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Nutritional Ecology of *Testudo hermanni hermanni* in the Albera, Catalonia

In this research project on the Albera tortoise (*Testudo hermanni hermanni,* the only native tortoise in Catalonia) the diet of the tortoises in the wild was studied, using the micro-histological technique for plant species determination from faecal samples (Fig. 1a, b). Besides this, the parasite load of the tortoises was assessed. The results will be useful for the evaluation and perhaps the adjustment of the nutrition of the tortoises in the Centre de Reproducció de Tortugues de l'Albera.

Material and Methods

The Albera tortoise and the CRT

Hermann's Tortoise, *Testudo hermanni* (Family Testudinidae), is a medium-sized terrestrial species (average carapace length ca. 130 to 180 mm), widespread in the European Mediterranean region. Two subspecies are currently recognized: *Testudo hermanni hermanni* (distribution: Western Europe from Spain through western and southern Italy); and *Testudo hermanni boettgeri* (distribution: Eastern Europe from north-eastern Italy to European Turkey). Most western populations of the species are in strong decline and have very restricted distributions, and captive breeding and reintroduction programs are necessary only for the most threatened populations (Bertolero *et al.* 2011).

For the western populations, a conservation genetic survey showed that, within the continental cluster (Albera in Spain,

Fig. 1a.
Wild Albera tortoise sitting on dry Oak (*Quercus suber*) leaves.
Photo B. Pfau

Var in France and continental Italy), the Albera tortoise had a rather low genetic variability and there had been a demographic bottleneck (Zenboudji *et al.* 2016). This population is in strong decline, and there are regular population surveys of the wild population, and special projects for the protection (Claparols Fabri *et al.* 2009, Dorado 2010, Paniagua Salazar *et al.* 2011, Villardell *et al.* 2012, Villardell-Bartino *et al.* 2015, Vidal 2019). For the conservation of these tortoises in the wild, the Paratge Natural d'Interès Nacional de l'Albera has been constituted in 1987, and the Centre de Reproducció de Tortugues de l'Albera (CRT) breeds and raises Albera tortoises for population reinforcement since, and coordinates the different projects for conservation (Capalleras *et al.* 2013, Vila de Vicente *et al.* 2017).

Collecting the samples

The plant and faeces samples were collected either during the population census together with the biologist Nil Torres Orriols, who visited some very good tortoise habitats on a regular basis to better monitor population structure and density (Bertolero *et al.* 2020), or during walks (Fig. 2a, b) in the activity time (usually 8:00 to 12:00) in tortoise habitats near the CRT in Garriguella. The tortoises were either seen directly, or they could be found when listening to the typical rustling noises of dry leaves or by following the tortoise trails to the hiding place.

Usually, wild tortoises defecate when caught and handled (Del Vecchio *et al.* 2013). For getting fresh faecal samples the tortoises which were found, each tortoise was placed in a separate, white, plastic pail and left there until defecation had taken place, which sometimes took a few hours. Afterwards, the animal was released immediately at the place it had been found.

For this research a special permit had been issued, and since the tortoises were collected in their main activity time in rather lush habitats, it was not necessary to replace the fluids they lost when defecating by injecting electrolyte solutions. None of the field workers had any contact with other tortoises besides the Albera tortoises, therefore the veterinarian in charge for the CRT tortoises did not recommend such a strict and elaborate biosecurity protocol as in other wild tortoise research projects (Marini *et al.* 2020, Martínez-Silvestre & Soler 2020).

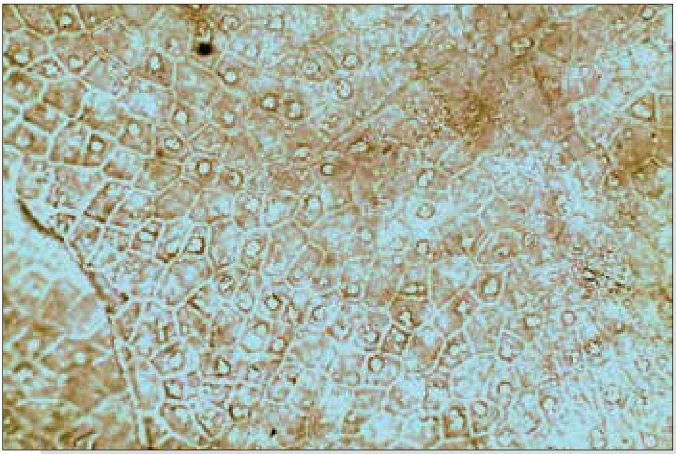

Fig. 1b.
Micro-histology of *Quercus suber* leaf epidermis.
Photo M. Loss

Fig. 2a.
The first author is searching for tortoises, and transporting the sampling equipment in a pail.
Photo N. Torres

