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The physiological consequences of ingesting a toxic plant (*Diplotaxis tenuifolia*) influence subsequent foraging decisions by sheep (*Ovis aries*)

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HIGHLIGHTS

- Toxins and nutrients interact affecting herbivores' ingestive experiences with toxic plants.
- The provision of a protein supplement reduced some of the toxic effects of glucosinolates.
- A strong aversion to the toxic plant developed when glucosinolates ingestion caused negative post-ingestive consequences.
- Negative previous experiences with the toxic plant have long lasting consequences on the foraging decisions of sheep.

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ABSTRACT

Toxins and nutrients interact and define herbivores' experiences with toxic plants. However, there are still open questions about the mechanisms by which nutrient-toxin interactions affect experience and as a consequence foraging decisions by consumers. This study provides a deeper insight into such mechanisms by using supplemental nutrients, a toxic plant typically avoided by herbivores (wild rocket; *Diplotaxis tenuifolia*), and a small ruminant (sheep; *Ovis aries*) as models. Thirty-six sheep were randomly assigned to four treatments ($n = 9$) where animals consumed: wild rocket ("DT"), wild rocket followed by a protein supplement ("DT + P"), wild rocket followed by a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M"), or alfalfa pellets in amounts that paired the ingestion of wild rocket by DT ("CTRL"). Towards the end of the phase of exposure (day 35), DT showed the lowest intake of wild rocket, as well as reduced levels of plasma thyroid hormones (T3 and T4), alanine aminotransferase, and a trend towards reduced hemoglobin relative to DT + P and DT + P + M. Total concentration of serum proteins and albumins were greater in sheep fed the protein supplements, which have probably elicited a protective effect on toxin ingestion. Foraging behavior was then evaluated in an experimental arena where animals could select among randomly distributed buckets containing a fixed amount of wild rocket or variable amounts of barley grain (a preferred food). Regardless of barley grain availability, DT showed lower intake and lower times spent eating wild rocket than DT + P and DT + P + M. Unexpectedly, CTRL (without previous experience with wild rocket) ingested amounts of wild rocket comparable to those observed by DT + P and DT + P + M. A negative feeding experience with wild rocket is needed for animals to display the typical pattern of aversion commonly observed in grazing conditions.

1. Introduction

Almost every plant produces chemical compounds, which are potentially toxic to herbivores [1]. Plant secondary metabolites (hereafter, "PSMs") have variable chemical composition and structure that define their impact on the digestion, physiology, and metabolism of consumers [2]. For instance, depending on their chemical structure and reactivity, polyphenolic compounds like condensed tannins affect digestion processes or are absorbed, exerting their toxic actions systemically [3]. PSMs can also render positive effects on animal health (e.g., [4]), but typically at low doses and when specific chemical and/or biological interactions occur after food ingestion [5,6]. Thus, intake of toxins can be harmful or beneficial to consumers depending on chemical composition, context and dose [7,8].

Plan secondary metabolites have been proposed to evolve as a chemical defense against herbivory [9]. In addition to their toxic effects

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on herbivorous insects and mammals, some PSMs provide orosensorial experiences (e.g., bitter, sour) that induce food rejection [10,11]. Many plants containing PSMs are nutritious and thus complete food rejection would represent lost opportunities to harvest needed nutrients from the feeding environment. Consequently, consumption of PSMs by herbivores is a regulated process where individuals maintain the level of PSMs ingestion within the limits of their detoxification capacity [12,13]. This regulation is possible because animals learn from the particular post-ingestive consequences of foods [14,15]. For instance, sheep learn to regulate intake of and preference for arrays of foods containing different plant toxins such that intake of macronutrients is similar to those animals fed the same foods but without toxins [16].

It has been widely shown that the chemical context in which PSMs occur influence their post-ingestive effects and as a consequence herbivores' foraging decisions. This is because the toxic effects of PSMs can be reduced by their interaction with other toxins [17] and/or nutrients [18] consumed in a meal. Ingesting foods with a variety of different PSMs, that act upon different organs and detoxification pathways, is likely to be less toxic than a large dose of any one toxin consumed individually [19,20]. However, this benefit is lost when detoxification pathways of PSMs overlap [21,22]. Finally, nutrients aid in detoxification processes and as a consequence they increase toxin tolerance and enhance intake of and preference for PSMs-containing foods [14,23]. Collectively, this analysis suggests that a better understanding of animals' behavioral responses to PSMs ingestion requires the knowledge of their digestive and/or physiological consequences and interactions with other toxins and nutrients in the feeding environment.

Our aim in the present study was to explore the role of experience with PSMs and nutrients on the foraging behavior of a mammalian herbivore by using a PSMs-containing plant typically avoided by grazers (wild rocket; *Diplotaxis tenuifolia*) and a small ruminant (sheep; *Ovis aries*) as a plant-herbivore interaction model. Wild rocket, as well as other members of the *Brassicaceae* family contains glucosinolates, a large family of sulphur-containing PSMs [24]. Glucosinolates are hydrolyzed by the enzyme myrosinase, mostly during severing and mastication of the plant tissue, leading to biologically active compounds like isothiocyanates, thiocyanates and nitriles, among others [25]. Isothiocyanates and thiocyanates confer a bitter and spicy flavor, and at moderate doses are known to cause intake depression, impaired thyroid function, fertility problems, and reduced growth in ruminants [26]. However, experience with glucosinolates can be improved by the manipulation of the animal's nutritional state. For instance, supplementation with iodine and copper has been shown to revert some of the deleterious effects of glucosinolates in calves, mainly because thiocyanates affect iodine uptake by the thyroid gland and excessive dietary sulphur affects cooper absorption [27]. Protein supplementation can also render a positive effect on animal performance because the digestive end-products of *Brassicaceae* ingestion are deficient in this nutrient [25] and because supplementary protein can favor detoxification processes in mammals [1]. We hypothesized that experience with wild rocket is determined by the physiological consequences of its ingestion, and that this experience will influence subsequent preference for this plant. Our objectives were to evaluate in sheep how some of the nutritional manipulations known to reduce the deleterious effects of thiocyanates influence: 1) some physiological parameters indicative of wild rocket toxicosis, and 2) feeding experience with wild rocket and subsequent foraging behavior.

2. Materials and methods

The study was conducted at the "Centro de Recursos Naturales Renovables de la Zona Semiárida" (CERZOS) located in Bahía Blanca (38° 44′ S; 62° 16′ W), Argentina, from February 2015 to June 2015. All experimental protocols fulfilled the animal welfare regulations of the Universidad Nacional del Sur (Bahía Blanca, Argentina) that follow National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) guidelines. Throughout the study, the sheep had free access to water.

