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SPECIAL ISSUE: HALF-CENTURY SPREADING VETERINARY SCIENCES

REVIEW ARTICLES

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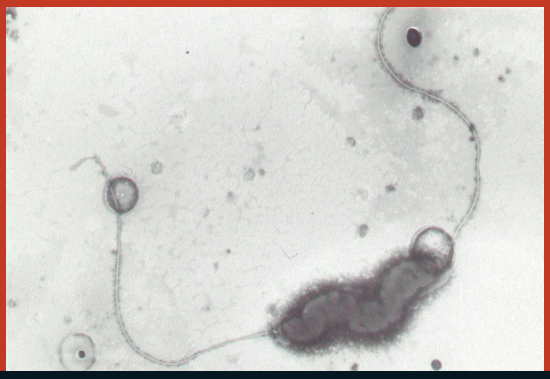

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In Memoriam

Dr. Daniel González Acuña (1962-2020)

Photograph: Elías Barticevic, INACH

For his incessant contribution to the knowledge of ectoparasites, wild animal health and veterinary medicine.

Editorial

2020: A challenging but extraordinary year for science

The past year marked the lives of many people around the world, not just those whose freedom was restricted or suffered the loss of a loved one, but all humanity. The COVID-19 pandemic produced by SARS-Cov-2 has shown once again that the constant expansion of the world's population and the degradation of ecosystems expose us to new threats. This situation is far from new, and what is more worrying is that it has been predicted for some time by the scientific community[1,2], which highlights the concern about the influence of science and evidence over decision-making. A concern that will remain once the pandemic has been controlled, and the rhythm of daily life has once again silenced the warning signs that today keep the world in suspense.

While coronaviruses have sparked interest among researchers, in December 2019 there were no scientists studying COVID-19, which is logical considering that no one knew about the disease. However, while these words are being written, nearly 90,000 scientific articles related to COVID-19 have been published and can be read on PubMed[3]. This is a reflection of the capacity of modern science to rapidly redirect efforts, allowing the establishment of faster and more efficient diagnostic tools that allow effective epidemiological control measures and adequate treatments, as well as methods to determine the health, social and cultural impact of the current pandemic.

This effort by society and the scientific world is unparalleled in the history of humanity and despite the fact that there is still much to do, we can say that it has paid off. Currently, it is possible to make the diagnosis in minutes, to know which of the different variants is the one that was contracted, and from these data to obtain a detailed vision of the origin and evolution of the disease. Every day more laboratories announce the approval of effective and safe vaccines by regulatory entities, which immediately begin to be used by governments in vaccination plans, focusing on the groups with the highest exposure and risk. Without going any further, two of the first vaccines to be approved for massive use in humans are mRNA vaccines. This technology had never been used before in a vaccine program, demonstrating unparalleled development capacity and allowed the first of these vaccines to enter phase 1 clinical trials only 66 days after the SARS-Cov-2 genome was known.

An important part of these scientific achievements have been recognised by the general population, and if there is something that we must highlight from the current situation, it is the new appreciation that general society has of science beyond the dissimilar results in the control of COVID-19 in different countries. However, this new evaluation has proven to be part of a two-sided coin, and just as there is a growing interest and appreciation for science, there is no less population that distrusts scientific results, a situation that only increases the challenges current and future that we have as scientists and humanity in general. The scientific community in general (scientists, scientific societies, government institutions, publishers, etc.), must accept part of the responsibility for this perception and misinformation which show that we must work to bring forward and humanise scientific work, because sharing our work and experience is now an essential task in the

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1 Cheng VC, Lau SK, Woo PC, Yuen KY. 2007. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 20, 660-694. https://doi.org/10.1128/CMR.00023-07

2 Fan Y, Zhao K, Shi ZL, Zhou P. 2019. Bat Coronaviruses in China. Viruses 11, 210. https://doi.org/10.3390/v11030210

3 https://pubmed.ncbi.nlm.nih.gov/?term=covid+19&sort=date

scientific career. In addition, we have to embrace the challenge of working together with the authorities, who must be open to the evidence and realise that science is an important (although not the only one) input on which to base decisions that will have an impact on thousands, if not millions of lives.

Another important lesson from this and other future emerging disease-related episodes is that the role of veterinary science should not be underestimated. Veterinary professionals constitute a workforce that provides crucial knowledge for public services that ensure the prevention and control of pandemics[4]. The increasing interaction between wild and domestic animals due to the expansion of agriculture and degradation of natural habitats in various parts of the world will continue to pose new and constant challenges to global health either for humans and animals. This scenario will require leadership from veterinary professionals and scientists dedicated to animal health, food safety, public health, ecology, and the various areas of biomedicine, in order to prevent future episodes as dramatic as those of this year 2020 that so much suffering has and will continue to cause in the near future.

Editorial Committee
Austral Journal of Veterinary Sciences

[4] Fathke RL, Rao S, Salman M. 2020. The COVID-19 pandemic: A time for veterinary leadership in one health. One Health 11, 100193. https://doi.org/10.1016/j.onehlt.2020.100193

Austral J Vet Sci **53, 1-8 (2021)**

REVIEW ARTICLE

In search of a combined brucellosis and tuberculosis vaccine for cattle

Gerhardt G. Schurig*, Stephen M. Boyle, Hamzeh Al Qublan, Nammalwar Sriranganathan

ABSTRACT. Bovine brucellosis is caused by *Brucella abortus*. The bacterial pathogen causes economic losses because it induces abortion in cattle. Vaccination of calves with live *B. abortus* strain 19 induces a certain level of protection but induces persistent antibodies against cell envelope lipopolysaccharide that make it difficult to Distinguish Infected from Vaccinated Animals (DIVA). Live vaccine *B. abortus* strain RB51 was developed to eliminate such interfering antibodies and therefore, facilitate the differentiation of infected from vaccinated animals and help in the eradication of the disease. Vaccination with strain RB51 induces levels of protection similar to strain 19 but neither of the two vaccines give complete protection. We have been working to enhance protection induced by strain RB51 vaccine. Protective *Brucella* antigens can be over-expressed in strain RB51 by introducing a plasmid containing the *leuB* gene and the genes encoding such antigens. To avoid the expression of antibiotic resistance genes, we produced a *leuB* deficient strain RB51 and introduced a plasmid containing the *leuB* gene and the genes to be over-expressed. This new strain maintains the plasmid and has induced significantly high protection levels in mice. In addition, it allowed the construction of an RB51 vaccine strain able to express *Mycobacterium bovis* protective antigens so that the vaccine could protect against brucellosis and tuberculosis simultaneously.

Key words: *Brucella*, *Mycobacterium*, vaccine, RB51, protection.

Animal brucellosis is a disease affecting various domestic and wild life species and is caused by an infection with bacteria belonging to the genus *Brucella*. The genus has several species and each species has a preference for specific animal hosts, for example, *B. abortus* mainly infects cattle, *B. melitensis* infects mainly goats and sheep and *B. suis* infects mainly pigs. These small Gram negative bacteria form a genetically coherent taxon which is related most closely to *Ochrobactrum* and more distantly to *Agrobacterium* and *Rhizobium* within the alpha-2 subgroup of the *Proteobacteriaceae*.

Most of the *Brucella* nomen species are transmissible to humans where it can cause serious acute and chronic disease with some cases resulting in death, making brucellosis an important zoonosis. *Brucella* is also a facultative intracellular parasite; the pathogenesis of brucellosis and the nature of the protective immune response are closely related to this property (Cheers 1984).

Because of the serious economic and medical consequences of brucellosis for both cattle farmers and humans in general, efforts have been made to prevent the infection through the use of vaccines (Nicoletti 1990). These were initially developed on an empirical basis, but with our current ability to manipulate the genome of the bacteria, more rational designs are being used for the development of better vaccines against the disease.

The lipopolysaccharide (LPS) molecule is closely associated with the phenotype of *Brucella* colonies in culture. *Brucella* can present itself upon culture with either a "smooth" or a "rough" colony morphology, with some strains presenting a "mucoid" phenotype (White and Wilson 1951). It is possible for smooth colonies to become rough spontaneously and some rough *Brucella* strains may revert to the smooth morphology making the degree of stability an important consideration in vaccine development. Virulence of *B. abortus* is associated with smooth morphology and therefore, *B. abortus* field strains producing acute and chronic brucellosis in cattle (and humans) are smooth. Smooth organisms have an LPS molecules containing a polysaccharide O-chain made from a homopolymer of perosamine (N-formyl-4-amino,4,6-dideoxy mannose), while rough organisms lack this O-chain on their LPS molecule; some strains possess only a greatly truncated portion of it (Caroff *et al* 1984, Moreno 1984). The O-chain of smooth *Brucella* is located on the surface of the organism. Essentially all animals infected with smooth strains respond immunologically to this O-chain by making antibodies against it. This is a very important point because, all serological tests used in the diagnosis of infected cattle are based on the detection of O-chain antibodies (Diaz R *et al* 1968, Nielsen *et al* 1988, Vemulapalli *et al* 2000). Also, it is very important to be aware that antibodies against the O-chain do not protect cattle against infection with *B. abortus* and in general, a clear role of any other *Brucella* antibodies in protection against cattle brucellosis has not been demonstrated. An effective immune response against *Brucella* infection in cattle requires a strong Cell Mediated Immune (CMI) response based on both, active T-helper1 and T-cytotoxic cells (Schurig *et al* 2002). The current understanding is that protection in cattle is highly dependent on the induction of these specific T lymphocytes and the concurrent activation of macrophages by T-helper1 cells secreting interferon-gamma (Schurig *et al* 2002, Vemulapalli *et al* 2000[c]).

The vaccine that has been most widely used to prevent bovine brucellosis is *B. abortus* strain 19 (Nicoletti 1990).

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This strain was first described in 1930 and was originally isolated from the milk of a Jersey cow as a virulent strain in 1923, but after being kept in the laboratory at room temperature for over a year, was found to have become attenuated (Buck 1930). This strain was able to induce a certain level of protective immunity in cattle. Effectiveness fluctuated depending on a variety of variables including age of vaccination, dose, route and prevalence of brucellosis in vaccinated herds (Nicoletti 1990). Strain 19 is an attenuated organism with a smooth morphology normally unable to grow in the presence of erythritol (Jones *et al* 1965). Although strain 19 is of low virulence for cattle, vaccination of pregnant cows can result in abortions. This event is rather rare, ranging from less than 1 up to 2.5% under field conditions and between 10-12% under experimentally controlled conditions (Mingle *et al* 1941, Moore 1950, Manthei 1952, Beckett and Mc Diarmid 1985, Tabynov *et al* 2016). Intravenous injections of pregnant cattle with strain 19 induces 100% abortions, while RB51 did induce up to 25% abortion (Palmer *et al* 1996). Strain 19 is pathogenic for humans and can lead to chronic infections if cases are not treated with appropriate antibiotics (Revich *et al* 1961, Wallach *et al* 2008). The most common route of human infections with strain 19 is accidental inoculation during vaccination of cattle. Strain 19 can also be found in the milk of cattle if vaccination is carried out during adulthood (Samartino *et al* 2000).

Since strain 19 is a smooth species of *Brucella*, the presence of LPS with O-chain on its surface is the reason for the appearance and persistence of O-chain antibodies in serum following administration of this vaccine (Diaz *et al* 1968, Nielsen *et al* 1988). Since these antibodies are the basis of all diagnostic tests, strain 19 vaccination prevents easy differentiation of vaccinated from infected animals and therefore, delays eradication efforts and leads to over condemnation of cattle. In order to decrease this problem to some extent, calves are vaccinated rather early in life since their antibody response to *Brucella* is weaker at that time. The problem becomes worse if calves are vaccinated for the first time later in life or are re-vaccinated for booster purposes since antibody levels will increase even more and be very persistent (Manthei 1952, Samartino *et al* 2000).

Several undesirable characteristics of strain 19, particularly its confusing effect on sero-diagnosis, led to the development of *B. abortus* strain RB51 vaccine (Schurig *et al* 1991). Strain RB51 is a highly attenuated mutant of *B. abortus* strain 2308. It is a very stable strain meaning that it does not change colony morphology upon multiple passages in culture or animals (Schurig *et al* 1991, Colby 1997). Strain RB51 has a rough colony morphology and is essentially devoid of O-chain (Schurig *et al* 1991). The *wboA* gene of strain RB51, a gene involved in the biosynthesis of the O-chain, is interrupted by an IS711 element impeding the production of O-chain (Vemulapalli *et al* 1996, 2000[a]). Since strain RB51 lacks O-chain, vaccinated cattle do not respond to the O-chain and do not develop antibodies to this antigen. Therefore, vaccinated animals remain serologically negative in all serological diagnostic tests. Furthermore, re-vaccination at any age does not induce O-chain antibodies in the animals allowing the application of "booster" vaccinations without affecting serological diagnostic tests necessary for disease eradication (Samartino *et al* 2000, Dorneles *et al* 2015). When used in cattle, one vaccination with strain RB51 induces protection levels similar to those induced by strain 19 and protection of the vaccinated animals can vary from 65% to 100% depending on the conditions prevailing in a specific herd or experiment (Schurig *et al* 2002). Revaccination with strain RB51 for a second time appears to increase immunity and represents an additional advantage of RB51 over strain 19. Since 1996, millions of cattle have been immunised with strain RB51 and reversion to a virulent form has never been observed testifying to its extreme stability.

As mentioned before, CMI responses play a critical role in resistance against intracellular bacterial infections (Schurig *et al* 2002, Vemulapalli *et al* 2000[c]). It is therefore critical that vaccines are presented to the immune system in a way that they induce a T cell response that can protect against the infection. Live bacterial vaccines are considered essential to induce an appropriate T cell mediated CMI protective response. Although the exact reasons for needing live organisms to induce the right response are debatable and are probably multiple, the synthesis of antigens by the live organisms during the immune response induction phase appears critical. Replication of the vaccine strain may be less critical than synthesis of new antigens since irradiated vaccines, where the bacteria do not replicate but are able to synthesize antigens after the irradiated vaccine application, are protective. Irradiated *Brucella* are able to induce protection through CMI responses while killed organisms are not (Montaraz and Winter 1968, Magnani *et al* 2009, Moustafa *et al* 2011). Since live bacteria are able to induce protective CMI responses, attenuated strains of bacteria are often used as live vaccines to protect against intracellular bacterial infections. Both *B. abortus* strain RB51 and strain 19 are live, attenuated vaccines able to induce protection while the same vaccines rendered metabolically inactive are not unless potent adjuvants are used (Montaraz and Winter 1968). This observation has important practical implications since live, attenuated vaccines have to be handled carefully (kept cold, use only shortly after reconstituting in the field, etc) to maintain their effectiveness.

In many cases live, attenuated vaccines do not provide high levels of protection, particularly if animals are confronted with high numbers of infectious bacteria (Manthei CA 1968). For example, a *Brucella* vaccinated cow will have a very high likelihood of being well protected against infection if she is exposed to a low number of field *B. abortus* bacteria (Manthei CA 1968). Not only does level of exposure affects the protective ability of the vaccine, vaccine dose will also affect the protective outcome (Manthei CA

1968, Confer *et al* 1985). In contrast, a cow may not be protected when exposed to the placenta and fetus of a *B. abortus* abortion where *Brucella* number in the hundreds of billions. Therefore, eradication of brucellosis not only depends on vaccination, it depends on a sustained vaccination program and good management where vaccinated animals are separated as much as possible from high level infection sources and where seropositive animals are consistently removed from the herd.

The fact that full protection in cattle is probably not achieved under most field conditions, made it relevant to work on approaches that could improve effectiveness of the current strain RB51 vaccine without changing its positive characteristics of being highly attenuated and leaving animals seronegative after one or multiple vaccinations. We hypothesized, some time ago, that over-expression (production of more than normal quantities) of a *Brucella* protective antigen by vaccine strain RB51 would result in enhancement of the vaccine's efficacy (Vemulapalli *et al* 2000[b], Vemulapalli *et al* 2002). Our studies demonstrated that we could over-express *B. abortus* Cu/Zn superoxide dismutase (SOD) protein in vaccine strain RB51(Vemulapalli *et al* 2002). SOD is considered to be one (He *et al* 2002) of probably many protective *Brucella* antigens and significantly increases the vaccine's protective capabilities in the murine model of brucellosis without altering the attenuation, stability or serological characteristics of the vaccine. This stimulated our interest in pursuing this approach to produce a more effective RB51 vaccine. Interestingly, even though homologous over-expression of Cu/Zn superoxide dismutase SOD enhanced protection significantly in mice, it did not enhanced protection against *B. abortus* infection in bison and elk (Olsen *et al* 2009, Nol *et al* 2016). In bison, the overexpressing RB51-SOD vaccine was actually less efficacious and was cleared faster from the vaccinated animals than the parenteral RB51 strain (Olson *et al* 2009). Faster clearance may have resulted in a weaker immune response and may explain the decrease in efficacy. This indicates that the murine model is not always an indicator of what may happen in cattle or other animal species making protection experiments in cattle crucial before a new vaccine can be called more effective in the target species.

Since homologous over-expression of a protective antigen can lead to a more effective vaccine, it was logical to think that expression by strain RB51 of a protective antigen derived from an unrelated infectious agent, for which protection is mediated by a CMI response, would protect against the unrelated disease as well as against infection with *Brucella*. Consistent with this hypothesis, protection against infection with *Neospora caninum* was demonstrated in the mouse model using a strain RB51 strain expressing parasite protective antigens (Rajasekaran *et al* 2008). Therefore, it may be possible to create strain RB51vaccines able to protect cattle against *Brucella* infection as well as protect against unrelated diseases simultaneously. Our approach to create a strain RB51 vaccine able to protect against *Brucella* and *Mycobacterium bovis* infections simultaneously is described below.

In order to use strain RB51 as a platform vaccine able to induce specific immune responses against a variety of homologous and/or heterologous antigens, a plasmid containing the gene encoding the foreign antigen along with an antibiotic resistance gene (as a plasmid marker) had been employed during our developmental work carried out in the past (McQuiston *et al* 1995, Vemulapalli *et al* 2000[c], Vemulapalli *et al* 2002). Because *Brucella spp*. have no naturally occurring plasmids, we adapted a broad host range plasmid that had been shown to replicate in *Brucella* (Kovach *et al* 1994). We eliminated extraneous plasmid DNA sequences and introduced a variety of promoters to allow for expression of genes derived from *Brucella* (homologous overexpression) as well as derived from other bacterial species (heterologous overexpression) and the resulting plasmid was designated as pNS or pLeuB (Seleem *et al* 2004, see figure 1).

The approach of using an antibiotic resistance gene as a selection marker has been criticized as not being environmentally safe because in the vaccinated animals, it has the potential to introduce the antibiotic resistance gene into normal and pathogenic flora. Therefore, expressing the protective antigens from a plasmid that is not dependent on an antibiotic resistance gene for selection of the desired strain, would be an advantage, as it would have a minimum environmental risk.

The *leuB* gene, encoding isopropyl malate dehydrogenase, is one of the four genes essential for the biosynthesis of leucine in *B. abortus* (Essenberg *et al* 1993). *B. abortus* is known to survive and replicate in nutrient-limited environments inside a host. Therefore, a *B. abortus* mutant lacking a gene for the biosynthesis of an essential amino acid like leucine is unlikely to survive in that environment including being able to replicate in minimal media (Essenberg *et al* 1993, Bacon *et a*l 1951, Bange *et al* 1996). The complementation of such an auxotroph with a plasmid carrying the wild-type *leuB* gene (encoding the enzyme necessary for the amino acid synthesis) would provide a means of selection and maintenance of this plasmid in *B. abortus* under minimum media conditions. Thus, we produced a *leuB* mutant in strain RB51 using allelic exchange (Rajasekaran *et al* 2008). The resultant *leuB* auxotroph (RB51leuB) cannot grow in leucine-deficient conditions but when complemented with a plasmid carrying the wild-type *B. abortus leuB* gene, the leucine deficiency of RB51*leuB* is eliminated (Rajasekaran *et al* 2008). Thus, the complemented RB51*leuB* strain can be used to over-express homologous and/or heterologous antigens and eliminate the environmental concerns related to antibiotic resistance. Importantly, the complemented RB51*leuB* strain retained the basic characteristics of the original strain RB51 which are attenuation, no induction

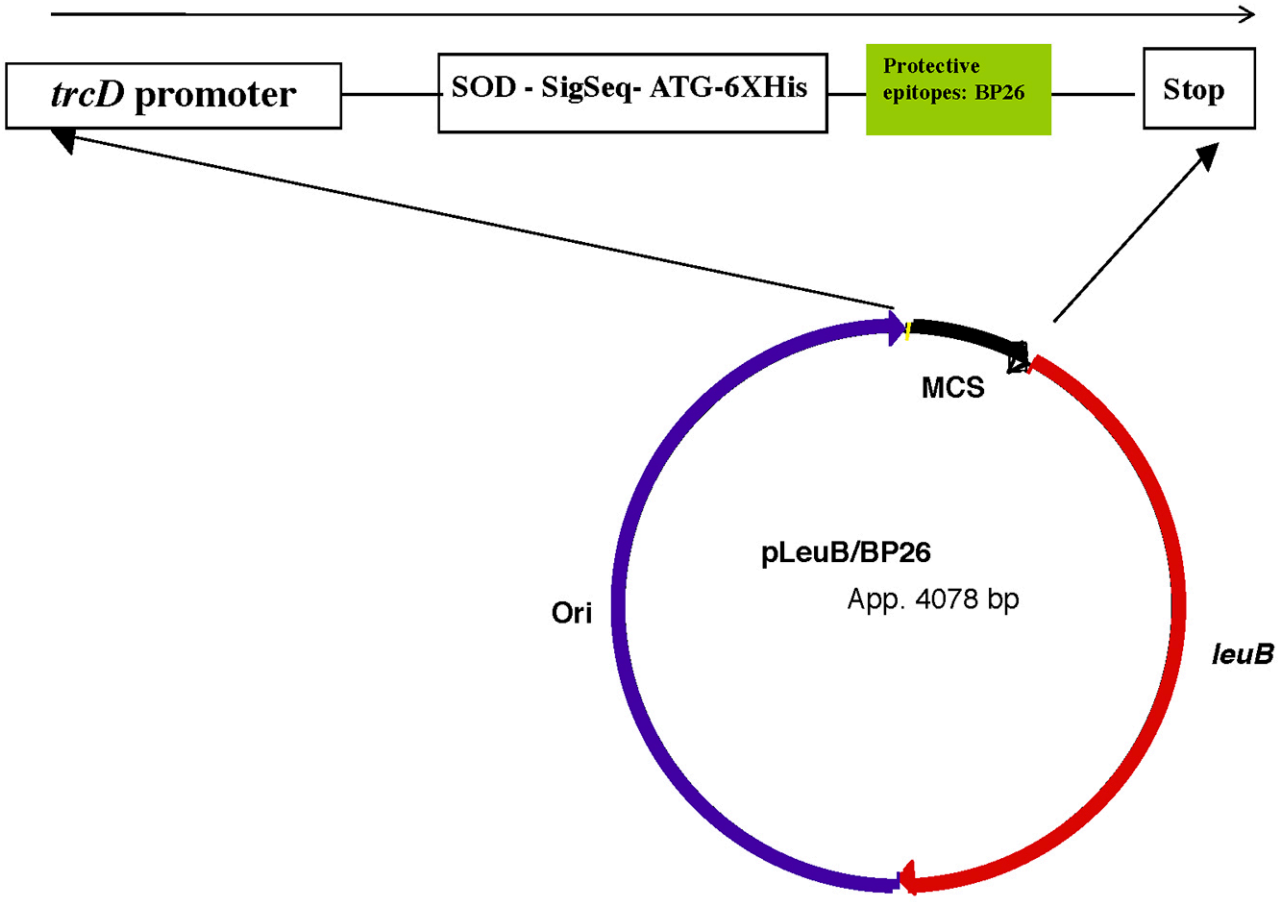

Figure 1. Schematic of pLeuB plasmid containing the leuB gene of *B. abortus*; a multiple cloning site (MCS) and an origin of replication (Ori). The trcD promoter is a very strong promoter as it is a hybrid between the lacZ and tryptophan promoter. The signal sequence (SigSeq) is from the SOD gene and followed by an ATG start codon and an in-frame histidine tag (6XHis) followed by a protective antigen from BP26 of *B. abortus*.

of O-chain antibodies and protection against *B. abortus* infection.

Using strain RB51*leuB* as a platform and pNSLeuB, an antibiotic-resistance marker free plasmid, we constructed several strains over-expressing homologous antigens. For example, strains RB51*leuB*/SOD (superoxide dismutase), RB51*leuB*/SOD/L7/L12 (ribosomal proteins) and RB51*leuB*/SOD/WboA (glycosyl transferase) were constructed to over-express the selected *Brucella* antigens: SOD alone, SOD and ribosomal protein L7/L12 or SOD and glycosyl transferase, respectively. The ability of these vaccine candidates to protect against a virulent *B. suis* challenge were evaluated in a mouse model. All vaccine groups protected mice significantly ($P<0.05$) when compared to the control group. Within the vaccine groups, the mice vaccinated with strain RB51*leuB*/SOD/WboA were significantly better protected than those that were vaccinated with either strain RB51*leuB*/SOD or RB51*leuB*/SOD/L7/L12. These results suggest that *Brucella* antigens can be over-expressed in strain RB51*leuB* and can elicit enhanced protective immune responses against brucellosis at least in the murine model of the disease (Rajasekaran *et al* 2011). The murine model for Brucellosis has been used by numerous authors to test protective abilities of vaccines (Montaraz and Winter 1968). Nevertheless, it is not clear if protective abilities observed in mice translate into protective abilities in cattle or other species. For example, work carried out with strain RB51 in mice indicating protection by strain RB51 was later demonstrated in cattle (Schurig *et al* 1991, Cheville *et al* 1993). On the other hand, RB51 overexpressing Cu/Zn superoxide dismutase showed higher protection than RB51 in mice but did not protect bison against Brucella challenge (Vemulapalli *et al* 2002, Olson *et al* 2009). It remains to be seen if similar results can be obtained in cattle.

Control of bovine tuberculosis is an important element in the control or eradication of human tuberculosis. Unfortunately, there is no practical vaccine available for the control of tuberculosis in cattle even so, the problem is very significant. To illustrate the dimension of the problem, long term data analysis of bovine tuberculosis in India suggests that approximately 7.3% of cattle are potentially infected. This means that there are 21.8 million

TB infected cattle, which amounts to more than all cattle in United States. This systematic analysis is even more alarming as approximately 10% of human tuberculosis is due to *M. bovis* infection. Therefore, control of bovine tuberculosis is an important component related to efforts of human tuberculosis eradication, especially in the developing world, as test and slaughter may not be an option due to economic and religious considerations (Srinivasan *et al* 2018).

Bacille Calmette Guerin (BCG) vaccine is the current TB vaccine used in humans. It is an attenuated strain of *M. bovis* (Behr *et al* 1997, Murray 2004). BCG was attenuated by serially passing a virulent *M. bovis* strain on ox-bile medium for 230 times in the laboratory. This process led to attenuation of the virulent *M. bovis* strain as indicated by self-limiting infection as well as partial resistance to reinfection with *M. bovis* (Takamura *et al* 2005). One of the serious disadvantages of BCG vaccine is that vaccinated humans and animals turn skin test positive. Therefore, BCG is rarely used in cattle to protect against bovine tuberculosis (Waters *et al* 2012). Recent, largescale field trials with cattle using very low doses of BCG demonstrated high levels of protection (vaccine efficacy between 85.7%-86.7%) without affecting their *ante mortem* skin or blood tests (Nugent *et al* 2018). This low dose approach may make vaccination of cattle with BCG more acceptable than in the past.

Following our previously defined strategy of over-expressing heterologous antigens in strain RB51, we speculated that expression of protective antigens from *M. bovis* in *B. abortus* strain RB51, a vaccine strain that is USDA approved and extensively used throughout the world, could make an effective dual vaccine able to protect against Brucellosis and Tuberculosis in cattle simultaneously. To prove this point, we selected the following protective *Mycobacterium* antigens: 85B, ESAT6 and Rv2660c for this purpose (Al Qublan, Dissertation, Virginia Tech, 2014). These genes were cloned and expressed in our auxotrophic *leuB* deletion mutant of strain RB51 (RB51*leuB*). As explained before, the advantage of using the RB51*leuB* deletion mutant is that one can have a plasmid encoding the protective antigens along with the *leuB* gene instead of a drug resistance gene for selection purposes

The reasons for selecting these antigens are outlined by characteristic that define them as protective antigens.

ANTIGEN 85B

Antigen 85B (Ag85B) is one of the most dominant protein antigens secreted by all mycobacterial species (Belisle *et al* 1997). Ag85B (30 kDa) belongs to the Ag85 complex, which is a family of three structurally related fibronectin-binding proteins (Ag85A, Ag85B and Ag85C) with mycolyl-transferase activity that is involved in the final stages of cell wall assembly. Ag85B protein is not only the major secretory protein of *M. tuberculosis* in broth culture, but it is also among the major proteins of all *M. bovis* proteins expressed and, it is a major stimulator of T-cell proliferation and IFN-γ production in most healthy-looking animals infected with *M. bovis*. Studies have shown that immunisation with plasmid DNA encoding Ag85B can stimulate strong cell-mediated immune response and confer significant protection to mice challenged with virulent *Mycobacterium* (Lozes *et al* 1997). All these findings suggest that Ag85B is a promising protective antigen for vaccine use in other animal species.

EARLY SECRETORY ANTIGENIC TARGET 6

Early secretory antigenic target 6 (ESAT6) is a protein encoded by the region of difference 1 (RD1) of the *M. bovis* genome. RD1 has been shown to be a major virulence factor involved with membrane-lysing activity (Smith *et al* 2008, Gao *et al* 2004). Although the exact function of ESAT6 has not been determined, studies have shown that deletion of the ESAT6 protein results in abrogation of the necrosis-inducing effect of tuberculosis on human monocyte-derived macrophages; suggesting that ESAT6 is involved with causing necrosis (Welin *et al* 2011). ESAT6 is a strongly recognized T-cell antigen in the first phase of infection and has demonstrated protective efficacy as a subunit vaccine in animal models (Brant *et al* 2000), a DNA vaccine (Qingtao *et al* 2013), and a recombinant BCG vaccine (Pym *et al* 2003).

RV2660C

Rv2660c is a newly recognised antigen of unknown function that was first reported in a gene expression profiling study by Betts and colleagues (Betts *et al* 2003). In their study, it was reported that in nutrient-starved cultures, expression of Rv2660c increased 100 to 300 fold making it the most strongly up-regulated of all nutrient starvation-induced genes identified. In another gene expression profiling study in a mouse model, it was found that Rv2660c was expressed at high levels during early and late stages of infection (Aagaard *et al* 2011). More importantly, Govender and colleagues reported that Rv2660c was preferentially recognized by patients with latent TB compared with those with active tuberculosis (Govender *et al* 2010). These findings suggest that Rv2660c is involved in latency and therefore provides a promising vaccine candidate for targeting tuberculosis infection as it transitions into latency.

Preliminary studies carried out in our laboratories, indicated that it was crucial that the DNA sequences of the protective *Mycobacterium* antigens to be cloned into our plasmid were synthesised using the codon usage of *Brucella*. The original ESAT6 sequence had a low codon optimization Index (COI) of 0.47 in *Brucella* and when optimized for strain RB51 expression, the COI changed to 0.95. Similarly, the Ag85b COI changed from 0.51 to

0.94 and Rv2660C COI changed from 0.29 to 0.93. These optimised sequences were used in the generation of the synthetic genes encoding the three protective antigens (Genscript NJ, USA). BamHI, BglII and XbaI sites were engineered into the sequences and the synthetic genes were then cloned into pLeub plasmid. Two strains were constructed one expressing 85B (RB51*leuB*/85B) and one expressing Rv2660c and ESAT6 as a fusion protein (RB51*leuB*/Rv2660c-ESAT6). The strain constructs were verified by PCR using specific primers for directional cloning.

After confirmation of expression of the tuberculosis protective antigens by Western blot, the above two vaccine constructs, RB51*leuB*/85B and RB51*leuB*/Rv2660c-ESAT6 were mixed in equal proportions and the vaccine denominated RB51TB and it was tested for its protective ability in one group of BALB/c mice. BCG immunisation testing was carried out in a second group of mice and PBS was used as the non-immunised control. The mice were vaccinated intraperitoneally (ip), boost vaccinated through the same route 4 weeks later and then challenged ip with *M. tuberculosis* four weeks after the booster vaccination. Four weeks after challenge, mice were killed and lungs and spleen were cultured to determine Colony Forming Units (CFUs) per organ. Vaccinated mice had significantly less CFUs in each cultured organ when compared to the non-vaccinated control group indicating good protection. There was no significant protection difference among the mice vaccinated with BCG and our strain RB51 vaccine constructs. These results clearly suggest that heterologous expression of protective tuberculosis antigens in strain RB51 can lead to protection against infection with *Mycobacterium* at least in the murine model. Mice were also protected against challenge with *B. abortus* indicating again that expression of heterologous antigens by strain RB51*leuB* does not alter its protective effect against brucellosis.

If the protective ability against mycobacterial infection in the murine model can be replicated in cattle, strain RB51TB vaccine could be used to protect cattle against brucellosis and tuberculosis simultaneously. In order for this vaccine to have practical application in the field, it not only has to protect against the two diseases, it should not induce positive tuberculin skin reactions in the vaccinated animals. If positive tuberculin reactions would be induced, the practical use of the vaccine would be limited.

In order to assess if RB51TB would induce positive tuberculin reactions after vaccination, eight tuberculin negative calves were immunised with RB51TB and booster vaccinated 4 months after the first vaccine application. A tuberculin test was carried out 4 months after the booster (unpublished). All cattle remained tuberculin negative suggesting that vaccination of cattle with RB51TB will not introduce problems related to tuberculosis and brucellosis diagnosis. One major challenge still remains, that is, to demonstrate that RB51TB protects cattle against tuberculosis. Such protection experiments, including safety and stability experiments, will be very costly and time consuming but will be necessary before approval to use this promising vaccine is granted.

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REVIEW ARTICLE

Dairy cow behaviour around calving: Its relationship with management practices and environmental conditions

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ABSTRACT. Calving is one of the most challenging and painful experiences for dairy cattle and a process that involves coping with physical and physiological changes, as well as environmental and management-related stressors. In recent years, it has been argued that the application of cow behaviour knowledge might facilitate their efficacious management during calving. This review aims to summarise and discuss current knowledge regarding the behavioural changes that occur around calving time. The relationship between calving behaviour, management practices, and environmental conditions in dairy cattle raised in intensive indoor production systems, as well as pasture-based systems, is also discussed. First, we briefly outline the process of parturition and the concept of maternal behaviour. We then describe behavioural changes that occur around parturition in normal and dystocic births and how variations in these behaviours can be used to predict normal or assisted calving in dairy cattle; particular emphasis is placed on the role of feeding, rumination, and lying behaviour. Finally, we review how management practices and environmental conditions can influence cow's behaviour at calving and discuss the importance of providing an environment that accommodates the behaviour they are motivated to perform. This review presents evidence that the time a cow is moved to the calving area, the type of group housing and the provision of a secluded area to calve, can impact the behavioral responses of dairy cows at calving. Evidence regarding the effects of exposure to environmental conditions such as heat during summer, and/or cold, wet and mud during winter can also have a negative impact on behaviour, suggesting potential benefits of providing cows with a protected area to calve. We conclude that a better understanding of the behaviour of parturient cows may help producers improve the care and management around calving time.

Key words: cattle, parturition, welfare, pasture.

INTRODUCTION

Calving is defined as the process of giving birth and, as in other mammals, it is a painful and stressful event for dairy cows (Maineau and Manteca 2011). Prolonged calving, delayed parturition, or severe assisted extraction of the calf at birth can result in a difficult birth, also called dystocia (Mee 2004). Research has shown the negative effect of calving difficulty on health, behaviour, and performance of dairy cows and higher neonatal mortality and morbidity in their calves (Mee 2008, Schuenemann *et al* 2011, Barrier *et al* 2013). In consequence, ensuring smooth parturition might be beneficial for the welfare of the cow and its calf.

The observation of behavioural changes is widely used to predict the onset of calving in dairy cows (Miedema *et al* 2011[a]). However, even experienced personnel may fail to detect calving time since perceptible behavioural changes do not occur for every cow or at a consistent time across calving (Ouellet *et al* 2016, Lange and Heuwieser 2017). Due to the current availability of technologies that facilitate behaviour monitoring, in recent years researchers have shown increased interest in the assessment of calving behaviour for predicting calving time (Ouellet *et al* 2016, Borchers *et al* 2017). Such sort of calving detection can be advantageous to ensure the provision of adequate supervision, timely human intervention (when difficulty arises), and early care to the newborn calf (Barrier *et al* 2012).

