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Cognition and behavior in sheep repetitively inoculated with aluminum adjuvant-containing vaccines or aluminum adjuvant only

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ABSTRACT

Aluminum (Al)-containing vaccines are common in sheep management and they have been associated with the Autoimmune/inflammatory Syndrome Induced by Adjuvants (ASIA syndrome). The aim of this study was to investigate cognitive and behavioral changes in sheep subjected to a protocol of repetitive inoculation with Al-containing products. Twenty-one lambs were assigned to three groups (n = 7 each): A (Control), B (Adjuvant-only), C (Vaccine). Group C was inoculated with commercial Al-containing vaccines; Group B received the equivalent dose of Al only (Alhydrogel®) and Group A received PBS. Sixteen inoculations were administered within a 349-day period. Ethologic changes were studied in late summer (7 inoculations) and mid-winter (16 inoculations). Animals in groups B and C exhibited behavioral changes: affiliative interactions were significantly reduced and aggressive interactions and stereotypies increased significantly. They also exhibited a significant increase in excitatory behavior and compulsive eating. In general, changes were more pronounced in the Vaccine group than they were in the Adjuvant-only group. Some changes were already significant in summer, after seven inoculations only. This study is the first to describe behavioral changes in sheep after having received repetitive injections of Al-containing products, explaining some of the clinical signs observed in ovine ASIA syndrome.

1. Introduction

Vaccines have contributed significantly to global health [1]. In veterinary medicine, vaccines have contributed to the eradication of infectious diseases such as rinderpest [2] and to the production of quality foodstuffs from animals [3]. Often, vaccines are coupled with an adjuvant which increases the vaccine's efficacy by promoting a fast, long-lasting, and effective immune reaction against the antigen [4]. Aluminum (Al) salts are one of the most widely used vaccine adjuvants because they provide unsurpassed performance and they are inexpensive [5]. In general, vaccines are considered reasonably safe [6], which has led to the perception that the benefits of immunization greatly exceed the risks, even if adverse effects occur occasionally [7]. However, the use of Al as an adjuvant has raised controversy in recent years: some researchers have maintained that, with the exception of some local acute reactions, Al poses no health risk [8,9], but others have reported a variety of adverse effects, and have called for further research on the subject [10,11].

In veterinary medicine, especially for farm animals, vaccination campaigns often are implemented to control emerging or re-emerging infectious diseases [12]. Between 2007 and 2010, several vaccination campaigns against bluetongue (BT) virus were undertaken in Europe [13]. BT is a viral, non-zoonotic, insect-borne, systemic disease of ruminants, which causes serious sanitary and economic problems, and is especially pernicious in sheep [14]. Historically, vaccination has been the most effective way of controlling BT [14]. Until the last decade, BT vaccines contained live, attenuated virus which could cause deleterious effects such as fetal malformations or even the re-emergence of BT

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Table 1
Commercial vaccines used in the experiment to inoculate lambs in Group C.Aluminum content was measured by inductively coupled plasma atomic emission spectrometry and is expressed as per dose.

Vaccine	Commercial name	Manufacturer	Antigen/s	dpi*	mg of Al#/dose
1	Heptavac P Plus	MSD Animal Health S.L.	*Pasteurella multocida, Mannheimia haemolytica, Clostridium spp.*	0, 23, 233	7.5
2	Autogenous vaccine	Exopol	*Staphylococcus aureus* spp. *anaerobius*	44, 69	1.644
3	Vanguard R	Zoetis	Rabies virus	98	1.025
4	Agalaxipra	Hipra	*Mycoplasma agalactiae*	129, 146	6.764
5	Ovivac CS	Hipra	*Chlamydophila abortus, Salmonella abortus ovis*	209, 233	5.6
6	Autogenous vaccine	Exopol	*Corynebacterium pseudotuberculosis*	254, 272	1.32
7	Bluevac-1	CZ Veterinaria S.A.	Bluetonge virus serotype 1	293, 329	4.18
8	Bluevac-4	CZ Veterinaria S.A.	Bluetongue virus serotype 4	293, 329	4.16

*****: dpi: days post first inoculation.
#: Al: Aluminum.

[15,16]. Since 2007, vaccines used in Europe have contained inactivated virus and Al hydroxide as an adjuvant [17,18]. By 2008, in Spain, several serotypes were circulating concurrently and, typically, two serotypes predominated in a given geographical area [19]. In response, throughout the country, a dual vaccination program was implemented that included two vaccines against the two serotypes circulating in each area. Animals received four vaccine doses within about a month, which culminated in an inoculation of 16 mg of Al per animal. After those vaccinations, a previously unreported syndrome was identified [20,21], which was characterized by an acute phase that included severe neurological symptoms with lesions of sterile meningoencephalitis, and a chronic phase that involved extreme weight loss and neurodegenerative lesions in the spinal cord. The chronic phase correlated with low environmental temperatures. All known ovine diseases were ruled out as a cause of the syndrome, and it was concluded that the process was associated with the use of Al-adjuvant containing vaccines, and has been included under the umbrella of the Autoimmune/inflammatory Syndrome Induced by Adjuvants (ASIA syndrome) [21,22]. In the chronic phase, sheep exhibited a variety of behavioral changes including periods of depression (i. e., lack of response to stimuli, stupor) and lethargy intermixed with periods of excitement, restlessness, polyphagia, and increased aggressiveness, especially compulsive wool biting between animals. Some of those behavioral changes were reproduced in a small cohort of repeatedly vaccinated lambs, but they were not fully characterized [21]. Similar behavioral changes have been observed in experimental laboratory animals that were inoculated with Al adjuvants [23].