2.1. Animals, treatments and exposure

Thirty-six 1-year-old Merino sheep wethers (*Ovis aries*; 40.2 ± 3.0 kg live weight [LW] [mean ± SD]) were brought from natural pasturelands to the study site, dewormed with a subcutaneous injection of ivermectin (0.2 mg/kg of LW; Ivomec, Merial, Argentina), and kept in a communal pen (30 × 20 m) for 15 days. During this period animals were fed alfalfa pellets ("alfalfa") at 0900 in amounts that satisfied their daily maintenance requirements [28].

Following the aforementioned adaptation period, all sheep were individually penned outdoors in adjacent wooden pens (2.5 m × 2.5 m) under a protective roof. Sheep were familiarized to these new experimental conditions for seven consecutive days. During this period they were fed alfalfa at 2.5% of LW/d and soybean meal at 0.4% of LW/d.

After familiarization, sheep were weighed and randomly assigned to one of four treatments balanced by LW ($n = 9$): 1) sheep exposed to wild rocket ("DT"), 2) sheep exposed to wild rocket and a protein supplement ("DT + P"), 3) sheep exposed to wild rocket and a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M"), and 4) sheep with no exposure to wild rocket ("CTRL"). Wild rocket was harvested from cultivated stands located at the study site every day at 0900, and chopped by hand to an average particle size of 5 cm immediately before feeding.

During the phase of exposure, all sheep were fed with an unrestricted amount of fresh-cut wild rocket from 1000 to 1200, except for CTRL. Sheep in this group received alfalfa in amounts that represented the average individual dry matter intake of wild rocket observed the day before in DT. Once the refused amounts of wild rocket were removed, DT + P were fed soybean meal at 0.4% of their LW, and DT + P + M were offered soybean meal at 0.4% of their LW previously mixed with 250 mg/kg of copper sulphate and 63 mg/kg of potassium iodine. Sheep in DT + P + M ingested an individual daily average amount of 39.8 ± 2.9 mg of copper sulphate and 10.0 ± 0.7 mg of potassium iodine (means ± SD), which are doses previously shown to reduce the negative post-ingestive effects of glucosinolates [26]. At 1500, all sheep were fed alfalfa at 2% of LW. Exposure lasted 35 days. All sheep were weighed at the end of exposure, which allowed for the estimation of daily LW gain (LWG).

2.2. Blood parameters

Blood samples were taken from 1400 to 1500 (i.e., before the alfalfa meal offer in the afternoon) on the last day of exposure (day 35). Two 10-mL samples (with or without heparin, Becton Dickinson Vacutainer System, New Jersey, United States) were collected per animal by puncture of the jugular vein. Samples with heparin were immediately submitted to the Veterinary Diagnostic Laboratory, Bahía Blanca, Argentina, for total blood cell count (ABX Micros 60 counter; ABX Diagnostics, Montpellier, France). Samples without heparin were allowed to clot for 45 min and then the serum was separated by centrifugation (2300 × *g* for 25 min; 4 °C) and stored at −20 °C until analyses. Triiodothyronine (T3) and Thyroxine (T4) serum concentrations were determined by immunochemoluminiscence using an automated analyzer (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA), and serum proteins were determined by fully automated cellulose acetate electrophoresis (Genio S; Interlab Srl, Rome, Italy). Liver function tests, including serum concentrations of aspartate transaminase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were conducted using an automated analyzer (Hitachi 902; Hitachi Ltd., Tokyo, Japan) and kits provided by Spinreact (Barcelona, Spain). Total serum thiocyanates were determined by the method of Bowler [29].

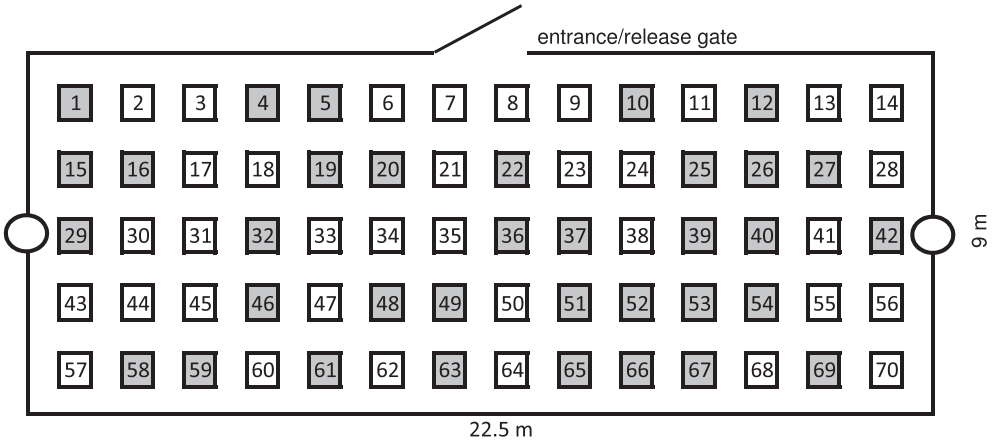

Fig. 1. A schematic representation of the experimental arena used in the study, representing its dimensions and placements of food buckets (squares), video cameras (circles), and main entrance. For illustrative purposes, one example of the random display of buckets containing wild rocket (e.g., gray squares; $N = 35$) and barley grain (e.g., white squares; $N = 35$) is presented.

2.3. Washout period and testing phase

The day after the exposure phase ended, sheep were returned to their communal pen and fed alfalfa at 0900 for 7 consecutive days in amounts that satisfied their daily maintenance requirements [28]. This washout period was conducted to allow sheep previously fed wild rocket to reduce their toxin load before the start of the testing sessions. Clearance time of thiocyanates is 2.7 days in humans [30].

Following the washout period, the foraging behavior of sheep with different experiences with wild rocket ingestion was evaluated in an experimental arena consisting of patches of wild rocket and patches of a highly palatable and familiar food. Sheep were tested in pairs selected at random within each treatment group and blocked by LW. Because groups were formed with 9 sheep, the individual in each group which showed during exposure the lowest intake of wild rocket was not included in the testing sessions. Sheep are gregarious animals and isolation normally affects their foraging behavior [31].

