sterile cups and data were compared for evaluating the interference of the ground on urinalysis' reliability. The colour of urine was light to medium yellow and there was no distinction between free-catch urine and urine collected from the ground considering this variable. Nevertheless, the transparency was affected by soil contamination, indeed the 10 free-catch urine samples were clear, whilst the same urine samples collected from the ground were slightly cloudy (9 samples) or cloudy (1 sample). The complete data are reported in Table 5.

No significant differences were detected for urinalysis, USG, uTP, uCrea and UPC values comparing the two sampling methods (Table 5). Seven samples resulted positive for dipstick proteins.

Regarding the microscopic sediment analysis, urine collected from the ground presented more soil contaminants (pollen, vegetable fibres, mold spore and bacteria) compared to urine collected in urine cups. Moreover, one epithelial cell and one granular cast were detected in a free-catch urine sample and in a sample of urine from the ground, respectively. However, the presence of the ground did not interfere with the identification of the biological elements (cells, crystals and casts), which were clearly distinguishable from the contaminants.

Statistical analysis of the sampling methods for uTP ($p = 1.0$), uCrea ($p = 0.4$) and UPC ($p = 0.9$) did not reveal significant differences. Additionally, when comparing the samples from the ground and the free-catch urines, the SDS-PAGE did not highlight any significant differences in the number of bands ($p= 0.46$). The most represented bands had a molecular mass (MM) of 97, 86, 70-69, 59, 38, 27, 21-22 and lower than 13 kDa (Figure 1).

To evaluate the influence of the sampling methods on the SDS-PAGE protein quantification (percentage and μg), a linear model was used, which showed non-significant values: the R^2 was 0.0003898 and p was 0.8195 for protein percentage and R^2 was 0.0003492 and p was 0.829 for protein μg. These results indicate that the sampling method did not interfere on the SDS-PAGE results.

Table 5 Dipstick, urine specific gravity, sediment analysis, urine total protein (uTP), urine creatinine (uCrea), and urine protein:creatinine ratio (UPC) in samples from 10 cattle, collected with a syringe from the ground and during spontaneous voiding. Data are reported as mean ± standard deviation or median and range (minimum-maximum value) depending on normal or not normal distribution, respectively (Fasoli, et al., 2021).

Variable	Free-catch urine	From the ground	*p*
USG	1.028 ± 0.006	1.028 ± 0.006	1.0
Dipstick			
pH	9 (8 - 9)	9 (8 - 9)	1.0
Leu (10^9/L)	0	0	-
Nit	0	0	-
Pro (g/L)	0.3 (0.0 - 1.0)	0.2 (0.0 – 0.3)	0.6
Glu (mmol/L)	0	0	-
Ket (mmol /L)	0	0	-
Bil (μmol/L)	0	0	-
Ery (cell/μL)	0	0	-
Uro (μmol/L)	0	0	-
Urine Chemistry			
uTP (g/L)	0.19 ± 0.05	0.19 ± 0.05	1.0
uCrea (g/L)	0.94 ± 0.27	0.94 ± 0.27	0.4
UPC	0.2 ± 0.02	0.2 ± 0.03	0.9
Sediment analysis			
Crystals (elements/50μL)	0 (0 – 10)	0 (0 - 4)	
SC (cell/50μL)	0	> 50	-
ESC (cell/50μL)	0 (0 – 1)	0	-
RBC (cell/50μL)	0	0	-
WBC (cell/50μL)	0	0	-
GC (cell/50μL)	0	0 (0 - 1)	-

Bil = urine bilirubin; Ery = urine erythrocytes; ESC = epithelial squamous cells; GC = granular casts; Glu = urine glucose; Ket = urine ketones; Leu = urine leukocytes; Nit = urine nitrate; Pro = urine protein; RBC = red blood cells; SC = soil contaminants; uCrea = urine creatinine; UPC = urine protein to urine creatinine ratio; Uro = urine urobilinogen; uTP = urine total proteins; USG = urine specific gravity; WBC = white blood cells.

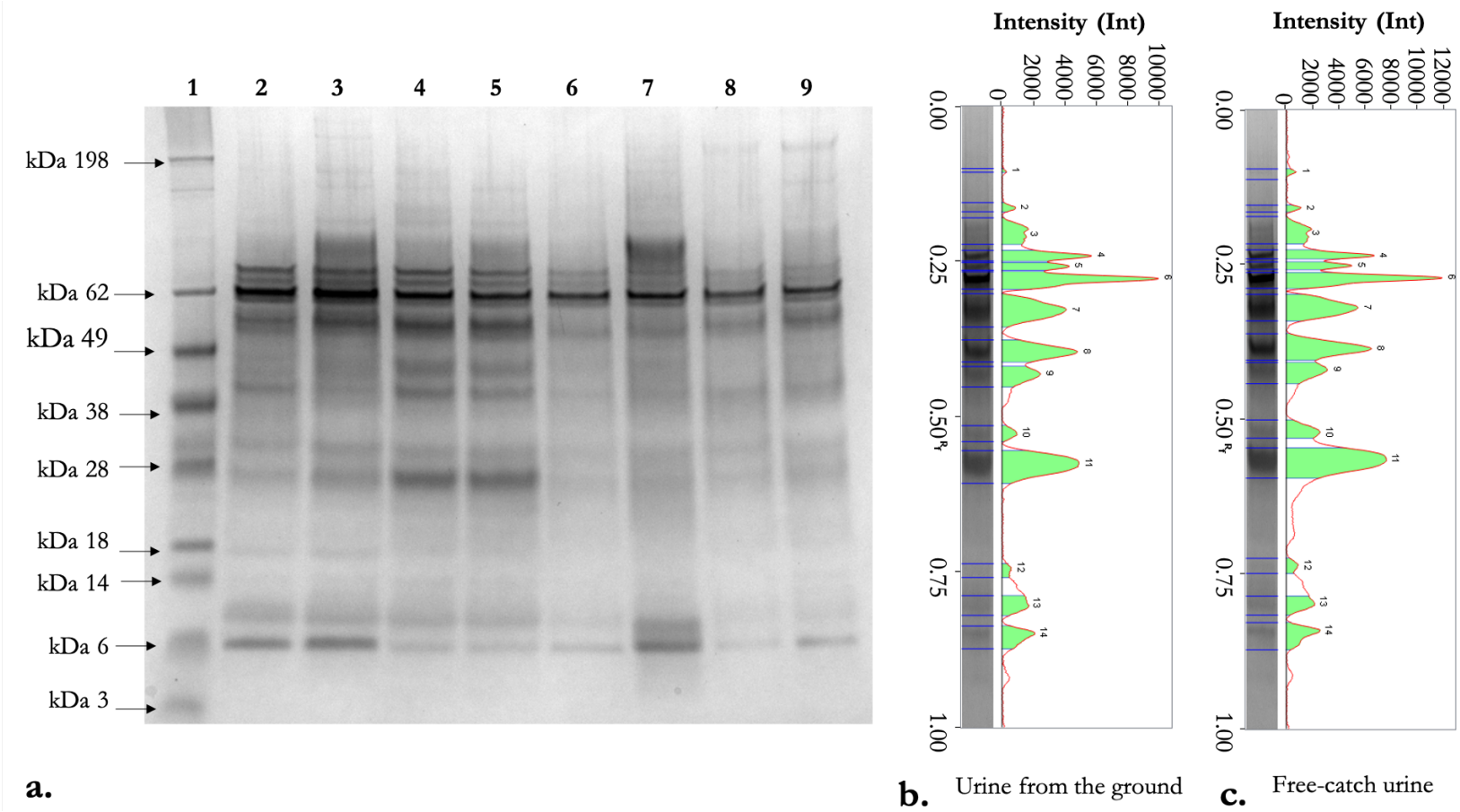


Figure 1 Representative SDS-PAGE gel and pherograms of urine samples collected from cattle using different methods. **a.** Lane 1 = molecular mass marker; lanes 2, 4, 6 and 8 = urine samples collected from the ground with a syringe; lanes 3, 5, 7, and 9 = the same urine samples collected by free-catch. **b.** Pherogram of the urine sample collected from the ground (lane 4). **c.** Pherogram of the same urine sample collected in sterile cup (lane 5) (Fasoli et al, 2021 – modified).

2. GIRAFFE

Despite the difficulties of collecting urine in sterile cups in this species, one sample was collected both at the start of the spontaneous voiding in a sterile urine cup and at the end of the voiding from the ground with a syringe. Complete data are reported in Table 6.

Table 6 Urine specific gravity (USG), dipstick results, sediment analysis, urine total protein (uTP), urine creatinine (uCrea), and urine protein:creatinine ratio (UPC) of the urine collected from giraffe ID 41 with a syringe from the ground and during spontaneous voiding.

Variable	Free-catch urine	From the ground
USG	1.034	1.034
Dipstick		
pH	9	9
Leu (10^9/L)	0	0
Nit	0	Trace
Pro (g/L)	0.30	0.30
Glu (mmol/L)	0	0
Ket (mmol /L)	0	0
Bil (μmol/L)	0	0
Ery (cell/μL)	0	0
Uro (μmol/L)	0	0
Urine Chemistry		
uTP (g/L)	0.16	0.13
uCrea (g/L)	1.9	1.8
UPC	0.09	0.07
Sediment analysis		
Crystals (elements/50μL)	0	0
SC (cell/50μL)	0	> 50
ESC (cell/50μL)	1	0
RBC (cell/50μL)	0	0
WBC (cell/50μL)	0	0
GC (cell/50μL)	0	0

Bil = urine bilirubin; Ery = urine erythrocytes; ESC = epithelial squamous cells; GC = granular casts; Glu = urine glucose; Ket = urine ketones; Leu = urine leukocytes; Nit = urine nitrate; Pro = urine protein; RBC = red blood cells; SC = soil contaminants; uCrea = urine creatinine; UPC = urine protein to urine creatinine ratio; Uro = urine urobilinogen; uTP = urine total proteins; USG = urine specific gravity; WBC = white blood cells.

The pherograms originated from these two aliquots from the same sample did not reveal any qualitative differences and, albeit quantitative differences are evident, the number and the molecular mass (MM) of protein bands were the same (Figure 2).

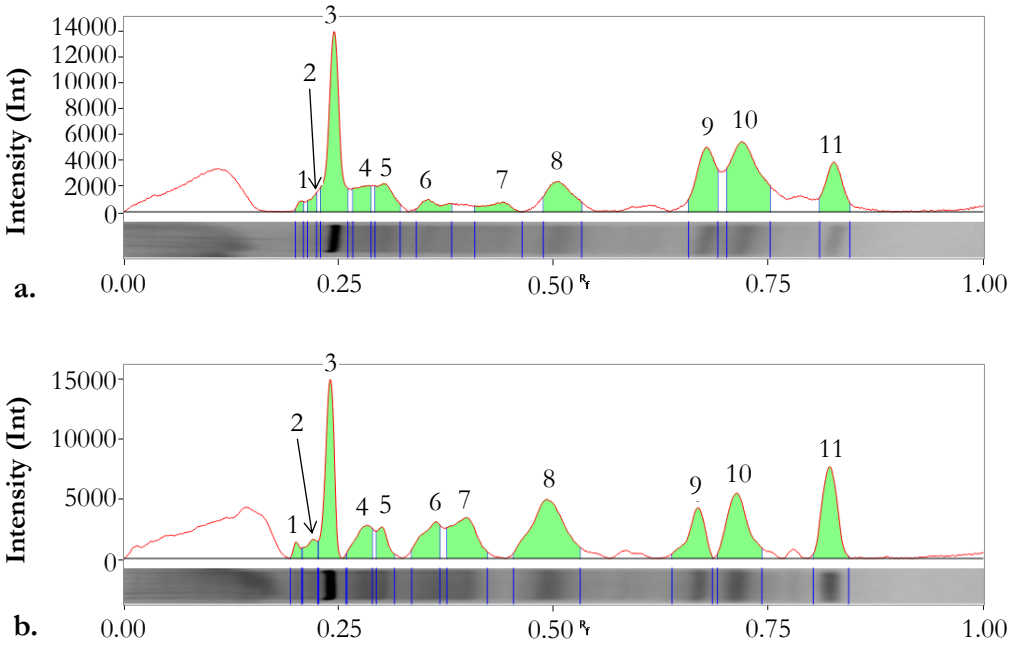


Figure 2 Representative pherograms of urine sample collected from giraffe ID 41 by different methods. **a.** Pherogram of the urine sample collected from the ground. **b.** Pherogram of the free-catch urine sample collecting in the urine sterile cup. The number on the peak indicates the band numbers.

DISCUSSION

The results obtained from bovine free-catch urines and urines sampled from the ground did not present statistical differences, suggesting that this technique could be considered reliable. Ideally, every laboratory test should be validated for the target species (Rideout, et al., 2018) and, even though this experiment cannot be considered as a validation, the protocol adopted has shown that this technique can be safely applied in cattle and can provide useful information about their health status using a non-invasive approach. Besides, the data obtained from the cows by using this method were comparable to data previously reported in cattle (Isani, et al., 2018; Hermann, et al., 2019; Ferlizza, et al., 2020b), confirming the reliability of this approach as non-invasive method for monitoring urinary and renal functionality.

The qualitative evaluation of proteome in urine collected from the ground highlighted that the electrophoretic profiles of the free-catch urines and those from the ground were not significantly different, and the bands were clearly visible and distinguishable. The linear model applied in quantitative evaluation of protein bands of bovine urines proved that the sampling method did not affect the result values which were reported either in percentage or in micrograms, giving a further confirmation of the reliability of the data obtained from the samples collected from the ground.

As far as giraffe urine is concerned, the results agreed with those obtained for bovine urine. However, it was only one sample and it was not possible to perform a statistical analysis.

The collection of urines from the ground/floor has been previously reported in two non-domestic species: the okapi and the giant panda (Glatston & Smith, 1980; Burrell, et al., 2017). Even in these studies, the authors reported that collecting urines via this method could trigger artifacts or undesired findings in urinalysis results (Glatston & Smith, 1980; Burrell, et al., 2017), therefore these occurrences should be carefully taken into consideration when interpreting the data. This is in accordance with the data obtained in the Experiment 1 of this thesis, since the microscopic examination revealed a moderate-to-elevated presence of bacteria in urine sampled from the ground due to environmental contamination, highlighting that this method is not without limitations. Therefore, since it is not possible to discriminate if the

bacteria originate from a true infection or derive from the soil, this sampling method is not suitable to diagnose urinary tract infection.

CONCLUSIONS

One of the most interesting aspects of this experiment was the use of non-invasive collecting method. The urine sampling from the ground can be considered as a useful and reliable tool; considering the inherent limitations the sampling of voided urines in sterile cups in giraffes shows, the possibility of collecting data about their health status by this technique is remarkable. In fact, it could help to manage the animal hosts in zoos as well as possible. Therefore, given this scenario, this sampling method has also been used in the Experiment 2.

EXPERIMENT 2

URINARY REFERENCE VALUES AND URINARY PROTEOME OF GIRAFFES[10]

[10] Part of the following data has been published in the paper: Fasoli S, Andreani G, Dondi F, Ferlizza E, Bellei E, Isani G. Urinary Reference Values and First Insight into the Urinary Proteome of Captive Giraffes. Animals (Basel). 2020 Sep 19;10(9):1696. doi: 10.3390/ani10091696.

INTRODUCTION

Nowadays, urinary reference values in giraffes have not been yet defined, albeit some studies which mainly focused on investigating the occurrence of uroliths were carried out (Wolfe, et al., 2000; Sullivan, et al., 2010; Sullivan, et al., 2013).

Reference interval, defined as "an interval contains all the possible values between and including an upper and lower limit" (Friedrich, et al., 2012), is needed to interpret the test results or to establish the health status of an individual, but for wild and zoo animals it might not always be possible to follow the recommended guidelines, mainly due to the difficulty to reach the high number of individuals required to define the reference intervals (Rideout, et al., 2018). In fact, the establishment of reference intervals may be not as straightforward as expected when wild or zoo animals are involved. Nevertheless, appropriate statistical methods and rigorous protocols could come to the aid of researchers or personnel working with wild animals, this way allowing to determine the reference intervals even in these species (Friedrich, et al., 2012). In fact, the knowledge of physiological and clinical parameters (in turn reflecting the different environments these animals come from and their different physiological needs) is a particularly important factor to take into consideration when trying to get an accurate picture of an animal's health in order to improve their husbandry and welfare (Hosey, et al., 2013a).

As previously stated, urinalysis is widely recognized as a useful tool in the routine health evaluation in Veterinary Medicine (Callens & Bartges, 2015; Piech & Wycislo, 2019), and different sampling techniques are even used in the wild species (see page 26).

Urine - which contains proteins derived from the ultrafiltration of plasma and from the urinary tract – could provide not only the entire set of proteins present in this biofluid, but also useful biological markers for kidney diseases and for other organ diseases (González-Buitrago, et al., 2007; Decramer, et al., 2008). Besides this information, the urinary proteome, and in generally the proteomes, can lead to the discovery of biological markers of disease presence, since the protein patterns of healthy and pathological individuals can be used as a comparison (González-Buitrago, et al., 2007). For instance, when a pathological condition affects the kidney, a change in the urinary proteome occurs: proteins with high and intermediate MM are abundant

when the glomeruli are involved, while high levels of low MM proteins are detected when tubules are affected (Hokamp, et al., 2018).

In this regard, some of the proteins identified in urines have been used as diagnostic tools to early diagnose renal diseases both in animals and humans. For instance, a decrease in uromodulin has been suggested as an index of tubular disfunction in dogs (De Loor, et al., 2013; Ferlizza, et al., 2020a) and cats (Ferlizza, et al., 2015); an increased in zinc-alpha-2-glycoprotein might be a potential biomarker for diabetic nephropathy in humans (Wang, et al., 2016), and albumin and clusterin have been used as biomarkers of Acute Kidney Injury (AKI) in humans (Vaidya, et al., 2008).

However, no biomarker could be evaluated without defining the physiological proteome of an organ or a biofluid; therefore, it is essential to investigate the latter in order to further define a specific biomarker of disease. Since the impact of biological sex on proteome has been studied through the years (Gianazza, et al., 2018), it is essential, especially for the female specimens, to detect the influence of all physiological changes (i.e., pregnancy, oestrus or lactation).

Given the paucity of data on urinalysis in giraffes, the aims of this study were to define the urinary reference values and to have a first insight into the urinary proteome of captive giraffes (*Giraffa camelopardalis*).

MATERIAL AND METHODS

1. ANIMAL STUDIED

One hundred and three urine samples were collected from 44 giraffes (*Giraffa camelopardalis*) hosted in four Italian zoos: Falconara Marittima (Parco Zoo Falconara, Falconara Marittima, AN, Italy), Fasano (ZooSafari Fasanolandia, Fasano, BR, Italy), Pistoia (Giardino Zoologico di Pistoia, PI, Italy) and Ravenna (Safari Ravenna, RA, Italy). These zoos are reported as Zoo A, B, C and D, respectively. One urine sample from each of 3 giraffes was collected in sterile urine cups. The studied group included giraffes with different age and sex (Table 7). The urine samples were collected from April 2018 to November 2019.

The animals hosted in the Zoo A were three giraffe males (one *Giraffa camelopardalis rothschildi*, one *Giraffa Camelopardalis reticulata* and one hybrid), ranging from 5 to 13 years of age. Their daily diet included hay, apple, carrots, bananas and mixed feeding. Fresh leaves were added third a week.

The Zoo B hosted 28 giraffes, 14 females and 14 males, ranging from 3 months of age to 21 years of age. The daily giraffe's diet included 7 kg of alfalfa, acacia brunches and 1.5 kg of corn and fava beans.

In Zoo C two females were hosted: mother and daughter, 20 and 7 years old, both *Giraffa camelopardalis rothschildi.* The diet of giraffes included alfalfa, 350 gr of apple, 500 kg of carrots, 2 kg of bran, 350 gr of bananas, cover liver oil and branches in summer.

The Zoo D hosted thirteen giraffes, 5 males and 8 females, ranging from 8 months of age to 20 years of age. Their diet included alfalfa, fruits and vegetable, and fresh grass and branches, when available.

Table 7 Studied group. Animal ID, sex, age (reported in separated columns considering the year of sampling) and zoo were indicated for each specimen.

Animal ID	Sex	Age		Zoo
		2018	2019	
1	F	3-y-o	4-y-o	B
2	M	1-y-o	2-y-o	B
3	M	7-y-o	-	B
4	M	6 m-o	-	B
5	M	5-y-o	-	A
6	F	-	3-y-o	B
7	M	1-y-o	2-y-o	D
8	F	-	13-y-o	B
9	F	-	20-y-o	C
10	M	3-y-o	-	B
11	M	3-m-o	-	B
12	F	3-y-o	4-y-o	B
13	M	10-y-o	11-y-o	D
14	F	-	4-y-o	D
15	F	-	N/A	D
16	F	-	N/A	D
17	F	-	N/A	D
18	F	-	N/A	D
19	F	-	7-y-o	C
20	F	17-y-o	18-y-o*	B
21	F	7-y-o*	-	B
22	M	5-y-o	-	D
23	M	16-y-o	-	B
24	F	20-y-o	21-y-o	B
25	M	3-y-o	-	D
26	F	5-y-o*	-	B
27	F	8-y-o	9-y-o	B
28	F	15-y-o	16-y-o	B
29	M	9-y-o	-	B
30	F	9-y-o*	-	B
31	F	-	11-y-o	D
32	M		1-y-7-m-o	B
33	F	-	N/A	D
34	F	-	8-m-o	D
35	M	1-y-o	2-y-o	B
36	F	14-y-o	15-y-o	B
37	F	13-y-o*	14-y-o	B
38	M	-	3-y-o	B
39	M	-	8-m-o	D
40	M	3-y-o	4-y-o	B
41	M	13-y-o	14-y-o	B
42	F	-	20-y-o	D
43	F	-	4-y-o	B
44	F	-	2-y-o	D

(*) urine samples excluded from the statistical analysis (pregnancy or post-partum);
A=Falconara; B= Fasano; C= Pistoia; D= Ravenna; N/A = not available.

2. SELECTION OF REFERENCE INDIVIDUALS

The reference individuals were selected following the criteria proposed by Friedrich et al. (2012) and reported in Table 8.

During this study, it was also possible to collect urine samples from four pregnant females. This diagnosis was done keeping in mind the birth of new calves or, knowing the exact date of mate, evaluating the changes in females (e.g., round abdomen). Complete data about the pregnant giraffes are reported in separated section at page 91.

Health status of giraffes was established considering the clinical history of animals and their physical examination. These criteria were applied both *a priori* and *a posteriori* and the giraffes affected by diseases that were diagnosed after urine sampling were ruled out statistical analysis. Regarding the age, giraffes were considered juvenile (< 12-m-o), subadult (12 m-o < 4 y-o), adult (4-9 y-o) and mature (> 9 y-o) in accordance to Muller (2018).

Table 8 Criteria for selection and exclusion of reference individuals, following the American Society for Veterinary Clinical Pathology reference interval guideline (Friedrich, et al., 2012).

Classification	Category	Subcategory
Selection criteria		
Biological	*Age*	Juvenile (< 12-m-o), subadult (12 m-o < 4 y-o), adult (4-9 y-o) and mature (> 9 y-o)
	Sex	Female, male
Clinical	*Health*	Physical examination
	History	Illness in the 4 weeks before and after the urine sampling
Exclusion criteria		
Physiological	-	Illness, pregnancy and post-partum period

3. PRE-ANALYTICAL PROCEDURES

In order to standardize the protocol and to decrease the possible bias in the results of the analysis, the pre-analytical procedures adopted in this study are reported in Table 9, following the guidelines proposed by Friedrich et al. (2012).

Table 9 Pre-analytical procedures and method used in giraffes' urine collection.

Pre-analytical procedures	Method used
Sampling collection	By a syringe without the needle, collecting the upper part of urine from the ground, immediately after spontaneous voiding.
Samples handling	Dipstick tests, urine specific gravity (USG) and microscopic sediment evaluation were performed within 3 hours after the collection.
Analytes stability	Urine samples were conserved in a portable fridge to avoid the deterioration of the biological samples.
Time of collection	In autumn and summer season from April 2018 to November 2019.
Patient preparation or handling	Not necessary, due to the non-invasive approach of the urine collection method.

4. ANALYTICAL PROCEDURES

About 5 mL of the giraffes' urines were collected with a syringe from the ground or cement, in the outdoor area of all zoos.

To limit soil contamination, only the upper part of the urine was collected, immediately after the spontaneous voiding. Urine samples were subjected to the following protocol: physical and chemical evaluation by visual inspection, urine sediment microscopic evaluation (100x and 400x), urine total protein (uTP), urine creatinine quantification (uCrea) and urine protein:creatinine ratio (UPC). On the supernatant were performed 1D-SDS-PAGE electrophoresis and the protein identification by mass spectrometry.

4.1 URINALYSIS

These analyses were performed within 3 hours from urine collection. Physical evaluation was carried out by visual inspection. Colour and turbidity of the urine were evaluated following the guideline reported in dogs and cats (Callens & Bartges, 2015; Piech & Wycislo, 2019).

The chemical evaluation was performed by visual inspection and with semi-quantitative dipstick test (KRUUSE VET-10 Urine Strips, JØRGEN KRUUSE A/S INTERNATIONAL, Denmark). The test consisted of a strip with nets for the semi-quantification of urobilinogen, glucose, bilirubin, ketones, specific gravity, blood, pH, protein, pH, nitrate and leukocytes. The colour of the strip changed when the sample

touched the net, depending on the urine composition. The chemical evaluation was performed by putting one drop of urine on each net and comparing the resulting colour change of strip with the coloured scale shown on the extern tag of the container after 60 seconds (Table 10).