Several validated tests have been used to assess behavioral and cognitive changes in sheep. Home pen observations have been used to quantify social and individual behaviors [24–27], whereas T-maze test, Open Field Test (OFT), and Novel Object Test (NOT) have been used to evaluate specific aspects of cognition. The T-maze test has been validated as a means of studying various aspects of spatial learning and working memory in sheep [28–31], OFT has been used to assess the response of an animal to a novel environment and isolation from the flock mates [28,32,33], and NOT has been applied to quantify the fear reactions of sheep [34,35]. The aim of this study was to investigate the behavioral and cognitive changes in sheep repeatedly inoculated with Al adjuvant-containing vaccines or Al adjuvant only.

2. Materials and methods

2.1. Experimental design

All experimental procedures were approved and licensed by the Ethical Committee of the University of Zaragoza (ref: PI15/14). Requirements of the Spanish Policy for Animal Protection (RD53/2013) and the European Union Directive 2010/63 on protection of experimental animals were always fulfilled. The study was carried out at the experimental research farm of the University of Zaragoza. Twenty-one unvaccinated, neutered, male purebred Rasa Aragonesa lambs were selected at 3 months of age from a pedigree flock of certified good health and assigned to one of three groups of seven animals each. Group A (Control) was inoculated with PBS, Group B (Adjuvant-only) was inoculated with the adjuvant only, and Group C (Vaccine) was inoculated with commercial vaccines that contained Al as adjuvant. The experiment ran from Feb 2015 to Feb 2016 (349 days), which included a full summer and a winter period.

2.2. Management of the animals

Housing and management conditions and diet were identical for all animals. The three groups were isolated from each other, but they occupied contiguous, identical pens within the same building. Pens were rectangular closed rooms, 7 × 3.5 x 6 m (length x width x height), open to the exterior of the building at one of the short walls through four closable windows. The daily ration was 1 kg of concentrate per animal, and straw and water *ad libitum*. Concentrate (Agroveco®, Zaragoza, Spain) contained 15.7% crude protein, 3.9% crude fat, 10.4% crude fiber, 9.5% crude ash, 0.22% sodium, and adequate proportions of mineral and vitamin supplements for growing lambs. Each day at 0830 h, concentrate was offered from a 2-m-long hopper that had two openings, which permitted all animals in a group to eat simultaneously. Minimum, maximum, and average ambient daily temperatures in the area were obtained from the Spanish Agency of Meteorology (AEMET). The average daily temperature was 25.5 °C in summer and 9.0 °C in winter.

2.3. Vaccination protocol

Lambs underwent an accelerated vaccination schedule, designed to inoculate -within an acceptable experimental time frame- an Al load equivalent to that a sheep under local management field conditions usually receives along 6–7 years. Animals in Group C (Vaccine) were inoculated with commercial vaccines against main ovine diseases (Table 1, Fig. 1), receiving 16 vaccine doses within 12 months and a total of 70.861 mg of Al. The recommended application procedures for each product were always followed. Animals in Group B (Adjuvant-only) were inoculated with Al-hydroxide only (Alhydrogel®, CZ Veterinaria, Spain), at concentrations that were identical to those of the corresponding commercial vaccine, measured by inductively coupled plasma atomic emission spectrometry (Table 1). Group A was inoculated with an identical volume of PBS. All inoculations were performed in the subcutaneous tissue of the area encompassing scapula and ribs, using individual, sterile disposable syringe and needle for each injection.

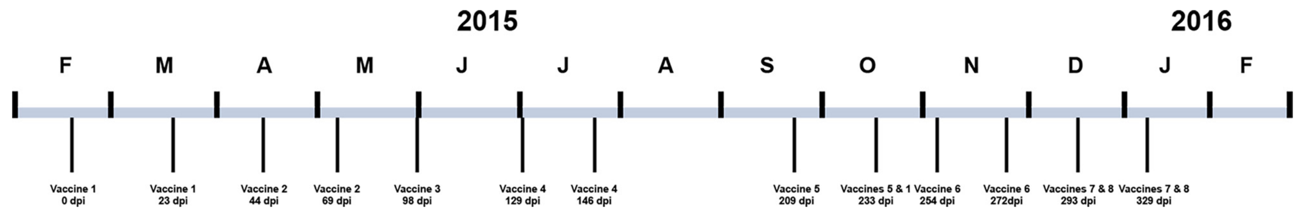

Fig. 1. Vaccination schedule. Each bar represents an inoculation date. In total, 16 inoculations were administered on one of 13 days. dpi: days post first inoculation.

2.4. Cognitive and behavioral assessments

Animals were subjected to two rounds of each of the tests described below. The first round began at 196 days post first inoculation (dpi), in September 2015 (late summer). The second round began at 336 dpi, in January 2016 (mid-winter; Supplementary table S1). In the summer round, all animals had been inoculated 7 times and, in the winter round, the animals had been inoculated 16 times.

