Departament de Ciència Animal i dels Aliments

Conditioned aversion to woody crops in small ruminants

Aversión condicionada a cultivos leñosos en pequeños rumiantes

Aversió condicionada a cultius llenyosos en petits remugants

DOCTORAL THESIS

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Tesis presentada por Carmen L. Manuelian Fusté, dirigida por la Dra. Elena Albanell Trullàs y el Dr. Gerardo Caja López del Departamento de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona, para la obtención del título de Doctor.

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Vº Bº

Dra. Elena Albanell Trullàs

Dr. Gerardo Caja López

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SCIENTIFIC DISSEMINATION

Part of this thesis has led to the following scientific and divulgative publications and presentations at international and national conferences.

Publications in international indexed peer-reviewed journals

Manuelian, C.L., Albanell, E., Salama, A.A.K., Caja, G. 2010. Conditioned aversion to olive tree leaves (*Olea europea* L.) in goats and sheep. Appl. Anim. Behav. Sci. 128:45-49.

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Albanell, E, Manuelian, C.L. 2012. Pastoreo selectivo en cultivos leñosos. Revista digital de divulgación científica UABDivulga (ISSN: 2014-6388).

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LIST OF ABBREVATIONS

AC	Autonomous Community
AV	Aversion
AV1	Group Aversion 1 with 200 mg LiCl/kg BW
AV2	Group Aversion 2 with 225 mg LiCl/kg BW
BW	Body weight
C	Control
C_{max}	Concentration at peak
CN	Casein
CP	Crude protein
CTA	Conditioned taste aversion
CTZ	Chemoreceptor trigger zone
DM	Dry matter
LiCl	Lithium chloride
NDF	Neutral detergent fibre
NPN	Non-protein Nitrogen
$T_{1/2}$	Plasma half-life
T_{max}	Time tat peak concentration
TP	True protein
TS	Total solids
WP	Whey protein

ABSTRACT

This thesis aimed to evaluate the suitability of conditioned taste aversion (CTA), based on the use of lithium chloride (LiCl), to prevent grazing damages in woody crops (i.e., olive groves and vineyards). The CTA is an associative learning behaviour in which an animal avoids consuming a particular feed previously paired with an inductor agent (i.e., LiCl). Currently, grazing sheep and goat for controlling ground cover in crops is not suitable because leaves and stems of woody plants are very palatable for them.

In Exp. 1, CTA against olive leaves (novel feed) was induced with a single LiCl dose (200 mg/kg BW) in goats (n = 10) and sheep (n = 10). Aversion was validated individually during the first 6 d and persistence for 4 mo. Ewes and does maintained complete aversion (intake 0 g) until d 23 and 53, respectively ($P < 0.05$) but their intake was markedly lower (ewes, 40 vs. 83 g; does, 24 vs. 60 g) than control animals throughout the experiment ($P < 0.05$). A single dose of LiCl was effective to induce CTA against olive leaf, does being more strongly averted than ewes.

In Exp. 2, CTA against olive leaves (novel feed) was induced in ewes of 3 breeds (Manchega, Lacaune and Ripollesa; n = 15 for each breed) using different LiCl doses (AV1 and AV2, 200 and 225 mg/kg BW). Ewes that consumed >10 g of olive leaves received a 2nd dose on d 9. Persistence was evaluated by a double-choice feeding assay with rye-grass trough 70 d. Effective aversion length varied by breed (Manchega < Lacaune = Ripollesa) for AV1, but no differences were detected for AV2. We concluded that the 225 mg LiCl/kg BW dose was effective for the 3 breeds.

In Exp. 3, long-term CTA (3 yr) against grapevine leaves (novel feed) was induced in ewes of 2 breeds (Lacaune and Manchega; n = 12 for each breed) with a single dose of LiCl (225 mg/kg BW). Effectiveness of CTA was validated under experimental (yr 1) and commercial vineyard conditions (yr 2 and 3). The CTA ewes showed a complete aversion against grapevines throughout yr 1 but a new LiCl dose were needed to maintain CTA during yr 2 and 3. Grazing CTA ewes reduced grass cover between vine lines by 68 and 44% (DM basis), yr 2 and 3, respectively. The use of CTA was effective for controlling ground cover in vineyards.

In Exp. 4, Li concentration was measured over 168 h (lactating does; n = 6) and 192 h (dry ewes; n = 6) in plasma, urine, faeces, and milk after a single LiCl dose of 200 and 225 mg/kg BW for does and ewes, respectively. Plasma Li concentrations peaked at 4 h in does and 12 h in ewes, according to their physiological stage. The calculated plasma half-lives were 40.3 and 30.9 h, for does and ewes, respectively. In goats, all Li administered was recovered at 96 h (urine, 92%; faeces, 6.5%; milk, 2.8%); however, the estimated clearance time established by faeces was 11 and 9 d for does and ewes, respectively. Consequently, the use of LiCl was considered as safe and suitable for inducing CTA in small ruminants.

In conclusion, induced CTA with LiCl in sheep and goats could be used as an alternative for controlling the ground cover in olive groves and vineyards, without expected damages to the trees and vines during grazing.

RESUMEN

El objetivo de la tesis fue evaluar la idoneidad de la aversión condicionada (CTA) con cloruro de litio (LiCl), para evitar daños en cultivos leñosos durante el pastoreo de las cubiertas vegetales. La CTA es un aprendizaje asociativo por el que un animal deja de consumir un alimento tras la administración de un agente inductor (LiCl). En la actualidad, los agricultores no permiten el pastoreo de las cubiertas en cultivos leñosos debido a la alta palatabilidad de estos.

En el Exp. 1, se creó CTA al olivo (alimento nuevo) con una sola dosis de LiCl (200 mg/kg PV) en cabras (n = 10) y ovejas (n = 10). La aversión se comprobó durante los primeros 6 d, y la persistencia durante 4 meses. Las ovejas y cabras rechazaron completamente el consumo de olivo durante 23 y 53 d ($P < 0.05$), respectivamente, y consumieron una cantidad inferior al control hasta el final del experimento (ovejas, 40 vs. 83 g; cabras, 24 vs. 60 g; $P < 0.05$). Una sola dosis de LiCl fue efectiva para inducir CTA al olivo, presentando mayor aversión las cabras que las ovejas.

En el Exp. 2, se creó aversión al olivo en 3 razas de ovejas (Manchega, Lacaune y Ripollesa; n = 15 por raza) utilizando diferentes dosis de LiCl (AV1 y AV2, 200 y 225 mg/kg PV). Las ovejas que consumieron > 10 g de olivo recibieron una segunda dosis el d 9. La persistencia de la CTA se evaluó ofreciendo como alimento alternativo rye-grass durante 70 d. La persistencia para AV1 varió en función de la raza (Manchega < Lacaune = Ripollesa), mientras que para AV2 no se detectaron diferencias. Los resultados indicaron que la dosis de 225 mg LiCl/kg PV fue igual de eficaz para las 3 razas.

En el Exp. 3, se creó CTA de larga duración (3 años) a la viña (alimento nuevo) en dos razas de ovejas (Lacaune y Manchega; n = 12 por raza) con una sola dosis de LiCl (225 mg/kg PV). La efectividad de la CTA fue validada en condiciones experimentales (año 1) y de viñedo comercial (año 2 y 3). Las ovejas CTA rechazaron totalmente consumir las hojas de vid durante el primer año, pero fue necesaria una segunda dosis de LiCl para mantener la CTA durante el segundo y tercer año. La cubierta vegetal de la viña se redujo un 68 y 44% (base MS) por el pastoreo en los años 2 y 3, respectivamente.

En el Exp. 4, se midió la concentración de Li durante 168 h (cabras en lactación, n = 6) y 192 h (ovejas secas, n = 6) en plasma, orina, heces, y leche tras la administración de una dosis de LiCl (200 y 225 mg/kg PV para cabras y ovejas, respectivamente). Las concentraciones plasmáticas de Li alcanzaron su máximo a las 4 h en cabras y 12 h en ovejas. La semivida de Li calculada en plasma fue de 40,3 y 30,9 h, en cabras y ovejas, respectivamente. En las cabras, se recupero todo el Li administrado a las 96 h (orina, 92%; heces, 6,5%; leche, 2,8%); sin embargo, el tiempo de eliminación estimado establecido por las heces fue del 11 y el 9 d en cabras y ovejas, respectivamente. Consecuentemente, el uso de LiCl se consideró seguro y adecuado para inducir CTA en los pequeños rumiantes.

En conclusión, la CTA inducida con LiCl en ovejas y cabras podría utilizarse como alternativa para el control de la cubierta vegetal en olivares y viñedos, sin dañarlos durante el pastoreo.

RESUM

L'objectiu de la tesi va ser avaluar la idoneïtat de l'aversió condicionada (CTA) amb clorur de liti (LiCl), per evitar danys en cultius llenyosos durant el pasturatge de les cobertes vegetals. La CTA és un aprenentatge associatiu pel qual un animal deixa de consumir un aliment gràcies a l'administració d'un agent inductor (LiCl). Actualment, els agricultors no permeten el pasturatge de les cobertes dels cultius llenyosos per l'alta palatabilitat d'aquests.

A l'Exp. 1, es va crear CTA a l'olivera (aliment nou) amb una sola dosi de LiCl (200 mg/kg PV) en cabres (n = 10) i ovelles (n = 10). L'aversió es va comprovar durant els primers 6 d, i la persistència durant 4 mesos. Les ovelles i cabres van rebutjar completament el consum d'olivera durant 23 i 53 d (P < 0.05), respectivament, i van consumir una quantitat inferior als animals control fins al final de l'experiment (ovelles, 40 vs. 83 g, cabres, 24 vs. 60 g; P < 0.05). Una sola dosi de LiCl va ser efectiva per induir CTA l'olivera, presentant major aversió les cabres que les ovelles.

A l'Exp. 2, es va crear aversió a l'olivera en 3 races d'ovelles (Manxega, Lacaune i Ripollesa; n = 15 per raça) utilitzant diferents dosis de LiCl (AV1 i AV2, 200 i 225 mg/kg PV). Les ovelles que van consumir > 10 g d'olivera van rebre una 2a dosi el d 9. La persistència de la CTA es va avaluar oferint com a aliment alternatiu rye-grass durant 70 d. La persistència per AV1 va variar en funció de la raça (Manxega < Lacaune = Ripollesa), mentre que per AV2 no es van detectar diferències. Els resultats van indicar que la dosi de 225 mg LiCl/kg PV va ser igual d'eficaç per les 3 races.

A l'Exp. 3, es va crear CTA de llarga durada (3 anys) a la vinya (aliment nou) en dues races d'ovelles (Lacaune i Manxega; n = 12 per raça) amb una sola dosi de LiCl (225 mg/kg PV). L'efectivitat de la CTA va ser validada en condicions experimentals (any 1) i de vinya comercial (any 2 i 3). Les ovelles CTA van rebutjar totalment consumir les fulles de vinya durant el 1er any, però va ser necessària una 2a dosi de LiCl per mantenir la CTA durant el 2n i 3er any. La coberta vegetal de la vinya es va reduir un 68 i 44% (base MS) com a conseqüència del pasturatge en els anys 2 i 3, respectivament.

A l'Exp. 4, es va mesurar la concentració de Li durant 168 h (cabres en lactació, n = 6) i 192 h (ovelles seques, n = 6) en plasma, orina, femta i llet després de l'administració d'una dosi de LiCl (200 i 225 mg/kg PV per cabres i ovelles, respectivament). Les concentracions plasmàtiques de Li arribar al seu màxim a les 4 h en cabres i 12 h en ovelles. La semivida de Li calculada en plasma va ser de 40,3 i 30,9 h, en cabres i ovelles, respectivament. En les cabres, es va recuperar tot el Li administrat a les 96 h (orina, 92%, femtes, 6,5%; llet, 2,8%); però, el temps d'eliminació estimat establert per la femta va ser del 11 i el 9 d en cabres i ovelles, respectivament. Conseqüentment, l'ús de LiCl es va considerar segur i adequat per induir CTA en els petits remugants.

En conclusió, la CTA induïda amb LiCl en ovelles i cabres podria utilitzar-se com a alternativa per al control de la coberta vegetal en oliveres i vinyes, sense danyar-los durant el pasturatge.

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CHAPTER 1. INTRODUCTION

1. INTRODUCTION

1.1. The relevance of woody crops in Spain

The total geographic surface of Spain is approximately 50.6 Mha, where the 33.4% are crop lands and the 19.8% pastures (MAGRAMA, 2013). Crop lands are mainly devoted to herbaceous crops (8.97 Mha; 52.8%), woody crops (4.56 Mha; 26.9%) and fallow lands (3.46 Mha; 20.3%; MAGRAMA, 2013). Near 33.7% of the Spanish crop land is submitted to Mediterranean climate conditions (MAGRAMA, 2013) which are characterized by short and mild winters, and dry and hot summers, being water the scarcest and most valuable resource and the main productivity factor.

Consequently, dry-farming systems are predominant in the majority of farms, combining olive (*Olea europea* L.) and vine (*Vitis vinifera* L.) in different proportions (Fernández-Zamudio and De Miguel, 2006). These woody species are frequently grown with marginal management and have survived the successive structural, social and economic changes which have taken place in these regions. Most of the oil and wine produced in Spain has Food Quality Certification and is appreciated by world consumers, being a key part of the spanish culture and economy.

1.1.1. Olive groves, olives and oil

Spain is the greatest world olive producer with 7,820,060 t in 2011 as shown in Fig. 1.1. (FAOSTAT, 2014). With Italy and Greece, it represents the 98% of olive production in Europe. The Spanish production is mainly located in the Autonomous Community (AC) of Andalucía (84%), the 94% of the harvest being intended to produce oil (MAGRAMA, 2013). During the harvest season of 2011-12, Spain produced 1,567,523 t of extra virgin olive oil (MAGRAMA, 2013).

In addition, Spain is the greatest world olive oil exporter. The Agencia de Información y Control Alimentarios (AICA) of the Ministerio de Agricultura, Alimentación y Medio Ambiente reported that Spain has exported 413,400 t of olive oil during January and February 2014. The main country of destination is Italy, followed by France, Portugal and United Kingdom. Also, Spain supplies olive oil to the United States of America, Australia, Brazil and Japan.

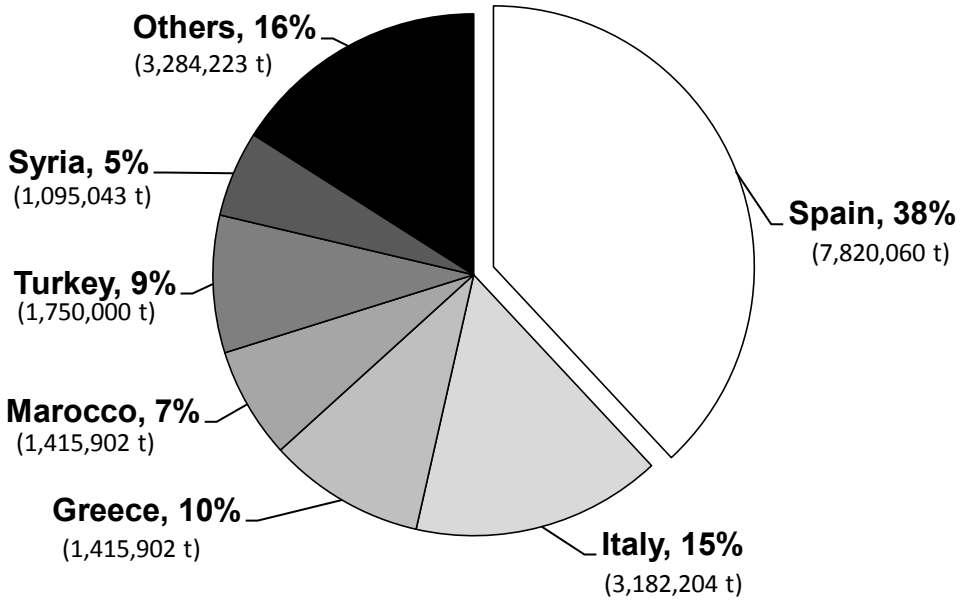

Figure 1.1. World's olive production by country in 2011 (FAOSTAT, 2014).

In Spain there are over 260 cultivated varieties of olive trees, although only 24 are used regularly in the production of oil. Each variety is traditionally linked to a specific growing area. The variety Picual is the most important and represents the 50% of the production in Spain, and it is the mainly variety cultivated in the AC of Andalucía. Its oil yield is the highest, approximately 27%, although the average is about 22%. The Cornicabra variety occupies the second-largest growing area, mainly in the AC of Castilla-La Mancha, and has an oil yield of 19%. The most common variety in the AC of Cataluña is the Arbequina, with a relatively high oil yield of 20.5% (Olive Oil from Spain, 2014).

1.1.2. Vineyards, grapes and wine

Regarding the world's grape production shown in Fig. 1.2., Spain is in 4th position with 5,809,315 t (FAOSTAT, 2014). Jointly with Italy and France, they harvest the 71.5% of the Europe's grapes production. The Spanish vineyards are located mainly in the AACC of Castilla-La Mancha (46.3%), Extremadura (8.7%) Castilla y León (7.9%), Valencia (7.5%) and Cataluña (5.9%), the 95.8% of the total harvest being devoted to produce wine (MAGRAMA, 2013). The last available data of fresh wine production (harvest season 2011-12) was 33,709,123 hL.

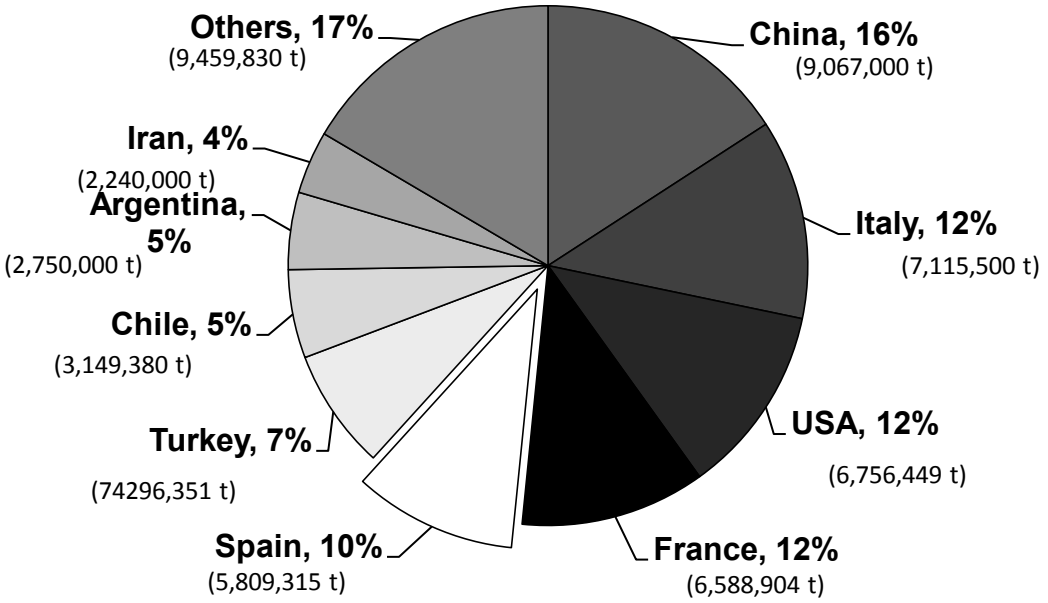

Figure 1.2. World's grape production in 2011 by country (FAOSTAT, 2014).

In addition, Spain competes with Italy for being the greatest world wine exporter. In 2013, Spain was the second greatest world wine exporter with 17,700,000 t. The main country of destination is Italy, followed by France, United Kingdom and Portugal. Spain also supplies wine to the United States of America, Russia, Canada and Japan (El vino en cifras, 2013).

The most common grape varieties in Spain, in order of importance of growing areas, are Tempranillo (20.9%), Bobal (7.5%), Garnacha, Monastrell, Pardina,

Macabeo and Palomino, all of them intended for wine production (El vino en cifras, 2014).

1.2. The relevance of small ruminants in Spain

The united Kingdom and Spain concentrate approximately half of the sheep population in the European Union, with 33.0 and 17.2%, respectively (Fig. 1.3.). Additionally, Greece and Spain account for almost the 57% of goat population with 33.6 and 21.4% of head, respectively (FAOSTAT, 2014; Fig. 1.4.).

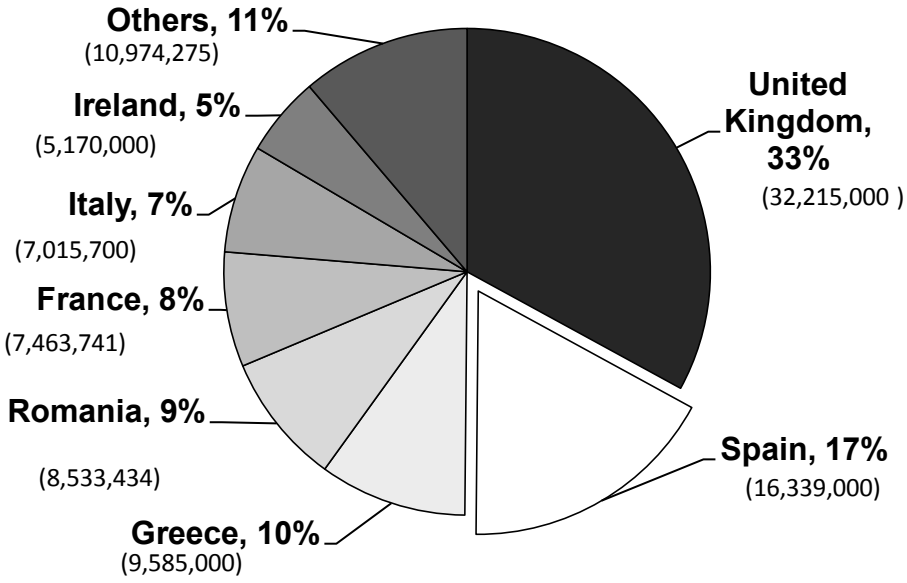

Figure 1.3. Europe Union's sheep census in 2012 by country (FAOSTAT, 2014).

One of the most important characteristics of sheep and goats farming is the ability to settle in areas where natural resources are scarce and agro-climatic conditions are extreme, as in the Spanish Mediterranean region. In that region, the traditional small ruminants farming, use as feed resources the by-products of cereal crops (straw and stalks), stubbles and fallow lands, as well as bushy pastures and the typical Dehesa (prairie under trees) that would otherwise sit idle and contribute to maintain the ecosystems (Correal and Sotomayor, 1998).

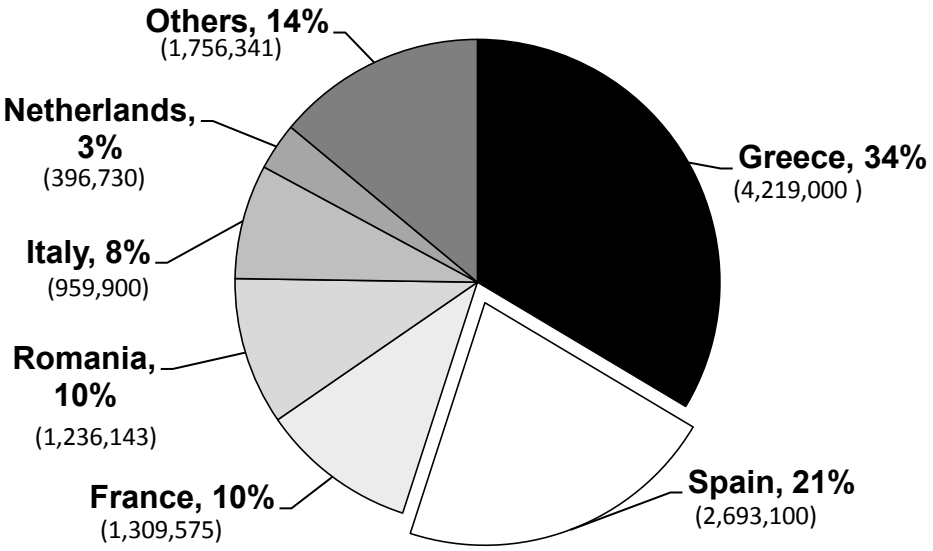

Figure 1.4. Europe Union's goat census in 2012 by country (FAOSTAT, 2014).

The low rainfall and its high variation from year to year, impacts on crop and pasture production, making difficult to manage the natural feed resources with the small ruminants and being easy to overgraze or undergraze them (Mangado et al., 2008). Spain is also the second greatest European country that produces ovine and goat meat with 122,003 t and 9,696 t, respectively (MAGRAMA, 2013). Additionally, Spain is the third European Union country producing sheep milk with 552,517 t, and the second producing goat milk, with 443,625 t (MAGRAMA, 2013).

Other environmental and socio-economic benefits derived from the small ruminants farms in the Mediterranean region are the following: the conservation and protection of natural areas and biodiversity through grazing, the control of biomass to avoid frequent forest fires extremely common in the Mediterranean area, the fixation of population in poor and disadvantaged rural areas that cannot support other agricultural activities, and the production of differentiated quality products (i.e., meats and cheeses) linked to specific areas, local breeds and traditional cultures, which help to increase the added value of production.