Urine specific gravity was analysed by refractometer (Giorgio Bormac, 41012 Modena, Italy). Urines were centrifuged at 1500 *g* for 10 minutes and their sediment evaluation was performed both under high (400x) and lower field (100x) by a microscope (Ferlizza, et al., 2015). For each sample, two drops of urine (~ 50 µL/drop), both unstained and stained with fuchsine solution (Samson Reagenz, Dr. Grogg Chemie AG, Stettlen-Deisswil, CH), were placed on microscope slides of 26 x 76 mm (BioSigma, VBS653 Microscope slide, Italy; APTACA Ref.13502, Microscope slides, Italy) and covered by 20 mm^2 coverslips (PRESTIGE, Micro Cover Glass). The sediment analysis was performed by identifying and counting the biological elements (i.e., cells, casts, crystals and debris) in the samples according to the guidelines reported in Table 4. The obtained urines' supernatants were stocked in different Eppendorf tubes at - 20° C and underwent the subsequent analysis within one month.

Table 10 Scale of KRUUSE VET-10 Urine Strips. h= hemolysis; nh= Non hemolysis.

	Negative	**±**	**+**	**++**	**+++**	**++++**
Urobilinogen [mg/dL(µmol/L)]	0.1-1(16)	-	2(33)	4(66)	8(131)	-
Glucose [mg/dL(mmol/L)]	Negative	100(5.5)	250(14)	500(28)	1000(55)	-
Ketones [mg/dL(mmol/L)]	Negative	5 (0.5)	15(1.5)	40(3.9)	100(10)	-
Blood (RBC/µL)	Negative		10^{h}	50^{h}	250^{h}	-
	Negative		10^{nh}	50^{nh}		-
pH	5-9					
Protein [mg/dL (g/L)]	Negative	Trace	30(0.3)	100(1.0)	300(3.0)	1000(10)
Leukocytes (WBC/µL)	Negative	-	25	75	500	-

4.2 URINE TOTAL PROTEIN AND CREATININE DETERMINATION

The analysis of the samples has been performed according to details reported in Paragraphs 3.1 and 3.2 of Experiment 1.

4.3 1D-SDS-PAGE ELECTROPHORESIS

After the thawing and the centrifugation at 3000 *g* for 10 minutes, urine supernatants were processed according to the protocols reported in Paragraphs 4 of the Experiment 1. Qualitative evaluation of protein profile was performed by visual inspection of pherograms and by the calculation of the total number of bands in each sample.

4.4 PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

One urine sample from each of 3 giraffes was concentrated with spin columns having a molecular mass cut-off of 3 kDa (Vivaspin 500, Sartorius, Goettingen, Germany), following the manufacturer's instructions, namely, the columns were filled with 500 μL of urine and centrifuged at 15000 *g* for 50 minutes at 10°C, reaching a final volume of 50 μL. After this process, 15.5 μg of each sample underwent SDS-PAGE on precast 12% polyacrylamide gel in reducing conditions with MES buffer (2-[N-morpholino-ethanesulfonic acid]) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing SDS and the gel was stained using Coomassie stain (Quick Coomassie Stain, Protein Ark, Sheffield, UK).

The bands were manually excised from the gel for subsequent identification using mass spectrometry. Protein identification was carried out following the data previously reported (Isani, et al., 2016; Bellei, et al., 2020). In essence, the bands underwent in-gel tryptic digestion; the digested dried samples were then re-suspended in 97% Water/3% ACN to which 1% formic acid was added, and were analysed using an UHPLC-ESI-QExactiveTM (Thermo Fisher Scientific, Reinach, Switzerland), composed of an UltiMate 3000 UHPLC System together with an ESI-QExactive Hybrid Quadrupole-OrbitrapTM mass spectrometer (LC-MS/MS-QO System).

Since the giraffe protein database is not annotated, a broader taxonomy, namely "all mammals", was selected in order for the identification to be based on

sequence homology. Protein-identification peak lists were generated using the Mascot search engine (http://mascot.cigs.unimo.it/mascot) against the UniProt database (UniProt.org), specifying the following parameters: mammalian taxonomy, trypsin enzyme, 1 max missed trypsin cleavage and Carbamidomethylation (C) as Fixed modifications, and Deamidated (NQ) and Oxidation (M) as Variable modifications, Monoisotopic Mass values, Unrestricted Protein mass, ± 10 ppm of Peptide mass tolerance and ± 0.02 Da of Fragment mass tolerance. Proteins with a score >80 or identified by at least two significant sequences were selected. The significant threshold in Mascot searches was set to obtain a false discovery rate <5% (5% probability of false matches for each protein with a score above 80). The biological processes, molecular functions and cellular components of the proteins identified were reported, according to Gene Ontology (GO) and UniProt.

5. STATISTICAL ANALYSIS

Statistical analysis was carried out using MedCalc Software version 19.3.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). Graphics were done using Tableau Software (© 2003-2020 TABLEAU SOFTWARE, LLC, A SALESFORCE COMPANY, Seattle, WA, USA) or MedCalc Software version 19.3.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020).

Selection of the reference individuals was done following the American Society of Veterinary Clinical Pathology (ASVCP) reference interval guideline (Friedrich, et al., 2012). As far as the biological criteria is concerned, animals were categorized according to their age group and sex. As far as the age is concerned, giraffes were considered juvenile (< 12-m-o), subadult (12 m-o < 4 y-o), adult (4-9 y-o) and mature (> 9 y-o) according to data reported in literature (Muller, 2018).

Out of 44 reference animals, 3 giraffes (pregnancy N=2; post-partum period N=1) were omitted from the statistical analysis, whereas only the urine samples collected during pregnancy of another 2 females were ruled out of the statistical analysis (Table 7). The mean values of the repeated measures (n=92) from the same reference giraffes (up to N=41) were calculated before carrying out the statistical analysis and the reference interval determination (Petrie & Watson, 2013). D'Agostino-Pearson test was completed for testing the normal distribution of the data previously displayed graphically using the frequency histograms. A p>0.05 was considered

indicative for normal distribution. The outliers were detected using the Tukey's test and the data not normally distributed were appropriately transformed when needed.

The uTP, uCrea, UPC and USG reference intervals were calculated using the Box-Cox transformation with robust methods (CLSI C28-A3) (Geffré, et al., 2011; Friedrich, et al., 2012). The 90% confidence intervals (CIs) of the lower limit (LL) and the upper limit (UL) were calculated using the Bootstrap method (10,000 iterations; random number seed: 978); the uCrea and the UPC were back-transformed after the Box-Cox transformation.

Considering sex and age as a source of differences, non-parametric tests were used, namely Mann-Whitney test and Kruskal-Willis test (Olea-Popelka & Rosen, 2019). A $p<0.05$ was set up as cut off. Regarding the 1D-SDS-PAGE electrophoresis results, the median and range (min-max) values of band numbers was calculated and sex and age groups were considered sources of differentiation.

Molecular mass protein bands interval recorded (3-166 kDa) was divided in 9 classes (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) by the software (MedCalc) and the relative frequency of each class was calculated for each subgroup. The relative frequency of each class was considered as its absolute frequency divided by the total number of class and expressed as percentage. As far as the sex is concerned, the relative frequencies of males and females were reported, as well as for the juvenile, subadult, adult and mature giraffes.

Regarding pregnancy, all variables were compared between pregnant and sexual mature females, which were considered as the female older than 5 years old.

RESULTS

1. ANIMAL STUDIED

The composition of the studied group is reported in Table 11 and Figure 3. Regarding the age, giraffes were considered juvenile (< 12-m-o), subadult (12 m-o < 4 y-o), adult (4-9 y-o) and mature (> 9 y-o) (Muller, 2018).

Table 11 Composition of the studied group. The number of the specimens that composed each subgroup is reported for male and female giraffes.

Class	Female	Male	Total (%)
Adult	9	4	13 (29.55)
Juvenile	1	4	5 (11.36)
Mature	8	4	12 (27.28)
N/A	5	0	5 (11.36)
Subadult	3	6	9 (20.45)
Total (%)	26 (59.09)	18 (40.91)	44 (100.00)

N/A = age not available.

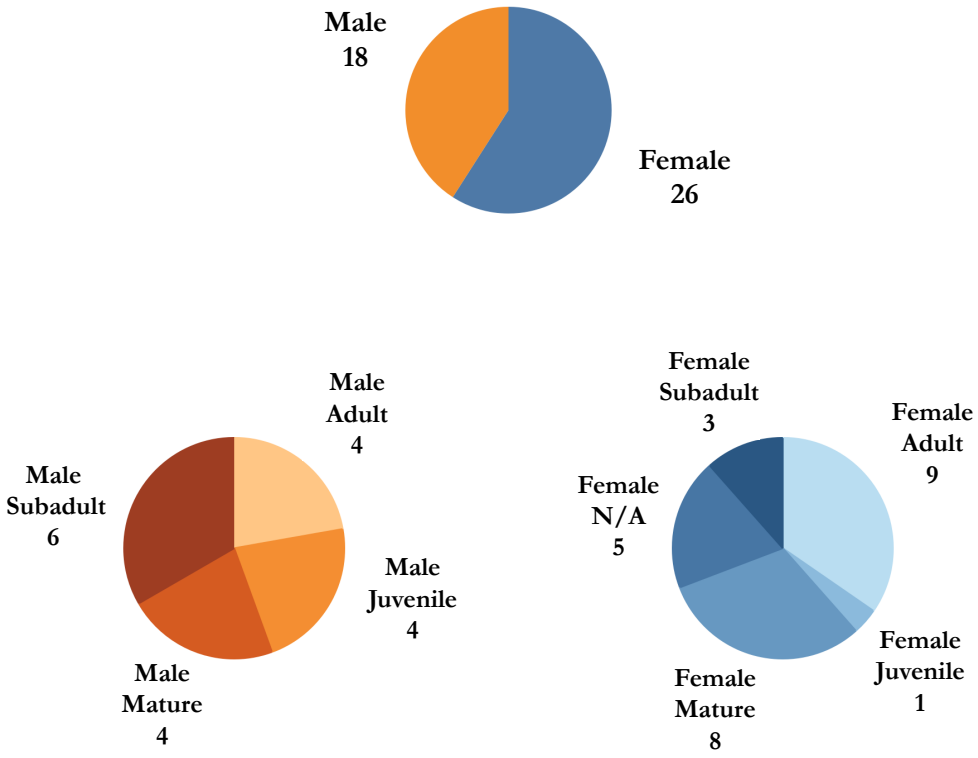


Figure 3 Graphical representation of the studied group (N = 44).

2. SELECTION OF REFERENCE INDIVIDUALS

According to the criteria used in this study for the selection of reference individuals (Friedrich, et al., 2012), three animals were ruled out the statistical analysis due to pregnancy (N=2) and postpartum period (N=1). Another two females (ID: 20; 37) were sampled both when pregnant (data excluded from the reference ranges) and when not pregnant (data included in the reference ranges). Before proceeding with the statistical analysis for establishing the reference intervals, normal distribution of each variables was analysed and the outliers were identified. Reference intervals were established for the USG, uTP, uCrea and UPC. Conversely, considering the sex and age, the too-small number of samples (< 20) of these subgroups did not permit to establish the reference intervals; therefore, only summary statistics were reported (see Table 15, Table 16, Table 17 and Table 18).

Data of urine specific gravity (USG) were normally distributed and *p value* was 0.3192. Tukey's test did not reveal outlier values. Considering the urine total protein (uTP), D'Agostino-Pearson test result was *p value* = 0.1595 and one outlier (ID 11) was identified. As far as urine creatinine (uCrea) and UPC is concerned, D'Agostino-Pearson test *p values* were 0.0040 and 0.0064 respectively, rejecting the normality. Consequently, it was necessary to apply logarithmic transformation in order to perform the Tukey's test, which is a test that can only be applied to normally distributed data (Friedrich, et al., 2012). No outliers were detected after this test for uCrea; conversely, one outlier (ID 36) was identified for UPC. Complete data are reported in Table 12.

Table 12 Tukey's test results and D'Agostino-Pearson test *p value* are reported for each variable. N indicates the number of specimens included in these analyses.

Variable	Unit	N	D'Agostino-Pearson test (*p*)	Outside values	Animal ID	Total outliers
USG	-	34	0.3192	None	-	0
uTP	mg/dL	40	0.1589	42.95	11	1
uCrea	mg/dL	41	0.2964[a]	None	-	0
UPC	-	40	0.5633[a]	0.19	36	1

a= Back-transformed after logarithmic transformation.

After the identification of outliers, it was decided to use a non-parametric method for determining the reference intervals since it was less affected by their presence (Friedrich, et al., 2012). Moreover, it was decided to use the robust method for the reference intervals calculation, which do not assume the normal distribution of

data considering the size of the samples, the studied species and the characteristics of the data (Friedrich, et al., 2012).

3. URINALYSIS

3.1. PHYSICAL-CHEMICAL URINE EVALUATION

All urine samples had a colour from light to medium yellow, while the turbidity was slightly cloudy to cloudy. The three samples collected in sterile urine cups appeared clear and even the colour ranged from light yellow to yellow.

The pH value, reported as median and range (min-max), was 9 (8-9). The dipstick test showed negative results in all samples analysed for leukocytes (Leu), glucose (Glu), ketones (Ket), urobilinogen (UBG) and bilirubin (Bil). Positive results were recorded in 12 giraffes for proteins (Pro) (30-100 mg/dL), in 5 specimens for erythrocytes (Ery) (10-50 RBC/μL) and in 12 animals for nitrite (Nit) (trace). Data of physical-chemical urine analysis of studied group are reported in Table 13.

Table 13 Urinary parameters and descriptive statistic. N: number of animals included in the statistical analysis (Fasoli, et al., 2020 – modified).

Variable	N	Mean	Median	SD	LL (90% CI range)	UL (90% CI range)
Bil	34	0.0	0.0	0.0	0.0	0.0
Ery (RBC/μL)	34	3	0	9	0	50
Glu (mmol/L)	34	0.0	0.0	0.0	0.0	0.0
Ket (mmol/L)	34	0.0	0.0	0.0	0.0	0.0
Leu (WBC/μL)	34	0.0	0.0	0.0	0.0	0.0
Nit	34	Neg	Neg	-	Neg	Trace
pH	34	9.0	9.0	0.5	8.0	9.0
Pro (mg/dL)	34	30.0	30.0	32.0	0.0	100.0
UBG (μmol/L)	34	0.0	0.0	0.0	0.0	0.0

Bil = urine bilirubin; Ery = urine erythrocytes; Glu = urine glucose; Ket = urine ketones; Leu = urine leukocytes; LL = lower limit; N = number of animals included in the statistical analysis; Neg = negative; Nit = urine nitrate; Pro = urine proteins; SD = standard deviation; UBG = urine urobilinogen; UL = upper limit.

3.2. URINE MICROSCOPIC SEDIMENT EVALUATION

The microscopic urine sediment evaluation showed rare cells (Figure 4) and soil contaminants (pollen, mold spore and fibres), due to the technique used for the urine sampling.

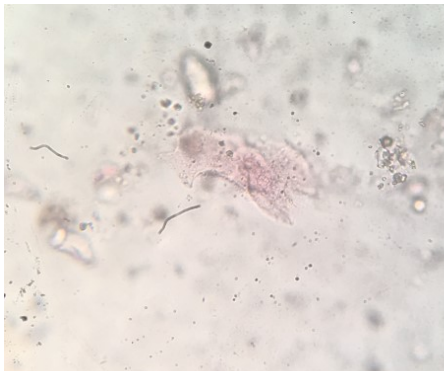


Figure 4 Squamous epithelial cells in giraffes' urine sediment.

4. REFERENCE INTERVALS

After the determination of data distribution and its representation in frequency histogram, urine specific gravity (USG), urine total protein (mg/dL) (uTP), urine creatinine (mg/dL) (uCrea) and urine protein:creatinine ratio (UPC) reference intervals were established using the robust method and considering the 90% confidence interval (Friedrich, et al., 2012). Complete data are reported in Table 14 and Figure 5, Figure 6, Figure 7 and Figure 8.

Due to the presence of the outliers (uTP: 42.95 mg/dL; UPC: 0.19), it was decided to perform a non-parametric method for determining the reference intervals since it was less affected by their presence (Friedrich, et al., 2012).

Table 14 Descriptive statistics and reference intervals of urine creatinine (mg/dL) (uCrea), urine protein:creatinine ratio (UPC), urine specific gravity (USG) and urine total proteins (mg/dL) (uTP). Mean, median, standard deviation (SD), lower limit (LL), upper limit (UL) and their 90% confidence interval (CI) range are reported. N indicates the number of reference animals included in the statistical analysis (Fasoli, et al., 2020 – modified).

Variable	N	Mean	Median	SD	LL (90% CI range)	UL (90% CI range)
uCrea (mg/dL)	41	145.23	154.62	93.56	39.59 (26.31 – 61.56)	357.95 (302.62– 425.80)
UPC	40	0.11	0.11	0.03	0.07 (0.07 – 0.08)	0.16 (0.15 – 0.17)
USG	34	1028	1.030	0.012	1006 (1001 – 1013)	1049 (1044 – 1053)
uTP (mg/dL)	40	15.78	17.58	8.78	4.54 (3.03 – 7.09)	35.31 (30.23 – 40.18)

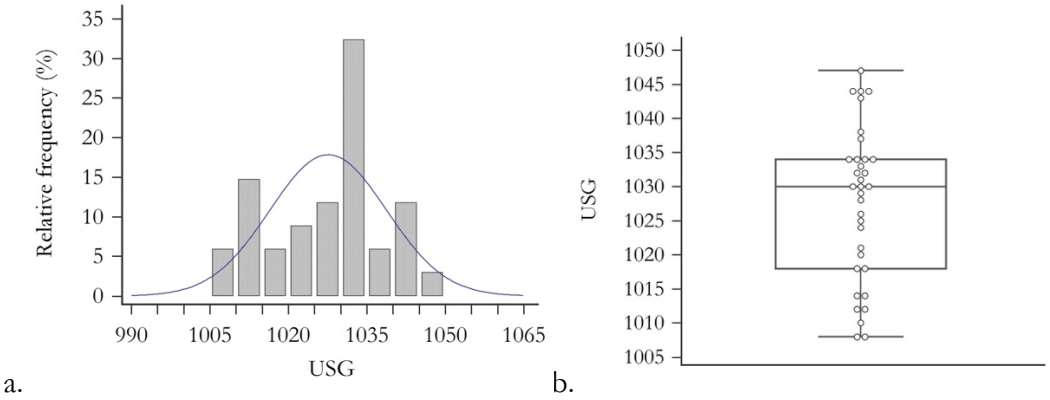


Figure 5 a. Frequency histograms of the urine specific gravity (USG) in giraffes. The blue line indicates the fitted distribution. b. Tukey test's Box-and-Whisker plot of USG (median, 25th and 75th percentiles, 95% CI of mean).

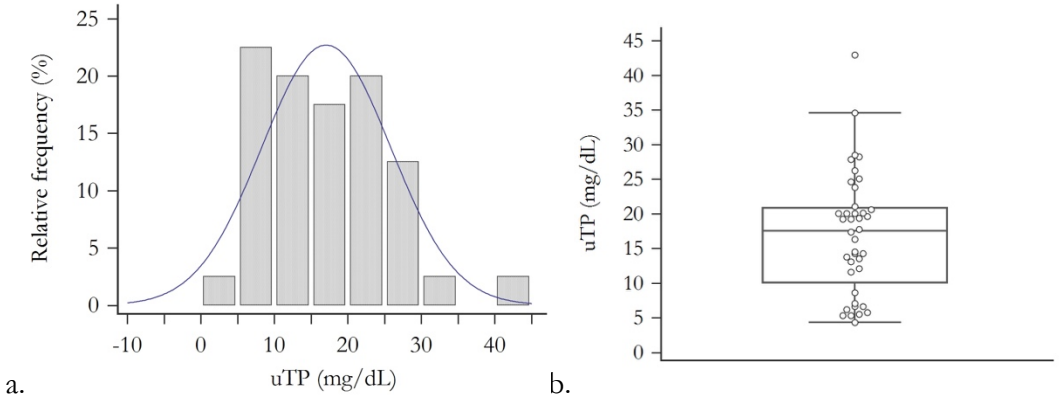


Figure 6 a. Frequency histograms of the urine total proteins (uTP) in giraffes. The blue line indicates the fitted distribution. b. Tukey test's Box-and-Whisker plot of uTP in studied group (median, 25th and 75th percentiles, 95% CI of mean). The circle indicates uTP value of ID 11.

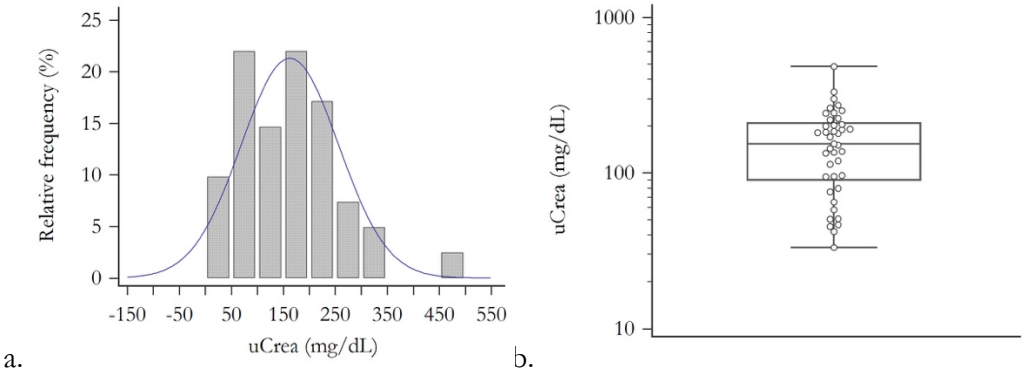


Figure 7 a. Frequency histograms of the uCrea in giraffes. The blue line indicates the fitted distribution. b. Tukey test's Box-and-Whisker plot of uCrea (median, 25th and 75th percentiles, 95% CI of mean) after logarithmic transformation.

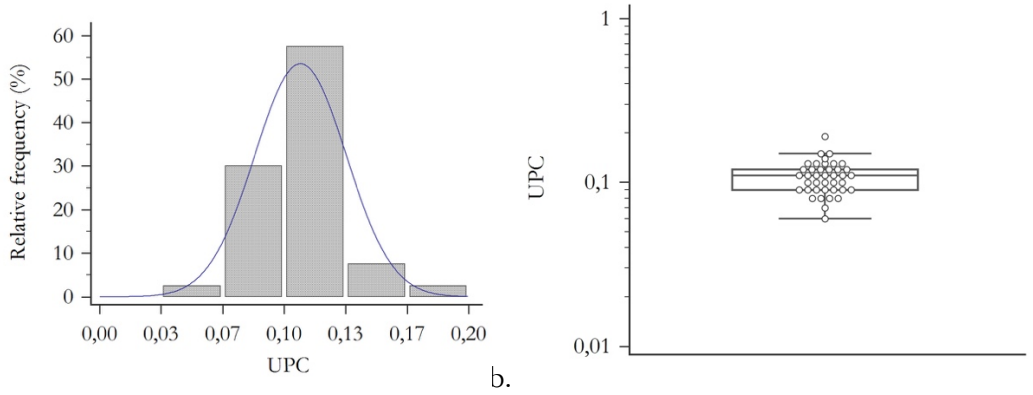


Figure 8 a. Frequency histograms of the UPC in giraffes. The blue line indicates the fitted distribution. b. Tukey test's Box-and-Whisker plot of UPC (median, 25th and 75th percentiles, 95% CI of mean) after logarithmic transformation.

4.1. SEX AND AGE

Considering the sex and the age classes, the limited number of giraffes did not permit to establish the reference intervals; therefore, median, minimum and maximum values for each subgroups are reported for USG (Table 15), uTP (Table 16), uCrea (Table 17) and UPC (Table 18).

Table 15 Median and range (min-max) of urine specific gravity (USG) are reported in male, female, juvenile, subadult, adult and mature giraffes. N indicates the number of reference animals included in the statistical analysis.

Subgroup	N	Median	Min - Max
Female	21	1028.00	1008.00 - 1047.00
Male	13	1033.00	1008.00 - 1044.00
Juvenile	2	1013.00	1012.00 - 1014.00
Subadult	7	1025.00	1010.00 - 1044.00
Adult	7	1030.00	1008.00 - 1043.00
Mature	13	1032.00	1008.00 - 1047.00

Table 16 Median and range (min – max) of uTP (mg/dL) are reported in female, male, juvenile, subadult, adult and mature giraffes. N indicates the number of specimens included in statistical analysis.

Subgroup	N	Median	Min – Max
Female	22	13.66	4.34 – 28.49
Male	18	19.85	5.35 – 42.95
Juvenile	5	17.39	6.18 – 42.95
Subadult	8	20.05	5.35 - 25.06
Adult	10	16.96	4.34 - 34.58
Mature	12	14.39	5.53 – 27.86

Table 17 Median and range (min – max) of uCrea (mg/dL) are reported in female, male, juvenile, subadult, adult and mature giraffes. N indicates the specimens included in statistical analysis.

Subgroup	N	Median	Min – Max
Female	23	136.05	33.29 – 273.23
Male	18	191.52	46.60 – 486.18
Juvenile	5	134.61	65.04 – 486.18
Subadult	9	183.53	46.60 – 273.23
Adult	10	168.41	33.29 – 301.51
Mature	12	127.98	45.47 – 333.10

Table 18 Median and range (min – max) of UPC are reported in female, male, juvenile, subadult, adult and mature giraffes. N indicates the specimens included in statistical analysis.