2.4.1. Cognitive test: T-maze

Each animal was subjected to the T-maze test on two consecutive days in summer and in winter rounds. The order in which lambs were tested alternated among the three groups, and the order was maintained in all tests. Tests were performed in a T-maze built of 1.4-m-high plastic panels, which had been validated for use with lambs [28]. A mirror clue and a loudspeaker were placed at floor level in the target zone in the left arm of the maze, which served as a social and a sound clue, respectively. Each animal was given up to 5 min to solve the maze. Video recordings of each test were blindly evaluated by a trained researcher (MPA) and latency, time taken to reach the target zone, time spent solving the maze, and the number of areas traversed was obtained.

2.4.2. Cognitive test: Open Field Test (OFT) and Novel Object Test (NOT)

Each animal was subjected to the OFT once in each of the two rounds. The order in which lambs were tested alternated among the three groups, and the order was maintained in all tests. The 4 x 4 m test arena built with 1.40 m high plastic panels, served as a novel environment for the animal that was completely isolated from other lambs. Water and food were offered from a bucket. Each animal was left in the test arena for 5 min, which was recorded with a videotape and a microphone. From the recordings, the observer (MPA) blindly calculated time the animal spent walking, exploring, standing, and trying to escape, among others parameters.

The NOT was performed in the same test arena. A blue plastic ball was lowered twice from the ceiling at the center of the arena and left there for 1 min. The observer recorded the distance between the lamb and the ball after 30 s, each time.

2.4.3. Behavioral tests: home pen individual and social behavior observations

All lambs were individually identified by numbers painted on their sides and rump. To record social and individual behaviors, a camera was placed at the top of each pen, and a videorecorder (Circontrol S.A., Spain) was set up in a room adjacent to the pens. In each of the two rounds, recordings were made for 12 h/day (0800 h – 2000 h) on seven consecutive days. After the experiment had concluded, a trained researcher (MPA) analyzed the videos consecutively in a blind manner.

The video data were quantified in two ways: Instantaneous sampling for individual behaviors, and continuous sampling for social behaviors. Instantaneous sampling involved quantifying 1 min from each 10 min of video, totaled 504 min per group and 1512 min per round. Continuous sampling involved quantifying three 2-h periods per day (0800 h – 1000 h, 1200 h – 1400 h and 1600 h – 1800 h) which totaled 42 h of observations per group and 126 h per round. The analysis of both individual and social behaviors involved documenting the number of events in which an animal displayed a specific behavior. Individual behaviors included feeding on concentrate, eating straw, resting, standing, and drinking. Social behaviors were assigned to one of three categories: i) affiliative interactions, ii) agonistic (aggressive) interactions and iii) stereotypies. Alternative text: A description of the individual and social behaviors evaluated is detailed in Supplementary Tables S2 and S3.

2.5. Hematology panel and welfare indicators

A standardized hematology panel was performed, and welfare and stress indicators including cortisol, creatine kinase (CK), lactate, glucose, non-esterified fatty acids (NEFA) and neutrophil/lymphocyte (N/L) ratio [36,24,37–39] were measured on the day after the recording period in each of the two rounds, away from recent handling of the animals (Supplementary Table S1). Blood samples were taken at 0800 h by jugular venipuncture, which required < 1 min per lamb. The leukocyte formula was estimated from blood Diff-Quick stained smears. The N/L ratio was calculated based on 100 leukocytes per sample. Cortisol concentration (nmol/L) in plasma was measured by enzyme immunoassay [40].

2.6. Statistical analysis

Comparisons were performed as follows: T-maze test: Unpaired comparisons between groups for each of the two days of the test in each round, and paired comparisons within groups between the two days of the test in each round. OFT: Unpaired comparisons between groups within each round of tests and paired comparisons within groups between the two rounds. NOT: Unpaired comparisons between groups for each of the two distances to the novel object in each of the rounds, and, within each group, paired comparisons between the two distances in each round. Behavior: Unpaired comparisons between groups within each round of tests and paired comparisons within groups between the two rounds, which were restricted to social behaviors because of seasonal influences on individual behaviors due to normal physiological seasonal variations (e.g., all groups drank more water in summer and ate more straw in winter). For most of the parameters in the hematology panel and the welfare indicators, unpaired comparisons between groups were performed for each round, and within-group paired comparisons between rounds were performed for cortisol, only.

A Shapiro-Wilk´s Test was used to confirm whether the data of the quantitative variables met the assumption of normality. A parametric test was used if the variable met the assumption, and a non-parametric test was used if it did not. Unpaired comparisons were performed using an ANOVA (A) Test (or Welch's *t*-test (We) if variances were not homogeneous based on a Levene's test) and a *post hoc* Duncan's test (parametric tests), or a Kruskal-Wallis (KW) test and a *post hoc* Dunn's test (non-parametric test). Paired comparisons were performed using Student's t (t) test for dependent samples (parametric test) or a Wilcoxon (W) test (non-parametric test). General lineal models (GLM) were developed to assess the influence of "Group" and "Round", and their paired interactions on the behavioral tests. All statistical analyses were performed using IBM SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). The alpha and beta levels were set at 0.050 and 0.200, respectively.