Fig. 2b.
The sample cups were brought back to the CRT and sometimes inspected by the specialists, here by Joan Budó.
Photo M. Loss

The faecal pellet was usually solid (Fig. 3) and could be collected using forceps. It was then put into a labelled (date, location, presence/absence of parasites and sex of the tortoise) transparent plastic cup with a lid which was stored in a freezer until analysis.

If nematodes were present, they often could be observed directly when crawling around inside the cup. (Fig. 4) When the samples were analysed the faecal pellet was crushed, and then it was obvious whether nematodes had been present or not. The nematode genus and species was not determined, and the parasite load of the tortoise could not be quantified.

The micro-histological technique

To better analyse plants in herbivore faeces the samples have to be macerated first. A common method for analysing herbivore diets has been described by Holechek (1982). Castellaro *et al.* (2007) showed that the plant epidermis in herbivore faecal samples can be identified to species by using form and size of the cells, the stomata and the trichomes, and this method is routinely used in the lab of Jordi Bartolomé at the

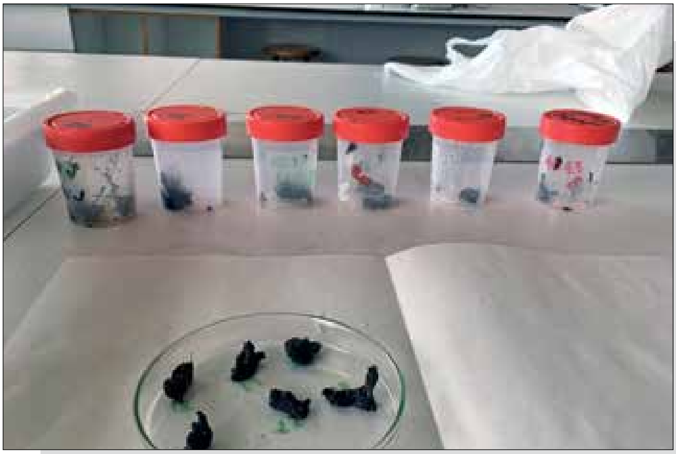

Fig. 3.
Faecal samples.
Photo M. Loss

Universitat Autònoma de Barcelona for the analysis of faecal samples of ungulates (Fig. 5). The lab already had reference collection of plant epidermis slides and training material (Fig. 6), but in this research project, this reference collection should be expanded to contain the tortoise food plants.

In the actual research project the sample to be analysed (either fresh plant leaves or tortoise faeces) was ground with a mortar to a homogenous paste, then ca. 0.5 g of this mass was filled into a test tube, covered with ca. 2 ml of 65% nitric acid, and incubated for 2 min in a thermostat in water at 80°C. Afterwards, the content of the test tube was poured into a larger beaker and mixed with 200 ml of distilled water, and then poured through two sieves, which were installed one above the other, the upper one with a mesh width of 0.5 mm and the lower one with 0.125 mm (Fig. 7). From the lower sieve, a small quantity of material was mounted on a microscopic slide with tree small drops of 50% glycerine, fixed with DPX or a similar mounting medium, and covered with a cover glass. (Fig. 8) From each sample, three slides were prepared as described by El Mouden *et al.* (2006) for their analysis of faecal samples of Moroccan *Testudo graeca graeca*.

Plant species identification

To aid with the determination an auxiliary table was created, and each plant

Fig. 4. Fresh faecal sample with nematodes in sample cup. Photo M. Loss

species was classified in groups with similar characteristics. We used, for example, the features of the stomata (roundish, or elongate, or small, or medium, or large, or with annexed cells or without them), and we used the trichomes (specular, or star-shaped, or with markedly round base, or single-cell, or multi-cell), and we also used the epidermis cells (roundish, or polygonal, or puzzle-piece-shaped, or with a thick cell wall, or with a thin cell wall…).