Understanding the behaviour of cows around the time of calving can improve the care and management (von Keyserlingk and Weary 2007) and helps to design facilities that meet the behavioural needs of the animals (Cook and Nordlund 2004). In the last 10 years, there has been a growing body of literature investigating the design and management of an appropriate calving site when provided in an indoor calving facility (i.e. Proudfoot *et al* 2014[a,b], Rørvang *et al* 2017[a], 2018[a]). However, limited research on this subject has been carried out in grazing systems where other considerations may need to be accounted for; for example, in seasonal-calving grazing systems the calving season is often associated with periods of inclement weather (i.e. cold, wet, and muddy conditions) during winter and early spring (Tucker *et al* 2007).

The aim of this review is to summarise and discuss current knowledge regarding the behavioural changes that occur around calving time. The relationship between calving behaviour, management practices, and environmental conditions in dairy cattle raised in both intensive indoor production and pasture-based systems is also discussed. First, we briefly outline the process of parturition and the concept of maternal behaviour. Secondly, we describe behavioural changes that occur around parturition in normal and dystocic births, and how variations in these behaviours

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can be used to predict normal or assisted calving in dairy cattle; particular emphasis is placed on the role of feeding, rumination and lying behaviour. Finally, we review how management practices and environmental conditions can influence the cow's behaviour at calving and discuss the importance of providing an environment that accommodates the behaviour they are motivated to perform.

PARTURITION PROCESS

Parturition is defined as the process of delivering a fully-grown foetus upon completion of the normal pregnancy period. In dairy cattle, the length of gestation ranges from 277 to 287 d depending on the breed, parity, twinning, and sex of the calf. For instance, in Holstein heifers and cows, gestation length averaged 278 and 279 d, respectively (Norman *et al* 2009). The process of parturition is traditionally divided into three phases, referred to as the stages of parturition, that move from one stage to the next (Wehrend *et al* 2006, Miedema *et al* 2011[a, b], Schuenemann *et al* 2011), ending with the delivery of the calf and the expulsion of the placenta (Noakes 2001, Schuenemann *et al* 2011).

The first stage, or dilation phase, comprises the dilation of the soft tissues of the birth canal (including the ligaments of the pelvis, cervix, and vulva), the onset of myometrial contractions, and the rotation of the fetus to its birthing position and its movement to the birth canal (Noakes 2001). It has been reported that this stage may be initiated as early as 24 h before the expulsion of the calf, but variation exists among cows (Jackson 2004). Regarding calving signs, relaxed pelvic ligaments, enlarged or tense udder, and viscous, bloody vaginal discharge are the most frequently reported signs during this stage (Proudfoot *et al* 2013). However, relaxed pelvic ligaments and suddenly enlarged udders may occur before parturition, or during the first and second stage as well (Noakes 2001). In general, the duration, frequency and amplitude of myometrial contractions increase and become more regular approximately 12 h before the onset of the second stage (Mainau and Manteca 2011). Finally, the first stage ends with the full dilatation of the cervix and the appearance of the amniotic sac outside the vulva.

The second stage or expulsion phase is characterised by the onset of rhythmic abdominal contractions, the dilation of the birth canal through the allantoic and amniotic sacs, and the progress of the calf through the birth canal. As parturition advances, the distension of the maternal birth canal causes great increases in the release of oxytocin from the posterior pituitary and this, in turn, accentuates the myometrial contractions (Noakes 2001). Schuenemann *et al* (2011) described this stage indicating that the cow adopts a recumbent position as the forces of the uterine and abdominal contractions help to expel the calf and remain in the same position until birth. The amniotic sac appears immediately before or after the onset of abdominal contractions and the calving progress - characterised by the appearance of the feet of the calf outside the vulva, followed by the nose and head (front presentation) or by the tail and pelvis of the calf (posterior presentation) - is evident every 15-20 min. As cows progress through the second stage of parturition, abdominal contractions are more frequent (between 3 and 9 abdominal contractions every 3 min). In cows experiencing normal eutocic calving, the mean time of the second stage has been reported to last between 60 and 110 min (60 min, Schuenemann *et al* 2011; 60 to 90 min, Proudfoot *et al* 2013; 110 min, Campler *et al* 2015). Also, several studies have reported that in primiparous cows the duration of the second stage is longer compared with multiparous cows (Meyer *et al* 2001, Noakes 2001, Schuenemann *et al* 2011). The longer duration of the expulsion phase has also been related to problems in the dam and its offspring (Mee 2004, Gundelach *et al* 2009). The second stage ends with the delivery of the calf or calves (Schuenemann *et al* 2011).

The third stage or expulsion of the placenta covers the period from birth until the expulsion of the fetal membranes (Noakes 2001), which normally should occur within the first 12-24 h after birth in both primiparous and multiparous cows (Schuenemann *et al* 2011). Finally, myometrial contractions persist, decreasing in amplitude but becoming more frequent and less regular in duration (Mainau and Manteca 2011).

MATERNAL BEHAVIOUR IN DAIRY COWS

In cattle, "maternal behaviour" is commonly used to describe the suite of behaviours expressed by the dam prior and after parturition that facilitate offspring survival and performance (Dwyer 2008, Chenoweth *et al* 2014). Activation of maternal behaviour is mediated by coordinated hormonal, neural and neuroendocrinal responses, in which hormones such as oestradiol, progesterone, prolactin, and oxytocin play a central role (Bridges 1984, Rosenblatt *et al* 1988). Furthermore, the expression of maternal behaviour is regulated by sensory stimuli (Rørvang *et al* 2018[a]) and experiential events over the female's lifetime (Bridges 2015).

Shortly before parturition, cows seek to isolate as a function of separating or hiding from other herd members and, therefore, give birth in a calm place (Lidfors *et al* 1994). Once the calf is born, cows invest most of their time licking the calf, a behaviour that stimulates the calf to stand, suckle, decrease heat loss and facilitate the establishment of the mother-offspring bond (von Keyserlingk and Weary 2007). Moreover, it has been shown that the expression of licking behaviour reduces the heart rate in the receiver cow (Laister *et al* 2011), suggesting a role in alleviating discomfort. Several studies report that older cows licked their newborn calves sooner compared with first parity cows (Le Niendre 1989, Jensen 2012, Campler

et al 2015), indicating that maternal experience is an important factor for cows' motivation to attend to their offspring and depends on parity number. Around 4 h after parturition, cows ingest the placenta and the amniotic fluids. It was proposed that placentophagia would have several functions, such as protection against predators, nutrient supply, immunological role, and accentuation of the hypoalgesia (Mainau and Manteca 2011). The latter might be caused by the presence of the placental opioid-enhancing factor, a molecule that potentiates the antinociception in the dam without disrupting the maternal behaviour. In cows, this analgesic effect was observed after the ingestion of amniotic fluids but not placenta (Pinheiro Machado *et al* 1997). Von Keyserlingk and Weary (2007) and more recently Rørvang *et al* (2018[a]), provide comprehensive reviews of the cattle maternal behaviour literature.

CHANGES IN THE BEHAVIOUR OF DAIRY COWS TO PREDICT CALVING TIME

In cattle, the first stage of parturition is marked by changes in feeding, rumination and lying behaviour. Also, tail raising, turning the head toward the abdomen, sniffing or licking the ground, stepping and an increased amount of time spent in lateral recumbency have been described as signs of imminent calving. It was suggested that these behavioural changes may be used to predict the onset and completion of this stage of parturition in cattle. Table 1 provides a summary of key behavioural changes observed before the onset of calving in indoor and pasture dairy systems. Next, we will discuss changes in the feeding, rumination and lying behaviour, as well as changes in activity and other behaviours observed prior and during the time of parturition, and how changes in these behaviours can be predictors of calving time in dairy cattle.

FEEDING BEHAVIOUR

Changes in dry matter intake (DMI) and feeding time on the day of calving have been well documented in dairy cows housed within intensive (indoor) group-housed systems (e.g., freestalls barns, compost barns). It has been reported that cows decreased their DMI by approximately 30% on the day of calving (Huzzey *et al* 2007, Proudfoot *et al* 2009, Schirmann *et al* 2013) compared to the previous day. This decrease occurred from 24 h to 6 h before parturition (Table 1). Time spent at the feed bunk on the day of calving follows a similar pattern as DMI (Huzzey *et al* 2007, Schirmann *et al* 2013, Büchel and Sundrum 2014), however, with a larger variation between individual cows (Miedema *et al* 2011[a], Jensen 2012).

Reduction in DMI and feeding time a few hours before calving may be due to a shift in the motivational priorities of the cow, as hypothesized by Proudfoot *et al* (2009), or due to pain and discomfort associated with the onset of parturition (Huzzey *et al* 2007). More recently, Neave *et al* (2017) reported that primiparous dairy cows consumed less feed than multiparous cows during the days around calving (d –1 to d +1). Additionally, primiparous cows spent more time at the feed bin and were more often replaced (i.e., cases in which a cow replaced another cow by physically making contact and taking her place at the feed bin) than multiparous cows in competitive interactions, reducing their ability to access the feed. This finding suggests that primiparous cows may shift their feeding times, visiting the feed bin when occupancy is lower, and thus avoiding competition with multiparous animals. Further research is necessary to understand how competitive interactions differentially affect the feeding behaviour of primiparous and multiparous animals as calving approaches.

Automated systems for measuring feed intake and feeding behaviour are more applicable to confinement housing systems where cows a receive total mixed ration, and thus it is not surprising that the majority of research has been conducted on cows housed in these types of systems. Feeding behaviour monitors are primarily used in research settings (DeVries *et al* 2003, Chapinal *et al* 2007), but commercially available feeding behaviour quantification methods have recently been developed and evaluated (Bikker *et al* 2014, Borchers *et al* 2016). Feeding behaviour monitoring is more challenging in grazing systems since visually observing grazing behaviour is time-consuming and open to the interpretation of the observer. Although there is some equipment available to identify grazing activity such as leg switch movement, jaw movement (Umemura *et al* 2009) and biting and chewing sounds (Laca and Wallis DeVries 2000), with additional information regarding bite mass and intake rate (Rutter *et al* 1997), most of these techniques are expensive and labour intensive. Delagarde and Lamberton (2015) using affordable sensor equipment (a portable accelerometer that records physical activity level) in their study and concluded that this type of technology is indeed a useful tool for the continuous automatic recording of grazing behaviour in dairy cows at pasture. To our knowledge, there are no electronic systems commercially available that will allow for detailed recording of the feeding behaviour of grazing cows, especially at calving time.

RUMINATION BEHAVIOUR

Changes in rumination behaviour have been evaluated as a signal of impending calving for cows housed in confinement (Soriani *et al* 2012, Schirmann *et al* 2013, Calamari *et al* 2014, Pahl *et al* 2014, Büchel and Sundrum 2014, Ouellet *et al* 2016, Borchers *et al* 2017). Soriani *et al* (2012) observed that rumination time declined progressively in the week before parturition, moreover, on the day of calving it decreased 3 h compared with the dry period. In concordance, Calamari *et al* (2014) found

Table 1. Behavioural changes observed before the onset of calving as reported for housed indoor or pasture dairy cows.

Behaviour	Behavioural measurement	Type of housing system	Result	Reference
Feeding	Dry matter intake	Indoor	↓ 24 h before calving	Huzzey *et al* 2007, Proudfoot *et al* 2009
			↓ 8 h before calving	Schirmann *et al* 2013
			↓ 6 h before calving	Büchel and Sundrum 2014
		Pasture	Not determined	
	Feeding time	Indoor	↓ 24 h before calving	Huzzey *et al* 2007, Proudfoot *et al* 2009
			↓ 8 h before calving	Schirmann *et al* 2013
		Pasture	Not determined	
Rumination	Rumination time	Indoor	↓ 24 h before calving	Soriani *et al* 2012, Calamari *et al* 2014
			↓ 8 h before calving	Borchers *et al* 2017
			↓ 6 h before calving	Büchel and Sundrum 2014, Ouellet *et al* 2016
			↓ 4 h before calving	Schirmann *et al* 2013, Pahl *et al* 2014
		Pasture	↓ 24 h before calving	Clark *et al* 2015
Lying	Lying time	Indoor	↓ 24 h before calving	Miedema *et al* 2011, Jensen 2012, Titler *et al* 2015, Ouellet *et al* 2016, Black and Krawczel, 2016
		Pasture	↓ 24 h before calving	Black and Krawczel, 2016, Rice *et al* 2017, Sepúlveda-Varas *et al* 2018, Hendriks *et al* 2019
	Transitions standing/ lying (lying bouts)	Indoor	↑ 4 h before calving	Jensen 2012
			↑ 6 h before calving	Miedema *et al* 2011, Ouellet *et al* 2016
			↑ 12 h before calving	Titler *et al* 2015
			↑ 24 h before calving	Black and Krawczel 2016
		Pasture	↑ 24 h before calving	Black and Krawczel 2016, Sepúlveda-Varas *et al* 2018, Hendriks *et al* 2019
			No difference	Rice *et al* 2017
Activity	Number of steps	Indoor	↑ 8 h before calving	Borches *et al* 2017,
			↑ 12 h before calving	Titler *et al* 2015
			↑ 24 h before calving	Black and Krawczel, 2016
		Pasture	↑ 24 h before calving	Black and Krawczel, 2016, Hendriks *et al* 2019
			No difference	Rice *et al* 2017
	Walking time	Indoor	↑ 24 h before calving	Miedema *et al* 2011
		Pasture	Not determined	
	Index activity	Indoor	↑ 6 h before calving	Jensen 2012
		Pasture	Not determined	
	Neck activity	Indoor	↓ 18 h before calving	Borches *et al* 2017
		Pasture	No difference	Clark *et al* 2015

↑ increase; ↓ decrease.

that the decrease in rumination time on the day of calving amounted to 70% on average of the value observed during the dry period. Table 1 shows that rumination time declines in the hours before parturition, supporting the hypothesis that rumination time measured on an hourly scale is a useful short-term predictor of calving.

Data on rumination behaviour of grazing dairy cows affected by the onset of calving is limited and the few available studies show results similar to those reported in dairy cattle in group-housed systems. In both confinement (Ouellet *et al* 2016) and pasture-based systems (Clark *et al* 2015), pregnant dairy cows ruminate less during morning and afternoon hours than in the evening and night in the days before parturition, which is in line with the diurnal normal rumination pattern of cows (Gregorini *et al* 2012). These results suggest that the reduction in rumination time during the period before calving might be independent of the type of production system (indoor versus pasture). Further investigations are needed to quantify the behavioural differences within a few hours before calving since they would be useful to identify predictors of the onset of calving in grazing cows.

Changes in rumination time at calving might be affected by parity, however, the literature on this topic is scarce. It was reported that primiparous Italian-Friesian dairy cows housed in a freestall barn spent less time ruminating than multiparous cows in the days before calving and at calving time, being this difference particularly pronounced in the week after parturition (Soriani *et al* 2012). These findings suggest that first lactation cows have more difficulties to cope with calving and the onset of lactation. Daily rumination time differs substantially between housed (Schirmann *et al* 2012) and pasture-based grazing animals (Gregorini *et al* 2012). Thus, future work should examine how rumination behaviour is affected by parity under pasture-based conditions.

In recent years, technology that allows rumination time to be easily quantified has become available (Clark *et al* 2015, Ouellet *et al* 2016, Borchers *et al* 2017), and their performance for automated calving prediction has been recently reviewed (Saint-Dizier and Chastant-Maillard 2018). Ouellet *et al* (2016) evaluated the performance of a 3-dimensional accelerometer attached to the ear tag to predict the onset of calving within 24 h, 12 h, and 6 h in housed dairy cows. The study showed that the reduction in rumination time was more accurate to predict calving within 6 h before parturition than 12 h or 24 h, however, with lower performance (63% sensitivity and specificity). Borchers *et al* (2017), using a collar consisting of an accelerometer and a microphone and applying three machine-learning techniques, found a greater level of sensitivity (72% to 79%) and specificity (80% to 89%) to predict calving within 8 h in housed dairy cows. In pasture-based dairy cows, Clark *et al* (2015) used a similar collar and achieved 70% sensitivity and 70% specificity in predicting the day of calving.

LYING BEHAVIOUR AND ACTIVITY

Reduction in lying time, more frequent posture changes and increase in walking activity are among others the most frequently reported behaviours in prepartum cows (Rørvang *et al* 2018[a]). During the last 24 h before parturition, cows managed in indoor housed conditions spent less time lying down compared with previous days, showing a greater number of shorter duration lying bouts - transitions between standing and lying - per day (Miedema *et al* 2011[a], Jensen 2012, Ouellet *et al* 2016). Similarly, cows on pasture also showed shorter lying times around calving (Rice *et al* 2017, Sepúlveda-Varas *et al* 2018, Hendriks *et al* 2019) (table 1).

An increased frequency of lying bouts has been observed in the 6 h prior to the onset of parturition (Miedema *et al* 2011[a], Jensen 2012, Ouellet *et al* 2016), reflecting the increased degree of restlessness (i.e., characterised by increased frequency of postural changes) (Miedema *et al* 2011[a], Jensen 2012) and the growing discomfort of the cow associated with the first stage of parturition (Ouellet *et al* 2016).

An increase in cow activity as calving approaches has been reported in animals housed in both confinement (Miedema *et al* 2011[a], Jensen 2012, Titler *et al* 2015, Black and Krawczel 2016, Borches *et al* 2017) and pasture settings (Black and Krawczel 2016, Hendriks *et al* 2019). However, comparison between studies is difficult because of the different methodological approaches to defining active behaviour. Miedema *et al* (2011) used continuous focal observations from video to quantify daily durations of walking and observed that cows walked for longer during the 24 h prior to calving compared to a 24-hour control period during late pregnancy. Jensen (2012) measured an activity index using an accelerometer attached to the cows' legs and reported that cows were more active the day before calving compared to 2-4 d before calving and, on an hourly scale, the level of activity increased throughout the 6 h prior to calving compared with the same time of day in the 3 preceding days. Titler *et al* (2015) monitored cow activity by counting steps using electronic data loggers and reported an increase in the number of steps within the 12 h immediately prior to calving. Borchers *et al* (2017) measured the prepartum activity using two types of devices. Firstly, they used data loggers and showed that the number of steps taken by the animals increased 8 h before calving, compared with a baseline period. Secondly, neck movements were measured using 3-axis accelerometers and it was observed that the overall neck activity decreased to its lowest value 18 h before calving, and then increased to its highest value 2 h before calving. More recently, the work of Hendriks *et al* (2019) showed an increased activity, measured by the number of steps taken using electronic data loggers, on the day of calving in grazing dairy cows.

Lying behaviour and activity at calving seem to be influenced by the housing system. Black and Krawczel

(2016) observed that cows lay down more frequently on pasture during the day of calving when compared to freestall conditions, suggesting that cows may find pasture a more comfortable surface for changing lying position compared to the mattresses commonly used in freestall calving pens. On pasture, the time required for grazing requires an increase in walking and, therefore, an increase in daily steps. In prepartum beef cows, it has been proposed that the increase in steps may also be due to cows walking in search for a safe place to calve, seeking isolation from the herd, or pacing due to discomfort (Duncan and Meyer 2019). Thus, step count differences may be detected in dairy cows kept in open grazing areas more readily than cows kept in free stalls barns because of differences in the environmental characteristics. On the contrary, another study found no change in lying bouts and numbers of steps during the days before calving in cows housed in the pasture which, according to the authors, may indicate that the pasture provides an adequate environment for cows to lie down (Rice *et al* 2017).

Differences in lying time (Miedema *et al* 2011[b], Titler *et al* 2015), frequency of transitions from lying to standing (Neave *et al* 2017) and activity (Titler *et al* 2015, Borchers *et al* 2017) have been reported between primiparous and multiparous cows in freestall housing conditions. Primiparous cows spent less time lying compared with multiparous cows during the final 24 h preceding calving (453 ± 44 min/d vs. 598 ± 40 min/d, respectively; Titler *et al* 2015) and 2 h prior to calving (Miedema *et al* 2011[b]). Authors speculated that this difference might be related to longer labour time and a higher number of contractions during parturition in primiparous cows (Miedema *et al* 2011[b], Schuenemann *et al* 2011). Primiparous cows also lay down more frequently, but for shorter periods, during the week before calving and during the period between the day before and after calving (Neave *et al* 2017) and became more active compared to multiparous cows in the last 6 h before the onset of parturition (Borchers *et al* 2017). It has been hypothesised that these behavioural differences related to parity may reflect an increased discomfort in primiparous cows as parturition approaches.

Relatively few studies looked at differences in lying behaviour of primiparous and multiparous dairy cows at calving in pasture-based systems. It was reported that first parity cows have lower daily lying times and higher frequency of lying bouts than multiparous cows during the first week after calving (Sepúlveda-Varas *et al* 2014). Moreover, increasing parity was associated with lower step counts during the period immediately before and after calving (Hendriks *et al* 2019). These findings indicate that parity number affects the frequency of lying behaviour and activity around parturition; thus, parity needs to be considered in the analyses of cow behaviour at calving time.

An advantage of measuring lying behaviour and activity is that the available technology is practical and affordable. Although increasing the number of validating sensors are available at the market which can automatically measure lying behaviour (McGowan *et al* 2007, Ledgerwood *et al* 2010, Mattachini *et al* 2013, Borchers *et al* 2016) and activity (Champion *et al* 1997, Robert *et al* 2009, Bikker *et al* 2014), few studies have attempted to evaluate the performance of these automated sensors to predict calving events using these measures. Titler *et al* (2015) used an activity index combining the number of steps, lying bouts, and standing time measured by an electronic data logger that was able to predict parturition with a time interval greater than 4 hours in 76% of the primiparous and multiparous Holstein cows evaluated in freestall barns. Using the same device, Ouellet *et al* (2016) evaluated lying time and lying bouts in housed cows (freestalls) to check the accuracy in calving prediction and found that a combination of these variables had a greater level of prediction accuracy within the next 6 h than considering the next 12 h and 24 h, but with high rates of false negatives and false positives (58% sensitivity, 61% specificity). Similarly, Borchers *et al* (2017) described calving prediction methods applying three machine-learning techniques to a combination of collected lying behavioural variables (numbers of steps, lying bouts and lying time) during the 14 prepartum days and achieved a sensitivity of 38% to 75% and a specificity of 88% to 91% in predicting the day of calving in housed dairy cows.

Since there are favourable results presented in the scientific literature on the automated detection of lying behaviours and activity to predict calving in dairy cows under confinement (Saint-Dizier and Chastant-Maillard 2018), future research should investigate whether the changes in daily lying time, lying bouts, or activity around the calving event could be used to predict the timing of calving in grazing cows.

OTHER BEHAVIOURS

In cattle, tail raising is mentioned as a sign of imminent calving (Owens *et al* 1985, Lidfors *et al* 1994, Wehrend *et al* 2006, Miedema *et al* 2011[a], Lange *et al* 2017). The frequency and duration of tail raising increase significantly the day before calving and peaks during the 6 h prior to parturition (Miedema *et al* 2011[a]). There is a growing consensus that raising the tail is one of the most consistent behavioural changes observed in the hours prior to parturition (Wehrend *et al* 2006, Miedema *et al* 2011[a], Lange *et al* 2017), making it a potentially useful indicator of calving. To date, several systems for calving prediction including inclinometers and accelerometers that detect tail raising are available (Mee *et al* 2019), but the performance of these marketed devices under field conditions still lack scientific support (SaintDizier and Chastant-Maillard 2015).

Jensen *et al* (2012), using continuous observation during the final 12 h prior to calving, reported that multiparous cows frequently turned their head toward their abdomens,

behaviour that increased during the final 2 h prior to calving and typically occurred during contractions. Pain and discomfort associated with abdominal contractions during the second stage of parturition (Mainau and Manteca 2011) likely explain that cows might be turning their head due to pain experienced during contractions as described by Jensen *et al* (2012), especially during the last hours before calving. On the contrary, Lange *et al* (2017) used repeated observations lasting 15 s every hour during the 24 h period before calving and observed that turning the head toward the abdomen did not occur often and did not increase before parturition in primiparous cows. These contradictory results may be explained by the different observational methods used since this behavioural sign lasts a few seconds and may be better detected during continuous observation rather than during repeated observations.

The literature also reports that cows display other behaviours before parturition such as stepping (Lange *et al* 2017), sniffing or licking at the ground (Miedema *et al* 2011[a]), and an increased amount of time spent in lateral recumbency (Schuenemann *et al* 2011). More recently, Lange *et al* (2017) built mathematical equations including different behaviours recorded via direct observations (i.e. tail raising, stepping, turning the head toward the abdomen, and lying lateral with abdominal contractions) to predict the second stage of parturition in dairy cows. These researchers found that the sensitivity and specificity were 69 and 88%, respectively, for predicting calving within 12 h for heifers (n = 29) examined from d 269 until calving and the positive predictive values were between 35% and 73% depending on the day of gestation, and concluded that predicting the second stage of calving by direct observation of plausible signs is imprecise and therefore not recommendable.

BEHAVIOURAL CHANGES ASSOCIATED WITH DYSTOCIA

Dystocia, defined as calving difficulty, can be the result of prolonged spontaneous calving (more than 2 hours in the second stage of parturition; Wehrend *et al* 2006, Kovács *et al* 2017) or severe assisted extraction of the calf by the farm staff (Mee 2004). Although detecting early behavioural changes associated with dystocia could serve as a useful tool to improve the handling of cattle experiencing a difficult delivery (Mee 2008), the literature is scarce. The lack of studies on pasture conditions might be likely due to the challenges associated with observing or recording calving events under field conditions.

Proudfoot *et al* (2009) found that cows with dystocia consumed approximately 2 kg dry matter less during the 48 h before calving compared with cows with eutocia (14.3 vs. 16.2 kg, respectively), and this difference increased to 2.6 kg in the 24 h before calving (8.3 vs. 10.9 kg/d). Cows with dystocia also reduced their feeding time before calving. The authors speculated that this reduction in feed intake could be due to the disproportion of the calf to dam size since larger calves could reduce the amount of space available in the rumen (Stanley *et al* 1993), or could be the result of pain associated with a large or malpositioned calf. In contrast, other studies reported that during the 6 to 12 h period preceding calving (Miedema *et al* 2011[b], Barrier *et al* 2012) or at calving time (Wehrend *et al* 2006) there are no changes in the feeding behaviour of cows with or without dystocia, most likely because the motivation to consume feed significantly decreases in the last hours prior to calving and, more drastically, at calving time (Huzzey *et al* 2005, 2007).

Focusing on rumination behaviour, cows with dystocia had lower rumination time than cows with normal calving within 8 h before parturition (13.2 ± 2.0 and 32.4 ± 2.3 min/4 h, respectively), and rumination time remained depressed for a longer period of time in dystocic cows than in eutocic cows (Kovács *et al* 2017). The fact that rumination activity is influenced by acute stress (Herskin *et al* 2004) suggest that cows with dystocia may have experienced a higher level of stress as parturition approached.

Changes in lying behaviour were also evaluated as early indicators of dystocia but with contradictory results. While some studies propose that lying frequency (i.e. number of lying or standing bouts) increased during parturition in assisted animals (Metz and Metz 1987), and this difference began during the 24 h before calving (Proudfoot *et al* 2009) or only during the final 2 h before calving (Miedema *et al* 2011[b]), compared to unassisted animals, others reported that the number of transitions from lying to standing and vice versa during the last 6 h before calving is similar between assisted and unassisted cows (Barrier *et al* 2012). More consistently, no differences in lying time have been found between cows with dystocia and cows with eutocia in the hours preceding calving (Proudfoot *et al* 2009, Barrier *et al* 2012, Miedema *et al* 2011[b]; Barraclough *et al* 2019).

Cows with dystocia display other behaviours during the hours leading up to calving compared with cows that calve without assistance. For instance, increased duration of tail raising was observed before calving in primiparous and multiparous cows that later experienced dystocia, and this was observed earlier in primiparous cows, from 4 h before calving, compared with only 2 h before calving in multiparous cows (Miedema *et al* 2011[b]). Similarly, assisted calving multiparous and primiparous cows raised their tail for a longer period of time compared to non-assisted calving cows during the last 6 h prior to calving (Barrier *et al* 2012). The latter study also found that dystocic cows lay in lateral recumbency for longer periods of time than cows calving naturally (Barrier *et al* 2012). These authors have argued that the expression of these behaviors is likely caused by acute pain experienced during the second stage of parturition when the calf enters the birth canal.

Most of the studies agree that when calving is imminent (over the last 24 h before calf expulsion), cows with

dystocia showed an increased restlessness compared to cows with eutocia (Metz and Metz 1987, Wehrend *et al* 2006, Proudfoot *et al* 2009, Miedema *et al* 2011[b], Barrier *et al* 2012). However, the definition of restlessness varies considerably between authors. Wehrend *et al* (2006) include behaviours such as rubbing against the wall, discharge of urine and scraping on the floor. Barrier *et al* (2012) assessed restlessness as being the total count of the bouts of standing, walking and of the changes in the weight distribution of the cow's body while in any lying posture. More often, frequent changes between standing and lying positions (i.e. standing or lying bouts) have been interpreted as signs of restlessness (Metz and Metz 1987, Proudfoot *et al* 2009, Miedema *et al* 2011[b]), and restlessness is commonly used to indicate discomfort and pain in animals. Indeed, dystocia is associated with high levels of pain (Mainau and Manteca 2011).

The above mentioned reports indicate that changes in the feed intake, feeding and rumination time, lying bouts, as well as tail raising behaviour, seem to be promising in the early detection of cows with a higher risk of dystocia. However, the majority of these studies should be considered preliminary due to the small sample sizes used (between 8 to 12 cows per group). Further research is needed to assess differences among parity, breeds and different housing conditions, including pasture-based systems.

MANAGEMENT PRACTICES AND ENVIRONMENTAL FACTORS AFFECTING BEHAVIOUR AT CALVING

It is well established that the provision of a comfortable environment around parturition minimises the risk of dystocia and enhances the subsequent health of the cow and calf (Mee 2004). In recent years, there has been an increased interest in housing and management practices to ease the calving process. Next, we discuss behavioural responses to common farm management practices and housing conditions including the time of moving a cow to the calving area, the social environment, and the use of a secluded area to calve. We also review the effects of cow exposure to environmental stressors such as hot and cold weather near to calving time on dairy cow´s behaviour, with particular emphasis on pasture-based systems.

TIME OF MOVING A COW TO THE CALVING SITE

Intensively managed dairy farms, cattle is generally moved to a calving area when calving is imminent, and this decision is often based on their due date and/or calving signs. Mee (2004) recommended moving cows to a calving area within 1 or 2 d before calving to allow animals to adapt to their new environment, social group, and diet. This management practice may be particularly important in heifers, where such stresses can negatively impact the calving process. Heuwiser *et al* (1987) reported that cows that were moved into a maternity pen 3 d before calving had lower blood concentrations of cortisol at calving compared with cows moved close to calving, suggesting a lower stress level in cows that had time to adapt to the new environment of the maternity pen. Additionally, Mee *et al* (2013) in a case-control study, using 30 dairy Irish herds, found that the herds with cows being transferred to the calving unit within 12 h before calving had a higher risk of perinatal mortality than herds moving cows 12 to 24 h, or more than 2 days before calving.

To date, only Proudfoot *et al* (2013) have studied the effect of moving cows from a group pen (bedded pack) to a calving area on the expression of normal calving behaviour, reporting that multiparous cows that were moved to an individual maternity pen closer to the time of calving (late first stage of parturition; moved about 2 h before calving and showing imminent signs of parturition, such as bloody mucous outside of the vulva and the start of abdominal contractions), experienced lower lying time and shorter lying bouts than cows moved with early signs of calving (early first stage of parturition; moved about 12 h before calving, showing signs of early first stage of parturition, such as raised tail, engorged, tense and leaky udder, and/or relaxed pelvic ligaments), or before any signs (moved on average 74 h before calving). These behavioural changes may be related to cows spending more time exploring their new environment to ensure that it is a safe place to give birth.

The timing of movement to a calving area is also a management practice that affects the ease of calving and health outcomes. Proudfoot *et al* (2013) showed that moving multiparous cows in the advanced first stage of parturition may interrupt and prolong the second stage by approximately 30 min. Also, cows with a longer second stage of parturition had higher inflammation levels (measured using haptoglobin) after calving compared with cows moved earlier. Cows experiencing prolonged labour may also be experiencing additional pain and restless behaviour (Mainau and Manteca 2011), increased need for assistance, and are at increased risk of dystocia (Schuenemann *et al* 2011, Barrier *et al* 2012). Similarly, Carrier *et al* (2006) reported a 2.5-fold increase in stillbirths in cows that were moved during the late part of the first stage of parturition. These findings suggest that moving a calving cow to an individual pen late in the birth process should be avoided as it represents a stressor capable of altering behaviour and affect their ability to calve normally.

Cows can also be moved too early to the calving area. In a recent study, farmers were asked about the moment when the cows and heifers were brought to the calving area, and an overall 42% of the producers said they brought their cows at the start of the prepartum period, 3 wk before their due date, while 25% said they moved their cows when the first signs of calving were detected (Villettaz-Robichaud *et al* 2016). However, first signs of calving could be anything from first to the second stage of labour (i.e. restlessness and

udder fill to bloody mucus, amniotic sac, or part of the calf emerging from the vagina). Field observations have also reported that cattle are at risk of developing postpartum health disorders such as ketosis and displaced abomasum and elevated blood non-esterified fatty acid concentrations when they remain in an individual calving pen for longer than 3 d (Cook and Nordlund 2004). It has been proposed that prolonged isolation may be stressful to cattle, which are naturally gregarious, increasing stress and the risk of disease (Sepúlveda-Varas *et al* 2013). To our knowledge, this approach has not been tested in dairy cows.

SOCIAL ENVIRONMENT

In intensive indoor dairy production systems, cows usually calve in individual calving pens or group calving pens, depending on the practices related to calving management of the farm (Cook and Nordlund 2004, Villettaz-Robichaud *et al* 2016). The use of individual calving pens has been recommended to protect the dam from potential social disturbances during calving and to reduce the risk of mismothering (Edwards 1983, Illmann and Špinka 1993). Edwards (1983) observed that when calving in groups, cows found it more difficult to maintain proximity to their calf and spent less time licking their calves compared with cows that calved alone. Also, approximately half of cows showed interest in an alien calf (a calf born from another cow which was not yet removed) before giving birth themselves. Interestingly, these behaviours did not produce the rejection of the cow's own calf, but on the contrary, could lead to the adoption of the alien calf (Illmann and Špinka 1993).

Larger commercial dairy farms are more likely to use group pens than individual pens (Villettaz-Robichaud *et al* 2016), which may not be consistent with the choice of the cows. Rørvang *et al* (2018[b]) examined whether periparturient multiparous dairy cows would voluntarily move into individual calving areas placed in a group calving location (referred to as motivation-based calving facilities). In this study, about half of the cows moved away from the group and calved in an individual calving area (34 of 66), with social factors having an important influence on the chance of a cow using the individual calving area; dominant cows were most likely to use the individual calving areas, whereas the presence of a calf born from another cow reduced the chance of cows calving inside the individual calving area.

In group calving areas, the choice of calving site may be also affected by the site of previous calving. In a study, a group of 10 cows was followed recording the location of the breaking of the amniotic sac as well as the place of birth and found that 9 out of the 10 cows calved within a distance of 1 cow length from where the previous calving took place, suggesting that the cows did not select calving site at random and potentially explained by the presence of amniotic fluid from other cows (Rørvang *et al* 2017[a]).

USE OF A SECLUDED AREA TO CALVE

The use of secluded areas in confinement calving settings has been the subject of considerable interest and revision over the last 5 years (Proudfoot *et al* 2014[a,b], Rørvang *et al* 2017[b], 2018[a,b]). Proudfoot *et al* (2014[a,b]) provided the first evidence that indoor-housed cows preferred a secluded environment to calve, particularly when they were housed individually and there was a high level of activity in the barn during the daytime. Rørvang *et al* (2017[b]) investigated dairy cows' preference regarding the degree of isolation at calving in an individual pen by offering different types (in terms of height, width or both) and levels of isolation (in terms of percentage coverage, 50% or 75%). Results showed that periparturient dairy cows had no preference for a specific calving pen design or level of isolation. However, cows experiencing a longer calving duration (longer second stage labour) were more likely to give birth in the most isolated individual calving pen (75% coverage) compared with those without prolonged labour, possibly explained by an increased level of restlessness and increased discomfort during calving that may have motivated them to seek further seclusion. In a follow-up study, researchers examined whether dairy cows would voluntarily isolate in an individual calving pen placed in a group calving setting were access to individual calving pens was free (with a gate permanently open) or for only one cow at a time (with a gate closed that a cow could push through) (Rørvang *et al* 2018[b]). Results showed that 50% of the cows calved in an individual pen, regardless of the presence of a functional gate in the pen or not. Cows housed with permanently open gates tended to be more likely to calve in the individual calving pens compared to cows housed with functional gates, suggesting that cows in labour and experiencing discomfort or pain, may have perceived the gates as obstacles rather than an advantage for isolating before calving.