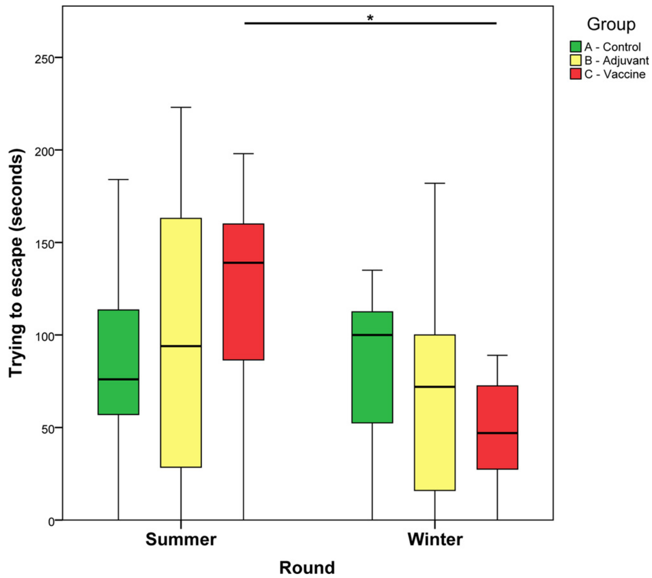

Fig. 2. Open Field Test (OFT). Trying to escape. Lambs in Group C (Vaccine, red) spent less time trying to escape the test arena in the winter round of tests (*p_t = 0.003). Statistical analysis was performed using Student's t-test for dependent samples. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are presented in Supplementary Table S14.

3. Results

3.1. Cognitive tests

3.1.1. T-maze test

There were no significant differences between groups in any of the two rounds (summer or winter). Within groups, there were no significant differences between the two consecutive days of testing in summer. In winter, however, Group C left the first area (latency) significantly (p_W = 0.027) earlier on the second day of testing (Supplementary Tables S4 – S11).

3.1.2. OFT and NOT

In neither summer nor winter did the three groups differ significantly between them in either of the two tests. In the OFT in winter, Group B lambs spent significantly (p_W = 0.043) more time exploring than they did in summer and Group C lambs spent significantly (p_t = 0.003) less time trying to escape (Fig. 2). In the NOT, in winter, Group B lambs were significantly (p_W = 0.043) farther from the novel object in the second exposure than they were in the first exposure (Supplementary Tables S12 – S19).

3.2. Behavioral tests

3.2.1. Individual behavior

In summer, Group C ate straw significantly (p_{KW} = 0.018) less frequently than did Group A lambs. Lambs in groups C and B spent significantly (p_A < 0.001) more time standing than did lambs in Group A. In winter, Group C and Group B lambs ate concentrate significantly (p_{KW} < 0.001) fewer times than did Group A lambs. Lambs in Group B rested less often (p_{KW} = 0.027) than did lambs in Group A and lambs in Group C and Group B stood significantly more often (p_A = 0.001) than did lambs in Group A (Fig. 3). The GLM indicated that the interaction effect between "Group" and "Round" was significant (p = 0.035) for standing (Supplementary Tables S20 - S25).

3.2.2. Social behavior

In summer, the level of affiliative interactions did not differ among groups (Fig. 4, Supplementary Table S26) but lambs in groups B and C exhibited significantly (p_{KW} < 0.001) more aggressive interactions (Fig. 5, Supplementary Table S27) and stereotypies (Fig. 6, Supplementary Table S28) than did lambs in Group A, and Group C lambs exhibited significantly (p_{KW} < 0.001) more stereotypies than did Group B lambs. In winter, Group C lambs exhibited significantly (p_{KW} < 0.001) fewer affiliations than did Group B lambs (Fig. 4, Supplementary Table S26). Furthermore, in winter, lambs in groups B and C showed significantly (p_{KW} < 0.001) fewer affiliations (Fig. 4, Supplementary Table S26), more aggressive interactions (Fig. 5, Supplementary Table S27) and stereotypies (Fig. 6, Supplementary Table S28) than did lambs in Group A.

In groups B and C, the frequency of affiliations was significantly lower (Group B: p_W = 0.045; Group C: p_W < 0.001; Fig. 4, Supplementary Table S26) and the frequency of aggressive interactions was significantly higher (Group B: p_W = 0.018; Group C: p_W = 0.003; Fig. 5, Supplementary Table S27) in winter than it was in summer. In Group C (p_W = 0.002) lambs exhibited significantly more stereotypies in winter than they did in summer (Fig. 6, Supplementary Table S28). In Group A, those three types of behaviors did not differ significantly between summer and winter (Figs. 4–6). The GLM indicated that the interaction effect between "Group" and "Round" was significant for affiliative interactions (p = 0.002) and aggressive interactions (p = 0.024) (Supplementary Table S29). By the end of the winter round (February 2016), animals in Group C exhibited wool biting, and five of seven lambs had multifocal areas of wool loss and depilation, normally in the rumps and withers (Supplementary Fig S1).

3.3. Hematology panel and welfare indicators

In summer, the hematology panels of the three groups did not differ significantly. In winter, the white blood cells (WBC; p_A = 0.047) and eosinophil number (p_{KW} = 0.016) were higher in Group C than they were in the other two groups. In summer, cortisol levels did not differ significantly between groups, however, in winter, cortisol levels were significantly higher in groups B and C than they were in Group A (p_A = 0.005). Furthermore, in Group A, but not in groups B and C, cortisol levels were significantly (p_t = 0.002) lower in winter than they were in summer (Fig. 7) (Supplementary Tables S30 - S31).