The preparations were examined with a microscope at 4x to 100 x magnification. A grid with three horizontal lines was projected on the viewing area, and for each grid field the number of the plant parts with certain characteristics, or if possible already according to plant species, was counted, stopping at the number of 100 pieces in total.

We found 15 recognisable plant groups: Asteraceae, Boraginaceae, Brassicaceae, Bryophyta, Cistaceae, Fabaceae, Fagaceae, Lamiaceae, Oleaceae, Plantaginaceae, Poaceae, Rosaceae, Urticaceae, "herbs which are not grasses", and "woody plants which were not grasses" (Loss 2020).

Actually, the groups of plants selected to be analysed were those that contained, at least, 100 fragments. All the plants present in the tortoise's faeces should be classified as "food plant".

For each variable (season, sex and presence/absence of parasites) a spreadsheet was generated, which listed the number of fragments of each plant group.

Results and Discussion

How to do nutrition research in tortoises and freshwater turtles

The simplest method to find out what the animals are eating is direct observation. This has been done in tortoises, too, see below in the results section, but since the

Fig. 5. Jordi Bartolomé actively helps with the laboratory work. Photo: M. Loss

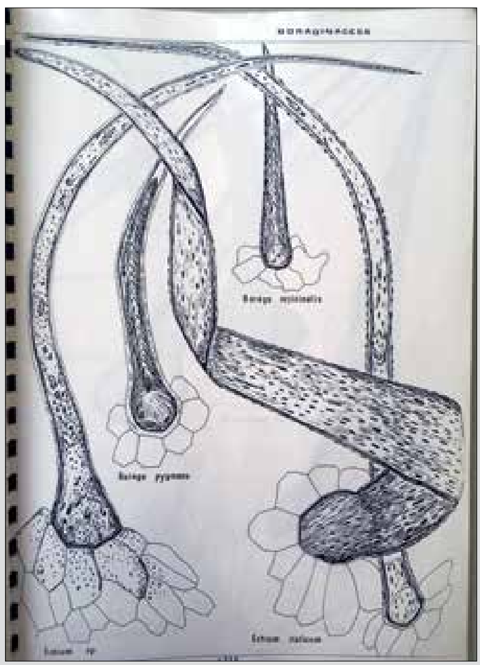

Fig. 6. Training material: How to identify Boaginaceae by their trichomes. Photo M. Loss

tortoises are shy and rather well camouflaged, and the observer will disturb them when getting too close, this method will often give incomplete results, and it is not suitable for finding out how much the tortoise has eaten of a certain plant.

It is a nice thought to analyse the contents of the next stop in the alimentary channel, the stomach. Stomach flushing has been used extensively for nutrition research in marine and freshwater turtles, but for tortoises this method is inapplicable. Besides this, turning the tortoise upside down for quite some time and washing the stomach contents out of the mouth would cause a lot of stress to the animals and this could harm them.

And so we changed viewing direction to what comes out. Tortoise faecal pellets may still show parts of the plant material (Fig. 9) or they may be fine-textured (Fig. 10). While in the stomach content the plant parts are still identifiable, most of them are broken down by microbial digestion during the long retention time in the intestinal tract (Fritz *et al.* 2010, Modica 2016) and the plant species are very difficult to determine directly in the untreated faecal smears.

It had been tried to analyse the plants which the tortoise has eaten by DNA barcoding the plant material in the faecal samples of *Testudo hermanni* and *Testudo graeca* on Mallorca, and comparing the obtained sequences with the information available in GenBank (Murat 2018, Vidal Ramón 2018), but due to the very incomplete datasets in GenBank many of the plant DNA could not be identified to genus or species level.