1.3. Woody crops production systems

With regard to soil cultivation practices, there are different ways to manage soil in woody crop lands. They vary from the most intensive and less environmentally friendly (all-arable) systems, to the traditional extensive or the more vegetal-animal integrated systems (sustainable agriculture).

1.3.1. All-arable systems

The predominant production system for woody crops is the all-arable farming. Ploughing buries the previous crop residues and weeds, modifying the soil structure and creating a clean surface without other plants concurrence (Fig. 1.5.a). Nevertheless, it reduces the soil biotic capacities for self-regeneration and, as a consequence, soil erosion is a major issue for woody crops, producing mid-term land degradation.

The use of tilling tools or heavy working equipment in the all-arable farming systems produce soil compactness, whose degree varies depending on the soil properties (texture, structure, moisture, and initial compactness; Håkansson et al., 1988; Hansen and Engelstad, 1999). They reduce the large soil pores (>30 μm), which are important for water and air retention and are the most easily occupied by plant roots, arthropods and earthworms (Håkansson et al., 1988). Earthworms are very important because of their role in soil processes, including aggregation, residues decomposition, nutrients mineralization (worms casts), aeration, water infiltration, as well as their positive influence in plant growth (Pommeresche and Løes, 2009). Another important aspect affected by soil compactness is the reduction in the water infiltration rate, which increases the potential risks of surface water pounding, water runoff, surface soil water logging and soil erosion (Håkansson et al., 1988).

Figure 1.5. a) Vineyard in all-arable systems. b) Vineyard with ground cover systems.

1.3.2. Ground cover systems

Ground cover between lines is an important step toward mitigation of soil erosion and degradation in woody crops (Fig. 1.5.b). Other soil benefits are the increase of water, organic carbon and nitrogen retentions (Alonso and Guzmán, 2006). It also contributes to maintain and increase the biodiversity of the environment, improving the natural equilibrium between crops pests and predators. The species used for ground cover can be spontaneous or cultivated. The species selected should be well adapted to the conditions of soil, climate and crop management. Woody crops on degraded and infertile soils can be favoured by using several legumes, as clover (*Trifolium* spp.) and vetch (*Vicia* spp., alone or with barley or oat) to fix atmospheric nitrogen (Guzmán and Foraster, 2007).

However, the growing plants of the ground cover may compete with the crop for nutrients (especially for nitrogen) and water, impacting on the vigour and the yield of the woody plant crop. Those detrimental effects could be desirable in some productive moments because they modify the phenology, health status, yield and quality of the crop, needing a careful management of the ground cover (Ibáñez et al., 2011).

The most important advantages and disadvantages of using ground cover in woody crops are listed in Table 1.1.

Table 1.1. Advantages and disadvantages of using ground cover in woody crops (Modified from Guzmán and Foraster, 2007).

Advantages	Disadvantages
Less water erosion and soil compactness	Compete by water and nutrients
Improve soil structure	Make difficult the harvesting of woody crops
Increase infiltration of rainwater	Increase the risk of fire in summer (if not controlled)
Increase of biomass	Increase the risk of frost in winter
Increase the biological activity of soil	Regrowth after being removed
Reduce the use of fertilizers	
Increase the N fixation (if legumes)	
Promote the biodiversity	
Help of pest and diseases control	
Provide feed for livestock	
Improve the landscape	

1.3.3. Sustainable agriculture systems

In the last years, more responsible and proactive actions for protecting earth's ecological balance have been encouraged. With the primary goals of profitability, social equity and conservation of natural resources (Fernández-Zamudio and De Miguel, 2006), the concept of sustainable agriculture farming system is used. It includes a wide range of techniques (free-range, low-input, holistic...) from which biodynamic and organic are currently the most relevant.

Biodynamic farming is considered a spiritual-ethical-ecological approach of agriculture developed by Rudolph Steiner. Farmers create a diversified and balanced within-farm ecosystem which boosts the health and fertility of the farm.

Self made preparation from fermented manure, minerals and herbs are used to restore and harmonize the vital life forces of the farm. In addition, biodynamic farmers work with the influences of the cosmos on soil, plant and animal health (Biodynamics Association, 2014).

Organic farming generally involves a diversity of crops, animals and methods where energy use and off-farm inputs are minimized. Management techniques include ground cover, green and animal manure, composting, mulching, crop rotation, direct seeding and reduced tillage, which maintain soil fertility. Instead of using chemical pesticides, ecological farmers use organic pest control, such as introducing beneficial insects to the field.

Spain has increased the organic agriculture surface since 2000 (Fig. 1.6.) (MAGRAMA, 2013). In 2012, 82,000 ha of organic olive groves were registered, which represents the 5.1% of the Spanish organic agriculture surface (MAGRAMA, 2013). The surface of vineyards registered as organic in 2012 were 168,039 ha, which represents the 10.5% of the Spanish organic agriculture surface (MAGRAMA, 2013).

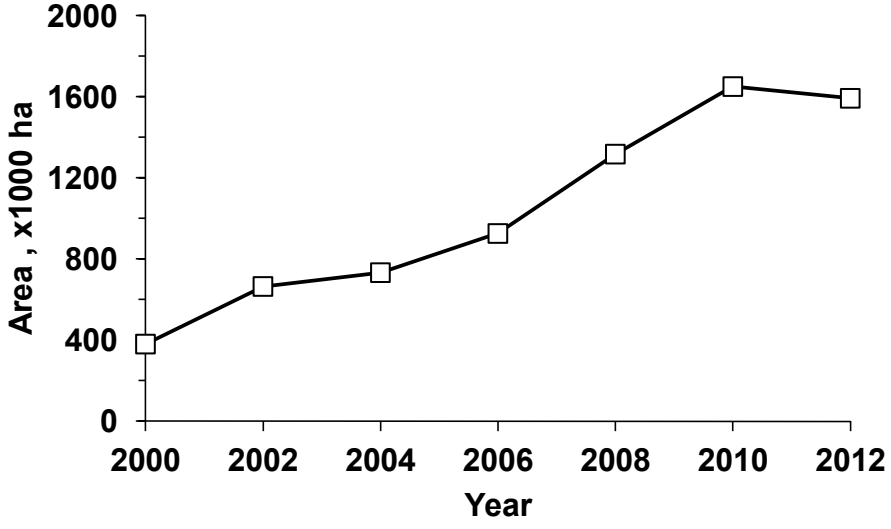

Figure 1.6. Organic agriculture surface evolution in Spain since 2000 (MAGRAMA, 2013).

1.4. Grazing in woody crops

Ground cover is usually controlled wiyh agrochemical products and tillage. However, grazing with small ruminants could be a more sustainable alternative with added benefits (Table 1.2.). Grazing is defined as the direct consumption of vegetation resources in the field by livestock. It is the most simple and inexpensive way to convert feed resources of low nutritional value for humans (plants) on high value animal products. Livestock only eat the aerial parts of the grass, keeping the roots and maintaining a ground cover length that protects the soil.

By grazing, livestock interacts at both vegetation and soil levels. Herbivores create and sustain sward heterogeneity (Tichit et al., 2005) and the spread of manure and urine improve the profile of nutrients and increase earthworm density and plant biomass (Murphy et al., 1995; Pommeresche and Løes, 2009). In addition, grazing controls the ground cover biomass better than tillage (Hatfield et al., 2007b). The optimal time to introduce livestock in a ground cover is when grass spikes are 10 cm high and when legumes are at 10% bloom (Guzmán and Foraster, 2007). The small ruminant hoof's shape and size churn and till up the soil instead of compressing it (Murphy et al., 1995), without affecting soil bulk density and compactness (Hamza and Anderson, 2005; Hatfield et al., 2007a). In addition, the use of ground cover minimizes the effect of animal trampling (Hamza and Anderson, 2005). Another important aspect is that earthworm density is affected by tractor passage; the low traffic (1 time/yr) increases their population 4 times compared to the conventional use of 5 times/yr (Hansen and Engelstad, 1999).

The main issue of grazing woody crops with livestock is that young leaves and sprouts are very palatable and attractive for them (Table 1.2.). As a consequence of their high acceptability, landowners do not allow to graze sheep and goat in order to prevent damages to the woody crops and production losses.

Table 1.2. Advantages and disadvantages of grazing ground covers with livestock.

Advantages	Disadvantages
Increase biodiversity	Possible damage/crop losses
Reduce ground cover	Compactness in high moisture soil and over-grazed areas
Decrease compactness	Investment and maintenance of the animals
Avoid erosion	Allocate the animals during the non-grazing periods
Spread manure and ground cover seeds	Lack of control of non-palatable weeds
Improve soil nutrient profile	Non-uniform use of the entire surface of the ground cover
Generate a new income/differentiated product	

1.4.1. Grazing olive groves

Sheep are compatible with olive groves because of their feeding behaviour. Traditionally, they were introduced seasonally in the olive groves to control ground cover; however, that practice is currently unemployed because landowners are worried about crop damages.

A study developed by the Asociación Andaluza de Ganadería Ecológica (AADGE) during 1997-98 in the region of Pedroches and La Sierra (Andalucia, Spain) gave advice for sheep grazing management in olive groves. Sheep prefer to eat grass rather than olive leaves and branches; however, in autumn they tended to consume olive branches looking for more nutrients. Therefore, a nutritive ground cover that supplies all required nutrients is really important in each moment (i.e., legume-grass mixture). After the harvest and pruning season (winter), it is also a good moment to graze sheep because wastes are a good complement of the ground cover (Guzmán and Foraster, 2007). The correct moment to remove animals from the olive groves is when olive tree sprouts appear, which also matches with the lowest ground cover biomass. The adequate seasons to allow sheep grazing in olive groves are early autumn (October and November), until harvesting, and late winter and spring (February to May) until ground cover dies. Some time adjustment has to be done regarding the precipitation rate and the use

of cultivated ground cover. The AADGE recommends an average grazing time of approximately 90 d, depending on the year, with an average stocking rate of 3-4 sheep/ha (Guzmán and Foraster, 2007).

1.4.2. Grazing vineyards

Sheep have a high appetence for grapevine leaves and they can eat them, damaging the vine (Emms, 2010). As a consequence, sheep have traditionally been used to graze vineyard's weeds and ground covers from October to April. During that period, vines are in a latency state; therefore, sheep should not damage the crop (Fig. 1.7.a).

On the other hand, due to their high appetence for grape leaves, sheep can be used for leaf plucking and pull out the excessive shoots, a practice which is usually manual. However, there is also a high risk of crop damage (Emms, 2010) because sheep grazing is done between 80% flowering and the veraison (when grape change the colour), which are very sensitive phenological stages for the vine. A close monitoring of the sheep is extremely important, being prepared to move the flock at any time (Fig. 1.7.b). Emms (2010) reported that, in New Zealand, delimited areas of 1-3 ha of vineyard were plucked by 100-300 sheep in 2-3 d.

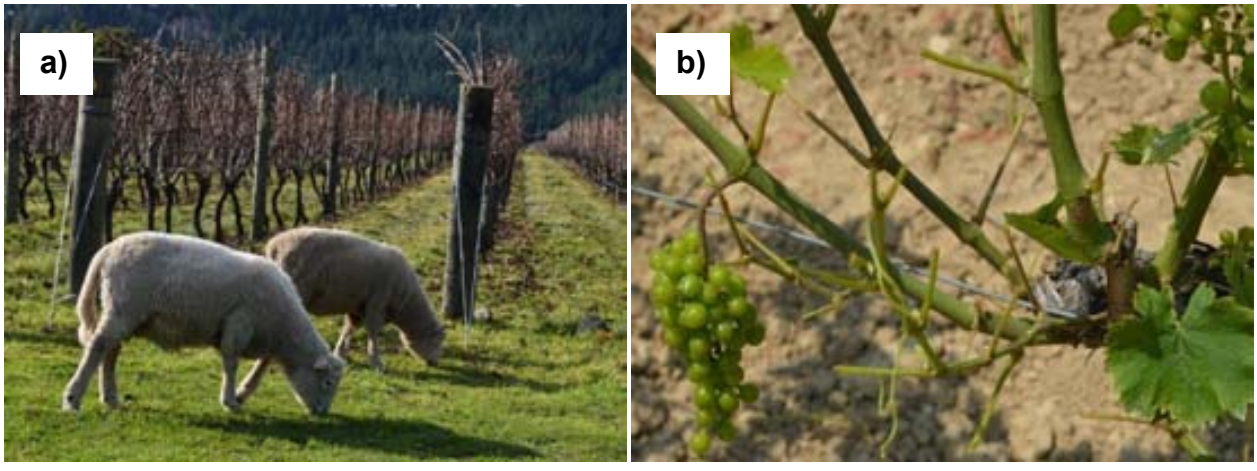

Figure 1.7. a) Sheep grazing vineyards in New Zealand during latency state of the crop in all-arable systems. b) Crop damages after using sheep for leaf plucking the vines.

Additionally, the vineyard spray calendar needs to be considered to avoid health risks for the animals and residues in animal products (Emms, 2010), such as pesticides and copper treatments to which sheep are specially sensitive.

1.5. Conditioned taste aversion in ruminants

1.5.1. Basis of ruminant feed selection

Ruminants have the ability to choose their diet from a large variety of plants species, cattle being less selective than sheep and goats. In pasture, development stage and nutritional value of the available vegetal species, determine the animal selection according to the concentration of nutrients (energy, protein, minerals, etc.) and toxins. Moreover, dietary requirements vary with animal age and physiological and environmental conditions. In addition, animals have the ability to modify their needs in case of deficiency or excess of a nutrient or a toxin. As a consequence, feed preferences vary throughout the animals life-span (Fig.1.8.a; Provenza, 1995).

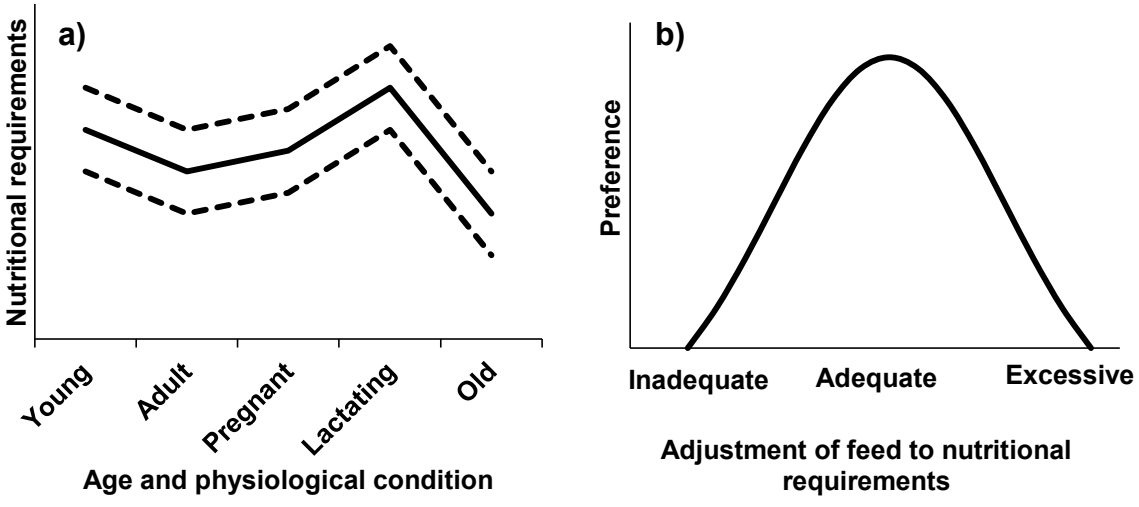

Figure 1.8. a) Animals nutrient requirements variation according to age and physiological condition (—, ideal requirements; ---, requirement adaptations to cope with the deficit or excess of toxins or nutrients. b) Food preferences evolution according to the adjustment of ingested feed to the animal nutritional requirements (Adapted from Provenza,1995).

To increase efficiency of nutritious feed choices and reducing toxicity risk, animals learn to select feeds through 3 main pathways (Provenza and Balph, 1988; Provenza et al., 1992; Mazorra et al., 2009):

The first way takes place around weaning, when offspring start eating new feeds. They learn through the impressions regarding the feed; this is known as imprinting. The second way is social learning, following social models from the herd/flock, especially from their mothers. It occurs mainly during the early stages of growth. The third way is based on self-learning by a trial-and-error process which takes place throughout the animal life-span as an adaptation measure to the variability of plants toxicity and nutritional quality over time and space. This third learning way seems to be more efficient and permanent than social learning (Thorhallsdottir et al., 1990a; Mirza and Provenza, 1994; Provenza et al., 1993a).

Self-learning is a consequence of the postingestive feedback which follows feed intake (Howery et al., 1998; Favreau et al., 2010). A positive feedback occurs when ingested feed provides adequate nutrients and satiety, and consequently the animal develops a preference for that feed. On the contrary, in a negative feedback the ingested feed involves an excess of nutrients or toxins which stimulate the emetic system, resulting in discomfort and developing rejection for that feed (Fig. 1.8.b; Provenza, 1995).

However, diet selection involves two processes: 1) The affective process, which is involuntary, explained above, and 2) The cognitive process, where the animal voluntarily associates the taste, appearance and smell of the feed with the postingestion feedbacks. Consequently, in future contact they can select or avoid the food “consciously” by the smell and appearance (Fig. 1.9.; Howery et al., 1998).

The emetic system (responsible for nausea and vomiting) activation is a key factor in the negative postingestive feedback. Therefore, by using a drug or another treatment which induces nausea or gastrointestinal illness, the animals’

feed preferences can be manipulated (Provenza et al., 1994b; Provenza, 1995; Bernstein and Koh, 2007). This process is known as conditioned taste aversion (CTA). It is based in a classical conditioning method, where the feed intake is the conditioned stimulus and the gastrointestinal discomfort is the unconditioned stimulus.

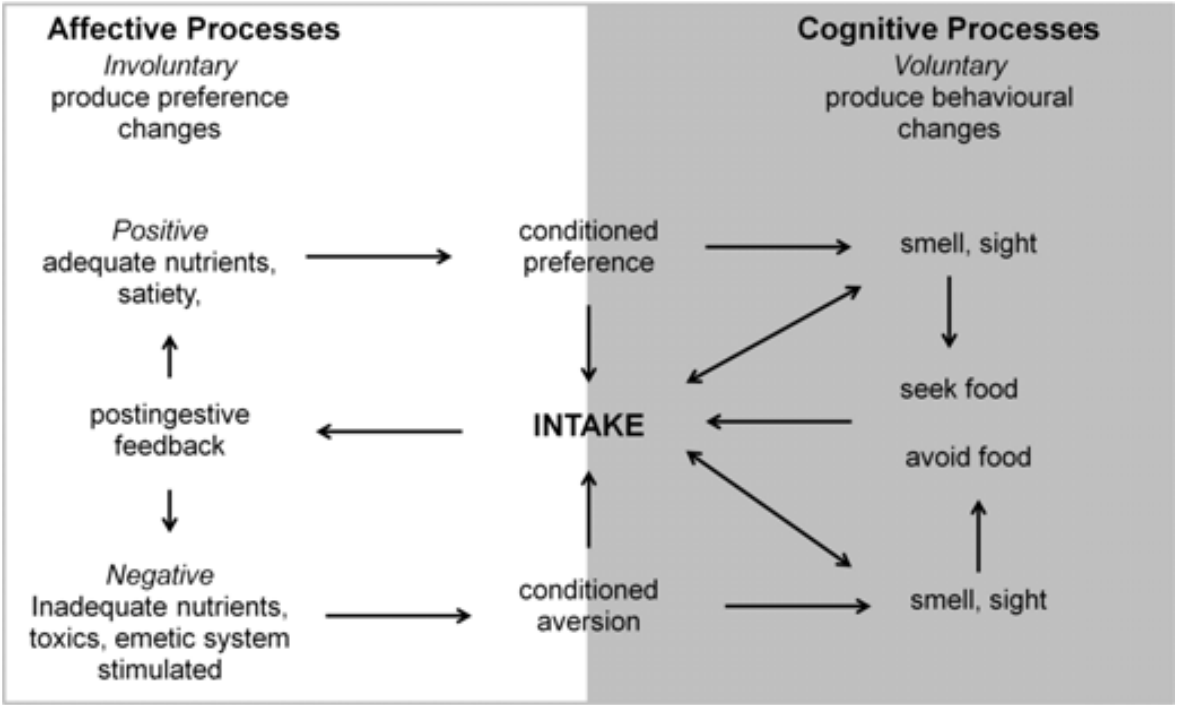

Figure 1.9. Schematic representation of the affective and cognitive processes in diet selection (adapted from Howery et al., 1998).

1.5.2. Conditioned taste aversion mechanism

The first CTA application studies began in the mid-seventies to prevent coyote attacks on sheep flocks. Since then, several methods have been tested to achieve CTA with different inductor agents, animal species and target feed as shown in Table 1.3. (Conover, 1995; Massei and Cowan, 2002). Although ciclophosphamide, thiabendazole and apomorphine induce CTA, lithium chloride (LiCl) is currently the most widely-used emetic with small ruminants for its stronger and longer effectiveness and persistence (Ralphs and Provenza, 1999).

Table 1.3. Summary of CTA studies results done with different models (target feed, averted species, inductor agent and results obtained (adapted from Conover (1995).

Target Feed	Species	Inductor agent	Results
Sheep	Canids	LiCl	-/+
	Coyotes	LiCl	-/+
Chickens	Raccoons	LiCl	+
Sea gulls and eggs	Foxes	LiCl	-
Eggs			
Odd colour and taste	Crows	Trimethacarb	+
		Landrin	+
	Mongooses	Carbachol	+
Avian	Raccoons	Emetine	-/+
		Estrogen	+
	Mammalian predators	LiCl	-
		Estrogen	+
	Predator guild	Trimethacarb	+
Turtle	Raccoons	LiCl	-
Honey and apiaries	Bears	LiCl	+
		Thiabendazole	+
Food handouts	Coyotes	LiCl	+
Rice seed	Blackbirds & cowbird	Methiocarb	+
Vegetables	Woodchucks	Emetine	-/+
Plants			
Palatable shrubs	Lambs	LiCl	+
Grain and feed	Horses	Apomorphine	-/+
	Ruminants	LiCl	+
Delphinium	Cattle	LiCl	+

1.5.2.1. Lithium chloride mechanism of action

The LiCl is a nonlethal gastrointestinal drug (Prien et al., 1971), water-soluble and easy to dose. It stimulates the chemoreceptor trigger zone area (CTZ) activating the emetic centre (Andrews and Horn, 2006), and generating CTA (Curtis et al., 1994) without other side-effects (Ralphs et al., 2001).

Provenza et al. (1994b) showed that blocking the CTZ receptors with an anti-emetic, the use of LiCl did not generate CTA in sheep, which demonstrated the important role of the emetic centre in CTA process. The CTZ is located in the area

postrema of the medulla oblongata on the floor of the 4th ventricle (Fig. 1.10.). The CTZ quimioreceptors (dopamine D_2, serotonin 5-HT_3, histamine H_1, opioids, acetylcholine and substance P) are easily stimulated by any potentially toxic substance in the blood, because the area postrema does not have brain-blood barrier. Additionally, spinal afferent and vagal digestive tract nerve pathways stimulates the area postrema and the emetic centre (Fig. 1.10.).

1.5.2.2. Lithium pharmacokinetics

Li is widely distributed on earth's crust. Trace amounts of Li are present in many minerals, in most rocks and soils, and in many natural waters. It is estimated to be from 20 to 70 mg/kg by weight of earth's crust (Kamienki-Consultant et al., 2004).

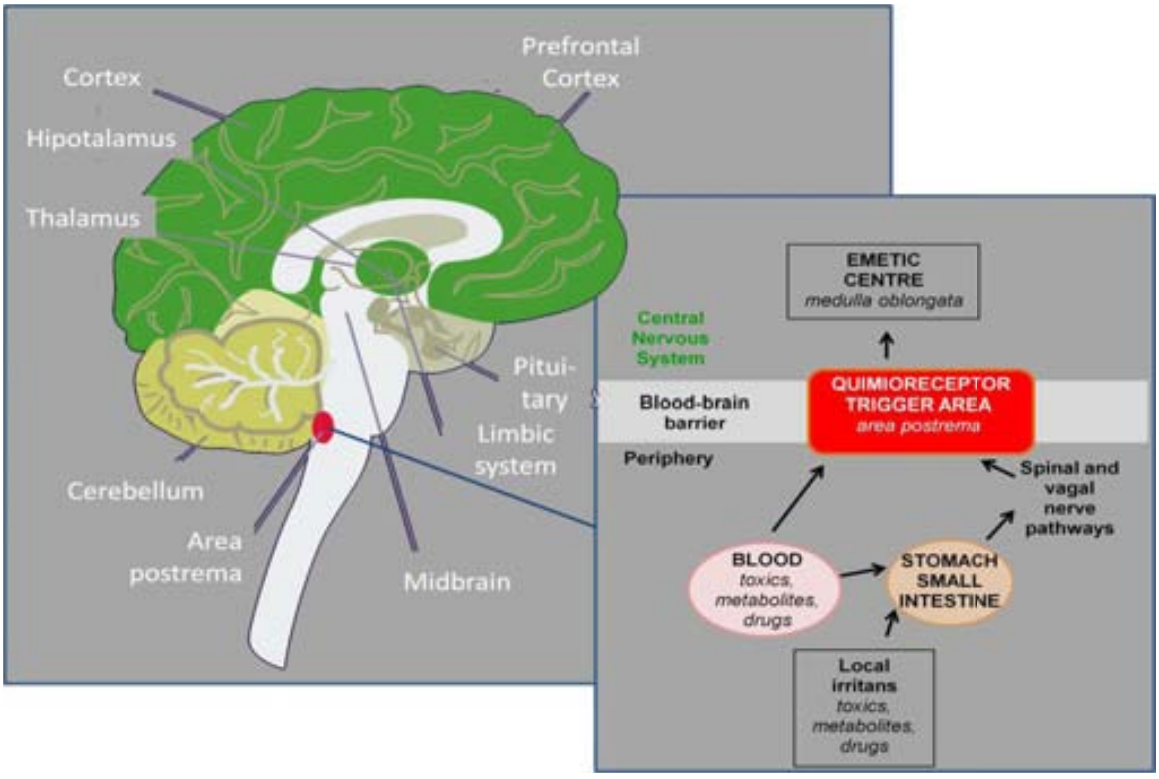

Figure 1.10. Anatomic position of the area postrema and schematic representation of the emetic centre stimulated by the quimioreceptor triggers area.