4. Discussion

This is the first scientific study to investigate cognitive and behavioral changes in sheep after inoculation with Al-containing products. Following compulsory vaccination campaigns against BT in the last decade, several behavioral changes were observed [20,21], which prompted research into the putative relationship between subcutaneously injected Al-containing vaccines and those changes. Here, we have used an experimental model to demonstrate that sheep repetitively inoculated with vaccines containing Al hydroxide or with Al hydroxide only exhibit behavioral changes similar to those reported previously for the ovine ASIA syndrome.

This experiment tries to reproduce in a short period of time, the clinical effects of successive inoculation of vaccines. Several vaccinations are administered annually to most flocks as preventative measures against a variety of infectious diseases, which sometimes involves the concurrent use of more than one vaccine [41]. Furthermore, most vaccinations are administered to sheep of all ages, e. g., an individual receives vaccine doses throughout its productive life. Sheep might receive additional vaccines if, for instance, there is an outbreak of a mandatory reportable infectious disease such as the BT outbreak in Europe [19]. In some flocks, sheep can receive a mean of 2–4 vaccines per year and a range of 14–28 vaccines in a 7 years life-span. Most sheep vaccines contain about 2 mg Al/ml, the normal dose is 2 ml, and

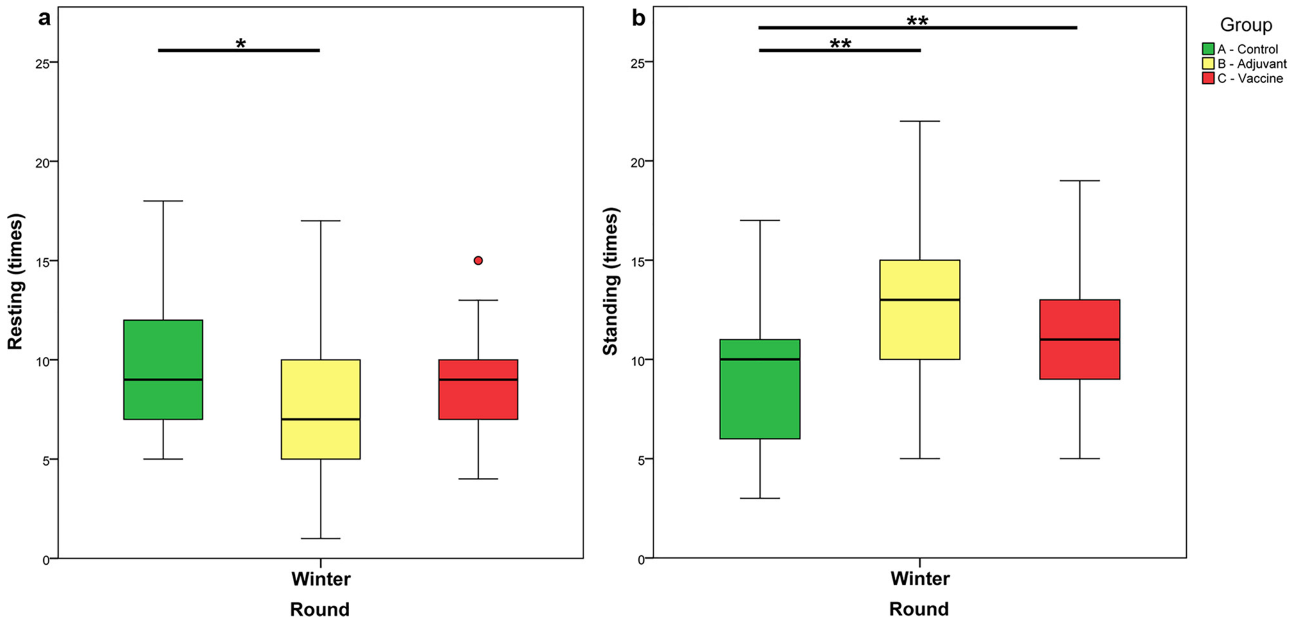

Fig. 3. Individual behavior: Resting (a) and standing (b) in the winter round of tests.
Group B (Adjuvant-only, yellow) lambs spent less time resting (*p_{KW} = 0.027) than Group A (Control, green) lambs. Group B (Adjuvant-only, yellow) lambs and Group C (Vaccine, red) lambs spent more time standing (**p_A < 0.001) than Group A (Control, green) lambs. Statistical comparisons were based on Kruskal-Wallis (KW) and *post hoc* Dunn's test for resting, and an ANOVA (A) test and *post hoc* Duncan's test for standing. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are presented in Supplementary Tables S22-S23.