Updated inventory of Albera tortoise food plants

The most detailed compilation of the food plants of the Albera tortoise had been

Fig. 7. Sieving the faecal material.
Photo M. Loss

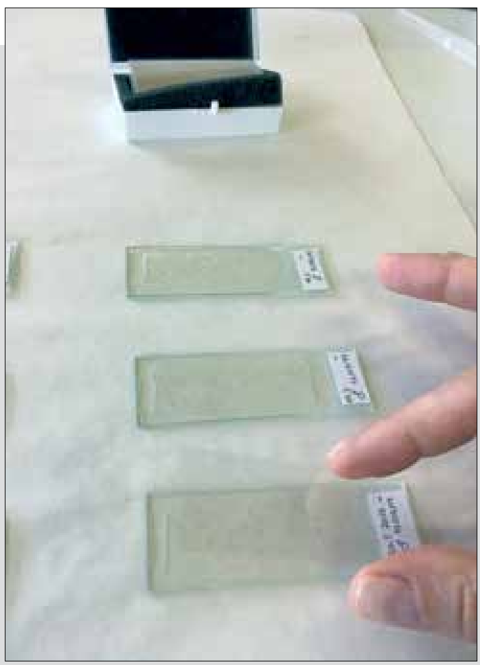

Fig. 8. Mounting the slides with coverglass.
Photo M. Loss

Fig. 9.
Dry faecal pellet of a *Testudo hermanni* (Mallorca).
Photo B. Pfau

Fig. 10.
The faecal pellets can be comparatively large (Menorca tortoise with faeces and an acorn of *Quercus pubescens* for size comparison).
Photo B. Pfau

published by Budó *et al.* (2009). They had noted down all their observations of tortoises feeding on leaves, flowers or fruits and they determined the plant species of each observation.

The actual nutritional analysis found coincidences in 9 plant families: Asteraceae, Brassicaceae, Fabaceae, Fagaceae, Oleaceae, Plantaginaceae, Poaceae, Rosaceae (Fig. 11a, b) and Urticaceae. The most eaten plant fragments were from the plant family Fabaceae (856 fragments) (Fig. 12a, b), followed by the Boraginaceae (443 fragments) (Fig. 13a, b), the Asteraceae (396 fragments), the Cistaceae (155 fragments) (Fig. 14a, b and the Brassicaceae (153 fragments).

Plant parts of the Fabaceae family were the preferred food of the tortoises, perhaps because of their high energy content and the easily assimilable minerals, like phosphorus, sodium, calcium (Tracy *et al.* 2006) and proteins (Lewis *et al.* 2005). Another reason for the preference might be the rather soft leaf structure (see for comparison Balsamo *et al.* 2004).

The second most eaten plants were from the Boraginaceae family, followed by the Asteraceae, which is similar to the results found by Muñoz-Miranda *et al.* (2009) in a reintroduced population of *Testudo hermanni hermanni* farther south in Catalonia (for an overview of the *Testudo hermanni* populations in Catalonia see Pfau & Budó 2019).

The species analysis of the Albera tortoise faeces revealed plant species that had not been observed to be eaten by Budó *et al.* (2009), mainly from the plant family Boraginaceae, but also from Bryophyta, Cistaceae, and also in small quantities from the Lamiaceae (Fig. 15a, b) family. On the other hand, the faecal samples did not reveal plant parts of the family Convolvulaceae (Fig. 16), Dipsaceae, Ericaceae, Geraniaceae, Malvaceae, Primulaceae and Ulmaceae, which had been observed to be eaten by the tortoises (Budó *et al.* 2009). A possible reason for this might be either the difficulty to identify many plant fragments with the micro-histology method or because these plants could have been more readily digested (Hatt *et al.* 2005). One example for identification difficulties is the vine *Clematis flammula*, which we expected in the faeces samples, because the tortoises are rather often seen feeding on them. A reason why we could not identify them in our faeces preparations could be the epidermis characters of this plant, which is soft, has no trichomes, and has a cell form which is also common in other plants.

In all we had a lot of plant fragments which we could not assign to a plant family, therefore we grouped them either in "soft herbs" or "woody herbs", both groups excluding grasses.

We did not find significant differences in the food plant spectrum as compared to the observations by Budó *et al.* (2009).