The experimental arena (Fig. 1) was built within the communal pen. It was delimited by a 1 m tall black canvas, and provided with an entrance/release gate (1.5 m wide). Inside the arena, seventy plastic buckets (20 × 20 cm × 15 cm height) were arranged in five lines of 14 buckets each. All buckets were separated by a distance of 1.5 m. During testing, half of the buckets contained a fixed amount of fresh wild rocket (16 g, as fed basis), while the other half of the buckets contained a variable amount of barley grain (hereafter, "barley"; a highly palatable and familiar feed). Fresh wild rocket was harvested and processed daily as described during the phase of exposure. The amounts of barley offered in each bucket were 4, 8, or 16 g (as fed basis). The reason to manipulate the level of barley availability was to increase the searching cost for finding the preferred alternative in the arena. Sheep display their learned preferences for unpalatable foods better with increased searching costs for palatable foods [32]. All pairs of sheep were exposed to each level of barley availability for periods of three consecutive days (i.e., testing sessions) in a random sequence. The amounts of barley used for each level of availability were selected based on previous work by Dumont et al. [33]. The presence of wild rocket and barley in the buckets was randomized every day.

Testing sessions started at 0800 and lasted 10 min for each pair of sheep, which entered into the arena in a random order across days. Sheep were free to move inside the experimental arena and to choose from which bucket they would eat. Once the testing session ended, the entrance/release gate was opened and the pair of sheep was walked back to the communal pen. Then, food refusals were collected and weighted, and fresh wild rocket and barley buckets were refilled according to the level of barley availability assigned to the upcoming pair of sheep. This procedure was repeated until all pairs of sheep finished their daily testing session. Testing sessions ended approximately at 1300. All sheep were fed alfalfa at 2% of their LW at 1700.

Two video cameras (Foscam FI8904W, ShenZhen Foscam Intelligent Technology Co., Shenzhen, China) were set in the experimental arena (Fig. 1) to obtain recordings at 30 frames/s (i.e., real-time) of sheep behavior during each testing session.

2.4. Chemical analyses of foods

All foods used during the study were sampled each time before feeding, composited for 7 days (during the exposure phase) or daily (during the testing phase), and then prepared for chemical analyses. Composited samples were dried at 60 °C for 48 h, ground using a Wiley Mill (1-mm mesh), and analyzed for crude protein ([34], Method 990.03), neutral detergent fiber (without the addition of sodium sulphide; [35]) and acid detergent fiber (using neutral detergent fiber residue; [34], Method 973.18) (Table 1). A subset of pooled samples of wild rocket was freeze-dried to constant weight and then ground using a Wiley Mill (1-mm mesh). These samples were used for total glucosinolates content determination [36] (Table 1).

Table 1
Chemical composition of foods used during exposure and testing phases.

	Exposure phase			Testing phase	
Item	Wild rocket	Soybean meal	Alfalfa pellets	Wild rocket	Barley grain
Dry matter, g/100 g	20.4 ± 3.3	92.0 ± 3.1	93.5 ± 2.6	16.5 ± 3.0	79.9 ± 2.1
Crude protein, g/100 g	16.1 ± 3.5	41.7 ± 0.7	17.6 ± 0.6	22.9 ± 3.5	7.2 ± 0.7
Neutral detergent fiber, g/100 g	48.9 ± 5.5	21.7 ± 1.4	57.0 ± 1.2	40.0 ± 3.9	21.4 ± 1.6
Acid detergent fiber, g/100 g	33.8 ± 5.1	6.6 ± 0.2	39.3 ± 0.6	26.0 ± 3.9	6.2 ± 0.3
Glucosinolates, μmol/g	37.2 ± 3.6	N.D.	N.D.	35.0 ± 2.5	N.D.

References: N.D.: not detected.

2.5. Statistical analyses

For a priori contrasts between treatments, CTRL was considered the control group for DT, DT the control group for DT + P, and DT + P the control group for DT + P + M. Other contrasts were statistically treated as post hoc and Tukey's HSD test was performed.

Statistical analyses were performed using the R environment [37]. Mixed effects models were evaluated during the selection process according to the procedure detailed in Zuur et al. [38]. Model diagnostics also included testing for normal distribution, homogeneity of variance, and linearity. Data that did not satisfy any of the latter properties were analyzed using a non-parametric test (Kruskal-Wallis test); for multiple contrasts package "PMCMR" was used [39]. Least square means and standard errors were obtained with the "lsmeans" package [40]. All data are reported as means ± 1 standard error of the mean (SEM).

Wild rocket and barley intake during the study were calculated as the difference between dry matter offered and dry matter refused (always expressed on a DM basis). Wild rocket and glucosinolates intake data during the exposure phase were analyzed with a mixed effects model [41]. The model included treatment, day, and treatment × day interaction as fixed effects, and sheep as random effects. Wild rocket and barley intake data during the testing phase were analyzed with a mixed effects model including treatment, level of barley availability, and treatment × level of barley availability interaction as fixed effects, and pair of sheep as random effects.

Video recordings during the testing phase were analyzed for the last session of each level of barley availability, when we consider sheep had the greatest level of exposure to the conditions of the test. Recorded activities of individual sheep were scan sampled at 10-s time intervals [42], and categorized as: 1) eating wild rocket, 2) eating barley, 3) searching (i.e., moving around buckets with the head down and/or visiting buckets but with no visible signs of ingestion), or 4) involvement in other activities ("idling"). Data from recorded activities were analyzed separately for each level of barley availability using Kruskal-Wallis test and averaging data from both sheep in the corresponding pair. The amount of visits (i.e., sheep eating from a given bucket) made to the same bucket containing either barley or wild rocket were also recorded. In this case, data were analyzed for each level of barley availability using Kruskal-Wallis test and averaging data from both sheep in the corresponding pair. We grouped data into total visits, first visit, second visit, and more than two visits (i.e., more visits).

Data from blood parameters, live weight, and live weight gain, were analyzed with a linear model using treatment as a fixed effect.

3. Results

3.1. Exposure phase

3.1.1. Dry matter intake of wild rocket, LW, and LWG

Dry matter intake of wild rocket increased at a faster rate across days of exposure for DT + P and DT + P + M than for DT ($F_{2,456} = 6.11$, $P = 0.024$; treatment × day interaction term). During the last three days of the exposure, dry matter intake of wild rocket was greater for DT + P and DT + P + M than for DT (185 and 186 versus 147 ± 12.9 g, respectively; $F_{2,24} = 3.76$, $P = 0.038$). There were no differences in the dry matter intake of DT + P and DT + P + M ($F_{1,24} = 0.01$, $P = 0.919$). Mean daily glucosinolates intake was greater for DT + P and DT + P + M than for DT (6.5 and 6.5 versus 5.1 ± 0.45 mmol/d, respectively; $F_{2,24} = 3.45$, $P = 0.048$).

