Mediterranean Agronomic Institute of Zaragoza

Alternatives to Lithium Chloride to induce conditioned taste aversion to olive leaves in sheep

19/06/2015

ELALOUANI AHMAD

Acknowledgment

At a time when a page is turned, I wanted to extend my sincere thanks to those who contributed to the writing of this thesis.

First of all, I want to express my sincere gratitude and appreciation to my supervisor Elena albanell Trullas, to Pr. Gerardo Caja and to Pr. Ahmed Salama researcher professors and to Carmen L. Manuelian Fusté PhD degree at the Department of animal and food science in the veterinary faculty of the Autonomous university of Barcelona. For the tenacity, skill and rigor with which they knew framed this study and the support provided to me throughout the development of this study.

Then, my thanks go to also wanted to all the teachers that I met on my carreer.

My thanks go to the distinguished jury members who were willing to consider this work; they find here the expressions of my gratitude and deep respect.

Finally, my thanks to all persons that support me nearly or farly in order to achieve this modest thesis.

List of tables

List of figures

List of Abbreviations

CTA: Conditioned Taste Aversion

T1: Treatment 1 that received 0.4 g tannins/ kg BW

T2: Treatment 2 that received 0.6 g tannins /kg BW

T0: Treatment 0 that received 0 g tannins/Kg BW

C: Control

Li: The treatment of 225 mg/ kg BW

LiCl: Lithium Chloride

Kg: Kilogram

NDF: Neutral Detergent Fiber

ADF: Fiber Acid Detergent

ADL: Lignin Acid Detergent

SPSS: Statistical Package for Social Science

LSD: Least Significant Difference

H : Value of Kruskal and Wallis.

Summary

This thesis aimed to evaluate the ability of Lithium Chloride alternatives as well as the poisonous plants and the secondary plants metabolites to establish conditioned taste aversion (CTA) to olive leaves in sheep. The CTA is an associative learning behaviour in which an animal avoids consuming a particular feed previously paired with an inductor agent.

The first objective was to determine which toxic plants of the Mediterranean area with an emetic effect are able to induce the CTA in sheep. The second objective was to study the commercial product Ellagitan rouge (AEB Ibérica) intended for enological use, in order to induce CTA to olive leaf in sheep as an alternative to the use of Lithium Chloride (LiCl). Finally the third objective was to compare the efficiency of a product rich in tannins, intended for tanning use (extract from *Acacia,* Proquip S.A.) when compared to LiCl, for inducing CTA to olive leaf in sheep.

The results obtained showed that amounts of 10g of *Hedera helixIvy* fruit (poisonous plant fruit) were not able to generate CTA in ewes. However, the commercial product Ellagitan rouge at doses of tannins (0.4 and 0.6 g tannin/kg BW) was able to create CTA to olive leaves in sheep (especially for Manchega ewes) but was not practical due to the application difficulties (use of esophageal tube). Nevertheless the lithium chloride was more efficient than tannins to create CTA to olive leaf in sheep.

Resumen

Esta tesis tuvo como objetivo evaluar la capacidad de las alternativas del cloruro de litio, así como plantas venenosas y metabolitos secundarios de las plantas, para establecer aversión condicionada (AC) a las hojas de olivo en ganado ovino. La AC es un comportamiento de aprendizaje asociativo en el que un animal deja de consumir un determinado alimento tras la administración de un agente inductor.

El primer objetivo fue determinar qué plantas tóxicas con características eméticas de la zona Mediterránea son capaces de inducir AC en ganado ovino. El segundo objetivo fue valorar el producto comercial Ellagitan rouge (AEB Ibérica) destinado para uso enológico, como alternativa a la utilización de cloruro de litio (LiCl) para crear AC a la hoja de olivo en ovejas. Por último, el tercer objetivo fue comparar la eficacia de un producto rico en taninos, utilizado en curtición (extracto de Acacia, PROQUIP SA) con el LiCl, para inducir a la AC a la hoja de olivo en el ganado ovino.

Los resultados obtenidos mostraron que 10 g de frutos de hiedra (*Hedera hélix*) potencialmente tóxicos, no fueron capaces de generar AC en ovejas. Sin embargo, el producto comercial Ellagitan rouge en dosis de taninos de 0,4 y 0,6 g/kg de peso vivo, fue capaz de crear AC (especialmente en ovejas de raza Manchega), pero no resultó práctico debido a sus dificultades de aplicación (uso de sonda esofágica). No obstante, el LiCl fue más eficiente que los taninos para crear AC a la hoja de olivo en ganado ovino.

Résumé

Cette thèse avait comme objectif l'évaluation de la capacité des alternatives au chlorure de lithium ainsi que les plantes toxiques et les métabolites secondaires des plantes à établir l'aversion conditionnée (AC) aux feuilles d'olivier chez les ovins. L'AC est un comportement d'apprentissage associatif dans lequel un animal évite de consommer un aliment particulier apres l'administration d'un agent inducteur.

Le premier objectif était de déterminer quelles plantes toxiques de la zone Mediterranenne avec des caractéristiques émetique sont capables d'induire l'aversion conditionnée chez les ovins. Le deuxième objectif étatit l'évaluation du produit comercial Ellagitan rouge (AEB Iberica) destiné aux usages enologiques, comme alternative a l'utilization du chlorure de litium (LiCl) pour créer AC à la feuille d'olive chez les brebis. En fin, le troisièmme objectif était la comparaison de l'efficaciét d'un produit riche en tannins, utilizé en curtition (Extrait d'Acacia, PROQUIP SA) avec LiCl, pour induire l'AC à la feuille d'olive chez les ovins.

Les résultats obtenus ont montré que 10 g de fruits de la lierre *(Hedera helix* L.), potentialement toxiques, n'ont été pas capables de génerer AC chez les brebis. Cependant, le produit comercial Ellagitan rouge en dose de tannins de 0.4 et 0.6 g/kg PV, était capable de créer l'AC (especialement chez les brebis de la race Manchega), mais s'avèrait non pratique du aux dificultés d'application (Utilization de la sonde esophagienne). En revancha, le LiCl était plus eficient que les tannins pour créer l'AC à la feuille d'olive chez les ovins.

Index

1. Introduction

The domestic ruminants develop dietary habits and foraging skills by learning early in life which allows them to select a wide variety of potential and available feed across the time and space (Provenza and Balph, 1987). Their preferences for, or aversions to, foods may result from trial and error learning based on cautious sampling and resultant nutritional and physiological consequences (Provenza and Balph, 1987). They can acquire aversions to feed that contain toxins by associating the flavor (taste and odor) of the feed with aversive postingestive feedback, which is apparently caused by stimulation of the emetic system. Basically, a positive feedback occurs when the ingested feed provides adequate nutrients and satiety, and consequently the animal develops a preference for that feed. Inversely, 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 (Provenza, 1995). This process is known as Conditioned Taste Aversion (CTA).

The CTA is based on a classical conditioning method, where the new feed is the conditioned stimulus and the gastrointestinal discomfort is the unconditioned stimulus (Dardou, 2007). For instance, the chemical compound of lithium chloride (LiCl) is an unconditioned stimulus and it is the most antiemetic drug used to induce CTA in ruminant (Provenza et al., 1994b). Additionally, this chemical compound acts on the vomiting center, causing discomfort to the animal that might be associated with the ingested feed and consequently induce CTA (Provenza, 1995; 1996). However, the problem with LiCl use at the commercial scale is related with the European legislation that is very restrictive with drug uses. Consequently, one of the possible solutions to this constraint is to find natural LiCl alternatives, e.g. poisonous plants and/or secondary plant metabolites.

Plant secondary compounds can act as inhibitors of digestion and/or toxins (Waterman, 1984). Additionally, the digestion inhibitors act directly on the animal gut and affect negatively the digestive processes by various ways and the toxins act specifically when they cross the cell membrane which leads to an important toxicity by its ingestion of small amounts (Waterman, 1984). Among the plant secondary metabolites that participate in the processes of diet selection, terpenes and tannins are widespread (Bruce et al., 2004). In terms of its uses to induce CTA, a lot of authors have made in evidence that, mammalian plant avoidance has been correlated with tannin content for many species (Robbins et al., 1987). In his studies about animal's behavior, Provenza showed that goats can learn to avoid eating plant tannins in their environment, combining the taste of food containing tannins, with aversive consequences post ingestive (Provenza et al., 1990). A study with lambs demonstrated that prior experience with tannins and terpene containing foods, influenced diet selection when several toxin-containing foods were offered (Villalba et al., 2004).

This study was designed to search natural and non-pharmacological alternatives to LiCl for induced CTA in sheep. Consequently, the experiments conducted aimed to demonstrate the ability of poisonous plant and secondary plant metabolites to induce CTA.

2. Conditioned taste aversion (CTA)

2.1. Definition

Pavlov (1927) observed that a presentation of a stimulus followed by a discomfort, showed a conditioned response to this stimulus when it was offered only to the animal. In 1945, Richter observed too this ability to associate two stimuli by associating feeding and discomfort. In fact, he observed that, if the consumption of poisoned bait did not lead to the death of the animal, this last avoided the consumption of the bait in the future presentation. The animal associated the bait with discomfort in order to survive. After the publications of Garcia, in 1955 a best interest to CTA was appeared. The CTA can be defined as a Pavlovian learning type where the new food serves as the conditional stimuli and the gastro intestinal discomfort serves as the unconditional stimuli (Domjan, 2000). It is a form of associative learning behavior in which an animal avoids consuming a feed previously paired with an illness effect (unconditioned stimulus) (Dardou, 2007).

2.2. Importance of Conditioned taste aversion

2.2.1. Feed selection and dietary learning

Grazing animals are always selective in what they eat; that is, they choose or harvest plant species, individual plants, or plant parts differently from random removal or from the average of what is available (Vallentine, 1990). In rangelands, the sheep are well known for feeding on a wide spectrum of plants and they possess some degree of nutritional wisdom which enables them to select foods that meet their nutritional needs and avoid those that cause toxicosis (Provenza et al., 1994a, b). It is considerably more difficult to identify feeding attractants in plants. In addition, small ruminants are constrained nutritionally to select diets of higher quality than large ruminants, and the efficiency with which small versus large ruminants ingest different plant parts and life forms may be different. The ruminants are able of detoxifying and eliminating many toxic plant compounds (Freeland and Janzen, 1974). The limitations of detoxification mechanisms may force herbivores to consume a variety of foods to avoid over-ingesting toxic compounds, to ingest small amounts of novel foods, and to sample foods continuously. Individual animals should prefer familiar to novel foods, and should be able to seek and ingest foods that rectify specific nutritional deficiencies (Provenza and Balph, 1987).

There are genetic, metabolic and morphological constraints on dietary learning. Plant olfactory and gustatory characteristics that ruminants have consistently associated with negative consequences may be genetically fixed, and it is often possible to explain avoidance of plant species and parts by herbivores in terms of deterrent phytochemicals and physical plant characteristics (Provenza and Balph, 1987). The social learning is another important factor influencing the ruminant's dietary learning and enables an inexperienced animal to avoid the inefficiency and risk of testing everything itself. The social learning theory predicts that the best models are nurturants (e.g. mother) and respected peers (e.g. dominant group member) (Provenza and Balph, 1987). For instance, the mother may greatly influence her offsprings' dietary habits. As a result, dietary learning may be more pronounced in early, as opposed to later, life and there may be a sensitive period that coincides with the transition from monogastric to ruminant (Provenza and Balph, 1987).

