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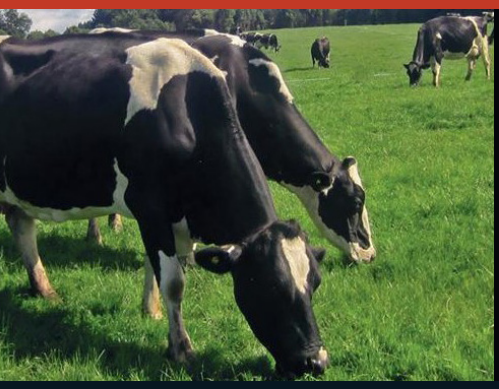

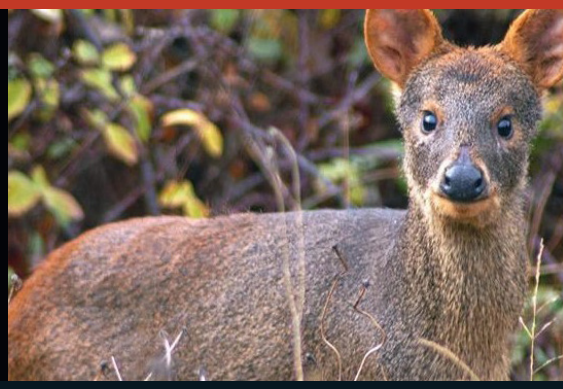

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ORIGINAL ARTICLE

Relationship between chronic diseases, hair cortisol concentration and welfare of housed dairy goats

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ABSTRACT. The aim of this study was to evaluate the relationship between seroprevalence of chronic diseases, hair cortisol concentration (HCC), and welfare of dairy goats housed throughout a productive cycle. Sixty multiparous dairy goats, over four years old, were selected. An animal welfare assessment was conducted using health indicators for goats, according to the AWIN protocol. Blood samples were also collected for haematology and determination of seroprevalence of chronic diseases, hair samples for determination of HCC, milk samples for chemical composition and somatic cell counts, and faecal samples for parasite load. Small Ruminant Lentivirus (SRLv) had a prevalence of 71.66%, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) of 5%, *Leptospira interrogans* of 40% and *Ovine Gammaherpesvirus* type 2 (OvHV-2) of 50%. The percentages of goats that tested positive for one, two or three diseases were 31.67%, 50% and 11.66% respectively. Haematological alterations included hyperproteinaemia (84.94 ± 1.58 g/L) and hyperfibrinogenaemia (6.11 ± 0.65 g/L) for those with one or two diseases, with significant differences being found ($P < 0.05$). The welfare indicators related to health and the number of diseases were poor body condition, poor coat, poor udder conformation, and mucosal lesions ($P < 0.05$). However, no significant differences were observed between HCC and the number of chronic diseases in dairy goats ($P > 0.05$). Higher concentrations of cortisol in hair were found at 150 days of lactation (16.65 ± 1.39 pg/mg) compared to the mating season (9.55 ± 0.04 pg/mg) ($P < 0.05$). No associations were found ($P > 0.05$) between the production, composition, and somatic cell counts in milk and cortisol concentrations and diseases. It was concluded that the presence of chronic diseases in goats did not influence hair cortisol concentrations, possibly due to an effect of adaptive tolerance to diseases, as occurs in other domestic species; however, there was an effect of the productive stage.

Keywords: chronic stress, caprine arthritis and encephalitis, paratuberculosis, leptospirosis

INTRODUCTION

The presence of chronic diseases in goat production can seriously threaten goat homeostasis, welfare, and productivity. These diseases produce inflammatory lesions in different organs and tissues, which reduce the productivity and well-being of animals (Licitra *et al.*, 2021). The disease tolerance of an animal is the ability to preserve homeostasis while limiting the detrimental impact of infection on health and performance without affecting the pathogen load *per se* (Nakov *et al.*, 2019). The main chronic infectious diseases of economic importance in intensive goat rearing systems are caprine arthritis and encephalitis, paratuberculosis, caseous lymphadenitis, leptospirosis and parasitosis caused by gastrointestinal nematodes (Muri *et al.*, 2016; Fontaine & Baird, 2008; Alberti *et al.*, 2012). When these diseases are not diagnosed and controlled in a timely manner, they represent a threat to health and well-being, negatively impacting production (Muri *et al.*, 2016; Di Cerbo *et al.*, 2010; Luna *et al.*, 2018). The presence of diseases in production animals is an indicator of deterioration in animal welfare, which implies that they experience continuous stress with physiological and behavioural responses, exerting an immunosuppressive effect. In livestock production, stress has been considered an inevitable reaction that occurs when animals are exposed daily to adverse environmental conditions, and is the cause of many unfavourable consequences, ranging from discomfort to death (Etim *et al.*, 2013).

Hair cortisol concentration (HCC) has been proposed as an indicator of chronic stress, as it is incorporated into the hair from the bloodstream and skin through passive diffusion during its growth stage, being a reliable long-term indicator to retrospectively evaluate the activity of the hypothalamic-pituitary-adrenal axis and the response to chronic stressors (González de la Vara *et al.*, 2011; Heimburge *et al.*, 2019; Russell *et al.*, 2012; Moya *et al.*, 2013). The stability of hair cortisol concentrations over time has been demonstrated, suggesting that it may be a reliable measure of long-term cortisol secretion (Davenport *et al.*,

2006; Stalder *et al*., 2012) and useful in humans and wild and domestic animals (Gow *et al*., 2010; Koren *et al*., 2002; Tekin *et al*., 2023; Casal *et al*., 2017). Although it is not completely clear whether HCC can serve as a retrospective indicator of the history of non-specific diseases in animals, preliminary studies in dairy cattle have reported higher concentrations of cortisol in the hair of sick cows (metritis, laminitis, and mastitis) than in clinically healthy cows (Comin *et al*., 2013). Other investigations in dairy cattle have not found the usefulness of HCC as an indicator of stress in dehorning, bronchopneumonia, or laminitis, considering these as acute processes (Braun *et al*., 2017; Braun, *et al*., 2019; Fischer-Tenhagen *et al*., 2018). In a study on hair cortisol concentrations in adult goats, no significant differences were observed between rough hair (poor nutrition) and normal hair (healthy goats) (Battini *et al*., 2015).

Welfare assessments of goat farms have gained relevance in recent decades, especially in intensive systems, because animals face greater exposure to stressors (Arsoy, 2020). Animal welfare assessment protocols have been developed at farm level, where they incorporate valid and reliable indicators that include direct indicators (measurable in the animal) and indirect indicators (measurable in the environment and in the management of livestock workers) (Caroprese *et al*., 2009; Battini, *et al*., 2015; Spigarelli *et al*., 2020).

Information on the impact of chronic diseases in dairy goats and their effects on welfare and productivity is limited compared with other domestic species. Therefore, it was hypothesised that chronic diseases in housed dairy goats would have a significant effect on hair cortisol concentrations as an indicator of chronic stress, producing a negative impact on the welfare and productivity of the goats. The aim of this study was to evaluate the relationship between the seroprevalence of chronic diseases, hair cortisol concentrations, and welfare of dairy goats housed throughout a productive cycle.

MATERIAL AND METHODS

Description of the productive unit

The present work was carried out in an intensive goat production unit located in Querétaro, Mexico, characterised by a temperate semi-dry climate, with summer rains (climate type BS1k (x')), altitude of 1920 m above sea level, north latitude 20° 54' 29'', west longitude 99° 55' 51'', average annual temperature of 17.6°C and average annual rainfall of 538 mm (INEGI, 2021). An evaluation instrument was used to assess the productive, nutritional, and health management of the animals. The goat farm had a history of clinical cases of caprine arthritis, encephalitis, paratuberculosis, and herpesvirus.

Animals and housing

This research project was evaluated and approved by the Institutional Subcommittee for the Care and Use of Experimental Animals (SICUAE.DC-2020/4-4, FMVZ, UNAM). This study was observational, analytical, and prospective (Noordzij *et al*., 2009). Initially, 60 dairy genotype goats, multiparous, over four years of age, average body weight of 56.45 ± 1.48 kg, were randomly selected. Each animal received a diet based on its nutritional requirements (NRC, 2007), consisting of alfalfa and oat hay, corn silage, and a vitamin-mineral mixture. The animals were housed in two pens connected to each other with a total area of 330 m^2, two automatic bowl drinkers, a linear feeder with

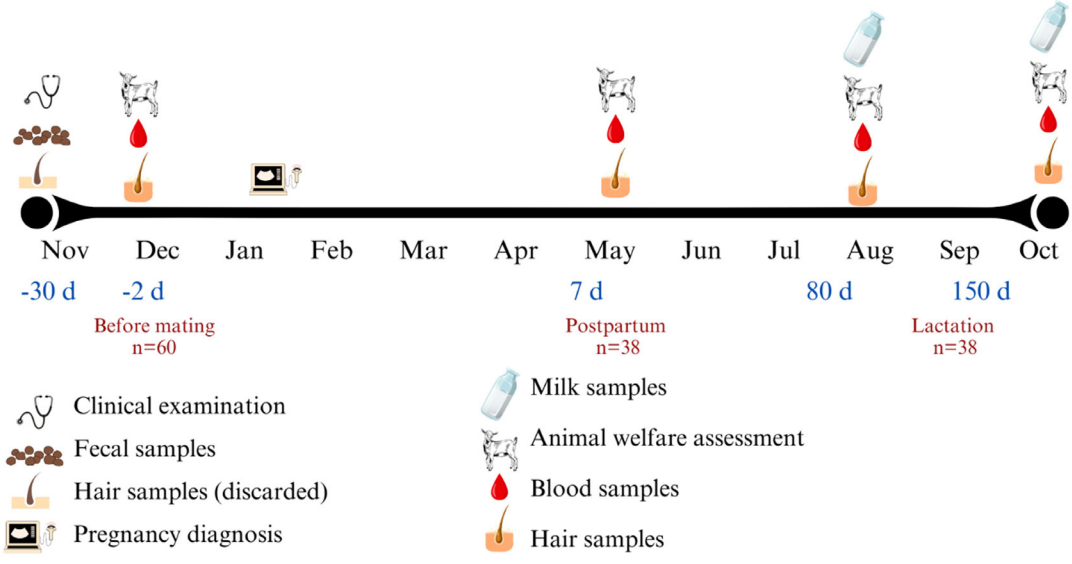

Figure 1.
Schematic representation of the experimental design for a productive cycle in housed dairy goats.

34 individual feeders, a dirt floor, and a metal shade. Goats were evaluated during different productive stages, including mating, gestation, postpartum, and lactation (Figure 1). Thirty days before starting the study, 10 g of faecal samples were collected rectally from each animal to determine parasite load using the McMaster technique (Figueroa-Castillo *et al*., 2015). A general clinical examination was performed for each of the selected animals (Duguna, 2016).

Reproductive management included synchronisation of oestrus with 150 µg intramuscular prostaglandin F2α (Lutalyse®, Zoetis), and mating was carried out 48 hours later. Forty-five days later, pregnancy was diagnosed using transrectal ultrasound (Mindray®). After birth, the kids were reared with their dams and weaned at 80 days. Milking was performed daily using a Flaco® carousel mechanical milking machine with 24 units, once a day at 07:00 h.

Health evaluation

Two days prior to mating, 10 ml of blood was collected from the jugular vein, and the serum was recovered by centrifugation and stored at -20°C until processing. The Luminex® Multiplex technique (Bio-plex 200 System) was used for the detection of Small Ruminant Lentivirus (recombinant proteins p16 and gp38), *Mycobacterium avium* subspecies *paratuberculosis* (PPA3 protoplasmic antigen), and *Brucella melitensis* (native hapten) (Nájera-Rivera *et al*., 2023). The Microscopic Agglutination technique (WHO, 2012) was performed using 70 µL of serum to identify 10 serovars of *Leptospira interrogans* (Autumnalis, Bratislava, Canicola, Gryppotyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Pyrogenes, Serdjo, and Tarassovi). We were provided with previous results of an outbreak of herpesvirus due to *ovine gammaherpesvirus* type 2, diagnosed using ELISA and PCR (Madrigal-Valencia *et al*., 2023). Goats were then grouped into one, two, and three disease groups and evaluated throughout the study.

For haematology, 4 ml of blood with EDTA anticoagulant was extracted 2 days before mating, 7 days postpartum, and at 80 and 150 days of lactation. The analytes determined were haematocrit, total protein, fibrinogen, total leukocyte count, and its differential (neutrophils, lymphocytes, monocytes, eosinophils, and basophils).

Hair cortisol analysis

The samples were recovered from the chest region, considering that it is one of the body areas that suffers less aggression from the environment and contamination (saliva, faeces, and urine). The hair of a 7 x 7 cm area of the chest region was shaved and discarded using an electric machine (Andis®) one month before starting the first sampling, since at that time the animals were housed in new pens for adaptation and observation. Hair sampling periods were performed by shaving the same chest area, 2 days before mating, 7 days postpartum, and at 80 and 150 days of lactation. The samples were stored in a dry room temperature bag and protected from sunlight until processing.

The HCC was determined using an adaptation of the cortisol extraction methodology proposed by Davenport *et al*. (2006) and Koren *et al*. (2002). In this process, 300 mg of hair was washed with 5 ml of isopropyl alcohol to remove any dirt, followed by drying at room temperature for 2 h. Subsequently, the hair was finely cut (approximately 0.2 cm) with surgical scissors, and 200 mg of hair was weighed on an analytical scale (Denver Instrument PI-314®). These samples were placed in 20 ml borosilicate bottles, and 10 ml of methanol was added. After shaking the samples for 3 h, they were left to rest overnight and finally shaken again for 3 h (Vortex Mixer, UltraCruz®). The supernatant was transferred to other borosilicate bottles and allowed to evaporate for 5–7 days at room temperature, keeping the samples in an extraction chamber. Cortisol adhering to the walls of the bottles was reconstituted with 200 µL of phosphate-buffered saline (PBS) and shaken for 3 min. Subsequently, a 1:20 dilution was carried out to determine hair cortisol (pg/mg) using the enzyme-linked immunosorbent assay (indirect ELISA) test in duplicate, using 50 µL of the diluted extract, following the instructions for the Arbor Assays Cortisol commercial kit (K003-H1/H5) DetectX ® (antibody cross-reactivity for prednisolone 7.8%, cortisone 1.21%, dexamethasone 18.8%, corticosterone 1.2%, progesterone, and oestradiol < 0.1%). The intra- and inter-assay coefficients of variation (CV) were 8.76% and 8.13%, respectively.

Animal welfare assessment

Individual evaluations of animal welfare were carried out 2 days before mating, 7 days postpartum, and 80 and 150 days of lactation, selecting indicators related to health: body condition score (-1: thin, 0: optimal, 1: fat), hair coat condition (0: good coat, -1: bad coat); for abscesses, lameness, nasal and ocular discharge, and faecal soiling, they were scored by assigning 0: absent and 1: present (AWIN, 2015). In addition, other indicators related to animal health were considered: coughing, lymphadenomegaly, udder disorders (asymmetry, wounds, and hardening), arthritis, mucosal lesions (oral and vaginal), and vaginal discharge, which were scored as 0 (absent) and 1 (present) (Table 1).

Milk analysis.

Milk production was recorded individually every 15 days from 80 to 150 days of lactation. To analyse milk composition, a 150 ml sample was collected in a glass container by manual milking from each teat at 80 and 150 days of lactation. Analytes measured included somatic cell count (cells/µL), using a DeLaval® automatic counter; determination of acidity (°Dornic) with an acidimeter; and milk composition, including percentage of fat, protein, lactose, and non-fatty solids, using a MilkoScan® infrared spectrophotometre. The reference values used for the chemical composition of goat milk were in accordance with Mexican standard NMX-F-728-COFOCALEC-2007.

Table 1.
Health indicators for the assessment animal welfare in housed dairy goats (AWIN, 2015).

Welfare indicator	Assessment criteria
Body condition score	Is visually assessed at the rear of individual goat, using a three-level scoring method.
Hair coat condition	Goats with poor hair coat condition (described as: matted, rough, scurfy, uneven, shaggy hair coat, frequently longer than normal) are recorded.
Abcesses	The presence of abscesses (ruptured or not) is recorded.
Lymphadenomegaly	Increase in the size of the lymph nodes, which are easy to observe and palpate, are recorded.
Udder disorders	Three characteristics are evaluated (asymmetry, wounds, and mammary gland hardening). The presence of one half of the udder that is at least 25% longer than the other is recorded.
Fecal soiling	The presence of soft fecal matter below the tail head and on both sides of the tail is visually assessed on individual goats, as a sign of diarrhea.
Coughing	One or a combination of the following: paroxismal coughing, respiratory distress including abdominal effort associated with breathing or wheezing. A single cough that may occur as part of a normal reflex when grazing not included.
Nasal discharge	The presence of any mucous or purulent discharge (white or yellowish) from the nose is visually assessed on individual goats.
Ocular discharge	The presence of clearly visible flow from one or two eyes is visually assessed on individual goats.
Vaginal discharge	The presence of any mucous or purulent discharge (white or yellowish) from the vaginal is visually assessed on individual goats.
Mucosal lesions	Ulcerative lesions of the oral and vaginal mucosa were recorded by evaluating each goat.
Lameness	Goats showing signs of severe lameness (based on abnormal gait, head nodding, spine curvature, kneeling) are recorded.
Arthritis	Articular inflammations in the front and hind legs of the goats were evaluated.