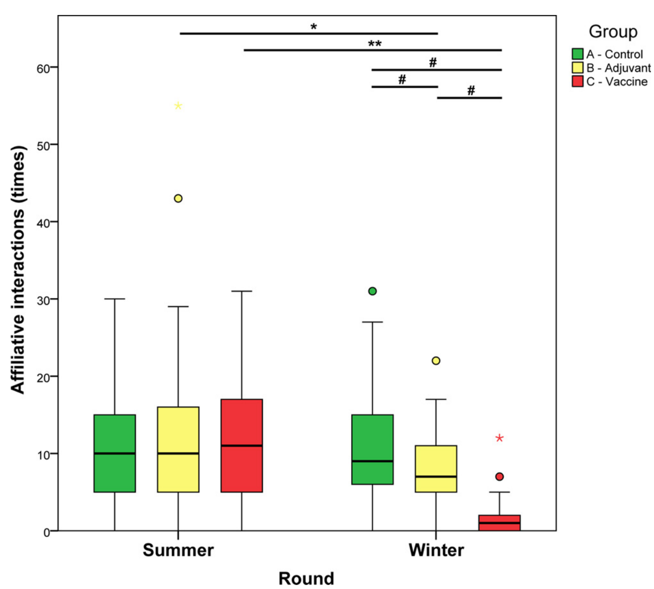

Fig. 4. Social behavior: Affiliative interactions. Group B (Adjuvant-only, yellow) performs less affiliative interactions in winter (*p_W = 0.045). Group C (Vaccine, red) performs less affiliative interactions in winter (**p_W < 0.001). Groups B (Adjuvant-only, yellow) and C (Vaccine, red) perform less affiliative interactions than Group A (Control, green) in the winter round ($^{\#}p_{KW}$ < 0.001). In addition, in the winter round, Group C (Vaccine) performs less affiliative interactions than Group B (Adjuvant-only, yellow) ($^{\#}p_{KW}$ < 0.001). Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn's test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Supplementary Table S26.

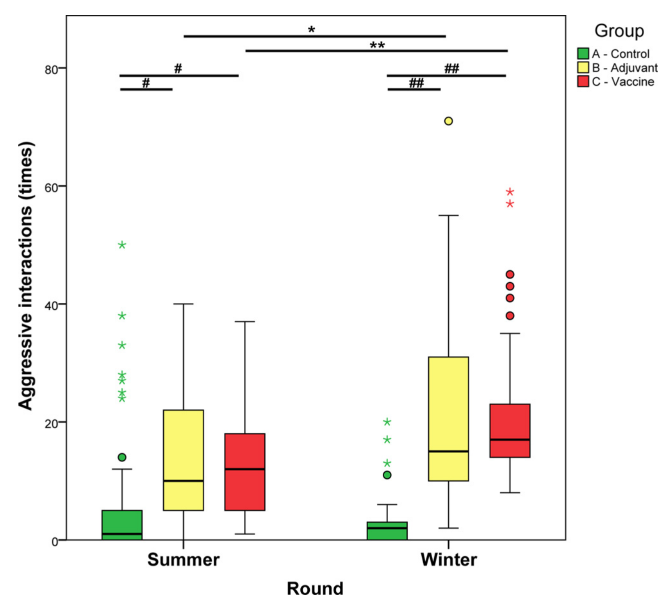

Fig. 5. Social behavior: Agonistic (aggressive) interactions. Group B (Adjuvant-only, yellow) performs more aggressive interactions in winter (*p_W = 0.018). Group C (Vaccine, red) performs more aggressive interactions in winter (**p_W = 0.003). Groups B (Adjuvant-only, yellow) and C (Vaccine, red) perform more aggressive interactions when comparing with Group A (control, green) both in the summer ($^{\#}p_{KW}$ < 0.001) and in the winter ($^{\#\#}p_{KW}$ < 0.001) rounds. Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn´s test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Supplementary Table S27.

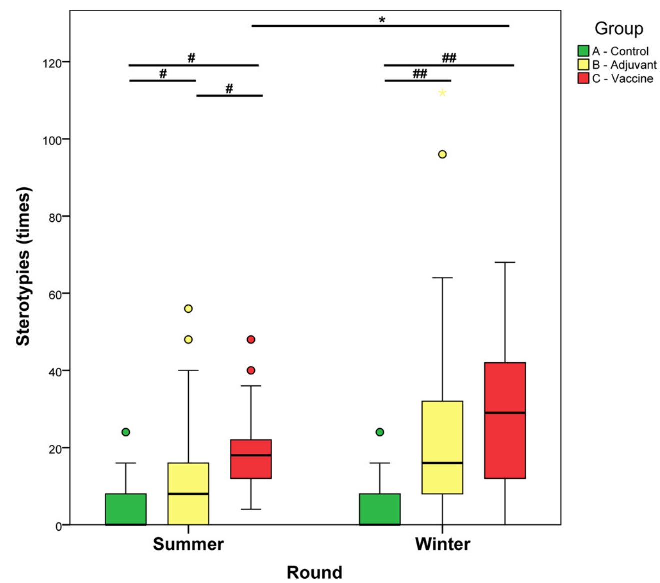

Fig. 6. Social behavior: Stereotypies. Group C (Vaccine, red) performed more stereotypies in the winter round (*p_W = 0.002). Groups B (Adjuvant-only, yellow) and C (Vaccine, red) performed more stereotypies than did Group A (Control, green) in the summer ($^{\#}p_{KW}$ < 0.001) and the winter ($^{\#\#}p_{KW}$ < 0.001) rounds. Furthermore, Group C (Vaccine, red) performed more stereotypies than did Group B in the summer round ($^{\#}p_{KW}$ < 0.001). Comparisons within groups were performed by Wilcoxon (W) test. Comparisons between groups were performed by Kruskal-Wallis (KW) test and *post hoc* Dunn's test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Supplementary Table S28.