Subgroup	N	Median	Min – Max
Female	22	0.11	0.07 – 0.19
Male	18	0.11	0.06 – 0.15
Juvenile	5	0.10	0.09 – 0.13
Subadult	8	0.11	0.08 – 0.13
Adult	10	0.11	0.08 – 0.15
Mature	12	0.11	0.06 – 0.19

Additionally, comparisons between the age classes and sex were performed to define possible influences. Due to the sample size, it was decided to apply a non-parametric test (Olea-Popelka & Rosen, 2019), namely Mann-Whitney test and Kruskal-Wallis test. The sex and age did not affect any of the parameters ($p > 0.05$), although the *p values* of uTP (0.0685) and uCrea (0.0551) referring to sex were borderline. Data are reported for USG, uTP, uCrea and UPC in Figure 9, Figure 10, Figure 11 and Figure 12 respectively.

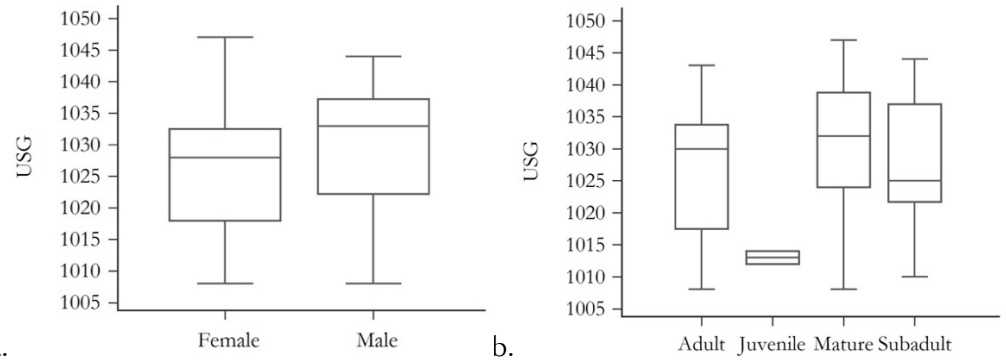


Figure 9 Comparison of USG values between males and females (a.) and among age classes (b.).

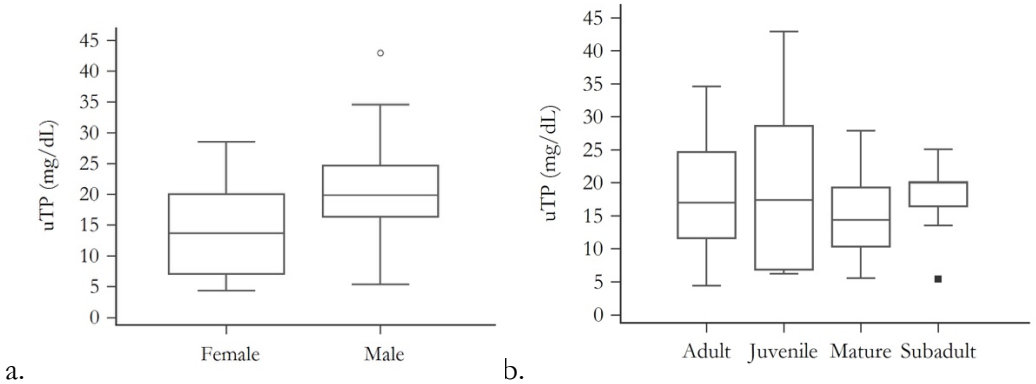


Figure 10 Comparison of uTP values between males and females (a.) and among age classes (b.). The circle and the square indicate the uTP values of ID 11 and 23, respectively.

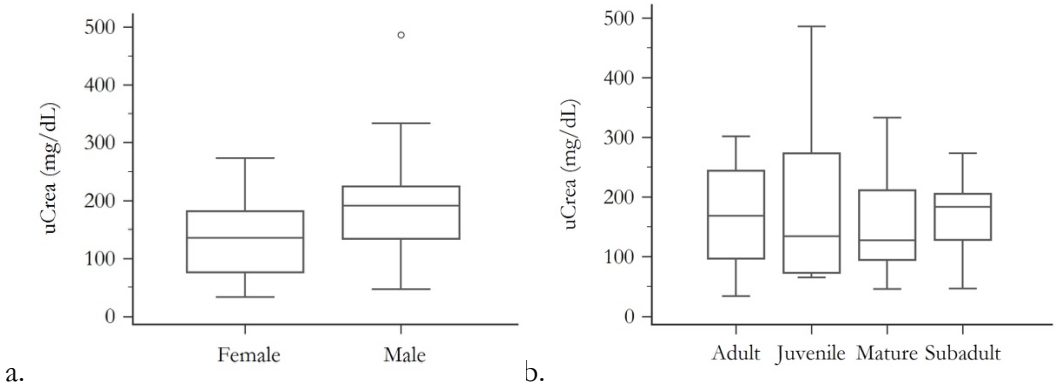


Figure 11 Comparison of uCrea values between males and females (a.) and among age classes (b.). The square indicates the uCrea value of ID 11.

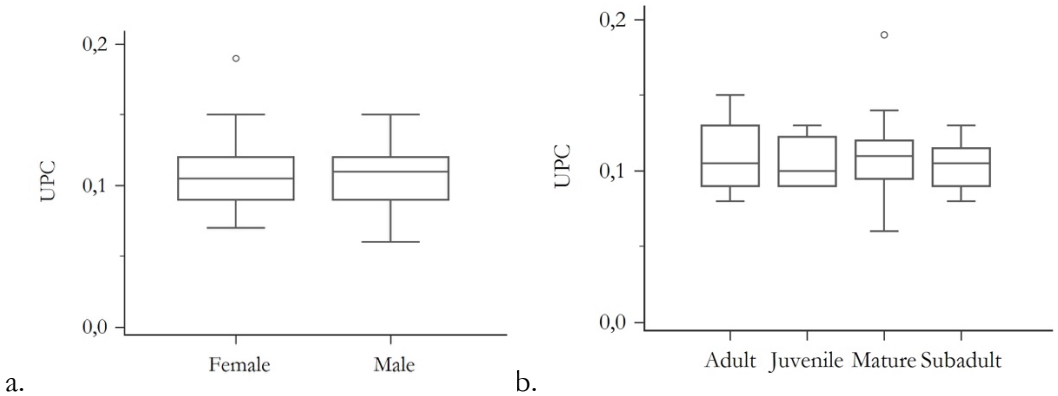


Figure 12 Comparison of UPC values between males and females (a.) and among age classes (b.). The circles indicate the UPC values of ID 36 (mature female).

5. 1D-SDS-PAGE ELECTROPHORESIS

Electrophoresis protein profiles allowed to detect a pattern of common proteins in giraffe urine. The most frequent protein bands in almost all of the samples analysed had an apparent molecular mass (MM) of 64, 50, 42, 34, 25, 18, 14 and 6 kDa. The band with MM of 64 kDa was present in all the analysed specimens, as well as the bands with low MM (18-6 kDa), which were well defined in all the urines examined. Conversely, the bands with MM included between 18 and 64 kDa and higher than 64 kDa appeared as traces in some samples. Representative gel and pherograms are reported in Figure 13 and Figure 14.

Moreover, it can be inferred that sex and age affected the urine proteome, especially the molecular mass (MM) expressed. Firstly, female giraffes expressed more bands with MM < 23 kDa than males. Secondly, only the mature males had a MM protein bands higher than 164 kDa.

Regarding the age, some differences were also reported. On one hand, the bands with MM lower than 23 kDa increased in specimens of up to 9 years of age and then decreased in the mature giraffes (Figure 15). On the other hand, the MM bands higher than 110 kDa appeared in mature specimens, but only mature male expressed those higher than 164 kDa (Figure 16).

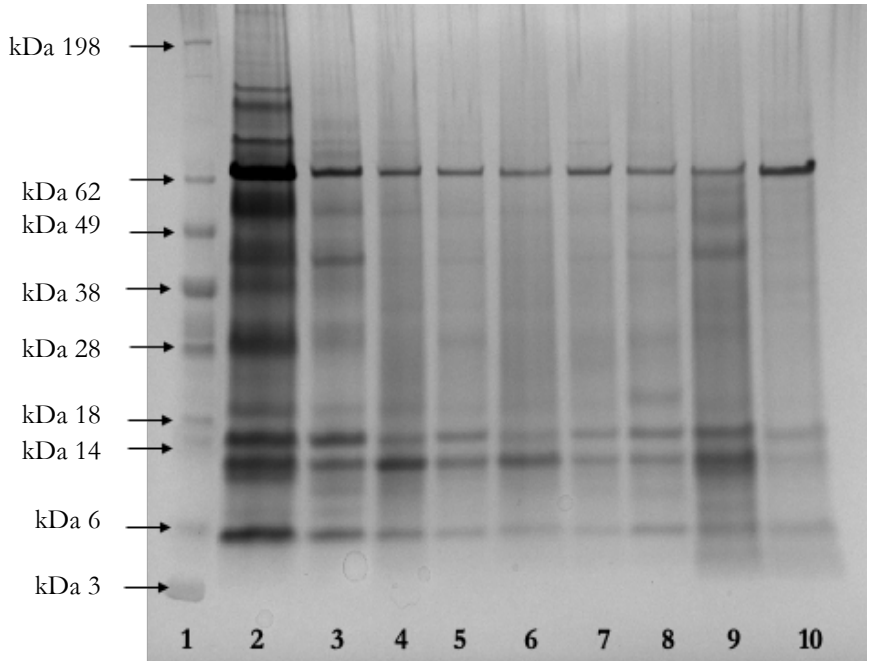


Figure 13 Representative gel; samples were run on 4-12 % gel and stained with silver staining (a.): lane 1: molecular mass marker; lane 2,3 and 8: urines from male giraffes; lane 4-7 and 9-10: urines from female giraffes.

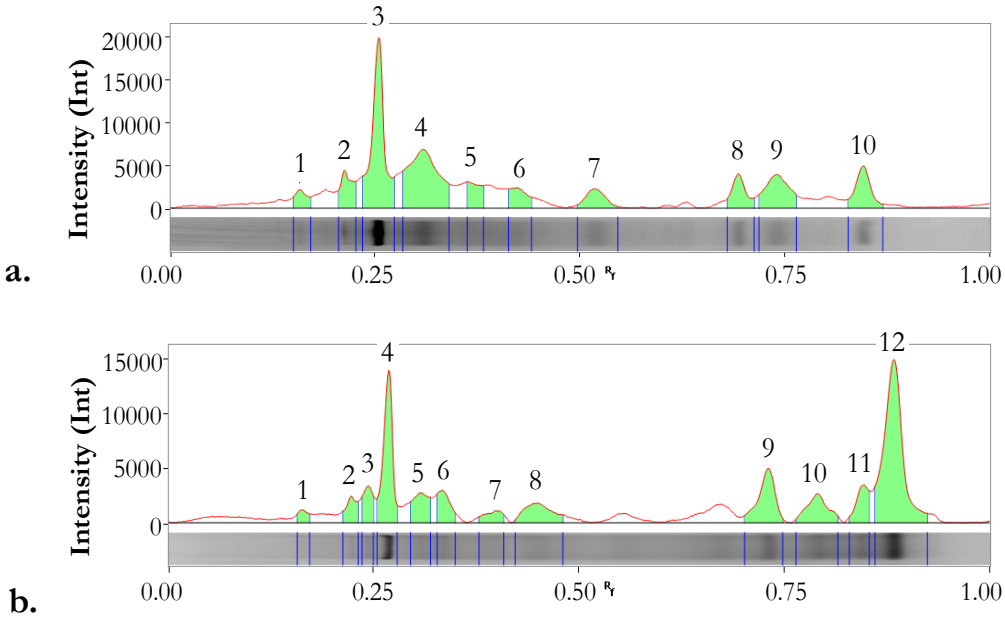


Figure 14 Representative pherogram of female (a.) and male (b.) of giraffe. Samples were run on 4-12 % gel and stained with silver staining.

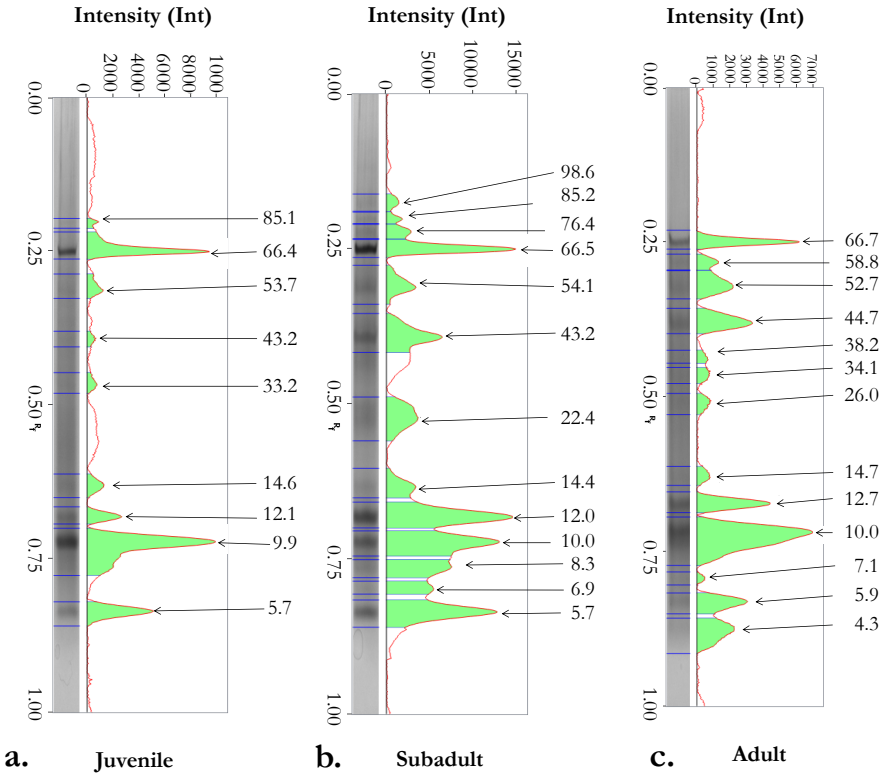


Figure 15 Pherograms of juvenile (a.), subadult (b.) and adult (c.) giraffes.

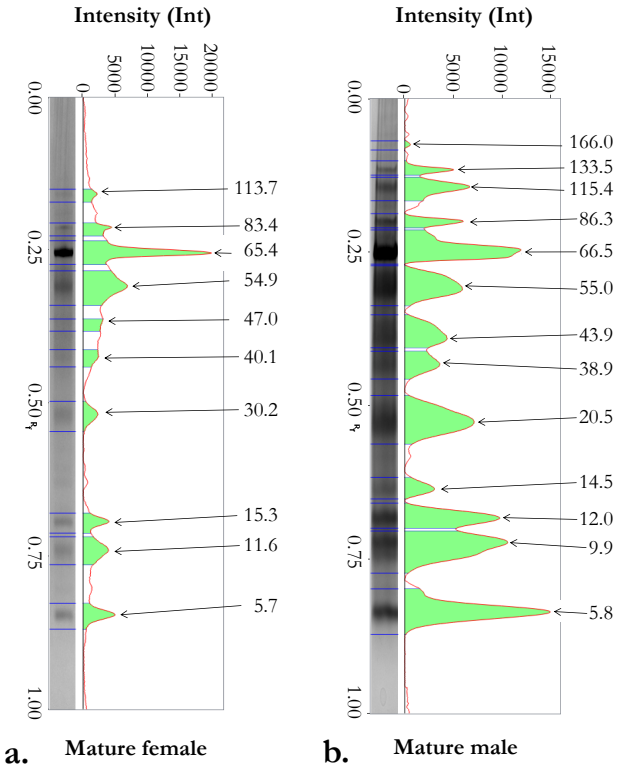


Figure 16 Pherograms of mature female (a.) and mature male (b.).

5.1. BAND NUMBER

After the representation of data distribution, the median and the range (minimum – maximum values) of bands number were calculated. Data are reported in Figure 17 and Table 19. Median and range (min-max) values of protein bands were 8 and 4-15, respectively. Additionally, comparisons among age classes and between males and females were performed with a non-parametric test to define possible influences, which were not found (sex: $p = 0.0671$, age: $p = 0.649950$) (Figure 18).

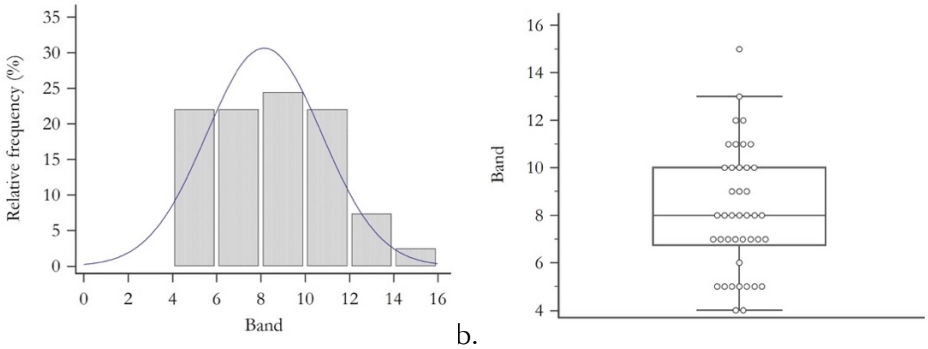
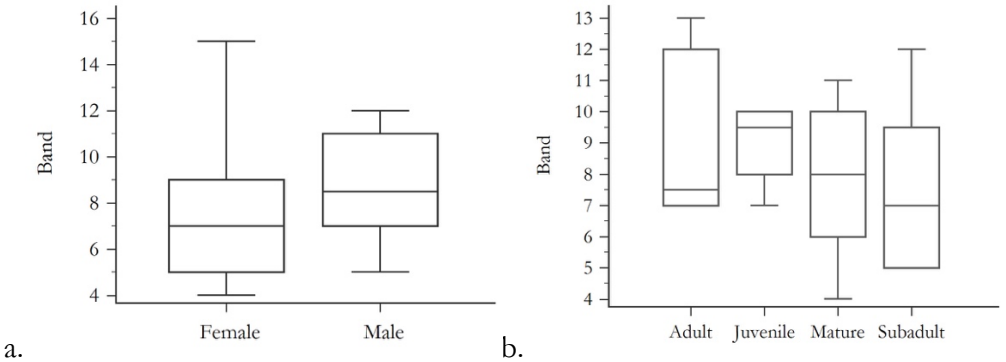


Figure 17 a. Frequency histograms of the band number in giraffes. b. Box-and-Whisker plot of band number (median, 25th and 75th percentiles, 95% CI of mean). The circle indicates the data of ID 16.

Table 19 Median and min – max values of the band number are reported in female, male, juvenile, subadult, adult and mature giraffes. N indicates the number of giraffes included in statistical analysis.

Band number	N	Median	Min – Max
Total	41	8	4 – 15
Female	23	7	4 - 15
Male	18	9	5 – 12
Juvenile	4	10	7 - 10
Subadult	12	7	5 - 12
Adult	6	8	7 - 13
Mature	14	8	4 – 11



a. b.

Figure 18 Comparison between band number in males and females (a.) and among age classes (b.).

5.2. MOLECULAR MASS PROTEIN BANDS

The MM interval (3-180 kDa) determined after SDS-PAGE was divided in 9 classes and their relative frequency was calculated for each studied group (Table 20 and Figure 19). Data about relative frequency of MM of all subgroups are reported in Table 20 and Figure 20.

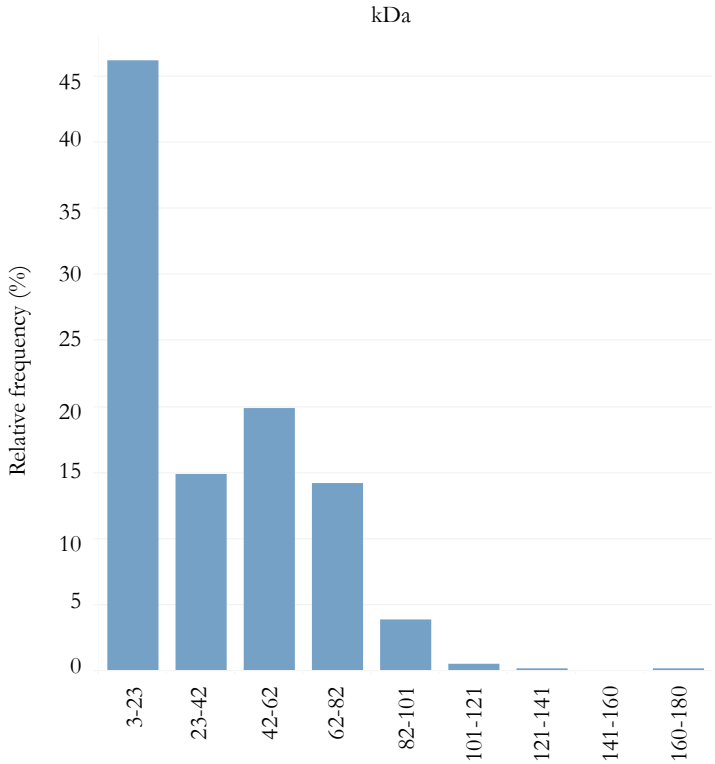


Figure 19 Graphical representation of the relative frequency (%) of MM classes (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) in the studied group.

Table 20 The relative frequency of the MM classes for all studied groups and subgroups is reported. N indicates the number of specimens included in each group or subgroup (Fasoli, et al., 2020).

Class	kDa	Relative frequency (%)						
		Total (N=41)	*Juvenile (N=4)*	*Subadult (N=12)*	*Adult (N=6)*	*Mature (N=14)*	*Male (N=18)*	*Female (N=23)*
1	3 – 23	46.22	46.67	50.42	50.57	42.79	42.75	49.64
2	23 - 42	14.92	11.67	15.97	13.79	17.31	15.24	14.60
3	42 - 62	19.89	18.33	15.97	22.99	21.15	19.70	20.07
4	62 - 82	14.18	18.33	13.45	11.49	12.98	15.99	12.41
5	82 - 101	3.87	5.00	4.20	1.15	3.37	4.83	2.92
6	101 - 121	0.55	0.00	0.00	0.00	1.44	0.74	0.36
7	121 – 141	0.18	0.00	0.00	0.00	0.48	0.37	0.00
8	141 – 160	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9	160 – 180	0.18	0.00	0.00	0.00	0.48	0.37	0.00

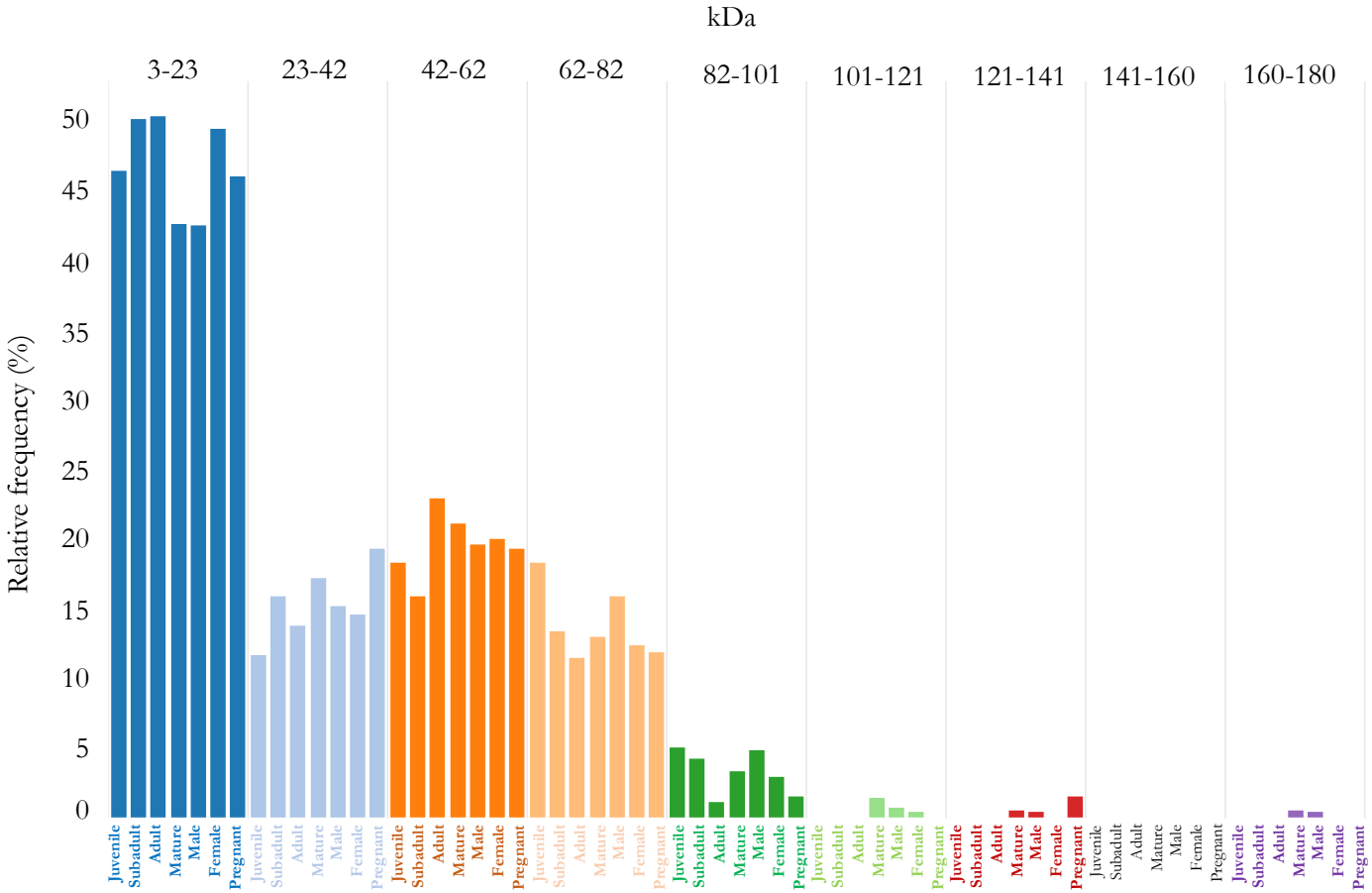


Figure 20 Graphical representation of the relative frequency (%) of MM classes (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) in the studied subgroup. Different colours represent the molecular mass classes (Fasoli, et al., 2020 - modified).