Live weight at the end of the exposure phase was similar between all groups (39.7, 39.6, 41.3, and 40.9 ± 1.0 kg, for CTRL, DT, DT + P, and DT + P + M, respectively; $F_{3,32} = 0.69$, $P = 0.564$). However, LWG was greater for DT + P and DT + P + M than for DT (41.7 and 79.9 versus −18.5 ± 17.1 g/d, respectively; $F_{2,32} = 8.64$, $P = 0.001$). Live weight gain was similar between DT + P and DT + P + M ($F_{1,32} = 0.72$, $P = 0.401$), and between CTRL (0.9 ± 17.1 g/d) and DT ($F_{1,32} = 0.04$, $P = 0.851$).

3.1.2. Blood parameters

Data from whole blood and serum analyses were summarized in Table 2. Red blood cell morphology and composition showed no differences between experimental groups ($P > 0.05$). However, there was a tendency for lower content of hemoglobin in the red blood cells of DT than for the rest of the groups.

Plasma T3 levels were significantly lower in DT than in the rest of the groups, whereas plasma T4 levels were lower in DT than in DT + P or DT + P + M. There was no difference in plasma T4 levels between CTRL and sheep in the rest of the groups.

The only liver enzyme that was affected by treatments was alanine aminotransferase (ALT). Plasma ALT levels were lower in DT than in the rest of the experimental groups.

Total plasma proteins content was lower for CTRL and DT than for DT + P and DT + P + M. Albumin concentration was higher for DT + P and DT + P + M than for DT, whereas there was no difference in albumin content between CTRL and sheep in the rest of the groups. The only plasma globulin affected by treatment was alpha-2 globulin, with greater concentrations for DT + P than for DT. No differences in alpha-2 globulin concentration was observed between the other experimental groups.

Total serum thiocyanates concentrations were the lowest for CTRL than sheep in the rest of treatments, whereas no differences were observed between DT, DT + P and DT + P + M.

3.2. Testing phase

3.2.1. Dry matter intake of wild rocket and barley

Fig. 2(a) summarizes wild rocket intake data in the experimental arena. Dry matter intake of wild rocket decreased gradually as barley availability increased ($F_{2,71} = 3.45$, $P = 0.037$). Sheep in DT showed

Table 2
Complete blood cell count, thyroid hormones, hepatic enzymes, and serum proteins of sheep ($n = 9$) fed during 35 days with wild rocket ("DT"), wild rocket followed by a protein supplement ("DT + P"), wild rocket followed by a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M"), or alfalfa pellets in amounts pairing ingestion of wild rocket by sheep in DT ("CTRL").

	Treatment					
Item	CTRL	DT	DT + P	DT + P + M	SEM	*P*-value
Red blood cells, 1 × 10^6/μL	10.87	10.39	10.98	10.87	0.33	0.609
Hemoglobin, g/dL	12.43	11.57	12.36	12.20	0.38	0.072
Packed cell volume, %	38.57	37.33	39.85	39.75	1.54	0.615
Mean cell volume, fL	37.42	35.48	36.49	37.86	1.45	0.660
MCH, pg	11.31	11.22	11.45	11.22	0.15	0.665
MCHC, g/dL	32.16	31.66	31.84	30.87	0.74	0.683
RDW	16.97	16.82	17.24	17.59	0.37	0.486
Leukocytes, 1 × 10^3/μL	6.55	6.82	6.95	7.48	0.41	0.453
Platelets, 1 × 10^3/μL	345.37	377.44	391.62	354.87	39.15	0.844
Triiodothyronine (T3), ng/mL	0.95[a]	0.62[b]	0.96[a]	0.82[a]	0.06	<0.001
Thyroxine (T4), ng/mL	62.8[a,b]	59.0[b]	70.1[a]	69.4[a]	3.90	0.048
ALP, U/L	233.60	267.44	293.75	250.60	31.47	0.601
ALT, U/L	11.12[a]	7.22[b]	9.87[a]	9.42[a]	0.82	0.016
AST, U/L	86.25	80.66	90.75	104.37	10.21	0.393
Total proteins, g/dL	6.19[b]	6.23[b]	6.56[a]	6.47[a]	0.09	0.028
Albumin, g/dL	4.26[a,b]	4.21[b]	4.37[a]	4.39[a]	0.06	0.042
Alpha-1 globulin, g/dL	0.095	0.099	0.121	0.128	0.017	0.226
Alpha-2 globulin, g/dL	0.75[a,b]	0.71[b]	0.84[a]	0.76[a,b]	0.03	0.047
Beta globulin, g/L	0.18	0.19	0.21	0.21	0.02	0.657
Total thiocyanates, μg/mL	5.06[b]	13.33[a]	18.19[a]	16.94[a]	2.18	<0.001

References: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase. For each item, means with no letters (a, b, c) or with letters in common are not different ($P > 0.05$).

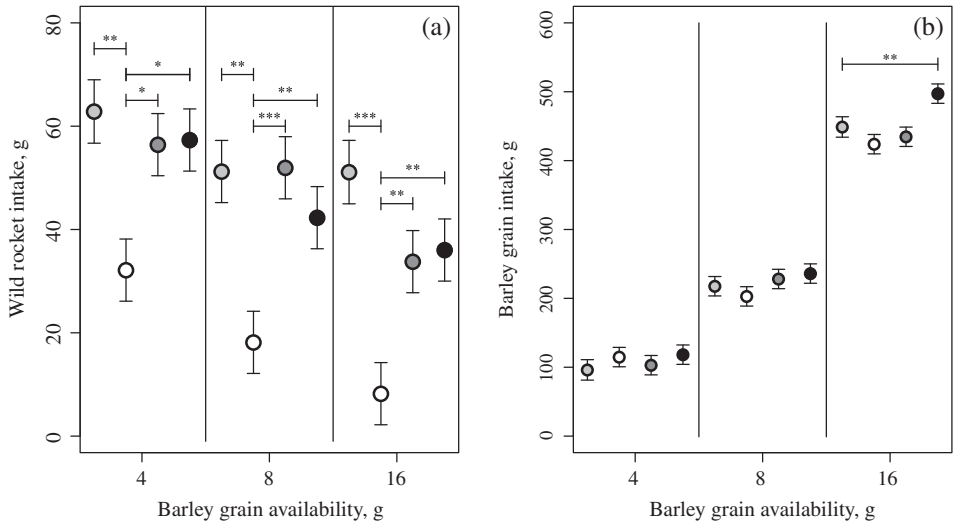

Fig. 2. Mean intake of wild rocket (a) and barley grain (b) by pairs of sheep ($n = 4$) previously fed with wild rocket ("DT", white dots), wild rocket followed by a protein supplement ("DT + P", gray dots), wild rocket followed by a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M", black dots), or alfalfa pellets in amounts pairing ingestion of wild rocket by sheep in DT ("CTRL", light gray dots). Data were collected during testing on an experimental arena where buckets containing a fixed amount of wild rocket or variable amounts of barley grain (4, 8, or 16 g) were randomly allocated. Dots represent least squares means values and error bars represent ±1 SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the lowest intake of wild rocket, irrespective of the level of barley availability ($F_{3,12} = 6.59$, $P = 0.007$). No differences in wild rocket intake were observed for CTRL, DT + P, or DT + P + M ($F_{2,12} = 0.22$, $P = 0.807$).