The Li upper geochemical value for natural Mediterranean soil is 115 mg/kg (Roca-Perez et al., 2010). In animals, Li is a trace element of unclear role and its body contents range in the order of 10^{-6} g/100g body weight (Georgievskii, 1982).

Currently, there is scarce information on the Li pharmacokinetics in small ruminants; however, it is widely studied in humans because it has been used for treating bipolar affective disorders, alcoholism, schizoaffective disorders and cluster headaches (Timmer and Sands, 1999). Li shows a narrow therapeutic ratio (relationship between toxic and effective dose) on humans (0.4 and 1.5 mmol/L; Parfitt 2005), being 37 mg LiCl/kg BW the recommended dose for prescription (Ralphs, 1999).

Li is handled in the human body similarly to Na and absorbed from the upper gastrointestinal tract in approximately 8 h (Timmer and Sands, 1999). It is distributed in the total body water space and excreted mainly by kidneys (90%), being faeces and breast milk excretion quite negligible in humans (Timmer and Sands, 1999; Parfitt, 2005). No bonding to serum proteins has been found (Timmer and Sands, 1999) and peak serum concentration (0.4-1 mmol/L) after a single dose is achieved between 0.5 and 3 h post-dosing, with an elimination half-life of 12 to 27 h following a first order elimination pattern (Parfitt, 2005; Timmer and Sands, 1999).

Differences with ruminants have been reported showing slower metabolization, being the serum Li-peak later in cows (Ralphs, 1999; 10-h) and sheep (Kauter and Godwin, 1995; 8-h; Mormède and Ledoux, 1980; 14 to 20-h) than in humans. These marked difference could be consequence of the dilution effect of the ruminal fluid (Mormède and Ledoux, 1980), the inhibition of the rumen contractions (Kauter and Godwin, 1995) and the slight Li recirculation (2%) by the saliva (Ulyatt, 1964), all of them delaying the absorption in the intestine. Li was detected in cow milk for 96 h after LiCl administration (Ralphs, 1999). However, it was also

found that milk Li residues were low and unable to create CTA in calves (Ralphs, 1999).

1.5.2.3. Factors modifying conditioned taste aversion

The ability of small ruminants to generate CTA, using LiCl as inducing agent, depends on many factors, which are described below. For a better understanding, they will be discussed separately, but interaction between them has to be considered.

Dose and number of LiCl administrations. Conditioned aversion has a close dose-effect relationship due to the degree of discomfort generated by the amount of LiCl administered, decreasing the feed intake as LiCl doses increases (Fig. 1.11.; du Toit et al., 1991; Egber et al., 1998; Massei and Cowan, 2002; Mazorra et al., 2006). Doses used to induce CTA range between 100 and 225 mg LiCl/kg BW, being lethal at 400 mg LiCl/kg BW (Egber et al., 1998).

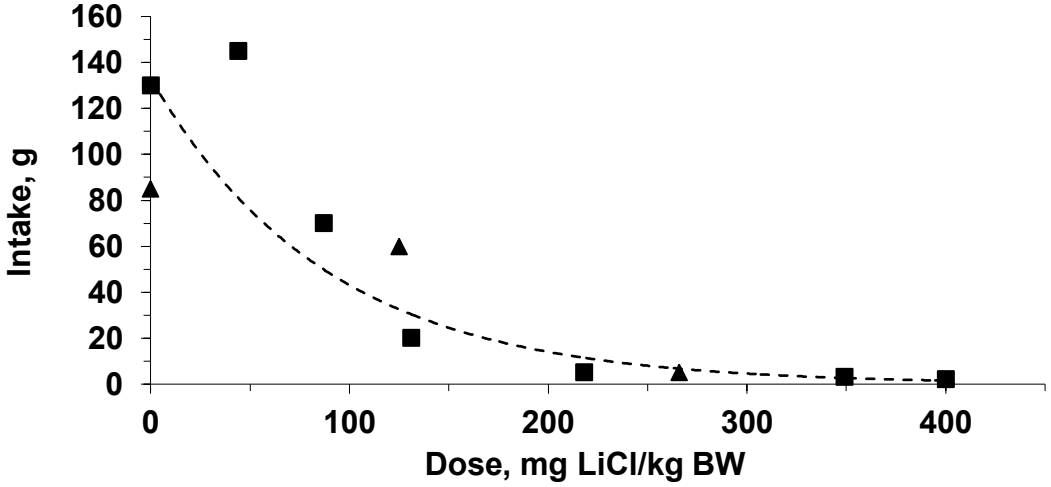

Figure 1.11. Feed intake reduction according to LiCl dose administered reported by du Toit et al. (1991; ■) Egber et al. (1998; ▲).

These doses are markedly greater than those used in humans. It is reported that lower doses induce partial CTA, which means that the animal will still

consume small amounts of the target feed (Table 1.4.). Greater doses will induce complete aversion, which means that animals will fully refuse to consume the target feed (Table 1.4.).

Table 1.4. Range of LiCl doses (mg/kg BW) used by different authors to induce feed aversion.

LiCl dose	Mazorra et al. (2006)	Burritt et al. (2013)
Low	100	125
Medium	150	150
High	225	175

In this range of doses, between 65 and 75% of sheep and goats show complete CTA after one single dose, the remaining animals needing a second dose (Burritt and Provenza, 1989a, 1996; Pfister et al., 1993; Gorniak et al., 2008).

Characteristics of the target feed and prior experience. The ability of small ruminants to generate CTA depends on the characteristics of the target feed and on their prior experience with it. Nevertheless, if the target feed provides an irreplaceable nutrient, CTA cannot be expected (Conover, 1995).

Small ruminants are afraid of new things and tend to avoid consuming any unfamiliar food (feed neophobia), which is a common behaviour in many vertebrate species (Provenza and Balph, 1988). Neophobia is an innate protective mechanism which allows animals to learn from the postingestive consequences of eating a new and potentially toxic feed before being harmed by it (Provenza and Balph, 1988; Van Tien et al., 1999). When a small ruminant consumes a new feed they start eating only a small amount; thereafter, if there is no negative feedback, they will increase the intake gradually until reaching the ingestibility threshold in their diet (Thorhallsdottir et al., 1987). Therefore, CTA is easier to be established for new feed, and it is strongest and more persistent (Burritt and Provenza, 1989b; Villalba and Provenza, 2000). On the other hand, creation of CTA for a familiar

feed is difficult, because it was related to a “learned safety” status (Ralphs, 1992). Usually, CTA is specific for each target feed, although there is evidence that it can be generalized in most cases to the plants of the same species (Ginane and Dumont, 2006).

In mixed diets, when animals become sick after eating both a familiar and a novel feed, they are able to generate CTA against the novel feed (Burritt and Provenza, 1991; Provenza, 1996); whereas if the diet consists of feeds of different novelty, they will avoid the newest. On the other hand, when the diet consists of different familiar feeds, the animal will avoid the feed consumed in excess or that one which made them sick in the past (Provenza, 1996). Additionally, animals previously treated with LiCl become more neophobic and more reluctant to eat novel feeds (Thorhallsdottir et al., 1987).

Proximity of the consequence. Malaise after CTA can be considered as a punishment for eating. Ruminants can learn with some delay between intake and induced sickness. It is reported that lambs could reduce novel feed intake when illness occurred within 4 h after a single LiCl dose. Repeated doses, a greater dose, or both, were required for reducing feed intake when a delay of 6 h or more was applied (Burritt and Provenza, 1991). On the other hand, when LiCl was administered to sheep 2, 1 or 0-h prior to eating the target feed, only the sheep which received the dose just before eating the target feed were able to establish a CTA (Provenza et al., 1993b). Therefore, LiCl has to be administered immediately after the animal consumes the target feed to induce the CTA.

Age and social facilitation. Mature animals are more reluctant to accept novel feed than young animals (Provenza and Balph, 1988). Moreover, young animals present more inquisitive character in sampling food and less stable long-term memory. Therefore, CTA created in mature animals would be more long-lasting than in young animals. It was reported that adult animals tend to avoid feeds which

cause gastrointestinal distress more than young animals (Provenza and Balph, 1988). Ralphs and Cheney (1993) also showed that young cattle require higher LiCl doses than mature cows to retain a strong CTA.

As indicated above, social models following are the second way for learning feed selective behaviour. Sheep preferences or avoidances can be modified by social facilitation (e.g., mother, siblings and close mates). Animals which have been averted to a target plant easily consume it again when they graze with non-averted animals, especially if no alternative feeds are offered (Thorhallsdottir et al., 1990b). Averted lambs grazing with non-averted lambs spent more time eating the target plant than if they grazed alone (Provenza and Burritt, 1991). The same pattern was observed in cows, where CTA was maintained for 2 and 3 yr, but they showed a rapid aversion breakdown when grazing together with non-averted cows (Lane et al., 1990; Ralphs, 1997). In addition, social facilitation can also be affected by age. Apparently, adults are less influenced in diet choice by social models than young animals (Provenza and Balph, 1988; Thorhallsdottir et al., 1990b).

A special social facilitation is the ewe-lamb pairing, although the influence of mothers' behaviour changes with the age of the lamb. Lambs younger than 8 wk of age are more attentive to their mothers' behaviour (Thorhallsdottir et al., 1990c), stay closer to them and are more influenced (Mirza and Provenza, 1990). Ewe-lamb facilitation could allow lambs to learn to avoid a target plant by grazing with their mothers (Mirza and Provenza, 1990, 1994; Thorhallsdottir et al., 1990a).

Alternative feed and persistence. Sheep have an inherent tendency to sample feeds; they will avoid a particular feed if it is always noxious, as it happens with most toxic plants. The aversion becomes weaker when there are no negative consequences every time they sample the averted food (Ralphs and Cheney, 1993; Thorhallsdottir et al., 1987). In some cases CTA animals never stopped eating the target plant completely and continued sampling small amounts of feed

during every trial (Thorhallsdottir et al., 1987). The availability of an alternative feed helps to avoid sampling the target feed and therefore to maintain CTA (Burritt and Provenza, 1990; Thorhallsdottir et al., 1990b).

To our knowledge, research studies on long-term (≥ 9 mo) CTA persistence are scarce. It has been reported that CTA persisted for 9 mo in sheep (Burritt and Provenza, 1990; Doran et al., 2009) and for 2 and 3 yr in cows (Lane et al., 1990; Ralphs, 1997). Burritt and Provenza (1991) reported that when animals consume 10 g or more, a re-enforcement dose is recommended.

Recapitulating, to promote long-term CTA, optimal conditions are the use of adult animals, novel target feeds and high doses of LiCl, as well as maintaining non-CTA animals grazing separately to avoid social facilitation (Ralphs, 1997).

CHAPTER 2. OBJECTIVES

2. OBJECTIVES

The general aim of this thesis was to evaluate the suitability of conditioned taste aversion (CTA) with LiCl, to prevent damages in woody crops when grazing small ruminants on ground covers.

The following specific objectives were proposed:

1. To develop a protocol to evaluate the response of CTA against olive tree and vines in small ruminants and their persistence in order to assess:
   - The effectiveness of a similar dose of LiCl in sheep and goats.
   - The effectiveness of different LiCl doses to induce a long-term persistency in sheep.
   - The CTA response of different breeds of sheep (i.e., Lacaune, Manchega and Ripollesa) to similar doses of LiCl.
   - The practical use of CTA sheep grazing in commercial vineyards for the production of cava.

2. To assess the Li kinetics and elimination after a single and effective LiCl dose in sheep and goats, in:
   - Lactating goats.
   - Dry sheep.

CHAPTER 3. EXPERIMENT 1

Conditioned aversion to olive tree leaves (*Olea europaea* L.) in goats and sheep

Applied Animal Behaviour Science 2010, 128:45-49.

3. EXPERIMENT 1

Conditioned aversion to olive tree leaves (*Olea europaea* L.) in goats and sheep

3.1. Abstract

With the aim of averting to olive tree leaves, lithium chloride (LiCl) was used in goats and sheep. A total of 10 dairy does (Murciano-Granadina breed) and 10 dairy ewes (Manchega breed) were used in 2 simultaneous experiments for comparing olive leaf intake in averted vs. control animals. Does and ewes were dry and non-pregnant and were randomly allocated into 4 experimental groups of 5 animals each. Animals had no previous contact with olive leaves. For aversion induction, all animals were penned in individual box stalls during 6 d, fed tall fescue hay ad libitum, and offered the olive leaves for 5 min (does) or 1 h (ewes). Aversion was induced by using a drenching gun for orally administering a solution of LiCl (200 mg/kg BW) in water, immediately following olive leaf consumption in the averted groups. Water alone was drenched in the control groups as a blank. Does learned faster to eat the olive leaves and developed stronger aversion to the LiCl treatment than ewes. After the aversion induction period, all the animals joined their respective flock or herd, grazed a cultivated pasture during the day and were complemented indoors with tall fescue hay during the night. Aversion memory was evaluated every 14 d via of 5 min lasting 144 days in the does, and 10 min lasting 130 days in the ewes. No LiCl was given during the aversion memory test period. No olive tree leaf consumption was detected until day 53 and 23, for does and ewes, respectively ($P < 0.05$). Olive tree leaf consumption in the averted groups was lower than in the control groups throughout the experiment ($P < 0.05$). Animal behaviour, when the olive leaves were offered, differed between averted and control groups during learning and memory test periods. Animals in the control group avidly ate the olive leaves, whereas animals in the averted groups strongly rejected the olive leaves. In conclusion, the LiCl conditioned aversion to olive tree leaves proved to be an efficient short-term method in sheep and goats under our experimental conditions. The method may be of special interest for implementing selective grazing and for avoiding the use of herbicides in organic cultures. Further research will be necessary before recommending its use in practice.

3.2. Introduction

Diet selection in small ruminants is the result of the animal physiological conditions and the chemical characteristics of the feeds (Provenza, 1996). Other factors for diet selection are mother, social and environment interactions (Burritt and Provenza, 1997; Mirza and Provenza, 1994; Thorhallsdottir et al., 1987) including feed aversion (du Toit et al., 1991; Provenza, 1995; Provenza et al., 1994a). Positive or negative consequences after eating some particular feed may create post-ingestive feedback that causes an increase or decrease in intake of that feed (Favreau et al., 2010). Provenza (1995) points out that learning can be used to condition ruminant feed selection in practice.

Intake decrease is the result of organoleptic characteristics (flavor, taste, texture) and post-ingestive effects (Provenza, 1996). The most used drug for conditioning aversion in ruminants is lithium chloride (LiCl) (Ralphs and Provenza, 1999; Ralphs et al., 2001), which is a safe (Prien et al., 1971), water-soluble and easy-to-dose product. After administration, LiCl produces indisposition because the emetic system is activated. Animals recover very quickly after LiCl treatment (Burritt and Provenza, 1989b; Provenza, 1995). Effective dose of LiCl in sheep and goats ranges between 150 and 200 mg/kg BW (du Toit et al., 1991; Egber et al., 1998). Aversion memory can persist long term (until 9 months) and can be re-established with a new dose of LiCl (Burritt and Provenza, 1990; Doran et al., 2009). Gorniak et al. (2008) showed, however, that persistence of aversion is associated with the possibility of choosing an alternative feed in goats.

Olive tree (*Olea europaea* L.) is an important crop in Spain, where it occupies nearly 2.5 × 106 ha and Spain, with more than 1 million t/year, is the first olive oil producer in the world (MARM, 2009). Cultivation practices in olive trees recommend the use of controlled plant cover between tree rows to prevent soil erosion, thus increasing the quality of soil by improving water, organic carbon and nitrate retention (Alonso and Guzmán, 2006). Goats and sheep, by means of

controlled grazing, may be an alternative for reducing plant cover during spring and increasing soil quality, instead of using farm machinery or herbicides. On the other hand, olive trees have slow growth and high economic value, their leaves and new branches being a very palatable feed for goats and sheep. For this reason landowners usually refuse to allow goat and sheep grazing on olive tree fields to prevent damages in the trees.

The objective of this experiment was to study whether LiCl-treated does and ewes can be averted from consuming olive tree leaves, used as a novel feed, under experimental farm conditions, and to evaluate the mid-term persistence of the achieved aversion.

3.3. Material & Methods

3.3.1. Animals and management

The experiment was conducted at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona (Bellaterra, Spain) during spring and early summer (March to July). The experimental procedures and animal care conditions were approved by the Ethical Committee of Animal and Human Experimentation (CEEAH, references 770 and 998) of the Universitat Autònoma de Barcelona.

A total of 10 Murciano-Granadina dairy does (34.7 ± 2.4 kg BW) and 10 Manchega dairy ewes (63.7 ± 3.5 kg BW) were used in 2 simultaneous experiments for comparing olive tree leaf intake in averted vs. control animals. Does and ewes were dry and non-pregnant, grazed during the day (6 h/day) in an Italian rye-grass (*Lolium multiflorum* Lam.) pasture and received tall fescue hay (*Festuca arundinacea* L.) as a complement in the shelter during the night.

Animals were penned in individual box stalls (1.1 m × 2.0 m) and randomly allocated into 4 groups of 5 animals each, to which experimental treatments were

applied. Treatments consisted of induced aversion (AV, LiCl treated) and control (C, water blank). All the animals were fed a basal diet of tall fescue hay ad libitum once a day (13:00 h). Water and a commercial block of vitamins and minerals (Multi-Block, Agraria Comarcal del Vallès, Les Franqueses, Barcelona, Spain) were freely available. Visual contact between animals of different treatments was avoided.

3.3.2. Olive tree leaves and feeds composition

Olive tree leaves (cv. Arbequina) were obtained at the end of the olive harvest season (January) from the cooperative “La Palma d’Ebre” (Tarragona, Spain), representing the most used cultivar in Catalonia (NE of Spain). The leaves were air-dried for 1 week and stored at room temperature until fed. Olive tree leaves and tall fescue hay offered during the experimental periods were sampled daily and a composite prepared and preserved at room temperature until analysis.

Dry matter was determined at 103ºC for 24 h and ash content was measured gravimetrically by igniting samples in a muffle furnace at 550ºC for 4 h (AOAC, 2003). The Dumas method (AOAC, 2003) with a Leco analyzer (Leco Corporation, St. Joseph, MI) was used for N determination and crude protein was calculated as percentage of N × 6.25. Neutral detergent fiber, acid detergent fiber and lignin were determined on an ash-free basis by the method of Van Soest (1982) using the Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium sulphite solutions. Ether extract was analyzed by the Soxhlet method (AOAC, 2003) using Soxhlet extractors of 250 mL (Fisher Scientific, Madrid, Spain) coupled to a water bath (Selecta Mod. 148, Abrera, Barcelona, Spain). Olive leaves and tall fescue hay chemical compositions are shown in Table 3.1.

Table 3.1 Chemical composition of olive leaves and tall fescue hay (DM basis).

Content (%)	Olive leaf	Fescue hay
Dry matter	83.6	90.1
Ether extract	6.0	2.3
Crude protein	8.8	10.6
Neutral detergent fibre	43.9	61.3
Acid detergent fibre	27.2	31.1
Lignin acid detergent	17.6	3.1
Ash	6.5	11.1

3.3.3. Conditioning aversion period

After 1 week of adaptation to confinement in the individual box stalls, aversion conditioning was carried out on day 1 of aversion period and verified for the next 5 d. Basal diet orts were removed (1000 h) and weighed daily. Immediately thereafter, 400 g of dry olive leaves were individually offered to the does for 5 min and to the ewes for 1 h. This was the first time the animals had been exposed to dry olive leaves (novel feed). Olive leaf intake was measured by weight difference, and animal eating behaviour and feed spillage, if occurred, were recorded during the aversion learning period. Animals consuming olive leaves in the aversion group received LiCl (Panreac, Castellar del Vallés, Barcelona, Spain), using a 200 mL drenching gun (Pimex, Abadiño, Vizcaya), immediately after olive leaf ingestion with 200 mg LiCl/kg BW dissolved in 100 mL of distilled water. Animals in the control groups received 100 mL of water as a blank to simulate the drenching effect. Using a drenching gun to administrate LiCl, instead of gelatin capsules as reported previously (Burritt and Provenza, 1990; du Toit et al., 1991), was preferred in this study because it was a safe routine procedure commonly used in the management of our sheep and goats. After the conditioning aversion period, all the animals joined their respective flock or herd and were grazed and complemented indoors as above indicated.

3.3.4. Aversion memory period

Persistency of the aversion was evaluated by memory tests of 5 min for 144 days and 10 min for 130 days in the averted does and ewes, respectively. Test days were: does (day 22, 23, 37, 53, 68, 86, 101, 115, 130 and 144) and ewes (day 23, 24, 42, 58, 72, 87, 101, 116 and 130). In these tests, experimental animals were individually restrained in the shelter before grazing (10:00 h) using head-lockers of the feed bunks. Cross consumption was prevented by leaving empty places between animals. Each animal was offered 400 g of olive leaves in plastic containers (30 cm × 30 cm × 15 cm). Intake was measured by weight difference with adjustments made for feed spillage. Differences in animal behaviour during the olive leaves offering time were also recorded. No LiCl was given to animals consuming olive leaves in either treatment during the memory test period.

3.3.5. Statistical analyses

Intake of olive leaves was analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC; Version 9.1) by specie (goat or sheep). The repeated factor was experimental days. Animals were a random factor nested within treatments. Even though sample sizes were small, the magnitude of the treatment effects was large, the SE small and the Mixed model procedure sufficiently robust to show the treatment effects and the day × treatment interactions. Significance was declared at $P < 0.05$. When F-ratio was significant, multiple mean comparisons, using the least significant difference (LSD), were used to test for differences between means.

3.4. Results & Discussion

3.4.1. Aversion conditioned learning in does

On the first day of aversion conditioning, there was no difference between the olive leaf intakes of the C and AV goat groups (Table 3.2.). Small ruminants, and especially goats, are neophobic to new feeds (Van Tien et al., 1999; Villalba and Provenza, 2000), which is why the C group ingested increasing amounts of olive leaves on successive days, indicating that does accepted the new feed. Does in the AV group rejected consuming olive leaves on the next day after the first LiCl dose (Table 3.2), although 1 doe regurgitated the LiCl and needed a second dose on day 3. Similar results were observed by Barbosa et al. (2008) with goats averted to mascagnia (*Mascagnia rigida* L.), a toxic plant used as a novel feed, and in which the aversion was conditioned by administering 100 mg/kg BW of LiCl. The authors stressed that 28% of goats (2 out of 7) needed a second dose to be effectively averted to mascagnia. Moreover, Gorniak et al. (2008) reported that goats were also averted from consuming leucaena (*Leucaena leucocephala*) with 130 mg LiCl/kg BW, although 30% of averted goats (2 out of 6) in their study needed a second dose.

Table 3.2. Olive leaves intake (g) during conditioning phase according to species and groups of animals (values are means ± SE).

Days	**Does**		**Ewes**	
	Control	Aversion	Control	Aversion
1	20.8 ± 11.0^{ax}	16.0 ± 2.0^{a}	9.6 ± 3.6^{ax}	24.0 ± 11.0^{a}
2	48.0 ± 22.0^{ay}	0.0 ± 0.0^{b}	8.4 ± 9.4^{ax}	0.0 ± 0.0^{b}
3	47.0 ± 21.0^{ay}	2.0 ± 2.0^{b}	8.0 ± 4.7^{ax}	0.0 ± 0.0^{b}
4	54.0 ± 25.0^{ay}	0.0 ± 0.0^{b}	33.6 ± 17.9^{ay}	0.0 ± 0.0^{b}
5	50.0 ± 18.0^{ay}	0.0 ± 0.0^{b}	101 ± 36.6^{az}	0.0 ± 0.0^{b}
6	48.0 ± 14.0^{ay}	0.0 ± 0.0^{b}	165.6 ± 23.2^{az}	0.0 ± 0.0^{b}

a,b: within a row in each species, values with different superscripts differ ($P < 0.05$). x,y,z: within a columns in each species, values with different superscripts differ ($P < 0.05$).

