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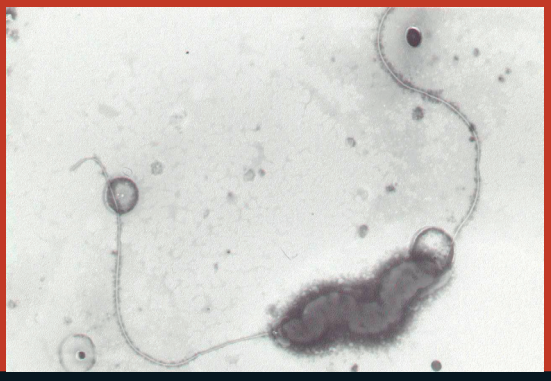

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ORIGINAL ARTICLE

Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in sheep flocks from three regions of Antioquia, Colombia

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ABSTRACT. Paratuberculosis or Johne's disease is a slow-developing infectious disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) affecting mainly domestic ruminants and producing a significant economic threat to livestock production systems. Although reports on paratuberculosis in small ruminants in Colombia are very scarce, the Colombian sheep industry has identified paratuberculosis as one of the causes of its low development. There have been reports of MAP infection in sheep flocks, mainly in the Cundiboyacense Plateau and the Bogotá savannah, but the prevalence of MAP infection in sheep and goat populations in Colombia is yet unknown. Therefore, the present study aimed to accurately estimate the prevalence of MAP infection at flock level in a sheep population of 24 flocks located in three regions of the province of Antioquia, Colombia. ELISA test as well as culture and direct qPCR were used as diagnostic tools. Overall, 456 blood serum samples were analysed and at least one seropositive animal was found in 17 (70% IC: 51.2-0.90) out of the 24 study flocks and, in total, 37 animals showed positive ELISA results (8% IC: 5.5-10.5). Regarding MAP direct detection, 90 faecal pools from the 24 flocks were cultured and subjected to qPCR diagnosis. Both direct qPCR and culture detected 25 (27.7%) and 64 (71.1%) faecal pools as MAP positive, respectively. More specifically, MAP positive pools were detected in 45.8% (IC: 24.3-67.3) and 83.3% (IC: 67.3-99.3) of the flocks by direct qPCR and culture, respectively. MAP infection is widespread in sheep flocks in the study regions and the combination of several diagnostic tests was necessary to achieve a more accurate and precise infection detection of this important pathogen.

Key words: Paratuberculosis, prevalence, Johne's disease, small ruminant.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is one of the most fastidious members of the *Mycobacterium* genus. It is the causal agent of Johne's disease (also known as paratuberculosis) which is an untreatable disease characterized by granulomatous enteritis, diarrhoea, loss of body weight and death (Chiodini 1993).

Although it is generally assumed that this infection occurs similarly in all domestic ruminant species, there is sufficient evidence to suggest that MAP infection in small ruminants is different to that in cattle, both in the clinical form as well as the MAP strains involved (Clarke 1997).

The disease is responsible for significant economic losses to livestock production worldwide (Sweeney 2011, Garcia and Shalloo 2015). Additionally, a zoonotic potential has been proposed since MAP has been consistently found in humans with Crohn's disease (Zarei-Kordshouli *et al* 2019). Furthermore, an increasing number of diseases such as Blau syndrome, type 1 diabetes, Hashimoto thyroiditis and multiple sclerosis have also been associated to MAP presence, reinforcing the zoonotic potential of this pathogen (Lee *et al* 2011, Sechi and Dow 2015).

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Some research on MAP infection in Colombia has been reported for small ruminants (Mogollón *et al* 1983, Mancipe *et al* 2009, Hernández *et al* 2017), but information on the presence and distribution of this infection is still scarce and the true prevalence (TP) of MAP infection in sheep populations in Colombia is clearly unknown. However, the Colombian sheep industry has identified paratuberculosis as one of the causes of the low development of the industry, limiting meat commercialization at national and international levels (Castellanos *et al* 2010). Unfortunately, there are currently no programs regarding the prevention and control of MAP infection for sheep populations in Colombia.

The lack of a prevalence estimate not only limits the capacity to assess the real impact of this important infectious disease, but also limits the capacity to allocate sufficient resources for its control precluding an adequate monitoring of the effectiveness of potential control measures. Since neither the Colombian sheep industry nor the Colombian government have estimates on the TP of MAP infected flocks, we aimed to accurately estimate the flock level prevalence of MAP infection and also to explore flock level risk factor associated with the presence of MAP antibodies in these sheep flocks located in the Antioquia province, Colombia.

MATERIAL AND METHODS

STATEMENT OF ANIMAL RIGHTS

The authors declare that the present study does not contain clinical studies or patient data. Informed consent

was obtained from all individual participants included in the study. The study was conducted according to the current law of animal protection in Colombia and was approved by the Ethics Committee for Animal Experimentation of the Universidad de Antioquia, Colombia (Act 111, May 2017).

ANIMAL POPULATION AND STUDY DESIGN

A cross sectional study was carried out using ELISA test as well as faecal culture and direct qPCR as diagnostic tools to assess MAP infection prevalence in this animal population and to explore the influence of multiple flock management practices associated with MAP seroprevalence (figure 1).

To avoid a possible selection bias and taking into account that in the province of Antioquia there were no official records of sheep flocks, a census to identify each sheep farm located in the study region was carried out before selecting the participants. According to this, the whole sheep population located in the Metropolitan Area, the Northern, and the Eastern region of the Province of Antioquia, Colombia, were the sample frame of the present study (n=2,479).

As a result of this search, 25 sheep farms were identified but one refused to participate in the study. All remaining sheep farmers agreed to participate, permitting the sampling and the interview (n=24) to take place. The Metropolitan Area region, the Northern region, and the Eastern region of the Province of Antioquia, Colombia were selected as the study regions due to their increase in sheep production in recent years and proximity to Medellín city, which is the nearest and main sheep consumption market.

The only inclusion criterion for sampling was that animals must be over one year of age. A representative sample of this animal population was taken following a multistage sampling procedure, in which a constant proportion of animals was taken from each flock (Dohoo *et al* 2010). The sample size calculation allowed an error of 5%, 95% confidence, and expected prevalence of 50%. According to this, the sample size was estimated at 384 animals. In each study participant flock, 20% of the animals over one year of age was randomly sampled. In those flocks with less than 20 animals over one year of age, only five animals were sampled in order to complete at least one single faecal pool. In total, 456 animals were sampled in selected flocks.

DATA AND SAMPLE COLLECTION

Between August and September 2017, each of the 24 sheep flocks was visited once for data and sample collection. Overall, blood and faecal samples were collected from

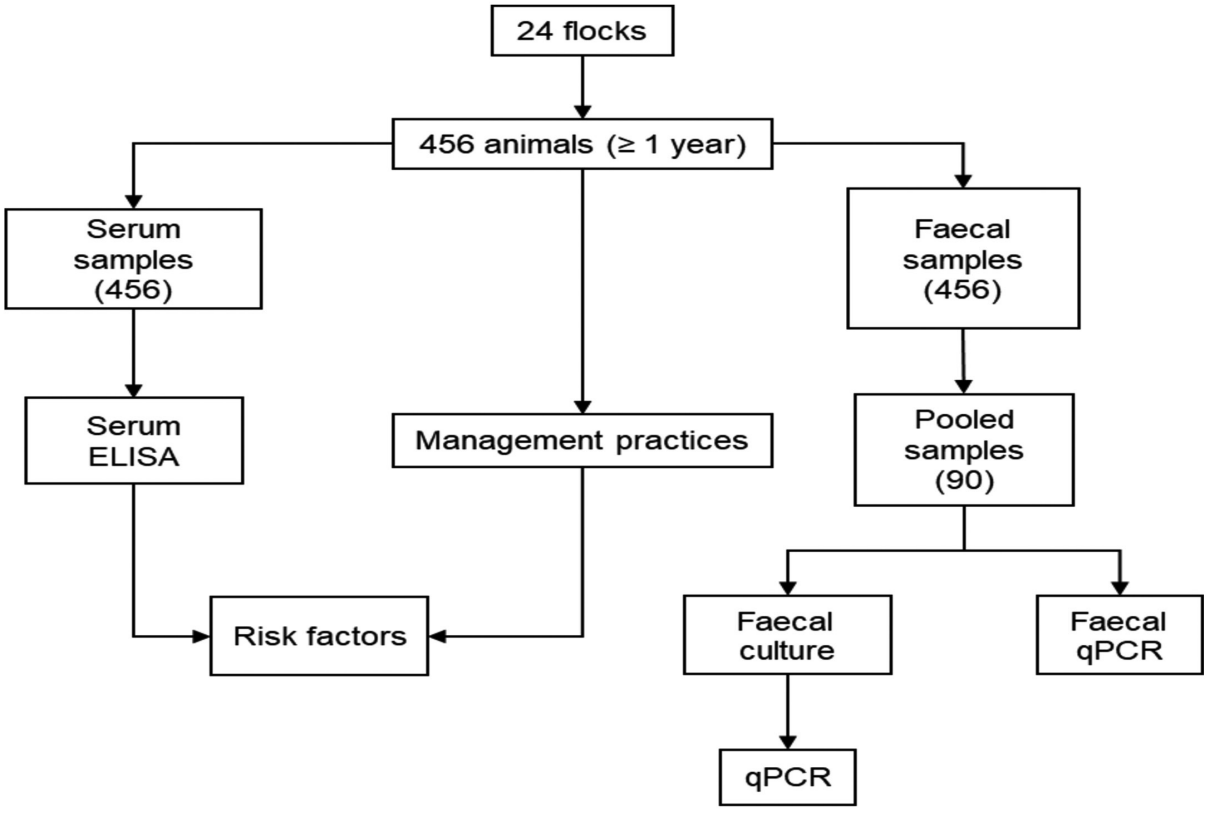

Figure 1. Overview of the study design for the determination of MAP prevalence in 24 sheep flocks of three regions of Antioquia, Colombia.

456 sheep of the 24 participating flocks. Available data regarding flock management practices were collected using a questionnaire, the same day in which blood and faecal samples were collected. The information was obtained from the flock manager or flock owner. All questionnaires included an introductory paragraph explaining the rationale and importance of the questions, how data was going to be used, and a confidentiality agreement. The questions were divided into three sections: 1) general information of the herd, 2) herd management practices, and 3) knowledge about the disease. Questions were read out to the farmer and answers were selected from multiple closed responses or otherwise written down. The questions searched for MAP infection transmission risk factors in ruminants, for example: shared roads between neighbouring flocks (Dhand 2007), presence of different species of ruminants in the same flock (Al-Majali *et al* 2008), co-grazing between cattle and goats or sheep (Çetinkaya *et al* 1997), community grazing, poor control of intestinal parasites (Angelidou *et al* 2014) and animal trade between related flocks (Marquetoux 2016).

DIAGNOSTIC TESTS

Enzyme-linked Immunosorbent Assay (ELISA). Blood samples were taken from the jugular vein using Vacuette® tubes of 7 mL without anticoagulant (Greiner Bio-one, Kremsmünster, Austria) and a single 21G x 1½'' needle per animal, after local cleaning and disinfection with antiseptic alcohol. After collection, the blood samples were left to stand at room temperature to allow clot retraction. Subsequently, each sample was centrifuged at 2000-2500 rpm for 3-5 minutes to ease the serum extraction. The serum obtained was kept refrigerated until arrival at the Diagnostic Unit of the Facultad de Ciencias Agrarias, Universidad de Antioquia in Medellin, Colombia, where it was frozen at -20 °C until analysis by ELISA in October 2017. The presence of antibodies against MAP in the blood serum samples was determined by ELISA using the commercial diagnostic kit CATTLETYPE® MAP Ab (Qiagen, Leipzig, Germany) according to manufacturer guidelines.

The test characteristics of the ELISA assays used in this study, which are licensed in Germany for the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in cattle, are sensitivity 59.1% and specificity 98.6%, determined by the Friederich Loeffler Institut, National Reference Laboratory for Paratuberculosis, using the reference panels for serum and milk of the NRL for paratuberculosis.

Culture. From each of the 456 study animals, a faecal sample (2-5 g) was taken with a new clean glove directly from the rectum. No animals had been vaccinated against MAP infection and researchers were unaware of their historical infectious status or the status at the time of sampling. The samples were kept refrigerated until arrival at the laboratory. To overcome the high costs of faecal culture, faecal samples of individual animals were pooled at the laboratory. Therefore, individual faecal samples of five animals from the same flock were pooled in a new sterile container (Fiorentino *et al* 2012, Mita 2016). Briefly, each faecal sample (2-5 g) from the study animals was homogenised in a sterile container. The pools (n=90) were frozen at –80°C, then shipped to the Laboratorio de Enfermedades Infecciosas, Instituto de Medicina Preventiva Veterinaria, Universidad Austral de Chile, Valdivia, Chile at 4°C allowing the slow defrosting of samples. On arrival, samples were immediately refrigerated and processed by both culture and direct MAP qPCR detection within the following hours. Pool faecal samples were processed in the BACTEC™ MGIT™ Para TB System (BD Diagnostic Systems, Franklin, NJ, USA), according to the manufacturer's instructions. Each inoculated MGIT tube was inserted into an MGIT 960 instrument (BD Diagnostic Systems, Franklin, NJ, USA) and incubated at 37°C for 49 days. Tubes signalling positive by day 49 were removed and confirmed for the presence of MAP by IS*900* qPCR. Tubes not signalling positive by that time were considered negative.

DNA extraction. A simple, efficient and low-cost method of harvesting MAP DNA based on mechanical cell disruption was used to extract DNA from direct faecal pool samples as well as from a positive MGIT culture tube (Salgado *et al* 2014). Briefly, a 200 µL aliquot of a bacterial suspension from pooled faecal samples or the medium of the MGIT tube was aseptically transferred to 1.5 mL centrifuge tubes, which were then centrifuged at 5,000 g for 5 min. The supernatant of each tube was discarded, and the opening of the tube was briefly touched with a clean soft paper tissue to remove the remaining liquid. The pellet was disrupted by pipetting with a mixture of 500 µL lysis buffer (2 mM EDTA, 400 mM NaCl, 10 mM Tris–HCL pH 8.0, 0.6% SDS) and 2 µL proteinase K (10 mg/mL), and then it was transferred to a bead beating tube (BioSpec Products Inc., Bartlesville, OK, USA) containing 200 µL of beads (0.1 mm zirconia/silica beads; BioSpec Products Inc., Bartlesville, OK, USA). The tubes were incubated at 56 °C for 2 h with shaking at 600 rpm. The tubes were then shaken in a cell Disrupter (MiniBeadbeater-8; Biospec Products) at 3,200 g for 60 sec and incubated on ice for 10 min. To remove foam and beads from the inner walls, the tubes were centrifuged at 5,000 × g for 30 sec. The samples were briefly vortexed to ensure that any DNA adhering to small solid particles was not lost when the lysate was transferred. All liquid contents from the bead-beating tube were transferred to 1.5-mL microcentrifuge tubes (Eppendorf tubes; Sigma-Aldrich) and 500 µL of 100% ethanol was added. The tubes were left standing for 2 min at room temperature before being vortexed for 5 seconds and centrifuged at 18,000 × g for 5 min. at 18 °C. The supernatant was discarded and the pellet was

washed once in 200 µL 70% ethanol by resuspension and centrifugation under the same conditions as mentioned above. Next, the pellet was resuspended in 50 µL of sterile distilled water. The tubes were placed in a dry heating block (Eppendorf; Germany) at 100 °C for 5 min. The solution was briefly centrifuged at full speed (16,000 × g for 30 sec) to remove any contaminating material. Finally, a 25-µL aliquot of the supernatant was placed into a new Eppendorf tube (Eppendorf tubes; Sigma-Aldrich) to be used as a template for qPCR.

Molecular detection and culture confirmation of MAP by qPCR. To detect MAP, either directly from faecal pools or confirmation of positive MGIT tubes, a qPCR protocol previously reported by Salgado *et al* (2013) was used. Briefly, the target was the insertion element IS*900*. The qPCR mixture included 5 µl DNA template, 10 µl TaqMan Universal Master Mix (Roche, Indianapolis, IN), 0.2 µM IS*900* primers, 0.1 µM probe (Roche, Indianapolis, IN), and water for a total volume of 20 µl. Primer sequences for IS*900*, which amplified a 63-nucleotide fragment of the IS*900* gene target, were 5'-GACGCGATGATCGAGGAG-3' (left) and 5'-GGGCATGCTCAGGATGAT-3' (right). The probe sequence was TCGCCGCC. The reactions were carried out in a Roche LightCycler System version 2.0 (Roche, Indianapolis, IN, USA) under the following standard conditions: one cycle at 95°C for 10 min; 45 cycles with three steps of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s; and a final cooling step at 40 °C for 30 s. Negative (Mix and Water PCR-grade) and positive (*Mycobacterium avium* subsp. *paratuberculosis* ATCC 19698) PCR controls were included.

STATISTICAL ANALYSIS

The information collected through the questionnaire and the ELISA test was first analysed descriptively and then analytically. These analyses were carried out using a bivariable and multivariable logistic regression, to explore the influence of multiple flock management practices to MAP seroprevalence. The calculation of the 95% confidence interval for the prevalence results was performed. Also, the calculation of the true prevalence was made using the WinEpi platform, available at www.winepi.net. An unconditional mixed-effects logistic regression analysis, grouped by flock to account for clustering, was also performed. The criteria of Hosmer-Lemeshow (P<0.25) was used to retain variables for the multivariable model. Statistical analysis was carried out using the Stata 12.0 software (StataCorp LP, College Station, Texas, USA). For the exploration of the influence of multiple flock managements practices to MAP seroprevalence, the unit of analysis was the individual and the case definition of a positive flock was the one that had at least one positive result to the ELISA test, culture or qPCR.

RESULTS

FLOCK CHARACTERISTICS

Flock characteristics and management practices explored and then considered as predictors for the risk factor assessment are presented in table 1. The study flocks were all pasture-based and mostly had an area of less than 2 hectares and the flock average population (> 1 year) was 155 animals. The flock distribution was composed mainly of small flocks with limited access to veterinary assistance. Katahdin, Dorper and Santa Ines were the predominant breeds. Most of the farms managed other ruminant species in their facilities, mainly cattle, and in 40.3% of the cases these species shared paddocks. A 61.1% of the flocks shared roads with neighbouring flocks and 52.6% spread slurry onto pastures. Purchase of animals was highly frequent in the study flocks. Most of the interviewed owners/managers reported not having heard about the disease before and not having seen paratuberculosis-symptomatic animals in their flocks in the last 2 years.

SEROPREVALENCE

Eight per cent (37/456, IC: 5.5-10.5) of the serum samples were positive, and at least one or more seropositive animals were detected in 70.8% (17/24, IC: 51.2-90.0) of the flocks. Positive ELISA results among regions were 70, 100, and 63.6% in the Metropolitan Area, Northern and Eastern region of Antioquia, respectively (table 2). The intra-flock seroprevalence ranged from 0 to 21.4% (data not shown).

FAECAL CULTURE

Overall, 78.8% (71 /90) of faecal pools showed positive results after confirmation by qPCR. MAP positive pools (one or more) were detected in 83.3% (IC: 67.3-99.3) of the flocks. The apparent prevalence values among different regions were 100, 67 and 72.7% in the Metropolitan Area, Northern and Eastern region, respectively (table 3).

qPCR OF FAECAL POOLS

qPCR informed 25 (27,7%) positive pools out of 90 faecal pools tested. Forty-six per cent (IC: 24.3-67.3) of the flocks showed MAP positive results in one or more of the analysed pools. The apparent prevalence among regions based on qPCR results of pools were 40, 100 and 36,4% in the Metropolitan Area, Northern and Eastern regions, respectively (table 3).

Table 1. Unconditional analysis of factors associated with the *Mycobacterium avium* subsp. *paratuberculosis* ELISA status in 24 flocks of three regions of Antioquia, Colombia.

Variable	Unit/Category	No of sampled animals	Distribution (%)*	No ELISA positive animals		P
				n	%	
Flock population (animals > 1 year)	≤70	107	23.4	14	13	
	71-140	71	15.5	4	5.6	0.086[a]
	141-210	57	12.5	4	7	0.191[a]
	211-280	90	19.7	7	7.7	0.172[a]
	>280	131	28.7	8	6.1	0.045[a]
Flock size (hectares)	< 2 has	226	49.5	16	7	
	≥ 3 ≤ 5 has	151	33.1	16	10.5	0.791
	> 6 has	79	17.3	5	6.3	
Presence of other ruminants	No	148	32.4	15	10.1	0.365
	Yes	308	67.5	22	7.1	
Sharing paddocks	No	272	59.6	17	6.2	0.128[a]
	Yes	184	40.3	20	10.8	
Sharing roads	No	177	38.8	11	6.2	0.196[a]
	Yes	279	61.1	26	9.3	
Spreading of manure on pastures	No	216	47.3	14	6.4	0.260
	Yes	240	52.6	23	9.5	
Use of dewormer	No	100	22	5	5	0.195[a]
	Yes	346	78	32	9.2	
Mobilization between flocks	No	239	52.4	21	8.7	0.480
	Yes	217	47.5	16	7.3	
Sheep purchase	No	240	52.6	17	7	0.515
	Yes	216	47.3	20	9.5	

[a]Variables used for the multivariable analysis ($P<0.25$).
*The distribution refers to the percentage that each value represents.

Table 2. Sheep-level seroprevalence of ovine paratuberculosis infection by ELISA test in three regions of the Province of Antioquia, Colombia.

Region	Flocks	Number of animals[a]	Tested animals	Positive animals by ELISA	Individual seroprevalence (%)	Positive flocks by ELISA	Flock seroprevalence (%)
Metropolitan Area	10	1012	194	13	6.7	7	70
Northern	3	323	62	5	8	3	100
Eastern	11	1144	200	19	9.5	7	63.6
Total	24	2479	456	37	8.11	17	70.8

[a]Animal over one year of age.

FACTORS ASSOCIATED WITH THE MAP SEROLOGICAL STATUS

Among the nine risk factors explored in the univariable analysis, four (flock population, sharing paddocks, sharing roads, use of dewormer) were associated with the seropositivity to MAP infection ($P\leq0.25$) and were eligible for their inclusion in the final model (table 1). However, no significant variables were obtained in the final model when the multivariable logistic regression analysis was carried out.

DISCUSSION

This study was carried out using a combination of direct and indirect diagnostic methods to detect MAP

Table 3. Flock-level prevalence of MAP infection in sheep by faecal culture and qPCR in three regions of Antioquia, Colombia.

Region	Flocks	Pools	Faecal culture			qPCR		
			Positive pools	Flock positive result[a]	Flock apparent prevalence	Positive pools	Flock Positive result[a]	Flock apparent Prevalence
Metropolitan Area	10	38	37	10	100	10	4	40
Northern	3	13	6	2	67	5	3	100
Eastern	11	39	28	8	72.7	10	4	36.4
Total	24	90	71	20	83.3	25	11	45.8

[a]Refers to flocks in which at least one pool resulted MAP positive by culture or PCR.

infection in sheep in the Metropolitan Area, Northern and the Eastern regions of the province of Antioquia, Colombia. The combination of ELISA, direct qPCR and faecal culture assured an accurate MAP infection detection in the study regions. To the author´s knowledge, this is the first epidemiological report on sheep MAP infection in Colombia, and one of the few studies in Latin America performed in small ruminants to estimate MAP prevalence and to explore management practices associated with MAP seropositivity.

The results show that antibodies against MAP are widespread in the study regions, which in general means that MAP infection had progressed significantly in this animal population, and the serological results indicate a significant proportion of individuals are in the late phase of this infection and, as such, efficient MAP shedders. The latter finding agrees with the results reported elsewhere in the same ruminant species (Attili *et al* 2011, Stau 2012, Moron-Cedillo *et al* 2013, Bauman *et al* 2016[a], Morales-Pablos *et al* 2020). Additionally, the observed flock-level prevalence of MAP infection in sheep flocks could be considered as high, in comparison with what has been reported by Bauman *et al* (2016[b]) in sheep flocks in Canada, using direct and indirect diagnostic methods (ELISA, bacterial culture and qPCR). According to a systematic review for Latin America and the Caribbean, the prevalence of this infection in sheep is 16% (7.9-24.1%) at an individual level, and the prevalence in sheep at the flock level was not reported due to the lack of studies that met the inclusion criteria; the high heterogeneity detected in overall prevalence estimations could be attributed to the high diversity in study design, the variable quality of measures, or the test used (Fernández-Silva *et al* 2014).