2.2.2. The mechanism of conditioned taste aversion

The mechanism responsible for the development of aversion is not well established, but it is suggested that animals learn which plants or food to eat and which to avoid through interactions between flavor (odor, taste and texture) and the post-ingestive consequences of nutrients and toxins (Provenza, 1996).

Empirically, the best way to teach an animal not to eat a particular plant is to pair eating the plant with nausea. Many toxins in plants cause food aversions by stimulating the emetic system of the midbrain and brain stem. Basically, the chemical compound of LiCl is used for causing aversions because it can be given in doses high enough to condition strong aversions without causing death or obvious signs of illness (du Toit et al., 1991). The gut-defense system is designed to automatically pair eating a food with gastrointestinal illness regardless of what the animal "thinks" caused the illness (Garcia et al., 1985). In fact, animals under deep anesthesia can also form aversions. The animal doesn't even have to be awake during the nausea to form an aversion to a food (Provenza et al., 1994a). When training livestock to avoid a food, it's very important that the target plant is novel, meaning the animal has never eaten the plant before (Ralphs, 1997). When animals are properly conditioned, aversions can persist for years. However, it is important to understand that aversions can diminish over time and various environmental and social conditions can rapidly accelerate the loss of a trained aversion.

Animals quickly learn to avoid a novel food when they experience nausea soon after eating a food. Thus, the antiemetic drug should be given to the animal immediately after it eats the food. Unfortunately if animals eat some of the target plant later when foraging on pasture, and don't experience illness, the positive feedback from nutrients may cause the animal to eat more of the target plant. During conditioning, animals are allowed to eat the target plant (the plant wanted to be avoided), then given a dose of antiemetic drug. Animals are usually trained in pens where access to foods can be controlled and where they can be watched to ensure that each animal eats the target plant (Burritt and Provenza, 1989a). The following scheme represents the affective and cognitive processes involved in diet selection (Figure 1; Manuelian, 2014).

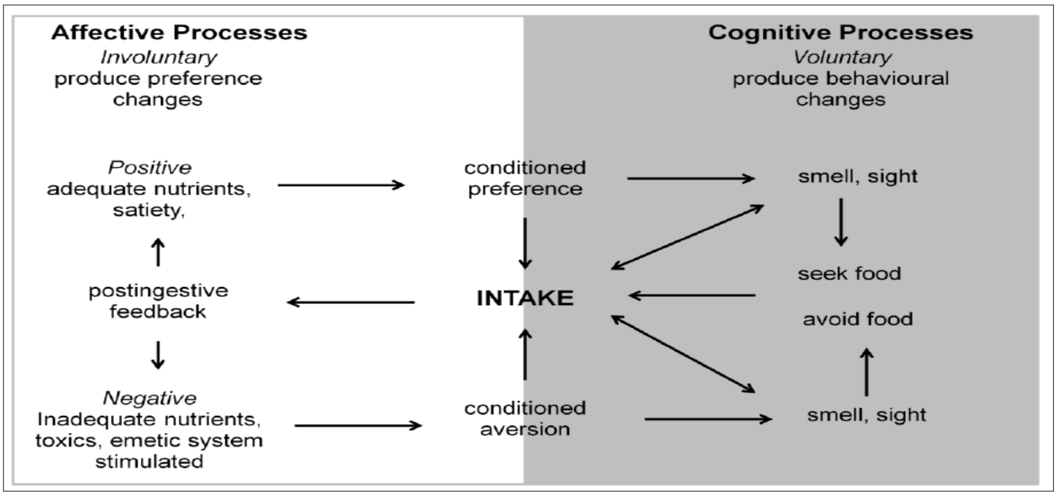


Figure 1. The mechanism of conditioned taste aversion (Manuelian, 2014).

2.3. The chemical repellents to create conditioned aversion taste

The chemical repellent is an unconditional stimulus for example an electric shock or a gastrointestinal discomfort (Dardou, 2007). The first conditioned aversion taste application studies began in the mid-seventies to prevent coyote attacks on sheep flocks (Conover, 1995). Since there, several methods have been tested to achieve CTA with different inductor agents, animal species and target feed as shown in table 1 (Conover, 1995). 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 (Provenza and Ralphs, 1999; Manuelian, 2014).

Table 1. Different mimics model illustrating the conditioning aversion using different chemical repellent (Conover, 1995; Manuelian et al, 2014).

Target feed		Species	Chemical repellent	Results
Plants	Palatable shrubs	Lambs	Lithium Chloride	Positive
	Grains and feed	Ruminants	Lithium Chloride	Positive
Eggs	Odd color and taste	Crows	Trimethacarb	Positive
larkspur		Cattle	Lithium Chloride	Positive
Sheep and lambs		Canids	Lithium Chloride	Mixed
		Coyotes	Lithium Chloride	Negative
Sheep		Olive leaves	Lithium chloride	Positive
Vegetables		Woodchucks	Emetine	Positive
		Racoons	Emetine	Mixed

Thorhallsdottir et al. (1987) found that (1) sheep learn to avoid palatable foods containing LiC1, but they continue to sample small quantities of foods that have been paired with illness and increase consumption when ingestion is no longer paired with illness, (2) the learned aversions persist for at least 2 months, and (3) sheep consume small quantities of novel foods initially. Furthermore, Burritt and Provenza (1991) found that when offered two foods, one familiar and one novel, lambs (1) are able to identify and avoid the novel food when it contains LiC1, and (2) associate adverse consequences with the novel food when the familiar food contains LiC1. On the same context, it has been approved that sheep have the ability to show CTA to olive leaf pairing with LiCl application (Manuelian, et al., 2014).

2.4. The variation factors of conditioned taste aversion

The ability to generate CTA, using chemical repellents, depends on many variation factors, which are described as following.

2.4.1. Dose and frequency of the chemical repellents

Conditioned aversion has a dose and effect relationship due to the degree of malaise generated by the amount of the chemical repellent administered, decreasing the feed ingestion as the chemical repellent doses increase (Manuelian, 2014).

2.4.2. Nutrient value and nutritional needs of the animal

Animals are more afraid to consume a novel food and risk becoming ill when they are nutritionally deficit and the food contains the needed nutrients. If the target feed provides an irreplaceable nutrient, CTA cannot be expected to change the animal behavior (Conover, 1995).

2.4.3. Neophobia

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) When an animal consumes a new feed it starts eating only a small amount; thereafter, if there is no negative feedback, it 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, 1989 b; 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).

2.4.4. The chemical repellent time administration

Ruminants can learn with some delay between intake and induced sickness. For instance, 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., 1994b). Therefore, the chemical repellent as the LiCl have to be administered immediately after the animal consumes the target feed in order to induce the CTA (Manuelian, 2014).

2.4.5. Age and social facilitation

Provenza y Balph (1988) reported that adult animals tend to avoid feeds which cause gastrointestinal distress more than young animals. Social facilitation is the most important factor preventing widespread application of aversive conditioning. When averted animals see other animals eat the target food they will sample it, and if there is no adverse reaction they will continue eating and extinguish the aversion. However, if averted animals can be grazed separately, aversions will persist. Aversive conditioning may provide an effective management tool to prevent animals from eating palatable poisonous plants that cause major economic loss. Additionally, it 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., 1990b), 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).

2.4.6. Alternative feed and persistence

The conditioned aversion becomes inefficient when there are no negative consequences after each animal ingestion of the averted food (Ralphs and Cheney, 1993; Thorhallsdottir et al., 1987). The availability of an alternative feed helps to avoid sampling the target feed and therefore to our knowledge, research studies on long-term (≥ 9 months) CTA persistence are recentely studied (Manuelian, 2014). A lot of authors reported that CTA persisted for 9 months in sheep (Burritt and Provenza, 1990; Doran et al., 2009; Manuelian, 2014) and for 2 and 3 yr in cows (Lane et al., 1990; Ralphs, 1997). In order to promote long-term CTA, the optimal conditions are the use of adult animals, novel target feeds and high doses of chemical repellent, as well as maintaining non-CTA animals grazing separately to avoid social facilitation (Ralphs, 1997).

2.5. The use of tannins to induce conditioned taste aversion

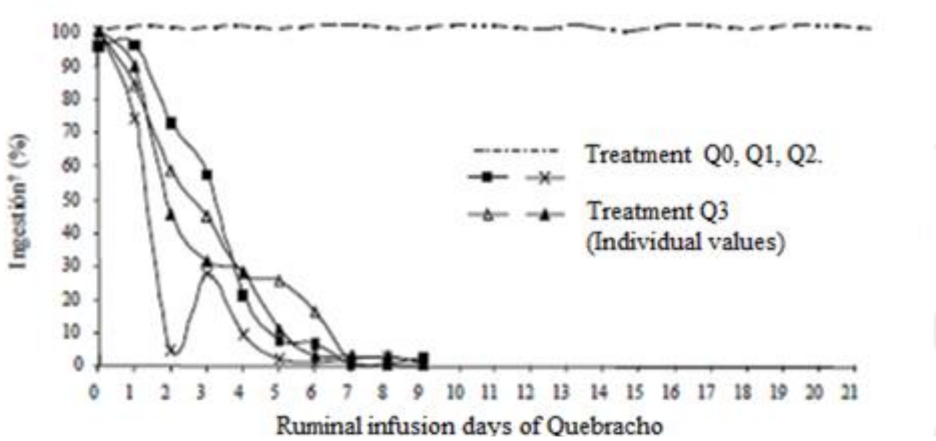
The word tannin refers to a heterogeneous group of polymeric phenolic compounds usually present in plants. The tannins act as an efficient defense mechanism against herbivores (Swain, 1979). The tannins occur commonly in both woody (about 80%) and herbaceous (about 15%) dicotyledonous plant species, and have been a topic of great interest to ecologists studying plant- herbivore interaction. This word was originally used to describe plant extracts used to tan animal leather (Haslam, 1998).

They exist in condensed and hydrolyzable forms. The hydrolyzable tannins are constituted by a nucleus composed by a glucid where its hydrohyl groups are esterified with phenolics acids as well as galic acid. The condensed tannins or proanthocianidins are hydroxyflavonoids polymers (Mcleod, 1974; Haslam 1994). The condensed tannins are more distributed in the nature than the hydrolyzable tannins and they are presented especially in leafs of trees, shrubs and legumes (Van soest, 1994; Aerst et al., 1999). The use of tannin-rich foods in animal production is a matter of interest, since through the years it has been demonstrated that tannins may exert both favorable and detrimental effects, when consumed by ruminants (Mueller-Hervey, 2006). They have negative impacts on intake and production: decreased nutrient utilization, particularly protein (Waghorn et al.,1994**)**, decreased palatability and consequently the amount of food ingested; decreased digestibility (Silanikove et al., 1996); volatile fatty acids production reduction, and decreased digestibility of organic matter and fiber (Ben Salem et al., 1997); damage of kidney and liver (Kumar and Singh., 1984); tissue damage in the rumen, intestine ulceration and morphological changes at the microvilli level (Hervás et al., 2003). Inversely, their positive impact may be involved; the ingestion of small amount of tannins can enhance the production and reproduction variables in terms of milk production, wool production and anthelmintics effect on the animal gut (Hervás, 2001).