Statistical analysis

SAS® statistical package (v. 9) was used to analyse the data. Univariate procedure was used for each variable collected to obtain measures of central tendency and dispersion. The Shapiro-Wilk normality test and Bartlett's test for homogeneity of variance were used. A completely randomised design was carried out using analysis of variance and Tukey's test to evaluate the differences between the productive stages. In addition, analysis of variance with repeated measurements over time (proc MIXED) was used to evaluate HCC and the number of diseases per productive stage; the level of significance was $P < 0.05$. The non-parametric chi-square test was used for the qualitative variables of the individual indicators related to health in different productive stages. To evaluate the degree of association between variables, Pearson and Spearman correlation analyses were performed, and the level of significance was set at $P < 0.05$.

RESULTS

Health evaluation

Table 2 shows the seroprevalences of Small Ruminant Lentivirus (SRLv), *Mycobacterium avium* subspecies *paratuberculosis* (MAP), *Brucella melitensis*, *Leptospira interrogans* and *Ovine Gammaherpesvirus* type 2 (OvHV-2) in housed dairy goats. The pathogenic serovars of *Leptospira interrogans* (Lepto) most frequently observed in dairy goats were Icterohaemorrhagiae (9/24), Hardjo (6/24), and Canicola (4/24). Table 3 presents the frequency of goats that were seropositive for one, two, or three chronic diseases, and their combinations. Only 38 goats were delivered, representing 63.33% of fertility. It should be noted that goats without disease did not become pregnant. Of the seropositive goats, 50% showed at least one clinical sign related to the diagnosed disease. Before mating, a low parasite load of 253.57 ± 87.46 eggs per gram of feces was recorded for strongylids, and 146.42 ± 49 oocysts per gram of feces for coccidia.

On the other hand, significant differences were observed by disease number and haematological alterations ($P < 0.05$), with one and two diseases were hyperproteinaemia (84.94 ± 1.58 g/L) and hyperfibrinogenaemia (6.11 ± 0.65 g/L), leukocytosis (13.95 ± 0.64 x 109/L) and neutrophilia (8.42 ± 0.44 x 109/L) were also observed for goats that had one disease.

Table 2.
Seroprevalence of chronic diseases in housed dairy goats (n=60).

Etiology	N° of goats	%
SRLv	43	71.66
MAP	3	5
Brucella melitensis	0	0
Leptospira interrogans *	24	40
OvHV-2	30	50

SRLv, Small Ruminant Lentivirus; MAP, *Mycobacterium avium* subspecies *paratuberculosis*; OvHV-2, *Ovine Gammaherpesvirus* type 2.
*Serial double microscopic agglutination test. A dilution cut-off of 1:100 was considered positive.

Table 3.
Frequency of dairy goats seropositive with one, two or three chronic diseases (n=60).

Diseases	Etiology	N° of goats	%
Without diseases	-	4	6.67
1	MAP	1	1.67
	SRLv	10	16.66
	OvHV-2	7	11.67
	Lepto	1	1.67
2	SRLv + Lepto	14	23.33
	SRLv + OvHV-2	12	20
	Lepto+ OvHV-2	4	6.66
3	SRLv + Lepto + OvHV-2	5	8.33
	SRLv + MAP + OvHV-2	2	3.33

SRLv, Small Ruminant Lentivirus; Lepto, *Leptospira interrogans*; OvHV-2, *Ovine Gammaherpesvirus* type 2; MAP, *Mycobacterium avium* subspecies *paratuberculosis*.

Hair cortisol analysis

Figure 2 shows the HCC in dairy goats at different productive stages. Highly significant differences ($P < 0.0001$) were observed by productive stage, finding a lower concentration before mating (9.55 ± 0.04 pg/mg) and the highest concentration was at 150 days of lactation (16.65 ± 1.39 pg/mg), which corresponds to the accumulation of

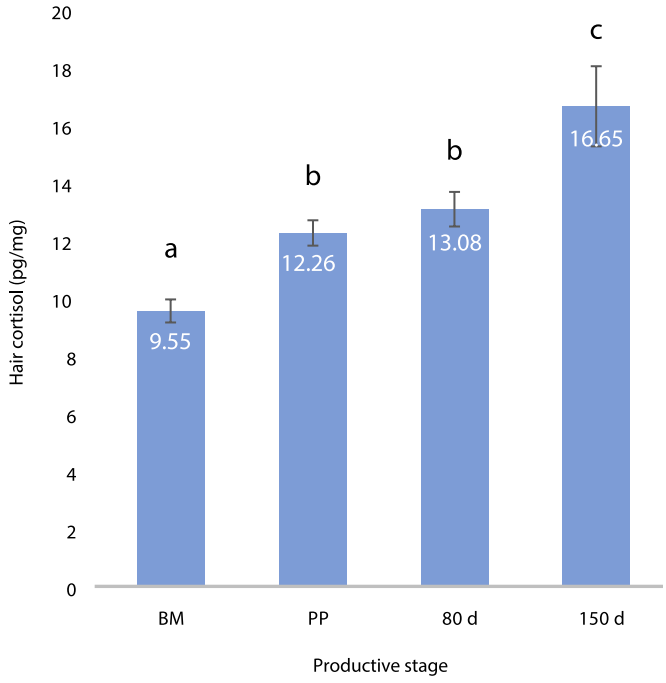

Figure 2.
Mean ± SE of hair cortisol concentrations (pg/mg) of housed dairy goats at different productive stages. BM, before mating (n = 60); PP, postpartum; 80 d and 150 d, days of lactation (n = 38). a, b, c: Different letters indicate differences between productive stages ($P < 0.001$).

cortisol from 81 to 150 days of lactation, during this period the goats were subjected to the stress of weaning (80 days postpartum) and mechanical milking. Cortisol concentrations in hair did not show significant differences ($P > 0.05$) in the number of diseases (Figure 3). At the mating period, goats with zero, one or three diseases recorded cortisol concentrations less than 10 pg/mg ($P > 0.05$) and goats with two diseases had an average of 12.18 ± 0.87 pg/mg ($P > 0.05$). In the postpartum period, the goats with two diseases recorded the highest concentrations of cortisol 15.57 ± 3.51 pg/mg compared to those with 1 or 3 diseases ($P > 0.05$).

Table 4 shows HCC and disease seroprevalence in housed dairy goats, no significant association was found ($P > 0.05$) with milk production (r = 0.2154, $P > 0.05$), chemical composition: fat (r = 0.1168, $P > 0.05$), protein (r = 0.1024, $P > 0.05$), lactose (r = 0.0863, $P > 0.05$) and non-fat solids (r = 0.0568, $P > 0.05$); and milk somatic cells (r = 0.0268, $P > 0.05$).

Animal welfare assessment

Individual evaluations of welfare related to health showed significant differences ($P < 0.05$) between the productive stages. In the two days period before mating, 38.3% of the animals (23 goats) had a poor body condition score (BCS) and coat; abscesses occurred in nine goats (15%), and locomotion problems associated with arthri-

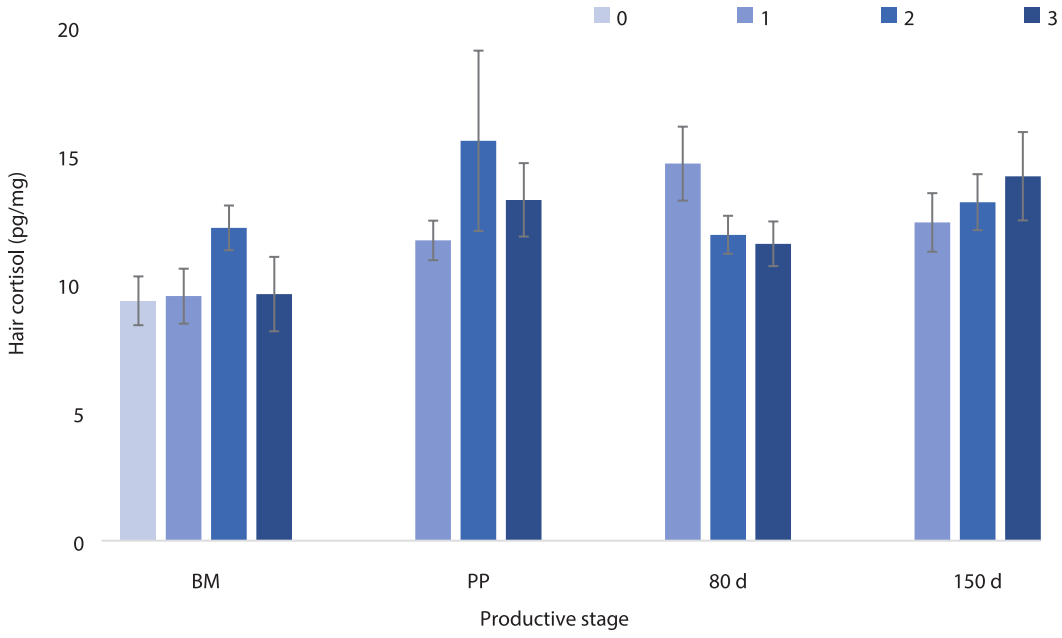

Figure 3.
Mean ± SE of hair cortisol concentrations (pg/mg) of housed dairy goats with different seropositive diseases and productive stages ($P > 0.05$). BM, before mating; PP, postpartum; 80 d and 150 d, days of lactation; 0, without disease; 1, one disease; 2, two diseases; 3, three diseases.

Table 4.
Milk production and chemical composition of milk in dairy goats housed according to the number of diseases.

	One disease (n = 13) (Mean ± SE)	Two diseases (n = 17) (Mean ± SE)	Three diseases (n = 5) (Mean ± SE)	r	*P-value*
Total milk yield (L)	186 ± 23.05	233 ± 21.48	192.81 ± 37.80	0.2154	> 0.05
Protein (%)	4.14 ± 0.24	4.31 ± 0.25	4.48 ± 0.47	0.1168	> 0.05
Fat (%)	4.62 ± 0.20	4.98 ± 0.29	5.01 ± 0.95	0.1024	> 0.05
Lactose (%)	4.06 ± 0.19	3.99 ± 0.13	4.10 ± 0.25	0.0863	> 0.05
Non fat solids (%)	9.12 ± 0.24	9.47 ± 0.27	9.55 ± 0.55	0.0568	> 0.05
SCC (cells/ µL)	824 ± 233.49	986 ± 136.08	925 ± 216.50	0.0268	> 0.05

SCC: somatic cells count

tis, mainly in the carpal region with the presence of mild to moderate lameness in six goats (10%). In addition, one case of ulcerative vulvovaginitis was observed, and 19 cases of ulcerative stomatitis were associated with *Ovine Gammaherpesvirus* type 2. Seven days postpartum, the animals presented with a poor coat condition (41%) and BCS (25%). At 80 days of lactation, 76% of the animals had a good BCS and coat condition, 8.33% of the goats had arthritis and hardening of the udder, and at 150 days of lactation, 27 goats showed mild to severe lesions in the oral mucosa. Figure 4 shows the frequencies of the different health indicators with the number of diseases, in which significant differences ($P < 0.05$) were found with poor BCS, poor udder conformation, and mucosal lesions in goats with the

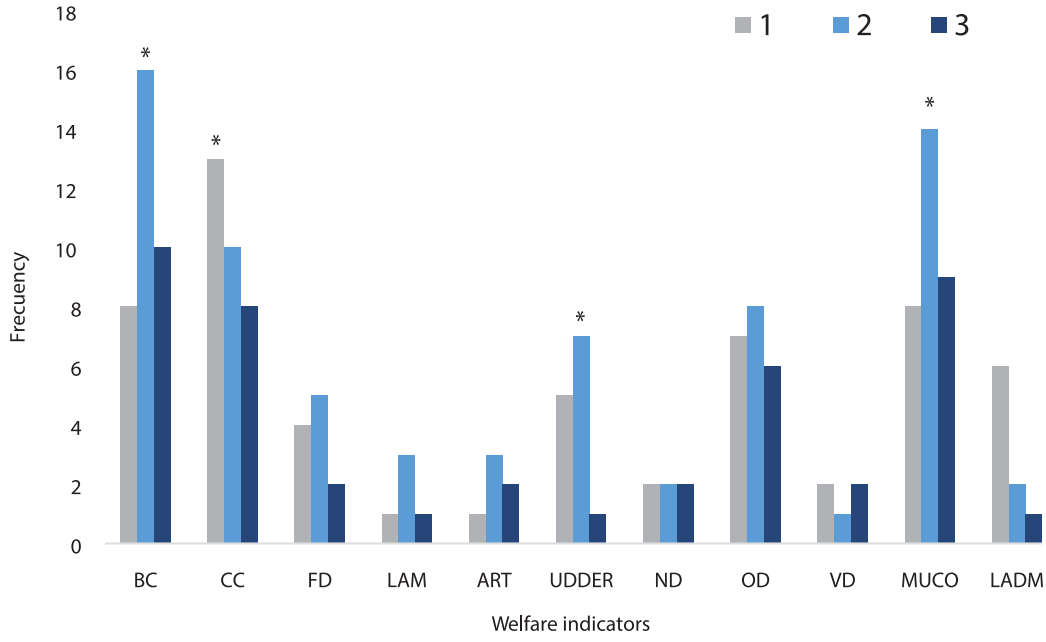

Figure 4.
Frequency of animal welfare indicators related to health in a productive cycle in housed dairy goats with different numbers of diseases: 1, one disease; 2, two diseases; 3, three diseases. BC, Thin body condition; CC, Poor coat condition; FD, Faecal dirt; LAM, Lameness; ART, Arthritis; UDDER, Udder characteristics; ND, Nasal discharge; OD, Ocular discharge; VD, Vaginal discharge; MUCO, Mucosal lesions; LADM, Lymphadenopathy.
*Significant difference within each indicator by number of diseases ($P < 0.05$)

two diseases. Animals positive for the two diseases had ulcerative stomatitis, with percentages of 30.3%, 33.3%, and 46.7% during the mating period, postpartum period, and 150 days of lactation, respectively ($\chi^2 = 14.595$, $P = 0.002$). Goats positive for the three diseases presented lameness and arthritis at 33.3% ($\chi^2 = 14.156$, $P = 0.048$) throughout the production cycle. Goats positive to one disease ($P < 0.05$) had a higher frequency of poor coat condition than those with two or three diseases.

DISCUSSION

In the present study, the prevalence of causal agents of chronic diseases in goats was 71.66% for SRLv, which was higher than that reported by Martínez *et al.* (2020), who obtained a seroprevalence of 22.3% for SRLv in goat farms in Mexico. Other studies carried out in Mexico reported a paratuberculosis prevalence of 9.87% (Guzmán *et al.*, 2016) and for *Leptospira interrogans* between 16.4% and 65.6%; the most frequent serovars were Icterohaemorrhagiae, Bratislava, Canicola, and Hardjo (Luna *et al.*, 2018), as in this study. It is important to mention that most epidemiological studies in goats have focused on detecting the prevalence of a single infectious agent; however, the interactions with other diseases and their effects on welfare and production are unknown. In the present study, HCC was not influenced by the prevalence of diseases, despite the fact that 50% of the animals showed signs of disease, mainly ulcerative stomatitis and less frequently, mammary hardening and arthritis. These were the main clinical alterations observed at 7 days postpartum and at 150 days of lactation.

Considering that stress describes non-specific responses of the body to all types of challenges (social, environmental, metabolic, immunological, etc.) that threaten homeostasis, individual variations in any contributor to the stress response can determine resilience or vulnerability to stress (Martínez-Miro *et al.*, 2016). Goats are well known for their resistance to diseases; compared to other domestic ruminants, they have a greater number of lymphocytes than neutrophils in the circulatory system, which suggests a good development of their immune system (Daramola *et al.*, 2005); however, there are few studies on this subject. In this research, no differences were found between the number of chronic diseases and hair cortisol concentrations; however, differences were found in the productive stage.

Currently, there are some investigations in dairy cows with different lesions that present with an increase in HCC, such as clinical endometritis (Burnett *et al.*, 2015), endometritis, mastitis, and lameness (Comin *et al.*, 2013). On the other hand, a study carried out in healthy calves and those with chronic bronchopneumonia did not find significant differences in hair cortisol concentrations (Braun *et al.*, 2019), suggesting that endogenous stress related to

the disease varies depending on the severity and that the measurement of cortisol in hair is not adequate for the detection of lower magnitude stress situations, such as those generated by subclinical disease.

The health indicators of the welfare protocol in dairy goats (AWIN, 2015) contributed to the detection of some lesions related to the diseases that were evaluated in this study, such as lameness, low body condition score, poor coat, and other indicators, such as hardening of the udder in the postpartum period (associated with SRLv), lymphadenomegaly (associated with abscesses), and lesions in the vaginal mucosa suggestive of *Caprine Herpesvirus* type 1, and in the oral mucosa due to *Ovine Gammaherpesvirus* type 2. Some of the observed injuries may be multifactorial in origin (physiological, nutritional, and seasonal status) and cannot be analysed alone. Arsoy (2020) identified different health indicators related to loss of welfare in dairy goat farms in Cyprus, the most frequent being the presence of abscesses, hoof overgrowth, lameness (arthritis due to SRLv), and mastitis; these indicators are related to pain behaviour and can reduce productivity and fertility in the flock. Battini *et al.* (2015), studied the characteristics of the coat condition in dairy goats (silky, shiny, rough, homogeneous, matted) also related to body condition, concluding that the coat condition is a practical, valid and reliable health indicator of goat welfare, that should be included in assessment protocols and be a useful tool for livestock producers. Studies carried out in goats and other ruminants (Di Cerbo *et al.*, 2010; Manfredi *et al.*, 2010; Waller, 2006) have mentioned that infection by gastrointestinal parasites can influence indicators of body condition and coat condition. However, in this study, the parasite load was low before mating, and no significant association between the parasite effect and body condition and coat were found.