Although no seropositive animals were identified in some flocks (data not shown) in this study. These animals were likely to be found in the early stages of infection and the levels of antibodies may have not been detectable (Nielsen 2010). The location and flock management practices also may limit the contact between neighbouring flocks and different animal species in these regions since the paddocks are distant to each other or the flocks are isolated, and the entry of other animals is not allowed. Nevertheless, MAP infection shows similar behaviour in the study regions, e.g. few seropositive animals being observed within each flock, but many flocks with at least one seropositive animals (Coelho *et al* 2007, Stau *et al* 2012, Morales-Pablos *et al* 2020, Khamassi Khbou *et al* 2020).

The lower rate of positive results obtained by qPCR in comparison to culture was expected as previously reported (Alinovi *et al* 2009, Plain *et al* 2014) for sheep. The different diagnostic sensitivity between qPCR and culture is mainly explained by the fact that the direct qPCR tool has to deal with faecal sample inhibitors for polymerase activity (Monteiro *et al* 1997, Thornton and Passen 2004) as well as intermittent MAP shedding (Whitlock *et al* 2000). On the other hand, culture can address these difficulties along the culture period due to the bacteria replication rate (Harris and Barletta 2001). It is well known that liquid media based on Middlebrook 7H9 Broth base such as BACTEC MGIT system has shown better results to grow several strains of *Mycobacterium avium* subsp. *paratuberculosis* than conventional media (HEYM) (Gumber and Whittington, 2007). However, the combination of both MAP detection tools improves the capacity of detection of infected flocks.

Even though some flocks showed only negative qPCR and culture results, it is also likely that the results for the tests did not necessarily mean that the animals were not infected, and it may be that the shedding phase has not started (infected animal in a noninfectious phase) yet (McKenna *et al* 2006, Nielsen 2010). The high MAP prevalence obtained in this study is consistent with what has been previously confirmed for cattle in the same region (Fernández-Silva *et al* 2011, Ramírez *et al* 2011, Correa-Valencia *et al* 2016, Correa-Valencia *et al* 2019). In addition, the absence of strategies, programs, or projects to prevent or control the infection and the historic management practices that allow the entrance and spread of the infection in the different animal productions help to explain the findings.

The small number of observations and the fit for several factors may explain the lack of association in

the multivariate linear or logistic regression analysis (Hackshaw 2008, Figueiredo *et al* 2013). Also, the number of positive and negative outcomes in the observed data influence the precision of the estimates of coefficients in the model. It has been suggested that the dataset should contain a minimum of 10(k+1) positive outcomes where k is the number of predictors in the model in order to adequately fit the model (Dohoo *et al* 2010). These reasons can explain why no significant variables were obtained in the final model.

In the last decade, the arrival of sheep in the study regions has occurred for purposes of restocking and genetic improvement due to the increase of lamb consumption in the region. However, these animals are purchased without quarantine or any diagnostic test. Most of these animals come from different provinces of Colombia where the prevalence of paratuberculosis infections is unknown. In the absence of biosecurity practices, a single animal (clinically or subclinically sick) is enough to infect a flock and disseminate MAP among other flocks. The paratuberculosis prevalence may increase as the biosecurity practices intended for the prevention and control of infection are not being implemented or executed in the flocks (Morales-Pablos *et al* 2020).

In conclusion, MAP infection is widespread in sheep flocks of the study regions and the combination of several diagnostic tests were necessary to achieve a more accurate diagnosis of MAP infection. Further studies including a larger sample size are needed to identify the risk factors associated with MAP infection in sheep in Colombia.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Metabolic response to water shortage in an isolated feral sheep population

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ABSTRACT. To establish metabolic responses for biochemical analytes related to freshwater shortage adaptation, a total of 376 blood samples were collected from feral sheep at the Socorro Island, Revillagigedo Archipelago. Comparisons were made between four sampling periods with repeated measurements at 0, 7, 14, and 21 d (94 blood serums: 84 females and 10 males). During the first week, the sheep received daily water *ad libitum*. During the second and third week, the sheep received 60% daily water in relation to the first 7 days intake. Analysis of variance was used to compare the mean values between sampling days. Stepwise regression analysis was used to evaluate the relationships between the biochemical analytes. The glucose (GLU), total cholesterol (COL-T), triglycerides (TAG), urea, albumin (ALB), total protein (PROT-T), sodium ion (Na^+), creatine kinase (CK), arginine vasopressin (AVP), and aldosterone (Aldo), were determined. With the exception of GLU, the COL-T, TAG, urea, ALB, PROT-T, Na^+, CK, AVP, and Aldo showed differences between sampling days with the higher values corresponding to 14 d with limited water intake. Negative correlations ($P<0.05$) between ALB with COL-T and TAG, were quantified. Positive correlations ($P<0.05$) between COL-T with TAG, and Aldo; between urea with PROT-T, between CK with ALB and PROT-T, between AVP with COL-T, TAG, urea, PROT-T and Aldo, and between Aldo with Na^+, were quantified. Results could help improve the accuracy of metabolic profiles used as a tool for evaluating dehydration indicators and to describe the physiological mechanisms employed by feral sheep to cope with limited availability of freshwater.

Key words: blood biochemistry, metabolic profile, feral sheep, resilience to water shortage.

INTRODUCTION

The limited availability of freshwater for livestock has been a limiting factor in ruminant physiology and productivity (Vosooghi-Postindoz *et al* 2018). Fortunately, small ruminants have several adaptive mechanisms to efficiently use their physiological water reserves (Casamassima *et al* 2016), in this way overcoming the stresses caused by water shortage and dehydration (Berihulay *et al* 2019). These mechanisms include decreasing body water losses in the urine and faeces. The urine water losses can be reduced by promoting water reabsorption at renal tubules (Rotondo *et al* 2016, Trepiccione *et al* 2019), whereas faecal water losses can be decreased by promoting water absorption at gastrointestinal level (Ames *et al* 2019). However, in both cases, the serum volume decreases due to the water uptake by cells (Trepiccione *et al* 2019). Hyperosmolality due to increased solute concentrations is, thus, commonly detected in water-restricted sheep (Pratt *et al* 2016). A remarkable example of these adaptive mechanisms started with the introduction and abandonment of 100 sheep (*Ovis aries* Linnaeus, 1758) by Australian settlers to the Socorro Island of the Revillagigedo Archipelago in 1869 (Salas de León *et al* 2015). In the absence of handling, sheep became feral and adapted to the insular environment (Hernández et al 2017). The prolonged isolation allowed the sheep population to develop traits adapted to the food and freshwater scarcity (Pickering *et al* 2013). In the absence of natural enemies, the sheep population grew to be about 5,000 individuals and they became the main disturbance agent of the island's ecological conditions (Hernández *et al* 2017). Thus, the Chamber of Deputies in Mexico approved the extermination of the feral sheep on the island (Ortiz *et al* 2016). To preserve the valuable germplasm of the Socorro Island sheep population, the Faculty of Veterinary Medicine of the University of Colima and the National Council of Science and Technology carried out a program of extraction and recovery (Hernández *et al* 2017). A thorough understanding of the adaptive physiological mechanisms to cope with limited water availability should be the starting point of all efforts to improve the resilience of domestic sheep to climate change. In this sense, the glucose (GLU), total cholesterol (COL-T), triglycerides (TAG), urea, albumin (ALB), total protein (PROT-T), sodium ion (Na^+), creatine kinase (CK), arginine vasopressin or antidiuretic hormone (AVP), and aldosterone (Aldo) are considered good indicators of dehydration. Thus, this study aimed to establish metabolic responses for biochemical analytes related to freshwater shortage adaptation, in feral sheep at the Socorro Island, Revillagigedo Archipelago, Mexico.

MATERIAL AND METHODS

ANIMALS

This experiment followed institutional and national guidelines for the care and use of animals. All experimental procedures were approved by the Committee of Ethical Review at Colima University (protocol approval number: 06/19; experimental period: October 2019). The animals

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belong to the feral sheep extraction and recovery program of the Socorro Island of the Revillagigedo Archipelago. The Socorro Island (figure 1), is a tropical volcanic island located in the Pacific Ocean (18°42'28" N, 111°02'49" W), 540 km south of Cabo San Lucas in Baja California Sur and 720 km west of Manzanillo, Colima, Mexico. It has an area of 132 km^2 and a maximum elevation of 1040 m. Climate is warm, sub-humid (Köppen Cfb) (Peel *et al* 2007). The average temperature is 22-27.5 °C. It is located in the path of tropical cyclones that occur from June to November with an average of 2.75 events/year, with precipitation of more than 400 mm during the season.

Currently, the livestock inventory is located on the animal husbandry experimental unit at the Faculty of Veterinary Medicine, University of Colima, Tecoman, Colima, Mexico (18°56'53" N; 103°53'50" W). The climate is warm, sub-humid (Köppen Cfb) with summer rains (Peel *et al* 2007). The average temperature is 26 °C with precipitation of 750 mm/year (Hernández *et al* 2017). The study was carried out by sampling and analysing 376 blood serums from total livestock inventory (94 feral sheep in maintenance: 84 females and 10 males), with initial body weight (average ± S.D.) of 45 ± 4 kg and 60 ± 6 kg, respectively, body condition score (2.20 ± 0.8) and age (14 ± 2 months). Feeding conditions of sheep in the island were maintained at the experimental housing unit. Animals were able to graze *Conocarpus* scrub, *Croton masonii* scrub, and *Pteridium-Dononaea* scrub, introduced from Socorro Island (Flores *et al* 2009). Previous to the study, the herd was dewormed with Ivermectin (1 mL/25 kg of live weight subcutaneously) followed by immunisations against *Clostridium*, *Pasteurella multocida* and *Mannheimia haemolytica* (BOBACT 8. SAGARPA B-0273-111; Intervet., Mexico City, Mexico) 2.5 mL/animal subcutaneously.

EXPERIMENTAL PROTOCOL

Comparisons were made between four sampling periods with repeated measurements at 0, 7, 14, and 21 d. During the first week, the sheep received daily water *ad libitum*. To determine the quantity of water for the second and third weeks, water intake was recorded during the first week. In the experiment, the sheep received 60% daily water in relation to the first 7 days intake; this was achieved by restricting water intake by 40% compared to the intake of the first week.

BLOOD COLLECTION AND DETERMINATION OF ANALYTES

Blood samples were collected by puncture of the jugular vein before morning feeding at 0, 7, 14, and 21 d with restricting water intake (figure 2).

For GLU, COL-T, TAG, urea, ALB, PROT-T, Na^+, and CK analysis, 8.5 mL of blood were collected into vacuum tubes with clot activator and serum separator gel (BD Vacutainer 367988; Becton-Dickinson Co., Franklin Lakes, United States). For AVP and Aldo, additional samples of 5 mL of blood were collected into vacuum tubes with EDTA-K_3 (BD Vacutainer 366352; Becton-Dickinson Co., Franklin Lakes, United States). To collect serum, blood samples were centrifuged at 1500 x *g* for 10 min by using a portable centrifuge (Porta-Spin C828; UNICO, Dayton, United States). Serum samples were separated using

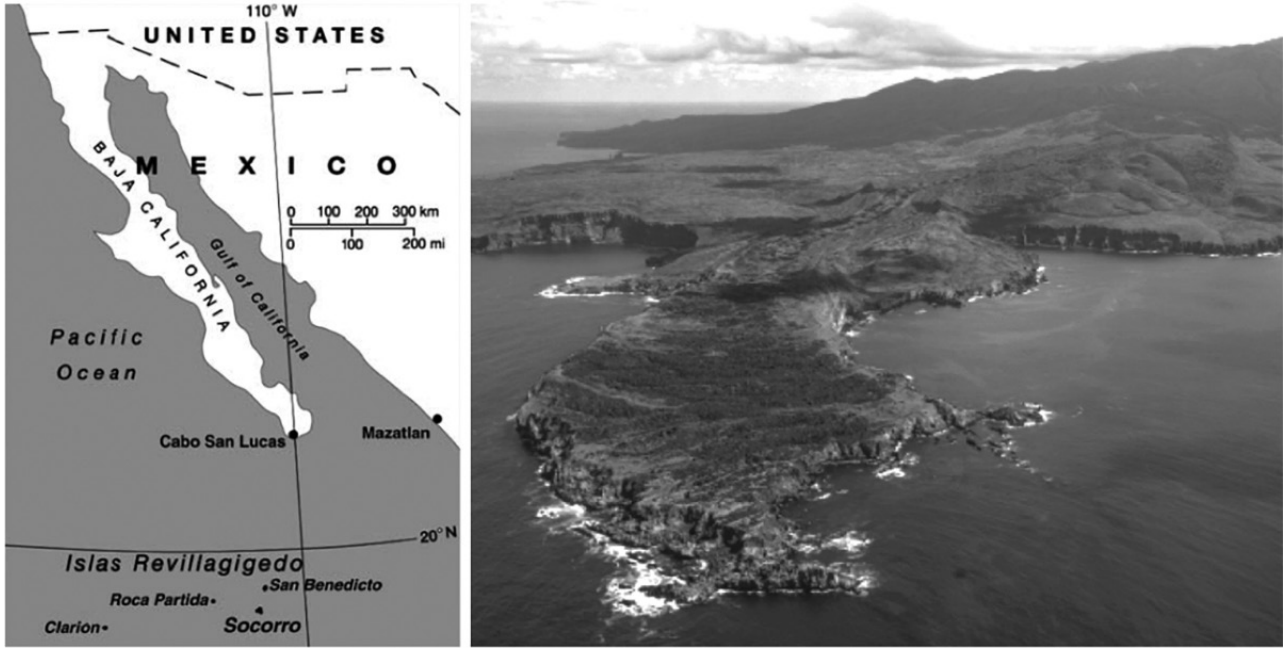

Figure 1. Location of the Socorro Island, Revillagigedo Archipelago, 720 km west of Manzanillo, Colima.

Figure 2. Feral sheep from the Socorro Island, Revillagigedo Archipelago.

1.5 mL tubes with lid (Tubes Safe-Lock 3810X; Eppendorf., Madrid, Spain) and transported at 4 °C in a portable cooler (Thermoelectric Cooler Car/Home M5644-710; Coleman Company, Kansas, United States) to the clinical laboratories at the Autonomous Metropolitan University and the University of Colima, where they were frozen at -20 °C until analysis. The concentration of each analyte was determined with a UV-Vis double beam spectrophotometer (Biochemistry Analyzer; KONTROLab, Guidonia, Italy) and the hormones were determined with a gamma counter (PC-RIA MAS; Stretec, Germany). Biochemical analytes, analytical method for each parameter, units in which the results were expressed, and corresponding commercial reagents, are described in (table 1).

Precision and reliability of the techniques were controlled using lyophilised control serum (SPINTROL NORMAL 1002100; Spinreact, Girona, Spain) and Assayed Multi-Sera AL 1027 (Randox Laboratories, Northern Ireland, United Kingdom). Hemolysis of serum was recorded on a qualitative scale of 0 (none) to 3 (dark). Samples showing haemolysis scores of 2 and above constituted less than 1% of all samples and did not introduce a significant bias in any of the tested models; thus, the influence of serum haemolysis was ignored.

STATISTICAL ANALYSIS

The data were described by average and S.D., and they were tested for normal distribution (Shapiro-Wilk test). The linear relationships between the biochemical analytes were identified by the use of a Pearson Correlation Coefficient matrix, and Stepwise regression analysis (PROC REG, SAS, System, v. 8.2, Cary, NC). Analysis of variance was used to compare the mean values between sampling days. A multiple comparison test of Tukey was performed when the effect of the sampling day was found to be significant (P<0.05). A diagnosis for outlier values was performed using robust multivariate outlier detection (OUTLIER; SAS, 2001). This macro calculates the robust Mahalanobis distance for each observation[1]. The following model was tested:

$$d_m(\mathrm{x},\overline{\mathrm{x}}) = \sqrt{(\mathrm{x}-\overline{\mathrm{x}})\sum\nolimits_x^{-1}(\mathrm{x}-\overline{\mathrm{x}})}$$

Where:
d_m (x, $\overline{\mathrm{x}}$) = robust Mahalanobis distance;
x = vector of the observation;
$\overline{\mathrm{x}}$ = vector average of the observations; and
$\sum_x^{-1}$ = variance-covariance matrix of the observations.

Regression diagnosis for main assumptions was performed (PROC UNIVARIATE; SAS, 2001). The linear functional form was visually checked by a normal plot. Shapiro-Wilk test was used to check the normality of residuals. Homoscedasticity was checked by plotting

[1] http://www.datavis.ca/sasmac/outlier.html, accessed May 10, 2020.

Table 1. Biochemical analytes, units, analytical methods, and corresponding commercial reagents.

Analyte*	Unit	Method	Reagent
Glucose (GLU)	mM	Colorimetric. Trinder[a]	1001190[1]
Total cholesterol (COL-T)	mM	Colorimetric. Liquid[b]	41020[1]
Triglycerides (TAG)	mM	Colorimetric. Liquid[c]	41032[1]
Urea	mM	Enzymatic[d]	1001333[1]
Albumin (ALB)	g/dL	Colorimetric. Bromocresol green	1001020[1]
Total protein (PROT-T)	g/dL	Colorimetric. Biuret	1001291[1]
Sodium ion (Na^+)	mM	Enzymatic. Galactosidase	1001385[1]
Creatine kinase (CK)	U/L	Enzymatic[e]	41250[1]
Arginine vasopressin (AVP)	pg/mL	Radioimmunoassay[f]	KIPERB319[2]
Aldosterone (Aldo)	pg/mL	Radioimmunoassay[g]	KIPZ0100[2]

*Official abbreviation of the International Union of Pure and Applied Chemistry (IUPAC); [a]Glucose Oxidase-Peroxidase; [b]Cholesterol Oxidase-Peroxidase; [c]Glycerol Phosphate Dehydrogenase-Peroxidase; [d]Urease-Glutamate Dehydrogenase; [e]Phosphocreatine NADPH; [f]sensitivity 20 pmol/L and intra-assay and inter-assay coefficient of variations were 5.6% and 6.1% respectively; [g]sensitivity 6 pg/mL and intra-assay and inter-assay coefficient of variations were 9.5% and 10.4% respectively; [1]Spinreact, Girona, Spain; [2]DIAsource ImmunoAssays, Ottignies-Louvain-la-Neuve, Belgium.

residual versus predicted values, and the Durbin-Watson test was employed to check for error uncorrelation.

RESULTS

The reference value, descriptive statistics for GLU, COL-T, TAG, urea, ALB, PROT-T, Na^+, CK, AVP, Aldo, and their comparison between sampling days with limited drinking water availability in feral sheep from Socorro Island, Revillagigedo Archipelago are shown in table 2.

As shown in table 2, the biochemical analytes quantified in feral sheep from Socorro Island, presented no differences between males and females. However, their concentrations were above the reference range of the sheep. Except for GLU, the serum concentration of the other biochemical analytes (combined males and females) showed differences ($P<0.05$) between sampling days, with the higher values corresponding to 14 d with limited water intake. Negative correlations ($P<0.05$) between ALB with COL-T and TAG, were quantified. Positive correlations ($P<0.05$) between COL-T with TAG, and Aldo; between urea with PROT-T, between CK with ALB and PROT-T, between AVP with COL-T, TAG, urea, PROT-T and Aldo, and between Aldo with Na^+, are shown in table 3.

DISCUSSION

Assessment of biochemical analytes sensitive to water shortage revealed that feral sheep from Socorro Island exhibited changes in serum concentrations of COL-T, TAG, urea, ALB, PROT-T, Na^+, CK, AVP, and Aldo under 60% water restriction. All sheep needed 14 d to adapt to the water shortage and the concentration of biochemical analytes was closer to the value recorded during the first week, at 21 d. This drop in biochemical analytes concentration suggests adaption to drinking water shortage at the end of the experimentation period. When small ruminants become dehydrated during water restriction, the serum volume decreases due to water uptake by tissue cells (Trepiccione *et al* 2019). In this sense, the urea molecules generate osmotic traction of water in the urinary tract (Weiner *et al* 2015), as water moves from areas of low concentration to those of high concentration to dilute urea in urine (Weiner 2017). Small ruminants such as the Awassi sheep, a Middle Eastern breed, have the ability to decrease urine volume and increased urea concentration, after being deprived of water for 5 d (Jaber *et al* 2004). Under conditions of drinking water shortage, however, the kidneys function is altered in Marwari sheep (Kataria and Kataria 2007) with slower glomerular filtration and higher urea reabsorption (Weiner and Verlander 2016), leading to increased blood concentrations of CK and urea (Weiner *et al* 2015), but without affecting GLU concentration (Abdelatif *et al* 2010), results similar to those found in this study (table 2). Reduced serum volume also increases concentrations of certain biochemical analytes (Berihulay *et al* 2019), and forces sheep to activate physiological mechanisms to adapt to dehydration and cope with limited drinking water availability (Mengistu *et al* 2016). Comisana sheep, a central and northern Sicily breed, under 60% water restriction for 40 d, increased their concentrations of ALB, COL-T, PROT-T, and TAG, without affecting your concentrations of GLU (Casamassima *et al* 2008). In another study by the same group, the Lacaune sheep, a South of France breed, under 60% water restriction for 28 d, increased their concentrations of CK and PROT-T (Casamassima *et al* 2016). Hamadeh *et al* (2006) suggested that concentrations of ALB, COL-T, CK, PROT-T, and urea are influenced by the consumption of water. In their study, they offered water to Awassi sheep every 3 d under

Table 2. Reference value, mean ± standard deviation, and comparison of different biochemical analytes of feral sheep from the Socorro Island, Revillagigedo Archipelago, during restricting water intake.

Analyte	Reference	Males n = 40 blood serums[1]	Females n = 336 blood serums[1]
Glucose (mM)	3.80 ± 0.33[†,a]	3.89 ± 0.28[a]	3.74 ± 0.24[a]
Total cholesterol (mM)	1.66 ± 0.31[†,a]	2.50 ± 0.11[b]	2.47 ± 0.09[b]
Triglycerides (mM)	0.33 ± 0.01[§,a]	0.40 ± 0.01[b]	0.39 ± 0.01[b]
Urea (mM)	7.70 ± 0.43[§,a]	8.63 ± 0.06[b]	8.61 ± 0.05[b]
Albumin (g/dL)	2.70 ± 0.11[†,a]	3.74 ± 0.48[b]	3.51 ± 0.37[b]
Total protein (g/dL)	7.20 ± 0.52[†,a]	8.87 ± 0.37[b]	8.71 ± 0.33[b]
Sodium ion (mM)	141.5 ± 4.1[†,a]	172.47 ± 22.11[b]	161.58 ± 17.29[b]
Creatine kinase (U/L)	10.3 ± 1.6[†,a]	13.20 ± 0.11[b]	13.17 ± 0.09[b]
Arginine vasopressin (pg/mL)	3.30 ± 1.37[¥,a]	8.53 ± 0.12[b]	8.50 ± 0.11[b]
Aldosterone (pg/mL)	20.10 ± 2.21[¶,a]	28.14 ± 1.15[b]	27.82 ± 1.01[b]

	Combined males and females n = 94 blood serums/sampling days Restricting water intake (d)			
	0	7	14	21
Glucose (mM)	3.75 ± 0.24[a]	3.76 ± 0.25[a]	3.76 ± 0.26[a]	3.75 ± 0.24[a]
Total cholesterol (mM)	2.37 ± 0.07[a]	2.49 ± 0.05[b]	2.58 ± 0.07[c]	2.45 ± 0.05[d]
Triglycerides (mM)	0.38 ± 0.01[a]	0.40 ± 0.01[b]	0.41 ± 0.01[c]	0.39 ± 0.01[d]
Urea (mM)	8.55 ± 0.03[a]	8.63 ± 0.02[b]	8.68 ± 0.04[c]	8.60 ± 0.02[d]
Albumin (g/dL)	3.04 ± 0.16[a]	3.63 ± 0.08[b]	4.04 ± 0.22[c]	3.42 ± 0.10[d]
Total protein (g/dL)	8.30 ± 0.15[a]	8.84 ± 0.12[b]	9.16 ± 0.14[c]	8.62 ± 0.14[d]
Sodium ion (mM)	140.00 ± 7.77[a]	167.02 ± 3.82[b]	186.27 ± 10.22[c]	157.68 ± 4.75[d]
Creatine kinase (U/L)	13.07 ± 0.07[a]	13.19 ± 0.05[b]	13.28 ± 0.07[c]	13.15 ± 0.05[d]
Arginine vasopressin (pg/mL)	8.40 ± 0.10[a]	8.52 ± 0.05[b]	8.62 ± 0.09[c]	8.49 ± 0.05[d]
Aldosterone (pg/mL)	26.78 ± 0.68[a]	28.09 ± 0.64[b]	28.96 ± 0.69[c]	27.59 ± 0.63[d]

[1]Data summarised by sheep across all the sampling days; [†](Kaneko *et al* 2008); [§](Lotfollahzadeh *et al* 2016); [¥](Mengistu *et al* 2016); [¶](Kataria and Kataria 2007); *significant differences were obtained between groups indicated with different letters; *P< 0 .05.