Fig. 2(b) summarizes barley intake data in the experimental arena. Barley intake increased with its availability in the arena ($F_{2,71} > 7.63$, $P < 0.001$). There was a treatment × level of barley availability interaction ($F_{6,71} > 4.27$, $P < 0.001$), mainly explained by a greater intake of barley by DT + P + M than by DT when the level of barley availability was 4 and 16 g.

3.2.2. Behavior

Fig. 3 summarizes data of the different activities measured in the experimental arena. Time spent eating wild rocket (Fig. 3a) decreased gradually as barley availability increased (Kruskal-Wallis test: Chi-squared$_2$ = 25.42, $P < 0.001$). Sheep in DT showed the lowest time spent eating wild rocket, irrespective of the level of barley availability in the arena (63.9, 26.8, 54.4, and 48.9 ± 4.78 s for CTRL, DT, DT + P, and DT + P + M, respectively; Kruskal-Wallis test: Chi-squared$_3$ = 12.62; $P = 0.005$).

Time spent eating barley (Fig. 3b) increased gradually as barley availability increased (Kruskal-Wallis test: Chi-squared$_2$ = 9.38, $P = 0.009$). However, there were no differences among groups regarding time spent eating barley (Kruskal-Wallis test: Chi-squared$_3$ = 6.78, $P = 0.079$).

Time spent searching (Fig. 3c) was greater when barley availability increased to 16 g (42.3, 41.0, and 62.2 ± 4.2 s, for 4, 8, and 16 g of barley availability, respectively; Kruskal-Wallis test: Chi-squared$_2$ = 11.72, $P = 0.003$). There were no differences among experimental groups regarding searching time during tests (Kruskal-Wallis test: Chi-squared$_3$ = 2.27, $P = 0.517$).

Time spent idling (Fig. 3d) was not affected by barley availability (Kruskal-Wallis test: Chi-squared$_2$ = 3.33, $P = 0.189$). Sheep in DT spent more time idling than sheep in the rest of the groups but only

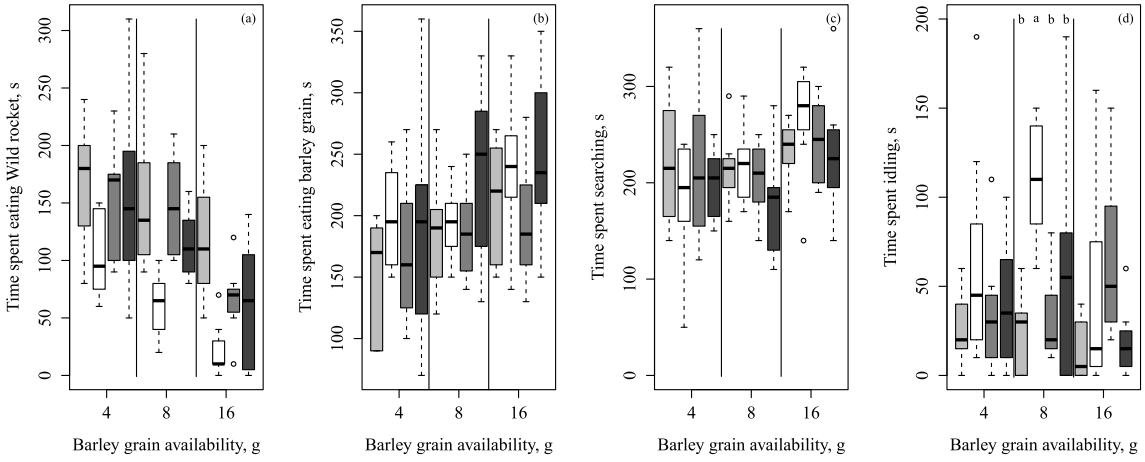

Fig. 3. Mean time spent eating wild rocket (a), mean time spent eating barley grain (b), mean time spent searching (c), and mean time spent idling (d) by individual sheep ($n = 8$) previously fed with wild rocket ("DT", white boxes), Wild rocket followed by a protein supplement ("DT + P", gray boxes), wild rocket followed by a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M", dark gray), or alfalfa pellets in amounts pairing ingestion of wild rocket by sheep in DT ("CTRL", light gray boxes). Data were collected during testing on an experimental arena where buckets containing a fixed amount of wild rocket or variable amounts of barley grain (4, 8, or 16 g) were randomly allocated. Box plots show the median (black line), the interquartile range (box), the minimum and maximum value within 1.5 times the interquartile range of the box (whiskers) and outliers (circles). Comparisons between treatments were done using the non-parametric Nemenyi's test. For each variable (i.e., activity) and level of availability of barley grain, boxes (representing treatments) that do not share letters in common differ significantly; $P < 0.05$.

when barley availability was 8 g (Kruskal-Wallis test: Chi-squared$_3$ = 11.30, $P = 0.004$).