According to the obtained results, we can conclude that goats are resilient in the presence of chronic diseases. They can maintain their productive performance when faced with different pathogens (Doeschl-Wilson *et al.*, 2021). Resilience to disease in production animals can be measured in terms of a reduction in health, physical condition, or productive performance. The magnitude of the reduction depends on different factors related to the host (resistance and tolerance) and the pathogen (virulence and burden on the animal) (Knap & Doeschl-Wilson, 2020). Furthermore, hair cortisol measurements in cattle could contribute to stress monitoring and animal well-being by assessing individual resilience to stressors (Ghassemi-Nejad *et al.*, 2019; Nair *et al.*, 2021). In this study, it is possible that the goats had an adaptive effect on the stress caused by diseases as a protective process by not maintaining permanently high cortisol levels in daily life (Heimburge *et al.*, 2019).

Regarding the production parameters, chemical composition, and somatic cell count in goat milk, no association was observed with the presence of diagnosed chronic diseases. Our findings differ from those obtained by Kaba *et al.* (2012), who indicated that SRLv-seropositive goats tend to have a lower percentage of fat, protein, and lactose; however, milk production and somatic cell counts were not affected. Another study carried out by Martínez-Navalón *et al.* (2013) mention that goats seropositive to SRLv tended to have shorter lactation periods, lower production and higher somatic cell count.

It was concluded that the seroprevalence of different diseases, with and without clinical manifestations, did not influence hair cortisol concentrations; however, differences were observed at the productive stage. Goats may have an adaptive effect on disease-induced stress as a protective process by not maintaining permanent high cortisol concentrations. It was found that welfare indicators such as coat condition, body condition score, udder characteristics, and mucosal lesions aided the detection of signs related to health problems in dairy goats in a practical manner. This is the first study carried out in housed dairy goats that investigated the effect of chronic infectious diseases, with hair cortisol concentrations in a productive cycle (from breeding to lactation), as well as the use of health-related welfare indicators, which allowed us to detect health problems in a practical, timely, and objective manner.

Conflict of interest

All authors declare that there is no conflict of interest.

Author contributions

IECA, AMTG, YMDH contributed to the conduct and design of the study. MSG, ADPM, JGPM, ADRC executed the experiment and analysed the blood, serum, fecal and hair samples. MSG analysed the data. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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ORIGINAL ARTICLE

Swimming performance of adults and copepodites of *Caligus rogercresseyi* against different water flow speeds in presence or absence of light and host fish attractants

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ABSTRACT. The present study was conducted to determine the swimming ability of adults and copepodites of *Caligus rogercresseyi* at three different water flow speeds, considering the presence or absence of attractants such as light and fish *(Salmo salar)*. A total of 360 gravid females, 360 adult males, and 720 copepodites were randomly selected from a sea lice hatchery and distributed into groups. Each group was placed in a plastic bucket within a tank, and exposed or not to two attractants (light or host fish) at different water flow speeds (0, 1 and 2 cm/s). The results showed higher migration of adults and copepodites in the presence of light than in its absence ($P < 0.05$); however, no significant differences related to sea lice swimming performance were found in the presence or absence of fish ($P > 0.05$). Water flow speed had no effect on either variable ($P > 0.05$). In the current study, adult *C. rogercresseyi* demonstrated superior swimming capabilities compared with copepodites.

Keywords: Caligidosis, salmon farming, migration behavior, swimming capability, *Salmo salar*.

INTRODUCTION

Caligidosis, caused by the ectoparasite *Caligus rogercresseyi* (Copepoda: *Caligidae*), is considered one of the most important health problems during the seawater fattening stage of Chilean salmon farming (Feest, 2015) and represents a serious threat to its profitability, mainly due to the cost related to its surveillance and control (Sánchez *et al*., 2015). The disease causes economic losses estimated at US $ 300 million a year, being approximately US $ 80 million used for treatments (Agusti *et al*., 2016). The parasite feeds on mucus and epidermal and dermal fish cells (Valenzuela, 2009). Massive infections produce wounds on fish skin, causing restlessness, stress, lack of appetite, decreased growth, and immunosuppression. In addition, this parasite can act as a vector for disease-causing microorganisms such as ISAv (Oelckers *et al*., 2014) and Rhabdovirus Ch01 (Økland *et al*., 2018). Although *C. rogercresseyi* is not considered a vector for *Piscirickettsia salmonis* (Labra *et al*., 2020), a bacterium that substantially affects salmon farming in Chile (Piscirickettsiosis), Arriagada *et al*. (2019) reported that the average abundance of adult *C. rogercresseyi* has been significantly associated with the cumulative mortality caused by this bacterium in both Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), suggesting a synergistic relationship between the two diseases.

González & Carvajal (2003) reported that the life cycle of *C. rogercresseyi* consists in eight stages of development. Nauplius I and II (approximately 0.45 – 0.48 mm) and copepodites (0.65 mm) are planktonic stages, with copepodites considered the infective stage. The parasitic phase begins when copepodites attach to the host and molt to chalimus I, which grows and molts to chalimus II, III, and IV until reaching the sexually mature adult stage, swimming freely over the host body surface.

In line with Genna *et al*. (2005), some environmental factors, such as light intensity, salinity, and host swimming speed, should influence the fixation of copepodites on the fish epidermis. In addition, Pino-Marambio *et al*. (2007) provided evidence that semiochemicals present in water mediate the parasite-host interaction between *C. rogercresseyi* and *S. salar*. On the other hand, Amundrud & Murray (2009) highlighted the importance of wind-driven circulation for larval sea lice transport and suggested that local environmental conditions have a considerable impact on the probability of sea lice infection spreading between wild and farmed fish populations.

The underlying hypothesis of this study was that adult *C. rogercresseyi* exhibits superior swimming capabilities compared to copepodites under identical laboratory conditions. Therefore, this study aimed to compare the swimming performances of *C. rogercresseyi* adults and copepodites under different water flow speeds, both in the presence and absence of light and fish (*S. salar*) as attractants.

†Deceased. This manuscript represents a posthumous publication of Dr. Gerold Sievers' work.

MATERIAL AND METHODS

Location

This study was conducted at the Quillaipe Experimental Station (41°33'20'' Lat, 72°44'00'' Long), Los Lagos Region, Chile, during the austral spring of 2017.

Aquarium, fish hosts and sea lice

An aquarium (126 × 36 × 52 cm) equipped with an external Eheim-Experience 250 filter pump (12 L/min) and an Aqua Design Amano© submersible oxygenator pump (50 L/min) (Aqua Design Amano Co., Ltd.) was filled with approximately 210 L of filtered seawater. A plastic bucket (34 × 25 × 27 cm) with a capacity of 23 L with a circular opening (6.6 cm diameter) was placed on the left side of the aquarium.

Ten juvenile Atlantic salmon (*S. salar*) were randomly chosen from a fish culture tank belonging to AQUADVISE (Quillaipe Experimental Station) and acclimated for 10 days before starting the trials. The mean total fish weight was 110 g, and the fish were maintained under a natural photoperiod of 12L:12D. Salinity (mean: 32 PSU), temperature (mean: 12.6 °C), and oxygen saturation (mean: 92.8%) were recorded daily. A feed ratio of 1.5% body weight, using commercial dry pellets, was considered.

A total of 360 gravid females, 360 adult males, and 720 copepodites were randomly selected from the AQUADVISE sea lice hatchery and divided into groups of 10 gravid females, 10 males, and 20 copepodites.

Experimental design

Adults and copepodites of *C. rogercresseyi* were challenged to swim from the plastic bucket to the aquarium through a circular opening during a 1-hour period under 12 different conditions: water flow speeds of 0, 1, and 2 cm/s, with variations including both the presence or absence of light and fish (*S. salar*) as attractants. Each set of 12 observations was replicated three times (R1, R2, and R3), resulting in a total of 36 observations. The overall experimental timeline spanned a total of six consecutive days.

Each observation started with the introduction of 20 adults (10 gravid females and 10 males) and 20 *C. rogercresseyi* copepodites into a plastic bucket within the aquarium, both filled with seawater. The first trial began with the presence of fish, followed by cleaning the aquarium and all its components to eliminate mucus from the water and start the trials in the absence of fish.

As illustrated in Figure 1, the water started to flow when suckled by a 70 µm filter located inside the plastic bucket, passing through the external filter-pump, and poured into the right side of the aquarium. Once there, the water flowed to the left side of the aquarium and passed through the 6.6 cm diameter opening of the plastic bucket, being suckled again by the 70 µm filter. An adjustable valve located in the external filter pump allowed different water flow speeds to be determined using a Gardena flow meter (L/min). The light was provided by an LED tube that was used to illuminate only the right side of the aquarium.

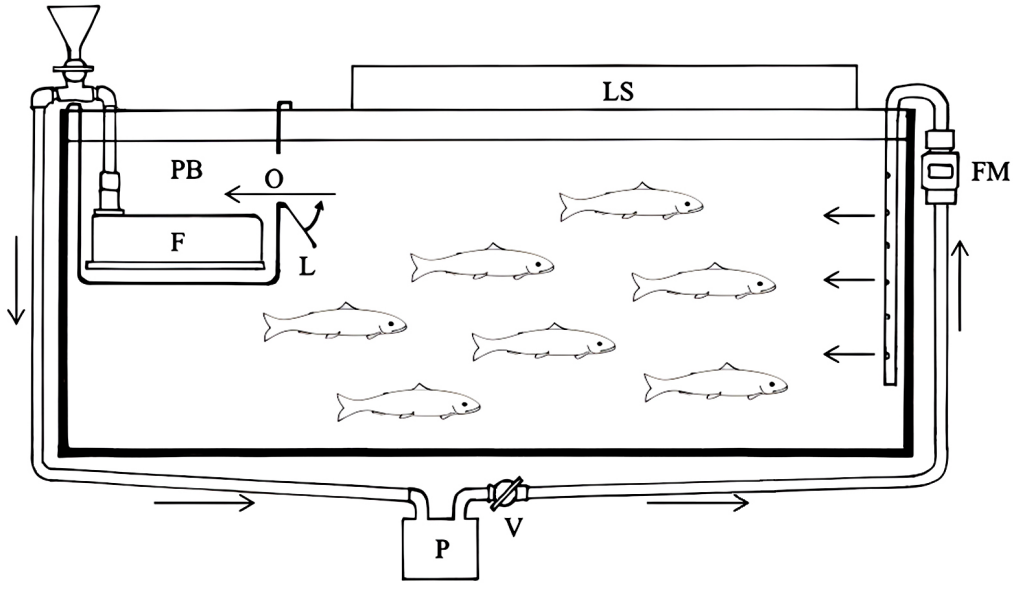

Figure 1.
Scheme of the aquarium, structure, and operational mechanisms. Water circulation is initiated by a 70 µm suction filter (F) located within the plastic bucket (PB). It then proceeds to the external filter pump (P), where a regulated flow is maintained by an adjustable valve (V) and monitored by a Gardena flow meter (FM) before being directed into the aquarium. Upon reaching the aquarium, the water cycles through the circular 6.6 cm diameter opening (O) in the plastic bucket (PB) and is subsequently drawn again by the 70 µm filter. The circular opening (O) was sealed at the end of each 1-hour observation period using the lid (L). LS represents the lighting system.

After 1 h, the circular opening of the bucket was sealed using a plastic plug, while the 70 µm filter continued to draw water from the bucket, allowing it to partially float.

Subsequently, the 70 µm filter and the walls of the plastic bucket were rinsed with fresh water to release the attached adults and copepodites into the remaining water of the bucket. This water was then passed through a 70 µm sieve to concentrate the adult parasites and copepodites. Adult parasites were carefully collected using tweezers and arranged neatly on paper to facilitate their counting. Copepodites were counted in pre-gridded petri dishes using a 10x magnification microscope. This process enabled the determination of the total number of adults and copepodites that had successfully migrated through the opening.

Statistical analysis

The analysis included a 3 × 2 × 2 factorial design, along with their respective interactions, as part of a negative binomial distribution model. The first factor comprised three levels of water speed (0, 1, and 2 cm/s), whereas the second and third factors represented the presence or absence of light and fish (*S. salar*) as attractants, respectively. The age of parasites (adults/copepodites) and sex were treated as categorical variables within the same model, allowing for a comprehensive assessment of their effects on migration dynamics.

Including "age" as a fixed effect could hinder the detection of statistically significant differences in parasite migration between adults and copepodites, as it would require estimating parameters associated with each level of this variable, consuming degrees of freedom and reducing statistical power. By considering "age" as a covariate, its effect on the number of migrating parasites is adjusted, allowing the model to concentrate on the relationships between other factors, such as water flow speed and the presence or absence of light and fish. On the other hand, the sex variable was treated as a covariate instead of a fixed factor due to the inability to determine the sex of the copepodites. This approach effectively controlled the variability associated with sex, allowing for the flexible manipulation of this variable without establishing specific levels for copepodites. By adopting this method, the analysis could isolate the effects of sex more accurately, thereby enhancing the robustness of statistical evaluation.

The time elapsed from parasite harvesting to the initiation of each trial was included as a numerical covariate, considering that a longer waiting time between the parasite harvest and the start of the trials could have affected the swimming capacity of the parasites. The variable "Day" was also included to account for the specific day on which each trial was conducted. This variable was treated as a random effect in the statistical model, allowing for the assessment of any potential temporal effects and slight environmental variations, such as changes in photoperiod, salinity, temperature, or oxygen saturation, that may influence the swimming performance of the parasites.

The replicates were treated as repeated measures given that each set of observations originated from the same experimental conditions. Consequently, the random effect associated with the variable "Day" also contributed to accounting for variability among the different replicates.

The normal distribution of the data was validated using the Shapiro-Wilk test from the SAS® statistical package version 9.3, with P-values < 0.05 for both adults and copepodites. PROC GLIMMIX in SAS® was employed, relying on both Poisson and negative binomial distributions. Ultimately, the results from the models based on negative binomial distribution were used.

RESULTS

Overall, a greater number of adults migrated than copepodites, particularly in the presence of light, regardless of the water speed tested. No differences were observed in the migration between adults and copepodites in relation to the presence or absence of fish (Table 1).

By analyzing each specific combination of variables separately, a higher migration of adults compared to copepodites was observed in the presence of light compared to its absence, especially when fish were present rather than absent, at water flow speeds of 0 and 1 cm/s (Table 2).

The probability of migration was significantly higher in the presence of light than in its absence for both adults ($P < 0.0001$) and copepodites ($P = 0.0015$). However, the migration probability for both adults and copepodites was not affected by the presence or absence of fish or by the different water speed conditions ($P = 0.1238$ in adults; $P = 0.3522$ in copepodites). The interaction between fish and water speed was significant only for copepodites ($P = 0.0033$) but not for adults ($P = 0.1059$) (Table 3).

There were no statistically significant differences in parasite migration according to the variable sex, regardless of the combination of the other factors analyzed ($P > 0.05$). Additionally, neither the time elapsed from parasite harvesting to the initiation of each trial nor the variable "Day" had a statistically significant effect on the migration of the parasites ($P > 0.05$ for both).

DISCUSSION

The infection process, which is crucial for the survival of all parasite species, requires each parasitic species to ensure temporal and spatial alignment of their infective stages in the presence of the host (Sievers, G., personal communication, July 21st, 2017). *C. rogercresseyi* follows a direct life cycle in fish, involving both obligate and free-living parasitic stages. Gravid females typically carry an average of 29 ± 3 eggs in each of their two egg strings (Jaramillo *et al*., 2015), and the infection dynamics of fish by *C. rogercresseyi* are influenced by the ability of copepodites to locate their host (Asencio, 2015). However, this process is time-limited by the endogenous energy

Table 1.
Ability to migrate (expressed as mean and median of the three replicates) in presence or absence of light, presence or absence of fish and three different water flow speed conditions for adults and copepodites of *C. rogercresseyi*.

	Light		Fish		Water flow speed			General
	presence	**absence**	**presence**	**absence**	**0 cm/s**	**1 cm/s**	**2 cm/s**	
n	18	18	18	18	12	12	12	36
Adults								
Mean	11.1	2	7.2	5.9	8	6.3	5.4	6.6
Median	13	1	6.5	6.5	7.5	5	3.5	6.5
Copepodites								
Mean	5.8	3.1	4.6	4.3	5.4	3.8	4.2	4.4
Median	5.5	3	3.5	4.5	5	3	4	4

cm/s = centimeters per second. In the "n" row, each value represents the total number of observations (each 1-hour trial) for each combination of variables. For example, the first value in the table (n=18; light presence) is obtained by multiplying by three (three replicates) the sum of: 2 trials with light at 0 cm/s (one with fish and one without fish), 2 trials with light at 1 cm/s (one with fish and one without fish), and 2 trials with light at 2 cm/s (one with fish and one without fish). This applies to each "n=18", according to its corresponding combination of variables. For "n=12", and using "Water flow speed=0 cm/s" as an example, it is obtained by multiplying by three the sum of: no light, no fish at 0 cm/s; no light, fish at 0 cm/s; light, no fish at 0 cm/s; and light, fish at 0 cm/s. This applies to each "n=12" based on its corresponding combination of variables. The other values in these columns reflect the number of adults and copepodites, expressed as both mean and median, that migrated from the plastic bucket to the aquarium during the total number of trials for each combination of variables "n". The value "n=36" in the "General" column represents the total number of possible combinations (12) multiplied by three, i.e., the total number of observations conducted during the study. The other values in this column represent the mean and median of the previous columns for both adults and copepodites.