The marked difference in intake between both AV and C goat groups ($P < 0.05$) in our results showed the effectiveness of LiCl to create aversion to olive tree leaves. At day 6 of the conditioning aversion period all treated goats showed aversion to olive leaves (Fig. 3.1.). Doe behaviour differed between groups during the offering time of olive leaves. Does in C group approached the feeding boxes and ate the olive leaves avidly, whereas does in AV group did not approach the feeding boxes or sniff the olive leaves but rejected them, trying to overturn the plastic containers. This behaviour was similar to that reported by Burritt & Provenza (1989a) in averted lambs.

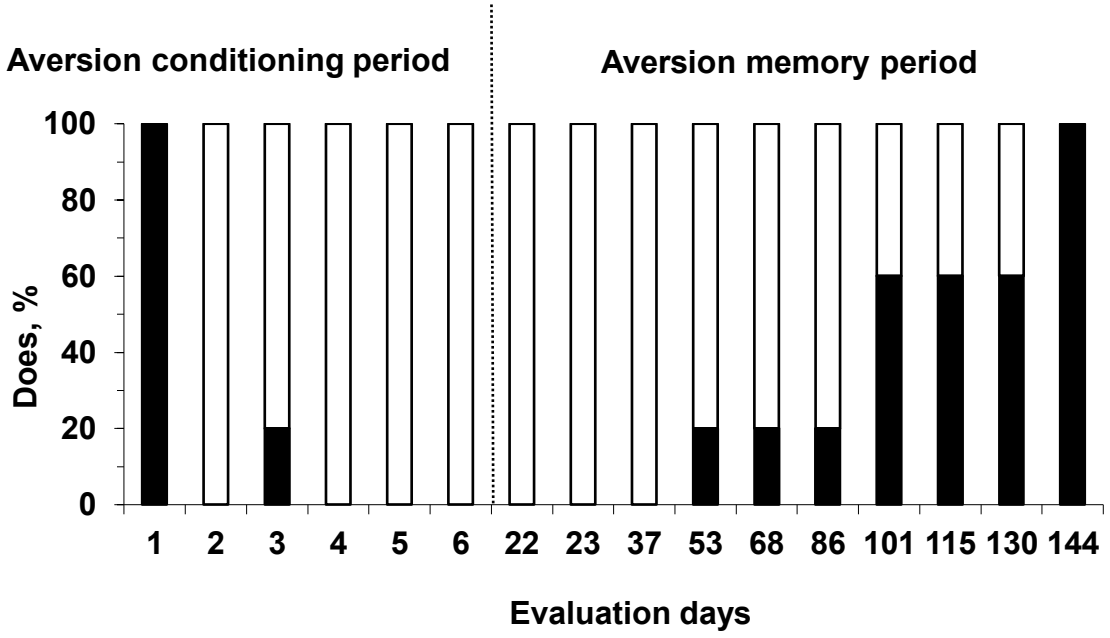

Figure 3.1. Percentage of averted does consuming olive leaf during the aversion conditioned experiment from March to July 2009 at the Experimental Farms of the Universitat Autònoma de Barcelona (Bellaterra, Spain) (□, does that did not eat olive leaves ; ■, does eating olive leaves).

3.4.2. Persistency of aversion conditioned memory in does

Aversion behaviour was observed in the AV goats until day 53 during the aversion memory test (Fig. 3.1.). On that day, the same doe which needed a second LiCl dose on day 3, started to eat olive leaves (16.0 g). From day 53 to 86, 80% of goats had total aversion to olive leaves. By day 130 the percentage of

does with total aversion had declined to 40%. A total of 3 does ate olive leaves on day 101, and on day 144 (end of the memory test study) all the does were eating olive leaves (Fig. 3.1.). However, intake differences between AV and C goat groups (Fig. 3.2.) remained significant throughout and at the end of the study (d 144), averaging 24 ± 12 g vs. 60 ± 5, respectively ($P < 0.05$).

The full aversion period in this study lasted longer than reported by Barbosa et al. (2008) in goats fed mascagnia, and could be a consequence of the greater dose of LiCl used during the conditioning aversion period in our study.

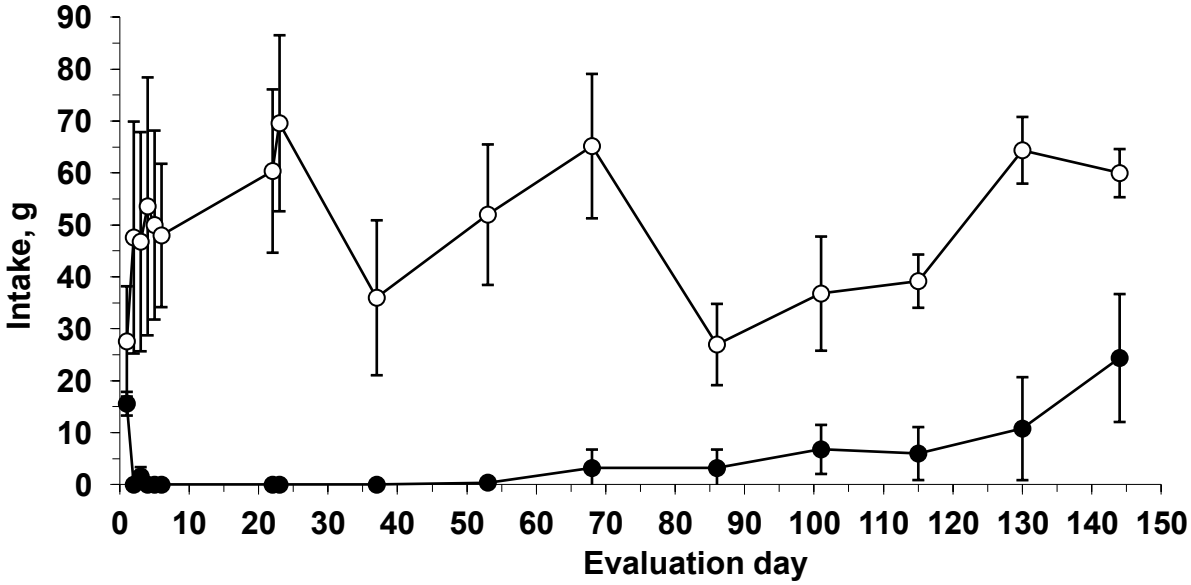

Figure 3.2. Intake of olive leaves by does during the aversion conditioned experiment according to treatment from March to July 2009 at the Experimental Farms of the Universitat Autònoma de Barcelona (Bellaterra, Spain) (○, control does; ●, averted does; vertical bars are ± SEM; differences are ($P < 0.05$).

3.4.3. Aversion conditioned learning in ewes

On the first day of aversion conditioning, there was no difference between the olive leave intake of the C and AV sheep groups. In addition, the C group ingested increasing amounts of olive leaves on successive days, indicating that they accept olive leaves, as observed in does (Table 3.2.). Ewes in the AV group rejected

consuming olive leaves on the next day after the first LiCl dose (Table 2) and none of them needed a second dose of LiCl.

Intake between C and AV sheep groups markedly differed ($P < 0.05$) and demonstrated the effectiveness of LiCl to create aversion in ewes. At the end of the conditioning aversion experiment (day 6), we observed a total aversion ($P < 0.05$) of the AV ewes to olive leaves (Fig. 3.3.). As previously indicated in goats, sheep results in our study were more marked than those reported by Burritt & Provenza (1989a) with lambs, which was attributed to the greater LiCl dose used in our study. Behaviour pattern of the averted ewes was similar to that observed in does.

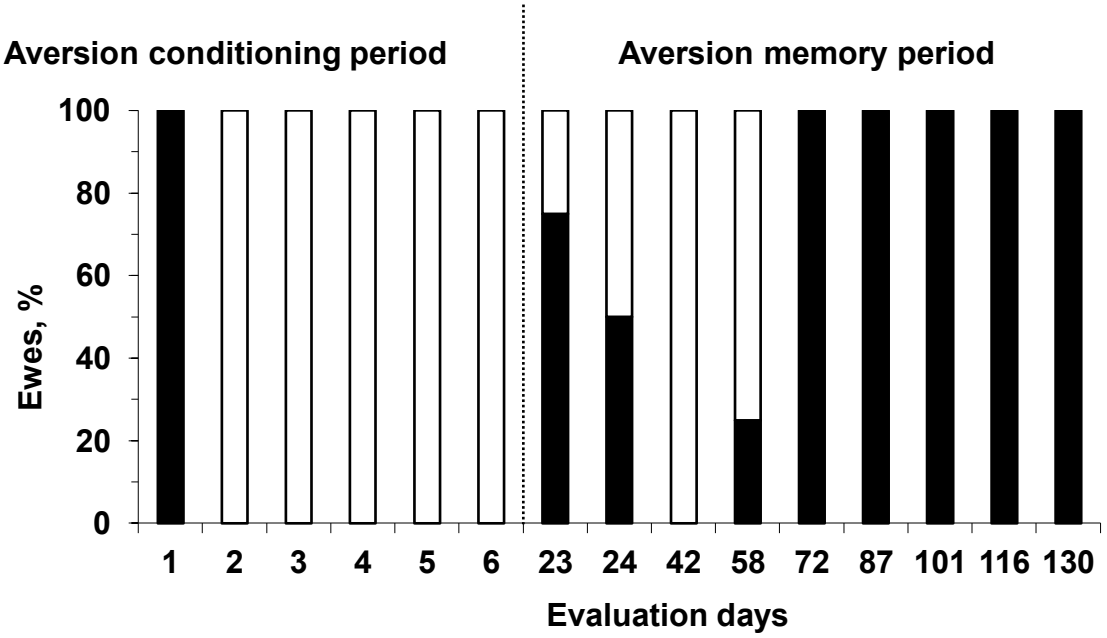

Figure 3.3. Percentage of averted ewes consuming olive leaf during the aversion conditioned experiment from March to July 2009 at the Experimental Farms of the Universitat Autònoma de Barcelona (Bellaterra, Spain) (□, ewes that did not eat olive leaves ; ■, ewes eating olive leaves).

3.4.4. Persistency of aversion conditioned memory in ewes

Eight days after the conditioning aversion experiment and before starting the aversion conditioned memory tests, 1 ewe of the AV group died from unknown reason. The necropsy done at the Service of Animal Pathology of the Universitat

Autònoma de Barcelona did not show lesions associated with LiCl administration or intoxication.

During the memory test experiment with ewes, a less marked aversion was observed than in does (Fig. 3.3.). Averted ewes sniffed olive leaves and tried to overturn the containers, or tasted olive leaves but rejected to ingest them. From day 87 to 130, ewes in AV group increased olive leaf consumption, however, difference in intake between C and AV groups was maintained until day 130 (83 ± 14 vs. 40 ± 20 g, respectively; $P < 0.05$; Fig. 3.4.). Our results agreed with those of Burritt & Provenza (1989a) in lambs, in which they reported that a partial aversive behaviour to wheat grain during 2 months was obtained by administering 150 mg LiCl/kg BW.

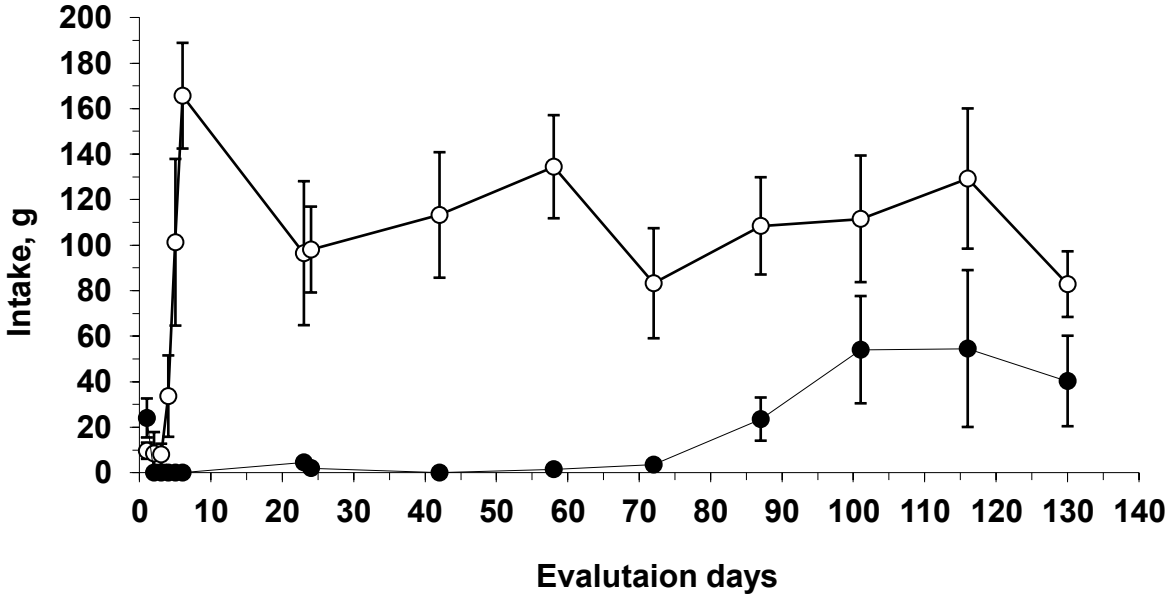

Figure 3.4. Intake of olive leaves by ewes during the aversion conditioned experiment according to treatment from March to July 2009 at the Experimental Farms of the Universitat Autònoma de Barcelona (Bellaterra, Spain) (○, control ewes; ●, averted ewes; vertical bars are ± SEM; differences are ($P < 0.05$).

The increase in the amount of olive leaves consumed by the AV group by the end of the study, indicated that the aversion created to the olive leaf lasted at least 4 months, and then started to be forgotten. This time is shorter than the 9 months

reported by Burritt & Provenza (1990) and Doran et al. (2009) during their pasture trials were animals could choose between averted and no averted plants. The difference between experiments may be a consequence of the conditions used in each case. In our study, animals could not choose an alternative feed to olive leaves during the aversion memory tests. Because olives leaves are palatable and do not have toxic effects in small ruminants, a post-ingestive negative feedback was not produced, consequently in the next test, animals increased their consumption and new ones from the AV group tried them. It is known that, even when AV animals reduced feed intake compared to C animals, extinction of the aversion occurs because there is no negative reinforcement for avoiding the averted feed (Provenza, 1995; Ralphs and Provenza, 1999). As reported by Burritt & Provenza (1990), it may be necessary to apply a new dose of LiCl for re-establishing the aversion to palatable plants.

Averted sheep, as well as goats, associated their indisposition to the olive leaf consumption rather than to the act of administering the LiCl dose to them by gun drenching, as reported by Burritt & Provenza (1989a). No decrease of olive leaves or tall fescue hay intake was recorded in C groups of does and ewes to which water was administered as a blank.

3.4.5. Does vs. ewes comparison

Neophobic behaviour in front of olive tree leaves, used as a new feed in our study, was more marked in ewes than in does. This was supported by the amount of olive leaves intake data (Table 3.2.) and by olive leaves intake pattern curves in the C groups of does (Fig. 3.2.) and ewes (Fig. 3.4.). Ewes needed more time daily than does (1 h vs. 15 min, respectively) to start consuming significant amounts of the novel feed and, in addition, ewes needed more days than does to reach an intake plateau (5 vs. 2 days, respectively) after which the olive leaves were consumed as a familiar feed.

The dose LiCl used in this study (200 mg/kg BW) was enough to create a total aversion in both species, but it seems that does remember the aversion easily and longer than ewes (Fig. 1 - 4.). Moreover, in this study the aversion response was more homogeneous in does than in ewes.

3.5. Conclusions

The results of this study further support the concept that feeding behaviour of small ruminants can be manipulated at short-term using conditioned feed aversion. Feed aversion may be rapidly and easily created in goats and sheep by using medium doses of LiCl. The persistence of the conditioned aversion, for the same dose (200 mg LiCl/kg of BW), proved to be longer in goats than in sheep. On the contrary, sheep were more neophobic than goats under our conditions.

Aversive conditioning may have special application in avoiding the use of herbicides in organic cultures (e.g., olive trees, other fruit trees and vineyards) and for allowing grazing in areas in which specific plants must be respected (e.g., protected and endangered species, toxic plants). Further studies are needed to evaluate seasonal forage preferences of ewes and does in actual olive groves as well as the persistence of aversions with sustained exposure to olive leaves.

CHAPTER 4. EXPERIMENT 2

Effect of breed and lithium chloride dose on the conditioned aversion to olive tree leaves (*Olea europaea* L.) of sheep

Applied Animal Behaviour Science 2014, 155:42-48.

4. EXPERIMENT 2

Effect of breed and lithium chloride dose on the conditioned aversion to olive tree leaves (*Olea europaea* L.) of sheep

4.1. Abstract

Grazing maybe an efficient tool for controlling weed cover in olive groves. However, olive leaves are very palatable for sheep, which damages the trees and compromised the later olive production. Lithium chloride (LiCl) is an effective agent for creating food aversion in ruminants through the activation of the emetic system. We investigated the response in three sheep breeds (Manchega, Lacaune and Ripollesa; N = 15 for each breed) to two doses of LiCl (AV1, 200; AV2, 225 mg/kg BW) for averting adult ewes to olive tree leaves compared to a control group (C, water blank). The aversion was reinforced on day nine in those ewes that consumed > 10 g of olive leaves. Persistence was evaluated by a double-choice feeding assay, where 100 g of olive leaves were offered side-by-side with 390 g of rye-grass (as fed), at several intervals across 70 days. Intake and persistence were compared between doses and breeds. Significant breed effects in the controls suggested a genetic component in neophobia (i.e., Ripollesa and Manchega were neophobic whereas Lacaune was not). Aversion was fully created with a single dose in all ewes, however, 20% of animals needed a reinforcement dose to strengthen the aversion (especially in Manchega ewes and AV1 dose). Total aversion persisted 54 days in AV2 and 33 days in AV1, whereas that differences only presented a tendency in Manchega breed (P = 0.058). Effective aversion length of AV1 vs. C varied by breed (Manchega < Lacaune = Ripollesa), but those differences were not detected in AV2 for which all the breeds showed less olive leaf intake until the end of the experiment (day 70, P < 0.001). In conclusion, breed affected aversion persistence at a 200 mg LiCl/kg BW dose. However, a dose of 225 mg LiCl/kg BW with a reinforced dose might mitigate the breed effect.

4.2. Introduction

The olive tree (*Olea europaea* L.) is an important crop in Spain which, with nearly 2.5 × 10^6 ha and more than 1 Mt/year of olive oil production, is the largest olive oil producer and exporter in the world (MAGRAMA, 2012). Olive grove pruning residues (branches with green leaves) are usually fed to ruminants in most producing countries because they are very palatable and nutritive (Nefzaoui,

1988). As a consequence of their high acceptability, landowners do not use to graze sheep and goat in olive groves in order to prevent damage to the trees and crop losses.

The use of a controlled green cover (i.e., natural or cultivated grass) under the olive trees is considered as a recommended cultural practice to prevent soil erosion, thus increasing water, organic carbon and nitrate retention (Alonso and Guzmán, 2006). Controlled grazing by small ruminants may be used as an alternative to reduce weeds land cover during spring, for conserving soil moisture, and to improve soil fertility, while avoiding the use of labour, machinery and herbicides.

Aversive conditioning is a form of associative learning behaviour in which an animal avoids consuming a food previously paired with a malaise–illness effect (unconditioned stimulus). It is considered a useful method for training livestock to avoid specific food or plants. The efficiency of conditioned aversion depends on food novelty (Burritt and Provenza, 1989b), product used to create the aversion (Conover, 1995; Zahorik et al., 1990), product dose (Egber et al., 1998), availability of an alternative feed (Gorniak et al., 2008; Thorhallsdottir et al., 1990b), animal species (Manuelian et al., 2010) and age (Thorhallsdottir et al., 1990c, 1987).

Conditioned aversion using lithium chloride (LiCl) has been used as a potential management tool to avoid palatable foods and/or poisonous plants in domestic livestock (Manuelian et al., 2010; Ralphs and Provenza, 1999; Ralphs et al., 2001). The LiCl is a water-soluble, easy-to-dose and safe (Prien et al., 1971) product for animal use. After administration, LiCl produces digestive malaise due to the activation of the emetic system (Provenza et al., 1994b) followed by a quick recovery thereafter (Burritt and Provenza, 1989b; Provenza, 1995). Recommended LiCl dose for sheep in practice is 200 mg/kg BW (du Toit et al., 1991; Egber et al., 1998) . Despite the significant variability reported in the

voluntary intake of food in the averted animals, there is no evidence of a breed effect.

The objective of this experiment was to evaluate the response of three sheep breeds to two LiCl doses for inducing conditioned aversion to olive tree leaves.

4.3. Material & Methods

The experiment was carried out at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona (Bellaterra, Spain) during late winter and spring. The experimental procedures and animal care conditions were approved by the Ethical Committee of Animal and Human Experimentation (CEEAH, reference 770) of the Universitat Autònoma de Barcelona.

4.3.1. Animals and management

A total of 15 Manchega dairy ewes (43.5 ± 0.9 kg BW), 15 Lacaune dairy ewes (54.7 ± 1.3 kg BW), and 15 Ripollesa meat ewes (45.0 ± 1.2 kg BW) were used in 3 parallel experiments for assessing the use of LiCl in conditioned aversion to olive leaves. Ewes were adult, dry and non-pregnant; and naïve to olive leaves. They grazed during the day (6 h/day) in an Italian rye-grass (*Lolium multiflorum* Lam.) pasture and received tall fescue hay (*Festuca arundinacea* L.) ad libitum in the shelter. Water and a commercial block of vitamins and minerals (Multi-Block, Agraria Comarcal del Vallès, Les Franqueses, Barcelona, Spain) were permanently available in the shelter.

Leaves of olive tree (cv. Arbequina) were obtained at the end of the harvest season (January) from an olive cooperative (La Palma d'Ebre, Tarragona, Spain). The cv. Arbequina represents the most used olive tree cultivar in Catalonia (NE of Spain). The leaves were air-dried for 1 week and stored at room temperature until fed. With the aim of offering the olive leaves in a double choice feeding assay,

Italian rye-grass was manually harvested every day from the grazing fields at vegetative phenological stage and stored at room temperature until used.

Samples of olive leaves, rye-grass and tall fescue hay used during the experiment were taken weekly, composited by treatment group, and stored under refrigeration (4ºC) until analysis. Figure 4.1. represents schematically the experimental design and the days were measurements were taken throughout the study.

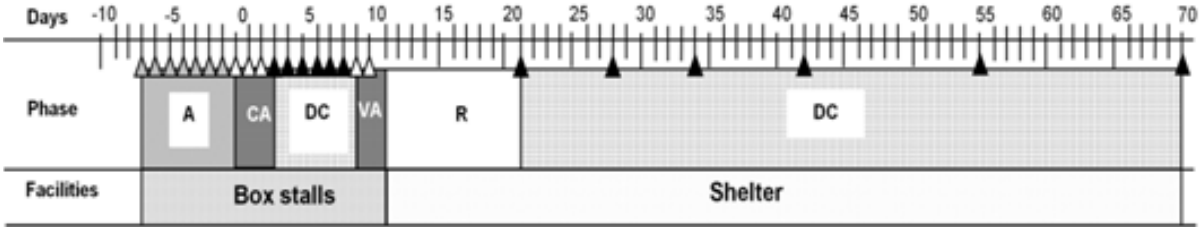

Figure 4.1. Synopsis of experimental design and measurements taken during the study. (A, adaptation period; CA, creation of conditioning aversion, DC, double-choice feeding test; VA, validation aversion; R, non experimental period; △ , measurement day in the adaptation period; △ , measurement day with lithium chloride (LiCl) administration; ▲, measurement day without LiCl administration).

4.3.2. Creation of conditioning aversion (learning period)

At the start of the experiment, ewes were individually penned in box stalls (1.1 m × 2.0 m) and randomly allocated into 3 groups of 5 animals for each breed, to which experimental treatments were applied. Visual contact between animals of different groups was avoided. Ewes received a basal diet of tall fescue hay ad libitum once a day and were adapted to the experimental facilities for 1-week prior to starting with the conditioned aversion creation.

Treatments consisted of 2 aversion conditioned groups of different LiCl doses (AV1, 200; AV2, 225 mg/kg BW) and a control group (C). Lithium chloride (Panreac, Castellar del Vallés, Barcelona, Spain) was orally administered in a water solution using a 200 mL drenching gun (Pimex, Abadiño, Vizcaya, Spain) as indicated by Manuelian et al. (2010). On the day of aversion creation (day 0),

fescue hay was removed 2 h before each ewe received 100 g olive leaves, as fed, for 1 h. Then, AV1 and AV2 ewes were conditioned by pairing the olive leaves (novel food) with the LiCl (unconditioned stimulus), whereas C ewes received 100 mL of water to equalize the drenching effect (blank). Olive leaf intake was measured by weight difference, and animal eating behaviour and olive leaf spillage, if occurring, was recorded. On the next 2 days (day 1 and 2) 100 g of olive leaves were offered for 1-h to each ewe to validate aversion. Validation was also done on days 9 and 10, after the double-choice test in the box stalls. Dosage of LiCl was repeated for any AV1 or AV2 ewe that consumed more than 10 g as fed of olive leaves at day 9, to reinforce their aversion.

Basal diet intake was recorded daily as a means of evaluating discomfort in the animals. Usual intake level was calculated according to the pre-treatment average consumption of fescue hay.

