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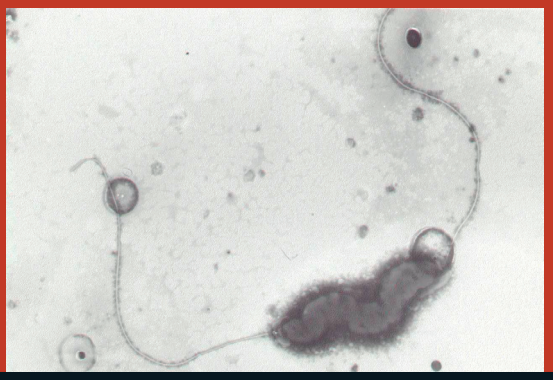

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Cover: Micrograph showing fluorescent merocyanine patterns on dog spermatozoa: opaque - low fluidity (spermatozoon on the left) or brilliant - high fluidity (spermatozoa on the right). Photograph captured by Alicia Alcantar-Rodríguez and provided by Ortega-Morales *et al*.

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Editorial

The magic number

Since the novel of Douglas Adams, *The Hitchhiker's Guide to the Galaxy* (1979), where the answer of a supercomputer to "the most important question", including life and the universe, is the number 42, no one has been so attached to a single number as scientists.

This is the purpose that we often assigned to P-values, an arbitrary threshold delimiting "statistical significance". Recently, more than 800 scientists from 50 countries signed a letter requesting the withdrawal of all concepts of Statistical Significance[1]. The dichotomized discussion involves the terms statistically "significant" and "non-significant", which depend on whether the P-value falls below or above this "magic" number, giving the idea that the items evaluated are categorically different.

Unfortunately, the false belief that crossing the threshold of statistical significance is enough to show that a result is 'real' has led scientists and us (journal editors) to privilege such results, thereby distorting the literature. Some could argue that an arbitrary but clear limit is better than an unclear one. Additionally, if we do not use statistical significance, then what should we be doing instead? Whatever the discussion might lead to in the future, what seems relevant now is to educate ourselves about statistical misconceptions, but further, we should consider the logic, the background knowledge, and the experimental design along with P value to conclude.

Austral Journal of Veterinary Sciences is not considering changing how it considers the statistical analysis in evaluation of papers at this time, but we are aware of this discussion in the scientific world since the conclusions will necessarily have an impact on what is currently considered suitable for publication.

Editorial Committee
Austral Journal of Veterinary Sciences

[1] https://www.nature.com/articles/d41586-019-00857-9

Austral J Vet Sci **51, 45-51 (2019)**
REVIEW

Electrochemotherapy in the treatment of neoplasms in dogs and cats

Marcelo M.M. Rangel[a*], Jean C.S. Luz[a], Krishna D. Oliveira[a], Javier Ojeda[b], Jennifer O. Freytag[a], Daniela O. Suzuki[c]

ABSTRACT. Electrochemotherapy (ECT) is a technique that combines chemotherapy with local application of specific electric pulses with the aim of increasing the permeability of the plasma membrane in a reversible way, improving the influx of chemotherapeutic drugs into the cytoplasm and potentiating their cytotoxic effects. This technique has broadened the range of possible treatments for oncological patients, either on its own or as adjuvant to surgical procedures. It is especially useful in tumors located in regions with only a small surgical safety margin, such as the limb extremities, skull, oral cavity, neck and perianal region, among others. ECT makes it feasible to perform procedures more conservatively, or even to perform otherwise infeasible procedures, by expanding the margins without removing healthy tissues. The objective of this paper is to provide a brief bibliographic review of the principles, applications and future possibilities of electrochemotherapy, helping to disseminate pertinent information about this relatively new technique for the treatment of cancer.

Key words: bleomycin, electroporation, veterinary oncology, electrochemotherapy.

MECHANISM OF ELECTROPORATION AND ELECTROCHEMOTHERAPY

Electroporation, or electropermeabilisation , consists of the exposure of cells to specific electrical pulses (1000 V/cm, 100 microseconds, 8 pulses at a 5 kHz frequency), which transiently increase the permeability of the plasma membrane, allowing access to the cytoplasm for molecules to which the membrane is normally impermeable or only slightly permeable (Mir *et al* 1996, Teissie *et al* 2005, Mir 2006). Under physiological conditions, the plasma membrane is subject to the so-called membrane resting potential, which remains balanced by pumps and ion channels, keeping the electrical potential of the membrane stable. Exposure of the cells to an external electric field produces an induced transmembrane potential difference, altering its resting potential and causing rearrangement of the molecules in the lipid bilayer, resulting in the formation of pores (Almers *et al* 2010, Kotnik *et al* 2010). Electroporation depends on the specific electrical parameters; the amplitude and duration of the electric field to which the cells are exposed are key points of the process (figure 1). At low amplitude and duration, there is no detectable effect on the plasma membrane in terms of its permeability, while at moderate amplitude and duration, electropermeabilisation occurs. In the pore model, the pores close after the end of exposure, causing the cells to remain viable. Excessive amplitudes and/or durations cause nonthermal irreversible electropermeabilisation , possibly due to nonclosure or late closure of the pores, resulting in a form of cell death called mitotic catastrophe. Mitotic catastrophe is cell death preceded by multinucleation that occurs during the metaphase (Kroemer *et al* 2009). Irreversible thermal electroporation can also occur when even higher amplitudes are applied, causing thermal damages to the cells (Cemazar *et al* 2015). An ultrastructural study showed some changes in the membranes, such as defects in the dynamic assembly of lipids and proteins after exposure to the electric field used in electroporation, i.e. proteins formed membrane agglomerates and hydrophilic pores. These defects in dynamic assembly would be responsible for the entry of molecules during electroporation (Spugnini *et al* 2007[c]).

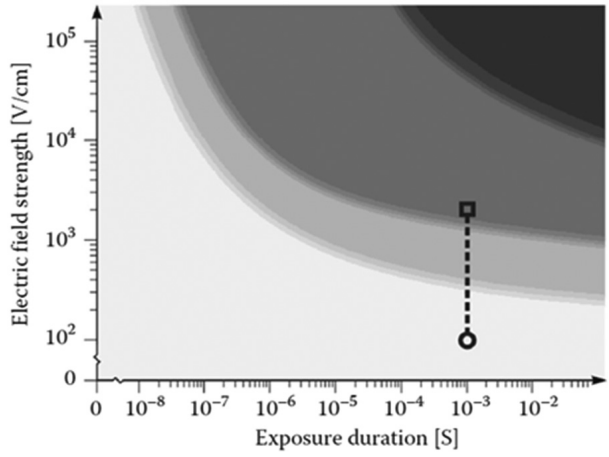

Figure 1. Electroporation and thermal effects caused by exposure of cells to electric fields. Graphics representing reversible and irreversible electroporation and thermal damage caused to cells due to the electric field strength ([V/cm]) and exposure duration ([s]) in seconds. Source: Cezamar, et al., 2015 (adapted).

ELECTROCHEMOTHERAPY

Electrochemotherapy (ECT) is a technique that combines chemotherapy with the application of such specific

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electrical pulses, allowing the entry of nonpermeating antineoplastic drugs (or those with low permeant potential) into cells (Mir 2006). The waveforms used in electrochemotherapy are square-wave mono- or biphasic pulses. The most common electric parameters used in electrochemotherapy are 8 pulses, 100 µs length, 1-5 kHz frequency and 1000 V/cm (for needle parallel electrodes) or 1300 V/cm (plate electrodes) of electric field intensity[1] (Spugnini *et al* 2007[a], Pavlin *et al* 2009, Tozon *et al* 2016, Maglietti *et al* 2016, Pierini *et al* 2016, Suzuki *et al* 2018, Lowe *et al* 2017). For this technique, bleomycin and cisplatin are the main drugs used (Sersa *et al* 2008, Escoffre and Rols 2012). Bleomycin is widely used in electrochemotherapy due to its high intrinsic cytotoxic potential and cell selectivity caused by the mechanism of mitotic cell death. Once inside the cell, bleomycin acts as an endonuclease, with the potential to promote single- and double-stranded ruptures in DNA, predominantly affecting cells in the process of proliferation (Orlowski *et al* 1988, MIR *et al* 1988, Mir 2006).

Although bleomycin can enter permeabilised cells using plasma membrane proteins, its capacity is highly restricted by the plasma membrane, which explains its limited action in these cells. This is because only some molecules are effectively internalised and reach the DNA (Mir *et al* 1996). Electropermeabilisation is a way of overcoming such barriers; *in vitro* studies report that electropermeabilisation potentiates the cytotoxic effect of the drugs by up to thousands of times (Poddevin 1991, Mir *et al* 1996, Gehl 2003, Orlowski *et al* 2016). ECT increases the absorption of antineoplastic drugs, which will remain internalised in the cell after membrane permeability is restored, maximizing their cytotoxicity. Another property of ECT is that its action is restricted to tissues exposed to the electrical pulses, reducing the risk of chemotherapeutic side effects (Spugnini and Porrello 2003, Gehl 2003, Miklavcic *et al* 2014, Tafuto *et al* 2015). Bleomycin is a drug that induces two mechanisms of cell death, depending on the number of molecules internalised after electropermeabilisation. At low concentrations of bleomycin, the cell dies after three doubling times (called mitotic death); at high concentrations, cell death occurs in a manner analogous to apoptosis (called mimetic apoptosis) (Tounekti *et al* 1993).

In order to reach the electroporation threshold, several aspects must be considered, such as physical factors (cell size and shape), biological features (cytoskeleton structure) and adequate electrical parameters. Hypothetically, however, it is possible to perform electroporation independently of the type of cell or tissue (Rols and Teissie 1992, Teissie *et al* 2005, Cemazar *et al* 2015, Sersa *et al* 2015).

Two factors must be taken into consideration for the efficiency of ECT. First, the chemotherapeutic drug can be applied either intratumorally, in which case immediate electropermeabilisation must be performed, or intravenously, with an interval of approximately 8 minutes until electropermeabilisation. The waiting time for the intravenous route allows the antineoplastic drug to reach its pharmacological peak in the tumor, remaining for approximately 20 to 25 minutes at an effective concentration according to the principles of the technique. The second factor is that the entire tumor, including any infiltration into adjacent tissue, must be exposed to a sufficient electric field to promote reversible electropermeabilisation, this being obtained by correct choice and placement of electrodes and application of electrical pulses with suitable parameters (Marty *et al* 2006, Domenge *et al* 1996). In the case of tumors with larger proportions due to abnormal vascularization, hindering proper distribution of the drug, both intravenous and intratumoral administration can be considered (Maglietti *et al* 2016).

Due to the stochastic nature of the process, access to the tumor through electrode placement and efficient distribution of the electric field could be more important factors than the chemosensitivity of the neoplasia to the drug (Gehl 2003, Mir 2006, Cemazar *et al* 2015).

In addition to increased membrane permeability, other mechanisms are described in relation to ECT, such as the effect of vascular lock and involvement of the immune response. According to Sersa *et al* (2008), electrochemotherapy promotes a process of vascular dysregulation throughout two distinct mechanisms. One of them is that the electrical pulses cause transient cytoskeleton damage and swelling of the vascular endothelium, which leads to the death of some tumor and vascular cells. Another is the endothelial cytotoxicity promoted by ECT. Both mechanisms reduce local blood flow, decreasing the oxygenation of the tumor, leading to hypoxia and subsequent necrosis of tumor cells (Spugnini *et al* 2006). Additionally, some studies have used intravital microscopy to confirm vasoconstriction induced by ECT in normal and tumoral blood vessels. Vascular lock (or vascular sequestration), induced transiently by electroporation alone and for a prolonged time by electrochemotherapy, keeps the antineoplastic accumulated and prevents it from rapidly exiting the tumor due to reduced blood flow (Jarm *et al* 2010).

Involvement of the immune system after electrochemotherapy is important for eliminating tumoral cells. Due to the heterogenicity of tumor cells (affecting the orientation, size and distribution of chemotherapy in the tumor), not all cells are eliminated by electrochemotherapy because some have not been effectively electroporated or internalized sufficient amounts of the chemotherapeutic drug (Miklavcic *et al* 2014, Sersa *et al* 1997). As with other physical methods, such as radiotherapy, when the fraction of remaining cells is low enough, they can be eliminated by the immune system. A similar situation was observed

[1] Tellado M, Maglietti F, Olaiz N, Michinski S, Marshall G. 2014. Electroquimioterapia como herramienta terapéutica en melanoma oral en caninos. Available at: http://vetoncologia.com/wp-content/uploads/2014/09/melanoma-oral-canino.pdf

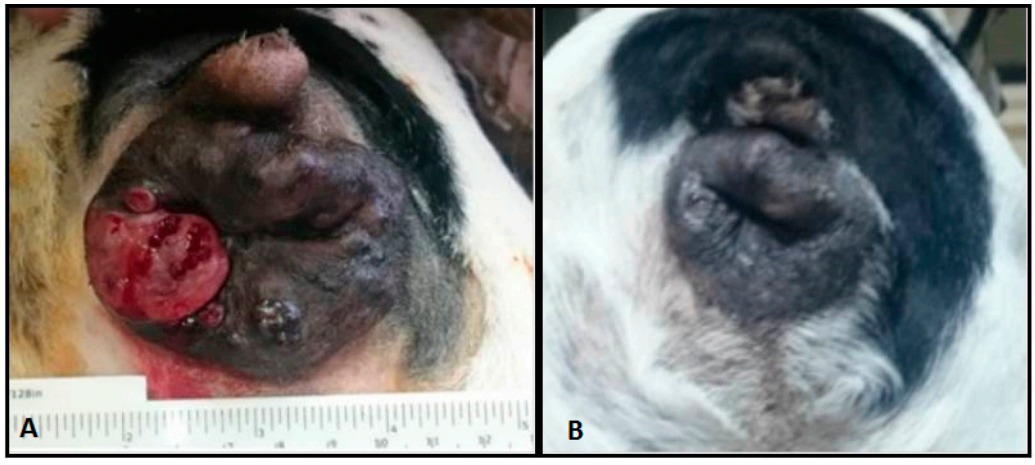

Figure 2. Anal hepatoid gland carcinoma in a dog treated with one session of electrochemotherapy (endovenous bleomycin 20 mg/m^2). Before ECT (A), 2 months after ECT (B).

in immunodeficient organisms, which had a significantly lower cure rate than immunocompetent individuals after electrochemotherapy treatment (Sersa *et al* 1997). This might occur due to exposure of immunogenic molecules, such as calreticulin, and the release of neoplastic antigens from the cells destroyed by the ECT, which can activate immune cells against the tumor (Calvet *et al* 2014, Gerlini *et al* 2013, Gerlini *et al* 2012, Miklavcic *et al* 2012).

CLINICAL RESULTS OF ELECTROCHEMOTHERAPY USE

Many studies have reported favorable results in different cancer types traditionally known for limited or no treatment option or for poor response to standard therapy (table 1). Additionally, ECT could be an option for unresectable tumors or those in areas with anatomical limitations that make them amenable to this treatment. For ECT evaluation, many oncologists use the criteria for solid tumors proposed by Veterinary comparative oncology group (VCOG) (Nguyen *et al* 2013): Compete response (CR) is defined as disappearance of all target lesions and pathologic LNs measuring < 10 mm along the short axis. Partial response (PR) consists of at least 30% reduction in the sum of the diameters of the target lesions. Progressive disease (PD) is defined as either the appearance of one or more new lesions or at least a 20% increase in the sum of the diameters of the lesions, taking as a reference the minimal sum on examination. Additionally, the sum must show an increase of 5 mm. For stable disease (SD), the evaluation must include tumors with less than 30% reduction (PR) or a 20% increase (PD) in the sum of the diameters of the target tumors, compared to the smallest sum of diameters during monitoring. Additionally, many studies include an overall response (CR+PR) to evaluate the study group.

In canines, favorable results are observed in skin tumors, especially mast cell and perianal tumors (figure 2). In the case of mast cell tumors, using the histological grade established by Patnaik *et al* (1984), two studies administering intratumoral cisplatin achieved complete remission in 62.6% of grade I and III tumors (25 cases) and in 78% (37 cases), including 7 grade I, 24 grade II and 6 grade III cases, with an average of 1,218 days until recurrence (Kodre *et al* 2009, Spugnini *et al* 2011). Another study using endovenous bleomycin in mast cell tumors reported complete objective remission when analysing 80 dogs; histological grade details were not included (Tozon *et al* 2016). However, ECT showed the greatest effectiveness in tumors < 2 cm. Lowe *et al* (2017) used a similar treatment, evaluating four groups and concluding that 51 dogs had 100% objective response and a recurrence time of 1,500 days. For perianal tumors, the objective response rate using intratumoral bleomycin or cisplatin was 92% when evaluated at 34 months (Tozon *et al* 2005) and 94% when evaluated at 14 months, with complete remission in 84.2% (Tozon *et al* 2010).

In cats, ECT has emerged as an effective treatment for squamous cell carcinomas (SCC), especially when the tumor invasion suggests aggressive treatments (figure 3). Several studies have reported that ECT can reach 100% objective response (73% complete remission + 27% partial response), with a median survival time of 452 days, for all stages of SCC considered together (Pierini *et al* 2016). Previous studies in SCC found an objective response rate of 87.5% (17 masses), with a recurrence rate of 22% observed in 2 to 8 years (Tozon *et al* 2014). Spugnini *et al* (2015) determined an 81% objective response rate in 21 cats with SCC. This study used a control group and compared the results with the ECT group. The median recurrence time was 30.5 month for the ECT group and 3.9 months for the control group.

In soft tissue sarcoma (STS) treatment using intratumoral bleomycin and cisplatin, a sample of 22 dogs had an objective response rate of 95% (Spugnini *et al*

Table 1. Comparative results of different tumors using electrochemotherapy.

Specie	Diagnosis	Cases (n)	Drug	Results	Reference
Dog	STS	22	Bleo (it)	CR: 90%	Spugnini *et al* 2007
Dog	Perianal tumor	12 (26 tumors)	Cis (it) Bleo (it)	OR. 92%	Tozon *et al* 2005
Dog	Perianal tumor	21 (66 tumors)	Cis (it) Bleo (it)	OR: 94%	Tozon *et al* 2010
Dog	MCT	51	Bleo (ev)	CR: 93-64%	Lowe *et al* 2016
Dog	MCT	25	Cis (L)	CR: 62,5%	Kodre *et al* 2009
Dog	MCT	37	Cis (it)	CR :78%	Spugnini *et al* 2011
Dog	CTVT	3	Bleo (it)	CR: 100%	Spugnini *et al* 2008
Dog	Nasal tumors	11	Bleo (ev)	CR: 27% OR: 91%	Maglietti *et al* 2017
Dog	MT	34	Bleo (it)	CR: 88,7%	Silveira *et al* 2010
Feline	STS	39	Bleo (it)	R: 4-19 months	Spugnini *et al* 2007
Feline	STS	64	CIs (it)	R: 666 days R: 180 days control group	Spugnini *et al* 2011
Feline	SCC	16 (17 tumors)	Bleo (ev)	CR:75%	Tozon *et al* 2014
Feline	SCC	12 (16 tumors)	Bleo (ev)	CR: 73%	Lowe *et al* 2016
Feline	SCC	26 cats	Bleo (iv)	CR: 81 %	Spugnini *et al* 2015
Feline/Dog	Lymphoma	2 dogs 4 felines	Bleo (it)	CR: 100%	
Feline/Dog	MT	140 dogs 36 cats	Bleo (iv)	R: 33%	Lowe 2016
Feline/Dog	MCT	80 dogs 20 cats	Cis (it), Bleo (it/ev)	CR: 100 % <2cms	Tozon *et al* 2016

SCC: Squamous cells carcinoma, MCT: Mast Cells Tumor, STS: Soft tissue sarcoma, CTVT: Canine transmissible venereal tumor, MT: Multiple tumors, Bleo: Bleomicina, CIS: Cisplatin, it: intratumoral, ev: endovenous.

Figure 3. Nasal squamous cell carcinoma in a cat treated with one session of electrochemotherapy (endovenous bleomycin 20 mg/m^2). Before ECT (A), 2 months after ECT (B).

2007[a]). On the other hand, two studies of feline STS in a total of 103 cats had overall responses rates of 70% and 54%, with recurrence times of 666 days and 570 days, respectively (Spugnini *et al* 2011[g], Spugnini *et al* 2007[b]). In both studies, tumors with smaller size had better responses. The evaluation of ECT in tumors with poor response to surgery or chemotherapy is very positive. Twelve dogs with melanoma treated with ECT using bleomycin IV reached an OR of 83.6% with a complete remission rate of 41.4%[2]. Maglietti *et al* (2017) used a single needle electrode for eleven dogs with intranasal tumors, achieving complete response in 27% and partial response in 67%. In this case, the survival time of the ECT group was significantly higher than that of the control group, with mean survival times of 16.86 months and 5.3 months, respectively. On the other hand, dogs with tumors that were chemotherapy resistant to standard protocols had been treated with ECT. In this case, three dogs with transmissible venereal tumors with a resistance to vincristine were treated with intratumoral bleomycin, achieving complete remission (Spugnini *et al* 2007). Similar results were reached in two dogs and four cats with cutaneous lymphoma (Spugnini *et al* 2008). Finally, Lowe (2016) treated different spontaneous tumors and achieved objective response in 66% of 176 patients.

Several points can be listed that support the applicability of the ECT, making it an exceptional tool in the treatment of skin and subcutaneous solid tumors. For example:

- High remission rate: complete remissions were obtained in 58.4% of tumors, independent of histological origin, and partial responses in 24.7% of nodules treated after a single session, according to the meta-analysis provided by Mali *et al* (2013) involving 1894 tumors;
- It not only treats the tumor but also acts on the adjacent margins, thus eliminating possible infiltrated tumor cells (Miklavcic *et al* 2012);
- It presents an increase in the response rate when reapplied at weekly intervals, on tumors that did not have complete remission with single application (Campana *et al* 2009);
- It stimulates the immune response against possible remaining cells after ECT (Miklavcic *et al* 2012);
- It is effective in areas previously treated by means of surgery or radiotherapy, and even to tumors that are refractory to chemotherapy (Miklavcic *et al* 2012);
- It does not cause immediate or delayed toxic effects to patients (Miklavcic *et al* 2012);
- It has a good cost-benefit relation regarding the technology and chemotherapy used, not requiring large investments (Marty *et al* 2006).

[2] Electroquimioterapia como herramienta terapéutica en melanoma oral en caninos. www.vetoncologia.com/wp-content/uploads/2014/09/melanoma-oral-canino.pdf. Retrieved:04 August 2018.

The technique has been developed to enable the use of ECT in tumors and internal cavities (Miklavcic *et al* 2012), and to imaging exams such as positron emission tomography, ultrasonography and magnetic resonance imaging, making it possible to locate such tumors and even position the electrodes in internal organs (Miklavcic *et al* 2010, Pavliha *et al* 2013). The exact localization of the tumor and the use of special needle electrodes allow the application of ECT in nonsuperficial tumors such as metastases of melanomas, sarcomas, bone tumors and even brain tumors (figure 2), or its use in the surgical treatment of internal organs or percutaneous treatment of the limbs. In these cases, the retraction of the electrodes could cause hemorrhage; however, the effect of vascular blocking would minimise this situation (Miklavcic *et al* 2010, Jarm *et al* 2010, Cemazar *et al* 2015). Techniques to avoid interferences on cardiac electrical activity are also being developed. The planning of the electric field is critical to the effectiveness of ECT and must be correlated with the site of application due to the heterogeneity of tissue conductivities, which may result in an irregular distribution of the electric field, and care must be taken not to affect adjacent organs (Domenge *et al* 1996, Deodhar *et al* 2011, Mali *et al* 2008, Suzuki *et al* 2018). Electrochemotherapy is under development. Currently, electroporation devices are being improved, and special types of electrodes for use in specific areas are being created as well as synchronisation techniques timing electrical pulses to the electrocardiogram for regions close to the heart. The use of guided laparoscopy is also being developed in addition to techniques for real-time monitoring of electroporation and distribution of the electric field, which allow improved control of the electrode positioning and an effective treatment of tumors even in areas of difficult access (Kranjc *et al* 2011, Mahmood *et al* 2011, Miklavcic *et al* 2012, Cemazar *et al* 2015).

Although electrochemotherapy has become a well-established treatment for malignancies of skin and nonskin origin, standard evaluations and higher-quality clinical data are needed. Therefore, a standardisation of terminology and reporting criteria is necessary to facilitate effective communication among researchers and appropriate comparison between different treatment technologies (Campana *et al* 2016).

CONCLUSION

ECT is a technique that increases the range of possible treatments for cancer patients, making it possible to perform more conservative procedures and even surgical procedures that had previously been technically unfeasible. The use of electrochemotherapy along with other therapies has shown encouraging results in dogs and cats with different types of cancer. Further research on this technique promises to contribute major advancements in the treatment of cancer.

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ORIGINAL ARTICLE

Evaluation of reduced amino acids diets added with protected protease on productive performance in 25-100 kg barrows

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ABSTRACT. The objective of this research was to evaluate the effect of adding protected protease to low-amino acids (AA) diets on the growth performance of barrows. Three decreasing levels of AA (protein levels), with or without the addition of protease were fed to 48 hybrid barrows (27.42±3.48 kg initial body weight). The experimental design was a completely randomised with a factorial arrangement of treatments. An analysis of variance was performed with GLM of SAS and the means comparison was performed with Tukey test ($P \leq 0.05$). The productive performance was not affected by addition of proteases in the diet at the three stages ($P > 0.05$). Only in growing barrows, the interaction of standard protein diet and protease reduced backfat thickness ($P \leq 0.05$). Protein level in finishing I barrows did not affect ($P > 0.05$) growth performance variables. Low-protein diets increased ($P \leq 0.05$) average daily gain, final body weight and fat-free lean gain in growing and finishing II barrows. Concentration of urea in plasma decreased with the reduction of CP and increased with the addition of protease ($P \leq 0.05$) at the three stages. In conclusion, low protein diets improved or maintained growth performance variables and reduced the plasma urea nitrogen, whereas supplementation with protease did not show any effect on productive performance.

Key words: protein content, swine, growth performance, carcass characteristics.

INTRODUCTION

The use of low-protein diets (LPD) in fattening pigs is a viable option for reducing nitrogen emission into the environment, which reduces the amount of greenhouse gases and soil contamination (Osada *et al* 2011). In addition, LPD enable to obtain a similar growth performance compared to standard diets when supplemented with crystalline AA (He *et al* 2016, Jiao *et al* 2016, Peng *et al* 2016). However, the reduction of more than 4-6% of crude protein (CP) in the diet affects growth performance and digestive enzymatic production (He *et al* 2016). Therefore, it is necessary to find alternative additives that compensate for the reduction of protein ingredients and the amount of CP in pig diets, increasing AA digestibility.