5.2.1. SEX

The relative frequency of the 9 MM classes showed differences between sexes (Table 20 and Figure 21). Indeed, it was observed that female giraffes never showed the protein bands with MM higher than 121 kDa and the frequencies of the MM bands between 3 and 23 kDa were higher in females (49.64 %) than males (42.75 %) (Figure 21).

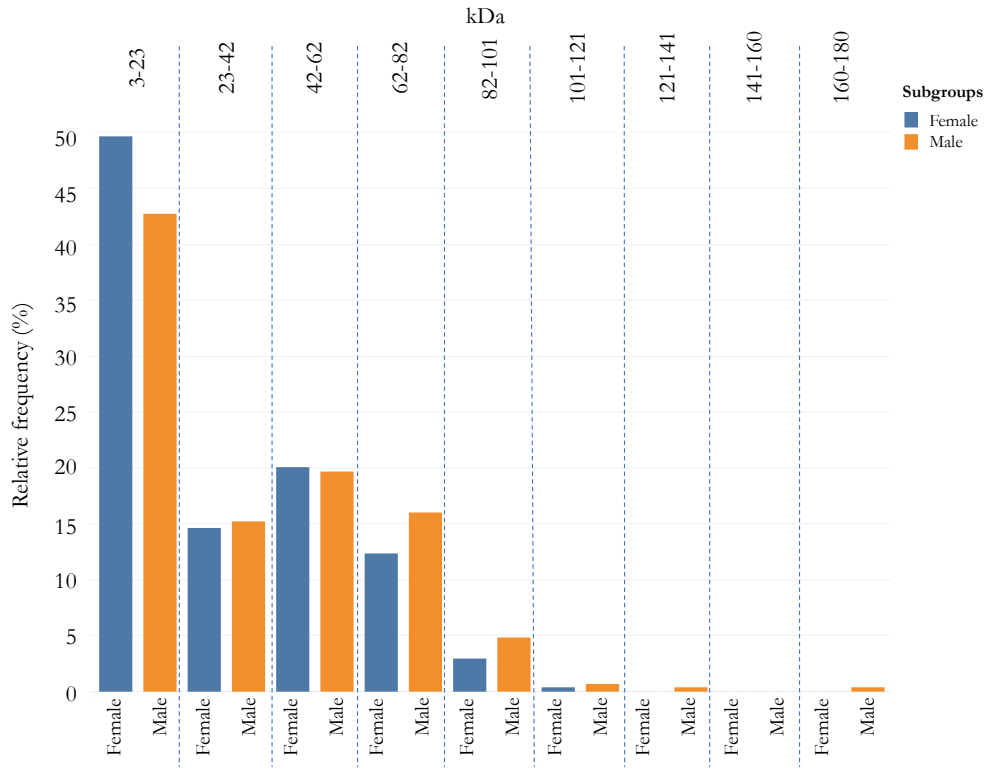


Figure 21 Graphical representation of relative frequency of the molecular mass classes (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) in female and male giraffes. Females did not express protein bands with MM higher that 121 kDa.

It may be speculated that the urine proteome could be influenced by physiological conditions, such as oestrus or mate season. Indeed, as reported in Figure 22, the number of bands of the female in oestrus was 15, when the median value in the other females was about 7 bands. Moreover, the MM of the bands in the urine of this female was about 97, 78, 75, 70, 62, 58, 47, 39, 27, 22, 17 and four bands lower than 14 kDa. Likely, similar findings were reported in dominant males. During the mating period, urines were collected from the two dominant males and the number of protein bands recorded in one of this samples was the highest of all specimens, namely

17 bands (Figure 23). Molecular mass of the protein bands in dominant males were about 166, 133, 115, 93, 86-87, 77, 73, 66, 62, 55, 48, 40-43, 37-38, 29, 20-23, 18, 14-15, 12, 9 and 6 kDa.

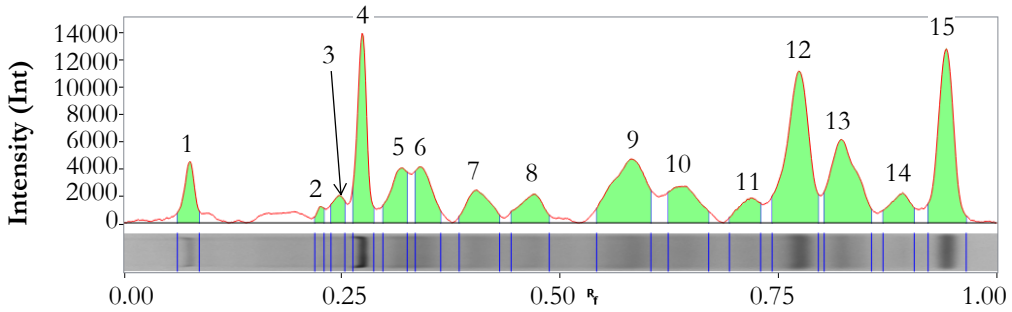


Figure 22 Pherogram of giraffe female (ID 16) during oestrus.

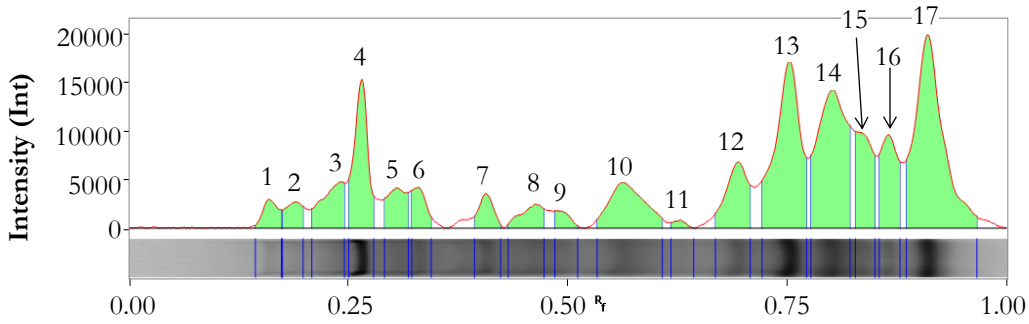


Figure 23 Pherograms of dominant male.

5.2.2. AGE

Regarding age groups, differences were found in the urine proteome among the four subgroups. Complete data are reported in Figure 24 and in Table 20.

The frequency of bands with MM comprised between 3 and 23 kDa increased with age (juvenile: 46.67 %, subadult: 50.42% and adult: 50.57 %) and then decreased after 9 years of age (mature: 42.79 %). The opposite trend was recorded for the bands in the range of 62 - 82 kDa. Interestingly, only mature animals presented bands with MM higher than 101 kDa. Moreover, it was discovered that only mature males expressed the bands with MM between 160 and 180 kDa (Table 20).

Within the juveniles' group, the urines of two giraffes, which were lactated by their mothers, was also analysed (Figure 25).

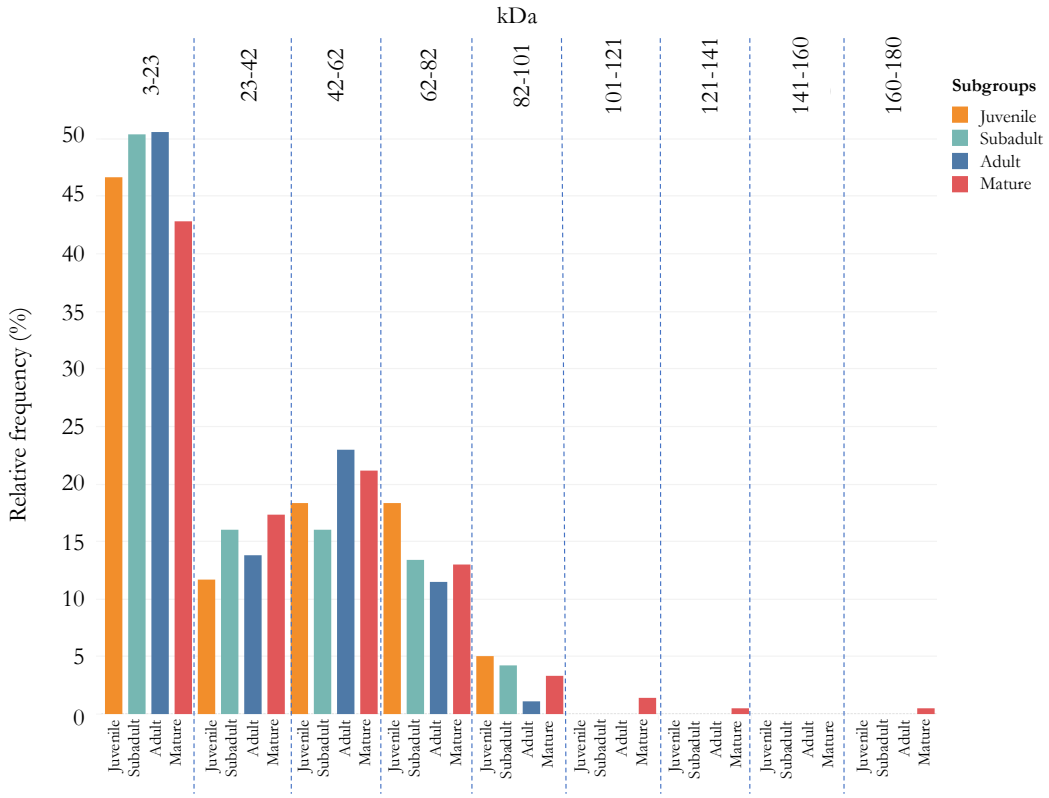


Figure 24 Graphical representation of the relative frequency of molecular mass (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) of age classes.

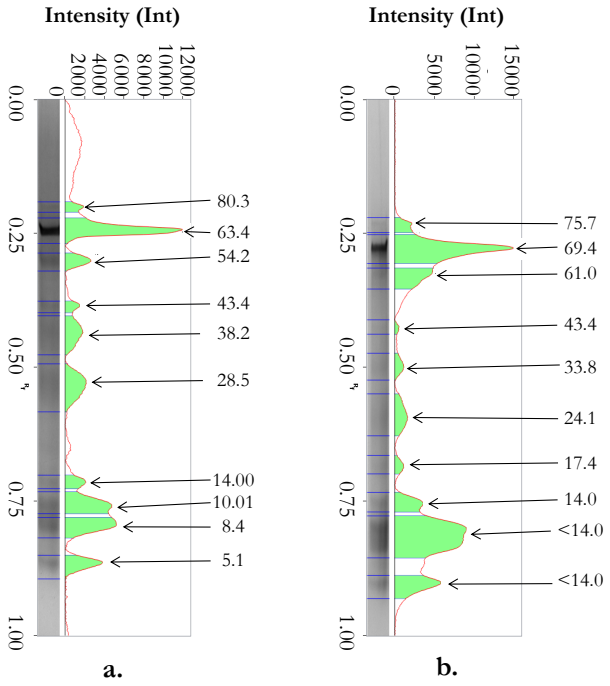


Figure 25 Pherograms of a 4-months-old giraffe 4 (a.) and a 6-months-old giraffe (b.).

6. PROTEIN IDENTIFICATION BY MASS SPECTROMETRY

The most represented protein bands were excised from the gel and were identified using mass spectrometry (Figure 26).

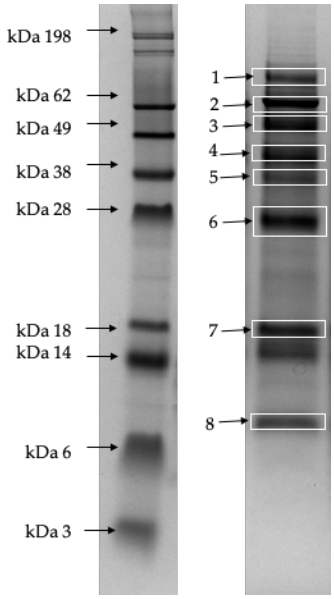


Figure 26 Left: molecular mass marker. Right: urine sample from giraffes. Numbers and arrows indicate the band excise for the protein identification by Mass Spectrometry as reported in Table 21 (Fasoli et al., 2020 – modified).

Starting from those with higher MM, the proteins identified were respectively uromodulin, lactotransferrin, serum albumin, acidic mammalian chitinase, alpha-1B-glycoprotein, clusterin, zinc-alpha-2-glycoprotein, pepsin A, actin cytoplasmic 1, haptoglobin, deoxyribonuclease-1, apolipoprotein, cathelicidin-1, lysozyme C-2, cathelicidin-1, ubiquitin and serine protease inhibitor Kazal-type.

Forty-three percent of the proteins identified were located in the extracellular region (serum albumin, lactotransferrin, acid mammalian chitinase, alpha-1B-glycoprotein, clusterin, zinc-alpha-2-glycoprotein, pepsin A, haptoglobin, deoxyribonuclease-1, apolipoprotein D, cathelicidin-1, and serine protease inhibitor Kazal-type); other proteins were present in the nucleus (13%) (clusterin, actin cytoplasmic 1, deoxyribonuclease-1, and ubiquitin), cytoplasm (7%) (acidic mammalian chitinase and ubiquitin), mitochondrion (3%) (clusterin), cytoskeleton (3%) (actin cytoplasmic 1), and endoplasmic reticulum (3%) (apolipoprotein D).

The most common molecular function of these proteins was binding to other molecules (39%) (serum albumin, uromodulin, lactotransferrin, actin cytoplasmic 1,

haptoglobin, deoxyribonuclease-1, and apolipoprotein D). Other proteins were enzymes (22%) (lactotransferrin, zinc-alpha-2-glycoprotein, pepsin A, deoxyribonuclease-1, and lysozyme C-2) or had regulatory functions (9%) (lactotransferrin and serine protease inhibitor Kazal-type).

Their biological processes are several, e.g., biological regulation, cell killing, immune system process, response to stimuli, cell population proliferation, cellular component organization, cellular process and developmental process.

The identification and the function of the proteins identified in the urine of giraffes are reported in Table 21, Table 22, and Figure 27. The biological processes, the molecular functions and the cellular components of the proteins identified were reported according to GO and UniProt.

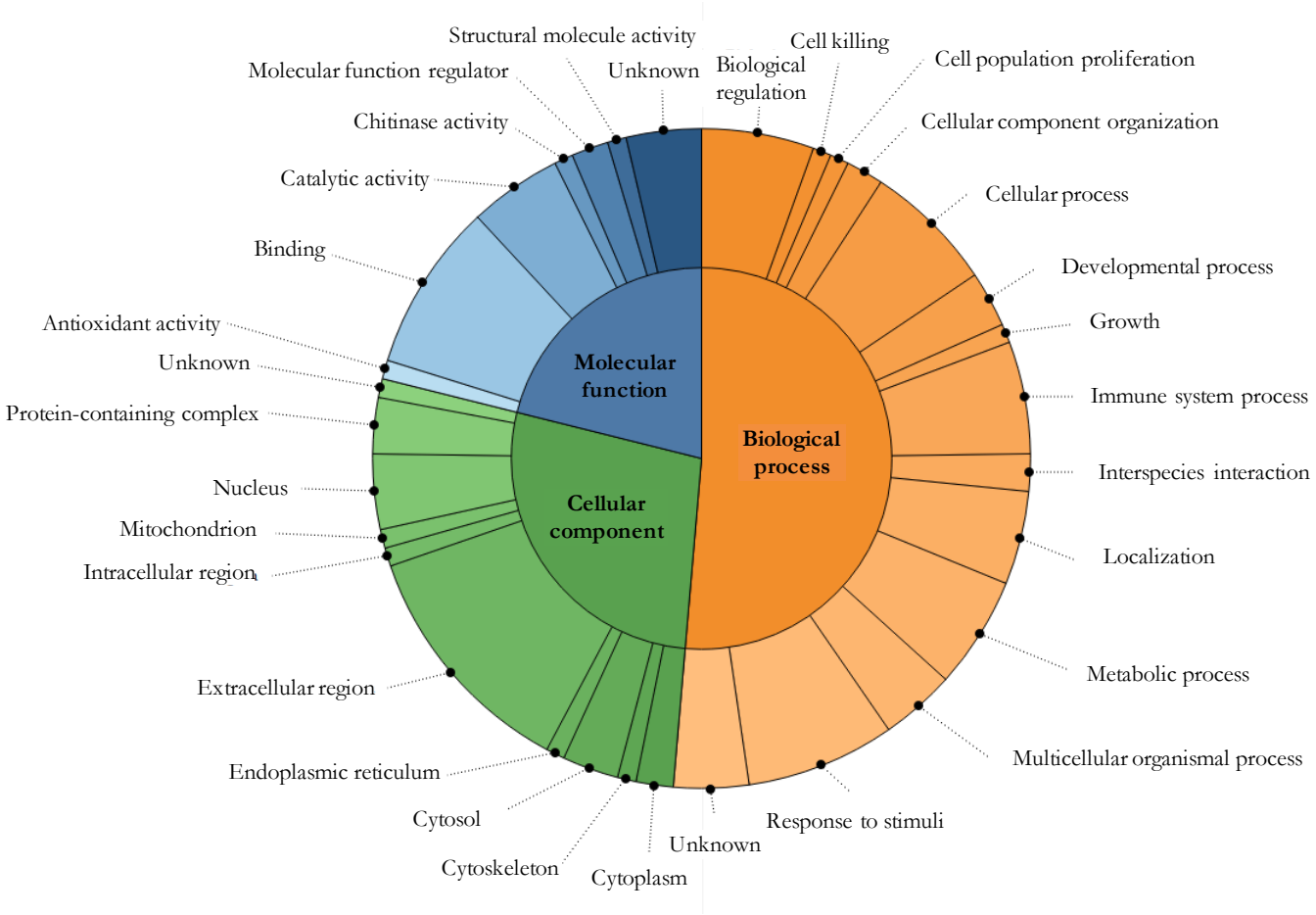


Figure 27 Graphical representation of the biological processes, the molecular functions and the cellular components of the protein identified in the urine of giraffes (Fasoli, et al., 2020 – modified).

Table 21 Identification of protein bands in giraffe urine by mass spectrometry (Fasoli, et al., 2020 – modified).

N	Database	Accession	Full Protein name	Species	Score	MM	Mass	Matched	Pep (sig)	Sequenc.	Seq (sig)	SC (%)
1	SwissProt	UROM_BOVIN	Uromodulin	*Bos taurus*	602	77	72646	55	35	18	14	20%
	SwissProt	TRFL_BOVIN	Lactotransferrin	*Bos taurus*	16	77	80002	8	2	8	2	9%
2	SwissProt	ALBU_BOVIN	Serum albumin	*Bos taurus*	1529	64	71244	180	104	46	30	50%
	SwissProt	ALBU_SHEEP	Serum albumin	*Ovis aries*	1450	64	71139	154	92	42	27	43%
3	SwissProt	CHIA_BOVIN	Acidic mammalian chitinase	*Bos taurus*	196	50	52780	16	8	10	5	15%
	SwissProt	A1BG_BOVIN	Alpha-1B-glycoprotein	*Bos taurus*	63	50	54091	11	6	5	5	6%
4	SwissProt	CLUS_BOVIN	Clusterin	*Bos taurus*	389	42	51651	32	21	15	9	21%
	SwissProt	ZA2G_BOVIN	Zinc-alpha-2-glycoprotein	*Bos taurus*	231	42	34059	19	12	9	8	21%
	SwissProt	PEPA_BOVIN	Pepsin A	*Bos taurus*	166	42	40320	31	17	8	6	9%
	SwissProt	ACTB_BOVIN	Actin, cytoplasmic 1	*Bos taurus*	50	42	42052	11	3	10	3	29%
5	SwissProt	HPT_CAPIB	Haptoglobin	*Capra ibex*	92	34	45411	12	6	10	5	18%
	SwissProt	DNAS1_PIG	Deoxyribonuclease-1	*Sus scrofa*	85	34	31834	11	4	5	3	19%
	SwissProt	APOD_BOVIN	Apolipoprotein D	*Bos taurus*	64	34	21616	12	5	5	3	20%
6	SwissProt	CTHL1_SHEEP	Cathelicidin-1	*Ovis aries*	48	25	18036	5	2	4	2	23%
7	SwissProt	LYSC2_BOVIN	Lysozyme C-2	*Bos taurus*	375	14	16749	26	16	7	4	51%
	SwissProt	CTHL1_SHEEP	Cathelicidin-1	*Ovis aries*	58	14	18036	9	5	7	5	38%
8	SwissProt	UBIQ_CAMDR	Ubiquitin	*Camelus dromedarius*	52	10	8560	9	3	7	2	80%
	SwissProt	ISK1_SHEEP	Serine protease inhibitor Kazal-type	*Ovis aries*	26	10	6483	3	1	2	1	23%

N: number of bands identified as reported Figure 26. Accession: Protein entry name from the UniProt knowledge database; Species: due to the absence of data regarding giraffes in the database, the protein was matched with other mammalian proteins; Score: the highest scores obtained using the Mascot search engine; MM: apparent molecular mass as predicted by the MM marker in the SDS-PAGE gels and expressed as kDa; Mass: theoretical MM reported in kDa; Peptides: total number of peptides matching the proteins identified; Pep (sig): total number of significant peptides matching the proteins identified; Sequence: total number of distinct sequences matching the proteins identified ; Seq (sig): total number of significant distinct sequences matching the proteins identified; SC: Sequence coverage.

Table 22 Function and biological classification of the proteins identified in giraffe urine. The biological processes, the molecular functions and the cellular components are reported according to GO and UniProt (Fasoli, et al., 2020 – modified).

Protein full name	Species	Biological Process	Molecular Function	Cellular Component
Serum albumin	*Bos taurus*	Cellular process, response to stimuli, biological regulation, localization	Binding	Extracellular region, protein-containing complex
Serum albumin	*Ovis aries*	-	Binding	Extracellular region
Uromodulin	*Bos taurus*	-	Binding	Intracellular region (secreted)
Lactotransferrin	*Bos taurus*	Immune system process, response to stimuli, developmental process, interspecies interaction, localization, biological regulation, cell killing, cellular process, multicellular organismal process	Regulation of molecular function, binding, catalytic activity	Extracellular region, protein-containing complex
Acidic mammalian chitinase	*Bos taurus*	Cellular process, immune system process, metabolic process, response to stimuli	Chitinase activity and chitin binding	Extracellular region, cytoplasm
Alpha-1B-glycoprotein	*Bos taurus*	-	-	Extracellular region
Clusterin	*Bos taurus*	Cellular and metabolic process, immune system process, cellular component organization, biological regulation, response to stimuli, localization, cell population proliferation	Protein binding	Cytosol, nucleus, mitochondrion, extracellular region, protein-containing complex
Zinc-alpha-2-glycoprotein	*Bos taurus*	Immune system process	-	Extracellular region
Pepsin A	*Bos taurus*	Metabolic process, multicellular organismal process	Catalytic activity	Extracellular region
Actin, cytoplasmic 1	*Bos taurus*	Cellular process, cellular component organization, localization, response to stimuli, developmental process, biological regulation	Binding, structural molecule activity	Cytoskeleton, cytosol and nucleus
Haptoglobin	*Capra ibex*	Response to stimuli, immune system process	Antioxidant activity and binding	Extracellular region
Deoxyribonuclease-1	*Sus scrofa*	Cellular process, metabolic process, immune system process, biological regulation	Binding, catalytic activity	Nucleus, extracellular region
Apolipoprotein D	*Bos taurus*	Developmental process, metabolic process, localization, biological regulation, multicellular organismal process, growth	Binding	Cytosol, endoplasmic reticulum, extracellular region
Cathelicidin-1	*Ovis aries*	Response to stimuli	-	Extracellular region
Lysozyme C-2	*Bos taurus*	Cellular process, response to stimuli, interspecies interaction, multicellular organismal process, metabolic process	Catalytic action	-
Ubiquitin	*Camelus dromedarius*	-	-	Nucleus, cytoplasm
Serine protease inhibitor Kazal-type	*Ovis aries*	-	Molecular function regulator	Extracellular region

DISCUSSION

1. REFERENCE INTERVALS

The selection of reference animals and the determination of intervals were carried out following the American Society of Veterinary Clinical Pathology (ASVCP) reference interval guidelines (Friedrich, et al., 2012). Inasmuch as the sample size and the distribution of the data, it was decided to use the robust method with 90% of confidence interval (Geffré, et al., 2011; Friedrich, et al., 2012). The health status of giraffes was assessed considering their clinical examination and history, due to the difficulty to perform blood sampling. During this study, animals did not show signs of disease, had a good body condition score and the zoo staff did not report behaviour abnormalities.