Effects of season, sex and parasites on the plant diet of the Albera tortoise

Effect of season

The samples were taken either in late spring (May / June) or in summer (July / August). We compared the number of plant

Fig. 11a. The Mediterranean blackberries (here *Rubus ulmifolius*) provide hiding places for the tortoises, and the leaves may be eaten. Photo B. Pfau

Fig. 11b. Micro-histology of *Rubus ulmifolius*, showing star-shaped trichomes. Photo M. Loss

Fig. 12a. A recently reintroduced tortoise (Menorca locale) in the Serra de Montsant eagerly eating clover, *Medicago sp*. Photo B. PFAU

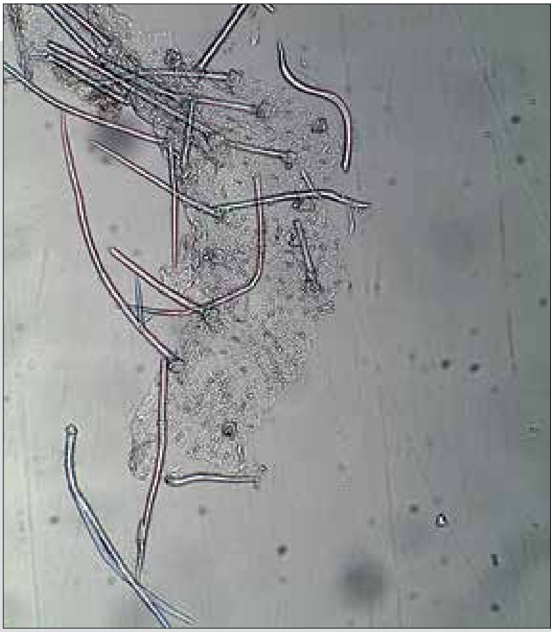

Fig. 12b. Micro-histology of *Medicago minima*, showing the long, single trichomes. Photo M. LOSS

Fig. 13a. *Borago officinalis*. Photo B. PFAU

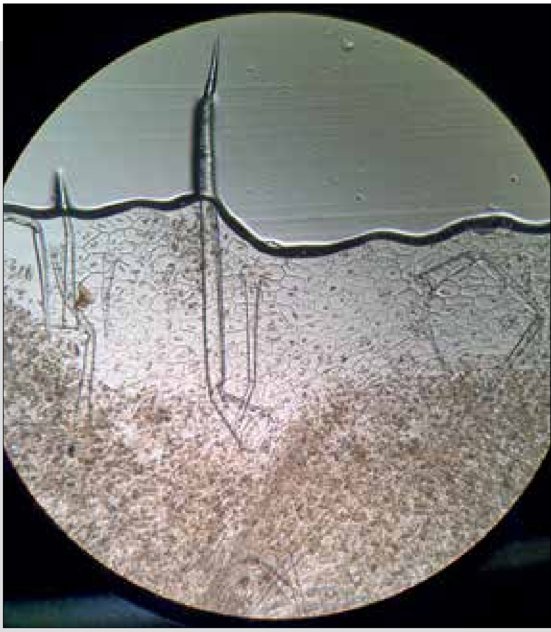

Fig. 13b. Micro-histology of *Borago officinalis*, showing the stout trichomes. Photo M. LOSS

Fig. 14a. *Cistus albidus*, named for the whitish tomentose hairs on the leaves. Photo B. Pfau

Fig. 14b. Micro-histology of the hairy surface of the *Cistus albidus* leaves. Photo M. Loss

Fig. 15a. *Lavandula sampaioana* is one of the most prominent Lamiaceae plants in the Albera. Photo B. Pfau

Fig. 15b. Micro-histology of the characteristic trichomes of *Lavandula sampaioana*. Photo M. Loss

fragments for each plant family between the spring and the summer samples and found significant (p-value) differences: In spring there were more Fabaceae (512 fragments, p-value 0,005) and Asteraceae (164 fragments, p-value 0,024) than in summer, and in summer there were more Boraginaceae (237 fragments, p-value 0,024) and Cistaceae (101 fragments, p-value 0,157) than in spring. In the other plant families, the differences were not statistically different (Fig. 17).

These differences coincide with the classification of the "spring" species as hemicryptophytes, which means that the leaves of these plants are mainly available to the tortoises in spring, while they wither in summer. Similar seasonal differences in tortoise diets had also been found in other tortoise biotopes (Bertolero *et al.* 2011, Del Vecchio *et al.* 2013, Muñoz-Miranda *et al.* 2009).