Addition of protease might improve availability of protein in pig diets through an increment on protein digestibility and amino acid (AA) availability in the gastrointestinal tract of pigs (Wang *et al* 2011, Guggenbuhl *et al* 2012). Nevertheless, in some cases, using protease in LPD for weaning, growing and finishing pigs did not improve growth performance or carcass characteristics variables (Reyna *et al* 2006, Zamora *et al* 2011). The variability in protease effectiveness was attributed to the age of pigs, the use of different types of ingredients, the source of protease (Adeola and Cowieson 2011), and the high degradation and inactivation of these enzymes in the gastrointestinal tract (Pan *et al* 2016). To surpass this variability of results using protease for pig diets, recent technological advances have developed coated (protected) protease (Xu *et al* 2017), which works under different conditions in the gastrointestinal tract (Pan *et al* 2016). Thus, using protected protease in pig diets may improve digestibility of AA which, in turn, is reflected in a better growth performance (Zuo *et al* 2015).

This improved protein and AA digestibility and availability in the gastrointestinal tract of pigs (Wang *et al* 2011, Guggenbuhl *et al* 2012) means that the supplementation of protected protease could compensate for the reduction of AA in diets for pigs when fed LPD. For these reasons, the objective of this research was to evaluate the addition of protected protease to low AA concentration (low-crude protein) diets in 25-100 kg barrows in terms of growth performance, carcass characteristics, and plasma urea nitrogen concentration.

MATERIAL AND METHODS

The experimental procedures were performed accordingly to the recommendations of the International Guiding Principles for Biomedical Research Involving Animals[1], and observing the standards for ethics, biosafety, and animal

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[1] CIOMS, Council for International Organizations of Medical Sciences. 2012. International Guiding Principles for Biomedical Research Involving Animals. http://www. cioms (Accessed January 23, 2018).

well-being of the Colegio de Postgraduados, Mexico, under the Official Mexican Regulation (Norma Oficial Mexicana, 1999) for the use of animals in experimentation.

The experiment was conducted in the Swine Unit of the Experimental Farm at the Colegio de Postgraduados, located in Montecillo, Estado de Mexico (98º 48' 27'' W and 19º 48' 23" N). The climate is temperate, semi-arid, with an average annual temperature of 15.9 °C, infrequent frosts, average annual rainfall of 686 mm and an altitude of 2241 m (García 1988).

Forty eight hybrid (Landrace × Yorkshire × Duroc; 27.42 ± 3.48 kg initial body weight and 60 days-old) barrows were used, housed in individual pens, each equipped with a single feeder and nipple drinker. Water and feed were provided *ad libitum*.

The experiment consisted in the evaluation of six treatments (T). Three decreasing levels of protein (standardized ileal digestible (SID) basis AA levels; control, medium, and low), with or without the addition of protease were evaluated (0.03% of protease was added, according to the amount indicated by the JEFO Company; Poultry Grow 250 ®, *Streptomyces griseus*, Type XIV, Puebla, Mexico) in pigs diet. Three stages were evaluated (growing, 25-50 kg body weight; finishing I, 50-75 kg; finishing II, 75-100 kg; tables 1-3 respectively), changing the feed for each stage accordingly to the average body weight of the pigs, trying to follow the recommendations of the NRC (2012) for each stage. Forty-eight barrows were distributed (each pig was considered a replicate) in a completely randomised design with a factorial arrangement of treatments; there were eight replicates (barrows) per treatment. The AA or CP (low-protein diets; LPD) reduction was achieved by decreasing the lysine content by 0.05% and 0.10% relative to control diet, with a proportionate reduction in concentrations of the remaining AA in the diet, trying to maintain the ratio relative to Lys (ensuring that all AA were supplied, at least the Lys proportion).

The nutritional values of the control treatment were established as recommended by the NRC (2012) to cover or exceed the requirements for each stage of growth. The diets for each stage were formulated with the Solver command[2] using the least-cost feed formulation method.

CHEMICAL ANALYSIS

Crude protein content of diets for each stage was determined by the Macrokjeldahl method (AOAC 2005), calcium and phosphorus content by atomic absorption spectrophotometry (Karl *et al* 1979) using a Perkin Elmer 4000 Model (Series Lambda 2, Perkin Elmer Inc., Norwalk, CT, USA). On the last day of the experiment, blood samples (5 mL; pre-prandial 08:00 h) were collected by vena cava puncture in live pigs, using Vacutainer tubes without anticoagulant (BD Vacutainer®), and stored at 4 °C. The blood samples were centrifuged (SIGMA 2-16 k, Germany) at 3500 *g* for 20 min to obtain blood serum. Serum samples were stored in Eppendorf tubes at –20 °C in a freezer (SANYO MDF-436, USA) until determination of plasma urea (Chaney and Marbach 1962).

VARIABLES

Growth performance variables were: average daily feed intake (ADFI), average daily gain (ADG), feed:gain ratio (FGR), fat-free lean gain (FFLG) and final body weight (BW). Carcass characteristics determined were: backfat thickness (BT), *longissimus* muscle area (LMA), lean meat percentage (LMP); and plasma urea nitrogen (PUN) concentration. The BT and LMA were measured using real-time ultrasound (SonoVet 600, MEDISON: Medison, Inc., Cypress, CA, USA) at the 10th rib on the first and last day of the experiment. The BT, LMA, initial and final BW data were used to determine FFLG and LMP, following the procedure indicated by Burson and Berg (2001): Lb. lean=5.7769+(0.401 x warm carcass wt., lbs)–(18.838 x 10th rib fat depth, in.)+(4.357 x 10th rib loin muscle area, sq. in.)+(1.006 x sex of pig) (barrow=1, gilt=2)).

STATISTICAL ANALYSIS

The experimental design was completely randomised with a factorial arrangement of treatments, where the factors were three CP levels and two protected protease levels (with and without). Shapiro-Wilk and Levene's test were used to check normal distribution and homogeneity of variance for all variables. Data were analyzed with the GLM procedure, and Tukey's test ($P \leq 0.05$) was used to compare treatment means (Statistical Analysis System 2010. Inc. Cary, NC, USA). The initial body weight was used as a covariate ($P \leq 0.05$).

RESULTS

LOW-PROTEIN DIETS

No differences ($P > 0.05$) were detected between treatments for FGR, LMP, BT and LMA due to the protein level in the diet for growing (table 4) and finishing II barrows (table 6). An improved response was observed in ADG, final BW, ADFI and FFLG in growing barrows fed 16.17 % and 15.49 % CP compared to 16.86 % CP ($P \leq 0.05$) (table 4). In finishing II barrows, values for ADG and final BW improved ($P \leq 0.05$) when using 12.08 % CP in the diet compared to 12.86 % and 13.63 % CP. The FFLG was greater ($P \leq 0.05$) when using 12.86 % and 12.08 % CP compared to control diet (13.63 % CP) (table 6). There were no effects ($P > 0.05$) of protein level on any growth performance variables in finishing I (50-75 kg) barrows (table 5).

[2] Microsoft Excel 2007

Table 1. Composition of diets for growing barrows (25-50 kg BW) fed low-protein diets supplemented with protease.

Ingredient, %	T1[¶]	T2	T3	T4	T5	T6
Sorghum grain (*Sorghum bicolor*)	77.55	77.51	79.35	79.31	81.11	81.06
Soybean (*Glycine max*) meal	18.66	18.66	16.90	16.91	15.19	15.20
Soybean oil	0.97	0.99	0.94	0.96	0.91	0.92
Biolys[†]	0.73	0.73	0.72	0.72	0.71	0.71
DL-Methionine	0.16	0.16	0.14	0.14	0.13	0.13
L-Threonine	0.10	0.10	0.09	0.09	0.09	0.09
Vitamins[§]	0.20	0.20	0.20	0.20	0.20	0.20
Minerals[Þ]	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Calcium carbonate	0.91	0.91	0.89	0.89	0.87	0.87
Calcium orthophosphate	0.26	0.26	0.29	0.30	0.33	0.33
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
Protease	0.00	0.03	0.00	0.03	0.00	0.03
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient composition calculated (SID AA)						
Metabolizable Energy (Mcal kg^{-1})	3.30	3.30	3.30	3.30	3.30	3.30
Crude Protein (%)	16.86	16.86	16.17	16.17	15.49	15.49
Lysine (%)	1.00	1.00	0.95	0.95	0.90	0.90
Threonine (%)	0.60	0.60	0.57	0.57	0.54	0.54
Tryptophan (%)	0.17	0.17	0.16	0.16	0.15	0.15
Phenialanine (%)	0.73	0.73	0.70	0.70	0.67	0.67
Arginine (%)	0.87	0.87	0.81	0.81	0.76	0.76
Histidine (%)	0.36	0.36	0.34	0.34	0.32	0.32
Isoleucine (%)	0.61	0.61	0.58	0.58	0.55	0.55
Leucine (%)	1.45	1.45	1.41	1.41	1.38	1.38
Valine (%)	0.76	0.76	0.73	0.73	0.69	0.69
Methionine + Cysteine (%)	0.57	0.57	0.54	0.54	0.51	0.51
Calcium (%)	0.67	0.67	0.67	0.67	0.67	0.67
Phosphorus (%)	0.40	0.40	0.40	0.40	0.40	0.40
Determined nutrient composition						
CP (%)	16.76	16.74	16.09	16.07	15.39	15.42
Calcium (%)	0.72	0.73	0.71	0.75	0.74	0.73
Phosphorus (%)	0.38	0.37	0.39	0.38	0.37	0.36

[†] Biolys, 50.7%; lysine. [¶]T, Treatment. [§]Supplied by kg: 5.0×106 IU vitamin A, 1.0×106 IU vitamin D3, 2.0×104 IU vitamin E; 2 g vitamin K3, 1 g tiamine, 5 g rivoflavin, 2 g pyridoxine, 25 g niacin, 15 g D-calcium panthotenate, 3 g folic acid, 225 g choline chloride, 0.3 g antioxidant, 15 mg vitamin B12 and 180 mg vitamin H-biotin. REKA® Lapisa Animal Nutrition. [Þ]Supplied by kg: 0.2 g Se, 0.1 g Co, 0.3 g I, 10 g Cu, 100 g Zn, 100 g Fe and 100 g Mn. REKA® Lapisa Animal Nutrition.

PROTEASE

The addition of protease to diets during three growth phases (growing, finishing I and finishing II) did not change (P>0.05) the growth performance and carcass characteristics of barrows (tables 4, 5 and 6, respectively).

In the growing stage, the control diet (16.86 % CP) with added protease reduced BT (P≤0.05); the protease interaction with the CP level had no effect (P>0.05) on other variables (table 4). There were no effects (P>0.05) of the interaction between protein level and protease addition on growth performance and carcass characteristics in finishing I and finishing II barrows (tables 5 and 6).

PLASMA UREA NITROGEN

The addition of dietary protease in the three phases of growth increased PUN concentration (P≤0.05) of barrows; while protein reduction reduced PUN concentration in all three phases (P≤0.05). In growing barrows, the lowest

Table 2. Composition of diets for finishing I barrows (50-75 kg BW) fed low-protein diets supplemented with protease.

Ingredient, %	T1[¶]	T2	T3	T4	T5	T6
Sorghum grain	81.80	81.80	83.88	83.83	85.91	85.86
Soybean meal	14.58	14.58	12.57	12.58	10.56	10.57
Soybean oil	0.91	0.91	0.86	0.87	0.82	0.84
Biolys[†]	0.64	0.64	0.64	0.64	0.65	0.65
DL-Methionine	0.10	0.10	0.09	0.09	0.08	0.08
L-Threonine	0.07	0.07	0.07	0.07	0.07	0.07
Vitamins[§]	0.20	0.20	0.20	0.20	0.20	0.20
Minerals[Þ]	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Calcium carbonate	0.87	0.87	0.84	0.84	0.82	0.82
Calcium orthophosphate	0.34	0.34	0.39	0.39	0.43	0.43
Phytases	0.01	0.01	0.01	0.01	0.01	0.01
Protease	0.00	0.03	0.00	0.03	0.00	0.03
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient composition calculated (SID AA)						
Metabolizable Energy (Mcal kg^{-1})	3.30	3.30	3.30	3.30	3.30	3.30
Crude Protein (%)	15.18	15.18	14.40	14.40	13.63	13.63
Lysine (%)	0.85	0.85	0.80	0.80	0.75	0.75
Threonine (%)	0.52	0.52	0.49	0.49	0.46	0.46
Tryptophan (%)	0.15	0.15	0.14	0.14	0.13	0.13
Phenialanine (%)	0.66	0.66	0.63	0.63	0.59	0.59
Arginine (%)	0.75	0.75	0.69	0.69	0.63	0.63
Histidine (%)	0.32	0.32	0.30	0.30	0.28	0.28
Isoleucine (%)	0.54	0.54	0.51	0.51	0.48	0.48
Leucine (%)	1.36	1.36	1.32	1.32	1.28	1.28
Valine (%)	0.68	0.68	0.64	0.64	0.60	0.60
Methionine+Cysteine (%)	0.48	0.48	0.45	0.45	0.42	0.42
Calcium (%)	0.62	0.62	0.62	0.62	0.62	0.62
Phosphorus (%)	0.33	0.33	0.33	0.33	0.33	0.33
Nutrient composition (evaluated in laboratory)						
Crude Protein (%)	15.12	15.16	14.24	14.32	13.57	13.52
Calcium (%)	0.71	0.72	0.75	0.73	0.70	0.73
Phosphorus (%)	0.36	0.37	0.38	0.37	0.34	0.39

[†] Biolys, 50.7%; lysine. [¶]T, Treatment. [§]Supplied by kg: 5.0×106 IU vitamin A, 1.0×106 IU vitamin D3, 2.0×104 IU vitamin E; 2 g vitamin K3, 1 g tiamine, 5 g rivoflavin, 2 g pyridoxine, 25 g niacin, 15 g D-calcium panthotenate, 3 g folic acid, 225 g choline chloride, 0.3 g antioxidant, 15 mg vitamin B12 and 180 mg vitamin H-biotin. REKA® Lapisa Animal Nutrition. [Þ]Supplied by kg: 0.2 g Se, 0.1 g Co, 0.3 g I, 10 g Cu, 100 g Zn, 100 g Fe and 100 g Mn. REKA® Lapisa Animal Nutrition.

concentration of this metabolite occurred with the lowest CP level (15.49 %) and the addition of protease ($P \leq 0.05$). In finishing I barrows, the lowest PUN concentration was also detected with the lowest CP concentration (13.63 %) regardless the addition or not of protease ($P \leq 0.05$). Finishing II barrows showed lower PUN ($P \leq 0.05$) with the lowest PC concentration (12.08 %) as in the previous growth stages.

DISCUSSION

The NRC (2012) does not establish a CP value for diets formulation, so, its concentration in the diet is the result of the AA level. The concentrations of lysine, methionine, threonine, tryptophan and other AA were reduced in the diets used in this study, trying to maintain the proportion of AA with respect to lysine (tables 1-3), which reduced the CP level of the diet. However, under the concept of minimum cost formulation of diets for pigs, in economic terms it is more feasible to exceed the recommendation

Table 3. Composition of diets for finishing II barrows (75-100 kg BW) fed low-protein diets supplemented with protease.

Ingredient, %	T1[¶]	T2	T3	T4	T5	T6
Sorghum grain	87.03	86.98	89.10	89.05	91.16	91.11
Soybean meal	10.41	10.42	8.40	8.41	6.39	6.40
Soybean oil	0.45	0.47	0.40	0.42	0.35	0.37
Biolys[†]	0.61	0.61	0.61	0.61	0.61	0.61
DL-Methionine	0.08	0.08	0.07	0.07	0.06	0.06
L-Threonine	0.07	0.07	0.06	0.06	0.06	0.06
Vitamins[§]	0.20	0.20	0.20	0.20	0.20	0.20
Minerals[Þ]	0.15	0.15	0.15	0.15	0.15	0.15
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Calcium carbonate	0.69	0.69	0.70	0.70	0.70	0.70
Phytase	0.01	0.01	0.01	0.01	0.01	0.01
Protease	0.00	0.03	0.00	0.03	0.00	0.03
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutrient composition calculated (SID AA)						
Metabolizable Energy (Mcal kg^{-1})	3.30	3.30	3.30	3.3	3.30	3.3
Crude Protein (%)	13.63	13.63	12.86	12.85	12.08	12.08
Lysine (%)	0.73	0.73	0.68	0.68	0.63	0.63
Threonine (%)	0.46	0.46	0.43	0.43	0.40	0.40
Tryptophan (%)	0.13	0.13	0.12	0.12	0.11	0.11
Phenialanine (%)	0.59	0.59	0.56	0.56	0.53	0.53
Arginine (%)	0.63	0.63	0.57	0.57	0.51	0.51
Histidine (%)	0.28	0.28	0.26	0.26	0.24	0.24
Isoleucine (%)	0.48	0.48	0.45	0.45	0.41	0.41
Leucine (%)	1.29	1.29	1.24	1.24	1.20	1.20
Valine (%)	0.60	0.60	0.56	0.56	0.52	0.52
Methionine + Cysteine (%)	0.42	0.42	0.39	0.39	0.36	0.36
Calcium (%)	0.52	0.52	0.52	0.52	0.52	0.52
Phosphorus (%)	0.32	0.32	0.32	0.32	0.31	0.31
Nutrient composition (evaluated in laboratory)						
Crude Protein (%)	13.43	13.41	12.92	12.85	12.01	12.10
Calcium (%)	0.52	0.54	0.51	0.53	0.52	0.53
Phosphorus (%)	0.37	0.36	0.39	0.33	0.34	0.36

[†] Biolys, 50.7%; lysine. [¶]T, Treatment. [§]Supplied by kg: 5.0×106 IU vitamin A, 1.0×106 IU vitamin D3, 2.0×104 IU vitamin E; 2 g vitamin K3, 1 g tiamine, 5 g rivoflavin, 2 g pyridoxine, 25 g niacin, 15 g D-calcium panthotenate, 3 g folic acid, 225 g choline chloride, 0.3 g antioxidant, 15 mg vitamin B12 and 180 mg vitamin H-biotin. REKA® Lapisa Animal Nutrition. [Þ]Supplied by kg: 0.2 g Se, 0.1 g Co, 0.3 g I, 10 g Cu, 100 g Zn, 100 g Fe and 100 g Mn. REKA® Lapisa Animal Nutrition.

of some AA (NRC 2012) than trying to accomplish the lower (goal) values (Dubeau *et al* 2011). In our study, the reduction of phenylalanine, arginine, isoleucine, leucine and valine was limited because the basal ingredients (sorghum grain-soybean meal) have a high concentration of these AA.

LOW-PROTEIN DIETS

The results indicate that it is feasible to improve or maintain growth performance variables when the amount of AA in barrow diets is reduced, as long as the lysine:AA ratio is maintained. Results of other studies showed that finishing pigs fed LPD, with an unchanged AA ratio per CP unit, had similar and even improved growth variables (Gallo *et al* 2014, Tous *et al* 2014).

In contrast, Qin *et al* (2015), Jiao *et al* (2016) and Peng *et al* (2016) reported that these growth variables were unaffected when CP content was reduced, but the requirement for the more limiting AA (Lys, Met, Trp, Thr) must be reached in the diet. The use of lower levels of AA in our experiment with a performance similar to that of the control protein diet, leads us to determine that

Table 4. Productive performance, carcass characteristics and plasma urea nitrogen concentration of growing barrows (25-50 kg) fed three levels of crude protein and two levels of protease.

% CP	Protease	ADFI (kg d^{-1})	ADG (kg d^{-1})	FGR	BWi (kg)	BWf (kg)	FFLG (kg d^{-1})	LMP (%)	BT (mm)	LMA (cm^2)	PUN (mg dL^{-1})
16.86	–	1.67	0.70	2.44	26.62	49.03	0.251	30.07	6.22^{a}	19.36	21.26^{b}
16.86	+	1.53	0.64	2.42	27.76	47.20	0.236	30.65	5.20^{b}	18.86	27.93^{a}
16.17	–	1.74	0.77	2.26	27.58	51.51	0.287	30.07	5.84^{ab}	19.86	18.73^{bc}
16.17	+	1.79	0.75	2.43	27.27	50.56	0.274	30.17	6.16^{a}	19.77	21.10^{b}
15.49	–	1.87	0.72	2.63	27.75	49.82	0.267	29.97	6.05^{ab}	19.37	12.43^{d}
15.49	+	1.85	0.76	2.44	27.47	50.93	0.279	29.90	5.87^{ab}	19.35	16.54^{c}
SEM		0.07	0.03	0.11	1.30	0.91	0.01	0.39	0.26	0.77	0.80
P value											
% CP × Protease		0.39	0.26	0.26	–	0.26	0.51	0.68	0.04	0.94	0.03
% CP		0.002	0.005	0.22	–	0.005	0.01	0.54	0.48	0.65	0.001
Protease		0.51	0.45	0.87	–	0.452	0.61	0.53	0.18	0.75	0.001

CP= Crude Protein; ADFI= average daily feed intake; ADG= average daily gain; FGR= feed:gain ratio; BWi= initial body weight; BWf= final body weight; FFLG= Fat free lean gain; LMP= lean meat percentage; BT= backfat thickness; LMA= *Longissimus* muscle area; PUN= plasma urea nitrogen concentration, SEM= standard error of the mean. ^{a,b,c,d} Means with different superscript differ ($P \leq 0.05$).

Table 5. Productive performance, carcass characteristics and plasma urea nitrogen concentration of finishing I barrows (50-75 kg) fed three levels of protein and two levels of protease.

% CP	Protease	ADFI (kg d^{-1})	ADG (kg d^{-1})	FGR	BWi (kg)	BWf (kg)	FFLG (kg d^{-1})	LMP (%)	BT (mm)	LMA (cm^2)	PUN (mg dL^{-1})
15.18	–	2.63	0.94	2.81	47.77	76.44	0.311	28.12	10.34	25.74	23.50^{b}
15.18	+	2.57	0.93	2.78	49.08	76.02	0.307	28.33	9.56	25.52	30.66^{a}
14.40	–	2.46	0.93	2.67	51.77	76.03	0.307	28.21	9.64	25.33	22.57^{b}
14.40	+	2.42	0.87	2.83	50.31	74.35	0.290	28.34	10.09	25.59	28.42^{a}
13.63	–	2.67	0.90	3.00	50.33	75.17	0.298	28.34	10.08	25.45	17.45^{c}
13.63	+	2.51	0.89	2.84	50.98	75.07	0.297	28.19	9.74	25.04	17.90^{c}
SEM		0.09	0.04	0.11	2.25	1.20	0.011	0.23	0.25	0.75	0.93
P value											
% CP × Protease		0.52	0.94	0.41	–	0.95	0.94	0.92	0.08	0.93	0.001
% CP		0.19	0.90	0.60	–	0.91	0.90	0.98	0.95	0.94	0.001
Protease		0.43	0.67	0.76	–	0.66	0.67	0.73	0.31	0.96	0.001

CP= Crude Protein; ADFI= average daily feed intake; ADG= average daily gain; FGR= feed:gain ratio; BWi= initial body weight; BWf= final body weight; FFLG= Fat free lean gain; LMP= lean meat percentage; BT= backfat thickness; LMA= *Longissimus* muscle area; PUN= plasma urea nitrogen concentration, SEM= standard error of the mean. ^{a,b,c,d} Means with different superscript differ ($P \leq 0.05$).

the nutritional requirement values coincide with the recommendations for pigs with low genetic potential for lean growth, when comparing CP and lysine concentrations evaluated in our study with the NRC (2012) and Brazilian tables (Rostagno *et al* 2017).

In the present research, the decrease in CP as a result of AA reduction showed beneficial or null effects on the growth performance. Gloaguen *et al* (2014) and González *et al* (2016) reported that a drastic reduction in dietary CP should not be made, because lowering CP by more than three percentage units affected ADG, ADFI, FGR, final BW and FFLG. The negative response to LPD may be attributed to AA deficiency, because CP reduction limits the concentration of AA and the amount of nitrogen necessary for the synthesis of non-essential AA (Gloaguen *et al* 2014).

Consistent with results of our study, Zamora *et al* (2011) and Qin *et al* (2015) confirmed that decreasing CP by less than two percentage units in diets for fattening pigs did not affect carcass characteristics. This may be a result of better balance of AA for protein synthesis and, therefore, AA are not required as an energy source, reflected by similar LMP values with different CP concentrations (Orlando *et al* 2007). However, González *et al* (2016) and Figueroa *et al* (2012) observed that in nursery and finishing pigs, decreasing dietary CP by more than three

Table 6. Productive performance, carcass characteristics and plasma urea nitrogen concentration of finishing II barrows (75-100 kg) fed with three levels of protein and two levels of protease.

% CP	Protease	ADFI (kg d^{-1})	ADG (kg d^{-1})	FGR	BWi (kg)	BWf (kg)	FFLG (kg d^{-1})	LMP (%)	BT (mm)	LMA (cm^2)	PUN (mg dL^{-1})
13.63	–	3.19	0.85	3.87	76.44	102.77	0.321	28.10	16.70	36.83	17.64[a]
13.63	+	3.59	0.96	3.74	76.02	106.32	0.357	28.12	14.52	36.02	19.47[a]
12.86	–	3.16	0.92	3.44	76.03	104.97	0.358	28.41	13.96	36.58	16.69[a]
12.86	+	3.39	0.88	3.91	74.35	103.72	0.336	28.32	15.85	37.30	18.97[a]
12.08	–	3.55	1.01	3.53	75.17	108.00	0.380	28.19	15.18	36.92	13.36[b]
12.08	+	3.51	1.02	3.44	75.07	108.20	0.365	27.69	16.33	36.26	17.51[a]
SEM		0.15	0.04	0.17	1.20	1.38	0.015	0.23	0.07	0.80	0.80
P value											
% CP × Protease		0.22	0.20	0.15	–	0.20	0.14	0.51	0.10	0.57	0.05
% CP		0.22	0.01	0.17	–	0.01	0.05	0.19	0.46	0.80	0.001
Protease		0.10	0.46	0.54	–	0.45	0.97	0.31	0.62	0.69	0.001

CP= Crude Protein; ADFI= average daily feed intake; ADG= average daily gain; FGR= feed:gain ratio; BWi= initial body weight; BWf= final body weight; FFLG= Fat free lean gain; LMP= lean meat percentage; BT= backfat thickness; LMA= *Longissimus* muscle area; PUN= plasma urea nitrogen concentration, SEM= standard error of the mean. [a,b,c,d] Means with different superscript differ ($P \leq 0.05$).

percentage units had a negative effect on BT, LMP and LMA. Reduction in FFLG and increase in BT in pigs fed LPD were attributed to the greater availability of energy for adipose tissue accretion, due to an imbalance between a greater amount of available energy and a deficiency of AA (Gómez *et al* 2002).