Table 3. Pearson Correlation Coefficients for different biochemical analytes of feral sheep from the Socorro Island, Revillagigedo Archipelago, during restricting water intake (n = 376 blood serums).

	COL-T[b]	TAG[c]	urea	ALB[d]	PROT-T[e]	Na^{+}[f]	CK[g]	AVP[h]	Aldo[i]
GLU[a]	0.04	0.03	0.04	0.03	0.03	0.03	0.04	0.06	0.03
	COL-T	0.33*	0.26	-0.75*	0.24	0.14	0.10	0.90*	0.33*
		TAG	0.14	-0.80*	0.09	0.13	0.11	0.84*	0.09
			urea	0.14	0.80*	0.09	0.20	0.82*	0.19
				ALB	0.07	0.09	0.60*	0.11	0.10
					PROT-T	0.10	0.79*	0.71*	0.12
						Na^{+}	0.19	0.13	0.76*
							CK	0.13	0.11
								AVP	0.84*
									Aldo

Official abbreviation of the International Union of Pure and Applied Chemistry (IUPAC); [a]Glucose; [b]Total cholesterol; [c]Triglycerides; [d]Albumin; [e]Total protein; [f]Sodium ion; [g]Creatine kinase; [h]Arginine vasopressin; [i]Aldosterone; *P<0.05

two physiological conditions, lactating and dry, over a 3-week-period, and an increase ALB (3.08 vs. 3.43 g/dL), COL-T (1.7 vs. 2.4 mM), PROT-T (7.39 vs. 7.86 g/dL), and urea (8.29 vs. 10.01 mM) was observed. Jaber *et al* (2004) also reported that water restriction increased COL-T (1.90 vs. 2.14 mM), PROT-T (7.59 vs. 8.03 g/dL), and urea (5.96 vs. 7.71 mM), and Ghanem *et al* (2008) reported that COL-T increased (1.61 vs. 2.06 mM).

The negative correlations ($P<0.05$) between ALB with COL-T and TAG presented in table 3, relate to the availability of acetyl-CoA. After its synthesis, the ALB resides in the smooth endoplasmic reticulum and Golgi bodies (Menzies *et al* 2016). The smooth endoplasmic reticulum is the compartment for lipid synthesis (Black *et al* 2002). There, the acetyl-CoA molecules are joined together to form COL-T and TAG (Kumar *et al* 2018). Thus, nascent ALB is expected to cleave the thioester bond of acetyl-CoA (Menzies *et al* 2016). It is plausible to think that this non-enzymatic acetylation of ALB is possible in the intracellular compartment using acetyl-CoA (Kumar *et al* 2018) and that this limits the availability of acetyl-CoA for the synthesis of COL-T and TAG (Morgan *et al* 2016). Positive correlations ($P<0.05$) between the different biochemical analytes presented in table 3 relate to two main mechanisms for conserving body water. The first mechanism involves osmoreceptors that detect increases in solute concentrations in the blood during dehydration (Prager-Khoutorsky 2017). These osmoreceptors send a neural signal to the hypothalamic supraoptic nucleus (Gizowski and Bourque 2018), to translate AVP from nine amino acids (aa) (Rotondo *et al* 2016). AVP stored and released by the neurohypophysis travels to the kidneys, binds to receptors in the distal or collecting tubules, and promotes antidiuresis (retention of water) (Pratt *et al* 2016), in the loop of Henle and the proximal tubule of the nephron (Bankir *et al* 2017). Water reabsorption varies, depending on the concentration of circulating AVP, which is usually determined by plasma osmolality, but also intracellular fluid volume (Trepiccione *et al* 2019). Hence, the serum volume decreases due to water uptake by cells (Trepiccione *et al* 2019) and COL-T, TAG, urea, PROT-T, and Aldo increase positively in relation to the AVP concentration (table 3).

The second mechanism involves baroreceptors that detect low blood pressure and low blood volume (Burlando *et al* 2019). In response, neurons send a signal to the juxtaglomerular cells of the kidneys to translate renin also called angiotensinogenase (Ames *et al* 2019), which hydrolyses angiotensinogen to angiotensin I (Patel *et al* 2017). Angiotensin I, is hydrolysed by the angiotensin-converting enzyme (ACE) forming angiotensin II (Burlando *et al* 2019), which stimulates the conversion of COL-T to Aldo (mineralocorticoid) in the adrenal gland (Ames *et al* 2019). Aldo conserves Na^+ in the renal distal convoluted tubule (Patel *et al* 2017), as can be seen in figure 3, where the results indicate that Aldo was positively correlated with Na^+, triggering reabsorption of water through osmosis. Figure 3 shows the linear regression model for these variables.

The Na^+ is the dominant extracellular cation and the main determinant of osmolality (Berihulay *et al* 2019), and therefore of water distribution between the extracellular and intracellular fluid compartments (Burlando *et al* 2019). This has also been reported by Hamadeh *et al* (2006) in Awassi sheep. In particular, the progressive increase in Aldo, in response to water restriction, leads to an increase in Na^+ concentration. Hence, many of the extrinsic regulators of renal function are intended to alter Na^+ excretion, preserve Na^+ balance, and regulate blood pressure (Trepiccione *et al* 2019).

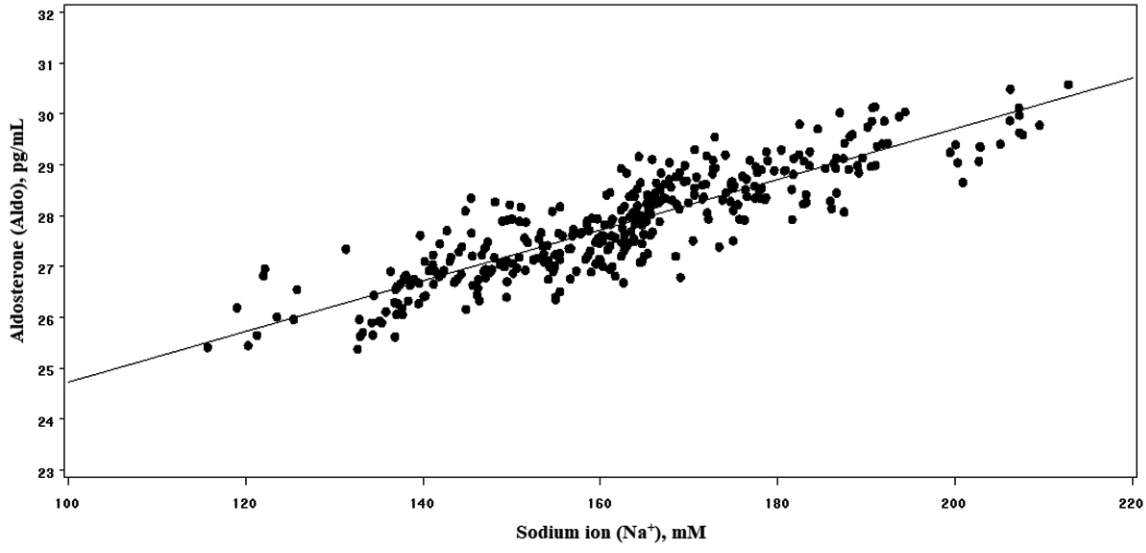

Figure 3. Relationship between aldosterone and sodium ion. Aldosterone (●); predicted response (—).

The feral sheep from Socorro Island of the Revillagigedo Archipelago needed 21 d to adapt to water shortage treatment. Results suggested that the feral sheep had high resilience to limited water availability, expressed in the concentration of different biochemical analytes considered as good indicators of dehydration. These results show the important role of water, as a limiting factor for sheep in low-water availability environments. The Pearson Correlation Coefficients could be used at the herd level, to detect physiological mechanisms to cope with water restriction. Understanding the physiological mechanisms associated to these values would help researchers to select animals that maintain their productive ability under environmental stressors such as drought.

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ORIGINAL ARTICLE

Aquatic pollution from anthropogenic discharges modulates gene expression in liver of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT. With the aim to characterise the biological impact of anthropogenic discharge in a river, gene expression in fish was evaluated as a biomarker for mixture effects of potential toxic compounds in the aquatic environment. Adult male rainbow trout (*Oncorhynchus mykiss*) were used as monitoring organisms. Within the German experimental set up, trout were exposed actively for 28 days in biologically purified sewage as well as in river water up- and downstream of the sewage treatment plant (STP) effluent. In a different approach, wild trout were captured in southern Chile near anthropogenic discharges. Fish were taken from river Maullin receiving sewage from a settlement and from river Pescado influenced by effluents from a fish farm. *In vivo* effects were assessed by RT-qPCR analyses of biomarker gene expression, vitellogenin (*vg*), metallothionein (*mt*) and cytochrome 1A (*cyp1A)* in liver samples with primers amplifying specific sequences previously confirmed by cloning and sequencing. The modulation of expression of marker genes involved in metal stress, reproduction and detoxifying systems in the liver of male rainbow trout revealed organismal response to anthropogenic contamination in two different study areas, such as Chile and Germany, thereby indicating a potential risk on the aquatic ecosystems.

Key words: anthropogenic discharges in river, gene expression, liver biomarkers, environmental exposure, *Oncorhynchus mykiss*.

INTRODUCTION

Currently, ecotoxicology faces a huge challenge in evaluating toxicity and how mixtures of chemical compounds can affect wild life in the aquatic environment (Holmstrup *et al* 2010, Kumar and Denslow 2017). Evaluation of biomarkers in exposed organisms can enhance characterisation of biological impact from aquatic pollution (Connon *et al* 2012). Endocrine disruptors (EDs) are natural or manmade substances that are able to interfere with the endocrine system and therefore represent a significant threat for aquatic organisms and human health (Rappaport and Smith 2010, Kortenkamp 2017). Many genes responsive to xenobiotic compounds have been reported in *in vitro* assays, but an effect of a particular chemical *in vitro* may not necessarily be predictive of its *in vivo* effects due to factors such as biodegradation and metabolism (Tyler *et al* 1998, Wilson *et al* 2016). However, analyses of robust molecular biomarkers in fish should facilitate diagnosis of sublethal adverse effects in organisms within the aquatic environment.

Besides heavy metals and dioxin-like compounds, estrogenic compounds are among the most studied contaminants because they are suspected to affect male fertility (Marlatt *et al* 2016). The latter include natural estrogen steroids, e.g. 17β-estradiol, phytoestrogens, and mycoestrogens, synthetic estrogens such as ethinylestradiol, as well as various industrial compounds and organic contaminants (Kagi 1993). On the other hand, the induction of vitellogenin (VG) synthesis in male fish has been widely used as a biomarker to detect estrogenic effects of xenoestrogens in the field (Schwaiger and Negele 1998). Vitellogenin (VG) is a precursor of the egg yolk protein which is synthesised physiologically in the liver of female fish during sexual maturation (Copeland *et al* 1986). The synthesis of VG is initiated by binding of an estrogen to a highly specific estrogen receptor in the hepatocytes (Mommsen and Walsh 1988, Benninghoff and Williams 2008). Males also produce VG under the influence of estrogenic compounds (Mommsen and Walsh 1988). An overexpression of VG has been observed in male carp as a consequence of exposure to estrogenic compounds (Valenzuela *et al* 2015) and is considered as part of a feminisation process in male fish (Hamilton *et al* 2014).

Another marker to evaluate the toxic effects of xenobiotics in the aquatic environment is Cytochrome-P450 (*cyp1*) (Buhler and Wang-Buhler 1998, Zhou *et al* 2010, Burkina *et al* 2017). This system plays a role in xenobiotic metabolism and some endogenous compound metabolism, such as steroids, fatty acids and prostaglandins (Arukwe 2002). Also *cyp1* is upregulated in response to dioxins and polycyclic aromatic hydrocarbons (PAH) (Buhler and Wang-Buhler 1998, Zhou *et al* 2010, Burkina *et al* 2017).

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Metallothioneins (MT) belong to a family of cysteine-rich low molecular weight proteins capable of binding both physiologically important natural and xenobiotic heavy metals. MT play a key role in homeostasis because they are involved in detoxification of potentially toxic metals (Chen *et al* 2007). MT overexpression was observed in response to heavy metals in mollusks and fish (Roesijadi 1994). Synthesis of MT is mainly induced by exposure to metals and to a less degree by hormones, cytokines and organic contaminants (Kagi 1993).

Chemical-analytical investigations are, in many cases, not suitable for identifying individual compounds because of the broad spectrum of anthropogenic substances and their often-discontinuous emission into the aquatic environment. While chemical analyses usually represent a snapshot, biomarker responses integrate a specific load over an extended time period. Furthermore, a biomarker response does not only point to a specific pollution of a water body, but also to the fact that the substances have been taken up and have led to an effect in the intact organism. Therefore, the use of specific biomarkers in fish represents a valuable tool for detecting aquatic pollution. In the present study two different experiments were carried out comparatively. The first one represents an active monitoring experiment using rainbow trout (*Oncorhynchus mykiss*) as test organisms. This study was performed at a sewage treatment plant (STP) and the respective receiving river in Bavaria, Germany. The location was chosen because at low flow rates during heat periods, the dilution ratio between waste water and river water can be 1:1. In such worst-case situations, a relatively high chemical load could be assumed. The second experiment was carried out in Patagonia, Chile. Within the frame of a passive monitoring program, adult male rainbow trout were captured in Maullin and Pescado river, located in Llanquihue Province, Región de Los Lagos, Chile. Fish were captured upstream and downstream of an anthropogenic emission including sewage treatment plant as well as fish farm effluents. The aim of this study was to reveal the biological effects of anthropogenic pollution by evaluating the transcriptional level of metallothionein (*mt),* vitellogenin (*vg)*, and cytochrome 1A (*cyp*1a) genes as molecular endpoints in the liver of male rainbow trout, reflecting organismal response in two different areas of the world.

MATERIAL AND METHODS

SAMPLE COLLECTION

The experiment in Germany was performed at a sewage treatment plant (STP) located in a small village in Bavaria, Germany. The STP has a catchment area of approximately 35,000 inhabitants. The investigations were carried out within the framework of an official water monitoring program of the federal state of Bavaria. In the exposure experiment, 4-years old adult male rainbow trout (*Oncorhynchus mykiss*) reared in unpolluted spring water under disease-controlled conditions, with body weight of 1441.44 g ± 212.68 and body length of 49.62 cm ± 2.39, were obtained from the breeding stock of the Bavarian Environment Agency and were used as test organisms. In each experimental setup, 20 fish were exposed in fish tanks (bypass exposure) to water of a small river upstream (US) and downstream (DS) of a sewage treatment plant (STP), as well as directly to the biologically purified sewage (D) for 28 days. A control group (C) was maintained in a tank receiving uncontaminated spring water. In all 4 experimental set ups, during the entire test period, the chemical-physical water parameters O^2, pH, temperature T°C and conductivity were measured continuously by using an online analytical system (IQ Sensor Net System 2020; WTW, Germany). Furthermore, the flow rates of the STP-effluent and the effluent receiving river were determined by means of a flow meter (Flowtronic, Typ Flo-Dar, Belgium) during the whole experiment. Figure 1 shows the bypass exposure tanks which were equipped with samplers and the online measuring systems.

The animal experiments were approved by the competent authority in the government of Upper Bavaria and were carried out according to the requirements of the German animal welfare legislation.

After the exposure period, fish were anaesthetised in MS222 (Tricaine, Pharmaq Ltd. 10g/100L) and subsequently killed by decapitation. After necropsy of fish, liver samples of 7 (US), 8 (DS), 7 (D), and 6 (C) individuals were removed and immediately submerged in Chomczynski solution for RNA preparation (Chomczynski and Sacchi 1987).

In the Chilean Patagonia, adult male rainbow trout were captured in the Maullin and Pescado rivers, both located in the Llanquihue Province, Región de Los Lagos. The fish were captured 1000 m upstream (US) and within 20 m downstream (DS) of discharge site. Conductivity was measured with Multiparameter Meter Model HI9829 (Hanna). Sampling sites were chosen because of accessibility in the field with the electric fishing device (Pottier *et al* 2019). Electric fishing preferable captures small fish expected to exhibit low mobility (Habera *et al* 2010). The source of contamination in the Maullin river was a sewage treatment plant discharge. Three male trout were captured upstream (US) and downstream (DS), respectively. In the Pescado river, which receives the effluent of a fish farm, six male trout were captured at each sampling site, upstream (US) and downstream (DS). Individual rainbow trouts were captured using electrofishing equipment (EFKO, model FEG 1000, 1 KW, 150-600 V). Fish were netted and submitted to lethal doses of 2-phenoxyethanol (1 mL/L) and euthanised by spinal section before tissues were removed. The liver was extracted and immediately submerged in RNAlater solution (Qiagen). The performed experiments complied with the Guidelines of the Comisión Nacional de Ciencia y Tecnología de Chile (CONICYT) and the Universidad Austral de Chile for the use of laboratory animals.

Figure 1. Fish tanks (bypass exposure) equipped with an online analytical system (IQ Sensor Net System 2020; WTW, Weilheim, Germany).

RT-qPCR ANALYSES

Total RNA was extracted with Chomczynski solution for RNA according to the suppliers' instructions (Winkler Ltd.). From total RNA cDNA was prepared with oligo-dT and M-MLV reverse transcriptase (Promega) according to standard procedures (Sambrook *et al* 1989). Specific primer pairs for rainbow trout genes were designed with Primer3 program for quantitative real-time PCR (qPCR) on cDNA template for metallothionein *mt*, vitellogenin *vg* and cytochrome 1A *cyp*1a (table 1). First, specificity of all amplicons was verified by cloning and sequencing. Next, all samples were processed in parallel. qPCR assay was conducted using Brilliant RII SYBRR Green, qPCR Master Mix (Agilent Technologies) according to the suppliers' instructions in a Mx3000 Real-Time Thermocycler. In a 40 cycles PCR reaction each cycle consisted in 20 s at 94 °C, 15 s at 55 °C and 15 s at 72 °C, followed by final heating to 95 °C revealing melting curves of unique amplification products. All analyses were performed in duplicate. The expression level of each gene was normalised to elongation factor 1a (*ef*1a) expression as reference gene with exon-spanning primers to control for genomic DNA contamination since no DNAse treatment of total RNA was included. RT-qPCR assays were analysed with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) via MxPro software (Stratagene) and expressed as Relative Quantity to Normalizer (Pfaffl 2001).

Table 1. Oligonucleotides for RT-qPCR analyses in *Oncorhynchus mykiss*.

Gene	acc number*	Oligonucleotides	Amplicon (bp)
Elongation factor 1α (*ef*1α)	NM001124339	omEF 1a s ctgaaggccggtatgatcgt omEF 1a a acattgtcaccaggcatggc	110
Metallothionein a (*mt*a)	M18103.1	omMTa s atcctgcaagtgctccaac omMTa a acaaaagctatgctcaagatgg	214
Vitellogenin (*vg*)	X92804.1	omVG s gggaagatgccaaggcagag omVG a agcggcttcatgaggtagt	176
Cytochrome 1A (*cyp* 1A)	AF015660.1	omCyp 1a s agtgatgagtttgggcaggt omCyp 1a a tcacggatgttgtccttgtc	178

*acc number: GenBank accession number.

STATISTICAL ANALYSES

The determined mRNA levels were presented as relative quantity to normalizer (dR) for three genes independently (*mt*, *vg*, *cyp*1a). The data from caged rainbow trout (C, n=6; US, n=7; D, n=7; DS, n=8) were analysed with One-way analysis of variance (ANOVA). For the data analyses of fish captured in Chile, from Maullin river (US, n=3; DS, n=3) and Pescado river (US, n=6; DS, n=6), the Unpaired Student's T-test was applied. Differences were considered significant at $P<0.05$.

RESULTS

CHEMICAL-PHYSICAL PARAMETERS AND FLOW RATES DURING THE EXPOSURE EXPERIMENTS

In figure 2 the chemical-physical data during the experiment are given. The continuous analysis of pH in the three test groups and the control approach revealed only slight deviations varying from 7.6 to 8.2. The water temperature varied depending on the trial site. The highest temperature, which was measured directly in the STP-effluent was in the range of 12.5°C. The temperature in the river upstream of the effluent was 7.4 °C, whereas downstream the mean temperature was determined to be 8.8 °C. The temperature of the spring water used to expose control fish was 10.3 °C. The conductivity varied between 1255.3 µs/cm in the STP-effluent and 722.4 µs/cm in the control water. In the river upstream of the STP-effluent discharge, the mean conductivity was 824 µs/cm, and downstream it was 975.4 µs/cm. The mean oxygen concentration in the control water was 9.3 mg/l, in the river upstream of the STP-effluent it was 10.5 mg/l and downstream it was 10 mg/l. The lowest oxygen concentration of 8.2 mg/l was analysed directly in the STP-effluent.

The continuous measurement of the flow rate in the STP-effluent revealed, with the exception of an increase in the first and third week of the experiment, a usually very stable flow rate (figure 3). The flow rate in the river downstream was in general higher than in the STP-effluent and displayed also 2 peaks in the first and third week accordingly.

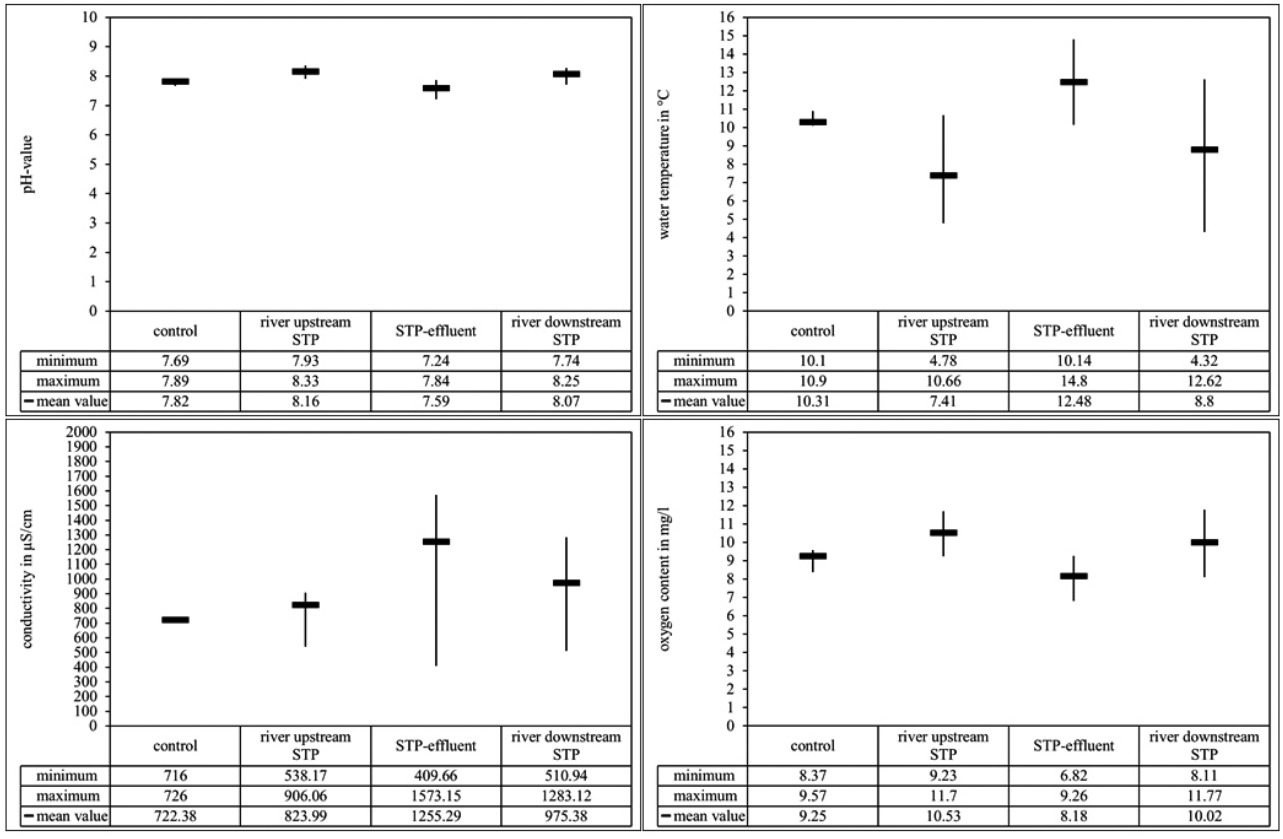
EFFECTS IN MALE RAINBOW TROUT EXPOSED TO STP EFFLUENTS AND CORRESPONDING RIVER WATER

In Bavaria, Germany, expression analyses of biomarker genes from caged male rainbow trout (figure 4) revealed that there was no significant difference in expression of *mta* gene between C, US, D and DS groups. No significant difference in the expression levels of *vg* gene was observed between control individuals and the other three groups. A significant decrease in expression of *vg* was detected in the DS group compared to the D group. Due to the fact that expression levels of *vg* in the US and D group revealed a high variability, no significant difference was shown between these two groups. A significant increase in the expression of *cyp1a* gene was observed in the US, D and DS groups in comparison to the control group C. Furthermore, the expression level of *cyp1a* gene was significantly lower in the DS group than in the US and D groups.