Table 2.
The Ability of *C. rogercresseyi* adults and copepodites to migrate (expressed as the median of the three replicates) in the presence or absence of light, presence or absence of fish, and three different water flow speed conditions. The values in this table represent the number of adults and copepodites that migrated from the plastic bucket to the aquarium, expressed as the median across the three replicates of each 1-hour trial for each possible combination of variables.

			Water flow speed		
	Light	**Fish**	**0 cm/s**	**1 cm/s**	**2 cm/s**
Adults	presence	presence	15	13	9
		absence	8	7	11
	absence	presence	3	0	0
		absence	2	0	2
Copepodites	presence	presence	10	9	3
		absence	4	3	9
	absence	presence	3	2	1
		absence	5	0	6

cm/s = centimeters per second.

reserves of copepodites, which constrain their capacity to find and infect a host (Boxaspen, 2006).

Amundrud & Murray (2009) emphasized how wind-driven circulation affects sea lice transport and infection spread between wild and farmed fish, highlighting the role of local environmental conditions. However, even with this taken into account, the limited quantity of infective forms produced by each female, the brief lifespan of the copepodites in plankton, and the widespread presence and expansion of this parasitosis may suggest that the infection process is highly successful and not contingent on external hazards.

Pino-Marambio *et al*. (2007) demonstrated that semiochemicals present in water play a mediating role in the parasite-host interaction between *C. rogercresseyi* and *S. salar*, similar to the relationship observed between *Lepeophtheirus salmonis* and its conspecific females and host fish. In the present study, the migration probability for both adults and copepodites of *C. rogercresseyi* was not influenced by the presence or absence of fish when considered as a single variable. However, the higher migration observed in adults and copepodites in the presence of light—particularly when fish were present rather than absent—at water flow speeds of 0 and 1 cm/s, supports the idea that semiochemicals play a significant role in the parasite-host interaction. The fact that this difference was only noted in the presence of light suggests that light acts as a primary stimulus for *C. rogercresseyi*, prompting the parasite to use its chemosensory systems to locate a suitable host to infect.

The finding that light emerged as a more influential factor than the presence of fish and water speed for both adult ($P < 0.05$) and copepodite migration ($P < 0.05$) supports the phototactic nature of sea lice, as previously documented by Genna *et al*. (2005). If both adults and copepodites of *C. rogercresseyi* are drawn to light, but adults display a greater proclivity for swimming and migration, this suggests that adult parasites may play a significant role in the infection process. Moreover, gravid females may potentially deposit egg strings on the surface of the host fish, with the nauplius I, nauplius II, and copepodite stages persisting within the fish's mucus until they molt into chalimus I, thereby establishing a permanent attachment to the host's scales using the frontal filament until reaching the sexually mature adult stage. This would mean that both the copepodite and the adult stage are infective. Nevertheless, for this to be possible, the time elapsed between the release of the egg strings by adult females and the emergence of the copepodites must be brief. This is particularly important given that the host's continuous mucus renewal may cause both egg strings and nauplius stages adhering to it to be expelled into the water, thus hindering copepodites from emerging within the mucus and initiating the infection process.

According to González & Carvajal (2003), under laboratory conditions, most larvae reach the nauplius II stage by day three of the planktonic phase, with the first copepodites beginning to emerge. By days 5–7, nearly all larvae had moulted into copepodites, which were ready to settle within approximately 5 days at 16.5 °C, 7 days at 9.8 °C, and 9 days at 12.1 °C. This suggests that, particularly during warmer summer temperatures, larvae may have sufficient time to moult into copepodites within the mucus and settle on the host using their hooked pair of antennae. Subsequently, during moulting, they extrude their frontal filaments to establish a permanent attachment to the fish, as previously described by González and Carvajal (2003). Further studies are necessary to experimentally validate this and to gain a more comprehensive understanding of the roles of adults and copepodites of *C. rogercresseyi* in fish infections.

C. rogercresseyi is a parasite found in free-living fishes, such as *Eleginops maclovinus*, *Odontesthes regia*, and *Paralichthys microps*. Initially, the parasite adapted to cultivated *O. mykiss*, subsequently extending its adaptation to *S. salar* (Carvajal *et al*., 1998; Bravo, 2003). This adaptation is particularly favoured by high fish stocking densities on farms, resulting in high prevalence and infection intensities (González *et al*., 2000). Furthermore, the close proximity of salmon farms has facilitated the development and spread of caligidosis in all geographical areas where this activity occurs (Bravo *et al*., 2008).

Caligidosis in Chile is primarily controlled by the application of chemical treatments to fish to diminish the population of adult parasites during their reproductive phase. However, this method of control has led to the emergence of parasite resistance to key drugs employed since the 1980s. The assumption that parasite sensitivity to various chemical products can be restored through the native fish fauna harbouring a sensitive parasite population seems implausible. In fact, the potential role of salmon farms as major reservoirs for resistant parasite strains raises concerns, suggesting that local fish fauna could be extensively infected by these resistant strains.

The superior migratory capacity of adults compared to copepodites reported in this study does not suggest that control strategies such as chemical treatments should focus on adult parasites. In fact, since both male and female *C. rogercresseyi* adults have the ability to detach from the host fish (González & Carvajal, 2003), they may be able to swim through the water column and locate new hosts when exposed to adverse environmental conditions. In contrast, the copepodite extrudes its frontal filament to permanently attach itself to the fish until it reaches the sexually mature adult stage and cannot detach from the host (González & Carvajal, 2003), which creates a significant opportunity for chemical treatments, especially during winter when adverse environmental conditions make the parasite more vulnerable.

In conclusion, light was identified as an attractant for both adult and copepodites of *C. rogercresseyi*, irrespective of the water speed (0, 1, or 2 cm/s) used. A higher number of adults migrated compared to copepodites, especially in the presence of light rather than absence. The presence of fish did not significantly affect sea lice migration behaviour ($P > 0.05$). Overall, adult *C. rogercresseyi* demonstrated superior swimming capabilities compared with copepodites

under identical laboratory conditions. Further research is needed to better understand the roles of *C. rogercresseyi* adults and copepodites in fish infections.

Competing interests

The authors declare that they have no competing interests.

Author contributions

G.S. contributed to the conception, design, and methodology of the study. All study materials were provided by G.S. and both authors worked together during the experimental trials. J.N. conducted the literature search, and the initial draft of the manuscript was authored by G.S. Both authors participated in the critical revision of the later version of the manuscript. G.S. designed Figure 1. As this is a posthumous article regarding the main author, J.N. was the only author who worked on the final version of the manuscript. However, no significant modifications were made to the latest version, which was jointly developed by both authors.

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SHORT COMMUNICATION

Molecular detection of haemobacteria in Colombian wild birds

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ABSTRACT. Colombia shows a high density and variety of bird species, making it one of the most diverse avian territories globally. Antioquia ranks among the top four provinces with the greatest variety of bird species, underscoring the importance of research efforts on the local bird fauna. Therefore, this study aimed to identify bacterial agents in the blood of wild birds from the municipality of Jardín (Antioquia, Colombia) by 16S qPCR sequencing. A descriptive cross-sectional study was conducted using non-probabilistic convenience sampling. Wild birds were captured using mist nets and blood samples were collected from each animal via puncture using sterile lancets in the brachial vein, and a drop of blood was collected on filter paper for qPCR analysis. The 16S gene in bacterial genomes was found in 13 out of 46 wild birds of the Passeriform and quasi-Passeriform orders, captured at three different locations within the study municipality at altitudes ranging from 1,665 to 2,034 m.a.s.l. Seven different bird species were recorded and four different haemobacteria were identified (i.e. Exiguobacterium spp., Escherichia coli, Stenotrophomonas spp., and Stenotrophomonas maltophilia). This study contributes to the knowledge in Colombia by identifying four different hemobacteria in wild birds. Further research is required on the health status of these birds and the attributable impacts on their populations and other related factors, including humans.

Keywords: Blood, *Exiguobacterium* spp., *Escherichia coli*; hemobacteria; *Stenotrophomonas* spp.; wild bird.

INTRODUCTION

Colombia, with its remarkable avian biodiversity, ranks among the most bird-diverse countries in the world. The province of Antioquia is one of the regions with the highest diversity of bird species, making essencial the study of the local avifauna (Vélez *et al.*, 2021). However, the high density of avian populations poses potential risks for the transmission of zoonotic diseases, highlighting the importance of the One Health approach. One Health emphasises the interconnectedness of human, animal, and environmental health, recognising that many diseases, particularly zoonoses, arise from human interactions with wildlife (Mackenzie & Jeggo, 2019). This interconnectedness is especially relevant in regions such as Colombia, where urban expansion and habitat conversion bring humans, domestic animals, and wildlife into increasingly close contact.

The risk of zoonotic bacterial transmission from wild birds is significant, particularly in areas in which human activity has altered the natural environment. Wild birds can carry a range of pathogens, including zoonotic bacteria, which may affect both humans and animals. Among these pathogens are *Escherichia coli*, *Salmonella* spp., Mycobacteria, *Pasteurella multocida*, and *Chlamydia psittaci*, which are known to cause severe illnesses in birds and can sometimes infect humans or domestic animals. *Escherichia coli*, that can cause septicemia, and diarrhea, respiratory distress, and lethargy (Bélanger *et al.*, 2011; Knöbl *et al.*, 2011; Borges *et al.*, 2017); *Salmonella* spp. which can lead to septicemia, and enteritis and weight loss (Pennycott *et al.*, 2006; Dos Santos *et al.*, 2020); Mycobacteria causing avian tuberculosis, which results in chronic illness and can be fatal due to weight loss, diarrhea, and respiratory issues (Gaukler *et al.*, 2009); *P. multocida*, responsible for fowl cholera, leading to acute septicemia or chronic infections in birds, causing from sudden death to chronic respiratory problems and joint infections (El-Demerdash *et al.*, 2023; Hashish *et al.*, 2023); and *C. psittaci* that can cause respiratory distress, diarrhea, and lethargy in birds, being also zoonotic (Liu *et al.*, 2019; Amery-Gale *et al.*, 2020; Stokes *et al.*, 2020).

The increasingly frequent interactions among humans, livestock, and wildlife in regions such as Antioquia increase the risk of zoonotic disease outbreaks, underlining the im-

portance of continuous monitoring and research on local bird populations. Understanding the prevalence of these bacteria in wild bird species is crucial not only for conservation efforts but also for public health and the economy. By monitoring the spread of these bacteria, conservationists and health professionals can work together to mitigate the risk of zoonotic transmission and protect both biodiversity and human health (Levin & Parker, 2012).

Given these challenges, employing a One Health approach—integrating the environmental, veterinary, and human health sectors—is essential. Such an approach enables early detection of zoonotic pathogens and helps prevent outbreaks that could impact wildlife conservation, public health, and the poultry industry (Chan *et al*., 2013; Naqvi *et al*., 2017). Therefore, this study sought to identify bacterial agents in wild bird in Jardín Antioquia) using molecular techniques to contribute to both wildlife conservation and public health efforts.

MATERIALS AND METHODS

Study area and bird sampling

A total of 46 wild birds of the Passeriform and quasi-Passeriform orders were captured in a natural area corresponding to a tropical montane cloud forest at six different locations within the study municipality at altitudes ranging from 1,665 to 2,053 m.a.s.l. in the municipality of Jardín (Southwest Antioquia, Colombia). Twenty different bird species were recorded (i.e. *Sporophila nigricollis*, *Myiozetetes similis*, *Zonotrichia capensis*, *Zimmerius chrysops*, *Stilpnia vitriolina*, *Thraupis episcopus*, *Tangara gyrola*, *Melanerpes rubricapillus*, *Tangara arthus*, *Eubucco bourcierii*, *Streptopelia decaocto*, *Atlapetes albinucha*, *Chlorophanes spiza*, *Momotus aequatorialis*, *Molothrus bonariensis*, *Ramphocelus dimidiatus*, *Elaenia flavogaster*, *Euphonia laniirostris*, *Elaenia frantzii*, and *Diglossa sittoides)*. Sampling was carried out for 2 weeks (17 July to 30 July 2022).

The capture processes followed the methodologies proposed by Ralph *et al*. (1996) and Álvarez *et al*. (2004), using monofilament nylon mist nets. After capture, the birds were sheltered in capture bags, where they remained for a maximum of 20 min post-capture. Subsequently, each bird was identified according to Hilty and Brown (2021), and Remsen *et al*. (2022).

Blood samples were collected from the brachial vein posterior to the left wing of each bird after cleaning with antiseptic alcohol. A puncture was made using sterile lancets, collecting a drop of blood on grade 3HW filter paper (65 g/m2) inside a Ziplock bag with a silica gel bead. It was immediately verified that each bird was in the condition to fly, and thereafter, it was released and returned to its habitat. The samples were labelled with consecutive numbers, species names, and identification of the study area, and transported to the laboratory where they were refrigerated at 4°C until analysis on 23 October 2022.

DNA extraction, 16S amplification, and sequencing

Two portions were extracted from each filter paper sample using a small hole punch. These portions were centrifuged with a PBS wash at 41,000 rpm × 120 min, and the supernatant was removed. The resulting sediment was resuspended in 450 µL of PBS. Total genomic DNA was extracted using the Blood or Body Fluids Spin protocol (QIAmp DNA Extraction Mini Kit ®, Qiagen, Germantown, MD), according to the manufacturer's instructions. The extracted DNA was quantified using spectrophotometry (Nanodrop), ensuring a concentration of at least 50 ng/µL, according to the method described by Xu *et al.* (2020).

The genetic material extracted from blood samples was subjected to qPCR for the detection of intraerythrocytic hemobacteria using specific primers (see Data availability statement) targeting 600 to 800 bp fragments of conserved regions of the 16S gene in bacterial genomes. Positive controls were provided by TestMol S.A.S laboratory, and DNase-RNase-free sterile water (Cat No.: 129114, Qiagen, Germany) was used as a negative control. Specific primers for Cytochrome B genes in mammals were used as internal controls for DNA extraction and qPCR (Pfeiffer *et al*., 2004). The use of these controls not only helped prevent contamination and ensure the validity of the results but also allowed for the replication of the experiment in future studies.

The qPCR assay was performed in a Mic qPCR Cycler 4 channel (BioMolecular Systems, Australia) using protocols standardised by the laboratory. MasterMix for real-time PCR (SYBR Green, Thermo Fisher Scientific®) was used with a final volume of 19 µL, including 5 µL of DNA. The thermal profile was run with an initial denaturation of 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 57°C, and 1 min at 72°C, and a final extension for 5 min at 72°C.

The amplification products were sent to the National Center for Genomic Sequencing (Macrogen®, Korea) for DNA purification and sequencing.

Molecular data analyses

A taxonomic name was designated for each sequence using BLAST (Altschul *et al*., 1990). To construct and edit the phylogenetic trees, sequences were aligned using ClustalW (Higgins *et al*., 1992) in BioEdit (Hall, 1999) to derive the consensus sequence. Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou & Nei, 1987) based on evolutionary distances computed via the Composite Maximum Likelihood (ML) method. MEGA version X (Kumar *et al*., 2018) was employed to perform both the phylogenetic and molecular evolutionary analyses. To refine the tree, the best-fit substitution model was evaluated as TN93 + I (Tamura & Nei, 1993) using the Find Best Fit substitution model tool. The robustness of the phylogenetic tree was tested using bootstrap analysis with 1,000 replicates, providing a measure of support for branching patterns. The resulting trees were visually edited and refined using MEGA X software to ensure clarity and accuracy.