4.3.3. Double-choice feeding test in individual box-stall pens

On day 3, a double-choice feeding test was carried out during six consecutive days to evaluate aversion food persistence. Aversion persistence was defined according to Massei and Cowan (2002) as the number of post-conditioning encounters after which the animal re-samples the averted feed (in our case, 10 g or more of olive leaves). Moreover, aversion was considered effective (LiCl effect remaining) when the averted ewes ate less olive leaves than the C ewes.

The double-choice feeding test consisted of recording the intake of two separated feeds offered at the same time with the same amount of dry matter. Olive leaves (averted feed, 100 g as fed) and Italian rye-grass (alternative feed, 390 g as fed) were offered for 1 h in individual plastic containers (38 cm × 26.5 cm × 15.5 cm; Araven, Zaragoza, Spain) placed side-by-side. Container positions were switched daily to prevent possible eating habits, since one position might be perceived as more convenient by the animal Meier et al. (2012). Differences in

animal behaviour throughout feeding tests and days elapsed until olive leaves were again eaten after aversion creation was also recorded. No additional LiCl doses were given.

After validating the conditioned aversion on day 9 and 10, all the ewes joined the Experimental farm's flock for grazing and were also supplemented indoors as previously indicated.

4.3.4. Double-choice feeding assay in the shelter

To extend the persistence study, double-choice feeding assays were also done in the barn using the head-lockers of the feed bunk for individual restraining. Each double-choice test lasted 30 min and cross consumption of feeds was prevented by leaving empty two places between ewes. Test-days recorded were: 21, 28, 34, 42, 55, and 70 days after the first LiCl dose on day 0 and the same protocol as explained in 4.3.3. was followed.

4.3.5. Feed composition analyses

Dry matter was determined at 103ºC for 24 h and ash content was measured gravimetrically by igniting samples in a muffle furnace at 550ºC for 4 h (AOAC, 2003). The Dumas method (AOAC, 2003) with a Leco analyzer (Leco Corporation, St. Joseph, MI) was used for N determination and crude protein was calculated as percentage of N × 6.25. Neutral detergent fibre, acid detergent fibre and lignin were determined on an ash-free basis by the method of Van Soest (1982) using the Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium sulphite solutions. Olive leaf, tall fescue hay and Italian rye-grass chemical compositions are shown in Table 4.1.

Table 4.1. Chemical composition of tall fescue hay, dry olive leaves and Italian rye-grass pature (DM basis). Values are means ± ES.

Content (%)	Fescue hay	Olive leaves	Rye-grass
Dry matter	89.57± 0.20	78.25 ± 0.03	20.02 ± 0.01
Crude protein	15.02 ± 0.21	9.33 ± 0.00	20.24 ± 1.32
Crude fibre	25.55 ± 0.04	16.49 ± 0.15	19.15 ± 0.74
Neutral detergent fibre	54.95 ± 0.15	40.61 ± 0.49	40.40 ± 0.52
Acid detergent fibre	28.60 ± 0.23	27.74 ± 0.63	21.64 ± 0.16
Lignin acid detergent	2.12 ± 0.20	15.71 ± 0.48	4.87 ± 0.04
Ash	9.87 ± 0.08	9.13 ± 0.04	13.37 ± 0.01

4.3.6. Statistical analyses

Data of olive leaf intake showed a non normal distribution that was not normalised using Box-Cox transformations (Box-Cox and Cox, 1964; Osborne, 2010). As a consequence, treatment and breed effects were analysed using a non parametric Kruskall-Wallis test of SPSS v. 19.0.0 of IBM (Chicago, IL, USA). Aversion persistency data were studied by survival analysis using the Kaplan-Meier non-parametric and log-rank (Mantel-Cox) tests of equality of SPSS across treatments and breeds. Tall fescue hay basal diet intake was analysed by metabolic body weight using a linear mixed model with repeated measure, and differences tested by LS Means of R 3.0.2. (R Core Team, 2013). Values are shown as mean ± SE or as median (for survival analyses), and significance was declared at $P < 0.05$ unless otherwise indicated.

4.4. Results

4.4.1. Neophobic behaviour

Intake of olive leaves showed marked differences between ewe breeds before applying the aversion treatments at day 0 ($H_2 = 27.9$; $P = 0.001$), and values were greater ($H_1 = 8.87$; $P = 0.003$) in Lacaune (87 ± 6 g) than in Manchega (60 ± 8 g), and being greater ($H_1 = 12.30$; $P = 0.001$) in Manchega than in Ripollesa (20 ± 3

g). Lacaune C ewes consumed almost all the olive leaves offered from the first day and their intake was similar thereafter (Fig. 4.2.a). Manchega C ewes gradually increased the intake of olive leaves during the first week and their intake steadied thereafter (Fig. 4.2.b). On the other hand, Ripollesa C ewes showed the lowest initial intake of olive leaves, which dramatically incremented during the first week. Moreover, Ripollesa C ewes showed the largest intake variability across the testing days with a similar plateau (Fig. 4.2.c) and final intake value equalling the other breeds (86 ± 4 g, on average; H_2 = 0.27; P = 0.873). Neophobia degree, expressed as the percentage of olive leaf refusal on the first day before applying the aversion treatments (day 0) with regard to the mean final intake value (day 70), were 1.1, 30.2 and 76.7% for Lacaune, Manchega and Ripollesa breeds, respectively.

4.4.2. Conditioning aversion learning

With the exception of one AV1 Manchega ewe, which spat out most of the dose on day 0 and received a second dose on day 2, AV1 and AV2 groups fully rejected consuming olive leaves on day 1 (H_2 = 42.03; P = 0.001) and day 2 (H_2 = 40.93; P = 0.001) after LiCl dosing (Fig. 4.2.).

Differences in ewe behaviour pattern between groups were observed after LiCl administration (day 1 and 2) in the box-stall pens. Averted ewes did not approach or sniff the containers with olive leaves and fully rejected consuming them. The basal diet intake decreased 34.9% in the treated ewes after LiCl administration on day 0 (AV1, 48.0 ± 6.1 g/kg $BW^{0.75}$; AV2, 45.2 ± 6.0 g/kg $BW^{0.75}$; P = 0.033). AV 1 ewes needed 1 day to recover the intake level, whereas AV2 ewes needed 2 days. On day 1 there were also differences in fescue hay intake between doses (AV1, 57.4 ± 4.1 g/kg $BW^{0.75}$; AV2, 44.2 ± 5.6 g/kg $BW^{0.75}$; P = 0.033). Additionally, the averted ewes showed drooped head and ears, and inactivity. Regarding breeds, both AV2 Manchega and Ripollesa ewes recovered usual intake level one day

later than AV1 ewes. However, AV1 and AV2 Lacaune ewes did not show that difference.

On day 3 and following days, intake of olive leaves in the box-stall pens varied according to breed and LiCl dose (Fig. 4.2.). On average, AV2 ewes of all breeds rejected consuming olive leaves until day 9, although one Manchega ewe (20%) showed slight olive leaf intake (2 to 12 g/day) at days 4, 6 and 9 and was reinforced. With regard to AV1, the Ripollesa ewes maintained a total aversion to olive leaves during the 9-day period but, three Manchega (60%) and two Lacaune (40%) ewes increased progressively the intake of olive leaves (>10 g/d), from 3 to 9 and 5 to 9 days, respectively. These ewes were reinforced with a second LiCl dose at day 9 and their full aversion was re-established on day 10. Nevertheless, aversion effects were still evident on day nine, where the AV1 ewes showed lower intake than C ewes in each breed (Manchega, H_1 = 5.54; P = 0.019; Lacaune, H_1 = 7.18; P = 0.005; Ripollesa, H_1 = 7.81; P = 0.005).

Differences in intake reduction of olive leaves in favour of AV2, when compared to AV1 doses, were only detected on day 3 (H_1 = 6.10; P = 0.013) and 7 (H_1 = 5.67; P = 0.017) in the Manchega ewes. Moreover, a tendency was also observed for days 4, 5, 6, 8 and 9 (P = 0.052 to 0.072) in that breed, and in the Lacaune ewes for days 6, 8 and 9 (P = 0.054 to 0.136). No differences between AV1 and AV2 doses were detected in the Ripollesa ewes averted groups (H_1 = 0.00; P = 1.000).

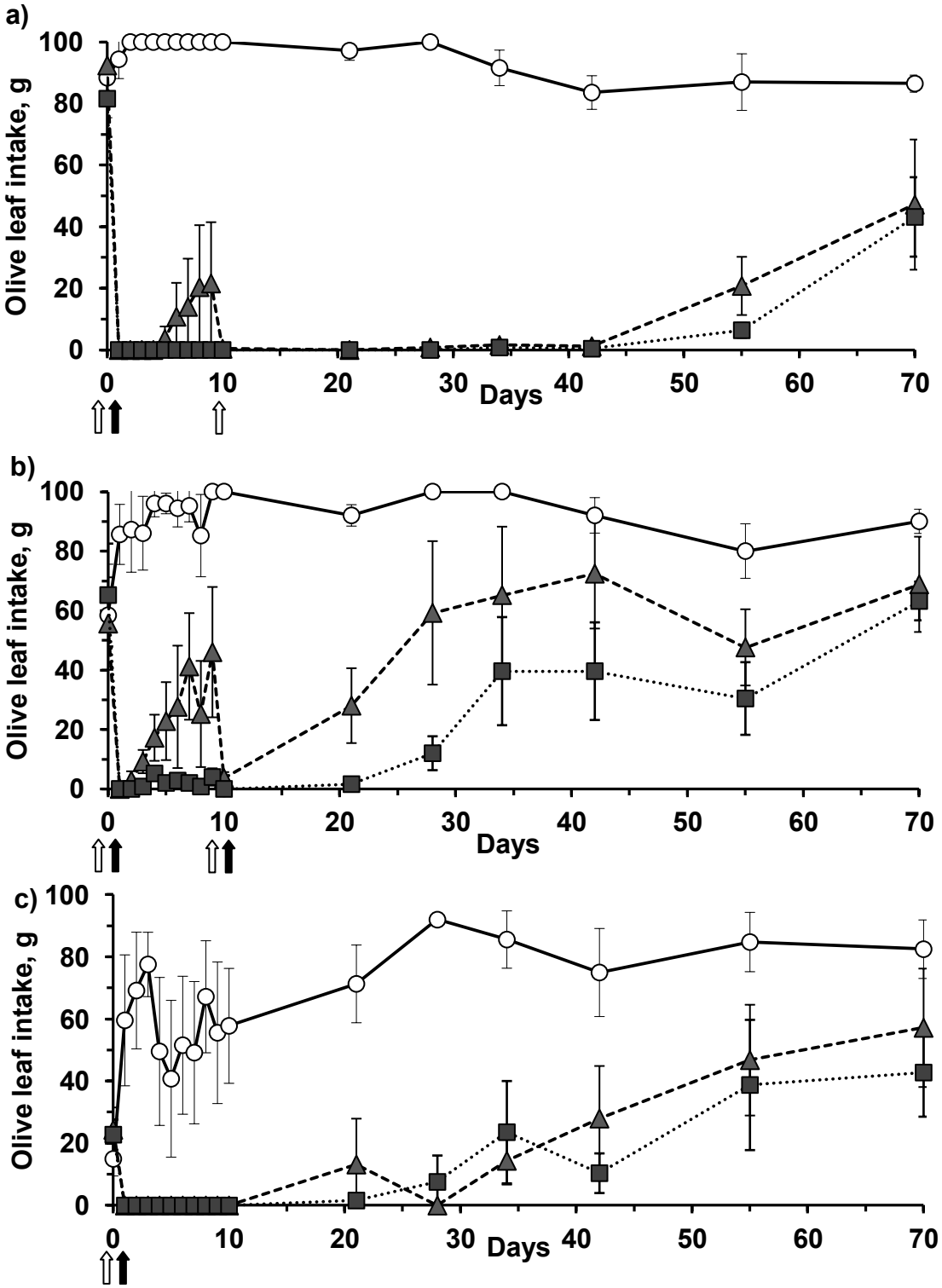

Figure 4.2. Intake of olive leaves during the aversion conditioned experiment according to treatment (○, control; ▲, AV1, 200 mg LiCl/kg BW; ■, AV2, 225 mg LiCl/kg BW) and sheep breed: (a) Lacaune, (b) Manchega, (c) Ripollesa. Application of LiCl treatment (⇧, AV1, 200 mg LiCl/kg BW; ⬆, AV2, 225 mg LiCl/kg BW). Values are means ± SE.

4.4.3. Persistence of the aversion: double-choice feeding assay

Appetence of rye-grass during the double-choice test, assessed by comparing the proportion of rye-grass eaten (ingested/offered × 100) averaged 92.2 ± 0.9% and did not differ by treatment (H_2 = 2.95; P = 0.229) or breed (H_2 = 2.06; P = 0.358) throughout the study. Persistence of aversion, evaluated using survival analysis as the days during which ewes refused consumption of the olive leaves after the first LiCl dose (aversion learning), tended to show a dose effect. On average, the aversion of AV2 ewes persisted longer (median survival time, 54 days; range, 28 to 80 days) than that of AV1 ewes (median survival time, 33 days; range, 16 to 51 days; X_1^2 = 3.35; P = 0.067). Nevertheless, when data were analyzed by breed, difference between AV1 and AV2 was only detected as a tendency in the Manchega ewes (X_1^2 = 3.58; P = 0.058; Fig. 4.3.).

With regard to effective aversion length, olive leaf intake of the averted ewes vs. C ewes, varied by LiCl dose, being 55 days for AV1 (intake at day 55, 38 ± 8 g vs. 84 ± 6 g; H_1 = 11.0; P = 0.001) and 70 for AV2 (intake at day 70, 49 ± 7 vs. 86 ± 4 g; H_1 = 16.1; P < 0.001), respectively. Moreover, effective aversion length of AV1 treated ewes vs. C ewes varied by breed: Manchega (intake day 34, 65 ± 23 g vs. 100 ± 0 g; H_1 = 3.72; P = 0.054; Fig. 4.3.b), Lacaune (intake day 55, 21 ± 9 g vs. 87 ± 18 g; H_1 = 3.75; P = 0.053; Fig. 4.3.a) and Ripollesa (intake day 55, 47 ± 18 g vs. 85 ± 10 g; H_1 = 3.23; P = 0.072; Fig. 4.3.c). No differences by breed were observed in effective aversion length for the AV2 dose vs. C: Manchega (intake day 70, 63 ± 7 g vs. 90 ± 5 g; H_1 = 5.0; P = 0.025; Fig. 4.3.b), Lacaune (intake day 70, 43 ± 13 g vs. 87 ± 3 g; H_1 = 7.25; P = 0.007; Fig. 4.3.a) and Ripollesa (intake day 70, 41 ± 15 g vs. 83 ± 11 g; H_1 = 3.84; P = 0.050; Fig. 4.3.c), respectively.

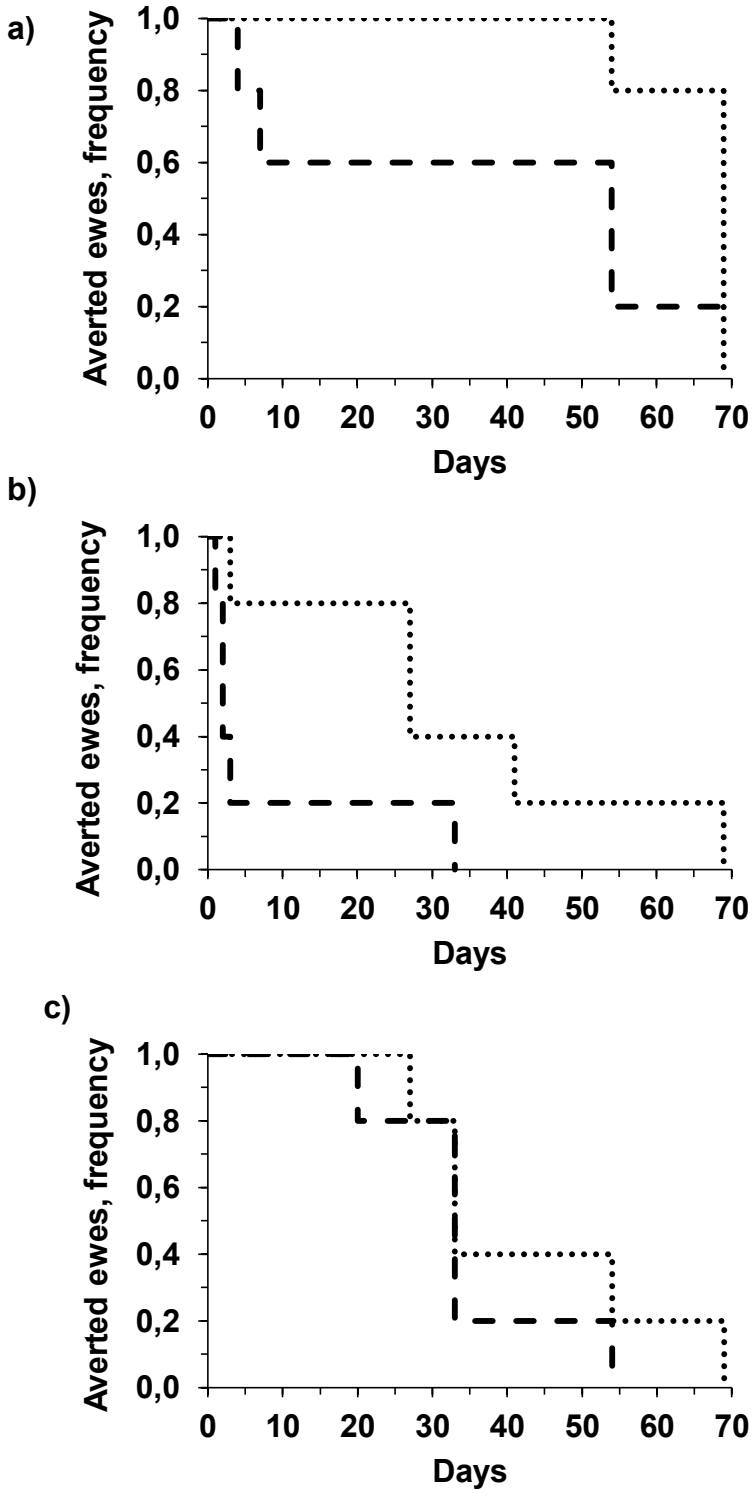

Figure 4.3. Frequency of averted ewes by groups (---, AV1, 200 mg LiCl/kg BW; •••, AV2, 225 mg LiCl/kg BW) and by sheep breed: (a) Lacaune, (b) Manchega, (c) Ripollesa.

4.5. Discussion

4.5.1. Neophobic behaviour

The avoidance of a new or unfamiliar feed is described as an innate protective mechanism (neophobic behaviour), which allows animals to learn from the post-ingestive consequences of eating a potentially toxic feed (Van Tien et al., 1999). With regard to the olive leaves used in our study, despite all the ewes being naïve, only Manchega and Ripollesa breed expressed neophobia on the first days. Intake of olive leaves gradually increased and steadied thereafter indicating the final acceptance of the new feed (Fig. 4.1.). Neophobic behaviour was previously reported in crossbred lambs and ewes offered unfamiliar feeds by Thorhallsdottir et al. (1987; rolled barley and rabbit pellets) and Pfister et al. (1993; corn). Moreover, Villalba et al. (2012) described a similar behaviour using unfamiliar flavours (coconut, cinnamon and onion) in crossbred lambs.

Villalba et al. (2009) observed the relationship between reluctance to eat novel foods and lamb's temperament in different breeds (white-faced Rambouillet-Columbia-Finn-Targhee or black-faced Suffolk crossbreds), and suggested that there is a genetic component in feeding neophobic behaviour. Interestingly, our Ripollesa ewes (local meat breed) were strongly neophobic, Manchega (medium yield dairy breed) were intermediate and Lacaune ewes (high yield dairy breed) did not express any neophobic behaviour, which may be a consequence of the degree of genetic selection applied in each breed. Olive leaf intake of the C ewe groups was high and similar in the 3 breeds at the end of the study, indicating that they were palatable for sheep.

4.5.2. Conditioning aversion learning

Aversion to olive leaves was fully established in the short term with a single dose of LiCl in most AV1 and in all AV2 ewes. A similar response to LiCl was

reported by Manuelian et al. (2010) in sheep and goats averted to olive leaves using a 200 mg LiCl/kg BW dose. The only Manchega ewe that was redosed (day 2) was as a consequence of the incomplete swallowing of the AV1 dose administered. Lower aversion rates (65 to 75%) to the first LiCl dose (130 to 200 mg LiCl/kg BW) were reported in small ruminants and the non-averted animals needed a new dose of LiCl on the following day to achieve the full aversion (Burritt and Provenza, 1996; Gorniak et al., 2008; Pfister et al., 1993).

Nefzaoui (1988) reported differences in composition and digestibility between green and dry olive tree pruning residues. On average, dry matter digestibility is greater in the green (56.9%) than in the dry leaves (48.2%). These moderate differences seem not to be enough to show differences in aversion behaviour. With this regard, other studies (C.L. Manuelian, unpublished data) showed that goats averted to dry olive leaves also refused to eat green olive leaves under on-field conditions.

4.5.3. Dose effect

Head droop, inactivity and decrease basal diet intake the days following LiCl administration was described by Pfister et al. (1993) as signs of discomfort. This behaviour pattern was more evident in the AV2 than in AV1 ewes, as supported by basal diet intake data. AV2 ewes needed more days to recover the daily intake after dosing, which suggested that their discomfort after the LiCl dose was higher than for the AV1 group. Moreover, a higher ratio of AV1 ewes needed to be reinforced to strengthen the aversion. These results complete and support the dose-dependent relationship found between feed intake and LiCl dose (du Toit et al., 1991; Egber et al., 1998), the aversion being greater with the higher LiCl dose (i.e., AV2 > AV1).

4.5.4. Breed effect

Breed was not previously reported as a factor modifying the persistence of conditioned aversion (Conover, 1995; Ralphs et al., 2001) but, our results supported the idea that breed has to be considered as a variation factor in aversion persistence studies. In practice, the short persistent breeds need to be reinforced with a second dose, as observed with the Manchega and Lacaune ewes in our study. On the contrary, Ripollesa ewes did not need reinforcement. Nevertheless, the extent of breed effect was also related to the LiCl dose. Differences in effectiveness of aversion between breeds were only detected with the AV1 dose, the AV2 ewes being more persistent regardless of the breed. Our results also suggested that breed differences in conditioned aversion food can be abolished when a high dose of LiCl is used.

4.6. Conclusions

The results of this study highlight the differences in neophobic food behaviour to olive leaves according to breed and further support the concept that food aversion persistence is dose dependent. Moreover, we proved for the first time that the acquisition of associative learning behaviour was different according to breed.

Finally, differences between breeds were greater at the low LiCl dose, indicating that sensitivity to LiCl depends on breed and dose. Consequently, persistence of the conditioned aversion to olive leaves in sheep was longer with the 225 mg LiCl/kg BW dose; additionally, fewer ewes needed to be re-enforced.

CHAPTER 5. EXPERIMENT 3

Creation and persistence of conditioned feed aversion to grape leaves for grazing sheep in vineyards (Descriptive study)

5. EXPERIMENT 3

Creation and persistence of conditioned feed aversion to grape leaves for grazing sheep in vineyards (Descriptive study)

5.1. Abstract

Conditioned taste aversion (CTA) is a useful tool to avoid grazing damages in vineyards. A 3-yr long-term study was carried out to evaluate the CTA and aversion persistence to grapevines by LiCl, under experimental and commercial conditions, using 24 ewes of 2 breeds (Manchega, n = 12; Lacaune, n = 12). The study included: CTA induction and validation under experimental conditions (barn and simulated vineyard) and grazing in commercial vineyards. Ewes were divided in 2 balanced groups by breed to which CTA (225 mg LiCl/kg BW) and control (C, water blank) treatments were applied. Validation (30 min/test) was done individually in the barn (d 1 to 3) and by group in a simulated vineyard (12 test-day during 1 yr). In the following 2 yr, CTA ewes rotationally grazed 2 commercial vineyard plots (yr 1: A, 5.6 ares, 3 h/d in mid-June; yr 2: B, 8.6 ares, 24 h/d in early-May) with spontaneous grass. The CTA ewes rejected the grapevine leaves whereas C ewes avidly ate them in the barn (0 vs. 95 ± 5 g/d) and in the simulated vineyard (0 vs. 1.47 ± 0.05 kg/d). The CTA ewes reduced grass between vine lines by 68 ± 8 and 44 ± 4% (DM basis) in the A and B vineyards, respectively. When grass was few palatable and scarce, CTA ewes started to ate grape leaves and sprouts needing LiCl re-inforcement doses (A, 100%; B, 50% ewes). In conclusion, CTA was an effective tool for controlling grass cover in vineyards.

5.2. Introduction

Grass cover in vineyards (i.e., natural or cultivated) increases biodiversity, proliferation of the natural enemies of grapevine common pests, improves soil fertility and reduces erosion (Ibáñez et al., 2011). It also contributes to control the vegetative development of the grapevines, obtaining a more balanced plant to grape growth (Ibáñez et al., 2011). Controlled grazing by sheep may be used as an alternative to manual, mechanical and chemical practices to reduce grass growth during spring, avoiding excessive nutrients and water competition with the crop. However, grape leaves and sprouts are very palatable for sheep, usually not

being allowed to graze in vineyards during spring and summer to prevent plant damages and grape losses.