DISCHARGE EFFECT ON MALE RAINBOW TROUT CAPTURED FROM MAULLIN AND PESCADO RIVER

In the Patagonian rivers (table 2), the sampling season was spring (november) for Maullin river which receives municipal wastewater, water temperature was similar US (14ºC) and DS (16ºC), conductivity was 52 µS/cm US and 35% higher at DS (80 µS/cm). A total of ten fish were captured, five at each site. From Maullin river the fish sampled from US had a mean size 25.2 cm ± 10.4, DS 21.2 cm ± 6.3. In Río Pescado, receiving effluents of a fish farm, during the winter (August) sampling season, the temperature at US (7ºC) was five degrees lower than on DS (12ºC) and conductivity varied from 66 µS/cm US to 84 µS/cm DS, an increase of 21%. Six fishes were captured at each site. From Pescado river captured fish mean size was US 9.3 cm ± 0.6, and DS 13.4 cm ± 3.8.

In the Maullin river (figure 5), no differences were observed in the expression of *mta* between the two



pH-value	control	river upstream STP	STP-effluent	river downstream STP
minimum	7.69	7.93	7.24	7.74
maximum	7.89	8.33	7.84	8.25
mean value	7.82	8.16	7.59	8.07

water temperature in °C	control	river upstream STP	STP-effluent	river downstream STP
minimum	10.1	4.78	10.14	4.32
maximum	10.9	10.66	14.8	12.62
mean value	10.31	7.41	12.48	8.8

conductivity in µS/cm	control	river upstream STP	STP-effluent	river downstream STP
minimum	716	538.17	409.66	510.94
maximum	726	906.06	1573.15	1283.12
mean value	722.38	823.99	1255.29	975.38

oxygen content in mg/l	control	river upstream STP	STP-effluent	river downstream STP
minimum	8.37	9.23	6.82	8.11
maximum	9.57	11.7	9.26	11.77
mean value	9.25	10.53	8.18	10.02

Figure 2. Online chemical-physical data during the 28-days experiment (pH, temperature T°C, conductivity and O^2) of biologically purified STP-effluent, river water upstream and downstream of STP-effluents and Control spring water (mean ± standard deviation).

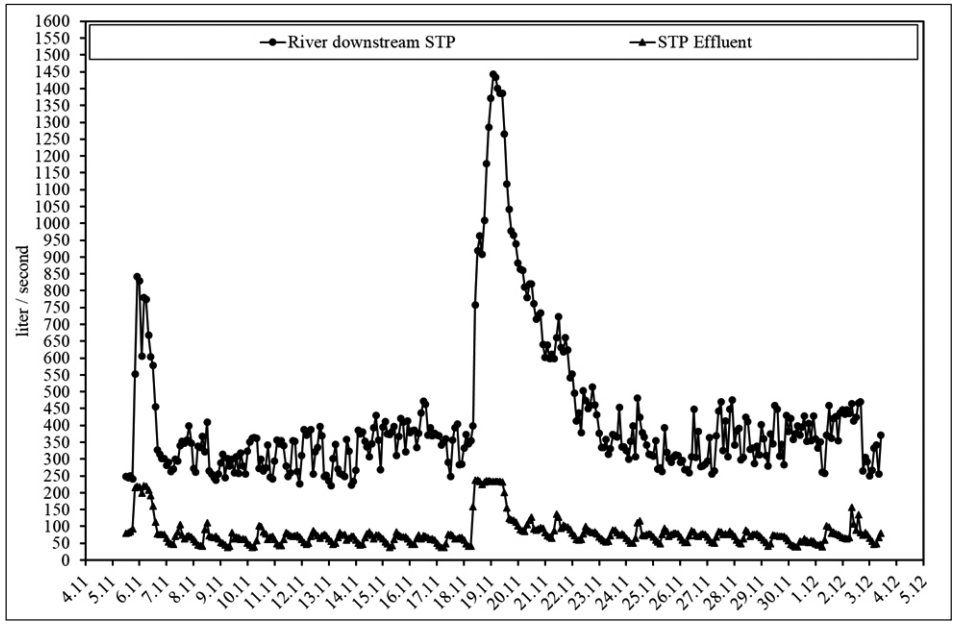


Figure 3. Daily average flow rates of the STP-effluent and the effluent receiving river during the 28-days exposure period.

Table 2. Biophysical data of *O. mykiss* caught in Patagonians rivers.

	Maullin River	Pescado River
Sampling season	spring (November)	winter (august)
Location	US DS	US DS
Water temperature (°C)	14 16	7 12
Water conductivity (µS/cm)	52 80	66 84
O. mykiss number (n)	5 5	6 6
Size (cm)	25.2±10.4 21.2±6.3	9.3±0.6 13.4±3.8

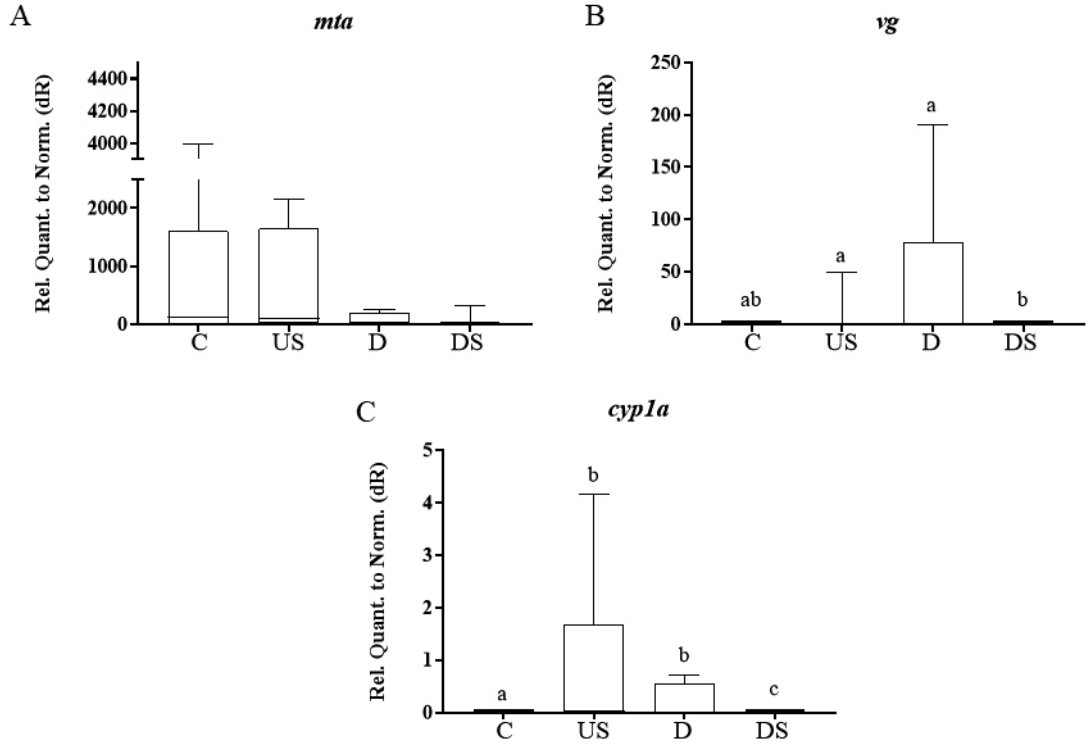


Figure 4. Sewage discharge effect on expression of marker genes in liver of caged male rainbow trout (Bavaria, Germany). mRNA levels of (A) metallothionein A (*mta*), (B) vitellogenin (*vg*) and (C) cytochrome 1A (*cyp1a*) in liver were measured using RT-qPCR and represented as relative quantity to normaliszer gene (dR). Analyses were performed in duplicate and all data were normalised for *ef1*a gene expression. Graphs depict gene expression data of C (control) n=6, US (upstream sewage discharge) n=7, D (at sewage discharge site) n=7 and DS (downstream sewage discharge) n=8. The box plot shows the median (middle line), the 75th and 25th percentiles and the maximum and minimum values (top and bottom of the whiskers). $P<0.05$ was considered statistically significant difference, indicated by different letters.

experimental groups. With regard to the expression of *vg* gene, at least one of the male trout captured downstream the discharge site showed a clear increase, whereas in trout captured further upstream very consistent low expression levels were detected. In fish captured downstream of the discharge, the level of expression of *cyp1a* significantly decreased in comparison to fish captured upstream of the discharge site.

In the Pescado river (figure 6), a comparison of *mta* expression in male rainbow trout captured up- and downstream of effluents from a fish farm revealed no significant differences between fish from the two sites, except for one individual from downstream that exhibited clearly upregulated *mta* expression. No clear differences in *vg* expression levels were established between fish captured upstream or downstream of a discharge site. Furthermore, in trout captured downstream the discharge site the expression of *cyp1a* tended to increase compared to individuals sampled upstream of the fish farm emission.

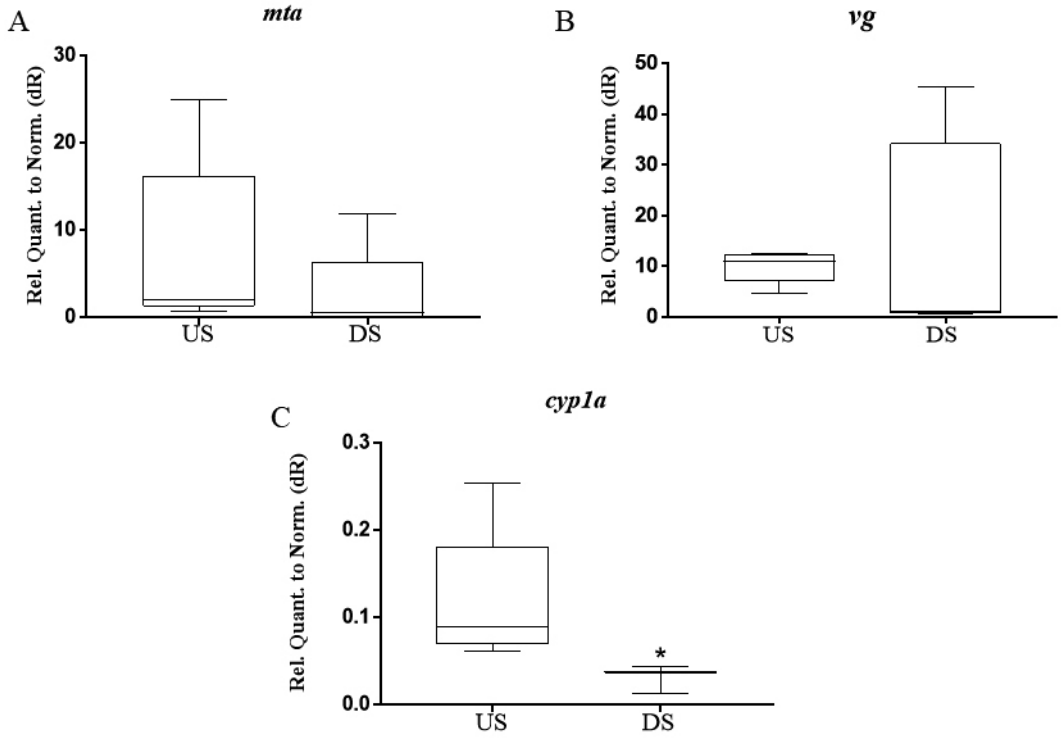


Figure 5. Discharge effect on expression of genes in liver of male rainbow trout captured in Maullin river. At Maullin river fish were captured US and DS of STP effluents. mRNA levels of (A) *mta*, (B) *vg* and (C) *cyp1a* in liver were measured using RT-qPCR. Analyses were performed in duplicate and all data were normalised for *ef1*a gene expression. Graphs depict relative quantity to Normalizer gene (dR) from individual adult male trout captured upstream of the discharge zone (US, n=3) and downstream discharge zone (DS, n=3). The box plot shows the median (middle line), the 75th and 25th percentiles and the maximum and minimum values (top and bottom of the whiskers). $P<0.05$ was considered statistically significant difference, indicated by different letters.

DISCUSSION

The present study was performed in order to implement a set of specific biomarkers on the transcriptional level of metallothionein, vitellogenin and cytochrome 1A. To prove the suitability of biomarkers, rainbow trout deriving from an exposure experiment in Bavaria, Germany, as well as individuals caught in two remote rivers in Patagonia, Chile, were examined comparatively.

Molecular endpoints in the liver of rainbow trout were used as biomarkers to assess the impact of anthropogenic discharge containing pollutant mixtures in one location in Bavaria, southern Germany, and in two rivers in southern Chile. Rainbow trout served as the model fish because of their wide distribution and sensitivity towards environmental pollution (Marlatt *et al* 2016, Martyniuk *et al* 2020).

Although farmed rainbow trout were used as test organisms which ensured a homogeneous test collective, in the caged fish from the experiments conducted in Germany a variability of the gene responses was obvious. The reason for the higher variability of expression levels observed predominantly in upstream compared to downstream samples is not clear. The variability of biomarker response could not be related to migration of experimental fish because trout were held in tanks and exposure took place via bypass. Possibly, the exposure to the effluent might pose constraint on the underlying variability in the real-world situation (Tyler *et al* 1998). Since *mta* was not affected, heavy metal stress seems to be unlikely (Ricketts *et al* 2015, Valenzuela *et al* 2015). In previous work, we observed a clear increase in the expression of *mt* in response to zinc in carp liver, with no concomitant significant change of *vg* expression. In a parallel experiment, a significant increase of *vg* expression in response to 17β-estradiol was not accompanied by changes in *mt* expression. This leads to the suggestion that specific stimuli elicit specific physiological responses, activating either the heavy metal response pathway or the estrogen receptor pathway, thereby validating the use of *mt* and *vg* as specific biomarker genes (Valenzuela *et al* 2015). Due to the fact that *vg* expression did not increase significantly in male fish exposed to STP-effluents at D compared to control, but was at least higher compared to fish exposed downstream of the effluents, estrogenic contaminants cannot be excluded. The continuous measurement of flow rates in our study revealed throughout the exposure period significantly higher flow rates in the river downstream of the discharge compared to the STP-effluent itself.

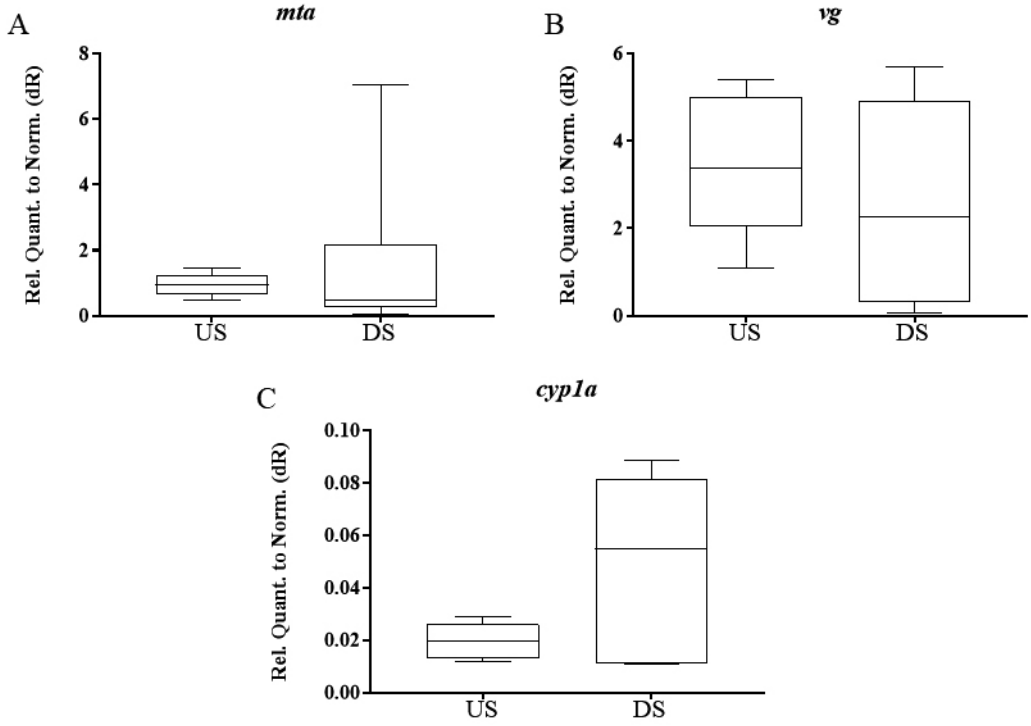


Figure 6. Discharge effect on expression of genes in liver of male rainbow trout captured in Pescado river. At Pescado river fish were captured US and DS of aquaculture effluent. mRNA levels of (A) *mta*, (B) *vg* and (C) *cyp1a* in liver were measured using RT-qPCR. Analyses were performed in duplicate and all data were normalized for *ef*1a gene expression. Graphs depict Relative Quantity to Normalizer gene (dR) from individual adult male trout upstream (US, n=6) and downstream of a discharge zone (DS, n=6). The box plot shows the median (middle line), the 75th and 25th percentiles and the maximum and minimum values (top and bottom of the whiskers). Considering $P<0.05$, no statistically significant difference was found.

As described elsewhere (Osachoff *et al* 2016), it can be assumed that, within the exposure period, dilution in the river might lead to concentrations under the detection level. The significant higher transcript level of *cyp1A* in samples from US and D as compared to controls implies an impact of compounds which are able to interact with the arylhydrogen receptor pathway and might reflect a dose dependent stress response (Zhou *et al* 2010, Ings *et al* 2011). The decrease of expression levels in fish exposed downstream of the discharge site might again be a consequence of higher dilution in the river.

At two remote field sites in southern Chile, for the first-time biomarker gene expression profiles in rainbow trout were used as representatives of physiological parameters to enhance insight into the effects of pollutants on wild trout from punctual release of sewage in Maullin river and discharge of aquaculture effluents in Pescado river. In the present study in Patagonia, fish were captured in the field in proximity of the discharge site, therefore, in contrast to the study in Germany with caged fish, mobility between sampling sites of free living individuals cannot be excluded. However, several studies have revealed that trout often remain stationary for long periods of time. A study in a New Zealand river that was performed over 72 days with recordings every 2 to 3 days, reported that half of the radio tagged wild rainbow trouts (40 individuals) were found to remain in their home area (Dedual and Jowett 1999). Specially, small individuals seem to move very little, as shown by radiotracking of hatchery raised trout of different size released in a Californian river (Cocherell *et al* 2010). Over a period of 9 weeks with weekly revision, small sized rainbow trout mainly remained in one location, with movements of less than about 500 m (Cocherell *et al* 2010). In accordance with these observations, mobility between US and DS seems very unlikely in the small rainbow trout sampled in the proximity of anthropogenic discharges in Patagonian rivers.

As far as we know, the data from the samplings at Maullin and Pescado rivers contribute biological base line information for wild trout in these areas, which might be important for further spatio-temporal monitoring schemes (Tattam *et al* 2016).

At Maullin river, apart from one exception, low levels of *mta*, and variable *vg* levels indicate little or no effect on genes regulated via heavy metal or estrogenic response elements present in *mta* or *vg* promoter regions, respectively (Kagi 1993, Zhou *et al* 2010). However, the nearly 10 times higher *vg* transcripts in one individual captured downstream of discharge site compared to all other samples might indicate triggering of a response

to estrogenic compounds in male trout, consistent with other *in vivo* and *in vitro* observations (Benninghoff and Williams 2008, Gagné *et al* 2013, Osachoff *et al* 2016). In this respect, it is known from other monitoring programs in Chile that e.g. pulp and paper production effluents lead to increased *vg* expression in *in situ* exposed rainbow trout (Chiang *et al* 2015).

At Pescado river, which receives effluents from a fish farm, a higher number of samples revealed a more consistent picture. Discharges from aquaculture facilities might include organic load such as faeces and unconsumed food as well as xenobiotics such as antibiotics and disinfectants (Nimptsch *et al* 2015). With regard to *mta* and *vg* expression no clear differences were found between fish captured upstream or downstream of discharge site. However, a nearly four times higher *cyp1A* transcript level in 50 percent of the fish caught at the discharge site might indicate a mobilisation of the xenobiotic-metabolising system (Burkina *et al* 2017). This could possibly be related to a contamination with toxic compounds, which are usually applied ectopically to limit propagation of highly infectious diseases impacting trout farms in southern Chile (Cárcamo *et al* 2017).

In the present study, three biomarkers indicative for adverse effects of contaminants on fish were evaluated and should be further strengthened by extending the panel of marker genes in future studies (Martyniuk *et al* 2020).

In recent years, the increasing concern on environmental impacts of anthropogenic contaminants in former pristine Patagonian rivers prompted toxicological studies in order to generate data on the presence and concentration of potential toxic substances (Alonso *et al* 2017). The importance of such data has been shown in other reports such as a study conducted as a whole-lake experiment in Canada, which demonstrated that chronic exposure to mixtures or even low levels of estrogen may exert dramatic effects on wild fish populations (Kidd *et al* 2007). Molecular biomarkers do not only play a pivotal role for the determination of water quality and detection of detrimental effects in complex mixtures (Triebskorn *et al* 2002, He *et al* 2017). Molecular biomarkers also can greatly support efficient monitoring of ameliorating protective measures within the frame of regular surveillance, as has been shown by means of decreasing *vg* concentrations in rainbow trout, indicating a reduction of estrogenic activity in STP-effluents and connected surface water as a consequence of advanced wastewater treatment (Henneberg and Triebskorn 2015).

In conclusion, the main outcome of this study is that different profiles of marker gene expression detected by RT-qPCR analyses in rainbow trout are suitable tools to indicate exposure to estrogenic or other xenobiotic compounds in the field. They can be used as a specific early warning system of a potential risk for the aquatic ecosystem. These data substantiate the importance of early alert biomarkers for biomonitoring and natural resource management such as Patagonian rivers.

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SHORT COMMUNICATION

Mycoplasma isolation in milk samples from dairy herds in Chile

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ABSTRACT. *Mycoplasma* bovine mastitis is a highly contagious disease, usually associated with clinical cases refractory to antibiotic treatment. The aim of this study was the isolation of *Mycoplasma* species in cattle milk samples from dairy herds in Chile. Bulk tank milk samples selected by convenience from 91 Holstein Friesian dairy herds located in Los Rios (66) and Los Lagos (25), the two most important dairy Regions in the country, were collected. Additionally, 100 individual milk samples from cows with a high incidence of clinical mastitis, refractory to antibiotic therapy, and negative bacteriological results for traditional mastitis pathogens, all from the Biobío Region and received in our diagnostic laboratory, were included. All samples were cultured for 10 days on PPLO medium. The differentiation of suspect colonies between genus *Mycoplasma* and *Acholeplasma* was performed by the digitonin test and a specific PCR. The species identification was performed by a *M. bovis* specific PCR and 16S rRNA sequencing. *Mycoplasma* was isolated from 3 (3.3%) bulk tank milk samples and 2 (2%) individual cow milk samples. All colonies were identified as *Mycoplasma* by the digitonin test and by a specific PCR. At species level, one strain isolated from a bulk tank milk sample was identified as *M. bovis*. The remaining two strains isolated from bulk tank milk samples were identified as *M. bovigenitalium*, while the two strains isolated from milk of individual cows were identified as *M. alkalescens*. These results show that not only *M. bovis* is present in Chilean dairy herds, but also other pathogenic species not previously described in Chile such as *M. bovigenitalium* and *M. alkalescens*, which pose a potential risk for dairy herds in southern Chile.