RESULTS AND DISCUSSION

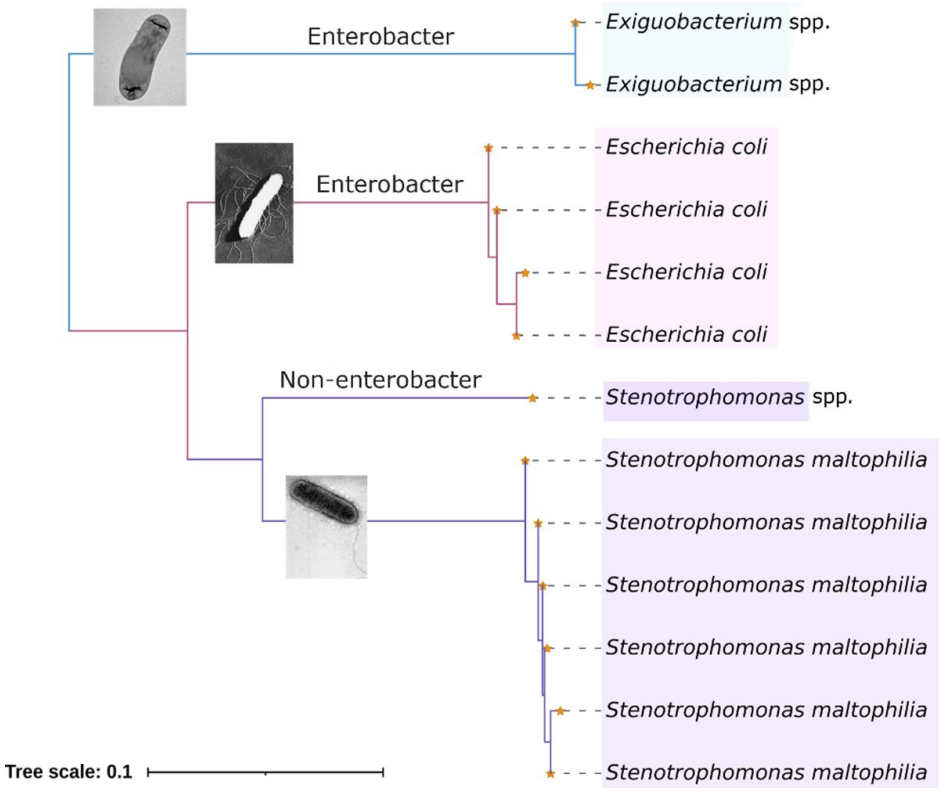
The objective of this study was to identify bacterial agents in the blood of wild birds through molecular approaches in an important area of native birds in Colombia. This area is renowned for its variety of ecosystems, and apart from threats linked to habitat, local bird populations might face susceptibility to infectious diseases, potentially exacerbating the challenges they face.

The 16S gene in bacterial genomes was found in 13 of 46 wild birds of the Passeriform and quasi-Passeriform orders. Positive birds were captured at three different locations within the study municipality, at altitudes ranging from 1,665 to 2,034 m.a.s.l. Four different haemobacteria were identified (i.e. *Exiguobacterium* spp., *E. coli*, *Stenotrophomonas* spp., and *Stenotrophomonas maltophilia*) (Figure 1) in seven different bird species recorded, with five adults and one juvenile (when possible, to determine the age group), as well as one male and one female (when possible, to determine the sex) (Table 1). No bird was recaptured.

Stenotrophomonas maltophilia, known for its pathogenicity in humans, can also impact wildlife, including wild birds. Affected individuals exhibit a range of symptoms including pneumonia, septicaemia, encephalitis, endocarditis, suppurative lymphadenitis, abscesses, and other disease syndromes (Brooke, 2012; Adegoke *et al*., 2017). This bacterium displays an open pan-genome, indicating extensive genetic variability across isolates from diverse environments (Xu *et al*., 2023). In South America, particularly in Peru, studies have identified multiple genotypes with varying resistance profiles, including significant resistance to trimethoprim/sulfamethoxazole and ceftazidime (Toledano *et al*., 2023) and to β-lactams and aminoglycosides in China (Li *et al*., 2023; Xu *et al*., 2023). This multidrug-resistant bacterium is also an opportunistic pathogen found in various environments such as water, rhizospheres, and other animals (Adegoke *et al*., 2017; Brooke, 2021). It possesses virulence factors, such as biofilm formation, motility, and antimicrobial resistance mechanisms, making it a concern for susceptible populations, including wild birds. In addition, studies have highlighted the ability of this bacterium to interact with other microorganisms, indicating its potential impact on diverse ecosystems (Brooke, 2021). While the research primarily focuses on clinical and environmental sources, the interaction of *S. maltophilia* with wild birds specifically is not directly addressed in the provided contexts. However, considering the species' adaptability and diverse

Table 1.
Characterization of the 13 study birds with positive molecular results to 16S gene of haemobacteria.

Cons.	Hemobacteria identified (% of compatibility in BLAST)	Altitude (m.a.s.l.)	Scientific name	Common name (in Spanish)	Common name (in English)	Sex	Age group
1	*Stenotrophomonas maltophilia* (100%)		*Stilpnia vitriolina*	Tángara rastrogera	Scrub tanager	ND	Adult
2	*Stenotrophomonas* spp. (100%)		*Thraupis episcopus*	Azulejo común	Blue-gray tanager	ND	Adult
3	*Stenotrophomonas* spp. (100%)		*Zimmerius chrysops*	Atrapamoscas caridorado	Golden-faced tyrannulet	ND	ND
4	*Stenotrophomonas maltophilia* (99%)	1,655	*Zonotrichia capensis*	Gorrión de montaña	Rufous-collared sparrow	ND	Juvenile
5	*Stenotrophomonas maltophilia* (97%)		*Tangara gyrola*	Tángara cabecirufa	Bay-headed tanager	ND	ND
6	*Stenotrophomonas maltophilia* (99%)		*Zimmerius chrysops*	Atrapamoscas caridorado	Golden-faced tyrannulet	ND	ND
7	*Escherichia coli* (97%)		*Zonotrichia capensis*	Gorrión de montaña	Rufous-collared sparrow	Male	ND
8	*Exiguobacterium* spp. (92%)		*Tangara arthus*	Tángara dorada	Golden tanager	ND	Adult
9	*Exiguobacterium* spp. (92%)	1,760	*Tangara gyrola*	Tángara cabecirufa	Bay-headed tanager	ND	ND
10	*Stenotrophomonas maltophilia* (99%)		*Eubucco bourcierii*	Torito	Red-headed barbet	Female	Adult
11	*Escherichia coli* (100%)		*Tangara arthus*	Tángara dorada	Golden tanager	ND	ND
12	*Escherichia coli* (97%)	2,034	*Thraupis episcopus*	Azulejo común	Blue-gray tanager	ND	Adult
13	*Escherichia coli* (100%)		*Stilpnia vitriolina*	Tángara rastrogera	Scrub tanager	ND	ND



Notes: Own work

Figure 1.
Phylogenetic tree of 16S nucleotide sequences (600-700 bp) obtained from the blood of wild birds. All ambiguous positions were removed for each pair of sequences (pairwise deletion option).

habitats, further investigation into the potential role of wild birds in the transmission or ecology of *S. maltophilia*, as well as the genus *Stenotrophomonas*, is mandatory.

Escherichia coli, a common bacterium in the gastrointestinal tract of warm-blooded animals, including wild birds, can significantly affect the gut microbiome. *E. coli* strains exhibit diverse circulating genotypes in South America and globally, with significant implications for public health. Recent studies have highlighted the prevalence of multidrug-resistant strains and their transmission pathways across various environments. Extended-spectrum β-lactamase (ESβL)-producing *E. coli*, particularly ST10, has been identified in South American llamas, indicating a global spread of these clones from wildlife to humans (Cárdenas-Arias *et al.*, 2023). In Bolivia, enterotoxigenic *E. coli* (ETEC) strains, notably ST218 and ST410, have been found in both clinical cases and environmental samples, emphasising the role of contaminated water as a reservoir for pathogenic strains (Calderón-Toledo *et al.*, 2023). Andean condors have been found to harbour critical priority *E. coli* strains with extensive resistance profiles, linking wildlife to the dissemination of antimicrobial resistance genes in ecosystems (Fuentes-Castillo *et al.*, 2020). Furthermore, the detection of critically important antimicrobial drug-resistant *E. coli* in various bird species suggests a potential for interspecies transmission of resistance, emphasising the need for increased research to understand the dynamics of drug-resistant bacteria in wildlife populations (Mukerji *et al.*, 2020; Murphy *et al.*, 2021; Rybak *et al.*, 2022).

The presence of virulence factors in *E. coli* strains from wild birds, such as those associated with pathogenicity, such as Shiga toxin-producing *E. coli* and atypical enteropathogenic *E. coli*, further emphasises the potential impact of *E. coli* on the gut microbiome of wild birds and the environment at large (Murphy *et al.*, 2021). In addition, the diversity of *E. coli* phylogroups within individual wild animals and communities reflects the broad distribution and genetic diversity of this bacterium, with implications for biodiversity conservation, agriculture, and public health, especially at the urban-wildland interface (Lagerstrom & Hadly, 2023). Although the focus on *E. coli* pathogenicity and resistance is crucial, it is equally important to consider the environmental and ecological factors that contribute to its spread, highlighting the need for integrated surveillance and intervention strategies.

The genus *Exiguobacterium* is part of the coryneform bacterial group, characterised by aerobic growth, non-spore-forming, irregularly shaped, gram-positive rods (Farrow *et al*., 1994). These bacteria have been found in a variety of habitats ranging from cold to hot environments (Vishnivetskaya *et al*., 2009). Although *Exiguobacterium* strains have been isolated from human clinical samples such as skin, wounds, and cerebrospinal fluid, their clinical relevance is not well understood (Hollis & Weaver, 1981). This genus is more commonly detected in water and soil, suggesting the possibility of sample contamination or environmental presence. The genetic diversity within the genus suggests a wide range of ecological niches, although specific circulating genotypes in South America remain underexplored compared to those in other regions.

The bird populations in Colombia play a crucial role in its ecosystems. According to the IUCN Red List of Threatened Species in 2023, 92 bird species are classified as vulnerable (VU), endangered (EN), or critically endangered (CR). Therefore, monitoring diseases that could impact these species, such as bacterial deseases diseases, is essential (Hollis & Weaver, 1981).

Nevertheless, substantial scientific knowledge gaps remain concerning the distribution of these pathogens and their biological interactions across various regions of Colombia. For instance, Antioquia Province boasts of a rich diversity of bird species and vectors, providing an ideal setting for studying the ecology of these pathogens (Pérez-Rodríguez *et al*., 2014).

There is an urgent need to assess whether the hemobacteria identified in this study pose a threat to the conservation of local bird species. This necessitates comprehensive sampling across a broader range of altitudes, encompassing diverse habitat types and timeframes. It is crucial to estimate the prevalence of these bacteria and other relevant pathogens in critical areas for native birds. This will provide valuable insights into the evolutionary and ecological dynamics of the disease in regions with high host and parasite diversity.

As haemobacterial screening becomes routine in wild bird populations, future research should focus on identifying factors influencing infection and transmission in the area.

DECLARATIONS

Competing interest statement

The authors declare that they have no conflicts of interest.

Ethical considerations

This study was approved by the Ethics and Bioethics Committee for Animal Experimentation of the Universidad Cooperativa de Colombia (Bioethical Concept No. BIO293, Act No. 21-109 of April 28, 2022). In addition, the ANLA granted the Universidad de Antioquia the "Framework permit for the collection of specimens of wild species of biological diversity for noncommercial scientific research purposes" (Resolution 0524 of May 27, 2014, and Resolution 1461 of December 3, 2014), supporting this research approach.

Author contributions

ACF and NMCV had the idea for the article and led the study conception and design. The samples were collected by ACF and AOAP. The molecular analysis of the samples was performed by ILJD, and data analysis by ACF. The literature search and data analysis, as well as the critical revision of the manuscript, were performed by all the authors. The first draft of the manuscript was written by AOAP and NMCV, and all the authors commented on previous versions of the manuscript. All the authors have read and approved the final manuscript.

Data availability statement

All data and materials are available with the corresponding author; the test conditions of the molecular processes are part of the intellectual property of the company TestMol© S.A.S. Dataset of obtained sequences is available at https://doi.org/10.5281/zenodo.14141016

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SHORT COMMUNICATION

Serological evidence of *Coxiella burnetii* in sheep herds from Lonquimay valley in the Chilean Andes

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ABSTRACT. *Coxiella burnetii* is the causative agent of Q Fever, a worldwide zoonotic disease that causes acute flu-like illness and chronic manifestations in humans in the form of endocarditis, hepatitis, and other symptoms. Domestic ruminants are the most important reservoirs of the bacteria, transmitting infections to humans during the calving/lambing season through direct contact with contaminated fetal tissues or inhalation of dust particles. The aim of this study was to provide serological evidence and estimate the individual true seroprevalence of *C. burnetii* exposure in sheep herds in Lonquimay, and to characterize farmers' knowledge of coxiellosis. A disease freedom survey was conducted in 30 sheep herds selected from the Indigenous Territorial Development Program database (PDTI). A total of 260 blood samples were tested using ELISA for *C. burnetii* antibody detection. Disease freedom and true animal-level prevalence were estimated, and a questionnaire was administered during farm visits to characterize farmers' zoonotic knowledge. A positive result was found in 3% (1) of the sampled herds, and the true animal prevalence (mTALP) was higher than previous unpublished estimations (mTALP 4.2%, 95% PPI 1.6%–9%). The estimated probability of the study sheep population not being free of *C. burnetii* was 34%. A lack of knowledge regarding *C. burnetii* or the consequences of Q Fever was detected, along with risky behaviors that could facilitate pathogen transmission. This study revealed evidence of exposure to *C. burnetii*, with low individual and herd-level prevalence. Initiatives to improve zoonotic knowledge among farmers need to be implemented in the short term.

Keywords: Q Fever; coxiellosis; sheep; prevalence; zoonoses; Chile.

INTRODUCTION

Coxiella burnetii is the etiologic agent of Q Fever, a zoonotic disease that usually manifests as asymptomatic cases or acute flu-like symptoms. A chronic form of the infection has been documented, presenting endocarditis, hepatitis, and lung fibrosis (Straily *et al*., 2017). Transmission to humans occurs mainly through direct contact with birth fluids and tissues from domestic ruminants (main reservoirs), consumption of raw milk or derived products, or inhalation of contaminated dust particles (Angelakis & Raoult, 2010). In animals, infection is mostly asymptomatic; however, reproductive disorders, such as abortion and low birth weight, may be observed (Palmer *et al*., 1983).

Q fever outbreaks have been reported in several countries, including Australia, the USA, Spain, France, Germany, Great Britain, and Switzerland (Bellini *et al*., 2014; Biggs *et al*., 2016; van Woerden *et al*., 2004). The largest reported outbreak occurred in The Netherlands between 2007-2010, associated with goat and sheep farming, with over 4,000 confirmed cases (Dijkstra *et al*., 2012; Schneeberger *et al*., 2014).

In Chile, the first reported outbreak occurred in 1998 when members of the Agricultural and Livestock Service (SAG) were infected through contact with imported sheep. This led to the declaration of Q Fever as an occupational disease, and its inclusion in the list of notifiable diseases (Ministerio de Agricultura, 2000). Throughout the following years, no data about *C. burnetii* presence in the country was published. In 2017, an outbreak of atypical pneumonia occurred in persons related to dairy farms in southern Chile (Ministerio de Salud, 2017), 20% of 357 suspected Q Fever cases were subsequently confirmed (Tapia *et al*., 2020). In response, research has been performed to update the disease status and provide insights into the infection risk (Cornejo *et al*., 2019; Hernández-Agudelo *et al*., 2023; Tapia *et al*., 2021; Weitzel *et al*., 2016). However, research has focused on dairy herds, leaving the situation for other ruminants unexplored. Nevertheless, SAG reported a seroprevalence of 0.041% in sheep herds near the zone in which the 2017 outbreak occurred; however, these data have not been published (Rosenfeld, 2022).

Lonquimay is a district located in the Araucanía Region in southern Chile, the country's third most important livestock region. Lonquimay has the second highest sheep population in the region, primarily destined for extensive fattening (Rojas *et al*., 2022). Situated in a high-al-

titude valley. To the east and southeast, it is contained by the Andes Mountain range and border with Argentina (Figure 1). The territory experiences harsh, cold winters that limit agricultural activities, which are mostly for basic subsistence. The temperature in winter can drop to -20°C, making the area nearly inaccessible, whereas in summer, can reach 30°C (Santibañez *et al*., 2017). An important characteristic is the use of state-owned high-elevation pastures for transhumance from December to May, which allows farmers to store the forage and use it to feed animals between May and December, when direct grazing is hindered by snow (Catrileo & Alvarado, 2009).

Due to the predominantly subsistence agriculture and significant poverty is expected that farmers' knowledge about zoonotic diseases is limited. Given this context, increasing the awareness of zoonotic and infectious diseases is crucial. The aim of this study was to provide serological evidence and estimate individual true seroprevalence of *Coxiella burnetii* exposure in sheep herds in Lonquimay and to characterize farmers' knowledge of coxiellosis.