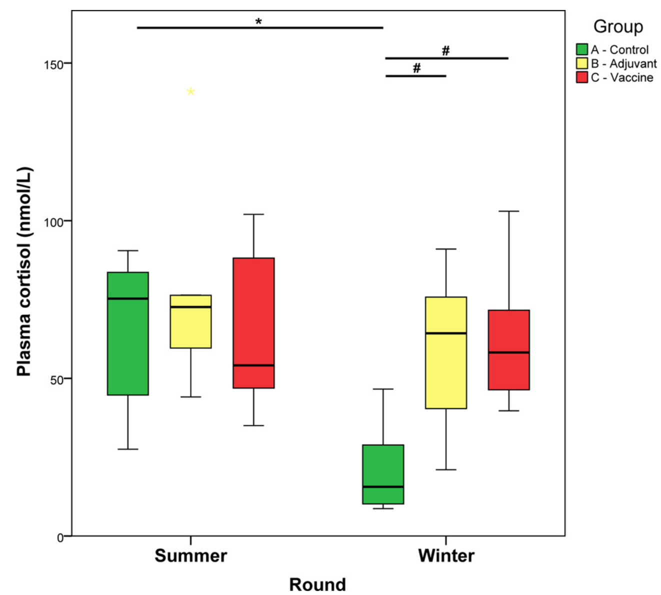

Fig. 7. Plasma cortisol levels. Group A (Control, green) shows a decrease in the cortisol levels (*p_t = 0.002) when comparing the winter and the summer round. Groups B (Adjuvant, yellow) and C (Vaccine, red) show higher cortisol levels than Group A (control, green) in the winter round ($^{\#}p_A$ = 0.005). Comparisons within groups were performed by Student's *t*-test (t). Comparisons between groups were performed by ANOVA (A) test and *post hoc* Duncan's test. Central bars indicate the median. The extremes of the boxes represent the first (Q1, lower) and third (Q3, upper) quartiles. Boxes represent the interquartile range (IQ = Q3-Q1), which indicates 50% of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 x IQ; lower: Q1 – 1.5 x IQ), and reflect the variability of the data outside Q1 and Q3. Data are shown in Supplementary Tables S30 and S31.

the vaccination schedule includes a primo-vaccination of two doses 3–4 weeks apart, followed by booster doses every 6–12 months. Al is a well-known neurotoxic metal [42,43] that can reach the central nervous system (CNS) in animal models through the parenteral route after intramuscular or subcutaneous inoculations [44,45,23], which might have contributed to the appearance of behavioral changes [23].

The cognitive and behavioral tests applied in our study have been used previously for studies in sheep and have been extensively validated [24–35]. To better understand the development of cognitive and behavioral changes, we performed two rounds of tests in the experiment: one in late summer, and the other in mid-winter. As previously indicated [21], the spontaneous behavioral changes observed in affected flocks are always most apparent under cold temperatures (i.e., winter conditions in the Northern hemisphere), an observation of difficult explanation to date. Furthermore, with that protocol, we were able to quantify the cumulative effect of vaccine doses in the development of the behavioral changes. The results of our experiment indicated that behavioral changes were most pronounced in winter; however, the changes might have been a response to reduced air temperatures, a by-product of the difference between seasons in the number of inoculations applied, or a combination of both. Likely, low temperatures act together with the cumulative impacts of multiple stressors such as vaccines and/or Al adjuvant, playing a role in the pathogenesis of the ovine ASIA syndrome.

The cognitive tests used in our study (T-maze test, OFT and NOT) did not detect significant differences between groups in either of the two rounds. Within the Vaccine lambs (Group C) and Adjuvant-only lambs (Group B), a few significant differences existed between the two rounds of tests. Among the Vaccine lambs, the most significant change was a reduction in the time spent trying to escape the test arena in the OFT in winter, which might reflect a reduction in social tendencies in the vaccinated lambs because, in this test, the animal tries to escape the isolation of the arena and return to the group [46]. In addition, Vaccine animals left the first area of the T-maze test (latency) earlier in the second day than they did on the first day of the winter round, although the time taken to solve the maze did not differ. The significant difference in latency between days might have been a product of the exceptionally long latency of the animals in the Vaccine Group on the first day of the test. In winter, lambs in the Adjuvant-only Group spent more time exploring (OFT) than they did in summer and they kept farther away from the novel object in the second exposure (NOT). Those changes might reflect a level of agitation or fear [47,33]. Collectively, the cognitive tests presented to the lambs indicated some changes in the Vaccine and Adjuvant-only groups, however; in general, vaccination or inoculation with the adjuvant only seemed not having a marked effect on the cognitive parameters evaluated by the different tests applied in the present study.