Key words: bovine mastitis, *Mycoplasma* mastitis, dairy herds.

INTRODUCTION

Bovine intramammary infection due to *Mycoplasma* is an emergent problem in the dairy industry of many countries. In recent years, an increase in *Mycoplasma* mastitis prevalence has been observed especially associated with large dairy herds (Fox *et al* 2003, Lysnyansky *et al* 2016, Nicholas *et al* 2016, Timonen *et al* 2017, Gille 2018, Abd El Tawad 2019). This type of infections cause major economic losses because are highly contagious, cannot be detected with conventional culture media, do not respond to antibiotic treatments, can affect multiple quarters, produce a large decrease in milk yield, and infected animals usually must be segregated or culled (Nicholas *et al* 2016). Common species of *Mycoplasma* isolated from intramammary infections in cows are *M. bovis, M. californicum, M. bovigenitalium, M. alkalescens* and *M. canadense.* However, *M. bovis* is the most frequent species and the one that produces the most severe clinical cases (Fox 2012). In cattle, *M. bovis* has also been associated with pneumonia, arthritis, otitis and reproductive disorders (Nicholas and Ayling 2003, Maunsell *et al* 2011).

Mycoplasmas are one of the smallest known microorganisms, they lack a cell wall and require special media for their *in-vitro* growth (Razin *et al* 1998). The routine bacteriological diagnosis procedure for *Mycoplasma* mastitis is the cultivation of milk samples on special media which are incubated for 7-10 days at 37 °C with 10% CO_2 (Hogan *et al* 1999). The morphological diagnosis is based on recognition of the typical "fried eggs" appearance of the *Mycoplasma* colonies, and its subsequent identification can be done by serological methods, PCR techniques, 16S rRNA gene sequencing, MALDI-TOF and whole genome sequencing (WGS) (Boonyayatra *et al* 2012, Nicholas *et al* 2016, Parker *et al* 2018).

Although the culture of milk samples has a low sensitivity, surveillance using bulk tank milk samples culture for *Mycoplasma* detection is essential in a *M*ycoplasma mastitis control program (Fox *et al* 2003, Wilson *et al* 2009, Fox 2012). The *Mycoplasma* mastitis prevalence, based on bulk tank milk culture, varies between countries, e.g. Belgium 1.5% (Passchyn *et al* 2012), Israel 2.7% (Lysnyansky *et al* 2016), USA 3.2% (APHIS-USDA. 2008), Japan 3.8 % (Murai and Higuchi 2019) and Grece 5.4% (Filiousis *et al* 2007). Sporadic outbreaks have been recently described in some countries but they may be underreported (Nicholas *et al* 2016).

In Chile, *M. bovis* had been previously reported in bulk tank milk samples by Sickles *et al* (2000) and later Bustos and Muñoz (2011) described *Mycoplasma* spp. in bulk tank milk samples in dairy herds of the Biobio Region. Despite clinical cases of bovine mastitis that are refractory to treatment and culture negative are common in Chilean dairy herds, no more data are available about the presence of *Mycoplasma* in bulk tank milk and cow milk samples. The aim of this study was the isolation of *Mycoplasma* species in cattle milk samples from dairy herds in Chile.

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MATERIAL AND METHODS

MILK SAMPLES COLLECTION

Ninety-one Holstein Friesian dairy herds from Los Rios (66) and Los Lagos (25), the two most important dairy Regions in the country, were selected by convenience. The herds had between 50 and 400 lactating cows and were providers of the "Sociedad Procesadora de Leche del Sur S.A" (PROLESUR). Most of them were managed under grazing-based systems with concentrate and silage as winter supplementation. Individual milk samples were collected from each bulk milk tank by trained personnel from the PROLESUR company in accordance with the recommendations of the National Mastitis Council, USA (Hogan *et al* 1999). All the samples were kept and transported at 4 °C to the laboratory within 8 h of collection for bacteriological examination. Additionally, 100 individual milk samples from cows with >500,000 cells/mL and negative bacteriological results for traditional mastitis pathogens were included. All these milk samples were received in our diagnostic laboratory and came from a dairy herd located in the Biobío Region, with a high incidence of clinical mastitis refractory to antibiotic therapy.

BACTERIOLOGICAL ANALYSIS

Immediately after arrival at the laboratory, 100 µL of each milk sample were plated onto the surface of PPLO Medium (Difco), supplemented with yeast extract, horse serum, salmon DNA, thallium acetate and penicillin and incubated at 37 °C with 10% CO_2 (Hogan *et al* 1999). The plates were read at 3, 7, 10 and 12 days of incubation using a stereoscopic microscope (40x), observing all the streaks made on the agar to detect *Mycoplasma* colonies. At the end of the incubation period, the plates with no growth were discarded and those growing small colonies with a "fried egg" appearance were considered suspicious of *Mycoplasma.* Then, the surrounding agar of the suspicious colonies was cut out and subcultured into PPLO broth supplemented as mentioned above, and incubated for 3 days at 37 °C with 10% CO_2, to obtain a pure culture. *M. bovis* ATCC 25025 strain was used as a positive control for all the tests.

For DNA extraction, 5 mL of a pure culture was centrifuged at 12,000 g for 30 seconds, the supernatant discarded, and the pellet resuspended in 150 µL of PBS, and processed with the commercial kit "AxyPrep Bacterial Genomic DNA Mini-Preparation Kit" (Axygen, Inc.) following the manufacturer instructions.

GENUS IDENTIFICATION

For the Genus differentiation between *Mycoplasma* and *Acholeplasma*, a common environmental organism, the digitonin disk test and a specific PCR test were used (Boonyayatra *et al* 2012). The digitonin disk test was performed using a sterile cotton swab moistened with pure culture broth of the suspicious colony, which was inoculated onto the entire surface of a PPLO agar plate. Afterwards, a digitonin disk (Udder Health System, Inc) was placed in the centre of the plate and incubated for 4 days at 37 °C with 10% CO_2. The presence of a growth inhibition zone surrounding the digitonin disk was visually checked. A clear inhibition zone >5 mm from the edge of the disk was considered as a positive test for the genus *Mycoplasma*. On the contrary, when the growth inhibition zone was <3 mm it was considered as a negative test indicating that the strain belongs to the genus *Acholeplasma*. In addition, a specific PCR test for genus differentiation was performed using the set of primers F2 5'-GTG(C/G)GG(A/C) TGGATCACCTCCT-3' and R2 5'-GCATCCACCA(A/T) A(A/T)AC(C/T)CTT-3' which targets the 16S-23S rRNA intergenic spacer region of *Mycoplasma* and R34 5'-CCACTGTGTGCCCTTTGTTCCT-3' of the 16S-23S rRNA intergenic spacer region of *Acholeplasma*. (Boonyayatra *et al* 2012). The amplification program was an initial denaturation cycle of 5 minutes at 94 ºC, followed by 35 cycles of 30 seconds at 94 ºC, 2 minutes at 55 ºC and 2 minutes at 72 ºC, with a final extension of 5 minutes at 72 ºC. Amplification products were separated by 2% agarose gel electrophoresis stained with ethidium bromide. The presence of two bands was considered a positive result for *Acholeplasma*, while *Mycoplasma* produces only one band.

SPECIES IDENTIFICATION

Once the suspect strains were confirmed as *Mycoplasma*, a specific PCR was performed for the identification of *M. bovis*. The primers mb-mp 1F 5'-TATTGGATCAACTGCTGGAT-3 'and mb-mp 1R 5'-AGATGCTCCACTTATCTTAG-3' were used for the amplification of the mb-mp 81 gene, as described by Foddai *et al* (2005). The thermocycler was set with an initial denaturation cycle of 5 minutes at 94 ºC, followed by 30 cycles of 1 minute at 94 ºC, 1 minute at 54 ºC and 1 minute at 72 ºC, with a final extension cycle of 10 minutes at 72 ºC. The amplification product was separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The presence of a band of approximately 447 bp was considered as *M. bovis*. Additionally, all strains were identified by sequencing the 16S rRNA gene, according to the protocol described by Botes *et al* (2005). PCR products of approximately 1048 bp in size were purified using the commercial kit Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Once the PCR products were purified, they were sequenced bidirectionally in MACROGEN (Korea). Consensus sequences were compared to sequences deposited at GenBank using the NCBI's nucleotide-nucleotide BLAST program. An identity above 98.6% was considered as the same *Mycoplasma* species (Kim *et al* 2014).

RESULTS AND DISCUSSION

Mycoplasma was isolated in 3 out of 91 (3.3%) bulk tank milk samples. Typical colonies were 300-400 µm in diameter, with a dense central nucleus, surrounded by a lighter peripheral area of growth, which gives it the characteristic "fried egg" appearance (figure 1). Additionally, *Mycoplasma* was also isolated in 2 out of 100 (2%) individual milk samples from cows with subclinical mastitis. All colonies were identified as belonging to the *Mycoplasma* genus by both the digitonin and PCR tests. These results show that *Mycoplasma* is present in dairy herds of southern Chile, however, it must be considered that its presence may be underestimated due to the low sensitivity of a single bulk tank culture. Despite this disadvantage, the quality of the *Mycoplasma* bulk milk tank culture can be improved by performing three consecutive cultures separated by 3-4 days (Gonzalez and Wilson 2002).

The three isolated strains from bulk tank milk samples were named J14, J35 and J71. Regarding the species identification, only strain J35 was identified as *M. bovis* by the specific PCR, which produced a band of approximately 447 bp (data not shown), and by partial sequencing of the 16S rRNA gene. Strains J14 and J71 were identified at the species level as *M. bovigenitalium* by partial sequencing of the 16S rRNA gene. The two strains isolated from cases of subclinical mastitis were named V49 and V61, both identified as *M. alkalescens* by partial sequencing of the 16S rRNA gene (table 1).

In Chile, *M. bovis* had been isolated in 5 out of 71 (7%) bulk milk tank samples of herds from Los Ríos and Los Lagos Regions, from different dairy farms (Sickles *et al*

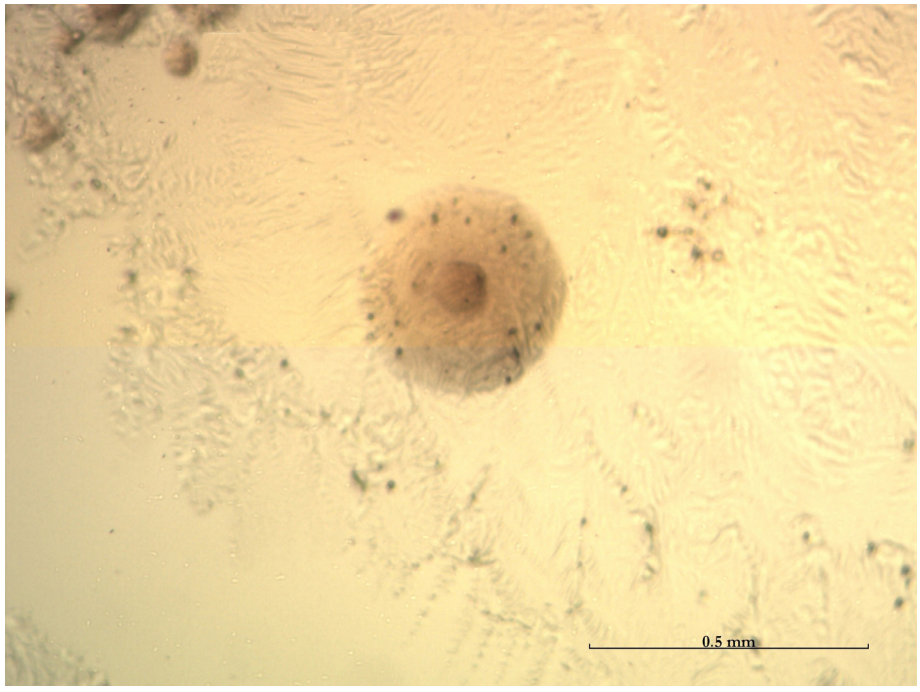

Figure 1. *Mycoplasma* colony on PPLO agar plate, with typical "egg fried" appearance (400x).

Table 1. Identification of the isolated mycoplasma strains.

Strain name	Source	Digitonin	PCR *Mycoplasma* spp.	PCR *M. bovis*	16s rRNA gene sequencing		
					Aligned strain	Access	Similarity
J14	BTM*	+	+	-	*M. bovigenitalium* NBRC 14862	AB680692.1	99%
J35	BTM*	+	+	+	*M. bovis* PG 45	NR102850.1	99%
J71	BTM*	+	+	-	*M. bovigenitalium* NBRC 14862	AB680692.1	99%
V49	SCM**	+	+	-	*M. alkalescens* PG51	NR025984.1	100%
V61	SCM**	+	+	-	*M. alkalescens* PG51	NR025984.1	100%

*Bulk tank milk.
**Subclinical mastitis.

2000). The percentage of *Mycoplasma* positive milk tanks was higher in this study, but the results are not comparable because in our study we used only samples from suppliers of PROLESUR company. Another Chilean study reports that *Mycoplasma* was isolated from 4 out of 11 (36%) bulk tank milk samples from the Biobío Region, but the strains isolated were not identified at species level (Bustos and Muñoz 2011). Although the percentage of *Mycoplasma*-positive bulk tanks found in our study was similar to those reported in other countries (Fox 2012), further studies are needed to find out the real prevalence of *Mycoplasma* in Chile.

We found only one bulk tank milk positive for *M. bovis*, which indicates that there is, at least, one cow with *Mycoplasma* mastitis in the herd, so it would be advisable to identify all infected animals and segregate or remove them from the herd (Fox 2012, Nicholas *et al* 2016). *M. bovis* causes more severe cases of intramammary infections due to *Mycoplasma*, and has been associated with respiratory infections, otitis, septic arthritis, and reproductive infections in cattle (Nicholas and Ayling 2003, Maunsell *et al* 2011). Intramammary infection due to *Mycoplasma bovis* is feared by dairy farmers and veterinarians because it is highly contagious, hard to control and there are still no vaccines available, as for other udder pathogens (Mella *et al* 2017).

In addition, this study describes for the first time in Chile the isolation of *M. bovigenitalium* and *M. alkalescens*. *M. bovigenitalium* has been documented as a cause of diseases in dairy herds, mainly associated to genital tract infections (Lysnyansky *et al* 2009) and cases of clinical mastitis (Baumgartner *et al* 2006, Lysnyansky *et al* 2016). It is one of the three most isolated *Mycoplasma* species from bulk milk tanks, with an isolation frequency that varies between 1% and 25% (Fox 2012). Occasionally, *M. bovigenitalium* and *M. alkalescens* can be isolated from milk samples, but not always their presence is associated with disease (Lysnyansky *et al* 2016). However, in our report *M. alkalescens* was isolated from cows with subclinical mastitis that had negative cultures results to other common mastitis pathogens, so it is likely to be the causative agent of intramammary infection in these animals.

In conclusion, *Mycoplasma* mastitis is a potential risk for dairy herds in southern Chile since, although in low percentages, the agents are present in the herd environment. It is important to note that not only *M. bovis* is present in these dairy herds, but also other pathogenic species not previously described in Chile such as *M. bovigenitalium* and *M. alkalescens*. Consequently, diagnostic laboratories should attempt to isolate *Mycoplasma* spp. when testing milk samples from cows with clinical mastitis that do not respond to antibiotic treatment.

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SHORT COMMUNICATION

Is the effectivity of copper ions treatment of milk enough to block *Mycobacterium avium* subsp. *paratuberculosis* infection in calves?

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ABSTRACT. Milk is an important transmission route of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) for dairy calves. Given its resistance to pasteurization, alternative milk treatments are needed to control MAP transmission via milk. The present study reports the evaluation of a novel milk decontamination treatment based on copper ions as a means of preventing infection in dairy calves. Ten newborn calves were assigned to one of two experimental groups (n=5) which were studied for 1 year. The first group was fed milk naturally contaminated with MAP and the second one received the same milk but after being treated with copper ions. In both groups, milk MAP load was estimated. The progression of the infection was monitored monthly and at the end of the study, calves were euthanised, and tissue samples were examined both grossly and by histopathology. The treatment of milk with copper ions significantly reduced the number of viable MAP. Faecal shedding of MAP was observed in both study groups, but the calves fed naturally contaminated milk began to shed MAP earlier. Only calves fed copper-treated milk showed histopathological evidence consistent with MAP infection. The latter offers more questions than answers, and maybe the presence of a more tolerant and virulent MAP strain could be the final answer to this situation.

Key words: MAP, milk, treatment, copper, calves, infection, control.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of a highly contagious chronic infectious disease known as paratuberculosis or Johne's disease. It affects mainly domestic and wild ruminants but also affects a wide range of non-ruminant animal species (Greig *et al* 1991, Harris and Barletta 2001) including humans (Chiodini *et al* 2012). MAP is highly resistant to adverse environmental conditions (low pH, high salt concentrations, and chlorine) (Whan *et al* 2001), being able to survive in the environment for more than a year (Salgado *et al* 2013) and can also survive pasteurisation (Grant *et al* 1996, Grant *et al* 2005).

Although the faecal-oral route is the main way of infection transmission (Sweeney 2011), the pathogen may also spread through colostrum and milk (Sweeney 1996) and newborns are at the greatest risk of infection, especially when consuming milk contaminated with MAP (Windsor and Whittington 2010, Sweeney 2011). Due to the above mentioned, the control of paratuberculosis infection is very challenging and many of the measures commonly adopted to prevent the newborn's infection (e.g. use of pasteurised milk or milk replacer) do not assure a pathogen-free milk diet (Grant *et al* 2005, Grant *et al* 2017).

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The latter invited us to explore other alternatives to control MAP infection or to decrease its load in colostrum and milk. We paid attention to the profuse evidence showing the antimicrobial properties of copper and copper alloys. Copper surfaces can eliminate bacterial pathogens, viruses and fungi (Faúndez *et al* 2004, Wilks *et al* 2005, Noyce *et al* 2006[a], Noyce *et al* 2006[b], Grass *et al* 2011). Recently, using an *in vitro* model, we showed that the use of a copper ions treatment protocol significantly reduced MAP load in PBS (Steuer *et al* 2018). Later, we observed that copper ions decreased viable MAP numbers in milk in a similar way (Steuer *et al* 2020). However, some MAP cells apparently either tolerate the effects of copper ions or repair sub-lethally damaged cells during subsequent liquid culture (Steuer *et al* 2018, Steuer *et al* 2020).

Due to the promising experimental results of the copper ions treatment, we aimed to estimate the effectiveness of this novel treatment in terms of its success to block infection transmission or the progression of MAP infection in newborn calves fed with MAP naturally contaminated milk and treated with copper ions.

MATERIAL AND METHODS

Between November 2017 and October 2018, a one-year longitudinal study was carried out in the Los Ríos Region, south of Chile. Based upon the animal welfare concern, since animals were exposed-infected and euthanised by the end of the experiment, the suitable facilities and the economic considerations, ten newborn calves were acquired from one small dairy herd (less than 100 animals), located in the Los Ríos region. This herd was selected because it had a history of confirmed negative paratuberculosis diagnostic results and absence of clinical cases. The 10 newborn calves were separated

from their mothers before they were able to nurse on their own, fed colostrum from their own dam (first day of life) in a hygienic environment, and kept in individual pens. Once calves had a dry hair coat and navel cord, according to international animal regulations regarding animal welfare (Albright *et al* 2007), they were transferred to an experimental farm (a completely separate farm also located in the Los Ríos region), where they were randomly separated in 2 experimental groups. Technical staff entered the pens rigorously wearing clean coveralls, boots, and gloves exclusively for each pen, as well as they also used exclusive items to keep the pen clean to eliminate the risk of cross-contamination.

In parallel, we managed the temporary use of 5 infected and infectious lactating cows from a herd with a history of confirmed high paratuberculosis infection rate and several clinical cases per year. These 5 infected and infectious cows were confirmed as MAP positive by faecal culture in the BACTEC-960 system and anti-MAP antibodies by an ELISA test and therefore selected as nurse dams, transferred to the experimental farm, and the milk collected was used to feed the calves. This naturally contaminated milk was sampled once a week for a total of 12 weeks (period of milk diet supply) in order to detect MAP and estimate its load.

After collecting the naturally contaminated milk used to feed calves in one of the groups, this milk was decontaminated using a copper treatment adapted from Steuer *et al* (2018) and modify to field conditions. Briefly, the treatment device consisted of a stainless-steel receptacle containing 20 L of milk naturally contaminated with MAP in which two high purity copper cylinders were immersed. The copper cylinders were stimulated with a low voltage (24V) electric current (3A) to quickly release copper ions for 8 minutes. The receptacle was carefully shaken during treatment to allow constant mixing.

The 10 newborn calves were randomly assigned to one of two experimental groups and each animal received a dairy diet of 4L/day for 12 weeks. Group A (n=5) received milk naturally contaminated with MAP and group B (n=5) was fed with the same MAP-infected milk but that was treated with copper ions. Each group of calves was kept in separated pens (without direct contact between groups) until the end of the study period (1 year). From the fourth month and until the end of the study period, the calves had access from their collective pens to a paddock (properly separated between pens), which guaranteed the minimum surface for each one in terms of animal welfare (minimum than 2.6 m^2 per animal) (Stull *et al* 2008). During the first 12 weeks, the calves were also offered hay, growth concentrate and water *ad libitum*. Finally, from 12 weeks onwards, the animals also received grass silage. From each calf, faecal samples were taken monthly and processed for culture as described below.

MAP detection and bacterial load in naturally contaminated milk samples, before and after treatment with copper ions, were estimated once a week during the first 12 weeks and once a month during the entire study period using the genome equivalent principle, according to a published protocol (Dzieciol *et al* 2010) based on the concentration of MAP DNA measured in a Nanoquant spectrophotometer (TECAN group, Männedorf, Schweiz) adjusted for a 10^8 dilution and the number of copies of the IS*900* target gene, and having the reference of the molecular weight of the genome of MAP ATCC strain 19698 to establish a standard curve for estimation of MAP numbers in the samples by Roche 2.0 real-time PCR, according to the equation shown by Steuer *et al* (2019).

The infectious process and the progression of MAP infection were evaluated *in vivo* before the first milk intake and then monthly by MAP faecal shedding (culture of faecal samples in the BACTEC-MGIT 960 liquid culture system, Becton Dickinson, Sparks, MD). Between 5 to 10 g of faecal material was obtained from all calves directly from the rectum using individual palpation sleeves. The samples were transported to the Laboratory of Infectious Diseases at the Institute of Preventive Veterinary Medicine, Faculty of Veterinary Sciences, Universidad Austral de Chile. Faecal samples were kept at room temperature until processing within the following 2 days.

To complement the bacteriological analyses and to determine if a positive culture corresponded to an active infection or to a transitory MAP passage through the digestive tract, a necropsy was performed at the end of the study period (12 months) on all calves to find lesions consistent with MAP infection in tissues, followed by a histopathological study of the samples obtained. The necropsy was blind as to the type of treatment of the animals. The calves were euthanised by an expert veterinarian pathologist using a retained projectile pistol, following the criteria of the Bioethics Committee of the Research and Development Department (DID) of the Universidad Austral de Chile (validation report Nº 263-2016). Ileum, mesenteric lymph nodes (MLN), and ileocecal valve were taken from each animal and placed in 10% formalin for subsequent histopathological and bacteriological analyses. Also, ileum and MLN samples were cultured in the BACTEC-MGIT 960 system for MAP detection, as mentioned previously.

After a minimum of 24 hours of fixation, the tissues were dehydrated through graduated alcohols and embedded in paraffin wax. Then, 5 mm thick sections were cut and stained with hematoxylin-eosin. Besides, serial sections of ileum samples (mucosa) were stained with Ziehl-Neelsen stain to observe the presence of acid-fast bacilli.

Differences in MAP load (using the $\log_{10}$ of the bacterial load estimates) between copper-treated and untreated milk samples and faecal samples between both calf groups were estimated using the Wilcoxon-Mann-Whitney test. Statistical analyses were run using the statistical software R version 3.6.3 (R core team 2020).

RESULTS AND DISCUSSION

The present study reports the application of a milk decontamination treatment based on copper ions at the field level, with a one-year follow-up of two groups of newborn calves that received different dairy diets, to evaluate the course of MAP infection.