2. URINALYSIS AND URINARY REFERENCE VALUES

As far as dipstick protein positivity is concern, it cannot be excluded that these results might be a false positive. Indeed, an alkaline pH (8-9, as for giraffes) could be responsible for dipstick protein positivity, as reported in sheep, goats, cows (Defontis, et al., 2013; Hermann, et al., 2019), Asian elephants (Wiedner, et al., 2009) and rhinoceroses (Haffey, et al., 2008). Likewise, the type of collection (e.g., from exam table or floor) could provoke a false positive reaction for protein (Jones, et al., 2012; Sink & Weinstein, 2012). Notably, the highest value of dipstick protein positivity (100 mg/dL) was detected in those urine samples wherein the median of uTP (mg/dL) was equal to 27.05 (20.06 – 28.12) and the pH was 9.00. Moreover, the assessment of UPC in these samples confirmed the absence of proteinuria; for this reason, it is reasonable to believe that this positivity might be caused by the alkaline urinary pH or by the type of collection. Therefore, it is advisable to use a quantitative analytical technique instead of a semi-quantity method for determining urine total protein, due to the high incidence of false positives in the dipstick test.

The dipstick erythrocyte positivity was recorded in 5 giraffes (10-50 RBC/μL), but none of the red blood cells were identified in the urine sediments of these specimens. Thus, an influence of contaminants on the dipstick erythrocytes net could be hypothesized; those, by reacting with the test-strip reagent, changed its colour and

revealed a dipstick erythrocytes positivity (Rao & Jones, 2008). As a matter of fact, this test has a high false-positive rate and, in humans, patients showing a positive dipstick test, a negative microscopic urinalysis and no symptoms are described as individuals with pseudo-haematuria (Rao & Jones, 2008). However, an USG lower than 1.007 could lead to erythrocyte lysis, resulting in negative microscopic finding (Rao & Jones, 2008). Only one of the 5 giraffes positive for erythrocytes had USG as 1.004, while the positivity outcomes were not confirmed by the other analysis (microscopic urine sediment evaluation) in the other 4 animals, further confirming their false positivity.

The dipstick nitrite positivity (trace) should be cautiously interpreted. In fact, nitrite measurement is not habitually reported in veterinary medicine and urine dipsticks are considered reliable for urine pH, glucose, ketones, bilirubin, occult blood, and protein (Piech & Wycislo, 2019).

The reference values established for giraffes were in accordance to the data previously reported in literature for domestic animals and, most of all, for ruminants (Braun & Lefebvre, 2008; Chew, et al., 2011b; Hermann, et al., 2019; Ferlizza, et al., 2020b).

The giraffes' USG echoed data reported in other herbivorous animals, both in non-domestic species, such as Asian elephants (Wiedner, et al., 2009), Bovidae and Antilocapridae (Wolfe, 2015) and captive rhinoceros (Haffey, et al., 2008) and in domestic animals, such as cows (Hermann, et al., 2019; Ferlizza, et al., 2020b), sheep and goats (Jones, et al., 2012). However, the results of USG should be interpreted carefully and evaluated repeatedly, since a single sample is not indicative of the urine concentration ability of the animal; in fact, low USG values, such as 1.003, have been reported in clinically healthy goats (Jones, et al., 2012).

Giraffe urine presented a low quantity of total proteins, just like other healthy ruminants (Isani, et al., 2018; Ferlizza, et al., 2020b). The interval established for uCrea was partially superimposable with previous intervals reported for giraffes (Sullivan, et al., 2010). The uCrea and the UPC upper limit of the reference interval were slightly lower than those reported in cattle (Hermann, et al., 2019). This difference might be explained by taking into account the extremely different husbandry between cows and giraffes. In fact, uCrea and UPC are variables that can both be affected by various factors that occur in cows but not in giraffes (e.g., the stage of lactation) (Hermann, et al., 2019).

However, these data could be representative only of captive giraffes since, in the wild, animals are faced with different environmental and nutritional conditions. This study was carried out on urine collected from animals having *ad libitum* access to water, so this might explain why their urine was less concentrated than expected. Moreover, the concentrate:hay ratio could influence the urinary values in giraffes (Sullivan, et al., 2010), and this aspect should also be taken into account when interpreting the urinalysis of this mammal.

The influences of sex and age were tested for urine total protein (uTP), urine creatinine (uCrea) and UPC and none statistical differences was determined. However, the comparison between sexes pointed out borderline *p values* of uTP and uCrea, suggesting that sex might influence these parameters. The median of uTP and uCrea in urine of giraffe males were higher than in females (uTP = females: 13.66 mg/dL; males: 19.85 mg/dL; uCrea = females: 136.05 mg/dL; males: 191.52 mg/dL), such as in urine of rat (Gautier, et al., 2014). Likewise, urinary creatinine is higher in men than in women (Thomas, et al., 2012) since the excretion of this metabolite is related to body mass (Tsuji, et al., 2017). In fact, the average weight of giraffe males is 1200 kg, while the average weight observed in females is 800 kg (Dagg, 2014e). Similarly, even though no statistical differences were reported for age, uCrea in juvenile (134.61 mg/dL) was lower than in subadult (183.53 mg/dL). These differences might be similarly explained by considering the correlation between urinary creatinine and muscle mass (Tsuji, et al., 2017). In fact, body weight is higher in subadults than in juveniles.

3. URINARY PROTEOME

3.1. 1D-SDS-PAGE ELECTROPHORESIS

The separation of the urinary proteome by 1D-SDS-PAGE electrophoresis has depicted a pattern of common proteins excreted in urine by this mammal. The urine proteins in giraffes had mainly a low MM and were present in small quantities. In fact, the most representative bands, which were present in all the specimens analysed, had an apparent MM of 64, 14, 10 and lower than 6 kDa.

Relative frequency of MM of protein bands pointed out differences, with age and sex as a source of variability (Table 20). As far as age is concerned, the relative frequency of bands included in the first kDa-class (3-23 kDa) increased with age

(juvenile: 46.67 %, subadult: 50.42 % and adult: 50.57 %). This trend might be explained considering what has been reported in male rats, where testosterone seemed to be one of the factors responsible for the increase of urinary excretion levels of the lower molecular mass proteins (Tsuji, et al., 2017). Even though the giraffe subgroups were mixed, the juvenile and subadult categories were majorly composed by male specimens (80% and 66% respectively).

Even sex highlighted differences between the urinary proteome of males and females. In fact, as reported in humans, gender influences the urinary protein pattern (Thongboonkerd, et al., 2006). Sex-dependent proteomic features were also reported in dogs, wherein multiple bands with a MM of 14-18 kDa appeared in urines of entire male dogs; these bands are caused by the prostate-specific protein (Miller, et al., 2014). Likewise, in humans, the urine of males highly expresses prostate-origin proteins (Guo, et al., 2015). The prostatic acid phosphatase is a glycoprotein with MM of 100 kDa reported in adult men and in cow (Muniyan, et al., 2013; Guo, et al., 2015), leading to suppose that this protein could be present in the bands with high MM (Figure 21) detected in mature male giraffes. However, the increase of high MM proteins has been reported in aged rats (Olukiran, et al., 2018), and it might justify the presence of these MM proteins in urines of mature giraffes.

It was discovered that intact and fragmented forms of lactoferrin were expressed in the urine of 2.5 or 5 weeks-old human infants, and the approximate MM of the most prominent fragments were 44, 38, 34, and 32 kDa (Goldman, et al., 1990). It might be supposed that the bands at 43, 38 and 33 kDa (Figure 25) detected in the urines of giraffe calves could be due to the presence of lactoferrin or its fragments.

Nevertheless, the low number of specimens for each subgroup involved in this study represented a limitation, albeit it allowed to get a first insight into the possible gender- and age-related differences and to confirm what has been reported in literature for other animals and humans.

3.2. Protein identification by mass spectrometry

Since the type of proteins excreted through urine ought to be informative of the animals' physiological status, their health and welfare, and their renal function, (Nkuipou-Kenfack, et al., 2017; Olukiran, et al., 2018; Boschetti, et al., 2019), hypothetical roles have been theorised for proteins in giraffe urine. The presence of

urinary proteins in giraffe, which have been proposed or gauged as renal biomarkers in other species, might be important to improve both the knowledge about renal physiology as well as to identify new biomarkers for diagnosing the renal impairment in this mammal.

The urinary proteins identified in this mammal were previously reported in other species, such as Arabian camels (Alhaider, et al., 2012), cats (Ferlizza, et al., 2015), cows (Bathla, et al., 2015), dogs (Brandt, et al., 2014; Miller, et al., 2014) and California sea lions (Neely, et al., 2018). Almost of all the proteins of giraffes have a binding and catalytic activity, they are located in extracellular region and are involved in cellular and metabolic process, biological regulation, response to stimuli and immune system process.

Notably, some of proteins detected in giraffes' urine prevent adhesion of bacteria in the epithelium lining the urinary tract, i.e., uromodulin, lactoferrin (or lactotransferrin) and cathelicidin (Zasloff, 2007). In fact, microbes (e.g. bacteria and fungi) have plasma membranes with negative charge, whereas lactoferrin and cathelicidin are positive-charged proteins (Zasloff, 2007; van Harten, et al., 2018; Lepanto, et al., 2019). Indeed, since some of the proteins present in giraffe urine are involved in the defence activities, it has been argued that these proteins might act as a protection against microbes.

Among the urinary proteins of giraffes, actin and ubiquitin are both involved in the aquaporin 2 trafficking and modulation (Noda, et al., 2004; Sasaki & Noda, 2007; Dugina, et al., 2009; Wu, et al., 2018). Moreover, when compared to other species, giraffes have high and variable levels of vasopressin, which in turn is responsible for the AQP2 expression (Damkjær, et al., 2015; Nawata & Pannabecker, 2018). Surprisingly, giraffes' RTM index (Paragraph 2.2.1) is closer to humans than camels, a species that lives in the arid environment and is able to highly concentrate urine (Maluf, 2002; Al-kahtani, et al., 2004; Reece, 2015b; Kihwele, et al., 2020; Veldhuis, et al., 2020). Despite this unexpected feature given their habitat, giraffes are able to highly concentrate urine and they rarely drink, even during the drought period (Dagg, 2014c).

In camels, an overexpression of cytoplasmatic proteins (including actin) has been interpreted as a conceivable adaptive mechanism for supplying with the drought (Warda, et al., 2014), and it might be reasonable to believe that, even in giraffes, a similar mechanism could occur. All these data considered, it might be supposed that

the giraffe ability to concentrate urine may also be due to complex biological processes that take place in their kidneys, in addition to the renal anatomical peculiarities described in Paragraph 2.2. However, further study is needed to corroborate this hypothesis. A short description of the function of the principal proteins identified in giraffe's urines is reported below.

3.2.1. ACIDIC MAMMALIAN CHITINASE

Mammals possess two active chitinases: chitotriosidase (Chit1) and acidic mammalian chitinase (AMCase), which are chitin-degrading enzymes (Mack, et al., 2015; Tabata, et al., 2018). These enzymes, which are mostly secreted by phagocytes, seem to play a crucial role in anti-infective defence and repair responses, and they probably play a role in asthma and other chronic diseases, too (Mack, et al., 2015).

Recently, a correlation between the feeding behaviour and acidic mammalian chitinase expression levels and chitinolytic activity of the enzyme, determining chitin digestibility (Tabata, et al., 2018) was found. In fact, acidic mammalian chitinase mRNA level in the stomach is strongly related to the feeding behaviours (Tabata, et al., 2018). The level of its mRNA is low in bovine four stomach, probably due to the fact that bacteria hosted in the bovine gastrointestinal tract supplement chitinases and may play an important role in chitin digestion, since insects may be present in the grass eaten by cattle (Tabata, et al., 2018).

Given these functions, it is reasonable to believe that this protein might play a similar role in the anti-infective defence and repair responses in giraffes. In fact, even giraffes are exposed to gamut of pathogens.

3.2.2. ACTIN CYTOPLASMIC 1

Actin is a globular protein forming filaments (F-actin), which is important for cell and intracellular movement, muscle contraction and many other functions and the actins are a family of highly conserved cytoskeletal proteins (Dugina, et al., 2009; Parker, et al., 2020). There are six actin isoforms in vertebrates, including the two cytoplasmic ones, which are ubiquitous and essential for cell survival, playing a role in cell attachment and contraction, cell activities and cell motility (Dugina, et al., 2009).

This protein – which is present in a band of 42 kDa - appears to be involved in the translocation of water channel aquaporin 2 (AQP2) and has been reported as an AQP2 binding protein, highlighting its importance in AQP2 trafficking (Noda, et al.,

2004; Sasaki & Noda, 2007; Dugina, et al., 2009). Indeed, F-actin depolymerization represents a critical step in the AQP2 trafficking induced by vasopressin - a peptide hormone that regulates this water channel of the kidney collecting duct - to the apical plasma membrane in collecting duct cells (Sasaki & Noda, 2007; Loo, et al., 2013). However, in physiological conditions, actin should not be detected in urine, which – conversely – could be identified after a 30 minutes long hypoxia (Lisowska-Myjak, 2010).

Notably, in giraffes, it might be speculated that this protein may be overexpressed, as it happens in camels (Warda, et al., 2014), justifying its presence in healthy animals. Thus, its identification might not be considered as a pathological finding. In camels, in fact, the increased expression of various cytoskeleton proteins (including actin) which promote intracellular trafficking and communication, led the authors to suggest this peculiarity as an adaptive characteristic to cope with alternative drought-rehydration periods that camels face, given their habitat (Warda, et al., 2014).

3.2.3. ALPHA-1B-GLYCOPROTEIN

Alpha-1B-glycoprotein is a protein with an apparent MM of 63 kDa, which is part of the immunoglobulin superfamily, given its high homology with the immunoglobulin heavy and light chain variable domain (Clerc, et al., 2016). This protein is mainly produced in the liver and its function is still unknown (Clerc, et al., 2016). Nevertheless, this protein is one of the candidates for molecular markers of mastitis in cattle and sheep (de Pontes, et al., 2017). Additionally, α-1B-glycoprotein is one of the proteins which have elevated expression in the urine of female patients with Cystitis/Painful Bladder Syndrome and its presence in urine appears to be associated with the occurrence of bladder cancer (Kreunin, et al., 2007; Goo, et al., 2010). Considering giraffes, it is difficult to hypothesize a role of this proteins in this species; thus, further studies are required to clarify its functions in giraffes' urinary proteome.

3.2.4. APOLIPOPROTEIN D

Apolipoprotein D is a cholesterol transport glycoprotein that, depending on its glycosylation, has a molecular mass from 19 and 32 kDa (Clerc, et al., 2016). This protein is produced more often in fibroblasts than in the liver and in the intestine, and a positive correlation between apolipoprotein D serum levels and age in females has been reported (Clerc, et al., 2016). Its serum concentration increases during disease or

inflammation; however, it has been identified in the urine of healthy humans (Holmquist, 1990; Clerc, et al., 2016). Moreover, apolipoprotein D is a member of the lipocalin superfamily, which is active in catabolism: it excretes cholesterol from peripheral tissues and transports cholesteryl esters to the liver (Zhou & Luo, 2020). Moreover, in the urine of humans affected by cell renal cell carcinoma, apolipoprotein D tended to decrease as the aggressiveness of renal cancer increased (Sandim, et al., 2016). Indeed, its expression levels was generally inversely related to the aggressiveness of different types of tumours (Sandim, et al., 2016). It might be hypothesized that this protein could be a physiological finding in giraffes, since it was identified also in healthy human urines.

3.2.5. CATHELICIDIN-1

Cathelicidins are one class of host defence peptides (HDPs), which have been reported in various animal phyla, and have antimicrobial and immunomodulatory functions (van Harten, et al., 2018).

These peptides have been found in numerous mammals and different members of this family have been identified among species, even though the α-helical cathelicidin peptide has been detected in every mammal studied, suggesting that - starting from a prototype - cathelicidin peptides might evolve quickly under the pressure of the microbes and environmental pathogens (Zanetti, 2005).

These peptides have an antimicrobial effect against various pathogens (i.e., Gram-positive and Gram-negative bacteria, fungi, parasites); indeed, their positive charge interacts with the negative charge of the bacteria's membranes, leading cell to die (van Harten, et al., 2018). However, it seems that cathelicidins have immunomodulatory properties instead of being bactericidal agents (van Harten, et al., 2018). In fact, cathelicidins can induce the degranulation of neutrophils and mast cells, the upregulation of inflammatory cytokines and cytokine receptors, the production of a range of chemokines and chemokine receptors, the migration of epithelial cells (influencing wound healing) and they can improve phagocytosis by opsonizing bacteria and upregulating bacterial recognition receptors (van Harten, et al., 2018).

Moreover, the detection of cathelicidin-1 in milk was associated with the presence of mastitis in ewes and this protein is implicated in alpha-herpesvirus

infections of the bovine respiratory system, which responds differently during an acute infection and the reactivation of viruses (Burucúa, et al., 2019; Katsafadou, et al., 2019).

However, low levels of cathelicidin are also present in human urine and this seems to have an epithelial origin; it is continuously synthesized by the tubular epithelium and released into the tubular lumen without being stored (Zasloff, 2007). Additionally, this protein has been identified in the urine of pregnant cows (Rawat, et al., 2016). It might be hypothesized that this protein could be a physiological finding in giraffes, especially for its activity against pathogens and its immunomodulatory function.

3.2.6. CLUSTERIN

Clusterin is a glycoprotein which has been purified from ram rete testis fluid, even though it is expressed in almost all tissues and found in all human fluids (Blaschuk, et al., 1983; Shannan, et al., 2006). In humans, this protein possesses two forms and the secreted one (sCLU) is a glycosylated dimeric protein of approximately 76–80 kDa, which – in the reducing condition – dissociates into subunits with molecular mass about 40 kDa (Blaschuk, et al., 1983; Shannan, et al., 2006).

Clusterin has been considered as a biomarker of Acute Kidney Injury (AKI), since its expression is stimulated in kidney of rats, dogs and primates after ischemia and reperfusion injury, even though no clinical study has demonstrated its use as a biomarker for early diagnosis or prognosis of AKI in humans (Vaidya, et al., 2008). However, it is involved in various physiological processes: apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodelling, lipid transportation, membrane recycling, and immune system regulation, which are important for carcinogenesis and tumour growth (Shannan, et al., 2006). In fact, clusterin upregulation has been reported in various human malignant tumours, including kidney tumour (Shannan, et al., 2006). This protein has been identified also in the urine of dogs (Brandt, et al., 2014). It might be hypothesized that this protein could be a physiological finding in giraffes, given its involvement in physiological processes.

3.2.7. DEOXYRIBONUCLEASE-1

The glycoprotein deoxyribonuclease-I acts as digestive enzyme and is mainly present in the pancreas and in the parotid glands (Fujihara, et al., 2012). Moreover, this enzyme – a DNA-degrading apoptotic endonuclease - mediates the DNA

fragmentation and is the most abundant apoptotic endonuclease in mammalian cells (Jang, et al., 2015). Its inactivation is protective against kidney injury and injuries to other organs (Jang, et al., 2015). Indeed, this protein, with molecular mass about 30 to 34 kDa, is activated in ischemia/reperfusion injury in the kidney of rats and it is known to mediate toxic, hypoxic and radiation injuries to the cell (Basnakian, et al., 2002; Jang, et al., 2015).

However, deoxyribonuclease-I can be detected in urine, wherein it seems to be extremely active (Fujihara, et al., 2012). Its total endonuclease activity is about 40% to 99% in most organs, including the kidney, and in the urine as well as blood (Jang, et al., 2015).

When comparing the tissue distribution of the mammalian deoxyribonuclease-I, it can be classified into pancreas, parotid, and pancreas–parotid (or mixed) type; this classification reflects the eating habits of the species: the omnivores have a pancreas-type isoform, rats and mice have a parotid-type, whilst the herbivores have pancreas–parotid-type (Fujihara, et al., 2012). Moreover, the different types of deoxyribonuclease-I reflect their different acid sensitivity; in fact, the pancreas-types are more sensitive to acidic conditions than the parotid-types and mixed types (Fujihara, et al., 2012). Especially the parotid-type is secreted into the small intestine in the pancreatic juice at neutral pH (Fujihara, et al., 2012). Additionally, a correlation between the tissue distribution of this protein and its ability to resist to proteolysis has been found, e.g., the parotid-type deoxyribonuclease-I maintains its digestion function activity, despite the simultaneous presence of trypsin and chymotrypsin in the pancreatic juice (Fujihara, et al., 2012).

Interestingly, deoxyribonuclease-I of giraffes matched with that of *Sus scrofa*, an omnivorous animal, which expresses this protein mainly in the pancreatic tissue, rather than both in pancreatic tissue and in the parotid gland as in *Bos taurus* (Fujihara, et al., 2012). Few distinctions between cows and giraffes have been highlighted by Gaastra et al. (1974): the authors investigated the primary structure of the giraffe's pancreatic ribonuclease and found out differences in amino acid sequence between the pancreatic ribonuclease of these two species (Gaastra, et al., 1974). Thus, it might be inferred that the enzymes in the pancreatic juice in giraffes are different from those in the pancreatic juice of cattle.

Although it's difficult to explain why the deoxyribonuclease-I was the only protein of giraffes matched with strictly non-herbivorous species, two hypotheses may be advanced. Firstly, the DNase I presents different actin-binding capacities among species and that of the pig is unaffected by G-actin, while, in cows, the G-actin inhibits the activity of the DNase I (Fujihara, et al., 2012). Given the presence of actin in giraffe's urine, it may be speculated that this characteristic of the DNase I would be necessary in order to guarantee an adequate function of both of these proteins. Secondly, it has been reported that the relatively small salivary glands might be a family trait of the giraffid (Sauer, et al., 2016), justifying a higher distribution of the DNase I in the pancreatic tissue rather than in the parotid gland.

3.2.8. HAPTOGLOBIN

Haptoglobin is an acute-phase glycoprotein, synthesized in the liver, where it is cleaved into light α chain (two variants α1 and α2) and a heavy β chain via disulphide bonds, with respective molecular mass of 9, 16 and 40 kDa (Clerc, et al., 2016). Being a scavenger of haemoglobin, its major function is to protect tissues from oxidative damage by capturing haemoglobin (Clerc, et al., 2016). Moreover, it is an important protein marker of acute-phase reactions in buffaloes, given its increase in the serum concentrations during inflammatory processes (de Pontes, et al., 2017). An increase in its levels in human patients' urines with renal cell carcinoma and breast cancer has also been found, which might be due to the ability of haptoglobin to bind to free haemoglobin, which promotes angiogenesis and carcinogenesis by releasing iron to a growing tumour (Sandim, et al., 2016). Haptoglobin is identified in the urine of California Sea lions (Neely, et al., 2018) and in urine of pregnant cows (Rawat, et al., 2016). It might be supposed that this protein acts in giraffes as it does in other species.

3.2.9. LACTOTRANSFERRIN

Lactotransferrin (or its short name lactoferrin)[11] is a glycoprotein, with a molecular mass about 80 kDa, which is able to bind to two ferric ions per molecule that strongly support innate immune defence against uncontrolled reactive oxygen species (ROS) production (Lepanto, et al., 2019). This protein is multifunctional and it has anti-bacterial, anti-biofilm, anti-fungal, anti-viral and anti-parasitic properties, has

[11] https://www.uniprot.org/uniprot/P24627

an immunomodulatory and anti-inflammatory actions and modulates the cell cycle, migration and differentiation to anti-cancer (Lepanto, et al., 2019). Being part of the natural immune system, it is localized in the mucosal surfaces, including the epithelia lining the urinary tract (Zasloff, 2007; Lepanto, et al., 2019).

Usually, the organisms that colonize the urinary tract are gram-negative microbes which require sufficient concentrations of iron to support growth (Zasloff, 2007). Hence, lactoferrin expresses its antimicrobial activity by restricting the availability of iron to microbes or by binding to their membrane, this way damaging microbes (Zasloff, 2007). Moreover, lactoferrin is produced by the kidney and may contribute to the immune defence in this organ by reducing the available free iron in the urine or by recovering free iron from urine, this way making it available for metabolic use (Åbrink, et al., 2000). Recently, lactoferrin has been suggested as an important modulator of innate immune response in the urinary tract (Patras, et al., 2019). Moreover, the isoform X1 of this protein has been identified in urine of pregnant cows (Rawat, et al., 2016). It might be assumed that, even in giraffes, this protein might act as a defence against microbes.

3.2.10. LYSOZYME C-2

Lysozyme is an enzyme with a molecular mass of 15 kDa, which has been identified in various human's body fluids (Tasca, et al., 2010). In the kidney, it is reabsorbed in the proximal tubules after filtration through the glomeruli (Tasca, et al., 2010). Lysozyme C belongs to pathways involved in neutrophil degranulation and is an antibacterial enzyme (Boileau & Gilmour, 2012; Neely, et al., 2018). Interestingly, it is highly concentrated in the mucosa of the true stomach of the ruminant-like species with differences in the time-dependence, pH-dependence and ionic strength-dependence of the rate of bacterial lysis, suggesting a response to the environmental-stimuli by the organism (Tabata, et al., 2018). This protein has been detected in the urine of California Sea Lions (Neely, et al., 2018), camels (Alhaider, et al., 2012) and dogs (Giori, et al., 2011; Brandt, et al., 2014). It might be supposed that this protein could have a role as an antibacterial enzyme in giraffes, too.

3.2.11. PEPSIN A

Pepsin is a gastric aspartic proteinase and a zymogen-derived protein with a crucial importance for the digestive processes (Gritti, et al., 2000; Konno, et al., 2000).