The mammalian plant avoidance has been correlated with tannin content for many plant species (Robbins et al., 1987). For instance, Provenza et al. (1990) showed that goats can learn to avoid eating plant tannins in their environment combining the taste of food containing tannins with aversive consequences post ingestive. Additionally, a study with lambs demonstrated that prior experience with tannin- and terpene containing foods influenced diet selection when several toxin-containing foods were offered (Villalba et al., 2004). Many works about the effect of moderate concentrations of tannins (< 50 g/kg DM) condensed or hydrolyzable) and not many which studying high concentrations (> 50 g/kg DM). Therefore, several authors indicate that ingestion of plant species with a high content of condensed tannins (generally above 50 g/ kgDM), reduced significantly the intake of metabolizable energy, due to a reduction in voluntary feed intake and degradation of organic matter (Barry and Duncan, 1984) (Figure 2).

Several mechanisms could explain the negative effect of high contents of tannins on voluntaryintake:

a) A reduction in the palatability of the plant containing the plant secondary compounds (McLeod, 1974).
b) A slowing of the dry matter digestion in the rumen (Waghorn et al., 1994).
c) Development of conditioned aversions induced after postprandial negative effects caused by the plant secondary compounds (Launchbaugh and Provenza, 1994; Provenza, 1995; Launchbaugh and Provenza, 2001).



Q0: 0 g quebracho/kg BW ; Q1: 0.5 g quebracho/ kg Bw ; Q2: 1.5 g quebracho /kg BW; Q3: 3 g quebracho/ kg BW

Figure 2. Effect of quebracho on feed ingestion in function of different doses of condensed tannins (Hervás, 2001).

The mechanism by which animals reduce feed intake containing tannins or other plant secondary compounds that may be toxic, is the identification of negative consequences of post-prandial ingestion and the subsequent development of conditioned aversions (Launchbaugh and Provenza, 1994; Provenza, 1995; Frutos et al., 1998; Launchbaugh et al, 2001). In the same context, Waghorn (1996) reported that the ruminants developed CTA against a feed that conatined a high content of condensed tannins (+60 g/kg DM).
The following tables (Table 2, 3 and 4) summarize the origin, the type of tannins, the animal species and the impact on the animal of tannins (CT: Condensed tannins and HT: Hydrolyzable tannins) (Hervás, 2001).

Table 2. Toxic effect of tannins administration (Hydrolyzable tannins = HT) (Hervás, 2001).

Tannins type	Administration form	Dose (g/KgBW)	Animal species	Clinic effect and animal lesion
HT	peritoneal	0.1	Sheep	Depression, anorexia, hepatic necrosis and nephrosis
HT	oral	0.9	Sheep	Without toxicity symptoms
HT	abomasal	> 1	Sheep	Hepatic necrosis and nephrosis
HT	oral	1	Sheep	Kidney and liver damage, abomasitis and duodenal enteritisis
HT	oral	1.1	Goats	Nephrosis, pancreatic necrosis and dermal necrosis
HT	oral	2-4.6	Sheep	Hepatic necrosis
HT	oral	3.4-8	Sheep	Depression,anorexia, dyspnea, cyanosis
HT	oral	8	Sheep	Increase of heart rate, decrease of ruminal motility and severe hepatic steatosis

Table 3. Impact of condensed tannins (CT) on nutritional value and zoo technical values on sheep (Barry et al, 1999)

% CT (DM)	Category	Observed impacts
< 2	Low	Without impact on voluntary feed intake and prevention of bloat
3-6	Benefit	Increase the production of wool, milk and amino acid absoption
> 7	Adverse	Decrease VFI and wool production, absorption of amino acid

Table 4. The tannins impact on the animal (Hervás, 2001).

Origin	Type of Tannins	Intake (g/kg DM)	Animal specie	Animal Impact
Holclus lanatus	CT	4.2	Sheep	Positive impact on body weight and wool
Tannic acid	HT	20	Sheep	Without impact on voluntary feed intake and production yield
Lotus corniculatus	CT	17	Sheep	Positive impact on ovulation and profilicity
		20		Increase the absorption of essential amino acids
		30		Increase the absorption of methionine and cystine
		34		Increase the milk yield and the average daily gain
		35		Decrease the apparent digestibility of gross protein
		20-40		Increase wool yield
		30-40		Increase the absoption of methionine and cystine
		44.5		Increase milk production
		< 50		Increase Nitrogen retention
Ceratonia siliqua	CT	50	Sheep	Decrease body weight
			Goats	Hard feces, pelleted, covered by mucous and blood
Quercus ilex	CT/HT	50	Sheep	Decrease Nitrogen retention
Quebracho	CT	50	Sheep	Decrease the apparent digestibility of the dry matter and NDF
Lotus pedenculatus	CT	> 50	Sheep	Decrease the voluntary feed intake
	CT	55	Sheep	Decrease the voluntary feed intake and ruminal fermentation rate
Calliandria callotryrsus	CT	60	Sheep	Decrease the number of cellulosic bacteria.
Lotus pedenculatus	CT	76-90	Sheep	Decrease the average daily gain of body weight
	CT	95		Decrease the number of cellulosic bacteria
Climedia hirta	HT	95	Goats	Decrease the voluntary feed intake
Quercus calliprinos	CT	95	Goats	Hard feces, pelleted, covered by mucous and blood
Leucaena	CT	129	Sheep	Decrease the Nitrogen retention
Pistacia lentiscus	CT	205	Goats	Hard feces, pelleted, covered by mucous and blood

CT: Condensed tannins; HT: Hydrolyzable tannins

3. Objectives

The objectives of the current study are highlighted as following:

The first objective was to determine which toxic plants of the Mediterranean area with an emetic effect are able to induce the conditioned taste aversion (CTA) in sheep.

The second objective was to study the commercial product Ellagitan rouge, intended for enological use (preparation based on ellagic and proanthocyanidinic tannin), in order to induce CTA to olive leaf in sheep as an alternative to the use of Lithium Chloride (LiCl).

The third objective was to compare the efficiency of a product rich in tannins, intended for tanning use (extract from *Acacia*) when compared to LiCl, for inducing CTA to olive leaf in sheep.

4. Material and Methods

4.1. Animals and management

The experiment was conducted at the Experimental Farm of the SGCE (Servei de Granges i Camps Experimentals) of the Autonomous University of Barcelona in Spain. The experimental protocol and animal care conditions were approved by the Ethical Committee of Animal and Human Experimentation (CEEAH, reference 2809).

In the experiment 1, it was used 2 Manchega ewes (58.9 ± 1.55 kg BW) that received 5 and 10 g of ivy (*Hedera helix* L.) fruits to each ewe. One of the two ewe was previously received a quantity of 1g of ivy fruits.

In the experiment 2, it was used a total of 12 dairy ewes: 6 Manchega ewes (74.42 ± 1.46 kg BW) and 6 Lacaune ewes (73.17 ± 2.72 kg BW), they were dry and non-pregnant. They received 1.7 ± 0.22 kg DM alfalfa hay/day and the water was freely available. The adaptation period, was conducted during 4 weeks and the distribution of ewes was in function of breed and treatment (Table 5). The ewes that did not received Ellagitan were T0, the ewes that received 1.5 g Ellagitan rouge/kg BW were T1 and the ewes treated with 2 g Ellagitan rouge/kg BW were T2.

Table 5. Distribution of ewes used in the experiment 2 in function of breed and treatment.

Breed	N	Treatment	g Ellagitan/kg BW	g tannins/kg BW
Lacaune	2	T0	0	0
Lacaune	2	T1	1.5	0.4
Lacaune	2	T2	2	0.6
Manchega	2	T0	0	0
Manchega	2	T1	1.5	0.4
Manchega	2	T2	2	0.6

The experiment 3 was conducted by using a total of 9 dairy ewes: 3 Manchega (76.4 ± 81.20 kg BW) and 7 Lacaune (77.8 ± 3.95 kg BW). The ewes were on the beginning of gestation. They were housed in individual boxes and received daily 1.5 ± 0.05 kg DM of alfalfa hay/day and the water was freely available. The adaptation period, was carried out during one week. The ewes were distributed in tree groups, the first group corresponded to the control, the second group corresponded to the tannin treatment and received 0.14 g mimosa extract/kg BW (0.1 g tannin/kg BW) and the third group corresponded to the lithium chloride (LiCl) group and received 225 mg LiCl/kg BW (Table 6).

Table 6. Ewes used in the experiment 3. Treatment with tannins and with LiCl.

Ewes (N)	Treatments	Dose
3	Control	---
3	Tannins	0.1 g tannin/kg BW
3	LiCl	225 mg LiCl/kg BW

During these experiments, the following parameters were measured:

- The ewe's behaviour only in the experiment 1
- Respiratory rate by observing thorax movements
- Environmental temperature by a digital thermometer installed on the farm
- Rectal temperature by using a digital thermometer (MT 1831 Lot 20/00, microlife medical science Asia Ltd. imp. Sanco health care SA)
- Heart rate by using a stethoscope (FC-201 Super Scope, Japan

Alfalfa hay intake was measured periodically by weight difference (quantity offered - quantity rejected).

4.2. Conditioning aversion period

In the experiment 1, the CTA creation was not carried out due to the large quantities of ivy fruits needed to induce the emetic system of the ewes.

In the experiment 2, after 4 weeks of animal adaptation to confinement, aversion conditioning was carried out. Alfalfa hay was removed 2 h before offering individually

100 g of olive leaf (50% dry and 50% fresh) during 15 min. This had been the first time that the ewes had been fed the novel feed.

Immediately after the olive leaf ingestion, the Ellagitan rouge (AEB Ibérica, Castellbisbal, Barcelona, España), was given to the ewes in function of the corresponded dose: T0, T1 and T2 where T0 corresponded to control ewes, T1 corresponded to the ewes that received 1.5 g Ellagitan rouge/kg BW and T2 corresponded to the ewes that received 2 g Ellagitan rouge/kg BW. The Ellagitan rouge used to conduct the experiment is a commercial product for enological use (preparation based on ellagic and proanthocyanidinic tannin), characterized by brown powder color, smell, pH of 4.27, specific weight of 0.4 ± 0.05 and LD50 of 2260 mg/kg (oral, rat). The Ellagitan rouge administration was made using an esophageal tube.

On the next days (2, 3, 4, 6, 8, 11, 12, 27 and 28 d) and (2, 3, 4, 6, 8, 11, 12, 15, 16, 32 and 33d) respectively for Lacaune and Manchega ewes, 100 g of olive leaf was offered during 15 minutes to each animal to validate aversion. Dosage of Ellagitan rouge was repeated for any ewe that consumed more than 30 g of olive leaf.

In the experiment 3, after one week of animal adaptation to confinement, aversion conditioning was carried out. During this period, the ewes received 1.5 ± 0.04 kg alfalfa hay/day. Alfalfa hay was removed 12 h before offering individually 100 g of olive leaf during 1h. This had been the first time that the ewes had been fed olive leaf which is the novel feed.

Treatments consisted of 2 aversion conditioned groups: LiCl and mimosa extract. The LiCl (Panreac, Castellar del Vallés, Barcelona, Spain) and mimosa extract (Proquip S.A., Vilanova del camí, Barcelona, Spain) was orally administered in a water solution using a 100 mL drenching gun (Pimex, Abadiño, Vizcaya, Spain) as indicated by Manuelian et al. (2010). Immediately after the olive leaf ingestion, 0.14 g mimosa extract/kg BW or 225 mg LiCl/kg BW was administer. On the next days (2, 3, 4, 8, 9 and 11 d), 100 g of olive leaf was offered during 20 min to each ewe to validate the aversion. Dosage of tannins was repeated for any ewe that consumed more than 20 g of olive leaf.