Under natural extensive pasture environment, cattle search for secure places to hide at calving to find protection from predators and bond with the calf (Rorvang *et al* 2018[b]). The early work of Lidfors *et al* (1994) reported that some dairy and beef cows isolated themselves before calving but this behaviour only occurred when suitable conditions were present, such as tall grass or tree cover with appropriate grazing sites nearby. Moreover, most cows made use of a shelter for calving when this was available. However, under an intensive pastoral grazing systems, prepartum group cows are commonly managed on small paddocks sizes (defined grazing areas) that are rotationally grazed in order to provide cows with access to a fresh allocation of pasture (Roche *et al* 2005); in these systems, the majority of calvings occur within the paddock, where there are fewer opportunities to separate and hide from the herd compared to an extensive, pasture-based system. A recent study evaluated dairy cow's preference for calving environment in group-housed in a

pasture setting (Edwards 2018). Cows had free access to a covered bedded-pack and to 2.1 ha of pasture subdivided into two different sections: open pasture and pasture with natural forage cover. Results showed that the bedded-pack barn (39%) and natural forage cover (35%) were most frequently selected for calving, compared with the pasture with no forage cover (26%). Similarly, a previous study reported that, when given a choice, most dairy cows chose to calve in a straw-covered yard rather than in a pasture paddock without forage cover (Edwards 1983). These results suggest that each cow has its preferences for the environment at calving, and the characteristics of the calving area in pasture-based systems may be of importance for determining whether a cow will use it or not at calving.

Collectively, these findings provide evidence that behavioural studies can contribute to understanding the needs and motivation of the periparturient cow to find a secluded area to give birth under both indoor-housed or pasture conditions and, therefore, may also promote animal welfare. However, more research is needed to better understand calving behaviour associated with the design of calving facilities in terms of size, shape and location of the secluded area.

ENVIRONMENTAL STRESSORS

In grazing production systems in temperate regions (e.g. Chile, New Zealand, and Ireland), dairy cows are kept on pasture all year round or at least for some part of the year. In these regions, cattle are sometimes exposed to adverse weather conditions particularly during summer and winter (Mee and Boyle 2020). In natural extensive environments, cattle will often have opportunities to find shade or shelter, but shade and shelter may not be available under intensive grazing dairy farming conditions (Webster *et al* 2015). Research has shown that exposure to inclement weather has negative effects on behaviour but most of the work to date has focused on lactating dairy cows, with little research during the prepartum period or when the process of parturition is imminent. Next, we will describe behavioural changes that may be associated with the exposure to hot and cold weather conditions in temperate regions and discuss the effect of providing shade or shelter during periods of adverse weather on the behaviour of dairy cows housed outdoors.

Hot conditions. Heat stress occurs when an animal's heat load is greater than its capacity to lose heat (Van laer *et al* 2014). Heat stress in cattle not only depends on air temperature, but on another weather factors as well, such as relative humidity, solar radiation, and air movement. For instance, moderate temperatures may already induce heat stress in conjunction with high humidity and intense solar radiation (e.g., 21°C and 75% humidity) (Van laer *et al* 2014, Webster *et al* 2015).

Behavioural coping strategies used by lactating cows to avoid a rise in body temperature include modified feeding (Kendall *et al* 2006, Karimi *et al* 2015) and grazing behaviour (Kendall *et al* 2006) (i.e. shifting feeding/grazing times to cooler periods during the day), increased water intake and time spent around a water source (Muller *et al* 1994, Schütz *et al* 2010[a]), reduced lying time (Tucker *et al* 2008, Schütz *et al* 2010[a]), decreased activity and movement (West 2003, Schütz *et al* 2008) and reduced rumination time (Soriani *et al* 2013). A study that evaluated the effects of heat stress (defined as temperature-humidity index, THI > 72, equivalent to 25°C at 50% relative humidity) on behavioural changes in prepartum dairy cows under confinement systems, showed that during the last 10 d before calving the cows adapt to heat stress through increasing meal size and reducing meal duration, as well as increasing standing times (Karimi *et al* 2015). Similarly, Paudyal *et al* (2016) reported that the average rumination time during the days prior to calving (d –14 to d –1) in healthy cows was lower in the hot season (monthly average THI ≥76) compared to the cool season (monthly average THI <76; 428 vs. 447 min/d), suggesting that variations in rumination behaviour can be explained by weather conditions.

A growing body of work has shown that the seeking of shade is also an important behaviour coping strategy for pasture lactating cows when exposed to hot summer conditions in temperate climates (Kendall *et al* 2006, Tucker *et al* 2008, Schütz *et al* 2008, 2009, 2010[a]). The work of Kendall *et al* (2006) showed that during mild summer conditions (average temperature 25°C) dairy cows preferred to stand, as opposed to lying, in shaded areas probably to maximize their surface area exposed to the environment to regulate body temperature. A study by Schütz *et al* (2008) found that dairy cows in warm conditions (air temperature >30°C) chose to stand beneath the shade rather than lying after experiencing 12 h of lying deprivation. Since dairy cattle are highly motivated to lie down (Jensen *et al* 2005), the authors suggested the motivation to find shade appeared strong relative to the motivation to undertake other valued behaviours such as resting. A follow-up study showed that cows preferred shade that provided more protection from solar radiation (50 and 99% blockage versus 25%) (Schütz *et al* 2009). In the latter study, the average ambient air temperature was 20°C (range: 13-24°C). Similar results were reported by Tucker *et al* (2008) who found that cows with access to a shade that provided more protection from solar radiation, spent more time using it than cows with access to a shade that provided less protection. In both studies, cows spent more time under the shade on days with higher levels of solar radiation and air temperature. Shade use peaked when solar radiation levels were highest (Tucker *et al* 2008, Schütz *et al* 2009). In a complementary study, the effect of the amount of shade was tested using 1 of 3 treatments for 5 d: access to 2.4 m^2 or 9.6 m^2 shade/cow, or no shade

during mild summer (Schütz *et al* 2010[a]). Cows that had more access to shade spent more than twice as much time in the shade and engaged in fewer aggressive interactions (defined as the contact between any part of one cow and another that resulted in immediate hoof movement), compared to the moment when less shade was available.

Edwards (2018) evaluated the effect of heat stress on calving location preference in dairy cows housed in pasture, where cows could choose to calve either in a covered bedded-pack barn or in a pasture paddock with natural forage. Results showed that cows were more likely to calve in the pasture when they were not experiencing heat-stressed conditions (defined as THI > 68, equivalent to 21°C at 75% relative humidity). On the contrary, cows preferred to calve inside the barn during heat-stress conditions due to better protection from solar radiation.

Current research concludes that heat-stressed dairy cows, under pasture-based systems, exhibit behavioural changes that indicate that animal welfare is compromised. Cows showed a high motivation to seek and use shade to reduce the impact of heat, but if the shade is insufficient in terms of space, they will increase aggressive behaviour. Furthermore, shade availability seems to be particularly important at calving, playing a role in determining where cattle chose to calve. However, little is known about the effect of heat stress on calving progress. A better understanding of the effects of shade availability, and other mitigation alternatives, on calving behaviour of dairy cows on pasture is needed.

Cold and wet conditions. In general, cattle are much better adapted to cope with low ambient temperatures than hot ambient environments. However, in temperate regions, exposure to winter weather that combines cold, wind and rain, causes cattle to increase heat losses to the surrounding environment, increasing energy requirements to maintain body temperature (Van laer *et al* 2014, Webster *et al* 2015).

Dairy cows usually change their behaviour to adapt to increased energy demand. For example, studies using non-lactating Holstein cows have reported that animals decrease food intake and time spent lying, stand with lowered heads, and adopt postures that might reduce heat loss when exposed to a combination of simulated and natural rain and wind in winter (Tucker *et al* 2007, mean air temperature 5°C, wind chill factor -10°C; Webster *et al* 2008, mean air temperature 3°C, wind chill factor -0.3°C; Schütz *et al* 2010[b], mean air temperature 10°C, wind chill factor -11°C). Since a high body condition helps insulates cows and to maintain more stable body temperatures, thinner cows changed their behaviour to reduce heat loss to a greater extent than well-conditioned cows under cold and wet conditions (Tucker *et al* 2007).

Another natural behavioural response of dairy cows to wet and windy conditions is seeking shelter. For example, Schütz *et al* (2010[b]), exposed non-lactating pregnant dairy cattle to one of four treatments (control, wind, rain, wind and rain) created with fans and sprinklers for 22 h, and assessed their motivation to use the shelter by creating a tradeoff between time spent feeding while exposed to the weather treatments and time spent in the shelter. Results showed that cows used the shelter approximately 50% of the time but with no difference between weather treatments. Those authors have argued that cows were motivated to use the shelters for other reasons than protection from the weather, possibly as a response to social isolation. This behaviour might be particularly important in the hours prior to calving. To our knowledge, this hypothesis has not been tested in grazing dairy cows under inclement weather.

Research has shown that temporarily managing dairy cows on wet and muddy surfaces has negative effects on lying behaviour (Chen *et al* 2017, Schütz *et al* 2018). Chen *et al* (2017), in a simulated stand-off situation exposed pregnant, nonlactating Holstein cows to three levels of soil moisture: 90 (dry), 74 (muddy), or 67% (very muddy) dry matter for 5 d each. Results showed that cows spent less time lying down in muddier conditions (dry, muddy, and very muddy treatments: 13, 12, and 9 h/24 h, respectively), and this response was more marked for heifers than for cows. When cattle chose to lie down on wetter soil, they limited the surface area exposed to their surroundings by tucking their legs beneath their bodies (Chen et al 2017). More recently, Schütz *et al* (2018) assessed the use and preference of pregnant, nonlactating Holstein cows for different wood chip surface types: clean and dry, dirty (contaminated with manure) and clean and wet. During 5 d of observation, cows were kept 18 h on woodchip surface and 6 h on pasture to allow for daily feed intake. Cows on the wet surface spent the least amount of time lying when restricted to one surface for 18 h (wet: 21%, dirty: 57%, clean: 64%), and spent more time lying when on pasture for 6 h (wet: 13%, dirty: 4%, clean: 3%), a time when ideally they should be grazing. Also, when given a choice, they clearly showed that they will avoid wet and dirty surfaces. This later research also suggests that wet surfaces not only influence the duration of rest but also the quality of rest. Cows on wet woodchips spent less time lying in a lateral position and with their heads supported, indicating reduced cow comfort and quality of rest on this surface (Schütz *et al* 2018).

Spring calving season predominates in grazing systems and the cows are sometimes moved from pasture to a separate area in periods of wet weather (Shütz *et al* 2019). For instance, some farms in southern Chile use "sacrifice" paddocks to keep prepartum cows (i.e. 3 wk prior to calving) during late winter and early spring. A "sacrifice" paddock can take the pressure off the rest of the farm by allowing grass cover to build up while vulnerable soils are wet. In these winter paddocks, depending on stocking density and usage, surfaces can become wet and muddy. In a recent study of our own, we followed Holstein dairy cows during the prepartum period (e.g., from 3 wk to 1 wk prior to calving) in the winter Chilean

season (Cartes *et al* 2019, mean 24/h air temperature 7°C, range = –2 to 16 °C). Cows were housed in pairs in 6 paddocks with (n=12) and without (n=12) access to a shelter with clean and dry wood chip bedding. Cows spent 60% of their daily time in the shelters and 75% of this time they were lying down. Furthermore, cows that had access to shelter during the prepartum period spent more time lying down during the wk 3 (706 min/d vs. 559 min/d) and wk 2 (742 min/d vs. 566 min/d) before calving compared to cows without shelter access. Shelter use in the wk prior to calving was similar between treatments. This study suggests that the provision of a protected area to rest during the weeks before calving might be beneficial for cow welfare.

In summary, cows can generally tolerate low air temperatures but they seek shelter in wet, windy and muddy conditions, suggesting that protection from inclement winter weather is important even in temperate regions. Also, newborn calves exposed to cold temperatures (<10°C), wet, and windy conditions show longer times to stand up than calves born during warm and dry weather (Diesch *et al* 2004). Therefore, the use of a shelter with dry and clean bedding can mitigate the negative effects of inclement weather and wet and muddy underfoot conditions in pasture-based systems. However, there is a lack of information about the effect of continued exposure to wet and muddy conditions throughout the prepartum period. During that time, lying down and getting up safely and comfortably may be of increasing importance due to the growing fetus, as well as on the day of calving when the cow becomes more restless (Lidfors *et al* 1994, Campler *et al* 2014). The potential effect of shelter availability in winter paddocks during the prepartum period on the ease of calving has not yet been investigated.

CONCLUSIONS

Behavioural changes could be used as predictors of normal birth. However, their use as a predictor of calving requiring assistance is still a challenge. Also, common farm management practices and housing conditions, such as the time of movement to the calving area, group housing or provision of a secluded area to calve can impact the behavioural responses of dairy cows at calving. Nevertheless, there is a lack of information regarding these subjects in grazing dairy cows. Exposure to adverse environmental conditions such as heat during the summer, or cold, wet and mud during winter can also have a negative impact on the behavior of cows housed outdoors, and studies on the behaviour of periparturient cows under such weather conditions are scarce. Further investigations on the effects of management practices and environmental conditions at calving time on cow calving behaviour are needed to better understand the behaviour of parturient cows and obtain information that might help producers to improve the care and management of these animals around calving time.

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REVIEW ARTICLE

Equine strangles: An update on disease control and prevention

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ABSTRACT. *Streptococcus equi spp. equi* (SEE) causes a disease in horses commonly referred to as strangles. Carrier or reservoir equids are important for the maintenance of the bacteria between epizootics and the initiation of outbreaks on premises, they also make the control and prevention of the disease more difficult. Disease outbreaks are common in many countries, affecting negatively equine health and causing major economic losses to the equine industry. This review describes general aspects of the disease caused by SEE in horses (clinical signs, pathogenesis, epidemiology, treatment, complications) and then focuses on prevention, control and eradication mechanisms.

Key words: *Streptococcus equi spp. equi*, lymphadenopathy, guttural pouch, empyema, strangles.

GENERAL BACKGROUND

Over the years our knowledge and understanding of equine infectious diseases have been increasing, but despite these advances, the control of many important equine infectious diseases remains challenging. Furthermore, a number of significant infectious diseases are (re-)emerging in many countries and regions, causing detrimental effects on animals' welfare and impacting negatively the equine industry.

The nature of the equine industry, where horses are regularly moved to and from competitions or between breeding farms, makes it difficult to control and prevent highly contagious infectious diseases. Incidents of disease introduction associated with national and international movement of horses are regularly reported[1] (Dominguez *et al* 2016, Moreira *et al* 2019, Pusterla *et al* 2011, Christmann and Pink 2017). Nevertheless, the spread of infectious and contagious diseases carried by animals with subclinical infection remains a challenge for national and international trade, in part because government prevention and control programs are mainly focused on exotic diseases, whereas the control and prevention of endemic diseases (e.g. equine influenza, equid herpesvirus-infection, contagious equine metritis, strangles) still needs more attention.

Streptococcus equi spp. equi (SEE) causes the disease commonly referred to as strangles (also paperas, gurma, gourme, Druse, etc.), which is a highly infectious and contagious bacterial infection that affects horses, donkeys and mules of any age. The disease has a worldwide distribution but outbreaks may occur when large numbers of horses are gathered together or re-introduction *after* population immunity has decreased (Dominguez *et al* 2016, Pusterla *et al* 2011, Boyle *et al* 2018). The bacterium is highly contagious and produces high morbidity and low mortality in susceptible populations previously free of disease. Transmission occurs via direct contact with infectious exudates and via fomite transmission. After infection, some animals keep harbouring the bacterium in their upper airways (more commonly in their guttural pouches). Carrier animals are important for maintenance of the bacteria between epizootics and initiation of outbreaks on premises, they also make the control and prevention of the disease more difficult (Boyle *et al* 2018, Ivens *et al* 2011, Boyle *et al* 2009). The disease causes major economic losses to the equine industry worldwide due to its prolonged course, extended recovery period and associated serious complications.

The review describes general aspects of the disease caused by SEE in horses (clinical signs, pathogenesis, epidemiology, treatment, complications) and then focuses on prevention, control and eradication mechanisms.

CLINICAL SIGNS

Infection with SEE can occur in horses of all ages, more severe clinical signs are seen commonly in younger horses (Sweeney *et al* 2005, Pusterla *et al* 2011) whereas older horses are often less severely affected and recover more rapidly, probably due to their immune status (Pusterla *et al* 2011, Boyle *et al* 2018).

Clinical signs vary among animals but are generally characterised by abrupt onset of pyrexia followed by pharyngitis and abscess formation in the mandibular and retropharyngeal lymph nodes (Boyle *et al* 2018, Sweeney *et al* 2005). The first signs, 3 to 14 days after exposure, are lethargy and fever (>40°C) (Waller 2014). Pharyngitis and lymphadenopathy develop in most horses, causing reluctance to eat, drink, abnormal position of the head (neck extension) and even upper airway obstruction (strangulation

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[1] Animal Health Trust. 2019. Equine influenza outbreaks reported in 2019. Available at: https://www.aht.org.uk/wp-content/uploads/2019/04/Equiflunet-outbreaks-to-15-April-2019.pdf

- strangles) necessitating a tracheostomy in severe cases (Mallicote 2015). Nasal discharge is common, only some horses develop cough (Mallicote 2015, Sweeney *et al* 2005, Boyle *et al* 2018). Inflammation of the larynx and pharynx makes this area sensitive to the touch, and palpation usually causes pain, stridor, gaging and cough. Although lymphadenopathy commonly develops in mandibular and retropharyngeal lymph nodes, parotid and cranial cervical lymph nodes can also be affected. Abscesses have a firm capsule and generally rupture 1 to 4 weeks after infection into the airway/guttural pouch (causing thick nasal discharge) or break through the skin (mandibular or parotid lymph nodes) (Judy and Chaffin 1999).

Complications like neuropraxia (laryngeal hemiplegia, dysphagia, or both) and damage to the recurrent laryngeal nerve with subsequent paralysis of the arytenoid cartilage may occur, contributing to breathing difficulty (Judy and Chaffin 1999, Mallicote 2015). Infection with SEE can spread occasionally to other locations, abscesses can form in multiple locations (abdomen, mammary gland, brain, etc.), a condition that is commonly called "bastard strangles". Also, SEE pneumonias and immune-mediated vasculitis leading to limb or head swelling have been described (Sweeney *et al* 2005).

PATHOPHYSIOLOGY AND IMMUNITY

SEE, a β-hemolytic Lancefield group C Streptococcus, is generally associated with disease in equids and is not considered a normal commensal of the respiratory tract. Its hyaluronic capsule and surface protein SeM enables SEE to evade phagocytosis, while many other cell surface antigens contribute to its virulence. Intensive research has been directed toward identifying cell surface antigens for developing better diagnostics and vaccines.

The bacteria enters the nose or mouth, then it attaches to and invades the cells within the tonsillar crypts of the lingual and palatine tonsils and the follicular epithelium of the pharyngeal and tubal tonsils, reaching the lymph nodes of the head and neck within 3 hours after infection (Timoney and Kumar 2008). There is no colonisation before penetration, thus a few hours after infection SEE is difficult to detect on the mucosal surface via culture but potentially visible within epithelial cells and subepithelial tonsillar follicles (Timoney and Kumar 2008). Hence, nasal or nasopharyngeal samples may be culture negative in the early stages of infection (Timoney and Kumar 2008, Boyle *et al* 2018). A few hours later, SEE is then translocated to the mandibular and retropharyngeal lymph nodes that drain the pharyngeal and tonsillar region. The interaction of complement with SEE causes migration of large numbers of polymorphonuclear neutrophils, causing abscessation, visible 3 to 5 days after SEE has entered the lymph nodes (Boyle *et al* 2018, Sweeney *et al* 2005).

SEE releases a hyaluronic acid capsule, antiphagocytic SeM protein, H factor binding Se18.9, Mac protein, and other undetermined antiphagocytic factors that modulate the proliferation and activity of neutrophils and macrophages (Timoney and Kumar 2008, Waller 2014), causing failure to phagocytose and kill the bacteria (Waller 2014, Boyle *et al* 2018). Relatively few organisms are present at the time of initial colonisation, but substantial bacterial propagation by the time of onset of fever has been detected (Mallicote 2015).

With lysis of the abscess capsule and evacuation of the contents, bacteria are eliminated. Nasal shedding of SEE begins 2-3 days after onset of fever and persists for 2-3 weeks in most animals. Shedding may persist much longer should infection persist in the guttural pouch or a sinus cavity (Chanter *et al* 1998, Newton *et al* 1997). Some animals with preexisting immunity and without clinical signs never exhibit detectable shedding (Boyle *et al* 2018).

Rupture of the abscesses allows for easy contamination of the environment and infection of other horses (Boyle *et al* 2018, Mallicote 2015).

Most horses recover from strangles over a period of weeks (~98%). Two to 3 weeks after infection mucosal and systemic immune responses are detected, coinciding with mucosal clearance of SEE (Galan and Timoney 1985, Boyle *et al* 2009). Long-term immunity to strangles is built in most horses after infection (approx. 75%), if not treated with antibiotics (Boyle *et al* 2018, *et al* 2002, Tiwari *et al* 2007, Galan and Timoney 1985). Just after the convalescent phase horses are resistant to re-infection (Boyle *et al* 2018, Galan and Timoney 1985).

Despite the development of antibody responses, some horses fail to clear all abscess material from their guttural pouches or sinuses (approx. 10%) and the residual purulent material forms chondroids that can remain in the horse for several years and even a lifetime (Newton *et al* 1997, Verheyen *et al* 2000). SEE persists in chondroids (visible) or in mucosal surface biofilms (invisible - inapparent) and is intermittently shed from carriers into the environment. Ongoing exposure to SEE due to the presence of carriers likely contributes to the maintenance of increased levels of immunity and extended strangles-free status within isolated herds of previously infected horses. Older horses, with waning immunity, and vaccinated animals have limited susceptibility to SEE and can develop a mild form of strangles ("atypical or catarrhal strangles"). Nevertheless, these animals still shed virulent SEE, able to cause severe disease in more susceptible horses (Sheoran *et al* 1997).

Recovered mares shed immunoglobulin Gb (IgGb) and IgA in their milk and colostrum, with specificities similar to those found in nasopharyngeal mucus of convalescent horses (Galan and Timoney 1987). Milk and colostral antibodies protect suckling foals during the first weeks and months of their lives (Boyle *et al* 2009).

SEE does not survives for long in the environment (surfaces exposed to direct sunlight), but it can persist for up to 1 month in sufficiently moist areas (Weese *et al* 2009). Transmission of SEE to naïve horses from acutely

or persistently infected ones is via direct contact (nose or mouth), or through contaminated drinking water, tack, and other fomites.

The persistent SEE infection status is critical to interepizootic transmission, the recurrence and the high incidence of disease, making it very challenging to control and prevent the disease around the world.

COMPLICATIONS

Most strangles cases progress as described above and resolve after rupture of the abscessed lymph nodes (Boyle *et al* 2018, Mallicote 2015, Waller 2014). However, complication rates vary with the duration and intensity of exposure to SEE, increasing up to 20% in some cases (Sweeney *et al* 1987, Piche 1984, Ivens *et al* 2011). Case fatality rates, although generally low, can be as high as 10% during farm outbreaks (Boyle *et al* 2018, Christmann and Pink 2017). Isolation of infectious horses is therefore critical in reducing the complication and case fatality rates (Boyle *et al* 2018).

After SEE infection various sequelae can occur: establishment of chronic carriers, spread of infection from the head and neck region to other locations (metastatic abscessation), and immune-mediated complications.

METASTATIC ABSCESSATION

Commonly referred to as "bastard strangles", it occurs when the infection spreads to lymph nodes or tissues distant from the lymph nodes of the head and neck. Diagnosis is reached with a history of exposure to SEE and laboratory results consistent with chronic infection, anaemia, fever that responds to penicillin, hyperfibrinogenemia, and hyperglobulinemia. Treatment requires long-term antimicrobial therapy, and appropriate local treatment or drainage of abscesses if possible. Nevertheless, bastard strangles often results in the death of patients, especially when lungs, lymphoid tissue including the spleen in the abdomen, liver, kidneys, or brain are involved (Waller 2014).

IMMUNE-MEDIATED COMPLICATIONS

Purpura haemorrhagica (aseptic necrotizing vasculitis). It is the most frequent type of immunologic complication (immune-mediated type III hypersensitivity reaction) in response to several different SEE antigens, including anti-SeM antibody responses that result in deposition of immune complexes in blood vessels causing severe vasculitis, substantial ventral oedema, and necrosis (Sweeney *et al* 1987, Mallicote 2015, Pusterla *et al* 2003). It occurs most frequently 3 to 4 weeks after strangles or the administration of strangles vaccine (Mallicote 2015). Animals hypersensitised to SEE antigens (titers greater than 1:1,600) are at increased risk of developing purpura haemorrhagica (Sweeney *et al* 2005). Treatment includes corticosteroids (dexamethasone) and supportive care (intravenous fluids, hydrotherapy, bandaging, etc.). Most horses can recover if given good veterinary care, but mortalities have been reported (8-25%) (Waller 2014, Pusterla *et al* 2003, Sweeney *et al* 1987, Heath *et al* 1991).

Myositis. Muscle infarctions, rhabdomyolysis with acute myonecrosis, and rhabdomyolysis with progressive atrophy after SEE infection are a relatively rare, localised immunologic complication that presents as various syndromes (Mallicote 2015). Although infarctions are a severe manifestation of purpura hemorrhagica, the mechanisms of rhabdomyolysis are not known, but inflammatory cascades as with streptococcal toxic shock syndrome or direct toxic effects of SEE in muscle tissue have been hypothesised (Boyle *et al* 2018). Widespread lymphocytic inflammatory infiltrates with marked change most evident in atrophied muscle samples have been reported in the histopathologic evaluation of muscle tissue (Mallicote 2015). Horses with myositis should be treated with corticosteroids, if there are signs consistent with concurrent infection, antibiotics are also indicated (Boyle *et al* 2018).

Myocarditis. Antigens of SEE were also reported to trigger inflammation of the myocardium, causing electrocardiographic abnormalities in convalescent horses (Boyle *et al* 2018, Mallicote 2015).

DIAGNOSIS

The diagnosis of SEE infection traditionally relied on bacterial culture out of material recovered from swabs, washes from the upper respiratory tract or abscess content. Advances in molecular technology (quantitative PCR or other PCR formats) have shown deficiencies in the culture test, proving that this method is no longer the gold-standard method for the detection of SEE, diagnosis of strangles or to detect carrier status. Serology is used to identify exposure, higher titers have been found in carrier animals and are also associated with a higher risk of developing immunomodulated complications. While still cited by some as the gold standard of diagnosis, culture results must be carefully interpreted, especially when used to screen samples collected from the upper respiratory tract.

CULTURE

Culture details are reported elsewhere (Mallicote 2015, Boyle *et al* 2018, Waller 2014). Briefly, samples are cultured in blood agar containing colistin and nalidixic acid. After overnight incubation, beta-haemolytic colonies of SEE are picked and used to inoculate Todd-Hewitt nutrient broth. Turbid cultures are also used to inoculate purple broth cultures containing trehalose, lactose, or sorbitol (SEE fails to ferment these sugars, whereas *S. zooepidemicus* ferments lactose and sorbitol, and *S. dysgalactiae* subsp. *S equisimilis* ferments trehalose) (Bannister *et al* 1985).

The isolation and identification of SEE is time consuming (at least 48h) and confounded by the presence of other beta-hemolytic bacteria (*S zooepidemicus*, *S equisimilis*) that tend to overgrow SEE in culture. In samples collected from the upper respiratory tract SEE has to outcompete other normal respiratory flora in order to be readily identified in culture. Thus, culture results must be carefully interpreted, especially when used to screen samples collected from the upper respiratory tract (Boyle *et al* 2018, Mallicote, 2015). The reporting delay could also have consequences for the spread of SEE if suspected animals are not isolated when samples are collected.

PCR ASSAYS

Different PCR-based tests have been developed. The first test that targeted the 50 region of the SeM gene was reported to be 3 times more sensitive than culture (Båverud *et al* 2007, Webb *et al* 2013). However, the region is highly variable, and some SEE strains even lack the target. Therefore, a variety of other sequences and qPCR formats were developed to assure even greater specificity and sensitivity (Webb *et al* 2013, Båverud *et al* 2007), also providing fast results (qPCR test can be completed within 1-2 hours, results may be available on the same day that samples arrive at the laboratory) (Boyle *et al* 2018, Webb *et al* 2013, Mallicote 2015, Waller 2014).

Recently, a triplex qPCR assay was developed (Webb 2013) that targets two SEE specific genes (eqbE, SEQ2190) and an internal control strain of *S. zooepidemicus* (within-assay control, to reduce false-negatives). This assay has an overall sensitivity and specificity of 93.9 and 96.9%, respectively, and is able to detect 10-fold fewer quantities of SEE than culture, regardless of the presence of contaminating bacteria (Webb *et al* 2013). Therefore, the triplex qPCR sets a new benchmark for quality control and sensitivity and is now regarded as the new gold-standard test for the detection of SEE (Waller 2014). Culture assays failed to identify 39.7% of qPCR-positive samples (Webb *et al* 2013). Historically, this poor sensitivity of culture assays and its failure to correctly identify qPCR-positive samples was excused by the claim that PCR does not distinguish between dead and live organisms, and so technically false-positive reactions affect the diagnostic value of PCRs with respect to detection of actual infection (Sweeney *et al* 2005). Although this is technically correct, as DNA does not persist on mucosal surfaces, experts emphasise that any culture-positive or qPCR-positive result should be taken seriously (Boyle *et al* 2018). Positive results of the clinical application of qPCR in the diagnosis and control of field outbreaks of strangles in several countries and management settings are considered a testament to the usefulness of qPCR over culture (Boyle *et al* 2018). Experts have also recommended the use of PCR testing of an endoscopically guided guttural pouch lavage for detection of SEE in subclinical infected carrier animals. Also, the visual detection of inflammation of the guttural pouch respiratory epithelium, as well as the presence of empyema, chondroids, or enlarged retropharyngeal lymph nodes on the floor of the guttural pouch, may suggest strangles even when the lavage is negative for SEE.

SEROLOGY

Indirect enzyme-linked immunosorbent assays (iELISA) are available to detect anti-SEE antibodies (anti-SeM, anti-antigen A (SEQ2190 N-Terminal Fragment) and -antigen B or C (SeM N-Terminal Fragment).

SeM-based-iELISAs were developed to detect antibody titers to SeM that peak about 5 weeks after exposure and remain high for at least 6 months (Timoney *et al* 2007, Galan and Timoney 1985, Galan and Timoney 1987).

The SeM antibody titer can be used to: 1) detect recent infection (4-fold or greater increase in titers, 10-14 days apart); 2) support the diagnosis of SEE-associated purpura hemorrhagica or metastatic abscessation (titer ≥ 1:12,800); and 3) identify high risk of developing purpura hemorrhagica (animals with titers >1:3,200 should not be vaccinated) (Boyle *et al* 2018, Boyle *et al* 2009).

Nevertheless, false positive results were associated with cross-reactivities between anti-SeM and anti-SzM40 antibodies, produced in response to the homologue protein to SeM expressed by *Streptococcus zooepidemicus* (Waller 2014). This was overcome with preincubation of the serum with heat-killed *S. zooepidemicus* which allowed to remove cross-reactive antibodies from the samples (Davidson 2008), but the step was not incorporated in full-length SEM assays (Waller 2014). Therefore, a SeM-specific titer should not be used to determine carrier status and a single value cannot be used as a measure of active infection or protection to reinfection (Boyle *et al* 2018). SeM titers wane over time (Boyle *et al* 2017, Sheoran *et al* 1997) and horses treated with antibiotics during an outbreak seem to mount a reduced immune response, remaining susceptible to reinfection (Piche 1984).

Dual antigen or combined antigen iELISAs were then developed to overcome the cross-reactivities with *S. zooepidemicus*. This combined iELISA test detect antibodies against antigen A (SEQ2190 N-Terminal Fragment; also known as Se75.3), antigen B or C (SeM N-Terminal Fragment) of SEE, if one or both (A and B or C) antibody results exceed the cutoff, the sample is considered positive (Robinson *et al* 2013, Knowles *et al* 2010). These tests were successfully used to determine the prevalence of exposure to SEE in horse populations in Lesotho (Ling *et al* 2011), UK (Knowles *et al* 2010, Waller 2014, Ivens *et al* 2011) and Sweden (Riihimaki *et al* 2017). The antigen A and C dual antigen iELISA test was compared to a commercial iELISA (IDvet) based on the full SeM protein, showing a similar sensitivity (89.9 vs 93.3%), but higher specificity (77.0 vs 99.3%), highlighting the application of this dual antigen anti-A and -C antigen test to identify

potential carriers before they can transmit the infection (Robinson *et al* 2013). Recently, this combined antigen A and C iELISA test was recommended to identify recent infection as early as 2 weeks after infection and exposed carrier animals without signs, provided currently available vaccines have not been used (Boyle *et al* 2018).

TREATMENT OF HORSES WITH STRANGLES

In animals with lymph node abscessation hot packing and topical softening agents can help in the development and maturation of the masses, speeding up the resolution (Sweeney *et al* 2005, Mallicote *et al* 2015). Surgical intervention of sufficiently mature and soft masses can be required when abscesses do not rupture spontaneously (Sweeney *et al* 2005, Boyle *et al* 2018). Once drainage occurs, daily lavage of the abscess with dilute povidone iodine solution should be instituted (Sweeney *et al* 2005).

The use of antibiotics in the treatment of SEE infected horses is still controversial. The bacterium is sensitive to many antibiotics, but reaching SEE in organised abscesses remains challenging. Penicillin is considered the drug of choice and antibiotic resistance has not yet been reported in SEE (Boyle *et al* 2018) but has begun to emerge in some strains of *S. equi spp. zooepidemicus* (Chalker *et al* 2012).

The treatment of isolated clinically healthy in-contact animals for 3 to 5 days can prevent the development of clinical signs, but it also prevents treated animals to develop SEE immunity (Sweeney 2005) and it delays abscess maturation, extending recovery times (Waller 2014, Boyle *et al* 2018). With severe cases, antibiotic treatment is often needed to reach clinical improvement, reducing fever, lethargy and upper airway obstruction (Mallicote 2015).

PREVENTION, CONTROL AND ERADICATION MECHANISMS

In many countries strangles is an endemic disease that generally comes in waves or outbreaks when naïve population is exposed to carrier animals. Historically, SEE was considered by veterinarians and the equine industry as a common pathogen that caused a limiting disease in young horses. Nevertheless, nowadays probably due to the increase in horse movements, waning immunity and other factors, strangles outbreaks are more common and frequent, reemerging even in countries where strangles have long been a rarity. The disease continues to cause major economic losses to the equine industry worldwide, by the direct effects of the disease (isolation, treatments, prolonged disease course, extended recovery period, serious complications) and indirectly by limiting horse movements and causing the cancellation of equestrian events.

Therefore, the authors believe national and international efforts should be directed to prevent and control the disease, starting by making the disease reportable and focusing on identifying and treating carrier animals and screening regularly possible exposure of moving equids. Shedding of the SEE from carrier animals enables the onward transmission of SEE and further outbreaks of disease. Identification and treatment of persistently infected carriers is critical to break the cycle of infection and eradicate SEE.

CONTROL DURING AND AFTER OUTBREAKS

Early identification of the infectious agent is essential to minimise the impact of the disease. Measuring rectal temperatures once or ideally twice daily aids in early identification of suspect outbreak cases.

Initial clinical signs of strangles (pyrexia, nasal discharge, and enlarged mandibular lymph nodes) vary among horses and are obviously not restricted to SEE infection. However, if infection is suspected, affected animals should be isolated and biosafety measures must be applied to minimise the transmission to in-contact animals. Confirmation of clinical cases can be achieved quickly by qPCR analysis of needle aspirates from enlarged or abscessed lymph nodes (Sweeney *et al* 2005). SEE rapidly invades (draining) lymph nodes of convalescent animals, hence in initial stages of disease negative results from qPCR analysis of nasal/retropharyngeal swabs or lavages can occur, and do not necessarily rule out SEE infection, especially if clinical signs suggest otherwise (Waller 2014, Boyle *et al* 2018).

General biosecurity measures are described in detail elsewhere (Waller 2014, Boyle *et al* 2018, Mallicote 2015). Nose to nose contact and contact with nasal discharge and purulent material from erupted abscesses must be avoided.

To minimise the spread of disease, horse movements should be stopped, and farms or equine facilities can be divided into 3 color-coded groups. The *red group* includes horses that have shown 1 or more clinical signs consistent with strangles. *Yellow/Orange/Amber group* horses are those that have had direct or indirect contact with an infected horse in the red group and may be incubating the infection. Horses in the *green group* have had no contact with infected or suspect animals. Body temperatures of horses in the yellow and green group should be measured twice daily to identify new cases and move them to the red group. Equipment used with horses can be colour coded as well to avoid cross contamination. Ideally, different equipment and personnel should be assigned to the different groups, if not possible, staff should always move from the lowest to the highest risk group (from green to red).