MATERIAL AND METHODS

Study population, design, and sample size estimation

The study population consisted of sheep herds from Lonquimay commune. This contains nearly 1300 sheep herds (Rojas *et al*., 2022), most of which use an extensive,

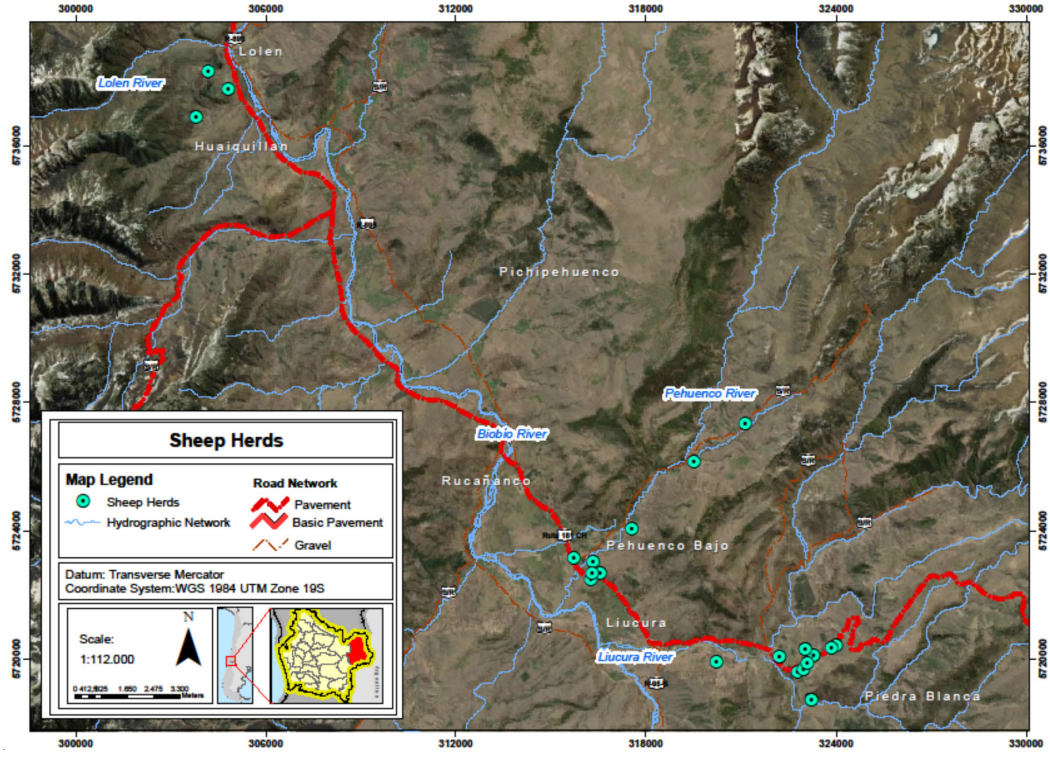

Figure 1.
Spatial distribution of sheep herds sampled to obtain serological evidence of *Coxiella burnetii* in Lonquimay.

self-subsistence fattening system; the lambs are sold mainly through non-established commercial channels.

A disease freedom survey was conducted between August 2022-August 2023. The required sample size was calculated using a one-stage sampling method for disease freedom assessment. Considering a population size of 1300 herds, expected herd-level prevalence of 10%, and 95% confidence level, a sample size of 29 herds was required. On every farm visited, 8-10 adult sheep were tested, depending on herd size. In herds smaller than 8 animals, all adult sheep were tested. Herds were selected from the Indigenous Territorial Development Program (PDTI) of the Agricultural Development National Institute (INDAP), based on their willingness to participate.

Sample collection and laboratory analysis

Five mL blood samples were collected by puncturing the jugular vein using additive-free vacutainers. The samples were kept cold until reception at the Veterinary Diagnostic Laboratory of the Universidad Católica de Temuco, Chile. Serum was obtained by centrifugation, and then frozen and stored until processing. A commercial ELISA test IDEXX Q

Fever Ab Kit (Maine, USA) was used to detect the presence of *C. burnetii* antibodies (IgG) following the manufacturer's instructions. This kit uses purified antigens from phases I and II of *C. burnetii*.

The ratio between the optical density (OD) of the samples (S) and the OD of the positive control (P) was calculated to obtain the ELISA results. The cut-off values for the S/P ratio were obtained from the test insert and interpreted as follows: samples with S/P% ≥ 40% were positive, samples with S/P% ≥ 30% and ≤ 40% were suspect, and samples with S/P% < 30% were negative. Suspect samples were considered to be positive.

Farmers' knowledge of Coxiellosis

Knowledge about *C. burnetii* infection was characterized through a survey (Table 1). The questionnaire included questions related to knowledge about coxiellosis, Q Fever, introduction and dissemination risk behaviors, biosecurity, and health events in humans, probably related to the suspicion of Q Fever.

Statistical Analysis

Sample size estimation was performed using the epi.ssdetect function from the R package "EpiR" (Stevenson *et al*., 2015). A Bayesian approach was used to assess i) the mean true animal-level prevalence (mTALP) corresponding to the distribution of the true prevalence in an average infected farm, and ii) an overall true animal-level prevalence (oTALP) corresponding to a fitted beta distribution across all infected herds. The model was constructed as follows:

$$y[i] \sim binomial(Ap[i], n[i]),$$
$$Ap[i] = Tp[i] \times Z[i] \times Se + (1 - Tp[i] \times Z[i]) \times (1 - Sp),$$
$$Z[i] \sim bernoulli(tau),$$
$$Tp[i] \sim beta(\alpha, \beta),$$
$$ATp[i] = Tp[i] \times Z[i],$$
$$Se \sim beta(\alpha, \beta),$$
$$Sp \sim beta(\alpha, \beta),$$
$$tau \sim beta(\alpha, \beta)$$

where *y[i]* is the number of positive animals in each infected herd, *Ap[i]* is the apparent prevalence in each herd, and *n[i]* is the sample taken in every farm. *Tp[i]* is the true prevalence for a truly infected herd. To obtain the actual true prevalence, a mixture of a point probability mass at zero and continuous beta distribution was used to model the probability of animal prevalence being zero as *ATp[i]=Tp[i]×Z[i]* (Verdugo *et al*., 2018). The prior values of the sensitivity (Se) and specificity (Sp) parameters of the ELISA kit were sourced from literature (Lurier *et al*., 2021); Se was set as beta(53.43, 86.43) and Sp as beta(100, 3.02). The prior distribution values of true individual animal prevalence were derived from an unofficial estimation of *C. burnetii* seropositivity in Chile, which is the most likely value (Rosenfeld, 2022). The value reported for sheep herds in Brazil was adopted as the upper level (Guatteo *et al*., 2011) and set as a beta distribution (beta(1.07, 19.21)); the herd-level prevalence *(tau)* was set as beta(1.39, 4.53) (Guatteo *et al*., 2011). Analysis was conducted using OpenBugs 3.2.3 (Lunn *et al*., 2009) through the R2Openbugs R package (Sturtz *et al*., 2005). The assessment of model convergence involved simulating three parallel chains and visual evaluation of the trace and density plots. Gelman-Rubin diagnostics were also used. The model was simulated for 25,000 iterations, with 5,000 discarded as a burn-in period.

The probability of absence of *C. burnetii* in sheep was estimated using the probability formula for surveys to substantiate freedom from disease proposed by Cameron and Baldock (1998), using Epitools-Epidemiologic Calculators (Sergeant, 2018) and making the following assumptions: animal-level prevalence, value obtained by model estimation ELISA Se 40% and Sp 99 % (Lurier *et al*., 2021); and error of 5 %.

Sensitivity analysis

For the sensitivity analysis, two sets of prior values were employed. In Set (A), the test performance was modified by fitting the beta distributions for both Se and Sp derived from the values provided by the test manufacturer. For Se, a confidence level greater than 95% and a mode of 99% were used, whereas for Sp, a confidence level greater than 95% and a mode of 97.3% were applied. In Set (B), the prior parameter for *tau* was represented by a diffuse beta distribution (1,1) (Table 2).

RESULTS AND DISCUSSION

Before the 2017 outbreak, information on *C. burnetii* status in animals and humans was scarce, and several studies have been conducted since then. Results from these studies have shown that *C. burnetii* is highly present in cattle (Hernández-Agudelo *et al*., 2023); however, no studies in sheep have been published to date. The present study found a lack of knowledge about general concepts related to zoonoses among sheep farmers (Table 1). Only 11%, 18%, and 14% of farmers, respectively, stated that they knew the meaning of "zoonotic disease", "coxiellosis" or "Q Fever", although 86% were aware that they could contract diseases from animals; 37% of farmers observed abortions in the last lambing season, only 7% declared having experienced flu-like symptoms in the same period, and 7% said that they did not seek medical help in response. None of the farmers stated that they had suffered from chronic diseases that could be related to chronic Q Fever.

Livestock farming in Lonquimay is characterized by small-scale, extensive practices oriented towards own consumption, with low levels of technification. Farmers in this region tend to be older and have lower education levels (Baez, 2005). These factors, combined with geographical isolation, infrequent veterinary medical attention, and difficult access to health services, may contribute to insufficient awareness of the health risks associated with animal production.

With respect to biosecurity, the questionnaire revealed

Table 1.
The outcomes of a questionnaire designed to evaluate zoonotic understanding and hazardous behaviors.

Question	% †
Do you raise animals of different species (cattle, sheep, goats)?	75
Have you heard about zoonotic diseases?	11
Have you heard about coxiellosis?	18
Have you heard the disease called Q Fever?	14
Do you know that you can get diseases from your farm animals?	86
Do you introduce animals from more than one origin, with unknown status, to your herd?	75
Do you implement a quarantine period before animal introduction?	10
Do you buy supplementary forage from other sheep herds?	64
Do you attend lambing?	39
Do you use personal protection elements when attending lambing?	57
Do you implement a disposal method for parturition membranes?	70
Do you carry out disinfection of animal housing?	75
Do you carry out peri-partum ewe isolation?	46
Last lambing season, did you observe abortion, stillbirth, or weak lamb birth?	36
Do you remember if you experienced flu-like symptoms in the last lambing season?	7
If you experienced flu-like symptoms, did you seek medical attention?	7
Do you suffer chronic illness compatible with suspicion of chronic Q fever?	0

†Percentages correspond to positive answers to the questions

management practices that could increase the risk of introduction and dissemination in a herd; 75% of farmers introduced animals from sources with unknown status, and only 10% used quarantine before introduction. However, this primarily involves the introduction of male breeding animals, which results in a lower impact on transmission (Amin *et al*., 2022).

Risk-related conducts for farmer security were also detected. For instance, 39% of farmers attend lambing and only 57% use protective equipment when attending lambing or other management practices. Nevertheless, 70% of farmers have a disposal method for parturition membranes, and 75% disinfect animal housing. In view of the lack of awareness observed in the survey, it was expected that risky behaviors would promote the transmission of *C. burnetii*. The 2017 outbreak highlighted the need to inform and educate farmers about the potential risks and consequences of *C. burnetii* infection.

Thirty (30) herds were tested, resulting in a total of 260 samples. At least one ELISA-positive animal was found in only one herd (3%) (CI95% = 0.36%-14%). In this herd, 5 animals tested positive (2% of all animals tested). The mTALP was estimated at 4.2% (95% PPI = 1.6-9%), while oTALP was 4.6% (95%PPI = 1.7-10%). *C. burnetii* presence was found to be higher in the population studied than has been reported in unofficial information from SAG, which, in the context of the 2017 Q Fever outbreak, reported a sheep seroprevalence of 0.041% from herds located near cases (Rosenfeld, 2022); however, this estimation is not fully comparable because no information is known about the study methodology used. Nevertheless, in South America, *C. burnetii* seroprevalence is highly variable, ranging from 0% to 66%, suggesting that the presence of *C. burnetii* depends vastly on the characteristics of the country and the specific zone (Epelboin *et al*., 2023).

Table 2.
Posterior distribution Median and 95 % PPI for mTALP and oTALP, Se and Sp with two sets of prior values for sensitivity analysis (Set A: Prior sensitivity 99%, Prior specificity 97.3%, Set B: Prior for tau Beta (1,1) 0.041%, sensitivity and specificity are the same as default model.

Parameter	Posterior distribution (Median (95% PPI))		
	Default Model	Set A	Set B
mTALP	4.2 (1.6-9)	4 (1.6-10)	4 (1.6-8)
oTALP	4.6 (1.7-10)	4.3 (1.7-10)	4.1 (1.6-8.5)

The low animal-level prevalence can be explained by the less likely introduction and transmission of *C. burnetii* within and between herds owing to the extensive productive system used, especially during the lambing season (Carrié *et al*., 2019), and the restricted animal trade, which mostly involves males for breeding (Baez, 2005) (Table 1). The environmental conditions were also considered. The lambing

season begins at the end of winter (September) and lasts until late October, when pastures are still covered with snow. During this season, the animals are mainly fed with stored forage, reducing the probability of *C. burnetii* being acquired from the soil. Although bacterial survival under conditions of extreme cold has been reported, the cold and humidity due to snow reduce dust and dryness, decreasing airborne transmission (EFSA, 2010). Another important aspect is ineffective reproductive management, resulting in a longer breeding season with a low concentration of births despite the relatively small herd sizes, leading to a lower risk of spreading and environmental contamination.

It is likely that the seroprevalence of *C. burnetii* is underestimated. This can be attributed to the selection of ELISA for diagnosis because of its low sensitivity (40%) when used as a screening method. This low sensitivity is caused by false negatives in infected animals due to long exposure periods, infected animals that are still in their incubation period, and infected animals with slow or failed humoral response (Berri *et al*., 2001).

Disease freedom probability estimation showed that the likelihood of the studied population not being free of *C. burnetii* is 34%. This suggests that even when a small number of positive samples were found, it is unlikely that the population is free from the disease.

In the sensitivity analysis, mTALP and oTALP show no significant variation between analysis with Se and Sp set with the manufacturer's values (Set A), and analysis with the herd level prevalence set with a diffuse beta distribution (1,1) (Set B) (Table 2). Across all models, mTALP and oTALP remained very similar, with overlapping PPI intervals. Model convergence ensures that the estimation obtained can be safely utilized for drawing inferences (Dodds & Vicini, 2004).

CONCLUSIONS

This study revealed evidence of exposure to *C. burnetii* in sheep herds in Lonquimay, indicating a low individual and herd-level prevalence. The characteristics of the production system, geography, and environment of the study area are likely to provide a low-risk scenario for bacterial transmission. Future studies are needed to estimate the prevalence of *C. burnetii* active infection in at-risk animals and to investigate in greater depth the factors contributing to this low-risk transmission scenario. Initiatives to improve zoonotic knowledge among farmers need to be implemented in the short term.

DECLARATIONS

Competing interest statement

The authors declare that they have no competing interests.

Ethical statement

All procedures were approved by the Universidad Católica de Temuco Ethical Research Committee on 29 December 2023, reference number: CEIUCT1229001/23).

Author contributions

OAV: supervision, conceptualization, methodology, data curation, formal analysis, funding acquisition, writing-review & editing. SN: investigation, validation. CA: investigation, validation, writing-review & editing. RP: investigation, resources.

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SHORT COMMUNICATION

First report of metals and metalloids on bone and claw tissues of Humboldt penguins (*Spheniscus humboldti*)

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ABSTRACT. Samples of bones (humerus) and claws of adult Humboldt penguins (*Spheniscus humboldti*) were opportunistically obtained from twenty-seven carcasses at two important nesting sites in northern Chile: Chañaral Island (CHI) and Pan de Azúcar Island (PAI). The concentrations of trace elements (Cu, Zn, Pb, Ni, Fe, Se, As, Br, Mn and Cr) were determined by X-ray fluorescence. The highest levels (mean ± standard deviation, µg/g dry weight) of Cu (26.57 ± 4.08), Zn (163.9 ± 42.7), Pb (1.86 ± 1.53), Ni (0.31 ± 0.03), Se (7.70 ± 4.87) and Cr (0.25 ± 0.24) were detected in bones, whereas the highest levels of Fe (3,162 ± 1,579), As (6.75 ± 4.21), Br (0.12 ± 0.06) and Mn (76.7 ± 47.9) were found in claws. In bones, Se and Ni levels were higher ($P < 0.05$) in CHI than in PAI. In claws, the contents of Pb, Fe, and Mn were higher at CHI than those at PAI, whereas only As exhibited higher contents at PAI than those found at CHI. The trace element content in the claws and bones found herein may be the result of either acute or chronic exposure to penguins, respectively. These findings may serve as a baseline for further studies to design adequate and opportune plans to protect this vulnerable species.

Keywords: Seabirds; trace elements; marine pollution; northern Chile; X-ray fluorescence.

INTRODUCTION

The Humboldt penguin (*Spheniscus humboldti*) naturally resides along the Pacific coast of South America, from Perú to Chile (De la Puente *et al*., 2013), with 23,800 mature individuals. In Chile, this species inhabits between Arica and Chiloé in colonies that are mostly concentrated between 21°S and 34°S (Wallace & Araya, 2015), with a population that ranged from 40,000 to 47,000 in the past (Schlosser *et al*., 2009), and has shown a remarkable decline, with a population of less than 5,000 pairs, where the presence of polluting waste is mentioned among the greatest threats to the species (Simeone *et al*., 2018).

Because it is a species classified as vulnerable by the IUCN Red List of Threatened Species in 2020 (https://www.iucnredlist.org), it is extremely difficult to obtain samples such as blood or internal organs, which are considered invasive to birds. Studies focusing on the assessment of metal contamination in the bones and claws of penguins remain quite limited because these birds are protected by law, and thus, these samples can only be obtained in cases where opportunistically dead bodies are available.

Trace elements can have toxic effects on living organisms (Rodríguez & Mandalunis, 2018). Heavy metals can occur naturally (e.g. volcans) at low levels in the environment, and in larger amounts, they can impact health (Newman, 2015). The occurrence of trace elements in aquatic ecosystems is mainly due to anthropogenic activities such as antifouling coatings, sewage discharge, waste incineration, coal combustion, oil spills, pipe corrosion, and solid waste disposal (Bargagli, 2000; Caccia *et al*., 2003; Duruibe *et al*., 2007; Zhang & Ma, 2011). In northern Chile, the massive development of mining activities has had a detrimental effect on coastal ecosystems due to elevated metal concentrations (Ramirez *et al*., 2005; Stauber *et al*., 2005). Numerous industries (e.g., mining, fishing, seaports) and cities (e.g., Arica, Iquique, Antofagasta, Chañaral) in northern Chile discharge waste materials into the sea, leading to increased levels of certain metals that are considered hazardous to ecosystem health and biota (Vermeer & Castilla, 1991; Cortés & Luna-Jorquera, 2011).