PROTEASE

Various reports (Zuo *et al* 2015, Pan *et al* 2016, Yu *et al* 2016) have demonstrated the efficacy of adding protected protease to pig diets, increasing CP digestibility and AA availability, improvements in the plasma concentration of total protein, increased pepsin enzyme activity in the stomach, as well as increased pancreatic amylase and trypsin. The combination of an increase in CP digestibility and lower consumption could reflect improvements in the growth performance of pigs fed protease (Mc Alpine *et al* 2012, O'Shea *et al* 2014). These changes resulted in a better productive performance in both weaning and nursery pigs, which does not have adequate protease production (Zuo *et al* 2015, Greiner *et al* 2016).

Previous research showed that supplementation of LPD with protease did not improve growth performance in fattening pigs (Morales *et al* 2002, Reyna *et al* 2006, Zamora *et al* 2011). Besides, Mc Alpine *et al* (2012) and O'Shea *et al* (2014) observed that the use of protected protease in pig diets could reduce ADG in response to a reduction of ADFI.

This phenomenon could be explained because the protease hydrolyses the protein in the small intestine, which releases components that can be absorbed, and increases the ileal digestibility of protein, increasing the amount of available N from the diet (Mc Alpine *et al* 2012, O'Shea *et al* 2014). Additional nutrients released could trigger a feedback mechanism to reduce feed intake because of a glucostatic and/or aminostatic response that could create nutrient imbalance in the gastrointestinal tract of pig (Nortey *et al* 2007).

PLASMA UREA NITROGEN

Decreasing dietary protein in pigs reduced the concentration of PUN (Qin *et al* 2015, Peng *et al* 2016). This lower PUN concentration is associated with a decrease in metabolic heat production associated with pig metabolism derived from lower amount of total nitrogen and hence, lower synthesis and excretion of urea originated by the AA excess when fed a standard CP diet, indicating better utilization of nitrogen by pigs fed LPD (Qin *et al* 2015). The increase in PUN concentration, which was observed in the present research when protease was added to the diet, may be due to greater digestibility of the CP diet and increased AA availability (Reyna *et al* 2006). The effect of the protease cannot be determined because reduction of protein never decreased growth performance of pigs. Thus, the extra amino acid available from the inclusion of the protease will be catabolized without further effect.

Based on the results of this study, we conclude that the reduction of the concentration of AA in the diet (low CP) improves or maintains growth performance and reduce the plasma urea nitrogen in 25-100 kg hybrid barrows compared to standard CP diets, however, the lysine:AA ratio in the diet must be maintained. The addition of protected protease to pig diets does not show any effect on productive performance, because reduction of protein never decreased growth performance of pigs.

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ORIGINAL ARTICLE

Comparison of yield characteristics of Damascus and Kilis goats in dry climatic conditions

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ABSTRACT. The aim of this study was to compare the reproductive traits, lactation milk yield, and body measurements of the Damascus (Shami) and Kilis goats raised as dairy goats in the dry climatic conditions of the Southeastern Anatolia region of Turkey. The study was perfomed using 596 Damascus goats and 82 Kilis goats between 3-5 years old. It was observed that the lactation milk yield, lactation period, withers height, and leg circumference of the Damascus goats (175.86 kg, 227.48 days, 72.67 cm, and 74.10 cm, respectively) were significantly higher ($P<0.05$) than those of the Kilis goats (107.48 kg, 170.39 days, 69.70 cm and 71.83 cm, respectively). This analysis indicated that the Damascus goat may be a good breed for dry climatic conditions. It is suggested that focusing on growing Damascus goats could increase productivity in the Southeastern Anatolian region of Turkey.

Key words: goat, body measurements, reproductive, lactation traits.

INTRODUCTION

As of 2017, although the total number of goats in Turkey (11,010,590 heads) was close to that of the entire European Union (12,615,362 heads), the annual milk production of Turkey (463,270 tons) is one-quarter of the volume of milk produced in the countries of the European Union (1,931,676 tons) (FAO 2017). The low goat milk production in Turkey is due to an overwhelming majority of its stock, as high as 98%, consisting of the hair goat (TUIK 2017), while only 2% are Cashmere goats (Angora) and dairy goats, such as the Damascus and Kilis goats (Tuncel and Bayındır 1983, Ilgar and Kırcan 2016). This is an indicator of the shortcomings of the milk yield-oriented goat breeding studies carried out in Turkey since the 1960s (Kaymakçı *et al* 2005, Atay 2016).

The Southeastern Anatolia region holds important potential for goat breeding. According to 2016 data, the region provides 22.6% of the goat stock and 24.1% of the goat milk production in Turkey (TUIK 2017).

In Turkey, the Damascus (Keskin 2000) and Kilis goats (Keskin and Tüney 2015) are bred in the Mediterranean and Southeastern Anatolia regions. The most prominent features of the Damascus goats include its adaption to high temperatures and suitability for breeding in plain areas (lowlands); it has an advantage over sheep in terms of its reproductive ability to benefit from arid pastures (Barıtçı and Adıgüzel 2017).

As in all economic activities, the goal of goat breeding is to earn profits. In order to increase profitability, it is necessary to increase fertility. An increase in goat farm production can be achieved by increasing the number of births and the number of kids at birth (Erten and Yılmaz 2013). Fertility is the most important yield trait in continuing the lineage, commencing milk production, and providing stock for meat production (Keskin *et al* 2016).

Reproductive traits are determined by environmental conditions, rather than genotypic factors. Therefore, the progress achieved with genetic selection alone is reduced. On the other hand, following some simple rules while selecting a breeding animal will contribute to an increase in reproductive yields, albeit at a slower pace than that of selection. While selecting breeding animals, giving priority to twin does or bucks and being careful to select animals among the progenies of twin parents, or selecting the breeding animals from families with high reproductive yield will lead to a slightly increased reproductive yield in the next generations (Çam *et al* 2012, Tozlu and Oflaz 2015).

In this study, the reproductive traits of the Damascus and Kilis goats of varying ages were investigated to determine their potential for use in the breeding studies carried out in the important goat-breeding region of Southeastern Anatolia in Turkey.

MATERIAL AND METHODS

The study was carried out on an experimental goat farm (37^0 50' 55'' N 40^0 39' 57'' E) in Bismil. The lowest altitude (546 m), Bismil district, in Diyarbakir province, where the study was carried out, was found to have moderate dry climate conditions during the study period. Diyarbakır is a city in the Southeastern Anatolia region of Turkey with an average temperature of 15.8 °C according to long-term meteorological data (1929-2016) (Anonymous 2017). The animals used in the study consisted of 3 to 5 year-old Damascus (n=596) and Kilis goats (n=82).

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The goats were grouped according to their milk yields and daily feed requirements were determined by taking their milk yields into account. Vetch fodder grown by the establishment was used as roughage and as a concentrate feed. The sheep and goat milk feed, a mixture also prepared by the establishment that contains 17.9% crude protein and 3 Mcal/Kg metabolic energy was comprised of barley, soybean meal, bran, mineral-vitamin premix, ground limestone, and salt (table 1). The mixture was provided twice daily, in the morning and evening. Fresh and clean water was provided for the animals. Milking was performed two times a day (early in the morning and towards the evening). Attention was given to taking an 8-hour break on average between two milking processes. The milking process was carried out with the aid of an automatic milking machine and all the data for milk were recorded in the herd registry system.

The births occurred from February to March. The live weights of the goats and kids after birth were determined using precision scales sensitive to 50 g. The kids were kept with the dams during a 15-day period after birth and then were separated from the dams and transferred to a separated kid pen. Within the pen, the kids were allowed to suckle the dams two times a day until weaning.

The milk records were obtained from the herd management program maintained at the goat farm.

Of the body measurements of interest in the study, withers heights (WH), body length (BL), chest depth (CD), and chest width (CW) were measured using a measurement stick, and chest girth (CG) and leg circumference (LC) were measured using a measuring tape (Cengiz *et al* 1989).

Birth rate, infertility rate, litter size, and single, twin or triplet birth rate, as reproductive features, were all calculated by the method proposed by Kaymakçı and Sonmez (1996).

Statistical evaluation was performed using SAS (2018). Multivariate Anova was used to evaluate the effect of gender, type of birth and age on birth weight. Independent t-test was used to obtain the differences between the goat breeds on birth weight.

Table 1. Composition and nutrient contents of the feed used in the research.

Ingredients	g/kg
Barley	646.9
Soyabean meal	131.0
Wheat bran	187.6
Mineral-vitamin premix	2.5
Ground limestone	24.5
Common salt	7.5
Chemical composition (DM basis)	%
Dry matter	89.0
Crude Protein	17.9
Crude fiber	7.8
Ether extract	2.4
Crude ash	3.9
Calcum	1.1
Phosphorus	0.6
Metabolizable energy (Mcal/kg)	3.0

RESULTS

Table 2 shows the descriptive values for reproductive traits obtained in this study. According to the table, there is no statistical difference in point of reproductive performance between goat breeds.

Table 3 shows the birth weights of the Damascus and Kilis kids. The birth weights of the male kids of both the Damascus and Kilis goats were significantly higher (P<0.05) than those of the female kids. The highest (*P*<0.05) birth weight was observed in single-birth type in Damascus goats, while the lowest birth weight was found in the 5-year age group. In Kilis goats, the highest (*P*<0.05) birth weight was found in single and twin births and in the age group of 3 years.

Table 4 shows the descriptive values for the lactation milk yield and lactation period of the Damascus and Kilis goats. The lactation milk yield of 5-year-old Damascus and Kilis goats and the length lactation period of 5-year-old Kilis goat were lower than the other age groups (*P*<0.05). The lactation milk yield and lactation period of the Damascus and Kilis goats were 175.86 kg and 107.48 kg, and 227.48 days and 170.39 days, respectively. It was found that the average milk yield and lactation period of Damascus goats were higher than Kilis goats (*P*<0.05).

Table 5 shows the descriptive values for the body measurements of the Damascus and Kilis goats As seen in Table 5, WH and LC of the Damascus goats were higher (*P*<0.05) than those of the Kilis goats.

As shown in table 6, in the Damascus goats, there were high correlations (*P*<0.01) between LW and CD (0.693), LW and CG (0.742), LW and LC (0.600), WH and LC (0.517), CW and CG (0.670), CW and LC (0.573), and CG and LC (0.572).

In the Kilis goats (table 7), there were high correlations (*P*<0.01) between LW and BL (0.660), LW and CD (0.566), LW and CW (0.629), LW and CG (0.673), LW and LC (0.597), BL and LC (0.485), CD and CW (0.496), CD and CG (0.707), CW and CG (0.739).

DISCUSSION

Almost all the reproductive traits of the Damascus goats were higher than those of the Kilis goats (table 2). The birth rate of the Damascus goats (99.30%) was similar to that reported by Keskin *et al* (2016) in Damascus goats, while the birth rate of the Kilis goats (93.90%) was higher than that reported in the same literature for the Kilis x Hair goat crossbreeds. The multiple birth rates of the Damascus and Kilis goats were lower (Gül *et al* 2010, Keskin *et al*

Table 2. The descriptive values for the reproductive traits of the Damascus and Kilis goats.

	Damascus		Kilis	
	n	%	n	%
Infertility rate	28	4.70	–	–
Gestation rate	568	95.30	82	100.00
Number of birth	564	99.30	77	93.90
Single kidding	303	53.35	47	57.32
Twin kidding	237	41.73	29	35.37
Triplet kidding	23	4.08	1	1.22
Quadruplet kidding	1	0.18	–	–
Livebirth	850		108	
Abortion	9	1.58	1	1.30
Stillbirth	4	0.70	4	4.88
Litter size		150.70		140.00

Table 3. The birth weights (kg) of the Damascus and Kilis kids.

		Damascus		Kilis	
		n	$\bar{X} \pm S_x$	n	$\bar{X} \pm S_x$
Sex	Female	423	3.50±0.03b	44	3.44±0.10b
	Male	427	3.89±0.04a	64	3.78±0.08a
Birth type	Single	303	3.91±0.04a	47	3.88±0.10a
	Twin	476	3.61±0.03b	58	3.49±0.07ab
	Triplet	69	3.37±0.08c	3	3.07±0.75b
	Quadruplet	4	3.67±0.24b	–	–
Age	3	253	3.89±0.22a	16	3.90±0.45a
	4	345	3.91±0.16a	68	3.42±0.13b
	5	252	3.64±0.22b	24	3.49±0.31b
Total		850	3.69±0.02A	108	3.64±0.66A

$\bar{X} \pm S_x$: mean ± standart error.
a, b, c: Different lower cases in the same column represent statistically significant differences (P<0.05).
A, B: Different capital letters in the same row represent statistically significant differences (P<0.05).

Table 4. The lactation milk yields (kg) and lactation period (day) of the Damascus and Kilis goats.

	Age	Damascus		Kilis	
		n	$\bar{X} \pm S_x$	n	$\bar{X} \pm S_x$
Lactation milk yield	3	224	176.26±24.49a	17	109.40±35.31a
	4	248	180.60±35.17a	49	112.46±59.58a
	5	124	165.68±27.29b	16	90.60±27.71b
Total		596	175.86±29.23A	82	107.48±50.04B
Lactation period	3	224	229.45±31.70a	17	174.78±47.57a
	4	248	225.76±39.80a	49	173.98±38.48a
	5	124	227.38±29.81a	16	155.23±20.83b
Total		596	227.48±33.57A	82	170.39±37.32B

a, b: Different lower cases in the same lines represent statistically significant differences (P<0.05).
A, B: Different capital letters in the same row represent statistically significant differences (P<0.05).

Table 5. Body measurements of the Damascus and Kilis goats.

	Age	Damascus		Kilis	
		n	$\bar{X} \pm S_x$	n	$\bar{X} \pm S_x$
Withers height (cm)	3	224	71.89±4.31	17	68.75±1.75
	4	248	73.30±2.68	49	70.06±3.43
	5	124	72.80±3.42	16	69.58±2.13
Total		596	72.67±3.42a	82	69.70±2.91b
Body length (cm)	3	224	74.89±3.63	17	73.58±2.22
	4	248	76.30±2.64	49	74.31±3.02
	5	124	73.10±7.25	16	72.83±2.71
Total		596	75.10±4.24	82	73.87±2.79
Chest depth (cm)	3	224	32.11±1.50	17	31.92±1.50
	4	248	33.45±1.79	49	32.78±1.33
	5	124	34.00±1.00	16	32.67±1.40
Total		596	33.06±1.68		32.58±1.37
Chest width (cm)	3	224	19.56±1.72	17	19.50±1.95
	4	248	21.35±2.04	49	20.56±1.97
	5	124	20.50±0.71	16	20.83±2.42
Total		596	20.50±1.85	82	20.40±2.04
Chest girth (cm)	3	224	90.44±5.13	17	89.92±5.99
	4	248	93.90±4.42	49	93.42±4.88
	5	124	95.10±3.01	16	93.00±4.87
Total		596	92.85±4.71	82	92.63±5.12
Leg circumference (cm)	3	224	73.06±2.74	17	70.50±1.67
	4	248	74.80±1.55	49	72.58±2.29
	5	124	74.60±1.82	16	70.92±3.41
Total		596	74.10±2.20a	82	71.83±2.54b

a, b: Different lower cases in the same lines represent statistically significant differences (P<0.05).

Table 6. The relationships between the live weight and body measurements of the Damascus goats.

	LW	WH	BL	CD	CW	CG
WH	0.447*					
BL	0.045	0.415*				
CD	0.693**	0.508*	0.227			
CW	0.511*	0.294	0.185	0.537*		
CG	0.742**	0.448*	0.055	0.547*	0.670**	
LC	0.600**	0.517**	0.195	0.441*	0.573**	0.572**

*: P<0.05. **: P<0.01.
LW: Live Weight; WH: Withers Height; BL: Body Length; CD: Chest Depth; CW: Chest Width; CG: Chest Girth; LC: Leg Circumference.

Table 7. The relationships between the live weight and body measurements of the Kilis goats.

	LW	WH	BL	CD	CW	CG
WH	0.213					
BL	0.660**	0.216				
CD	0.566**	0.186	0.235			
CW	0.629**	–0.120	0.279	0.496**		
CG	0.673**	–0.042	0.305	0.707**	0.739**	
LC	0.597**	0.424*	0.485**	0.323	0.421*	0.399*

*: P<0.05. **: P<0.01.

2016) or higher (Keskin and Biçer 1997, Keskin 2000) than those reported in some studies of the Damascus and Kilis x Hair goat crossbreeds. In this study, the litter sizes of Damascus and Kilis goats (150.7% and 140.3%) were lower than those reported by Keskin *et al* (2016) in Damascus goats and Kilis x Hair goat crossbreeds (180% and 162.5%), but higher than those reported by Kutlu (1990) in Akkeçi x Kilis and Saanen x Kilis goat crossbreeds (124.2% and 132.9%). The Damascus goat is recommended as a fertile goat breed in view of its good adaptability (Barıtçı and Adıgüzel 2017); the favorable results obtained in this study and also previous studies show that it has a higher litter size than that of the Nubian, Pygmy, American Alpine, French Alpine, Saanen, and Toggenburg goats (Amoah *et al* 1996), which are among the most fertile goats in the world.

A comparison of birth weights of the Damascus and Kilis kids (table 3), in terms of their sex and birth types, revealed that the birth weights of the male kids were significantly higher ($P<0.05$) than those of the female kids, and the birth weights of the kids from single births were slightly higher than those of the kids from multiple births. This result is compatible with those reported in other literature (Şimşek *et al* 2007, Akbaş *et al* 2013, Keskin *et al* 2016). The mean birth weights of the Damascus and Kilis goats were higher than those of the Saanen X Hair goat F_1 and G_1 crossbreeds (Şimşek *et al* 2007), Saanen (Akbaş *et al* 2013), Damascus, and Kilis x Hair goat crossbreeds (Keskin *et al* 2016). A comparison of birth weights with respect to the ages of the dams showed that the birth weights of kids of 3- and 4-year-old dams in Damascus and the birth weights of kids of 3-year-old dams in Kilis goats were slightly higher than kids of the dams at other ages.

The data showed (table 4) that the lactation milk yield and lactation period of the Damascus and Kilis goats were lower than those reported by Keskin (2000), Keskin *et al* (2004), Özuyanık (2004), Kaymakçı *et al* (2005) and Keskin *et al* (2016) in Damascus goats.

Table 5 shows the descriptive values for the body measurements of the Damascus and Kilis goats. The average WH of the Damascus and Kilis goats were higher than those reported by Aktepe (2009) in Kilis goats and by Bingöl *et al* (2011) in Norduz goats, but similar to those reported by Barıtçı and Adıgüzel (2017) in Damascus and by Ünalan and Ceyhan (2017) in Kilis goats. The average BL of the Damascus and Kilis goats (75.10 cm and 73.87 cm, respectively) were higher than those of Kilis goats (Aktepe, 2009, Alızadehasl and Ünal 2011, Ünalan and Ceyhan 2017), and similar to those of Damascus goats (Barıtçı and Adıgüzel 2017). The average CD of the Damascus and Kilis goats (33.06 cm and 32.58 cm, respectively) were higher than those reported by Aktepe (2009), Alızadehasl and Ünal (2011), and Ünalan and Ceyhan (2017) in Kilis goats, by Bingöl *et al* (2011) in Norduz goats, and by Karakuş (2016) in Hair goats and Saanen goats. The average CW of the Damascus and Kilis goats (20.50 cm and 20.40 cm, respectively) were higher than those reported by Karakuş (2016) in Hair goats and Saanen goats and by Barıtçı and Adıgüzel (2017) in Damascus goats, but similar to those reported by Bingöl *et al* (2011) and Ünalan and Ceyhan (2017) in Kilis goats. The LC of the Damascus and Kilis goats (74.10 cm and 71.83 cm, respectively) were higher than those of the Norduz goats (Bingöl *et al* 2011).

As seen in Table 6, in the Damascus goats, there were high correlations ($P<0.01$) between LW and CD (0.693), LW and CG (0.742), and LW and LC (0.600), which agree with results from the studies that reported high correlations ($P<0.01$) between the live weights and body measurements of the Sanen, Kilis, and Hair goats (Pesmen and Yardimci 2008, Çam *et al* 2012, Alızadehasl and Ünal 2011).

As shown in table 7, the high and significant correlation ($P<0.01$) between CW and CG, CD and CG, and CW and CG in the Kilis goats agrees with results reported in the scientific literature (Cam *et al* 2010, Sarıyel 2013, Karakuş 2016).

It is concluded that the litter size, lactation milk yield, lactation period, WH and, LC of the Damascus goats were significantly higher than those of the Kilis goats. This analysis indicates that the Damascus goat may be better than Kilis goat breed for the dry climate of the important goat-breeding region of the Southeastern Anatolia region in Turkey. Based on the findings in that particular region of Turkey, it may be beneficial to compare the raising of Damascus goats in other parts of Turkey, or the world, with similar climates.

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ORIGINAL ARTICLE

The effect of ginger on testis of Broiler breeders

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ABSTRACT. The rhizome of ginger is a fresh or dried organ of the plant *Zingiber officinale*, which has been used as a medicine since ancient times. The present study was conducted to examine the effects of ginger on testicular histomorphometry in roosters of broiler chicken flocks. Eighty single-day rooster of broiler chickens belonging to 308 Ross breed was used in the present study. The chickens were divided into two groups with 40 broiler roosters (experimental and control groups). Initially, the rhizome of ginger was powdered; 1 g/kg of ginger powder was added to the ration of the group treated with ginger from the beginning of the breeding season. The blood samples were taken from each chick at 20 weeks of age. The samples were stained with H&E. Data was expressed as mean ± SD. T-test was used to analyse and compare the difference between the control is also an experimental groups using SPSS 9.0 software. The testosterone level, weight of the testicle, thickness of seminal tube and number of spermatids in the experimental group compared to the control group increased significantly $P<0.05$. The most important difference between the control and the intervention group treated with ginger was the number and density of spermatids and spermatozoids cells in the lumen area of the seminal tubes. The present study obtained positive results for the efficacy of using ginger in roosters of broiler chick flocks.

Key words: ginger, histomorphometry, roosters, testis, testosterone.

INTRODUCTION

Promoting fertility indices is of particular importance in poultry industry, and there are always numerous studies being conducted on the principles of management and nutrition in order to achieve the highest quality. Nowadays, extensive research is being conducted on using medicinal plants in the field of sexual disability treatment; according to books on traditional medication, it is possible to increase fertility and eliminate such issues as hormonal imbalance, sexual disability (sexual dysfunction), low sperm count, sperm motility, prostate inflammation, and varicocele through using medicinal plants (Ebisch *et al* 2007). The rhizome of ginger is a fresh or dried organ of the plant *Zingiber officinale*, which has been used as a medicine since ancient times. This plant populates a large area, from East Asia to tropical Australia. The most important compounds of ginger are shogoals, gingerols, ginkels, jranil, gingerols, pyrogallols, and zinjiburon (Bhattaria *et al* 2001 and Gupta *et al* 2001). Regarding the anti-inflammatory effects of this plant, numerous reports have indicated that the active compounds of this plant, namely gingerols, shogoals and curcumin, have the potential to inhibit the production of prostaglandins, nitric oxide and even the interleukins involved in inflammation. The ginger and its compounds have been shown to have anti-vomiting, anti-thrombosis, liver, and anti-inflammatory properties. It also has antioxidant and androgenic activities. Due to the antioxidant nature of ginger, it has a desirable effect on sperm incompatibility treatment and poor sperm function (Garolla *et al* (2005). Additionally, and more specifically, the enzymes producing these substances as inflammatory mediators are inhibited by the active ingredients of ginger, which is one of the main components of these compounds and has antioxidant and antibacterial properties. It has been reported that gingerols, shogoals, and zinjiburon compounds of ginger have antioxidant properties (Dalia 2010). The effects of ginger on the body include the removal of free radicals (Altman and Marcussen 2001), spermatogenesis (Mir heydar *et al* 1996) and increasing sexual desire (Zargari 1999). It has also been reported that testosterone is needed to maintain the spermatogonia and inhibit sperm cell apoptosis[1]. Research has shown that ginger has beneficial compounds that are of great help in sperm production. Therefore, lack of these compounds can lead to malformation of the cell membrane (Sharma *et al* 1996). Research has also shown that in chickens taking 100 mg ginger daily for one month increases sperm parameters, testicular antioxidant enzymes, and testosterone levels (Koracevic *et al* 2001). Therefore, the present study was conducted to examine the effects of ginger on testicular histomorphometry in roosters of broiler breeder.

MATERIAL AND METHODS

Eighty single-day rooster of broiler chickens belonging to 308 Ross breed was used in the present study. To this end, a broiler farm in the private sector located in the city

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of Tabriz was used, a farm which was quite homogenous in terms of geographical and environmental conditions. The room was disinfected after being washed with high pressure water. All the chambers, doors, and windows were covered, and finally the saloon was charged with formaldehyde gas to zero the microbial load of the environment. The temperature of the room was about 32 °C during the first day, decreasing by one degree for every three days until it reached 21 °C at the 27th day of the breeding season, and this degree remained constant for all groups until the end of the breeding season. A mixture of natural daylight and artificial lighting was used to provide the required lighting in the present study. All chickens were fed in the same way in terms of the components of the dietary formula and their dosages (table 1). The diet of the chicks consisted of the same components used in the current approach of the majority of countries in order to achieve the highest possible practicality and use for the present research. The lighting program was regulated at 10-15 lux with 12-17 hours duration per day. The study complies with the notification protocol of the Islamic Republic of Iran Veterinary Office and the changes required by the geographical area of the breeding farms.

Table 1. Control basal - diet fed to broiler breeder rooster (NRC 1994).