A useful tool to avoid animals to consume a particular feed is conditioned taste aversion (CTA). The CTA is an associative learning behaviour in which an animal avoids consuming a particular food (conditioned stimulus) previously paired with an inductor agent (e.g., lithium chloride, LiCl) as a consequence of a malaise-illness feed-back effect (unconditioned stimulus). The LiCl is a water-soluble salt, easy-to-dose and a safe product for domestic livestock use (Ralphs and Provenza, 1999; Manuelian et al., 2010, 2014; Burritt et al., 2013) which produces gastrointestinal malaise due to the activation of the emetic system followed by a quick full recovery thereafter (Provenza, 1995; Manuelian et al., 2014).

With regard to aversion persistency, Burritt and Provenza (1990) and Doran et al. (2009) reported that CTA persisted in sheep throughout the grazing season (3-4 mo) being re-established nearly to a complete aversion with a single LiCl dose at the next grazing season (9 mo later; Burritt and Provenza 1990). Nevertheless, Lane et al. (1990) and Ralphs (1997) reported 2 and 3 yr CTA persistence, respectively, in grazing cows without the use of reinforcing doses.

The objective of this work was to study the suitability of using CTA against grapevines for grazing sheep on grass covered commercial vineyards and to evaluate its persistence at long-term in 2 breeds of dairy ewes.

5.3. Material & Methods

The research was carried out at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals) of the Universitat Autònoma de Barcelona (Bellaterra, Spain) and at 2 commercial vineyards located at the Penedès county (A, Caves Gramona; B, Caves Recaredo; Sant Sadurni d'Anoia, Barcelona, Spain) throughout 3 yr (May 2011 to June 2013). The experimental procedures and animal care conditions were approved by the Ethical Committee of Animal and

Human Experimentation (CEEAH, reference 770) of the Universitat Autònoma de Barcelona.

5.3.1. Animals and management

A total of 24 adult ewes of 2 dairy breeds (Manchega, n = 12, 67.5 ± 2.8 kg BW; Lacaune, n = 12, 66.2 ± 2.5 kg BW) from the SGCE and naïve to vines were used. Ewes were dry and open, managed under semi-intensive conditions (grazing during the day and sheltered during the night) and supplemented with hay and concentrates according to their requirements. Ewes were grouped at the start of the experiment in 2 balanced groups according to breed (6 ewes each) to which the experimental treatments (CTA and control) were applied and fed a basal diet of alfalfa hay ad libitum.

5.3.2. Conditioned taste aversion induction

Ewes were individually submitted to the experimental CTA treatments using the restraining facilities of the barn (head-lockers). Visual contact between animals of different groups was avoided during the tests.

On the first experimental day (d 0), basal diet was removed 2 h before individually offering 100 g of grape leaves for 30 min. All animals consuming more than 10 g of leaves were treated as CTA ewes, by administering 225 mg LiCl/kg BW in a hypertonic solution, or as control ewes (C) receiving 100 mL of tap water to equalize by the drenching effect. The LiCl (Lithium chloride pure 98%, Panreac, Castellar del Vallés, Barcelona, Spain) was dissolved in distilled water and dosed by using a 200 mL drenching gun (Pimex, Abadiño, Vizcaya, Spain), as indicated by Manuelian et al. (2014). On the following 3 d (d 1, 2 and 3), the same amount of grape leaves was offered to all ewes for 30 min to assess the effectiveness of the CTA created.

5.3.3. Simulated vineyard grazing

The CTA created against grapevine leaves under barn conditions was validated on a vineyard like conditions created at the SCGE. With this aim, a surface of 99 m2 (11.0 x 9.0 m) was delimited on a Italian ryegrass (Lolium multiflorum Lam.) prairie in which 2 rows of vines (length, 2.0 m; separation, 2.8 m; height above ground level, 0.7 m) trellis were made with metal posts and wood clamps. Grapevine green leaves and sprouts (2 kg as fed) were fixed on the trellis simulating a typical vineyard of the Penedès county. Ryegrass was in different vegetative stages according to the season (May to June and October) but grass height at sheep entrance was maintained at approximately 20 cm throughout the experiment. Intake of grapevine leaves intake of each group was measured by weight differences.

A total of 12 test-sessions, in which ewe groups were allowed to graze alone during 30 min, were done during the first year (5, 11, 19, 27, 33, 40, 46, 55, 62, 68, 150 and 375 days) after CTA induction.

5.3.4. Grapevines

Grapevine leaves and sprouts (var. Tempranillo), used for the CTA induction and validation experiments at the SCGE farm, were obtained through May to October from canopy green pruning from the experimental vineyard of INCAVI (Institut Català de la Vinya i el Vi, Sant Sadurní d'Anoia, Barcelona, Spain). Grapevine material was collected on the week previous to the test and stored under refrigeration (4 ºC) until used. A composite of the grapevine leaves and sprouts used during the experiment was done and stored under refrigeration for analysis.

5.3.5. Grazing on commercial vineyards

A vineyard. The CTA induced ewes (n = 12) were moved to a commercial vineyard (A, Caves Gramona, Sant Sadurni d'Anoia, Penedès County, Barcelona, Spain) after the first year (days 401 to 410 after CTA induction) for a descriptive grazing study. From a parcel of the vineyard (0.25 ha var. Tempranillo) 10 plots of 5.6 ares (width × long, 2.8 × 20 m) were selected and enclosed by an electric net fence (Lacme Secur; La Fleche, France). Each plot included a grass covered row between trellis of 2.8 m width and the corresponding half rows of grapevines. The ewes were allowed to graze each plot for 3 h/d during 10 days (401 to 410 days post learning). Ewes were fed after grazing with alfalfa pellets (0.5 kg/ewe, as fed) and barley straw ad libitum. Ewe behaviour during grazing was continuously monitored by 2 observers and if one ewe consumed occasionally more than 4 bites of grapevine leaves it was reinforced for CTA with a new LiCl dose given *in situ*.

To determine the grass cover reduction done by the ewes, 4 squares (25 × 25 cm) randomly chosen in each plot which were clipped and weighted before and after grazing. A sample of the grass cover before grazing was collected for dry matter analysis. Damages in the vineyard (broken branches and clusters, defoliation, grape eating) were visually observed by one viticulture expert after each grazing.

B vineyard. At the next spring (yr 3), the previous CTA ewes were submitted to a new CTA persistence test (as described previously) in the SCGE farm and 6 Lacaune ewes refusing to consume grapevines were selected for the on-field experiment. The ewes were moved to a new commercial vineyard (Caves Recaredo, Sant Sadurni d'Anoia, Penedes County) with spontaneous grass cover for a descriptive grazing study during mid May and early June.

From a parcel of the vineyard (4 ha var. Tempranillo) 4 plots of 2.2 ares (width × long, 6 × 36 m) were selected by homogeneity and enclosed by portable metal

fences (long x height, 3.55 × 2.00 m). Each plot consisted of 2 grass covered rows with the corresponding trellis (ewes were able to pass under the trellis) and grapevine row in the middle and half rows of grapevines in both sides. Ewes remained in the plots during 24 h/day for 3 to 5 days in 2 periods (late May and early June) according to ground cover offer with free access to a water tank. Visual monitoring was done twice a day (0900 and 1700 h). Ewes which were observed to eat grape leaves and sprouts (> 4 bites) were re-dosed with LiCl. The sheep returned to the SGCE farm between the grazing periods where they were again evaluated for CTA persistency.

Before start grazing, grass cover biodiversity was measured by performing 3 parallel diagonal transepts in each plot with a total of 52 lecture-points every 1.5 m and the Shannon index was calculated according Magurran (1988). Biomass and chemical composition before and after grazing were calculated by clipping and weighing the grass of 8 squares (25 × 25 cm) following the 2 diagonal transepts of the plots. To avoid reducing grass offer, samples before grazing were taken from the transepts of the row next to the tested plot, whereas samples after grazing were taken from the grazed row. Damages in the grapevines after grazing were also controlled visually by a viticulture expert.

5.3.6. Sample composition analyses

Dry matter was determined at 103ºC for 24 h and ash content was measured gravimetrically by igniting samples in a muffle furnace at 550 ºC for 4 h (AOAC 2003). The Dumas method (AOAC 2003) by using a Leco analyzer (Leco Corporation, St. Joseph, MI) was used for N determination and crude protein was calculated as percentage of N × 6.25. Neutral detergent fibre, acid detergent fibre and lignin were determined on an ash-free basis by the method of Van Soest (1982) using the Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium sulphite solutions. Chemical

composition of grapevine leaves (CTA induction), Italian rye-grass (simulated vineyard) and spontaneous grass cover (commercial vineyard B) are shown in Table 5.1.

Table 5.1. Chemical composition of grape leaves, Italian rye-grass and ground cover of commercial A vineyard (DM basis). Values are means ± ES.

Content (%)	Grapevine leaves	Rye-grass	Ground cover A
Dry matter	20.92	20.02	41.84
Crude protein	20.98	20.24	10.10
Crude fibre	14.88	19.15	27.53
Neutral detergent fibre	29.68	40.40	54.83
Acid detergent fibre	18.44	21.64	31.32
Lignin acid detergent	4.26	4.87	4.74
Ash	8.56	13.37	9.81

5.3.7. Statistical analyses

Data of grapevine leaves intake in CTA induction and in simulated vineyard showed a non normal distribution and were unable to normalise using the Box-Cox transformation (Box-Cox and Cox, 1964; Osborne, 2010). As a consequence, the CTA treatment effects were analysed using a non parametric U de Mann-Whitney test within and without breed and their differences separated at $P < 0.05$ by using SPSS v. 19.0.0 of IBM (Chicago, IL, USA).

5.4. Results

5.4.1. Conditioned taste aversion induction and validation

Despite being all the ewes naive for grapevines, the Lacaune ewes started consuming grapevines from the first day, the Manchega ewes needing 1 day more to start consuming the grapevine leaves. As a consequence LiCl administration was delayed in the Manchega ewes. The consumption of grape leaves of Lacaune C ewes triplicated (317%) from day 0 to 1 (29 ± 13 vs. 92 ± 4 g, respectively; $P <$

0.001; Fig. 5.1.a) and steadied thereafter, whereas Manchega C ewes increased 163 and 131% the consumption of grapevines from day 0 to 2 (day 0, 51 ± 17; day 1, 71 ± 11 g; day 2, 99 ± 1 g; $P < 0.05$; Fig. 5.1.a). No difference in grapevine leaves intake was found between the C ewes of both breeds after day 2 ($P > 0.05$).

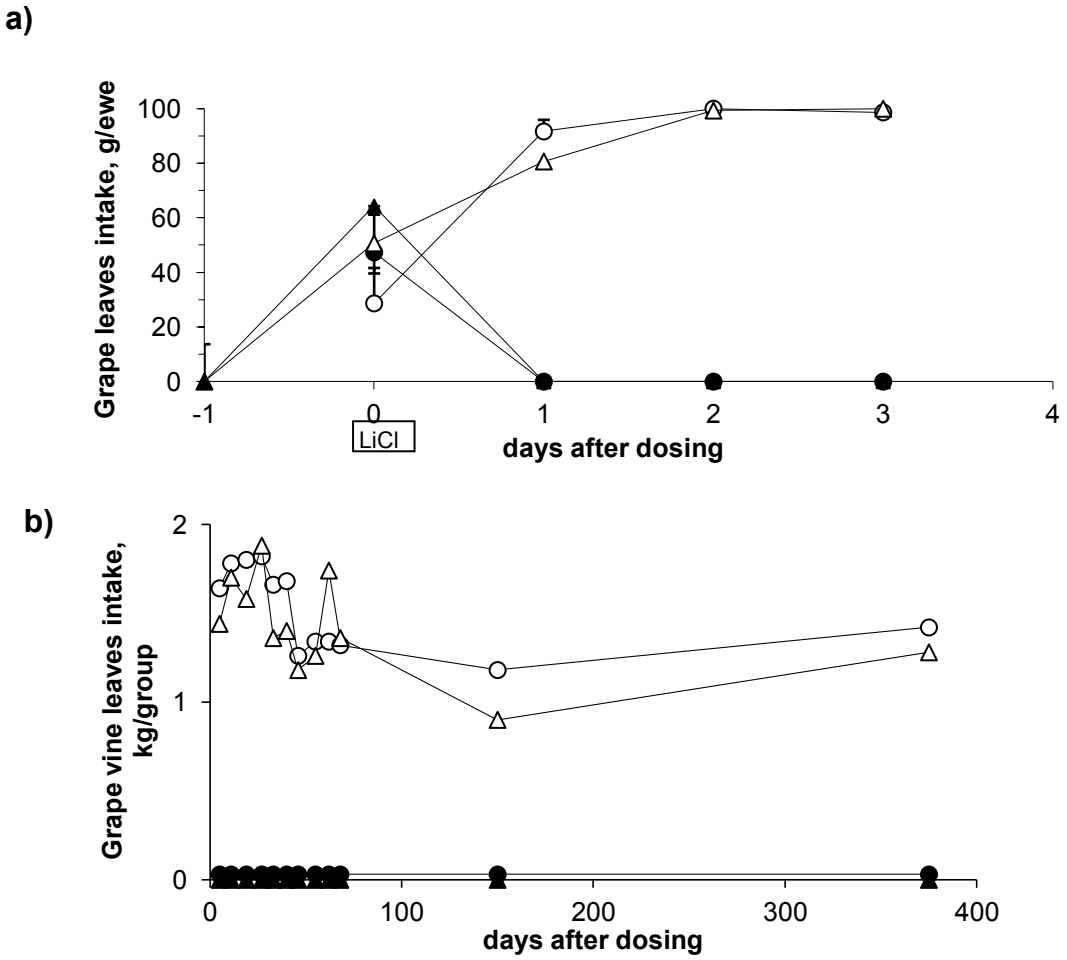

Figure 5.1. a) Intake of grapevine leaves during the conditioned taste aversion induction according to treatment. The LiCl day administration is indicated. Values are means ± SE. b) Intake of grapevine leaves during the simulated vineyard experiment. (○, Control Lacaune; ●, CTA Lacaune; △, Control Manchega; ▲, CTA Manchega).

On the other hand, all CTA ewes of both breeds fully rejected consuming grapevine leaves on the following days after LiCl dosing ($P < 0.001$; Fig. 5.1.a) and

showed refusal behaviour (i.e., butting) against the plastic containers in which the grapevine leaves were offered.

In all simulated vineyard grazing tests, the ewes of both treatments and breeds showed a similar intake pattern, the CTA ewes fully rejecting to consume the grapevine leaves ($P < 0.001$; Fig 5.1.b) and not approaching the trellis frames with the grapevines, whereas the C ewes showed a great attraction to grapevine leaves which more than 65% offered were eaten avidly ($P > 0.05$; Fig. 5.1.b).

5.4.2. Grazing on commercial vineyards

A vineyard. All CTA induced ewes of both breeds maintained the aversion to grapevine leaves on day 400 after inducing CTA without needing to be re-dosed before moving to the commercial A vineyard on day 401.

After grazing for 30 h (3 h/day during 10 days), the 12 CTA ewes reduced the grass cover between vine rows by 68 ± 8% DM. Nevertheless, after day 403 at which the grass cover was scarce and low quality (i.e., mature shot spontaneous grass), some ewes started to bite grapevine leaves and sprouts. The Lacaune ewes started first than the Manchega ewes (Table 5.2.) but, at last, all ewes needed to be reinforced because they consumed more than 4 bites/ewe (Table 5.2.). However, no relevant damages in the vineyard were observed, the grapes being not damaged.

B vineyard. Before moving to the B vineyard all Lacaune CTA ewes showed a complete aversion towards grapevine leaves (intake 0 g, Table 5.3., day B), however, in the CTA persistence checking during the resting period, 3 ewes needed to be reinforced to re-establish a complete aversion, that showing the greater intake needing 2 re-enforcement doses (Table 5.3.).

Table 5.2. Bites number and LiCl re-inforced dose administration during the experiment in the A commercial vineyard during year 1 (Lc, Lacaune; Mn, Manchega).

Ewe		Days after inducing aversion									
No.	Breed	401	402	403	404	405	406	407	408	409	410
1	Lc	0	0	1	10*	0	0	0	0	0	0
2	Lc	0	0	0	0	10*	0	0	0	0	0
3	Lc	0	0	0	1	0	11*	0	0	0	0
4	Lc	0	0	0	1	3	0	10*	0	0	0
5	Lc	0	0	0	0	8*	0	0	0	0	0
6	Lc	0	0	7*	0	0	1	0	0	0	0
7	Mn	0	0	0	0	0	0	10*	0	0	0
8	Mn	0	0	0	0	0	0	9*	0	0	0
9	Mn	0	0	0	0	0	0	0	10*	0	0
10	Mn	0	0	1	0	0	0	8*	0	0	0
11	Mn	0	0	0	0	0	0	10*	0	0	0
12	Mn	0	0	0	0	0	0	7*	0	0	0

*Ewes redosed with 225 mg LiCl/kg BW after showing an intake of grapevine leaves greater than 10 g/ewe.

Table 5.3. Intake of grapevine leaves (g) of the conditioned taste averted Lacaune ewes during the checking of the aversion persistence before grazing and after receiving a LiCl re-enforcing dose during the resting days between grazing periods in the B commercial vineyard.

Ewe No.	Before grazing	After grazing			
	April 30	May 22	May 23	May 30	June 4
1	0	36*	0	0	0
2	0	0	0	0	0
3	0	0	0	0	30*
4	0	0	0	0	0
5	0	72*	0	0	30*
6	0	0	0	0	0

*Ewes redosed with 225 mg LiCl/kg BW after showing an intake of grapevine leaves greater than 10 g/ewe.

The Shannon index before grazing was 2.44 ± 0.10 (Table 5.4.). Botanical composition showed a greater presence of legumes than grasses the most frequent species being *Medicago minima* (19.0 ± 6.9%), *Psoralea bituminosa* (9.6

± 2.1%), *Bromus madritensis* (17.4 ± 1.8%) and *Daucus carota* (17.1 ± 3.4%) as shown in Table 5.4.

Table 5.4. Botanical composition and biodiversity (Shannon's index) of each plot of the commercial vineyard grazed in the B commercial vineyard.

	Botanical composition			Biodiversity
	% Fabaceae	% Poaceae	% Others species	Shannon's index[1]
Plot 1	22.5	28.3	49.5	2.48
Plot 2	24.3	24.4	51.3	2.19
Plot 3	14.4	30.6	55.0	2.60
Plot 4	17.5	32.5	50.0	2.53
Overall	19.7 ± 2.6	29.0 ± 2.0	51.5 ± 1.4	2.44 ± 0.10

[1]According to Magurran (1988).

Grass cover reduction was 44.0 ± 4.0% DM on average, leaving on the ground the more fibrous and less nutritive part of the plants, as shown by the chemical composition (Table 5.5.).

Ewes started to consume grapevine leaves on the last grazing day in plot 3. No other patch showed damages in the crop.

Table 5.5. Chemical composition (DM basis) of the commercial vineyard B before and after grazing each plot and ground cover (GC) reduction (% of DM).

	Plot 1		Plot 2		Plot 3		Plot 4		Overall	
Item, %	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Dry matter	29.9	45.8	25.0	60.0	28.9	40.1	30.4	37.7	28.6 ± 1.4	45.9 ± 5.8
Crude protein	10.5	8.1	12.5	6.4	13.8	8.2	8.9	8.4	11.4 ± 1.2	7.8 ± 0.5
Crude fibre	27.9	33.0	28.8	38.5	30.0	35.9	31.4	32.9	29.5 ± 0.9	35.1 ± 1.5
Neutral detergent fibre	51.4	56.5	48.0	65.2	34.9	55.1	50.5	53.5	46.2 ± 4.4	57.6 ± 3.0
Acid detergent fibre	24.6	30.4	29.2	40.8	24.2	35.8	34.0	36.4	28.0 ± 2.7	35.9 ± 2.5
Lignin acid detergent	2.5	3.6	4.5	7.6	4.1	7.6	5.7	6.1	4.2 ± 0.8	6.2 ± 1.1
Ash	8.6	10.3	9.3	8.8	9.5	8.5	8.2	8.7	8.9 ± 0.3	9.1 ± 0.5
GC reduction	–38.3		–42.5		–54.0		–41.0		–44.0 ± 4.0	

5.5. Discussion

5.5.1. Neophobic feed behaviour and breed differences

The avoidance of a new or unfamiliar feed is described as an innate protective mechanism (neophobic behaviour), which allows animals to learn from the post-ingestive consequences of eating a potentially toxic feed (Van Tien et al., 1999). With regard to the grapevine leaves, both breeds (Manchega and Lacaune) expressed neophobia on the first days, increasing gradually their intake and steadying thereafter, which indicated the final acceptation of the new feed. Despite all the ewes being naïve, Manchega ewes showed greater degree of neaphobia than Lacaune ewes, needing more days to start consuming grapevine leaves and to steady their intake. Neophobic behaviour breed differences was previously reported by Manuelian et al. (2014) using olive leaves, however, only Manchega and Ripollesa breed expressed neophobia on the first days. Additionally, Villalba et al. (2009) observed the relationship between reluctance to eat novel foods and lamb's temperament in different breeds (white-faced Rambouillet-Columbia-Finn-Targhee or black-faced Suffolk crossbreds) and suggested that there is a genetic component in feeding neophobic behaviour.

5.5.2. Conditioned taste aversion induction and validation

Grape leaves aversion was completely established with a single dose (225 mg LiCl/kg BW) the first day, which agreed with the results of Manuelian et al. (2010, 2014) when inducing CTA against olive leaves in sheep. Moreover, no intake nor behaviour differences were observed in the CTA trained ewes by breed, which supported the adequacy of using the 225 mg LiCl/kg BW dose, as recommended by Manuelian et al. (2014), for abolishing the possible breed interactions.

5.5.3. Re-establishing the CTA

Complete CTA was recovered in most ewes (except in one) the day after administering the re-inforced dose. This effect was stronger than that observed by Burritt and Provenza (1990) who obtained a mild aversion when used a lower reinforcing dose (160 mg LiCl/kg BW). The fact that the ewe needing 2 doses in the B vineyard was that showing a greater intake of vineyard, supported the idea that the amount of feed consumed before applying the LiCl dose could be directly related with the strength and persistence of the CTA (Massei and Cowan, 2002). On the other hand, not all ewes needed to be re-inforced in the B vineyard, which may be consequence of a great individual variability for maintaining an effective CTA and the need of permanently monitoring the whole group of CTA ewes.

5.5.4. Long-term CTA in vineyards

To our knowledge, scarce information on long-term CTA persistence is currently available. It has been reported that CTA persisted for 9 months in sheep (Burritt and Provenza, 1990; Doran et al., 2009) and for 2 and 3 years in cows (Lane et al., 1990; Ralphs, 1997). In our study the CTA was able to persist in most ewes during 3 grazing seasons (3 years). Our results agreed with those of Burritt and Provenza (1990), where the averted sheep to true mountain-mahogany (*Cercocarpus montanus*) took fewer bites than the control sheep (19 vs. 64 bites, respectively) 9 month later the aversion induction: Moreover, Doran et al. (2009) showed a strong aversion of sheep towards grape leaves 9 month after induction.

The availability and quality of the grass cover feed helps to avoid eating the target feed and therefore to maintain CTA (Burritt and Provenza, 1990; Thorhallsdottir et al., 1990). As reported by Ralphs and Cheney (1993) and Thorhallsdottir et al. (1987), the CTA become weaker when there are no negative consequences every time of eating bouts the averted food, which facilitates the

loss of CTA. In both commercial vineyard experiments, ewes started resuming grape leaves when the grass cover was scarce.

5.6. Conclusions

The CTA to grapevine leaves can be successfully established with an initial dose of 225 mg LiCl/kg BW, being necessary an annual reinforcement for maintaining it. Because of the individual variability, it may be necessary in practice to reject animals that do not learn CTA easily. Finally, to prevent the occurrence of vineyard damages, the permanent monitoring of the flock behavior and grass availability are key aspects. Our results highlight the use of CTA flocks for selective grazing in vineyards, as an alternative to the use of herbicides and/or machinery control systems for GC.

CHAPTER 6. EXPERIMENT 4

Kinetics of lithium as a LiCl dose suitable for conditioned taste aversion in lactating goats and dry sheep

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6. EXPERIMENT 4

Kinetics of lithium as a LiCl dose suitable for conditioned taste aversion in lactating goats and dry sheep

6.1. Abstract

Lithium chloride (LiCl) is widely used for inducing conditioned taste aversion (CTA) in livestock to avoid that the animals eat toxic plants and to graze groundcover on valuable crops. However, pharmacokinetic studies of LiCl at effective CTA doses are lacking. With this aim, 6 Murciano-Grandina dairy does at late lactation and 6 dry Manchega dairy ewes, were orally dosed with 200 and 225 mg LiCl/kg BW, respectively. Does were placed in metabolism cages whereas ewes were group fed in pens. Lithium was measured over 168 h (does) and 192 h (ewes) at predefined intervals in plasma, urine, feces, and milk using Flame Atomic Absorption Spectroscopy. Plasma Li concentrations reached a maximum at 4 h in does (13.4 ± 1.35 mg Li/L) and 12 h in ewes (17.7 ± 0.8 mg Li/L). The calculated plasma half-lives were 40.3 ± 3.8 h and 30.9 ± 2.1 h for does and ewes, respectively. In goats, all Li administered was recovered at 96 h (urine, 92 ± 4%; feces, 6.5 ± 1.3%; milk, 2.8 ± 0.4%); however the estimated clearance time established by feces were 11 and 9 d for does and ewes, respectively. Additionally, maximum Li excretion in doe milk was 15.6 ± 0.5 mg/L which was approximately half of the calculated effective dose for a 5 kg BW sucking kid. In conclusion, Li kinetics in goats and sheep were similar to cattle and elimination took longer than in monogastric species. The low concentration of Li in feces, urine and milk, as well as the complete elimination of Li from the body after 1.5 wk, allows us to conclude that LiCl is safe and suitable for inducing CTA in ruminants.