Metal (loids) such as Pb, Cu, Fe, Mn, As, Ni and Zn may be sequestered in bones, which become a concern in animal's health as these metals can interfere with calcium homeostasis, inhibit bone-forming cells (osteoblasts), stimulate bone absorption cells (osteoclasts) and alter the mineralization process. This can lead to decreased bone density, increased risk of fractures and impaired skeletal development in growing animals (Newman, 2015; Rodríguez & Mandalunis, 2018; Ciosek *et al*., 2023). Non-essential elements (e.g., Pb, Cd, Ag, Ti, and As) are extremely toxic with no biological functions, whereas essential elements (e.g., Cu, Fe, Cr, Se,

Br, Ni, Zn and Mn) are required in very small amounts because they perform vital functions for the maintenance of animal life (McCall *et al*., 2014; Nordberg & Nordberg, 2016). Some metals (e.g. Pb, Cu, Cr, Ni, and Zn) and metalloids (e.g. As) can induce severe adverse effects in birds, even an increase in the mortality rate (Newman, 2015). Commonly, Zn accumulation in birds has been linked to binding to metallothionein, but it can also accumulate in the muscles and bones (Wastney *et al*., 2000). Some less-known metals, such as Mn, can accumulate in the bone, cartilage, and tissues that are dense with mitochondria, and overexposure to Mn can cause serious health problems in birds (Sarnowski & Kellam, 2023). At high concentrations in biota, Ni is both toxic and carcinogenic (Newman, 2015), and wild birds from polluted environments can accumulate in bone higher Ni concentrations than other internal tissues (Outridge & Scheuhammer, 1993). Adverse effects of Se (a non-metal) in wild aquatic birds have been linked to pollution of the aquatic environment by anthropogenic activities, including impaired reproduction, reduced growth, histopathological lesions, among others (Hoffman, 2002). There is no available data on the non-metal Br levels in avian wildlife, but some evidence indicates that Br can alter the metabolism of rats and broilers with subsequent deleterious effects (Du Toit & Casey, 2010; Pavelka, 2004).

Human nails have been shown to be valuable biomarkers for heavy metal exposure, providing insight into long-term exposure due to their slow and continuous growth (Sukumar & Subramanian, 2007). Other studies have revealed that elements such as As, Cd, Hg and Pb accumulate in nails, reflecting both environmental and dietary exposure (Guallar *et al*., 2002; Shokoohi *et al*., 2022). In birds, claws are similar to human nails, making them a useful bioindicator of heavy metal contamination. Mercury (Hg) concentrations in the talons of bald eagles (*Haliaeetus leucocephalus*) correlated significantly with levels in other tissues, suggesting that talons may serve as a non-lethal alternative for contaminant monitoring (Hopkins *et al*., 2007).

Despite the potential risks posed by metal contamination to seabirds, to the best of our knowledge, no studies have been conducted on bones or claws of Humboldt penguins. Consequently, the primary goals of this study were to evaluate, for the first time, the concentrations of selected trace elements in adult Humboldt penguins and to compare these levels with those in the existing literature. By acquainting these objectives, this work contributes to filling a gap in trace element accumulation in the claw and bone of Humboldt penguins, adding light to the potential risks to this vulnerable species and their habitats.

MATERIALS AND METHODS

Bone and claw samples of Humboldt penguin carcasses were collected opportunistically from Pan de Azucar and Chañaral Islands (Figure S1, Supplementary material). Pan de Azúcar Island, situated 1km away from the coastline is a site on the northern coast of Chile highly impacted by mining activities (Celis *et al*., 2014), whereas Chañaral Island is an isolated place, with little anthropogenic influence, 7 km away from the coastline and 100 km north of Coquimbo Bay. Both islands are important sites where Humboldt penguins nest. Bone (left femur) and claw samples of adult Humboldt penguin carcasses were carefully and opportunistically collected in December 2015 (nesting period of the species). Disposable plastic gloves were used for handling and storing samples. The bones (n=27) and claws (n=27) were collected from multiple individuals across the penguin colonies, as it was not feasible to assess metal contamination in each individual separately, but rather as a collection of that species. To maintain sample integrity, clean plastic containers and sealed plastic bags (Ziploc®) were used, where each sample was stored separately to avoid cross-contamination between samples.

First, the bones and claws were cleaned with alcohol swabs to ensure the removal of any external contamination (Mateo-Lomba *et al*., 2022). After cleaning, the samples were thoroughly rinsed (Milli-Q water) and left to dry at room temperature before element analysis.

Recently, low-cost X-ray fluorescence (XRF) has been used in wildlife and ecotoxicological studies with promissory results (Specht *et al*., 2019; Hampton *et al*., 2021; Celis *et al*., 2022). Thus, a portable XRF Niton XL3t GOLDD+ (Thermo-Fisher Scientific, Omaha, USA) was used to determine the burden of chemical elements in the samples. The blank was a certified 99.99% silicon dioxide (SiO2) analysed for every 20 samples. Precision and accuracy were verified using international reference standards Rare Earth Ore "CGL 124" (USZ-42 Mongolia Central Geological Laboratory), with precision > 98 % and accuracy within 95–99 %. The QA/QC detection limits (µg/g) were as follows: 1.43 for Cu, 8.72 for Zn, 0.26 for Pb, 0.07 for Ni, 23.52 for Fe, 0.39 for Se, 9.27 for As, 0.07 for Br, 0.04 for Mn and 0.16 for Cr. The uncertainty of the measurements (µg/g) were for bones as follows: Cu (1.08 ± 0.35), Zn (9.37 ± 3.01), Pb (1.47 ± 1.30), Ni (0.06 ± 0.01), Fe (105.96 ± 356.71), Se (4.15 ± 8.08), As (6.82 ± 5.28), Br (0.0024 ± 0.0014), Mn (0.13 ± 256.71) and Cr (0.43 ± 0.42); for claws were Cu (0.84 ± 0.10), Zn (6.08 ± 3.98), Pb (0.40 ± 0.38), Ni (0.04 ± 0.01), Fe (34.38 ± 36.52), Se (2.09 ± 6.23), As (9.12 ± 6.44), Br (0.004 ± 0.002), Mn (0.07 ± 0.11) and Cr (0.15 ± 0.10).

Nonparametric statistical methodologies were employed because the data did not meet the assumptions of normality and homoscedasticity even after applying a log transformation. The differences among the element levels were determined using Kruskal-Wallis analysis. Post hoc tests were conducted with critical differences in the mean rank. To estimate the relationship between the trace element concentrations in the bones and claws, ordinary least squares regression was used. The uncertainty value (σ) for the portable XRF was determined using the following equation: $\sigma = (C/NET)\ (BGK/t)^{0.5}$, where C represents the element concentration, BKG is the estimated background count obtained

through fitting, t is the measurement time, and NET is the net element count derived from the Gaussian function in the fitting process (Zhang *et al*., 2021). For bones and claws, Spearman rank correlation coefficients were calculated to determine the relationship between the trace element levels in bones and claws. Statistical significance was set at $P < 0.05$. The data were analysed (t-Student) using SPSS 27.0.

RESULTS AND DISCUSSION

Information on the levels of trace elements in Humboldt penguins is fragmentary; only a few studies have focused on the bones of penguins worldwide, whereas no studies have been conducted on penguin claws (Espejo *et al*., 2017). The concentrations of Cu, Zn, Pb, Ni, Fe, Se, As, Br, Mn and Cr are shown in Table 1, which were detected in both matrices and locations studied. The highest levels of Cu, Zn, Pb, Ni, Se and Cr were detected in bones, whereas the highest contents of Fe, As, Br and Mn were found in claws. The highest mean concentrations detected corresponded to Fe (3,162.2 µg/g) at Chañaral Island (CHI), whereas Br exhibited the lowest levels (0.05 µg/g) at Pan de Azúcar Island (PAI). In bones, the levels of Se and Ni were significantly higher at CHI than those levels found at PAI, whereas Cu, Zn, Pb, Fe, As, Br, Mn and Cr contents showed no statistical differences. In the claws, the levels of Pb, Fe, and Mn were higher in CHI than in PAI ($P < 0.05$), whereas As values were not ($P < 0.05$, Table 1).

At PAI the levels of Zn, Pb and Mn in the bones were higher than those levels found in the claws, while As and Br contents showed the opposite ($P < 0.05$). At CHI, the concentrations of Cu, Ni and Se found in the bones were statistically higher than those detected in the claws, but Fe levels were higher in claws than in bones ($P < 0.05$). These findings in bones and claws of this species from the two studied geographical areas may be linked to different anthropogenic sources, since Pan de Azúcar Island presents major human activities (e.g. mining, industries) than Chañaral Island (Celis *et al*., 2014).

Significant relationships were found between the concentrations of the trace elements (Figure 1). In both bone and claw samples, a positive correlation was noted between Cu-Zn, Cu-Ni, Cu-As, Zn-Se, Fe-Mn, Fe-Cr, Se-As and Mn-Cr ($P < 0.05$). Only in bones was a significant positive correlation noted for Cu, Zn, and Ni with Cr, and for Ni with Pb, Fe, and Mn. Similar findings were noted for Se with Cu, Pb, Ni, Zn-As, and As with Cu in the claws (Figure 1). Similarly, Squadrone *et al*. (2018) observed a positive correlation between Cu-Ni and Fe-Cr in feathers of African penguins (*Spheniscus demersus*). Another study by Celis *et al*. (2015) reported a significant positive correlation between Cu-As, Zn-As and Cu-Zn in excreta of Adélie penguins (*Pygoscelis adeliae*) from Antarctica. Probably, the positive correlations indicate that the elements for all the study colonies are of the same source. On the other hand, negative correlations ($P < 0.05$) were found between Cu-Br, Ni-Br, Fe-Se, As-Mn and Se-Mn, in both bones and claws.

The contents of As, Cu, Pb, Mn, Se and Cr in the bones found herein are higher, the Ni contents are lower, while the Zn levels are within the range of those levels detected in bones of penguins of the genus *Pygoscelis* from different locations of Antarctica (Table S1, Supplementary material). The comparison with others aquatic birds revealed that our Ni and Pb levels in bones are lower than Ni (10.5-36.1 µg/g dw) and Pb (32.4-59 µg/g dw) levels, whereas our Cu levels are higher than Cu (4-8.2 µg/g dw) levels reported by van Eeden

Table 1.
Concentrations of chemical elements (µg/g, d.w) in bones and claws of Humboldt penguins sampled at nesting sites from Pan de Azúcar Island (PAI, n=23) and Chañaral Island (CHI, n=4).

	Bones		Claws	
	PAI	**CHI**	**PAI**	**CHI**
Cu	23.17 ± 6.80 a	26.57 ± 4.08 a *	21.50 ± 5.76 a	17.18 ± 2.23 a **
Zn	163.9 ± 42.7 a *	159.0 ± 26.7 a	128.3 ± 69.9 a **	130.6 ± 72.1 a
Pb	1.86 ± 1.53 a *	1.64 ± 0.42 a	0.49 ± 0.39 a **	1.23 ± 0.62 b
Ni	0.27 ± 0.03 a	0.31 ± 0.03 b *	0.22 ± 0.04 a	0.26 ± 0.03 a **
Fe	2,247.1 ± 2,281.6 a	1,272.3 ± 566.1 a *	1,361.8 ± 1,037.3 a	3,162.2 ± 1,579.1 b **
Se	1.95 ± 5.05 a	7.70 ± 4.87 b *	2.53 ± 5.5 a	0.38 ± 3.05 a **
As	3.53 ± 3.01 a *	4.33 ± 1.31 a	6.75 ± 4.21 a **	1.98 ± 1.83 b
Br	0.05 ± 0.03 a *	0.08 ± 0.05 a	0.12 ± 0.06 a **	0.09 ± 0.04 a
Mn	45.1 ± 53.4 a *	30.57 ± 14.93 a	19.86 ± 32.35 a **	76.7 ± 47.9 b
Cr	0.25 ± 0.24 a	0.23 ± 0.06 a	0.14 ± 0.08 a	0.24 ± 0.11 a

Data are shown as mean ± standard deviation. Different letters between collecting sites for the same biological matrix indicate significance at $P < 0.05$. Differences between bones and claws for the same location are indicated with an asterisk ($P < 0.05$).

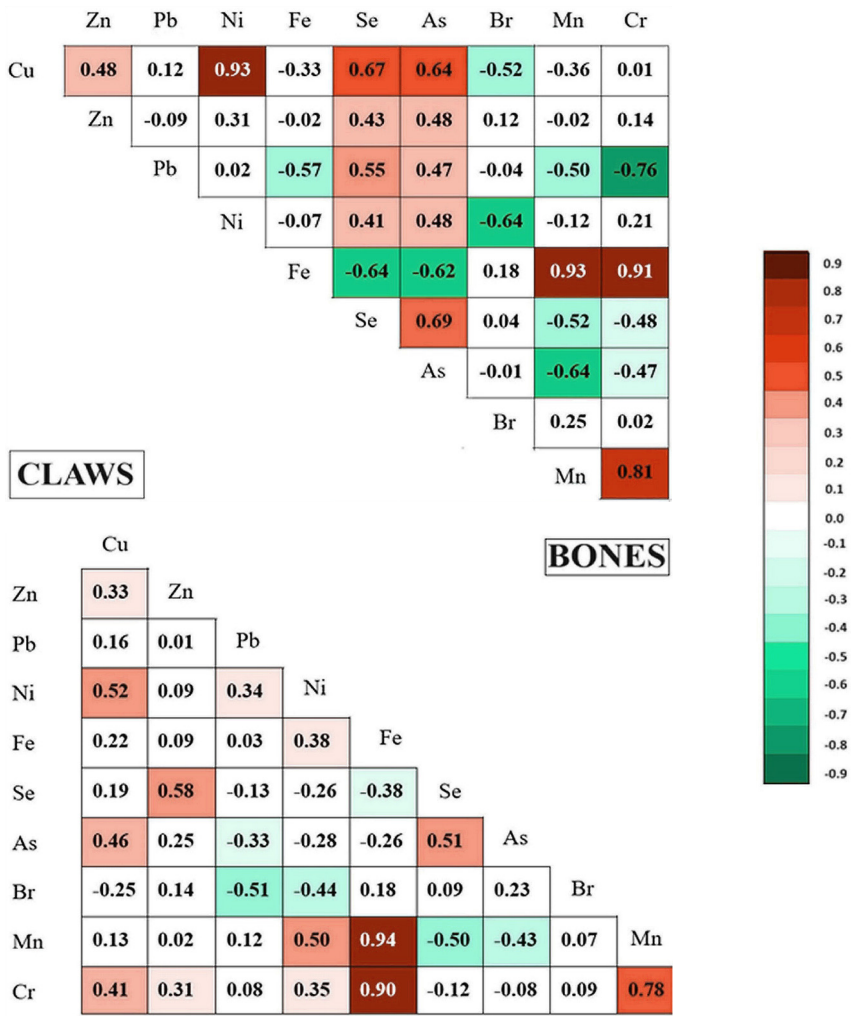

Figure 1.
Correlation matrix among the concentrations of selected trace elements.

and Schoonbee (1996) in bird bones from a metal-polluted wetland in South Africa. Our mean Fe levels in bones were 79.4 times higher than the mean Fe levels measured in the bone (tarsometatarsus) of diving ducks (*Aythya ferina*) from the Baltic Sea (Kalisinska *et al*., 2007). The higher As, Cu, Pb, Mn, Se and Cr concentrations in bones detected herein could be explained by the major copper-mining activities occurring in northern Chile (Vermeer & Castilla, 1991; Ramírez *et al*., 2005; Cortés & Luna-Jorquera, 2011), where the nesting sites focus of this study are located.

Pollutants in bones accumulate during the lifetime of the organism; therefore, metals in Humboldt penguin bones may be considered an indicator of long-term exposure, as stated by Barbosa *et al*. (2013) in Antarctic penguins. Our Pb levels are < 10 µg/g dw, a threshold known to be toxic to birds (Scheuhammer, 1987), which suggests very small biological effects in the Humboldt penguin. However, there was a difference between Pb levels in penguins from Chañaral Island (a colony with almost no contact with humans) and penguins from Pan de Azúcar Island (a site with mining and human presence), possibly due to the indirect effects of human impact on Humboldt penguins. Metal (loid) intoxication has a negative impact on human health, as long/short term exposure to high Zn, As, Cu, Cr, Pb, Ni, and Fe concentrations alters the bone remodelling process, leading to the development of different bone pathologies (Rodríguez & Mandalunis, 2018). Chronic exposure to metals may pose a threat to penguins (Espejo *et al*., 2017a) and humans (Newman, 2015). When an organism is exposed to metal contamination, claws tend to accumulate trace elements from six months to 1.5 years, whereas bones are more representative in terms of years or even decades of exposure (Rabinowitz, 1991; Gutiérrez-González *et al*., 2019). The accumulation of metal (loids) in the bone, although not causing any problems, can trigger the reappearance of chronic toxicity by mobilisation of these elements in the body (Silbergeld *et al*., 1988). The data on bones and claws found herein can be useful for future research to deter-

mine if there are differences in the temporality of exposure and accumulation dynamics. Trends observed in claws and bones over time would result from either acute or chronic exposure to penguins, respectively, an issue that needs more attention.