It is the principal acid protease of the stomach, it is synthesized in the gastric mucosa and secreted into the lumen as pepsinogen (Tang, 2013). The latter is converted to pepsin once it comes in contact with the stomach acid environment, changing its molecular mass from 42 kDa (pepsinogen) to 35 kDa (pepsin) (Gritti, et al., 2000; Tang, 2013). In giraffes, the abomasum is the organ most similar to the stomach of non-ruminants, whose walls secrete the digestive juices (Dagg, 2014e). In fact, the abomasum is the only stomach compartment with glandular mucosa able to secrete pepsin (contained in the digestive juices) (Ducharme, et al., 2017).

The peptic secretion can be assessed by quantifying the pepsinogens in various substrates, including urine (Gritti, et al., 2000). Only the pepsinogen A is present in urine in physiological conditions, due to the fact that just a part of it is metabolized by the kidney, whereas the pepsinogen is completely reabsorbed and metabolized (Gritti, et al., 2000; ten Kate, et al., 1989). As far as giraffes are concerned, it is reasonable to believe that this protein might be a physiological finding in urine.

3.2.12. SERINE PROTEASE INHIBITOR KAZAL-TYPE

Serine peptidase inhibitor Kazal type 1 (SPINK1), or tumour-associated trypsin inhibitor (TATI), is a protease inhibitor acting during inflammation, cell proliferation and cancer pathogenesis, which has been isolated from various tissue, including urothelium (Rink, et al., 2013). SPINK1 binds to trypsin, inhibiting its activity and it seems to protect the pancreas from prematurely activated trypsinogen (Wang & Xu, 2010). Low concentration of this protease is expressed in some healthy tissues, as renal and bladder epithelium (Shariat, et al., 2005). However, it also seems to be involved in self-regulation of acinar cell phagocytosis, proliferation and growth of a variety of cell lines, in the response to inflammatory stimuli or injury (Wang & Xu, 2010). It has been confirmed that its loss is associated with features of biologically aggressive urothelial carcinoma of the bladder (Rink, et al., 2013). This protein could be part of the physiological urinary protein pattern even in giraffes.

3.2.13. SERUM ALBUMIN

Albumin, being 35-50% of total circulating proteins, is the most abundant protein found in serum (Eckersall, 2008). Bovine serum albumin is a non-glycosylated protein with a MM of 66.4 kDa (Eckersall, 2008). This protein is synthesized in the hepatocyte cytoplasm and, once secreted into the circulation, is modified by a covalent,

irreversible and nonenzymic glycation of lysine residues (Eckersall, 2008). Indeed, albumin catabolism takes place in various tissues (especially muscle, liver, and kidney) where – following atrial natriuretic peptide concentration – a certain rate of it enters cells by pinocytosis and is degraded by protease action (Eckersall, 2008).

Albumin's function is to regulate the osmotic pressure and it is responsible for the 80% of the colloid osmotic pressure; moreover, it is involved in the molecular transport due to its ability to bind to numerous metabolites, preventing their loss through the kidney (Eckersall, 2008).

In dogs, cats and humans with renal disease, the concentration of albumin increases due to glomerular damage, and this protein is a considered as biomarker of Acute Kidney Injury (Vaidya, et al., 2008; Grauer, 2011). However, low concentrations/traces of albumin have been reported in the urine of healthy humans (Zhao, et al., 2017) and healthy animals, such as camels (Alhaider, et al., 2012), dogs (Giori, et al., 2011; Brandt, et al., 2014; Miller, et al., 2014), California sea lions (Neely, et al., 2018) and cows (Pyo, et al., 2003; Ferlizza, et al., 2020b). Even in giraffes, it might be speculated that this protein could be a physiological finding in urine of healthy animals.

3.2.14. UBIQUITIN

Ubiquitin is a small and regulatory protein, which can be attached to other proteins as a post-translational modification and is responsible for cellular proteins degradation (Hegde, 2010; Leestemaker & Ovaa, 2017). Ubiquitination of proteins is a multi-step process and involves enzymes of three different classes: E1, E2, E3 enzyme (Leestemaker & Ovaa, 2017). Indeed, ubiquitin is covalently bound to the target protein through an enzymatic cascade: first a ubiquitin-activating enzyme (E1), then a conjugating enzyme (E2) and finally the protein ligase E3 (Mendes, et al., 2020). E1 activates the mature ubiquitin, then this active form is transferred to the E2, where this complex is associated with the E3, which transfers the ubiquitin moiety to the target protein (Mendes, et al., 2020). Since the E3 enzymes have specific domains, they are the most specific to a given substrate, in fact they ligate ubiquitin to only one or more than one substrate, making the ubiquitin-conjugation machinery highly specific (Hegde, 2010).

In this connection, it has been reported that one regulatory protein, the E3 ubiquitin ligase CHIP, is highly expressed in the collecting duct and modulates the function of aquaporin-2 (AQP2) by interacting with it and by directly ubiquitylating this water channel in vitro (Wu, et al., 2018). CHIP, in fact, is a protein able to ubiquitinate unfolded proteins, assisting in the proteasome-mediated degradation of proteins, and is modulated in abundance by vasopressin (AVP) (Hegde, 2010; Wu, et al., 2018). Therefore, it might be inferred that ubiquitin (and the resulting ubiquitylation) is involved in water handling and urine concentration.

Considering giraffes, it is interesting to note that vasopressin is a hormone present in high, though variable, concentrations in giraffe plasma (Damkjær, et al., 2015). Moreover, vasopressin is one of the hormones that regulates the functions of the distal convoluted tubule (DCT) and cortical collecting duct (CCD), which play unique and diverse roles in water and electrolyte handling, and - as previously reported - AVP modulated the CHIP (Wu, et al., 2018). Therefore, it might be speculated that the ability of giraffes to concentrate their urine might involve ubiquitin, considering its role in the modulation of AQP 2 and the resulting water absorbability. However, further studies are needed to corroborate this hypothesis and to confirm the role of the ubiquitin in giraffe urine.

As far as other animals are considered, the ubiquitin A-52 ribosomal protein fusion product 1 has been reported in canine urine proteome (Brandt, et al., 2014).

3.2.15. UROMODULIN

Uromodulin is the most abundant protein in urine of healthy animals (Nkuipou-Kenfack, et al., 2017). Originated from the renal thick ascending limb (TAL) of the loop of Henle, uromodulin or Tamm-Horsfall protein is a highly glycosylated protein (MW > 1 million Da), which can be dissociated (e.g., by sodium dodecyl sulfate) into monomeric subunits with a MM of 95 kDa (Wu, et al., 2018).

Uromodulin is involved in various processes: e.g., water homeostasis, blood pressure regulation or inhibition of the kidney stones formation (Devuyst, et al., 2017). Considering giraffes, the latter is especially remarkable, since - in vitro - uromodulin seems to inhibit crystal aggregation of calcium oxalate (CaOx) or calcium phosphate (CaP), frequently described in giraffes, and stabilizes the calcium channel by reducing the calcium excretion in the distal convoluted tubule (Devuyst, et al., 2017; Jones, et

al., 2018). Additionally, a possible involvement of uromodulin in the modulation of sodium transport has been suggested, since its absence leads to the reduction of the Na^+-K^+-Cl^- transporter (NKCC2) activity and to the impaired NaCl reabsorption at TAL of the loop of Henle (Graham, et al., 2017). Moreover, at the luminal membrane of this site, uromodulin provides a water barrier, which may play a role in ion transport to maintain countercurrent gradients in the interstitium (Graham, et al., 2017).

This protein has been proposed as a biomarker of pregnancy in cows and/or of tubular disfunction in dog (De Loor, et al., 2013; Bathla, et al., 2015; Ferlizza, et al., 2020a). In humans, uromodulin has been suggested as a biomarker for congenital disorders, tubular function and estimated glomerular filtration rate (eGFR), acute kidney injury or kidney stones (Devuyst, et al., 2017). Moreover, the increase of its fragments was reported in advanced age and uromodulin's synthesis deregulation appears to be associated with chronic kidney diseases (Nkuipou-Kenfack, et al., 2017). Furthermore, this protein prevents the adhesion of bacteria in the epithelium lining the urinary tract (Zasloff, 2007).

Uromodulin has been reported in dogs (Miller, et al., 2014; Ferlizza, et al., 2020a) and cows (Rawat, et al., 2016). Considering giraffes, it might be speculated that even in this species, uromodulin may have similar roles as in other animals, especially its function in the water homeostasis and in the inhibition of crystal aggregation, which could be particularly important since the frequency of uroliths in giraffes and their habitat.

3.2.16. ZINC-ALPHA-2-GLYCOPROTEIN

Zinc-alpha-2-glycoprotein (ZAG) is a 40 kDa protein secreted in urine and in other body fluids (Hassan, et al., 2008). Produced in the liver, the sequence and the structure of ZAG are similar to the class I MHC molecules, reflecting its role in immunoregulation and in lipid catabolism (Hassan, et al., 2008; Clerc, et al., 2016). Moreover, androgens regulate the transcription of ZAG; this sheds light on its direct role in tumour progression as well as other tumour-proliferating proteins (e.g., apolipoprotein D) (Hassan, et al., 2008). An increase in zinc-alpha-2-glycoprotein could be considered as a biomarker for diabetic nephropathy and a suggested one for female breast and male prostatic carcinomas (Clerc, et al., 2016; Wang, et al., 2016). This protein has been identified in urine of pregnant cows (Rawat, et al., 2016). It

might be speculated that ZAG could have similar immunomodulatory role even in giraffe urine.

PREGNANCY

Despite the main limit of this section and the fact that the analyses were performed on very few samples, these data might give a first insight into the pregnancy-related changes of the urinary proteome.

Giraffe females become sexually mature at 3-4 years of age and they continue cycling as long as they are pregnant (Dagg, 2014f). Their gestation period is about 446-457 days long (~ 15 months) (Dagg, 2014f). About 3 weeks after parturition, females come into oestrus, even if they are lactating the calves (Dagg, 2014f).

1. RESULT

During the sampling period, 11 urines were collected from 4 pregnant females and 1 urine sample was collected from one female about 15 days after parturition. Moreover, the urines of 2 of the 4 giraffes sampled were collected both when they were pregnant (6 samples) and non-pregnant (7 samples). Data are reported in Table 23.

Table 23 Animal, sex, age and conditions.

Animal ID	Sex	Age (years)	Conditions
20	Female	8	10 months-pregnant
21	Female	7	~ 15 days after parturition
26	Female	5	1 month-pregnant
30	Female	9	2.5 month-pregnant
37	Female	13	3 month-pregnant

1.1. URINALYSIS, UTP, UCREA AND UPC

The physical-chemical examination did not reveal differences among these giraffes and with the data of the whole group. In fact, their data were superimposable with the urinary parameters established. Even the urinalysis of the same giraffes did not reveal any differences between the pregnant and non-pregnant conditions.

Differently, the UPC results appeared higher than the references established in one specimen. The UPC mean values of the female ID 37, a 3-months-pregnant giraffe, was 0.24, whereas the upper limit (90% CI) established for giraffe was 0.16 (0.15 – 0.17). Complete data of physical-chemical urine analysis, uTP (mg/dL), uCrea (mg/dL) and UPC are reported in Table 24.

Table 24 Mean, median, standard deviation (SD), range (min-max) of dipstick test results, urine specific gravity (USG), urine creatinine (uCrea), urine total protein (uTP) and UPC of pregnant giraffes (N=4; n=11); complete data of female ID 21 are also reported (Fasoli, et al., 2020 - modified).

Variable	Mean	Median	DS	Min	Max	ID 21
Bil	0	0	0	0	0	0
Ery (RBC/μL)	1	0	2	0	5	0
Glu (mmol/L)	0	0	0	0	0	0
Ket (mmol/L)	0	0	0	0	0	0
Leu (WBC/μL)	0	0	0	0	0	0
Nit	Neg	Neg	Neg	Neg	Trace	Neg
pH	9	9	0.5	8	9	9
Pro (mg/dL)	0	0	15	0	30	0
UBG (μmol/L)	0.1	0.1	0	0.1	0.1	0.1
USG	1.020	1.018	0.014	1.008	1.035	1.028
uTP (mg/dL)	12.93	10.48	7.33	7.51	23.25	9.29
uCrea (mg/dL)	114.14	104.65	72.42	41.06	206.21	126.30
UPC	0.14	0.11	0.07	0.10	0.24	0.07

Bil = urine bilirubin; Ery = urine erythrocytes; Glu = urine glucose; Ket = urine ketones; Leu = urine leukocytes; Nit = urine nitrate; Pro = urine proteins; UBG = urine urobilinogen; uCrea = urine creatinine; USG = urine specific gravity; uTP = urine total protein.

1.2. 1D-SDS-PAGE ELECTROPHORESIS

Pregnant giraffes expressed protein bands similar to those of other specimens, even though some differences were detected. The median (min-max) of the number of bands were 10 (5-12). Pregnant giraffes did not show the MM bands comprised between 101 and 121 kDa. The relative frequency of those between 23 - 42 kDa and 62 – 82 kDa increased, while the relative frequency of the bands included in the range of 3-23 kDa decreased (Table 25, Figure 28 and Figure 29). Moreover, the protein bands in the range of 121-141 kDa appeared only when females were pregnant. Additionally, as reported in Figure 29, the number of bands in the gel's region between 18 and 62 kDa increased during pregnancy. In fact, the non-pregnant female showed 4 protein bands, whilst the other two females (3 and 10 months pregnant respectively) both expressed 6 protein bands. The MM of these 6 protein bands were 57-59, 50, 47-48, 40-41, 32-35, 22-27 kDa. On the contrary, the MM of bands of the non-pregnant giraffe were 54, 46, 29 and 20 kDa. Even the bands with MM lower than 18 kDa increased, switching from three to four bands.

As far as the ID 21 giraffe is concerned, the total number of bands was 12, and in the gel zone between 62 and 18 kDa, there were 5 bands in this samples. The apparent MM of these bands were about 60, 57, 49, 34 and 26 kDa. Complete data are reported in Table 25, and Figure 30.

Table 25 Relative frequency of the 9 molecular mass classes for pregnant, ID 21 (post-partum) and female (as females older than 5 years old).

kDa classes	Interval (kDa)	Relative frequency (%)		
		Female (N=11)	*Pregnant (N=4)*	ID 21
1	3 – 23	47.33	46.27	34.78
2	23 - 42	19.08	19.40	13.04
3	42 - 62	19.85	19.40	17.39
4	62 - 82	11.45	11.94	21.74
5	82 - 101	1.53	1.49	13.04
6	101 - 121	0.76	0.00	0.00
7	121 – 141	0.00	1.49	0.00
8	141 – 160	0.00	0.00	0.00
9	160 – 180	0.00	0.00	0.00

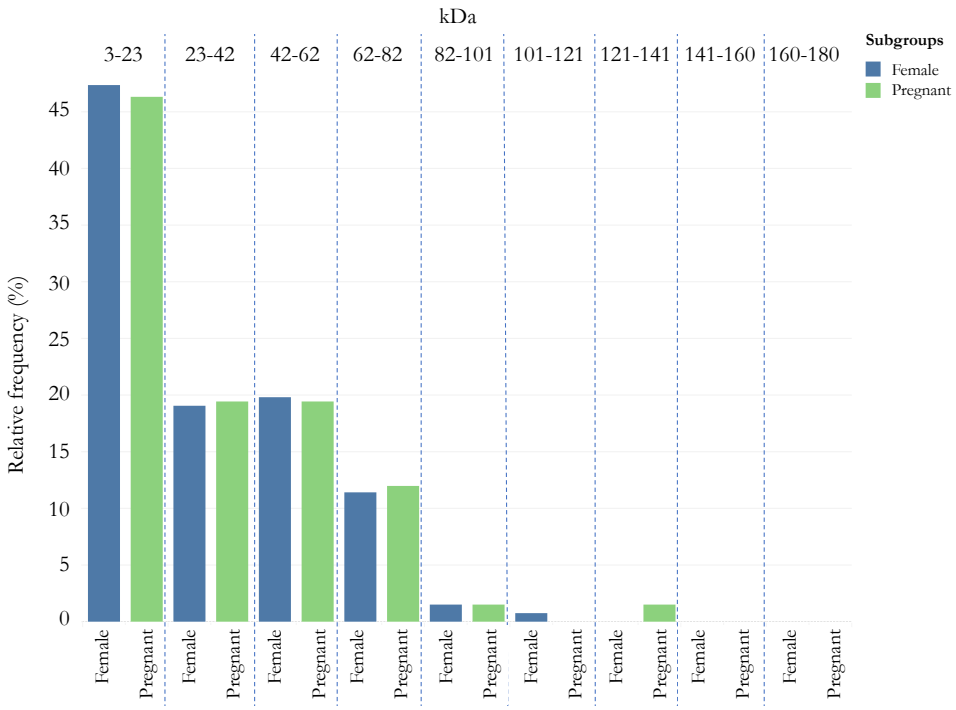


Figure 28 Graphical representation of the relative frequency of molecular mass classes (1 = 3 – 23 kDa; 2 = 23 – 42 kDa; 3 = 42 – 62 kDa; 4 = 62 – 82 kDa; 5 = 82 – 101 kDa; 6= 101 – 121 kDa; 7 = 121 – 141 kDa; 8 = 141 – 160 kDa; 9 = 160 – 180 kDa) in females (as females older than 5 years old) and pregnant females.

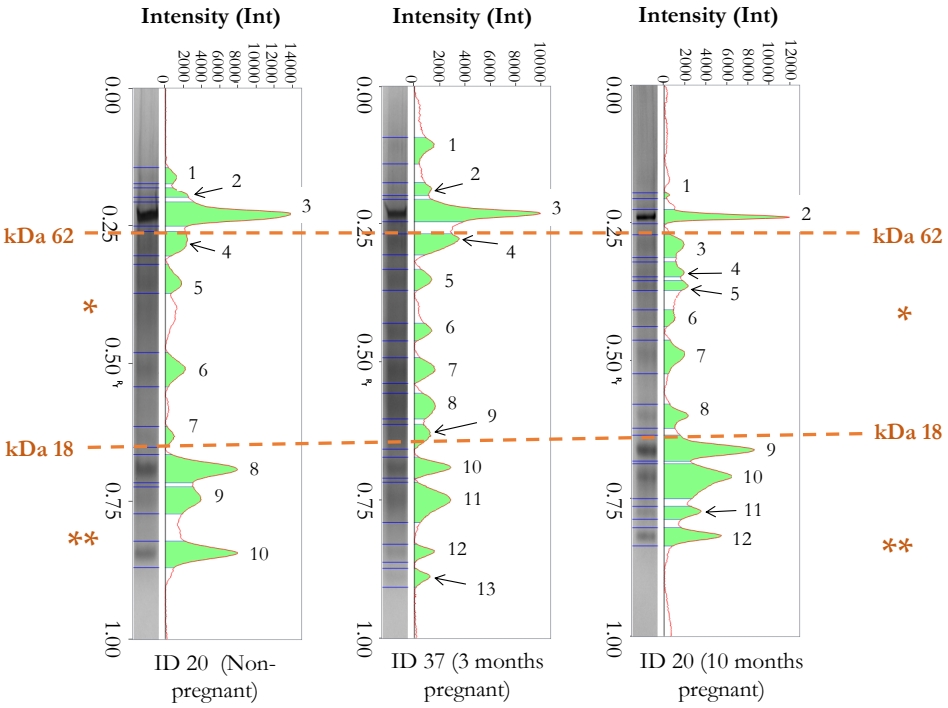


Figure 29 Pherograms of non-pregnant female (ID 20), 3 months pregnant female (ID 37) and 10 months pregnant female (ID 20). One asterisk (*) indicates the gel zone between 18 and 62 kDa and two asterisks (**) indicate the gel zone between 3 and 18 kDa (Fasoli et al, 2020 – modified).

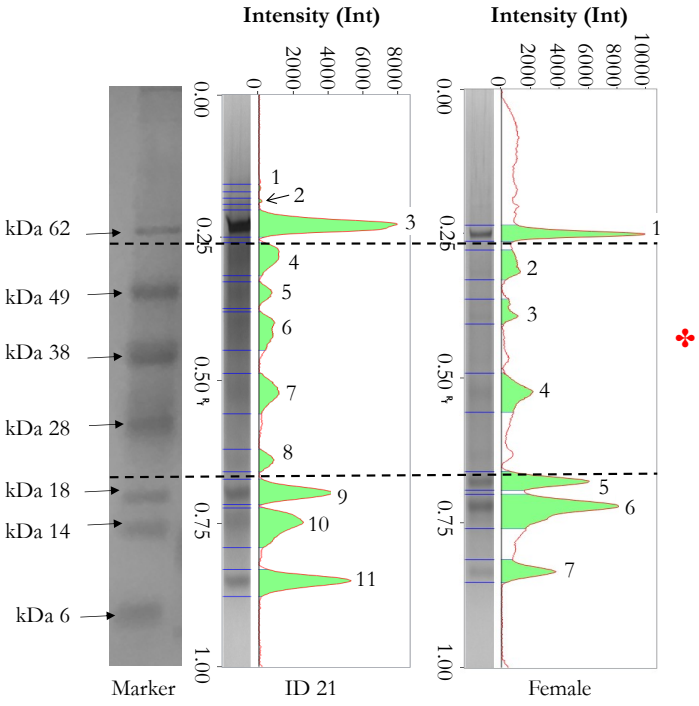


Figure 30 Pherograms of ID 21 and other female (> 5 years-old). One asterisk (✤) indicates the gel zone between 18 and 62 kDa.

2. DISCUSSION

Regarding the data about urinalysis, no difference has been reported and the data of pregnant giraffes and ID 21 were in line with the data reported for the other studied animals.

However, regarding the urine total protein (uTP), urine creatinine (uCrea) and UPC, mean values of pregnant females fell into the reference intervals established, except for one specimen (ID 37), whose UPC mean value was 0.24. This giraffe was a 3-months-pregnant female and, as reported in healthy women, proteinuria increased during pregnancy (Phillips, et al., 2017); consequently, it is reasonable to assume that urine total protein may affect the urine protein:creatinine ratio, showing an abnormal value. Indeed, the UPC mean value of the same female after pregnancy (0.14) fell into the reference limits established.

The urinary proteome of pregnant giraffes was a challenge to analyse. It might be speculated that the increase in relative frequency of proteins between 23 and 42 kDa could be due to haptoglobin, cathelicidin and bovine pregnancy-associated protein. As reported in cows, haptoglobin and cathelicidin (proteins identified in giraffe urines in this study) increase during the early days of gestation, whilst other proteins appear only when the animal is pregnant, such as the bovine pregnancy-associated protein (Pyo, et al., 2003; Rawat, et al., 2016). Another possible reason could be the presence of one group of proteins named pregnancy associated glycoproteins (PAGs). Despite the absence of their identification in urine until now, some PAGs might be the cause of the qualitative change in the urine proteome of pregnant giraffes.

Interestingly, some of these proteins are used for early pregnancy diagnosis in bovines (Balhara, et al., 2013) and more endeavours should be carried out to identify the proteins in pregnant giraffe urines and to develop likewise tools such that diagnose gestation in this wild mammal.

Considering ID 21, the MMs of some proteins detected in urine of this giraffe were similar to MMs of the proteins of pregnant females. Moreover, it may be speculated that the protein with MM of 60 kDa might be the bovine pregnancy serum protein (PSP60), a protein purified from extracts of bovine foetal cotyledons (Mialon, et al., 1933). This protein was detectable in the bovine maternal blood until 87-105 days post-partum, although the decrease of its concentration started before the calving

and it might be correlated to the decline in the binucleate cell count (Mialon, et al., 1933). However, the overall data of this giraffe might be interpreted considering that some of the putative proteins in pregnant giraffes' urines - e.g., the pregnancy associated glycoproteins (PAGs) or bovine pregnancy serum protein (PSP60) - are proteins whose concentration remains high for weeks after calving (Mialon, et al., 1933; Balhara, et al., 2013).

As previously reported, the main limit of this section lies in the narrow number of urine samples analysed. However, it is important to underline that the studies conducted on unconventional species (such as endangered species) could be informative and relevant even when carried out on small-sample size (Olea-Popelka & Rosen, 2019). Anyway, further studies are required and recommended to explore this research area.

CONCLUSIONS

Establishing the urinary reference values of giraffes can be considered a starting point for clinical applications and giraffe's husbandry, and it allows to improve the knowledge of their physiology.

The identification of the most relevant proteins of the giraffe proteome has highlighted the importance of defence against microbes and concentration of urine process. The presence of proteins in giraffe's urinary proteome, similar to those detected in other species, leads to hypothesize that healthy mammals share common physiological processes. Remarkably, some of the proteins identified in giraffes are reported in scientific literature as biomarkers for renal diseases or pregnancy in other species. The research into these aspects may encourage to study either the identification of possible new urinary biomarkers or to confirm the presence of those already validated in other species in giraffes. However, the paucity of studies on the urinary proteome in non-domestic herbivorous has made the interpretations of the results of this section demanding and difficult.

Finally, the possibility of detecting the pregnancy-related changes of urinary proteome might be an interesting opportunity to improve the husbandry of female giraffes, with the aim to help the personnel monitor pregnant giraffes' health status as the pregnancy progresses, without sedation or restrain. However, additional studies are suggested to further analyse and confirm the urinary proteome in pregnant giraffes, since this research provided a first insight into their urinary proteins.