A significantly ($P<0.01$) higher MAP load was observed in the untreated milk samples when compared to the copper-treated ones. Bacterial load estimation by genome equivalent principle on naturally contaminated milk samples showed a MAP load between $5.2x10^2$ and $6.46 x10^4$ DNA copies (genome equivalents) per mL of milk. In most of the copper-treated milk samples, no presence of MAP DNA was detected except for two occasions. Therefore, we dare to speak of viable MAP in untreated copper milk, because there is convincing published evidence showing that copper produces significant damage on bacterial DNA, making it non-amplifiable (Steuer *et al* 2018, Warnes *et al* 2010, Weaver *et al* 2010).

We cannot rule out that feedstuff could have been contaminated with faecal material from infected animals. As for MAP faecal shedding, we did not observe significant MAP faecal load differences between groups. All calves shed MAP sporadically in their faeces at least once during the follow-up period. The latter does not necessarily mean an active infection, but also a transient passage of the pathogen through the intestine (Corbett *et al* 2019). However, the group of calves naturally exposed to MAP infection (group A) began to shed earlier (when they were still consuming milk diet) than the other group. This agrees with Mitchell *et al* (2012), who concluded that natural infections may have a much shorter time to shedding than generally assumed. On the other hand, group B showed positive faecal results from the fifth month of the study.

No calves had positive cultures for MAP from fresh tissues collected at necropsy. However, calves from both groups had gross pathology evidence consistent with a MAP infection (table 1), e.g. enlarged lymph nodes adjacent to the last third of the jejunum and ileum (figure 1A) lymphangitis in the ileum's serosa (figure 1B), red and edematous ileocecal valve (figure 1 C), and thickening and folding of the ileal mucosa (figure 1D). Interestingly, only calves from group B had Langhans-type cells in the lamina propria of the jejunum and ileum (table 2; figures 2A and 2B). Although it was not possible to demonstrate the presence of acid-fast bacilli in any of the tissues sampled, the presence of Langhans type giant cells in the lamina propria at any level of the gastrointestinal tract should make us suspect a MAP infection, even if no acid-fast bacilli are found (Manning and Collins 2001). The latter could be interpreted as a paucibacillary infection, caused by consumption of a dairy diet containing MAP loads significantly lower than a highly faecal-contaminated environment from infected animals. This point agrees with Mitchell et al (2012), who concluded that the initial MAP dose is the most important factor in infection progression. Also, Mortier *et al* (2014) observed that in young calves, a high inoculation dose resulted in more pronounced lesions than a low inoculation dose.

Another possible explanation is what Mehtar *et al* (2008) described when *Mycobacterium tuberculosis* was exposed to copper, and some strains that resist this treatment were those that showed more virulence in experimental laboratory animals. Supporting the latter, Wolschendorf *et al* (2011) also demonstrated that copper resistance is associated with pathogen virulence. Having the above mentioned in mind, maybe the copper concentrations and/or the exposure time (8 min) may not have been enough to eliminate MAP in raw milk. Interestingly, what we know as a fact is that we previously demonstrated in PBS and also in milk, that although copper ions are highly effective in reducing MAP numbers, some MAP cells survived (Steuer *et al* 2018, Steuer *et al* 2020).

Although the number of calves in the present study represented the most challenging task to fulfil, we combined

Table 1. Anatomopathological findings.

Group	Calf	Thickening and folding of the ileal mucosa	Enlarged mesenteric lymph nodes	Lymphangitis in the ileum's serosa	Edematous ileocecal valve
A	270817	Yes	Yes	Not observed	Yes
A	050917	Yes	Yes	Yes	Yes
A	300917	Yes	Yes	Yes	Not observed
A	011017	Yes	Yes	Not observed	Yes
A	021017	Yes	Yes	Yes	Not observed
B	311017	Yes	Yes	Not observed	Not observed
B	011117	Yes	Yes	Not observed	Not observed
B	021117	Yes	Yes	Not observed	Not observed
B	031117	Yes	Yes	Yes	Not observed
B	041117	Yes	Yes	Not observed	Not observed

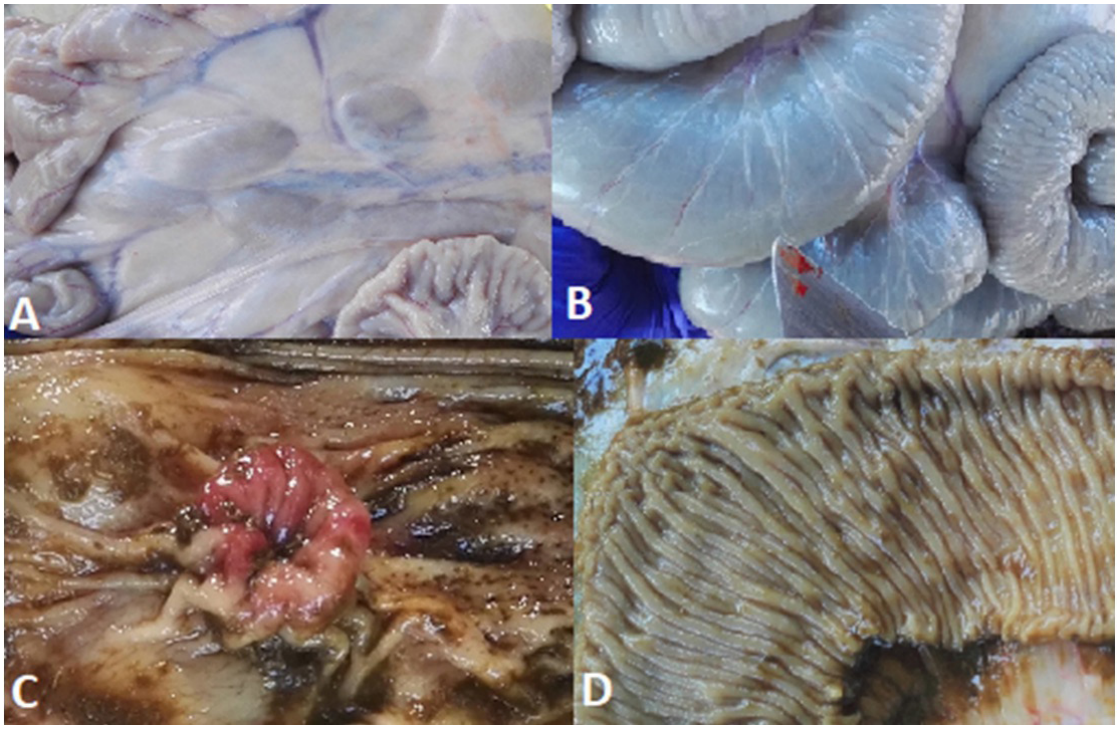

Figure 1. Findings observed after post-mortem examination of calves from groups A and B. A: enlarged lymph nodes adjacent to the last third of the jejunum and ileum, calf #031117, group B. B: lymphangitis in the ileum's serosa, calf #300917, group A. C: reddened and edematous ileocecal valve, calf #270817, group A. D: thickening and folding of the ileal mucosa, calf #011017, group B.

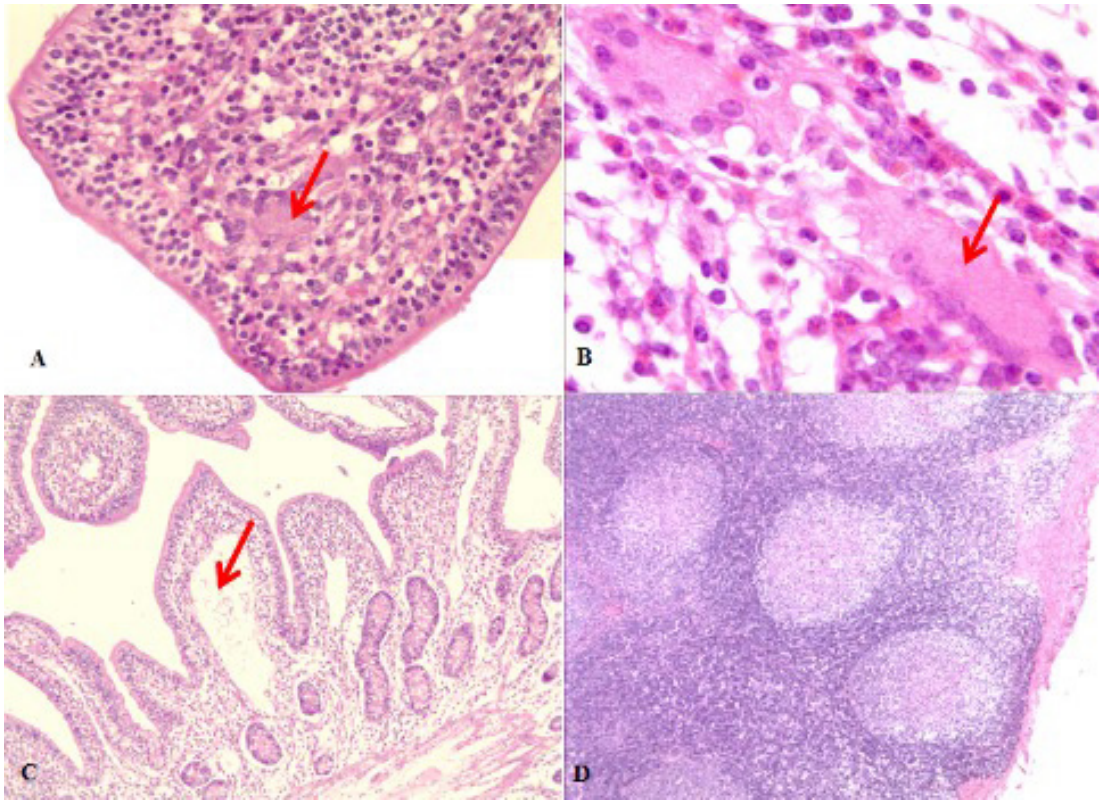

Figure 2. Histopathological findings of the tissue samples collected from all groups of calves. **A**: Langerhans-type giant cell on lamina propria of intestinal villi, calf #041117, group B, 40X HE. **B**: a more detailed Langerhans-type giant cell on deep lamina propria, calf #021117, group B (B), 60X HE. **C**: lymphangitis and lymphatic vessels dilatation in the center of the intestinal villi. Calf #031117, group B, 10X HE. **D**: activation of cortical germinal centers in mesenteric lymph node. Calf #021017, group B, 10X HE.

Table 2. Histopathological findings.

Group	Calf	Intestine	Mesenteric lymph node	Ileo-cecal valve	Ileo-cecal lyphm node	Ziehl-Nielsen
A	021017	Eosinophils (+++)	–	Eosinophils (++)	Active germ centers (++)	–
A	270817	Eosinophils (++)	Active germ centers (++)	Eosinophils (+) Active Peyer's patches germ centers (++)	Active germ centers (++)	–
A	050917	Eosinophils (++) Active Peyer's patches germ centers (+)	Active germ centers (+)	Eosinophils (+) Active Peyer's patches germ centers (++)	Active germ centers (+)	–
A	300917	Eosinophils (+) Active Peyer's patches germ centers (+)	Active germ centers (+)	Eosinophils (+) Active Peyer's patches germ centers (++)	Active germ centers (++)	–
A	011017	Eosinophils (++) Active Peyer's patches germ centers (+) Macrophages in Peyer's patches (+)	Active germ centers (+)	Eosinophils (+) Active Peyer's patches germ centers (+)	Active germ centers (+)	–
B	311017	Eosinophils (+) Lymphatic vessel dilation in lamina propria propia (+) Langhans–type giant cells (+)	Active germ centers (+)	Eosinophils (+) Active Peyer's patches germ centers (++)	Active germ centers (++)	–
B	011117	Eosinophils (+) Lymphatic vessel dilation in lamina propria (+) Macrophages in Peyer's patches (+)	Active germ centers (+)	Eosinophils (+)	Active germ centers (++)	–
B	021117	Langhans–type giant cells (+) Lymphatic vessel dilation in lamina propria (++) Macrophages in Peyer's patches (+) Eosinophils (+)	Active germ centers (++)	Eosinophils (+) Active Peyer's patches germ centers (++)	Active germ centers (++)	–
B	031117	Eosinophils (++) Langhans–type giant cells (+) Lymphatic vessel dilation in lamina propria (++)	Active germ centers (++)	Eosinophils (+) Langhans–type giant cells (+)	Active germ centers (++)	–
B	041117	Eosinophils (++)	Active germ centers (++)	Eosinophils (++)	Active germ centers (++)	–

statistic strength for the analysis with animal welfare for experimental animals.

Therefore, given the findings shown in the present study, we cannot judge the effectiveness of copper ions to block infection transmission and progression without first exploring if copper treatments are selecting for more virulent MAP strains, as described with many antibiotic drugs (Beceiro *et al* 2013).

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SHORT COMMUNICATION

Molecular characterisation and antibiotic sensitivity profile of *Pasteurella multocida* isolated from poultry farms in Malaysia

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ABSTRACT. Fowl cholera has caused significant economic losses in many poultry producing countries worldwide. In Malaysia, outbreaks of fowl cholera are frequently reported and encountered in different types of poultry productions. The objective of this study was to characterise 13 avian *Pasteurella multocida*, isolated from fowl cholera outbreaks in Central Peninsular Malaysia in the period between 2000 and 2018. The isolates were subjected to multiplex polymerase chain reaction (PCR) for capsular serotyping, disc diffusion method for antimicrobial susceptibility profiles, and molecular genotyping using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The capsular serotyping showed all 13 *Pasteurella multocida* isolates belonging to capsular serotype A. The antimicrobial susceptibility showed several multidrug resistance strains among the *P. multocida* isolates. All the isolates were resistant to erythromycin (100%), streptomycin (68%), tetracycline (37%), enrofloxacin (37%), florfenicol (23%), penicillin G (14%), gentamicin (14%), and amoxicillin (14%). The PFGE analysis clustered the isolates into three clones. Group A included isolates with a similarity of 87% from the year 2000, 2013, and 2018. Three sequence types were identified using MLST typing namely, ST129, ST231, and ST355. The ST355 was assigned for the first time in the Rural Industries Research and Development Corporation (RIRDC) database. Besides, ST129 has been reported in India, China, and Sri Lanka, which highlights the possibility of transmission between Asian countries. This study provides an insight into epidemiological information of *Pasteurella multocida* that causes fowl cholera outbreaks in the central region of Peninsular Malaysia.

Key words: antimicrobial susceptibility, MLST, *Pasteurella multocida*, PFGE.

INTRODUCTION

Pasteurella multocida (*P. multocida*) is a Gram-negative bacterium that can cause a wide range of diseases in animals, such as fowl cholera in poultry, haemorrhagic septicemia in bovine, and atrophic rhinitis in swine (Wilkie *et al* 2012, Wilson and Ho 2013). Fowl cholera is an epizootic, highly contagious avian disease that could affect several avian species including commercial chickens (Botzler 1991). Since it was discovered, it has caused significant economic losses in the poultry industry worldwide (Harper *et al* 2006). The disease may occur as an acute septicaemia form with high morbidity and mortality (up to 100%), or as a localised chronic infection (Heddleston *et al* 1964, Harper *et al* 2006). Sudden death for a large number of birds is usually the first clinical sign in acute fowl cholera (Glisson *et al* 2013). *Pasteurella multocida* can currently be subdivided into four subspecies: subsp *multocida*, subsp *gallicida*, subsp *septica*, and subsp *tigris*. All subspecies, excluding tigris, have been isolated from fowl cholera outbreaks (Harper *et al* 2006). Serotype A is the dominant serotype of *P. multocida* causing fowl cholera while serotypes B, D, and F have been less reported to cause disease in poultry (Wilkie *et al* 2012). Outbreaks were reported in Asia and all over the world (Wang *et al* 2013, Jones *et al* 2013, Singh *et al* 2013). Antibiotics are widely used in the treatment of *P. multocida* infections in poultry, which have increased antibiotic resistance (Murray 1992). A study conducted in Brazil showed 19.64 % of *P. multocida* strains isolated from chicken and turkey farms were multidrug-resistant to three or more drugs in different categories using the disc diffusion method (Furian *et al* 2016). A number of epidemiological studies were conducted to investigate the distribution of *P. multocida* strains in several countries (Sarangi *et al* 2016, Li *et al* 2018, Peng *et al* 2018). Pulsed-field gel electrophoresis (PFGE) is a genotyping technique that analyses bacterial chromosomes using restriction enzyme into DNA fragments. The PFGE fragments pattern can be used to study the strain variation and evolution (Gunawardana *et al* 2000). PFGE also has been used to study outbreaks of fowl cholera in poultry (Kardos and Kiss 2005, Sellyei *et al* 2017). However, multilocus sequence typing (MLST) is the current gold standard typing method for *P. multocida*, which uses seven housekeeping genes to characterise and study the global distribution of *P. multocida* sequence types (STs) (Kardos and Kiss 2005, Subaaharan *et al* 2010, Singh *et al* 2013).

In Malaysia, avian *P. multocida* outbreaks were frequently reported in commercial and backyard farms (Arumugam *et al* 2011, Nafizah *et al* 2014, Khoo *et al* 2017). However, information on *P. multocida* serogroups, antibiotic resistance profile, and molecular genotyping are poorly investigated. Therefore, the aim of this study is to molecular characterise *P. multocida* isolates from fowl cholera outbreaks submitted to the Laboratory of Bacteriology at Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia. The isolates undergo multiplex

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PCR serotyping to determine the serogroup as well as the disc diffusion method to determine the resistance profile of the isolates. Additionally, genotyping was performed using PFGE and MLST to study the variation and evolution of the *P. multocida* isolates.

MATERIAL AND METHODS

BACTERIAL ISOLATES

Thirteen *P. multocida* subspecies *multocida* isolates from the Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia were analysed in this study (table 1). The samples were submitted for diagnostic purposes, between the years 2000 to 2018 from fowl cholera outbreaks in backyard chicken farms located in Selangor, Malaysia. The bacteria isolates were obtained from chicken internal organs including liver, spleen, and lungs, and were subject to biochemical identification. The samples were cultured onto 5% blood agar (OXOID, UK). Suspected colonies showing *P. multocida* colony morphology, were subjected to biochemical tests, namely, oxidase, indole, Triple Sugar Iron (TSI), Sulfide Indole Motility (SIM), citrate, urease reactions and Ornithine Decarboxylase Test (ODC), trehalose, mannitol, D-sorbitol, and dulcitol.

MOLECULAR IDENTIFICATION

Genomic DNA was extracted using the boiling method. The bacteria were boiled at 97 °C for 10 min, then placed in an ice container for 5 min, then centrifugated for 10 min at room temperature. The isolates were confirmed as *P. multocida* using PCR targeting the KMT1 gene as described by Townsend *et al* (1998). *P. multocida* ATCC 12945 was used as a positive control.

MULTIPLEX PCR CAPSULAR TYPING

The isolates were subjected to capsular serotyping using the primers designed by Townsend et al (2001). Genomic DNA was extracted using the boiling method. The multiplex PCR was carried out with a final reaction volume of 50µl, 30 PCR cycles; 95°C for 45 sec, 56.2°C for 45 sec, 72°C for 45 sec. The PCR product was visualised using a UV transilluminator.

ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility test was carried out using the disc diffusion method following the Clinical and Laboratory Standards Institute standards (CLSI) VET01- A4 (4th ed.) and the M45 (3rd ed.). Two replicates were performed for each isolate against eight antibiotics, namely, streptomycin 10µg, amoxicillin 10µg, tetracycline 30µg, gentamicin 10µg, erythromycin 15µg, penicillin G 10u, enrofloxacin 5µg, and florfenicol 30µg. The bacteria suspensions were cultured on Mueller-Hinton agar then the antibiotic where placed on the plates. After 24 hours of incubation, the average zone of inhibition was measured and interpreted. The *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control.

Table 1. Antibiotic resistance profile for each *P. multocida* isolated from fowl cholera outbreaks in poultry farms in Malaysia in the period of 2000 to 2018. *Pasteurella multocida* isolated from fowl cholera outbreaks in poultry farms in Malaysia in the period of 2000 to 2018.

Number	Year	Sample	PFGE	ST	Antibiotics resistance profile ‡)
1	2000	PM201	A3	129	E
2	2000	PM202	A3	-	E
3	2000	PM203	A3	-	E
4	2000	PM204	A3	-	E
5	2013	PM205	A1	129	ST, ENR, TE, E
6	2013	PM206	A2	-	ST, ENR, TE, E. AMX, CN, P
7	2014	PM207	B	129	ST, ENR, TE, E, AMX, CN, P, FFC
8	2016	PM208	C1	355	ST, E
9	2016	PM209	C2	-	ST, E
10	2016	PM210	C3	-	ST, E
11	2016	PM211	C3	-	E
12	2018	PM212	A1	-	ST, ENR, TE, E, FFC
13	2018	PM213	A1	231	ST, ENR, TE, E, FFC

‡) List of antibiotics the isolates were resistant against, according to M45 (3rd ed.) and VET01- A4 (4th ed.) of the CLSI standard. ST= Streptomycin, AMX= Amoxicillin, TE= Tetracycline, CN= Gentamicin, E= Erythromycin, P= Penicillin G, ENR= Enrofloxacin, FFC= Florfenicol.

PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

A single colony from each isolate was cultured in brain heart infusion broth (BHI) (OXOID, UK) and incubated at 37°C for 24 hr. The culture was then mixed to cell suspension buffer and adjusted to 0.6-0.7 of McFarland standard. The cell mixtures were pipetted into CHEF disposable plug moulds (Bio-Rad Laboratories, USA) and allowed to solidify for 10 min at 4 °C. The plugs were transported into 2 ml cell lysis buffer and incubated in a water bath at 56 °C for 17 hr with one hour shaking at 100 rpm. The plugs were washed two times with deionised water for 10 min each at 50 °C, followed by 5 times washing with the TE buffer. A small slice of each plug was placed in a 2 ml tube containing 200 µl per-restriction mixture for 15 min at 37 °C. Then, a restriction mixture containing the ApaI enzyme was added and incubated at 37 °C for 2 hr. Finally, the restriction mixture was removed, and 0.5 TBE buffer was added for 5-10 min. The electrophoresis was performed using the following conditions; initial switch time 1 sec, final switching time 40 sec, a constant voltage of 6 V, and an angle of 120. With a total running time of 23 hr and 14°C running temperature. BioNumerics 6.6 software was used to analyse the *P. multocida* PFGE profiles. The dendrogram was created with 1% optimization and 1% tolerance using the Dice similarity coefficient, by unweighted paired group method of arithmetic averages (UPGMA). Strains with more than three differences in DNA fragments and a similarity of <85% were classified into different PFGE types (Van Belkum *et al* 2007).

MULTILOCUS SEQUENCE TYPING

One isolate representing each year was characterised via the MLST scheme. Briefly, PCR amplification was carried out for seven housekeeping genes, using the primers designed by Subaaharan *et al* (2010), then the sequences were analysed using the *P. multocida* MLST RIRDC database[1] to get the *P. multocida* sequence type (ST).

RESULTS AND DISCUSSION

Pasteurella multocida is a significant pathogen that causes epizootic diseases in animals including fowl cholera in poultry. Outbreaks of fowl cholera have been reported all over the world in both wild and domestic birds (Botzler 1991, Kardos and Kiss 2005). To enhance our understanding of the variation and transmission of the bacterial strains, many techniques were used to study the population structure of *P. multocida* worldwide (Gunawardana *et al* 2000, Subaaharan *et al* 2010). In this study, thirteen isolates confirmed to be *P. multocida* were found to belong to capsular serotype A.

The isolates showed an overall high level of antibiotic resistance, five isolates were multidrug resistance (MDR) (PM205, PM206, PM207, PM212, and PM213), as shown in table 1. Also, the PM207 isolate was found to be resistant to all antibiotics tested, namely erythromycin, streptomycin, tetracycline, enrofloxacin, florfenicol, penicillin G, gentamicin, and amoxicillin, which belonged to six classes of antibiotics. MDR strains of *P. multocida* have been reported in the USA, Germany, Spain, and recently reported in China MDR strains of *P. multocida* against florfenicol, chloramphenicol, ofloxacin tetracycline streptomycin, kanamycin, and sulfamethoxazole (Li et al 2018; Zhu et al 2020). Overall, the isolates were resistances to erythromycin (100%), streptomycin (68%), tetracycline (37%), enrofloxacin (37%), florfenicol (23%), penicillin G (14%), gentamicin (14%), and amoxicillin (14%). Similar findings were reported in a study of avian *P. multocida* in Mississippi, whereby the isolates were resistant 78% against erythromycin, 78% streptomycin, and 46% penicillin (Jones et al 2013). In this study, the high resistance against erythromycin and streptomycin was reported. It is high likely that these occur due to excessive usage of antibiotics in poultry farms in Malaysia. In addition, these antibiotics are also used as growth promoter in poultry feeds in Malaysia (Hassali et al 2018). Besides, *P. multocida* isolates may also have acquired resistance genes from other Gram-negative bacteria (Wilson and Ho 2013).