Ingredients (%)	Roosters
Maize	66.00
Soybean meal (44% CP)	11.0
Wheat bran	18.37
Vitamin-Mineral mix	0.5
Calcium carbonate	1.5
Oysters shell	0.5
Dicakium phosphate	1.4
DL-methionine	0.08
L-lysine HCL	0.07
Salt	0.2
Sodium bicarbonate	0.15
Natuzyme-P*	0.03
Toxin binder*	0.1
Formycine Cold*	0.1
Nutritional analysis	2700
ME, kcal kg^{-1}	13.5
Crude protein (%)	1.1
Calcium (%)	0.4
Available Phosphorus (%)	0.16
Sodium (%)	0.18
Chloride (%)	0.6
Lysine (%)	0.3
Methionine (%)	3.15
Ether extract (%)	4.2
Fibre (%)	1.80
Linoleic acid (%)	

*Natuzyme-p, containing phytase: 1,000,000 U/g. ß glucanse: 700U/g, α-amylase: 700U/g, cellulose: 6000U/g, pectinase: 700U/g, xylanse: 10.000U/g, lipase:30 U/g, and protease: 3000 U/g, Toxin binder contains natural hydrated sodium calcium aluminium silicats; Formycine Gold is a broad spectrum disinfectat feed additive that includes ammonia, formaldehyde, propionic acid and sodium bentonite. The basal - diet (control) supplied per kg: 3mg of vitamin A and 37 mg of vitamin E.

IMPLEMENTATION OF THE TREATMENT

Initially, the rhizome of ginger was powdered; 1 g/kg of ginger powder (0.1% of the base rations) was added to the ration of the group treated with ginger from the beginning of the breeding season. The total period of ginger treatment was 16 weeks. A total number of 80 single day the chickens (308 Ross Breed) were raised to 20 weeks of age. They were then examined and tested. Blood samples of 1-2 ml were taken from each chicken. The blood samples were then referred to a specialized poultry laboratory for testing.

Blood samples were taken from the vein underneath the wings of the chicks at the end of 20th week before the slaughter to measure the blood testosterone concentration. The serum of blood samples was separated and centrifuged at 1372 g-force for 15 minutes. The separated serum was transferred into 2 mm microtubes by a sampler. Microtubes were immediately transferred to a freezer (20± 2 °C) and kept there until required experiments were carried out. The chickens blood serum samples were tested by ELISA (ELx808^{TM}, Absorbance Reader, BioTek, USA) and serum testosterone levels were measured by RIA kits (Immunotech, Marseille, France).The testicles were fully removed from the body and weighed with a highly sensitive digital scale after abdominal dissection. The testicles were then placed in dishes containing formalin fixer to prepare the tissue removal process. The samples were put in a 10% buffer formalin solution, and 6-µm thick paraffin blocks were stained with the general method of hematoxylin and Eosin (H&E) stain after passing the autotechnicom steps (Bancroft and Gamble 2008). Histomorphometry variables of the weight of the testicle, thickness of seminal tube and the number of spermatids with the same cross-section were measured underneath a Nikon optical microscope made in Japan (Model: E200 Eclipse, Eye lens: 10X - 20mm, Magnification: 8 unit-1500 unit) having a linear gradient and a 40 × lens. Spermatozoid density was comparatively calculated in each slide, which is quite clear in photomicrographs. A Nikon camera microscope manufactured in Japan (5-megapixel color CCD) was then used to prepare the corresponding Konica photomicrograph micrograph, and the obtained results were used for statistical analysis.

STATISTICAL ANALYSIS

Data was expressed as mean ± SD. T-test was used to analyze and compare the difference between the control and treatment groups using SPSS 9.0 (SPSS Inc, Chicago IL, USA).

RESULTS

The obtained mean level of testosterone hormone in the treatment group increased significantly as a result of using ginger in comparison with the control group at the level of $P<0.05$ (table 2). According to table 3, the obtained mean level of testis weight to the body significantly increased with ginger in the treatment group compared with the control group at the level of $P<0.05$ (table 3). The obtained mean level of the thickness of seminal tube significantly increased with ginger in the treatment group compared with the control group at the level of $P<0.05$ (table 3). The obtained mean weight of the testicle significantly increased with ginger in the treatment group compared with the control group at the level of $P<0.05$ (table 3).The obtained mean number of spermatids significantly increased with ginger in the treatment group compared with the control group at the level of $P<0.05$ (table 3). Photomicrographs 1-6 also indicate that the lumen of control group chicks is almost empty of spermatozoids and the number of spermatozoa has significantly increased in the lumen of chicks treated with ginger.

In the observations of testicular tissue at the age of 20 weeks, high density of generic cells was observed in epithelium and lumen space of seminal tubes. The development of blood vessels was more significant at this age and the interstitial spaces of the vessels of the group treated with ginger turned out to be both sharper and thicker in comparison with the control group (figures 1A and 1B). The most important change observed in the testicles of the poultry at the age of 20 weeks was the presence of spherical and spatial nuclear spermatocyte cells in the internal part of the epithelium. Also, spermatozoids were observed in the luminal space of seminal tubes. Unlike mammals, these cells were stretched and bent, and long tails were seen. In chicks with 20 weeks of age, generic cells were observed all through the seminal tube. The most important difference between groups was the number and density of spermatozoids cells in the lumen area of the seminal tubes at treatment group than control group (Figures 1C and 1D). The significance of this difference is presented in table 3.

DISCUSSION

Khaki *et al* (2008) reported that ginger extract increases the level of testosterone and the weight of testicles. The results of Johari *et al* (2009) indicated that the weight of testes increases along with the increase of ginger dose in the test group. The concentration of testosterone in the group treated with ginger increases. Testosterone stimulates the spermatogenesis and divides the FSH by cooperating with the hormone of the spermatogonia cells. Shogaols and gingerols are irritant androgens and have the potential to increase testicular weight and increase testosterone hormone. Morakinyo *et al* (2008) indicated that ginger extract inhibited the production of released radicals and reduced lipid peroxidation significantly by maintaining antioxidant activity of superoxide dismutase enzymes, catalase and glutathione peroxidase. It also increased the number of sperms in comparison with the control group. Shariatzadeh *et al* (2016) reported to compare the levels of testosterone in different groups. Bordbar *et al* (2013) indicated that ginger extract increased the volume of seminal tubes, the number of sperms and the level of testosterone in the infertile mice. Ginger plant seems to stimulate reproduction, following the induction of infertility by the Busulfan drug. Khaki *et al* (2008) reported

Table 2. The comparison of mean biochemical parameter obtained from testis of control group and treatment group receiving ginger* (n=20).

Parameter	Control group	Treatment group
Testosterone level (Ng/ml)	3.644 ± 0.89a	5.930 ± 0.331b

*Values are means ± SD.
Different literals in the same row indicate statistical difference ($P<0.05$)

Table 3. The comparison of mean morphometric parameters obtained from testis of control group and treatment group receiving ginger* (n=20).

Parameteres	Control group	Treatment group
Testicle weight (gr)	230/83 ± 0/413[a]	303/53 ± 33/33[b]
Thickness of seminal tube (μm)	17.74 ± 0.383[a]	21.3 ± 0.263[b]
Number of spermatozoids	123.4 ± 11.07[a]	158.2 ± 4.24[b]

*Values are means ± SD.
Different literals in the same row indicate statistical difference ($P<0.05$).

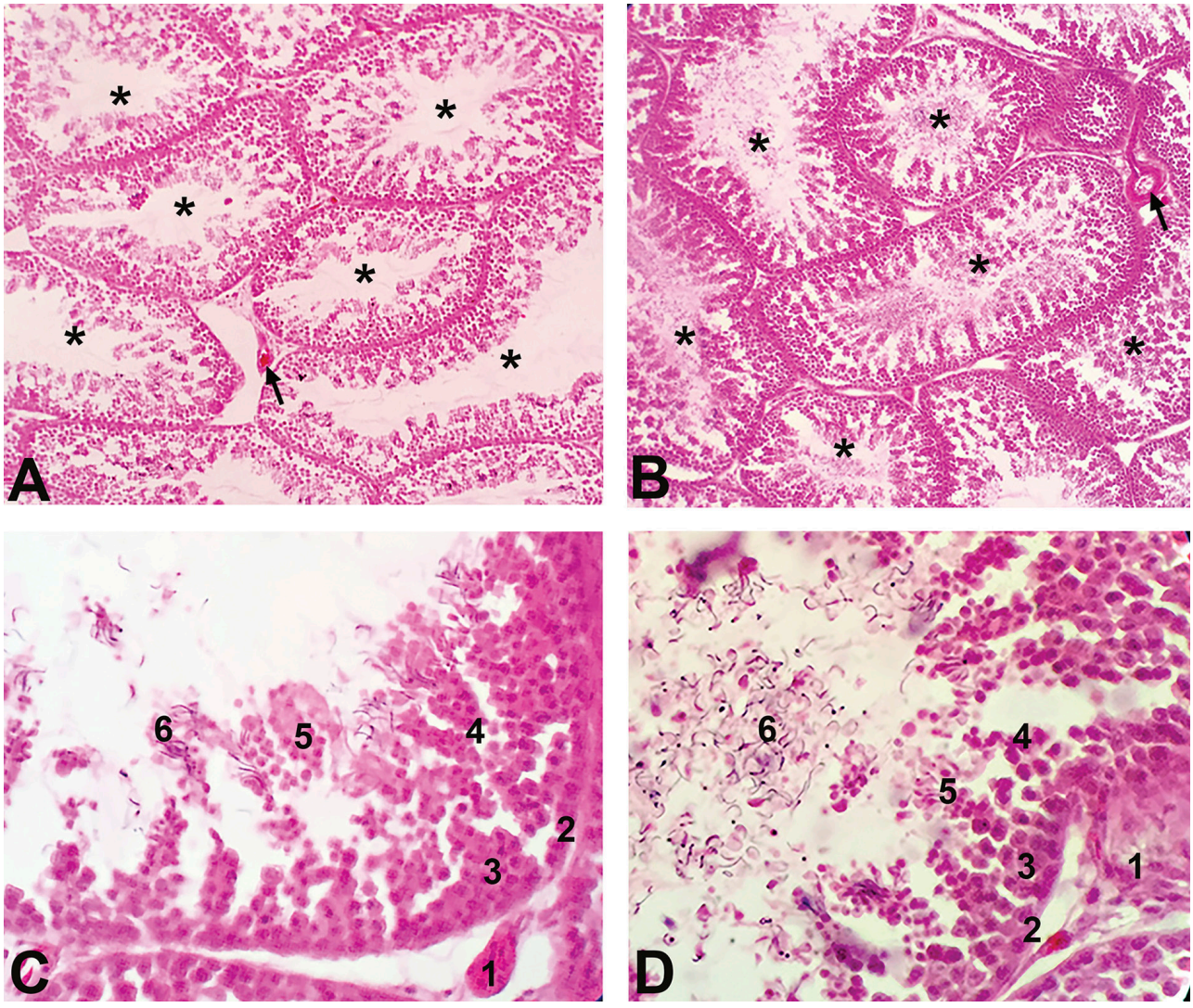

Figure 1. Histological structure of the testis of broiler chiken. A) Normal group B) Treatment group. Seminiferous tubule (star), Capillary (arrow) (H&E sainting, 10×). C) Normal group D) Treatment group. 1: Leydig cell; 2: spermatogonium; 3: primary spermatocyte; 4: secondry spermatocyte; 5: spermatid; 6: spermatozoid (H&E sainting, 100×).

that using 100 mg of ginger brings about a significant increase in the viability of sperm. Khani *et al* (2012) argued that using ginger in the ration of poultry increases the serum levels of superoxide dismutase enzymes, catalase, and glutathione peroxidase, which are considered to be important antioxidant enzymes. There was a significant difference, indicating the efficacy of ginger on testicular tissue, between the control and treatment groups of the present study in terms of the number of spermatids and spermatozoids at 20 weeks of age. According to the results of the testosterone test, there was a significant difference between the control and treatment groups in terms of blood contents, indicating the efficacy of ginger in increasing the level of testosterone. The findings of the present study are consistent with the results of Khaki *et al* (2008), Bordbar *et al* (2013), Shariatzadeh *et al* (2016) and Johari *et al* (2009). Also, the results of this study showed an increase in testicular weight, which is consistent with the results of Johari *et al* (2009) and Khaki *et al* (2008). There was also a significant difference between the two groups in terms of the number of spermatozoa cells. This points to the effect of ginger on increasing testosterone and spermatogenesis in testicular tissue. There was also a significant difference between the two groups in the present study in terms of the number of spermatozoids in the lumen space. The number of spermatozoids is related to the spermatogenesis process, which is also influenced by the testosterone hormone. It can be concluded that the effects of ginger include increased testosterone, which accelerates and activates the process of spermatogenesis in the testes, which is consistent with the results of Morakinyo *et al* (2008) and Bordbar *et al* (2013). Increased diameter of the vessels in the testicular interstitial space in the ginger group is one of the main differences between

the two groups in terms of changes in the structure of the tissue. Increased diameter of the vessels increases and accelerates blood flow and the level of testosterone hormone, which results in improved spermatogenesis in the testicle and, finally, increased fertility.

The positive results using ginger in roosters of broiler chick flocks found in the present study can have significant value in the economic cycle of poultry industry, including agricultural industry, veterinary administration, colleges, meat industry, and industries related to the processing of meat products, since fertility is one of the most important factors in poultry industry and huge costs are paid annually to improve the quality and quantity of fertility.

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ORIGINAL ARTICLE

The effect of non-traditional cooling on dog sperm cryosurvival and ability to perform the acrosome reaction

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ABSTRACT. The objective of this study was to assess cryosurvival, plasma membrane fluidity, and capability of cryopreserved dog (*Canis lupus familiaris*) spermatozoa, cooled to –5 °C before freezing, to perform the acrosome reaction under the effect of progesterone and calcium ionophore. In the first experiment, fresh spermatozoa diluted in Tyrode's medium plus albumin, lactate, and pyruvate (TALP) were incubated at 38 °C in 5% CO_2 in air, with progesterone or calcium ionophore added at 2, 4, and 6 h after incubation and sampled 30 min later to assess the acrosome reaction. In the second experiment, diluted sperm were packaged in plastic straws, cooled to either +5 °C or –5 °C and cryopreserved. Progressive motility, plasma membrane integrity and fluidity, capacitation status and acrosome integrity were assessed before and after freeze-thawing. After thawing, sperm were assessed, resuspended in TALP and incubated to assess the acrosome reaction. Parameters for sperm cryosurvival were similar in sperm cooled to either +5 °C or –5 °C, except in the percentage of hyper-fluid membranes which was lower ($P<0.05$) in sperm cooled to –5 °C. There were no differences in the percentages of frozen-thawed spermatozoa with acrosome reaction, induced by progesterone or calcium ionophore, between cooling treatments. In conclusion, cooling of dog spermatozoa to –5 °C did not improve sperm cryosurvival but had a positive effect on plasma membrane fluidity.

Key words: dog semen, freeze-thawing, progesterone, calcium ionophore, membrane fluidity.

INTRODUCTION

Sperm cryopreservation results in different advantages in dog breeding, such as the movement of genetic material among different geographic regions, thus increasing genetic variability and the use of the best males (Eilts 2005). However, cryopreservation invariably reduces the fertilising capacity of spermatozoa (Watson 1995), therefore, the research aiming to elucidate the mechanisms responsible for damage to sperm physiology caused by low temperatures is of great importance (Eilts 2005). A phenomenon that sperm have to face during cryopreservation is the phase transition of lipids that comprise the plasma membrane. As the temperature decreases, the lipids progressively change from a liquid-crystalline phase to a gel phase. Consequently the membrane loses elasticity (Watson 1995). Most lipids undergo this change at temperatures above zero degrees, however, there is evidence that some additional phase transitions could occur below zero degrees (Crowe *et al* 1989). The lipid composition of the plasma membrane (cholesterol and unsaturated fatty acids) is directly related to the motility of the sperm and the fluidity of the membrane; the presence of unsaturated fatty acids gives the membrane greater fluidity. Lucio *et al* (2017) have reported the presence of both saturated (palmitic and stearic) and unsaturated fatty acids (arachidonic and oleic) in dog spermatozoa.

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In addition, freezing affects the physical states of membrane lipids due to changes in the hydration level. Membrane phase transition may cause different effects on the membrane conformational disorder in the frozen state when the ice nucleation occurs at different sub-zero temperatures (Balasubramanian *et al* 2009).

Thus, spermatozoa are subjected to volume changes (i.e., intracellular water flow for ice formation), while their plasma membrane is suffering conformational rearrangements –changes in fluidity– due to lipid phase transitions. One approach that could assist the sperm plasma membrane to manage those stressors in order to maintain its viability is to extend the cooling of spermatozoa to sub-zero temperatures (–5 °C) before freezing. In this way, the occurrence of severe changes in plasma membrane fluidity could be reduced. Some researchers have been exploring the effect of cooling to sub-zero temperatures on sperm cryosurvival (Garzon-Perez *et al* 2010, Contreras-Mendez and Medrano 2016, Alcantar-Rodriguez and Medrano 2017). However, they have obtained either positive or null effects on sperm cryosurvival.

Assessment of the sperm fertilising capacity by measuring the ability of the cell to suffer the acrosome reaction is considered a valuable test in fresh and cryopreserved spermatozoa (Graham and Foote 1987, Whitfield and Parkinson 1992). The objective of this work was to test further the effect of cooling to –5 °C before freezing on dog sperm cryosurvival, plasma membrane fluidity and the sperm capability to carry out the acrosome reaction, comparatively employing 2 inductors: progesterone (P_4) and calcium ionophore (CI). In this work, 2 cooling protocols were compared by inducing the acrosome reaction on cryopreserved dog spermatozoa as a measure of the *in vitro* fertilising capacity of spermatozoa.

MATERIAL AND METHODS

All experiments comply with the guidelines of the Institutional Subcommittee for the Care of Animals in Experimentation from the National Autonomous University of Mexico (Faculty of Superior Studies, Cuautitlan). Semen assessment and cryopreservation were carried out in the laboratory of Animal Reproduction (UIM-FESC), National Autonomous University of Mexico.

SEMEN COLLECTION AND PROCESSING

Semen was collected by manual stimulation from 3 dogs (first experiment, 6 ejaculates per male: 1 Shih-tzu and 2 mixed races, 3 to 5 years of age) and 5 dogs (second experiment, 3 ejaculates per male: 3 Belgian and 2 German Shepherd, 2 to 8 years of age) once a week from the same male, from January to August 2016 (20°N). Only 1 ejaculate from the corresponding dog was collected per each day of work, and each individual ejaculate was treated separately. Only ejaculates showing at least 80% progressive motility and 85% viability were included in these experiments.

Each ejaculate was diluted 1:1 (v/v) in a Tris/citric acid/glucose solution (TRIS 198.2 mM, citric acid 72.9 mM, glucose 8.9 mM, and kanamycin 0.83 mg/ml) to be transported at approximately 28 °C. Semen arrived at the laboratory within 30 min of collection.

SEMEN ASSESSMENT

Immediately after collection, semen was macroscopically assessed for volume, colour, consistency and presence of strange materials (hair, blood). Once in the laboratory, semen in the transport solution was left at room temperature (18 – 23 °C) for 45 min to adjust, then an aliquot was taken and diluted 1:6 (v/v) in phosphate-buffered saline PBS at 34 °C. Ten min later, sperm were microscopically assessed for progressive motility, sperm concentration, viability, sperm morphology, plasma membrane integrity, acrosome integrity, capacitation status and plasma membrane fluidity.

Progressive motility, the percentage of sperm showing progressive and linear movement was subjectively assessed under light microscopy (Leica DMLS) using the 20x objective.

Sperm concentration was estimated from a 1:200 dilution (semen: formaldehyde saline solution v/v) with the aid of the Neubauer chamber using the 40x objective.

Viability was assessed by counting live (non-stained) and dead (stained) spermatozoa (at least 200 hundred) in smears stained with eosin-nigrosine using the 100x objective. Sperm morphology was assessed by counting normal and abnormal (primary and secondary) cells (at least 200 hundred) in smears stained with eosin-nigrosine using the 100x objective (Feldman and Nelson 1996).

Plasma membrane integrity was assessed as follows: 50 µl of diluted spermatozoa (in PBS 1+5 v/v) were added to 5 µl of SYBR14 (100 nmol/L) and mixed. Immediately, 5 µl of propidium iodide (PI, 12 µmol/L) was added and mixed again for 10 s. Finally, 5 µl of glutaraldehyde (0.4%) was added to immobilise the spermatozoa (Garner and Johnson 1995). The percentage of live cells (SYBR14-positive and PI-negative) was calculated after counting 200 spermatozoa under fluorescence microscopy (Leica DMLS) using the 100x objective.

Acrosome integrity was assessed as follows: diluted sperm were smeared on a slide and air-dried, and then cells were permeabilised in alcohol for 60 min. Then, 50 µl of fluorescein-conjugated Pisum sativum agglutinin (PSA-FITC) lectin (L0770 Sigma St. Louis MO, USA) was spread on the slide and left in the dark for 10 min. Immediately, the slide was gently washed with distilled water and air-dried (Medrano *et al* 2009). One drop of an antifade solution (DABCO 220 mM in glycerol, (D-2522 Sigma St. Louis MO USA) was put on the slide, and a coverslip was positioned on top. The percentage of cells showing a smooth and well-defined acrosome was calculated after counting 200 spermatozoa under fluorescence microscopy using the 100x objective.

Capacitation status was assessed by the chlortetracycline (CTC) assay as follows: 100 µl of diluted sperm (in PBS 1+5 v/v) were added to 100 µl of CTC solution (pH 7.8), mixed for 30 s and 20 µl of glutaraldehyde (0.2%) were added to immobilise the sperm (Green and Watson, 2001). CTC-stained sperm were mixed (1:1 v/v) with antifade solution on a slide, and a coverslip was positioned on top. Percentages of cells showing any of the CTC patterns were calculated after counting 200 spermatozoa under fluorescence microscopy using the 100x objective: F, with uniform fluorescence over the whole head (non-capacitated acrosome-intact spermatozoa); B, with fluorescence-free band in the post-acrosomal region (capacitated acrosome-intact spermatozoa); or AR, with almost no fluorescence over the whole head except for a band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa) (Rota *et al* 1999).

To assess sperm plasma membrane fluidity, a Merocyanine 540 assay was carried out as follows: a stock solution of Merocyanine (5 mM) in dimethyl sulphoxide (DMSO, 154938 Sigma St. Louis MO, USA) was prepared and stored at room temperature (23 °C) protected from the light until use. Then, a working solution of Merocyanine (40 µM) in PBS (495 µL PBS + 5 µL Merocyanine (5 mM) in DMSO) was freshly prepared before use. One hundred and forty microlitres of sperm in PBS were added to 10 µL of the Merocyanine working solution, mixed, and left for 1 min to interact. Then, 22 µL of glutaraldehyde (0.4%) were added to fix the sperm. One drop of this mix and 1 drop of antifade solution (DABCO 220 mM in glycerol/PBS) were put on a warm glass slide, and a glass cover slide was positioned on top. Gentle pressure

was applied to the cover slide, with the aid of absorbent paper to eliminate the excess liquid. Percentages of cells showing either of the Merocyanine patterns, opaque (low fluidity) or brilliant (high fluidity - high-binding cells), were calculated after counting 200 spermatozoa under fluorescence microscopy (Leica DMLS) using the 100x objective (Steckler *et al* 2015).

EXPERIMENTAL DESIGN

Experiment 1. Validation of the use of 2 inductors of the acrosome reaction on fresh-incubated dog spermatozoa. This experimental stage was carried out to identify the optimum incubation time for each of the inductors of the acrosome reaction to produce the highest proportion of acrosome-reacted spermatozoa. Fresh spermatozoa, diluted in Tyrode's medium plus albumin, lactate and pyruvate (TALP), were incubated during 6.5 h, employing 2 inductors of the acrosome reaction: CI and progesterone (P4). Semen was collected, diluted and transported as mentioned. Immediately after adjustment at room temperature, diluted semen was centrifuged at 750 g for 5 min, supernatant was removed and TALP medium was added to reach 400 x10^6 sperm/ml. Sperm in TALP were further diluted to reach either 100 x10^6 sperm/ml (for CI experiment) or 75 x10^6 sperm/ml (for P4 experiment). Immediately, sperm suspension was split in aliquots of 200 µl each, which were incubated at 38 °C in 5% CO2 in air, and sampled when required. Either CI (2.5 µM, Sigma-Aldrich, USA) in Tyrode's medium (Szász *et al* 2000) or P4 (10 µg/ml, Sigma-Aldrich, USA) in Tyrode's medium (Cheng *et al* 2005) were added at 2, 4 and 6 h of incubation and left for 30 min to interact before sampling. Progressive motility, plasma membrane integrity, capacitation status and acrosome integrity were assessed at 0, 2, 2.5, 4, 4.5, 6 and 6.5 h of incubation in the control and treated groups (figure 1).

Eighteen ejaculates from 3 dogs (6 from each male; 3 per each AR inductor) were used in this stage.

Experiment 2. Sperm cryopreservation and post-thawing incubation. This experimental stage was carried out to assess both sperm cryosurvival after cooling to 2 pre-freeze temperatures (+5 °C vs. –5 °C) and the capacity of frozen-thawed spermatozoa to perform the acrosome reaction employing calcium ionophore A23187 and progesterone (figure 2). Semen was collected, diluted and transported as previously mentioned. Immediately after adjustment at room temperature, diluted semen was centrifuged at 750 g for 5 min, supernatant was removed and egg yolk-Tris (EYT) medium containing 3% glycerol (Peña and Linde Forsberg 2000) was added to reach 400 x10^6 sperm/ml. Fifteen ejaculates (6 straws: 3 for each cooling temperature) from 5 dogs were used in this stage.

Diluted sperm were slowly cooled from 23 °C to 5 °C in approximately 2 h (0.17 °C/min). Then, EYT medium

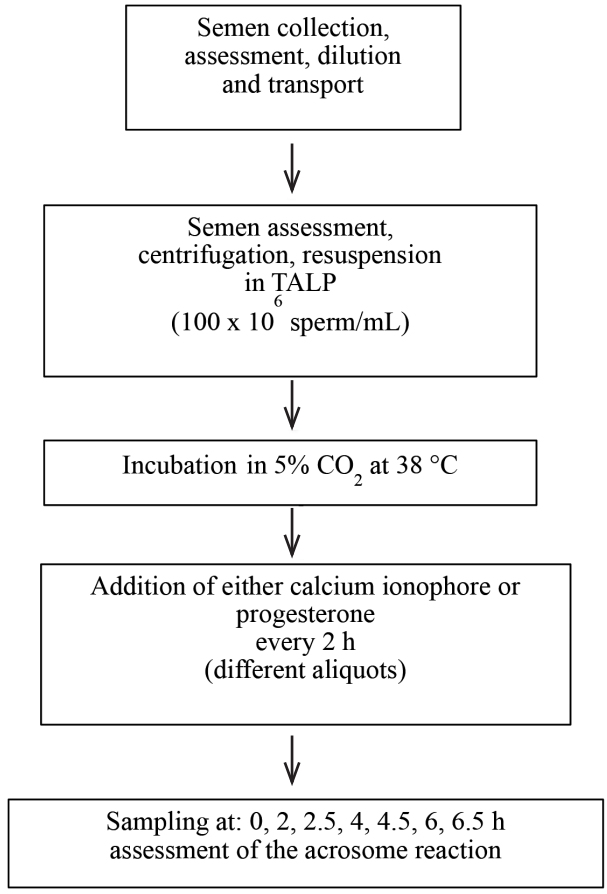

Figure 1. Flow chart of Experiment 1. Validation of the use of 2 inductors of the acrosome reaction on fresh-incubated dog spermatozoa.

containing 7% glycerol was added to reach a final concentration of 200 x10^6 sperm/ml and 5% glycerol, and diluted sperm was packaged into 0.5 ml plastic straws, which remained in equilibrium for 30 min before freezing. Half of the straws at +5 °C (control) were frozen in 4 cm of nitrogen vapour over liquid nitrogen levels for 15 min and stored in liquid nitrogen. The other half at +5 °C were further cooled to –5 °C at approximately 0.04 °C/min (experimental), frozen and stored as mentioned. To cool the straws to sub-zero temperatures, an insulated box filled with crushed saline ice (10% w/v) at –12 °C was used. This method had been previously validated in our laboratory (Alcantar-Rodriguez and Medrano 2017). Temperature was carefully monitored with the aid of a thermocouple (HANNA Instruments, USA) positioned inside a monitor straw containing EYT diluent (5% glycerol). Readouts were stored in a computer using a special software (HANNA Instruments, USA).