6.2. Introduction

Grazing to control ground cover in woody crops is more environmentally friendly than mechanical and chemical cultivation practices. Small ruminants produce low soil compaction and their manure improves soil fertility (Pommeresche and Løes, 2009) but crop sprouts (e.g., vines, fruit trees) are very palatable for them. Modifying their grazing preference by conditioned taste aversion (CTA) with lithium chloride (LiCl) at an effective dose may allow sheep and goats to graze in woody crops (Provenza, 1995; Ralphs et al., 2001). The effective LiCl dose for small ruminants ranges between 125 and 250 mg LiCl/kg BW (du Toit et al., 1991;

Egber et al., 1998; Mazorra et al., 2006) and varies by species and breed (Manuelian et al., 2014, 2010).

Humans handle Li similarly to Na. The Li is absorbed in approximately 8 h (Timmer and Sands, 1999) and is distributed in the total body water space. Li excretion is mainly by urine, with only negligible levels found in faeces (Timmer and Sands, 1999) and milk (Parfitt, 2005). No bonding to serum proteins has been found and peak serum concentration (0.4 to 1 mmol/L) after a single dose is achieved between 0.5 and 3 h post-dosing (Parfitt, 2005; Timmer and Sands, 1999). Excretion follows a first order elimination pattern with a plasma half-life of 12 to 27 h (Parfitt, 2005; Timmer and Sands, 1999).

Compared to monogastrics, ruminants showed a slower Li metabolism and a delayed serum Li-peak as reported in cows (10 h, Ralphs, 1999) and sheep (14 to 20 h, Mormède and Ledoux, 1980; 8 h, Kauter and Godwin, 1995). Moreover, Li in cow's milk lasted for 96 h after LiCl administration, and the milk Li residues were low and unable to induce CTA in calves (Ralphs, 1999).

We are not aware of any study of pharmacokinetics of LiCl at effective CTA doses in small ruminants. Therefore, the aim of this study was to assess the Li kinetics and excretion after an effective CTA dose in goats (200 mg LiCl/kg BW) and sheep (250 mg LiCl/kg BW) and establish clearance times to be used in practice.

6.3. Material & Methods

The experiment was done at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona, Bellaterra, Spain). The experimental procedures and animal care conditions were approved by the Ethical Committee of Animal and Human Experimentation (CEEAH, references 999 and 1001).

6.3.1. Experiment 1

Six open multiparous Murciano-Granadina dairy goats (45.0 ± 1.6 kg BW) with healthy and symmetrical udders, were used for assessing the kinetics and excretion of a 200 mg LiCl/kg BW dose. Does were adult, non-pregnant and at late lactation (180 d in milk; 1.5 ± 0.1 L/d) and had a 1 week pre-experimental period for adaptation to the diet and to metabolic cages. Does were fed ad libitum once-a-day (10.00 h) with a total mixed ration (composition: 60.4% dehydrated alfalfa hay, 15% barley whole grain, 9.1% sugar beet pulp, 7.5% corn meal, 3% soybean meal, 3% sunflower meal, 1% molasses, 0.6% NaCl, 0.2% NaHCO3 and 0.2% vitamins A, E and D3; chemical composition: 90.7% DM, 17.2% CP, 30.9% NDF). Mineralized salt blocks (Na, 36.74%; Ca, 0.32%; Mg, 1.09%; Zn, 5 g/kg; Mn, 1.5 g/kg; S, 0.91 g/kg; Fe, 0.30 g/kg; I, 75 mg/kg; Co, 50 mg/kg; Se, 25 mg/kg, as fed; Ovi Bloc, Sal Cupido, Terrassa, Spain) and clean water were freely available in each metabolic cage. Feed intake and water consumption were recorded daily throughout the experiment. Feed spillage or water wastage, if occurring, were taken into account.

Goats were milked once-a-day (0800 h) with a portable milking machine (vacuum, 42 kPa; pulsation, 90 pulses/min and 66% ratio; Westfalia Separator Ibérica, Granollers, Barcelona, Spain) provided with recording conical jars (2 L ± 5%). Milking routine included postdipping in an iodine solution (P3-io shield; Ecolab Hispano-Portuguesa, Sant Joan Despí, Spain).

On the first experimental day, does were orally administered a single LiCl dose (200 mg LiCl/kg BW; Panreac, Castellar del Vallès, Spain) as a distilled water solution (2.25% w/v) using a 70 mL drenching gun (Lavelvage, Verrières-le-Buisson, France) following the morning milking. To avoid inducing aversion against their diet, total mixed ration was offered 4 h later.

Blood samples were taken from the jugular vein into plastic vacuum tubes (Vacutainer-6 mL; sodium heparin, 17 IU/mL; BD Diagnostics, San Agustin de

Guadalix, Spain) prior to the first milking and dosing (time 0) and, subsequently, at 9 time points (4, 6, 12, 24, 48, 72, 96, 120 and 168 h). Plasma was obtained by centrifugation of whole blood for 15 min at 1,500 × g at 4°C, and stored frozen at − 20°C until Li analysis.

Individual milk, faeces and urine total production were collected, recorded and sampled on the previous day, the dosing day (0 to 12 and 12 to 24), and daily (48, 72, 96 and 120 h) until day 7 (168 h). For milk, a sample at 144 h was also collected. Fresh faeces were sampled (10%), dried at 60°C for 48 h and stored at room temperature until analysis. Also precise-time faecal samples (approximately 10 g) were taken directly from the rectum at the same times as above indicated and directly stored at − 20°C until Li analysis. For milk and urine, 50 mL samples were stored − 20°C until Li analysis. Moreover, a 100 mL milk sample of each goat was preserved with 2 antimicrobial tablets (Bronopol Broad Spectrum Microtabs II; D & F Control Systems, Norwood, MA, USA) and stored at 4°C until composition analysis.

6.3.2. Experiment 2

Six adult, dry and non-pregnant Manchega dairy ewes (55.5 ± 3.1 kg BW) were used for assessing the kinetics of a 225 mg LiCl/kg BW dose. Ewes were sheltered, as a group, in a straw bedded pen and fed once-a-day with tall fescue hay (Festuca arundinacea L.; 89.6% DM, 15.0% CP, 55.0% NDF) ad libitum. Mineralized salt blocks (composition indicated above) and clean water were permanently available.

Ewes were orally administered a single 225 mg LiCl/kg BW dose at 0800 h and fed 4 h later, according to the procedures previously indicated for goats. Faeces and blood sampling were done after individual restraining by using feeders with head-lockers. Blood samples were taken from the jugular vein prior to dosing (time 0) and, subsequently, at 9 time points (4, 6, 12, 24, 48, 72, 96, 120 and 192 h).

Individual precise-time faecal samples (approximately 10 g) were directly collected from the rectum faeces on the previous day, the dosing day (0 to 12 and 12 to 24), and daily (48, 72, 96, 120, 144, 168 h) until day 8 (192 h). Blood samples were processed for plasma as above indicated. Plasma and faeces were stored frozen (− 20°C) until Li analysis.

6.3.3. Feed analyses

A composite of the diets used in both trials was ground through a 1 mm stainless steel screen, and analyzed for DM, ADF and NDF after adding amylase and sodium sulphite solutions, and ash content according to analytical standard methods (AOAC, 2003). The Dumas method (AOAC, 2003) with a Leco analyzer (Leco Corp., St. Joseph, MI) was used for N determinations and CP was calculated.

6.3.4. Milk composition analyses

Milk samples were analyzed by near-infrared spectrometry (Foss NIRSystems 5000; Foss Electric, Hillerød, Denmark) for total solids (TS), fat, milk crude protein (CP = N × 6.38), true protein (TP), and casein (CN) contents. Milk whey protein (WP) was calculated as WP = TP – CN. Milk NPN was calculated as NPN = CP – TP.

6.3.5. Lithium analyses

Lithium concentration was measured by Flame Atomic Absorption Spectroscopy (FAAS) using an AAnalyst800 spectrometer (operating conditions: 670.8 nm, air flow at 17 L/min and acetylene at 2 L/min) provided with an AS800autosampler (both from Perkin-Elmer, Norwalk, CT, USA), at the Instituto de Investigación en Recursos Cinegéticos (Ciudad Real, Spain). Briefly, subsamples of faeces were dried at 60ºC on a plastic Petri dish until constant weight, and then homogenized with a ball mixer mill (MM400 Retsch, Haan, Germany). Samples of 0.4-0.5 g were

digested with 3 mL of nitric acid (69%, AnalaR Normapur Prolabo, Llinars del Vallés, Spain) in Pyrex tubes in a heating block (Micro, Selecta, Abrera, Spain) with an electronic controller (RAT-2, Selecta). Tubes were heated for 5 h at 50°C, then 5 h at 100°C, and finally left at room temperature overnight. The following day, 2 mL of H2O2 (30% v/v Suprapur, Merck, Whitehouse Station, NJ) was added and the tubes were sonicated for 60 s. Tubes were then heated for another 45 min at 90°C and then for 2 h at 120°C. Finally, digests were diluted to 50 mL Milli-Q grade Type 1 water and stored at 4°C until analysis. Every sample set (20 faeces samples) included one blank to provide quality control data. Spiked samples for 2 Li concentrations (2 and 15 µg/g DM) and per triplicate were used to calculate mean recoveries (and SD) which ranged from 83.4% (± 14%) to 79.5% (± 5.3%) for the low and high dose, respectively. Repeatability (in terms of relative standard deviation, 2.5% RSD), was considered satisfactory. Results of Li concentration in faeces were expressed on DM.

Samples of milk, urine and plasma were treated in a similar manner. Initially they were diluted 1:10 with Milli-Q grade Type 1 water, but additional dilutions were performed when necessary. Recovery was calculated for all 3 kinds of samples using 4 different Li concentrations (range, 0.2 to 2.0 µg per sample), and varied from 98.1% (±1.3%) to 108.3% (±2.1%) for milk, from 78.6% (±20.9%) to 102.4% (±6.7%) for urine, and from 99.2% (6.7%) to 120.9% (16.9%) for plasma.

6.3.6. Pharmacokinetic calculations

All Li concentrations presented were corrected for the basal Li concentration. Clearance time of LiCl was obtained from a linear regression of the Li concentration values after the peak, transformed as ln, following the European Medicines Agency's guidelines (EMEA, 2014). Statistical tolerance limit within a 95% level of confidence (95% CI) was used as suggested by EMEA (2014). The calculation of individual plasma half-life ($T_{1/2}$) was performed using the PK Solutions computer program (Farrier, 1997). The peak concentration (C_{max}) and

time to peak concentration (T_{max}) were read from the plotted concentration-time curve of each analyte.

6.3.7. Statistical analysis

Basal daily values of intake (feed and water), milk (yield and composition) and excreta production (faeces and urine) were calculated as averages during the pre-experimental period. Lithium concentration in pooled faecal, precise-time faecal samples, intake (feed and water), milk (yield and composition) and excreta production (faeces and urine) were analyzed in R v.3.0.2. (R Core Team, 2013) using one-way ANOVA with repeated measures. Multiple comparisons were done with Tukey's test. Data were presented as means and SE. Significance was declared at $P < 0.05$, unless otherwise indicated.

6.4. Results

6.4.1. Experiment 1

Feed intake of lactating goats during the pre-treatment period (1.99 ± 0.11 kg DM/d, on average) decreased by half over two days after the LiCl administration (day 1, 0.93 ± 0.15 kg DM; day 2, 0.97 ± 0.29 kg DM; $P < 0.001$), as expected. Moreover, water intake (pre-treatment, 4.73 ± 0.47 L/d) tended to decrease similarly only for day 1 (2.33 ± 0.67 L; $P = 0.1003$), despite the goats not showing signs of apparent malaise after dosing. No differences ($P > 0.05$) in feed and water intake were detected thereafter until the end of the experiment (day 7).

Production of faeces and urine showed an attenuated and delayed pattern according to intakes, faeces (0.36 ± 0.10 kg DM; $P = 0.003$) and urine (0.73 ± 0.13 L; $P = 0.001$) production decreasing on d 2, with regard to the pre-treatment values (0.68 ± 0.01 kg DM/d and 1.67 ± 0.27 L/d). Milk yield (1.45 ± 0.13 L/d), milk fat content (4.47 ± 0.06%) and milk TS (12.63 ± 0.12%) did not vary by effect of

LiCl administration (P > 0.05). Milk CP (pre-treatment value, 3.67 ± 0.09%) decreased on day 3 and 4 (3.31 ± 0.10 and 3.34 ± 0.08%, respectively; P < 0.01). Milk CN (pre-treatment value 3.09 ± 0.08%) decreased on day 2 (2.75 ± 0.17%; P = 0.045) and milk TP on day 3 (2.84 ± 0.06%, P = 0.0433).

Evolution of Li concentration in the plasma of lactating goats after administering a single dose of LiCl showed a positive skewed distribution as shown in Fig.6.1a. Basal value was 0.2 ± 0.7 mg Li/L, peaked at 4 h post-administration (C_{max}, 13.4 ± 1.4 mg Li/L) with no differences between 4 and 24 h post-administration values (on average, 12.7 ± 0.6 mg Li/L; P > 0.05) and slowly declined thereafter, reaching the pre-treatment basal level at 120 h (0.1 ± 0.3 mg Li/L; P = 1.000). The $T_{1/2}$ value was 40.3 ± 3.8 h, on average.

Concentration of Li in milk samples (Fig. 6.1.a) showed a similar pattern and order of magnitude to those of plasma, although its basal value before dosing was below the analytical limit of detection. Li concentration peaked at 12 h post-administration (C_{max}, 15.6 ± 0.5 mg Li/L), with no differences between 12 and 24 h (on average, 15.4 ± 0.4 mg Li/L; P = 0.999) and decreased thereafter. No differences were observed after 96 h, the mean milk Li concentration being on average 2.0 ± 0.9 mg Li/L (P = 0.187).

Basal Li concentration in urine was 0.9 ± 0.1 mg Li/L (a similar order to the initial and final values in plasma), but markedly increased after dosing (× 200; Fig. 6.1.b). Peak was delayed to 24 h (C_{max}, 494.3 ± 23.2 mg Li/L), with no differences between 24 and 48 h (on average, 470.8 ± 22.8 mg Li/L; P = 0.760) and decreased thereafter. With regard to the tail of the distribution, urine Li concentration at 120 h did not differ (40.1 ± 19.3 mg Li/L; P = 0.879).

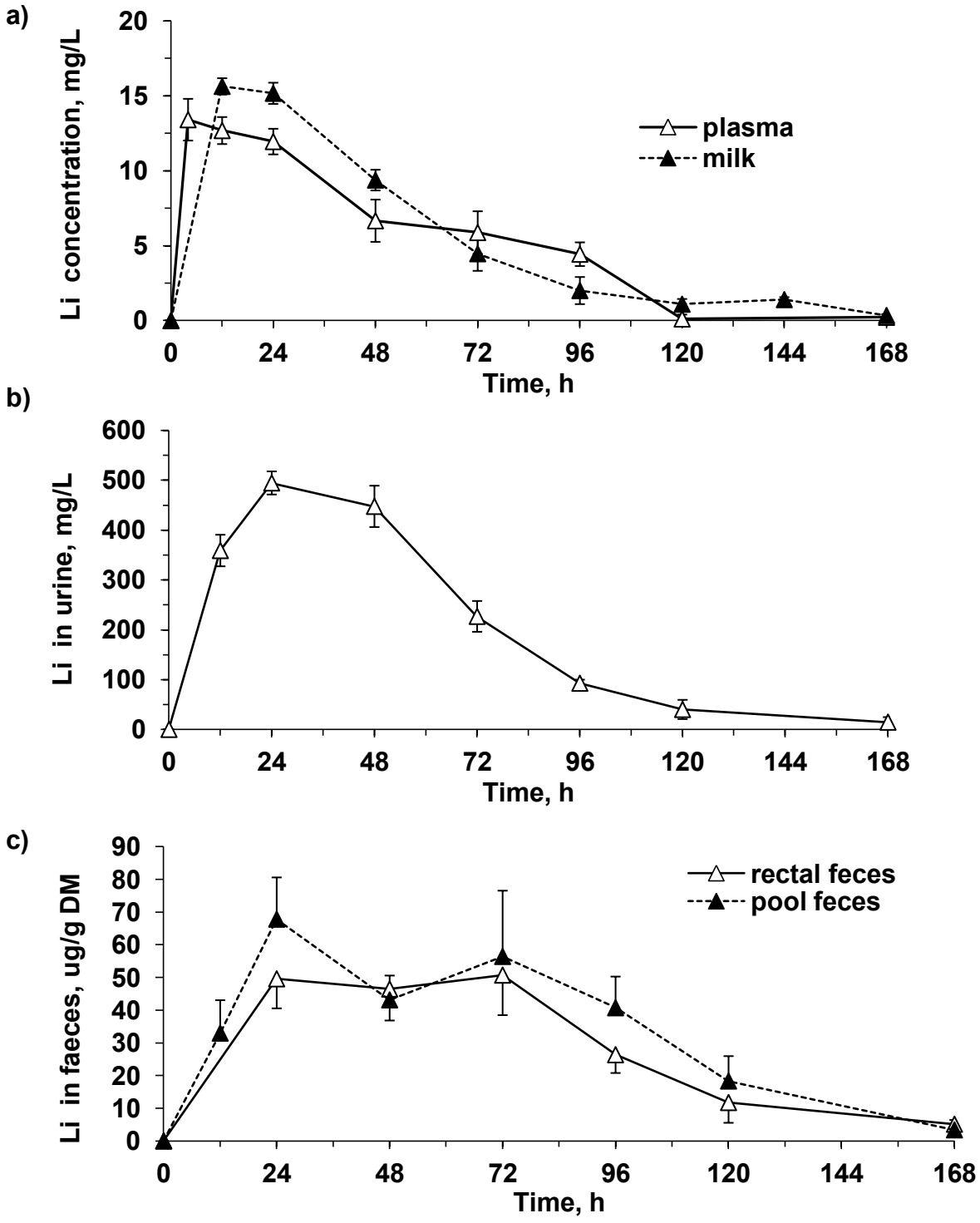

Figure 6.1. Li concentration in plasma and milk (a), urine (b), and rectal faeces and pool faeces (c) of goats after a single LiCl dose (200 mg/kg BW). Each point represents a mean (n = 6); bars indicate the SE.

Li concentration in faeces did not vary ($P > 0.05$) by effect of the sampling procedure (pooled samples of time-intervals vs. precise-time samples), showing an extended skewed distribution with a plateau of 52.4 ± 4.0 µg Li/g DM, on average, between 24 and 72 h (Fig. 6.1.c). Basal concentration was 1.5 ± 0.2 µg Li/g DM, on average, which did not differ from the values at 120 h (15.03 ± 4.2 µg Li/g DM, on average; $P > 0.05$).

Recovery of the administered Li dose grew quadratically ($r^2 = 0.99$) according to time elapsed after administration, 66 ± 6% and 102 ± 5% being recovered after 48 and 96 h, respectively. On average, most Li was excreted by urine (92 ± 4%; $P < 0.001$) when compared to faeces (6.5 ± 1.3%) and milk (2.8 ± 0.4%). The excretion pattern was maintained for the whole experimental period, the values ranging between 88 to 93% for urine, 4 and 12% to faeces, and 1 to 4% for milk.

Clearance time of the Li administered dose in goats (200 mg LiCl/kg BW), calculated as the superior limit with a 95% CI, agreeing with EMEA (2014), varied between 6 and 11 d according to the type of excreta considered (Fig. 6.2.), being 5.8 d (139 h; Fig. 6.2.a) for milk, 10.4 d (249 h respectively; Fig. 6.2.b) for urine and 11.0 d (265; Fig. 6.2.c) for faeces.

6.4.2. Experiment 2

Evolution of Li concentration in the plasma of dry ewes after administering a single dose of LiCl (225 mg LiCl/kg BW) showed a positive skewed distribution, as shown in Fig. 6.3.a. Basal value was 1.4 ± 0.7 mg Li/L, peaked at 12 h post-administration (C_{max},17.7 ± 0.8 mg Li/L; $P < 0.001$; Fig. 6.3.a) and slowly declined thereafter, reaching the pre-treatment basal level at 96 h (1.9 ± 0.82 mg Li/L; $P = 0.302$). The $T_{1/2}$ was 30.9 ± 2.1 h, on average.

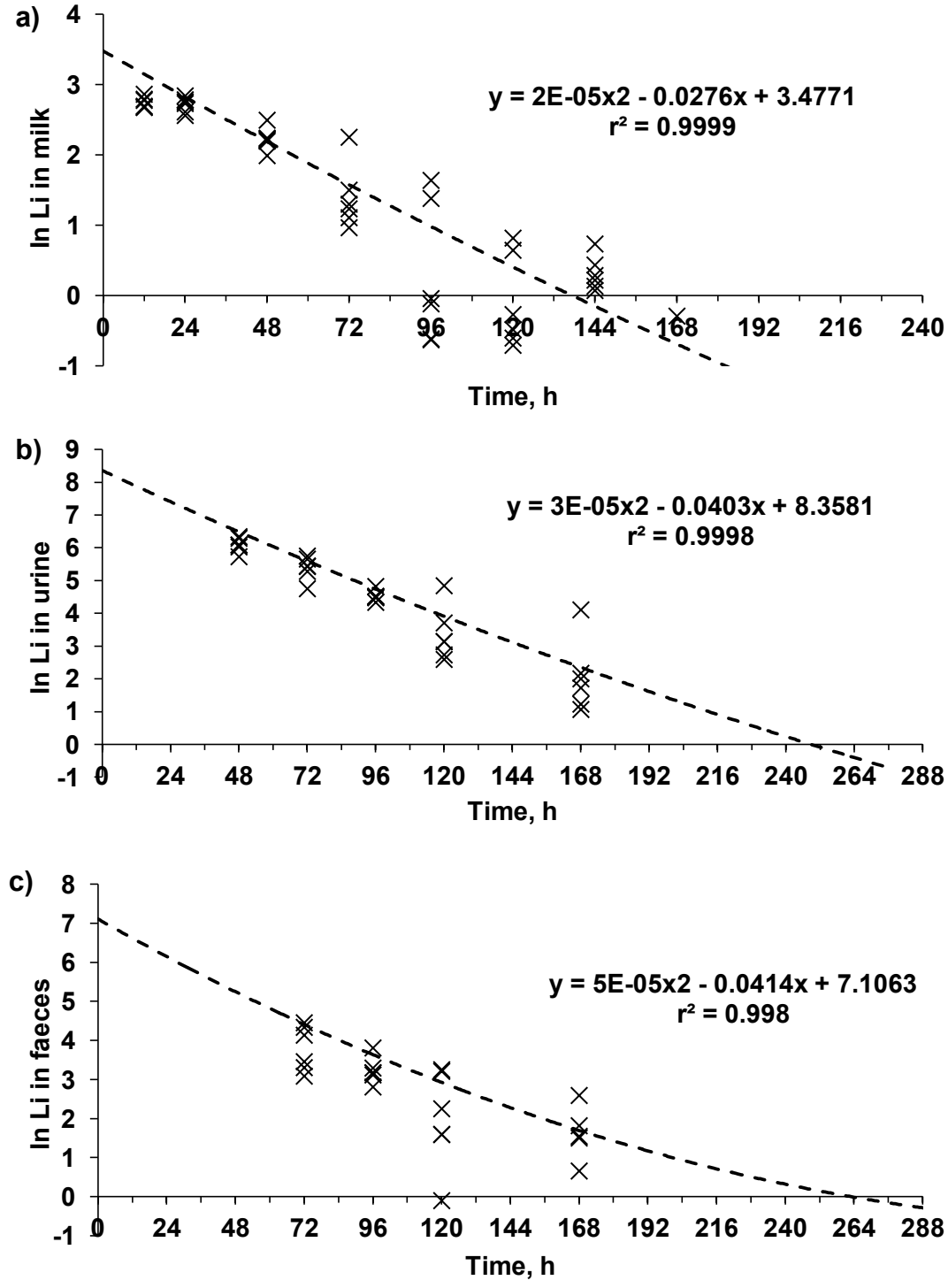

Figure 6.2 Clearance time calculation for Li excretion (a, milk; b, urine; c, faeces) in goats according to EMEA (2014) guidelines (95% confidence superior limit) after a single dose of 200 mg LiCl/kg BW. Each point represents an animal.