Fig. 4 summarizes data and shows multiple comparisons for the number of visits made by sheep to each bucket containing either barley or wild rocket. Sheep in DT showed the lowest number of visits to buckets containing wild rocket, which was consistent among the different levels of barley availability assayed (Kruskal-Wallis test: Chi-squared$_3$ = 14.15, $P = 0.003$; 17.06, $P < 0.001$; and 17.95, $P < 0.001$; for 4, 8, and 16 g of barley availability, respectively). There were no differences between CTRL, DT + P, and DT + P + M regarding the total number of visits made to buckets with wild rocket ($P > 0.950$). Sheep in DT ate from less buckets with wild rocket (i.e., first visits) than sheep in the rest of the treatments, and this pattern was consistent among different levels of barley availability (Kruskal-Wallis test: Chi-squared$_3$ = 11.48, $P = 0.009$; 18.85, $P < 0.001$; and 21.06, $P < 0.001$; for 4, 8, and 16 g of barley availability, respectively). There were no differences among CTRL, DT + P, and DT + P + M for the number of first visits made to buckets containing wild rocket ($P > 0.947$ for all contrasts). Sheep in DT made less second visits to the same bucket than sheep in the rest of the treatments when levels of barley availability were 8 and 16 g (Kruskal-Wallis test: Chi-squared$_3$ = 15.86, $P = 0.001$; and 29.50, $P < 0.001$; respectively). When the level of barley availability was 4 g only DT + P showed greater number of second visits than DT. There were no differences among CTRL, DT + P, and DT + P + M for the number of second visits made to buckets with wild rocket ($P > 0.892$ for all contrasts). More than two visits to the same bucket containing wild rocket were less likely for DT than for sheep in the rest of the groups but only when the level of barley availability was 16 g (Kruskal-Wallis test: Chi-squared$_3$ = 31.46, $P < 0.001$). When the level of barley availability was 4 g, there were no differences among

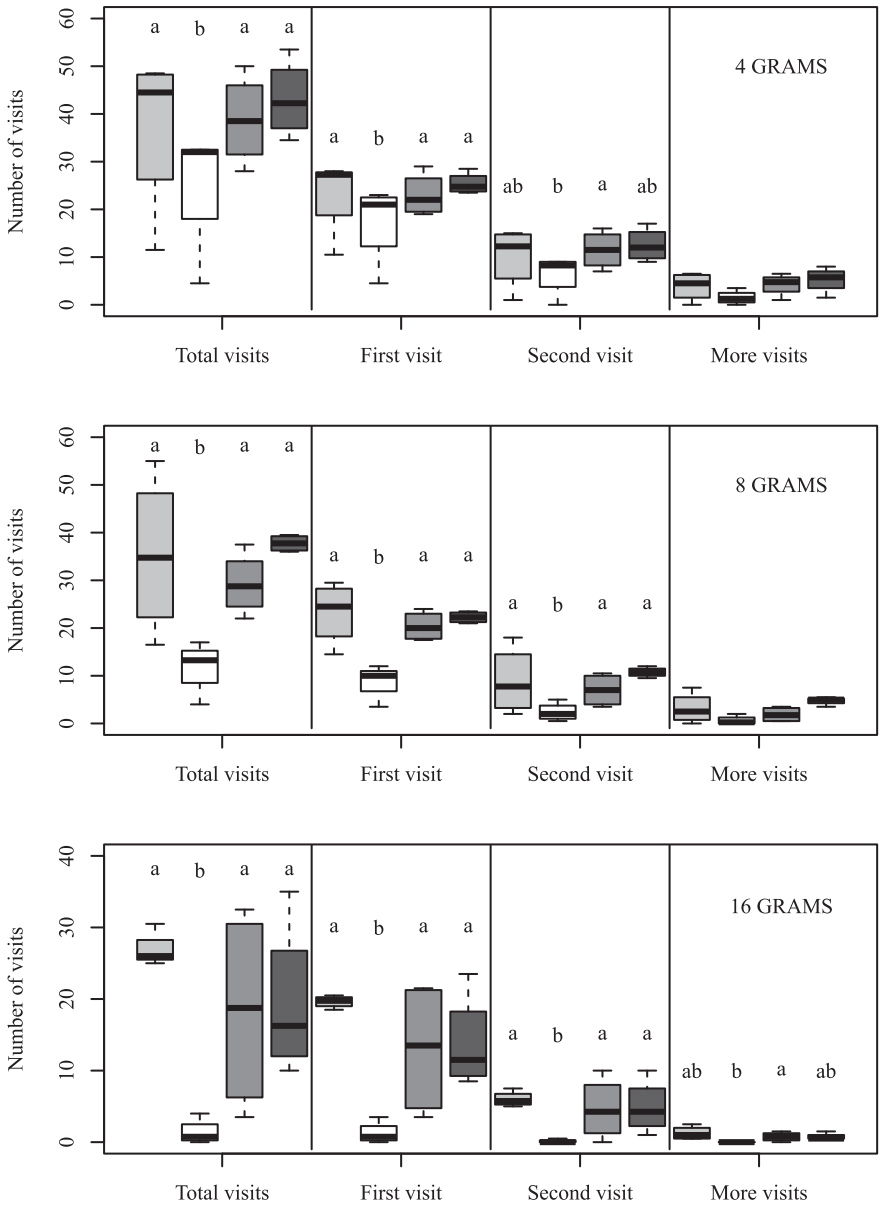

Fig. 4. Total number of visits, number of first visits, number of second visits, and number of more than two visits made to buckets containing wild rocket during testing phase by pairs of sheep ($n = 4$) previously fed with wild rocket ("DT", white boxes), wild rocket followed by a protein supplement ("DT + P", gray boxes), wild rocket followed by a protein supplement + a mineral supplement containing iodine and copper ("DT + P + M", dark gray), or alfalfa pellets in amounts pairing ingestion of wild rocket by sheep in DT ("CTRL", light gray boxes). Figures are segregated for the different levels of barley grain availability (4, 8, or 16 g) offered in separated buckets. Box plots show the median (black line), the interquartile range (box), the minimum and maximum value within 1.5 times the interquartile range of the box (whiskers) and outliers (circles). Comparisons between treatments were done using the non-parametric Nemenyi's test. For each variable (i.e., number of visits) and level of availability of barley grain, boxes (representing treatments) that do not share letters in common differ significantly; $P < 0.05$.

experimental groups regarding more than two visits to the same bucket containing wild rocket (Kruskal-Wallis test: Chi-squared$_3$ = 5.73, P = 0.125). However, when the level of barley availability was 8 g DT + P showed a greater number of more than two visits to the same bucket containing wild rocket than DT. There were no differences between CTRL, DT + P, and DT + P + M regarding the number of more than two visits to the same bucket containing wild rocket ($P > 0.960$ for all contrasts).

There were no differences among treatments for the total number of visits, number of first visits, number of second visits, and number of more than two visits made to buckets containing barley at any level of barley availability ($P > 0.701$, $P > 0.650$, $P > 0.507$, and $P > 0.307$; respectively).