Screening by dual antigen ELISA A and C iELISA to identify SEE-persistently-infected-horses can commence at least three weeks after the resolution of the last clinical case. Horses in the yellow and green groups must be included in the screening, otherwise carrier horses, which remained subclinical or were exposed before and did not get infected, will remain undetected and stay as a source of future re-infections. All horses from the red group and those from the yellow and green group that tested positive by

iELISA should be examined further. Ideally, upper airway with guttural pouch endoscopy should be performed, followed by the collection of bilateral guttural pouch washes (Waller 2014). If not possible, nasopharyngeal swabbing/washing samples should be collected (Boyle *et al* 2018, Waller 2014). Samples should be analysed by qPCR to maximise sensitivity. Identified carriers should be treated as described in figure 1.

PREVENTION - ERADICATION

Identification and treatment of carrier animals. Horses that are SEE carriers are persistently infected but seem clinically normal and generally remain unaffected during strangles outbreaks. In most farms, once clinical signs of affected animals resolve, the screening of unaffected animals is considered unnecessary but it should not be the case, as it is increasing the chances of keeping possible carrier animals and a future infection source in the population. Nevertheless, screening by dual antigen A and C iELISA has been successful in identifying persistently infected horses (Boyle *et al* 2009, Riihimaki *et al* 2017, Robinson *et al* 2013).

Serology for identification of infected animals. To prevent the entry of carrier animals into a herd, before entry horses should stay in quarantine and serology of blood samples collected on arrival should be performed (Waller 2014). If negative, the analysis should be repeated 14 days later to identify horses that have been incubating the infection and seroconvert after arrival. If the second sample is still negative, it should be safe to enter the new arrivals into the herd. If positive, on day zero or 14, further diagnostics are required (Waller 2014). Endoscopy of the upper airway and guttural pouches should be examined to identify obvious signs of persistent infection (empyema, chondroids, follicular hyperplasia, etc.). During this procedure, (saline wash) samples from both guttural pouches should be taken for qPCR analysis. If visually the airways are normal and qPCR analysis results are negative, it should be safe to introduce the horse into the herd. If qPCR samples test positive or chondroids are visible on endoscopy, the horse should be treated to eliminate the carrier status before entry into the premises (Waller 2014).

Treatment of carrier animals. Once carrier animals have been identified, elimination of SEE from the guttural pouches should be accomplished by endoscopic guttural pouch lavage. If large numbers of chondroids are present, surgical hyovertebrotomy and ventral drainage through the Viborg triangle can be performed, but the procedure carries inherent risks of general anesthesia and surgical dissection around major blood vessels and nerves, plus the contamination with SEE of the hospital environment (Waller 2014).

For guttural pouch lavages, horses are sedated to facilitate drainage of flush material, if chondroids are present, they can be removed with basket tools passed through the biopsy channel of the endoscope (Waller 2014, Verheyen e *et al* 2000). Lavages with isotonic saline or polyionic fluid using rigid or indwelling catheters or with a suction pump attached to the endoscope should be repeated until negative qPCR results are obtained.

Topical instillation of acetylcysteine solution (20%, weight/volume) into guttural pouches can be performed to assist empyema treatment. Once the lavage is finished,

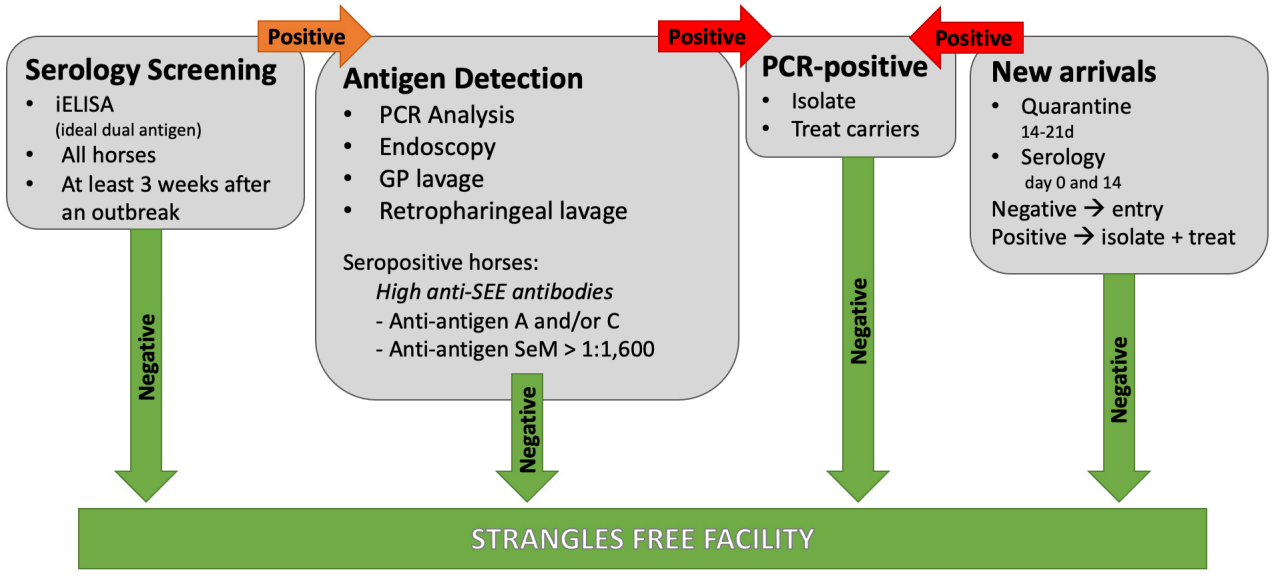

Figure 1. Diagram of procedures to reach a strangles free facility. SEE: *Streptococcus equi* subspecies *equi*; GP: guttural pouch; iELISA: indirect enzyme-linked immunosorbent assays; PCR: Polymerase C Reaction; d: days.

topical benzylpenicillin mixture can be instilled into the guttural pouches to facilitate SEE elimination Waller 2014, Boyle *et al* 2018). The administration of systemic antibiotics may further improve treatment success (Waller 2014, Boyle *et al* 2018).

In two-weeks intervals the guttural pouches should be resampled for qPCR analysis to confirm SEE elimination. Two to 3 qPCR negative results, two weeks apart, are considered sufficient to confirm elimination of carrier status.

Vaccination. Ideally, vaccines should provide adequate levels of protection against circulating SEE strains with a long duration of immunity, especially for horses that travel and are exposed to other horses from different origins. They should also be safe to be administered and capable to differentiate infected animals from vaccinated ones, which enables normal movement of vaccinated horses but also permits the identification of vaccinated horses that were exposed to and are protected from SEE (differentiate infected from vaccinated animals - DIVA) (Waller 2014).

–Killed and cell extract vaccines: These are the first types of vaccines that were developed. Nowadays, commercially available ones which are administered by the intramuscular route, include Equivac S (Zoetis New Zealand), Strepguard (MSD Animal Health), and Strepvax II (Boehringer Ingelheim). Little is known about the real efficacy of these vaccines. However, they were reported to reduce the severity and frequency of strangles but adverse reactions were common, the protection conferred was short-lived and DIVA is not possible (Hoffman *et al* 1991).

–Live-attenuated vaccines: There is one live-attenuated aroA deletion mutant available in Europe based on an isolate from Holland (Equilis StrepE, Europe; MSD Animal Health) (Kelly *et al* 2006, Jacobs *et al* 2000). Submucosal and intramuscular (IM) injection protects from lymph node abscessation (Jacobs *et al* 2000), but adverse reactions from IM injections made this administration route less practicable (Kelly *et al* 2006, Jacobs *et al* 2000, Kemp-Symonds *et al* 2007). The vaccine contains the same genetic material as virulent strains of SEE, and therefore it has no DIVA properties.

Another live-attenuated vaccine is the Pinnacle IN (Zoetis), available in North America and some other countries. It is for intranasal administration, based on the CF32 strain isolated in the USA (New York 1981) and attenuated with nitrosoguanidine. Lymph node abscess formation and bacterial shedding after vaccination were reported, it caused severe adverse effects when injected intramuscularly and it does not permits DIVA (Waller 2014). Pinnacle IN vaccine-like SEE strains were isolated in strangles cases in New Zealand, suggesting an increased sensitivity to vaccine reactions and possible revert to virulence (Patty and Cursons 2014). Efficacy has not been reported, but Zoetis claims that following experimental challenge live-attenuated vaccines for strangles can confer significant levels of protection. However, they also lack DIVA properties and adverse reactions occur (Waller 2014).

Sequence analysis of the SeM gene identified differences between strains of SEE (Parkinson *et al* 2011, Anzai *et al* 2005, Kelly *et al* 2006, Patty and Cursons 2014, Ivens *et al* 2011), suggesting that the population of SEE is changing over time as the organism is evolving with dominations of SeM-9 strains in the UK (Parkinson *et al* 2011, Ivens *et al* 2011). The vaccines Pinnacle and Equilis were derived from strains that are distantly related to dominant SeM-9 strains. It is likely that antibody responses cross-react among SEE strains (Galan and Timoney 1988) but the level of protection conferred by the vaccines against circulating SEE remains unknown (Waller 2014).

–Subunit vaccines: Recombinant SEE proteins have also been produced to generate vaccines. They are safer as they only contain target proteins and no SEE DNA, making it possible to DIVA.

Nevertheless, vaccination of horses with three different subunit vaccines (recombinant SeM; SEE-specific proteins; SEE adhesin proteins) did not protect against *in vivo* challenge with SEE (Sheoran *et al* 2002, Timoney *et al* 2007), despite the generation of serum antibody response. The inclusion of immunoglobulin-cleaving proteins IdeE and IdeE2 in subunit vaccines were found to be important for effective protection (Guss *et al* 2009). A developed vaccine (known as Strangvac), based on a SeM-9 SEE strain isolated in Sweden (Guss *et al* 2009), included immunoglobulin-cleaving proteins IdeE and IdeE2 but not SeM or SEQ2190 proteins, to reach DIVA properties. The strain used is more closely related to the circulating strains in Europe (UK) and was found to provide protection against *in vivo* challenge (Guss *et al* 2009).

CONCLUSIONS

Strangles outbreaks are common in many countries and they continue to affect equine health negatively and cause major economic losses to the equine industry worldwide.

National and international efforts should be directed to establish effective and on-going disease surveillance together with the identification and treatment of persistently infected carriers.

Developed diagnostic tests (qPCR and serology) have greatly improved over the years and should become available and accessible worldwide to assist the identification of persistently infected equids to prevent future outbreaks.

Although strangles vaccination is used in many equine facilities to reach herd immunity, conferred protection by available vaccines is limited and their antibody response cannot be differentiated by existing serology tests. Further research is needed to generate and test effective vaccines and to use diagnostic testing alongside vaccinations that provide adequate herd immunity.

For now, we believe that the surveillance of outbreaks as well as the testing and treating of SEE carriers can reach disease control and prevention and will lead to the break of the cycle of infection, and, eventually, to disease eradication.

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REVIEW ARTICLE

Gurltia paralysans: a neglected parasite of domestic cats

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ABSTRACT. *Gurltia paralysans* (order Strongylida; family Angiostrongylidae) is a metastrongyloid parasite that causes chronic meningomyelitis in domestic cats in South America. The geographic distribution of *G. paralysans* includes rural and peri-urban areas of Chile and Argentina. However, feline gurltiosis has recently been reported in other South American countries, including Uruguay, Colombia, and Brazil, and was also recently reported in Tenerife, Canary Islands (Spain). Feline gurltiosis is increasingly detected in domestic cats in southern Chile and its apparent geographic range is also increasing, together with an awareness of the disease among veterinarians. The life cycle of the parasite is unknown, but is probably indirect, involving gastropods as the intermediate host, as in other metastrongyloid nematode species. The clinical signs of *G. paralysans* infection include progressive pelvic limb ataxia, paraparesis, paraplegia, faecal or urinary incontinence, and/or tail paralysis. A definitive diagnosis of feline gurltiosis is still challenging and only possible with necropsy, when adult *G. paralysans* nematodes are detected within the spinal cord vasculature, together with macroscopic lesions, and characteristic morphological features. A semi-nested PCR method was recently developed for the *in vivo* diagnosis of this neglected parasite. Current treatment options include macrocyclic lactones and mylbemicn oxime, but the prognosis is poor in severe cases. In this article, we review *G. paralysans* infection in cats, focusing on the diagnosis shortcomings and the future directions of research into its biology and the associated neurological disease. Comprehensive updates on the epidemiology and clinical features, diagnosis, treatment, and prevention of feline gurltiosis are provided.

Key words: *Gurltia*, feline, nematode, spinal cord.

INTRODUCTION

Gurltia paralysans (Nematoda; order Strongylida; superfamily Metastrongyloidea; family Angiostrongylidae) is a metastrongyloid parasite that causes chronic meningomyelitis in domestic cats (Wolffhügel 1933, Levin 1968, Bowman *et al* 2002, Gómez *et al* 2010, Moroni *et al* 2012). The geographic distribution of *G. paralysans* includes areas of Chile, Argentina, Uruguay, Colombia, and Brazil (Guerrero *et al* 2011, Gómez-Alzate *et al* 2011, Rivero *et al* 2011, Moroni *et al* 2012, Melo Neto *et al* 2019) (figure 1), but recently has been isolated in the Canary Islands, Spain (Udiz-Rodríguez *et al* 2018). The nematode can be found in the leptomeningeal veins and the parenchyma of the spinal cord of the feline host, and the infection has been associated to progressive paraparesis, paraplegia, faecal or urinary incontinence, and/or tail paralysis (Gómez *et al* 2010, Moroni *et al* 2012, Mieres *et al* 2013). The life cycle of the parasite is unknown, but is likely to be heteroxenous, as other metastrongyloid nematodes. The diagnosis of feline gurltiosis is challenging and, so far, only possible by necropsy (Wolffhügel 1933, Wolffhügel 1934, Moroni *et al* 2012, Muñoz *et al* 2017).

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HISTORICAL PERSPECTIVE

Gurltia paralysans was first reported in the early 1930s in Chile by Kurt Wolfgang Wolffhügel (1869-1951), a German scientist, naturalist, and parasitologist (Wolffhügel 1934, Bowman *et al* 2002). Wolffhügel diagnosed eleven cases of domestic cats, studying their pathological lesions and extracting adult nematodes from their spinal cords. All affected animals came from the Provinces of Llanquihue and Puerto Varas, Southern Chile (latitude 41° south). The genus *Gurltia* was named after Ernst Friedrich Gurlt (1794-1882), a German veterinary anatomist and teratologist. Wolffhügel called the disease "*paraplejia cruralis parasitaria felis*" and initially placed the nematode as genus *Hemostrongylus*, later called *Angiostrongylus*.

MORPHOLOGICAL CHARACTERISTICS

The measurements of male and female specimens reported in the literature are summarised in table 1. Males of *G. paralysans* have a body length of 12-18 mm and are 0.072 mm wide just anterior to the bursa, with a 0.026-0.032 mm wide in the cephalic region (Wolffhügel 1934, Moroni *et al* 2012, Rodríguez 2013, Muñoz *et al* 2017). The oesophagus is 0.368-0.0392 mm long, and the oesophago-intestinal junction is 0.008 mm long. The nerve ring is 0.148-0.164 mm in diameter and the excretory pore occurs 0.232-0.240 mm from the anterior end (Muñoz *et al* 2017) (figure 2, table 1). No cervical papillae are reported. The spicules are 0.65-0.902 mm long, curved in lateral view, and the tip has a main stem and a single-pointed branch surrounded by a bluntly rounded membrane (Wolffhügel 1934, Moroni

Table 1. Measurements of morphological characters of male and female *Gurltia paralysans* reported in the literature.

Characters	Male				Female			
	Wolffhügel 1934	Moroni *et al* 2012 (n = 4)	Rodríguez 2013 (n = 7)	Muñoz *et al* 2017 (n = 7)	Wolffhügel 1934	Moroni *et al* 2012 (n = 6)	Rodríguez 2013 (n = 14)	Muñoz *et al* 2017 (n = 2)
Total length	12	13-18	10.20-16.35	14-15	20.5-23.0	23-30	23.55-36.06	27-28
Maximum width	0.07	0.1	0.075-0.078	0.072	—	0.1	0.113-0.150	0.082-0.088
Oesophagus length	0.360-0.432	0.4	0.225-0.338	0.036-0.039	—	0.6	0.226-0.338	0.444-0.468
Nerve ring	—	—	—	0.148-0.164	—	—	—	0.132
Nerve ring/ anterior end distance	—	—	—	0.075-0.114	—	—	—	—
Excretory pore	—	—	—	0.232-0.240				0.236
Anal/end distance	—	—	—	—	—	0.03	0.001-0.038	—
Anal/vulva distance	—	—	—	—	—	0.13	—	—
Vulva/end distance	—	—	—	—	0.150	—	0.112-0.171	0.102-0.112
Spicules length	0.65	0.8	0.756-0.902	0.722-0.816	—	—	—	—
Spicule width	—	0.003	—	—	—	—	—	—
Gubernaculum length	—	—	0.037-0.039	0.062	—	—	—	—
Bursa width	—	0.13	0.076-0.150	—	—	—	—	—
Bursa length	—	0.066	0.038-0.039	—	—	—	—	—
Egg length	—	—	—	—	0.05-0.065	—	—	0.040-0.72
Egg width					0.039-0.054			0.026-0.048

All measurements are given in millimetres (mm).

et al 2012, Rodríguez 2013, Muñoz *et al* 2017). The male gubernaculum is 0.062 mm long, slender, curved, and tapering distally in lateral view (Moroni *et al* 2012, Rodríguez 2013, Muñoz *et al* 2017). The bursal lobes are symmetrical and the bursal rays are arranged with the ventral rays, rays 2 and 3, fused to a common stem and only separated distally. The lateral rays have a common stem and the anterolateral ray (ray 4) branches off first and is longer than the other 2 laterals (rays 5 and 6), which share a common stem. The external dorsal ray (ray 8) is shorter than the lateral rays and appears segmented in mature specimens; the dorsal rays (rays 9 and 10) have thick stems, with some variation in length between the 2 male specimens examined, and only divide into 2 small branches at the distal tip. The genital cone has a ventral cone-shaped membrane (Muñoz *et al* 2017). Females have a body length of 20.5-36.06 mm and a width of 0.082-0.088 mm just anterior to the vulva (Wolffhügel 1934, Moroni *et al* 2012, Rodríguez 2013, Muñoz *et al* 2017). The cephalic region is 0.032-0.036 mm wide. The oesophagus is 0.444-0.468 mm long and the oesophago-intestinal valve is 0.008-0.012 mm in diameter. The nerve ring is 0.132 mm in diameter and the excretory pore occurs 0.236 mm from the anterior end. The vulva opens 0.102-0.112 mm from the tail tip, and the posterior margin of the vulva has a folded flap in mature specimens (figure 2). No didelphic or prodelphic infundibula or sphincters are visible. The uteri join to form a vagina 1.44 mm from the vulval opening. The eggs in the uterus and vagina are 0.040-072 mm × 0.026-0.048 mm in size, depending on maturity. Scanning electron microscopic images have revealed the presence of double submedian cephalic papillae, amphids, and a lip with a tooth at the anterior end of the parasite (Muñoz *et al* 2017).

Figure 1. Geographic localisation of reports of natural infections of *Gurltia paralysans* in South America.

LIFE CYCLE OF *G. PARALYSANS*

The transmission or life cycle of *G. paralysans* is mostly unknown. Adult males and females and eggs have been found in the veins from the subarachnoid space of the host spinal cord (Moroni *et al* 2012). No eggs or larvae were found in the faeces, blood, bronchial lavage, or other body fluids of eight infected cats (Peña 2014). As other closely related metastrongyloid species infecting felines, terrestrial gastropods as intermediate hosts (IH) and/or paratenic hosts (PH) have been hypothesised in the life cycle of *G. paralysans*. Thus, cats may become infected by consuming *G. paralysans*-carrying gastropods (i.e., slugs and snails) or a PH with an infective third-stage larvae (L3), including insects, frogs, toads, lizards, birds, and rodents (Moroni *et al* 2012, Melo Neto *et al* 2019) (figure 3). In a recent study, 835 terrestrial gastropods, including members of the Fam. Arionidae, Limacidae, Helicidae, and Milacida, were collected during August 2015 and November 2016 in Valdivia, Southern Chile, close to reported cases of gurltiosis (Sepúlveda 2018). All gastropods were subjected to enzymatic digestion to isolate *G. paralysans* larvae. Ten per cent of the gastropod samples were analysed with semi-nested PCR targeting the 18S ribosomal RNA (rRNA) gene, and 2.6% were analysed with histopathology (Sepúlveda 2018). However, the results of the study indicated the absence of *G. paralysans* by the three methods used. Thus, the molluscan species analysed may not act as IH, and further studies are required to evaluate the role of other species of aquatic gastropods in this geographic area (Sepúlveda 2018).

In a hypothetical migration route, a mollusc or PH is ingested by a cat. The infective larvae (L3) migrate via the mucosae of the digestive system to the veins or lymphatic system of the abdominal viscera and then via connections of the azygos or caval venous system with thoracic, lumbar or sacral intervertebral veins to reach the vertebral venous plexus (VVP) (figure 3). The VVP is in direct communication with the cranial venous system, and because no valves exist in either of them, the blood may flow cranially or caudally, depending on the pressure relationship (Gómez & Freeman 2004). The valveless VVP

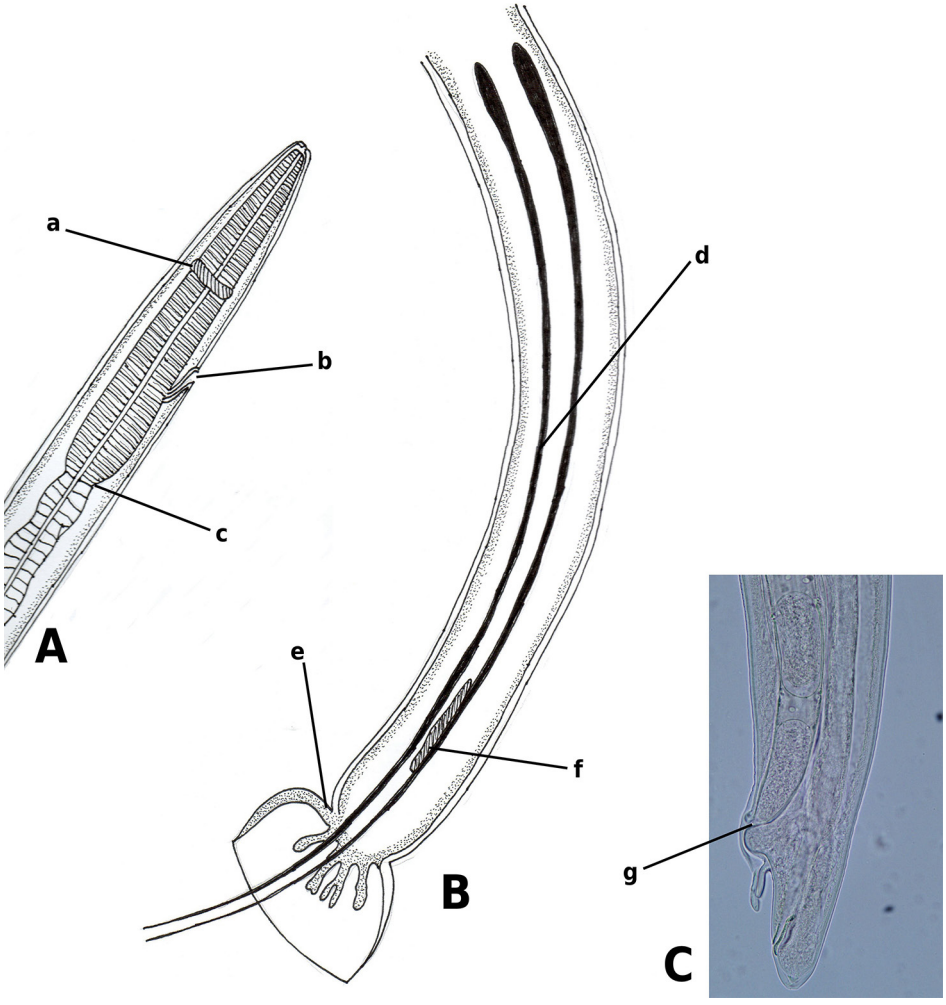

Figure 2. Schematic morphology of *Gurltia paralysans*. Adult (A), cranial end. Note the nerve ring (a), anal (excretory) pore (b), and oesophago-intestinal junction (c). Male adult (B), caudal end, showing the spicules (d), bursal rays (e), and gubernaculum (f). Photograph of a female adult larva (C), caudal end, showing the vulva and anus (g). (Illustration and photograph by Marcelo Gómez).

is probably used by *G. paralysans* to reach the spinal subarachnoid space or even the brain (Katchanov and Nawa 2010, Moroni *et al* 2012). In spinal schistosomiasis in humans, the dissemination of the parasite occurs via the intestinal veins to the VVP (Shahlaie *et al* 2005). Spinal schistosomiasis usually involves the lower thoracic and lumbosacral spine, probably because the VVP connects the intra-abdominal veins with those of the lower spine (Paz *et al* 2002). Consistent with this, the presence of adult specimens or eggs of *G. paralysans* within the ventral VVP (video 1)[1] and the basivertebral veins located in the vertebral bodies (figure 4) has been observed (unpublished data). These venous connections could also explain the presence of eggs and adult *G. paralysans* in distant places, such as the cerebrum and the anterior chamber of the eye, in more recent reports (Figueroa 2017, Udiz-Rodríguez *et al* 2018, Melo Neto *et al* 2019). The adaptation of this nematode to migrate exclusively into the venous system may be associated with abiotic factors in the vein environment, such as chemical (CO_2 concentration, O_2 concentration) and physical factors (temperature, mechanics), nutrients, etc (Read & Sharping 1995). Critical gaps for future research in the migratory pathway of *G. paralysans* include the vein tropism of adults and the neuroanatomical localisation to the subarachnoid veins of the spinal cord.

To date, all reports of feline gurltiosis have shown that domestic cats are predominantly affected. However, recent reports from Brazil have described the isolation of *G. paralysans* from the lumbar spinal cord segments of adult wild cats, including the northern tiger cat (*Leopardus triginus*) and a female margay (*L. wieddi*) (Oliveira 2015, Dazzi *et al* 2020). Although the huiña or kodkod (*L. guigna*) and Geoffroy's cat (*L. geoffroyi*) have been

[1] Available at www.australjvs.cl/ajvs

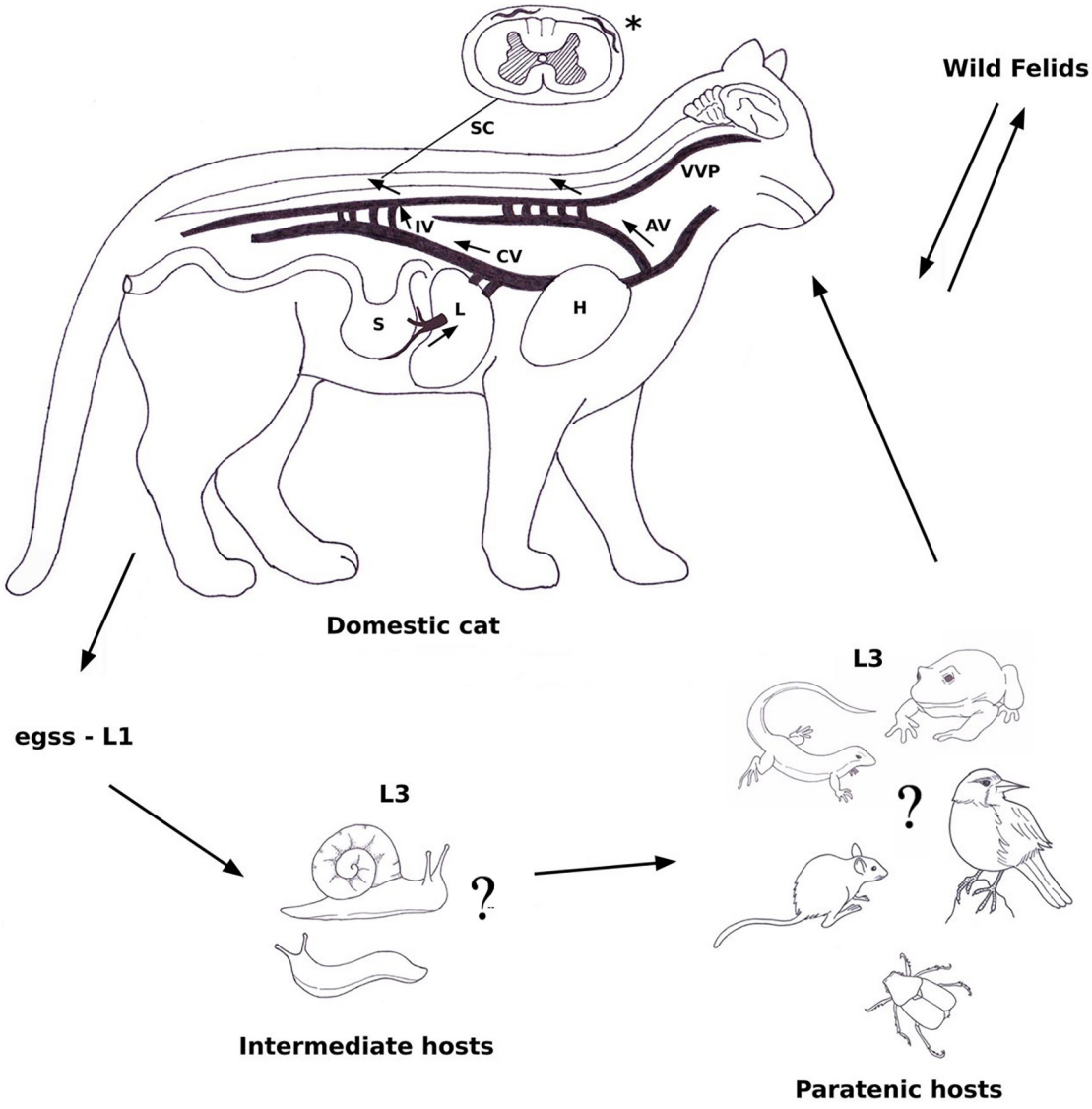

Figure 3. Proposed life cycle and migration pathways of *Gurltia paralysans*. Domestic cats or wild felids acquire the L3 larvae by ingesting an infected intermediate host (gastropods) or paratenic host (lizards, rodents, amphibians, birds, or insects). Infective larvae penetrate the stomach and enter the hepatic portal system and then the caudal vena cava and/or the azygous venous system. From those vein systems, the larvae migrate to the spinal cord via the intervertebral veins and the vertebral venous plexus. The larvae invade the veins of the subarachnoid space of the spinal cord, where they mature (asterisk) and lay eggs. It is still unknown how domestic cats eliminate the eggs or first larval stage (L1), or how the intermediate host becomes infected with L1. AV: azygos vein; CV: caudal vena cava; IV: intervertebral veins; H: heart; L: liver; S: stomach; SC: spinal cord; VVP: vertebral venous plexus; L1: first-stage larvae; L3: third-stage larvae. (Illustration by Marcelo Gómez).

suggested as possible definitive hosts, neither larval stages nor clinical cases have been diagnosed yet in these wild felids (Muñoz *et al* 2017). Further molecular studies are necessary to determine the role of wild cats as hosts and the transmission to other species.

EPIDEMIOLOGY

The first cases of feline gurltiosis were reported in domestic cats from areas of the Valdivian rainforest in southern Chile (Wolffhügel 1933). In Chile, cases has been reported in the La Araucanía, Los Ríos, and Los Lagos regions (Gómez *et al* 2010, Moroni *et al* 2012). Since then, cases have been reported in Argentina (Guerrero *et al* 2011, Bono *et al* 2016), Uruguay (Rivero *et al* 2011), Colombia (Gómez *et al* 2011), and Brazil (Togni *et al* 2013, Melo Neto *et al* 2019) (figure 1). In Chile, cases of feline gurltiosis have predominantly been diagnosed in rural areas, but more specific spatial distribution in this environment is poorly known (Wolffhügel 1933, Gómez

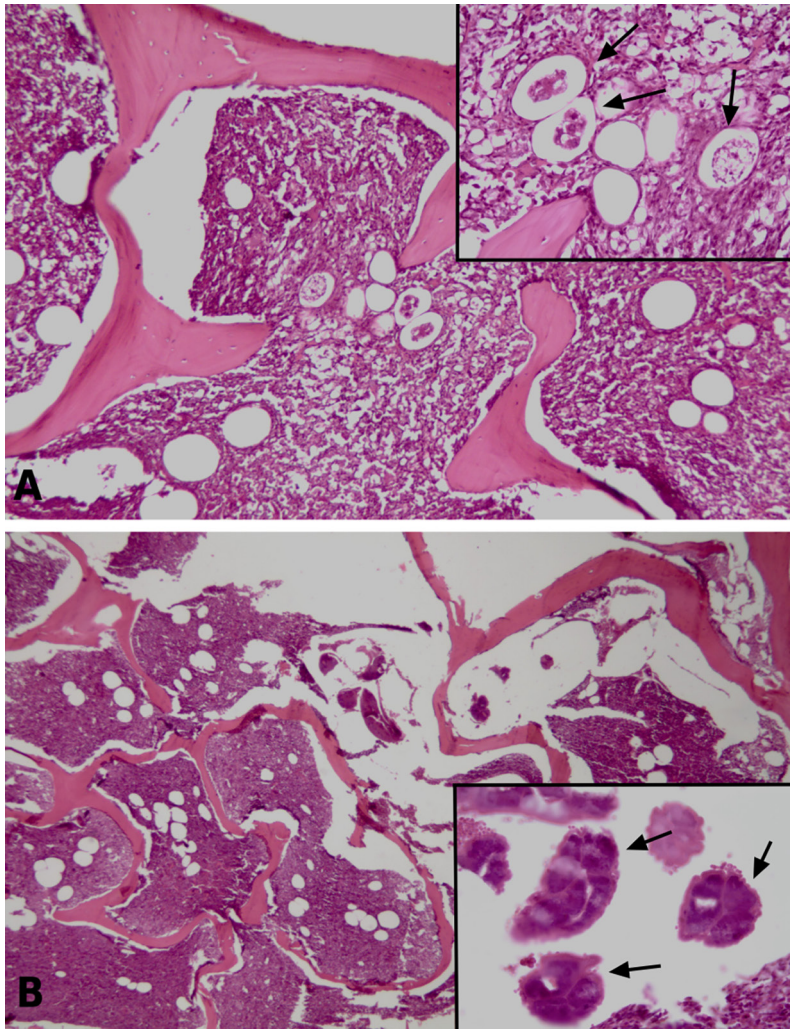

Figure 4. Presence of embryonated eggs (cross-section) (arrows) (A) and fragments of an adult specimen (arrows) (B) of *Gurltia paralysans* in the spongiosa of a lumbar vertebral body from an affected domestic cat. Inserts show augmented views (40×).

et al 2010, Moroni *et al* 2012, Mieres *et al* 2013). The disease has been diagnosed in three different regions in the southern part of Chile: the Araucanía region (e.g., Lastarria), the Los Rios region (e.g., Punucapa, Niebla, Paillaco, and Futrono), and the Los Lagos region (e.g., Futrono, Pichirropulli, and Ancud) (Moroni *et al* 2012). In Colombia, six cases of gurltiosis in domestic cats were reported in Antioquia (municipalities Tarso and Amagá) with signs of pelvic limb weakness and spinal hyperesthesia (Alzate *et al* 2011). In Argentina, in 2011, one cat with *G. paralysans* was reported in the Baradero area of the province of Buenos Aires and three cases in rural areas of the Santa Fé province, in the districts of Las Colonias, San Cristobal, and Castellanos (Guerrero *et al* 2011, Bono *et al* 2016). In Uruguay, two cases of feline parasitic meningomyelitis due to *G. paralysans* were described between 2008 and 2009 in the rural area of Fray Bentos (Rivero *et al* 2011). This parasitosis has been recognised in Brazil since the mid-1990s, under the local name "bambeira", "derrengado", or "renga" (Togni *et al* 2013). In 2013, four cats with *G. paralysans* infections were reported in the state of Rio Grande do Sul (Togni *et al* 2013), and eleven cases were recently found in the state of Pernambuco (municipalities of Caetés and Capoeiras), northeast Brazil (Melo Neto *et al* 2019). Also in Brazil, two wild cats (*L. wieddii* and *L. triginus*) were found in the state of Parana (Chapecó) with classical post-mortem spinal cord lesions of feline gurltiosis (Oliveira 2015, Dazzi *et al* 2020). More recently, the first case outside of America was reported in Tenerife (Canary Islands, Spain) (Udiz-Rodriguez *et al* 2018). In 1993, a cat with neurological and necropsy findings in the lumbar spine compatible with feline gurltiosis was reported in the United States (Bowman *et al* 2002). Both cases of *G. paralysans,* in the Canary Islands and in the US, may have resulted from the introduction of *G. paralysans*-infected domestic cats from endemic areas of South America or the importation of infected IH, as

reported for *Angiostrongylus cantonensis* introduced to Canary island (Foronda *et al* 2010), although further research is still required.