EXPERIMENT 3

FIRST INSIGHTS INTO THE URINARY METABOLOME OF CAPTIVE GIRAFFES BY PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY[12]

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URINARY METABOLOME OF GIRAFFE

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FIRST INSIGHTS INTO URINARY METABOLOME OF CAPTIVE GIRAFFES BY PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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Abstract: The urine from 35 giraffes was studied by untargeted 1H-NMR, with the purpose of obtaining, for the first time, a fingerprint of its metabolome. The metabolome, as downstream of the transcriptome and proteome, has been considered as the most representative approach to monitor the relationships between animal physiological features and environment. Thirty-nine molecules were unambiguously quantified, able to give information about diet, proteins digestion, energy generation, and gut-microbial co-metabolism. The samples collected allowed study of the effects of age and sex on the giraffe urinary metabolome. In addition, preliminary information about how sampling procedure and pregnancy could affect a giraffe's urinary metabolome was obtained. Such work could trigger the setting up of methods to non-invasively study the health status of giraffes, which is utterly needed, considering that anesthetic-related complications make their immobilization a very risky practice.

Keywords: captive giraffes; urine; metabolomics; 1H-NMR

INTRODUCTION

According to the International Union for Conservation of Nature (IUCN), giraffe (*Giraffa camelopardalis*) is declared a vulnerable species (Muller, et al., 2018). Moreover, different measures have been taken to monitor and protect giraffe population. For example, the International Union for Conservation of Nature (IUCN) Species Survival Commission (SSC) Giraffe and Okapi Specialist Group (GOSG) was established with the aim of studying and guaranteeing the conservation needs of this species[13]. In addition, from November 26, 2019, giraffes are included in Appendix II of the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) to improve its protection, subjecting it to strict regulation[14].

Zoos represent a significant part of the protection strategy for giraffes, with projects explicitly aimed at protecting endangered species and pursuing high standards of animal welfare (Paul-Murphy & Molter, 2019). In these structures, however, giraffes may be subjected to sources of stress that reverberate negatively on individual and social behaviors (Hosey, et al., 2013a). Causes of stress could be represented by the presence of visitors and attendants (Normando, et al., 2018). Among the efforts that have been made to reduce the stressors, some are devoted to developing protocols to evaluate their general health status that do not involve immobilization, but are based on indirect methods (Hosey, et al., 2013a). In fact, giraffes are particularly prone to anesthetic-related complications and death, due to their unique cardiovascular system, making immobilization a risky practice (Dagg, 2014g; Gage, 2019).

The possibility of obtaining information from urine collected from the ground seems particularly attractive from this point of view, but the literature on this type of sampling is absent for giraffes and it has been only reported in okapi (Glatston & Smith, 1980). Indeed giraffes have been studied more for their iconic height and the mechanisms existing at the cardiovascular level to counterbalance the consequent state of primary hypertension (Agaba, et al., 2016; Hargens, et al., 1987; Zhang, 2012).

Among the completely unexplored characteristics of giraffe urine is its metabolome, the ensemble of low weight molecules produced by the cellular metabolism. Studies carried out by liquid chromatography–mass spectrometry

[13] https://www.giraffidsg.org/
[14] https://www.cites.org/

(LC/MS) or by proton nuclear magnetic resonance spectroscopy (1H-NMR) on humans and other animals suggest that the giraffe's urinary metabolome may be particularly informative about the general health of the animal. In horse urine, molecules revealing the action of the intestinal microbiota were found in micromolar concentrations (Patel, et al., 2012; Laghi, et al., 2018). Molecular patterns of the urinary metabolome linked to inflammatory processes have been identified in humans (Barbara, et al., 2017). Urinary profile responses to the calorie content of the diet were identified in rat (Kok, et al., 2018). The effects of heat stress were studied in cattle by metabolomic profiling of urine (Liao, et al., 2018). Indeed, the use of urine as a source of biological data in giraffes could be a suitable alternative, due to its non-invasive approach that could avoid the immobilization of animals.

Among the analytical platforms capable of fulfilling the requirements, proton nuclear magnetic resonance spectroscopy (1H-NMR) has been widely used for the investigation of urine metabolomes, taking advantage of its high reproducibility and minimal sample preparation.

In the present study, we wanted to verify the feasibility of 1H-NMR based metabolomic studies focusing on the urine of giraffes. For this purpose, we characterized the molecular profile of healthy giraffes held in captivity to obtain preliminary quantitative values that could be applied for the diagnosis of diseases affecting this animal. Moreover, the samples collected gave the opportunity to have a first insight about the influence of important physiological factors, such as the sex and age of the subjects, on the urinary metabolomic profile.

Materials and Methods

1. Compliance with ethical requirements

All the procedures related to animals respected the Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes (Article 1, Paragraph 1, Letter b) and the Italian legislation (D. Lgs. n. 26/2014, Article 2, Paragraph 1, Letter b).

2. Sample collection

A total of 35 captive giraffes (*Giraffa camelopardalis*) were involved in the current study. Based on physical examinations, giraffes did not show symptoms of diseases both before and during the urine sampling period. The giraffes were housed in five Italian zoos: Zoosafari Fasanolandia (FA) (N = 11), Safari Ravenna (RA) (N = 4), Giardino Zoologico di Pistoia (PT) (N = 1), Parco Natura Viva (VR) (N = 4), and Parco Faunistico Le Cornelle (BG) (N = 15).

The details for each giraffe are reported in Table 1. Their age ranged from a minimum of 6 months to a maximum of 20 years. The giraffes were categorized in 3 age classes: Young (from 6 months to 6 years old, N = 14), Adult (from 6 to 15 years old, N = 16), and Old (older than 15, N = 9), according to the following information. In female giraffes the first birth is at about 6.4 years old, even if sexual maturity is reached at 3–4 years (Bercovitch & Berry, 2010; Bercovitch & Berry, 2015). Giraffe males are considered as adults when older than 6 years old, according to Lee et al. (Lee, et al., 2017).

The samples were collected between 10:00 a.m. and 2:00 p.m., in connection to the daily activities of the keepers. Urine samples were collected with a syringe from the ground. To limit the soil contaminants, only the upper part of the urine was collected, immediately after the spontaneous voiding, before it was absorbed by the soil. A sample from one male was also collected directly into a sterile beaker, preventing the sample from touching the ground. Four urine samples were collected from two females during and after pregnancy. After collection, the urine samples were centrifuged at 1500 ×g for 10 min, to further remove potential ground contaminants, and the supernatants were frozen at −80 °C.

Table 1. Animal information (Zhu, et al., 2020b).

Sample ID	Name	Sex	Age (years)	Zoo
N.01	Ronny	Male	14	FA
N.02	Nicole	Female	14	FA
N.03	Giulietta	Female	17	FA
N.04	Marcello	Male	9	FA
N.05	Italia	Female	8	FA
N.06	Carlos	Male	2	RA
N.07	Daniele	Male	11	RA
N.08	Cleopatra	Female	20	PT
N.09	Alto	Male	2	FA
N.10	Congo	Male	0.3	FA
N.11	Roberto	Male	0.6	RA
N.12	Martina	Female	0.6	RA
N.13	Linda	Female	16	BG
N.14	Sandy	Female	16	BG
N.15	Raffa	Female	7	BG
N.16	Telete	Female	2	BG
N.17	Rusman	Male	16	BG
N.18	Akuna	Female	10	BG
N.19	Ciokwe	Male	5	BG
N.20	Miro	Male	9	BG
N.21	Lucia	Female	16	BG
N.22	Nuvola	Female	7	BG
N.23	Sahel	Female	2	BG
N.24	Russel	Male	16	BG
N.25	Ramiro	Male	3	BG
N.26	Madiba	Male	6	BG
N.27	Nasanta	Female	2	BG
N.28	Macchia	Male	5	VR
N.29	Secondo	Male	11	VR
N.30	Akasha	Male	7	VR
N.31	Quarto	Male	9	VR
N.32	Luna	Female	15	FA
N.33	Kenya	Female	20	FA
N.34	Alessia	Female	4	FA
N.35	Mina	Female	14	FA

3. METABOLOMIC ANALYSIS

We prepared urine samples for NMR by thawing and centrifuging them for 15 min at 18,630 ×g at 4 °C. We added the supernatant (350 μL) to bi-distilled water (350 μL) and to a D2O solution (200 μL) of TSP (3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid) 10 mM and of NaN3 2 mM. A 1M phosphate buffer had been used to set the D2O solution to pH of 7.00 ± 0.02. After a further centrifugation, we recorded 1H-NMR spectra at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy), at a frequency of 600.13 MHz, equipped with Topspin software (Ver. 3.5).

According to Zhu et al. (Zhu, et al., 2019), we suppressed the signals from broad resonances using a CPMG- (Carr-Purcell-Meiboom-Gill) filter composed of 400 echoes with a of 400 s and a 180° pulse of 24 s, for a total filter of 330 ms. We also applied pre-saturation, to reduce the signal from water. We employed Topspin software to apply a line broadening of 0.3 Hz and to adjust the phase of each spectrum. We set the recycle delay to 5 s, by considering the relaxation time of the protons under investigation. We employed R computational language (R Development Core Team, 2011) for any further processing of spectra, quantification of molecules, and data mining, with custom scripts.

We aligned the spectra by using the TSP signal as a reference (−0.017 ppm). We adjusted the baseline of each spectrum by distinguishing irregularities of the baseline from genuine signals, according to the "rolling ball" idea (Kneen & Annegarn, 1996), implemented in the R package "baseline" (Liland, et al., 2010). We performed the assignment of the signals by comparing chemical shift and multiplicity with the libraries (Ver. 10) of Chenomx software (Chenomx Inc., Canada, v. 8.3).

According to Dieterle et al. (Dieterle, et al., 2006), water intake behavior can change the dilution of urine as much as five times, obscuring any trend in metabolite concentrations. We removed this confounding factor by calculating, for each sample, the ratio between the area of TSP peak and the intensity of the spectrum. This allowed us to estimate the dilution of each sample and to select the one with the mostly representative dilution. We used this sample as a reference by quantifying the molecules from the added TSP. We then normalized the other samples towards the reference by probabilistic quotient normalization (PQN) (Dieterle, et al., 2006).

4. STATISTICAL ANALYSIS

We conducted the statistical analysis in R computational language (R Development Core Team, 2011) and we refined the artwork by GIMP (version 2.10, www.gimp.org). Prior to univariate analysis, we transformed the data to normality by BoxCox transformation (Box & Cox, 1964). To investigate the effects of sex on urinary metabolites, we considered only adult, non-pregnant giraffes. This allowed us to reduce potential interferences due to different age classes. We then highlighted any difference by t-test. To investigate age related effects, by removing sex effect, we applied a two-way ANOVA test followed by Tukey- HSD, by taking advantage of the "aov" function of the R package "stats" (Chambers, et al., 1992). For the above statistical tests, we accepted a cut-off p-value of 0.05.

In agreement with Bazzano et al. (Bazzano, et al., 2018), we highlighted any trend characterizing the samples with robust principal component analysis (rPCA) models (Hubert, et al., 2005), using the molecules accepted by univariate analysis as a base. We took advantage of the PcaHubert algorithm implemented in the "rrcov" package. The algorithm grants robustness with a two-steps approach. In the first step outlying samples are detected according to their distance from the others along and orthogonally to the PCA plane. A second step determines the optimal number of principal components (PCs). The main features of each rPCA model are summarized by a scoreplot and by a Pearson correlation plot. The former is the projection of the samples in the PC space and highlights the underlying structure of the data. The latter relates the concentration of each variable to the components of the model.

RESULTS

1. URINARY METABOLITES IDENTIFICATION BY UNTARGETED 1H-NMR

A representative spectrum of the metabolites identified in the giraffe's urine is reported in Figure 1. In this study, we identified 39 molecules (Table S1). These molecules mainly pertain to the classes of amino acids and derivatives and organic acids and derivatives. Hippurate (30.63%), creatinine (25.17%), and phenylacetylglycine (12.64%) were the most represented metabolites.

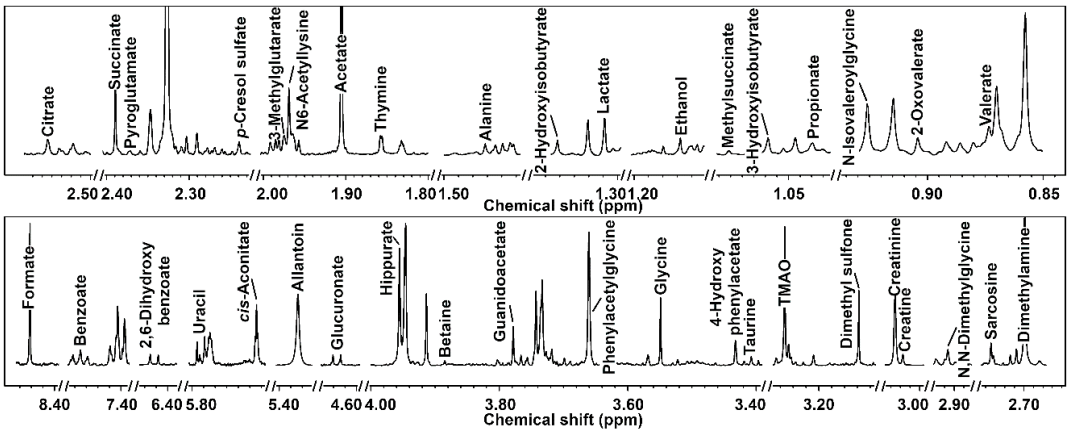

Figure 1. Portions of 1H-NMR spectra, representative of all the spectra obtained in this study. Each molecule's name appears over the NMR peak used for its quantification. To ease the visual inspection of each portion, a different spectrum with a convenient signal-to-noise ratio has been selected (Zhu, et al., 2020b).

2. EFFECTS OF SAMPLING PROCEDURE AND LOCATION

To check the potential influence of the different sampling methods, we wanted to collect pairs of samples during the same voiding, one directly and one from the ground. Unfortunately, we only succeeded in this task for one individual (Ronny). Among 39 quantified compounds, four molecules showed a variation of concentration higher than 50%, namely p-cresol sulfate, citrate, glycine, and benzoate. 1H-NMR signals for these compounds are reported in Figure 2. In detail, benzoate and glycine were more concentrated in the urine collected from the ground, while citrate and p-cresol sulfate showed the opposite trend. Overall, the 39 molecules showed a median difference between the two samples of 4.8%. As these observations were based only on one pair of samples from a single individual, we decided not to exclude these molecules from the subsequent analyses.

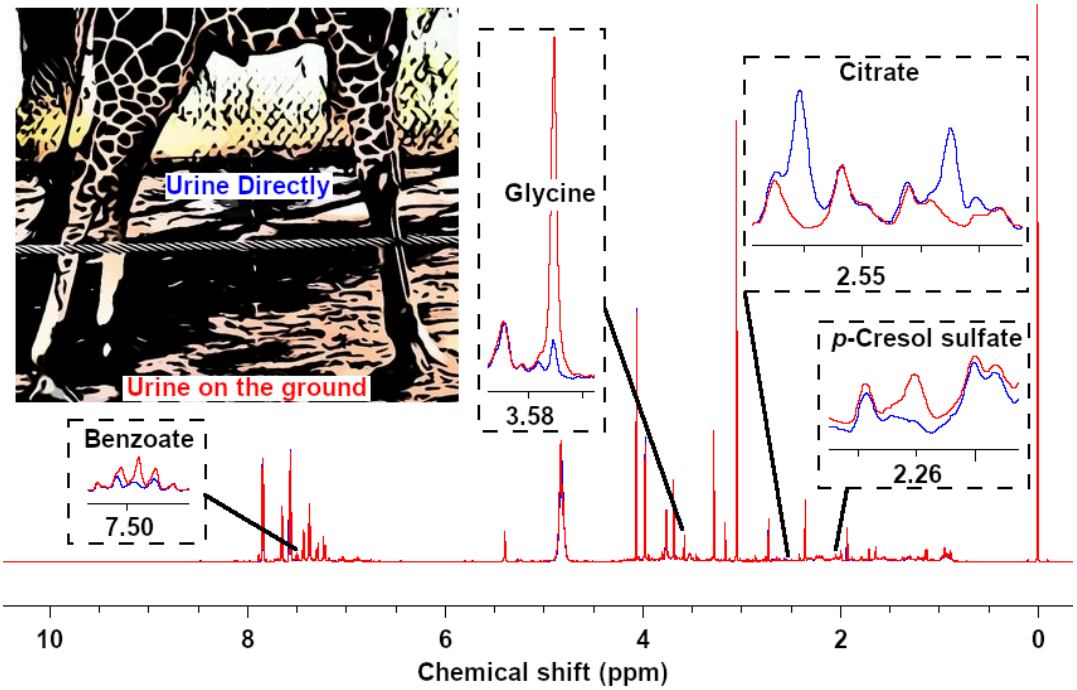

Figure 2. Representative sections of two spectra obtained from analyzing urine from the same giraffe (Ronny), collected directly (blue line) and from the ground (red line) during one urination, respectively (Zhu, et al., 2020b).

To obtain hints about the potential effects of location on the metabolome of giraffe urine, we selected the samples from the locations BG (Parco Faunistico Le Cornelle) and FA (Zoosafari Fasanolandia), where most of the samples had been collected, and we set up a three-way ANOVA analysis aiming at excluding any effect related to gender or age. None of the molecules quantified appeared as significantly different in relation to zoo, so this variable was not considered in the subsequent analyses.

3. Sex affects the giraffe urine molecular profile

To obtain preliminary data on the effect of sex on the urinary metabolome, we focused on samples collected from adult, non-pregnant individuals. Six molecules were found to be significantly ($p < 0.05$) affected by sex, as shown in Table 2.

Table 2. Metabolite concentrations (mmol/L, median (IQR)) in the adult group were significantly ($p < 0.05$) affected by sex, as assessed by t-test (Zhu, et al., 2020b).

	Female (6)	Male (7)	Trend	*P* value
Acetate	2.04 (5.23×10^{-1})	1.33 (9.04×10^{-1})	↓	0.034
Hippurate	13.50 (10.70)	19.30 (19.50)	↑	0.047
Lactate	2.77×10^{-1} (8.90×10^{-2})	1.28×10^{-1} (7.35×10^{-2})	↓	0.003
Phenylacetylglycine	7.82 (2.41)	15.20 (5.53)	↑	0.014
Succinate	2.48×10^{-1} (3.00×10^{-2})	1.66×10^{-1} (8.80×10^{-2})	↓	0.006
Thymine	1.77×10^{-1} (4.94×10^{-2})	2.86×10^{-1} (1.79×10^{-1})	↑	0.043

To have an overall view of the data, a robust principal component analysis (rPCA) model was calculated on their concentration, as shown in Figure 3.

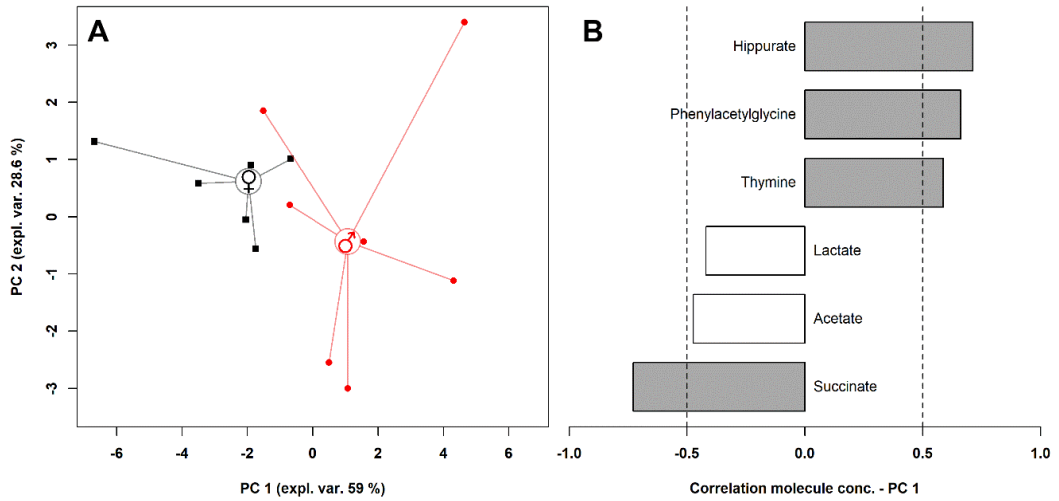

Figure 3. rPCA model calculated on the concentration of the significantly different molecules between male and female giraffes. The scoreplot (A) represents with squares and circles females and males, respectively. The median of each sample group is represented by wide circles. The loading plot (B) reports the correlation between the importance of each substance over principal component 1 and its concentration. Gray bars highlight significant correlations ($p < 0.05$) (Zhu, et al., 2020b).

Three principal components (PCs) were accepted by the algorithm to depict the overall data features. PC 1, accounting for 59% of the variance thus represented, indeed significantly summarized the peculiarities connected to sex ($p < 0.05$), with female and male individuals appearing respectively at low and high PC scores. Among these molecules, hippurate, phenylacetylglycine, and thymine were more abundant in the urine of male individuals, while lactate, acetate, and succinate were more concentrated in the females' urine.

4. Effect of age on the urinary metabolome

Age was found to significantly affect ($p < 0.05$) the concentration of three urinary metabolites, namely formate, alanine, and valerate, (Figure 4).

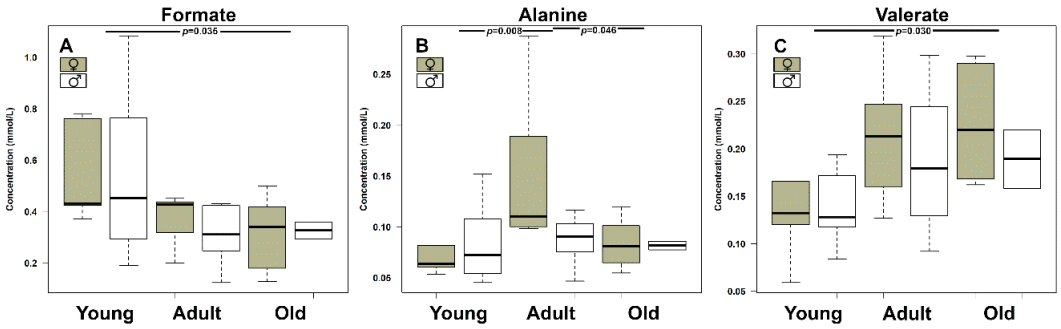

Figure 4. Boxplots showing the concentration of molecules significantly ($p < 0.05$) affected by age, as assessed by two-way ANOVA followed by Tukey post-hoc test (Zhu, et al., 2020b).

To understand if their evolution was part of a trend spanning over the entire life of the giraffe, these molecules were used as a base for an rPCA model (Figure 5). Three PCs were accepted by the algorithm to depict the overall data features. PC 1, accounting for 44.1% of the variance thus represented, summarized effectively the peculiarities connected to age ($p < 0.05$), with Young, Adult, and Old individuals appearing respectively at low, intermediate, and high PC scores. Among these molecules, formate and alanine were more abundant in young individuals, while valerate showed an opposite trend.

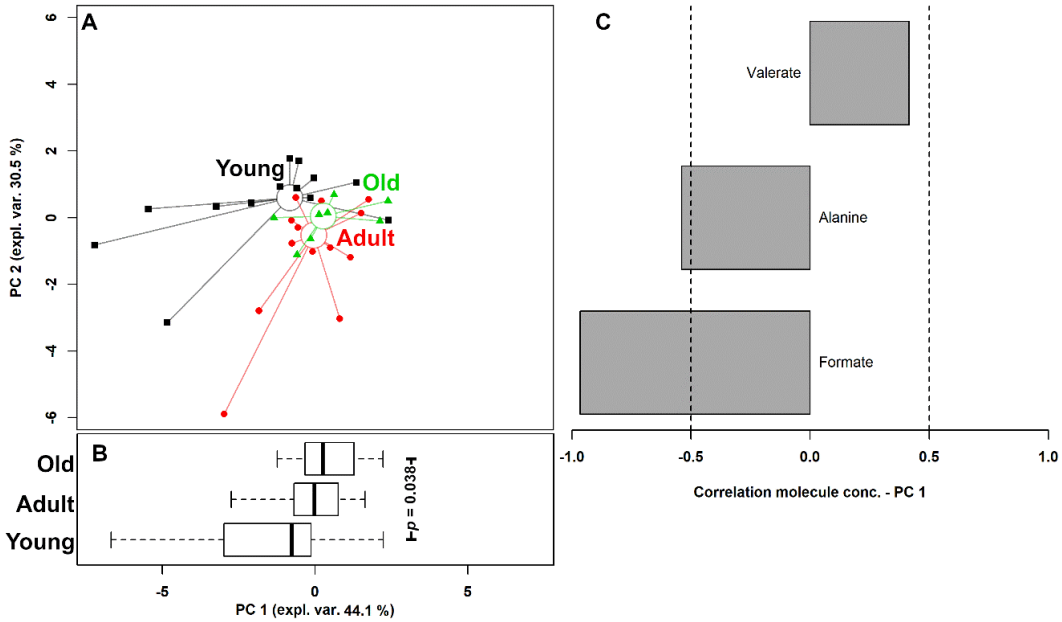

Figure 5. rPCA model of the concentration of the molecules showing a significant difference among the giraffes grouped by age. The scoreplot (A) shows the samples from the three groups with squares (Young), circles (Adult), and triangles (Old). The median of each sample group is represented by wide circles. The boxplot (B) summarizes the positions of the samples along PC1 and compares them by two-way ANOVA, followed by Tukey post-hoc test. The loading plot (C) reports the correlation between the importance of each substance over PC 1 and its concentration. Gray bars highlight significant correlations ($p < 0.05$) (Zhu, et al., 2020b).

5. Pregnancy Related Urinary Metabolome

Urine samples were obtained from two female giraffes during and after pregnancy (Table S2). Despite the limited number of samples, it was possible to observe a variation of five metabolites during the pregnancy. These molecules showed consistent trends in the samples from both giraffes. All these molecules showed a relevant increase in concentration during the pregnancy, except for phenylacetylglycine, as shown in Table 3.