4. Discussion

Results from this study support the hypothesis that the interaction between PSMs and nutrients influence consumers' physiology and as a consequence preference for a PSMs-containing plants like wild rocket. The nutritional supplements used in the present study (particularly the high protein meal) attenuated the deleterious effects of thiocyanates, as observed on some blood parameters related to thyroid gland functioning and red blood cell profiles. Sheep fed soybean meal during exposure showed greater intake of wild rocket, spent more time foraging and visited more sites containing this plant in the experimental arena than sheep which experienced wild rocket in the absence of a protein supplement. Unexpectedly, sheep naive to wild rocket (CTRL) showed levels of intake of this plant and behavioral responses during testing comparable to those observed in sheep which experienced wild rocket under supplementation. This response highlights the importance of a prior negative experience with wild rocket for the development of an aversive response to this plant during grazing.

4.1. Physiological consequences as a source of post-ingestive experience with wild rocket

During exposure, DT + P and DT + P + M showed a faster increase in wild rocket acceptance than sheep in DT. Ruminants are typically reluctant at accepting novel foods, i.e., they are neophobic, a protective mechanisms which probably evolved to reduce the likelihood of poisoning in novel feeding environments [43]. The transition from novel to familiar involves a process of generalization over familiar cues (e.g., familiar flavors) present in novel feeds [44] as well as learning about the post-ingestive consequences of the novel food [45,16]. Foods that provide chemicals required by the animal are increasingly accepted and become preferred, whereas those foods with chemicals that disrupt the animal's homeostasis are avoided [15].

In wild rocket, the end-products of glucosinolates after hydrolysis are bitter and spicy, orosensorial dimensions that are innately rejected by herbivores [46]. Nevertheless, DT + P and DT + P + M at the end of exposure showed greater intake of wild rocket than DT even though they experienced the highest levels of glucosinolates intake. Positive post-ingestive experiences from nutrients can override initial rejection of salient and bitter flavors [47]. Rats develop preference for previously rejected flavors when these flavors are paired with intragastric infusions of carbohydrates [48].

In the present experiment, protein supplementation (soybean meal) improved intake of wild rocket during exposure, whereas no additional advantage on intake or physiological responses was observed when minerals (iodine and cooper) were added to the protein concentrate. Protein supplementation in ruminants increase intake of plant species of high fiber content or with a high energy to protein content ratio [49]. Wild rocket, as well as other plants in the *Brassicaceae* family, presents high content of the sulphur-containing amino acid *S*-methyl-cysteine sulphoxide (SMCO) [50]. During ruminal fermentation, SMCO is hydrolyzed to dimethyl disulphide (DMDS), a compound that can inactivate proteins by blocking sulphydryl groups [51]. Considering the potential inactivation of wild rocket proteins, and given the fact that plants in the *Brassicaceae* family typically contain high levels of soluble carbohydrates [52], it is possible to predict an enhanced intake of wild rocket by sheep in response to protein supplementation. DMDS is also known to cause hemolytic anemia which is diagnosed by the occurrence of Heinz-Ehrlich bodies and reduced hemoglobin levels [53]. We observed a tendency for a reduced hemoglobin concentration in DT which could be explained as the result of DMDS toxicity. Similarly, sheep fed a mustard- (*Brassica juncea*) based meal showed lower hemoglobin concentration than sheep fed a groundnut- (DMDS free) based meal [54].

In addition to the positive effects of protein on wild rocket intake, it was also observed a clear improvement in thyroid gland function. Sheep in DT had lower concentration of triiodothyronine (T3) than CTRL. However, animals fed wild rocket and soybean meal (DT + P and DT + P + M) displayed greater concentrations of T3 and thyroxine (T4) than DT; furthermore, these concentrations were comparable to those observed in CTRL. Thiocyanates (end-products of glucosinolates hydrolysis) are known to generate thyroid disturbances characterized by depressed levels of plasma T3 and T4 [55]. This is because thiocyanates inhibit iodine uptake by the thyroid gland [56]. Iodine supplementation in ruminants fed foods high in glucosinolates content has been shown to increase plasmatic concentration of T3 and T4 [57]. Nevertheless, no additional benefits on these hormones over protein supplementation were observed by iodine supplementation in animals consuming wild rocket. Thus, our results suggest that protein supplementation alone was responsible for returning concentrations of thyroid hormones to levels observed in CTRL. In contrast to our study, previous research exploring the effect of glucosinolates on consumers used foods of high protein content as glucosinolates source, i.e., mustard cake [57], mustard meal [27], which made it unlikely for the occurrence of a protein deficiency in the experimental animals. Moreover, daily glucosinolates ingestion in previous studies (e.g., [27]) was greater than glucosinolates intake in the present study, making iodine supplementation in the former scenario a more effective means for reducing the negative impacts of these toxins on thyroid function.

Glucosinolates intake does not appear to produce deleterious effects on liver morphology and function in ruminants [58,59,60], but see [61]. Consistent with these findings, we found no differences in alkaline phosphatase (ALP) and aspartate aminotransferase (AST) concentration between CTRL and DT. However, alanine aminotransferase (ALT) concentrations were the lowest for DT. Cattle fed *Festuca arundinacea* Schreb (tall fescue) containing alkaloids show lower levels of AST and ALT than cattle fed tall fescue with low levels of alkaloids [62] or without alkaloids [63]. Similarly, Cox-Ganser et al. [64] found higher levels of AST in lambs fed grass-clover pastures than in lambs fed Brassica pastures. Low ALT levels may indicate a reduced concentration of this enzyme in hepatocytes or a reduced liver tissue mass [62]. However, unlike ALP and AST, ALT is not considered an accurate diagnostic tool for liver disease in ruminants [65].

Protein supplementation in this study was effective at attenuating some of the negative post-ingestive effects of wild rocket ingestion. Total serum proteins and albumin concentrations were lower in DT than in DT + P and DT + P + M. A reduction in serum protein content for DT could have been induced by the toxic effects of wild rocket. For instance, sheep fed Brassica pastures show lower levels of albumin than sheep fed grass pastures [64]. However, our results suggest that modulation of serum proteins level was mainly explained by the greater protein intake of supplemented sheep, since sheep that did not eat wild rocket (CTRL) showed reduced levels of serum proteins when compared to DT + P and DT + P + M. Once thiocyanates enter the blood stream they partially bind to albumins [66]. Increased albumins due to protein supplementation (serum albumin is a good indicator of sheep protein status) [67] might have increased binding of thiocyanates in plasma which in turn reduced the biological activity of the toxin [68]. Protein

supplementation also aids in the process of detoxification of a wide array of toxins [1]. Foley and Moore [46] suggested that detoxification and excretion of toxins are the most important variables affecting ingestion of toxic plants, which is consistent with the increase in wild rocket intake by supplemented animals in our study. Because we did not find evidence of a lower level of total serum thiocyanates in DT + P and DT + P + M when compared to DT, we suggest that the reduction of the biological activity of the toxin is a more likely explanation for the positive effects of protein supplementation than the increased excretion of the toxin.