The disease has been associated with rural settings and rainforest areas, with humid ecosystems and abundant vegetation (Wolffhügel 1933, Gómez *et al* 2009, Rivero *et al* 2011, Togni *et al* 2013, Melo Neto *et al* 2019). The prevalence of the nematode in South America is unknown, but is likely to be underestimated and the disease under-diagnosed (Muñoz *et al* 2017). Modelling studies have indicated that southern Chile and Argentina, and the areas in Brazil, Uruguay, and Colombia, where gurltiosis has been diagnosed, are regions at clear risk of the spread of angiostrongyloid nematodes based on their climatic suitability (Morgan *et al* 2009). No seasonal occurrence pattern has yet been reported for feline gurltiosis, which is detected in all seasons of the year (Gómez *et al* 2010, Moroni *et al* 2011, Rivero *et al* 2011). In one report, cats with gurltiosis were all co-infected with *Aelurostrongylus abstrusus*, despite none of them showing respiratory symptoms (Mieres *et al* 2013). This could indicate that cats infected with *G. paralysans* and *A. abstrusus* could share IH or PH. Factors affecting the distribution of gastropod species are important in determining whether the life cycle of *G. paralysans* can be completed and the potential contact with suitable hosts. Previous studies have indicated that the range of metastrongyloid parasites (i.e., *Angiostrongylus vasorum*) has expanded into new countries and regions (Traversa *et al* 2010, Maksimov *et al* 2017, Lange *et al* 2018). Models have been used to predict the distribution of *A. vasorum* and the risk of infection based on climatic variables and their effects on the survival rates of the intermediate hosts (Maksimov *et al* 2017). Similar modeling information is required for *G. paralysans* to predict the distribution range. The causes of the apparent re-emergence of metastrongyloid parasitoses in domestic animals are still unknown, but several factors may explain the recent increases in reports of feline gurltiosis in several countries (Traversa *et al* 2010, Melo Neto *et al* 2019). These include global warming, changes in the seasonal population dynamics of vectors, and massive movement of animals (Traversa *et al* 2010, Maksimov *et al* 2017). Further investigations on this neglected field are needed, including identifying the exposure by serological/molecular prevalence investigations, specific local and global geographic ranges of feline gurltiosis and determining the epidemiological and climatic factors that allow the establishment of *G. paralysans* infection.

PATHOLOGICAL LESIONS

The necropsy findings in infected domestic cats include diffuse submeningeal congestion of the lumbar, sacral, and coccygeal spinal cord segments (Gómez *et al* 2010, Moroni *et al* 2012). Several intravascular nematode larvae and pre-adult stages can be identified histologically in the meningeal veins of the spinal cord, associated with congestion, thrombosis, and thickening of the subarachnoidal vessels (Gómez *et al* 2010, Moroni *et al* 2012, Mieres *et al* 2013, Togni *et al* 2013, Moroni *et al* 2017). Studies have reported mild smooth-muscle hypertrophy, moderate adventitial fibroplasia, and marked subintimal fibrosis of the spinal cord venules (phlebosclerosis) (Togni *et al* 2013). In some specimens, concentric thickening of the venule wall may produce stenosis of the vessel lumen (Togni *et al* 2013). Intraluminal papillary projections with an arboriform appearance to the interior of the dilated venules have been interpreted as varicose lesions (venular varices) (Moroni *et al* 2012, Togni *et al* 2013). Sections of normal or dilated and tortuous varicose venules may contain thrombi with various levels of organisation (Togni *et al* 2013). The spinal cord parenchyma may show multiple haemorrhages and extensive foci of malacia, with gitter cells and adjacent gliosis (Togni *et al* 2013). Lymphocytes, intermingled with fewer macrophages, primarily infiltrate the meninges, forming a perivascular pattern. Mature eosinophils scattered randomly within the leptomeninges have also been observed, which are consistent with extensive spinal leptomeningitis and thrombophlebitis (Moroni *et al* 2012, Togni *et al* 2013). Some animals may also show granulomatous leptomeningitis or suppurative leptomeningitis (Togni *et al* 2013). White-matter lesions in the spinal cord segments may have variable degrees of Wallerian degeneration, characterised by the distension of the myelin sheath diameter, irregular axons, axonal swelling, bulbous axonal fragmentation (caused by the presence of axonal spheroids), microcavitation, and focal areas of mineralisation (Moroni *et al* 2012, Togni *et al* 2013, Moroni *et al* 2017). Varicose venules can also be observed in the white matter of the spinal cord, but are associated with recesses in the meninges (Togni *et al* 2013). The activation of glial and endothelial cells and immune cell infiltration, visualised with immunohistochemical markers (i.e. GFAP, CNPase, factor VIII, CD3, and CD45R) in affected spinal cord samples, indicate gliosis and chronic inflammatory spinal cord lesions subsequent to the ischemia caused by parasitic vascular injury (Vienenkotter *et al* 2015, Jara 2018). The predominant cellular infiltrate in the affected spinal cord is of the mononuclear type, indicating the chronic nature of the lesions (Jara 2018). A recent study analysed the presence of histopathological lesions in the cerebrum, cerebellum, and brainstem in 13 feline patients with post-mortem spinal lesions due to *G. paralysans* (Figueroa 2017). Congestion and hyperaemia were observed in the peripheral blood vessels of both the dorsal and ventral zones of the cerebrum in the 13 cats. In 7 cases, mononuclear cell infiltrate was observed around the choroid plexus, the third and fourth ventricles, and associated blood vessels. Six cats showed thickening of the meninges and 2 showed perivascular neutrophilic inflammatory infiltrate at the level of the cerebral subarachnoid space (Figueroa 2017). Similar findings of leptomeningeal vascular congestion, varices,

and perivascular cellular infiltrate were observed in the encephalons (frontal, temporal, and occipital cortices) of 11 cats infected with *G. paralysans* in Brazil (Melo Neto *et al* 2019). However, no clinical cases of feline gurltiosis have been observed with clinical cerebral, cerebellar, or brainstem syndromes.

Histological samples from ten *G. paralysans*-infected cats were analysed at the hepatic level. All samples showed signs of periportal hepatic degeneration, periportal inflammatory infiltration, comprising neutrophilic and mononuclear infiltration, indicating direct injury to the liver (Verscheure 2014). However, the possible mechanisms by which intravascular parasites could cause this type of injury pattern in the liver remains unclear. *Angiostrongylus vasorum* can induce moderate liver parenchymal parasitic hepatitis and lesions such as interstitial hemorrhage disseminated inflammatory cells in the portobiliar space or around centrolobular veins attributable to larval nematode migration (Rinaldi *et al* 2014, Cook *et al* 2015). In kidneys, gurltiosis has been associated to the presence of hyaline protein deposits inside Bowman's capsule (in 8 out of 10 cases), the thickening of Bowman's capsule in five cases, and the presence of interstitial inflammatory infiltrate, consisting of neutrophils and eosinophils (in 4 out of 10 cases) (Verscheure 2014). These findings are compatible with glomerulonephritis, which could have been immune-mediated by the deposition of immune complexes of the host (Verscheure 2014).

Further studies are required to understand the role of the immune pathogenesis of the disease, such as interleukins, cytokines, and host cells (e.g., eosinophils, neutrophils, plasma cells, T cells) and how *G. paralysans* regulates feline endothelial functions (e.g. via excretory or secretory antigens) allowing the parasite to use intravascular habitat.

CLINICAL SIGNS

The most common clinical manifestation of feline gurltiosis is chronic and progressive ambulatory paraplegia (Gómez *et al* 2010, Gómez *et al* 2011, Rivero *et al* 2011, Mieres *et al* 2013, Togni *et al* 2013). Based on 19 cases, the duration of the clinical signs ranges from 2 weeks to 48 months (Mieres *et al* 2013, Rivero *et al* 2011, Togni *et al* 2013, Bono *et al* 2016, Moroni *et al* 2016). Other clinical signs include pelvic limb ataxia, pelvic limb proprioceptive deficit, pelvic limb tremor, pelvic limb muscle atrophy, tail trembling, tail atony, and faecal and urinary incontinence (Gómez *et al* 2010, 2011, Gómez *et al* 2011, Guerrero *et al* 2011, Rivero *et al* 2011, Moroni *et al* 2012, Mieres *et al* 2013, Togni *et al* 2013, MeloNeto *et al* 2019). The neurological signs are associated with the neuroanatomical lesions observed at necropsy and in histopathological specimens (Mieres *et al* 2013). The associated haematological abnormalities included non-regenerative anaemia and low mean corpuscular haemoglobin concentrations (hypochromia) (Rojas 2011, Mieres *et al* 2013) indicating chronic inflammatory disease or chronic blood loss (Gredal 2011). The eosinophilia associated with parasitism has commonly been reported in domestic animals, but is not a common finding in cats with gurltiosis (Mieres *et al* 2013), which has also been reported in dogs with neural angiostrongylosis (Bourque 2002). No signs of coagulopathy have been observed in naturally infected cats. However, high levels of urea in the blood have been reported, probably arising from neurogenic urinary dysfunction (Mieres *et al* 2013). A bronchial lavage analysis of five naturally *G. paralysans*-infected cats, showed the absence of larval stages and eggs (Peña 2014). Ocular lesions (uveitis, chorioretinitis, posterior synechiae, corneal oedema) have recently been reported to be associated with the presence of a motile adult specimen of *G. paralysans* in the anterior chamber of the eye in a domestic cat (Udiz-Rodríguez *et al* 2018).

DIAGNOSIS

Imaging studies by computed tomography, myelography, and magnetic resonance imaging (MRI), indicate that *G. paralysans* induce lesions in the thoracolumbar, lumbar, and sacral regions, suggesting diffuse inflammatory spinal cord lesions (Gómez *et al* 2010, Mieres *et al* 2013, Togni *et al* 2013). Myelograms show the retention of columns of contrast medium in the thoracolumbar region (Mieres *et al* 2013). The intramedullary accumulation of contrast medium, similar to the pattern for myelomalacia, in the thoracolumbar spinal cord segment has been reported in infected cats (Guerrero *et al* 2011), similar to myelographic examinations of dogs with *A. vasorum* infections (Lun *et al* 2012). However, the myelographic evaluations in dogs with intramedullary parasitic infections attributed to *Spirocerca lupi* are normal (Chai *et al* 2008). MRI images of affected cats show multiple nodular areas of hyperintensity in the periphery of the spinal cord, which could also represent slow venous flow within the perimedullary veins (Mieres *et al* 2013) (figure 5). Spinal cord lesions with nodular or granular aspects have also been detected in *S. mansoni* infections in humans (Nobre *et al* 2001). Isointensity on T1-weighted images and hyperintensity on T2-weighted images, associated with acute haemorrhagic spinal cord lesions, have been described in dogs infected with *A. vasorum* and *Schistosoma* (Nobre *et al* 2001, Wessmann *et al* 2006). Spinal cord enlargement on T1-weighted and T2-weighted images is a common finding in dogs and humans with nematode-associated myelopathy (Kanpittaya *et al* 2000, Jabbour *et al* 2011).

A definitive diagnosis of feline gurltiosis can only be made by post-mortem examination, demonstrating the presence of nematodes in the spinal cord vasculature (Gómez *et al* 2010, Guerrero *et al* 2011). A clinical history of chronic paraparesis or paraplegia (including signs of symmetric or asymmetric pelvic limb ataxia, tail paralysis and faecal or urinary incontinence) in potentially

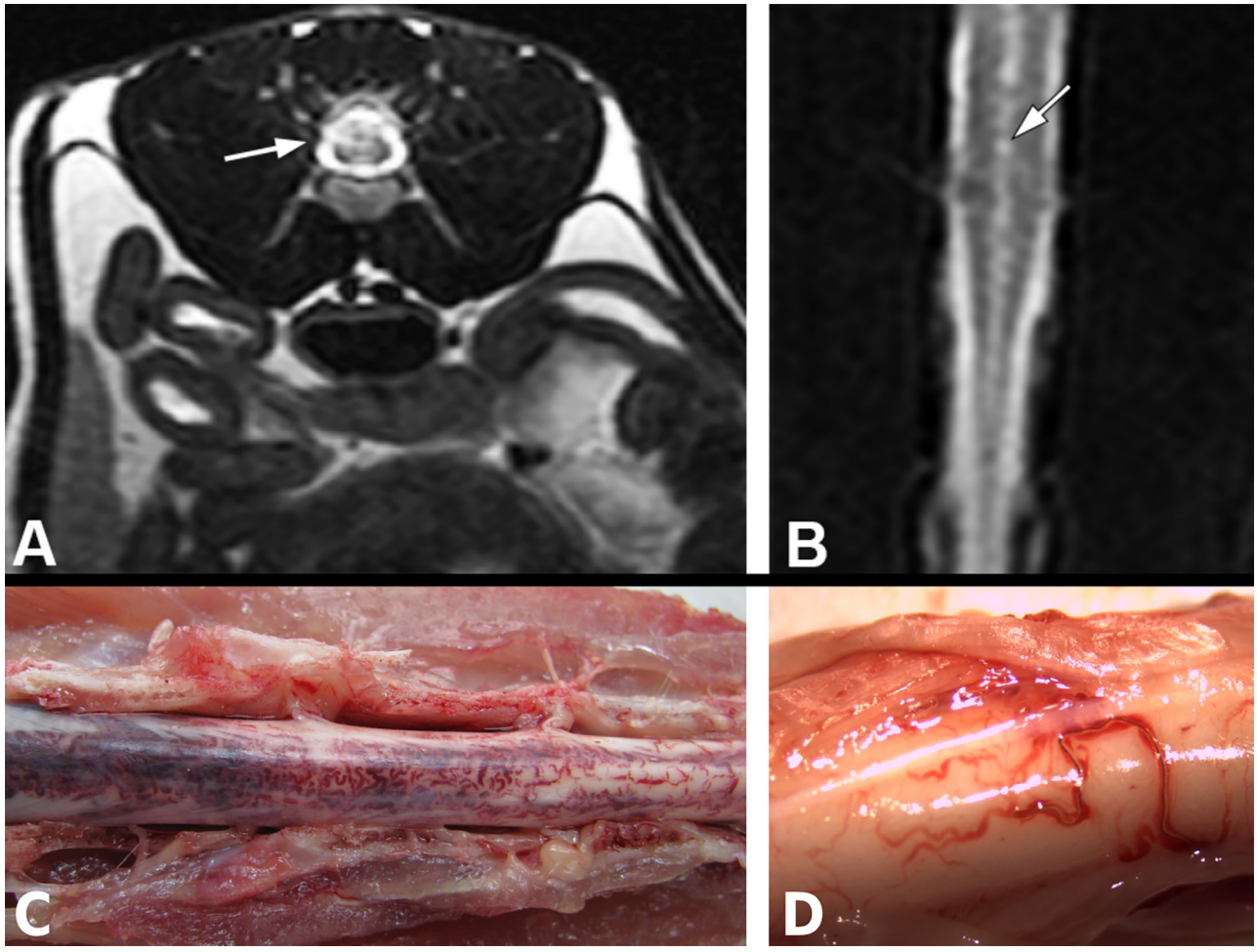

Figure 5. Magnetic resonance image of a cat with myelopathy due to *Gurltia paralysans* infection. Transverse T2W image showing hyperintense area in the spinal cord at level 3 (arrow) (A). Dorsal T2W image of a cat with vascular venous congestion and venous stasis of the dorsal spinal vein at the conus medullaris (arrow) (B). Macroscopic image of the lumbar spinal cord of a domestic cat with *G. paralysans* infection, demonstrating severe submeningeal vein congestion (C). Macroscopic image of the spinal cord of a domestic cat showing an adult nematode of *G. paralysans* inside a leptomeningeal vein (D).

endemic areas, laboratory findings (cerebrospinal fluid [CSF], haemography, faecal examination), and imaging findings are necessary to exclude other myelopathies and to establish a presumptive diagnosis of feline gurltiosis (Mieres *et al* 2013).

A cross-reactivity of *G. paralysans* and *A. vasorum* using a commercial serological test developed for the diagnosis of canine angiostrongylosis in domestic dogs has been recently evaluated for the use in domestic cats with gurltiosis. Preliminary results showed that the Angio Detect Test™ (IDEXX Laboratories), a rapid test designed to detect circulating antigen based on *A. vasorum*-specific antibodies, can be used as an effective test for cats displaying clinical signs of *G. paralysans* infection (Gómez *et al* 2020).

Novel molecular techniques for *G. paralysans* have been recently developed that could facilitate the clinical diagnosis of this nematode in infected animals (see below).

MOLECULAR CHARACTERISATION OF *G. PARALYSANS*

Members of the genus *Gurltia* are morphologically similar to those of the closely related genera of the Family Angiostrongylidae (Moroni *et al* 2012). Using sequences of the 28S rRNA D2–D3 region, the complete internal transcribed spacer 1 (ITS1) and ITS2 of the 5.8S rRNA, and the partial 18S rRNA gene (Gómez *et al* 2013, Hermosilla *et al* 2013, Muñoz *et al* 2017), confirmed that *G. paralysans* is a member of the family Angiostrongylidae and are a distinct taxonomic genus in the superfamily Metastrongyloidea (Muñoz *et al* 2017). A phylogenetic analysis showed that *G. paralysans* is most closely related to *A. vasorum* and *Filaroides martis* (figure 6).

Targeting a 717 bp conserved nucleotide sequences of the D2-D3 region of the 28S rRNA gene , a semi-nested PCR method to detect *G. paralysans* and differentiate from these other nematodes has been developed (Hermosilla

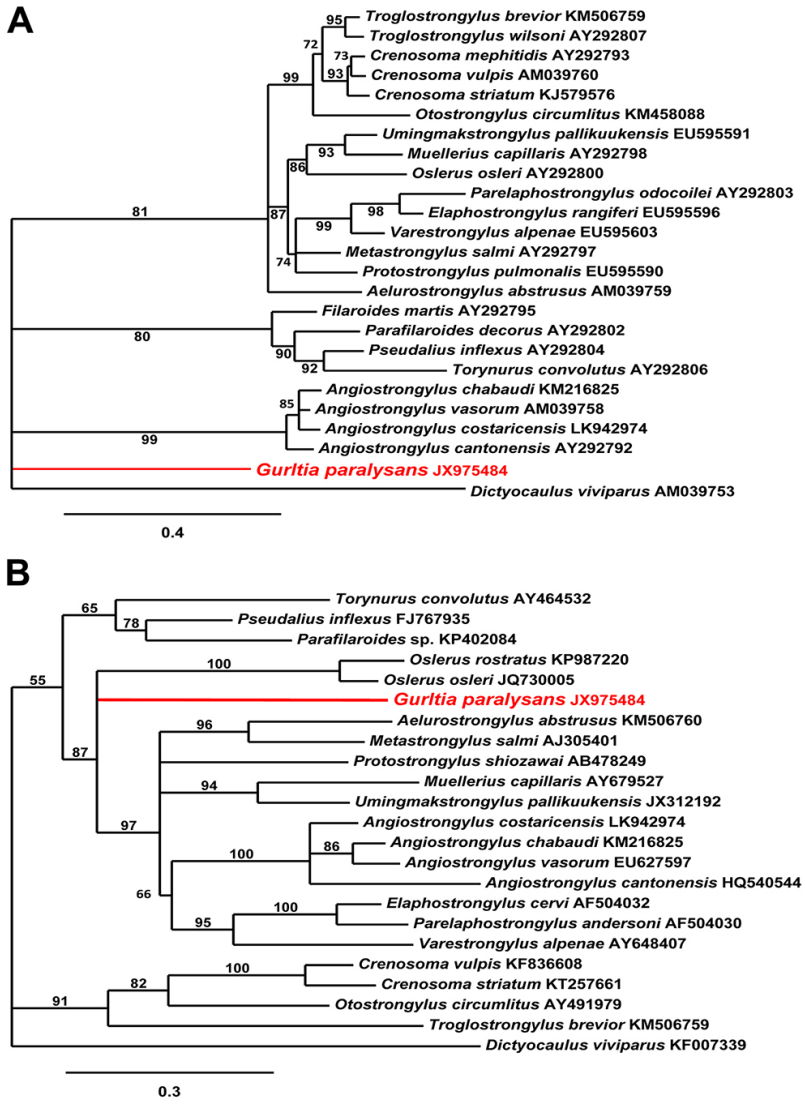

Figure 6. Phylogenetic relationships of *Gurltia paralysans* (red) and other metastrongyloid nematodes. Phylogenetic analyses were based on 28S rRNA D2–D3 (A) and ITS rDNA sequences (B) (taken from Muñoz *et al* 2017).

et al 2013, Muñoz *et al* 2017) (figure 7). The D2-D3 region of the parasite can be used as a molecular marker, and that this PCR is an effective diagnostic method for the identification of infected cats using serum and blood samples (Hermosilla *et al* 2013, Muñoz *et al* 2017). This PCR method also allows the detection of the larval stages of *G. paralysans* in potential IH, and can be used in epidemiological prevalence studies in domestic cats. Using CSF samples, DNA fragments of *G. paralysans* has been successfully detected in 4 out of 6 CSF samples (figure 7) (López *et al* 2020).

TREATMENT, PREVENTION, AND CONTROL

The administration of four doses of ivermectin (0.2-0.4 mg/kg) at 1-weekly intervals have satisfactory outcomes in mild or moderate cases (Gómez *et al* 2012). Ricobendazole (20 mg/kg) and ivermectin has been also used combined in four adult cats with suspected feline gurltiosis (chronic ambulatory paraparesis) in an endemic area in Argentina (Guerrero *et al* 2011). The cats were observed for 5 weeks after their treatment to assess their clinical evolution. The preliminary results indicated no progression of the clinical condition, suggesting that some antiparasitic effect was achieved. However, no randomised trials of antihelminthics have been conducted for feline gurltiosis. Fenbendazole, milbemycin oxime, or moxidectin combined with imidacloprid may reduce the risk of *G. paralysans* infection, as they do for other related nematode species, such as *A. vasorum*, but further research is required.

No prepatent period has been observed for *G. paralysans in vivo*, and the monthly administration of drugs

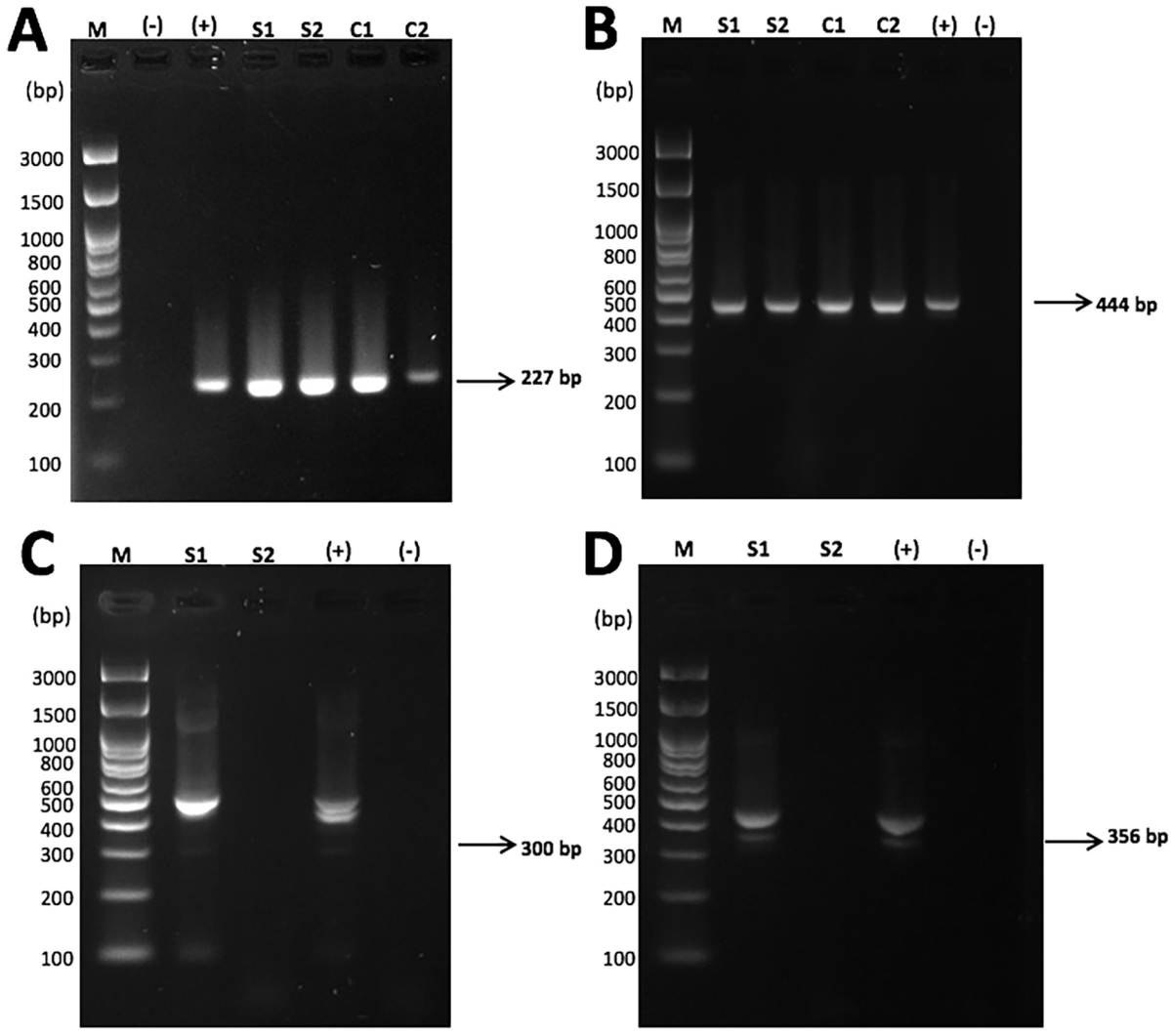

Figure 7. Molecular detection of *Gurltia paralysans* in the serum and cerebrospinal fluid of infected and uninfected domestic cats. A) Amplification of *IRBP* gene as the internal control in infected and uninfected domestic cats. PCR amplified a 227-bp fragment. B) Amplification of a sequence conserved in Metastrongylidae in serum and cerebrospinal fluid samples from infected and uninfected domestics cats, using the AaGp28Ss1 and AaGp28Sa1 universal primers. PCR product of 444 bp. C) Semi-nested *Aelurostrongylus abstrusus* PCR using specific AaGp28Sa1/Gp28Sa3 primers. PCR product of 300 bp. D) Semi-nested *G. paralysans* PCR using specific AaGp28Ss1/Gp28Sa3 primers. PCR product of 356 bp. C1 and C2, cerebrospinal fluid of domestic cats. In this case, only serum samples are shown in C and D. S1, infected cat. S2, uninfected cat. (+) the positive control and (–) the negative control. M, molecular size marker (100 bp ladder). The PCR products were run on a 2% agarose gel. Animals also showed the presence of *G. paralysans* specimen upon *post-mortem* examination.

containing macrocyclic lactone (i.e. ivermectin, selamectin, or milbemycin) might be considered a suitable prophylactic treatment for the prevention of *G. paralysans* infection in known endemic areas. The prevention of the infection by limiting access to PH or IH by maintaining cats indoor should be considered in endemic areas.

CONCLUSIONS

Gurltia paralysans is a metastrongyloid parasite that may cause chronic meningomyelitis in domestic cats in South America. The geographic distribution of *G. paralysans* includes rural and peri-urban areas. The life cycle of the parasite is still unknown, but is probably indirect, as in other metastrongyloid nematodes. Further research is required to clarify the infection of IH and the transmission to PH. The clinical signs of *G. paralysans* infection include progressive pelvic limb ataxia, paraparesis, paraplegia, faecal or urinary incontinence, and/or tail paralysis. The definitive *in vivo* diagnosis remains challenging, but the disease could be tentatively identified by clinical signs, haemography, CSF analysis, and imaging studies (CT and

MRI). The PCR is currently a complementary diagnostic method, using serum and/or CSF samples. The definitive diagnosis still remains post-mortem based on the presence of larvae in the spinal cord. Preliminary reports suggest that the administration of macrocyclic lactones drugs may be useful in treating mild cases. Veterinarians and owners should be warned of the environmental control of definitive and intermediate hosts as an effective approach to reducing the likelihood of infection.

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REVIEW ARTICLE

Animal models of chronic pain. Are naturally occurring diseases a potential model for translational research?

Daniel E. Herzberg, Hedie A. Bustamante*

ABSTRACT. Despite the vast amount of molecular data obtained from classical pain studies, there is an ongoing translational pain model crisis reflected by the reduced amount of new effective and safe compounds developed to treat chronic pain in humans. Naturally occurring chronic pain in animals may offer some advantages over induced models of chronic pain, including a natural development of the condition that induces pain, the heterogenicity of the population that affects, and the chronologic age in which they develop, among others. The identification and study of naturally occurring painful diseases that resemble a particular chronic painful condition in humans has been proposed as a potential tool to investigate the molecular mechanisms and thus, accelerating drug development at the preclinical and clinical level. Currently, certain types of chronic pain in companion and large animals have gained attention as potential translational models of chronic pain. Examples of these include canine and feline osteoarthritis, neoplastic diseases as osteosarcoma and bovine and equine lameness. The present review describes the limitations of animal models of chronic pain and briefly enters in how naturally occurring pain models could represent a translational approach to chronic pain.

Key words: chronic pain, animal models, translational.

INTRODUCTION

Chronic pain (CP) develops from multiple clinical conditions defined by longstanding pain that adversely impacts the quality of life (Gereau IV *et al* 2014), affecting at least 20-30% of the general population and individuals of all ages, races and genders. The International Association for the Study of Pain (IASP) defines chronic pain as "pain that lasts or recurs for more than three months" (Treede *et al* 2015). This definition has evolved in the last few years from a unidimensional to a multidimensional state, including sensitive, cognitive and emotional components (Kumar and Elavarasi 2016). Chronic pain has many special features, but one of the most prominent originates from a complex dysfunction of the nervous system and lasts after the normal healing period. It is paroxistic and it lacks any adaptative function, associated to biochemical and phenotypical changes in the nociceptive pathway (Ueda 2008). Interestingly, the global burden of pain is large and growing. The IASP estimates that 1 in 5 patients experience pain and that 1 in 10 patients are diagnosed with CP yearly, with chronic pain being the most common cause for treating medical care (Enright and Goucke 2016). Moreover, there is agreement that in low- and middle-income countries CP is poorly managed with more than 80% of patients not receiving proper treatment (Jackson *et al* 2016). Finally, during 2019, the IASP published the classification of chronic pain for the International Classification of Diseases (ICD-11), which will improve recognition and diagnosis of different clinical conditions, thus establishing chronic pain as a health condition (Treede *et al* 2019).

The transition between acute and chronic pain is another key component in the pain paradigm. Woolf *et al* (2010) mentioned that the physiopathological differences between acute and chronic must be thoroughly defined before developing potential new treatments. Recently, concepts as "*adaptative*" and "*non-adaptative*" have been described and used in order to obtain a better classification of chronic pain (Adrian *et al* 2017). The development of a "*non-adaptative*" pain is always related to neuronal and glial plasticity that leads to central sensitization and maintains the painful sensation after the healing period (Woolf 2011). This plasticity in the Central Nervous System (SNC) has also been associated with clinical features of pain, including the exaggerated pain response after a painful stimuli (*Hyperalgesia*); and the painful response after a non-painful stimuli (*Allodynia*). Furthermore, chronic pain has been classified according to its origin in *nociceptive*, *neuropathic* and *nociplastic*.

Nociceptive pain includes inflammatory conditions such as osteoarthritis and it can be defined as "pain that arises from actual or threatened damage to non-neuronal tissue and is due to the activation of nociceptors". Neuropathic pain includes conditions such as diabetic neuropathy, chemotherapy induced neuropathy, phantom limb pain and it can be defined as "pain caused by a lesion or disease of the somatosensory system". Nociplastic pain was recently introduced by the IASP Council and includes fibromyalgia. Nociplastic pain can be defined as "pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing pain". Although most of the painful conditions mentioned earlier have been studied in human patients, they commonly occur in

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animals. This finding has led to a new area of research in which naturally occurring diseases could confirm and/or elucidate previously confirmed mechanisms in rodents. The present review describes the limitations of animal models of chronic pain and briefly enters in how naturally occurring pain models could represent a translational approach to chronic pain.

EXPERIMENTAL PAIN MODELS

A continuous increase in the used of animal models to study biological processes and disease has been noted since the early 1900s (Mogil 2009) and pain transmission and abnormal pain processing has been the focus for research since the late 19th century. Moreover, the study of pain has extensively relied on preclinical animal models in order to determine the sensory and psychological complexities of this condition (Burma *et al* 2017). Animal models of pain have been mainly developed in order to recreate a pathological painful condition aiming to identified a mechanistic explanation of pain biology and the identification of therapeutic targets to develop new compounds to safely alleviate pain (Gregory *et al* 2013).

In order to obtain accurate data that can be effectively translated to humans, an animal model of pain must encompass a sufficient high face validity (the capacity of the model to recreate all possible signs and symptoms of the disease that supposed to model) and predictive validity (the capacity of a model to be sensitive to an analgesic compound and insensitive to a non-analgesic compound) (Mogil 2009). Nonetheless, in recent years it has become clear that the face and predictive validity of current pain models is not sufficient to fully complete the task they were implemented for originally (Mao 2009, Vierck *et al* 2008, Quessy 2010, Clark 2016). Similarly, the credibility of efficacy data obtained from animal models of pain has been questioned (Mogil *et al* 2010).

Although animal models of pain have proven successful in elucidating pathophysiological mechanisms of chronic pain, the impact of these models in drug development has been disappointing, with failure rates in the clinical phase of around 90 to 95% (Arrowsmith 2012). In 2014, the reported likelihood of approval of an experimental analgesic drug to pass to Phase I trial was only 10.7% (Hay *et al* 2014). This report remarks the fact that no novel analgesics compounds have been developed since the 70's to safely and effectively treat chronic pain.

Several strategies have been proposed in order to improve this "*translational gap*" between scientific data and new compound development. These strategies include the higher refinement of the current models, the development of new and more accurate models, the replacement of evoked measure behaviour for operant behaviour measurement, and least but not last to include the measurement of variables that better reflect "*quality of life*" (Blackburn-Munro 2004, Mogil 2009, Quessy 2010, Clark 2016). It is important to consider that the limitations here described by any means overshadow the discoveries and advances made from these models, which according to Burma *et al* (2017) have been instrumental in advancing our understanding of the mechanistic under-pinning's of pain states and developing and testing new analgesic compounds.

According to Mogil (2009), an animal pain model must encompass three components: a *subject*, an *assay* and a *measured outcome.*

THE SUBJECTS

Many animal species, including laboratory animals such as rats, mouse, rabbits, small companion animals, large animals, and exotic species have been used to study the underlying mechanisms of chronic pain. During the '60's and 70's, mouse, rats, dogs, cats, and rabbits were the most used animals for pain studies. With the arrival of a new century, the development of transgenic mice which were incorporated in pain research (Mogil 2009). However, there are some behavioural inconvenients in mouse, such as difficulties in conditioning and other technicisms that make rats continue in the first place for pain studies (Wilson and Mogil 2001) and to the present-day rats have been employed in the vast majority of pain studies. Nonetheless, a recent search in the Pubmed database using the terms "pain"/"chronic pain" and "rat"/"mice" revealed that during 2019 a total of 1,590 papers used rats and 1,559 papers used mice as experimental models. Regarding chronic pain, 526 and 467 papers described the used of rats and mice, respectively. The highly extensive use of rodents for pain research is based mainly in the similarities in the neuroanatomy and physiology across mammalian species (Burma *et al* 2017). In order to reduce response variability, rodents employed in pain studies are frequently restricted to a certain breed, young age and one sex. Nonetheless, chronic pain states in humans are more frequent to occur in median-age or older, mainly women patients. Based on this, several authors have argued that restricting experimental animals to a certain age and gender do not represent the whole population to which the painful condition is modelled (Klinck *et al* 2017). Growing evidence has shown age-dependent differences in behavioural pain responses (Weyer *et al* 2016). Also, sexual dimorphism has an enormous impact on the development of chronic pain (Burma *et al* 2017).