Li concentration in faeces collected from rectum at precise-times showed a skewed distribution with a basal concentration of 2.7 ± 0.6 µg Li/g DM, peaked at 24 h (C_{max}, 765.1 ± 126.4 µg Li/g DM) with no differences between 24 and 48 h post-administration values (on average, 691.7 ± 90.1 µg Li/g DW; $P > 0.05$) and slowly declined thereafter, reaching the pre-treatment basal level at 96 h (75.3 ± 18.93 µg Li/g DW; $P = 0.994$; Fig. 6.3.b). Clearance time of the Li administered dose in ewes (225 mg LiCl/kg BW), calculated according to EMEA (2014) from faeces samples, was 8.8 d (210 h, Fig. 6.4.).

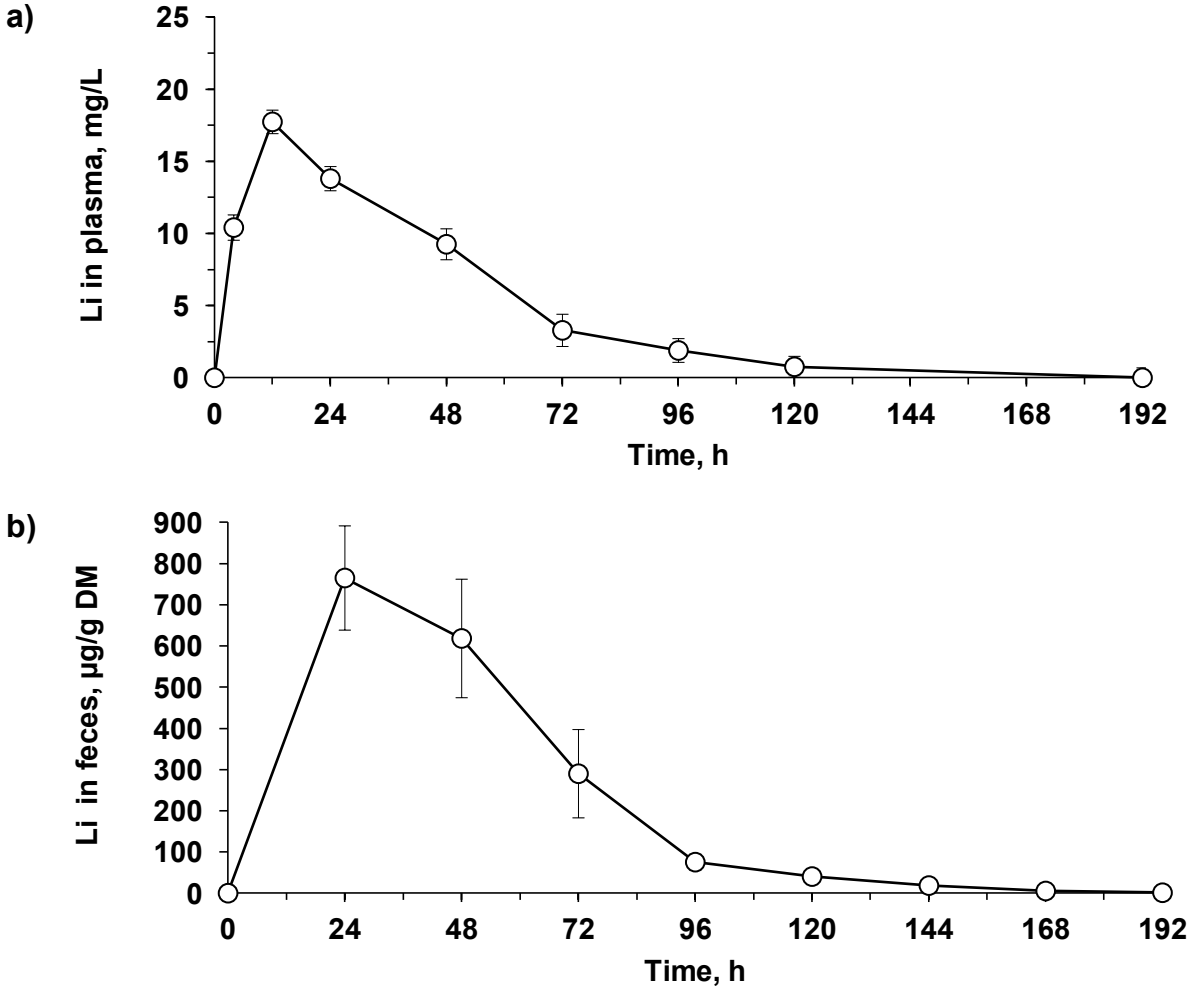

Figure 6.3. Li concentration in plasma (a) and faeces (b) of ewes after a single LiCl dose (225 mg/kg BW). Each point represents a mean (n = 6); bars indicate the SE.

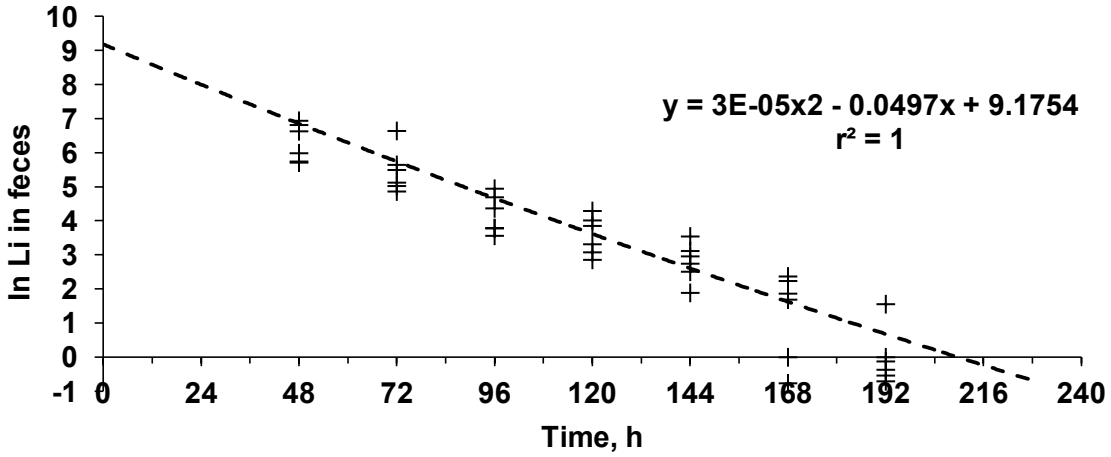

Figure 6.4. Clearance time calculation for Li faecal excretion in ewes according to EMEA (2014) guidelines (95% confidence superior limit) after a single dose of 225 mg LiCl/kg BW. Each point represents an animal.

6.5. Discussion

Administration of 200 mg LiCl/ BW in goat and 225 mg LiCl/kg BW in sheep caused a mild gastrointestinal discomfort for a short time, as shown by the immediate decrease in food and water intake and posterior recovery on the following day. Intake reduction and days to recover feed intake observed in the goats of Exp. 1 were greater than those observed by Manuelian et al. (2014; 33.4% and 1 day) in sheep using the same dose of LiCl (200 mg/kg BW). Other signs of apparent malaise (head droop and inactivity), as previously reported in sheep (Manuelian et al., 2014; Pfister et al., 1993), were not observed in our goats.

The decrease in faeces was observed only on day 2, whereas feed intake was reduced on day 1 and 2. Urine production on day 2 also tended to decrease similarly to water intake on day 1 (− 50%). Those findings are a result of the rumen capacity to buffer the daily intake variations.

Plasma Li concentrations followed a similar distribution in both Exp. 1 and 2 on sheep and goat despite being dry and lactating, respectively. Basal values started

similarly, and C_{max} varied according to the dose used, as reported by Ralphs (1999) in cows (11.9 to 19.3 mg Li/L). Provenza et al. (1993) studied the change of Li concentration in the plasma of sheep dosed with 150 mg Li/kg BW through a rumen cannula, reporting values of Li in plasma at 3 h approximately 1.5 times lower than those obtained in our data. Nevertheless, according to the kinetic pattern of Li shown in our results, the maximum Li concentration should appear later (near 12 h). On the other hand, T_{max} values after dosing observed in our goats and sheep agreed with those reported in cows (Ralphs, 1999; 10 h) and sheep (Mormède and Ledoux, 1980; 14 to 20 h; Kauter and Godwin, 1995; 8 h). Compared to monogastrics (humans and rats), in which Li pharmacokinetic is the reference, the T_{max} values range between 0.5 and 3 h (Timmer and Sands, 1999; Parfitt, 2005). These values are markedly earlier than in ruminants as a consequence of the dilution effect of the ruminal fluid (Mormède and Ledoux, 1980) and the inhibition of the rumen contractions (Kauter and Godwin, 1995). The big difference between ruminants and monogastrics could be a consequence of the slow absorption and the slight recirculation (2%) of Li by saliva (Ulyatt, 1964).

Even though the LiCl dose was lower in goats than in sheep, Li concentration in goat plasma peaked earlier than in sheep. Sheep recovered the basal level (day 4) earlier than goats (day 5), which may indicate a faster Li metabolism in sheep than in goats. Consequently, sheep had Li $T_{1/2}$ shorter than goats, the values being close to those reported by Mormède and Ledoux (1980) in sheep (36 ± 9 h) and Hatfield et al. (2001) in horses (43.5 h). As observed for T_{max}, expected $T_{1/2}$ was later than in monogastrics which ranged between 11.2 and 24 h in plasma (Timmer and Sands, 1999; Hatfield et al., 2001; Parfitt, 2005).

To our knowledge, scarce information on Li concentration in milk is currently available. The C_{max} obtained in goat milk was of a similar magnitude to plasma, and approximately 4 times higher than the values reported by Ralphs (1999) in cow milk after cows received the same dose. Moreover, taking into account their respective milk yield, cows excreted 12 times less Li in milk than goats on the first

day (Ralphs, 1999). However, goats and cows recovered their basal levels at the same time (4 d), the milk compartment being the first one to return to its basal value after dosing. Values of T_{max} in milk agreed with those reported by Ralphs (1999) in cows (10 h), regardless of the dose used, and were similar to those observed in our results for Li in plasma.

Considering that, according to du Toit et al. (1991) and Launchbaugh and Provenza (1994), a minimum dose of 100 mg LiCl/kg BW (Li, 16.4%) is necessary to induce a mild aversion in ruminants, it can be calculated that a sucking goat kid (5 kg BW) would require 82 mg of Li to be averted by the milk of its mother. Therefore, taking into account the Li administered (1,5 g Li/doe) and the above mentioned excretion by milk (2.8% Li in 96 h), the estimated Li intake by the kid during the whole excretion period would be approximately half of the calculated effective dose (41.3 mg Li). Although we advised against the use of suckling goats for inducing aversion, no adverse effects are expected if the kids consumed their milk immediately after dosing. These results were consistent with those of Ralphs (1999) who reported that nursing behaviour was not affected in calves sucking from cows averted by a single dose of 200 mg LiCl/kg BW.

Urine was the main way of excretion of the administered Li, as observed in our results in lactating goats when compared to plasma and milk, although their basal values were similar. The Li concentration in urine peaked and started to decline later than plasma and milk concentrations, recovering its basal values at the same time as plasma did. Ulyatt (1964), using doses of 250 to 500 mg Li/sheep, reported that Li concentrations in rumen decreased to 60% at 6 h after dosing, recycling by saliva being lower than 2% and excretion mainly done through the kidney.

Li concentration in goat faeces showed an extended plateau exceeding those of plasma and urine, as a consequence of the large size and long permanence time in the reticulorumen compartment of the forestomachs. Results of Li excretion

obtained with the 2 techniques used for sampling faeces were similar, showing that both of them could be used to determine Li. In practice, rectum sampling was easy and less exposed to possible contamination.

Although basal Li value in sheep and goat faeces were similar, it increased dramatically (× 10) during the first 24 h in sheep. Consequently, it took longer to recover basal value. The higher Li concentration after dosing, especially at C_{max}, could be explained by the higher dose administered and the physiological status. Goats were at late lactation, which allowed Li excretion by milk, whereas sheep were dry, having only faeces and urine as ways of excretion.

Our study showed that the Li orally administered to sheep and goat reached the basal concentration in plasma after 4 and 5 d, respectively, being mainly excreted by urine. Although little information about Li excretion is available in ruminants (Mormède and Ledoux, 1980; Ulyatt, 1964), the fractionation of Li removal was consistent with that reported in humans (Parfitt, 2005) and dogs (Radomski et al., 1950).

Despite there being the possibility of Li entering the cells via the Na channels and not being pumped out of the cells by the NKA (Na+/K+-ATPase) transporters, a number of Na-dependent permeases can use Li instead of Na and prevent sequestration within the cells (Timmer and Sands, 1999). On the other hand, addition of Li to in vitro rumen cultures did not alter the fermentation of cellulose (Hubbert et al., 1958).

We recovered the full administered dose at 96 h in goats and we estimated that it would happen at the same time in sheep. The different clearance time calculated for goats showed faeces excretion as the longest. Consequently, the recommended clearance time for LiCl in livestock should be taken from feces to prevent any possible Li residues.

6.6. Conclusions

The dose of LiCl administered to induce CTA in small ruminants is completely excreted in a few days, mainly by urine. Although Li is widely distributed on the Earth's crust, to avoid any possible contamination in organic crops with CTA small ruminants, clearance time of 9 and 11 d for sheep and goats, respectively, are recommended. The small amount of Li excreted milk is not enough to produce milk aversion. The only detected side-effect of administering LiCl is a slight decrease in basal diet and water intake that animals overcome in 2 d. The useful application of CTA with LiCl, which allows animals to graze in organic crops and remove ground cover, and the results observed in the present study lead us to recommend that international regulatory agencies (e.g., European Food Safety Authority) should consider the benefits of authorizing this practice in livestock animals.

CHAPTER 7. GENERAL DISCUSSION

7. GENERAL DISCUSSION

This section discusses jointly the main results obtained from the experiments performed to evaluate CTA towards olive tree leaves and grapevine leaves in sheep and goats.

7.1. Conditioning taste aversion learning period

In all experiments, the learning period corresponded to the time required to induce and confirm CTA after the administration of LiCl (6 days for Exp. 1; 3 days for Exp. 2; and 4 days for Exp. 3). Therefore, when a single dose was not effective to induce CTA during this period, redosing was administered, as used by other authors (Burritt and Provenza, 1996; Gorniak et al., 2008; Pfister et al., 1993).

Dose effect. Despite using different LiCl CTA doses (200 and 225 mg LiCl/kg BW), all animals fully rejected consuming olive leaves and grapevines the day after LiCl administration, independently of the dose used. Exceptions were 1 doe in Exp. 1, and 1 ewe in Exp. 2, which did not swallow the LiCl dose completely. On average, 5% of the animals (does, 1 out of 15; ewes, 1 out of 35) dosed with 200 to 225 mg LiCl/kg BW needed to be redosed to ensure adequate CTA. Other authors reported higher redosing rates (25 to 35%) using 130 to 200 mg LiCl/kg BW to establish CTA after LiCl administration (Burritt and Provenza, 1996; Gorniak et al., 2008; Pfister et al., 1993).

Species and breed effect. In Exp. 1 to 3, there were no differences in inducing CTA according to the used species (sheep or goat) or sheep breed (Lacaune, Manchega or Ripollesa). Animals established CTA against the target feed after a single LiCl dose, with the exception of the 2 animals indicated above.

7.2. Conditioned taste aversion behaviour

Marked differences in target feed intake and feeding behaviour were observed between C and AV groups during the testing time of Exp. 1 to 3. After overcoming feed neophobia, animals from C groups approached the target feed (olive leaves and grapevines in boxes or simulated vineyard) and ate it avidly. That feeding behaviour proved the high apetence of olive leaves and grapevines by small ruminants.

On the other hand, CTA induced animals refused to approach the feeding boxes or grapevines, sniffed the leaves but rejected eating them, or consumed less than 10 g or 4 bites of the target feed. The described feed behaviour was similar to that reported by Thorhallsdottir et al. (1987) who showed that, in some cases, CTA animals never stopped eating the target plant completely.

One day after LiCl administration most CTA animals showed head droop, inactivity and decreased intake of the basal diet, which suggested signs of malaise and discomfort as reported by Pfister et al. (1993). On average, the CTA animals overcame the discomfort observed within 2 d, as shown in the intake of basal diet in our results (Exp. 2 and 4).

Dose effect. In Exp. 2, where different LiCl doses were tested, all averted ewes showed the same CTA behaviour previously described, independently of the LiCl doses used. Nevertheless, the discomfort signs were more evident in ewes which received the higher dose (AV2, 225 mg LiCl/kg BW dose) than in those who received the lower dose (AV1, 200 mg LiCl/kg BW).

Species and breed effect. In Exp. 1 to 3, there were no differences in the feeding behaviour of the CTA individuals according to species (sheep and goat) and breed used (Lacaune, Manchega and Ripollesa), all of them markedly refusing the target feed.

7.3. Persistence of conditioned taste aversion

The complete CTA memory or persistence was defined according to Massei and Cowan (2002) as the number of postinducing days after which the animal resume consuming the target feed (i.e., more than 10 g or 4 bites). However, a complete long-term CTA persistence is difficult to achieve in sheep and goats, mainly due to their innate feed sampling behaviour (Thorhallsdottir et al., 1987). Consequently, effective CTA memory was considered when the averted ewes markedly consumed less target feed than the control animals.

Dose effect. In Exp. 2, ewes which received the lower CTA dose (AV1, 200 mg LiCl/kg BW) were the first to resume olive leaves after establishing CTA (learning period). Therefore, a higher ratio of AV1 ewes needed to be reinforced to strengthen the CTA on day 9. On the other hand, ewes which received the higher dose (AV2, 225 mg LiCl/kg BW dose) showed a longer complete and effective CTA persistence.

We hypothesised that dose effect differences may be explained by the results obtained in the kinetics experiment (Exp. 4), where ewes receiving the 225 mg LiCl/kg BW showed higher Li plasma concentration than goats receiving 200 mg LiCl/kg BW; and by the lower basal diet intake observed during the first days of the learning period of Exp. 2.

Ewes which received the 200 mg LiCl/kg dose showed a similar complete CTA persistence against olive leaves in Exp. 1 and 2. Nevertheless, effective persistence was shorter in the Exp. 2 than in Exp. 1, the CTA ewes of Exp. 2 resuming the olive leaves intake earlier despite being exposed to an alternative feed (Exp. 2; Italian rye-grass). These findings disagree with those reported by Burritt and Provenza (1990) and Thorhallsdottir et al. (1990b) for whom the use of an alternative feed makes the CTA behaviour stronger. Our results may be a consequence of the disposition of the feeding boxes during the double-choice test; the target and alternative boxes were placed side-by-side, which facilitated

animals to mix both feeds, and made it difficult to discriminate the olive leaves. Moreover, ruminants are able to discriminate plants by shape, colour and height (Bailey et al., 1996), which suggested that it could be easier for them to discriminate the target feed from the alternative feed under simulated or true on-field conditions.

Ewes which received the 225 mg LiCl/kg BW dose in Exp. 2 and 3 showed different length of complete CTA persistence (54 vs. 375 days, respectively). In Exp. 3, ewes resume eating grapevines in yr 2, when the ground cover was scarce. To strengthen the CTA in these animals, a re-inforcing LiCl dose was administered to 100% ewes (12 out of 12) in yr 2 and 50% ewes (3 out of 6) in yr 3. As reported by Provenza (1995) and Ralphs and Provenza (1999), the extinction of the CTA occurs because there is no negative feedback to avoid the target feed. In this way, a new LiCl administration could re-establish or strengthen CTA (Burritt and Provenza, 1990), as shown in our data. These results also agree with Burritt and Provenza (1990) and Doran et al. (2009) who obtained CTA during one grazing season and strengthened the CTA in the following season with a new LiCl dose; nevertheless, they did not study the CTA longer than 9 months. On the other hand, Lane at el (1990) and Ralphs (1997) reported that CTA cows against larkspur (*Delphinium* spp.), a highly palatable and toxic plant, maintained the aversion for 2 and 3 yr without the need to strengthen. Any accidental consumption of larkspur may act as an CTA inductor agent because of its toxic effect.

These results support the dose-dependent relationship found between feed intake and LiCl dose (du Toit et al., 1991; Egber et al., 1998).

Breed effect. Regarding CTA persistence, sheep breed differences were found for the 200 mg LiCl/kg BW dose (Exp. 2), being the CTA persistence shorter in Manchega than in Lacaune, and the Lacaune similar to Ripollesa. The breed differences were abolished by using the 225 mg LiCl/kg BW dose, as confirmed in

Exp. 3 where Lacaune and Manchega ewes showed the same CTA persistence. Breed effect was not considered a variation factor in previous CTA studies (Conover, 1995; Ralphs et al., 2001).

Species effect. Differences between species (sheep vs. goats) receiving the same CTA dose (200 mg LiCl/kg BW) were detected in Exp. 1. Complete CTA persisted longer in goat than in sheep; however, effective CTA persistence was similar in both species although the resume of olive leaves consumption was more gradual in goats than in sheep. We hypothesised that the slower Li metabolism in goats, supported by the results of the Li kinetics experiments (Exp. 4), may be the reason for this species being more sensitive than sheep to the same LiCl dose.

7.4. Neophobic feed behaviour

The increasing consumption of olive leaves and grapevine leaves observed in control groups during the first experimental days of Exp. 1 (ewes and does, olive leaves), Exp. 2 (ewes, olive leaves) and Exp. 3 (ewes, grapevines) showed the innate neophobic feed behaviour of small ruminants previously reported by Thorhallsdottir et al. (1987) and Pfister et al. (1993).

Breed effect. Although all individuals were naïve to both target feeds, Lacaune ewes only expressed neophobia against grapevines, indicating a non previously described breed effect. The neophobic behaviour was consistent throughout all 2 studies (Exp. 2 and 3), showing differences between breeds (Ripollesa > Manchega > Lacaune).

Species effect. Despite both species, ewes and does, were naïve to the olive leaves in Exp. 1, different degree of feed neophobia was observed between species. Ewes needed more testing time (60 vs. 10 min) and days (1 vs. 2 days) than goats to really incorporate the novel feed to their diet.

7.5. Implications of using CTA animals

The results obtained in this Thesis allowed recommending some key management practices when training livestock to CTA for grazing the ground cover of woody crops. They are the following:

Animals selection. Choose a small group of adult, non-pregnant, dry ewes and/or does which are naïve (never eating before) to the target plant (woody crop edible parts). Lactating animals can be used if their milk is withdrawn in the 6 d following LiCl administration.

Recommended LiCl dose. Induce long-term CTA with the recommended dose of 200 and 225 mg LiCl/kg BW for goat and sheep, respectively. On average, animals will need 2 or 3 d to overcome the discomfort produced by LiCl administration. The CTA should be confirmed individually and the animal re-dosed if needed. Do not reinforce the same animal more than 3 times in the same season.

Conditioned taste aversion induction and confirmation. Offer the animals the target feed as the unique feed source during 24 h, in order to assure its acceptability and intake on the treatment day. On the following day, offer 200 g of the target feed individually for 30 min, and administer the recommended LiCl dose if consumption is > 10 g.

Pasture management and persistence. Wait between 9 and 11 d to move the animals to the selected crop to respect the LiCl clearance time. Ground cover should be abundant, nutritive and palatable for grazing animals to prevent that they resume consuming the target feed. Allow the CTA animals to graze a delimited patch of the crop area to obtain a more uniform grass reduction, and move them to another patch when grasses measure less than 5 cm height. A close monitoring of the animals is recommended to detect any accidental consumption of the target feed. The CTA could persist easily for 1 yr, being necessary only to strengthen the aversion once a year.

CHAPTER 8. FINAL CONCLUSIONS

8. FINAL CONCLUSIONS

The conclusions obtained in this thesis are:

1. Naïve goats and sheep to woody crops can establish CTA against it with one single dose of LiCl, if the proper administration is ensured.
2. The discomfort generated by LiCl administration in sheep and goat was overcome in 2 days.
3. Persistence of CTA, number of reinforced administrations to strengthen the aversion and degree of discomfort varied according to the LiCl dose used.
4. The recommended dose to obtain a long-lasting CTA was 200 mg and 225 mg LiCl/kg BW for goats and sheep, respectively.
5. When applying the recommended dose, re-dosing LiCl could be necessary in some animals to ensure CTA at mid and long term (> 1 yr).
6. Sensitivity to LiCl depended on the species (goat > sheep) and breed (Lacaune = Ripollesa > Manchega) used. Nevertheless, breed differences could be abolished using the recommended dose.
7. Neophobic feed behaviour also showed differences between species (sheep > goats) and breed (Ripollesa > Manchega > Lacaune).
8. The recommended LiCl doses for small ruminants were fully excreted, mainly through urine, in approximately 96 h.
9. The LiCl excreted by milk was low and insufficient to generate CTA in suckling off-spring (i.e., kids).
10. Calculated clearance time for the recommended LiCl doses were 9 and 11 days for goats and sheep, respectively.
11. Ewes treated at the recommended LiCl dose were effective in controlling grass cover in commercial vineyards, without appreciable damages in the woody crop.
12. To minimize potential damages in the woody crops during grazing with CTA small ruminants, a palatable ground cover and a careful follow-up of the animals needs to be ensured.

CHAPTER 9. REFERENCES

9. REFERENCES

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