The PFGE phylogenetic tree clustered the isolates into three groups shown in table 1. Group A has an 87.1% similarity among isolates from different years (2000, 2013, and 2018). Group B showed 85% similarity, including isolates from the year 2016. PFGE type C has one isolate that belongs to the year 2014, as shown in figure 1. The high similarity of 87.1% in group A suggests that these isolates were isolated from related outbreaks of the same strains of *P. multocida* and had developed or acquired resistant genes from other Gram-negative bacteria over the years. For instance, PFGE was used to investigate the epidemiology of strains *P. multocida* isolated from two cases of fowl cholera outbreaks in the region of eastern Hungary. The results showed high genetic relatedness among the isolates and suggested that the second outbreak was recurrent of the same strain of *P. multocida* (Kardos and Kiss 2005).

MLST typing is the gold standard typing method for *P. multocida* (Subaaharan *et al* 2010). Five isolates were typed using MLST, and three STs were detected, namely, ST129 from three isolates, ST231, and ST355, as shown in table 1. Besides, ST355 was identified for the first time in the MLST database in this study. Interestingly, ST129 was recently reported in several epidemiological studies in Asia. For instance, a study was published in 2013 in China reported many outbreaks of fowl cholera involving *P. multocida* ST129 (n=40) (Wang *et al* 2013). Moreover, another study in 2016 in China reported a high occurrence (91%) of ST129 as well (Wang *et al*

[1] http://pubmlst.org/pmultocida_rirdc/

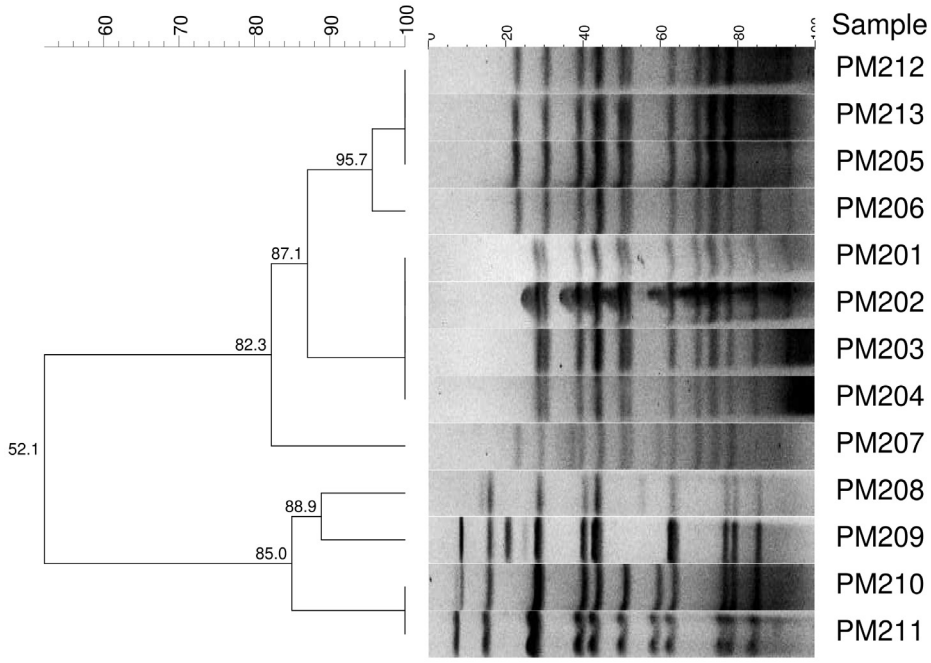

Figure 1. PFGE phylogenetic tree of *P. multocida* isolates using BioNumerics 6.6. The dendrogram shows high similarity (82.3%) between the years (2000, 2013, 2014, and 2018) and high similarly among the isolates from the year 2016.

2016). A recently published study in China reported that 84% of avian *P. multocida* isolates were ST129, and they suggested that ST129 is a significant and high virulence ST of *P. multocida* in southwestern China, causing fowl cholera infections in many types of poultry species (Li *et al* 2018). The study also found that most of the ST129 isolates exhibited multidrug resistance for antibiotics, including amoxicillin, tetracycline, florfenicol, and streptomycin, which is very similar to the resistance pattern findings for the ST129 isolates in this study (table 1). Furthermore, *P. multocida* ST129 was also reported in India in fowl cholera outbreaks (Sarangi *et al* 2016). And in Iran from chicken fowl cholera outbreak according to the RIRDC MLST database[2]. On the other hand, the ST129 was also reported to cause hemorrhagic septicemia in bovine in Sri Lanka (Hotchkiss *et al* 2011), as well as infection in pigs according to the RIRDC MLST database[3]. As a result, ST129 was suggested as adaptable to many types of hosts (Hotchkiss *et al* 2011, Peng *et al* 2018). Although the distribution of the ST129 was discovered in India, it was suggested as a result of possible transmission from China and Sri Lanka (Sarangi *et al* 2016). In the current study, ST129 was identified in several fowl cholera cases among poultry in Malaysia and had a relatively similar antibiotic profile to the ST129 isolates reported in southwestern China (Li *et al* 2018). This finding shows the high possibility of transmission of ST129 from or to China into Malaysia. Besides, *P. multocida* was proved to spread between different countries. In a study of the fowl cholera outbreaks in Denmark and Sweden, it shows that migrating birds had spread a strain of *P. multocida*, which caused several fowl cholera outbreaks among these countries (Christensen and Bisgaard 2000, Petersen *et al* 2001). Regarding our study, Malaysia is a significant winter home for many species of migratory birds coming from the north due to its stable weather (DeCandido *et al* 2004). Thus, increasing the possibility of transmission of new strains into the country.

MLST is an effective tool to study *P. multocida* genotype variation and evolution (Subaaharan *et al* 2010). *Pasteurella multocida* ST231 belongs to the CC129 and shared six alleles (*est, pmi, Zwf, mdh, gdh, pgi*) with ST129 single locus variant (SLV) besides sharing a relatively similar antibiogram (table 1), which suggested that it evolved from ST129. Another study had published a complete genome sequence of *P. multocida* serotype A, isolated from haemorrhagic septicemia infection in buffaloes in Malaysia under the accession No. CP007205 in NCBI GenBank (Jabeen *et al* 2017). Based on the analysis of the RIRDC MLST database, this strain belongs to ST201, which also belongs to CC129. This isolate is SLV with ST129, this finding suggested that it evolved from ST129 and caused haemorrhagic septicemia infection in buffaloes

[2] https://pubmlst.org/bigsdb?page=info&db=pubmlst_pmultocida_isolates&id=984

[3] https://pubmlst.org/bigsdb?db=pubmlst_pmultocida_isolates&l=1&page=profiles

in Malaysia. Haemorrhagic septicemia was reported to be endemic and of significant economic importance in many countries, including Malaysia (Benkirane and De Alwis 2002). The investigation provides significant insights into the epidemiological importance of CC129 in Malaysia. In contrast, recently published in China, there is a report on a double-locus variant (DLV) genotype from the CC129 identified as the first hypervirulent and multi-antimicrobial resistant avian *P. multocida* (ST342) (Zhu *et al* 2020). In India, SLV from CC129 (ST280) was also reported causing fowl cholera outbreaks (Sarangi *et al* 2016). These findings highlight the threat of *P. multocida* CC129 as ubiquitous and causing infections in many animal host species in Asian countries.

Overall, this study provides important epidemiological data on the diversity of *P. multocida* causing fowl cholera infection in Malaysia and highlighted the high potential of transmission of the same sequence type among the nearby countries. This study also reported the CC129 is a big threat to the poultry industry in Malaysia, and as a wildly reported CC causing fowl cholera in south Asian countries. The high antibiotic resistance shown among isolates in this study warrants the prudent use of antimicrobial agents in the poultry production in Malaysia.

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Austral J Vet Sci **53, 127-131 (2021)**

SHORT COMMUNICATION

Chemical immobilisation of the wild Patagonian otter (*Lontra provocax*) and the North American mink (*Neovison vison*)

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ABSTRACT. The Patagonian otter (*Lontra provocax*) is an endangered species endemic to southern Chile and Argentina. Most of its distribution range has recently been occupied by the American mink (*Neovison vison*). As part of a long-term study on the impact of mink in Patagonia, we assessed five reversible anaesthetic combination protocols in different doses on wild *L. provocax* and *N. vison*, and described the occurrence of any adverse effects. We assessed 16 anaesthetic procedures with a combination of ketamine-medetomidine (KET-MED; 6.0±2.8-0.05±0.01 mg/kg IM, respectively) or ketamine-dexmedetomidine (KET-DEX; 4.1±0.9-0.02±0.004 mg/kg IM) in *L. provocax* and 23 anaesthetic procedures with KET-MED (13.3±4-0.1±0.04 mg/kg IM), KET-DEX (4.8±0.3-0.024±0.001 mg/kg IM) in a low dose of ketamine (LDK) or KET-DEX (10.2±0.9-0.025±0.002 mg/kg IM) in a high dose of ketamine (HDK) in *N. vison*. Reversal was accomplished using atipamezole at 5 times the dose of MED or 10 times the dose of DEX. All anaesthetic combinations produced complete immobilisation and rapid anaesthetic induction, except for two otters anaesthetised with KET-MED which exhibited a longer time to initial effect. Hypothermia was commonly observed at the end of the anaesthetic procedures. Due to the hypoxemia presented in four otters at the beginning of anaesthesia, it is recommended to use additional oxygen when possible.

Key words: Anaesthesia, atipamezole, mustelid, reversal.

INTRODUCTION

The Patagonian otter (*Lontra provocax*) is an endangered species native to Southern Chile and Argentina (Larivière 1999) and the American mink (*Neovison vison*) an invasive species in Chile since the 1960s (Medina 1997). Recently undertaken research involving the capture of these two species has required anaesthesia (Soto-Azat *et al* 2006).

Ketamine (KET) combined with medetomidine (MED) followed by antagonism with atipamezole (ATI) is a well-documented safe and efficient anaesthetic protocol to be used in carnivores (Jalanka and Roeken 1990), including mustelids, and recommended especially under field conditions (Spelman *et al* 1994, Spelman 1999). However, bradycardia, hypotension, hypoxemia and hypothermia have been described as major adverse effects (Spelman 1999, Fernandez-Moran *et al* 2001, Soto-Azat *et al* 2006). This study aimed to evaluate five anaesthetic combination protocols based on different doses of 3 drugs on wild *L. provocax* and *N. vison*, using KET-MED and KET-DEX, both antagonised with ATI, and to describe the main adverse effects observed.

MATERIAL AND METHODS

A total of 14 *L. provocax* and 23 *N. vison* were captured in southern Chile between 2004 and 2013 (permit granted by Subsecretaria de Pesca Nº2286 and 448). Otters were captured with soft-catch leghold traps, and minks with box traps. After estimating the weight of each animal, they were physically restrained and injected with the anaesthetic combination administered by hand with a 1 ml syringe and a 23G needle (Soto-Azat *et al* 2006). Anaesthetic protocols were divided into five groups: 1) four otters anaesthetised with a combination of KET-MED at a dose of 6.0±2.8-0.05±0.01 mg/kg IM (two otters were anaesthetised twice; capture and subsequent radiotransmitter implantation), 2) 10 otters anaesthetised with a combination of KET-DEX at a dose of 4.1±0.9-0.02±0.004 mg/kg IM, 3) 10 minks anaesthetised with a combination of KET-MED at a dose of 13.3±4-0.1±0.04 mg/kg IM, 4) six minks anaesthetised with a combination of KET-DEX at a dose of 4.8±0.3-0.024±0.001 mg/kg IM (low dose of ketamine: LDK), and 5) seven minks anaesthetised with a combination of KET-DEX at a dose of 10.2±0.9-0.025±0.002 mg/kg IM (high dose of ketamine: HDK). Reversal was accomplished using ATI at five times the dose of MED or 10 times the dose of DEX, respectively. Details of anaesthetised individuals are provided in tables 1 and 2.

Anaesthetic variables were recorded, including time to initial effect, recumbency time, loss of pedal reflex and reversal time. To evaluate the anaesthetic depth, we examined animals for sonorous stimulus response, jaw relaxation, interdigital toe pinch and level of alertness. The degree of muscular relaxation was characterised as "adequate" or "inadequate". Physiological parameters monitored were rectal temperature measured with a digital thermometer, cardiac rate measured with a stethoscope, respiratory rate, capillary refill time and arterial haemoglobin oxygen saturation (SPO_2) measured with a pulse oximeter, however, in the combination of KET-DEX with LDK in minks it was not possible to measure SPO_2. All parameters were monitored at 5 min intervals for 30 min, following Soto-Azat *et al* (2006).

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Table 1. Summary of chemical immobilisation in wild otters (9 females and 5 males) anaesthetised intramuscularly with ketamine-medetomidine or ketamine-dexmedetomidine and reversed with atipamezole.

Parameters with KET-MED	n	Mean	SD	Range
Weight (kg)	4	11.5	2.04	10-14.5
Ketamine dose (mg/kg)	6	6.0	2.8	3.4-10.0
Medetomidine dose (mg/kg)	6	0.05	0.01	0.03-0.06
Initial effect (min)	6	4.9	4.3	1.2-10
Recumbency (min)	6	5.7	3.08	2.4-10
Reversal times (min)	6	18	12.5	5-37
Body temperature	6	37.3	0.7	35.8-40
Heart rate	6	86.1	9.1	73-93.2
Respiratory rate	6	22.3	3.3	17.5-25.6
SpO_2	3	86	7.21	80-94
Parameters with KET-DEX	n	Mean	SD	Range
Weight (kg)	10	6.3	2.12	3-8.74
Ketamine dose (mg/kg)	10	4.1	0.9	2.9-5.7
Dexmedetomidine dose (mg/kg)	10	0.02	0.004	0.01-0.03
Initial effect (min)	10	2.8	1.3	1.4-5.5
Recumbency (min)	10	5.9	3.4	2.4-13
Reversal times (min)	10	65.4	58.4	8-180
Body temperature	10	37.1	0.7	32.2-39.1
Heart rate	10	107.3	22	84-161
Respiratory rate	10	38	16	23.3-52.66
SpO_2	3	84.2	4.3	70-96

RESULTS AND DISCUSSION

The anaesthetic induction was rapid and smooth for all individuals, except for two otters anaesthetised with KET-MED which exhibited a longer time to initial effect (10 min), however, this was shorter than the time reported by Bauquier *et al* (2010). The average time to initial effect for otters was 4.9 and 2.8 min for KET-MED and KET-DEX, respectively. The recumbency time was similar for both combinations. The average time to initial effect for minks was 1.7, 2.2 and 1.3 min for KET-MED, LDK KET-DEX and HDK KET-DEX, respectively. The recumbency time for minks was 6.2 min with LDK KET-DEX (table 2). The anaesthetic recovery following ATI administration was smooth and calm, as previously reported in other species of otters (Fernandez-Moran *et al* 2001, Soto-Azat *et al* 2006). The average time to total recovery in otters was 18.0 and 65.4 min for KET-MED and KET-DEX, respectively. In three otters anaesthetised with KET-DEX recovery lasted longer than 1 hour. The recovery in minks was evaluated only with the combination KET-DEX. Average total recovery time was 22.1 and 28.0 min with LDK and HDK, respectively (these animals were released for home range studies). The other minks were euthanised following legal recommendations.

Muscle relaxation was classified as adequate for both anaesthetic protocols and both species. Capillary refill time in all cases fell within normal ranges (1-3 seconds).

For the KET-MED combination, the rectal temperature average was 37.3 and 37.4 ºC in otters and minks, respectively. Temperature continuously decreased in all studied individuals. Further, four animals showed moderate hypothermia (34-36 °C) at the end of the anaesthetic procedure. Rectal temperature also decreased in all individuals anaesthetised with the KET-DEX combination. The most affected were minks with HDK, decreasing to 35.3 ºC average (figure 1). Rectal temperature average in otters under KET-DEX was 37.1 ºC. In minks with LDK the average was 36.7 ºC and in minks with HDK it was 35.4 ºC (figure 1). Anaesthetic related hypothermia usually occurs due to depression of the hypothalamic thermoregulatory centre, and as core body heat redistributes to the skin surface through anaesthetic-induced vasodilation (Matsukawa *et al* 1995, Taguchi and Kurz 2005). Although we tried

Table 2. Summary of chemical immobilisation in minks (10 females and 13 males) anaesthetised intramuscularly with ketamine-medetomidine or ketamine-dexmedetomidine and reversed with atipamezole.

Parameters with KET-MED	n	Mean	SD	Range
Weight (kg)	10	0.740	0.2	0.4-1.1
Ketamine dose (mg/kg)	10	13.3	4	9.1-20
Medetomidine dose (mg/kg)	10	0.1	0.04	0.09-0.2
Initial effect (min)	10	1.7	0.8	1-3.3
Recumbency (min)	8	3.3	2.1	1.3-7.4
Reversal times (min)	–	–	–	–
Body temperature	10	37.4	0.7	34.1-39.7
Heart rate	10	130	4	80-196
Respiratory rate	10	39.5	6	24-83
SpO_2	9	93	1.2	80-100
Parameters with KET-DEX (LDK)	**n**	**Mean**	**SD**	**Range**
Weight (kg)	6	0.560	0.2	0.375-1
Ketamine dose (mg/kg)	6	4.8	0.3	4.2-5
Dexmedetomidine dose (mg/kg)	6	0.024	0.001	0.021-0.025
Initial effect (min)	6	2.21	1.1	1-4
Recumbency (min)	6	6.2	4.6	2-13.3
Reversal times (min)	6	22.1	20	2.3-60
Body temperature	6	36.7	1.13	32.5-40.4
Heart rate	6	149	13	120-196
Respiratory rate	6	50	6.2	32-80
SpO_2	–	–	–	–
Parameters with KET-DEX (HDK)	**n**	**Mean**	**SD**	**Range**
Weight (kg)	7	0.635	0.2	0.380-1
Ketamine dose (mg/kg)	7	10.2	0.9	8.5-11
Dexmedetomidine dose (mg/kg)	7	0.025	0.002	0.021-0.028
Initial effect (min)	7	1.32	0.47	1-2
Recumbency (min)	7	2.85	0.69	2-4
Reversal times (min)	7	28	24.2	1.5-60
Body temperature	7	35.4	1.2	32-39
Heart rate	7	130	15	72-196
Respiratory rate	7	36	4.7	60-20
SpO_2	1	91	0.9	90-92

to prevent hypothermia by using hot water bottles, the temperature decreased under desirable levels in most cases. Fournier-Chambrillon *et al* (2003) described a decrease in temperature as the major adverse effect using KET-MED, but placing the animals on a warmed table was an effective way to correct this problem. This situation occurred in both species in our study. Quick reversal with ATI seems to be a key factor to control this adverse effect. However, the ideal would be to increase the temperature to normal ranges and then reverse it, but our field conditions with no access to electricity prevented this scenario.

For the KET-MED combination, cardiac rate in otters was below expected when compared to the previously reported heart rates (100-180 beats/min) for *L. canadensis* under inhalation anaesthesia with isofluorane (Spelman *et al* 1993). Some individuals had mild bradycardia with stable heartbeat, other individuals had a mild tendency to decrease cardiac rate, with an average of 86 beats/min.

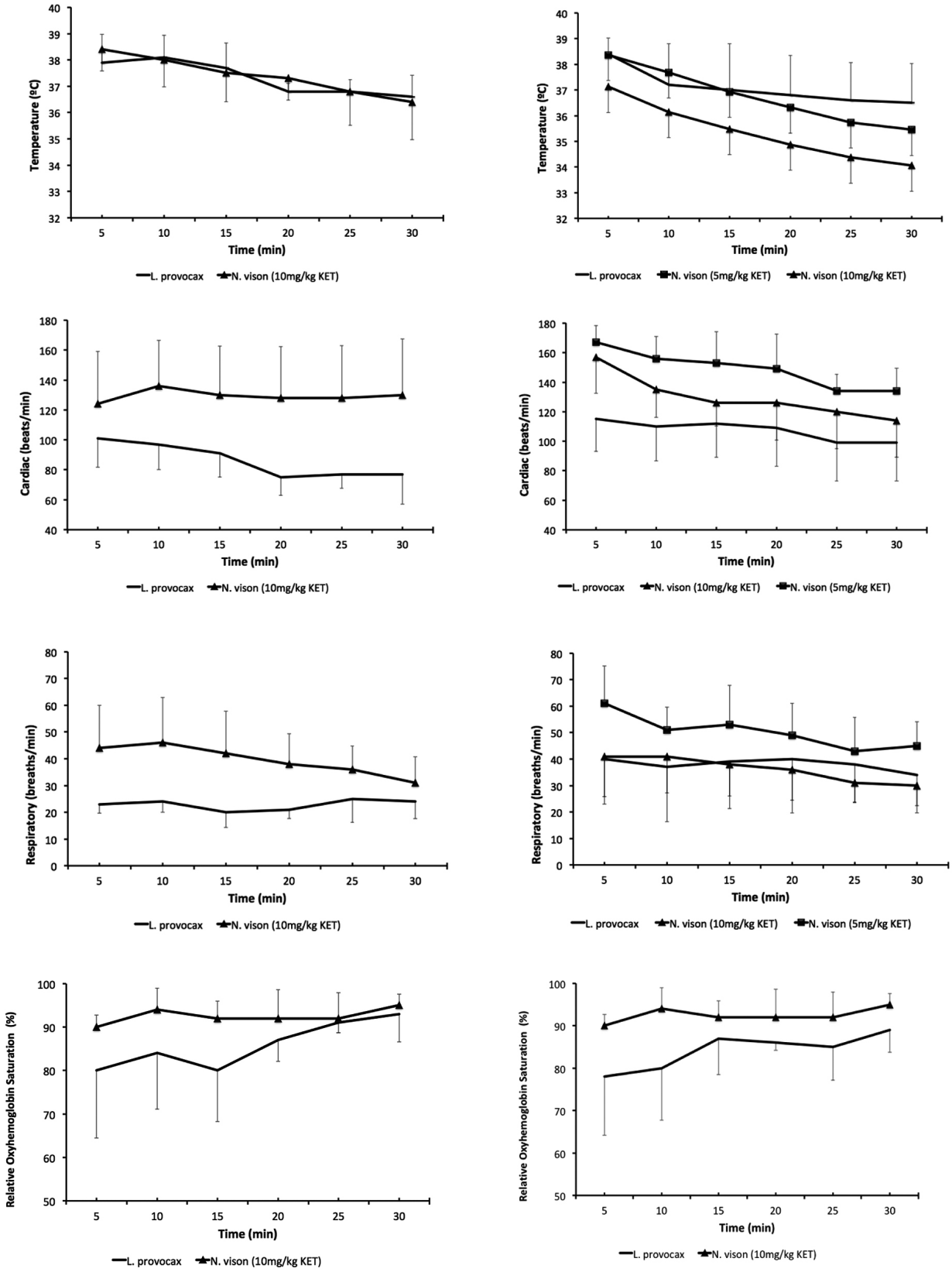

Figure 1. Means and SD (standard deviation) for rectal temperature, cardiac rate, respiratory rate and relative oxyhaemoglobin saturation at 5 min intervals for otters (*L. provocax*) and minks (*N. vison*) anesthetised with Ketamine-medetomidine (first column) and with Ketamine-dexmedetomidine (second column), the latter combination in two different doses of Ketamine in the case of minks. Single line: *L. provocax*; line with square: *N. vison* 5mg/kg KET; line with triangle: *N. vison* 10mg/kg KET.

Heart rate in minks averaged 130 beats/min and there was a pattern of maintenance of cardiac activity with a small range of variation. However, four minks had some cardiac frequencies below the expected range. With the KET-DEX protocol only one otter had values of cardiac rate below 100 beats/min, with an average of 107 beats/min in otters, 149 beats/min in minks with LDK and 130 beats/min in minks with HDK. The cardiac rate was generally stable for all animals under both anaesthetic protocols, with a slight tendency to decrease, however, animals under KET-DEX had values closer to the expected, according to previously reported values (figure 1).