Studies on trace metals in vertebrate animals are useful for extrapolating their potential effects in humans (Newman, 2015). It is estimated that there will be an increasing demand for trace elements in the manufacture of new products (e.g. solar panels, wind turbines, and electronic devices) in the near future, with a 300% increase in the demand for chemical elements such as Pb and Ni, among others (Erel *et al*., 2021). This raises the concern that the increasing use of various toxic metals may result in high concentrations in humans, predominantly in populations that are not fortunate enough to live in regulated and monitored regions. For this reason, the increased use of metals must be accompanied by the maximum recycling of metals and consideration of environmental and toxicological aspects in the selection of metals for industrial use (Babayigit *et al*., 2018). This study proved that penguin bone may be used to monitor trace element contamination in aquatic ecosystems. Further research is required in this regard.

In conclusion, this study adds novel data regarding the accumulation of trace elements in penguin bones and it is the first study to measure the levels of trace elements in penguin claws. Most of the elements studied herein in the bones of Humboldt penguins were higher than those previously reported in bones of different penguin species elsewhere. Our findings add valuable data on trace element accumulation in the Humboldt penguin on the northern coast of Chile. Also, this study proved that X-ray fluorescence is a useful low-cost analytical procedure for assessing trace elements in the bones and claws of penguins. Because claws are a continuously growing tissue, the question of whether claws can provide reliable spatial and temporal data on trace metal contamination in penguins is an issue that needs to be further investigated. Considering that Humboldt penguins are vulnerable species which face a dramatic population decline, this study adds valuable information that can help to elucidate whether metal contamination affects this species. Further studies are needed to better understand any possible cause in order to be able to implement measures to reverse such a decline.

DECLARATIONS

Competing interest statement

The authors declare that they have no conflicts of interest.

Ethics statement

This study was approved by the Research and Animal Ethics Committee of the Faculty of Veterinary Sciences, Universidad de Concepción, Chile.

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Author contributions

Adesina provided the concepts, data analysis, and writing; Espejo worked with data collection and analysis; Celis worked with writing of the manuscript; Sandoval collaborated with data analysis; Specht revised the manuscript and analyzed the data.

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CASE REPORT

Subepiglottic cyst with aspiration pneumonia in a Japanese Black calf

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ABSTRACT. A four-month-old Japanese Black calf with stridor was diagnosed with a subepiglottic cyst and aspiration pneumonia. Despite treatment, the calf died 17 days after hospital admission. The present case suggests a link between the presence of the subepiglottic cyst and the development of aspiration pneumonia.

Keywords: aspiration pneumonia, calf, endoscopy, subepiglottic cyst.

Subepiglottic cysts have been reported as a cause of upper airway obstruction (Koch & Tate, 1978), and numerous cases of subepiglottic cysts in horses have been documented with established diagnostic and therapeutic procedures in horses (Tulleners, 1991; Hobo *et al*., 1994; Dougherty & Palmer, 2008; Salz *et al*., 2013). Conversely, a few subepiglottic cysts have been reported in cattle (Mattoon *et al*., 1991; Kirmizigül *et al*., 2008; Yoneshige *et al*., 2022), with no complications beyond wheezing, respiratory noise, or dyspnea. Consequently, the significance of diagnosing and treating subglottic cysts in cattle and their prognosis remains inadequately addressed. Herein, the authors describe a case of a subepiglottic cyst with aspiration pneumonia in a Japanese Black calf.

A four-month-old Japanese Black male calf (107 kg), initially diagnosed with stridor by a local veterinarian, was presented to the Animal Hospital for Large Animals at Kitasato University. The calf was treated with a mixture of penicillin (10,000 IU/kg) and streptomycin (12.5 mg/kg) (Meiji Seika Pharma Co., Ltd., Japan) and dexamethasone (5 mg/head) (ZENOAQ, Japan) for one week prior to hospitalization, but the stridor persisted, and the general condition deteriorated. Upon examination, the calf exhibited anorexia, dysphagia, pulmonic murmur, and wheezing sounds. Ultrasonography (MyLabOne VET; Esaote Europe B.V., Netherlands) revealed pulmonary consolidations in the right third and fourth intercostal spaces. An endoscopic examination (endoscope VQ5112B; Olympus Corporation, Japan) through the nasal cavity, revealed a cyst-like structure on the subepiglottis (Figure 1) that, obstructed the trachea on inspiration (Figure 1A), and was pushed outwards by the closed epiglottis while breathing and obstructed the airway on exhalation (Figure 1B). Subsequently, bronchoalveolar lavage was performed, and the fluid was subjected to bacterial culture by outsourcing to a company (Meiji Seika Pharma Co.,

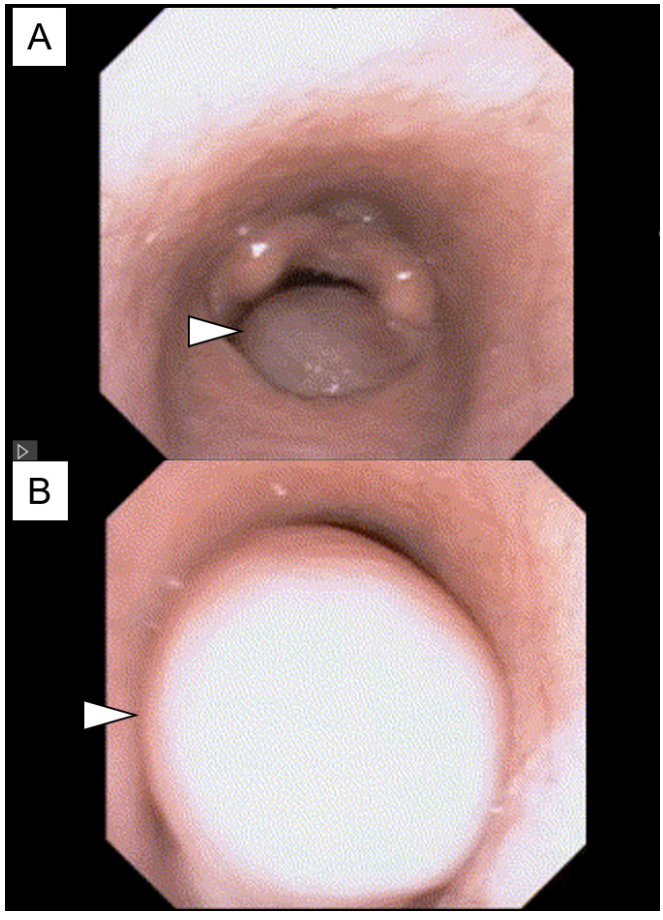

Figure 1.
Subepiglottic cyst observed by endoscopy. A: on inspiration, a cyst-like structure in the subglottis obstructs the trachea; B: on exhalation, the cyst-like structure appears large in the foreground, pushed outwards by the closed epiglottis. Arrowhead: the cyst-like structure.

Ltd., Japan), isolating *Truperella pyogenes*. Drug susceptibility testing showed that isolates were susceptible to penicillin, ampicillin, cefazolin, ceftiofur, tylosin, doxorubicin, tiamulin, tianfenicol, florfenicol, enrofloxacin, norfloxacin, and marbofloxacin.

Based on the results of clinical examinations, the case was diagnosed as laryngitis and pneumonia with the cyst. The treatment was then started with dexamethasone (2 mg/day) and marbofloxacin (2 mg/kg, Marbocyl; Meiji Animal Health, Japan), and a mixture of procaterol (1 mL, MEPTIN Inhalation Solution 0.01%; Otsuka Pharmaceutical Co., Ltd., Japan), acetylcysteine (1 mL, Mucofilin 20% Inhalant Solution; Eisai Co., Ltd., Japan), and tyloxapol (2 mL, Arevel; Alfresa Pharma Corporation, Japan) in 3 mL of physiological saline for inhalation via nebulizer. Although the wheezing decreased with treatment, the pneumonia signs progressed, and the patient died 17 days after admission.

Necropsy revealed a 3 cm diameter subepiglottic cyst with a grayish-white spot (approximately 3 mm in diameter) on the top of the cyst white-beige spot (Figure 2A). The cyst was filled and taut. The thin cyst wall (approximately 1 mm thick) adjacent to laryngopharyngeal mucosa (Figure 2B), contained a cloudy aqueous solution (approximately 14 mL). The epithelium lining the cyst (Figure 2C) was histologically similar to submucosal gland ductular epithelium (Figure 2D). The cloudy fluid collection within the cyst was aseptically collected using a sterilized needle and a 10 mL syringe for bacterial culture, and *T. pyogenes* was isolated, likewise the procedure for the bronchoalveolar lavage fluid collected on the first day of hospitalization. Aspiration pneumonia was also observed in the anterior lobe of the right lung, with plant materials suggesting aspiration of feed, foreign body multinucleated giant cells, bacterial colonies, and fibrinosuppurative bronchopneumonia (Figure 3).

In the present case, the subepiglottic cyst was likely originated from the dilation of submucosal gland duct, as the histological features matched those of submucosal gland ductular epithelium without villi. Similar histological char-

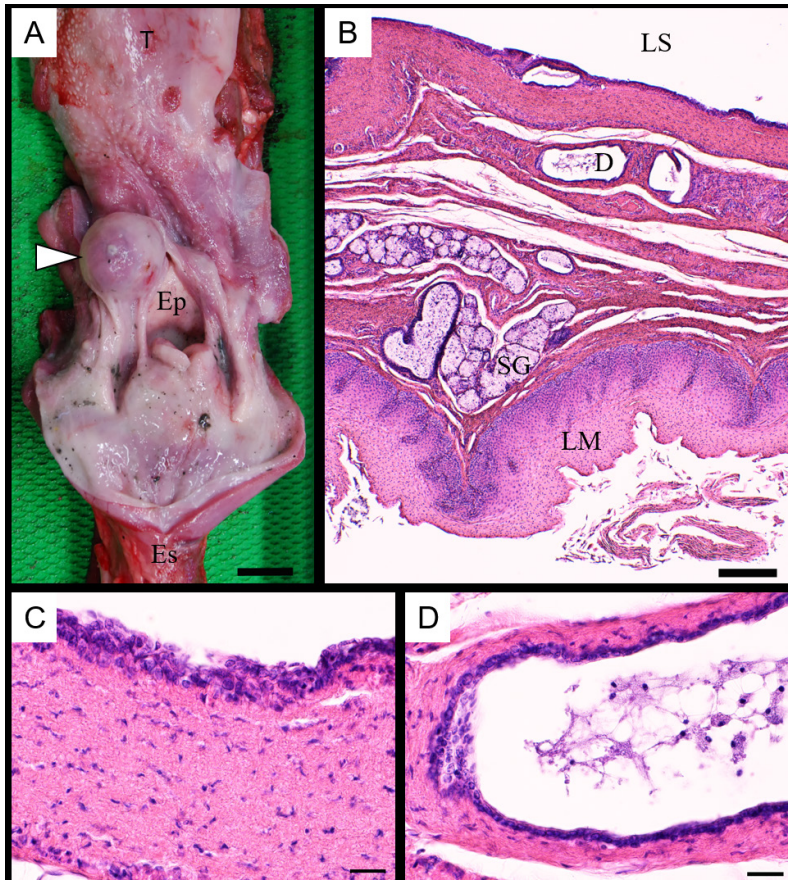

Figure 2.
Subepiglottic cyst. A: the cyst is observed under the epiglottis (bar = 2 cm); B: cyst wall adjacent to the mucosal lining (bar = 250 μm); C: epithelium lining the cyst (bar = 25 μm); D: submucosal glandular duct: the cyst epithelium resembles the ductular epithelium of the submucosal glands (bar = 25 μm).
Arrowhead: cyst; D: duct of salivary gland; Ep: epiglottis; Es: esophagus; LS: luminal surface of the cyst; LM: laryngopharyngeal mucosa; SG: salivary gland; T: tongue.

acteristics have been reported in calves (Yoneshige *et al*., 2022) and dogs (McCally *et al*., 2012). Therefore, it is suggested that submucosal gland is a major origin of animal subepiglottic cysts.

Aspiration pneumonia secondary to the subepiglottic cyst was deemed the cause of death. Surgical removal of the cyst was not performed due to the calf's compromised condition, likely exacerbated by aspiration pneumonia. Early diagnosis and surgical intervention are crucial for a favorable prognosis in cases of subepiglottic cysts. Endoscopy is the gold standard for evaluating the upper respiratory tract in horses (Desmaizieres *et al*., 2009) and to diagnose subepiglottic cysts (Tulleners, 1991; Salz *et al*., 2013). In addition, it has been proven that X-ray (Mattoon *et al*., 1991), ultrasonographic examination, and computer tomography (Yoneshige *et al*., 2022) are useful for diagnosing subepiglottic cysts in calves. Previous cases have shown favorable outcomes following surgical intervention in cattle and other species (Mattoon *et al*., 1991; Hobo *et al*., 1994; McCally *et al*., 2012; Salz *et al*., 2013; Yoneshige *et al*., 2022). In the present case, the presence of the cyst was confirmed by endoscopy and the patient was diagnosed with a subepiglottic cyst before death and could not be removed surgically owing the compromised condition of the patient meaning that these imaging tests should have been performed before our clinical visit. Therefore, subepiglottic cysts should be considered in the differential diagnosis for calves presenting with wheezing, and appropriate diagnostic procedures should be conducted early to prevent general condition deterioration or concomitant diseases.

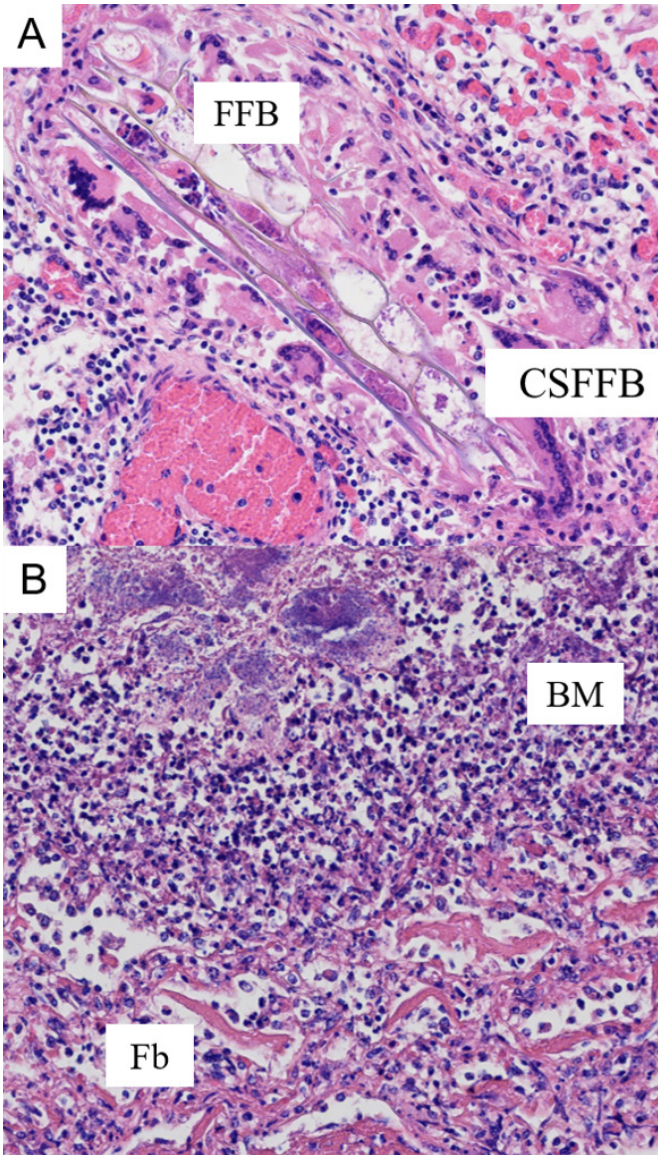

Figure 3.
Aspiration pneumonia in the anterior lobe of the right lung. A: multinucleated giant cells surround the fiber-like foreign body; B: bacterial masses and fibrin are present in the lung parenchyma. FFB: fiber-like foreign body, CSFFB: cells surround a fiber-like foreign body, BM: bacterial mass, Fb: fibrin.

In this case, it was impossible to determine whether the primary disease was a subepiglottic cyst or aspiration pneumonia. In horses, it has been reported that subepiglottic cysts may less frequently be associated with aspiration pneumonia (Koch & Tate, 1978). On the other hand, cattle may be at higher risk for aspiration pneumonia than horses, since rumen contents are a major cause of aspiration pneumonia (Panciera & Confer, 2010). This patient presented persistent wheezing as the main clinical sign, and although the ultrasound examination on the initial day of hospitalization confirmed pneumonia lesions, the clinical symptoms, such as bronchial breath sound or cough, were not clear. During the period of hospitalization and treatment, the general condition of the case deteriorated, and the animal died. Moreover, *T. pyogenes* was isolated from both bronchoalveolar lavage and cyst fluid which may suggest a link between the subepiglottic cyst and aspiration pneumonia, although the sequence of events remains unclear. White-beige patches on the surface of the cyst may indicate that bacteria isolated from the cyst have invaded.

In conclusion, this is the first report of a subepiglottic cyst and aspiration pneumonia in a Japanese Black calf. Further investigation is needed to elucidate the relationship between these conditions.

DECLARATIONS

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Competing Interest

The authors declare no competing interest.

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