Olive leaf and alfalfa hay intake were measured by calculating the difference between the quantity offered and the quantity rejected. After each treatment application, the biological values (heart rate, respiratory rate and body temperature) of each ewe were measured at 2, 4, 6, 8 and 24 h after treatment and the environmental temperature was measured twice a day, on the morning and the evening.

4.3. Chemical analysis

4.3.1. Olive tree leaf and feeds composition

The novel feed was olive tree leaf (50% dry and 50% fresh). The fresh olive leaves were obtained from an abundant olive tree in the forest around the farm of the Veterinary Faculty of Barcelona. The dry olive leaves (cv. Arbequina) were obtained from the cooperative "La Palma d'Ebre" (Tarragona, Spain), representing the most used cultivar in Catalonia. The dry leaves were air-dried and stored in the cold chamber.

The olive tree leaf and alfalfa hay offered to the ewes during the experimental period were sampled daily and preserved in a fridge at 4ºC until its chemical composition 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. The Kjeldahl method (Kjeltec 8400, Foss,Hillerod, Denmark) 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 using the Fiber Analyzer incubator (ANKOM Technology, Macedon, NY, USA). The feed chemical composition of basal diet and olives leaf is summarized Table 7.

Table 7. Olive leaf and alfalfa hay chemical composition (DM basis).

Content %	Olive leaf	Alfalfa hay
Dry matter	82.3	87.5
Crude Fiber	15.9	29.0
NDF	39.6	41.0
ADF	25.2	29.0
ADL	13.2	6.4
Crude Protein	11.2	19.4
Ash	8.5	11.8

4.3.2. Determination of total phenolics

Total phenols (TP) were extracted using methanol 70%. The TP extract was treated with polyvinylpolypyrrolidone as a phenolics-binding agent. Total phenolics (TP) were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965), using tannic acid as standard. Absorbance was read at 760 nm.

4.3.3. Determination of condensed tannins

The Acid Butanol Assay for proanthocyanidins is widely used to determine condensed tannins (CT). Although the assay is simple and gives good indication of the presence of condensed tannins, chemical characteristics of the tannins such as position of the interflavan bond and oxygenation pattern affect color yield significantly. For example, color yield with quebracho tannin is much lower than color yield with procyanidins such as Sorghum tannin because the interflavan bond in quebracho is not readily broken (Hermingway and Karchewy, 1989).

Condensed tannins were analyzed by the acid-butanol assay (Waterman and Mole 1994). An aliquot of the extract was mixed with butanol:HCl (95:5), 2% ferric ammonium sulfate and boiled for 50 minutes in a bath. A control with butanol:water (95:5) was used as a comparison. Absorbance was read at 550 nm and condensed tannins were determined using quebracho and cyanidin as a standard.

The TP and CT contents of the commercial products used for create CTA are summarized in the Table 8.

Table 8. Tannin and phenol content of Ellagitan and Mimosa extract using different standard (TP = Total phenols; CT = Condensed tannins)

	% TP	% TP©	% CTq	% CTc	% CT©
Ellagitan rouge	70	65	27.8	2.8	---
Mimosa extact	75	----	71.4	7.2	72.5

q: quebracho as standard ; c: cianidin as standard; ©: commercial value

4.4. Statistical analysis

The statistical analysis in terms of descriptive statistics, Kruskal-Wallis non parametric ANOVA (the data of the olive leaf intake were not distributed normally) of the olive leaf intake and biological values was made using the Statistical Package for Social Sciences software (SPSS, version 9.2, IBM, Chicago, USA). The repeated factor was experimental days. Animals were a random factor nested within treatments. Significance was declared at $P < 0.05$. When F-ratio was significant, multiple mean comparisons, using the Fisher Least Significant Difference (LSD), were used to test the differences between means.

The mathematical model equation used to study the olive leaf intake was:

$$\mathbf{Y_{ijk} = \mu + T_i + B_j + A_k + e_{ijkl}}$$

Where:

$\mathbf{Y_{ijk}}$: Olive leaf intake or biological value

$\mathbf{\mu}$: Population means

$\mathbf{T_i}$: Fixed effect of treatment

$\mathbf{B_j}$: Fixed effect of breed

$\mathbf{A_k}$: Animal random factor

$\mathbf{e_{ijkl}}$: Experimental error

5. Results and Discussion

5.1. Experiment 1. Toxic plants of the Mediterranean area with emetic effects

After exploring the bibliography, 22 toxic plants with emetic effects were found (Table 24, Annex). Among these 22 species, 7 were possible to be localized in Barcelona province (Table 9). Finally, for their abundance in the area around the veterinary faculty in Bellaterra, only 2 plants were chosen: broom (*Genista tinctoria* L.) and ivy (*Hedera helix* L.*).*

Tabla 9. The emetic effects and characteristics of several toxic plants species

Specie	Commonly name	Spanish name	Characteristics	Effects
Anagyris foetida L.	Bean trefoil	Hediondo	Flowers in autumn and in winter. Contains alkaloids, cystine and malate acid	Leaves are purgative, seeds are emetic
Genista tinctoria L.	Dyer's broom	Genista	Flowers between May and July. Leafs contain luteolina	Purgative and emetic
Hedera helix L.	Ivy	Hiedra	It grows in rocky places. Fruits contain hederine	Hederina is purgative and emetic
Thapsia villosa L.	Villous deadly carrot	Zumillo	Contain resin	Purgative and emetic
Scrophularia aquatica L.	Figworts	Escrofularia	Contain a glycoterpenoid (verbascosaponin A)	Purgative and emetic
Gratiola officinalis L.	Hedgehyssop	Alarda	Contain glucosydes and graciolinagraciosoline	Purgative and emetic
Narcissus pseudonarcissus L.	Narcissus	Narciso	Contain cristalizable alkaloids (narcisine)	Emetic and toxic

Unfortunately, the time of harvesting the broom fruits was past and they had fallen on the ground and could not get them in sufficient quantities. Consequently, it was decided to conduct the trial using ivy fruits. This plant is a species of ivy native from rainforests of western, central and southern Europe, North Africa and Asia, from India to Japan. Besides containing inositol, carotene, formic acid, malic hederotanico, chlorogenic, it is a toxic plant that leads to coma and produced vomiting. The fruits contain hederin which acts as an emetic and purgative also causing the meninges and diarrhea.

During the experiment 1, it was recorded and observed the biological values and the ewes' behavior that received 5 g (previously received 1 g) and 10 g of ivy fruits. According to the trial results in terms of body temperature, respiration rate and behavior of the ewes, it was observed that the doses 1, 5 and 10 g of ivy fruits were not able to generate a discomfort or any alteration to the ewes (Figure 3). Possibly, one of the reasons of no effects of the ivy fruit amounts used in this experiment, was the experience acquired by the ewes during pasturing, ivy fruits possible was familiar around the forest and being habituated to ingest this plant for several times. In order to get emetic effects it was needed to use large quantities of ivy fruits and this possibility was excluded.

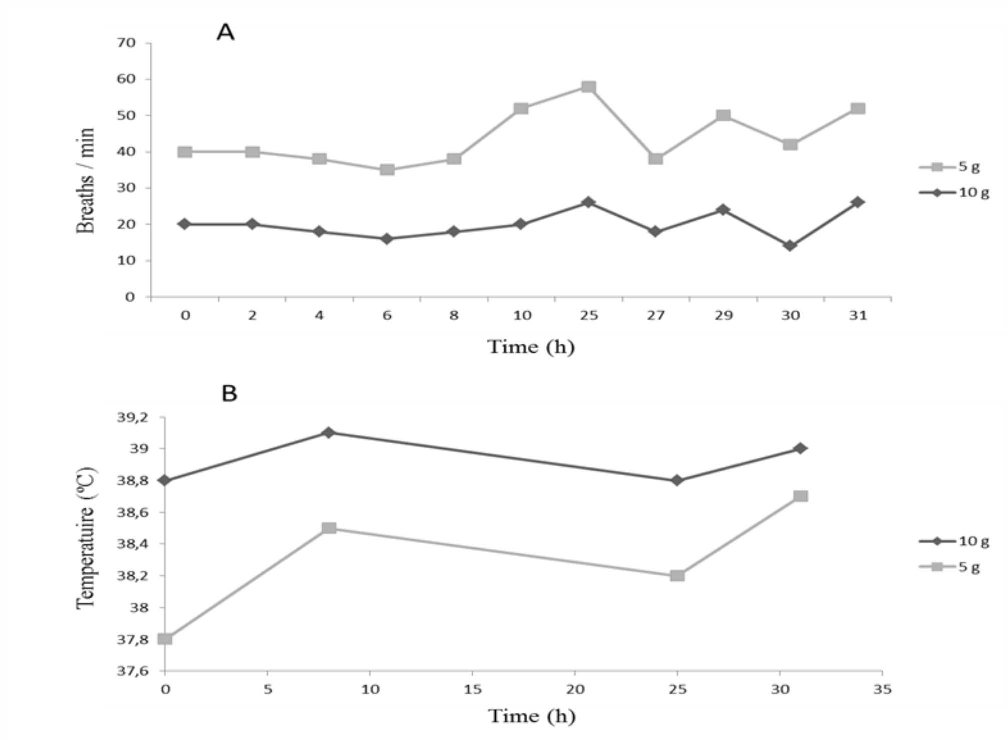


Figure 3. Respiration rate (A) and body temperature (B) of the ewes that consumed 5 g and 10 g of ivy fruits.

5.2. Experiment 2. Conditioned taste aversion to olive leaf using a commercial tannin product for enological use in sheep

5.2.1. Biological values

The respiration rate of Manchega (MN) and Lacaune (LC) ewes used in the experiment 2, showed a variability that oscillated between 15 and 30 breaths/min before and after the treatment application on day 24 (Figure 4). The aberrant value observed on day 28, was explained mainly by the increase of the environmental temperature. The respiratory rate mean was 23.0 ± 0.6 breaths/min and 23.7 ± 0.3 breaths/min for Lacaune and Manchega respectively.