Thawing was performed by immersing straws (3 per treatment) in water at 38 °C for 30 s. The content of each straw was poured in dry tubes into the water bath (4.5 l volume). Each straw was assessed separately. Variables

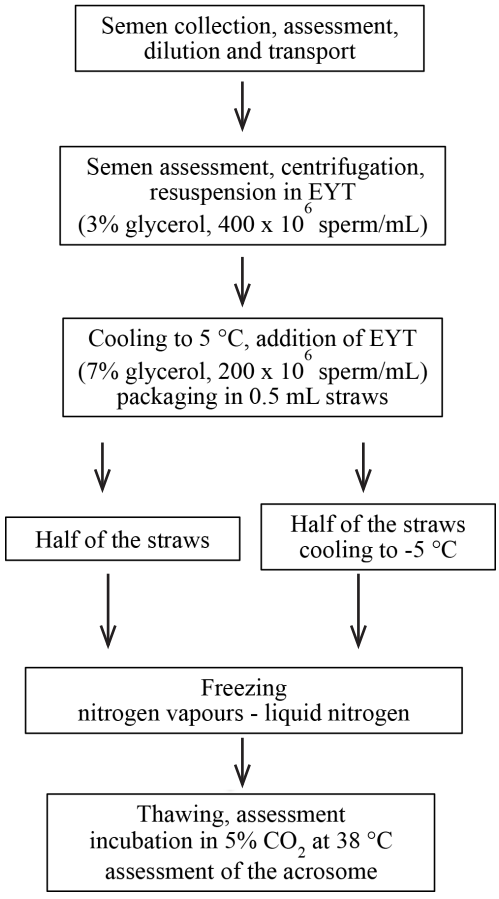

Figure 2. Flow chart of Exeriment 2. Sperm cryopreservation and post-thawing incubation.

were progressive motility, viability, plasma membrane integrity, acrosome integrity, capacitation status, and plasma membrane fluidity.

Induction of the acrosome reaction on cryopreserved dog spermatozoa. Straws (3 per treatment) were thawed, and sperm was assessed as mentioned. Then, diluted sperm from the 3 straws were pooled and centrifuged at 300 g for 4 min, supernatant was removed and the pellet was resuspended in TALP to reach $75x10^6$ sperm/ml. Sperm suspention was split into 5 aliquots of 200 µl each that were incubated at 38 °C in 5% CO_2 in air, and sampled at (i) 0 h – control, (ii) 4 h – control and (iii) 4:30 h – control, CI and P_4. CI and P_4 were added after 4 h of incubation and left for 30 min to interact with the sperm. Then, sperm acrosome and plasma membrane integrity were assessed.

STATISTICAL ANALYSIS

Data from the first experiment (incubation times and the effect of AR inductors) was analysed using the Friedman test of analysis of variance (ANOVA, repeated measures), Wilcoxon matched pair test and the "t" test (Snedecor and Cochran 1989). Data from the second experiment (sperm cryopreservation and post-thawing incubation) was analysed by (i) ANOVA (fresh semen variables), with variables expressed in percentages arcsine-transformed to normalise them, (ii) "t" test (frozen-thawed sperm, cooled before freezing at either +5 or –5 °C) and (iii) Friedman ANOVA (incubation times of frozen-thawed sperm). To compare treatments (Control, CI, and P_4) at 4:30 h of incubation, ANOVA and Tukey tests were used as well as the software SPSS v15.0 (2006, Chicago, USA).CITAR ATRAS

RESULTS

EXPERIMENT 1.VALIDATION OF THE USE OF 2 INDUCTORS OF THE ACROSOME REACTION ON FRESH-INCUBATED DOG SPERMATOZOA

Stage 1. Calcium ionophore A23187 (CI). Sperm motility decreased constantly as incubation progressed. At 2.5 and 6.5 h of incubation, there were no differences between the control and CI, however, at 4.5 h of incubation they were different ($P<0.05$) (figure 3). The number of plasma membrane-intact spermatozoa decreased constantly as incubation progressed; there were no differences between the control and CI at any of the incubation times (figure 3). The number of acrosome-intact spermatozoa decreased as incubation progressed; there were no differences between 4 and 4.5 h as well as between 6 and 6.5 h. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P<0.05$) between the control and CI 3 (figure 3).

The number of non-capacitated, acrosome-intact spermatozoa (CTC, F Pattern) decreased constantly as incubation progressed. At 2.5 and 4.5 h of incubation, there were differences ($P<0.05$) between the control and CI, however, at 6.5 h of incubation, there was no difference (figure 4). Number of capacitated, acrosome-intact spermatozoa (CTC, B Pattern) increased constantly as incubation progressed, reaching top values at 4 and 4.5 h and decreasing at 6.5 h of incubation ($P<0.05$). At 2.5 h of incubation, there was no difference between the control and CI, however, at 4.5 and 6.5 h of incubation, they were different ($P<0.05$) (figure 4). The number of acrosome-reacted spermatozoa (CTC, AR Pattern) increased constantly as incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P<0.05$) between the control and CI (figure 4).

Stage 2. Progesterone (P4). Sperm motility decreased constantly as incubation progressed. At 2.5 and 6.5 h of incubation, and there were no differences between the control and P4, however, at 4.5 h of incubation they were different ($P<0.05$) (figure 5). The number of plasma membrane-intact spermatozoa decreased constantly as incubation progressed; there were no differences between the control and P_4 at any of the incubation times (figure 5). The number of acrosome-intact spermatozoa decreased as

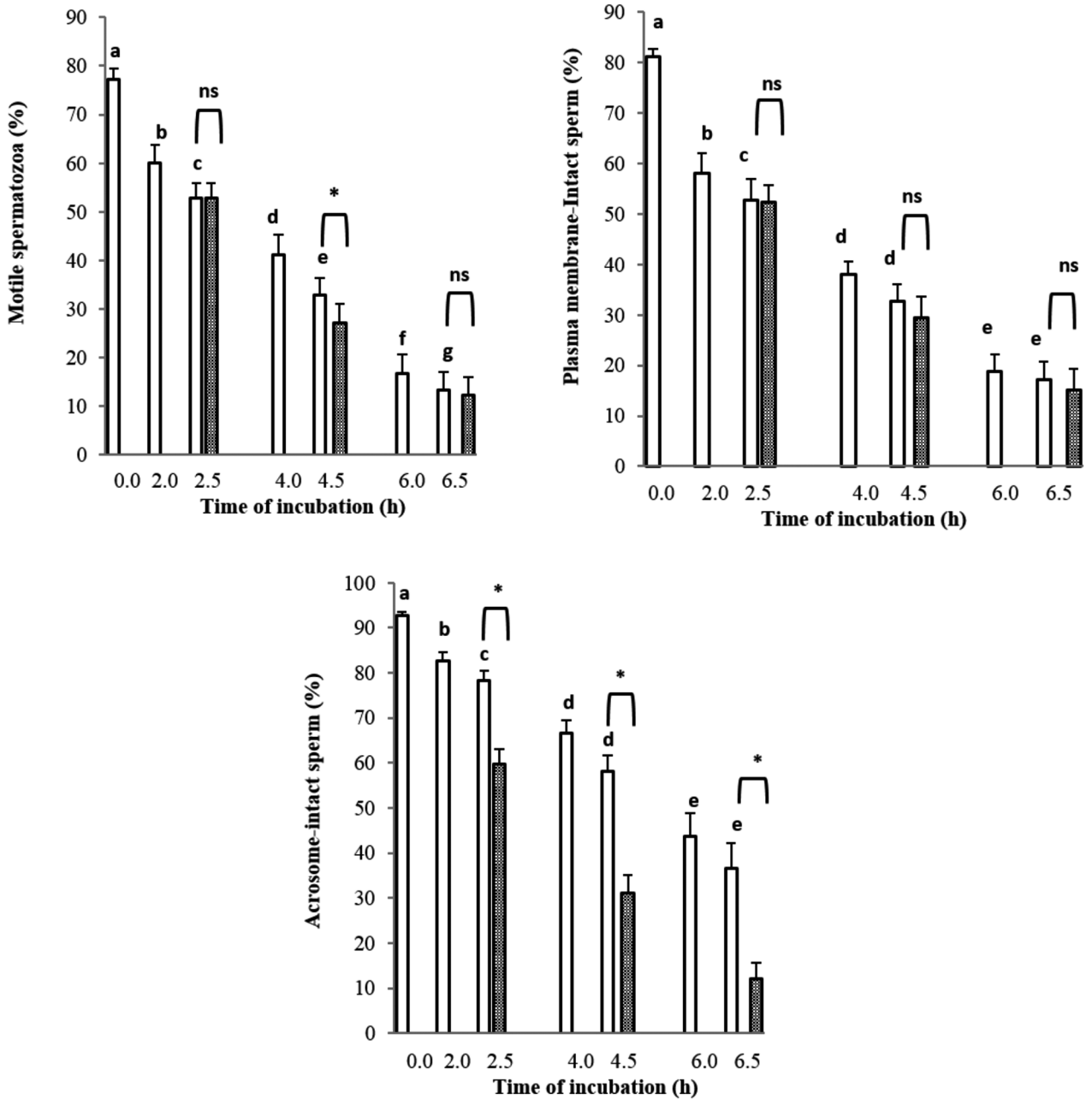

Figure 3. Motile, plasma membrane-intact, and acrosome-intact dog spermatozoa incubated at 37 °C during 6.5 h under the effect of calcium ionophore. Values are means + SEM.

Control: open bars (a-g), calcium ionophore: shadowed bars.

Control vs calcium ionophore: *P<0.05, ns = non significant.

incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences (P<0.05) between the control and P_4 (figure 5).

Number of non-capacitated, acrosome-intact spermatozoa (CTC, F Pattern) decreased constantly as incubation progressed; there were significant differences (P<0.05) between the control and P_4 at 2.5, 4.5 and 6.5 h of incubation (figure 6). The number of capacitated, acrosome-intact spermatozoa (CTC, B Pattern) increased constantly as incubation progressed, reaching top values at 4 and 4.5 h and decreasing at 6.5 h of incubation (P<0.05). At 2.5 h of incubation, there was no difference between the control and P_4; however, at 4.5 and 6.5 h of incubation, they were different (P<0.05) (figure 6). The number of acrosome-reacted spermatozoa (CTC, AR Pattern) increased constantly as incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences (P<0.05) between the control and P_4 (figure 6).

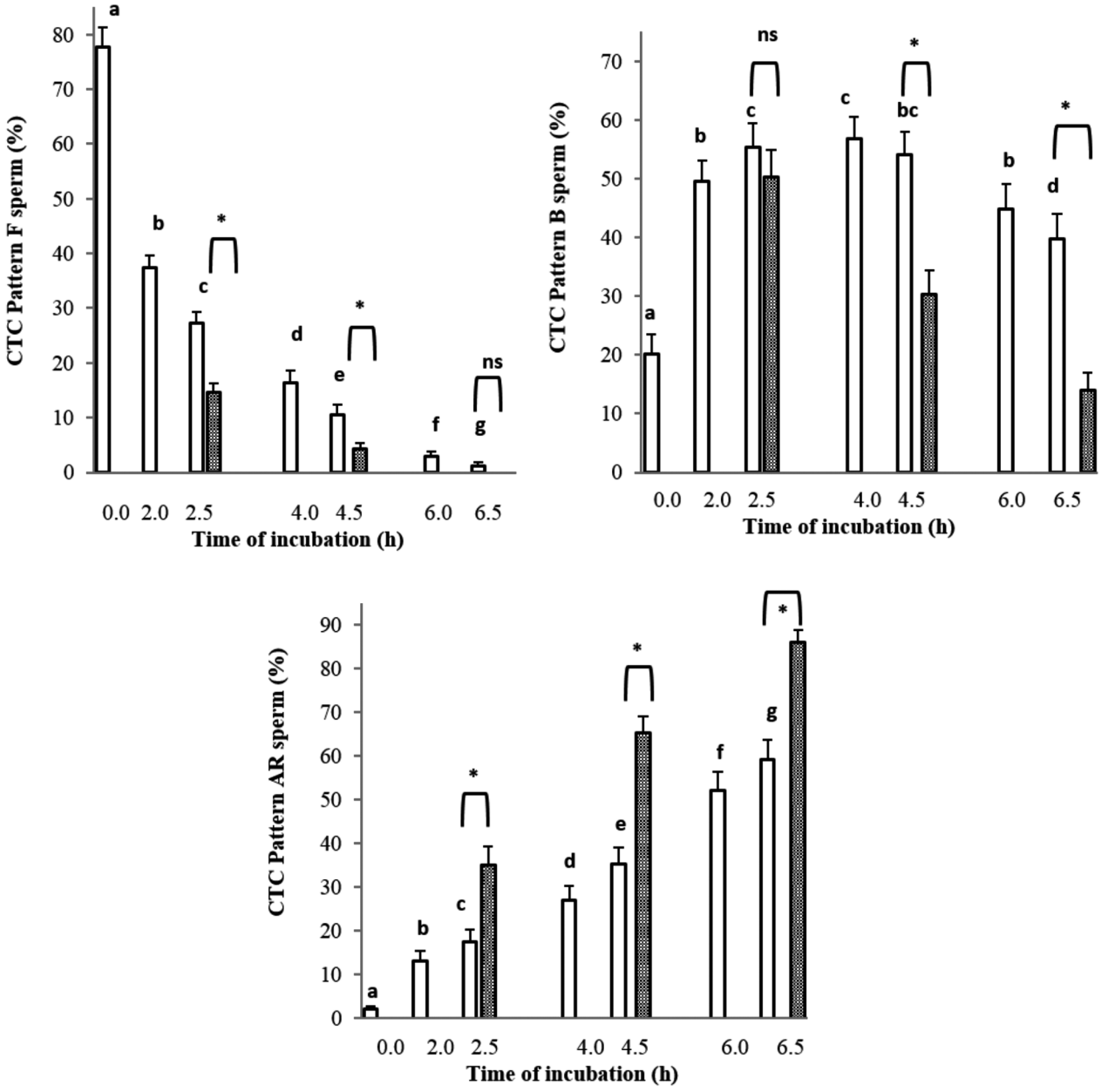

Figure 4. Capacitation status of dog spermatozoa incubated at 37 °C during 6.5 h under the effect of calcium ionophore. Values are means + SEM.

Control: open bars (a-g), calcium ionophore: shadowed bars.

Control vs calcium ionophore: **P*<0.05, ns = non significant.

EXPERIMENT 2. SPERM CRYOPRESERVATION AND POST-THAWING INCUBATION

Stage 1. Sperm cryosurvival. After thawing, there was significant difference (*P*<0.05) in the percentage of Merocyanine high-binding (hyper-fluidity) cells between sperm cooled to either +5 °C (69.5 ± 3.30 Mean ± SEM) or –5 °C (63.8 ± 3.00 Mean ± SEM). There were no differences between cooling temperatures in the other sperm characteristics (table 1).

Stage 2. Induction of the acrosome reaction on cryopreserved dog spermatozoa. The number of acrosome-intact spermatozoa from +5 °C and –5 °C cooling treatments decreased as incubation progressed. At 4.5 h of incubation in each cooling treatment, there were differences between the control and the experimental groups (CI and P_4), but there was no difference between CI and P_4. The number of plasma membrane-intact spermatozoa from +5 °C and –5 °C cooling treatments decreased significantly (*P*<0.05) as incubation progressed. However, at 4.5 h of incubation,

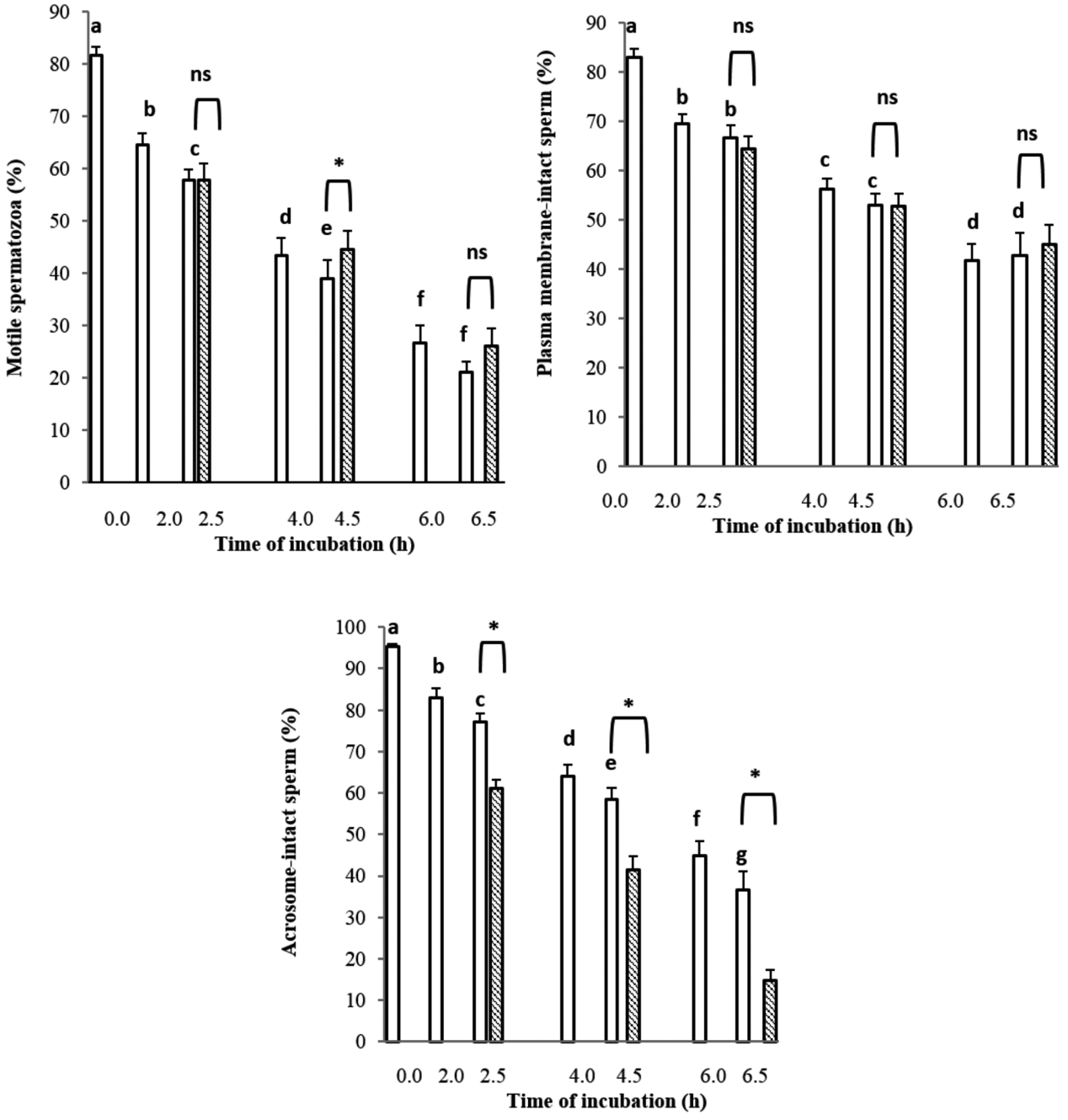

Figure 5. Motile, plasma membrane-intact, and acrosome-intact dog spermatozoa incubated at 38 °C in 5% CO_2 in air during 6.5 h under the effect of Progesterone. Values are means + SEM.

Control: open bars (a-g), Progesterone: shadowed bars.

Control vs Progesterone: *P<0.05, ns = non significant.

there were no differences between the control, CI and P_4 of each cooling treatment. Figure 7 comparatively shows the percentages of acrosome-intact and plasma membrane-intact spermatozoa from +5 °C and –5 °C cooling treatments. There were no differences in any pair of values (+5 °C vs. –5 °C) incubated at the same time with an inductor of the acrosome reaction (CI & P_4) or without (control).

DISCUSSION

This work was conducted to compare the effect of 2 pre-freeze cooling temperatures, +5 °C vs. 5 °C, on the *in vitro* fertilising capacity of cryopreserved dog spermatozoa assessed by the sperm capacity to perform the acrosome reaction, CI and P_4. In the first experiment, the induction

Table 1. Effect of cooling to +5 °C and –5 °C before freezing on dog sperm cryosurvival.

Cooling target temperature	Progressive motility (%)	MC540 high-binding cells (%)	Acrosome integrity (%)	Capacitation status (CTC patters %)			Plasma membrane integrity (%)
				F	B	AR	
+5 °C	30.0 ± 3.09	69.5 ± 3.30[a]	80.9 ± 0.99	11.4 ± 1.21	73.4 ± 1.29	15.2 ± 0.82	34.0 ± 2.68
–5 °C	28.7 ± 2.15	63.8 ± 3.00[b]	82.3 ± 0.95	12.5 ± 0.90	73.4 ± 1.00	14.1 ± 0.91	36.3 ± 2.79

Values are Means ± SEM. Different letters in columns indicate significant differences ($p<0.05$). Progressive motility, visual; plasma membrane fluidity, merocyanine (MC540); acrosome integrity, PSA-FITC; capacitation status, CTC assay; plasma membrane integrity, SYBR14/PI.

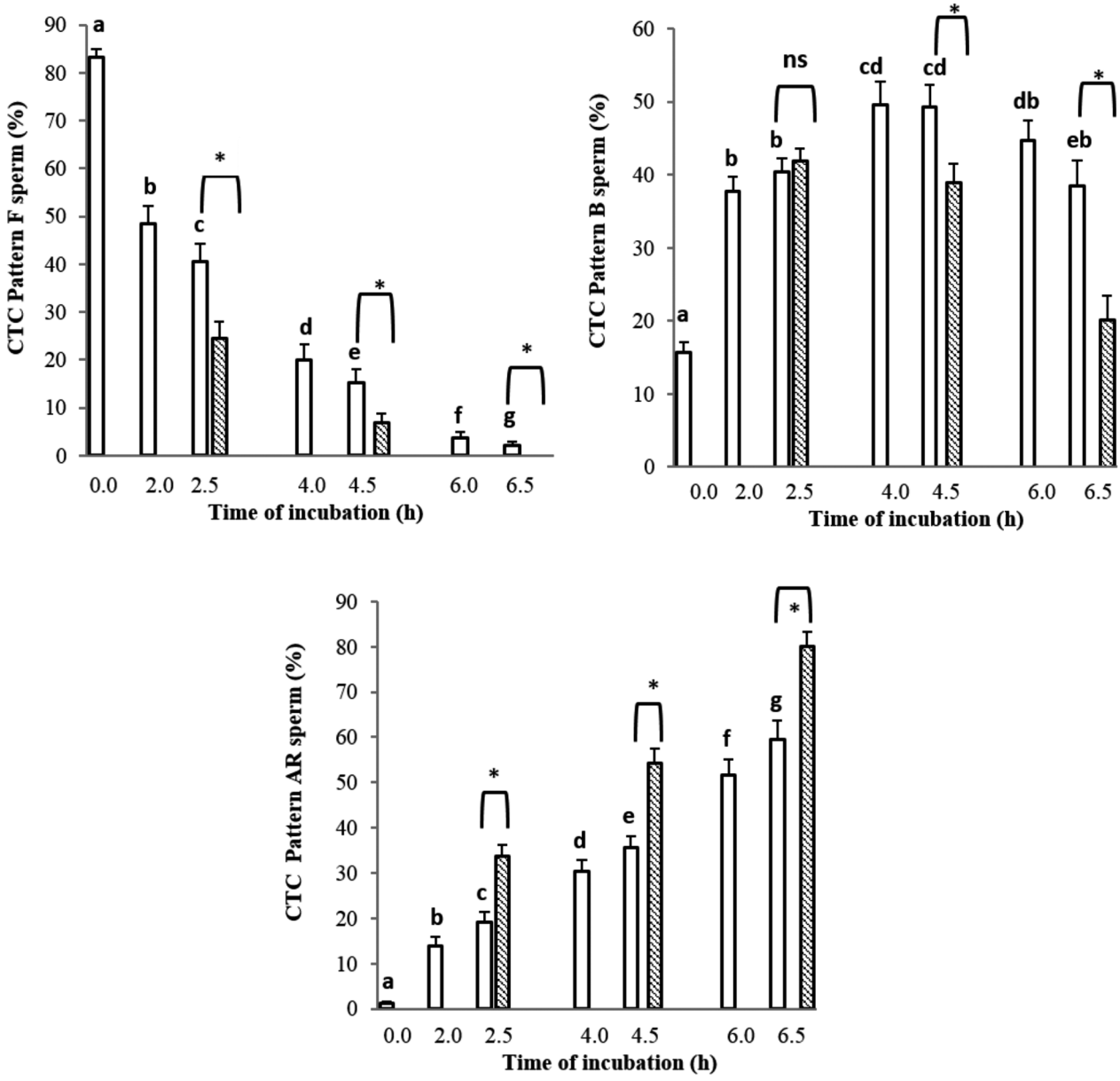

Figure 6. Capacitation status of dog spermatozoa incubated at 38 °C in 5% CO_2 in air during 6.5 h under the effect of Progesterone. Values are means + SEM.

Control: open bars (a-g), Progesterone: shadowed bars.

Control vs Progesterone: *$P<0.05$, ns = non significant.