In the tortoise nutritional analysis in Italy (Del Vecchio *et al.* 2013) grasses (Poaceae) were a major part of the diet in spring, which could not be confirmed in our analyses. Meek (1989; 2010) supposed that the grasses were avoided because of their low content in nutrients and humidity, even as they are readily available. For Desert tortoises (*Gopherus agassizii*) it was even supposed that the uptake of grasses would have a negative influence on the water and nitrogen balance (Nagy *et al.* 1998, Henen 2002).

Fig. 16. *Tuberaria guttata* and *Convolvulus arvensis* are readily eaten by the tortoises, but their remains could not be identified from the faecal samples with micro-histology. Photo B. Pfau

Observations on the diet of *Homopus (=Chersobius) signatus* in South African spring showed that only 50-60% of the faecal volume could be identified to plant family level (Loehr 2002), which is similar to our results. Since tortoises prefer to eat plant flowers if available (see also Jennings 2002) a follow-up survey of the diet of the Albera tortoise should include a pollen analysis, too.

Effect of sex

We did not find significant differences in consumption of different plant families between the sexes. One, rather curious, result is that we found Urticaceae (all in all 20 fragments) only in the faeces samples of females. Other small differences can be attributed to differences in seasonal activity of the sexes, for example when comparing the fragment number for Cistaceae in all the faeces samples (133 in the male's samples and 18 in the female's samples) or for Brassicaceae (103 in the male's samples and 23 in the female's samples).

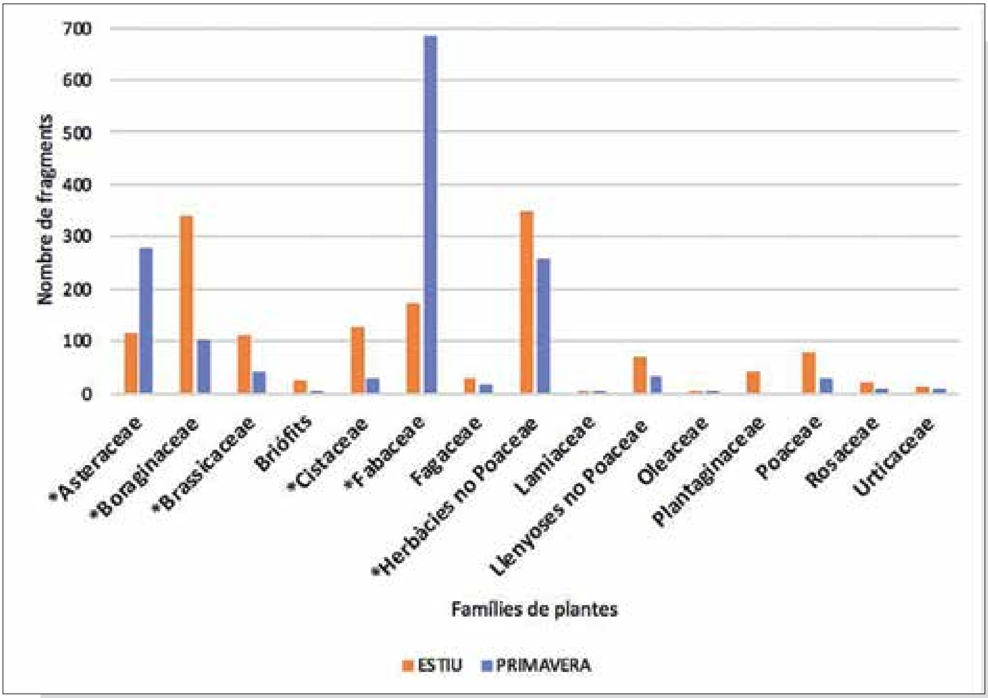

Fig. 17. Diagram showing the differences of the plant families eaten in summer (estiu) and spring (primavera),screenshot taken from Loss (2020)

Effect of parasites

Mediterranean tortoises (*Testudo hermanni hermanni*) are usually infested with nematodes, mostly Pharyngodonidae (Oxyuroidea) and Atractidae (Cosmocercoidea) (Gagno 2005). The nematodes themselves are not found in each faecal sample, and also the eggs are shed intermittently, and low nematode loads even seem beneficial to the growth of captive juvenile tortoises (Brosda 2011).

In wild tortoises, hindgut parasites (oxyuridae) can be beneficial (in high quality habitats nematode infestation was positively correlated with growth rates) or they may have negative impacts (in low quality habitats), as discussed by Rodriguez-Caro *et al.* (2020) in *Testudo graeca* in southern Spain.