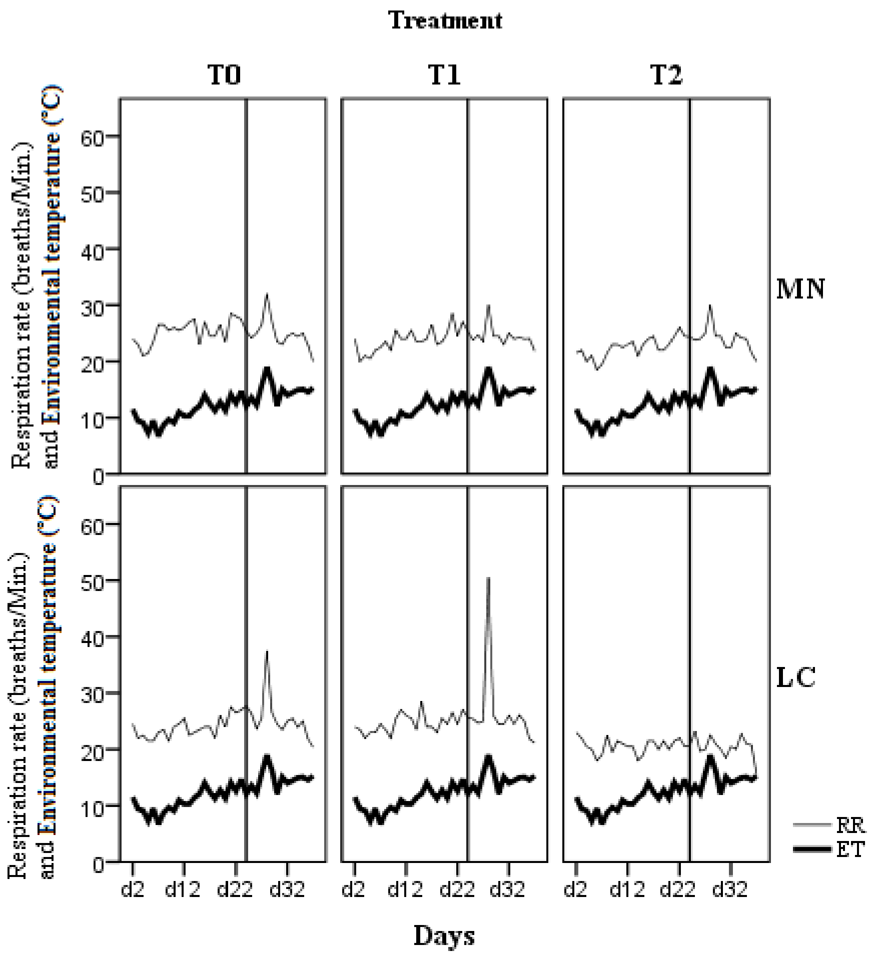


Figure 4. Environmental temperature and respiration rate of ewes treated with Ellagitan rouge (T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW).

The analysis of variance of the ewe's respiration rate showed that the factor breed had not a significant effect on the respiration rate variation ($P > 0.05$). Inversely, the factors treatment, period and the environmental temperature, had a significant effect on the respiration rate variation ($P < 0.05$). Although there was significant difference between the respiration rate of the adaptation period (24.3 ± 0.3) and the experimental period (22.9 ± 0.3) did not affect negatively the health of the treated ewes.

The Fisher LSD test showed that the mean respiration rate and the body temperature of control ewes (T0) were not significantly different to ewes of treatment T1 (1.5 g Ellagitan rouge/kg BW). Nevertheless, ewes T2 (2 g Ellagitan rouge/kg BW) presented respiration rate and body temperature significantly different to ewes T0 and T1 treatment (Table 10).

Table 10. Respiration rate, body temperature and heart rate means by treatment (values are means ± SE; T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW)

Treatment	N	Respiration rate (breaths/min)	Body temperature (ºC)	Heart rate (bpm)
T0	4	25.27 ± 0.44^{b}	38.62 ± 0.02^{b}	75.19 ± 0.84^{b}
T1	4	25.55 ± 0.44^{b}	38.61 ± 0.02^{b}	67.27 ± 0.84^{a}
T2	4	22.29 ± 0.44^{a}	38.37 ± 0.02^{a}	68.03 ± 0.84^{a}

Values with different superscripts differ ($P < 0.05$)

The body temperature values showed a variability of values that oscillated between 38.5 and 40.5ºC before and after the treatments application (day 24). The tannins application seems not influence negatively the body temperature in ewes treated. The mean body temperature was 38.5 ± 0.05 ºC and 38.6 ± 0.07 ºC for Lacaune and Manchega respectively. Figure 5 represents the environmental temperature and the body temperature of Manchega and Lacaune ewes throughout the study.

The analysis of variance of the ewe's body temperature showed that the breed, treatment period and the environmental temperature had a significant effect on the body temperature variation ($P < 0.05$). Although there was significant difference between the body temperature of the adaptation period (38.6 ± 0.02 ºC) and the experimental period (38.5 ± 0.02 ºC) did not affect negatively the health.

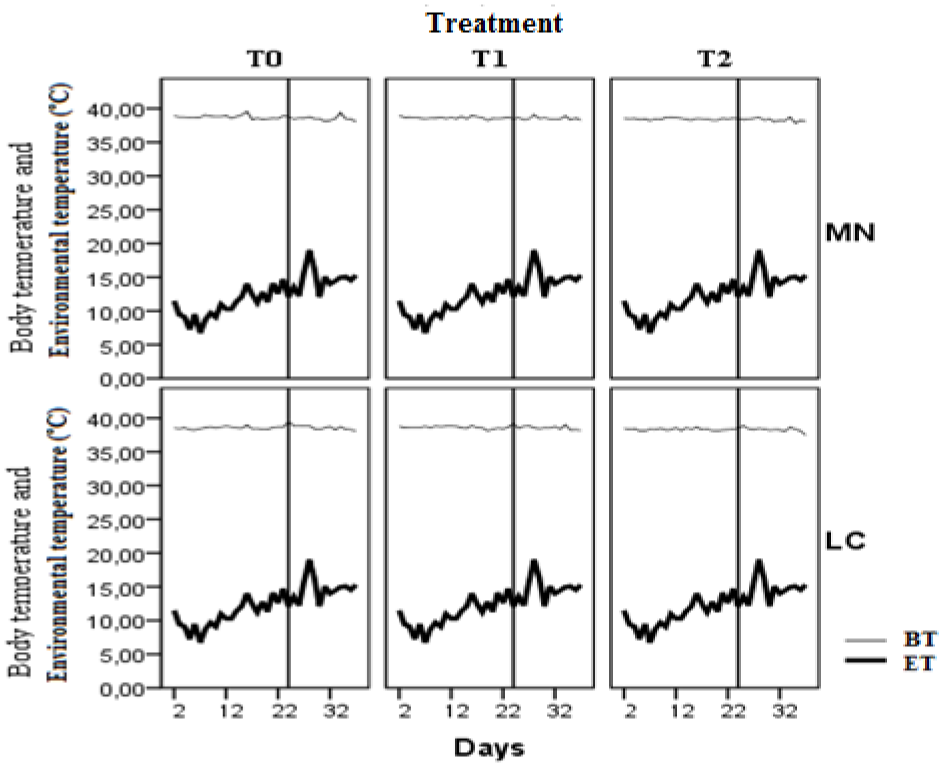


Figure 5. Environmental and body temperature of ewes treated with Ellagitan rouge (T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW).

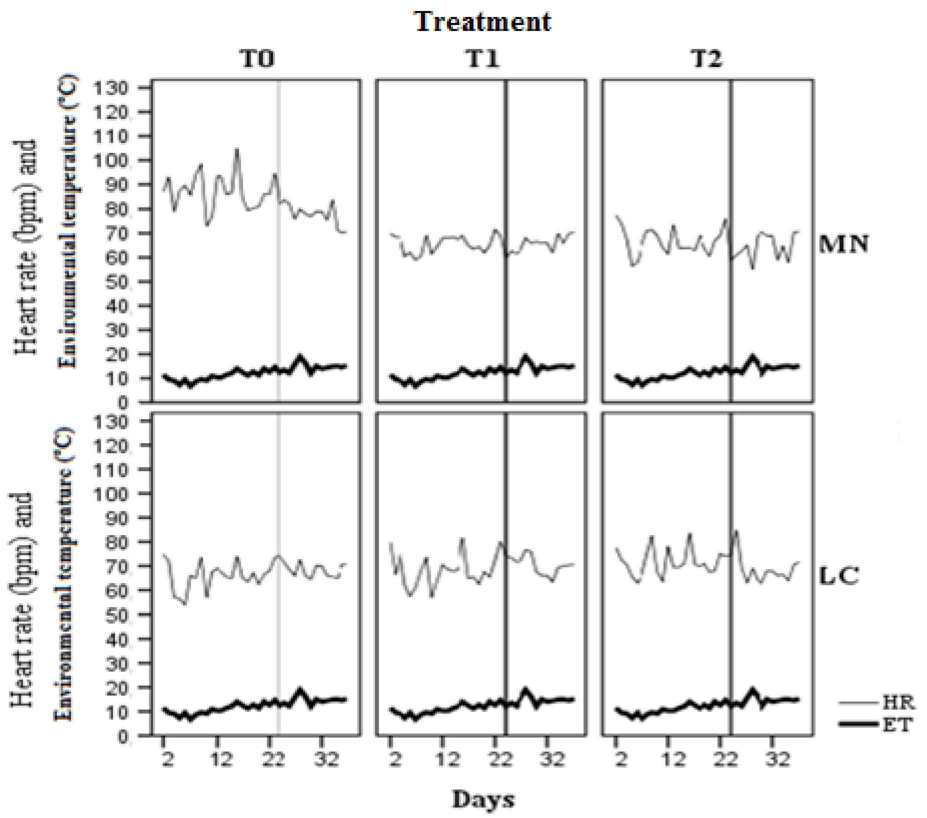


Figure 6. Environmental temperature and heart rate of ewes treated with Ellagitan rouge (T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW).

The heart rate of ewes during the adaptation and experimental period showed a normal variation before and after the tannins administration (55 ± 0.6 to 105 ± 0.6 bpm) which was different to the normal values ranged between 60 to 90 bpm, reported by Meseguer (2006). The analysis of variance of the ewe's heart rate showed that the factor period had not a significant effect on the heart rate variation ($P > 0.05$). Inversely, the breed, treatment and the environmental temperature had a significant effect on the heart rate variation ($P < 0.05$).

The difference between the mean heart rate of the adaptation period (70.7 ± 0.6 bpm) and experimental period (69.3 ± 0.8 bpm) was not significant. However, the mean heart rate of T0 was statistically different to T1 and T2 treatment (Table 10). The possible reason of this contradiction was the highest values of heart rate in two control Lacaune ewes (54 to 105 bpm).

5.2.2. Conditioning aversion learning

The first day of the CTA treatment in Manchega ewes, there was a decrease of 66.3% of olive leaf intake in T1 ewes (0.6 g tannins/kg BW) and an important decrease of 94.0% in T2 ewes (0.6 g tannins/kg BW). On the next days, ewes in both treatments (T1 and T2 group) increased olive leaf consumption, however, difference in intake between T0 and T2 group was maintained until day 33 (Figure 7 and Table 11). The ewes of the treatment T2 were not redosed and only one ewe of the treatment 1 was redosed on day 6.

For the Lacaune ewes, the first day of CTA treatment, it was observed that ewes of T1 and T2 showed an important decrease (100%) of olive leaf intake in relationship with the control ewes (Figure 8). Unfortunately, on the next days, it was observed that the Lacaune ewes treated showed a progressive increase in their intake of olive leaf (Table 11) until the end of the experiment, despite that T1 and T2 Lacaune ewes were reinforced with a new tannin dose on day 8. Probably in Lacaune ewes the tannin dose tested (T1 and T2) was insufficient for averting olive leaf.

Many studies support the existence of an inverse relationship between tannin concentration and feed intake in ruminants (Kumar and Vaithiyanathan, 1990; Launchbaugh and Provenza, 1994; Provenza, 1995; Frutos et al., 1998; Launchbaugh et al, 2001; Hervás, 2001; Villalba et al., 2010). For instance, condensed tannins above

3% may act as feeding deterrent (Provenza, 1995). Our study results were similar to Hervás results (2001) reporting the negative effect of tannins on the feed intake in sheep using a dose of 0.28, 1.14 and 2.28 g condensed tannins/kg BW.