Home pen observations of individual behaviors identified several significant behavioral changes, which were most pronounced in winter. Lambs in the Vaccine and Adjuvant-only groups spent more time standing or walking and lambs in the Adjuvant-only Group spent less time lying down than did the lambs in the Control Group in the winter round. Differences between groups in time spent standing were already apparent in the summer round which entailed seven inoculations, only. The changes in the treatment groups reflect restless or excitatory behavior [48] because resting patterns can be used to identify social stress in animal husbandry [49]. Sheep exhibit a consistent and synchronous pattern of activity and resting [49,50], which the inoculations appeared to have altered. Those changes were similar to some of the symptoms reported after the application of BT vaccines in 2008–2010 [21]. In our study, in winter, Vaccine and Adjuvant-only groups fed on concentrate fewer times than did the Control Group. Given that the amount of

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concentrate offered to all groups in either summer or in winter was the same, and was consumed shortly after it was presented, the reduced frequency probably reflects prolonged bouts of feeding at the hopper, which is symptomatic of polyphagia or compulsive eating. Polyphagia was a symptom in animals that exhibited the chronic phase of the ovine ASIA syndrome after having received the BT vaccinations [21]. It is uncertain why the Vaccine lambs in our study ate straw less frequently than did the Control lambs in summer, but not in winter. Probably, that difference was not associated with the vaccination procedures.

In our study, home pen observations of social behaviors demonstrated several significant changes in the behavior of Vaccine and Adjuvant-only groups. In winter, affiliative interactions among the Adjuvant-only and the Vaccine groups were much less frequent than they were in summer. In winter, but not in summer, Vaccine and Adjuvant-only groups exhibited significantly fewer affiliative interactions than did the Control Group. In winter, Vaccine animals engaged in very few affiliative interactions, which was even lower than Adjuvant-only lambs. In general, sheep are gregarious, and have a strong drive to be in the company of flock mates [51]. Rasa Aragonesa is an autochthonous breed that is particularly gregarious, and a reduction in affiliative interactions by an individual is uncommon and readily detected by an observer. A reduction in affiliative interactions might indicate a deleterious effect on animal welfare [52]. In our study, in summer and winter, the frequencies of aggressive interactions and stereotypies were significantly higher in Vaccine and Adjuvant-only groups than they were in the Control Group. Furthermore, in the treatment groups, the frequency of those behaviors was significantly higher in winter than it was in summer. Wool loss and depilation, which were caused by wool biting, occurred in the Vaccine Group, only, in winter. Apparently, these types of behavioral changes in sheep had occurred at a very early stage of the inoculation protocol because they were already significantly higher in the treatment groups than they were in the Control Group in the summer round, after only seven inoculations had been administered. Aggressive behaviors are often associated with hierarchical interactions and the dominance of some individuals over others, and stereotypies are repetitive behaviors that are associated with a worsened environmental condition [53]. Increases in those behaviors have been correlated with poor welfare status [36], which might have been also reflected by the reduction in affiliative interactions. Aggressions (including wool biting) and stereotypies in sheep can be associated with housing, isolation [27], and other management factors including diet [25,54,26]. In our study, all experimental groups were maintained in the same manner, which included long-term confinement to a limited space. In the Control Group, however, the frequencies of aggressive interactions and stereotypies were always very low and did not differ between summer and winter, which we interpreted as the normal basal levels. These results in the Control Group rule out other known causes for these behavioral changes and link them to the treatments applied in the other groups. In our opinion, all these behavioral changes exhibited by the Vaccine and Adjuvant-only lambs in our study are of outmost importance, as they are the first scientific explanation of some of the previously observed behavioral changes in flocks affected by the chronic phase of ovine ASIA syndrome [21]. Indeed, these changes can be undoubtedly detected by veterinarians and farmers in field conditions but they have never been scientifically linked to vaccination and/or Al inoculations.

The reduction in the cortisol levels in the Control Group in our study reflected a previously-described seasonal variation in sheep between September and February [55]. The Vaccine and Adjuvant-only lambs did not exhibit a similar reduction, therefore differing significantly with the control in winter. Cortisol is a good indicator of stress in animals that are exposed to adverse situations [56,57], and reflects the stimulation of the hypothalamic-pituitary-adrenal gland axis [58]. Our study suggests that stress levels were higher in the Vaccine and Adjuvant-only animals than they were in the Control Group in winter. The increase in the WBC and eosinophils in the vaccinated animals might indicate an increase in stress. In humans, stress, either physical or emotional, is one of the main causes of an elevated WBC, which has been demonstrated in mice [59]. In ruminants, an elevated WBC has been associated with handling, which can be stressful [60]. In our study, conditions were not stressful, which suggests that the vaccination was responsible for the increase in the WBC in the Vaccine Group. Al-containing vaccines induce a pro inflammatory effect through the activation of the inflammasome [61], which can lead to leukocytosis among other effects [62]. Therefore, the repetitive inoculation of vaccines or Al adjuvant only could induce a persistent proinflammatory status in our animals that contributed to the behavioral changes observed. Although the pathogenesis for this effect is still to be elucidated, autoimmune reactions generated along the course of a chronic, active inflammation may play an important role [11,22].

Pathology performed at the end of the experimental period showed that Vaccine and Adjuvant-only inoculated animals presented persistent subcutaneous granulomas at the inoculation site with active migration of Al-laden macrophages to the regional lymph nodes. These granulomas likely act as a continuous and non-specific inflammatory stimulus and they could have somehow contributed to the behavioral changes observed in the present work. A comprehensive characterization of the granulomas will be described elsewhere [63].

5. Conclusions

This is the first scientific study to demonstrate behavioral changes in sheep after repetitive inoculation with commercial vaccines that contain Al hydroxide or the equivalent amount in Al only. In addition, it provides the first scientific explanation for some of the changes reported in spontaneous cases of ovine ASIA syndrome [21].

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2018.10.019.

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