Testudo hermanni hermanni kept in semi-captivity in France and having high parasitic loads preferred plants of the Ranunculaceae family, which are known “natural vermifuges” due to their ranunculine content to plants of the Asteraceae family, which do not contain any vermicide substances (Longepierre & Grenot 1999).

We tried find out whether tortoises with higher parasitic load (nematodes seen in the sample containers) would selectively take up other plants than the tortoises with low parasitic load (no parasites, even with microscopic analysis, in the faeces samples), but the comparison of the samples whith and without visible parasites did not show any significant differences.

Other nutritional components in the diet of *Testudo hermanni*

Morin (2015) summarized the nutritional intake and the nutritional requirements of herbivore tortoises like *Testudo hermanni*. Being “herbivorous” does not mean that they eat nothing but plant parts”, but their protein requirements of about 20-25% (dry weight) could be met by plant leaf food only (Donoghue 2006), since the most eaten food plants which we identified are in this protein range, and also the Romaine lettuce ("laitue romaine" in Morin 2015), which is often fed to the breeding group tortoises in the CRT, is specified to have 24% protein and a 1:1,1 Ca/P ratio (see below).

It might be possible that especially growing tortoises or gravid females need protein with a more complete amino acid composition and therefore search for animal food (Gagno *et al.* 2012, Budó & Mascort 2001), and female tortoises are also often observed to eat calcium-rich soil, mollusc shells, or bones (Dordević & Golubović 2013, Moore & Dornburg 2014, Sullivan & Cahill 2019), obviously to satisfy their calcium needs. Veterinarians recommend a calcium:phosphorus ratio of 2:1, and many of the plants in the diet of the Albera tortoise are in this range, see for example Krüger (undated), but Romaine lettuce is insufficient.

Tortoises are often seen eating dry or fresh faeces of herbivorous livestock like cows, or wild herbivores like rabbits, but also faeces of carnivores (Soler & Martínez-Silvestre 2011) or even humans. Eating dry faeces might meet their mineral needs, but eating fresh faeces could also be a source of humidity, enzymes and beneficial gut microorganisms (McBee, 1971).

Conclusions and next steps

The plant list in Budó *et al.* (2009) was essentially confirmed, additionally fragments of plants of the families Boraginaceae, Bryophyta, Cistaceae and, in small quantities, Lamiaceae were found. The Fabaceae plants were eaten the most, followed by the Boraginaceae (leaves), while in the reference publication the second place on the list was occupied by the Asteraceae.

For the most important food plants a poster exhibition in the meeting room of the CRT will be created (Fig. 18).

There were differences in the plant spectrum between spring and summer, as expected. In spring the most eaten plants belonged to the families Fabaceae and Asteraceae, while in summer the Boraginaceae were among the most eaten plants.

The analysis did not show significant differences in the diet between the sexes, and there was no obvious effect of the parasite load on preferences of certain, potentially deworming, food plants. Maybe better result would have been obtained with a greater number of samples.

The results will be used to improve the nutrition of the tortoises in the Centre de Reproducció de Tortugues de l'Albera. We will especially analyse the protein, dietary fibre and secondary phytochemicals content of the food for the tortoise breeding group (Fig. 19) in more detail and perhaps find a way of supplementation if necessary.

Acknowledgements

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Bertolero, A., J. Budó & N. Torres (2020): Característiques poblacionals de