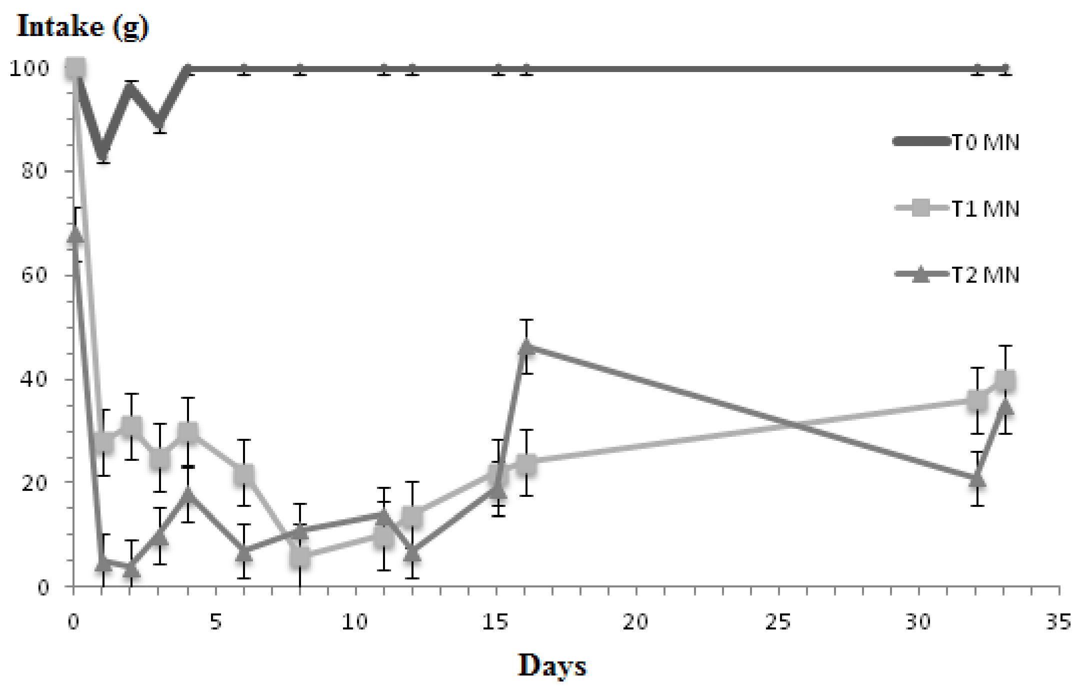


Figure 7. Intake of olive leaves of Manchega ewes treated with Ellagitan rouge according to treatment (T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW) Values are means ± SE.

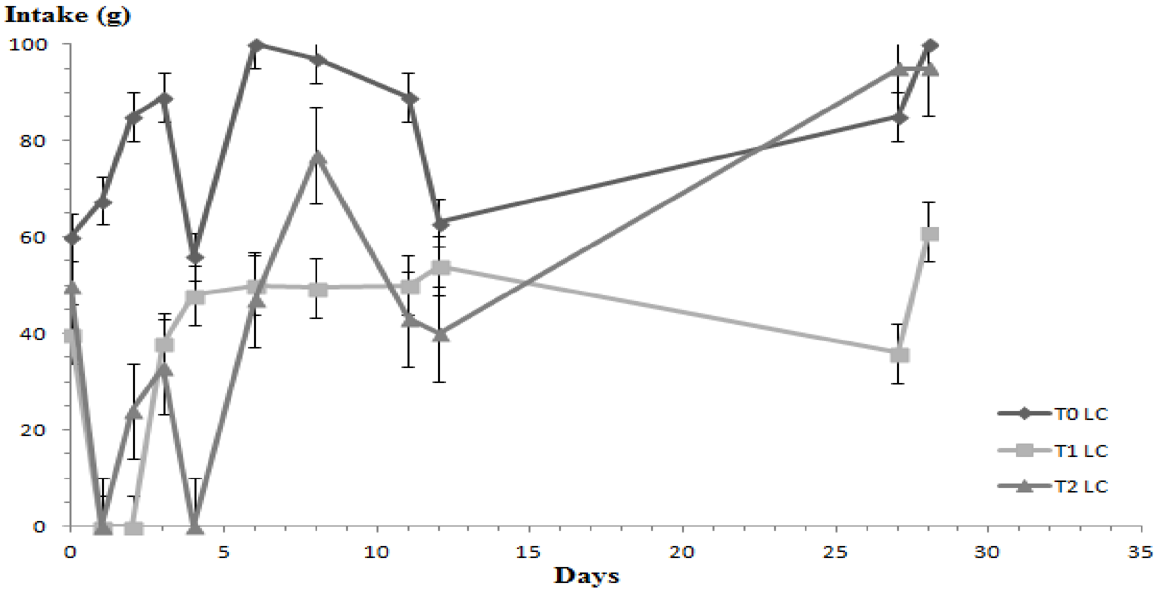


Figure 8. Intake of olive leaf of Lacaune ewes treated with Ellagitan rouge according to treatment (T0, Control; T1, 1.5 g Ellagitan/kg BW; T2, 2 g Ellagitan/kg BW) Values are means ± SE.

On the first day of conditioning aversion period, there was no difference between the olive leaf intakes of the control and the treatments T1 and T2 (Table 11). On the second day corresponded to the following day after the tannins application, the Manchega and Lacaune ewes decreased significantly their intake of olive leaf in relationship with the control. On the next days, the treated ewes decreased their intake of olive leaf after the first tannins dose only for Manchega ewes. Howerver, the Lacaune ewes showed a variability in their intake until the end of the experiment where it was significantly similar to the control intake (Table 11).

Table 11. Olive leaf intake (g) during conditioning period according to breed and treatments (values are means ± SE).

	Manchega			Lacaune		
Day	Control	T1	T2	Control	T1	T2
0	100 ± 0^{ay}	100 ± 0^{a}	68 ± 0^{a}	60 ± 40^{ay}	40 ± 0^{a}	50 ± 30^{a}
1	83 ± 15^{ax}	28 ± 28^{b}	5 ± 14^{b}	68 ± 28^{ax}	0 ± 0^{b}	0 ± 0^{b}
2	96 ± 4^{ax}	31 ± 7^{b}	4 ± 3^{c}	85 ± 9^{ax}	0 ± 0^{b}	24 ± 8^{c}
3	89 ± 3^{ax}	25 ± 1^{b}	10 ± 4^{b}	89 ± 9^{ax}	38 ± 38^{a}	33 ± 33^{a}
4	100 ± 0^{ax}	30 ± 24^{a}	18 ± 10^{b}	56 ± 6^{ay}	48 ± 44^{a}	0 ± 0^{b}
6	100 ± 0^{ax}	22 ± 20^{a}	7 ± 18^{a}	100 ± 0^{ax}	50 ± 50^{a}	47 ± 47^{a}
8	100 ± 0^{ax}	6 ± 6^{b}	11 ± 7^{b}	97 ± 3^{ax}	50 ± 49^{b}	77 ± 13^{b}
12	100 ± 0^{ax}	14 ± 8^{b}	7 ± 0^{b}	63 ± 7^{ay}	54 ± 50^{a}	40 ± 32^{a}
27				85 ± 5^{a}	36 ± 26^{a}	95 ± 1^{a}
28				100 ± 0^{a}	61 ± 39^{a}	95 ± 5^{a}
32	100 ± 0^{a}	36 ± 8^{b}	21 ± 5^{b}			
33	100 ± 0^{a}	40 ± 10^{b}	35 ± 15^{b}			

a,b: within a row in each breed, values with different superscripts differ ($P < 0.05$). x,y: Within a column in each species, values with different superscript differ ($P < 0.05$).

5.3. Experiment 3. Conditioned taste aversion to olive leaf using a commercial tannin product for tanning use in sheep

According to the experiment 2, it was observed that the tannins were able to induce CTA to the olive leaf especially for Manchega ewes. The higher volumes needed implicated its dosage using the esophageal tube, which results non-practical. For this reason, it was decided to search a product with a higher tannins concentration and find the compatible volume with the drenching gun.

5.3.1. Biological values

Figure 9 summarizes the evolution of body temperature of control ewes (C) and ewes treated (T) before and after the tannins application on day 11. The body temperature variation did not differ from the normal body temperature values (38.5 ºC and 40.5 ºC). Consequently, the administration of 0.14 g mimosa extract/kg BW (0.1 g tannin/kg BW) to the ewes did not influence negatively their body temperature. The difference between the mean body temperature of the ewes treated with tannins and the control was not statistically significant (38.53 ± 0.19 ºC and 38.45 ± 0.19 ºC respectively; $P > 0.05$).

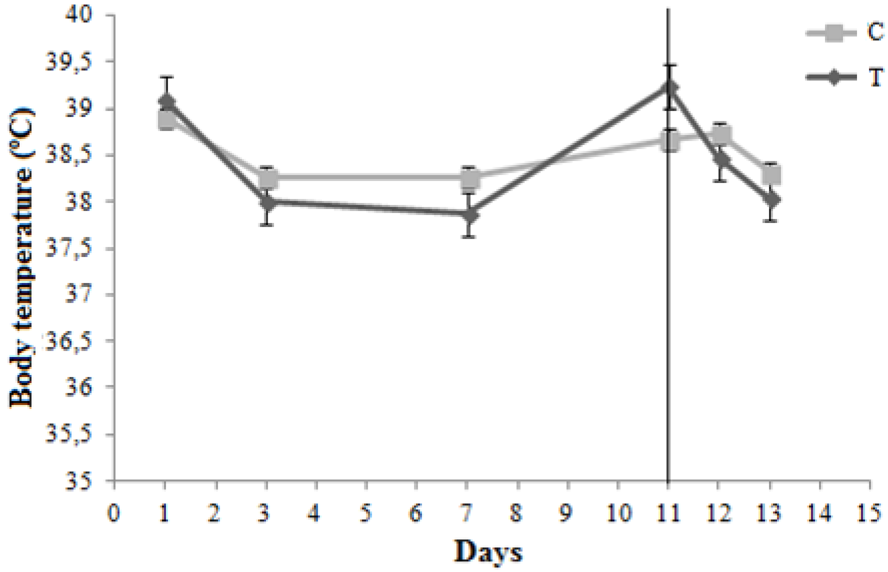


Figure 9. Body temperature of ewes before and after tannin treatment on day 11 according to treatment (C, control; T, 0.1g tannin/kg BW). Values are means ± SE.

Figure 10 summarizes the evolution of ewes' body temperature after the LiCl dose (day 1). It was observed that the administration of 225 mg LiCl/kg BW to the ewes did not influence negatively the body temperature (ranged 38.5 to 40.5 ºC).

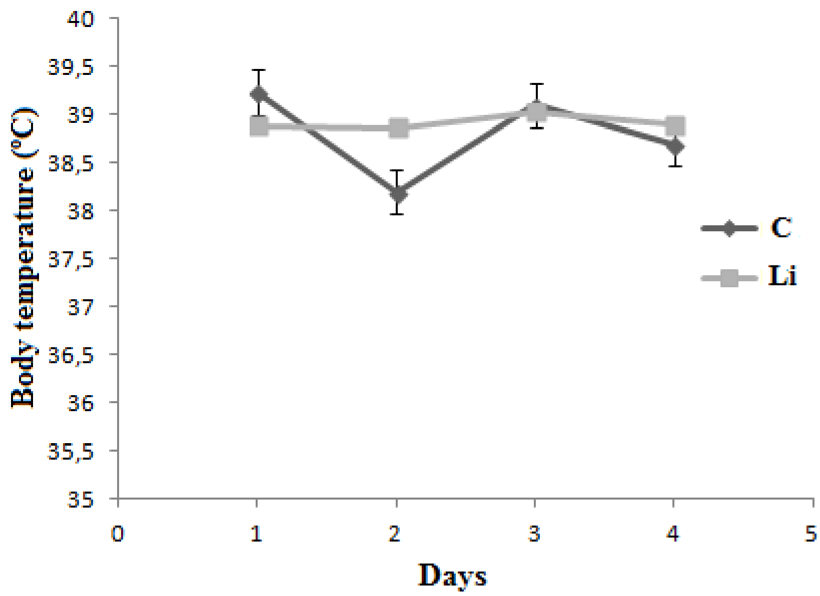


Figure 10. Body temperature of ewes after LiCl treatment on day 1 according to treatment (C, control; Li, 225 mg LiCl/kg BW). Values are means ± SE.