THE ASSAYS

Several assays have been developed over the years, as pain is not a unitary phenomenon (Mogil 2009). Most assays offer the possibility to explore the pain system under controlled settings (Sunil kumar Reddy *et al* 2012). Similarly, assays have been classified in different manners. Walker *et al* (1999), classified the assays as somatic (acute nociceptive and pathological) and visceral. According

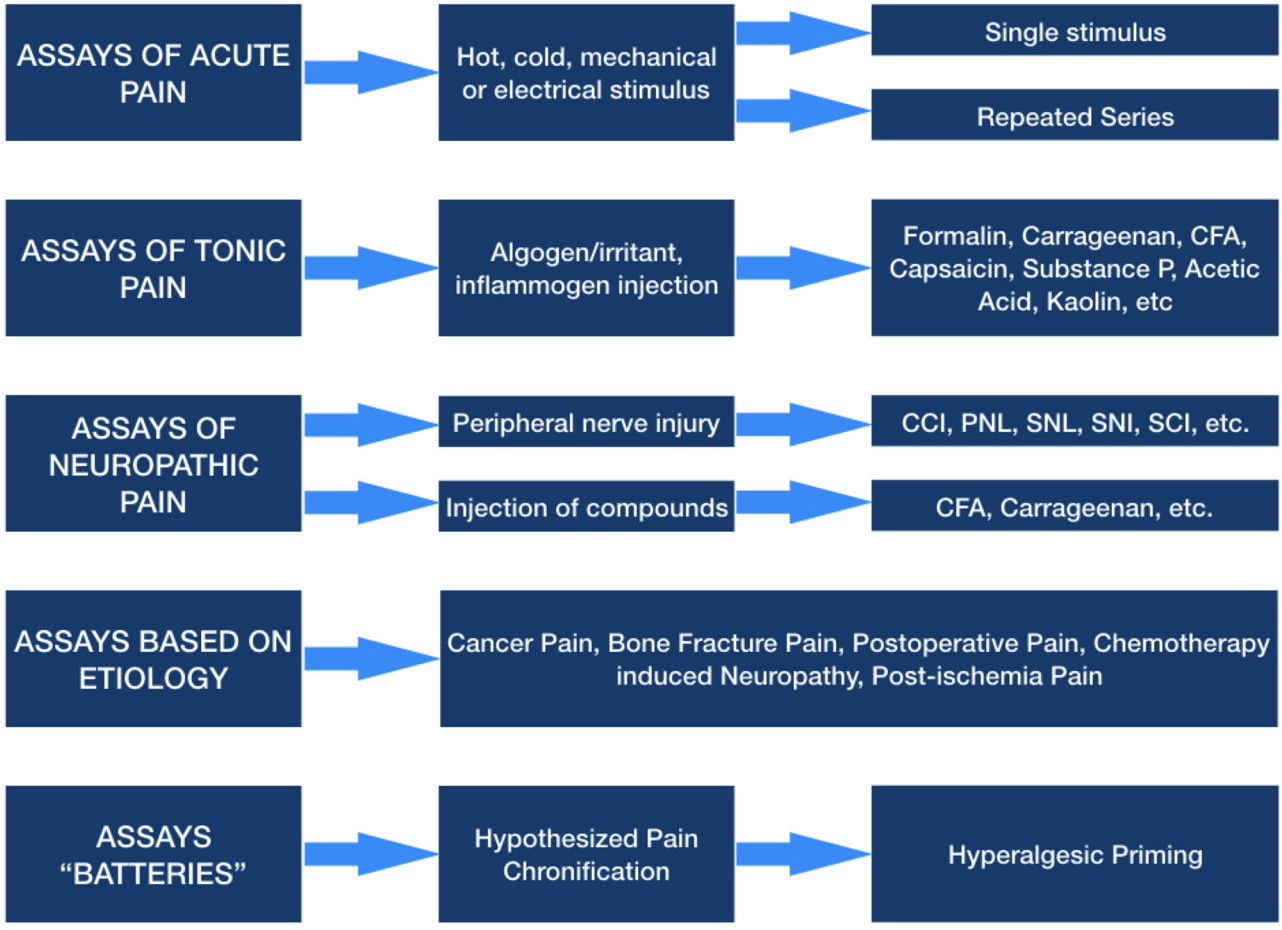
to Mogil (2018), chronic neuropathic pain assays were developed as a response to the short duration of pain that the traditional models originated at that time. Furthermore, Sunil kumar Reddy *et al* (2012) mentioned that, mechanistically, the most important categories should include both peripheral and central sensitization, since both of them occur during pain.

Recently, Mogil (2018) described different assays for pain evaluation, which are summarised in figure 1. This description classifies the existing assays according to the most common features described over the years. For the purpose of this review, we will briefly describe the most common assays of tonic pain and neuropathic pain.

ASSAYS OF TONIC PAIN

Inflammatory pain models used different irritants injected into the skin, paw, muscle, joint, and visceral organs. Several of these substances can induce both an acute and chronic inflammatory response (Sandkühler 2009, Boyce-Rustay *et al* 2010). Moreover, they have been backwardly validated by the effectiveness of opioids and non-anti-inflammatory drugs in controlling pain induced after their administration (Ren and Dubner 1999, Radhakrishnan *et al* 2003, Wilson *et al* 2006, Boyce-Rustay *et al* 2010, Gregory *et al* 2013).

Formalin (0.5 to 5% formaldehyde) injection induces inflammation and pain, with two temporal phases of different duration and underlying mechanisms (Tjølsen *et al* 1992). The acute phase (Phase I) is short lasting, mainly mediated by activation of the transient receptor potential cation channel (TRPV) (McNamara *et al* 2007). After a brief quiescent period, the acute phase is followed by a continuous and longer lasting phase (Phase II) that is supposedly mediated by instauration of a central sensitization state due to the continuous nociceptive transmission from type C fibres. Phase I of formalin test can be attenuated by local anaesthetics, while morphine, NMDA antagonist and gabapentin inhibit Phase II, but not Phase I (Dubuisson and Dennis 1977, Matthies and Franklin 1992, Abbadie *et al* 1997, McNamara *et al* 2007). Carrageenan is a water-extractable polysaccharide derived from the marine plants *Gigartina aciculaire* and *Gigartina pistillata*. Carrageenan injection promotes the instauration of an acute inflammatory



CFA: Complete Freund's Adjuvant; CCI: Chronic Constriction Injury; PNL: Partial Nerve Ligation; SNL: Spinal Nerve Ligation; SNI: Spinal Nerve Injury; SCI; Spinal Cord Injury.

Figure 1. Summary of current assays used in animal models of pain (Summarised from Mogil (2018).

state that converts into chronic after approximately 2 weeks. Carrageenan can induce mechanical and thermal hyperalgesia in the injured site, as well as in the non-affected area, both mediated by peripheral and central sensitization, respectively (Radhakrishnan *et al* 2003). Carrageenan injection can also enhance avoidance, spontaneous and guarding pain behaviours as well as a reduction in the weight bearing force of an affected limb (Radhakrishnan *et al* 2004, Gregory *et al* 2013). Complete Freund's adjuvant (CFA) is an emulsion of heat-killed bacteria that includes *Mycobacterium butyricum* or *Mycobacterium tuberculosis* in sterile mineral oil (Swingle and Grant 1977). CFA injection induces an immune-mediated chronic inflammation of the surrounding tissue. This chronic inflammation is longer than that induced by carrageenan, nonetheless, clinical features mechanical and thermal primary and secondary hyperalgesia are well identified after CFA administration. Moreover, CFA injection modifies the behaviour of animals during the conditioned place preference (CPP) and conditioned place avoidance (CPA) tests. CFA injection has been also used to model chronic inflammatory joint diseases such as in rheumatoid arthritis (Shippenberg *et al* 1988, Ren and Dubner 1999 Gregory *et al* 2013, Parvathy and Masocha 2013).

ASSAYS OF NEUROPATHIC PAIN

Neuropathic pain (NP) often remains as one of the most challenging conditions to treat (Colloca *et al* 2017). The traditional translational approach has consisted of multiple attempts to understand mechanisms in animal models and then apply these data into the clinic (Bouhassira and Attal 2016). Neuropathic pain models have been developed to mimic painful pathological conditions such as nerve trauma, nerve compression, low back pain or diabetic neuropathy (Mogil 2009). However, animal models for nociplastic conditions such as fibromyalgia have not been developed (Yezierski and Hansson 2018). Peripheral nervous system (PNS) damage and its associated pain have been mainly modelled using the surgical intervention of a peripheral nerve, with the most common being the sciatic nerve and its branches (Colleoni and Sacerdote 2010, Gregory *et al* 2013, Challa 2015). Several animal models of NP using mechanical peripheral nerve injury to induce changes such as allodynia and hyperalgesia are currently described (Dowdall *et al* 2005). The most used models are nerve transection (NT) (Wall *et al* 1979), chronic constriction injury (CCI) (Bennett and Xie 1988), partial sciatic ligation (PNL) (Seltzer *et al* 1990), spinal nerve ligation (SNL)(Kim and Chung 1992), spare nerve injury of the tibial and peroneal nerve (SNI) (Decosterd and Woolf 2000), and inflammation (Maves *et al* 1993). All these different models are well characterised and all of them have advantages and disadvantages as well as limited ability to precisely model the clinical condition. Nevertheless, these models have provided the basis for the mechanistic understanding that we have achieved to date on NP syndromes (Ossipov *et al* 2006).

THE MEASURED OUTCOME

Bedside pain models recreate a previously determined painful condition, nonetheless, they may also define a measurable and clinically translatable indicator (Klinck *et al* 2017). One of the most criticised aspects in pain research in the low capacity to identify and clinically use a specific, measurable, easy to score and translatable outcome (Blackburn-Munro 2004). Outcome measures are designed to evaluate multiple parts of the pain experience and can be broadly categorised in evoked and non-evoked measures.

Evoked responses during pain evaluate the behavioural response after heat, cold, mechanical, or electrical stimulation. Most of these behaviours are associated to spinal reflexes (i.e. limb withdrawal), spino-bulbospinal reflexes (i.e. jumping and abdominal stretching) and innate behaviour (vocalisation, licking, scratching, biting, guarding) and can also be observed in decerebrated animals (Matthies and Franklin 1992). Evoked measures are easy to perform and provide a quantifiable outcome. Interestingly, they have become the most frequent type of outcome reported in pain studies (Mogil 2009). Currently, an increasing debate about the capacity of this outcome to score the whole context of pain experience is underway (Vierck *et al* 2008). First, evoked pain reflexes have been useful to investigate the underlying mechanisms of pain hypersensitivity (hyperalgesia and/or allodynia), but not pain itself (Clark 2016). Secondly, evoked measures do not consider the cognitive and emotional aspect of pain (Mogil 2009, Quessy 2010); and thirdly, evoked tests are incapable to measure spontaneous pain states, which is by far the most frequent painful sensation reported by humans patients (Backonja and Stacey 2004). Furthermore, allodynia in human patients is frequently triggered by a dynamic component which differs from the mechanical allodynia evaluated using static pressure von Fray filaments in the affected tissue of rodents (Samuelsson *et al* 2005).

Non-evoked (operant) measures evaluate animal behaviours that require a functional spinal-cerebrospinal integration (Mogil 2009). Operant conditioning is based on the psychological theory in which learning occurs when a response to a stimulus is reinforced (learning theory) (Mogil 2018). If a positive reinforcement follows the response to a specific stimulus, then the response becomes more probable to occur thereafter. If a negative reinforcement occurs, or a reward is removed, the response becomes less probable. In this case, individuals may perform behaviours that terminate the exposure to the painful stimulus or increase the administration of analgesics (Mogil 2018). Positive reinforcement has been criticised by some researchers, mainly because if the pain response of the individuals is systematically reinforced in an early stage, it could

be possible that the pain behaviour would continue after the original stimulus is terminated (Fordyce *et al* 1973).

Some studies using operant measures of pain behaviour have used paradigms such as condition place preference (CPP) and condition place avoidance (CPA) (Sufka 1994; Ding *et al* 2005). During CPP testing, a treatment group is allowed to gain access to an analgesic compound during the evaluation period and accordingly, the time spent in the analgesic administering chamber would indicate a preference. In contrast, during a CPA test, individuals are subsequently evaluated by an observer in order to discriminate the preference for a chamber in were no nociceptive stimulation occurs (Gregory *et al* 2013). These results could be used as indicators of aversiveness, which is clinically relevant because people with chronic pain markedly avoid painful stimulus (Mogil 2009).

Non-evoked measures have continuously demonstrated to be more concordant with clinical results than evoked measures. NP models evaluated using non-evoked measures have demonstrated a clear hypersensitivity for cold stimulation and not by heat, which is consistent with the clinical impression that cold allodynia is more common than heat allodynia after mechanical nerve damage. In contrast, evoked measures constantly demonstrate the opposite, with a notorious hypersensitivity to heat (Vierck *et al* 2005, Leffler and Hansson 2008).

An important limitation of these non-evoked paradigms is that they require significant learning with extensive training (Li 2013). Nonetheless, fast learning procedures have been implemented, allowing to perform CCP testing after a 3-day pre-conditioning habituation (Okun *et al* 2011). Similarly, in non-evoked measurement, one motivating factor could be associated with behavioural response (Mogil 2009). Additionally, some authors have criticised that analgesic compounds could also play a role affecting the mental state of a painful animal which can then modify an operant behaviour (Gregory 2013).

One of the most common features of chronic pain is the development of spontaneous pain. Several behaviours have been evaluated for identifying spontaneous pain, including aggression, bite force, food intake, locomotion activity, rearing, struggling, weight bearing, posture and gait alteration (Mogil and Crager 2004). Similarly, other studies have focused on the use of more sophisticated techniques such as ultrasonic vocalisation and facial grimace scale (Han *et al* 2005, Sotocina *et al* 2011). Nonetheless, evaluating chronic pain using only behavioural indicators has proven complicated. Rodents do not manifest pain in a consistent way that could allow an evaluator to differentiate between mild and severe pain (Roughan and Flecknell 2003). Many of the proposed behaviours are not specific, some of them are affected by pain but do not represent ongoing pain. In some cases, the frequency of presentation of the behaviour is not frequent enough or highly variable among individuals (Graham and Hampshire 2016). Similarly, electroencephalography, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have gathered great attention in the last couple of years regarding its ability to identify specific areas of the brain that become activated during a painful experience.

A different approach to obtain outcome measures is the use of methods of quantitative sensory testing (QST), which is a diagnostic tool that allows determining the sensitive perception evoked in response to a defined sensory stimulus. QST has been extensively used in human and rodents. The sensory modalities include small fibre sensory function, such as thermal detection/pain threshold and pinprick sensitivity, and large fibre sensory function, such as mechanical and vibration detection thresholds (Themistocleous *et al* 2018).

In summary, animal pain testing faces at least six main criticisms (Mogil *et al* 2010). These criticism include: a) the exaggerated emphasis on endpoints as withdrawal reflexes as dependent measurement; b) the lack of evaluating states that accompany chronic pain as sequelae or other comorbidities; c) the fact that most models are highly artificial including the administration of inflammatory mediators and surgical nerve damage; d) most primary symptoms of human chronic pain are spontaneous; e) the mismatch in the epidemiological prevalence of chronic pain in the human population and the usual choices of animal models and f) differences in the standard design between animal experiments and human clinical trials (Vierck *et al* 2008, Vierck *et al* 2002, Brennan *et al* 1996, Scholz *et al* 2009, Rice *et al* 2008).

NATURALLY OCCURRING PAIN MODELS

Naturally occurring diseases and painful conditions that affect animals might better reflect the complex genetic, environmental and physiological variation present in humans (Kol *et al* 2015). Moreover, previous reports have strongly suggested the use of veterinary conditions as translational pain models (Lascelles *et al* 2018). A letter to the editor by Quessy (2010) in the prestigious *The Journal of Pain* mentioned at that time the urgency for a translational research agenda with a focus on natural diseases and validated, relevant outcome measures, suggesting that clinical trials in animals with natural disease may improve the predictive veracity for drug candidate selection, praising the role of Veterinary Medicine. Even more, Kol *et al* (2015) proposed that companion animals would play an important role in defining translational medicine, suggesting the need for identifying naturally occurring diseases with potential for accelerating translation. Moreover, Kol *et al* (2015) and Lascelles (2018) go one step forward and suggest a new paradigm in drug testing and clinical trials, incorporating a II phase veterinary clinical trial between the preclinical research and human clinical trials. Nonetheless, this intermediate step would require an important change in the form in which traditional pain research has been performed over the years. These changes

would include experimental design (number of animals, replicability, reproducibility, among others) and ethical concerns that would limit large-scale studies.

Interestingly, in the last 5-10 years, Veterinary Medicine has stepped forward and have responded to the challenge, identifying and better defining naturally occurring conditions with potential translation, including the field of pain. Painful conditions commonly occur in veterinary patients including dogs and cats, horses, cattle, sheep, and swine. In most animals, these painful conditions develop naturally and usually over a long period of time, which better resembles the painful condition in human patients with chronic pain. A different asset to using translational pain models that occur naturally is the phylogenetical proximity between some of these species and humans (Obrien *et al* 2008, Hoeppner *et al* 2014). Lascelles *et al* (2018) mention that naturally occurring models may better reflect the complex genetic, environmental, temporal and physiological influences present in humans.

Some examples of painful conditions include tail docking (amputation), dehorning, castration, and lameness in cattle and sheep; osteoarthritis, denervation, chronic ligament injuries, and lumbar pain in horses; osteochondrosis, tail docking, castration in pigs; amputation, osteoarthritis, osteosarcoma, carcinoma, intervertebral disk disease, degenerative lumbosacral stenosis in dogs, and diabetic neuropathy, inflammatory mammary carcinoma, osteoarthritis, amputation, interstitial cystitis and inflammatory bowel disease in cats (Klink *et al* 2017).

Nonetheless, scientific evidence discussing how these conditions could represent a translational approach has only been reported for feline interstitial cystitis (Buffington 2001), feline diabetes mellitus (Mizisin *et al* 2002), osteoarthritis (McCoy 2015) and various tumours in dogs (Peña *et al* 2003, Brown *et al* 2009) and cats (Pérez-Alenza *et al* 2004), lameness in dairy cattle and horses (Bustamante *et al* 2015, Meneses *et al* 2018, Rodriguez *et al* 2018, Herzberg *et al* 2019), tail docking in dairy cows (Troncoso *et al* 2018). According to Klinck *et al* (2017), this relatively limited evidence could be indicative of the complexity in the design and interpretation of studies in which the number of recruited subjects is reduced. Additionally, as in experimental animal models, limitation exist in naturally occurring conditions, which confirms that predictive results should be analysed with caution. Nonetheless, we strongly believe these limitations represent an opportunity to develop novel research strategies, including the confirmation of previously described pathophysiological mechanisms using a genomic and proteomic approach.

Interestingly, the vast majority of the test used in experimental models have been evaluated in naturally occurring models, including thermal and mechanical thresholds, activity monitoring, operant testing, fMRI, PET and electroencephalography. Troncoso *et al* (2018) used a QST battery in order to confirm the presence of chronic pain in long-term tail docked dairy cows. Probably the most studied and well-defined translational model of chronic pain is companion animals' osteoarthritis. Lascelles *et al* (2018) confirmed that spontaneous painful osteoarthritis in companion animals offers translational potential.

Recently, Lascelles *et al* (2019) discussed the priorities and future research for measurement of chronic pain in companion animals. This interesting review concludes that improvements must be made in the development and standardisation of clinical metrology instruments, including partnering with owners; in better defining and evaluating health-related quality of life (HRQoL), in the measurement of animals that suffer chronic pain; advance in computational gait analysis instrumentation; further studies in QST methodology; advance in the definition of nociceptive withdrawal reflex in companion animals; obtain outcome measures for cancer pain and define the effect of placebo on outcome measures. Moreover, preliminary findings should be taken with caution, considering that several of the concerns represented in traditional pain models could also be present in naturally occurring painful conditions. These considerations led to probably the most important conclusion: collaboration is the path forward in chronic pain diagnosing and management in both human and veterinary medicine.

Finally, here we have described the current limitations of animal models of chronic pain and briefly explained how naturally occurring pain models could represent a translational approach. We consider that Veterinary Medicine must play an important role in the finding, development, confirmation and applicability of potential translational models that would help solve human and non-human animals' medical conditions under the concepts of *one health, one welfare and one pain*.

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REVIEW ARTICLE

Infectious bronchitis virus variants in chickens: evolution, surveillance, control and prevention

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ABSTRACT. Infectious bronchitis is a disease of the upper respiratory tract of chickens caused by a Gammacoronavirus (infectious bronchitis virus, IBV). Severe economic losses are caused by IBV due to a reduction in egg production and/or egg quality in layers in addition to poor feed conversion and increased condemnations in broiler chickens. The extreme variability of this virus is in part due to its RNA genome, which predisposes it to mutations and generates genetic variation. In addition, recombination events add to the variability of this virus. IBV variability was first described in 1956 by Jungherr. Since then, dozens of serotypes and hundreds of genotypes have been reported. Variant IBV strains are those that, can escape from the immunity generated by conventional strains, despite not being fully different from conventional strains affecting a geographic region. At the genomic level, these differences can be equal or greater than 5% of the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient, reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence. The main goal of surveillance is to assist the development of efficient preventative measures in the field. This review aims to critically analyse the literature related to IBV variability and judiciously comment and discuss on how to better prevent this poultry endemic disease.

Key words: IBV, variants, variability, evolution, surveillance.

INTRODUCTION

Understanding factors that might play a role in the generation of infectious bronchitis variants is crucial in order to perform surveillance, prevent their occurrence and establish prevention strategies. These factors involve inherent viral characteristics, management, vaccines and vaccination, immunity and concomitant infectious diseases. The purpose of this review is to merge research findings with clinical observations and laboratory expertise on the generation, diagnostic, surveillance and prevention of infectious bronchitis variant strains in commercial chicken production.

The infectious bronchitis coronavirus (IBV) is the causative agent of one of the most economically important diseases in modern poultry production, infectious bronchitis (IB), in chickens. The disease was first described by Schalk and Hawn in 1931. Nowadays the disease is endemic in most of the countries that possess a developed commercial poultry industry. Its global economic impact has been estimated as the second most damaging poultry disease after highly pathogenic avian influenza (TAFS-Forum 2011). The clinical picture is associated with upper respiratory tract infection characterised by conjunctivitis, tracheitis and loss of ciliary movement in the trachea. In addition, reproductive effects such as drop in egg production and alterations in the egg internal and external quality can be seen in layer and breeder birds. If layer chicks get exposed early in their lives the virus can infect the oviduct, altering its normal development and inducing false layers (Broadfoot *et al* 1956, Crinion and Hofstad 1972, Gallardo *et al* 2019). Some IBV strains have kidney tropism inducing nephritis and urate deposition (Winterfield and Albassam 1984, Albassam *et al* 1986) while others have been associated to enteric disorders (El-Houadfi *et al* 1986, Hauck *et al* 2016). Secondary infections are common in meat-type birds affected by IBV inducing airsacculitis and increasing condemnations at the processing plant.

INFECTIOUS BRONCHITIS VIRUS

IBV is a single-stranded, positive sense RNA virus of the *Coronaviridae* family, genus *Gammacoronavirus*[1]. The viral genome comprises two untranslated regions (UTR's) in its 5' and 3' ends, two overlapping reading frames (ORF's) encoding the structural polyproteins 1a and 1ab, and the region encoding the main structural proteins i.e. spike (S), envelope (E), membrane (M) and nucleocapsid (N). Finally, two accessory genes ORF 3 and ORF 5 encode for proteins 3a, 3b and 5a and 5b (Ziebuhr *et al* 2000). The S protein is located on the surface of the virus external membrane. During binding to the host cell, the S protein is cleaved on a cleavage site rich in basic bases into the amino-terminal S1 (~535 amino acids) and the carboxy-terminal S2 (~627 amino acids) (Cavanagh *et al* 1986). The S1 portion of the S protein of IBV is responsible for viral attachment to host cells, virus variability and eliciting neutralizing antibodies in

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[1] http://www.talk.ictvonline.org/taxonomy/

chickens (Cavanagh 1983, Cavanagh and Davis 1986, Kusters *et al* 1987, Casais *et al* 2003). The S1 displays the most genetic and phenotypic variability among different IBV strains (Cavanagh 1983, Cavanagh and Davis 1986, Kusters *et al* 1987) and is therefore the best target to assess variability. Variant IBV strains are those that, despite not being fully genetically different from conventional strains affecting a geographic region, are phenotypically different and escape from the immunity generated by conventional serotype-specific vaccines. At the genomic level, these differences can be greater or equal to 5% in the hypervariable region of the S1 gene. These variant strains are usually restricted to geographic regions and most of the time are transient (Gallardo *et al* 2016), reason why diagnostics and epidemiological surveillance are crucial to determine their existence and persistence. This strategy helps planning preventative measures in the field.

IBV EVOLUTION

Viral evolution depends on two separated and independent mechanisms described by Mayr (Mayr 1988): (a) Generation of diversity, in which genetic/phenotypic variants are generated and serve as material for (b) selection, the virions generated after the replication process are released in the environment, and the survivors will serve as the genetic pool for subsequent generations (Gallardo *et al* 2010). IBV variability is generated by insertions, deletions and point mutations in addition to recombination events (Toro *et al* 2012). Jungherr and collaborators reported viral variability between IBV isolates for the first time in 1956. The described serological variations differentiated Massachusetts and Connecticut IBV serotypes (Jungherr *et al* 1956). IBV selection has been proved previously by different research groups (McKinley *et al* 2008, van Santen and Toro 2008). The rapid evolution ability is what makes IBV highly successful in the environment and is the reason why IBV continues to spread and circumvent vaccination programs used in the poultry industry (Toro *et al* 2012). Nowadays, we recognise the existence of dozens of IBV serotypes and even more genotypes and variants. Variants have been detected all over the world: Latin America, associated with kidney lesions (Hidalgo *et al* 1986); Africa, associated with swollen head syndrome (Morley and Thomson 1984); Egypt, associated with enteric disease (El-Houadfi *et al* 1986). In Asia, variants have been present since at least 1979 (Lohr 1988). In Australia, where evolution has been independent from other countries in the world (Ignjatovic *et al* 2006), variant IBV strains have been isolated since 1960 (Cumming 1963).

The introduction of exotic genotypes into a geographic region or country can increase the local genetic pool of IBV strains. This situation increases the chances of genetic recombination with local IBV strains and potentially generates IBV variants. The introduction of these IBV strains can be accidental, via contaminated poultry products and lack of biosecurity, and/or premeditated. The most common premeditated introduction is caused when vaccines from exotic genotypes are introduced as a tool for controlling IBV outbreaks caused by variant strains. There are several examples of generation of variant IBV strains after the premeditated introduction of exotic genotypes. This review will focus on two cases. The first example is the event reported by Lee and Jackwood, where they describe the generation of the IBV genotype GA98 after the introduction and use of the DE072 vaccine. The IBV GA98 was the causal agent of extensive and costly outbreaks of IB in broilers in the state of Georgia (Lee and Jackwood 2001). The second example is the introduction of the IBV vaccine type 793B, 4/91 or 1/96, a UK-originated variant, to countries in Europe (Franzo *et al* 2014, Moreno *et al* 2017), Middle East (Tatar-Kis *et al* 2014) and Latin America (Sesti *et al* 2014, de Wit *et al* 2017). The reported consequences in Italy and Spain have been the recombination between local strains and the introduced vaccine (Moreno *et al* 2017).

VACCINES AND THEIR USE

Vaccines capable of inducing cross protection against different genotypes of IBV are of paramount economic and practical importance (Gelb 2018). Some commercially available vaccines are heterogenous, meaning that the predominant subpopulations in the vaccine are diverse and do not induce protective immune responses in chickens (Ghetas *et al* 2014). The best example of these heterogeneous vaccines is the ArkDPI vaccine. ArkDPI was originally isolated from the Delmarva peninsula and partially attenuated at the University of Delaware by 50 passages in embryonated eggs (Gelb and Cloud 1983). Then, the strain was distributed to different vaccine companies where they were further attenuated to produce different vaccines. The heterogeneity of the S gene in vaccines like ArkDPI might play the role of a virulent strain in terms of genetic variability, resulting in the emergence of new variants after replication, generation of diversity and selection. In addition, recombination of vaccine strains with local strains might also play a role. ArkDPI vaccines showing higher heterogeneity have been associated with respiratory signs and tracheal damage (Ndegwa *et al* 2012). Studies in variant IBV variability have shown different levels of heterogeneity in different IBV genotypes (Gallardo *et al* 2016). It is important to understand the heterogeneity of variant IBV strains before selecting them as vaccine candidates. Vaccine homology will also play a role. Heterologous vaccines will either provide no protection or, at best, partial protection. If partial protection is achieved, it will ameliorate clinical signs but will not reduce shedding of challenge/field virus. High loads of wild-type IBV strains combined with vaccine viruses creates the perfect scenario for variant virus generation. In summary, as it has been reported in the literature, live

attenuated vaccine usage has a major role in the genetic profile of IB strains isolated in the field (Jackwood and Lee 2017).

Other than vaccine selection, vaccine application is crucial to avoid variant IBV generation. Currently available live attenuated IBV vaccines are mostly applied in the hatchery at day of age and by spray or drinking water between 10 to 15 days of age in broilers and at least 3 to 4 times in layers before the laying onset. Massive application strategies for IBV vaccination are partially inefficient and usually result in vaccination failure (Jordan 2017). An example of partly ineffective vaccination methods are spray cabinets. Although the spray vaccination processing does not seem to damage the IBV virions, there is a significant titer reduction, most likely associated with the mechanical force applied to the virus particle during the vaccination process (Roh 2014). In addition, viruses are greatly wasted in the environment, especially when volumes as low as 7 ml per chick box are used (Jordan 2016). These issues are responsible for poor coverage during spray cabinet vaccination. Similar issues happen when using drinking water vaccination in the field, where poor vaccine coverage allows vaccine recirculation and rolling reactions. These rolling reactions allow the virus to mutate in every replication cycle while poor coverage allows the entrance of field viruses into the flock, creating recombination opportunities and subsequently generation of variant strains. All these problems are aggravated if half or quarter doses are applied.

POPULATION IMMUNE STATUS

Population immune status plays a crucial role in the evolution of IBV, not only because of the effect of vaccination in selective pressure but also because of immunodepression that will allow the virus to evolve freely in the affected population. Numerous epidemiological studies using conventional and molecular virology techniques have demonstrated the capabilities of IBV to circumvent vaccination programs which have been implemented since 1950 (Toro *et al* 2012). In particular, it is interesting to analyze Ark-serotype attenuated vaccines, where viral populations different from the vaccine emerge after vaccination causing disease and poor immune responses in vaccinated chicken flocks (McKinley *et al* 2008, Toro *et al* 2012). In the absence of immune pressure due to vaccination, IBV nucleotide changes in the S1 gene occur at a rate of 10^{-3} substitutions per site per year (Cavanagh *et al* 1998). GA98, which emerged due to the use of DE072 vaccine, showed mutation rates of 10^{-2} substitutions per site per year in the hypervariable region (HVR) of S1(Lee *et al* 2001). Immunocompetent chickens show normal immune responses that are able to restrict viral replication and limit generation of genetic variants for selection. In chickens showing less than optimal immune responses, the vaccine viruses are able to replicate in a larger number of individuals and viral populations different from the challenge strain may become predominant (Gallardo *et al* 2012, Toro *et al* 2012). This phenomenon can particularly happen when chickens are infected by highly prevalent and ubiquitous immunosuppressant viruses, i.e. chicken anemia (CAV) and infectious bursal disease virus (IBDV). Infectious bronchitis infection course in CAV or IBDV immunosuppressed flocks is longer, the clinical signs are more severe and the virus is able to persist for longer periods in the environment, facilitating diversity generation and subsequently emergent variant IBV strains (Gallardo *et al* 2012).

DETECTION, SURVEILLANCE, CONTROL AND PREVENTION

DETECTION AND SURVEILLANCE OF IBV

While we are very informed about the IBV strains affecting chickens in the U.S. and most of the countries in the E.U. due to continuous surveillance, there is lack of information in several countries in the world. In countries where surveillance is performed, most of the genotypes detected are not indigenous variants (de Wit *et al* 2010), but rather genotypes derived from the evolution of prevalent viruses and vaccines used in the region. Poor diagnostic and surveillance capabilities are a reality in most Latin American and African countries and, where these tools are available, it is very common to see errors in technique and result interpretation (unpublished data). Accurate diagnosis and targeted surveillance is crucial to adequately prevent and control IBV variant rise and its detrimental effects (Gallardo *et al* 2016). Other than orienting in the vaccine selection and vaccination strategy, accurate diagnosis will drive efforts to impede variant generation. IBV diagnostics are based on: (1) detection of the viral genome by molecular methods, such as reverse transcriptase polymerase chain reaction (RT-PCR) or RT quantitative PCR (RT-qPCR), targeting conserved genes such as N or M (Gallardo *et al* 2010, de Quadros 2012) and (2) conventional virology methods, such as virus isolation, serology and virus neutralization (Villegas and Alvarado 2008). A combination of these techniques is used in several laboratories around the world for diagnosing the disease. For surveillance, serum neutralisation in embryonated eggs, haemagglutination inhibition and sequencing of a portion or full length S gene amplified by RT-PCR are the most commonly used techniques (Villegas and Alvarado 2008, Gallardo *et al* 2010). Serum neutralization is used to type IBV using serotype-specific antibodies. However, hyperimmune sera are not readily available when new IBV variants arise. In addition, serum neutralisation test is expensive due to the use of a large number of SPF eggs. A similar problem is observed with HI, where specific reagents are needed. In addition, HI requires the treatment of the IBV isolate with neuraminidase since IBV does not have haemagglutination

capabilities naturally. Nowadays, the most commonly used technique is the RT-PCR to amplify the HVR of the S1 gene followed by sequencing of the amplicon, with or without prior virus isolation (Gallardo *et al* 2010). Virus isolation, if successful, increases the number of viral particles and consequently the amount of template for the polymerase reaction. The drawback of previous virus isolation is that *in vitro* replication allows viral mutations in the IBV genome, increasing variability during the virus adaptation to the embryonated egg. RT-PCR directly from chicken specimens followed by sequencing, even though less sensitive, is the best method to perform surveillance. This methodology will provide genetic information about the wild-type virus without the variability added during virus isolation. The nucleotide heterogeneity of S1 is largely contained within three different HVR's. Those HVR's are located in amino acids 38 to 67, 91 to 141 and 274 to 387 (Cavanagh *et al* 1988). Complete or partial analysis of the S1 has been used to determine IBV genotypes. Currently, nearly 100 genotypes have been recognised for causing impact on the commercial poultry industry. Some are restricted to specific geographic areas (Gallardo *et al* 2016).

The size of the S1 fragment amplified by RT-PCR for surveillance is of major importance. While using the full S1 is the best choice because it allows the analysis of all three HVR's, this technique is less sensitive than amplifying a shorter fragment of S1 and requires a considerable amount of well-preserved viral RNA in the sample. On the other hand, while amplification of small segments (between 300 and 500 bp) increases the test sensitivity, short sequences overlook variations in other portions of the HVR's or recombination sites (Moreno *et al* 2017). While not as accurate as using the full S1 gene, the amplification of a segment of 750 nucleotides has been proven to be adequate for IBV surveillance and evolution studies *et al* 2012). Inconsistencies in phylogenetic analyses makes IBV genotyping and characterisation difficult. Valastro proposed a classification system using full S1 sequences (Valastro *et al* 2016). The reasoning behind this classification is that there is lack of consistency when using only one or two of the three HVR's of the S1gene in the analysis. In addition, the spatial component of the characterisation shows evidence that most of the current strains originated from old lineage IBV's and their rapid evolution assures the discovery of new genetic variants in the future. Full genome characterisations are useful when available. The information provided by whole genome sequencing should confirm the partial or complete analysis of the S gene and provide further information on other genes. Variability of the outer viral proteins such as the spike and envelope are of particular interest.

SURVEILLANCE INTERPRETATION

The routinely performed genotypic surveillance of IBV involves molecular amplification and sequencing of a portion of S1 or the complete S1 gene. These sequences are curated and compared with sequences located in databases such as GenBank. The sequences should be compared in phylogenetic trees with the most commonly used vaccines and strains isolated in the geographic area of interest. The size of the segments compared are of major importance. Short sequences do not account for all HVR's and might provide inaccurately high homologies when compared to other sequences in the database, leading to wrong interpretations. In addition, comparing segments of different lengths can generate high identities with low query coverages, resulting in biased homologies that need to be considered in the interpretation. If the coverage is low, the confidence of the analysis might be at risk. The detection of a genetic variant does not imply that this virus is the causal agent of problems in the field. Surveillance data should be correlated with clinical signs, pathology, management, field data and vaccination records. Not all variants acting in a geographic region require the preparation of autogenous vaccines since a percentage of them are transient and restricted to a limited geographic area. It is important, other than performing molecular epidemiology using phylogenetic trees, to associate these trees with pathological observations in the field, perform virus isolation and biological characterisation in chickens. If the viruses are highly pathogenic or cause severe productive losses, vaccine protection studies are recommended. The objective is to test protection elicited by commercially available vaccines. If the protection provided by the available vaccines is not adequate, studies to assess variability of the detected variant should be performed to determine if formulating a homologous vaccine to prevent outbreaks is safe (Gallardo *et al* 2016). Sometimes IBV variants that are not clearly associated with disease are detected. In this case, it is recommended to continue with close epidemiological surveillance. Generation of databases for specific regions is desired, since they allow for better interpretation of results (Gallardo *et al* 2016).