Table 3. Urinary metabolites (mmol/L) affected by pregnancy consistently across the two giraffes observed (Zhu, et al., 2020b).

	Giulietta		**Nicole**	
	Not Pregnant	**Pregnant** [1]	**Not Pregnant**	**Pregnant**
Phenylacetylglycine	10.20	3.52 ↓	10.40	5.02 ↓
Benzoate	2.14	3.88 ↑	2.46	12.22 ↑
Glycine	1.06	3.04 ↑	1.79	11.65 ↑
Taurine	1.75×10^{-1}	2.93×10^{-1} ↑	7.98×10^{-2}	1.33×10^{-1} ↑
p-Cresol sulfate	1.46×10^{-2}	2.15×10^{-2} ↑	6.37×10^{-2}	3.50×10^{-1} ↑

[1] For readability, only molecules changing by more than 40% for both giraffes are shown.

DISCUSSION

The present paper describes one of the first studies ever devoted to the urinary metabolome of nonfarmed animals, and the very first focusing on the giraffe metabolome. Due to such paucity of studies on the topic, a key point that needs to be addressed before giraffe urine can be used for metabolomics studies is the possibility of relying on samples collected from the ground. Several aspects, in fact, make the collection of urine directly from the individual during urination highly impractical. To obtain a first insight on this point, we managed to collect the same urine sample either at the start of a spontaneous voiding or from the ground with a syringe at the end the voiding. The corresponding 1H-NMR spectra were highly superimposable, except for four molecules, namely benzoate, citrate, p-cresol sulfate, and glycine. The fact that the non-volatile glycine showed the greatest differences gave hints that the discrepancies could be mainly connected to dynamic variations in composition during urination, in agreement with Sink and Weinstein (Sink & Weinstein, 2012; Tang, 2013; Theodorou & France, 2009). Modifications induced by the collection method could therefore be considered a confounding factor of lower entities than inhomogeneity in the composition of urine during voiding.

The 39 molecules identified give information about protein digestion, diet, gut-microbial co-metabolism, and energy production. Their quantitative observation therefore offers a handy perspective of the health status of giraffes, through a quintessentially non-invasive sampling method.

Comparisons with the urinary metabolome of other animals are also possible, giving indirect information about the differences in metabolism. An example of this possibility is offered by allantoin. This molecule is the fourth most concentrated in giraffe urine (Table S1), identically to yak (*Bos grunniens*) (Zhu, et al., 2019) and horse (Zhu, et al., 2018). Differently from these strictly herbivorous animals, this molecule is the most concentrated in the urine of the giant panda (Zhu, et al., 2020a), even if the giant panda consumes an amount of vegetables in relation to body weight (as much as 30%) much higher than ruminants or horses, which should lead to the lowest concentration of urinary allantoin (Chen, et al., 1991). This apparent contradiction leads to speculate that the main mechanism determining the concentration of allantoin

in the urine of the above-mentioned animals is likely to be its renal reabsorption, which is very effective in strictly herbivorous animals (Chen, et al., 1991).

1. Sex affects the giraffe urine molecular profile

In the current study acetate, succinate, and lactate concentrations appeared to be significantly higher in female giraffe urine, while hippurate, phenylacetylglycine, and thymine were more concentrated in male urine. For acetate, two of the authors of the present paper identified a similar situation in horse urine (Zhu, et al., 2018). For the other molecules, indirect connections with published findings can be devised. There is an abundance of references, focusing on humans, showing that exercise leads to higher concentrations of acetate, succinate, and lactate in urine, and lower concentrations of thymine and hippurate (Enea, et al., 2010; Mukherjee, et al., 2014; Sheedy, et al., 2014). Ginnett et al. showed that female giraffes spend more time walking, foraging, feeding, and traveling than males (Ginnett & Demment, 1997). The two observations seem to suggest that the sex-related differences observed in the urine of males and females may be partly due to the different daily activities. Contrary to the previously reported molecules, phenylacetylglycine is mainly a co-metabolite of gut microorganisms, derived from valine, leucine, phenylalanine, lysine, or ornithine (Mayneris-Perxachs, et al., 2016). Its different concentration in relation to sex may therefore reflect peculiarities in gut microbiota profiles or different foraging behaviors, similarly to what was recently observed in the giant panda (Zhu, et al., 2020a). Ginnett et al., in fact, demonstrated that males prefer larger bites than females, with potential consequences on the food, and in turn urine, metabolome profile (Ginnett & Demment, 1997). It is tantalizing to speculate that the length of the neck, which is higher in males (Dagg, 2014g), may play a role too. In fact, Schußler and Greven (Schußler & Greven, 2017) found an allometric direct relationship between rumen-to-mouth distance and the duration of rumination intercycles, influencing in turn the digestive action of ruminal microorganisms.

2. Effect of age

By removing the gender effect by two-way ANOVA, it was possible to focus on the effect of age. In parallel with previous studies in rats and humans (Schnackenberg, et al., 27-30 August 2007; Slupsky, et al., 2007), formate and alanine

were negatively related to age. The trend observed for formate is very likely related to the gut microbiome. In fact, in the gut microbiota of the juvenile giraffes there is a prevalence of *Bacteroides* and *Acinetobacter* genera, responsible for the degradation of starch and cellulose to formate (Theodorou & France, 2009), while in the gut of adult giraffes other genera tend to prevail, such as *Treponema* (Schmidt, et al., 2018).

The concentration of amino acids in urine has been consistently linked to the turnover of muscle amino acids (Soupart, 1959; Zhu, et al., 2018), with urinary concentration of alanine specifically related to exercise (Pechlivanis, et al., 2010). Therefore, the difference in the concentration of alanine could be ascribed to a variation of daily activity intensity along age.

3. Effect of pregnancy

Early identification of pregnant giraffes with maximum accuracy is an important issue for optimizing their management. Although some diagnostic methods (e.g., ultrasonography) have been described in domestic animals (Karen, et al., 2004), their application to wild or captive animals is hindered by practical reasons. Metabolomics approaches seem in principle promising for setting up diagnostic methods that might be more convenient in specific contexts, due to the possibility to quantify a high number of molecules at the same time. However, previous studies performed in domestic animals were focused on serum (Sun, et al., 2017; Kenéz, et al., 2016), a sub-optimal sample from the point of view of non-invasivity. Therefore, despite the restricted number of samples analyzed in the present study, the obtained data can provide a preliminary urinary fingerprint of pregnancy in giraffes.

Taurine is an important amino acid during pregnancy and lactation, because it satisfies the needs of both the fetus and suckling infant. In our research, taurine excretion through urine increased during early pregnancy, consistent with human studies (Diaz, et al., 2013). Taurine is rarely found in plants (Bouckenooghe, et al., 2006), so that herbivores cannot obtain a sufficient amount taurine from the diet. Remarkably, in ruminants the urinary taurine concentration is strongly diet-dependent, as can be inferred from the works of Bristow et al. on cows fed with maize silage compared to free grazing cows (Bristow, et al., 1992). Diet is therefore likely to trigger biosynthetic pathways, such as the one leading to taurine from methionine (Dasarathy, et al., 2010). Moreover, a specific pathway, converting homocysteine to taurine and

glycine through cysteine, is known to become effective in early pregnancy (Dasarathy, et al., 2010). This latter mechanism is a likely reason for the increasing trend of taurine excretion we found in the present work.

A further contribution to urine metabolome profile modifications may be due to changes in the gut microbiota. In fact, among the molecules showing the greatest changes we found p-cresol sulfate and phenylacetylglycine, mainly described as gut microorganism co-metabolites (Mayneris-Perxachs, et al., 2016; Patel, et al., 2012), absorbed at the intestinal level and then expelled through urine. Interestingly, the change in the concentration of both has been related, in humans, with alterations in the microbiota profile linked to inflammatory states (Barbara, et al., 2017; Sarosiek, et al., 2016), in which pregnancy is known to play a role (Edwards, et al., 2017). Despite the very limited number of cases here, these observations support the compelling possibility to use the urine metabolome to gain specific information about giraffe inflammatory status during pregnancy, as modulated by the gut microbiota.

CONCLUSIONS

This work represents a primer in giving quantitative information about the urinary metabolome of captive giraffes, as detected by untargeted 1H-NMR. Foraging behaviors and daily activity could be considered as one of the main reasons for the differences we highlighted that are linked to sex and age. A preliminary observation conducted on two female giraffes suggests that 1H-NMR based metabolomics could be conveniently applied to monitor modifications occurring during pregnancy, some of which are potentially related to inflammatory status triggered by modification of the microbiota profile.

Author Contributions: C.Z., L.L., S.F., and G.I. conceived and designed the research. C.Z. and L.L. performed metabolomics analysis. S.F. collected the samples. C.Z., L.L., S.F., and G.I. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: The following are available online at www.mdpi.com/2218-1989/10/4/157/s1

Table S1: Concentration (mmol/L, median (IQR)) of the molecules quantified by 1H-NMR in all the samples studied in the present investigation, sorted by abundance (Zhu, et al., 2020b).

Molecule	Concentration
Creatinine	20.9 (2.00)
Hippurate	19.1 (5.81)
Phenylacetylglycine	8.95 (2.13)
Allantoin	6.87 (2.46)
Benzoate	2.13 ($7.38x10^{-1}$)
Acetate	1.49 ($7.27 x10^{-1}$)
Glycine	1.15 ($6.96 x10^{-1}$)
Glucuronate	$8.68x10^{-1}$ ($2.10x10^{-1}$)
Trimethylamine N-oxide	$7.90x10^{-1}$ ($6.22x10^{-1}$)
Dimethyl sulfone	$7.40x10^{-1}$ ($3.11x10^{-1}$)
Guanidoacetate	$7.24x10^{-1}$ ($1.59x10^{-1}$)
4-Hydroxyphenylacetate	$5.84x10^{-1}$ ($2.89x10^{-1}$)
Dimethylamine	$5.14x10^{-1}$ ($3.10x10^{-1}$)
N6-Acetyllysine	$4.95x10^{-1}$ ($1.20x10^{-1}$)
3-Methylglutarate	$4.24x10^{-1}$ ($1.08x10^{-1}$)
Formate	$4.00x10^{-1}$ ($1.56x10^{-1}$)
N-Isovaleroylglycine	$2.90x10^{-1}$ ($5.40x10^{-2}$)
cis-Aconitate	$2.77x10^{-1}$ ($5.18x10^{-2}$)
Pyroglutamate	$2.62x10^{-1}$ ($8.77x10^{-2}$)
Propionate	$2.58x10^{-1}$ ($1.38x10^{-1}$)
Taurine	$2.02x10^{-1}$ ($1.93x10^{-1}$)
Thymine	$1.81x10^{-1}$ ($7.96x10^{-2}$)
Lactate	$1.75x10^{-1}$ ($1.16x10^{-1}$)
Valerate	$1.66x10^{-1}$ ($6.32x10^{-2}$)
Betaine	$1.64x10^{-1}$ ($3.87x10^{-2}$)
3-Hydroxyisobutyrate	$1.50x10^{-1}$ ($1.93x10^{-2}$)
Succinate	$1.49x10^{-1}$ ($7.69x10^{-2}$)
Creatine	$1.45x10^{-1}$ ($3.64x10^{-2}$)
Ethanol	$1.17x10^{-1}$ ($4.65x10^{-2}$)
2-Oxovalerate	$1.14x10^{-1}$ ($2.84x10^{-2}$)
Sarcosine	$1.08x10^{-1}$ ($4.99x10^{-2}$)
2,6-Dihydroxybenzoate	$1.02x10^{-1}$ ($2.65x10^{-2}$)
Alanine	$8.55x10^{-2}$ ($2.68x10^{-2}$)
Citrate	$6.56x10^{-2}$ ($2.14x10^{-2}$)
N,N-Dimethylglycine	$6.27x10^{-2}$ ($6.07x10^{-2}$)
2-Hydroxyisobutyrate	$5.81x10^{-2}$ ($4.12x10^{-2}$)
Uracil	$5.55x10^{-2}$ ($1.83x10^{-2}$)
p-Cresol sulfate	$4.86x10^{-2}$ ($4.10x10^{-2}$)
Methylsuccinate	$2.43x10^{-2}$ ($7.11x10^{-3}$)

Table S2. Concentration (mmol/L) of the molecules quantified by 1H-NMR in the samples collected during and after pregnancy (Zhu, et al., 2020b).

	Giulietta		Nicole	
	Not Pregnant	**Pregnant**	**Not Pregnant**	**Pregnant**
Formate	2.91×10^{-1}	3.03×10^{-1}	2.00×10^{-1}	2.82×10^{-1}
Benzoate	2.14	3.88	2.46	12.22
2,6-Dihydroxybenzoate	9.83×10^{-2}	1.82×10^{-1}	1.10×10^{-1}	1.07×10^{-1}
Uracil	6.27×10^{-2}	8.18×10^{-2}	8.60×10^{-2}	5.76×10^{-2}
cis-Aconitate	2.72×10^{-1}	2.55×10^{-1}	2.19×10^{-1}	2.09×10^{-1}
Allantoin	6.76	9.86	9.87	6.53
Glucuronate	7.18×10^{-1}	4.70×10^{-1}	3.70×10^{-1}	3.64×10^{-1}
Hippurate	24.7	21.6	23.00	8.11
Betaine	2.01×10^{-1}	1.46×10^{-1}	1.29×10^{-1}	1.24×10^{-1}
Guanidoacetate	8.07×10^{-1}	7.54×10^{-1}	4.95×10^{-1}	7.05×10^{-1}
Phenylacetylglycine	10.20	3.52	10.40	5.02
Glycine	1.06	3.04	1.79	11.65
Acetoacetate	4.27×10^{-2}	3.70×10^{-2}	4.33×10^{-3}	8.08×10^{-3}
4-Hydroxyphenylacetate	3.55×10^{-1}	3.62×10^{-1}	6.19×10^{-1}	7.81×10^{-1}
Taurine	1.75×10^{-1}	2.93×10^{-1}	7.98×10^{-2}	1.33×10^{-1}
Trimethylamine N-oxide	1.36	1.38	1.52	1.44
Dimethyl sulfone	9.76×10^{-1}	1.09	4.64×10^{-1}	1.17
Creatinine	13.1	15.80	12.90	14.10
Creatine	1.17×10^{-1}	9.12×10^{-2}	1.27×10^{-1}	1.22×10^{-1}
N,N-Dimethylglycine	6.22×10^{-2}	9.52×10^{-2}	2.49×10^{-2}	1.28×10^{-1}
Sarcosine	1.74×10^{-1}	1.66×10^{-1}	1.12×10^{-1}	2.01×10^{-1}
Dimethylamine	5.16×10^{-1}	9.48×10^{-1}	8.00×10^{-1}	9.72×10^{-1}
Citrate	3.14×10^{-2}	2.70×10^{-2}	3.82×10^{-2}	4.07×10^{-2}
Succinate	1.32×10^{-1}	5.26×10^{-2}	1.98×10^{-1}	1.56×10^{-1}
Pyroglutamate	3.28×10^{-1}	3.24×10^{-1}	2.64×10^{-1}	2.29×10^{-1}
p-Cresol	1.46×10^{-2}	2.15×10^{-2}	6.37×10^{-2}	3.50×10^{-1}
3-Methylglutarate	4.59×10^{-1}	4.66×10^{-1}	4.93×10^{-1}	4.12×10^{-1}
N6-Acetyllysine	5.71×10^{-1}	5.95×10^{-1}	5.46×10^{-1}	4.70×10^{-1}
Acetate	1.94	1.47	1.57	3.97
Thymine	1.73×10^{-1}	1.36×10^{-1}	1.58×10^{-1}	1.40×10^{-1}
Alanine	6.47×10^{-2}	6.65×10^{-2}	1.86×10^{-1}	1.85×10^{-1}
2-Hydroxyisobutyrate	1.04×10^{-1}	4.72×10^{-2}	3.23×10^{-2}	3.25×10^{-2}
Lactate	6.83×10^{-2}	1.52×10^{-1}	2.12×10^{-1}	1.46×10^{-1}
Ethanol	1.17×10^{-1}	1.09×10^{-1}	1.15×10^{-1}	1.41×10^{-1}
Methylsuccinate	1.34×10^{-2}	1.62×10^{-2}	1.58×10^{-2}	2.24×10^{-2}
3-Hydroxyisobutyrate	1.57×10^{-1}	1.52×10^{-1}	1.64×10^{-1}	1.42×10^{-1}
Propionate	3.04×10^{-1}	1.71×10^{-1}	1.48×10^{-1}	6.24×10^{-1}
N-Isovaleroylglycine	2.92×10^{-1}	2.68×10^{-1}	3.12×10^{-1}	2.77×10^{-1}
2-Oxovalerate	9.38×10^{-2}	1.35×10^{-1}	9.90×10^{-2}	1.25×10^{-1}
Valerate	1.68×10^{-1}	1.63×10^{-1}	2.45×10^{-1}	1.98×10^{-1}

GENERAL DISCUSSION

This research project achieved the following aims: the evaluation of the reliability of a non-invasive sampling method from the ground by a syringe and its application in giraffes; the establishment of the urinary reference values and the definition of preliminary urinary proteome and metabolome.

The reliability of non-invasive approach to collect urine (proved both by the analysis in the Experiment 1 and the data of giraffe's urine sample collected either in sterile urine cup and with a syringe from the ground in the Experiment 1 and 3) makes this technique applicable, irrespective of the giraffe's husbandry. Most of all, it helps zoo personnel to pursue the welfare of animals, either by guaranteeing early diagnosis of urinary and renal diseases, or by reducing anaesthesia-related complications.

Despite their fascinating cardiovascular adaptations and imposing stature, which have intrigued scientists throughout history (Agaba, et al., 2016), giraffes possess some features in common with the other herbivores.

The urinary reference intervals determined in giraffes are similar to those reported in healthy cows (Isani, et al., 2018; Hermann, et al., 2019; Ferlizza, et al., 2020b).

The metabolites identified in giraffes' urine share a similarity with those reported in other species. For instance, in the urinary metabolome of the yak (*Bos grunniens*), some of the most concentrated metabolites were creatinine, hippurate and allantoin (Zhu, et al., 2019), similar to giraffes. Creatinine was also reported in sheep's, camels', goats' and horses' urine (Li, et al., 2011; Escalona, et al., 2015; Ahamad, et al., 2017; Zhu, et al., 2018; Contreras-Jodar, et al., 2019). Hippurate was also found in the urine of sheep and horses (Li, et al., 2011; Escalona, et al., 2015; Zhu, et al., 2018). Phenylacetylglycine was described in urinary metabolome of sheep and horses (Li, et al., 2011; Escalona, et al., 2015). Allantoin was reported in goats and horses (Escalona, et al., 2015; Zhu, et al., 2018; Contreras-Jodar, et al., 2019). Glycine was reported in goats', horses' and cows' urine (Escalona, et al., 2015; Ahamad, et al., 2017; Zhu, et al., 2018; Contreras-Jodar, et al., 2019).

Some of the metabolites identified in giraffe urine are well-known components of the digestive process in rumen and, more generally, of the energy metabolism. Formate is a metabolite originated from the degradation of amide and cellulose by

Bacteroides, an anaerobic bacteria genus also reported in giraffes (Fuller, 2004; Theodorou & France, 2009; Schmidt, et al., 2018). Glycine and alanine produce acetyl-CoA via pyruvate, which is an important player in metabolic processes, as well as succinate, one intermediate of the tricarboxylic acid cycle (McDonald, et al., 2010a). In turn, pyruvate plays an important role in the ruminal digestion, since it leads to the major end products of rumen carbohydrate digestion: acetic, propionic and butyric acids, carbon dioxide and methane (McDonald, et al., 2010a; McDonald, et al., 2010b). In fact, just like other ruminants, in giraffes' guts, microbes ferment plants in order to generate volatile fatty acids, which serve as the main energy source (Agaba, et al., 2016).

Moreover, the majority of the proteins identified in giraffes was also previously reported in the urine of other herbivores, such as camels (Alhaider, et al., 2012) and cows (Bathla, et al., 2015), proposing the existence of a shared set of proteins in the urine of healthy herbivores.

Therefore, an overall viewpoint of all the proteomic and metabolomic data obtained in this thesis leads to consider giraffes just like other domestic ruminants. However, the paucity of studies on exotic animals has severely limited the possibility of comparing the giraffes with other exotic species. Nevertheless, among non-domestic animals that – in different manners – might possess some features in common with giraffes, particular attention should be given to *Camelus dromedarius*, with whom giraffes share some of the characteristics of their urinary proteins and the presence of unique genetic changes involved in the immune response (Alhaider, et al., 2012; Hoter, et al., 2019).

A recent research on giraffe's genome revealed, through comparative analyses, that some genes exhibit unique genetic changes in this species and probably contributed to giraffe's unique features (Agaba, et al., 2016). Interestingly, among the genes that exhibited positive selection in giraffe, some were enriched in natural killer cell activation and immune response (Agaba, et al., 2016). This is particularly remarkably in the view of the results reported in the Experiment 2 of this thesis (Table 26).

Table 26 Immune-related biological processes of proteins identified in giraffe's urine (Experiment 2) and immune-related biological processes wherein genes exhibiting positive selection in giraffes are involved; [1]: (Agaba, et al., 2016).

Biological processes of proteins identified in giraffe's urine in this study	Biological processes wherein genes exhibiting positive selection in giraffe are involved [1]
Cell killing Immune system process Response to stimuli	B cell mediated immunity Cellular defence response Immune system process Macrophage activation Natural killer cell activation Response to stimulus

Even in *C. dromedarius* the genes involved in immune responses were found superior in terms of accelerated evolution compared to their homologs in cattle species (Hoter, et al., 2019). Moreover, it has been suggested an unusual immune system in the camelids; particularly in *C. dromedarius*, the identified urinary proteins were mainly located in the extracellular region and were involved in the immune response (Alhaider, et al., 2012; Warda, et al., 2014). Therefore, it may be speculated that an excellent efficiency of the immune system might be considered as a trait that giraffes share with camels.

Another common point between giraffe and *C. dromedarius* is the presence of clusterin in both of their urinary proteome, which leads to hypothesise that this protein could have a role in these animals' ability to deal with the drought.

Clusterin, in fact, is a protein involved in various physiological processes (Shannan, et al., 2006). A study on camelid genomes, which also analysed the expression of genes related to cell protection in renal medulla, has highlighted that the expression level of the gene *clusterin* dramatically increased (by about 8.9-fold) and it showed the highest transcription level in the renal medulla in camels under water restriction conditions, leading the authors to suggest that this gene may play a major role in the cytoprotection of the renal medulla during water restriction (Wu, et al., 2014).

Notably, even the genes encoding the epithelial Na^+ channel (ENaC), responsible for the reabsorption of sodium in the kidney, were upregulated in the renal cortex and medulla of camels under water restriction conditions, leading the authors to hypothesize that camels regulate the activity of this channel to cope with different physiological water requirements (Wu, et al., 2014). Since the ENaC is regulated by

ubiquitylation (Ronzaud & Staub, 2014) and given the presence of ubiquitin in the giraffe urinary proteome, it might be speculated that, even in giraffes, a similar process enabling them to cope with the drought might be in place.

Overall, this similarity may be understandable if one considers the habitat where giraffes and camels live (Dagg, 2014c; Abdalla, 2020). Giraffe, in fact, seldom drinks even in semi-desert areas, and the Arabian camel (*C. dromedarius*) is able to efficiently excrete highly concentrated urine (Dagg, 2014c; Hoter, et al., 2019). It is reasonable to theorise that these two mammals might share physiological adaptations which enable them to withstand the shortage of water in their habitat. Therefore, further studies are encouraged, given the paucity of the knowledge on renal physiology of giraffes.

Considering these common points between camels and giraffes, future researchers are encouraged to shed light on the mechanisms responsible for water reservation in giraffes. Moreover, more findings might confirm if giraffes do possess the same adaptations of camels. They might give meaningful information about how giraffes respond to the drought and the mechanisms settled up to reabsorb water when the animals don't have the possibility to have frequent access to a water source.

GENERAL CONCLUSIONS

Urines collected from the ground could be considered a useful method for monitoring the urinary and renal function in giraffes. Reliable results were obtained and they allowed to collect information about giraffe's health status without sedation and anaesthesia. However, it is important to highlight that this approach is not free of limitations. Ground or soil contaminants might affect the results, especially if they are obtained through dipstick test; to reduce this, the collection should be performed in a cautious manner.

Establishing the urinary reference values allowed to improve the physiological knowledge about this mammal and could be considered a useful tool for clinical pathologists, veterinarians and researchers specialized in Zoo Animal Medicine. Urinary proteome revealed a specific protein pattern that is partially superimposable with previous findings in other species. Proteins identified in giraffes' urine could be involved in the defence against pathogens and in the regulation of the urine concentrations. However, it must be highlighted that the data were obtained on urine collected from captive giraffes, which have *ad libitum* access to water. This aspect might explain why their urine were not much concentrated.

The study of urinary metabolome permitted the identification and quantification of interesting metabolites, which gave information about giraffe's physiology, e.g., diet or protein digestion, daily activities and the way they eat, which in turn reflected changes in molecule concentration. The differences in the urinary metabolome between females and males depicted how these data might be helpful to obtain information about this species. Likewise, the influence of age, as well as pregnancy, revealed important new data that were obtained by a non-invasive technique, proposing alternative strategies to investigate the physiological features of wild/zoo mammals.

Research should be encouraged since it would be interesting to perform these experiments in wild animals, to confirm and expand what was discovered on captive giraffes.

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