The Fisher LSD test showed that the mean body temperature of the ewes treated with 225 mg LiCl/kg BW (38.9 ± 0.2 ºC) and the control ewes (38.8 ± 0.2 ºC) were not significantly different ($P > 0.05$).

5.3.2. Conditioning aversion learning

During the first day of CTA period, it was observed that the ewes treated with tannins (0.1 g tannin/ kg BW) decreased their intake of olive leaf about 35% (69.8 ± 4.9 g) in relation with control (86.1 ± 2.8 g). Nevertheless, the ewes treated with LiCl (225 mg LiCl/kg BW) refused consume the olive leaf (10.9 ± 10.6 g). In the next days, the ewes treated with tannins increased their olive leaf intake until day 11 where they ingested the 96% of olive leaf offered. Inversely, the ewes treated with LiCl fully rejected consuming olive leaves until the end of the experiment (day 11).

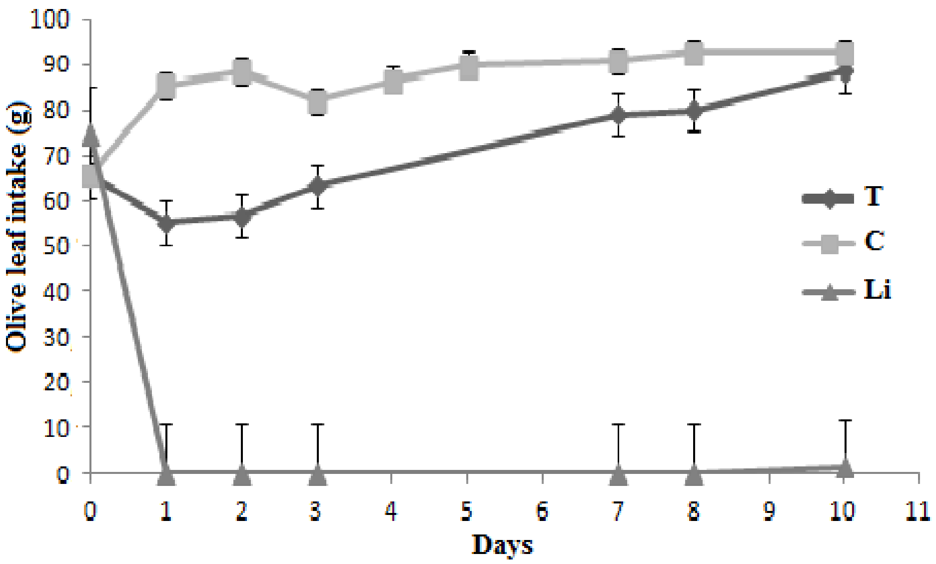


Figure 11. Intake of olive leaves during the aversion conditioned experiment according to treatment (C, control; T, 0.1 g tannin/ kg BW; Li, 225 mg LiCl/kg BW). Values are means ± SE.

The comparison of olive leaves intake means between the treatments showed that the treatment with tannins (69.76 ± 4.85g) and the treatment with LiCl (10.86±10.64 g) were statistically different. Inversely, the treatment control (86.07±2.77 g) and the treatment with tannins were not statistically different (H=15.58; *P*=0.0004) (Table 12). The dose of tannins used was not able to decrease the olive leave intake of the ewes in relationship with the control when the ewes treated with 0.1 g tannins/kg BW ingested 65.1% of the control intake on the beginning of the experiment. Unfortunately, the intake of the ewes treated with tannins was 95.4% of the control intake at the end of the experiment (Table 12).

The olive leaf intake of the ewes that received 225 mg/kg BW of LiCl was 0% of the control intake on the beginning of the experiment and still 0% of the control intake at the end of the experiment. Consequently, the LiCl was more efficient to create conditioned aversion to olive leaf than tannins.

Table 12. Olive leaf intake (g) during conditioning period according to treatments (values are means ± SE).

Day	Control	Tannins	LiCl
1	66.0 ± 17.7[a]	65.3 ± 11.6[a]	74.7 ± 25.3[a]
2	85.3 ± 7.1[a]	55.3 ± 23.1[a]	0.0 ± 0.0[b]
3	88.7 ± 1.8[a]	55.7 ± 20.1[a]	0.0 ± 0.0[b]
4	82.0 ± 6.9[a]	63.3 ± 20.8[a]	0.0 ± 0.0[b]
8	90.7 ± 4.1[a]	79.0 ± 11.4[a]	0.0 ± 0.0[b]
9	92.7 ± 1.7[a]	80.0 ±11.0[a]	0.0 ± 0.0[b]
11	92.7 ± 3.71[a]	88.7 ± 6.6[a]	0.0 ± 0.7[b]

Values with different superscripts differ ($P < 0.05$)

In comparison with the LiCl, the dose of tannins used was not able to establish a persistent and observable CTA to olive leaves in sheep during the experimental period. Manuelian et al. (2014) showed that LiCl was able to establish efficiently the CTA to olive leaves in sheep. In conclusion the dose of tannins used was able to decrease the olive leaves intake but not to establish the conditioned taste aversion to olive leaves in sheep.

5.3.3. The neophobic behaviour

The neophobia degree is expressed as the percentage of olive leaves refusal on the first day before applying the inductor agent (tannins or LiCl). On the first day of the CTA period, it was observed that the Manchega ewes didn't ingest the olive leaves when it was given for the first time. This behavior was explained mainly by the neophobia that the ewes acquired, in order to avoid a possible toxicity caused by the novel feed ingestion. On the contrary, the Lacaune ewes consumed the olive leaves offered when it was given for the first time. Consequently, the Manchega ewes were more neophobic than Launce ewes and needed more contact and time to ingest the novel feed.

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 (Manuelian, 2014). With regard to the olive leaves used in our study, only Manchega breed expressed neophobia on the first days. Intake of olive leaves gradually increased and steadied thereafter indicating the final acceptance of the new feed. Neophobic behaviour was previously reported in crossbred lambs and ewes offered unfamiliar feeds (rolled barley and rabbit pellets) by Thorhallsdottir et al. (1987). Moreover, Villalba et al. (2012) described a similar behaviour using unfamiliar flavours (coconut, cinnamon and onion) in crossbred lambs.

6. Conclusions

The results of this study highlight the following results:

- 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 and the neophobic behavior of sheep depends on the breed.
- Amounts of 10g of Ivy fruit (poisonous plant fruit) was not able to generate any alteration or discomfort to the ewes.
- The Ellagitan rouge at doses of tannins (0.4 and 0.6 g tannin/kg BW) was able to create conditioned taste aversion to olive leaves in sheep (especially for Manchega ewes) but was not practical due to the application difficulties (use of esophageal tube).
- The doses of tannins between 0.4 and 0.6 g tannin/kg BW, did not affect the health parameters of the treated ewes in terms of respiration rate, body temperature and heart rate.
- The lithium chloride was more efficient than tannins to create conditioned taste aversion to olive leaf in sheep.

7. Bibliographic References

Aerts RJ, Barry TN, Mc Nabb WC 1999. Polyphenols and agriculture: Beneficial effects of proanthocianidin in forages. Agriculture, Ecosystems and Environment, 75: 1-12.

Barry TN, Duncan SJ 1984. The role of condensed tannins in the nutritional value of lotus pendunculatus for sheep.1. Voluntary intake. Bri. J. Nutri, 51: 485-491.

Ben Salem H, Nefzaoui A, Ben Salem L, Tisserand JL 1997**.** Intake, digestibility in sheep given fresh or air-dried *Acacia cynophylla* Lindl. foliage. Ann. Zootech., 46: 361-374.

Bruce A, Kimball BA, Nolte DL 2004. Herbivore experience with plant defense compounds influences acquisition of new flavor aversions. Appl. Anim. Behav. Sci., 91: 17–34.

Burritt EA, Provenza, FD, 1989a. Food Aversion Learning: Conditioning Lambs to Avoid a Palatable shrub (*Cercocarpus montanus*). J. Anim. Sci., 67: 650–653.

Burritt EA, Provenza, FD, 1989b. Food Aversion Learning: Ability of Lambs to Distinguish Safe from Harmful Foods. J. Anim. Sci., 67: 1732–1739.

Burritt EA, Provenza, FD, 1991. Ability of lambs to learn with a delay between food ingestion and consequences given meals containing novel and familiar foods. Appl. Anim. Behav. Sci., 32: 179–189.

Conover MR 1995. Behavioral Principles Governing Conditioned Food Aversions Based on Deception, In: Mason, R. (Ed.), National Wildlife Research Center Repellents Conference. Denver, Colorado, 29–40.

Dardou D 2007. L’aversion olfactive potentialisée par le goût au cours du vieillissement chez le rat: étude comportementale et immunocytochimique. PhD Thesis, l’université de Bourgogne, Bourgogne. France.

Domjan M 2000. “The essential of conditioning and learning. Second Edition.

Doran MP, George MR, Harper JH, Ingram RS, Laca EA, Larson S, Mcgourty GT 2009. Vines and ovines: using sheep with a trained aversion to grape leaves for spring vineyard floor management, in: Book of Abstracts of the 60th Annual Meeting of the European Association for Animal Production. Barcelona, Spain, p. 325.

Du Toit, JT, Provenza FD, Nastis A 1991. Conditioned taste aversions: how sick must a ruminant get before it learns about toxicity in foods? Appl. Anim. Behav. Sci., 30: 35-46.

Font Quer P 1983. Plantas Medicinales. Editorial Labor S.A. Barcelona. España.

Freeland WJ and Janzen DH 1974. Strategies in herbivory by mammals: the role of plant secondary compounds. Am. Nat., 108: 269-289.

Frutos P, Duncan AJ, Kyriazaki I and Gorden IJ 1998. Learned aversion towards oxalic acid containing foods by goats: Does rumen adaptation to oxalic acid influence diet selection?. J. Chem. Ecol., 24:383-397.

Garcia J, Kimeldorf D, Koelling RA 1955. Conditioned aversion to saccharin resulting from exposureto gamma radiation. Science, 122: 157-158.

Garcia J, Lasiter PS, Bermùdez-Rattoni F, Deems DA 1985. A general theory of aversions learning. In "Experimental Assessments and Clinical Applications of Conditioned Food Aversions" Ed: M.S. Braveman and P.Bronstein. New York Acdamy of Sciences. New York.

Ginane C, Dumont B 2006. Generalization of conditioned food aversions in grazing sheep and its implications for food categorization. Behav. Processes ,73: 178–86.

Haslam E 1998. Practical polyphenols. From Structure to Molecular Recognition and Physiological Action; Cambridge University Press: Cambridge, UK, 1998.