CONTROL OF IMMUNOSUPPRESSIVE VIRAL DISEASES

Immunosuppression in poultry can be caused by several factors, including stress, nutritional deficiencies, mycotoxins and viral diseases (Hoerr 2010). The main viral immunosuppressive diseases reportedly linked with IBV cases in the field are IBDV and CAV. IBV cases in broiler chickens usually occur between 35 and 49 days of age (figure 1) and are potentially associated with immune deficiencies (Toro *et al* 2006, Gallardo *et al* 2016). As a confirmatory experiment, bursa of Fabricius samples were collected from a representative number of flocks in a broiler integrator in the U.S. Each bursa was divided in half, one half was used for IBDV viral load measurement by RT-qPCR and the other half was used for bursal histomorphometry to evaluate lymphoid depletion (Gallardo 2018) (figure 2).

Results showed a peak of IBD viral load between 25 to 30 days of age, followed by a peak of lymphoid depletion in the bursa around day 35 of age. These results correlate with the reported age of highest incidence of IBV cases in broiler chickens (figure 1) (Gallardo *et al* 2016). This correlation was previously reported by Toro *et al* (2006) where they measured lymphoid depletion scores of bursa and thymus sections in 322 cases of diagnosed IB between 1997 and 2002 in Alabama. In that project, peaks of lymphoid depletion associated with IBDV and CAV in bursa and thymus, respectively, were found in broilers between 30 and 40 days of age (Toro *et al* 2006). IBDV and CAV immunosuppression can be prevented by vaccination strategies. An efficient vaccination strategy for IBDV prevention must involve hyperimmunisation of breeders using live attenuated or recombinant and inactivated vaccines in order to protect chickens until approximately 3 to 4 weeks of age. In addition, vaccination of the progeny with recombinant, live attenuated or antigen-antibody complex IBDV vaccines provide appropriate protection until approximately 5 to 6 weeks of age in broilers (Gallardo 2018). For CAV, an effective preventative strategy is to generate adequate immune response in the breeders to impede vertical transmission and to transfer maternal antibodies to the chicks. For that, an adequate vaccine should be selected and the induced immunity should be

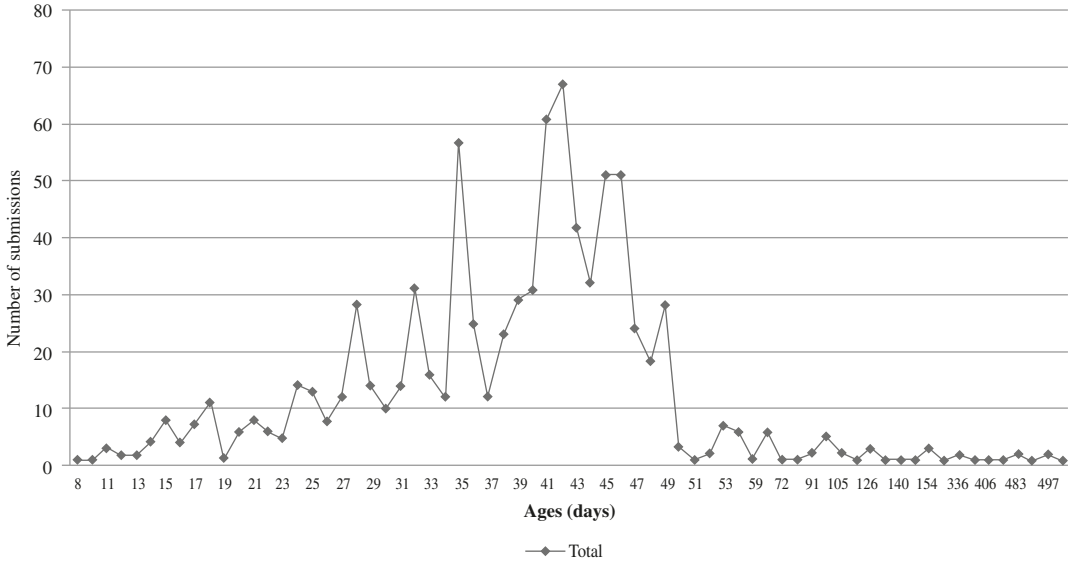

Figure 1. Age of occurrence of IBV respiratory cases in broilers and layers (n:1444) in California between 1997 and 2012.

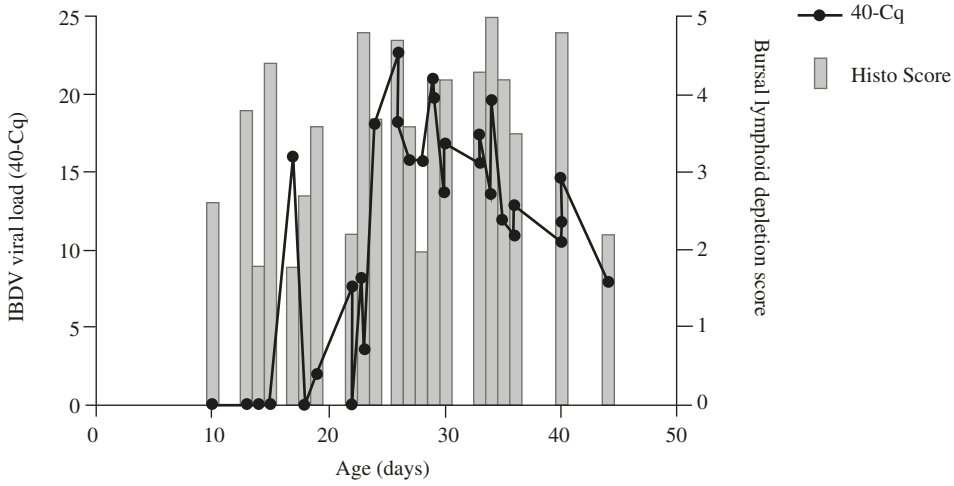

Figure 2. Bursa of Fabricius lymphocyte depletion scores from 0 to 5. (0: no lymphoid depletion to 5: highest level of lymphoid depletion) and viral load in bursas expressed as 40-Cq. These data were obtained from commercial broiler chickens at different days of age starting at 10 days and ending at 43 days.

periodically assessed (Gallardo 2018). There are commercial ELISA kits available for CAV antibody assessments. Serum samples diluted 1:100 allow clear dispersion of the data points (Toro 2015). The results should be analysed considering S/N ratio limit values of 0.2 and 0.8. Values below 0.2 represent protective titers, values between 0.2 and 0.8 represent low titers and values above 0.8 represent non-protected chickens or negative titers (Toro 2015). Even though it has not been reported as a direct cause of increased IBV cases, Marek's disease virus (MDV) can cause immunosuppression, affecting both humoral and cellular immune responses (Gimeno and Schat 2018). The MDV immunosuppression pathogenesis is very complex, poorly understood and in many cases under diagnosed. MDV vaccination protects against some aspects of viral immunosuppression but certainly not all (Gimeno and Schat 2018).

IBV MANAGEMENT

Proper management is a key component of a disease-free flock. Chickens can be exposed to stressors and infectious diseases that impair innate and acquired immunity, eroding general health and welfare (Hoerr 2010). Any stress caused by poor management can affect the immune system and the consequences can be linked to upper respiratory tract infections (Hoerr 2010). An example of poor management is poor ventilation. If intensive productive units are poorly ventilated, the incidence of respiratory diseases increases. Increased particulate matter and ammonia in the air (Shepherd *et al* 2015, Zhao *et al* 2015), in addition to the increased concentration of Gram-negative bacteria in the environment (Zhao *et al* 2016), can cause irritation, inflammation and changes in the microbiota, inducing complex upper respiratory diseases (figure 3) (Gallardo 2018). Addressing the presence of risk factors and understanding their interaction is essential for a successful management and consequently optimal health and welfare. In addition, genetics and nutrition have a major role in an efficient production system (Hoerr 2010). Food-borne mycotoxins and suboptimal nutrition can diminish immune responses, particularly the innate immunity to pathogens (Hoerr 2010). Biosecurity is the first barrier for pathogen introduction in poultry flocks. Lack of or flawed biosecurity facilitate the introduction of IBV, immunosuppressive pathogens (CAV, IBDV, MDV, reoviruses and adenoviruses) and other respiratory viruses such as avian influenza, Newcastle disease and avian metapneumoviruses. These upper respiratory tract viruses affect the ciliated epithelium and mucous glands of sinuses and tracheas, predisposing the entrance of more pathogens.

In conclusion, IBV is highly variable and variants arise because of the constant evolution of this virus. There are factors that predispose the virus to more variability, such as the introduction of exotic genotypes, poor vaccine selection and application and immunosuppressive diseases.

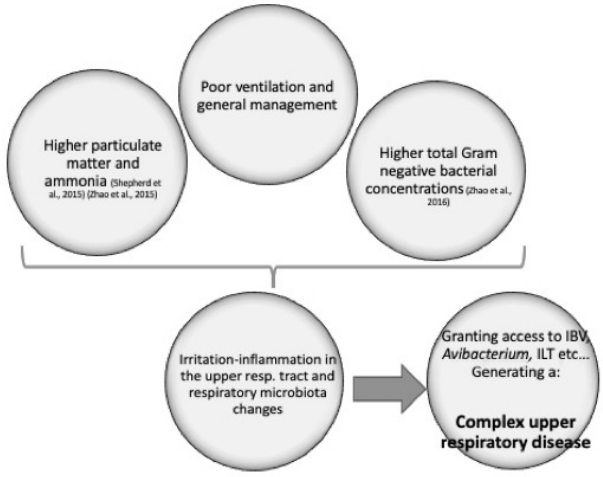

Figure 3. Effects of poor management in the air quality and upper respiratory tract of chickens, pathogenesis of complex upper respiratory disease.

Diagnosis, detection and surveillance are necessary to investigate and properly analyse the circulating IBV genotypes. This understanding depends on appropriate interpretation of results and assists the development of suitable preventative strategies and vaccination decisions. In addition, good practices, management and adequate control of immunosuppressive diseases help preventing the arise of variant IBV strains. Prevention of endemic diseases in poultry is based on properly using the available diagnostic and surveillance tools and interpreting the results obtained. When these tools are not properly used, misleading information is shared, and deceptive epidemiological data is reported. These events can affect future preventative strategies and predictions on IBV evolution.

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REVIEW ARTICLE

Checklist and state of knowledge of helminths in wild birds from Chile: an update

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ABSTRACT. Helminths are an important component of biodiversity with over 24,000 species parasitising wild birds globally, with this figure on the rise given the growing interest in wildlife parasitology. The present study aimed to establish an updated baseline of the helminthological surveys on wild birds from Chile. Available publications were reviewed to build a parasite-host association checklist and also to discuss the state of knowledge regarding these parasites. A total of 92 publications were found between the years 1892 and 2019. Regarding helminth parasites, 174 taxa belonging to 3 phyla and 37 families were recorded, 114 taxa were identified at species level, with the rest remaining incompletely described. Also, 4 taxa corresponded to new genera and 16 to new at species for science. The most reported parasites were platyhelminthes (53.9%) followed by nematodes (36.2%) and acanthocephalans (9.2%). Sixty-five avian species from 19 orders have been recorded as hosts, with most of them having been studied only once (64.6%). Out of these, the order Charadriiformes had the highest number of publications (n=23). In the case of the avian species present in the country, 14.2% of native, 40% of endemic and 22.2% of exotic species have been recorded hosting helminths. Regarding heteroxenous parasites, only 2 species have had their life cycles elucidated. Among the methodologies used for parasitic identification, 48.9% of the studies used morphological tools, 5.4% used molecular tools and 4.3% used both tools. For that reason, there are evident gaps in the data concerning the hosts sampled, methodologies and issues related to the biology of parasites such as life cycles, among others. In this sense, the need for specialists and cooperative research becomes indispensable to improve our understanding of helminths.

Key words: wildlife, parasitology, helminths, Chile.

INTRODUCTION

Helminths are defined as metazoan parasites including phyla Platyhelminthes (class Cestoda and Trematoda), Nematoda and Acanthocephala, all of them having been reported parasitising wild birds around the world (Wobeser 2008, Roberts *et al* 2013). Around 24,000 helminth species have been estimated as infecting birds, although this figure could underestimate the real number of parasitic species for this host group (Carlson *et al* 2019). Thus, these organisms should also be considered as an important component of the biodiversity from any territory (Poulin and Morand 2004).

Helminth parasites can be found not only in the digestive tract but potentially in all tissues, organs, and cavities, depending on the preference for every taxon in every host (Wobeser 2008). The complexity of life cycles varies between different species, with direct or monoxenous life cycles (1 host) and indirect or heteroxenous life cycles (2 or more hosts) (Roberts *et al* 2013).

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Parasites from wild birds have been widely studied in Europe and North America providing a vast knowledge of taxonomy, disease, and ecology (Woberser 2008). In South America, the helminth fauna of wild birds have been mostly studied in countries such as Brazil and Argentina with several species and life cycles elucidated thanks to the constant surveys of several groups of local and international parasitologists interested in those hosts (e.g. Lopes *et al* 2017[a,b], Drago and Lunaschi 2015, Hernández-Orts *et al* 2019). Notwithstanding the above, the knowledge of helminth parasites in wild birds from Chile is fragmentary, with several avian species without records of its helminth fauna, which could be related to the reduced number of parasitologists performing active research in the country (Hinojosa-Sáez and González-Acuña 2005). The last review on helminth parasites in wild birds from Chile was published by Hinojosa-Sáez and González-Acuña (2005) who reported a total of 49 taxa from all phyla of metazoan parasites parasitising 30 avian species.

Based on a literature review, this study aimed known the number of helminth species parasitising wild birds in Chile and provide a concise description and discussion of the state of knowledge related to parasites, hosts, and methodology used by authors, among others.

MATERIAL AND METHODS

The present study included literature on helminth fauna of wild birds from Chile and it considered both native and introduced species. A time period between the years 1800 and December 2019 was established. Articles and books were searched through the databases of NCBI,

Scholar Google, WOS (Web of Science), Scopus and Biodiversity Library (https://www.biodiversitylibrary.org). The following keywords were used in those platforms: helminth, tapeworm, fluke, trematode, Digenea, Nematoda, Acanthocephala, Cestoda, parasite, and avian, using the operator OR between these words together with the word Chile next to the operator AND. Undergraduate theses, technical reports, and scientific meetings do not constitute formal publications (grey literature) and were thus not considered in the present work. Surveys whose location was only stated as "Patagonia", "South America" or "Tierra del Fuego" were also included in this study. The only requirement for those cases was that sampled birds had to be distributed in the country.

The topics considered in every article and book were the following: publications: year and type of publication (book, scientific article); parasites: species, state(s) of development and type of parasitism (natural, experimental); hosts: species, organ(s) parasitised, locality and origin (wild, zoo, rehabilitation centre). Besides, the type of life cycle (direct, indirect or unknown) for recorded helminths was established according to Yamaguti (1958, 1961, 1963), Khalil *et al* (1994), Anderson (2000) and Moravec (2009). For any new taxa recorded in Chile, a revision of local literature was made to establish its state of knowledge. Additional topics were the area of knowledge, the methodology used for the identification of parasites and the helminthological collections.

The classification of helminths is based on Khalil *et al* (1994), Gibson *et al* (2002), Jones *et al* (2005), Bray *et al* (2008), Anderson *et al* (2009), Amin (2013), Roberts *et al* (2013), Waeschenbach *et al* (2017) and the databases Global Cestode Database (https://tapeworms.uconn.edu/) and WoRMS (http://www.marinespecies.org/). The taxonomy of avian hosts and also their native, introduced or endemic status followed Avibase (https://avibase.bsc-eoc.org/avibase.jsp?lang=EN) and Martínez and González (2017). Meanwhile, conservation status for every host was based on the IUCN Red List (https://www.iucnredlist.org/).

A checklist was constructed indicating taxonomy and species of parasites and related hosts ordered alphabetically, state(s) of development for each helminth taxa and organ(s) parasitised, locality and region as a roman number between parenthesis ordered geographically from North to South (i.e., XV= Arica y Parinacota region; I= Tarapacá region; II= Antofagasta region; III= Atacama region; IV= Coquimbo region; V= Valparaíso region; RM= Metropolitan region; VI= Libertador General Bernardo O'Higgins region; VII= Maule region; VIII= Bío-Bío region; XVI= Ñuble region; IX= Araucanía region; XIV= Los Ríos region; X= Los Lagos region; XI= Aysén del General Carlos Ibáñez del Campo region; XII= Magallanes y de la Antártica Chilena region), and finally the respective reference. Any requested information not stated by authors was indicated as "Ni" ("Not indicated").

RESULTS

PUBLICATIONS

A total of 92 publications (88 scientific articles and 4 books) reporting helminths in wild birds from Chile have been published between the years 1896 and December 2019. During that period, and considering the 3 centuries (1800-1899; 1900-1999; 2000-2019), the number of publications per period was 1, 44 and 47, respectively. The highest number of publications was registered between 2011 and 2019, with 26 publications (figure 1).

HELMINTHS TAXA

One hundred and seventy-four taxa of helminths organised in 3 phyla, 6 classes, 9 orders, 37 families and 95 genera have been found parasitising wild birds in Chile. Out of these taxa, 114 were classified at species level with 4 of them constituting new genera, while 16 are new species. Another 47 helminths were identified at genus level only, 9 at family level, 3 at class and 1 at phylum level. Furthermore, 23 out 47 genera have only been recorded up to this taxonomic level in the country without a specific identification. Other two taxa correspond to accidental parasites of birds: *Anisakis* sp. and *Pseudoterranova* sp. A checklist of parasite-host associations is shown in supplementary table S1[1].

When analysing taxa for every phylum, 36.2% (63/174) belong to Nematoda, 54.6% (95/174) to Platyhelminthes (52 taxa belonging to class Cestoda and 43 taxa to class Trematoda) and 9.2% (16/174) to Acanthocephala. Also, 63% (58/92) of publications recorded platyhelminthes, 57.6% (53/92) nematodes and 18.5% (17/92) acanthocephalans. Only 33.7% (31/92) recorded mixed infections between different phyla. About the type of mixed infections, 91.3% (84/92) of the publications recorded natural mixed infections, 14.1% (13/92) experimental mixed infections and 4.4% (4/92) had no details on the type of infection.

HOSTS

A total of 65 species from 19 orders and 32 families of birds were recorded as being parasitised by helminths. Also, 2 birds were not identified at species level, i.e. *Larus* sp. and *Anas* sp. A total of 63 bird species were catalogued as native, with 4 of them being also considered as endemic species, while another 2 were catalogued as exotic species. Consequently, and considering the number of avian species present in Chile, 14.2% (63/443) of native species, 40% (4/10) of endemic species and 22.2% (2/9) of exotic species have been recorded harbouring helminths to date. With regard to the conservation status of hosts, 57

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

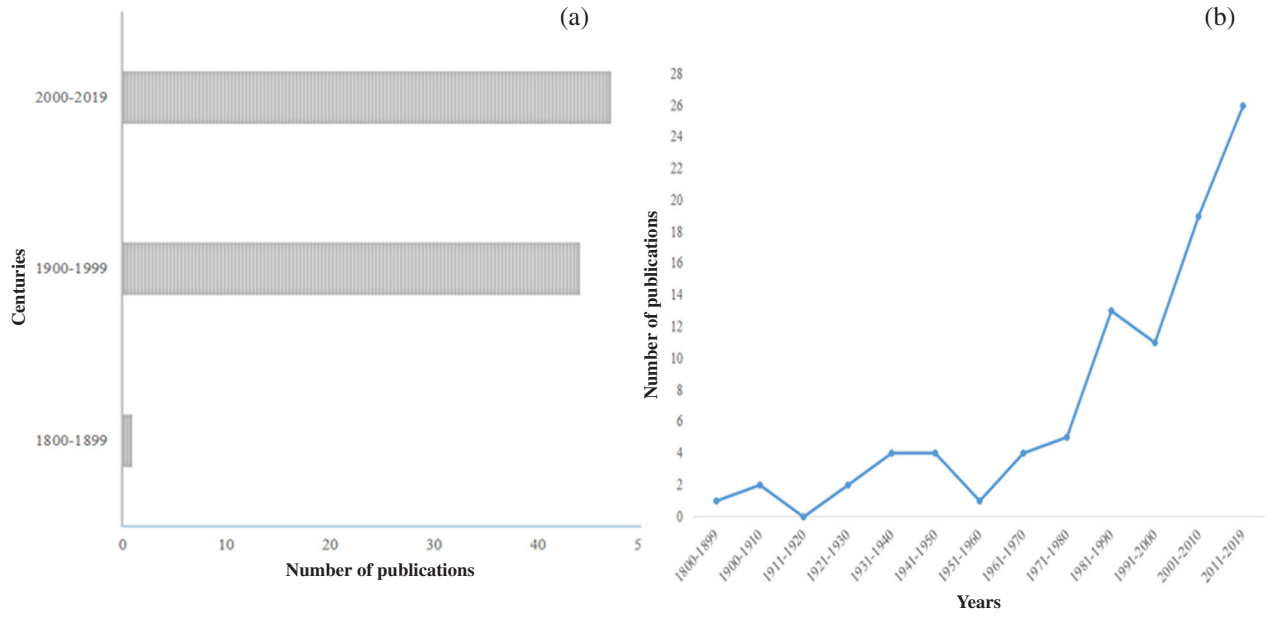

Figure 1. Time scale showing trends in publications regarding helminth fauna in wild birds from Chile. (a) Data organised by centuries from 1800 to the present, and (b) data organised by decades from the year 1900, with only one group for the seventeenth century.

species were classified as Least concern (LC), 3 as Near threatened (NT) and 5 as Vulnerable (VU).

The avian order with the highest number of publications was Charadriiformes (n= 23) followed by Sphenisciformes (n= 14), Passeriformes (n= 11) and Suliformes (n= 10). In the case of the number of species of hosts studied, the order Passeriformes denoted the highest number of publications (13 out 153 species) followed by Charadriiformes (10 out of 81 species). Regarding the number of publications for every avian species, kelp gull (*Larus dominicanus*) recorded the highest number (n= 17) followed by Brown-hooded gull (*Chroicocephalus maculipennis*) (n= 10) and the Neotropic cormorant (*Phalacrocorax brasilianus*) (n= 8). Despite the above-mentioned information, 64.6% (42/65) of avian hosts have been studied only once.

When considering the origin of birds, 79.4% (73/92) of publications were based on wild birds, 7.6% (7/92) came from wildlife rehabilitation centres, 4.4% (4/92) from zoos and 9.8% (9/92) of publications did not detail the source.

The analysis of publications covering the subject of infection sites showed that 60.9% (56/92) of them recorded parasites from the digestive system, 2.2% (2/92) from the respiratory system and 6.5% (6/92) from other organs/sites (e.g. articulations, blood, bursa of Fabricius, coelomic cavity, pancreas and tendons), where as 34.8% (32/92) did not detail the organ/tissue.

LIFE CYCLES

Seventy-four out 114 species have indirect life cycles, 20 have direct life cycles and 20 have unknown life cycles. Two out of 74 heteroxenous species have had their life cycles elucidated in Chile: *Profilicollis altmani* and *Profilicollis antarcticus*. Additionally, there are other 3 heteroxenous helminth species with incomplete knowledge of their life cycles: *Dibothriocephalus dendriticus*, *Stephanoprora uruguayense* and *Tylodelphys* cf. *destructor*. Considering the 47 parasitic taxa identified only to genus level, 43 of them would have indirect and 4 direct life cycles.

PARASITES BY REGION

Avian hosts from 12 out of 16 regions of Chile have been recorded as parasitised by helminths. The most studied regions were Los Ríos region (19.6% of publications, 18/92), Ñuble and Biobío regions (16.3%, 15/92 each one), and Los Lagos region (10.9%, 10/92). In contrast, wild birds from Tarapacá (I), Atacama (III), Libertador General Bernardo O'Higgins (VI) and Aysén del General Carlos Ibáñez del Campo (XI) regions have not been surveyed for helminths yet. Furthermore, in 15.2% (14/92) of publications, the authors did not record the locality of collection of birds.

ADDITIONAL INFORMATION

Although all the publications were based on taxonomy, some of them considered additional focuses, e.g. 6.5% (6/92) considered pathology and 2.2% (2/92) had an ecological overview. These views were always combined with taxonomy (8.7%, 8/92).

METHODOLOGIES

The results on the methodology used showed that 48.9% (45/92) of publications used only morphological tools, while 5.4% (5/92) of surveys used molecular tools with 4 out 5 studies using it together with morphological description. Also, one study was based on the sole use of molecular tools and another used Giemsa stain. Other techniques used with the morphological descriptions of isolated helminths were histopathological analysis with hematoxylin-eosin stain (6.5%, 6/92) and coproparasitological techniques (4.4%, 4/92). In contrast, 44.6% (41/92) of studies did not detail the technique used to identify the parasites. All surveys, except for 1 publication which used only Giemsa stain for blood smear examination, were based on necropsy of birds.

HELMINTHOLOGICAL COLLECTIONS

Only 45.7% (42/92) of the studies deposited parasites in a helminthological collection, 21.7% (20/92) of publications did so in a national collection, 19.6% (18/92) placed them in an international collection, and 4.4% (4/92) placed them in both collections. Additionally, only one study out 5, which used molecular tools, detailed the sequences of the parasites deposited in GenBank.

DISCUSSION

Since the review by Hinojosa-Sáez and González-Acuña (2005) that was published 14 years ago, a substantial increase in the helminthological knowledge of birds has been recorded until 2019; 49 taxa in the former review versus 174 taxa in this work. This prominent increase could be the consequence of a higher number of publications, i.e. 42 additional scientific articles since 2005, plus another 21 studies that were not considered in the former review (see Lönnberg 1896, Fuhrmann 1908[a, b]; Baylis 1928, 1932, Duthoit 1931, Baudet 1937, Porter 1937, Tagle 1942, 1966, Rausch and Morgan 1947, Schuurmans-Stekhoven 1950, Dubois and Rausch 1960, Yamaguti 1963, Szidat 1969, Forrester *et al* 1978, Torres *et al* 1981[a], 1982[b], Bartlett and Greiner 1986, McDonald 1988, Wong and Anderson 1991).

The first study reporting helminths in a Chilean wild bird was made by Lönnberg (1896) and the last by Oyarzún-Ruiz *et al* (2019). On a time scale, the number of publications from the year 2000 to present is higher than that registered during the past century (47 vs 43), which is noteworthy because in 19 years there have been more articles published than in one century. This could be related to a major interest of researchers in this group of hosts.

The increase in the number of species is remarkable as well, from 34 in the former review to 115 species up to 2019. Also, 6 new species were described after the former review was published in the year 2005. In contrast, although there are 16 new species for science described to date, 2 have questionable taxonomic validity, i.e. *Contracaecum macronectidis* (Schuurmans-Stekhoven 1950) and *Taenia diaphoracantha* (Fuhrmann 1908[b]). The first species is the only record of a helminth species for Southern giant-petrel (*Macronectes giganteus*) from Chile, however, the description was brief and based on one immature worm only, therefore, additional surveys are needed to collect and describe mature worms which are necessary for an accurate identification (see Anderson *et al* 2009). For the second species, isolated from a Southern rockhopper penguin (*Eudyptes chrysocome*), it must be noted that the genus *Taenia* only parasitises mammals as definitive hosts (Khalil *et al* 1994). Also, the genera *Tetrabothrius* and *Parorchites* are the only tapeworms recorded infecting penguins (Brandão *et al* 2014). The description for *T. diaphoracantha* provided by Fuhrmann (1908[b]) detailed several hooks which could be related to genus *Parorchites*, which is also the only penguin tapeworm with a rostellum (Khalil *et al* 1994). However, this genus has not been recorded in this penguin, *E. chrysocome*. On the other hand, the figure of an adult *Streptocara* sp. from Adelie penguin (*Pygoscelis adeliae*) by Fredes *et al* (2008) resembles specimens of *Stegophorus* spp. with some features that are typical for this genus (e.g. well-developed helmet-like structure with several denticles, thin and long vestibule, and deirids at vestibule-esophagus union) which are absent in *Streptocara* (see Yamaguti 1961, Anderson *et al* 2009). For this reason, a revision of the material is needed to establish the identity of these worms.

Furthermore, 23 genera have been recorded only once in Chile, highlighting the need of reviewing the available material and carry out additional surveys to classify these parasites and to determine if they correspond to new species or previously described taxa in other countries, thus amplifying its geographic range to our country.

Anisakis sp. and *Pseudoterranova* sp. are parasites whose definitive hosts are marine mammals such as cetaceans and pinnipeds meanwhile fishes act as intermediate hosts (Anderson 2000). These nematodes occasionally are found in the stomachs or intestines of wild birds (George-Nascimento and Carvajal 1980, Torres *et al* 2005) which get parasitised through predation of fishes. Only larval stages are found in birds because these nematodes mature exclusively in the former mammals, therefore, birds are considered to be accidental hosts (Anderson 2000).

When considering the total native avifauna from Chile (443 species) (Martínez and González 2017, MMA 2018), it is found that only a few species have been studied for helminthological fauna (14.2%). Even exotic species lack attention (22.2%), e.g. the house sparrow (*Passer domesticus*) and the ring-necked pheasant (*Phasianus colchicus*) have no published articles regarding endoparasitic fauna. Some possible explanations could be the few groups of parasitologists working on helminths in wildlife in the country. Besides, several avian hosts have restricted distributions in rugged terrain, such as different birds

that are only present in the highlands (e.g., puna hawk, *Geranoaetus poecilochrous*) or in Patagonia (e.g., lesser rhea, *Rhea pennata pennata*) (Martínez and González 2017). Furthermore, 10 avian species are considered endemic in Chile (Martínez and González 2017) and 4 out of them have been studied for helminths. Those studies on endemic species have identified helminth species that seem to be restricted to Chile, with no further records out of the country, e.g., *Navonia pterodromae* from pink-footed shearwater (*Ardenna creatopus*) and Juan Fernández petrel (*Pterodroma externa*) (Diaz *et al* 2007), and *Aploparaksis tinamoui* from Chilean tinamou (*Nothoprocta perdicaria*) (Olsen 1970, Rubilar *et al* 1996), therefore, they might be considered as endemic parasites. In this way, helminthological studies in the rest of the endemic species could provide similar results, however, the threatened state of conservation of these host populations and their recondite habitats (Martínez and González 2017, MMA 2018) limit the efforts of sampling.

In the case of the avian orders and species, the order Charadriiformes and the kelp gull concentrated most of the published studies, a result similar to that suggested by Hinojosa-Sáez and González-Acuña (2005). In fact, most publications covering this avian order correspond to those on kelp gull (17 out of 23). This could be associated to the wide geographical distribution and population size of this bird in the country, a species with no conservation issues and, consequently, allowed to be captured according to the Hunting Law of the State (SAG 2018).

There are other cases in which certain birds have been surveyed but no helminths have been isolated, such as the Chilean pigeon (*Patagioenas araucana*) and the invasive monk parakeet (*Myiopsitta monachus*) (Arriagada *et al* 2010, Briceño *et al* 2017). This absence could be the consequence of factors such as the age of birds, necropsy procedure focused only on gastrointestinal parasites or small sample size. Thus, additional surveys including a complete parasitic necropsy are encouraged to determine their helminth fauna in the country. Also, there is an erroneous report by Fuhrmann (1908[a]) who indicated helminths in a great black-hawk (*Buteogallus urubitinga*) coming from Chile. However, this species is not distributed in the country but it is found in Central America and Northern Argentina (Ferguson-Lees and Christie 2005).

With regard to infection site in hosts, only three helminth species were recorded as extra-intestinal parasites, i.e. *Cyathostoma* (*C.*) *phenisci* infecting the respiratory system (Baudet 1937, Oyarzún-Ruiz *et al* 2015) and *Pelecitus circularis* and *Pelecitus fulicaeatrae* in the joints of birds (Bartlett and Greiner 1986, González-Acuña *et al* 2017). The reduced record for extra-intestinal parasites could be related to the non-sampling of such organs at the moment of necropsy. For example, *Prosthogonimus ovatus*, a fluke from the bursa of Fabricius in the Neotropic cormorant, and the avian schistosomes in the blood vessels of kelp gull have been recorded in Argentina (Drago and Lunaschi 2015, Brant *et al* 2017) but not in Chile. At first, it could be considered that these helminths are not present in the country. However, all studies related to these two birds in Chile have focused only on digestive parasites. In consequence, their presence in the national territory cannot be discarded. Thus, future surveys should consider all organs and tissues as possible habitats of parasites, providing data to increase the number of host-parasite associations and helminth biodiversity for Chile.

As for the species with indirect life cycles, only 2 have known life cycles in the country: *Profilicollis altmani* with marine birds (Franklin's gull *Leucophaeus pipixcan*, grey gull *Leucophaeus modestus*, silvery grebe *Podiceps occipitalis*, whimbrel *Numenius phaeopus*, brown-hooded gull and kelp gull) as definitive hosts (Riquelme *et al* 2006, González-Acuña *et al* 2017, Rodríguez *et al* 2017[a]) and sandy-shore molecrab *Emerita analoga* as intermediate host (Jerez *et al* 2010, Rodríguez *et al* 2017[a]); *Profilicollis antarcticus* with marine birds (imperial shag *Leucocarbo atriceps* and kelp gull) as definitive hosts (Torres *et al* 1991[b], 1992, Rodríguez *et al* 2017[a]). On the other hand, there are examples of incomplete known life cycles, e.g. *Stephanoprora uruguayense* whose definitive hosts in Chile are piscivorous birds (major grebe *Podiceps major*, Peruvian pelican *Pelecanus thagus*, brown-hooded gull and kelp gull), and second intermediate hosts are galaxiid fishes (Torres *et al* 1982[a], 1983b, 1991b, 1992, 1993, Viozzi *et al* 2008). However, the first intermediate host remains unknown. In Argentina, this life cycle was elucidated by Ostrowski de Núñez (2007), indicating the aquatic snail *Heleobia parchappei* as the first intermediate host. This genus of snail is also present in Chile (Valdovinos 1999), therefore, surveying these mollusks could elucidate the life cycle in the country. Another example is *Dibothriocephalus dendriticus* whose definitive hosts are gulls (brown-hooded gull and kelp gull) and teleostean fishes (galaxiids, perchs and salmonids) as second intermediate hosts (Muñoz and Olmos 2008), however, the first intermediate host has not been reported. Torres *et al* (2007) recorded the copepod crustacean *Mesocyclops longisetus* as the first intermediate host for another broad tapeworm, *Dibothriocephalus latus*, in Southern Chile. Thus, a relative to these crustaceans, probably, could act as a suitable host as well.

In some cases, there is incomplete identification of larval or adult stages belonging to the same genus, e.g. *Wardium* spp. and *Confluaria* spp. The former has been recorded as larvae in the brine shrimp (*Artemia persimilis*) and adult tapeworm in austral thrushes (*Turdus falcklandii*). Nevertheless, in both cases there is no species identification (Llanos-Soto *et al* 2019, Redón *et al* 2019), therefore, a relationship between both remains uncertain. Furthermore, shorebirds act as definitive hosts as well (Khalil *et al* 1994). For the second example, this tapeworm has been isolated in the same brine shrimp as *Confluaria podicipina*, which use grebes as definitive hosts (Redón *et al* 2019). However, in the silvery grebe from Chile, this

group has been identified only to genus level (González-Acuña *et al* 2017). Besides, there are records of larval stages of helminths which use birds as definitive hosts (DH), however, these remain unknown in Chile, e.g. third larval stages of *Contracaecum multipapillatum* (herons and pelicans as DH) in the flathead grey mullet (*Mugil cephalus*) (Fernández 1987, Anderson 2000), cystacanths of *Profilicollis chasmagnathi* (aquatic birds as DH) in estuarine crabs (Rodríguez *et al* 2017[b], Hernández-Orts *et al* 2019) and cysticercoids of *Fimbriarioides* sp. (waterfowl and shorebirds as DH) in brine shrimps (Khalil *et al* 1994, Redón *et al* 2019). Hereafter, additional surveys on wild hosts incorporating a precise identification of worms through the use of morphological and molecular tools, and also experimental infections, could elucidate the hosts and features of these unknown life cycles in the country (e.g. Ostrowski de Núñez 2007, Rodríguez *et al* 2017[a]).

There is an evident lack of knowledge for newly described species in avian hosts regarding their life cycles in the country with only the avian hosts known to date and no data on the intermediate hosts (heteroxenous life cycles). However, there is a particular case with the possible larval stage isolated reported by Redón *et al* (2019) who found the larval stage of *Flamingolepis* in brine shrimp. Hence, this crustacean could act as an intermediate host for *Flamingolepis chileno*, the only species recorded for this genus in Chile (see supplementary table S[1]).

Although there were other areas of science considered in conjunction with helminthological research, these were just a few; only 2 studies were focused on ecology and other 6 had a pathological focus. In consequence, and considering the importance for understanding the effects of parasitism on wild bird populations (Wobeser 2008), there is a poor understanding of the diverse interactions between helminth fauna and wild birds in Chile. This situation highlights the obvious need for specialists and cooperative work on these and other fields of research, a situation mentioned previously by Hinojosa-Sáez and González-Acuña (2005).