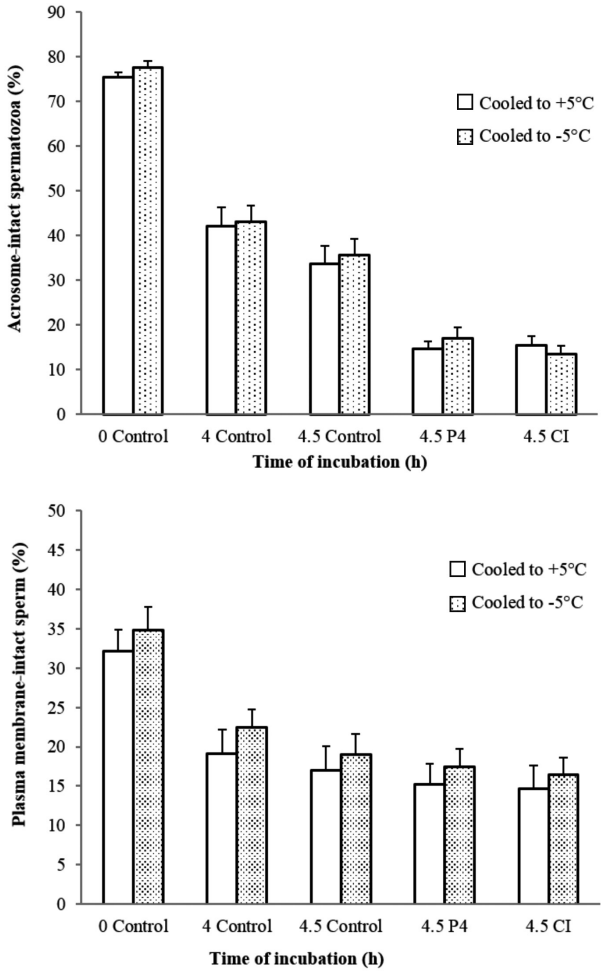

Figure 7. Acrosome-intact and plasma membrane-intact dog spermatozoa after freeze-thawing and post-thaw incubation for 4.5 h at 38 °C in 5% CO_2 in air, without or with Progesterone (P4)/Calcium ionophore, to induce the acrosome reaction. Sperm were colled to two target temperatures before freezing: +5 °C or –5 °C Values are means ± SEM.

of acrosome reaction in fresh-incubated spermatozoa using CI and P_4 was validated. Taking into account CTC capacitation patterns (i.e., B and AR) and the acrosome status, 4 and 4.5 h of incubation were chosen as the optimun time of incubation for the sperm to display the acrosome reaction. Thus, in the next experiments, those times of incubation were used.

The second experiment assessed sperm cryosurvival. One important finding was that after thawing, there was a significant difference in the number of Merocyanine high-binding (hyper-fluidity) cells between sperm cooled to either +5 °C or –5 °C, with the value of the former larger than that of the latter. This observation agrees with the proposed hypothesis that cooling of spermatozoa to sub-zero temperatures, around the freezing point (of diluted spermatozoa), favours sperm plasma membrane reorganisation after lipid phase transition takes place, thus avoiding an excessive increase of membrane fluidity (Watson 1995, Holt 2000). We are aware that this hypothesis disagrees with the general concept that an increase in membrane fluidity before freezing favours sperm cryosurvival (Giraud *et al* 2000, Aboagla and Terada 2003). Increased plasma membrane fluidity may benefit some sperm characteristics, such as motility (Giraud *et al* 2000), but also seems to be related to the incidence of premature sperm capacitation (Watson 1995). In physiological conditions, plasma membrane fluidity increases during sperm capacitation to prepare sperm membranes to suffer the acrosome reaction and facilitates the sperm-oocyte interaction (Flesch and Gadella 2000); however, in cryopreserved sperm, hyper-fluidity shortens the window of sperm fertility (Watson 1995). Studying the cryopreservation of dog spermatozoa, Alcantar-Rodriguez and Medrano (2017) found no differences in sperm quality and plasma membrane fluidity between sperm cooled to either +5 °C or –5 °C. In that work, approximately 52% of spermatozoa were classified as Merocyanine high-binding cells (hyper-fluidity). In contrast, in our work, values were 69.5 and 63.8% for +5 °C and –5 °C, respectively. In the study by Alcantar-Rodriguez and Medrano (2017), sperm were frozen on the next day after collection. In contrast, in our work sperm were collected and frozen on the same day. Thus, it could be the long storage at +5 °C and the stress of cryopreservation itself that minimised any positive effect of cooling to sub-zero temperatures on dog sperm fluidity. Plasma membrane fluidity may be modified during cryopreservation by both removal of cholesterol from the plasma membrane and by lipid peroxidation (Moein-Vaziri *et al* 2014), however, in our work, we were not able to discriminate between these 2 mechanisms.

Regarding the induction of the acrosome reaction on frozen-thawed spermatozoa, there were no differences between CI and P_4 or between cooling temperatures (+5 °C or –5 °C) in the proportion of spermatozoa that carried out that process. CI and P_4 have been previously used to induce the acrosome reaction in fresh and cryopreserved dog spermatozoa (Szász *et al* 2000, Cheng *et al* 2005), however, to our knowledge, CI and P4 have not been comparatively used in frozen-thawed dog spermatozoa. In our work, both induced the AR after 4.5 h of incubation; thus, either of them may be employed for that purpose in future research.

In this work, we partially proved our hypothesis that cooling of spermatozoa to –5 °C favours sperm plasma membrane reorganisation, thus avoiding an excessive increase of plasma membrane fluidity. The membrane phase behaviour of different cellular types (human prostate tumour cells, porcine smooth muscle cells and human dermal fibroblasts) during freezing seems to depend on the ice nucleation temperature (Balasubramanian *et al* 2009). In these cellular types, membrane phase transition causes different effects on the membrane conformational disorder in the frozen state when the ice nucleation occurs at either –3 °C (favours dehydration) or –10 °C (intracellular ice formation). Balasubramanian *et al* (2009) reported that ice nucleation at –6 °C (between temperature for dehydration

and that for intracellular ice formation) enables a fraction of cellular and membrane bound water to stay in the cell and thus promotes cell viability. This may be the mechanism by which cooling to –5 °C before freezing favours sperm cryosurvival.

At this point, we do not know whether cooling to sub-zero temperatures improves the fertilising capacity of frozen-thawed dog spermatozoa. A test of fertility by artificial insemination employing cryopreserved spermatozoa would provide further insights on that issue. It should also be considered that semen from different dog breeds may show variations in sperm cryosurvival when freeze-thawing protocols are modified (Yu *et al* 2002).

In conclusion, cooling to –5 °C did not improve dog sperm cryosurvival but produced a positive effect of plasma membrane fluidity, and the amount of Merocyanine high-binding (hyper-fluidity) cells was smaller than that of sperm cooled to +5 °C.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Faecal shedding of campylobacteria among domestic and wild animals from an urban coastal area

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ABSTRACT. *Campylobacter jejuni* and *Campylobacter coli* are the leading cause of food-related diarrhea worldwide. However, other campylobacteria such as other *Campylobacter* spp., *Arcobacter* spp. and *Helicobacter* spp. are also recognised as emerging pathogens, although they are not frequently isolated by traditional culturing methods. Moreover, *Campylobacter* spp. have become increasingly resistant to antibiotics due to antibiotic usage in animal and human medicine. It has been suggested that pet ownership increases the risk for campylobacteriosis. However, environmental factors such as temperature, humidity, and poor sanitation conditions have also been associated with the highest rates of shedding among animals. This study assessed the faecal shedding of campylobacteria in an urban coastal area among 68 (66.0%) domestic animals, mainly dogs (n=61), and 35 (33.9%) wild animals belonging to 13 species and eight orders. None of them had symptoms of gastroenteritis and campylobacteria were detected by PCR in 21 samples (20.4%), while only six (5.8%) were detected by culturing, i.e. *Campylobacter upsaliensis* (n=4, dogs), *C. jejuni* (n=1, dog) and *Arcobacter butzleri* (n=1, chicken). None of the isolates was resistant to ciprofloxacin, but two were resistant to erythromycin, i.e. *A. butzleri* (MIC=8 µg/ml) and *C. upsaliensis* (MIC=128 µg/ml). Regarding the virulence factors, only one isolate of *C. jejuni* was positive for *cdtC* and *cadF* genes and one isolate of *A. butzleri* was positive for *cadF* and *ciaB* genes. This is the first study to assess the faecal shedding of campylobacteria in animals from the urban coastal area of Valparaíso, Chile. Although *C. jejuni* and other emerging campylobacteria were detected mainly from pet dogs and in a low rate, further investigations are needed to assess the potential transmission of these zoonotic bacteria or their antibiotic resistance between pets and owners.

Key words: *Arcobacter, Campylobacter*, domestic and wild animals, *Helicobacter.*

INTRODUCTION

Campylobacteria is a group bacteria that share morphologic and ecologic characteristics, i.e. they are Gram negative curved rods that often colonise the digestive tract of their hosts, despite the fact that they belong to different taxonomic groups such as the genera *Campylobacter, Helicobacter, Arcobacter, Lawsonia and Anaerobiospirillum* (On 1996). Among them, the genera *Campylobacter, Helicobacter*, and *Arcobacter* have been more frequently associated with gastroenteritis in humans and animals (Bascuñana *et al* 2011, Collado and Figueras, 2011). Bacteria from the genus *Campylobacter* spp. have been worldwide recognised as the leading cause of acute bacterial gastroenteritis in humans (Ghosh *et al* 2014). The incidence rate of campylobacteriosis in the United States had been estimated in 14.3 per 100,000 population in 2012, causing approximately 1 million illnesses and 75 deaths annually, while in Europe, 246,307 cases were reported during 2016 according to EFSA (Crim *et al* 2014, EFSA, 2016). The vast majority of human gastroenteritis are caused by *C. jejuni* (80% - 85%) and *C. coli* (10%-15%) (Bullman *et al* 2012). However, data increasingly suggest that other campylobacteria such as emerging *Campylobacter* spp. together with *Arcobacter* spp. and *Helicobacter* spp have been underestimated as the cause of human and animal disease for several reasons including the capacity for isolation and differentiation of the methods used (Collado *et al* 2013, Bascuñana *et al* 2011, Bullman *et al* 2012, Leahy *et al* 2017). The detection of virulence associated genes has meant a considerable progress to determine the pathogenic potential of campylobacteria. In this regard, the presence of several virulence factors have been linked to motility, adhesion capacity, cell invasion and production of cytotoxins that could explain the pathogenic capacity of campylobacteria in the gastrointestinal tract (Khoshbakht *et al* 2013, Levican *et al* 2014). There is a need to demonstrate the link between the presence of virulence factors and pathogenicity, which is key to better understand bacteriosis in order to develop and implement more effective therapies against infection (Lapierre 2013). On the other hand, a constant monitoring of antimicrobial susceptibility among *Campylobacter* isolates has been recommended due to the current emergence of isolates resistant to antibiotics including fluoroquinolones, tetracycline and erythromycin, which are the treatment of choice (Garcia *et al* 2009).

Campylobacteria infection is transmitted via direct contact with faeces or by cross contamination through

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food, as well as by raw or undercooked meat or raw milk, while the main type of food involved in relation to public health is poultry and pork (Frasao *et al* 2017). However, other sources of transmission that have been recognised are the consumption of contaminated water as well as the contact with a wide variety of domestic and wild animals, especially cats, dogs, cattle, sheep, pigs and birds (Tresierra *et al* 2006, Leahy *et al* 2017). In fact, pet ownership can significantly increase the risk for human *Campylobacter* infection, because it has been estimated that about 6% of human enteric campylobacteriosis is transmitted from pets, while these animals also represent a potential source of dissemination of antimicrobial resistance due to their close contact with humans (Rossi *et al* 2008).

In Latin American countries, some studies have shown variable rates between 9% and 20% of faecal shedding among domestic and wild animals (Tresierra *et al* 2006, Fernández *et al* 2011). In those studies, the highest values have been associated with a high environmental temperature and humidity and poor sanitation conditions, as well as to the possible contact between wild animals and humans or poultry (Fernández *et al* 2011, Tresierra *et al* 2006). Furthermore, the animals with the highest values have been those belonging to the orders Galliformes and Primates (Tresierra *et al* 2006).

Faecal shedding among domestic animals has been shown to range from 20% to 75%, and such results depend not only on the detection method but also on the population of animals sampled (Leahy *et al* 2017). For instance, the lower end of this range corresponds to pet dogs, and the higher, to dogs in shelters or kennels. In fact, remarkable differences have been observed between studies including stray dogs or pet dogs (Toledo *et al* 2015).

Valparaíso region is a coastal urban area located in the center of Chile, which presents a Mediterranean Climate[1]. The average annual temperature in Valparaíso is 58.0°F (14.4°C), ranging from 64.0°F (17.8°C) in January to 53.0°F (11.7°C) in July, while the average annual amount of precipitation is 19.9" (505.5 mm)[1]. Considering that no previous studies on *Campylobacter* or other emerging campylobacteria have been conducted in this geographic region, the aim of this study is to determine the prevalence of faecal shedding of these zoonotic bacteria of public health concern among domestic and wild animals from the Valparaíso region (Chile) and to determine the presence of potential virulence factors and/or antibiotic resistance among the isolates.

[1] http://www.weatherbase.com/weather/weathersummary.php3?s=85558&cityname=Valparaiso%2C+Valparaiso%2C+Chile&units=

MATERIAL AND METHODS

SAMPLE COLLECTION

A total of 103 faecal samples from domestic and wild animals were collected between September and November 2016 (table 1). Domestic animals corresponded to pets hospitalised at the Santo Tomás Veterinary Clinical Hospital, Valparaíso, Chile. The wild animals analysed were those treated at the Animal Rescue Foundation Ñamku, Concón, Valparaíso, Chile[2]. This foundation is dedicated to recover the health conditions of wild animals that have been rescued from risk situations generated by humans. All animals were sampled upon arrival to their respective center. However, all of those animals that had been treated with antimicrobials, as well as those that did not shed stools or present difficulty in issuing depositions, were excluded from this study. This study was approved by the ethics committee of the Medical Faculty of the Universidad Andrés Bello, Chile.

The samples corresponded to animal fresh depositions shed by spontaneous evacuation during the time established for their collection, considering only one sample per animal regardless of the time they stay in the center. The animals were not handled directly and the whole procedure was carried out with the help and supervision of the personnel in charge of the animals. For collection, clean plastic films were placed in the floor of the cage and the samples were taken using wooden sticks and deposited in clean sterile flasks for culturing immediately after issued. For DNA extraction, an aliquot of stools was deposited into a tube containing RNA later™ (Sigma, USA) and stored refrigerated at 4 °C, as recommended by Gray *et al* (2012). RNA was used later because it has performed as a good DNA preservation method, preserving DNA in samples refrigerated for at least 1 month with the added benefit of lower PCR inhibition (Gray *et al* 2012). Afterwards, samples were transported and then processed for culturing on the same day, while for DNA extraction, samples were stored refrigerated and processed weekly.

BACTERIAL DETECTION AND IDENTIFICATION

For the direct bacterial detection from faeces by PCR, total DNA was extracted from samples conserved in RNA later within the week of collection. To do this, 100 µL of each sample were washed twice in phosphate buffered saline (PBS) pH 7.4, 0.01 M (Winkler, Chile). DNA was then extracted using the commercial kit Instagene matrix® (Biorad, USA), according to the manufacturer's instructions for bacteria. The presence of stool derived PCR inhibitors was assessed as described by Holland *et al* (2000). In brief, a set of ten DNA samples were randomly

[2] http://namkufundacion.cl

selected and used to amplify the 16S rRNA gene by using commercial kit GoTaq Green Master Mix (Promega, USA) and universal primers PA and PH in PCR reactions. The PCR products were then subjected to electrophoresis in 1% agarose gel (Sigma, USA), stained with SYBR® Safe DNA Gel Stain (Thermo Fisher, USA) and visualised in UV transilluminator. As the ten samples yielded a strong positive amplicon, no inhibition was assumed.

The samples were submitted to genus-specific PCR for *Campylobacter* (Linton *et al* 1996), *Arcobacter* (Harmon and Wesley 1997), and *Helicobacter* (Moyaert *et al* 2008), using the commercial kit GoTaq Green Master Mix (Promega, USA) with the concentrations of primers and conditions indicated by their respective authors (table S1)[3]. In the case of samples positive for genera *Campylobacter* and *Arcobacter*, a second PCR was carried out for the detection of specific species associated with human infections: *C. jejuni, C. coli, C. ureolyticus, C. upsaliensis*, *C. concisus*, *A. butzleri* and *A. cryaerophilus* (Bullman *et al* 2012, Collado *et al* 2013, Figueras *et al* 2014) (Table S1). In all cases, PCR products were then subjected to electrophoresis in 1% agarose gel (Sigma, USA), stained with SYBR® Safe DNA Gel Stain (Thermo Fisher, USA) and visualized in UV transilluminator.

For culturing, samples were directly seeded onto *Campylobacter* Charcoal Deoxycholate Agar supplemented with Cefoperazone and Amphotericin B (CCDA, Liofilchem, Italy), and incubated for 48h to 72h at 37 ºC under microaerobic conditions (~O_2 5-7%, CO_2 5-10%, N_2 85%, H_2 3%) using the Anaerocult C ® (Merck, USA) generator system into an appropriate anaerobic jar. Presumptive colonies of campylobacteria were submitted to Gram stain to observe the characteristic morphology (Gram negative curved or s-shaped rods) and re-streaked on Trypticase Soy Agar supplemented with 5% sheep blood (BA) and incubated as described above.

PHENOTYPIC AND MOLECULAR CHARACTERISATION

Phenotypic characterisation of these colonies was carried out by using biochemical tests including catalase, oxidase, hydrolysis of hippurate, and susceptibility to nalidixic acid and cefalotin (Table S2) as described by Fernández *et al* (2016).

For the molecular characterisation, total DNA was extracted from the isolated colonies with the Instagene Matrix™ (Biorad, USA) kit according to the instructions of the manufacturer. Genus and species specific molecular identification of this colonies were carried out as described above. The presence of potential virulence associated genes was determined by PCR in all of the isolates identified as *Campylobacter* spp. or *Arcobacte*r spp. The tested genes were selected on the basis of their prevalence in previous studies as well as on their putative role in pathogenesis for *Campylobacter* spp. (*cadF,* associated with adhesion and cell invasion; *iamA,* invasion associated marker; *pldA,* phospholipase A associated with lysis of erythrocytes; *cdtA, cdtB* and *cdtC,* encoding the subunits A, B and C of the Citolethal Distending Toxin) and *Arcobacter* spp. (*cj1349*, encoding a fibronectin binding protein implicated in adhesion; *cadF,* associated with adhesion and cell invasion; *ciaB*, encoding a putative invasion protein) (Khoshbakht *et al* 2013, Levican *et al* 2013). PCR reactions were carried out using the commercial kit GoTaq Green Master Mix (Promega, USA), with the concentrations of primers and conditions described in the literature (Khoshbakht *et al* 2013, Levican *et al* 2013) (table S2)[4]. The PCR products were then subjected to electrophoresis in 1% agarose gel (Sigma, USA), and then stained with SYBR® Safe DNA Gel Stain (Thermo Fisher, USA) and visualised in UV transilluminator. The control strains used in all the phenotypic and molecular tests were the type strains of *C. jejuni* subsp. *jejuni* DSM 4688^T (= ATCC 33560^T), *C. coli* DSM 4689^T (= ATCC 33559^T), and *A. butzleri* LMG 10828^T.

The susceptibility to antimicrobials was carried out with the Kirby-Bauer method using the MH-F medium (Müeller Hinton Agar supplemented with 5% defibrinated horse blood and β-NAD 20 mg/L, Liofilmchem, Italy) for ampicillin, amoxicillin/clavulanic acid, gentamicin, ciprofloxacin, nalidixic acid, erythromycin, azithromycin, and tetracycline, using the strain *C. jejuni* subsp *jejuni* DSM 4688^T (= ATCC 33560^T) as control. All plates were incubated at 37° C for 48 hours under microaerophilic conditions (~O_2 5-7%, CO_2 5-10%, N_2 85%, H_2 3%) using the Anaerocult® C (Merck, USA) generator system into an appropriate anaerobic jar. In parallel, the minimum inhibitory concentration (MIC) was determined by the double dilution in agar method from 0.125 µg/mL to 256 µg/mL, for erythromycin and ciprofloxacin as previously described (Fernández *et al* 2016). Cut offs for interpretation of Kirby-Bauer and MIC results were obtained from the recommendations of the *Comité de l'Antibiogramme* of the *Société Française de Microbiologie* (2017).

RESULTS

A total of 103 samples were collected, of which 74 (71.8%) were obtained from domestic animals, mainly dogs (n=61). On the other hand, 29 (28.2%) wild animals were sampled which belonged to 13 species and eight orders (table 1). None of these animals had symptoms of gastroenteritis.

Overall, 21 samples were PCR-positive to *Campylobacter* spp. (n=8, 38.1%), *Helicobacter* spp. (n=8, 38.1%), and *Arcobacter* spp. (n=5, 23.8%). Among the *Campylobacter* species, *C. upsaliensis* (n=4, 50%), *C. jejuni* (n=1, 12.5%),

[3] Available at: www.australjvs.cl/ajvs

[4] Available at: www.australjvs.cl/ajvs

and *C. coli* (n=1, 12.5%), were identified, while only *A. butzleri* (n=1, 20%) was identified among *Arcobacter* spp. positive samples. Animals belonging to four species were positive for campylobacteria tested by genus specific PCR, i.e. domestic dog (*Canis lupus familiaris*, Order Carnivora), chicken (*Gallus gallus domesticus*, Order Galliformes), Southern sea lion (*Otaria flavescens*, order Carnivora), and Magellanic penguin (*Spheniscus magallanicus*, Order Sphenisciformes). Only six samples (5.8%) were positive for campylobacteria by culturing, which corresponded to five *Campylobacter* and one *Arcobacter* (table 2). The predominant species isolated was *C. upsaliensis* (n=4) from dog faeces (table 1).

The PCR detection of virulence-associated genes demonstrated the presence of only the *cdtC* and *cadF* genes in the *C. jejuni* 191101 isolate recovered from dog, and the *ciaB* and *cadF* genes in the *A. butzleri* 191103 isolate from chicken, while all other isolates were negative for all tested genes.

All isolates were susceptible to ciprofloxacin, while one isolate of *C. upsaliensis* (261004) isolated from dog was resistant to erythromycin (MIC=128 µg/mL), azithromycin, amoxicillin-clavulanic acid and tetracycline, and the remaining *Campylobacter* isolates were susceptible to all antimicrobials tested (table 3). On the other hand, the *A. butzleri* isolate (191103) from chicken also showed resistance to erythromycin (MIC=8 µg/mL).

DISCUSSION

The faecal shedding of campylobacteria observed in wild animals (3/29, 10.3%) and domestic animals (18/74, 24.3%) was relatively low considering previous studies in different animals in South America and other geographic locations (Fernández *et al* 2011). Regarding wild animals, our study included individuals belonging to 13 species from eight orders which mainly corresponded to birds, and among them, only two southern sea lions and a Magellanic penguin were PCR-positive for campylobacteria (table 1). A previous study on faecal shedding of *Campylobacter* in wild animals from the Peruvian Amazon region obtained a 11% of shedding by culturing, the main source being the orders of Galliformes and Primates (Tresierra *et al* 2006). The positivity found in that study was explained

Table 1. Campylobacteria detected in faeces of domestic and wild animals by PCR and culturing.

Species	Order	Common name	n (%)	Direct detection by PCR (n)	Detection by culturing (n)
Domestic animals (n=74)					
Canis lupus familiaris	Carnivora	Domestic dog	61 (59.2)	*C. upsaliensis* (4); *C. jejuni* (1) *Campylobacter* spp. (2) *Arcobacter* spp. (3) *Helicobacter* spp. (5)	*C. upsaliensis* (4) *C. jejuni* (1)
Felis silvestris catus	Carnivora	Domestic cat	7 (6.8)		
Gallus gallus domesticus	Galliformes	Chicken	6 (5.8)	*A. butzleri* (1); *Helicobacter* spp.(2)	*A. butzleri* (1)
Wild animals (n=29)					
Otaria flavescens	Carnivora	Southern sea lion	2 (1.9)	*A. butzleri* (1); *Helicobacter* spp.(1)	
Bubo virginianus	Strigiformes	Magellanic horned owl	6 (5.8)		
Glaucidium nanum	Strigiformes	Austral pygmy owl	1 (1.0)		
Tyto alba	Strigiformes	Barn owl	2 (1.9)		
Spheniscus magallanicus	Sphenisciformes	Magellanic penguin	3 (2.9)	*C. coli* (1)	
Anas platyrhynchos	Anseriformes	Mallard	5 (4.9)		
Larus dominicanus	Charadriiformes	Pacific gull	3 (2.9)		
Vanellus chilensis	Charadriiformes	Southern lapwing	1 (1.0)		
Vultur gryphus	Accipitriformes	Andean condor	2 (1.9)		
Geranoaetus polyosoma	Accipitriformes	Red backed owl	1 (1.0)		
Milvago chimango	Falconiformes	Chimango caracara	1 (1.0)		
Falco peregrinus	Falconiformes	Peregrin falcon	1 (1.0)		
Turdus falcklandii	Passeriformes	Austral thrush	1 (1.0)		
Total (%)			103 (100%)	21 (20%)	6 (5.8%)

Table 2. Phenotipic and molecular identification of campylobacteria isolated in this study.

Isolate	Catalase	Oxidase	Hypurate	Nalidixic acid	Cefalotin	Species id. by PCR
261004	–	+	–	R	S	*C. upsaliensis*
071104	–	+	–	R	S	*C. upsaliensis*
181111	–	+	–	R	S	*C. upsaliensis*
181108	–	+	–	R	S	*C. upsaliensis*
191101	+	+	+	S	R	*C. jejuni*
191103[a]	–	+	–	R	R	*A. butzleri*
Control	+	+	+	S	R	*C. jejuni*

R=Resistant, S=Susceptible
[a]The strain 191103 was isolated from chicken while all others were isolated from dogs.

Table 3. Antimicrobial susceptibility of the isolates recovered in this study.