Haslam E 1994. Complexation and oxydative tranformation of polyphenols. Polyphenols, Palma de Mallorca. España.

Hervas G 2001. Los taninos de quebracho en la nutrición de las ovejas: Efecto sobre la fermentación en el rumen, digestibilidad, toxicidad y utilización como protectores frentes a la degradación ruminal. PhD thesis.Universidad de León. Spain

Hervás G, Frutos P, Giráldez FJ, Mantecón AR, Del Pino MCA 2003. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim. Feed Sci. Technol., 109: 65-78.

Kumar R, Singh M 1984**.** Tannins: Their adverse role in ruminant nutrition. J. Agric. Food Chem., 32: 447-453.

Kumar R, Vathiyanathan S 1990. Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Anim. Feed Sci., 30: 21-38.

Lane MA, Ralphs MH, Olsen JO, Provenza FD, Pfister JA 1990. Conditioned taste aversion: potential for reducing cattle loss to larkspur. J. Range Manag., 43: 127–131.

Launchbaugh KL, and Provenza FD 1994. The effect of flavor concentration and toxin doses on formation and generalization of flavor aversions in lambs. J. Anim. Sc., 72: 10-13.

Launchbaugh KL, Provenza FD 2001. Herbivore response to anti-quality factors in forages. J. R. Mang., 54: 431-440.

Manuelian CL, Albanella E, Rovai M , Salama AAK, Caja G 2014 .Effect of breed and lithium chloride dose on the conditioned aversion to olive tree leaves (Olea europaea L.) of sheep. Appl. Anim. Behav. Sci., 155: 42–48.

Manuelian CL 2014. Conditioned aversion to woody crops in small ruminants. PhD thesis, Facultat de Veterinaria, Universitat Autònoma de Barcelona (Bellaterra, Spain).

Mcleod MN 1974. Plant tannins- their role in forage quality. Nutrition abstract and reviews, 44: 803-812.

Meseguer PJ 2006. Manual de propedéutica y biopatología clínicas veterinarias. Veterinaria, Diagnostico, Zaragoza.

Mirza SN, Provenza FD 1990. Preference of the mother affects selection and avoidance of foods by lambs differing in age. Appl. Anim. Behav. Sci., 28: 255–263.

Mirza SN, Provenza FD, 1994. Socially induced food avoidance in lambs: direct or indirect maternal influence? J. Anim. Sci., 72: 899–902.

Mueller-Hervey I 2006 .Unravelling the conundrum of tannins in animal nutrition and health. J. Sci.Food Agric., 86: 2010-2037.

Pavlov IP 1927. Conditioned reflexes. Oxford university press, Oxford, UK.

Peronny S. 2005. La perception gustative et la consommation des tannins chez le Maki (*Lemur catta*), Musé National d'Histoire Naturelle, Paris, France.

Provenza FD 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. J. Range Manage, 48: 2-17.

Provenza FD 1996. Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. J. Anim. Sci., 74: 2010-2020.

ProvenzaFD, Balph DF 1987. Diet Learning by Domestic Ruminants: Theory, Evidence and Practical Implications Appl. Anim. Behav. Sci., 18: 211-232.

Provenza FD, Balph DF 1988. Development of dietary choice in livestock on rangelands and its implications for management. J. Anim. Sci., 66: 2356–2368.

Provenza FD, Burrit EA, Clausen TP, Bryant JP, Reichrdt BP, Distel RA 1990. Conditioned flavor aversion, A mechanism for goats to avoid condensed tannins in blackbrush. American naturalist, 136: 810-828.

Provenza FD, Lynch JJ, Nolan JV 1993. Temporal contiguity between food ingestion and toxicosis affects the acquisition of food aversions in sheep. Appl. Anim. Behav. Sci., 38: 269-281.

Provenza FD, Lynch JJ, Nolan JV 1994a. Food aversion conditioned in anesthetized sheep. Physiol. Behav., 55:429–432.

Provenza FD, Ortega-Reyes L, Scott CB, Lynch JJ, Burritt EA 1994b. Antiemetic drugs attenuate food aversions in sheep. J. Anim. Sci., 72:1989–94.

Provenza FD, Ralphs MH 1999. Conditioned food aversions: principles and practices, with special reference to social facilitation. Proc. Nutr. Soc., 58:813–20.

Ralphs MH 1992. Continued food aversion: training livestock to avoid eating poisonous plants. J. Range Manag., 45: 46–51.

Ralphs MH, Cheney CD 1993. Influence of cattle age, lithium chloride dose level, and food type in the retention of food aversions. J. Anim. Sci., 71:373–379.

Ralphs MH 1997. Persistence of aversions to larkspur in naive and native cattle. J. Range Manag., 50 :367–370.

Robbins CT, Hanley TA, Hagerman AE, Hjeljord O, Baker DL, Schwartz CC, Mautz WW 1987. Role of tannins in defending plants against ruminants – reduction in protein availability. Ecology, 68: 98–107.

Silanikove N, Gilboa N, Perevolotsky A, Nitsan Z 1996**.** Goats fed tannin-containing leaves do not exhibit toxic syndromes. Small Rum. Res., 21:195-201.

Singleton VL, Rossi JAJr 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic., 16: 144-158.

Swain T 1979. Tannins and lignin. Herbivores, their interaction with secondary plant metabolites. 637-683. New York Academic Press.

Thorhallsdottir AG, Provenza FD, Balph DF 1987. Food Aversion Learning in Lambs with or without a Mother: Discrimination, Novelty and Persistence. Appl. Anim. Behav. Sci., 18:327–340.

Thorhallsdottir AG, Provenza FD, Balph DF 1990a. Ability of lambs to learn about novel foods while observing or participating with social models. Appl. Anim. Behav. Sci., 25: 25-33.

Thorhallsdottir, A.G., Provenza, F.D., Balph, D.F., 1990b. The role of the mother in the intake of harmful foods by lambs. Appl. Anim. Behav. Sci., 25: 35–44.

Vallentine JF 1990. Grazing Management. Academic Press, San Diego, USA.

Van Soest PJ 1994. Nutritional Ecology of the ruminant. Cornel University Press. Ithaca. New York,USA

Villalba JJ, Provenza FD 2000. Roles of novelty, generalization, and postingestive feedback in the recognition of foods by lambs. J. Anim. Sci., 78:3060–3069.

Villalba JJ, Provenza FD, Han GD 2004. Experience influences diet mixing by herbivores: implications for plant biochemical diversity. Oikos., 107:100–109.

Villalba JJ, Provenza FD,Hall JO, Lisonbee LD 2010. Selection of tannins by sheep in response to gastrointestinal nematode infection. J. Anim. Sci., 88: 2189-2198.

Villalba JJ, Catanese F, Provenza FD, Distel RA 2012. Relationships between early experience to dietary diversity, acceptance of novel flavors, and open field behavior in sheep. Physiol. Behav., 105: 181–187.

Waghorn GC, Shelton ID, McNabb WC, McCutcheon SN 1994 . Effects of condensed tannins in *Lotus pedunculatus* on its nutritive value for sheep. 2. Nitrogenous aspects. J. Agric. Sci.,123:109-119.

Waghorn GC 1996. Condensed tannins and nutrient absortion from the small intestine. In: Proceedings of the Canadian Society of Animal Science, 175-194.

Waterman PG, Mole S 1994. Analysis of phenolic plant metabolites. Backwell Publications, Oxford. UK.

Waterman PG, Ross JAM, Mckey DB 1984. Factors affecting some phenolic compounds, and digestibility, and nitrogen content of the mature leaves of *Barteria fistulosa* (Passifloraceae). J. Chem. Ecol., 10: 387-401.

8. Annex

Table 13. Characteristics of toxic plants and its effects on the animal (Font Quer, 1983)

Specie	Taxonomy	Localization	Caracteristics	Effects
Pokeweed	*Phytolacca americana* L.	Spain meadows USA	Perennial herb, bitter leaves and discoid fruits. Flowers in summer and matures in autumn.	Purgative and emetic effects. Toxic in overdose
Marvel of Peru	*Merabilis japala* L.	Mexico	Ornamental specie. Cultivated in summer and collected in autumn. Betaine of nicotinic acid called trigonelline	Purgative effects
Black mustard	*Brassica negra koch* L.	Spain	Collected in summer. Contains between 20 and 30% of fatty oil and glycosides called sinigrinina	Emetic effect
Heartsease	*Viola tricolor* L.	Spain (Pyrenees)	Flowers in lowlands and the end of winter and the beginning of spring	The presence of saponnins has resolved the epidermal problems Purgative and emetic in overdose
Sempervivum	*Sempervivum tecturum* L.	European mountains	Houseleek species Tannins, formic acid Antimicrobial, immunomodulatory and hepatoprotective	Emetic effect
Bean trefoil	*Anagyris foetida* L.	Spain (Catalunya)	Flowers in autumn and in winter Contains alkaloids (anagyrine) cystine, malate acid	The leaves are purgative and the seeds are emetic

Dyers' bugloss	*Alkanna tinctoria tausch* L.	Mediterran-ean region.	Flowers between April and Juny and Cultivated in roads and sand contains alcanina and aucusico acid.	Purgative and emetic effect
Dyer's broom	*Genista tinctoria* L.	Spain	Flowers between May and July. Leaves contain luteolina	Purgative and emetic depends on the part and the dose manipulated
Carqueja	*Genistella trdentada sampoyo* L.	Spain Morocco	The composition is unknowen It is used against the influenza	Purgative
Liquorice	*Glycyrrhiza glabra* L.	Spain	Flowers until July The root contains glicirricina and saponnins It is used againt the liver problems	
Holly	*Ilex aquifolium* L.	Spain	Flowers between April and Juny Contain caffetannic acid dextrosa and wax	The fruits are purgative and the leafs are diuretic and laxative
Ivy	*Hederá hélix* L.	Spain	It grows in rocky places and the fruits contain hedrerine	Hedrerina is purgative and emetic
Deadly carrots	*Thapsia garganica* L.	Spain Portugal	Tapsin resin contains tapsicos acids, isovaleric.	The resin is irritating and purgative
villous deadly carrot	*Tapsia villosa* L.	Spain	Contain resin	Purgative and emetic.
sowbread	*Cyclamen balearicum wilkomm* L.	Spain	Contain culamine and glycosidic saponin with a high haemolytic power	Purgative and emetic
Coris	*Coris mosnpiliensis* L.	Spain	The composition is unknowen	The roots are emetics.

bog bilberry	*Vaccinium uliginosum* L.	Spain	Contain malic acid, benzoic acid	Emetic and the fruits cause headache
Escrofularia	*Scrophularia aquatica* L.	Spain	The composition is litelly unknowen	Purgative and emetic
Hedgehyssop	*Gratiola officinalis* L.	Spain	Contain glucosydes and graciolinagraciosoline	Purgative and emetic
White bryony	*Bryania dioica* L.	Spain	Contain Glucosydes brionine, brionidine	Emetic High doses damges the Adosis alta daña los riñones
Squill bulbs	*Urginea maritima backer* L.	Mediterranean Basin	Onion-like liliaceae the bulbs contain bufadienolides (raticide activity) and cardiac glycosides (medicinal properties as cardiotonics)	Toxic bulb
Narcissus	Narcissus pseudo narcissus L.	Spain	Contain cristalizable alkaloids called narcisine	Emetic and toxic