Plant Family / Group	Common name	Potential food plant	Common name
Asteraceae	Compositae: Aster, daisy and sunflower family	*Crepis bursifolia* *Urospermum dalechampii*	Italian Hawksbeard Smooth Golden Fleece
Boraginaceae	Forget-me-not family	*Borago officinalis* *Echium vulgare*	Starflower Viper's bugloss
Brassicaceae	Cruciferae: Mustard and cabbage family	*Lobularia maritima*	Sweet alyssum
Bryophyta	Mosses		
Cistaceae	Rock-rose family	*Cistus monspeliensis* *Tuberaria guttata*	Montpellier cistus Annual rock-rose
Convolvulaceae	Morning-glory family	*Convolvulus arvensis*	Field bindweed
Crassulaceae	Stonecrop family	*Umbilicus rupestris*	Wall pennywort
Dipsaceae	Teasel family	*Scabiosa atropurpurea*	Mourning bride
Ericaceae	Heather family	*Arbutus unedo*	Strawberry tree
Fabaceae	Leguminosae: Pea and bean family	*Lupinus angustifolius* *Psoralea bituminosa*	Narrow-leaved lupin Arabian pea
Fagaceae	Oak, chestnut and beech family	*Quercus suber*	Cork oak
Geraniaceae	Cranesbills family	*Geranium molle*	Dove's-foot cranesbill
Lamiaceae	Labiatae: Mint, deadnettle and sage family	*Salvia pratensis*	Meadow sage
Malvaceae	Mallows	*Malva sylvestris*	Common mallow
Oleaceae	Olive family	*Olea europaea*	Olive tree
Plantaginaceae	Plantain family	*Plantago lanceolata*	Narrowleaf plantain
Poaceae	Gramineae: Grasses	*Festuca ovina*	sheep fescue
Primulaceae	Primrose family	*Anagallis arvensis*	Red pimpernel
Ranunculaceae	Buttercup family	*Clematis flammula*	Fragrant virgin's bower
Rosaceae	Rose family	*Rubus ulmifolius*	Elmleaf blackberry
Ulmaceae	Elm family	*Celtis australis*	European nettle tree
Urticaceae	Nettle family	*Parietaria sp.*	Pellitory
Vitaceae	Grapevine family	*Vitis vinifera*	Grapevine

Table 1. Plant families and some examples of potential food plants

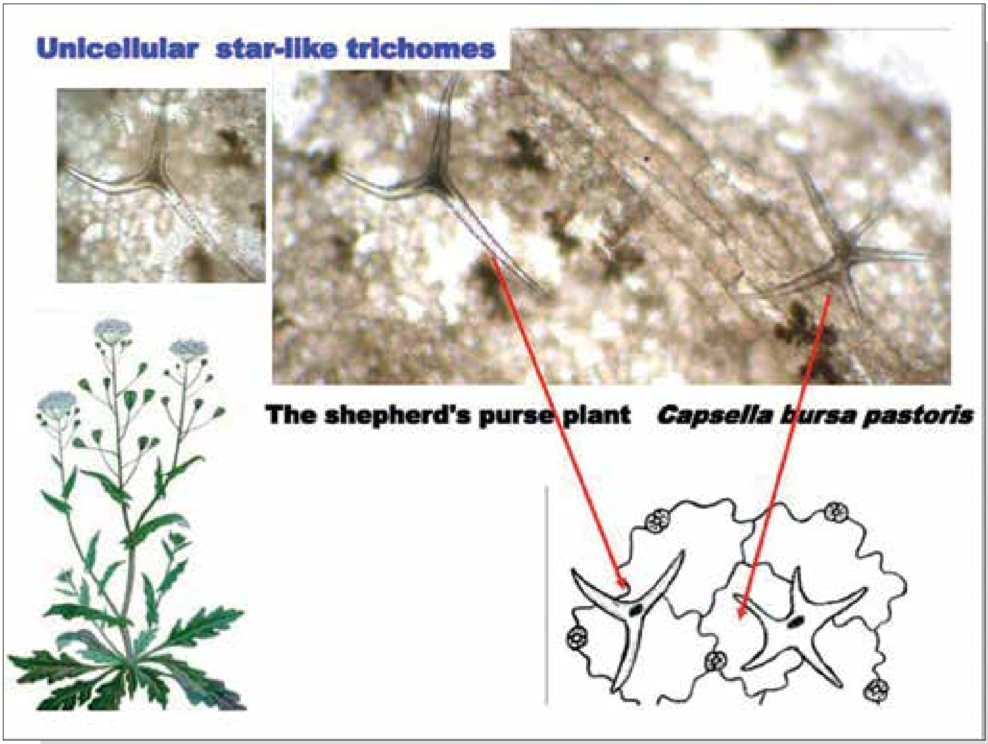

Fig. 18. Poster for the exhibition in the CRT meeting room. Photo M. Loss

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Fig. 19. A female Albera tortoise of the breeding group is sitting in the food dish.
Photo B. PFAU

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