Respiratory rate for the KET-MED combination averaged 22 breaths/min in otters, within the expected normal range. Minks had a mean of 39 breaths/min and seven individuals presented pronounced tachypnea at the beginning of the procedure. With the KET-DEX combination, three otters and seven minks had pronounced tachypnea at the beginning of anaesthesia: the otters presented 64-68 breaths/min, and the minks had 44-60 breaths/min. In both species, respiratory rate stabilised in the second or third monitoring period, which subsequently decreased to normal values in all cases (figure 1). Respiratory rate under both protocols showed to be fairly stable, starting with tachypnea in some cases but tending to decrease to normal parameters. Respiratory rate may be higher using KET-DEX than KET-MED, possibly due to fewer adverse effects of DEX on the respiratory system.

The average values for SpO2 were above 80%. Two otters under KET-MED and two otters under KET-DEX anaesthesia showed lower SpO2 values at the beginning of the anaesthetic procedure, possibly due to artefact movements and imprecise measurements during the monitor's adjustment on the tongue. Nevertheless, it is recommended to supplement oxygen if SpO2 drops below 90% when possible (figure 1).

Several adverse effects have been described using KET alone, especially hyperthermia, rigidity and convulsion (Reuther and Brandes 1984). Alternatively, a protocol with butorphanol/midazolam/medetomidine works well in seals and sea lions (Spelman 2004). In this study, the five anaesthetic combinations with KET-MED and KET-DEX lead to a rapid induction and complete inmobilisation with the advantage of being reversed with ATI. However, at low doses of KET, anaesthesia was not as deep as desirable at the beginning and at the end of the procedure. Doses <5 mg/kg of KET may not induce reliable immobilisation. Arnemo *et al* (1994) recommended doses of KET between 6.5 and 11.8 mg/kg, especially for painful procedures. Anaesthesia depth scores demonstrated that these two anaesthetic combinations can be used for short procedures, but supplementary anaesthesia is necessary for more complex procedures such as surgeries. Both anaesthetic combinations worked well. In the KET-MED combination, we used 2mg/kg more KET than *Bauquier et al* (2010), because they used a low level of anaesthesia and in two cases they had to administer additional doses of ketamine. Taking all results together, we recommend the use of 8.0 mg/kg of KET and 0.025 mg/kg of DEX IM in otters, and 10 mg/kg of KET and 0.025 mg/kg of DEX IM in minks for field anaesthesia of short procedures (<30min) with reversal by ATI 0.5 mg/kg IM.

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SHORT COMMUNICATION

Detection of antimicrobial resistant *Salmonella enterica* strains in samples of ground hedgehogs (*Atelerix albiventris*) reared as pets in the urban area of Santiago, Chile

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ABSTRACT. The breeding of exotic pets has become a popular practice in Chile and, within this group of animals, small mammals such as guinea pigs and hedgehogs have gained importance due to their docile behaviour. The most common exotic hedgehog species in Chile is the African pygmy hedgehog (*Atelerix albiventris*). It has been reported that these pets are reservoirs of some zoonotic pathogens, among which *Salmonella enterica* constitutes an important threat for the owners. This study aimed to detect the presence of *Salmonella* strains in faeces from hedgehogs (*Atelerix albiventris*) admitted to a veterinary clinic in Santiago, Chile, and to characterise the antimicrobial susceptibility of the isolated strains. From 200 animals sampled, *S. enterica* was detected in 5 hedgehogs, corresponding to serotypes Muenchen (2), Infantis (2) and IV43:z4,z23:- (1). Furthermore, phenotypic antimicrobial resistance was determined in all subsp. *enterica* isolates. These results suggest that in Chile these exotic pets constitute a potential hazard for public health, therefore, supporting educational campaigns about basic biosecurity measures is necessary, mostly aimed at pet owners and risk groups.

Key words: hedgehogs, *Salmonella*, antimicrobial resistance, Chile.

INTRODUCTION

Non-traditional pets have become very popular in developed and developing countries. Apart from exotic reptiles, amphibians and fishes, unconventional pets include a variety of species of mammals, such as non-human primates, ferrets, prairie dogs, and hedgehogs (Hoelzer *et al* 2011).

Hedgehogs are native to Europe, Asia and Africa, and include multiple species, although two of them are generally reared as pets: the European hedgehog (*Erinaceus europaeus*), and the African hedgehog (*Atelerix* spp. and *Hemiechinus* spp.) (Riley and Chomel 2005). These animals are omnivores, with a diet composed of insects, slugs, worms, snails, fungi, fruits, and certain vegetables like spinaches, carrots and broccoli (Santana *et al* 2010). Among the hedgehogs, *A. albiventris* is becoming the most popular pet in Chile and around the world, with increasing populations in captivity (Fredes and Román 2004, Okada *et al* 2018). Since 2007, the importation of these animals to Chile has not been allowed and all the individuals legally commercialised have been bred in the country. However, there is no record of the reproduction, number and distribution of these animals housed as pets.

With regard to the risks associated with their breeding, some studies have determined that these mammals could act as hosts of zoonotic pathogens, including *Salmonella* spp., *Yersinia* spp. and *Mycobacterium* spp., among others (Riley and Chomel 2005, Santana *et al* 2010, Keeble and Koterwas 2020). *Salmonella enterica* is an enteric pathogen widely distributed in nature, which causes infection in a range of hosts including humans and other mammals, birds and reptiles (Marus *et al* 2019). More than 2,600 serotypes have been described within this species, including some host-restricted and other host-generalist serotypes. When it comes to zoonotic infections, *Salmonella* serotypes are commonly transmitted by consumption of contaminated animal and plant-derived food. This usually results in self-limiting gastroenteritis (Ferrari *et al* 2019), although patients with some risk factors, such as infants, immunocompromised individuals, and the elderly have shown extra-intestinal infections that can cause meningitis, sepsis and even death (Gordon 2008). In the last 30 years, infection with this bacterium in humans accounts for the most common notifiable outbreaks of any infectious disease (Smith *et al* 2014). In Chile, official records show a similar trend in which *Salmonella* represents the major cause of foodborne disease outbreaks among cases with a confirmed diagnosis (Olea *et al* 2012).

In non-outbreak, individual and domestic contexts of non-typhoidal *Salmonella* infections, other transmission pathways may lead to disease in humans, such as the direct or indirect exposure to pets (Younus *et al* 2010, Braun *et al* 2015, MacDonald *et al* 2018). These cases appear sporadically and it mostly affects only a few people, being less studied and reported, and therefore constituting much more common events than is believed (Boore *et al* 2015).

Hedgehogs in captivity represent a public health risk due to the epidemiological evidence that links bacterial detection in these animals, which generally appear as asymptomatic carriers with a high *Salmonella* load, with a disease in humans (Riley and Chomel 2005, Kagambega *et al* 2013, Anderson *et al* 2017, MacDonald *et al* 2018).

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Also, antimicrobial resistant phenotypes have been described in pathogens isolated from the gastrointestinal tract of hedgehogs, generating concern about the potential transmission of these genetic traits to the gut microbiota of owners (Zare and Ghorbani-Choboghlo 2015).

The objective of this work was to report the isolation of zoonotic *Salmonella* serotypes in African pygmy hedgehogs (*A. albiventrix*) kept as pets in the Metropolitan Region from Chile, and characterise antimicrobial-resistance phenotypes in these isolates.

MATERIAL AND METHODS

SAMPLES

Between March 2017 and July 2018, 200 animals (103 males and 97 females) were sampled upon admission to a veterinary clinic specialising in exotic animals, located in the urban area of the city of Santiago, Chile. This sample size represented 10% of all hedgehogs registered in the clinic. For sampling, animals were weighted and maintained in individual cages until they defecated. Then, fresh faeces were immediately collected with sterile swabs, immersed into Cary Blair transport medium and stored at 4 °C for analyses at the laboratory (Fresno *et al* 2013). The age was registered from clinical records or by directly asking to owners. Each animal was sampled only once.

The inclusion criteria corresponded to animals that had not been treated with antimicrobials two weeks prior to sampling. Before the sampling procedure, their owners signed an informed consent that had been approved by the Bioethics Committee (14-2017 VETUCH) of the Faculty of Veterinary and Animal Sciences, University of Chile.

BACTERIOLOGICAL ISOLATION

Once at the laboratory, faeces were processed as reported previously (Fresno *et al* 2013). Briefly, swabs were inoculated into 5 mL of buffered peptone water (Difco®) supplemented with 20 mg/mL of Novobiocin (Sigma®) (Jensen *et al* 2003) and then incubated at 37 °C for 24 hours. Later, 100 µL of the bacterial suspension was seeded into Rappaport Vassilidis semisolid agar (MSRV, Oxoid®) supplemented with 20 mg/mL of Novobiocin, and incubated for 24-48 hours at 41.5 °C. Cultures with bacterial growth were plated in Xylosa Lysine Deoxycolate agar (XLD, Difco ®) and incubated at 37 °C for 24 hours. Suspicious colonies of *Salmonella* spp. were confirmed by the detection of the *invA* gene in a PCR test (Malorny *et al.* 2003) and then were serotyped at the Chilean Institute of Public Health, the national reference laboratory, according to the Kauffman-White scheme[1].

ANTIBIOTIC RESISTANCE PHENOTYPES

The antimicrobial susceptibility was evaluated by the disk diffusion method (Kirby Bauer), according to the standards recommended by the Clinical Laboratory Standards Institute (CLSI 2012) and the National Antimicrobial Resistance Monitoring System (NARMS 2011). The following antimicrobials, which are of frequent use in veterinary and human medicine, were evaluated: enrofloxacin (10µg), amoxicillin + clavulanic acid (20/10µg), tetracycline (30µg), ampicillin (10µg), cefadroxil (30µg), azithromycin (15µg), ceftriaxone (30µg), kanamycin (30µg), nalidixic acid (30µg), streptomycin (10µg), ceftiofur. *Escherichia coli* ATCC 25922 was used as a control strain. The multi-drug resistance (MDR) condition was determined by the simultaneous resistance to three or more antimicrobial classes.

ANALYSES OF RESULTS

Associations between bacteriological results and data from hosts (sex, weight, and age) were explored with ANOVA and categorical data analyses using Infostat (v2010) software.

RESULTS AND DISCUSSION

From 200 samples analysed, a total of 5 animals resulted positive for (2.5%) *S. enterica* isolation in their faeces. These detections included subspecies *enterica*, with serotypes Muenchen (2) and Infantis (2), and subspecies *houtenae,* with the serotype 43:z4,z23:- (1). All *S. enterica* subsp. *enterica* strains showed antimicrobial resistance phenotypes, including a multi-drug resistant *S. enterica* ser. Muenchen isolate (table 1). In contrast, the *S. enterica* subsp. *houtenae* isolate was pansusceptible. No associations ($P>0.05$) were detected between the variables analysed.

The results obtained from the study confirm the presence of antimicrobial resistant *S. enterica* strains in hedgehogs, representing potential risk for owners in Chile. Studies from other countries have reported infections by *Salmonella* spp. in humans who kept hedgehogs as pets[2] (Riley and Chomel 2005, Chomel *et al* 2007). Although there is no evidence of similar cases in Chile, it is possible that animal to human transmission is occurring. In general, such zoonotic events are underreported since the disease generally appears individually rather than collectively in the exposed population and also because of the self-limiting nature of the disease (Morse *et al* 2012).

In the last years, *S. enterica* ser. Muenchen has been related to foodborne outbreaks linked to turtle meat, pigs and plant-derived foods in Australia, Europe and North America (Jackson *et al* 2013, Bonardi *et al* 2016, Draper

[1] Grimont PA, Weill FX. 2007. Antigenic formulae of the *Salmonella* serovars. 9th ed. WHO Collaborating Center for Reference and Research on Salmonella, Institut Pasteur, Paris, France. Available at https://www.pasteur.fr/sites/default/files/veng_0.pdf.

[2] CDC. 2019. Outbreak of *Salmonella* infections linked to pet hedgehogs. Available at https://www.cdc.gov/salmonella/typhimurium-09-20/index.html.

Table 1. Description of *Salmonella enterica* isolates, animal hosts data, and antimicrobial resistance phenotypes.

Salmonella serotype	Sex	Weight (gr)	Age	Antimicrobial resistance*											
				AMC	AMP	TE	CFR	EFT	ENR	CN	SXT	AZN	CRO	K	NA
Muenchen	F	231	3 m	–	–	–	I	–	–	–	–	–	–	–	–
Muenchen	M	302	1.8 y	–	R	R	–	R	–	–	–	–	–	–	–
Infantis	F	304	2.2 y	–	–	–	–	R	–	–	–	–	–	–	–
Infantis	F	365	4.2 y	–	–	–	I	–	–	–	–	–	–	–	–
IV 43: z4, z23:–	F	348	2.7 y	–	–	–	–	–	–	–	–	–	–	–	–

*AMC, Amoxicillin/ Clavulanic acid; AMP, Ampicillin; TE, Tetracycline; CFR, Cefadroxil; EFT, Ceftiofur; ENR, enrofloxacin; CN, Gentamicin; SXT, Sulfamethoxazole /Trimethoprim; AZN, Azithromicin; CRO, Ceftriaxone; CIP, Ciprofloxacin; K, Kanamycin; NA, Nalidixic acid. Underlined and non-underlined abbreviations correspond to critically important and highly important antimicrobials, respectively (WHO 2019). R, Resistant, I, Intermediate, – Susceptible.

et al 2017, Schielke *et al* 2017), respectively. These reports suggest a diversity of sources when causing disease in humans. This contrasts with the other serotype found, *S. enterica* ser. Infantis, which is an emerging pathogen generally associated to outbreaks by consumption of poultry products (Aviv *et al* 2019). The last isolate, the IV 43: z4, z23:- serotype, belongs to the subsp. *houtenae*, and has been mostly detected in reptiles. Despite its lower pathogenic potential when compared with subsp. *enterica* serotypes, extra-intestinal infections in children have been described (Lamas *et al* 2018).

The specific transmission pathways by which hedgehogs from this study resulted colonised with different *Salmonella* serotypes are unknown. They might become infected in pet stores or breeders, or after being sold, due to co-habitation with other pets or by consumption of contaminated food offered by their owners to these mammals. Furthermore, the access to other kind of food or preys, is also frequent, especially in some household systems with a partial confinement. In these situations, the potential consumption of insects, lizards or other commodities and animals, could allow their contamination with diverse *Salmonella* serotypes, as has been described in this study and elsewhere (Kagambega *et al* 2013).

Antimicrobial resistance is an emerging threat to public health at a global scale, in which major drivers for selection of resistant agents are the misuse and overuse of antimicrobials, both in the human and animal context (Millanao *et al* 2018). In this regard, the World Health Organization categorised these drugs according to their therapeutic relevance, trying to preserve their effectiveness for human medicine. In this study, we found resistance phenotypes against critically important (ampicillin and ceftiofur) and highly important antimicrobials (tetracycline) (WHO 2019) in both Muenchen and Infantis serotypes (table 1). The resistance against ceftiofur is of particular concern, because the bacterium could be potentially carrying the plasmid encoded *blaCMY-2* gene, that also confers resistance against ceftriaxone (Alcaine *et al* 2005), an antimicrobial recommended for the treatment of extra-intestinal infections of *Salmonella* in humans (Yang *et al* 2016). Such bacterial traits might be circumstantially detected in hedgehogs after the consumption of contaminated food or may represent the *in vivo* selection of resistant clones because of antimicrobial-based therapies used in these animals. Although our exclusion criteria for sampling was the absence of treatments of this type during the last two weeks prior to sampling, persistent infections with drug-resistant bacteria might also be occurring (Pignon and Mayer 2011). Whatever the source of pathogens in pets, a zoonotic transmission to people, especially young children, the elderly and immunocompromised patients (Lawson *et al* 2018), may have severe clinical outcomes (Anderson *et al* 2017), especially in extra-intestinal infections which require an antibiotic-based therapy (Ichimi *et al* 2018). The pet hedgehog ownership is not recommended in these risk groups (Keeble and Koterwas 2020).

Non-specific clinical signs of salmonellosis have been reported in hedgehogs, such as anorexia, diarrhea and weight loss (Riley and Chomel 2005, Keeble and Koterwas 2020). However, all positive animals detected in this work were asymptomatic, a condition that predominates in previous reports (Craig *et al* 1997, Riley and Chomel 2005, Santana *et al* 2010, Hoelzer *et al* 2011, Lawson *et al* 2018), suggesting that sampling of animals in order to detect *Salmonella* carriers should not be constrained by their clinical status. In this regard, owners must be aware about risks presented by the handle of apparently healthy animals. Some basic biosecurity measures could be recommended by veterinarians to hedgehog owners in the context of veterinary practice, such as washing hands after touching them or cleaning its enclosures and supervising that children play safely, avoiding kisses and snuggles with these animals[1].

This work constitutes the first description of *Salmonella* infection in hedgehogs from South America. In other regions, a range of *S. enterica* prevalence fluctuating between 0% (Handeland *et al* 2002) and 96% (Kagambega

et al 2013) has been reported both in free-living and domesticated hedgehogs. A 2.5% infection rate detected in pets sampled for this study suggests a low exposure to *Salmonella* that probably represents adequate management in most cases of domestic ownership. However, hedgehogs may shed *Salmonella* spp. intermittently, especially during exposure to stress from inadequate nutrition, housing and care, as has been reported in other pets and livestock (Hoelzer *et al* 2011). In this study we sampled each animal only once, and bacterial infection data may be underestimated. Other considerations regarding our results could be the limitations in the diagnostic sensitivity of isolation procedures, and the unknown number of hedgehogs and husbandry conditions of these animals living in Chile.

Finally, the presence of multidrug resistant *Salmonella* strains was confirmed in hedgehogs in Chile. Further studies should address possible animal to human transmission events, which have been documented several times in other regions. The zoonotic risk and the impacts on natural ecosystems if these animals are released, are major factors to be considered when establishing public policies for their pet ownership.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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The subheading "Introduction" is written on the next page following the Abstract and Resumen. In the following line, indented by 5 spaces, the context of the study is briefly presented without an extensive revision of the theme, and only citing the most relevant references. The hypothesis and objectives of the study must be clearly and concisely presented.

Material and methods

Separated by one space from the previous section, this section should contain sufficient detail to allow others to repeat the study. When the first reference in the text is made to medications or chemicals, the generic name, dose and route of administration should be indicated. For specialised equipment, the brand, model and manufacturer's name must be indicated. Studies involving animals or humans must mention the appropriate Bioethical Committee Certification. Details of all statistical methods used must be given at the end of this section under the sub-heading "Statistical analysis" and should include adequate detail to allow readers to determine precisely how data have been analysed and the units that are used to express the results (mathematical mean, standard deviation, standard error of the mean, mediums, ranges or confidence limits, etc.). The use of parametric (Chi-square, student's t-test, ANOVA, etc.) or non-parametric (Wilcoxon, Kruskal-Wallis etc.) analyses must be indicated. The name, version and sources of computational statistical analysis programs must be identified, e.g. SPSS 9.0 (SPSS Inc, Chicago IL, USA).

Results

Separated by one space from the previous section, this section should contain a concise and logical description of the results obtained without discussion or reference to other work. The results can be supported by tables and/or figures that present the pertinent data without repetition, and data presented in tables and figures should not be repeated in the text. In the case of Original articles only, this section and the Discussion are separated.

Discussion

This section should evaluate and interpret the results and relate these to other relevant results. The results should not be repeated and new results must not be presented in this section. Care should be taken to ensure that the discussion is developed in a logical and concise manner, and conclusions are reached, as well as a discussion of their relevance. Conclusions that are not directly supported by the data of the study or other unpublished studies should not be presented.

Acknowledgements

This section should be brief, and should only include people or institutions that have made a direct contribution, provided necessary

material or have provided the facilities for the study's development. The source of funding should be indicated in this section.

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The accuracy of the reference section is the responsibility of the authors and references must be verified against the original article. Please ensure that all articles cited in the text are included in the reference list and vice versa. In the main text, citations should be listed in parentheses in chronological order, citing authors' names, and using *et al* after the first author's name where there are more than two (e.g. Smith 1994, Castro and Martínez 1996, Weiss *et al* 2002).

All lines after the first line of each entry in the reference list should be indented 0.5 cm from the left margin (hanging indentation). The reference list must be ordered alphabetically according to the first author's name, and all authors' names and initials must be included. When no author is given, use the term "Anonymous" in both text and reference list. References with the same author, single or with coauthors, should be listed in chronological order. If there were more than five authors, *et al* must be used after the fifth one. The letters a, b, c, etc. should be appended as a superscript when more than one work is cited from the same author within the same year. Author names should appear with the initials and first letter of the surname in upper caps and the remainder of the surname in lower caps, with no periods between initials. Journal title abbreviations and names of books must be in italics. For journals, ISI abbreviations must be used. The following examples can be used as a guide:

For journal articles:

Mella C, Medina G, Flores-Martin S, Toledo Z, Simaluiza RJ, *et al*. 2016. Interaction between zoonotic bacteria and free living amoebas. A new angle of an epidemiological polyhedron of public health importance?. *Arch Med Vet* 48, 1-10.

Neverauskas CE, Nasir A, Reichel MP. 2015. Prevalence and distribution of *Neospora caninum* in water buffalo (*Bubalus bubalis*) and cattle in the Northern Territory of Australia. *Parasitol Int* 64, 392-396.

For books, chapters in books or occasional publications:

Leeson S, Summers JD. 2005. *Commercial poultry nutrition*. 3rd ed. Nottingham University Press, Nottingham, UK.

Larson V. 2009. Complications of chemotherapeutics agents. In: Silverstein D, Hopper K (eds). *Small Animal Critical Care Medicine*. Saunders Elsevier, St Louis, Mo, USA, Pp 817-820.

WHO, World Health Organization. 1972. International Drug Monitoring: The role of national centres. *Tech Rep Ser* WHO Nº 48.

SAG, Servicio Agrícola y Ganadero, Chile. 1996. Resolución Exenta Nº 3599 del 29 de noviembre de 2006.

For softwares:

SAS, Statistical Analysis System. 2000. *SAS version 6.0*. SAS Institute Inc., Cary, NC, USA.

R Core Team. 2014. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.

For articles and proceedings published in regular series:

Zimbelman RB, Rhoads RP, Rhoads ML, Duff GC, Baumgard LH, Collier RJ. 2009. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. *Proceedings of the 24th Southwest Nutrition and Management Conference*, Tempe, Arizona, USA, Pp 158-169.

For PhD and MSc dissertations:

Lindberg A. 2002. Epidemiology and eradication of bovine virus diarrhea virus infections. *PhD Dissertation,* Swedish University of Agricultural Sciences, Uppsala, Sweden.

Minimise the citation of abstracts as references. Authors are specifically discouraged from citing "unpublished data" or "personal communication", unless this information exists in written form, in which case the text should be referred to as a footnote, but this should not appear in the list of references. References to papers which have been accepted but not published should be cited as "in press", whereas manuscripts which have been submitted for publication but not accepted should be referred to as "unpublished data".

Web pages should not be included as references. If required, web page addresses should be written as footnotes, including date of consultation.

COMPLEMENTARY INSTRUCTIONS

Tables

The titles to tables and figures should be self-explanatory. The number of tables should be kept to a minimum and presented on separate pages with their respective titles at the top. Information in tables must not be repeated in the text. Tables must be numbered consecutively with Arabic numbers in the order in which they are referred to in the text. The brief title to the table should indicate the contents of the table and should be understandable without reference to the text. Each column of each table must have a short or abbreviated heading. Only column headings and general titles should be separated with horizontal lines. Data columns should be separated by spaces and not vertical lines. When additional explanatory information is required, this should appear at the foot of the table. Explanatory information for non-standard abbreviations and units should appear within parentheses. If superscripts are used to indicate significant differences between values, use a, b, c. Minimise the number of digits in each column. Indicate a zero value as 0. Table widths should not exceed 80 mm for one column or 170 mm for two columns.

Figures

Figures should be submitted on separate pages, with their respective titles in English at the bottom and numbered consecutively using Arabic numerals in the order they are referred to in the text, e.g. Figure 1, not Fig. 1. Figures include all illustrations that are not Tables, e.g. graphs, radiographies, ecographies, electrocardiograms, photographs, etc. Figures must be vertically oriented and be accompanied by a short descriptive caption that contains an explanation for all markers, lines and symbols used but no abbreviations. If the figure contains sections, these should be labelled as a, b, c, etc. in the top right corner and must be described in the caption. Figures may be one or two column-widths (80 or 170 mm, respectively). The authorship of non-original figures must be acknowledged, and

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Authors are expected to consider carefully the list and order of authors before submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only before the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the corresponding author: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors after the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended.

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