4.2. Impact of previous experiences with wild rocket on foraging behavior of sheep

Both groups of sheep offered the protein supplement during exposure showed greater intake of wild rocket in the foraging arena than animals which experienced the plant without supplementation. Supplemented animals displayed an increase in wild rocket intake of 71, 221, and 250% relative to DT when availability of barley grain in the testing trials was 4, 8, or 16 g, respectively. Several toxic plants like wild rocket are also nutritious and this explains why an amelioration in toxicity leads to an increase in preference for such plants (e.g., [69]). However, a closer interaction between nutrients and toxins is needed in order to ameliorate the negative impact of toxins [23]. In natural foraging conditions, when there is a simultaneous availability of nutritive plants and toxic plants, it is unlikely that herbivores will eat both foods in the same sequence as observed in this study (see also [1]). When free to choose, herbivores display optimal foraging decisions based on maximization of nutrient intake rate [70], as well as minimization of toxins loads in the diet [71]. Therefore, herbivores focus on those plants and patches that offer high nutrient – low toxin intake rates, being the inclusion of plant species with high concentration of chemical defenses or lower content of nutrients dependent upon the depletion of the preferred plant species (see [32]). The selective intake of nutritious plants in natural settings and the asynchronous and sporadic sampling of unpalatable species likely enhance negative experiences with toxic plants, which explains the typical patterns of avoidance observed in grazing animals [12,22]. Shaw et al. [72] observed that only lambs forced to eat *Artemisia tridentata* (a toxic shrub) along with highly nutritious herbs increased intake and showed greater use of the shrub in a subsequent preference trial.

Foraging behavior was affected by previous experience with wild rocket. Sheep exposed to DT + P and DT + P + M spent a greater amount of time eating wild rocket than DT; although, time spent searching was similar among groups as well as time spent eating barley grain. Catanese et al. [32] observed that sheep fed a low-quality food in close temporal association with ingestion of a protein supplement ("CS+") spent more time eating the low-nutritious hay in a subsequent foraging trial than sheep that were fed the same hay but without the protein supplement ("CS−"). However, unlike the present study, sheep in CS+ spent less time foraging a preferred resource (*Medicago sativa* hay) than sheep in CS−. This discrepancy between results could be explained by differences in the experimental settings in which testing took place between studies. In Catanese et al. [32] the use of a "U-shaped corridor" allowed for a clearer substitution between activities (eating the low-quality food or searching and eating the high-quality food) than the use of the experimental arena in the present study.

Sheep exposed to DT showed a lower amount of total visits, as well as first visits, to buckets containing wild rocket than sheep in the rest of the groups. Sheep graze in a sequence in which preferred plants' parts (e.g., leaves) are eaten first, then they continue grazing the remaining parts of preferred plants (e.g., pseudostems), and once preferred plants are depleted they start grazing less preferred plants [73]. Thus, increased number of visits to the same bucket with wild rocket (i.e., similar to a second grazing) could be interpreted as an increased motivation to eat this plant in the presence of a higher quality alternative. Sheep previously fed wild rocket and the protein supplement were more likely to make more than a single visit to the buckets containing wild rocket than DT when barley grain availability increased. These results are in contrast to previous findings with low-quality foods where greater preference for the low-quality food previously paired with a protein supplement becomes more evident as availability of the preferred high-quality food declines [32]. These results highlight differences between foods which are unpalatable due to their poor nutritional value from those which are unpalatable due to the presence of toxins. Even when protein supplementation can improve digestion of foods poor in nutrients [74], the nutrient supply by this type of foods per se is still low. In contrast, the reduction of PSMs' activity by protein supplementation could provide animals with full access to a very nutritious food, as it is the case for wild rocket. In this sense, following conditioning a high-quality food should act as a better substitute for a low-nutritious food than for a highly nutritious food provided with toxins. This encourages further studies exploring animals' foraging decisions when exposed to foods that vary in the nutritional and/or toxicological properties.

Sheep that were not previously exposed to wild rocket showed greater intake of this plant during testing sessions than DT. A possible explanation is that during testing phase CTRL ingested small amounts of wild rocket that were insufficient to cause negative post-ingestive consequences and to develop an aversion to this plant. For instance, the highest individual intake of wild rocket by CTRL during testing phase was on average only a 21.5% of wild rocket intake during exposure phase by DT + P and DT + P + M. Food preferences and aversions reside along a continuum that depends on the amount of nutrients and/or toxins ingested [15]. Sheep develop preferences for foods associated to low doses of high-energy substances (i.e., propionate or acetate) and aversions to foods associated to high doses of these same high-energy substances [75]. A complementary piece of evidence for this suggestion comes from DT + P and DT + P + M, which showed levels of ingestion of wild rocket during testing sessions similar to CTRL. Catanese et al. [17] observed that sheep exposed to alkaloid-containing tall fescue and a tannin-rich legume ("Treatment") that attenuates the negative post-ingestive effects of alkaloids ingest more tall fescue during exposure than sheep exposed to the tall fescue with alkaloids alone ("Control"). However, during a trial in which all sheep were fed with tall fescue with alkaloids only, sheep in Treatment showed the lowest intake of tall fescue. As the authors suggested, a contrast between the situation in which the toxic plant was experienced (with a supplement that ameliorates the negative effect of toxins) and the situation in which the toxic plant was later ingested (without the protective effect of the supplement) can explain the stronger aversion developed by sheep in Treatment to the toxic plant during testing. In our work, DT + P and DT + P + M expressed their previous positive experience with wild rocket and, similar to sheep in CTRL, this could have been possible by the low ingestion of wild rocket during the testing phase. This argumentation highlight the need of further research to elucidate how experiences with a toxic plant in a "safe" context are influenced by different environmental situations in which the toxic plant can be found during foraging (e.g., without the "medicine" or with alternative foods providing non-complementary nutrients or toxins).

In conclusion, our study suggests that nutrient-toxins interactions have the potential to attenuate the negative post-ingestive effects of toxic plants on consumers and enhance preference for these chemically defended plants in mixtures of plant species. Protein supplementation improved several physiological parameters in sheep consuming wild rocket and as a consequence the experience of the animals with this toxic food. In turn, such positive experiences increased preference for wild rocket in a foraging arena that also presented foraging sites with a high-quality food as an alternative for selection. Our results also suggest that a negative feeding experience with wild rocket is needed for animals to display the typical pattern of aversion to this plant commonly observed in grazing conditions.

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