	C. upsaliensis 261004	*C. upsaliensis* 071104	*C. upsaliensis* 181111	*C. upsaliensis* 181108	*C. jejuni* 191101	*A. butzleri* 191103
Kirby-Bauer method						
Erythromycin	R	S	S	S	S	R
Ciprofloxacin	S	S	S	S	S	S
Gentamicin	S	S	S	S	S	S
Tetracyclin	R	S	S	S	S	S
Amoxicilin/Clavulanic acid	R	S	S	S	S	S
Azithromicin	R	S	S	S	S	S
MIC (µg/mL)						
Erythromycin	128	1	1	2	1	8
Ciprofloxacin	0.5	0.5	0.5	0.5	0.5	0.5

R=Resistant, S=Susceptible.

by the high temperature and humidity as well as poor sanitation conditions in the peri-urban areas were the animals sampled live (Tresierra *et al* 2006). Considering this, the lower temperature and humidity present in the urban coastal area where this study was conducted could explain the lower incidence observed. Regarding campylobacteria shedding in penguins and sea lions, *C. coli, A. butzler*i and *Helicobacter* spp. were the species detected in the present study. *Campylobacter* spp. has already been detected from marine mammals and seabirds in Antarctic and subantarctic region. However, the species most commonly reported corresponded to *C. lari* or related species (García-Peña *et al* 2010, 2017). In studies conducted in Europe, the overall detection of *Campylobacter* among wild animals has ranged from 6% to 43% and the differences have been attributed mainly to the feeding habits, diet, and preferred habitats, but also to other factors such as migration patterns, lifespan, or differerent life stages like breeding, migration, molting and wintering (Waldenstrom *et al* 2002, Antilles *et al* 2013, Krawiec *et al* 2017). In this same line, it has been highlighted that those animals living or feeding at watery habitats such as water edges or in shallow waters of habitats that commonly harbour *Campylobacter* spp. may present a high overall prevalence (Waldenstrom *et al* 2002).

Most of wild animals sampled here corresponded to raptors and scavenging birds admitted at the Animal Rescue Foundation Ñamku, Valparaíso (Chile) which were all negative for campylobacteria. On the contrary, Molina-Lopez *et al* (2014), studied the presence of *Campylobacter* spp. and *Salmonella* spp. among faecal samples of 121 raptors admitted to the Wildlife Rehabilitation Centre of Torreferrusa (Spain), and 9 out of them (7.4%) were positive for *Campylobacter* spp., demonstrating that these bacteria as well as other enteropathogens may be present in the wildlife admitted to rehabilitation centres, therefore, their zoonotic risk for the staff and general population must be considered (Molina-Lopez *et al* 2014).

It should be noted that due to the difficulty in the access to this kind of samples, the present study included only a few individuals of the different species sampled and this could explain the low prevalence observed. Therefore, further studies aimed to clarify whether this is the true tendency for faecal shedding of campylobacteria among animals in

this geographical zone and to determine the factors behind this tendency are needed. Despite of this, the presence of stool derived PCR inhibitors after the extraction process was not observed in this study. Moreover, sampling was performed once for each animal and upon their arrival to the veterinary clinical or rescue foundation. Thus, cross contamination with campylobacteria due to their contact with humans or other animals was avoided. However, another limitation of this study is that the sensitivity of PCR methods used was not determined. Therefore, we are not able to state whether negative samples are due to the absence of campylobacteria or they are negative due to a bacterial load in the sample which was under the detection limit of the method.

The faecal shedding among domestic animals (24.3%) was also low, but in previous studies it ranged from 20% to 75% depending on the detection method and on the population sampled (Leahy *et al* 2017). The low end of this range (20%) corresponds to pet dogs, while the high end (75%) corresponds to dogs in shelters or kennels (Leahy *et al* 2017). In fact, remarkable differences have been observed between studies including stray dogs or pet dogs (Toledo *et al* 2015). Therefore, it is possible that the overall low prevalence of campylobacteria among pets in this study could also be due to the fact that they belong to known owners who provide them good standards of care.

By culturing, a lower isolation rate (5.8%) in comparison with PCR detection was obtained. Moreover, *Helicobacter* spp. were not isolated, even though they were detected by PCR in 3 animals. The lower performance of culturing could be attributed to the overgrowth of accompanying bacteria that hampered the recognition of campylobacteria, as well as the possible presence of Viable But Non-Culturable bacteria (VBNC) as suggested by Bullman *et al* (2012).

Only isolates belonging to *Campylobacter* spp. and *Arcobacter* spp. were obtained and, as expected, *C. upsaliensis* was the most commonly isolated species from dogs (Rossi *et al* 2008, Leahy *et al* 2017). Although the role of this species as human pathogen is not clearly established, in some cases it was associated with gastroenteritis (Holmerg *et al* 2015). Moreover, its prevalence among humans could have been underestimated due to the fact that methods used in the public health laboratories have been developed to detect *C. jejuni* and *C. coli* (Holmerg *et al* 2015). Regarding the major pathogen *C. jejuni*, the isolate 191101 of this species was recovered from a dog, confirming that pets could be a reservoir for this species. As previously suggested, *C. jejuni* could be transmitted to dogs by a food source or by their owner and there is a need for further study on this (Tamborini *et al* 2012). On the other hand, evidence suggests that the transmission of *C. jejuni* from dogs to humans can also occur through the contact with faeces of infected dogs, therefore, its shedding represents a potential threat to public health (Tamborini *et al* 2012, Leahy *et al* 2017).

The presence of potential virulence associated genes was determined by PCR in all of the isolates of *Campylobacter* spp. and *Arcobacte*r spp. Only the *cdtC* and *cadF* genes were detected in the *C. jejuni* isolated from dog and the *ciaB* and *cadF* genes in the *A. butzleri* isolated from chicken. Both species have been associated with gastroenteritis in humans, therefore, it was expected the presence of putative virulence genes. On the contrary, it is possible that negative results for the other tested genes were due to differences in DNA sequences of those genes among strains because the primers have been designed on the basis of the representative strains *C. jejuni* 81-176 and *A. butzleri* RM4018 (Levican *et al* 2013, Iglesias-Torrens *et al* 2018). In this regard, Iglesias-Torrens *et al* (2018) have observed a higher diversity of *cdtA* alleles among *C. jejuni* strains found in wild birds compared to broilers or humans. The authors hypothesize that these differences among sequences of certain *cdt* alleles could be linked to the ability to colonize different hosts.

None of the isolates obtained in this work was resistant to ciprofloxacin, even though a resistance to this antibiotic has been previously observed among isolates from animals and humans (Garcia *et al* 2010, Carbonero *et al* 2012, Toledo *et al* 2015, Lapierre *et al* 2016). On the contrary, two isolates were resistant to erythromycin, i.e. *A. butzleri* 191103 (MIC=8 µg/mL) and *C. upsaliensis* 261004 (MIC=128 µg/mL). Moreover, the *C. upsaliensis* isolate was also resistant to azithromycin, amoxicillin-clavulanic acid and tetracycline. Multidrug resistance (MDR) has been previously observed among isolates from humans and broilers. However, the most frequent MDR profile has been the resistance to tetracyclyne, ciprofloxacin, naludixic acid and ampicillin, while erythromycin and gentamicin are considered the most efficient antibiotics against *Campylobacter* isolates from dogs (Iglesias-Torrens *et al* 2018). In contrast, Tsai *et al* (2007) observed a higher rate of resistance to azithromycin (93.9%), clindamycin (87.9%), erythromycin (81.8%), tetracycline (78.8%) and a lower rate to ciprofloxacin (18.2%) among stray dogs from Taiwan. The authors suggested that the differences in resistance between strains from stray dogs compared with those from humans or broilers may reflect the differences in antimicrobial use between pet animal veterinary medical practice and human medical practice (Tsai *et al* 2007).

In conclusion, in this study it was posible to observe the shedding of *C. jejuni* and other emerging campylobacteria in faeces mainly obtained from pet dogs. This shedding was lower than that found in previous reports. Despite the fact that a low number of individuals different than dogs was sampled, the low shedding observed could be explained by different factors such as the weather conditions, and the good standards provided by their owners. However, the presence of these bacteria warrants future studies to assess the potential transmission of these zoonotic bacteria to owners, or even the transmission of the observed antibiotic resistance to other bacteria present in their microbiome.

In this sense, under a ONE HEALTH approach, it is also necessary to design education programs aimed at owners in order to avoid this transmission of campylobacteria from pet to owners or vice versa.

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ORIGINAL ARTICLE

Burnout syndrome prevalence in veterinarians working in Chile

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ABSTRACT. Burnout Syndrome is a psycho-emotional syndrome that affects workers in any activity or profession. In recent years, veterinarians have been described as one of the most affected professionals, which has motivated the development of this research. The primary objective of this work was to determine the prevalence of Burnout Syndrome in veterinarians working in Chile. We evaluated the possible correlation of Burnout Syndrome with socio-demographic variables. The Socio-Demographic Characterization Survey and the Maslach Burnout Syndrome Inventory-General Survey (MBI) were applied to 521 participants, who were contacted through the Veterinary Medical Association of Chile (COLMEVET) and social networks. Fisher and Chi-square statistical tests and correspondence analysis were used to determine the association among variables. The prevalence of Burnout Syndrome in Chilean veterinarians was 24% (124/521). There was statistical significance between Burnout Syndrome and the variables 'years in employment' and 'monthly salary'. There is a high prevalence of Burnout Syndrome in veterinarians working in Chile that was related to the variables 'years in employment' and 'monthly salary'. Prevention using psychological therapy could reduce the incidence of symptoms linked to adaptive difficulties, cognitive discrepancies, psychological discomfort, and emotional regulation, promoting health improvement and resistance to Burnout Syndrome in Chilean veterinarians. This research corresponds to the first exploratory study related to the subject in Chile.

Key words: Burnout Syndrome, veterinarians, Maslach, Chile.

INTRODUCTION

The International Labor Organization (ILO) concludes that work-related stress is one of the pathologies of the future, which is increasing progressively and globally from an already large base rate. In Europe alone, approximately 40 million people are affected by this syndrome[1]. Burnout Syndrome, first described in the 1970s, is a group of work-related symptoms and signs that usually occur in people with no history of psychological or psychiatric disorders (Maslach and Leiter 1997, Maslach *et al* 2001). Understanding that it is not an entity other than work-related stress, it is defined as a psycho-emotional syndrome, affecting workers who are immersed in chronically deficient work environments. This results in physical and emotional exhaustion (fatigue), loss of empathy (depersonalization) and an inadequate capacity for self-assessment (low personal fulfilment)[2]. Emotional exhaustion refers to feelings of being emotionally fatigued by one's contact with other people. Depersonalization denotes an excessively detached response toward the recipients of one's service or care. Low personal fulfilment refers to a decline in the person's self-feelings of competence and goal achievement at work (Bakker *et al* 2014). It is produced by a discrepancy between the expectations and the ideals of the employee and the actual requirements of the position. Burnout Syndrome's symptoms develop gradually and are usually absent when entering a new type of employment (Moss *et al* 2016). The risk factors associated with this syndrome can be divided into four categories: (1) personal characteristics, (2) organizational factors, (3) quality of work relationships, and (4) exposure to end-of-life problems (Poncet *et al* 2007).

During the last decade, Burnout Syndrome has become a public health problem, with a prevalence of between 4% and 7% of the active working population (Maslach *et al* 2001). Diagnoses continue without being included in clinical classification systems, such as the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) or the International Statistical Classification of Diseases and Related Health Problems (ICD-10) (Bauernhofer *et al* 2018). There is also no consensus on the definition of Burnout Syndrome and its main symptoms (Shirom and Melamed, 2006). Moreover, the diagnosis is superimposed on the symptoms with other diagnoses, especially chronic fatigue[3] and depression (Ahola *et al* 2014).

The veterinary proffesion is considered to be one of the most vulnerable jobs in terms of job stress, as postulated

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by the ILO[4]. Further, work stress has been the primary factor associated with high suicide rates among veterinarians (Acero 2004). A series of factors that are related to occupational fatigue, such as the euthanasia factor (a procedure that involves a high psychological strain), have been recognized in the practice of veterinary medicine (Nett *et al* 2015, Scotney *et al* 2015). Other factors include wage gaps between human surgeon doctors and veterinarians (Lee 2013), access to drugs, university debt, and high intra-disciplinary competition (Acero 2004). Likewise, veterinarians are subject to social vulnerability and ignorance within the profession regarding how they defend themselves when they are judged[5]. Capó[2], Acero (2004) and Lee (2013) agree that the most vulnerable group of veterinarians is small animal practitioners. Chile is not free or unaware of this situation within the veterinary practice, and this is why we aim to carry out a first exploratory study of Burnout Syndrome in Chilean veterinarians. The hypothesis used in the present investigation was that Burnout Syndrome exists among veterinarians in Chile and it is associated with socio-demographic and labor variables.

MATERIAL AND METHODS

The study used a descriptive quantitative method, with a transversal descriptive design. The study sample included veterinarians who work in Chile. There were no exclusion criteria for gender, age, marital status, work area, years of experience, or salary. The sole exclusion criteria employed in the present study was having a history of psychological treatment related to work (prior to entering the current job) since people affected by the burnout are psychologically healthy (Maslach *et al* 2001). This study was approved by the Santo Tomás University Ethics Committee (Resolution 016-17).

The sample size was determined using the infinite population formula[6] because the number of veterinarians in Chile was unknown, and an infinite population with 95% confidence interval was considered. The minimum sample size was 385. The total sample size was 618 participants, of which 521 met the inclusion criteria. All participants accepted and signed the informed consent form. They were enrolled with the collaboration of the Veterinary Medical Association of Chile (COLMEVET) and through social networks (emails, publications in forums of Chilean veterinarians). Participants completed a socio-demographic survey (gender, age, salary, years in employment, civil status, labor area) (table 1). The variables considered were: (1) gender (woman, man), (2) age (<33 years, between 33 and 55 years of age, > 55 years of age), (3) monthly salary (≤100,000 Chilean Pesos (i1), 100,001-300,000 Chilean Pesos (i2), 300,001-500,000 Chilean Pesos (i3), 500,001-900,000 Chilean Pesos (i4), 900,001-1,200,000 Chilean Pesos (i5), and ≥1,200,001 Chilean Pesos (i6)), (4) years in employment (<ten years in employment, ten-twenty five years in employment, >twenty five years in employment), (5) marital status (married, divorced, separated, single), and (6) labor area (small animal practice (SAP), large animal practice (LAP), public health (PH), animal production (AP), government entities (GE), food and food safety (FS), and another area (AA).

Work-related Burnout Syndrome was measured with the Maslach Burnout Syndrome Inventory-General Survey (MBI) using Google forms. The questionnaire was previously evaluated by a panel of experts that included a veterinarian and two psychologists who ensured that the digital version used retained the characteristics of the paper inventory. This version of the instrument was validated in Chile[7] and comprises a survey with the three-dimensional approach (table 1) validated by Pando *et al* (2015). The MBI consists of 22 questions (Maslach and Jackson 1981). These questions evaluate the prevalence of work-related

Table 1. Socio-demographic groups.

Variable	Group
Age group	< 33 years, ≥33 years, ≤55 years, > 55 years
Gender	Woman, man
Civil status	Single, married/civil union, separated, divorced, widow/widower
Years in employment	<10 years, 10-25 years, >25 years
Labor area	Small animal practice (SAP), Large animal practice (LAP), Public health (PH), Animal production (AP), Government entities (GE), Food and food safety (FS) and another area (AA).
Salary per month	≤100,000 Chilean Pesos (i1); 100,001-300,000 Chilean Pesos (i2); 300,001-500,000 Chilean Pesos (i3); 500,001-900,000 Chilean Pesos (i4); 900,001-1,200,000 Chilean Pesos (i5) and ≥1,200,001 Chilean Pesos (i6).

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Table 2. Maslach inventory survey for burnout classification.

Appearance evaluated	Items to evaluate	Indications of burnout
Emotional exhaustion	1-2-3-6-8-13-14-16-20	More then 26
Depersonalization	5-10-11-15-22	More then 9
Personal fulfillment	4-7-12-17-18-19-21	Less then 34

experiences on a scale of 0 (never) to 6 (every day). The inventory comprises three dimensions of the Burnout Syndrome, which are exhaustion, cynicism, and professional inefficacy. The role of work-related fatigue or exhaustion has been considered to be at the core of Burnout Syndrome (Maslach *et al* 2001). For a participant to be recognised with Burnout Syndrome, they must present altered results in all areas evaluated: 1) emotional exhaustion: a score of more than 26 points, 2) depersonalization: more than 9 points, and 3) personal accomplishment: less than 34 points (table 2). The scoring was taken from the Maslach questionnaire specified as values associated with burnout (Maslach *et al* 1996).

STATISTICAL ANALYSES

The data were arranged in frequency distribution tables, percentages (prevalences) were calculated, and the Fisher exact test and Chi-square tests were applied to determine the association between socio-demographic variables and Burnout Syndrome among the different groups analysed. Subsequently, a correspondence analysis was used to graphically demonstrate, in a two-dimensional space, the multivariate relationship between the variables that were significantly associated. The Odds Ratio (OR) calculation was performed for the dichotomous variables. The softwares used were GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA) and R-Commander 3.4.4 (Bell Laboratories, Murray Hill, New Jersey, USA).

RESULTS

Out of the 521 participants included in the analysis, 124 recorded sufficient scores in the MBI for meeting the criteria for Burnout Syndrome, equivalent to a prevalence of 24%. The results showed a statistically significant association between 1) the presence of Burnout Syndrome and years of employment, and 2) the existence of Burnout Syndrome and salary. Regarding the sex variable, there was a tendency to associate with the female sex but it was not statistically significant (tables 3 and 4).

AGE GROUP

Within the 124 participants that were affected by Burnout Syndrome, the distribution by age groups was as follows: There were 83 participants (66.9%) in the group between 33 and 55 years of age, 39 participants (31.5%) were under 33 years of age, and the remaining 2 participants (1.6%) were older than 55 years of age. Out of the 397 participants without Burnout Syndrome, 274 participants (69%) were in the group between 33 and 55 years of age, 111 participants (27.9%) were under 33 years of age, and the remaining 12 participants (3.1%) were older than 55 years of age. There were no significant statistical differences between these groups (table 5).

Table 3. Relationship between burnout and variables (Chi-square test).

Variable	*P*-value
Gender	0.061
Age	0.56
Civil status	0.92
Years in employment	0.006 **
Labor area	0.125
Salary	0.002 **

Table 4. Relationship between burnout with other variables (Fisher exact test).

Variable	*P*-value
Gender	0.068

GENDER

Amongst the 124 participants affected with Burnout Syndrome, the participants were distributed by gender as follows: 97 participants were women (78.2%), and 27 participants were men (21.8%). Out of the 397 participants without Burnout Syndrome, 276 were women (69.5%), and 121 (30.5%) were men. Although a trend was observed, there were no significant statistical differences (table 6) or did not show a significant statistical difference (table 7).

MARITAL STATUS

The number of people belonging to each marital status group within the total group of 124 participants with Burnout Syndrome was: Married 33 (26.6%), divorced 3 (2.4%), separated 3 (2.4%), single 85 (68.6%), and widowed 0

Table 5. Statistical significance between variables according to age group.

Contingency table burnout * age group					
Count		Age group			Total
		< 33 years age	33-55 years age	> 55 years age	
Burnout	0	111	274	12	397
	1	39	83	2	124
Total		150	357	14	521

Chi-square test			
	Value	gl	Asymptotic meaning (bilateral)
Chi-square Pearson	1.159[a]	2	0.560
Likelihood ratio	1.234	2	0.539
Linear association by linear	0.919	1	0.338
N valid cases	521		

a: 1 box (16.7%) has an expected lower frequency than 5. The minimum expected frequency is 3.33.

Table 6. Statistical significance between variables according to gender.

Contingency table burnout * gender				
Count		Gender		Total
		Man	Woman	
Burnout	0	121	276	397
	1	27	97	124
Total		148	373	521

Chi-square test					
	Value	gl	Asymptotic meaning (bilateral)	Exact meaning (bilateral)	Exact meaning (unilateral)
Chi-square Pearson	3.520[a]	1	0.061		
Correction for continuity[b]	3.105	1	0.078		
Likelihood ratio	3.656	1	0.056		
Fisher's exact statistic				0.068	0.037
Linear association by linear	3.513	1	0.061		
N valid cases	521				

a. 0 boxes (.0%) have an expected lower frequency than 5. The minimum expected frequency is 35.22.
b. Calculated only for a 2x2 table.

Table 7. Odds Ratio (OR) between both gender categories.

	OR	CI (95%)	Association
Burnout x Gender	1.5750	0.9775-2.5378	No significant statistical differences

(0%). The number of participants for each marital status within the 397 participants without Burnout Syndrome was: Married 103 (26%), divorced 13 (3.3%), separated 14 participants (3.5%), single 266 (67%), and widowed 1 (0.2%). Significant statistical differences were not observed between these groups (table 8).

YEARS IN EMPLOYMENT

Within the 124 participants affected with Burnout Syndrome, 101 participants (81.5%) had less than ten years of employment, 22 participants (17.7%) had ten to twenty-five years of employment, and 1 participant (0.8%) had more than twenty-five years of employment. Of the 397

Table 8. Statistical significance between variables according to marital status.

Contingency table burnout * marital status

Count		Marital status					Total
		Married	Divorced	Separated	Single	Widowed	
Burnout	0	103	13	14	266	1	397
	1	33	3	3	85	0	124
Total		136	16	17	351	1	521

Chi-square test

	Value	gl	Asymptotic meaning
Chi-square Pearson	0.942[a]	4	0.918
Likelihood ratio	1.210	4	0.876
Linear association by linear	0.002	1	0.967
N valid cases	521		

a: 4 boxes (40.0%) have an expected lower frequency than 5. The minimum expected frequency is .24.

without burnout, 265 participants (66.8%) had less than ten years in employment, 121 participants (30.4%) had a range of ten-twenty five years of employment, and only 11 participants (2.8%) had more than twenty-five years of employment. Significant statistical differences (*P*=0,006) were found between the groups (tables 9 and 10).

WORK AREA

Of the affected group with Burnout Syndrome, 95 (76.6%) participants worked in SAP, 1 (0.8%) worked in LAP, 3 (2.7%) worked in PH, 4 (3%) worked in AP, 7 (5.6%) worked in GE, 6 (4.8%) worked in FS, and 8 (6.5%) participants worked in AA. For the group without burnout, 260 (65.5%) worked in SAP group, 13 (3.2%) worked in the LAP group, 15 (3.8%) worked in PH, 17 (4.3%) worked in AP, 28 (7%) participants worked in GE, 11 (2.8%) participants worked in FS, and 53 (13.4%) participants worked in AA. There were no significant statistical differences between work area groups (table 11).

Table 9. Statistical significance between variables according to years in employment.

Contingency table burnout * years in employment

Count		Years in employment			Total
		< 10 years (el1)	10-25 years (el2)	> 25 years (el3)	
Burnout	0	265	121	11	397
	1	101	22	1	124
Total		366	143	12	521

Chi-square test

	Value	gl	Asymptotic meaning
Chi-square Pearson	10.074[a]	2	0.006
Likelihood ratio	10.929	2	0.004
Linear association by linear	9.926	1	0.002
N valid cases	521		

a: 1 box (16.7%) has an expected lower frequency than 5. The minimum expected frequency is 2.86.

Table 10. Statistical significance between groups according to years in employment.

Contingency table burnout * years in employment (Fisher exact test)

	10-25	>25
<10	0.0220**	0.3171
10-25	1.0000	1.0000

** Significant statistical differences.

MONTHLY SALARY

The percentage of participants belonging to affected groups with Burnout Syndrome were as follows: i1 with 3 participants (2.4%), i2 with 15 participants (12.1%), i3 with 36 participants (29%), i4 with 41 participants (33%), i5 with 16 participants (13%), and i6 with 13 participants (10.5%). The group without Burnout Syndrome comprising of 397 participants, distributed as follows: i1 with 12 participants (3%), i2 with 49 participants (12.3%), i3 with 70 participants (17.6%), i4 with 115 participants (29%), i5 with 41 participants (10.3%), and i6 with 110 participants (27.8%). Significant statistical differences (*P*=0.002) were found between these groups (tables 12 and 13).

Table 11. Statistical significance between variables according to labor area.

Contingency table burnout * labor area									
Count		Labor area							Total
		SAP	LAP	PH	AP	GE	FS	AA	
Burnout	0	260	13	15	17	28	11	53	397
	1	95	1	3	4	7	6	8	124
Total		355	14	18	21	35	17	61	521

Small animal practice (SAP), large animal practice (LAP), public health (PH), animal production (AP), government entities (GE), food and food safety (FS) and another area (AA).

Chi-square test			
	Value	gl	Asymptotic meaning
Chi-square Pearson	9.981[a]	6	0.125
Likelihood ratio	11.021	6	0.088
Linear association by linear	3.979	1	0.046
N valid cases	521		

a: 4 boxes (28.6%) have an expected lower frequency than 5. The minimum expected frequency is 3.33.

Table 12. Statistical significance between variables according to monthly salary.

Contingency table burnout * monthly salary								
Count		Monthly salary						Total
		i1	i2	i3	i4	i5	i6	
Burnout	0	12	49	70	115	41	110	397
	1	3	15	36	41	16	13	124
Total		15	64	106	156	57	123	521

≤100,000 Chilean Pesos (i1); 100,001-300,000 Chilean Pesos (i2); 300,001-500,000 Chilean Pesos (i3); 500,001-900,000 Chilean Pesos (i4); 900,001-1,200,000 Chilean Pesos (i5) and ≥1,200,001 Chilean Pesos (i6).

Chi-square test			
	Value	gl	Asymptotic meaning
Chi-square Pearson	19.136[a]	5	0.002
Likelihood ratio	20.878	5	0.001
Linear association by linear	7.893	1	0.005
N valid cases	521		

a:1 box (8.3%) has an expected lower frequency than 5. The minimum expected frequency is 3.57.

Table 13. Statistical significance between groups according to monthly salary.

Contingency table burnout * monthly salary (Fisher exact test)					
	i2	i3	i4	i5	i6
i1	1.0000	0.5653	1.0000	0.7548	0.4039
i2	1.0000	0.3203	0.8687	0.6908	0.0589
i3	0.3203	1.0000	0.3586	0.6176	0.0008**
i4	0.8687	0.3586	1.0000	0.6176	0.0064**
i5	0.6908	0.6176	0.6176	1.0000	0.0200**

** Significant statistical differences.

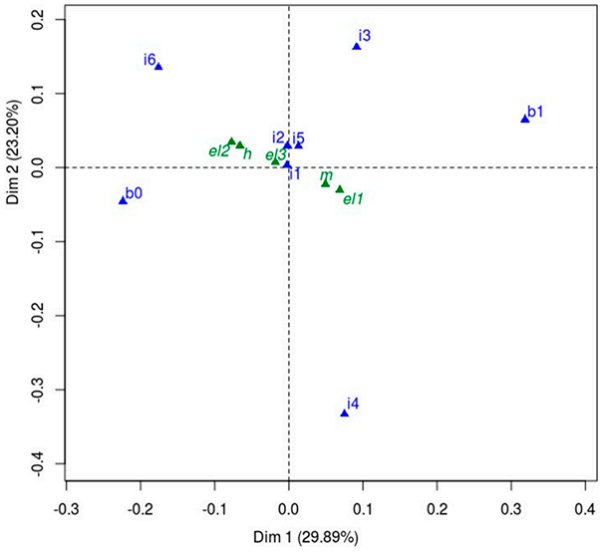

Figure 1. Correspondence analysis with active variables: Burnout Syndrome (without Burnout Syndrome (b0)/ with Burnout Syndrome (b1)), years in employment (el1 to el3), and a monthly salary (i1 to i6). Illustrative variables: gender (woman (m)/man (h)); years in employment (el1 to el3).