The present review showed that most studies were based on necropsy of birds except for 1 publication which used blood smears for detecting microfilariae (see Forrester *et al* 1977). Thus, the limits imposed by accessing carcasses in the non-studied birds could be bypassed through their capture with mist nets or sampling captive birds in rehabilitation centres (Lutz *et al* 2017), without the need to euthanize specimens. In those cases, the collection of faeces would allow the description of digestive, urinary and respiratory helminths through coprological techniques such as simple flotation or modified Baermann technique. However, the limitations of these techniques could be related to the specific identification of parasitic structures, i.e. species, for example (Smith *et al* 2007). Also, the screening of blood smears should be considered to look for microfilariae, a group of nematodes parasitising various orders of birds around the globe (Bartlett 2008) but with minimal attention here in Chile.

Another taxonomy-related issue recently discussed is the deposition of organisms in accessible collections such as museums, which is also applicable to parasites (Krell 2016). A not negligible percentage of surveys did not specify if isolated helminths were placed in a helminthological collection (53.3%), including some cases of new species such as *Variolepis fernandensis* (Nybelin 1929), *Notocotylus tachyeretis* (Duthoit 1931) and *C.* (*C.*) *phenisci* (Baudet 1937). Moreover, there was a case where material belonged to a private collection (e.g., *Tetrabothrius* (*Neotetrabothrius*) *eudyptidis* (Lönnberg 1896)). The deposition in accessible collections is important particularly for new species, species with conflictive identification as stated in the present review with the helminths *T. diaphoracantha* and *C. macronectidis*, and parasites not completely identified as in the case of the 23 taxa recorded only to genus level, for example. These collections would allow researchers to review material and solve those taxonomical conflicts. In Chile, the only available collection allowing the deposition of helminths is the Museum of Zoology at the Universidad de Concepción, Concepción (see https://www.naturalesudec.cl/zoologia-museo/). For these reasons, we suggest that researchers interested in parasitology should deposit holotypes and paratypes of parasites in this public national collection.

Since parasites are an important component of the biodiversity, although generally neglected by non-parasitologists, research programs should consider parasites as an overall goal in regions considered as hotspots of biodiversity (Poulin and Morand 2004, Mariaux and Georgiev 2018).

As a final recommendation, and considering that there is a conspicuous lack of information mostly related to the methodology, forthcoming researches should fill these voids of data to allow future comprehensive analysis and comparisons related to different species of helminths.

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REVIEW ARTICLE

The biological basis of smoltification in Atlantic salmon

Francisco J. Morera[a*], Marcos Castro-Guarda[a], Daniela Nualart[b], Gabriel Espinosa[a], Jose L. Muñoz[c], Luis Vargas-Chacoff[b,d]

ABSTRACT. Chile is the second-largest producer of Atlantic salmon in the world, and the Chilean salmon production accounts for 27% of the world's production. One important step of the productive cycle in freshwater is the smoltification process that prepares the fish for the marine life stage. This review describes the biological basis of smoltification in Atlantic salmon, with particular attention on branchial osmoregulatory adaptations. We also discuss some of the infectious diseases and problems in smoltification (two of the main causes of losses in Chilean aquaculture) that could be related from a physiological point of view.

Key words: Chilean salmon culture, smoltification, Atlantic salmon.

GENERAL BACKGROUND

Aquaculture provides the world's growing population with both food and livelihoods (FAO 2020). Currently, aquaculture is the fastest-growing sector in the animal production industry worldwide, with an annual increase of 7.5% since 1970 (FAO 2020). According to the Food and Agriculture Organization (FAO) of the United Nations, aquaculture's contribution to total fish production has risen steadily in all continents, and Chile is one of the top-ten aquaculture producers. These ten countries contributed to 88% of the entire world's production by mass. Moreover, China is the major producer country by far (FAO 2020). In 2019, the value of seafood exported by China was more than USD 20 billion while Chile exported about USD 5 billion of seafood in the same year[1].

Chilean aquaculture production relies heavily on salmonids farming (salmon and trout), accounting for 84% of total Chilean aquaculture production and practically 100% of all farmed fish. According to Subpesca-Chile, in 2019 Atlantic salmon (*Salmo salar*) was the main species farmed in Chile, accounting for 58.7% of total production, followed by Pacific salmon (*Oncorhynchus kisutch*) with 36.2%, and Rainbow trout (*Oncorhynchus mykiss*) with 5.1% (Subpesca Chile 2020).

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[1] https://thefishsite.com/articles/rabo

Chile is the second-largest producer of Atlantic salmon in the world, with an annual average volume close to 800,000 tons between 2014[2] and 2019[3]. Chilean production of salmon species amounts for 27% of the world's production, while Norway continues to lead with 52% of the share (Iversen *et al* 2020). However, after the massive ISA virus infection in 2007, the Chilean government and the salmon industry put significant efforts into basic and applied research on many of the key aspects of salmon production to maintain Chile in a leading position worldwide (Olson and Criddle 2008, Martini Costa 2019, Iversen *et al* 2020).

SUMMARY OF THE SALMON AQUACULTURE PROCESS

In the last 40 years, the global salmon farming industry has switched from a small-scale operation to a mass-production scheme (Bjørndal and Aarland 1999, McLeod *et al* 2006, Olson and Criddle 2008). The high level of industrialisation of salmon aquaculture is significant due to the incremental success of strategies to adapt the salmon wildlife cycle to a large-scale farming setting.

Wild salmonids begin their life in freshwater. Adult salmons spawn in freshwater, their eggs hatch into alevins, and then they begin their development into fry and parr. At this stage, environmental cues initiate the smoltification process, preparing the fish for downstream migration and entrance into seawater, where they will grow up as a marine, predatory species (Björnsson and Bradley 2007, Björnsson *et al* 2011). This anadromous strategy confers reproductive and developmental advantages to salmon because it enables them to utilise a relatively safe environment provided by freshwater for reproduction, whereas juvenile migration towards the ocean allows

[2] https://www.salmonchile.cl/en/production/

[3] https://www.salmonexpert.cl/article/industria-salmonicultora-se-acerca-al-milln-de-toneladas-de-produccin-anual/

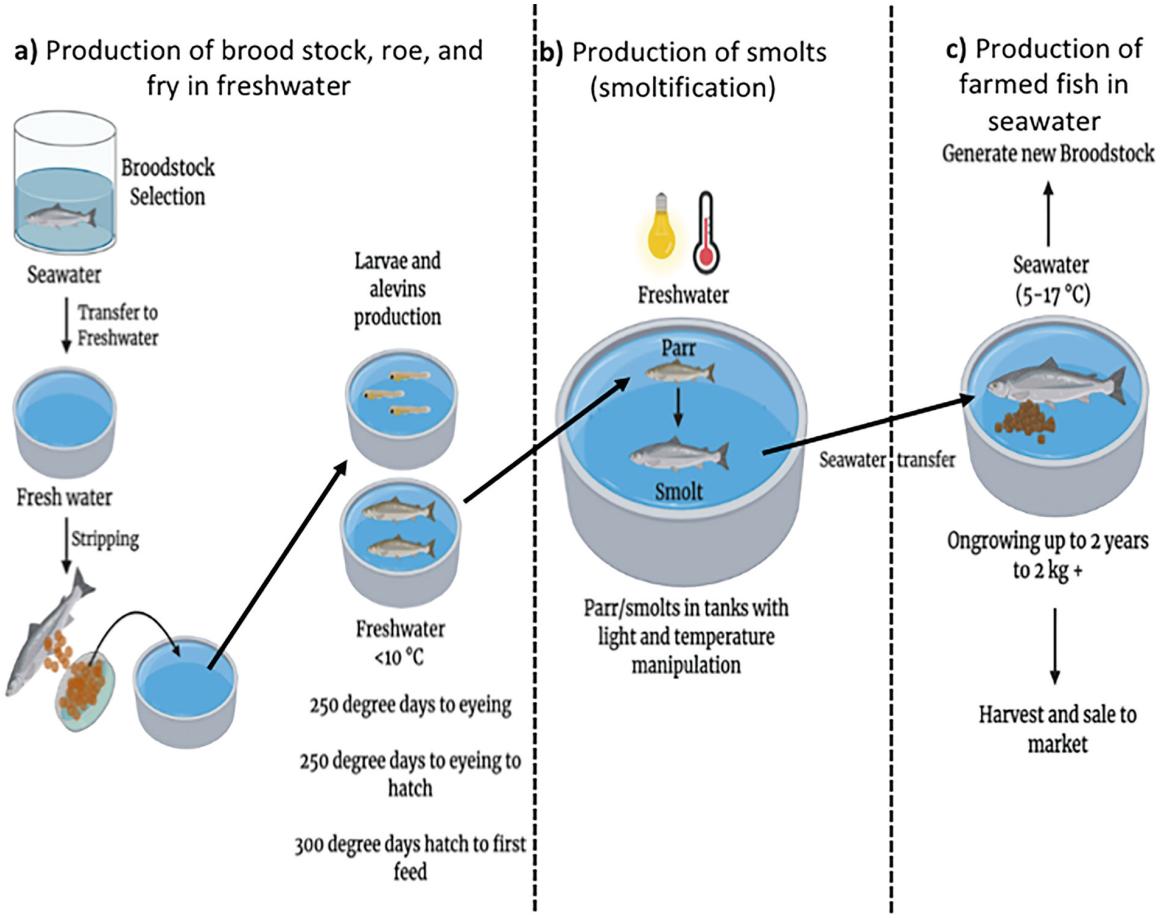

Figure 1. Summary of the salmon production process.

them to feed on a rich supply of fish and other marine organisms (Stefansson *et al* 2008). Thus, smoltification represents the key turning point in the anadromous life cycle of Atlantic salmon (McCormick 2009, Björnsson *et al* 2011).

The salmon production process was developed considering the biological background of this typically anadromous life cycle. The salmon production process can be divided into three steps (see figure 1): a) production of broodstock, roe, and fry in freshwater; b) production of smolts (smoltification) in freshwater; and c) growing farmed fish in seawater (Asheim *et al* 2011, Asche and Bjorndal 2011).

The production cycle of farmed salmon takes about three years on average. During the first year of production, the eggs are fertilised and the fish develop and grow to approximately 100 grams in a controlled freshwater environment (figure 1a and 1b) (Bergheim *et al* 2009). Subsequently, fish are transferred to seawater cages, where they continue growing until approximately 4-5 kg for 14-24 months (figure 1c). After reaching harvesting size, fish are transported to primary processing plants where they are slaughtered and gutted (Mowi 2020).

SMOLTIFICATION IN THE SALMON PRODUCTION CYCLE

In the productive salmon chain, the industrial smoltification phase (figure 1b) correlates directly with production efficiency because smolt quality impacts the indicator "yield per smolt" (harvest weight per smolt released). "Yield per smolt" is calculated as the fraction between harvest weight for each smolt transferred to seawater. This parameter is influenced by premature mortality rates, disease, temperature and growth attributes. The Faroe Islands reached a higher yield per smolt in the world: 4.87 kg in 2013. In Norway, the average yield per smolt was estimated at 3.71 kg, and in Chile, it was 3.58 kg in the same year.

The average yield per smolt for the Chilean salmon industry in 2013 was estimated at only 3.58 kg (Mowi 2020), and during 2015 it reached a minimum value of only 3.0kg. After improving the production strategy, productivity reached a record level of over 4.4-4.5kg per smolt during 2018, according to Aquabech[4].

[4] https://www.fishfarmingexpert.com/article/chile-production-has-reached-record-levels/

In the yearling production system, smoltification occurs following the seasonal yearling signals (natural photoperiod and temperature) rather than being dictated by the regular artificial programming of salmon. To start the smoltification, the salmon must reach a size-related threshold to respond to the ambiental signals. During the yearling smoltification, temperatures are not expected to be a problem since ambiental water supply is used following the species' natural smoltification period.

In the under-yearling production system, smoltification must be induced artificially through photomanipulation as the most common strategy, and the process takes place at a time of the year that is different to that dictated by regular biological, but artificially induced, programming. In this system, smoltification is induced by an artificial "winter signal" (alternating light and dark cycles of 12 hours), typically during late summer, when ambient temperatures are at their highest. In a typical photomanipulation program, the recommendation is to avoid a temperature drop during the transition from the first to the second period (Staurnes *et al* 2001).

BIOLOGICAL BASIS OF PHYSIOLOGICAL CHANGES THAT OCCUR DURING SMOLTIFICATION

In nature, smoltification, also called parr-smolt transformation, is a complex adaptation process driven by the endocrine system that consists of several independent but coordinated developmental changes in the biochemistry, physiology, morphology, and behaviour of juvenile salmon (McCormick 2013). These changes possess a high energetic cost for the fish and correlate with decreased defenses related to the immune system (Pontigo *et al* 2016): however, this process prepares the fish for downstream migration and transition to the marine life stage (Björnsson and Bradley 2007, Stefansson *et al* 2008). Important components of the parr-smolt transformation are i) environmental cues, primarily photoperiod and temperature (Björnsson and Bradley 2007); ii) endocrine control of smoltification (Björnsson *et al* 2011), and iii) physiological changes in osmoregulation that allow the smolt to thrive in high-salt environments (Clarke *et al* 1996, McCormick 2013).

ENVIRONMENTAL REGULATION OF SMOLTIFICATION

Photoperiod and seasonal temperature fluctuations are two important environmental cues that work together to transform Atlantic salmon parrs into smolts (Clarke *et al* 1996). In the Northern hemisphere, smoltification in wild salmon is complete by spring, when a rising temperature of 8-10 °C initiates wild smolt migration to seawater (Jonsson and Ruud-Hansen 1985, Clarke *et al* 1996). The mechanism by which photoperiodic information is translated into a neuroendocrine response in teleosts has not been fully elucidated. Different preparatory photoperiods show differential gill NKA activity and expression patterns in ionocytes (van Rijn *et al* 2020). Melatonin secretion by the pineal gland of salmonids can be directly stimulated by photoperiod (Falcón *et al* 2007). Also, elevated temperature increases melatonin secretion, and the salmon pineal gland could be working as a photoperiod and temperature sensor (Nisembaum *et al* 2020).

HORMONAL REGULATION OF SMOLTIFICATION

Parr-smolt transformation engages several endocrine signaling systems (see figure 2) (Björnsson *et al* 2011). After salmons have reached a size- or growth-related threshold, the light–brain–pituitary axis is stimulated by photoperiod and seasonal temperature, resulting in simultaneous increments of growth hormone (GH), cortisol, and thyroid hormones (McCormick 2001). Besides its growth related functions, GH modulates intermediary metabolism and osmoregulatory mechanisms in fish by stimulating somatomedin activity, such as insulin-like growth factors IGF-1 and IGF-2 (McCormick *et al* 1991, Madsen *et al* 1995, McCormick 1996). GH and cortisol interact to control hyperosmoregulatory mechanisms in gills, gut, and kidneys, promoting increased salinity tolerance and changes in growth (weight to length ratio) and intermediary metabolism. In gills, cortisol and the GH/IGF-1 axis promote differentiation of salt-secreting ionocytes (see next section for details), a process that requires upregulation of three major osmoregulatory membrane transporters: the sodium-potassium ATPase (NKA), the sodium-potassium-2 chloride cotransporter 1 (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR) (Hwang *et al* 2011).

Lastly, thyroid hormones regulate olfactory imprinting, metabolism, morphological changes such as silvering, and possibly behaviour (McCormick 2013), whereas prolactin in smoltification is thought to be a general inhibitor of most aspects of smolt development (Sakamoto and McCormick 2006). An increase in thyroid hormones (plasma T4) is detected in hatchery smolt after release and in wild smolt during migration (Iwata *et al* 2003, McCormick *et al* 2003). Also, plasma T4 increases after smolts are exposed to water with different chemical compositions (Hoffnagle and Fivizzani 1990) or during entry into estuarine environments (McCormick *et al* 2013).

OSMOREGULATORY CHANGES IN GILLS DURING SMOLTIFICATION

As mentioned above, salmonids begin their life cycle in freshwater, where they are hyperosmotic to the external medium. Osmotic pressure favours water entry into the body and the loss of salt by diffusion across the gill. To compensate for this passive flow of water and ions to maintain homeostasis, the fish eliminates excess water as diluted urine and obtains salts from food in the intestine

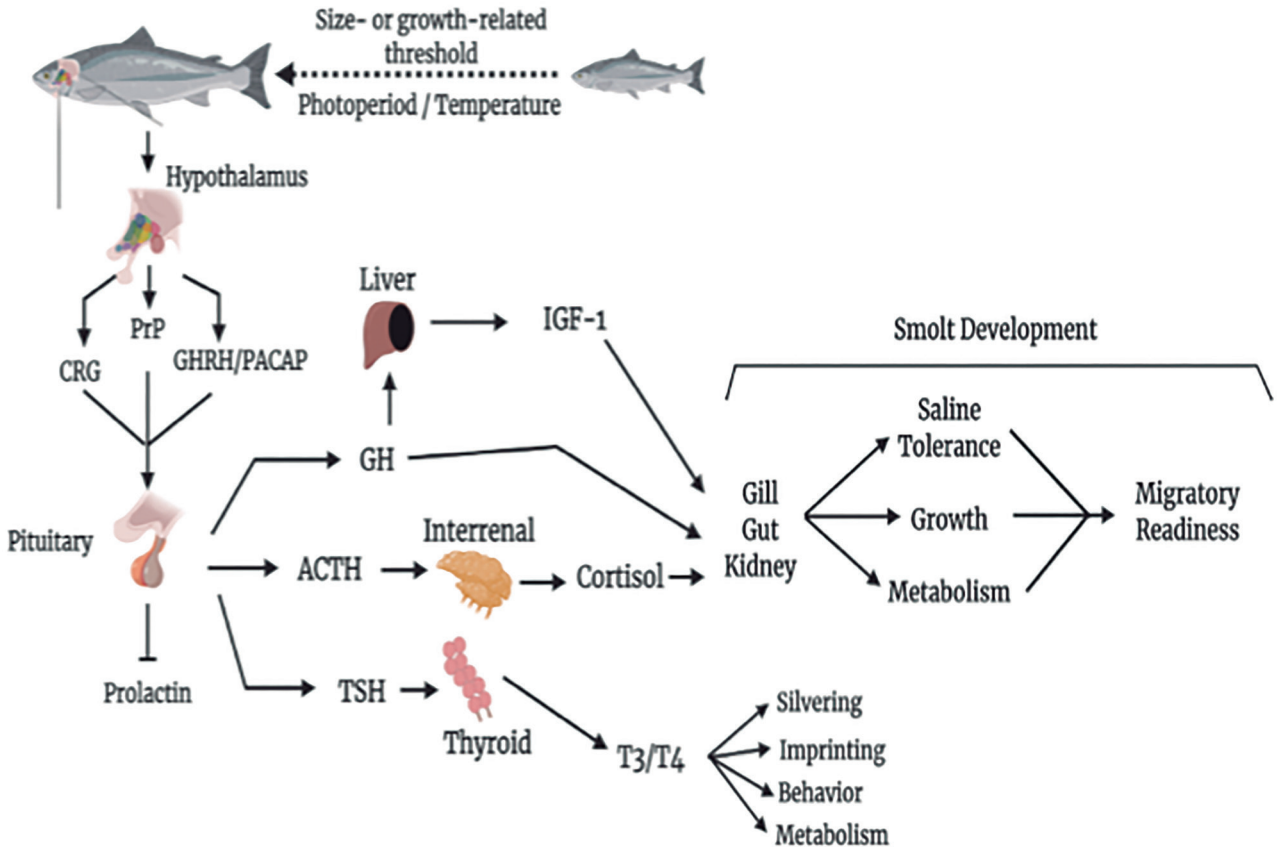

Figure 2. Neuroendocrine control of smoltification (modified from McCormick 2013). Increased response of the light-brain-pituitary axis stimulates circulating levels of growth hormone (GH), cortisol, and thyroid hormones. This response is triggered when the fish reach a size- or growth-related threshold to initiate smoltification in response to photoperiod/temperature stimuli. GH and cortisol interact to control hyperosmoregulatory mechanisms in the gill, gut, and kidney, resulting in increased salinity tolerance, as well as changes in growth and metabolism. CRF: corticotropin-releasing factor; PrP: prolactin-releasing peptide; GHRH: growth hormone-releasing hormone; PACAP: pituitary adenylate cyclase-activating peptide; ACTH: adrenocorticotropic hormone; TSH: thyroid-stimulating hormone.

and water by active uptake through the gill's epithelium. As they move into seawater, the osmotic gradient is reversed because the internal fluids of salmonids are approximately one-third the osmolarity of seawater, and they become hypoosmotic relative to the external medium. Accordingly, salmons lose water and gain salts by passive diffusion. As compensatory mechanisms, they drink seawater, reduce their urine production, and actively secrete salts across the gill's epithelium through specialised cells called ionocytes, mitochondria-rich (MR) cells, or chloride cells (Clarke *et al* 1996).

One of the key events in osmoregulatory changes during the parr-smolt transformation involves fine-tuning of the ion-transporting machinery in the gill epithelia (Tipsmark *et al* 2008). Ion transport is primarily carried out by MR cells (Madsen *et al* 2015) and requires expression changes in NKA (D'Cotta *et al* 2000), NKCC1, and other critical proteins at the cell surface, such as CFTR and claudins (Hirose *et al* 2003, Hiroi *et al* 2005). In most euryhaline teleosts, upregulation of gill NKA (Kamiya and Utida 1969, Morgan *et al* 1997, Seidelin *et al* 2000) and NKCC1 (Pelis *et al* 2001, Tipsmark *et al* 2002, Wu *et al* 2003) are associated with seawater acclimation.

NaCl secretion by teleost gills, necessary in seawater life, is accomplished via secondary active transport of Cl^- and passive transport of Na^+ (figure 3). The driving force for active transport is provided by NKA, which maintains intracellular Na^+ at low levels and intracellular K^+ at high levels, compared to the extracellular medium (Marshall and Grosell 2006). This NaCl secretion mechanism needs an additional condition to work in seawater: a thermodynamic requirement to recycle K^+ out via conductive pathways (potassium channels, figure 3). The molecule responsible for this K^+ transport is still unknown in salmonids, but several K^+ channels may be involved in the function of MR cells in other teleosts (Marshall and Grosell 2006). One plausible candidate is the inward-rectifying K^+ channel (eKir), highly expressed in gills of the seawater-acclimated Japanese eel (Suzuki *et al* 1999). Another candidate is a large-conductance, calcium-activated K^+ channel (called BK for "Big K^+"), whose expression was recently detected in gills from the teleost fish *Porichthys notatus* (Rohmann

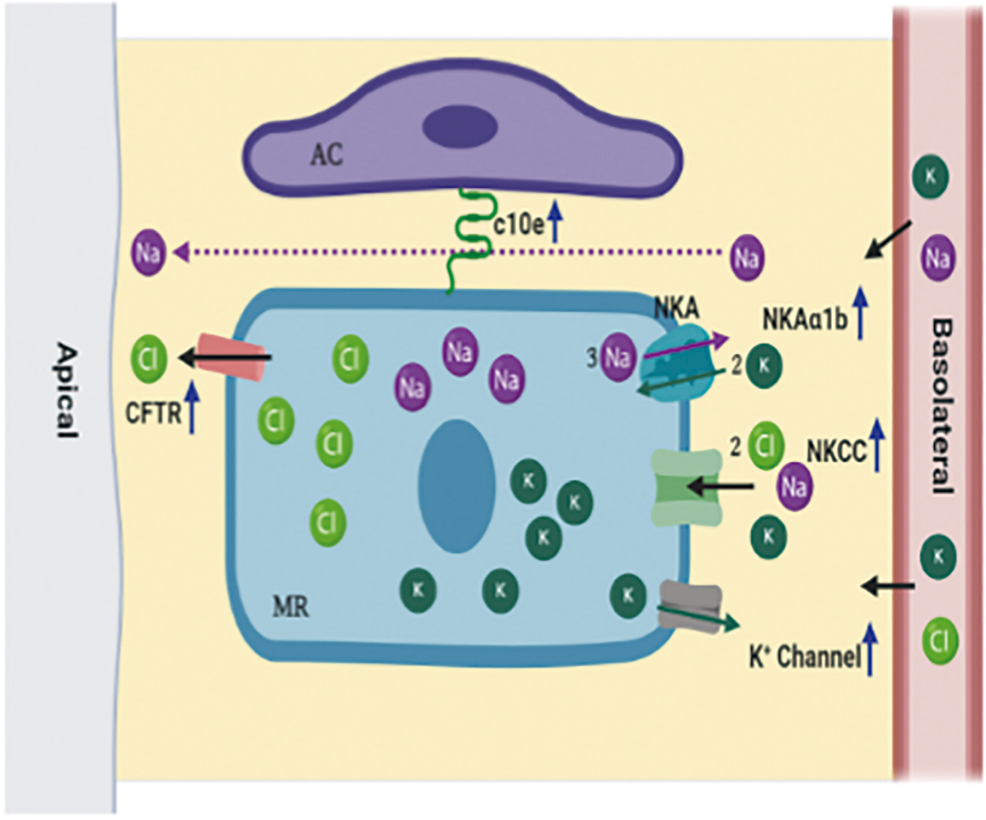

Figure 3. Model of NaCl secretion in the gill epithelium after seawater acclimation (Modified from Marshall and Grosell, 2006). A leaky paracellular shunt is formed (Claudin 10e mediated) between MR and AC that allows Na+ to be passively secreted. In MR cells Cl– enters via the Na+, K+, 2Cl co-transporter (NKCC) driven by the Na+ gradient, which is maintained by Na+, K+-ATPase (NKA). Cl– accumulates above its electrochemical equilibrium intracellularly, and exits through the CFTR type anion channels at the apical membrane. The K+ ion is also accumulated intracellularly until it exits through K+ channels. During smolt development, the amount of NKAa1b subunit increases. After exposure to SW, NKAa1b increases even more. NKCC, CFTR, and claudin 10e are also upregulated in FW during smolt development and increased further after exposure to SW.

NKA: Na+/K+-ATPase; NKCC: Na+/K+/Cl- cotransporter; CFTR: cystic fibrosis transmembrane regulator; MR: mitochondria-rich cell; AC: accessory cell.

et al 2009). Also, an inward-rectifying K^+ channel (Kir1.1 or ROMK for Renal Outer Medullary K^+ channel) and a BK channel were detected in MR cells from Mozambique tilapia (Furukawa *et al* 2012) and, more recently, in the gills from rainbow trout and Atlantic salmon by our research team (Loncoman *et al* 2015).

The capacity to measure the activity or the expression of different molecular components of this salt secretion machinery has provided useful tools for examining smolt development, and it will be discussed with greater detail in the next section.

MARKERS OF SMOLTIFICATION

Historically, research on smoltification has focused on understanding smolt development in the aquaculture industry and, in particular, the need to control the timing and quality of smolt for the transfer of juveniles from freshwater into ocean net pens (McCormick 2013). Knowing exactly when the smolts are ready to be relocated to seawater is crucial for the productivity of salmon farmers. Increased tolerance to salinity is often measured by either increased survival or by lower plasma electrolytes and osmolality after direct transfer from freshwater to high salinity (a "seawater challenge" as an example of a salinity tolerance test) (Clarke *et al* 1996, McCormick 2013). The main disadvantage of salinity tolerance tests is that they fail to distinguish between seawater tolerance and transient adaptation: since some fish may show increased survival but ultimately fail to adapt to long-term seawater exposure (Iremonger 2008).

Currently, the use of molecular markers of smoltification (see table 1) are used to accurately determine the transfer time to seawater since many physiological

Table 1. Common laboratory methods to test smoltification.

Name	Technique	Assay	References
Gill NKA activity	Spectrophotometry	Coupled assay to quantify ADP generated by ATPase activity by detecting the disappearance of NADH at 360nm	(McCormick 1993)
Gill NKA activity	Spectrophotometry	Detection of Pi generated by ATPase activity at 312nm[1] or 578nm[2]	[1](Zaugg 1982) [2](Flik *et al* 1983)
NKA subunit mRNA expression	Real-Time PCR	PCR primers for specific detection of mRNA from NKA subunits a1a and a1b	(McCormick *et al* 2009)
NKA protein expression	Immunohistochemistry	Antibodies against the NKA protein	(McCormick *et al* 2013)

changes produced during the parr-smolt transformation are detrimental to continued life in freshwater, and they tend to revert relatively fast if the fish are not able to enter seawater environments before smoltification is complete (the "smolt window") (Stefansson *et al* 2008). In most instances, the salmon industry uses a single enzymatic marker for smoltification: the enzymatic activity of gill NKA, which activity increases during the transition from parr to smolt (McCormick *et al* 2013, Zaugg 1982, McCormick *et al* 2009, Nilsen *et al* 2007). This protein activity is often considered an indicator of smolt development (McCormick 1993, Clarke *et al* 1996). The Chilean salmon industry uses NKA enzymatic activity in gills as a main marker to determine the timing of smolt transfer to seawater.

Many scientific publications describe that some of the salt secretion machinery`s critical molecules expressed in the gills of salmonids correlate with seawater adaptation. Atlantic salmon expresses two major NKAα isoforms in distinct gill ionocytes. NKAα1a is the most abundant isoform in freshwater, whereas NKAα1b predominates in seawater (McCormick *et al* 2009). Gill mRNA levels of NKAα1b increase during Atlantic salmon smolting, whereas NKAα1a mRNA decreases (Nilsen *et al* 2007). Also, co-transporters (NKCC1), aquaporins, and ion channels (CFTR, ROMK, and BK) are expressed in gills covary with salinity adaptation (Tipsmark *et al* 2010, Furukawa *et al* 2012; McCormick 2013, Loncoman *et al* 2018). Tipsmark *et al* (2008) showed that Claudin10e mRNA levels, a family of membrane proteins that form tight junctions and thus determine transepithelial resistance and ion permeability, also increase during smolt development and after exposure to seawater. All these molecules are considered potential molecular markers of smoltification, but there is no available evidence to determine its true predictive value in decreasing fish mortality in seawater. Some of these alternative markers are being implemented in Chile for research purposes (Loncoman *et al* 2015, Loncoman *et al* 2018, Vargas-Lagos *et al* 2018), except for the NKA subunits that some laboratories in Norway and Chile measure as a service (see table 1); but the salmon industry has not adopted these markers for routine tests, probably due to the lack of quantitative data about any advantage over NKA measurements alone.

The search for new possible markers for smoltification is ongoing using all available techniques, even high throughput analyses such as transcriptomics (Houde *et al* 2020, Versen *et al* 2020, West *et al* 2020) and small RNA sequencing (Shwe *et al* 2020).

SMOLT-WINDOW OR SMOLTIFICATION WINDOW

In industrial salmon production, survival and growth performance in seawater are the two major manifestations of smoltification. However, if smolts are prevented from reaching seawater exposure, several of the preparatory changes associated with marine life are reverted, a process known as desmoltification in Atlantic salmon or parr-reversion in Pacific salmonids (figure 4) (Hoar 1988, Duston *et al* 1991, Stefansson *et al* 1998). This natural reverting process closes the "smolt-window" during which smolts can enter and quickly adapt to seawater. Conceptually, the smolt-window (or smoltification window) is considered the period when physiological conditions to be transferred into seawater are at their optimal peak (Sharron 2015). The smolt-window is operationally associated only with the increase in gill NKA enzyme activity (figure 4).

The smolt window duration is in the range of 300-400 degree-days (d°C) (Stefansson *et al* 1998, McCormick *et al* 1999, Stefansson *et al* 2008). In the salmon farming industry, the decision to transfer smolt into seawater is taken using a threshold value of NKA activity, without any consideration of its dynamic properties, i.e. whether NKA activity is on the rise (smoltification phase), reaching its peak, or in decline (desmoltification phase). Zydlewski and Zydlewski (2012) show that gill NKA activity measurements are predictive of performance during the first few days of acclimation but, after transfer, fish grew at the same rate, with no differences in fish size and growth rate, among groups with initially different gill NKA activities. They concluded that gill NKA expression in freshwater at the peak of smolting

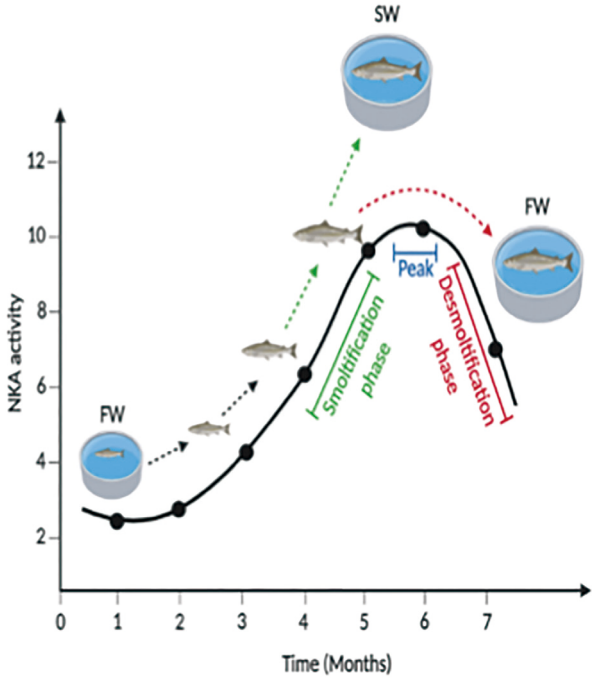

Figure 4. Changes in gill NKA activity levels during the smolt development of Atlantic salmon (according to McCormick 2013).

does not predict long-term growth in seawater (Zydlewski and Zydlewski 2012).

CURRENT CHALLENGES IN CHILEAN SALMON PRODUCTION: ARE SMOLTIFICATION AND INFECTIOUS DISEASES RELATED?

Aquaculture farming in Chile has experienced a high-speed growth, mainly due to favourable geographical and environmental conditions, but this fast growth, together with confinement systems and the presence of native life, increases the risk of infectious disease outbreaks. The 2019 annual reports from SERNAPESCA (SERNAPESCA 2019) describes the principal causes of mortality in the Chilean salmon aquaculture. This report pointed out that two of the leading causes are "unadapted" fish (fish with problems in osmoregulation by deficient smoltification, 11.2%) and infectious diseases (23.9%).

Historically, smoltification problems are the other leading cause of economic losses in Chilean salmon aquaculture because the amount of "delayers and unadapted" fish at the seawater stage is still significant, with an 11.2% in 2019, being the first one the infectious diseases (23.9%) as we previously mentioned (SERNAPESCA 2019). These groups consist of fish seeded in seawater during a suboptimal developmental phase of smoltification that prevents adaptation to their new environment. The "unadapted" are fish that do not adapt to the marine environment early on and die after entering the sea, mainly due to osmoregulatory problems. The "delayers" are fish that adapted relatively well after entering the sea but subsequently present impaired growth and productive performance[5].

In recent years, some Chilean smoltification facilities have adopted the Norwegian production standards from their company owners, and they claim that the problems regarding smoltification have been solved.

It is particularly surprising that despite the research focused on smoltification, very little has been studied regarding the impact of this transformation on the salmon`s immune system. Several earlier studies in smolts and post-smolts of Atlantic salmon produced evidence for a possibly weakened immunity. Decreased plasma lysozyme, IgM levels, and leucocyte levels were observed (Muona and Soivio 1992, Melingen *et al* 1995). In the last years, some groups have started to show some data of association between the increase in infectious diseases that occurs after transfer to seawater with an alteration in the immune response during smoltification. Post-smolts show a weak response against viral (Moore *et al.* 2017; Nuñez-Ortiz *et al.* 2018; Jensen *et al.* 2019) and also, their skin barrier to infection (Karlsen *et al.* 2018) or gut immune functions (Wang *et al.* 2020) are weak during the first post-smolt period.

Recent transcriptomics analysis have demonstrated repressed expression of genes associated with the immune system during smoltification and after seawater transfer (Johansson *et al* 2016, Krasnov *et al* 2016); opening the possibility of a biological relationship between two of the most important causes of death in Chilean aquaculture (infectious diseases and problems in smoltification) and a warning about the need for more scientific research regarding this relationship between two different and disconnected fields of study.

FUTURE PERSPECTIVES

Smoltification in salmonids continues to be a field of study in constant progress: new transporters are being discovered that change their expression during this process (Fleming *et al* 2019, Koltenyuk *et al* 2020, McKay *et al* 2020), and the parr-smolt transformation process is characterised in new strains of salmon in culture (van Rijn *et al* 2020), or wild salmon (Bernard *et al* 2019). Even different aspects of the process are evaluated (Nemova *et al* 2020), and the biological character of the process continues in development (Striberny *et al* 2021).

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[5] https://www.salmonexpert.cl/article/pathovet-da-un-paso-en-la-prediccin-de-peces-desadaptados-y-rezagados/

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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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A proof will be sent to the corresponding author for proofreading in PDF format, and must be returned within the specified time, otherwise the Editor reserves the right to carefully proof-read the article but without assuming responsibility for errors, to continue with the publication process. Alterations to the proof that do not correspond to minor errors will be charged to the authors. Neither the Editor nor the Publisher accept any responsibility for printed errors that had not been indicated by the authors.

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