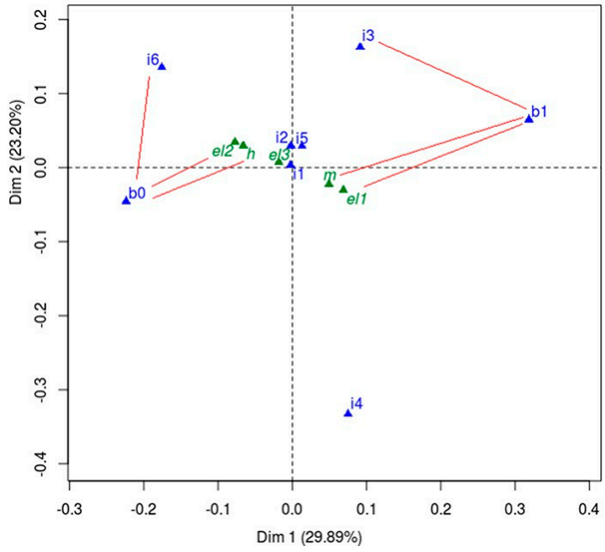

Figure 2. Interpretation correspondence analysis between variables in the study. Active variables: Burnout Syndrome (without Burnout Syndrome (b0)/ with Burnout Syndrome (b1)), years in employment (el1 to el3), and a monthly salary (i1 to i6). Illustrative variables: gender (woman (m)/man (h)); years in employment (el1 to el3).

Subsequently, correspondence analysis was carried out considering as active variables: Burnout Syndrome (without Burnout Syndrome (b0)/with Burnout Syndrome (b1)), years in employment (el1 to el3), and salary (i1 to i6). With this analysis, 53.09% of the total variability of the variable system was explained. Dimension 1 explained 29.89% of the total variance of the system. Dimension 2 explained 23.20% of the variance of the system. This analysis showed the association between the presence of Burnout Syndrome (b1) with few years of employment (el1) and lower salary (i3). The associative tendency with the female gender (m) was also visualized with b1 (figure 1). In contrast, the lack of Burnout Syndrome (b0) is associated with a higher salary (i6), average years of employment (el2), and is a tendency associated with the masculine gender (h) (figure 2).

DISCUSSION

The ILO considers the veterinary proffesion as one of the most vulnerable professions (together with health and education) to suffering from Burnout Syndrome. Our results showed Burnout Syndrome prevalence at a rate of 24%. This rate can be considered high when comparing with other studies[8] (Ordenes 2004).

In the correspondence analysis (figure 2), it is possible to see how it relates to the presence of burnout with professional experience and lower remuneration. These data agree with the analysis of the different socio-demographic variables; the veterinarians most affected by Burnout Syndrome are those with lower years of employment and lower remuneration. This result is in agreement with the conclusions of other researchers, such as Apiquian (2007), who reported that people with little work experience are the ones at higher risk (Apiquian 2007). Similarly, Nett *et al* (2015) conclude that veterinarians who have less than twenty years of work are the most affected by psychological pathologies. Hatch *et al* (2011) also propose that an influential factor in the development of Burnout Syndrome is being a recent graduate with scarce work experience. The observed relationship between burnout (less than 10 years of work experience) and professional experience (tables 8 and 9) could be explained by the effect of experiencing less safety in the work environment, a situation that would diminish over time. In contrast, Kipperman *et al* (2017) concluded that there is no statistical relationship between Burnout Syndrome and years of employment. However, this result was derived from a survey of personal appreciation and not from a validated instrument. Hence, such a finding is methodologically restricted in terms of its generalisation.

The years of employment are associated with a better employability (Acero 2004, Lee 2013), however, the Chilean scenario is very different from the USA work field. The Association of American Veterinary Medical

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Colleges (AAVMC)[9] concluded that employability reached 98% in the second year, while in Chile the employability only reaches 73% in the second year. The work field is also oversaturated with proffesionals, resulting in lower salaries. In the present investigation, the last (highest) income segment (il6) is the one with lower burnout levels when compared to any of the other segments (table 12). The data seems to point towards a significant jump in that level of income, since enough money would mean not to suffer the syndrome, which could be related to income levels that allow the coverage of basic needs and even allow benefits that the lower income segments cannot achieve. The reason the difference between the categories il1-il2 and il6 is not significant is that for this comparison there were only 15 and 64 people in categories il1 and il2, respectively. Lee (2013) does not refer directly to income but does recognise that those participants who have higher salaries have a low prevalence of burnout. The Office for National Statistics (ONS) concluded that there is a correlation between wealth and salary with the happiness of people[10]. According to ONS, a higher salary is related to lower levels of anxiety[5]. These findings are in accordance with the results of the present investigation, where it is evident that the highest salaries are found amongst those who reported less burnout.

One of the variables in which a significant statistical difference was not demonstrated in the current investigation (although an association tendency exists) was being a woman with an indication of burnout (table 4, figure 2). There are international publications where it is postulated that women make up a risk group for the development of work stress and Burnout Syndrome (Pando *et al* 2015). In this regard, there are some studies in veterinary medicine from New Zealand (Gardner and Hini 2006) and Australia (Hatch *et al* 2011) that conclude that women are a risk group for the development of work stress and associated psychological pathologies at work. However, in the present investigation it was not possible to establish significant statistical differences for this variable despite the observed trend.

In the present investigation, there are limitations such as the one that corresponds to the MBI, a validated but limited instrument since it focuses mainly on emotional aspects and not on cognitive ones[11] (Reijula *et al* 2003, Shirom 2003, Bria *et al* 2014). The results obtained in this study should be interpreted with caution; taking these results as an opportunity for improvement to prevent Burnout Syndrome.

Affected workers, universities, and professional associations must participate actively in the prevention of Burnout Syndrome since a large number of students describe financial and environmental expectations about the work context that do not contradict reality until they are very advanced in their studies. The adaptation difficulties associated with this discrepancy between professional ideals and the reality of work can be an essential factor in the challenges of adaptation and psychological distress[10] (Wanous *et al* 1992). This aspect of the adaptive complexity presents a high correlation with the symptoms related to stress and this could be remedied with an early approach of the student to the labor reality, as well as training in emotional and cognitive management related to psychic discomfort. Psychology has developed several techniques that could be integrated, both in the curricular network of universities and at work. These techniques could significantly reduce the incidence of symptoms related to adaptive difficulties, cognitive discrepancies, mental discomfort, and emotional regulation, promoting health and the resilience of future veterinarians (Bakker *et al* 2014).

It is concluded that Veterinarians working in Chile present a high prevalence of Burnout Syndrome, which is related to low monthly salary and less than ten years of employment, therefore, the hypothesis generated for this investigation is accepted. Prevention in the workplace and with undergraduate students could reduce the incidence of symptoms linked to adaptive difficulties, cognitive discrepancies, psychological discomfort, and emotional regulation, promoting health and the resilience of future veterinarians.

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SHORT COMMUNICATION

Hemicastration in Chilean rodeo stallions in competition

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ABSTRACT. The objectives of this study were: a) establish the frequency, age and reason for hemicastration, b) determine the most frequently removed testicle and c) compare the athletic performance between unilaterally castrated and intact Chilean rodeo stallions. Two hundred and sixteen Chilean Horse stallions participating in the 2016 Chilean rodeo qualifying rounds and National Championship (CRNC) were evaluated. Owners and/or riders were interrogated about age, hemicastration, age at hemicastration, removed testicle, reason and effects of hemicastration on athletic performance of stallions. The statistical analysis was descriptive and the results were expressed in means and percentages. Chi-square test was used to compare the performance between unilaterally castrated and intact stallions. Performance was qualified based on qualification to the CRNC and ultimately participation in the Champion series. Fifty-three percent of the stallions were unilaterally castrated (47% left testicle; 53% right testicle) (P>0.05). Mean age of hemicastration was 6.97±2.24 years. Main reasons reported for hemicastration were: traumatic (76%), increased volume (13%) and preventive (11%), but not all of them with medical confirmation. Seven owners or riders were unaware of the reason of hemicastration. Forty-six percent of the qualified stallions to the CRNC (P>0.05) and 29% of the qualified to the Champion series were unilaterally castrated (P=0.0122). It was concluded that half of the 2016 elite Chilean Horse stallions were unilaterally castrated, without preference for a testicle, after the age of initiation of sporting activity. A 43.5% of hemicastration in Chilean rodeo stallions are performed preventively or based on subjective assessments of owners and riders without veterinary diagnosis, however, it is unclear that hemicastration affected the athletic performance in these cases.

Key words: hemicastration, stallions, Chilean rodeo.

INTRODUCTION

The main use of the Chilean horse is the Chilean rodeo (García *et al* 1997), a sport carried out in a circular arena with a sand surface, where two horses with their respective riders must herd a steer at canter. Then, one of the horses performing a lateral canter tries to stop the steer pressing it with its chest against a padded surface (Montory 2016). The Chilean rodeo is an annual competition in which the regional rodeo associations of Chile perform competitions with the objective of achieving the sporting requirements to participate in the qualifying rounds. In the qualifying rounds they compete in order to be part of the 256 horses that participate in the Chilean Rodeo National Championship, a competition that lasts three days and is held once a year. On the last day, the 78 best horses participate in the Champion series of the Chilean Rodeo National Championship of Chile (Arancio 2016).

In the Chilean rodeo the stallions could suffer testicular trauma during the lateral movement at canter, associated with the anatomical position of the testicles and the muscles of the thighs (Apaoblaza 2015). In addition, the risk of suffering a testicular traumatism is greater considering that it has been reported that the testicular size in Chilean stallions is proportionally greater than expected for a pony (Muñoz-Alonzo *et al* 2016), and similar to the testicular size of Dutch warmblood stallions or a draft stallions at 4 years of age (Parlevliet *et al* 1994, Stout and Colenbrander 2011). Muñoz-Alonzo *et al* (2016), reported that many of the stallions participating in the Chilean rodeo are unilaterally castrated with the purpose of improving their performance. Because there are few studies regarding hemicastration in the Chilean Horse stallions (Apaoblaza 2015, Muñoz-Alonzo *et al* 2016), the objectives of this study were to: a) establish the frequency, age and reason for hemicastration, b) determine the most frequently removed testicle and c) compare the athletic performance between unilaterally castrated and intact Chilean rodeo stallions participating in the 2016 Chilean rodeo qualifying rounds and National Championship.

MATERIAL AND METHODS

The study was conducted between February and April 2016. Two hundred and sixteen Chilean Horse stallions were evaluated, from 5 to 20 years old, which correspond to all the stallions participating in the 2016 Chilean rodeo qualifying rounds and National Championship. All these stallions were considered elite horses for participating in the high classes. Individual identification of stallions participating in each event was taken from the website[1] of the Chilean Rodeo Federation. The owner or rider of each stallion was consulted personally for: name and age of the stallion and if it was unilaterally castrated. If the stallion

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[1] http://www.caballoyrodeo.cl/portal_rodeo/stat/port/resultados.html

was unilaterally castrated, the owner or rider was consulted for: age of the stallion at the time of hemicastration, the side of removed testicle, the reason for the procedure and if they perceived any effect on athletic performance after hemicastration. Then, in all the stallions, the same evaluator made a visual inspection of the testicular area to verify if it was unilaterally castrated and the side of the removed testicle. Once the 2016 Chilean Rodeo National Championship was finished, the results of the participating stallions were obtained from the website[2] of the Chilean Rodeo Federation. Within all the horses participating in the qualifying rounds of the 2016 competition, the stallions qualified for the Chilean Rodeo National Championship (n=68) were considered as high performance stallions. Within these high performance individuals, another group of 24 stallions was additionally identified, comprised by the individuals classified to the Champion series of this Championship.

The procedure used in the study was approved by the Ethics Committee of the Faculty of Veterinary Sciences of the Universidad of Concepción.

STATISTICS ANALYSIS

The statistical analysis of the frequency of hemicastration, of the most removed testicle, age and reasons of hemicastration was descriptive. The results were expressed as percentages, mean and standard deviation. Chi-square test was used to compare athletic performance between unilaterally castrated and intact stallions in three moments: participation in the 2016 qualifying rounds, qualification to the 2016 Chilean Rodeo National Championship and to the Champion series of the 2016 Chilean Rodeo National Championship.

RESULTS AND DISCUSSION

Out of the participating stallions in the 2016 Chilean rodeo qualifying rounds and National Championship: 115 (53.24%) were unilaterally castrated and 101 (46.76%) were intact (included four cryptorchids). The mean age of these was 10.9±2.24 years old. There are no studies on the frequency or prevalence of hemicastration in horse population of other breeds or equestrian sports. This could be due to that no other sport equine populations performs lateral canter in the way that the Chilean Horse does. Also, Chilean Horse Stallions have been reported with a high relative testicular size (Muñoz-Alonzo *et al* 2016). Most of the reports in the literature, correspond to statistical information on the hemicastration of stallions with unilateral testicular lesions (de Ban 1970, Maxwell 2005) or cryptorchidism performed in clinical centers (Cox *et al* 1979, Varner and Schumacher 1998, Mueller and Parks 1999, Searle *et al* 1999, Lu 2005, Marshall *et al* 2007, Hartman *et al* 2015, Huppes *et al* 2017). There is only one study conducted on 507 Chilean Horse stallions in training and or competition, which reported that 13.8% had been submitted to hemicastration (Apaoblaza 2015). The difference in the frequency of hemicastration between this study and the Apaoblaza (2015) study, could be attributed to the age difference of the evaluated stallions and because in the Apaoblaza (2015) study not all stallions were in competition.

Out of the unilaterally castrated stallions, 46.96% had the left testicle removed and 53.04% had the right testicle removed ($P>0.05$). The absence of statistical differences in relation to the most frequently removed testicle, suggests that there is no predisposition to problems in a certain testicle. This coincides with the study by Muñoz-Alonzo *et al* (2016), in addition, these researchers reported that in the Chilean Horse stallions there are no significant differences in the size of both testicles in intact stallions.

In relation to age, only 94 owners or riders knew the age of hemicastration. Table 1 shows the number and percentage of unilaterally castrated stallions, by age. The mean age of hemicastration in Chilean Horse stallions was 6.97±2.24 years old, suggesting that removal was done shortly after they start training for the praxis of Chilean rodeo, but it is more frequent in the first years of competition. This coincides with another study that reported that hemicastration occurs at the beginning of the training or in the first years of the sports activity (Marshall *et al* 2007).

Regarding the reasons of hemicastration, seven owners or riders of unilaterally castrated stallions were unaware of the reason of hemicastration. Reported reasons hemicastration (n=108) were: 64 testicular contusion (59.26%), 14 testicular size increase (12.96%), 12 preventive

Table 1. Number and percentage of Chilean rodeo stallions, by age of hemicastration (n=94).

Age of hemicastration (years of age)	Number (n)	Percentage (%)
3	3	3.19
4	6	6.38
5	10	10.64
6	30	31.91
7	15	15.96
8	12	12.77
9	5	5.32
10	5	5.32
11	5	5.32
13	2	2.13
15	1	1.06

[2] http://www.caballoyrodeo.cl/portal_rodeo/stat/port/resultados.html

(11.11%), 10 testicular ascent (9.26%) and eight because discomfort when cantering (7.41%). However, 50 (43.5%) stallions were unilaterally castrated without a veterinary diagnosis, including 12 stallions unilaterally castrated preventively. Only 65 out of 115 owners or riders were aware of the diagnosis that the veterinarian had made at the time of surgery (36 varicocele, 22 testicular trauma and seven testicular torsions). Surgically, the conditions in which hemicastration is indicated are: varicocele, testicular trauma, testicular tumors, hydrocele, orchitis, periorchitis, and most cases of scrotal hernia (Trotter 1993). This result raises doubts if hemicastration was indicated or not in the 38 stallions whose actual reason is unknown. Testicular contusion and testicular ascent are both attributable to testicular trauma, considering that it is the most frequent testicular alteration in stallions and that it manifests with increased testicular sensitivity and testicular ascent due to the retraction of the cremaster muscle (Varner and Schumacher 1999). In the Chilean Horse stallions, cantering discomfort can also be attributed to testicular trauma (Apaoblaza 2015), especially considering the large testicular size in relation to body size in Chilean Horse stallions (Muñoz-Alonzo *et al* 2016) already mentioned. However, in the Chilean rodeo stallions the possibility that some musculoskeletal injuries or pathologies could be erroneously attributed by owners or riders to testicular trauma, should not be ruled out. In fact, a study is available, based on clinical records from three veterinary practitioners, reporting musculoskeletal injuries in 114 Chilean rodeo horses and pointing out that 38.6% of the lameness involves the hindlimbs, with the most frequent lesion being unilateral tarsal osteoarthrosis (Mora-Carreno *et al* 2014). Because there is no similar background in other equestrian sports, an interesting aspect within the results was the high percentage of stallions that are unilaterally castrated in a preventive manner, probably to avoid a removal surgery while the stallions are in competition. A percentage slightly lower than 20% reported by Apaoblaza (2015) in Chilean Horse stallions reflect the low perception of risk associated with this surgery, even when there are described complications related to castrations such as anesthetic risks, hemorrhages, infections and evisceration (de Band 1970, Moll *et al* 1995, Searle *et al* 1999; Green 2001, Hartman *et al* 2015, Huppes *et al* 2017). Also, there seems to be a perception by owners that hemicastration would not affect reproductive performance. Despite the compensatory testicular hypertrophy of the residual testicle, the daily sperm production of a single testicle is significantly lower compared to an intact stallion (Hoagland *et al* 1986, McCormick *et al* 2012), which potentially reduces the number of doses that can be obtained from an ejaculate for artificial insemination.

According to the perception of the owners or riders, the athletic performance of the stallions improved after hemicastration in 95.95% of the cases that was expected in stallions with unilateral testicular problems. However, in 43.5% of cases a diagnosis had not been made by a veterinarian, so the signs or symptoms attributed by the owner or rider to a unilateral testicular problem could have corresponded to a non-diagnosed musculoskeletal injury. Therefore, in these cases, rest or light exercise and the administration of non-steroidal anti-inflammatory drugs, after hemicastration (Searle 1999, Green 2001), could have reduced or temporarily eliminated the signs of musculoskeletal impairment erroneously attributed to unilateral testicular problems.

In relation to athletic performance, out of the 68 stallions that participated in the 2016 Chilean Rodeo National Championship, 45.59% of them were unilaterally castrated and 54.41% were intact ($P>0.05$). Within the unilaterally castrated, three individuals were preventively unilaterally castrated and ten individuals had no veterinary diagnosis associated to the hemicastration. Out of the 24 stallions that qualified for the Champion series of the 2016 Chilean Rodeo National Championship, 7 (29.16%) were unilaterally castrated and 17 (70.83%) were intact ($P=0.0122$). No preventively unilaterally castrated stallions were present and three individuals had been unilaterally castrated without veterinary diagnosis. The significant difference found between the frequencies of unilaterally castrated and intact stallions in the Champion series was unexpected, because in the previous phase of the competition there was no difference between those frequencies. To explain this finding related to the low presence of unilaterally castrated stallions in the high classes of Chilean rodeo competition, we propose that the presence of pre-existing musculoskeletal injuries or pathologies that erroneously led to the hemicastration of the stallions, could reappear and negatively affect athletic performance at times of greater physical exigency, as could happen in the stallions participating in the Champion series.

The main limitations of this study were that some owners or riders did not know the reason and/or age of hemicastration of their stallion and that they did not have a definitive diagnosis of the supposed testicular problems made by a veterinarian, demonstrating a deficiency of medical records in the private veterinary practice in Chile.

The results allow us to conclude that the frequency of hemicastration in elite Chilean rodeo stallions is close to 50%, without a tendency for right or left testicle removal and that the age of hemicastration coincides with the start of competitive sports activity or is slightly later. On the other hand, the main reasons of hemicastration are traumatisms. However, an important percentage is done in a preventive manner or are based on subjective assessments of owners and riders without veterinary diagnosis. Nevertheless, it is unclear that hemicastration affects the athletic performance when it has been carried out preventively or without a veterinary diagnosis. Therefore, hemicastration should only be performed on damaged testicles after veterinary diagnosis.

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Manuscripts should be submitted to the Editor via the on-line platform www.australjvs.cl and must include:

- An electronic version of the text (MS Word format), tables (preferably in MS Word format), charts (Excel format), figures and photographs (TIFF, 300 dpi)
- A cover letter to the Editor signed by the corresponding author should include the following:

A declarative statement that the manuscript represents new information that has not been previously submitted or published elsewhere; or an explanation of any previous publication or presentation of all or parts of the manuscript.

A declarative statement that all authors of the paper have read and approved the final version of the manuscript submitted and that all have made substantive contribuionts to the work. The statement must include the email address of all the authors.

Specification of the type of manuscript that is being submitted.

A description of how the information provided in the manuscript is original, new, timely, significant, and relevant to the readers of *Austral J Vet Sci*.

- The manuscripts must be original, unpublished and may not be considered for publication in another journal.
- Manuscripts that do not conform to editorial requirements will be returned without review.
- For manuscripts describing studies involving animals or humans, the appropriate Bioethical Committee Certification must be mentioned under Material and Methods.
- Referees of Austral Journal of Veterinary Sciences will aid the Editorial Committee to determine whether the manuscript fulfills publication requirements. The authors must suggest at least three referees. All articles submitted for publication will be assessed by two referees. The referees will be selected by the Editorial Committee, and may or may not include those nominated by the authors. In the case of a disagreement between the referee's reports, a third referee will aid the Editorial Committee to reach a decision. Referees are obliged to keep all information from the articles confidential, including unpublished information. Authors should state any potential conflicts of interest at the time of submission of the manuscript. Such information will not alter established editorial and review policies but will assist the editorial staff in avoiding any potential conflicts that could give the appearance of a biased review.
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PREPARATION AND FORM OF MANUSCRIPTS

Type of articles

Review articles: provide expert summaries of current knowledge in a particular field of veterinary science, and do not necessarily have a set format. Authors should consult with the Editor before initiating a review. The Editorial Committee may solicit an expert to prepare a review, which will also be refereed and edited. Reviews must not exceed 30 pages in length, including tables, figures and references.

Scientific articles: report new advances in veterinary science based on original research. The format must include abstract, introduction, material and methods, results, discussion, acknowledgements (when pertinent) and references. The maximum length of the manuscript is 20 pages, including tables, figures and references.

Short communications: briefly inform of an advance, experimental result, new methodology, with the following format: abstract, introduction, material and methods, results and discussion (combined), acknowledgements (when pertinent) and references. The maximum length of the manuscript is 12 pages, including tables, figures and references.

Case report: is a brief note that describes preliminary findings and contributes significantly to the understanding of the Veterinary Science. The maximum length is 1,300 words which includes the main body of the text and cites. An abstract of 50 words is required, plus 15 references and two tables or figures, or one of each. Acknowledgements can be included (when pertinent). Subtitles must not be used to divide the main body of the text.

JOURNAL STYLE AND LAYOUT

General presentation: Manuscripts must be written using 12 point Times New Roman font with one and a half-line spacing, on one side only of letter paper (21.5 x 27.9 cm) using 2 cm margins on all sides. Pages must be numbered consecutively in the top right corner, and lines must be numbered on the left,

starting with number one, on all pages. The main body of the text must be indented.

Headings must be in upper case, left-justified on a separate line with no full stop following, e.g. MATERIAL AND METHODS. Only the first letter of sub-headings is capitalised. Primary sub-headings (e.g. Experimental design) should be left-justified; secondary sub-headings are left-justified and italicised. Do not use underlining and do not number sub-headings or itemised lists.

In the text, numbers must be written in numerals. When a sentence begins with a number or when necessary for clarity, this should be written in words. A decimal point must be preceded by a number (e.g. 0.5 not .5). All measurements must be reported in SI units (www.nist.gov/pml/pubs/sp811/) unless it is normal practice in a discipline to use derivatives (e.g. the Curie international unit). Dates must be formatted as 07 September, 1954 in the text, but they may be abbreviated in tables and figures. Use the 24-hour clock for times of day (e. g. 13:00 h). Chemical nomenclature must be expressed using the Biochemical Society Standards (Biochem J 209, 1-27, 1983), generic names (in lower caps) must be used for medications. If brands and sources of medications need to be included, this should be included as a foot-note. Enzymes must be identified at first mention, in accordance with the Enzyme Commission of the International Union of Biochemistry. Latin terminology and abbreviations commonly used in scientific literature, such as *in vitro*, *in vivo*, *ad libitum* must be italicised. Scientific names of animal species should be mentioned once in the text, complete and in brackets, subsequently only the common name should be used. Probability values must be presented as $P<0.05$ or $P<0.01$. Standard deviation, standard error of the mean and confidence intervals are abbreviated as follows: SD, SEM and CI, respectively.

Title

Title must be short, specific and informative. The title is centred in bold, starting at line 10 without using trade names or abbreviations. Only the first letter is capitalised. Scientific names of animal species must be mentioned in the tittle, in brackets, only in the case of non-domestic species.

Author's names and addresses

Author's names are written underneath the title, separated by a space. Use full name and separate authors by commas, as in the example: Christopher A. Westwood, Edward G. Bramley, Ian J. Lean. Superscript letters should be used after each author's name to identify the section, department, service or institute, city and country of the author where the work was conducted. The corresponding author is indicated using the superscript letter followed by an asterisk, with the telephone, mailing and email addresses indicated in the footnote.

Footnotes

These are used to indicate a web address (URL) and to define abbreviations used in table titles, commercial brands, the name and address of companies. They must be indicated with numbers.

Abstract

The second page must contain an abstract of no more than 250 words that describes the objectives of the study or research, the material and methods used, the principal results and the most important conclusions. Non-standard abbreviations must not be used. On a separate line, left-justified, and separated by a space, up to four Key words should be identified. The use of key words containing more than two words (a phrase) must be avoided.

Introduction

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.

References

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

when appropriate, authorisation to reproduce these figures must be provided.

Changes to authorship

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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